AVIAN TELOMERE DYNAMICS

Christopher Graham Foote

This thesis is submitted for the degree of Doctor of Philosophy

Division of Ecology & Evolutionary Biology
Faculty of Biomedical & Life Sciences
University of Glasgow

September 2008

© Chris Foote 2008

CANDIDATE'S DECLARATION

I declare that the work recorded in this thesis is entirely my own unless otherwise stated, and that it is of my own composition. No part of this work has been submitted for any other degree.

Christopher Foote
September 2008

Thesis Summary

Telomeres, the repetitive DNA sequences that cap eukaryotic chromosomes, are thought to play an important role in linking life conditions and senescence. In vertebrate somatic cells, telomeres shorten at each cell division, and the rate at which they do so has been linked to cellular and organismal senescence. Although telomeres generally shorten with age in vertebrates, in most species studied there is considerable variation between same age individuals. In this thesis, I examined the telomere dynamics of various avian species, investigating both the causes of variation in telomere length among individuals and what effect this variation has on attributes such as survival rates.

Previous studies have shown that most telomere loss occurs in young individuals and it thus makes sense that early life conditions are responsible for much of the inter-individual variation in telomere length. I investigated this idea by studying chick telomere dynamics in a wild population of lesser black-backed gulls *Larus fuscus*. There was considerable variation in hatching telomere length among individuals and much of this variation was related to circumstances during embryonic growth. Larger hatchlings had shorter telomere lengths, suggesting that embryonic growth rate could have affected telomere attrition. Independent of this trend, males had longer telomeres at hatching than females. Although telomere length did decrease with age post-hatching, these initial variations remained consistent during the initial post-hatching period.

The relationship between early life conditions and telomere length was investigated further with a longitudinal study of telomere length in chicks of the European shag *Phalacrocorax aritotelis*. A previous study on this population of birds had shown that telomere length declines with age within individuals over a period of several years. However no change in telomere length was detected over a period of 11-13 days during the chick period. Body size had no effect on telomere length, but males did have longer telomere than females.

These initial chapters investigate telomere length in chicks; however there are very few studies that investigate telomere length over the entire lifespan of long-lived species. I thus next examined the telomere dynamics of two species of long-lived seabird, the

northern and southern giant petrels (*Macronectes* spp.). In both giant petrel species, telomeres were shorter in adults than chicks, but there was no trend for adult telomere length to decrease with age. In southern giant petrels, there was a significant relationship (independent of age and sex) between an individuals telomere length and whether it was still alive 8 years after it was initially sampled. This relationship was not present in northern giant petrels, possibly due to a smaller sample size. The results thus support both the idea that most telomere loss occurs in young individuals and that telomere length may be an indicator of life expectancy.

Various methods exist to measure telomeres. As the number of taxa whose telomere dynamics are being studied increases, it becomes increasingly important to know which methods are the best to use and to what extent these methods are applicable across species. These questions were investigated in relation to work conducted on the telomere dynamics of the blue-footed booby *Sula nebouxxi*. Both the TRF and qPCR techniques were used to measure booby telomeres, but problems arose with both methods. It is possible that these problems occurred because blue-footed boobies have a particularly large amount of interstitial telomeric DNA, although a more detailed analysis of booby telomeres would be necessary to determine this. These findings suggest that standardised methods to measure telomeres cannot necessarily be applied to every new species whose telomere dynamics are studied.

The evidence presented here suggests that the study of telomere dynamics can be a very powerful tool for behavioural ecologists. It now seems possible that telomeres might provide both a way of measuring the long-term costs of early life-conditions and a way to measure the quality of an individual. However, further research is still needed to fill in the considerable gaps in our knowledge and fully exploit the potential telomeres have for behavioural ecology.

Acknowledgements

This work would not have been possible without the help of many people. Most obvious are those who provided blood samples and other data, which formed the basis of three of the chapters in this thesis; thanks to Francis Daunt, Maggie Hall, Richard Phillips and Roxana Torres for this.

In my own field-work, Gunnar Hallgrímsson and Sveinn Kári Valdimarsson gave invaluable assistance and advice and the Sandgerdi Marine Centre provided excellent facilities. Back in the lab, Kate Griffiths and Aileen Adams provided much help with various lab techniques, while Pierre Bize, Winnie Boner and Francois Criscuolo gave a lot of their time helping me with qPCR's in an attempt to stop chapter six happening. Special thanks to Lizzie Gault who more or less kept her patience while being asked a huge number of telomere related questions over the course of my PhD.

A huge number of other people in the department gave me invaluable advice or help of some sort over the course of my PhD; thanks to Maria Bogdanova, Anaid Diaz, Britt Heidinger, Chris Donaldson, Mike Fisher, Davina Hill, Miran Kim, Tony Robertson, Clare Stamper, Patrick Walsh, Roni Wright and particularly Lilliana D'Alba. Thanks also to my family for general supportiveness, especially my sister for providing an excellent role model in how to complete a PhD thesis on time.

Finally, thanks to my supervisors Pat Monaghan and Lubna Nasir for their constant support and advice, even during the rather long periods when everything was going wrong.

This PhD was funded by a NERC studentship grant.

Contents

List of figures

List of tables

Chapter 1: General Introduction

This chapter will review the literature on telomeres in reference to their structure and function at the cellular level, their connection to cellular and organismal ageing and the link they provide between ageing and organismal attributes. The various methods used to measure telomere lengths will also be briefly reviewed.

1. Telomere structure and function

1.1 What are telomeres?

Telomeres are the ends of eukaryote chromosomes. The sequence and structure of telomeres is highly conserved (Henderson 1995); in vertebrates, telomeres comprise several kilobase pairs of double stranded DNA containing the repetitive sequence TTAGGG (Meyne *et al.* 1989), which ends in 100-200 bases of single-stranded TTAGGG at the 3' end, known as the 3' overhang (Makarov *et al.* 1997; Wright *et al.* 1997).

The exact structure of telomeres is unknown and varies between taxa, but the 3' overhang seems to be crucial to telomere function (Blackburn 2005). It was at one time thought that the overhang was simply a by-product of the way DNA is replicated, but in fact there is a wide range of machinery in the cell dedicated to producing this overhang (Huffmann *et al.* 2000). The overhang is folded round to form a t-loop, which 'tucks in' to the double stranded part of the telomere (Griffiths *et al.* 1999; fig. 1.1). It is this 'tucking in' that is the key feature of the t-loop; the size of the loop does not seem to be important, merely reflecting the overall length of the telomere (Rahman *et al.* 2008).

Various proteins, collectively known as shelterin, play a key role in shaping the structure of vertebrate telomeres (reviewed in de Lange 2005; fig. 1.2). Six shelterin proteins have been identified in vertebrates: TRF1, TRF2, POT1, TIN2, TPP1 and Rap1. Non-shelterin proteins also play important roles in telomere function, but shelterin proteins are distinguished from these as they are found nowhere but at chromosome ends and have no other known roles.

TRF1, TRF2 and POT1 can all directly recognize telomeric TTAGGG repeats with very high specificity (Bianchi *et al.* 1999; Loayza *et al.* 2004; Court *et al.* 2005). POT1 is involved in processing the 5' end of the telomere in order to form the 3' overhang (Hockemeyer *et al.* 2005; Sfeir *et al.* 2005), although exactly how it does this is not known. TRF1 and TRF2 then help form the overhang into a t-loop. *In vitro*, TRF1 has the ability to bend, loop and pair telomeric DNA, suggesting that it might be the protein that shapes the 3' overhang into a t-loop (Bianchi *et al.* 1997, 1999). TRF2 also mediates t-loop formation *in vitro* (Griffith *et al.* 1999; Stansel *et al.* 2001).

TIN2, TPP1 and Rap1 do not directly interact with the telomeres, but instead help to connect the other shelterin proteins (fig. 1.2). TIN2 is the linchpin of the shelterin complex, tethering TPP1 to POT1 and TRF1 to TRF2 (Liu *et al.* 2004a; Ye *et al.* 2004). Rap1 is closely associated with the actions of TRF2 (Li *et al.* 2000).

The structure of the shelterin complex is conserved in the vertebrate species that have been studied (de Lange 2005). In non-vertebrate species, the exact structure and function of the proteins associated with telomeres varies. However, proteins similar to those found in shelterin have been found in several non-vertebrate taxa (reviewed in de Lange 2005).

1.2 Telomere function

Telomeres have several important functions in eukaryotic cells. Telomeres were first discovered when Barbara McClintock, working on maize, and Herman Muller, working on *Drosophilia melanogaster*, both noticed that broken chromosome ends always fused with each other, but that natural chromosome ends never did (McClintock 1938, 1942; Muller 1938). More recent work has shown that telomeres are protected from the machinery that repairs DNA breaks. Telomeres thus provide a way for cells to distinguish between the natural ends of chromosomes and breaks in the chromosome that require repairing (Chan & Blackburn 2004). Telomeres also play a role in the alignment and segregation of chromosomes during meiosis (Blackburn 2005).

Perhaps the most interesting function of telomeres is the protection they provide against the erosion of the terminal parts of chromosomes that occurs at each cell division. Some erosion of the chromosome during cell replication is inevitable due to the incomplete

replication of the terminal parts of the DNA strand, known as the end replication problem (Watson 1972; Olovnikov 1973; fig. 1.3). Without the telomere repeats as protection the coding parts of the chromosome would be lost. It is likely that telomeres initially evolved as a solution to this problem (Nosek *et al.* 2006). This gradual shortening of telomere repeats over time is related to the most interesting function of telomeres, their connection to cell senescence.

1.3 Cell senescence

A senescent cell is a previously proliferative cell that has ceased to divide. In cultured human fibroblasts, the number of senescent cells gradually increases as the number of divisions undergone increases; eventually all cells in the population will cease to divide, usually within 40-60 population doublings (Hayflick 1965, 2003). This limit on cell replication is known as the 'Hayflick limit', after the author of the first set of experiments that demonstrated this phenomenon. The exact number of replications cells can undergo varies according to the tissue and species they were taken from. The 'Hayflick limit' does not apply to all cell types; in humans only somatic cells show limited *in vitro* replication. Stem cells can replicate indefinitely, as can cancer cells (reviewed in Campisi & d'Agga di Fagagna 2007).

1.4 Telomere shortening and cell senescence

There are various pathways through which a cell can become senescent, but one of the main ways is through telomere-dependant cell senescence, sometimes termed mortality 1 or M1 senescence (Harley *et al.* 1990). Telomeres will shorten at each cell division due to the end replication problem (section 1.2). When a telomere has shortened to a certain critical length a DNA damage response (DDR) is triggered similar to that caused by double strand DNA breaks. The DDR is mediated by various proteins, particularly ATM kinase, that activate the genes p53 and p21. Activation of these genes results in a permanent arrest of the cell-cycle and the cell becomes senescent (d'Adda di Fagagna *et al.* 2003; Takai *et al.* 2003; Herbig *et al.* 2004). Telomere shortening thus provides an explanation for the 'Hayflick limit' on cell replication *in vitro* (section 1.3).

The key factor in triggering senescence seems to be the shortening of the telomere to the extent where it can no longer form into a loop. The inhibition of the shelterin protein TRF2 (section 1.1), in cells grown *in vitro*, results in activation of a DDR and senescence

(Takai *et al.* 2003; Celli & de Lange 2005). Similarly, transfection of short G-rich telomeric oligonucleotides into a human cell (mimicking the effect of the t-loop being opened) triggers p53 dependant cell-cycle arrest (Saretzki *et al.* 1999).

1.5 Causes of telomere shortening

1.5a The end replication problem

As discussed in section 1.2, some telomere loss is inevitable at each cell division due to the end replication problem. Initially it was thought that this was the cause of all telomere attrition and thus that telomere loss occurred at a constant rate at each cell division. This gave rise to the idea of telomeres as a 'mitotic clock' that kept track of the number of divisions a cell has undergone (von Zglinicki 2003). However, in cultured human fibroblasts, there is considerable heterogeneity in both the number of divisions cells can undergo (Smith & Hayflick 1973; Hayflick 2003) and in the rate of telomere shortening at each cell division (Martin-Ruiz *et al.* 2004). Clearly this is inconsistent with the idea of telomeres as a 'mitotic clock'. Instead factors other than the end replication problem must also contribute to telomere attrition.

1.5b Oxidative stress and telomere loss

Reactive oxygen species (ROS) are produced by the mitochondria as an unavoidable by-product of energy production. Their presence in the cell causes damage to DNA, which will build up over time (Packer & Fuehr 1977; Fraga *et al.* 1990). This damage plays a significant role in telomere shortening (von Zglincki & Schewe 1998; von Zglinicki 2000).

Oxidative stress damages DNA bases or deletes them altogether. Some of these bases will be repaired or replaced, others will not. If a base in the telomere region is damaged and not repaired before the next cell division, DNA replication will be terminated at this point and any telomere repeats beyond will be lost (von Zglinicki 2003). Thus, oxidative stress results in the loss of more telomeric DNA than would be the case due to the end replication problem alone. The smallest amount of telomere loss seen in cultured human fibroblast cells is 10-20bp per cell division (von Zglinicki 2002), suggesting that this is the lower limit of telomere loss set by the end replication problem. The difference between this and the average loss in human fibroblasts of 50-100bp is primarily due to oxidative stress (von Zglinicki *et al.* 2000).

Cells can defend themselves against the effects of oxidative stress through antioxidants. Different types of cell have varying levels of antioxidant defences. Human fibroblast strains that have high antioxidant levels have a lower rate of telomere shortening than strains with low antioxidant levels (von Zglinicki *et al.* 2000). Increasing the levels of antioxidant enzymes in a cell increases its replicative lifespan and reduces its rate of telomere shortening (Furumoto *et al.* 1998; Serra *et al.* 2003). Conversely, increasing the stress cells are under or interfering with their antioxidant defences accelerates telomere shortening and decreases cell lifespan (Kurz *et al.* 2004; Richter & von Zglinicki 2007). At a given level of oxidative stress the damage caused to DNA varies considerably, purely due to chance. This stochastic element of oxidative damage could explain the variation in the rate of telomere shortening between populations of cloned cells *in vitro* and between individuals *in vivo* (von Zglinicki 2003).

Telomere loss due to oxidative stress may actually be an adaptive mechanism (Jennings *et al.* 2000; von Zglinicki 2003). The build up of damage to DNA caused by ROS can eventually lead to a cell becoming cancerous (Campisi *et al.* 2001; Campisi 2003). The longer a cell has been actively dividing and incurring damage, the greater this risk becomes. By using telomeres to set a limit on a cells lifespan the chances of this occurring are much reduced. Crucially, telomeres are more sensitive to oxidative damage than the rest of the genome, due to the presence of many GGG triplets which are a major target of ROS (Kruk *et al.* 1995; Petersen *et al.* 1998). Telomeres also have a much lower rate of damage repair than other parts of the genome. Petersen *et al.* (1998) showed that when single base pair DNA damage was induced, it was almost completely repaired within twenty-four hours in all parts of the genome except the telomeres, where it was not repaired for the whole nineteen-day duration of their experiment. Thus, as damage accumulates in the important, coding areas of the chromosomes, it will be accumulating at a faster rate in the telomeres. By the time damage in the main body of the chromosomes has reached dangerous levels, the telomeres will already have become short enough to trigger senescence. Telomeres can thus be thought of as sentinels, detecting the level of oxidative damage in the genome and shutting down the cell if this level becomes dangerously high (von Zglinicki 2002). The end replication problem still sets an outer limit on a cells replicative lifespan but this will rarely be reached.

1.6 Telomerase and the restoration of telomeres

Although telomere loss at cell division is unavoidable, the damage does not have to be permanent. The reverse transcriptase telomerase is capable of replicating telomere repeats. First discovered in the ciliate *Tetrahymena* (Greider & Blackburn 1985), telomerase is widespread among eukaryotes. Its expression slows or prevents telomere shortening and extends the lifespan of a cell (Bodnar *et al.* 1998).

Telomerase consists of a catalytic protein component (TERT) and an RNA component (TER). TERT can fold around the 3' overhang at the end of the telomere and TER is then used to create a template from which telomeric DNA repeats can be synthesised in the 5' to 3' direction, elongating the telomere (fig 1.4). Shorter telomeres are more likely to be elongated than longer telomeres, which seems to be due to the action of shelterin proteins (section 1.1) particularly POT1 (Liu *et al.* 2004b). Longer telomeres attract more shelterin, thus increasing the amount of POT1 loaded on to the 3' overhang. This decreases the chance of TERT attaching itself to the chromosome, preventing telomere elongation. Shorter telomeres have less POT1 loaded onto the overhang, which increases the chance of TERT attaching to the chromosome and elongating the telomere (fig. 1.5). If shelterin is inhibited, then the rate of telomere elongation by telomerase increases (Loayza & de Lange 2003).

Telomerase is expressed in stem and germ cells and telomere shortening is thus very limited in these cells (Mantell & Greider 1994; Blackburn 2005). Clearly this is crucial, as it prevents any telomere loss being passed on to offspring (Schaetzlein *et al.* 2004). The level of telomerase expression in somatic cells varies among species. For example, telomerase is down-regulated in most somatic cells in humans (Kim *et al.* 1994) but not in mice (Prowse & Greider 1995). In species where telomerase is expressed in somatic cells, exact levels vary between tissue types. For example, in birds telomerase is expressed in both post-mitotic cells (such as liver and brain cells) and mitotic cells (like intestinal cells and stem cells), but is expressed at a higher level in the mitotic cells (Haussmann *et al.* 2007).

The existence of an enzyme that can prevent telomere shortening begs the question: why do all cells not express telomerase and thus prevent telomere-driven cell senescence? The answer seems to be linked to the idea of telomeres as sentinels detecting the level of

oxidative stress (section 1.5b). If telomere shortening is prevented then this function of telomeres will be lost, leading to the risk of oxidative damage in the rest of the genome becoming dangerously high. This will increase the risk of cells becoming tumourous (Campisi *et al.* 2001; von Zglinicki 2003). Indeed telomerase is strongly linked to cancer, being expressed in more than 85% of human cancers (Kim *et al.* 1994; Shay & Bacchetti 1997). It has been suggested that telomerase expression is down-regulated in long-lived species (like humans) but not in short lived species (like mice) as short lived species will not live long enough for high cancer rates to become an issue. However in the few avian species studied, telomerase levels in adult tissues were higher in long-lived species than short-lived ones (Haussmann *et al.* 2004, 2007). Alternatively, it has been suggested that species with a large body mass have a higher risk of cancer than species with a small body mass, and therefore down-regulate telomerase. Such a relationship seems to be present in rodents; species with a large body mass down-regulate telomerase more than those with a smaller body mass (Seluanov *et al.* 2007; Gorbunov & Seluanov 2008).

Thus, telomere length maintenance is in a state of equilibrium between telomere loss and re-addition; there is a trade-off between increasing longevity by reducing the rate of cell senescence and increasing longevity by reducing the risk of tumours developing. The best way to manage this trade-off will vary between cell types and between species.

Although telomerase is the main route through which telomeres are elongated, it is not the only one. Lengthening of telomeres in the absence of telomerase has been noted in several tumour cell lines and has been termed ALT, for alternative lengthening of telomeres (Murnane *et al.* 1994; Bryan *et al.* 1995). ALT is thought to occur by recombination of telomeres (Dunham 2000; Bechter *et al.* 2004), but little is known about its occurrence in normal cells.

1.7 Telomere shortening at the cellular level

When discussing telomere shortening, it is often convenient to refer to the length of telomeres in a cell as though they are all identical. In fact, every telomere in a cell varies in length. There is a relatively conserved pattern to the length of each individual telomere within a cell (Graakjaer *et al.* 2003; Britt-Compton *et al.* 2006). Within this pattern there is a considerable amount of individual variation, resulting in a unique ‘telomere profile’ for every individual. Some of this variation seems to have a genetic basis: of the two alleles of

each chromosome a human parent has, there is more correlation between parent and child in the allele the parent passes on than in the allele they do not (Graakjaer *et al.* 2006). As well as the initial telomere length, the rate of telomere loss also varies between different telomeres in a cell. Not all of the variation in telomere profiles can be due to genetics; the correlation between the telomere profiles of two elderly adults, even monozygotic twins, never exceeds 0.8 (Graakjaer *et al.* 2003).

The differences in telomere length between chromosomes may have a functional purpose. In *C.elegans* and cultured yeast and human cells the distance between the end of the telomere and distal genes effects the expression of these genes (Gottschling *et al.* 1990; Baur *et al.* 2001; Joeng *et al.* 2004).

The difference between telomere lengths within a cell raises the question of which telomere it is that triggers cell senescence; is it the first telomere to shorten below a critical length or are there certain telomeres which determine when senescence is triggered? Although there have been several conflicting answers to this question (e.g. Martens *et al.* 2000; Hemann *et al.* 2001), the consensus now seems to be that a subset of short telomeres in the cell is enough to cause cell senescence (Zou *et al.* 2004; Capper *et al.* 2007).

As well as variation within a cell, telomere lengths and the rate of telomere shortening also differs between cell types. Post-mitotic tissues, such as adult human and mouse brain cells, suffer little or no telomere shortening (Cherif *et al.* 2003; Nakamura *et al.* 2007). In mitotic cells, the degree of telomere loss will depend on the levels of oxidative stress, anitoxidant defences and telomerase expression (Richter & von Zglinicki 2007).

1.8 Telomere shortening and senescence at the organismal level

A key assumption in linking telomere dynamics with lifespan is that cell senescence contributes to organismal ageing. *In vitro* studies cannot provide this evidence, as they cannot show the effect of senescent cells on tissue function (Rubin 2002). However, detecting senescent cells *in vivo* is notoriously difficult (Baird 2006), so there are a limited number of studies linking senescent cells with organismal ageing. What studies there are seem to support the link between cell senescence and ageing. Crucially, senescent cells seem to be rare in young individuals and increase in number with age (Dimri *et al.* 1995;

Herbig *et al.* 2006). Senescent cells are also found at sites of chronic age related pathology, as would be expected if they are involved in ageing (Chang & Harley 1995; Vasile *et al.* 2001; Price *et al.* 2002). The changed phenotype of senescent cells also links them with ageing. For example, senescent cells upregulate the genes for extracellular-matrix degrading enzymes and inflammatory cytokines (Shelton *et al.* 1999; Chang *et al.* 2000; Campisi & d'Adda di Fagagna 2007). An increasing proportion of cells with this expression profile will be likely to have a negative effect on tissue function (Funk *et al.* 2000; Parrinello *et al.* 2005; Baird 2006). Given this fairly strong connection between cellular and organismal senescence, it seems reasonable to link telomere shortening with organismal ageing (Monaghan & Haussmann 2006).

It should be empathised that telomere shortening is not the only mechanism through which cells can become senescent. It has been suggested that ROS may activate specific signalling pathways that cause cell senescence in a telomere-independent way (Finkel & Holbrook 2000). Other, non-genome related, stresses can also result in cell senescence (reviewed in Campisi & d'Adda di Fagagna 2007).

2. Measuring telomeres

Several methods have been developed to measure telomere lengths. This section will briefly outline how each technique works and their advantages and disadvantages. The main division is between those techniques that measure the average telomere length in a cell population (TRF, Q-PCR, T-OLA) and those that measure individual telomeres in a particular cell or chromosome (Q-FISH, Flow-FISH, STELA). The TRF and Q-PCR methods are the best methods to use when comparing overall telomere length between individuals of the same species (Nakagawa *et al.* 2004). For this reason, these two techniques were used to make all telomere measurements discussed in this thesis (a more detailed description of the TRF and qPCR protocols can be found in chapters 2 and 6 respectively).

2.1 Telomere restriction fragment (TRF) analysis

TRF analysis (Harley *et al.* 1990) measures the average telomere length in a cell population. Restriction enzymes are used to cut the telomeres away from the rest of the chromosome and these telomere fragments are then separated out by size on an electrophoresis gel. TRF's are then transferred to a nylon membrane and hybridised with a digoxigenin labelled probe. A chemiluminescent detection system is used to visualize the TRF and autoradiographs of it are taken. An example of an autoradiograph is shown in fig. 1.6. There are variations on this basic technique, which can have significant effects on TRF values. TRF fragments can be separated either by constant field gel electrophoresis (CFGE) or by pulse-field gel electrophoresis (PFGE). CFGE cannot separate fragments bigger than 30kb. Thus, if a species has a significant number of telomeres longer than this, PFGE should be used to separate the fragments as TRF values will otherwise be artificially low. Another variation on the technique is to carry out extraction, digestion and electrophoresis of the samples in agarose plugs; this has the benefit of reducing DNA degradation, which can artificially increase the number of short telomere fragments (Haussmann & Mauck 2008a).

TRF analysis is the most widely used method of measuring telomere lengths. It requires no specialist equipment and results are thus fairly easy to generate. However, relatively large amounts of time (3-5 days) and DNA (0.5-10µg) are required. Analysis of

the autoradiograph smears can also be somewhat subjective, even with the help of computer software (Haussmann & Mauck 2008). More seriously, there can be significant variation (up to 5%) between TRFs from the same individual depending on the choice of restriction enzymes used (suggesting the presence of subtelomeric restriction site polymorphisms and/or subtelomeric length polymorphisms) (Cawthon 2002).

2.2 Quantitative polymerase chain reaction (Q-PCR) assay

The Q-PCR assay (Cawthon 2002) measures the relative amount of telomere repeats in a cell population. For each sample the ratio of telomere repeat copy number to single gene (i.e. a gene that does not vary in size between individuals) copy number is calculated. This ratio is then compared to that of an arbitrary reference sample – the amount it differs by (the T/S ratio) is proportional to the average telomere length of the sample.

The main advantage of the Q-PCR method over TRF analysis is that it is much quicker to carry out and requires less DNA. Additional primers do need to be developed to amplify the single copy gene. Any gene that does not vary in copy number among individuals can be used, so the method should be suitable even for species where the genetic information available is limited.

There are two main problems with the Q-PCR method. Firstly, while the TRF method supplies the size of the average telomere length in base pairs, Q-PCR assays only provide a ratio that is meaningless except in relation to the reference DNA sample. This is not a problem when comparing between individuals within the same study, but it does make it difficult to compare between studies and especially between species. One solution is to analyse several samples with both the TRF and Q-PCR techniques and from this calculate how T/S ratio relates to telomere length. The second problem with Q-PCR assays is that in species that have interstitial telomeric repeats (e.g. avian chromosomes contain telomeric repeats near the centromeres – Venkatesan & Price 1998), these will be measured in addition to terminal telomeric repeats (Nakagawa *et al.* 2004). This is not a problem when measuring the rate of telomere change within an individual, as the amount of interstitial telomeric repeats will not vary over time, but it could be a problem in cross-sectional analyses.

2.3 Telomeric-oligonucleotide ligation assays (T-OLA)

T-OLA can be used to measure the length of the 3' overhang by ligating telomeric repeat oligonucleotides that have hybridised to the overhang (Cimino-Reale *et al.* 2001). In humans, the size of the 3' overhang is proportional to both the size of the telomere as a whole (Rahman *et al.* 2008) and the rate of telomere shortening (Huffmann *et al.* 2000). If this is true of non-human species, then T-OLA could become a useful way to measure telomeres.

2.4 Fluorescence *in situ* hybridisation (FISH) methods

As their name suggests, FISH methods utilise fluorescent dyes to visualize telomeres, which are then quantified by measuring light intensity. FISH methods can be used to measure telomere lengths in single cells. Flow cytometry FISH methods (Flow-FISH, Rufer *et al.* 1998), can be used to measure the average telomere length in a single cell. Quantitative FISH (Q-FISH, Zijlmans *et al.* 1997) is able to measure the lengths of individual telomeres within a cell.

The FISH methods, especially Q-FISH, require specialised equipment and are thus not a realistic option for most research groups (Sedivy *et al.* 2003). Q-FISH can also only visualise telomeres in metaphase chromosomes which limits its use to proliferating cells. Even then, it is difficult to generate adequate numbers of metaphase chromosomes from tissues such as blood. For these reasons, the FISH methods are of limited use for ecologists interested in comparing telomere lengths of many different individuals.

2.5 Single telomere length analysis (STELA)

STELA is a PCR based method for measuring individual telomere lengths (Baird *et al.* 2003). It works by attaching a linker or 'telorette' to the end of the 3' overhang – in addition to a telomere specific sequence, the telorette ends in a 20bp non-complementary 'tail'. The telorette is then ligated to the complementary, C-rich, 5' end of the chromosome, effectively tagging the telomere with the non-complementary tail. PCR is then performed using two primers; one that is identical to the tail and another that specifies to a sequence in the subtelomeric region.

Because of this need for a primer designed specifically for the subtelomeric region, new primers need to be designed not only for different species, but for different

chromosomes within a species. This limits the use of STELA to species whose genetic sequences are well characterised (Nakagawa *et al.* 2004). However, unlike Q-FISH its use is not limited to metaphase chromosomes and it requires less specialist equipment, so in the long-term it may be the best way to measure individual chromosomes.

3. Telomere length and organismal attributes

3.1 Variation in telomere dynamics between species

Cross species comparison of telomere dynamics is difficult, primarily because so few species have been studied. Among those species that have been examined, there is considerable variation in both absolute telomere lengths and the rate of telomere loss.

3.1a Variation in absolute telomere lengths

There is considerable variation in telomere length between species, ranging from as low as 20bp in some cilliates up to as high as 150,000bp in some mouse strains (Louis & Vershinin 2005). However, there seems to be little correlation between absolute telomere length and the lifespan of a species. Among the primates, humans have the shortest telomeres but the longest lifespan (Kakuo *et al.* 1999), while in various strains of mice studied, there was no link between lifespan and telomere length (Hemann & Greider 2000). This lack of relationship has been found in a wide variety of other vertebrate species (Vleck *et al.* 2003). The exceptions to this trend are pine trees, where the longest living species have the longest telomere lengths (Flanary & Kletetschka 2005).

3.1b Variation in the rate of telomere loss

A link does seem to exist between the lifespan of a species and its rate of telomere loss. The proliferative lifespan of cells in culture is positively linked to the lifespan of the species they are taken from (Rhome 1981). The lifespan of individual cells may also be greater in long-lived species, which will result in a lower rate of cell division and less telomere loss (Rhome 1981).

At the organismal level, there does seem to be a general trend for longer-lived species to have a lower rate of telomere loss. In a variety of avian species studied, telomere loss was generally greater in short-lived than long-lived species (fig. 1.7). In the pine tree *Pinus longaeva*, one of the longest living species on the planet, no telomere shortening was seen with age (Flanary & Kletetschka 2005). There are exceptions to this trend. No telomere loss with age was seen in either of two species of sea urchins with very different lifespans (Francis *et al.* 2006), suggesting that telomere dynamics are not an important determinant of longevity in these species.

3.2 Reasons for species variation

3.2a Telomerase expression

In the few avian species studied, telomerase expression positively correlated with lifespan (Haussmann *et al.* 2004, 2007). This may account for the lower rate of telomere loss seen in the long-lived species compared to the short-lived species (Haussmann *et al.* 2003). Telomerase levels also correlate with lifespan in pine trees (Flanary & Kletetschka 2005).

However, telomerase levels cannot explain differences in telomere dynamics among mammalian species. Humans express no telomerase in adult somatic cells (Kim *et al.* 1994), while mice express high levels (Prowse & Greider 1995). Clearly telomerase levels do not correlate with the rate of telomere loss or lifespan in these species.

3.2b Oxidative stress levels

It is well established that birds tend to have a longer lifespan than mammals of a similar size, despite birds having relatively higher metabolic rates and levels of energy expenditure, both of which are generally thought to reduce lifespan (Holmes & Austad 1995). This seems to be because birds both produce fewer molecules of ROS per molecule of oxygen consumed, and that they are less susceptible to the ROS that are produced (Ogburn *et al.* 1998; Holmes *et al.* 2001). Two labs independently found significantly lower production of H_2O_2 (a common ROS) in pigeons compared with similar sized Norway rats (Barja *et al.* 1994; Ku & Sohal 1993). The increased resistance to ROS seen in birds could be due to increased levels of antioxidant enzymes (Holmes *et al.* 2001). The structure of avian cells may also make them more resistant to oxidative stress than mammalian cells. Mitochondrial membranes in pigeon liver cells have greater resistance to lipid peroxidation than do rat liver cells (Pamplona *et al.* 1996). This relationship between lifespan and vulnerability to oxidative stress may be quite widespread (Sohal *et al.* 1990; Ku & Sohal 1993; Barja & Herrero 2000). Given the established link between oxidative stress and telomere loss (section 1.5b), this provides further evidence for a link between lifespan and telomere dynamics.

3.3 Problems with cross-species studies

There are various problems associated with cross-species studies of telomere dynamics. The rate of telomere loss varies between different tissue types, so care must be taken when comparing species whose telomere dynamics have been examined in different tissues.

Comparisons of telomere lengths across species will also be inaccurate if the individuals are not age-matched. Most studies use the average rate of change in telomere length per year as a measure of the relative rate of telomere loss (e.g. Haussmann *et al.* 2003; Hall *et al.* 2004). However, the rate of telomere loss varies with age in most species studied (e.g. Zeichner *et al.* 1999; Hall *et al.* 2004; Pauliny *et al.* 2006), so using the rate of telomere loss per year to compare between them is fairly arbitrary. Telomere loss also varies considerably within individuals of the same species, further clouding cross-species comparisons. However, this variation between individuals could represent the most interesting aspect of telomere dynamics.

3.4 Variation between individuals of the same species

3.4a Variation with age

If telomeres are linked to organismal senescence, then one might expect to see them shorten with age. In mammals, the proliferative potential of cells in culture is sometimes negatively correlated with the age of the donor (Rohme 1981). This relationship is not always present (Cristofalo *et al.* 2004), although this is perhaps inevitable given the large amount of variability in the rate of telomere shortening seen *in vitro*. Telomere length itself is a much better predictor of the replicative potential of cultured cells (Allsopp *et al.* 1992).

A trend for telomeres to shorten with age *in vivo* has been found in many species (table 1.1). However, as the table shows, this trend is far from universal. No change in telomere length with age is seen in *Drosophilia melanogaster* (Walter *et al.* 2007) or the two species of sea urchin that have been studied (Francis *et al.* 2006), while no change with age is seen in adult European shags and wandering albatross (Hall *et al.* 2004). In the case of Leach's storm petrels (Haussmann *et al.* 2003) and the bristlecone pine *Pinus longaeva* (Flanary & Kletetschka 2005), telomere length seems to increase with age. The lack of the expected negative relationship between age and telomere length in these species could be due to high levels of telomerase expression; both Leach's storm petrels and bristlecone pines are known to express telomerase in adult somatic tissues (Flanary & Kletetschka 2005; Haussmann *et al.* 2007). Alternatively the lack of relationship may be due to the differential survival of individuals with very long telomeres; indeed, Haussmann and Mauck (2008b) recently suggested this is true of the Leach's storm petrel.

A more powerful way of looking at the change in telomere length with age is through longitudinal studies. However, very few longitudinal studies of telomere length have been conducted, primarily because of the difficulty of gathering several tissue samples from the same individual. Longitudinal studies also limit the range of tissues that can be studied; for obvious reasons, longitudinal studies on telomeres in the liver or brain are hard to come by. Longitudinal studies therefore tend to examine telomere dynamics in blood cells. Such studies that have been carried out have generally shown a reduction in telomere length with age. In humans (Zeichner *et al.* 1999), mice (Kotrshchal *et al.* 2007), domestic cats (Brümmendorf *et al.* 2002), the European shag (Hall *et al.* 2004) and dunlins (Pauliny *et al.* 2006) there was a general trend for telomeres to shorten within individuals as they grew older.

3.4b Telomere length and individual lifespan

If telomere length is linked to ageing within individuals, we would expect to see a correlation between telomere length and lifespan within species. In humans, peripheral blood leukocyte (PBL) telomere length predicted mortality in individuals over the age of 60 (Cawthon *et al.* 2003). Other studies have failed to replicate this finding (Martin-Ruiz *et al.* 2005; Bischoff *et al.* 2006). However, these studies used an older cohort of individuals, so it could be that there is no correlation between telomere length and mortality in the very old (Baird 2006). Nakamura *et al.* (2007) found that in patients over 90 years old at their time of death, telomere lengths of white and grey brain cells had longer telomeres than in those individuals dying at younger ages. This suggests that individuals with longer telomeres have a greater chance of surviving to this age.

This apparent increase in mortality in individuals with shorter telomeres may be related to an increased chance of contracting age-related diseases. In a study of 190 same age individuals, those who reported heart disease had shorter PBL telomeres than those who did not (Starr *et al.* 2007). Similarly, Benetos *et al.* (2001) found that men with shorter PBL telomere lengths showed increased symptoms of heart disease. There has even been the suggestion of a connection between telomere length and mood disorders (Simon *et al.* 2006), although another study found no correlation between cognitive decline and telomere length in the very old (Harris *et al.* 2006).

Significantly, all of these studies on humans are cross-sectional and so cannot make a link between lifespan and the rate of telomere shortening, which is probably more important than absolute telomere length. There are few longitudinal studies on humans, primarily because of the difficulty of obtaining repeat blood samples from the same individual. This suggests that work on non-human species could be fruitful. However, little work has been done to link telomere length and mortality in non-human species. A link between the rate of telomere loss and lifespan has been shown in rats (Jennings *et al.* 1999), while in a cross sectional study, lifespan was linked to telomere length in dunlins, but not in sand martins (Pauliny *et al.* 2006).

3.4c Variation between same age individuals

In all the species listed in table 1.1, there is considerable variation in telomere length between individuals of the same age (e.g. fig. 1.8). Longitudinal studies have also shown a significant variation in the rate of telomere loss between individuals followed over the same time period (Zeichner *et al.* 1999; Hall *et al.* 2004; Pauliny *et al.* 2006). Some of this variation is to be expected due to the stochastic nature of telomere shortening, but not all of it. This variation between same-age individuals suggests that telomeres can probably not be used for estimating an individual's chronological age, as was once hoped (e.g. Juola *et al.* 2006). More interestingly, it suggests that rather than being a measure of chronological age, telomeres may provide an estimate of the biological age of an individual (Nakagawa *et al.* 2004); if two individuals of the same age have very different telomere lengths, then it suggests that the individual with shorter telomeres is biologically older. Thus, by examining the causes of the variation in telomere length between individuals, we may be able to examine the long term effects of differing life history strategies (Monaghan & Haussmann 2006).

3.5 Causes of variation between same age individuals

A considerable number of factors have been linked to inter-individual variation in telomere dynamics. These range from 'inbuilt' factors, such as sex and heritability, to environmental factors such as growth rates and stress levels.

3.5a Paternally inherited

In humans, initial telomere length is partly inherited (Nawrot *et al.* 2004; Nordfjäll *et al.* 2005; Andrew *et al.* 2006; Njajou *et al.* 2007). Heritability has been estimated to be as

much as 44% in populations with shared environmental influences (Njajou *et al.* 2007). The exact mechanism of heritability is somewhat unclear. Nawrot *et al.* (2004) found a correlation in telomere length between fathers and their daughters and between mothers and their offspring of both sexes. They did not see such a correlation between fathers and sons, which led them to suggest that telomere length is an x-chromosome linked trait. However, other studies have found a correlation between telomere lengths of fathers and their offspring, but not between telomere lengths of mothers and their offspring, which would suggest telomere length is paternally inherited (Nordfjäll *et al.* 2005; Njajou *et al.* 2007). Baird *et al.* (2006) showed that telomere lengths in male germline cells range in size from 8.8kb to over 16kb, despite expressing telomerase at high levels (and so presumably avoiding telomere attrition). This suggests that a large amount of the variation in telomere length in humans could be due to inbuilt differences in paternal zygotic telomere length. Paternal age has also been linked with offspring telomere length (Unryn *et al.* 2005; Njajou *et al.* 2007); surprisingly, the older the father is, the longer the telomeres of his offspring are likely to be.

The degree of inheritance in species other than humans has not been extensively studied. However, the assumption is that some degree of heritability is found in most species.

3.5b Sex

Differences in average telomere length between the sexes have been found in several species, with the general trend being for males to have shorter telomeres than females. This might be expected, given that males generally have shorter lifespans than females (at least in vertebrates). Males have shorter telomeres than females in humans (Benetos *et al.* 2001), rats (Cherif *et al.* 2003) and the ant *Lasius niger* (Jemielity *et al.* 2007). Sex differences in some species may be inbuilt. In humans however, there is no difference between the sexes at birth; telomere length diverges between the sexes with age (Nawrot *et al.* 2004). Thus, it is likely that the different life histories of males and females play a role in their differing telomere lengths (Kotrschal *et al.* 2007). For example, it has been suggested that the higher rate of telomere shortening and shorter lifespan in male rats compared to female rats is due to their higher levels of oxidative stress (Tarry-Adkins *et al.* 2006).

3.5c Early life conditions

There are considerable theoretical reasons to expect telomere loss to be at its greatest during early growth. Cell turnover will be very high at this point and oxidative stress levels are also likely to be higher. Both cross-sectional and longitudinal studies seem to support this idea. In humans, telomere loss is fastest in infants and slows considerably in adults (Frenck *et al.* 1998; Zeichner *et al.* 1999). The same relationship has been found in longitudinal studies of the domestic cat (Brümmendorf *et al.* 2002). In both the European shag and the wandering albatross there is no trend for telomeres to shorten in adults but there is a trend for telomeres to be longer in chicks than in adults (Hall *et al.* 2004). In dunlins, the average rate of telomere loss within individuals was over twice as high in young birds as in older birds (Pauliny *et al.* 2006). Clearly, these studies suggest that the conditions experienced by individuals early in life are particularly important in terms of telomere dynamics; however, as yet, little work has been done to test this idea. In the European shag, chicks hatching late in the breeding season were shown to have a greater rate of telomere loss between chick and adult life stages than those individuals born earlier in the season (Hall *et al.* 2004). Most other studies looking at the effects of early life condition have concentrated on variation in growth rates.

3.5d Growth rate

Given that most telomere loss occurs early in life, it follows that the rate at which an individual grows will be a key determinant of its rate of telomere loss. Growth rates have been linked to lifespan for some time, both between species (Rollo 2002) and within species (Metcalfe & Monaghan 2001, 2003). A link between growth rate and telomere loss has so far only been shown in rats and the European shag. Rats that underwent growth retardation had longer telomeres in the liver and kidney as adults and had a longer lifespan. Conversely, rats that underwent a period of accelerated catch-up growth following a period of poor foetal nutrition had shorter adult kidney and liver telomeres and a shorter lifespan (Jennings *et al.* 1999). In European shags measured as chicks and again as adults, individuals laying down a large tissue mass for their body size showed the greatest rate of telomere loss (Hall *et al.* 2004).

3.5e Stress

Although conditions during early life seem to be particularly crucial in determining rates of telomere loss, this does not mean that conditions experienced by adults are not important.

A key factor in the variation of telomere loss between adult individuals is the amount of stress they experience. Stress in this context can refer to anything from reproductive stress to psychological stress. In female house mice (*Mus musculus*) sampled as infants and at 6 months old, those individuals exposed to reproductive stress showed a higher rate of telomere loss than non-stressed controls. In male house mice, individuals exposed to crowding stress had a higher rate of telomere loss than controls (Kotrschal *et al.* 2007). In humans, psychological stress has been linked to telomere shortening. PBL telomere lengths were analysed in mothers caring for a chronically ill child (and therefore presumably highly stressed). The duration of care (and thus the chronicity of the stress) correlated with higher levels of oxidative stress, lower levels of telomerase and lower telomere lengths. Interestingly, the level of stress the women perceived themselves to be under also correlated with their telomere lengths (Epel *et al.* 2004). Telomere length and telomerase levels have also been linked to other measures of stress in humans, such as smoking, high blood pressure and obesity (e.g. Valdes *et al.* 2005; Epel *et al.* 2006), although not all studies have found such links (e.g. Bischoff *et al.* 2006)

3.6 Telomere length as a measure of individual quality

Given the link between telomere length and cell senescence, we would expect to see the established connection between telomere length and mortality and disease (see section 3.4b). Interestingly, several studies suggest that telomere length may also be linked to other measures of individual quality. In a cross-sectional study, sand martins with longer telomeres had greater lifetime reproductive success than those with shorter telomeres (Pauliny *et al.* 2006). In tree swallows, telomere length at one year old predicts an individual's chance of surviving to the next breeding season. This relationship continues for at least three breeding seasons (Haussmann *et al.* 2005). Any mortality at such a young age (tree swallows can live up to 8 years) is presumably not due to senescence, suggesting that telomere length is linked to other factors affecting mortality. In the nematode worm *C.elegans*, longer telomere lengths have been linked to both increased longevity and a greater resistance to heat stress (Joeng *et al.* 2004, although see Raices *et al.* 2005). Adult *C.elegans* consist entirely of post-mitotic cells and thus experience no telomere derived cell senescence (Raices *et al.* 2005); any effect of telomere length must therefore be acting through a different mechanism. It has been suggested that the length of telomeres may affect levels of gene expression in *C.elegans* (Joeng *et al.* 2004). In general though, the exact way telomeres can affect factors other than cell senescence is not known. It is

possible that telomere length merely correlates with these measures of individual quality, rather than having a direct effect on them (Haussmann *et al.* 2005). Even if this is the case, studies of inter-individual differences in telomere dynamics would be even more interesting. More research on what other factors telomere length is linked to, in a variety of species, is clearly needed (Monaghan & Haussmann 2006).

4. Thesis content

There is substantial evidence linking telomere dynamics with the long term effects of different life history strategies. One thing that has been mostly missing from previous research, however, is work on species in their natural environments. The majority of studies on telomeres have been carried out on humans or captive species, such as rats. Examining species such as these, whose current lifestyles may be substantially different to their 'natural' lifestyles, could give misleading results. The substantially longer telomeres of inbred captive mice compared to wild mice (Hemann & Greider 2000), is just one demonstration of the potential problems of working with captive species. The work presented in this thesis examines the telomere dynamics of several wild avian populations. Broadening the range of organisms whose telomere dynamics have been studied will in itself help expand our understanding of telomere dynamics (Monaghan & Haussmann 2006). Of the species studied in this thesis, only the European shag has previously been studied in relation to its telomere dynamics (Hall *et al.* 2004).

The next chapter (**chapter 2**) provides more detail on the TRF protocol that was used to measure telomeres in the work described in the remaining chapters. The following two chapters both detail work investigating telomere length in relation to early development. Previous research has suggested that telomere loss is likely to be highest during early growth (section 3.5c). However, little work has been done to investigate the relationship between early life conditions and telomere dynamics. In **chapter 3**, inter-individual variation in telomere length in relation to early development is investigated in chicks of the lesser black-backed gull *Larus fuscus*. **Chapter 4** also investigates variation in telomere length among young individuals, with a longitudinal study of telomere loss in chicks of the European shag *Phalacrocorax aristotelis*. Very few studies have been able to investigate telomere dynamics in long-lived species across a wide age-range, primarily due to the difficulty of obtaining such information. **Chapter 5** presents the results of such a study; the telomere dynamics of a population of giant petrels (*Macronectes* spp.) containing a wide range of known age individuals are examined. Analysis of such data allows the exploration of the relationship between telomere length and age (section 3.4a). In addition the relationship between telomere length and survival is examined; it has previously been suggested in other avian species that individuals with longer telomeres

have better survival rates than individuals with shorter telomeres (section 3.6). Finally, **chapter 6** discusses the potential problems that may be encountered when measuring telomere lengths in relation to work done on the blue-footed booby *Sula nebouxii*.

Blood samples and other field data used to produce the work described in chapters 4 - 6 was kindly provided by other researchers; European shag data was provided by Maggie Hall, blue-footed booby data by Roxana Torres and giant petrel data by Francis Daunt and Richard Phillips. Telomere assays and PCR sexing were conducted in association with Lubna Nasir, Elizabeth Gault, Kate Griffiths, Winnie Boner and Pierre Bize. Data analysis and interpretation was carried out in association with Pat Monaghan.

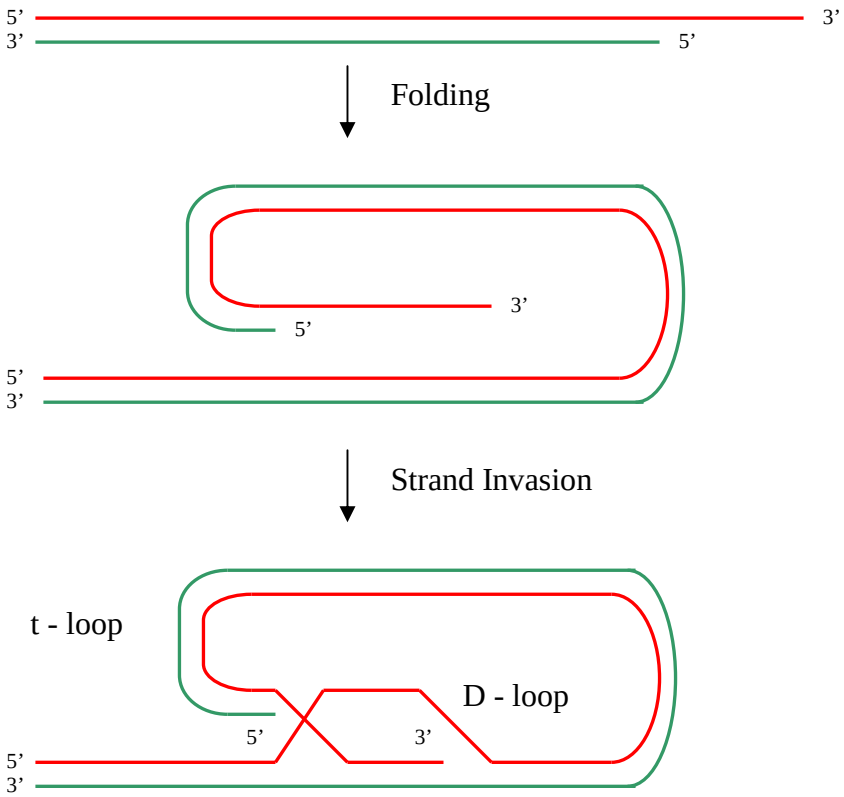


Fig. 1.1: Demonstration of the basic telomere structure. The top diagram shows the 3’ overhang formed at the end of the telomere. The telomere is then folded over (middle diagram) and the overhang is ‘tucked in’ to the main strand (the area where this tucking in occurs is known as a D-loop - bottom diagram), allowing the end of the telomere to form a t-loop. Figure adapted from de Lange (2005).

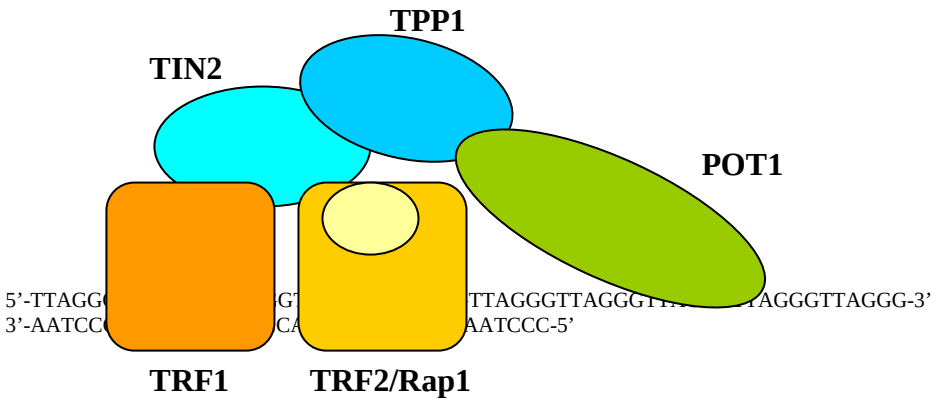


Fig. 1.2: Schematic rendering of the shelterin complex attached to the end of a telomere. TRF1, TRF2 and POT1 all directly attach to the telomere, while TIN2, TPP1 and Rap1 bind the complex together. Figure adapted from de Lange (2005).

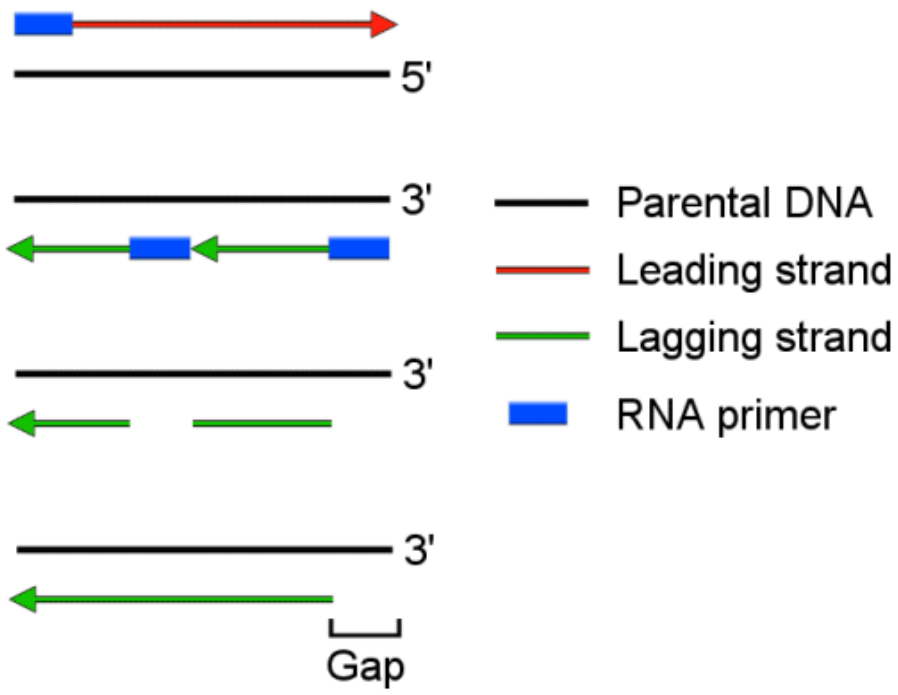


Fig. 1.3: Demonstration of the end replication problem. The leading DNA strand (5’ to 3’ direction) is easily synthesised by DNA polymerase. However, DNA polymerase cannot synthesise in the 3’ to 5’ direction. This means that when the lagging DNA strand (3’ to 5’ direction) is replicated, the DNA polymerase has to attach itself to an RNA primer in order to function. The DNA is then synthesised in short segments known as Okazaki fragments. At the end of the chromosome there is no DNA left for the RNA primer to attach to, so the terminal part of the DNA strand cannot be replicated.

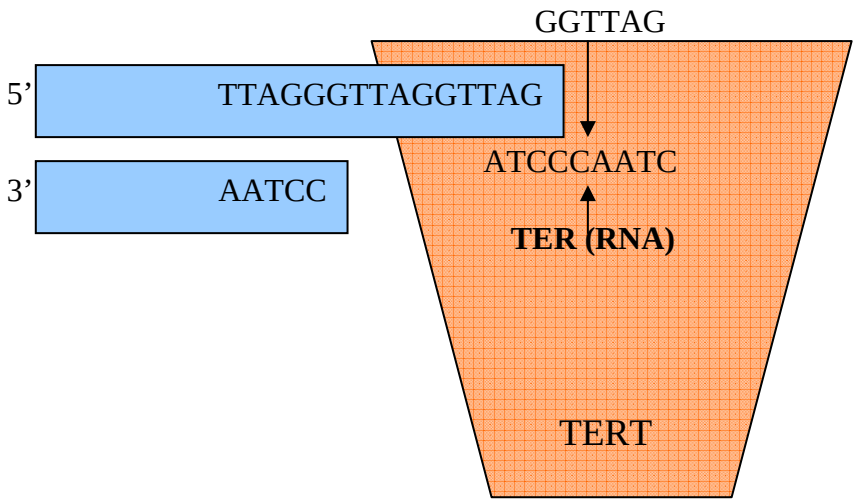


Fig. 1.4: Schematic representation of telomere elongation by telomerase. Blue rectangles represent the telomeres, the orange shape telomerase. The last few nucleotides in the 3’ overhang pair with the complementary sequence in the RNA component of telomerase. This chromosomal end is elongated by polymerization of dGTP, dTTP and dATP, using the RNA as a template. The extended telomere un-pairs from telomerase and the 3’-5’ lagging strand is synthesised by primase-polymerase. The telomere is now eligible for another round of elongation.

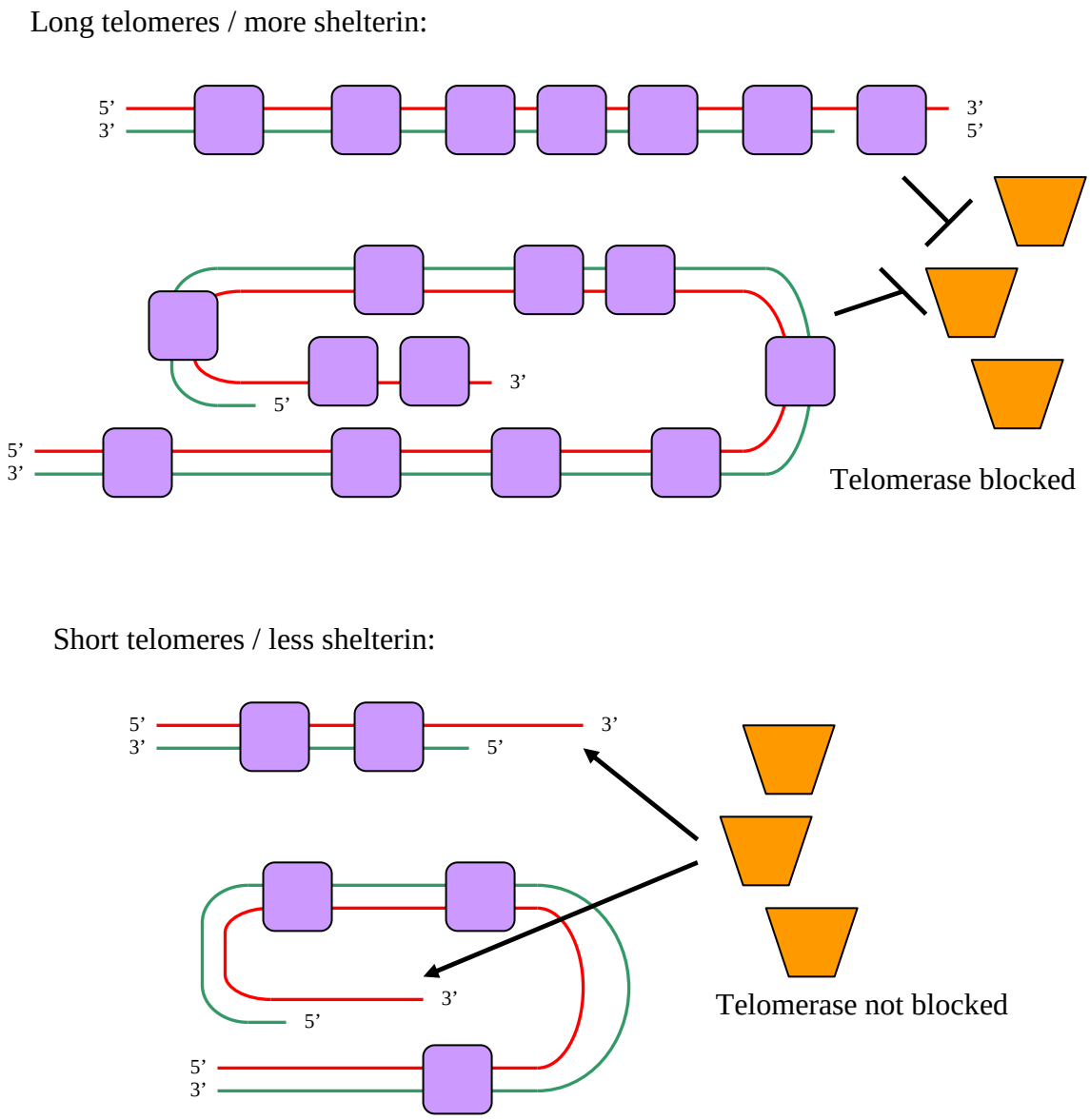


Fig 1.5: Schematic representation of the effect of shelterin on telomere elongation. Long telomeres (top diagram) attract lots of shelterin (purple blocks), meaning that telomerase (orange blocks) is blocked from attaching to the 3’ overhang. Shorter telomeres (bottom diagram) have less shelterin attached, increasing the chance of telomerase being able to attach to the 3’ overhang. Figure adapted from de Lange (2005).

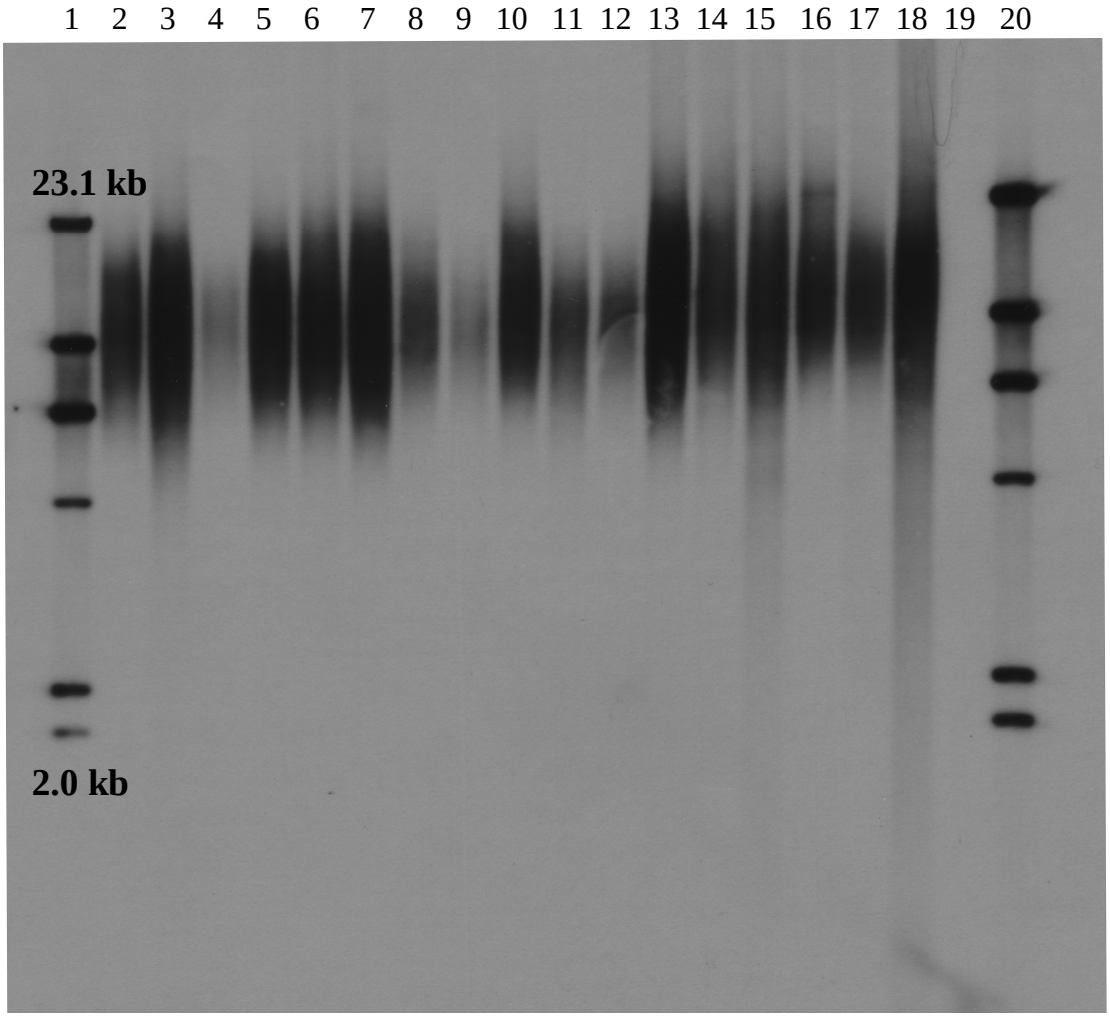


Fig. 1.6: Example of an autoradiograph produced by TRF analysis (with constant field gel electrophoresis). Lanes 1 and 20 contain a molecular size marker, lanes 2 – 19 each represent a different sample (lane 19 is blank). Increased intensity of the smear at a given molecular weight means there are more telomere fragments of this size in the cell population.

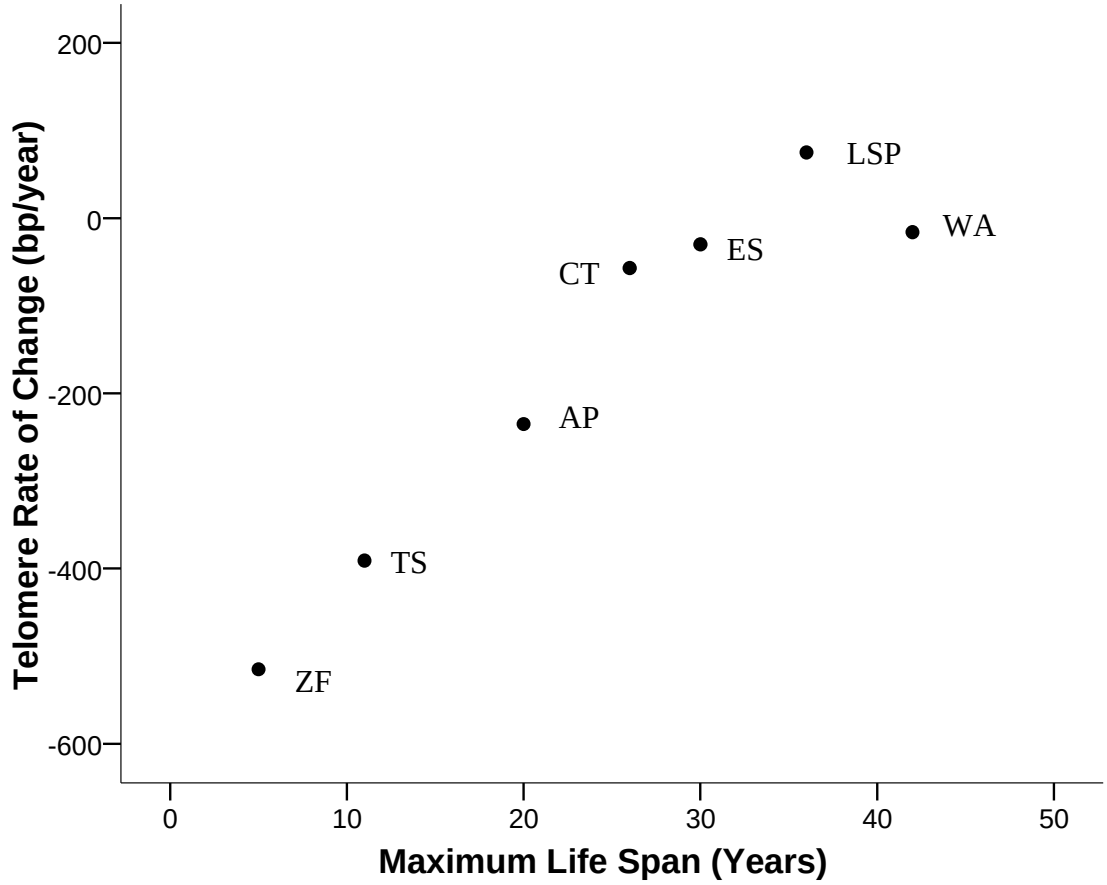


Fig. 1.7: The relationship between maximum lifespan and the mean rate of telomere change (base pairs lost or gained per year) in various avian species. Species key: ZF = zebra finch (*Taeniopygia guttata*), TS = tree swallow (*Tachycineta bicolor*), AP = Adélie penguin (*Pygoscelis adeliae*), CT = common tern (*Sterna hirundo*), ES = European shag (*Phalacrocorax aristotelis*), LSP = Leach's storm-petrel (*Oceanodroma leucorhoai)*, WA = wandering albatross (*Diomedea exulans*). Data taken from Vleck *et al.* 2003 and Hall *et al.* 2004.

Table 1.1: Examples of vertebrate species whose telomere dynamics have been studied in relation to age.

Species	Cell types examined	Telomere loss with age?	Reference
Humans	Peripheral blood leukocytes (PBLs), fibroblasts, pancreas, white and grey matter	Yes (extent of loss varies between tissue types)	Ishii *et al.* (2006) Nakamura *et al.* (2007) Okuda *et al.* (2002) Baird (2006 – review of studies on PBLs)
Rat (*Mus musculus)*	Liver, kidney, brain, liver, pancreas	Yes (except brain)	Cherif *et al.* (2003)
Domestic cat (*Felix domesticus*)	PBLs	Yes	Brummendorf *et al.* (2002)
Domestic chicken (*Gallus domesticus)*	Red blood cells	Yes	Delaney *et al.* (2000)
Domestic dog (various breeds)	Various	Yes	Nasir *et al.* (2001)
Donkey (*Equus asinus*)	PBLs	Yes	Argyle *et al.* (2003)
Horse (*Equus equus*)	Fibroblasts	Yes	Argyle *et al.* (2003)
European shag (*Phalacrocorax aristotelis)*	Red blood cells	Yes (but not within adults)	Hall *et al.* (2004)
Wandering albatross (*Diomedea exulans*)	Red blood cells	Yes (but not within adults)	Hall *et al.* (2004)
Frigate bird (*Fegata minor*)	Red blood cells	Yes	Juola *et al.* (2006)

Zebra finch (*Taeniopygia guttata*)	Red blood cells	Yes	Haussmann *et al.* (2003)
Adélie penguin (*Pygoscelis adeliae*)	Red blood cells	Yes	Haussmann *et al.* (2003)
Tree swallow (*Tachycineta bicolor*)	Red blood cells	Yes	Haussmann *et al.* (2003)
Common tern (*Sterna hirundo*)	Red blood cells	Yes	Haussmann *et al.* (2003)
Leach's storm petrel (*Oceanodroma leucorhoai*)	Red blood cells	No (telomere length increases with age)	Haussmann *et al.* (2003)
Sandmartin *(Riparia riparia*)	Red blood cells	Yes	Pauliny *et al.* (2006)
Dunlin *(Calidris alpina)*	Red blood cells	Yes	Pauliny *et al.* (2006)

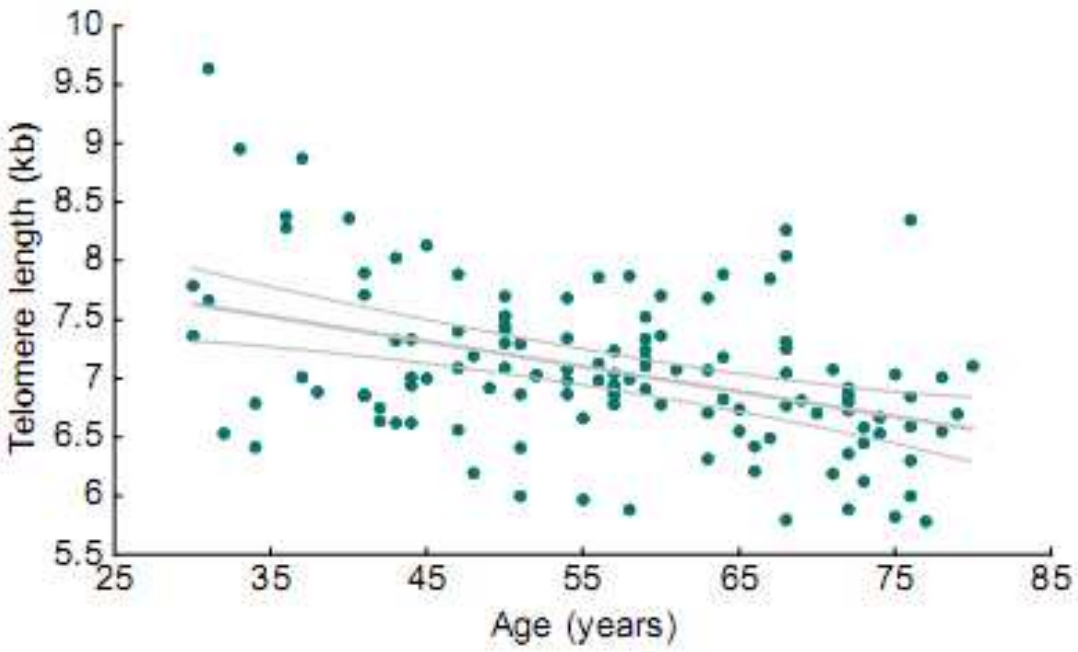


Fig. 1.8: The relationship between white blood cell telomere length and age in a random sample of humans. Although there is a trend for telomere length to shorten with age, there is considerable variation between same-age individuals. Figure taken from Unryn *et al.* (2005)

Chapter 2: Materials and Methods

Materials used

1. General Chemicals

Brilliant®SYBR®Green QPCR Master Mix – Stratagene

Blocking reagent – Roche

Bromophenol Blue – Institute of Comparative Medicine

Chloroform - Sigma

CPSD chemiluminescence substrate – Roche

DIG Easy Hyb – Roche

Ethidium bromide – Sigma

Hydrochloric acid – BDH

Maleic acid (M0375) – Sigma

Phenol: chloroform: isoamyl alcohol (25:24:1) – Sigma

Sodium acetate buffer solution – Sigma

Sodium chloride – Sigma

Sodium dodecyl sulphate (SDS) - Sigma

Sodium hydroxide pellets – BDH lab supplies

TE buffer – Qiagen

Tris base – Sigma

Tri-sodium citrate – BDH lab supplies

Tween®-20 (polyoxyethylene sorbitan nonolaurate) – Sigma

100% pure ethanol – Fisher Scientific

2. Complete kits

DNeasy Blood and Tissue Kit – Qiagen

3. Restriction Enzymes

Hind III – New England Biolabs

Hinf I – Invitrogen

Msp I – New England Biolabs

Proteinase K, recombinant PCR grade - Roche

Rsa I - Invitrogen

4. Molecular Size Standards

CHEF DNA size standard (48.0 – 8.0 kb) – BIO-RAD

DIG labelled DNA molecular weight marker (23.1 – 2.0 kb) – Roche

1 kb DNA ladder (12,126 – 75 bp) – GIBCOBRL Life Technologies

5. Primers and Probes

Anti-digoxigenin-AP fab fragments – Roche

Digoxigenin (DIG) labelled telomere probe $(TTAGGG)_7$ – Roche

^{32}P γ-ATP labelled telomere probe $(C_2TA_2)_4$ – Roche

GAPDH-F (5'-AACCAGCCAAGTACGATGACAT–3') – VH Bio Ltd

GAPDH-R (5'-CCATCAGCAGCAGCCTTCA -3') – VH Bio Ltd

Tel1b (5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3')

Tel2b (5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3')

– VH Bio Ltd

6. Equipment

6.1 Major Equipment

Constant-field gel tank – BIO-RAD

Centrifuge - Eppendorf 5417R

Mechanical shaker – Stuart Scientific

Optimal cross-linker – Spectrolinker™ XL-1000, Spectronics Corporation

Oven – Hybridiser HB-1D, Techne

Pulse field gel tank – CHEF-DR®II Pulsed Field Electrophoresis Systems

QPCR machine - MX3000P®, Strategene

Spectrophotometer – ND-1000, Nanodrop®

UV transluminator - Universal Hood, BIO-RAD

Video graphic printer – UP-890CE, Sony Corporation

X-ray film processor – Compact X4 Automatic, Xograph Imaging Systems

37°C Water bath – Grant Instruments

6.2 Consumables

ABgene® PCR plates – Thermo Scientific

Agarose - Seakem

Amersham Hybond-N^+ membrane for nucleic acid transfer - GE Healthcare

Amersham Hyperfilm ECL (chemiluminescence film) – GE Healthcare

Chromatography paper – Whatman International

'Clear Seal Diamond' optically clear heat sealing film – Thermo Scientific

Paper towels

7. Buffers and Solutions

BLB buffer: 1% SDS, 50mM Tris, 50mM EDTA, dH_2O. This was stored at room temperature.

10X Blocking buffer: 10g blocking reagent, 100ml 1X maleic acid (maleic acid heated to 65°C in order to dissolve blocking reagent). This was autoclaved and stored at 4°C.

1X Denaturation buffer: 0.5M NaOH, 1.5M NaCl, dH_2O. This was stored at room temperature.

10X Detection buffer: 100mM Tris-HCL (12.1g Tris base, adjusted to pH 9.5 with conc. HCL), 100mM NaCl, sterile H_2O. This was autoclaved and stored at room temperature.

0.5M EDTA pH8: 17.9g EDTA, 100ml dH_2O. Adjusted to pH 8.0 with NaOH pellets and made up to 100ml. This was autoclaved and stored at 4°C.

0.25M HCL: 21.55 ml conc. HCL, 978.45 ml dH_20. This was stored at room temperature.

10X Maleic Acid: 116.1g maleic acid, 87.6g NaCl, sterile H_2O. Adjusted to pH 7.5 with NaOH pellets and made up to 1L. This was autoclaved and stored at room temperature.

2.5M NaCl: 14.6g NaCl, 100ml dH_2O. This was autoclaved and stored at room temperature.

1X Neutralization buffer: 0.5M TRIS-HCL (60.5g Tris base, pH adjusted to 7.5 with conc. HCL), 3M NaCl, dH_2O. This was stored at room temperature.

Nuclei lysis buffer: 2mM EDTA pH 8.0, 0.4M NaCl, 10mM Tris, dH_2O. This was stored at room temperature.

10% SDS: 10g solid SDS**,** 100ml dH_2O. This was stored at room temperature.

20X SSC: 3M NaCl, 0.3M Tri-sodium citrate, dH_2O. This was stored at room temperature.

Stringency wash I: 2X SSC, 0.1% SDS, dH_2O. This was stored at room temperature.

Stringency wash II: 0.2X SSC, 0.1% SDS, dH_2O. This was stored at room temperature.

50 X TAE buffer: 2M Tris base, 50mM EDTA, 1M glacial acetic acid, dH_2O. Adjusted to pH 8.15 using glacial acetic acid. This was stored at room temperature.

10X TBE buffer: 0.09M Tris Borate**,** 0.002M EDTA, dH_2O. This was stored at room temperature.

TE buffer: 10mM Tris-HCL, 1mM EDTA, dH_2O. This was stored at room temperature.

1M Tris-HCL pH8: 12.1g Tris base, 1 litre dH_2O. Adjusted to pH 8.0 with conc. HCL. This was stored at room temperature.

1X Wash buffer: 1X maleic acid, 0.3% Tween®20, sterile H_2O. This was stored at room temperature.

Methods

To prevent unnecessary repetition, this section includes a detailed description of the protocols that are referenced throughout this thesis. Protocols that were only used in one chapter are described in detail within that chapter.

1. Manual DNA extraction

10-20 µl of blood/buffer mix (buffer used varied depending on species) was added to a 1.5ml sterile eppendorf. To this, 250µl of nuclei lysis buffer, 100µl of 10% SDS and 10µl of proteinase K (10µg/ml) was added and the mixture was incubated overnight at 37°C. During incubation samples were continuously agitated. Samples were then deproteinised by the addition of an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). Samples were centrifuged at 14,000rpm for 10 minutes at room temperature to separate them into organic and aqueous phases. The upper, aqueous phase was transferred to a sterile 1.5ml eppendorf. DNA was then precipitated out by the addition of 1/10 volume of 3M sodium acetate and 3 volumes of molecular biology grade 100% ethanol. The mixture was incubated for an hour at -20°C to help precipitation. The samples were then centrifuged at 14,000rpm for 10 minutes at 4°C, resulting in pelleted DNA. The supernatant was removed and the pellet was washed in 70% ethanol. The pellet was allowed to air dry for 5 minutes and was then re-suspended in 40µl of TE buffer and stored at -20°C.

2. Determination of DNA concentration and quality

2.1 Determination by spectrophotometry

DNA samples were diluted 1:20 by adding 95µl of dH_2O to 5µl of the sample. Optical density readings were taken at 260nm and 280nm, using 100µl of dH_2O as a control. This gives a measure of the quantity of DNA in the sample. The purity of the DNA can also be estimated from the ratio of the optical density readings taken at 260nm and 280nm (260/280 ratio). The 260/280 ratio of a pure preparation of DNA is generally accepted to be around 1.8. Substantially lower values than this suggest the presence of proteins or other contaminants such as phenol, but for our purposes a ratio of 1.5 or higher was adequate.

2.2 Determination by gel electrophoresis

This method was used in addition to spectrophotometry, in order to ensure that the DNA had not suffered significant amounts of degradation. A 1% gel was made by adding 0.5g of agarose to 50ml of 1X TBE buffer and heating in a microwave for around 2 minutes. After being allowed to cool slightly, 2.5µl of 100mg/ml ethidium bromide was added. The gel was then poured into a casting tray containing a comb with the requisite number of wells and was left to set for around 20 minutes. DNA samples were diluted to a concentration of 100ng/µl with TE buffer. 2.5µl of this dilution (25ng of DNA) was mixed with 1µl of bromophenol blue loading buffer and added to the wells of the gel using a micropipette. Gels were run at 150V for approximately 10 minutes. The gel was then removed from the electrophoresis unit and the DNA was visualized using a UV transluminator (BIO-RAD) and photographed using a photographic unit (Sony). Figure 2.1 shows a representative photo of such a gel.

3. Telomere restriction fragment (TRF) analysis

3.1 Digestion of DNA with Hinf1/Rsa1

The restriction endonucleases *Hinf* I and *Rsa* I were selected because they do not contain recognition sequences that will cut within the telomeric repeat sequences. The use of these enzymes allows for complete digestion of non-telomeric DNA, leaving only telomeric DNA intact. This remaining telomeric DNA is referred to as a Telomere Restriction Fragment (TRF). By calculating the mean TRF length in the digested DNA sample we can make an estimate of the average telomere length in the initial cell population.

For each sample a mixture was made up containing 10µl of DNA diluted to 100ng/µl (1µg DNA), 7µl of dH20, 2.5µl of*Hinf*1 buffer, 2.5µl of *Rsa*1 buffer, 0.25µl of *Hinf*1 and 0.25µl of *Rsa*1. The mixture was incubated for 12-18 hours in a 37°C waterbath.

The success of the digest was confirmed by running part of each digest out on an electrophoresis gel. The gel was prepared and visualised in the same way as described in section 2.2. The gel was run at 150V for approximately 20 minutes. Figure 2.2 shows an example of such a gel.

If digests were not immediately used, they were stored at -20°C for up to one month.

3.2 Constant field agarose gel electrophoresis and Southern blot

Digested DNA was separated out on a 0.8% agarose gel made by dissolving 2g of agarose in 250ml of 1X TAE buffer. 3µl of bromophenol blue loading dye was added to each digested DNA sample and 20µl of this mixture was added to each lane. Two marker lanes containing a DIG labelled DNA molecular weight marker (23.1 kb – 2.0 kb, Roche) were also run on each gel. Each marker lane contained 10µl of a mixture containing the marker, dH_2O and bromophenol blue loading dye in a 5:5:1 ratio. The gel was run at 150V for approximately 3.5 hours.

The digested DNA was transferred to a positively charged nylon membrane (Amersham) by use of the Southern blot technique (Southern 1975) following a standard protocol (Current protocols in molecular biology). The gel was first immersed in 0.25M HCL for 5-10 minutes. Completion of this step was indicated by the colour of the bromophenol blue loading dye changing from blue to yellow. The gel was then twice washed in 1X denaturation buffer for 15 minutes. During this step, the bromophenol blue loading dye regained its original colour. Finally the gel was twice washed in 1X neutralisation buffer for 15 minutes. Between each wash stage, the gel was briefly rinsed in H_2O. All of these pre-treatment steps were carried out at room temperature and with gentle agitation using a mechanical shaker (Stuart Scientific).

The Southern transfer itself was set up as follows: the treated gel was placed on a 'wick' of chromatography paper, both ends of which were immersed in a reservoir of 20X SSC. On top of the gel was placed the nylon membrane, which had been washed in 2X SSC. Two more layers of chromatography paper, also washed in 2X SSC, were placed on top of the membrane. Finally paper towels were placed on top to a depth of approximately 10cm. The DNA was transferred from gel to membrane by capillary action, with the 20X SSC acting as a transfer buffer. Gels were flipped before Southern transfer to reduce the chance of irregularities in the upper surface of the gel causing an uneven transfer of DNA. Transfer was carried out overnight and the DNA was then UV cross-linked to the membrane using a trans-illuminator (Spectrolinker™ XL-1000, Spectronics Corporation). After being washed with 2X SSC, the membrane was then stored at 4°C ready for the probe hybridisation and chemiluminescent detection stage.

3.3 Hybridisation and chemiluminescent detection

All hybridisation steps were carried out using a Techne Hybridiser HB-1D hybridisation oven (Techne) and standard hybridisation flasks on a rotary mount. Membranes were pre-hybridised for 1 hour at 42°C in 15ml of DIG Easy Hyb (Roche). This solution was replaced with 10ml of DIG Easy Hyb containing 2.5µl of digoxigenin (DIG) labelled telomere probe $(TTAGGG)_7$. Hybridisation was then carried out at 42°C for 3 hours.

Following hybridisation, the membrane underwent various wash stages to prepare it for chemiluminescent detection. All wash stages were carried out at room temperature unless otherwise stated, and with gentle agitation of the membrane.

Firstly the membrane was washed in stringency wash I for 2 x 5 minutes at room temperature and then in stringency wash II for 2 x 15 minutes at 50°C (using the same apparatus as in the hybridisation stage). Blots were then washed for 5 minutes in 1X wash buffer and then for 30 minutes in freshly prepared 1X blocking buffer. The membrane was then incubated with a DIG specific antibody (750 units/ml Anti-Digoxigenin, Fab fragments) covalently coupled with alkaline phosphatase (Anti-DIG-AP) (Roche). An Anti-DIG-AP working solution was prepared at a concentration of 75mU/ml (1:10,000) in 1X blocking buffer. Before preparation of the working solution the Anti-DIG-AP was centrifuged for 5 minutes at 13,000 rpm to avoid background signal being generated by aggregated antibody. The blot was washed in the Anti-DIG-AP working solution for 30 minutes.

The membrane was then washed for 2 x 15 minutes in 1X wash buffer and finally for 5 minutes in 1X detection buffer. The detection itself was carried out using the chemiluminescent alkaline phosphate substrate CPSD (Roche). Excess detection buffer was removed from the blot by briefly placing it on a piece of chromatography paper, DNA side up. The blot was then placed on an acetate sheet and 3ml of substrate solution (CPSD diluted 1:100 with 1X detection buffer) was pipetted onto the DNA side of the damp membrane. Another acetate sheet was then placed over the blot, and any air bubbles were carefully removed. The blot was then incubated for 5 minutes at room temperature. Excess liquid was removed and the two acetate sheets sealed with tape. The sealed membrane was then incubated for 10 minutes at 37°C to increase the chemiluninescent signal. The membranes were then used to generate autoradiographs. The exposure times varied from 1

to 30 minutes according to what generated the optimum image quality for analysis. Figure 2.3 shows a representative TRF gel and how the mean TRF length is calculated from it.

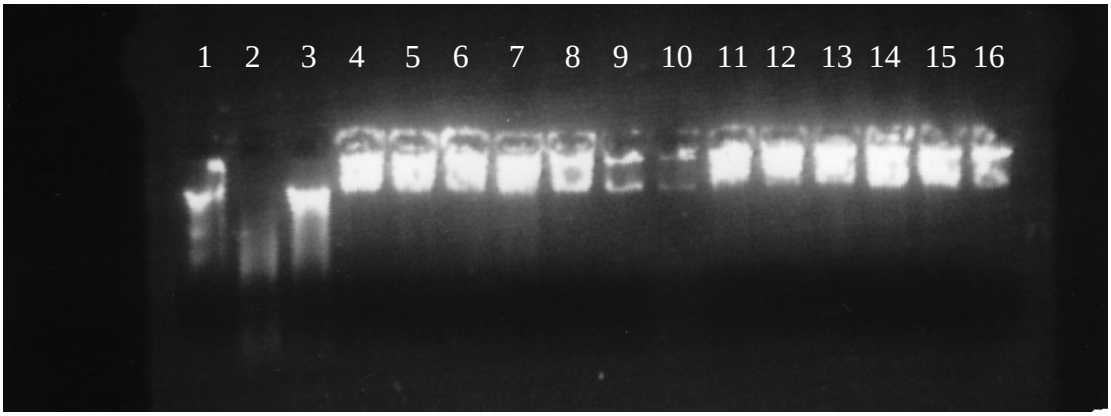


Figure 2.1: Representative electrophoresis gel of intact genomic DNA (in this case, DNA from giant petrels). DNA in lanes 4 – 16 consists of a single tight band, showing that the DNA is in good condition. Conversely, the DNA in lanes 1 – 3 has much more of a diffuse band, suggesting that the DNA has significantly degraded during extraction or storage. Such samples would be discarded and the DNA re-extracted. In some cases, the quality of the re-extracted DNA would be good enough to use; in other cases DNA continued to be degraded in every re-extraction attempted. In these cases, the blood sample itself was clearly degraded and the sample was omitted from the study. See individual chapters for details on how many such samples there were.

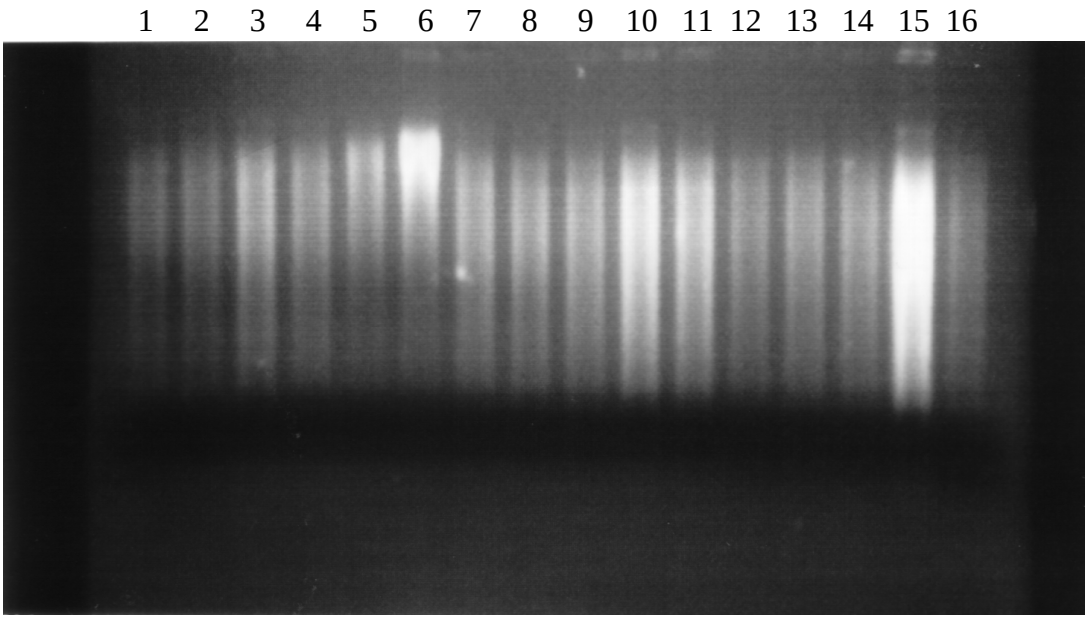


Figure 2.2: Representative electrophoresis gel of digested genomic DNA (in this case DNA from giant petrels). Fully digested DNA will produce a clean smear, as in lanes 1-5 and 7-16. Conversely, in lane 6 DNA is banded towards the top of the smear, indicating that digestion was not successful. If digestion was unsuccessful, the sample was re-digested; in none of the studies described in this thesis did a sample have to be permanently omitted because of an unsuccessful digestion (i.e. all such samples were successfully re-digested).

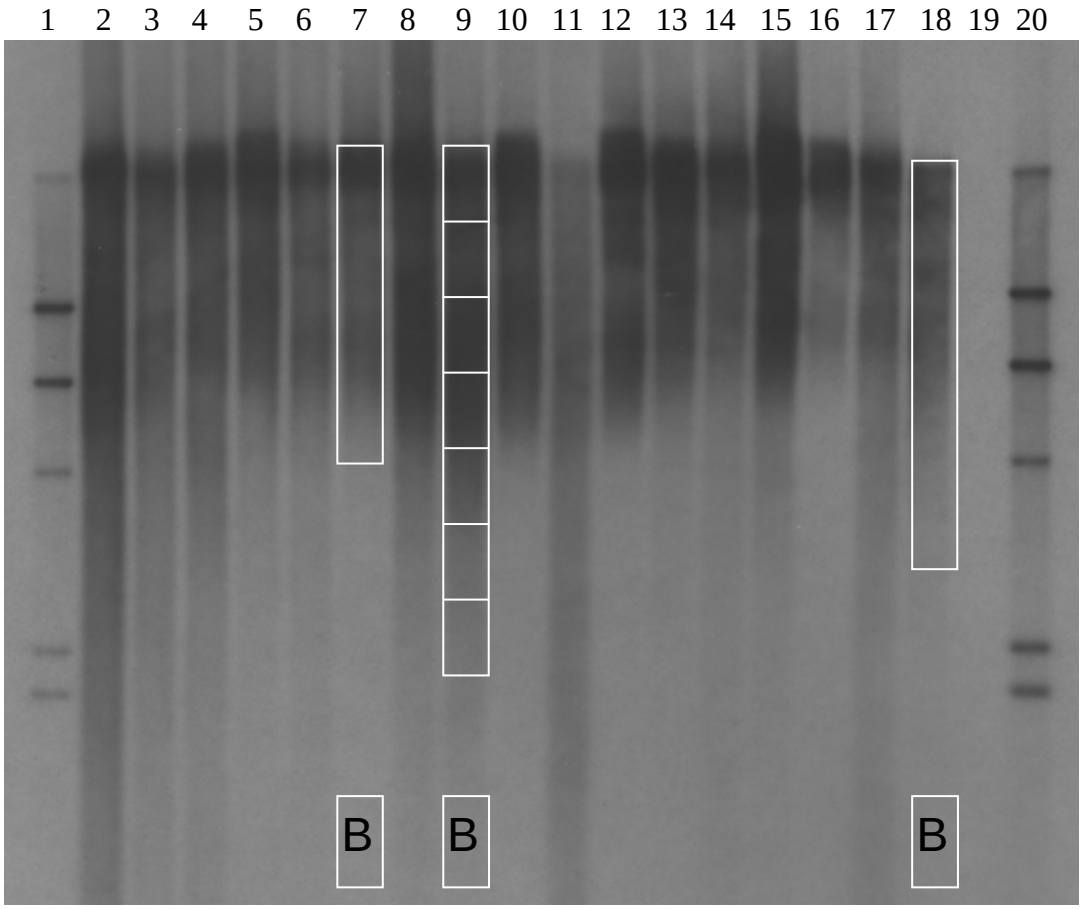


Figure 2.3: Example of a TRF gel and how mean TRF is calculated from it (gel contains lesser black-backed gull DNA). First and last lanes contain a Dig-labelled marker (23.1kb-2.0kb), the rest of the lanes contain gull samples. The white boxes in lanes 7, 9 and 18 demonstrate the 'analysis window' used to calculate mean TRF. The analysis window always began at the largest marker and continued until the intensity in the window was the same as the background intensity. The size of the analysis window thus varied for each lane. Carrying out the analysis in this way ensured that we took into account changes in the proportion of short telomere fragments between samples; this can have a significant effect on mean TRF (Haussmann & Mauck 2008a). The analysis window was split into several equal size boxes (as shown in lane 9) to give the intensity at different fragment sizes. Mean TRF length per lane was then calculated using the formula: mean TRF length = $\sum(OD_i) / \sum(OD_i / L_i)$ where OD_i is signal intensity and L_i is DNA size (Kb) at position $_i$. The background intensity was calculated from an area at the bottom of each lane (white box marked 'B'); this was subtracted from signal intensity before each calculation.

Chapter 3: Telomere dynamics in relation to early growth conditions in the wild in the lesser black-backed gull *Larus fuscus*

Abstract

There has recently been much interest in the long-term effects of early growth conditions. Telomeres, the repetitive DNA sequences that cap eukaryotic chromosomes, are potentially an excellent tool for studying such effects. Telomeres shorten at each cell division and considerable evidence links the rate at which they do so with cellular and organismal senescence. Previous research has shown that telomere loss is greatest during early life, so conditions during this time will significantly affect telomere attrition and senescence rates. However, relatively little is known about the pattern of telomere loss under natural conditions. I examined telomere dynamics during growth under natural conditions in the lesser black-backed gull *Larus fuscus*. Although telomere length significantly decreased with age during the chick period, there was a considerable amount of inter-individual variation in telomere length. Much of this variation was related to circumstances during embryonic growth. Larger hatchlings had shorter telomere lengths, suggesting that embryonic growth rate could have affected telomere attrition. Independent of this trend, males had longer telomeres at hatching than females. The variation in hatching telomere length caused by embryonic growth conditions remained consistent during the initial post-hatching period.

Introduction

Conditions during growth and development have profound implications for an organism's phenotypic development and subsequent life history (Bateson 2003; Gluckman & Hanson 2006; Monaghan 2008). There has been much recent interest in the mechanisms that mediate such effects, and in particular how the pattern and pace of organismal growth is linked to the rate of degeneration later in life (Arendt 1997, 2003; Jennings *et al.* 2000; Metcalfe & Monaghan 2001, 2003; Barker *et al.* 2005; Barker 2006). Telomeres, the repeat nucleic acid sequences that cap eukaryotic chromosomes, are thought to play an important role in linking the patterns of growth and degeneration (Campisi *et al.* 2001; Chan & Blackburn 2004; Monaghan & Haussmann 2006). In somatic cells, telomeres shorten at each cell division, eventually reaching a critical length that triggers cell senescence (Campisi *et al.* 2001). Initial telomere length is partly determined by genetic factors (Nordfjäll *et al.* 2005) but environmental factors, particularly oxidative stress, also affect the rate of telomere loss, at least *in vitro* (von Zglinicki 2002; Richter & von Zglinicki 2007).

Previous studies on both mammalian and avian species have shown that most telomere loss occurs early in life (Frenck *et al.* 1998; Zeichner *et al.* 1999; Hall *et al.* 2004; Pauliny *et al.* 2006). This suggests that factors affecting individuals during early growth will significantly affect telomere loss and, potentially, the pattern of ageing (Monaghan & Haussmann 2006). In mammals, post natal growth has been linked to telomere attrition. In male rats, accelerated post-natal growth, induced as a catch-up response to an episode of poor foetal nutrition, resulted in a shortening of both kidney telomeres and lifespan (Jennings *et al.* 1999). However, we know relatively little about the pattern of telomere loss during growth under natural conditions.

Birds are a particularly interesting group to examine, as there is considerable inter-individual variation in growth patterns in many wild populations (Starck & Ricklefs 1998). Variation in telomere length among same age chicks has been shown in several wild avian populations (Haussmann *et al.* 2003; Hall *et al.* 2004; Haussmann *et al.* 2005; Juola *et al.* 2006; Pauliny *et al.* 2006), but the proximate cause and functional significance, if any, of this variation is unknown. In the European shag, *Phalacrocorax aristotelis*, chicks laying down higher tissue mass for their body size and chicks born late in the season both showed

a higher rate of red blood cell telomere attrition when measured as chicks and again as adults (Hall *et al.* 2004). This suggests that the growth conditions experienced by chicks could be responsible for some of the variation seen in telomere length. To date, no studies have examined the relationship between avian pre-hatching growth conditions and telomere length; given that a substantial amount of growth occurs before hatching it is possible that conditions during the pre-hatching phase may also be an important determinant of telomere loss.

In this paper we examine the effect of early growth conditions on telomere dynamics under natural conditions in the lesser black-backed gull, *Larus fuscus*. The lesser black-backed gull is an interesting species for this type of study, as there is considerable inter-individual variation in early growth conditions in relation to position in the clutch (lesser black-back gulls lay three eggs which differ in size and contents and hatch asynchronously – Royle 2000; Royle *et al.* 2001), sex (males grow faster than females – Griffiths 1992; Nager *et al.* 1999; Nager *et al.* 2000b), parental and egg quality, laying date and local environmental conditions (Bolton 1991; Nager *et al.* 2000a; Blount *et al.* 2002; Verboven *et al.* 2003).

Materials and methods

Study site and sampling

Field work was carried out at a breeding colony in Sandgerdi, Iceland (64°2' N, 22°41' W) during the 2005 breeding season. Nests for use in the study were selected at random throughout the breeding season (range of laying dates: 143 – 166 days in julian calendar, mean laying date 153.6 days). All nests came from the same area of the colony and contained three egg clutches. Nests were checked regularly and eggs were marked to determine both laying and hatching order. First, second and third laid eggs are referred to as A, B and C eggs respectively. The length and width of each egg was measured and egg volume was calculated according to the formula: egg volume (cm^3) = 0.000476 x length (mm) x $width^2$ (mm) (Bolton *et al.* 1992). Chicks were measured (body mass, wing length and head-bill length) within 24 hours of hatching and a small blood sample taken by superficial venipuncture of the brachial vein; chicks were marked and, where possible, re-measured and a second blood sample taken at 10 days of age. Initially, 85 chicks from at least 57 different broods (1 complete brood of three chicks, 18 pairs of siblings, 39 chicks

with no siblings sampled and 7 chicks of unknown parentage) were sampled; 34 of these chicks were re-sampled (from at least 27 broods: 1 complete brood, 2 pairs of siblings, 24 chicks with no siblings sampled and 3 chicks of unknown parentage). Chick sex was established using a PCR based method (Griffiths *et al.* 1998). The blood was separated into plasma and red cells by centrifugation within 4 hours of collection, as it was felt this would better aid storage of the samples. The red cells had BLB buffer (1% SDS, 50mM Tris, 50mM EDTA) added to them in a 1:1 ratio within 4 hours of collection and were stored at room temperature until DNA extraction.

Measurement of telomere restriction fragments (TRFs)

DNA was extracted from red blood cells, which are nucleated in avian species. Samples were digested with proteinase K before DNA extraction by a standard phenol-chloroform-ethanol-precipitation method. DNA was checked for degradation by 1% agarose gel electrophoresis. DNA was successfully extracted in this way from the 119 samples discussed in this chapter. However, non-degraded DNA could not be extracted from an additional 24 samples collected in the field; these samples were discarded from the study. Laying dates of these samples were spread across a wide range (144 – 157 days) and there was no other reason to believe that the omission of these blood samples would introduce a sampling bias to our study.

Approximately 1µg of DNA from each sample was digested with the restriction enzymes *Hinf*I and *Rsa*I for 16h at 37°C. Digested DNA samples were separated on a non-denaturating 0.8% agarose gel at 150V for 3hrs. Two marker lanes (23.1 – 2.0Kb) were run on each gel. DNA was transferred from the gel to a nitro-cellulose Hybond N+ membrane (Amersham, UK) by Southern blot. The membrane was hybridised with a digoxigenin (DIG) labelled telomere probe $(TTAGGG)_7$ (Roche) for 3 hours at 42°C. A chemiluminescent detection system (Roche) followed by exposure to autoradiography film was used to visualize the TRFs. Having scanned the images, the intensity of TRF smears at different molecular sizes was calculated using TotalLab software (Photoretix). Mean TRF length was calculated using the formula: mean TRF length = $\sum (OD_i) / \sum (OD_i / L_i)$ where OD_i is signal intensity and L_i is DNA size (Kb) at position $_i$. The background intensity was subtracted from signal intensity before each calculation. Mean TRF length is referred to as telomere length for the rest of this chapter. Analysis was carried out blind with respect to

age, sex and structural size. See chapter 2 for a more detailed description of the TRF protocol.

Figure 3.1 shows a representative TRF gel. For the majority of samples, a clean DNA smear was observed indicating that, if interstitial banding was occurring, it did not hinder our analysis of mean TRF length. The black bands at the top of each lane in figure 3.1 are due to the larger telomere fragments not being separated out during gel electrophoresis (sometimes termed the limit of mobility – Haussmann & Mauck 2008a). Ideally, we would have used pulse-field gel electrophoresis to separate these bands, but we were limited in our capacity to do so when these experiments were carried out. However we feel that, given the small size of the bands and the low mean TRF length of the gulls, the use of standard gel electrophoresis will have had a limited effect on mean TRF length.

To control for inter-gel variability, we ran a control sample (a randomly chosen gull sample) on every gel. Due to lack of DNA, one such control sample was run on 6 of our gels (mean TRF length values for this sample ranged from 10.97 – 11.62kb, coefficient of variation (CV) = 2.01%) and a different control sample was run on the remaining 6 gels (mean TRF length values ranged from 7.74 – 8.48kb, CV = 3.13%). If our samples are separated according to which control sample they were run alongside, then there is no difference between the two groups in terms of hatching telomere length ($t = 0.994$, $df = 83$, $P = 0.323$) or day 10 telomere length ($t = 0.460$, $df = 32$, $P = 0.648$). Samples from the same individual taken at hatching and 10 days old were always run on the same gel, so inter-gel variability was not an issue for longitudinal samples. Siblings were not run on the same gel, but the use of the control samples should have ensured that this was not important.

Statistical analysis

Analysis of factors linked to hatching telomere length was done using generalised linear mixed models, in which nest identity was included as a random factor to take account of the non-independence of chicks from the same brood (Crawley 2002). Chicks of unknown parentage were excluded from the GLMM analysis to avoid the presence of unknown siblings confounding our results (there were 7 such chicks). In addition 10 chicks were excluded as their sex was unknown (as there was not enough DNA left after TRF analysis to carry out the PCR sexing technique). In order to examine the maternal and

environmental factors influencing hatching telomere length, a candidate set of models was created and the 'best' model selected using the AIC criterion (Burnham & Anderson 2002; Johnson & Olmland 2004). The following factors were potentially available for inclusion in our model set – sex, hatching head-bill length, egg volume, egg position and laying date. Duration of embryonic growth (i.e. time between the onset of incubation – which in first laid eggs does not occur until the second egg in the clutch is laid - and hatching) was not included as a potential explanatory variable, as there was little inter-individual variation in this (CV = 4.32%) and there was no indication that this was linked to telomere length (fig. 3.2).

Potentially, we could have created a candidate set of models containing all combinations of the other five variables; however, this would be theoretically unsound given that the number of models required (31) would be very high relative to our sample size (Burnham & Anderson 2002). There was no *a priori* way to reduce the number of variables included in our models as, based on past work, it was legitimate to expect any or all of the variables to be linked to telomere length. Equally, initial exploratory data analysis provided no obvious way to eliminate variables from our model set (with the exception of duration of embryonic growth). Given this, we felt the best way to create a candidate model set was to begin with a global model containing all five variables and to then backward drop variables, beginning with the least-significant. Table 3.1 shows the five models created using this method. Based on their Akaike weights, none of these models could be determined to be the 'best' model (as weight < 0.9), so model averaging (from all five models in table 3.1) was used to produce an estimate of effect size for each variable (Burnham & Anderson 2002; Johnson & Olmland 2004).

Analysis of the factors influencing telomere loss at ten days old was done in the same way. Due to relatively high chick mortality in the study (only 14% of our chicks survived until 20 days old, which is considerably lower than previous years in this colony - pers. comm. G. Hallgrímsson), the sample size at ten days old was lower than at hatching (85 chicks at hatching, 34 at ten days old). There was no significant difference in hatching telomere length between chicks that survived till 10 days old and chicks that did not ($t = 1.629$, $df = 83$, $P = 0.107$, mean difference = 0.54 ± 0.33 kb). The initial variables included in the global model were hatching telomere length, sex, hatching head-bill length, instantaneous increase in head-bill length and egg volume. Egg position was not included

as there was no difference between A, B and C eggs in terms of day 10 telomere length ($F_{2, 31}$ = 0.835, P = 0.443) or in the magnitude of telomere loss (absolute loss: $F_{2, 20}$ = 0.311, P = 0.736; percentage loss: $F_{2, 20}$ = 0.338, P = 0.717). Twelve of our 34 chicks were excluded from the subsequent GLMM analysis as we did not have data on hatching telomere length (11 chicks) or egg volume (1 chick) for them. Parental identity was not included as a random factor in this analysis, as only 2 pairs of siblings were present in our reduced sample (if parental identity was included in the global model as a random factor, estimates of the covariance parameters were not significant - P > 0.10). Table 3.2 shows the six candidate models created by backward dropping the least significant variable in each successive model. Based on their Akaike weights, none of the candidate models could be considered to be the 'best'; as before an average estimate of effect size was calculated for each variable.

We used head-bill length as a measure of body size in all analyses, as body mass is affected by the residual yolk mass and so is not a good measure of size in recently hatched chicks. Instantaneous growth ((ln(2nd measurement) – ln(1st measurement)) / time) was used, rather than absolute or proportional growth, as it provides a better way of capturing the exponential increase in growth that is occurring at this point in the chick period. Means are quoted ± 1 standard error of the mean.

Results

Hatching telomere length

There was considerable variation in hatching telomere length (mean = 8.34 ± 0.17 kb, CV = 18.40%, n = 85). Hatching telomere length showed relatively low repeatability within broods (repeatability analysis – as in Lessells & Boag 1987: r = 0.383, $F_{18, 20}$ = 2.346, P = 0.034), suggesting that siblings were not more likely to have similar telomere lengths than non-siblings. Based on GLMM model averaging (see table 3.1), larger sized hatchlings (as indicated by their longer hatching head-bill length) tended to have shorter telomeres (average effect size = -0.18 ± 0.11, 95% CI: -0.40, 0.05). This average effect size is the equivalent of a 1.7 kb (20.38 % of mean telomere length) difference between the smallest and largest chicks in our sample. Independent of this effect (i.e. when controlling for hatchling size – see table 3.1), female chicks had shorter hatching telomere lengths than males (average difference = 0.77 ± 0.40 kb, 95% CI: 1.55, -0.02). Egg volume had no clear

effect on telomere length (average effect size = 0.025 ± 0.020, 95% CI: 0.064, -0.014). Egg position had no effect on hatching telomere length (average difference between A and C chicks = 0.10 ± 0.17 kb, average difference between B and C chicks = 0.03 ± 0.16 kb) and neither did laying date (average effect size = 0.001). None of these latter three variables were significant in a model with no other variables included (egg position: P = 0.376; laying date: P = 0.144; egg volume: P = 0.203).

Change in telomere length

At 10 days of age, there was still substantial variation in telomere length (mean = 7.12 ± 0.21 kb, CV = 17.00%, n = 34). Within individuals, telomere length declined significantly between hatching and 10 days old (paired t test: t = 3.360, df = 22, P = 0.001, n = 23, mean loss = 0.95 ± 0.26 kb). It was not possible to determine if telomere loss was more similar within than between broods, as in only three of the broods included in this analysis did we have telomere loss data for more than one chick.

In order to see whether telomere length at 10 days was predicted by the conditions experienced by chicks before this point, a GLM analysis was conducted with telomere length at 10 days as the dependent variable, and hatching telomere length, laying date, sex, hatching head-bill size, egg volume and instantaneous increase in head-bill length as independent variables. Chicks with longer telomeres at hatching had longer telomeres at 10 days old (average effect size = 0.52 ± 0.22, 95% CI: 0.94, 0.09). As at hatching, males had longer telomeres than females at 10 days old (model with sex and no other variables: $F_{1, 20}$ = 6.518, P = 0.019). However, sex is not an important variable in our model set (table 3.2; difference between the sexes based on model averaging = 0.02 ± 0.20 kb). This suggests that rather than being an effect of sex, the higher telomere length of males at day 10 is a reflection of their larger hatching telomere length. There was also no difference in telomere loss between the sexes (absolute difference: t = 1.027, df = 22, P = 0.316, mean difference = 0.53 ± 0.52 kb; proportional difference: t = 0.882, df = 22, P = 0.388, mean difference = 5.31 ± 6.02 %). Growth rate during the first 10 days after hatching (as indicated by the instantaneous increase in head-bill length) had no effect on telomere length at 10 days (average effect size = 42.16 ± 59.16). The same was true if body mass was used as a measure of growth instead of head-bill length. Hatching head-bill length (average effect size = 0.05 ± 0.11), egg volume (average effect size = 0.025 ± 0.032) and laying date (average effect size = 0.02 ± 0.04) all had no relationship with telomere length at 10 days.

None of these variables had a significant relationship with telomere length at 10 days in a model with no other variables included (head-bill growth rate: P = 0.626; hatching head-bill length: P = 0.219; egg volume: P = 0.298; laying date: P = 0.599).

Discussion

Considerable variation was seen in both the telomere length of chicks of the same age and in the rate of telomere loss within individuals during the first ten days of life. Substantial variation between same age individuals has also been found in the other birds in which this has been studied (Haussmann *et al.* 2003; Hall *et al.* 2004; Haussmann *et al.* 2005; Juola *et al.* 2006; Pauliny *et al.* 2006). In lesser black-backed gulls studied here, this variation seems to be linked to pre-hatching growth conditions. Larger hatchlings (based on head-bill length) had shorter telomere lengths. Previous work has shown a negative correlation between telomere loss and post-natal growth (Hall *et al.* 2004; Jennings *et al.* 1999); our study suggests that there might be a similar relationship between telomere loss and embryonic growth. This could perhaps be due to a higher rate of cell division and/or higher levels of oxidative stress. However, hatchling size provides only an indirect measure of embryonic growth; further, experimental, work will be necessary to determine if the rate of embryonic growth is directly responsible for this relationship. In addition, parental quality is likely to affect chick size. It is not known how adult telomere length correlates with adult quality, but, given that telomere length is likely to be at least partly heritable, parental quality may represent a confounding factor to our results.

Independent of this trend, males had longer telomeres than females at hatching. This sex difference was maintained at 10 days old, with telomere length in males and females differing by the same degree as at hatching, although this was due to males having a longer hatching telomere length rather than any specific effect of sex (there was no significant interaction between sex and hatching telomere length). This is, to our knowledge, the first time a sex difference in telomere length has been shown in birds, although males have been shown to have shorter telomeres than females in humans (Benetos *et al.* 2001; Nawrot *et al.* 2004) and rats (Cherif *et al.* 2003).

In previous longitudinal studies of telomere length (Zeichner *et al.* 1999; Hall *et al.* 2004; Pauliny *et al.* 2006; Kotrschal *et al.* 2007), a significant decrease was seen within

individuals over time. These studies measured telomere loss over a period of months or years; our study demonstrates that, in young birds, a significant difference can be seen over a much smaller period of time, in this case just ten days. Indeed the average telomere loss (11.39% of mean hatching telomere length) seen in this ten day period is much larger than that seen over a period of years in other bird species; in dunlins (*Calidris alpina*) measured as chicks and again at three years of age, an average decrease of 3.24% of initial telomere length was found (although this was based on only four individuals; Pauliny *et al.* 2006). Such a large decrease in gull chick telomeres in such a short period of time supports the idea that most telomere loss occurs in young animals (Zeichner *et al.* 1999; Hall *et al.* 2004; Baerlocher *et al.* 2007).

Inter-individual differences in telomere length were consistent over the first 10 days of life; individuals with longer telomeres at hatching had longer telomeres at 10 days. However, there was no relationship between pre-hatching growth conditions (hatchling size, egg volume, laying date and egg position) and the change in telomere length that occurred during the 10 days after hatching. Similarly growth rate (in terms of increase in structural size) had no effect on the change in telomere length post-hatching. However, these findings could be a result of our low sample size of 10 day old individuals.

Although many studies have shown that there is significant variation in telomere length between same-age individuals, very few have looked at the causes of this variation. There are considerable theoretical reasons to believe that growth conditions will be one of the biggest factors affecting telomere loss (Metcalfe & Monaghan 2003; Monaghan & Haussmann 2006). Previous studies have shown a link between post-natal growth and telomere loss (Hall *et al.* 2004; Jennings *et al.* 1999), but embryonic growth, which is usually the period of highest cell turnover, may be even more important in determining the rate of telomere loss. Our study is the first, to our knowledge, to suggest such a relationship between embryonic growth and telomere loss (although it should be stressed that other factors, such as parental quality, could be responsible for our results). We have also shown that the inter-individual differences in telomere length caused by embryonic growth conditions are consistent, at least in the short term. What is now needed is further work to ascertain if these inter-individual differences remain consistent into adulthood, and if so, what significance these differences have in terms of an individual's life-history.

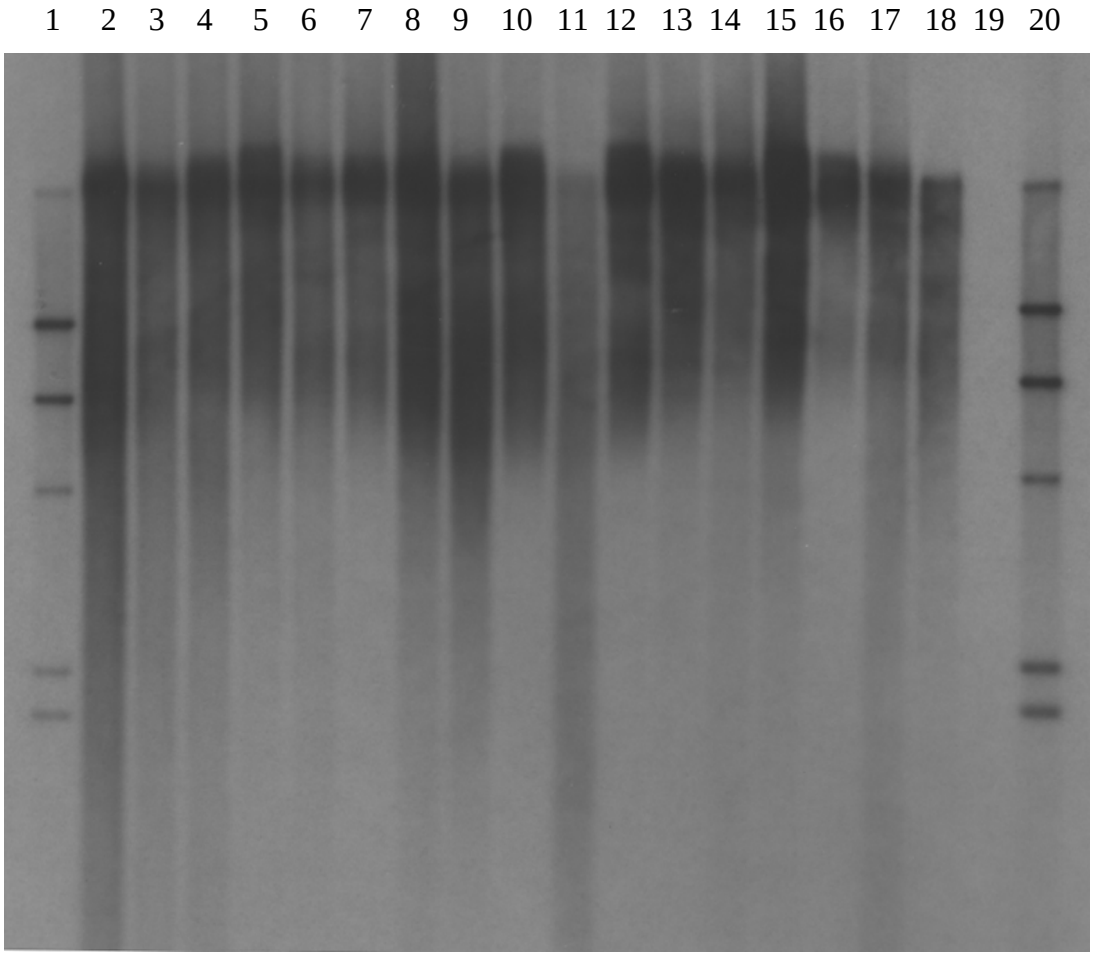


Figure 3.1: Representative gull TRF gel. Lanes 1 and 20 contain markers, lanes 2 - 18 contain gull samples (lane 19 is blank).

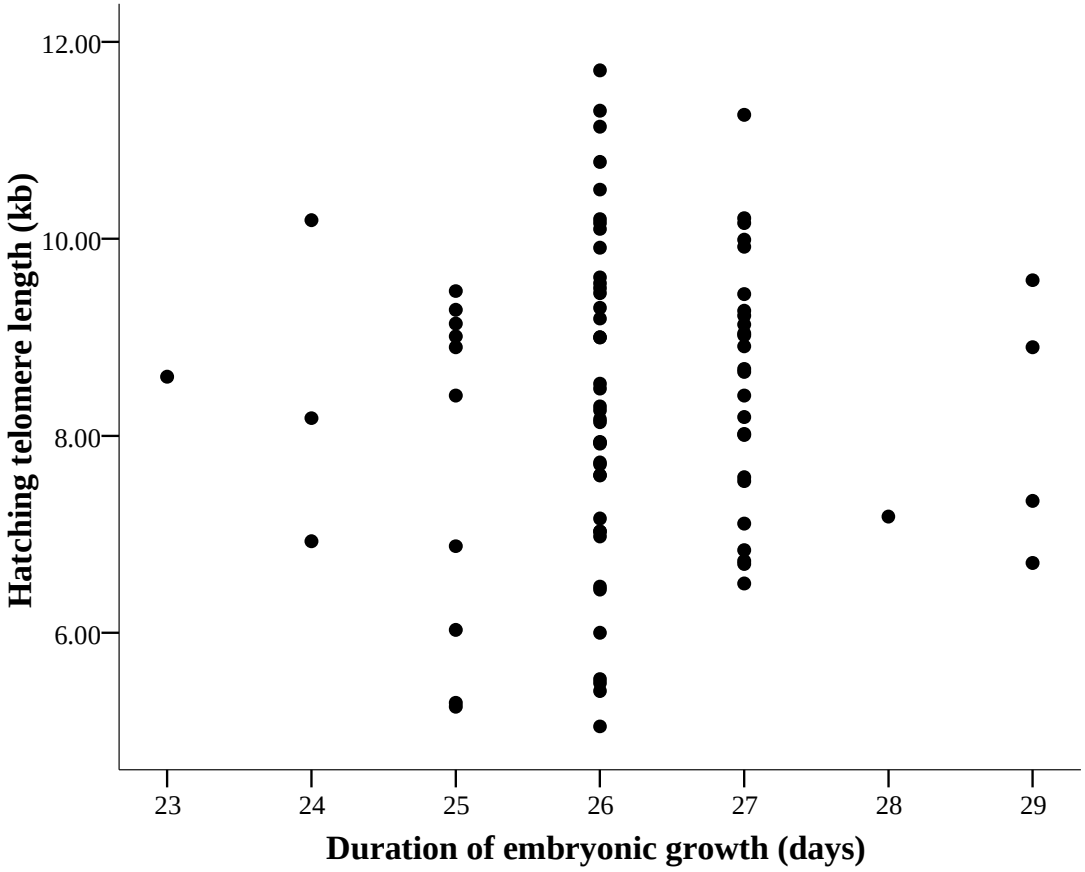


Figure 3.2: Relationship between the duration of embryonic growth (i.e. time between the onset of incubation and hatching) and hatching telomere length (r^2 = 0.041, n = 85).

Table 3.1: Akaike's second order information criteria (AIC_c) of the GLMM's of hatching telomere length in relation to 5 other variables (n = 68). Parental identity was included as a random factor in all models. See methods for details of how the model set was constructed. The lower the AIC_c score of a model the 'better' it is; the 'delta AIC_c' column shows the difference in AIC between the best model and every other model. The Akaike weight gives a measure of how likely a given model is to be the best model in the set. AIC_c was used rather than AIC due to the relatively small sample size (Burnham & Anderson 2002). Any models containing interaction terms had considerably larger AICc values and so were not included in the candidate model set. Effect sizes (see results) were calculated based on model averaging of all the models shown in the table (Burnham & Anderson 2002; Johnson & Olmland 2004).

Variables included in model	**AICc**	**Delta AIC_c (Δ_i)**	**Akaike weight (W_i)**
Sex and head-bill length	259.91	0	0.30
Sex	260.00	0.09	0.29
Sex, head-bill length, egg volume and egg position	260.19	0.28	0.26
Sex, head-bill length and egg volume	261.86	1.95	0.11
Sex, head-bill length, egg volume, egg position and laying date	264.10	4.19	0.04

Table 3.2: Akaike's second order information criteria (AIC_c) of the GLMM's of day 10 telomere length in relation to 6 other variables (n = 22). See methods for details of how the model set was constructed. The lower the AIC_c score of a model the 'better' it is; the 'delta AIC_c' column shows the difference in AIC between the best model and every other model. The Akaike weight gives a measure of how likely it is that a given model is the best model in the set. AIC_c was used rather than AIC due to the relatively small sample size (Burnham & Anderson 2002). Any models containing interaction terms had considerably larger AICc values and so were not included in the candidate model set.

Variables included in model	**AICc**	**Delta AIC_c (Δ_i)**	**Akaike weight (W_i)**
Hatching TRF, egg volume and instantaneous growth	60.96	0	0.46
Hatching TRF, egg volume, instantaneous growth and hatching head-bill length	62.32	1.36	0.23
Hatching TRF, egg volume, instantaneous growth, hatching head-bill length, laying date and sex	62.64	1.68	0.20
Hatching TRF, egg volume, instantaneous growth, hatching head-bill length and laying date	64.47	3.51	0.08
Hatching TRF	66.61	5.64	0.03
Hatching TRF and egg volume	70.82	9.86	0

Chapter 4: A longitudinal study of telomere loss in chicks of the European shag *Phalacrocorax aristotelis*

Abstract

Telomere length varies considerably among same-age individuals. Given this, longitudinal studies represent the best way to follow the change in telomere length over time. However, very few such studies have been conducted, particularly on wild populations. We conducted a longitudinal study of telomere length in chicks from a wild population of European shags *Phalacrocorax aristotelis*. Individuals were sampled twice during the chick period, with an interval of 11 or 13 days between samplings. We also examined how body size and sex affected telomere length. Within individuals, telomere length did not decrease with age. There was also no trend for telomeres to shorten with age cross-sectionally. Body size had no effect on telomere length, but males had longer telomeres than females. Previous longitudinal studies have demonstrated a decline in telomere length within individuals over a period of months or years. Our results show that such a decline cannot necessarily be detected over a much shorter time period. This suggests that the time between sampling is crucial to the success of longitudinal studies of telomere length.

Introduction

Previous studies on telomere dynamics have shown that there is a considerable amount of inter-individual variation in telomere length among individuals of the same age (e.g. Frenck *et al.* 1998; Cherif *et al.* 2003; Haussmann *et al.* 2003; Hall *et al.* 2004; Unryn *et al.* 2005; Juola *et al.* 2006; Pauliny *et al.* 2006). This presents a problem with cross-sectional studies of telomere length; it is difficult to examine the change in telomere length over time when there is so much variation among individuals of the same age. Longitudinal studies can avoid these problems by following the change in telomere length over time within an individual. However, very few longitudinal studies of telomere dynamics have been conducted and even fewer have examined wild populations (Hall *et al.* 2004; Pauliny *et al.* 2006), rather than humans or laboratory animals (Zeichner *et al.* 1999; Brümmendorf *et al.* 2002; Kotrschal *et al.* 2007).

One of the few longitudinal studies conducted on a wild avian population was on the long-lived seabird the European shag *Phalacrocorax aristotelis* (Hall *et al.* 2004). Telomere length decreased in individuals sampled as chicks and again as adults. In this chapter we present additional longitudinal data from the same population of shags. We sampled individuals twice during the chick period, in order to determine whether a decrease in telomere length is evident over this much shorter time period. Cross-sectional data on telomere dynamics (including data from this population of shags) have suggested that most telomere loss occurs in young individuals (Frenck *et al.* 1998; Hall *et al.* 2004; Pauliny *et al.* 2006). However, the exact point this telomere loss occurs is not known; is it during the chick stage (and if so, when during the chick phase is telomere loss at its greatest?) or does the loss occur in newly fledged adults, before they return to breed? Determining this would be very difficult with cross-sectional data, but by looking at chicks longitudinally we might be able to determine the amount of telomere loss (if any) occurring during the chick stage.

In addition to a lack of longitudinal studies of telomere length, there are also very few studies that have attempted to explain why telomere length varies so much among individuals. Some of this variation seems to be genetically inherited (Nordfjäll *et al.* 2005; Njajou *et al.* 2007). However, *in vitro* studies on human cells have suggested environmental factors, such as oxidative stress, also affect telomere shortening rates (von

Zglinicki 2002; Richter & von Zglinicki 2007). Thus, one would expect that factors that affect oxidative stress levels, such as growth rates and stress levels, will also affect telomere length; there are studies which support this idea (Jennings *et al.* 1999; Epel *et al.* 2004; Kotrschal *et al.* 2007). In shags, the rate of telomere loss within individuals correlates with initial telomere length, laying date and the relative amount of body mass laid down as a chick (Hall *et al.* 2004). It is thus interesting to see if the rate of telomere loss within chicks (if there is any) correlates with the same variables. It is also interesting to examine which variables, if any, correlate with telomere length cross-sectionally, across a substantial sample of similar age individuals.

Materials and methods

Study site and sampling

Field work was carried out at a breeding colony on the Isle of May (Firth of Forth, Scotland 56°11'N, 02°33'W) during the 2003 breeding season. All blood samples and body size data were collected by Margaret E. Hall. Chicks were marked after hatching; as shag chicks are altrical we were still able to determine parental identity and number of siblings for each chick (brood size was 2 or 3 chicks). 31 chicks were sampled, from 19 different broods (2 broods of 3 chicks, 8 pairs of siblings and 9 chicks with no siblings sampled). 26 of these chicks had two blood samples taken from them, the remaining 5 were only sampled once. Samples were taken either 11 or 13 days apart. The exact age of the chicks was unknown but we were able to make an estimation of age from their wing length (using a regression equation based on known age chicks from 1997 and 1998: 23.1703 - 14.4043 x (ln (-ln (wing length -13.9063) + ln (263.9751))). We were able to test the accuracy of these estimates for each chick sampled twice by comparing the interval between the two estimated ages and the actual interval between blood samplings. The predicted measurement interval was correct for 9 chicks (35%), incorrect by 1 day for 11 chicks (42%) and incorrect by 2 days for 6 chicks (23%). Estimated ages of the chicks ranged from 12 – 26 days old when the first sample was taken and 23 – 39 days old when the second sample was taken. Blood from the 5 chicks sampled once was taken during the second time period. At the same time as the second blood sample was taken, measurements were also made of body mass, wing length, head-bill length and tarsus length. Wing length was also measured at the time of the first blood sample. Blood was stored in 90% ethanol

at -20°C until DNA extraction. Chick sex was established using a PCR based method (Griffiths *et al.* 1998).

Measurement of telomere restriction fragment (TRFs)

DNA was extracted from red blood cells, which are nucleated in avian species. Samples were digested with proteinase K before DNA extraction by a standard phenol-chloroform-ethanol-precipitation method. DNA was checked for degradation by 1% agarose gel electrophoresis. DNA was successfully extracted in this way from the 57 samples discussed in this chapter. However, non-degraded DNA could not be extracted from an additional 3 samples collected in the field (all of these were the first blood sample taken from chicks whose second blood sample is included in this study). These samples were discarded from the study. There was no reason to believe that the omission of these blood samples would introduce a sampling bias to our study.

Approximately 1µg of DNA from each sample was digested with the restriction enzymes *Hinf*I and *Rsa*I for 16h at 37°C. Digested DNA samples were separated on a non-denaturating 0.8% agarose gel at 150V for 3hrs. Two marker lanes (23.1 – 2.0Kb) were run on each gel. DNA was transferred from the gel to a nitro-cellulose Hybond N+ membrane (Amersham, UK) by Southern blot. The membrane was hybridised with a digoxigenin (DIG) labelled telomere probe $(TTAGGG)_7$ (Roche) for 3 hours at 42°C. A chemiluminescent detection system (Roche) followed by exposure to autoradiography film was used to visualize the TRFs. Having scanned the images, the intensity of TRF smears at different molecular sizes was calculated using TotalLab software (Photoretix). Mean TRF length was calculated using the formula: mean TRF length = $\sum (OD_i) / \sum(OD_i / L_i)$ where OD_i is signal intensity and L_i is DNA size (Kb) at position $_i$. The background intensity was subtracted from signal intensity before each calculation. Mean TRF length is referred to as telomere length for the rest of this chapter. Analysis was carried out blind with respect to age, sex and structural size. See chapter 2 for a more detailed description of how TRF measurements were carried out.

Figure 4.1 shows a representative TRF gel. A clean DNA smear was observed in the majority of samples (>95%), suggesting that, if present, interstitial bands did not significantly affect our analysis. To control for inter-gel variation, 46 of the 57 samples were run twice on different gels (we did not have enough DNA to take two measurements

from the remaining samples). The average of the two calculated telomere lengths was used in our analysis. There was a very high repeatability between the two values calculated for each sample (mean difference between repeats = 0.378 ± 0.041 kb – equivalent to 3.3% of the overall mean TRF of 11.34 kb; repeatability analysis: r = 0.849, $F_{46, 45}$ = 12.206, P < 0.001; paired t test between repeats: t = 0.263, df = 45, P = 0.794). Samples from the same individual taken at different time periods were run together on the same gels (i.e. the two samples were run alongside each other on two different gels). This will further decrease the effects of inter-gel variability on our longitudinal analysis. Siblings were not run on the same gels. It is possible this might affect our analysis of the similarity of telomere lengths among siblings (see results); however the high repeatability of samples run on different gels makes this unlikely.

Statistical analysis

We produced a measurement of body size using the first factor of a principal component analysis (PCA) containing wing length, body mass and head-bill length. We excluded tarsus length from the PCA as it had a low variability compared to the other measurements (coefficient of variations (CV): tarsus length = 2.7%, body mass = 13.9%, wing length = 13%, head-bill length = 5.5%) and so would be less useful in determining how body size varies between individuals. Analysis of factors linked to telomere length at the second sampling was done using generalised linear mixed models, in which nest identity was included as a random factor to take account of the non-independence of chicks from the same brood (Crawley 2002). We analysed the second telomere sample in this way, rather than the first telomere sample, as we had both a larger sample size and more variables at this time point. Sex, age, body size score (from PCA) and all possible interactions were included in the models. Initially sex, age and body size were included in the model. Non-significant terms, beginning with the least significant, were then sequentially removed from the model. All means are quoted ± 1 standard error.

Results

Change in telomere length

There was no difference in proportional telomere loss between chicks with a brood size of 2 or 3 (t = 0.805, df = 14, P = 0.434, n = 16) or between chicks sampled 11 or 13 days apart (t = 0.476, df = 14, P = 0.641, n = 16) – to avoid pseudo-replication one chick from

each brood was chosen at random for use in these analyses, hence the reduced sample size. We therefore did not separate chicks according to brood size or time between sampling in our analysis of telomere loss. There was no significant decrease in telomere length between the two time periods our chicks were sampled (paired t test: $t = 0.352$, $df = 25$, $P = 0.728$, $n = 26$; correlation between first and second TRFs: $r^2 = 0.917$; mean change in telomere length = 0.025 ± 0.073 kb).

Factors affecting telomere length

Mean TRF at the second sampling was 11.35 ± 0.13 kb, CV = 6.41%. Siblings do not have more similar telomere lengths than non-siblings (within brood repeatability analysis: $r = 0.018$, $F_{9, 12} = 1.039$, $P = 0.464$). There was no difference in telomere length between chicks with a brood size of 2 or 3 ($t = 0.859$, $df = 29$, $P = 0.560$), so we did not include brood size in our analysis.

In a stepwise regression analysis of GLMM models containing sex, body size and age (tables 4.1a, b and c), sex has a significant effect on telomere length (males have longer telomeres than females: average difference = 0.72 ± 0.23 kb; fig. 4.2) but body size and age do not. If the increase in wing length between the first and second sample (the only measurement of structural size for which we have two measurements) was included in the model (containing age and sex) instead of body size it was also not significant (absolute increase per day: $F_{1, 22} = 0.228$, $P = 0.638$; proportional increase per day: $F_{1, 22} = 1.045$, $P = 0.318$). Body size was not significant if included in a model on its own ($P = 0.122$) and neither was age ($P = 0.324$). To increase the age range over which we were examining telomere length, we included both values of telomere length we had for each individual (increasing the age range from 16 to 27 days). We then a) included all telomere length values in a GLMM with chick ID as a random factor (in addition to nest identity) and b) randomly included one of the two telomere length values we had from each individual, such that half of our values came from the 1st sampling period and half from the 2nd period. There was still no relationship between age and telomere length in either analysis (a – $F_{1, 53} = 0.851$, $P = 0.360$; b – $F_{1, 24} = 0.001$, $P = 0.974$; fig.4.3 – in both cases random factor(s) $P < 0.001$).

Discussion

We found no detectable change in telomere length within individuals sampled at two different times during the chick period. There was also no relationship between age and telomere length in a cross-sectional multivariate analysis, further suggesting that there is no obvious change in telomere length during the short period of time we studied the chicks. This contrasts with the data presented by Hall *et al.* (2004). Over a much longer time period, they found that telomere length did decrease with age and, furthermore, that most of this telomere loss seemed to occur during the chick period. Thus, rather than suggesting that no telomere loss is occurring at all in shag chicks, our results suggest that no change is occurring during the short period of time we followed them over. Note that this does not necessarily mean that no telomere loss occurred during this time period; it could be that there was just not enough to detect with the methods we were using. A method that tracks changes in the length of individual telomeres (such as Q-FISH) might show telomere loss which methods examining telomere loss in a population of cells (such as the TRF method) cannot.

In chapter 3, we carried out a longitudinal analysis of telomere length in chicks of the lesser-black backed gull (*Larus fuscus*) over a similar time period to the shags in this chapter (10 days in the gulls, 11-13 days in the shags). Interestingly, in the gulls we did detect a significant amount of telomere loss over this short time period. What is the cause of this apparent difference in the rate of telomere loss between shag and gull chicks? Shags have a longer chick period than gulls (7 weeks compared to 5) so one would expect growth rates to be generally slower. The gulls were also sampled at an earlier stage in their development than the shags when we would expect a higher rate of growth (gulls sampled during first third of their developmental period, shags around the second third of theirs). Comparison of the increase in wing length supports the idea that the gull chicks were growing faster than the shag chicks during the time between samplings (average proportional increase in wing length per day: gulls = 19.4%, shags = 9.3%). However, this argument assumes a connection between growth rate and telomere loss. We could find no such connection in either our gull or shag chicks, but Hall *et al.* (2004) did find a weak relationship between the relative amount of tissue mass laid down by shag chicks and the length of their telomeres as adults. It is possible that there are other reasons behind the difference in the rate of telomere shortening between the two species.

In our cross-sectional analysis, male shags had shorter telomeres than females. It is notable that no such sex difference was seen in the study carried out by Hall *et al.* (2004). They looked at a considerably larger age range of individuals than we did, but conversely had fewer individuals of the same age. Thus, it is possible that a sex difference can only be seen if a substantial sample size of (approximately) same age individuals is studied. Alternatively, it could be that the difference in the sexes is temporary and is seen only in chicks, not in adults. Whether this sex difference is 'inbuilt' or is due to a difference in conditions experienced by males and females is unknown. The direction of the sex difference might seem surprising given that male shag chicks grow at a faster rate than females (Daunt *et al.* 2001). However, this finding is consistent with the results from our study on lesser black-backed gull chicks, where males also had longer telomeres than females. Conversely, in the few other species where a sex difference in telomere length has been shown, such as humans (Benetos *et al.* 2001; Nawrot *et al.* 2004) and rats (Cherif *et al.* 2003), females have longer telomeres than males. This suggests the possibility that sex differences in telomere dynamics differ between birds and mammals.

There was no direct effect of body size on telomere length (although males are larger than females and have longer telomeres). This is perhaps surprising given that Hall *et al.* (2004) found a weak connection between telomere length and growth rate (in terms of relative tissue mass laid down). However, this was a longitudinal study carried out over a long time period (individuals sampled as chicks and then as adults). It is thus possible that such an effect cannot be seen in a cross-sectional study carried out over a much shorter time period.

Given the considerable amount of inter-individual variation in telomere length seen in most species studied, longitudinal analysis is clearly the best way to examine the factors affecting telomere loss. Previous longitudinal studies of telomere length (Zeichner *et al.* 1999; Hall *et al.* 2004; Pauliny *et al.* 2006; Kotrschal *et al.* 2007) have measured the decline in telomere length over a period of months or years. Our study on lesser black-back gulls showed that a significant difference in telomere length can be seen over a much shorter period, in that case just 10 days. However, our results here show that this might not always be the case, as we could detect no telomere loss over a similar time period, even though it is likely that such a loss is occurring. Clearly the time between samplings is a crucial part of any longitudinal study. Too much time between samples and it is difficult to

ascertain exactly when any telomere loss took place. Too little time and one risks the amount of telomere loss being too small to measure.

Table 4.1: General linear mixed models with telomere length as the dependent variable and age, body size and sex as explanatory variables. Nest identity was included in the models as a random effect ($P < 0.001$ in all models). The least significant variable was removed from each model, until only one variable was remaining.

a)

Variable	Numerator df	Denominator df	F	P
Age	1	27	0.842	0.367
Body Size	1	27	1.063	0.312
Sex	1	27	4.547	0.042

Dependent Variable: mean TRF length at 2nd sample (n = 31)

b)

Variable	Numerator df	Denominator df	F	P
Body Size	1	28	0.266	0.610
Sex	1	28	6.320	0.018

Dependent Variable: mean TRF length at 2nd sample (n = 31)

c)

Variable	Numerator df	Denominator df	F	P
Sex	1	29	9.412	0.005

Dependent Variable: mean TRF length at 2nd sample (n = 31)

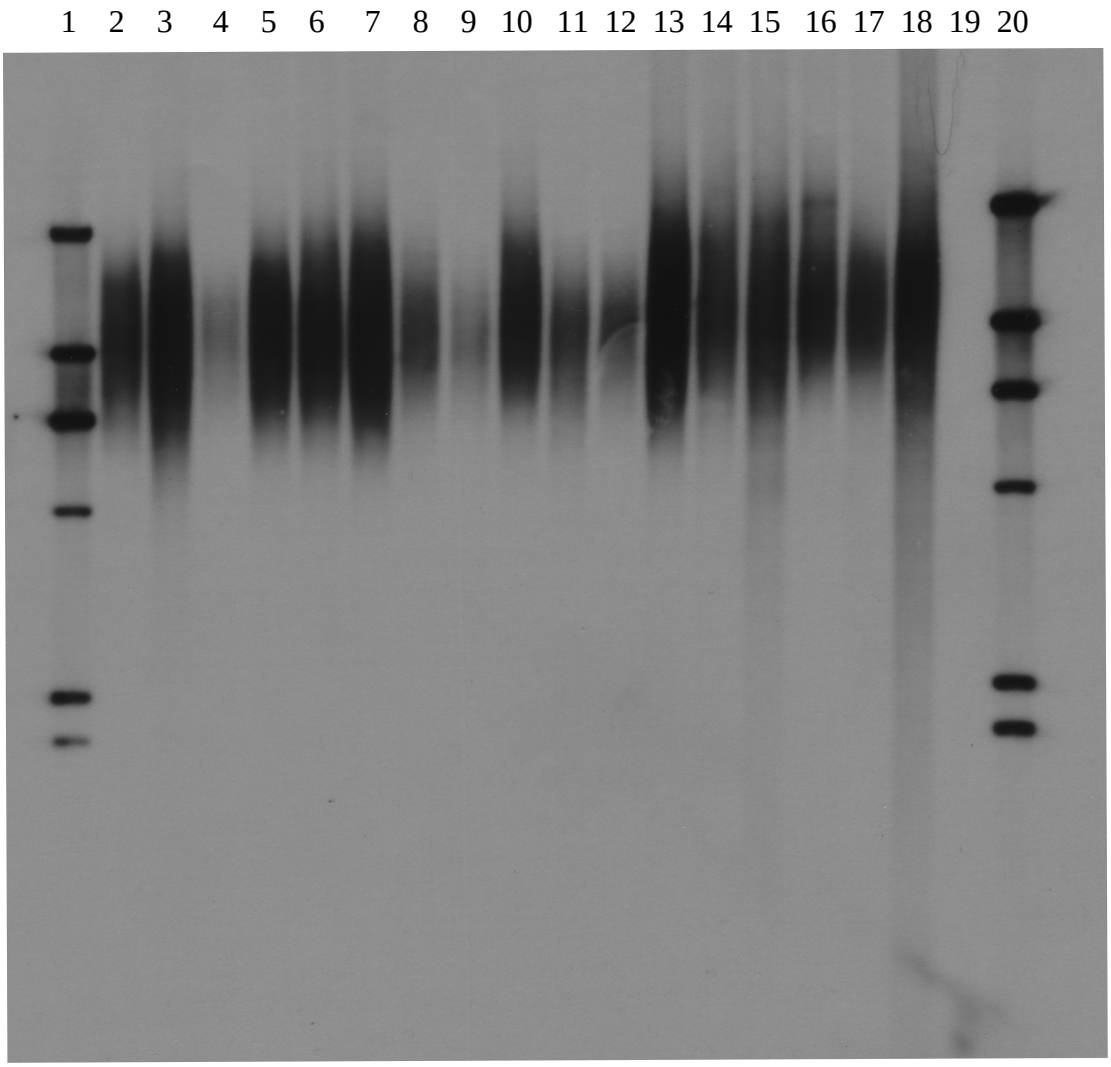


Figure 4.1: Example of a shag TRF gel. Lanes 1 and 20 contain markers, lanes 2 - 18 contain shag samples (lane 19 is blank).

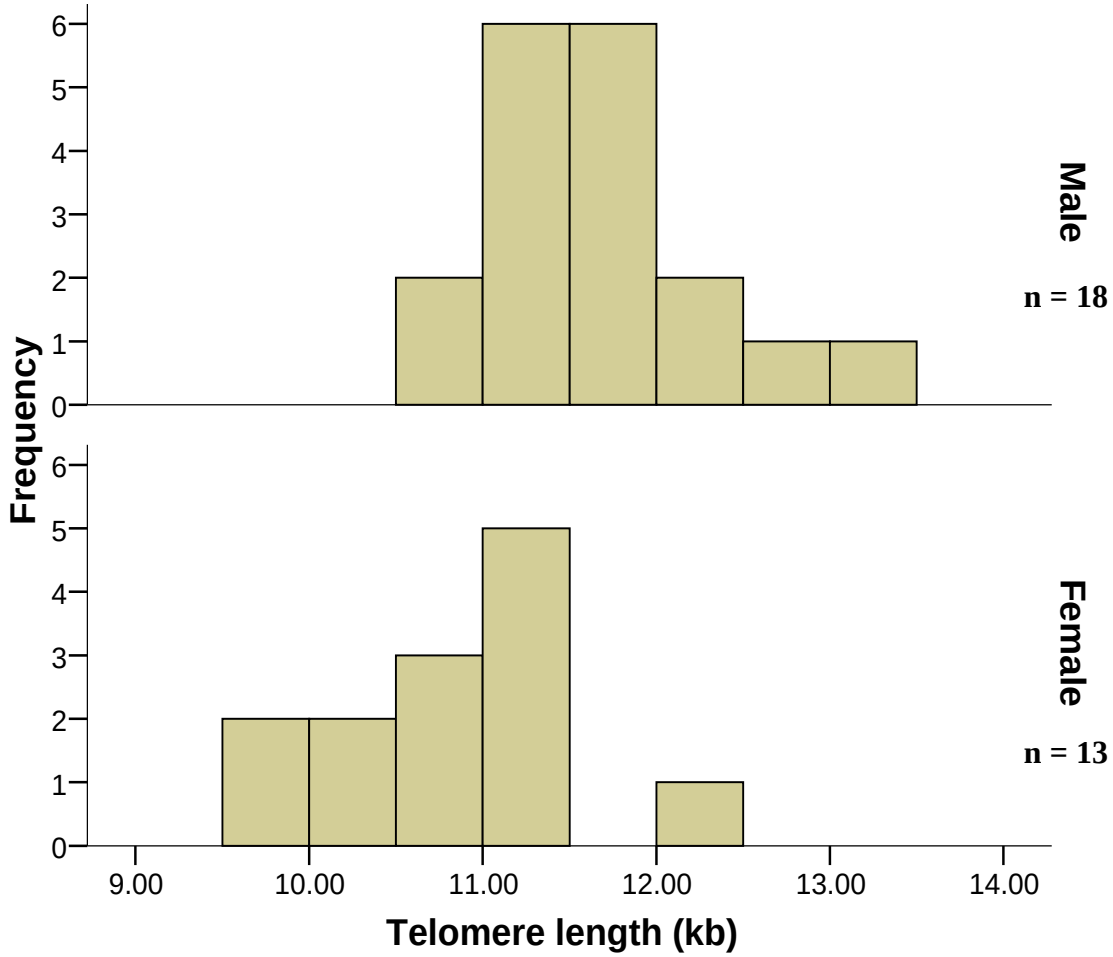


Figure 4.2: Histogram of telomere length (at second sampling) separated by sex. Males (top panel) have longer telomeres than females (bottom panel).

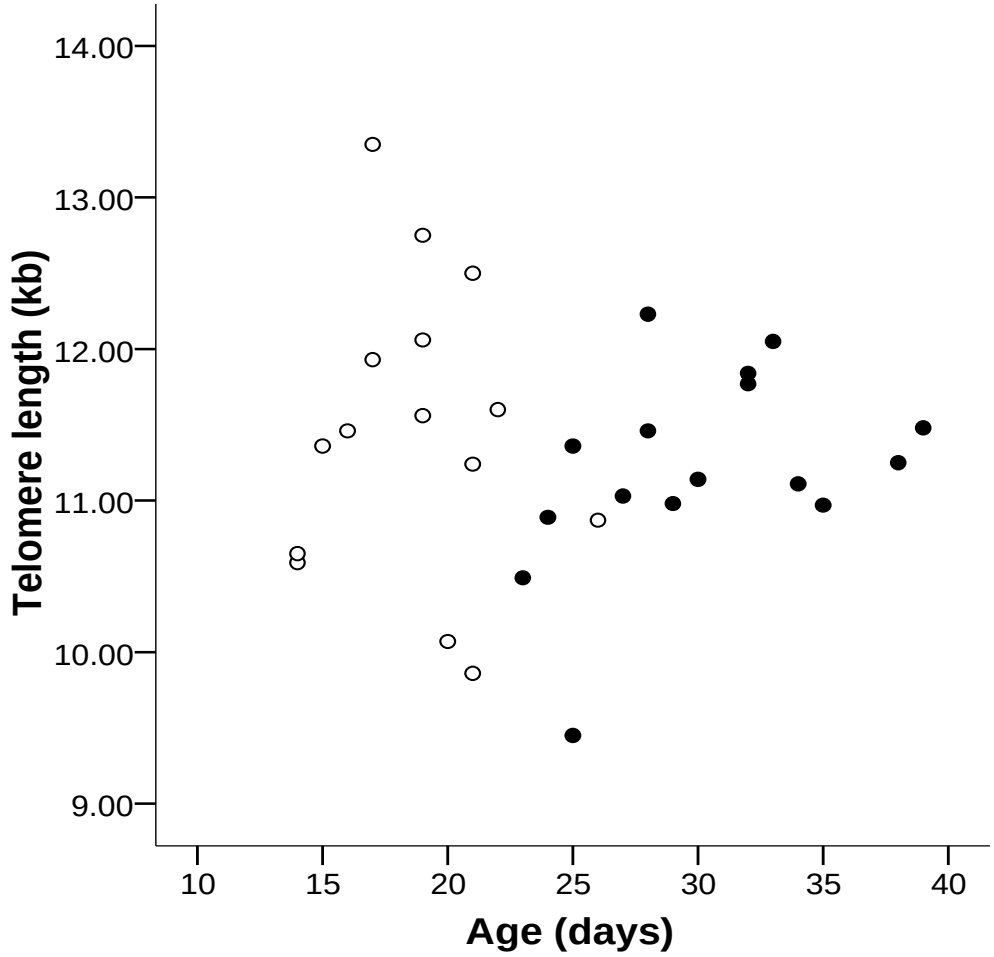


Figure 4.3: Telomere length in relation to age (n = 31). The data set shown was created by randomly including one of the two telomere length values we had from each individual, such that half of our values came from the 1st sampling period (open circles) and half from the 2nd period (closed circles). In a GLMM analysis, age had no relationship with telomere length.

Chapter 5: Telomere dynamics in relation to age and survival in the southern giant petrel *Macronectes giganteus* and the northern giant petrel *Macronectes halli*

Abstract

There are very few studies that examine telomere dynamics across the lifespan of a long-lived species. The few studies that have been carried out have shown that telomere length decreases with age, although not at a constant rate. In this study we examine the telomere dynamics of two species of long-lived seabird, the northern and southern giant petrels (*Macronectes* spp.). The petrel population we used has been studied for many decades by the British Antarctic Survey (BAS) and so the age of even very old individuals was known. In addition, BAS provided data on the survival of our study individuals in the 8 years after they were initially sampled. This allowed us to determine if there was a relationship between telomere length and survival, as has recently been found in other species. In both giant petrel species, telomeres were shorter in adults than chicks, but there was no trend for adult telomere length to decrease with age. Males had shorter telomeres than females in both species. In southern giant petrels, there was a significant relationship (independent of age and sex) between an individuals telomere length and whether it was still alive 8 years after it was initially sampled. This relationship was not present in northern giant petrels, possibly due to a smaller sample size. Our results thus support both the idea that most telomere loss occurs in young individuals and that telomere length may be an indicator of life expectancy.

Introduction

Very few studies have examined the change in telomere length across the lifespan of long-lived species. This is primarily because there are very few wild populations where the age of very old individuals is known. However, there are avian populations that have been studied for sufficient time that their age structure is well characterised. The few (cross-sectional) studies that have examined the telomere dynamics of such populations have found a general trend for telomeres to shorten with age (Haussmann *et al.* 2003; Hall *et al.* 2004; Juola *et al.* 2006). There is some variation within this trend. In the wandering albatross *Diomedea exulans* and European shag *Phalacrocorax aristotelis*, there is no trend for telomeres to shorten with age among adult individuals (Hall *et al.* 2004). This is not the case in adélie penguins *Pygoscelis adeliae*, common terns *Sterna hirundo*, tree swallows *Tachycineta bicolor* (Haussmann *et al.* 2003) or frigate birds *Fregata minor* (Juola *et al.* 2006). More exceptionally, in Leach's storm petrel *Oceanodroma leucorhoai* telomere length apparently increases with age (Haussmann *et al.* 2003), although this may be a consequence of differential survival in relation to telomere length (Haussmann & Mauck 2008b). It is also interesting to note that none of these studies have found a difference in telomere dynamics between the sexes; sex differences have been found in several mammal species (Benetos *et al.* 2001; Cherif *et al.* 2003; Nawrot *et al.* 2004).

Although age clearly explains some of the variation in telomere length among individuals, it does not explain all of it; in all of the species mentioned above, considerable variation in telomere length is seen among same-age individuals. Recent evidence has suggested that, rather than just being a function of age, telomere length is a measure of individual state. For example, in sand martins (*Riparia riparia*), individuals with longer telomeres have greater life-time reproductive success (Pauliny *et al.* 2006). Telomere length also seems to be a predictor of survival, even in young individuals who would not be undergoing senescence. Tree swallows with longer telomeres at one year old are more likely to survive until the next breeding season (Haussmann *et al.* 2005). It is not known if telomere length directly affects survival or if it correlates with other measures of individual state. Clearly though, the idea of telomere length as an indicator of individual state is an interesting one and deserving of study in other species.

In this chapter, we look at the relationship between telomere length, age and survival in two species of long-lived sea bird, the northern giant petrel *Macronectes halli* and the southern giant petrel *Macronectes giganteus*. The giant petrel population we have data from has been studied since the 1960's, so we have data from a wide range of known-age individuals. Information on the telomere dynamics of two more long-lived species is valuable in itself, given the rarity of such data. It is interesting to see how telomere length varies with age in this population and how telomere dynamics vary between the sexes, if at all. We were also provided with information on the survival of individuals for 8 years after sampling, allowing us to investigate the relationship between telomere length and survival in this population. This data set also provides an opportunity to examine what differences, if any, exist in the telomere dynamics of two very closely related species. Northern and southern giant petrels share very similar lifestyles and are phylogenetically very close. Indeed they were thought to be one species until very recently (Bourne & Warham 1966). If telomere length is primarily affected by genetics and/or lifestyle we would predict that northern and southern giant petrels would have very similar telomere dynamics.

Materials and methods

Study site and sampling

All individuals were sampled in 2000 at a breeding colony on Bird Island, South Georgia (54°00' N, 38°03' S). All blood samples and other field data were collected by Francis Daunt. Long term data on these birds was provided by Richard Phillips (BAS). Thirty-seven adult northern giant petrels and 47 adult southern giant petrels were sampled. Apart from one northern giant petrel, the age of these individuals was known as the result of a long term banding programme. Ages of these individuals ranged from 12 – 29 years in the northern giant petrels and 12 – 40 years old in the southern giant petrels. Age ranges were the same for both sexes. We also sampled 10 northern giant petrel chicks and 16 southern giant petrel chicks. Giant petrel's lay one egg each breeding season, so all chicks came from different nests. Blood was taken by superficial venipuncture of the brachial vein and stored in 90% ethanol at -20°C until DNA extraction. Sex of adult individuals was determined in the field based on body size (a method which has > 90% accuracy – Copello *et al.* 2006), while chick sex was determined using a PCR based method (Griffiths *et al.* 1998).

Survival data for adult individuals was collected in the 8 breeding seasons after initial sampling, based on the assumption that if an individual was not seen at the breeding ground it had not survived the winter. Giant petrels have a very high level of breeding site fidelity (Hunter 1984), so this seems to be a reasonable assumption; 51 of the 83 adults sampled (61.4%) died during this 8 year period (survival after 8 years: northern males: 10 dead, 11 alive; northern females: 6 dead, 9 alive; southern males: 18 dead, 9 alive; southern females: 17 dead, 3 alive). We did not possess any post-fledging data on the chicks sampled, so we were unable to include these individuals in the survival analysis.

Measurement of telomere restriction fragments (TRFs)

DNA was extracted from red blood cells, which are nucleated in avian species. Samples were digested with proteinase K before DNA extraction by a standard phenol-chloroform-ethanol-precipitation method. DNA was checked for degradation by 1% agarose gel electrophoresis. DNA was successfully extracted from all of the blood samples collected in the field.

Approximately 1µg of DNA from each sample was digested with the restriction enzymes *Hinf* I and *Rsa* I for 16h at 37°C. Digested DNA samples were separated on a non-denaturating 0.8% agarose gel at 150V for 3hrs. Two marker lanes (23.1 – 2.0Kb) were run on each gel. DNA was transferred from the gel to a nitro-cellulose Hybond N+ membrane (Amersham, UK) by Southern blot. The membrane was hybridised with a digoxigenin (DIG) labelled telomere probe $(TTAGGG)_7$ (Roche) for 3 hours at 42°C. A chemiluminescent detection system (Roche) followed by exposure to autoradiography film was used to visualize the TRFs. See chapter 2 for a more detailed description of the TRF protocol. Figure 5.1 shows a representative TRF gel. A clean DNA smear was observed in the majority of samples, suggesting that interstitial banding was not a significant problem.

Throughout this thesis telomere length has been estimated from TRF gels by calculating the mean TRF length. This is the 'standard' way of analysing TRF gels, but recently it has been suggested that other methods may be superior (Haussmann & Mauck 2008a). In particular, it has been suggested that the most important thing to consider when analysing TRF gels are the shortest telomere fragments in the smear. Although the mean TRF method does take into account the shortest telomeres (see chapter 2), we decided in this chapter to try another method of analysis in addition to the mean TRF, concentrating

just on the proportion of short telomeres in each TRF smear. For both methods, having scanned the images, the intensity of TRF smears at different molecular sizes was calculated using TotalLab software (Photoretix). The mean TRF length was calculated using the formula: mean TRF length = $\sum (OD_i) / \sum(OD_i / L_i)$ where OD_i is signal intensity and L_i is DNA size (Kb) at position $_i$. The background intensity was subtracted from signal intensity before each calculation. For the sake of clarity, the mean TRF length will be referred to as 'telomere length' for the rest of this chapter. We examined how the proportion of short telomere fragments varied between individuals by defining fragments between 5.0 - 2.3 kb in size to be 'short'. Inevitably, any attempt to separate 'short' telomeres from 'long' or 'medium' size telomeres will involve an arbitrary division; however, from examination of our gels we felt that making this division at 5 kb meant that only the shortest telomeres in the smear were being included in the analysis, while still ensuring that there was substantial variation between individuals (i.e. there were not many individuals with 0% 'short' telomeres). We calculated the signal intensity of these short fragments as a proportion of the total signal intensity between 23.1 – 2.3 kb. The background intensity was subtracted from signal intensity before each calculation. The lower size limit was set to 2.3 kb as no sample produced a smear containing telomere fragments shorter than this. The upper size limit was set at 23.1 kb because a constant field electrophoresis gel cannot resolve fragments larger than this. For the rest of this chapter, the proportion of telomere fragments between 5.0 – 2.3 kb in size will be referred to as 'the proportion of short telomeres'. All analysis was carried out blind with respect to species, age and sex.

To control for inter-gel variability, 90 of the 110 samples were run on two different gels. The average of these two values was used in our analysis. Measurements of the mean TRF length were highly repeatable (mean difference = 0.35 ± 0.03 kb, equivalent of 3.91% of the overall mean TRF of 8.70 kb; repeatability analysis: $r = 0.921$, $F_{89, 90} = 24.297$, $P < 0.001$). Measurements of the proportion of short telomeres were less repeatable (suggesting that this method of analysing TRF gels might be less reliable than the mean TRF method), but still consistent (mean difference = 3.82 ± 0.36 %, equivalent to 29.6% of the overall mean of 12.89%; repeatability analysis: $r = 0.739$, $F_{89, 90} = 6.674$, $P < 0.001$).

Statistical analysis

The relationship of telomere length with age and sex was examined using general linear models (GLM) containing age as a covariate, sex as a fixed factor and either mean TRF

length or the proportion of short telomeres as the dependent variable. The exact age of chicks was not known so they were all classified as being 0 years old. For this reason, when chicks were analysed separately from adults age was not included in the GLM. The relationship between telomere length and survival was examined using a GLM containing age, sex and survival (i.e. if an individual was alive or dead in the 2008 breeding season), with mean TRF length or the proportion of short telomeres as the dependent variable. Data on the proportion of short telomeres was not arc-sin transformed as it was already normally distributed (as no individual had a proportion of short telomeres below 2%). All means are quoted ± 1 standard error.

Results

Northern giant petrels

Northern giant petrels showed considerable inter-individual variation in telomere length (mean TRF = 8.78 ± 0.14 kb, coefficient of variation (CV) = 10.7%, n = 47). In a GLM model, age had a significant negative relationship with telomere length ($F_{1, 43}$ = 42.313, P < 0.001, slope = -0.073 ± 0.011, n = 46; fig. 5.2a). Sex had a marginally significant effect on telomere length ($F_{1, 43}$ = 3.842, P = 0.056, n = 46), with males tending to have shorter telomeres than females (mean difference = 0.413 ± 0.211 kb, 95% CI: 0.838, -0.012).

When only adult individuals were included in the GLM, age no longer had any effect on telomere length ($F_{1, 33}$ = 0.684, P = 0.414, slope = -0.014 ± 0.018, n = 36). In contrast, sex did have a significant effect on telomere length when only adults were included in the analysis, with males having shorter telomeres than females ($F_{1, 33}$ = 9.375, P = 0.004, n = 36, mean difference = 0.604 ± 0.197 kb; fig.5.3a). Telomere length still did not have a significant relationship with age if the sexes were considered separately (males only: $F_{1, 19}$ = 0.103, P = 0.752, n = 21; females only: $F_{1, 13}$ = 0.668, P = 0.428, n = 15). It was not possible to examine sex differences in the chicks, as our sample contained only 2 female northern giant petrel chicks.

Results were very similar when the difference in the proportion of short telomeres was examined (mean proportion of short telomeres = 12.12 ± 0.85 %, CV = 47.86%, n = 47). Older individuals had more short telomeres than young individuals ($F_{1, 43}$ = 28.602, P < 0.001, slope = 0.406 ± 0.076, n = 46; fig. 5.2b), although conversely there was no

significant difference between the sexes ($F_{1, 43}$ = 2.567, P = 0.116, n = 46). If only adults were included in the analysis, age was not related to the proportion of short telomeres ($F_{1, 33}$ = 0.312, P = 0.580, slope = 0.078 ± 0.139). This was still the case if males and females were analysed separately (males only: $F_{1, 19}$ = 0.168, P = 0.686; females only: $F_{1, 13}$ = 2.030, P = 0.178). Males were found to have a significantly larger proportion of short telomeres than females ($F_{1, 33}$ = 4.869, P = 0.034; mean difference = 3.46 ± 1.57 %).

In a GLM containing age, sex and survival (i.e. was an individual still alive in 2008), telomere length varied significantly between the sexes, but age and survival did not (table 5.1a). This was still true if age (as it was not significant) was removed from the model (table 5.1b) or if survival was included in a model on its own (t = 0.232, df = 34, P = 0.803). If the sexes were separated, survival was still not a significant factor, with or without age included in the model (P > 0.20 in all cases). If the proportion of short telomeres was used as the dependent variable, the results were the same (table 5.2a, b; survival in a model on its own: t = 0.168, df = 34, P = 0.867). Table 5.3 shows the average mean TRF length and the average proportion of short telomeres for individuals that were alive or dead in 2008, separated by sex. If the sexes were separated, survival was still not a significant factor, with or without age included in the model (P > 0.15 in all cases).

Southern giant petrels

Similar to the northern giant petrels, southern giant petrels showed a substantial amount of inter-individual variation in telomere length (mean TRF = 8.65 ± 0.15 kb, CV = 13.54%, n = 63). Older individuals had shorter telomere lengths than younger individuals ($F_{1, 60}$ = 54.550, P < 0.001, slope = -0.062 ± 0.008, n = 63; fig. 5.4a). Sex had no significant effect on telomere length ($F_{1, 60}$ = 3.119, P = 0.082, n = 63), but there was a trend for males to have shorter telomeres than females (mean difference = 0.378 ± 0.214 kb, 95% CI: 0.805, -0.050).

If only adult individuals were included in a GLM containing age and sex, age had no effect on telomere length (males and females: $F_{1, 44}$ = 2.029, P = 0.161, slope = 0.017 ± 0.012, n = 47; males only: $F_{1, 25}$ = 0.629, P = 0.435, n = 27; females only: $F_{1, 18}$ = 1.655, P = 0.215, n = 20). No significant difference between the sexes was seen in this model ($F_{1, 44}$ = 2.430, P = 0.126, n = 47); however if sex was included in a GLM on its own there was a significant difference between males and females ($F_{1, 45}$ = 4.971, P = 0.031, mean

difference = 0.428 ± 0.192 kb; fig. 5.3b). In southern giant petrel chicks, males had significantly shorter telomeres than females (t = 2.649, df = 14, P = 0.019, mean difference = 1.01 ± 0.380 kb, n = 16).

There was considerable inter-individual variation in the proportion of short telomeres in southern giant petrels (mean proportion of short telomeres = 13.47 ± 0.80 %, CV = 46.97%, n = 63). Older individuals had significantly more short telomeres than young individuals ($F_{1, 60}$ = 37.442, P < 0.001, slope = 0.298 ± 0.049; fig. 5.4b), although this was not true if only adults were examined (males and females: $F_{1, 44}$ = 0.140, P = 0.710, slope = 0.031 ± 0.082; males only: $F_{1, 25}$ = 0.003, P = 0.957; females only: $F_{1, 18}$ = 0.257, P = 0.618). Males had a higher proportion of short telomeres than females, both when adults and chicks were examined together and when adults and chicks were examined separately (chicks and adults: $F_{1, 60}$ = 4.486, P = 0.038, mean difference = 2.63 ± 1.24 %; adults: $F_{1, 44}$ = 6.774, P = 0.013, mean difference = 3.60 ± 1.38 %; chicks: t = 1.381, df = 14, P = 0.189, mean difference = 1.75 ± 1.27 %).

In a GLM containing age, sex and survival (i.e. was an individual still alive in 2008), sex had a significant effect on telomere length, while survival was very close to significant (table 5.4a). As age was not significant we removed it from the model; both sex and survival then had a significant relationship with telomere length (table 5.4b); individuals that survived had longer telomeres than those that did not (average difference = 0.47 ± 0.21 kb). There was no significant interaction between sex and survival (table 5.4c). If the proportion of short telomeres is used as the dependent variable in the GLMs then the results are similar; sex and survival have a significant relationship with the proportion of short telomeres, while age does not (table 5.5). Individuals that survived until 2008 had more short telomeres than individuals that did not survive (average difference = 3.36 ± 1.48 %). There was no interaction between sex and survival (P = 0.838). Table 5.6 shows the average mean TRF length and the average proportion of short telomeres for individuals that were alive or dead in 2008, separated by sex.

Difference between the species

When the TRF values for both species were compared (including species, sex and age), there was no significant difference in telomere length between northern and southern giant petrels ($F_{1, 105}$ = 0.113, P = 0.738, n = 109). This is still true if chicks and adults are

analysed separately (adults: $F_{1, 79} = 2.416$, P = 0.124, n = 83; chicks: $F_{1, 23} = 0.513$, P = 0.481, n = 26). There was also no difference between the species in terms of the proportion of short telomeres (adults and chicks: $F_{1, 105} = 0.343$, P = 0.560; adults: $F_{1, 79} = 3.182$, P = 0.078; chicks: $F_{1, 23} = 1.678$, P = 0.208).

Discussion

In both northern and southern giant petrels, adults had shorter telomeres than chicks (in terms of both the mean TRF length and the proportion of short telomeres). Conversely, there was no detectable decline in telomere length with age among adults. In this, giant petrels are similar to other long-lived avian species, such as the wandering albatross and the European shag (Hall *et al.* 2004), although this is not a universal pattern (Haussmann *et al.* 2003; Juola *et al.* 2006). Our results thus support the idea that most telomere loss occurs in young individuals, although we cannot say how much of this telomere loss occurs during the chick period and how much during the years after fledging before an individual returns to the breeding ground. Note also that in a cross-sectional study such as this one, a lack of detectable telomere loss with age does not mean no such telomere loss is occurring. A small trend for telomeres to shorten with age in adults could easily be obscured by the substantial amount of variation in telomere length between same age individuals. This is particularly true if individuals with longer telomeres have a better chance of surviving to older ages, as has been suggested in Leach's storm petrel (Haussmann & Mauck 2008b). Ideally, data on telomere loss would be collected longitudinally, following individuals throughout their lives. However, in very long-lived species like the giant petrels, such data are very difficult to collect (although such data has been gathered on the Alpine swift *Alpus melba* – P. Bize Pers. Comm.).

The effect of sex on telomere length varied depending on the species, the method used to analyse telomere length (mean TRF length or proportion of short telomeres) and if adults or chicks were analysed separately or together. However, when there was a sex difference, it was always the case that males had shorter telomeres than females. To our knowledge, this is the first time any sort of sex difference has been seen in a study examining a wide age range of individuals in an avian species, although males do have shorter telomeres than females in adult humans (Benetos *et al.* 2001; Nawrot *et al.* 2004) and rats (Cherif *et al.* 2003).

In previous chapters, we have shown that in chicks of the lesser black-backed gull and European shag, males have longer telomeres than females. Why is there a sex difference in the opposite direction in giant petrels? Possibly it could be due to the inclusion of adults in the analysis (only chicks were examined in the shags and gulls); in rats and humans, a sex difference is seen only in adults, not in infants (Cherif *et al.* 2003; Nawrot *et al.* 2004), suggesting that sex differences might change with age. Indeed in southern petrels, when the telomere length of chicks and adults were examined separately, there was a sex difference in chicks but not in adults (although this was not the case for the proportion of short telomeres). However, it is possible that there is some fundamental difference in the way sex effects telomere length in the gulls and shags and how it affects telomere length in the giant petrels. This difference could possibly be due to the degree of sexual dimorphism (or the degree of difference in the pattern of growth between the sexes). Unlike most other seabird species (such as lesser black-backed gulls or wandering albatrosses), giant petrels show considerable size differences between the sexes; indeed they have been described as the 'most sexually dimorphic of seabirds' (Croxall 1982). Female body mass is only 80% that of males (Hunter 1987) and there are differences in structural size beyond this; for example, head-bill length can be used to sex fledglings and adults (Gonzalez-Solis 2004; Copello *et al.* 2006). It is possible that this larger body size and the faster growth rates necessary to achieve it result in male giant petrels having shorter telomeres than females. This would be consistent with humans, where the larger sex has the shorter telomeres. However, the European shag shows a considerable amount of sexual dimorphism (Daunt *et al.* 2001) and yet males have longer telomeres than females (at least in chicks). There are also sex differences in diet in both giant petrel species, with females tending to have a more marine-based diet while males feed more on scavenged carrion (Hunter 1987; Gonzalez-Solis *et al.* 2000); however it is difficult to see what effect this could have on telomere dynamics.

There was no difference in telomere length between northern and southern giant petrels, nor was there any difference in the way age affected telomere length. There was some difference between the species in the way sex affected telomere length (e.g. there was no sex difference in mean TRF in adult southern petrels, there was in adult northern petrels), but the overall trend in both species was for males to have shorter telomeres (although we could not examine sex differences in northern petrel chicks). This lack of difference is perhaps not surprising given how closely related the species are in terms of

phylogeny, habitat, size and general lifestyle (Bourne & Warham 1966; Hunter 1987). What differences there are, such as differences in size, feeding habits and breeding periods, tend to be small. Indeed, differences in diet and body size are stronger between the sexes than between the species (Hunter 1987). It is possible that this could be a general trend across taxa; closely related species that possess very similar life-histories, will have similar telomere dynamics.

Previous studies have found a relationship between telomere length and survival in several avian species (Haussmann *et al.* 2005; Pauliny *et al.* 2006), humans (Cawthon *et al.* 2003) and *C.elegans* (Joeng *et al.* 2004). Our data provides some evidence to support the idea of a similar relationship in southern giant petrels. In southern giant petrels, individuals with shorter telomeres (in terms of mean TRF length or proportion of short telomeres) were less likely to survive over the 8 years after we sampled them (independent of age and sex). There was no relationship between telomere length and survival in the northern giant petrels. This could be due to the incomplete nature of the data as many of the individuals we sampled were still alive. Indeed, more southern petrels from our sample had died during this 8 year period than northern petrels (74.5 % of southern petrels had died, 44.4 % of northern petrels had died) and we had a larger initial sample of southern petrels. Thus, it is possible that we did not see a relationship between telomere length and survival in northern petrels because of this smaller sample size; perhaps when we know the lifespan of all the individuals in our study such a relationship will become apparent. Given that telomere length did not shorten with age in adult southern petrels, the relationship between telomere length and survival is not just a case of older individuals with shorter telomeres dying, as has been seen in humans (Cawthon *et al.* 2003). Instead, the link between telomere length and survival must be due to a mechanism other than telomere dependent senescence, as it was in tree swallows (Haussmann *et al.* 2005). Whether telomere length is causally linked to survival in southern petrels, or merely correlates with it we cannot say, but certainly our results support the idea that telomere length could be an indicator of individual quality.

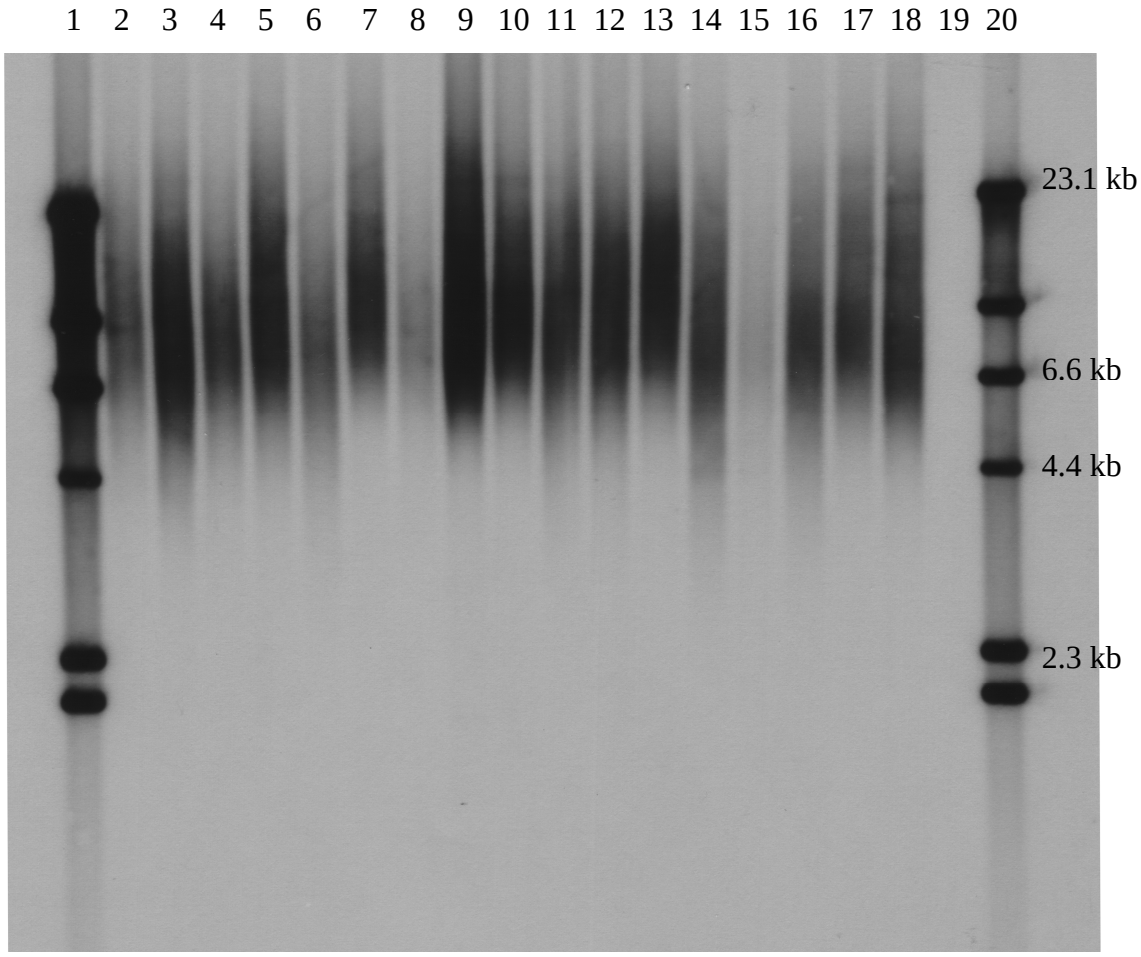


Figure 5.1: Example of a petrel TRF gel. Lanes 1 and 20 contain a size marker, lanes 2 - 18 contain a mixture of northern and southern giant petrel adults and chicks (lane 19 is blank).

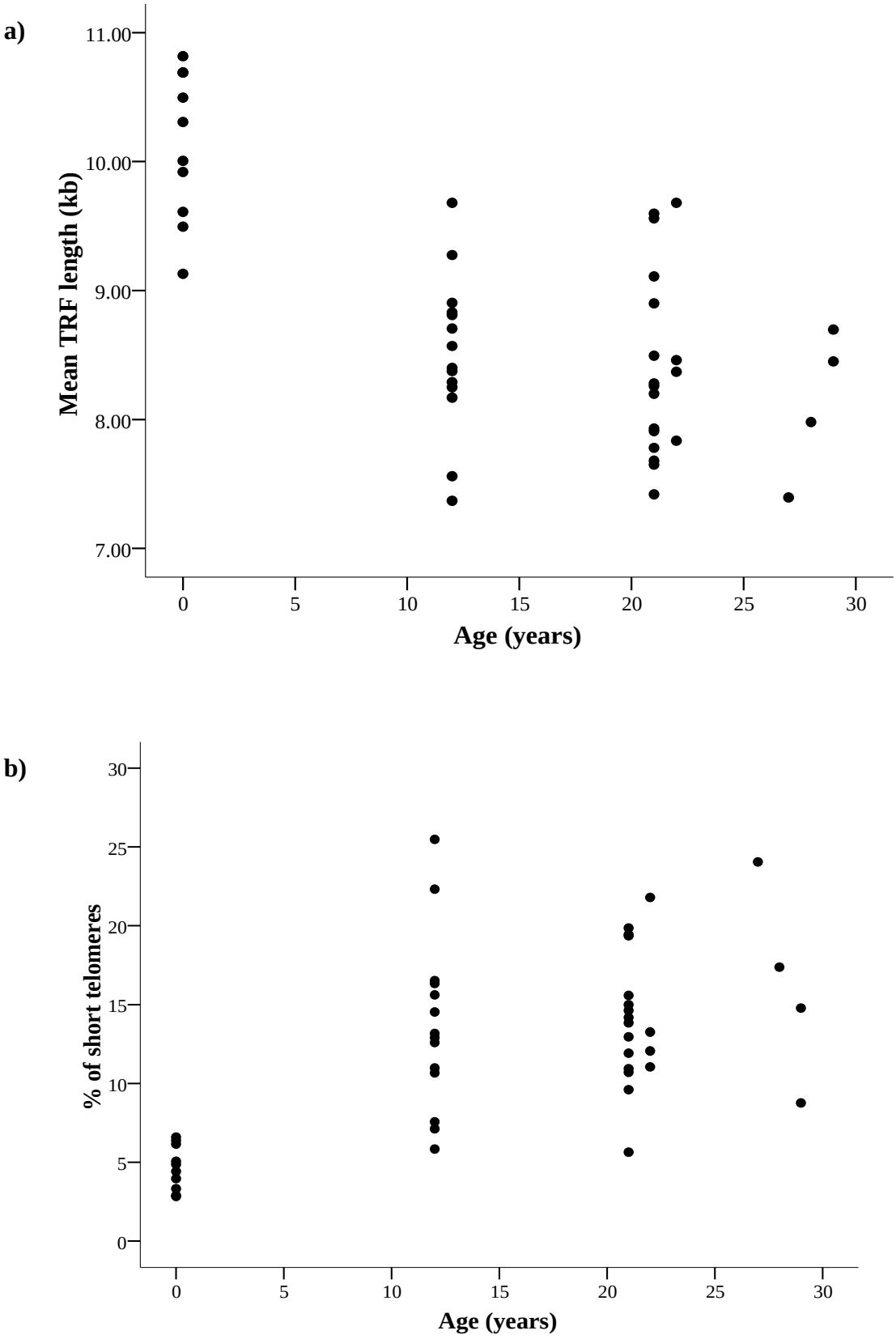


Figure 5.2: Age in relation to a) mean TRF length and b) proportion of telomere fragments shorter than 5 kb, in northern giant petrels (n = 46).

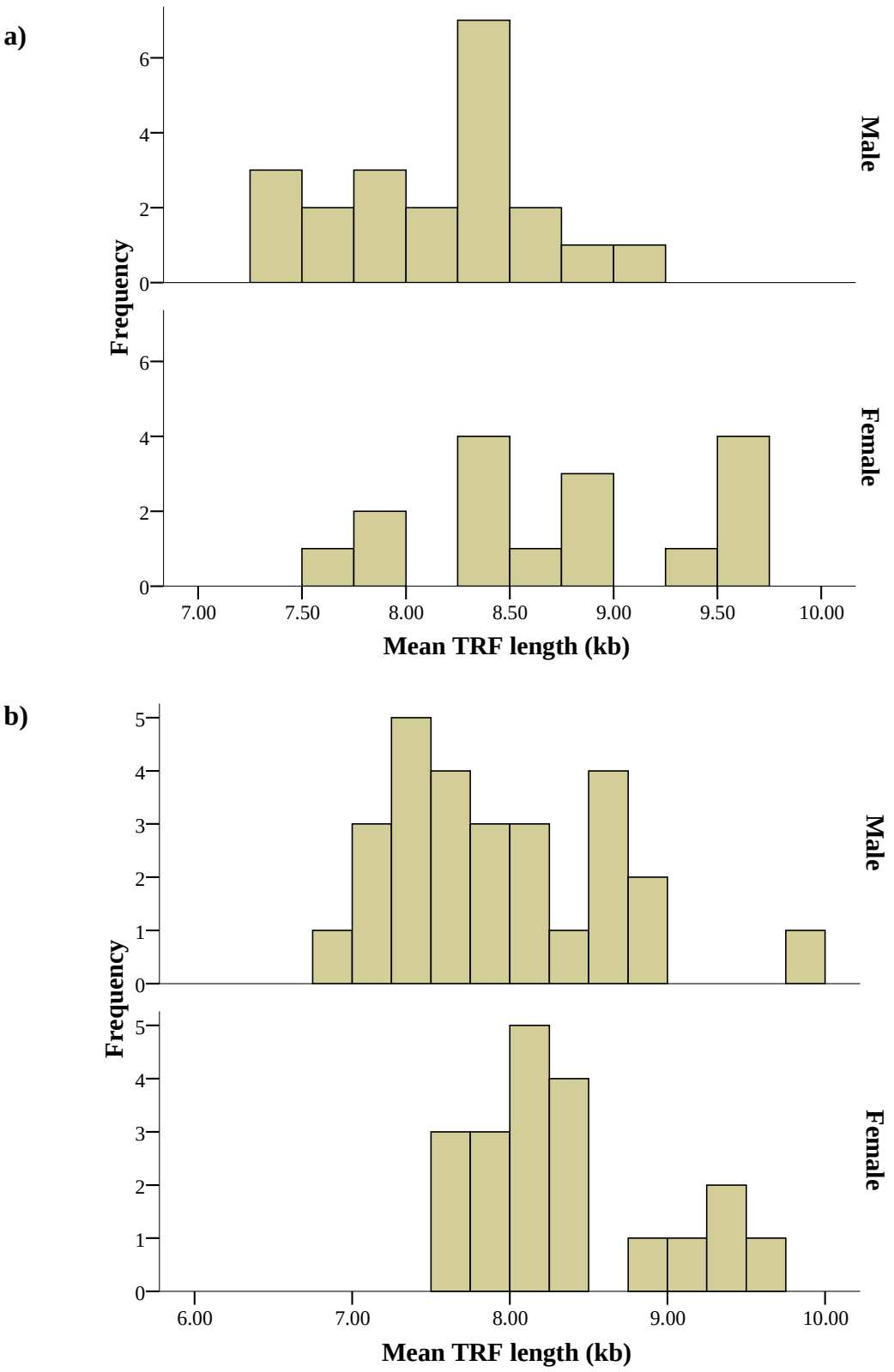


Fig. 5.3: Distribution of adult mean TRF lengths for a) northern giant petrels (n = 36), and b) southern giant petrels (n = 47), separated by sex. In both species, females tended to have significantly longer telomeres than males.

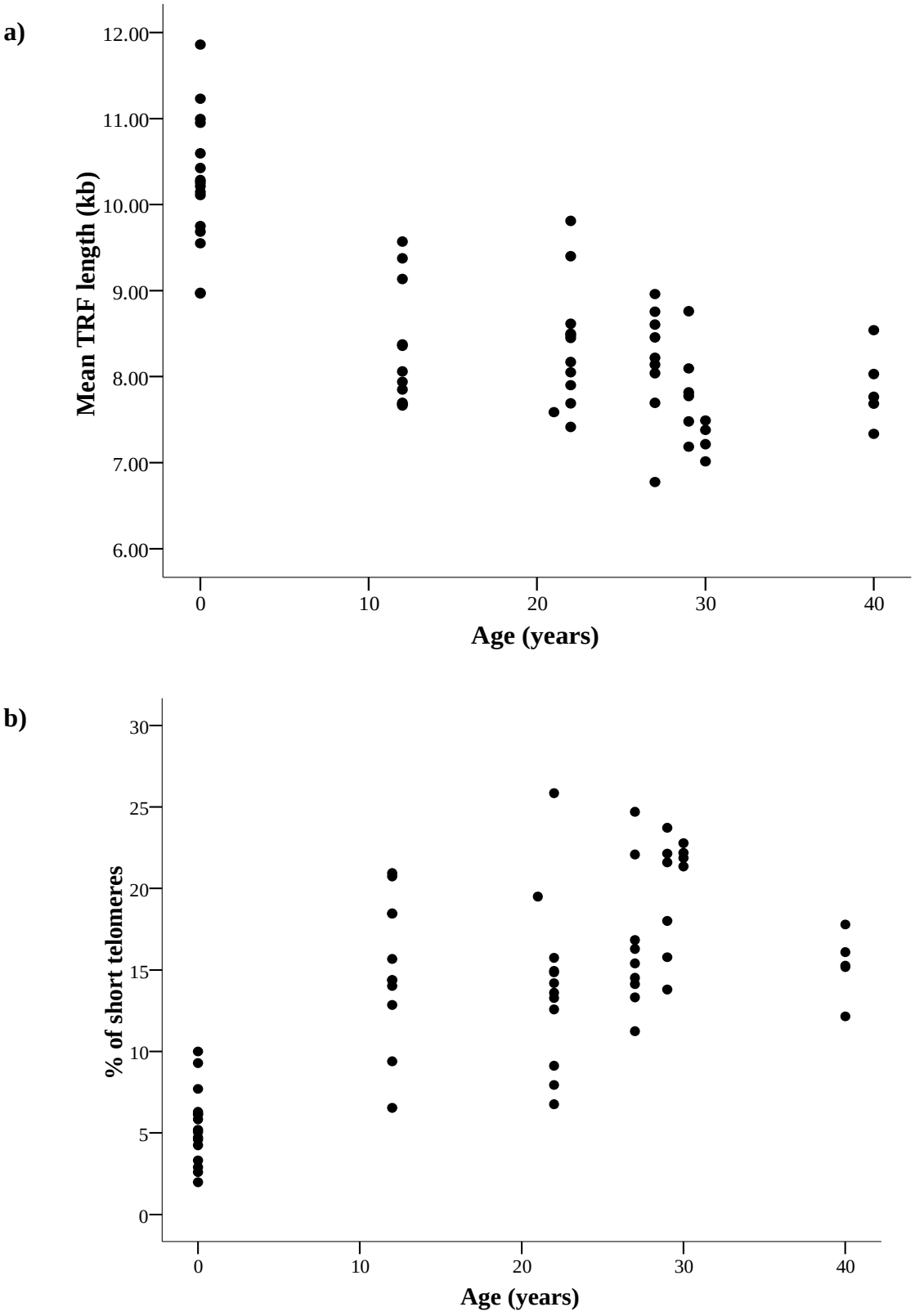


Fig. 5.4: Age in relation to a) mean TRF length and b) proportion of telomere fragments shorter than 5 kb, in southern giant petrels (n = 63).

Table 5.1: GLM's of telomere length in relation to a) survival (i.e. was an individual still alive in 2008), sex and age and b) survival and sex, in adult northern giant petrels (n = 36).

a)

Dependent Variable: Mean TRF length

Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Survival	.052	1	.052	.148	.703
Sex	3.227	1	3.227	9.243	.005
Age	.283	1	.283	.812	.374
Error	11.171	32	.349		

b)

Dependent Variable: Mean TRF length

Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Survival	.001	1	.001	.002	.961
Sex	3.242	1	3.242	9.341	.004
Error	11.454	33	.347		

Table 5.2: GLM's of the proportion of short telomeres in relation to a) survival (i.e. did an individual survive until 2008), age and sex and b) sex and survival, in adult northern giant petrels (n = 36).

a)

Dependent Variable: % of telomere fragments below 5kb

Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Survival	1.809	1	1.809	.082	.776
Age	8.498	1	8.498	.386	.539
Sex	105.614	1	105.614	4.793	.036
Error	705.112	32	22.035		

b)

Dependent Variable: % of telomere fragments below 5kb

Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Survival	.002	1	.002	.000	.992
Sex	106.107	1	106.107	4.907	.034
Error	713.610	33	21.625		

Table 5.3: Average telomere length and mean proportion of short telomeres for adult northern giant petrels, separated first by sex and then by whether an individual was dead or alive in the 2008 breeding season. Means are quoted ± 1 s.e.

	Male		Female	
	Dead (n = 10)	**Alive (n = 11)**	**Dead (n = 6)**	**Alive (n = 9)**
Telomere length	8.31 ± 0.16 kb	8.02 ± 0.13 kb	8.50 ± 0.30kb	8.95 ± 0.21 kb
% of short telomeres	14.28 ± 1.26 %	16.76 ± 1.35 %	14.28 ± 1.63 %	10.62 ± 1.61 %

Table 5.4: GLM's of telomere length in relation to survival (i.e. was an individual still alive in 2008), sex and age in adult southern giant petrels (n = 47). Age was not close to significant in model A and so was removed from model B. Model C shows that there is no interaction between sex and survival.

a)

Dependent Variable: Mean TRF length

Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Survival	1.332	1	1.332	3.399	.072
Sex	1.700	1	1.700	4.338	.043
Age	.301	1	.301	.769	.386
Error	16.853	43	.392		

b)

Dependent Variable: Mean TRF length

Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Survival	1.870	1	1.870	4.795	.034
Sex	2.898	1	2.898	7.432	.009
Error	17.154	44	.390		

c)

Dependent Variable: Mean TRF length

Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Survival	1.317	1	1.317	3.314	.076
Sex	1.494	1	1.494	3.759	.059
Survival – Sex Interaction	.066	1	.066	.165	.687
Error	17.089	43	.397		

Table 5.5: GLM of the proportion of short telomeres in relation to survival (i.e. did an individual survive until 2008), age and sex in adult southern giant petrels (n = 47).

Dependent Variable: % of telomere fragments below 5kb

Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Survival	88.418	1	88.418	5.112	.029
Sex	185.774	1	185.774	10.741	.002
Age	1.257	1	1.257	.073	.789
Error	743.698	43	17.295		

Table 5.6: Average telomere length and mean proportion of short telomeres for adult southern giant petrels, separated first by sex and then by whether an individual was dead or alive in the 2008 breeding season. Means are quoted ± 1 s.e.

	Male		Female	
	Dead (n = 18)	**Alive (n = 9)**	**Dead (n = 17)**	**Alive (n = 3)**
Telomere length	7.75 ± 0.14 kb	8.27 ± 0.25 kb	8.30 ± 0.15 kb	8.64 ± 0.39 kb
% of short telomeres	18.85 ± 0.91 %	15.80 ± 1.62 %	14.60 ± 1.00 %	10.91 ± 2.20 %

Chapter 6: Measurement of telomeres in the blue-footed booby *Sula nebouxii*

Abstract

Recently there has been much debate about the best way to measure telomere lengths; using the 'traditional' telomere restriction fragment (TRF) length analysis or using a qPCR based method. More generally, as an increasing number of species are utilised in studies of telomere dynamics, the question arises of how well such standardised methods for measuring telomeres can be applied across taxa. These issues were examined in relation to work done on the telomere dynamics of the blue-footed booby *Sula nebouxii*. TRF analysis could not be successfully applied to blue-footed boobies because of the presence of substantial banding in the TRF smear. Using a qPCR based protocol did produce usable measurements of relative telomere length. However, there was a suggestion that the PCR was not amplifying one clear product, as it should, casting doubt on the reliability of the results produced. It is possible that these problems occurred because blue-footed boobies have a particularly large amount of interstitial telomeric DNA, although a more detailed analysis of boobie telomeres would be necessary to determine this. These findings demonstrate that the standard protocols for measuring telomeres can not necessarily be applied to every new species whose telomere dynamics are studied.

Introduction

Several different protocols have been developed for the measurement of telomeres. The 'traditional' method is telomere restriction fragment (TRF) analysis (Harley *et al.* 1990). This is the method that has been used throughout this thesis. However, a method of measuring telomeres using quantitative PCR (qPCR) has been suggested as superior to the TRF method, as it requires both less DNA and less time than the TRF method (Cawthon 2002; see chapter 1 for a more detailed description of the costs and benefits of the two methods). Criscuolo *et al.* (2008 – see appendix) have recently shown that the qPCR method, initially developed for the measurement of human telomeres, can also be applied to avian species and that the results obtained correlate with those produced by the TRF method. Researchers planning to carry out research on telomere lengths thus have to decide on which method is the best to use.

Related to the debate on how best to measure telomeres, is the question of whether these standardised protocols will necessarily work with every new species whose telomere dynamics are studied. The general assumption has been that the methods that have been developed to measure telomeres will be applicable across taxa. However this assumption might just reflect that these are methods originally developed by researchers working on a limited set of species (humans and a few laboratory species). Alternatively, given that telomere structure is so conserved across taxa, we might expect telomere measurement protocols to be easily transferable across species; this has been the case in the species that have been studied so far. However, as ecologists utilise telomere measurements in an increasing variety of species, it is important to outline the potential problems that might occur when measuring telomeres in a new species. This chapter explores some of these issues in relation to work we did on the telomere dynamics of the blue-footed booby *Sula nebouxii*. We encountered various problems in adapting standard protocols for measuring telomeres (both TRF and qPCR) to this species. We outline these problems and suggest what they might mean in relation to the study of telomere dynamics in novel species.

Materials and methods

Study site and sampling

Field work was carried out at a breeding colony on Isla Isabel, off the Pacific coast of Mexico (21°52'N, 105°54'W). All blood samples and field data were collected by Roxana

Torres. Sixty-seven adult blue-footed boobies were sampled, ranging in age from 4 – 19 years old (49 males, 22 female). Four chicks were also sampled. Blood was taken from all individuals by superficial venipuncture of the brachial vein and stored in 2% EDTA, initially at 4°C at the field site but at -20°C upon arrival at the University of Glasgow (which was within two weeks). Adult sex was determined from physical characteristics (body size, foot colour and eye colour are all consistently different between males and females, allowing reliable sexing – Nelson 1978) while chick sex was determined using a PCR based method (Griffiths *et al.* 1998). Body mass was also measured in 25 of the adult individuals.

Measurement of TRFs

DNA was extracted from red blood cells, which are nucleated in avian species, using a method adapted from Kanai *et al.* (1994). Samples were digested with proteinase K before extraction by a chloroform-ethanol-precipitation method. DNA was checked for degradation by 1% gel electrophoresis (fig. 6.1).

Approximately 1µg of DNA from each sample was digested with the restriction enzymes *Hinf*I and *Rsa*I for 16h at 37°C. Digested DNA samples were separated on a non-denaturating 0.8% agarose gel at 150V for 3hrs. Two marker lanes (23.1 – 2.0Kb) were run on each gel. DNA was transferred from the gel to a nitro-cellulose Hybond N+ membrane (Amersham, UK) by Southern blot. The membrane was hybridised with a digoxigenin (DIG) labelled telomere probe $(TTAGGG)_7$ (Roche) for 3 hours at 42°C. A chemiluminescent detection system (Roche) followed by exposure to autoradiography film was used to visualize the TRFs. See chapter 2 for a more detailed description of the TRF protocol.

Figure 6.2 shows a representative TRF gel produced using this method. The lack of a clear smear indicated that blue-footed boobie TRFs could not be successfully analysed using this method; the banding would make it impossible to accurately estimate mean TRF size. The same problem had been encountered when measuring TRFs from the zebra finch *Taeniopygia guttata* (pers. comm. E.A. Gault). In that case, the problem was solved by extracting the DNA using the DNeasy Blood and Tissue Kit (Qiagen). Although the DNA we had extracted by chloroform-ethanol-precipitation did not appear to be degraded (fig. 6.1), the DNA was re-extracted using the DNeasy kit, following the manufacturer's

protocol. TRFs were then re-measured as previously. However, the TRF smears still exhibited substantial banding (fig. 6.3)

Standard constant-field gel electrophoresis is unable to resolve telomere fragments larger than 23 kb; bigger fragments than this will move through the gel in a size independent manner. Utilising pulse-field gel electrophoresis (PFGE) removes this limitation. PFGE differs to standard gel electrophoresis in that, rather than a constant voltage running in only one direction, the voltage is periodically switched between three different directions (one running through the gels central axis and two running at 120° either side of the gel). The pulses of voltage in each direction are of equal time which results in a net forward migration of the DNA fragments. Larger fragments of DNA are slower to react to a change in voltage direction and so will migrate down the gel at a slower pace. PFGE thus allows even very large DNA fragments to be separated by size. We felt that modifying our TRF technique to use PFGE might solve the problem of the banding. DNA was digested overnight at 37°C using three restriction enzymes: *Hinf* I, *Hind* III and *Msp* I. Digested samples were run on a 0.8% agarose gel at 3.5 V/cm for 24 hours (initial switch time: 0.5 seconds, final switch time: 7 seconds). Two different markers were run on each gel (48.0 – 8.0 kb and 23.1 – 2.0 kb). Following electrophoresis, gels were hybridised overnight at 37°C with a ^{32}P γ-ATP labelled telomeric probe $(C_2TA_2)_4$. Visualization of the TRFs was carried out in the same way as before. Figure 6.4 shows a representative blue-footed boobie pulse-field TRF gel. The banding was still present, suggesting that the problem was not being caused by the use of constant-field gel electrophoresis.

Measurement of telomeres using qPCR

Given the failure of the TRF assay to provide usable data in the case of blue-footed boobies, we decided to use an alternative, qPCR based, method of measuring telomeres. A protocol to measure telomeres using qPCR was first described by Cawthon (2002) for use on human telomeres, and was recently applied to the measurement of avian telomeres (Criscuolo *et al.* 2008). The qPCR assay works by calculating the number of PCR cycles it takes for the TTAGGG sequence in a given DNA sample to accumulate enough products to pass a set threshold; this is known as its C_t value. Samples with small C_t values have more of the TTAGGG sequence than samples with larger C_t values, and so must have longer telomeres. To control for variations in initial DNA quantity, the C_t value of a single

copy gene is also calculated and the ratio of these two values is used to calculate relative telomere lengths.

PCRs were performed in a MX3000P® QPCR system (Strategene). Telomere primers used in the blue-footed boobie assay were: Tel1b (5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3') and Tel2b (5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'). The single copy control gene used was glyceraldehyde-3-phosphatase dehydrogenase (GAPDH). This gene was amplified using the following primers: GAPDH-F (5'-AACCAGCCAAGTACGATGACAT–3') and GAPDH-R (5'-CCATCAGCAGCAGCCTTCA -3'). These primers are specific to the GAPDH gene in the zebra finch, but also amplify the blue-footed boobie GAPDH gene. All primers were supplied by VH Bio Ltd. qPCR for both telomeres and GAPDH was done using an initial DNA quantity of 20ng per reaction. The tel1b and tel2b primers were used at a concentration of 100 nM, while the GAPDH primers were used at a concentration of 300 nM. In both cases the final volume in each reaction was 25µl, containing 12.5µl of Brilliant®SYBR®Green QPCR Master Mix (Stratagene). Telomere PCR conditions were 10 min at 95°C followed by 30 cycles of 1 min at 56°C and 1 min at 95°C. Conditions for GAPDH PCR were 10 min at 95°C, followed by 45 cycles of 1 min at 60°C and 1 min at 95°C. Amplification of telomeres and GAPDH were performed on different plates, due to the differing conditions of the two reactions (comparison between them was still possible as the same standards were run on both plates – see below).

Each 96-well plate contained a serial dilution (40ng, 20ng, 10ng, 5ng, 2.5ng of DNA per well), run in triplicate, of DNA from the same reference boobie sample. This was used to generate a reference curve to control for the amplifying efficiency of the qPCR (accepted range 100 ± 15 %, Stratagene). In addition, a 'golden sample' (DNA from one boobie sample) was run in triplicate on every plate. This sample served two purposes. Firstly it was used to determine the threshold C_t value; this was set at the point where amplification of the product in this sample was occurring at an exponential rate. Secondly, the golden sample was used as a reference to calculate the relative telomere values of the other samples: relative telomere length of a sample (T/S ratio) = $2^{(\Delta Ct \text{ of control sample} - \Delta Ct \text{ of reference sample})}$, where $\Delta C_t = C_t$ telomere – C_t control gene. The T/S ratio of the control

sample was always 1.0; the T/S ratio of an individual with longer telomeres than the reference individual was < 1.0, while the T/S ratio of an individual with telomeres shorter than the reference individual was > 1.0. The two C_t values of the golden sample always varied slightly from plate to plate due to variations in the PCR reaction; values for the telomere C_t varied from 14.38 – 15.25 (mean = 14.66) and for the GAPDH C_t from 30.07 – 30.92 (mean = 30.46). We felt that this represented an acceptable level of intra-plate variation (within 1 C_t). Two sets of plates were rejected from the analysis as either the telomere or GAPDH C_t value of the golden sample were too far outside of this range (one with a telomere C_t value of 15.72, another with a GAPDH Ct value of 31.61). The samples on these plates were re-run.

On both telomere and GAPDH plates, all samples were run in triplicate. C_t values were highly repeatable (C_t^{GAPDH}: repeatability analysis - $r = 0.802$, $F_{128, 242} = 12.435$, $P < 0.001$, mean (± 1 s.e.) coefficient of variation (CV) = 0.84 ± 0.05 %; $C_t^{telomere}$: repeatability analysis - $r = 0.874$, $F_{128, 241} = 20.787$, $P < 0.001$, mean CV = 1.72 ± 0.09 %). This level of intra-plate repeatability in the blue-footed boobie assay compares well with intra-plate repeatability in other avian species (Criscuolo *et al.* 2008). In addition, the T/S ratio of 44 of the samples was recalculated on a second plate (again, each sample on a plate was run in triplicate) while a further 7 samples had their T/S ratios recalculated on two more plates. The T/S ratios had a fairly high repeatability between plates (repeatability analysis: $r = 0.670$, $F_{50, 58} = 5.371$, $P < 0.001$; mean difference in T/S ratio between plates run twice = 0.40 ± 0.06).

Although C_t values were repeatable, and the amplifying efficiency of the qPCR was within an acceptable range, other diagnostics of how well the assay was working were not so favourable. This assay utilises Brilliant®SYBR®Green to detect the PCR product being amplified. Brilliant®SYBR®Green will detect any double stranded DNA product amplified by the PCR reaction, so an important assumption made with this assay is that no product other than GAPDH/telomere DNA is being amplified; if an additional product was being amplified Ct values would be artificially lowered. To control for this possibility, a dissociation curve was produced at the end of each PCR run by measuring the temperature at which the PCR product melted. If only one product was amplified by the reaction (as should be the case) then only one peak would be present in the resultant dissociation curve.

This was the case for our GAPDH assays (fig. 6.5a). However, the dissociation curve produced for our telomere assays suggested the possibility that another product was being amplified in addition to TTAGGG repeats (fig. 6.5b). To further investigate this problem we ran out a small amount of the PCR product from some of our telomere assays on an electrophoresis gel, along with the product produced by the same assay carried out on DNA from Alpine swifts *Apus melba* (which have already been shown to work correctly with the qPCR assay – Criscuolo *et al.* 2008). The gel (fig. 6.6) showed that there was no specific additional product, but that the product that was being amplified was more diffuse than the amplified product from the Alpine swift assay. The dissociation curve was the same for every boobie sample we ran through the assay. This means that any change in telomere C_t values caused by the lack of one clear amplification product will be the same for each individual; relative telomere lengths (which are what this assay provides) will not be affected. Therefore it is potentially reasonable to suggest that the data gathered is usable, albeit while taking into account the possible flaws in the qPCR assay when applied to blue-footed boobies.

Results

There was significant variation in T/S ratio among individuals in our sample (mean T/S ratio = 1.29 ± 0.058, CV = 37.98%). In a GLM containing age and sex, there was no trend for telomere length to decrease with age ($F_{1, 68}$ = 0.108, P = 0.744, n = 71; fig. 6.7). Similarly, T/S ratio did not significantly differ between the sexes ($F_{1, 68}$ = 2.823, P = 0.097, n = 71). When body mass (which we only had a measure of for 25 of our individuals, all of whom were males) was added to the GLM, age was still not significant ($F_{1, 22}$ = 2.418, P = 0.134) and body mass also showed no correlation with T/S ratio ($F_{1, 22}$ = 0.055, P = 0.817, n = 25).

Discussion

In the three other avian species whose telomere dynamics have been studied in this thesis, measurement of telomere lengths was very straightforward, with the standard protocol for measuring TRF lengths (Harley *et al.* 1990) producing good results. In the case of blue-footed boobies, measurement of TRFs proved impossible due to the presence of substantial banding in the TRF smears. Similarly, although we were able to produce measurements of relative telomere lengths using the qPCR assay, the dissociation curves for the

amplification of telomeric DNA suggests the results may be unreliable. This contrasts with the relative ease with which the basic telomere qPCR protocol (Cawthon 2002) has been adapted to other avian species (Criscuolo *et al.* 2008). Why do blue-footed boobies appear to differ so much from other avian species?

It is theoretically possible that the sequence of telomeric DNA in blue-footed boobies differs to that in other avian species and that this is the cause of the problems encountered when measuring their telomeres. However, given the very high conservation of the TTAGGG repeat among those vertebrates whose telomeric regions have been sequenced (Meyne *et al.* 1989; Henderson 1995), this is highly unlikely to be the case. A more likely explanation is that blue-footed boobies may have substantially more interstitial telomeric DNA than most avian species. Interstitial telomeric sequences seem to occur in all avian species (Venkatesan & Price 1998) and the qPCR assay will amplify DNA from these regions in addition to DNA from the telomeres themselves (Nakagawa *et al.* 2004). If blue-footed boobies have a particularly high proportion of telomeric DNA in interstitial regions than this could cause the lack of one clearly defined product being amplified during the qPCR (fig. 6.6). The presence of interstitial telomeric DNA is not normally a problem when using the TRF method (Nakagawa *et al.* 2004), as the amount of such DNA is too small to be detected. However, if boobies have a particularly large amount of interstitial DNA, then this could be detected in the TRFs; the bands seen in the boobie TRF smears might thus be caused by interstitial telomeric DNA. Obviously, this is just speculation; there is no evidence that boobies have more interstitial telomeric DNA then other avian species. To determine if this was the case we would need to analyse individual boobie telomeres, using a technique such as Q-FISH (Zijlmans *et al.* 1997; see chapter 1). Such work is outside the scope of this study.

It is possible that our qPCR data, despite its flaws, still provides an accurate measurement of relative telomere lengths, but there is inconclusive support for this idea. For example, we found no relationship between age and telomere length. Had we found a negative relationship between telomere length and age (the relationship generally seen in most species) one might have said that this gives us greater confidence in our results. However, given that all but 4 of the individuals in our sample were adults, it might be suggested that a lack of relationship between age and telomere length is unsurprising, as this has been seen in other species (e.g. Hall *et al.* 2004). More generally, it is notable that,

even with all the problems we had applying the qPCR protocol to blue-footed boobies, we still produced potentially usable results, which was not the case with the TRF method. It is also notable that the same problems seem to have affected both of the two main methods used to measure telomeres (TRF and qPCR). This suggests that if a problem is encountered using one method, then switching to the other method will not necessarily solve the problem. Paradoxically however, this might provide further evidence that the two methods are measuring essentially the same thing (as suggested by Criscuolo *et al.* 2008) and so results gathered using one method can be compared to results obtained using the other method with confidence.

More generally, our experience with the blue-footed booby demonstrates that standard protocols for measuring telomeres will not necessarily work smoothly with every new species they are applied to. However, the fact that, to our knowledge, blue-footed boobies are the first species in which such problems have been encountered suggests that such instances will be rare. Despite this, it might still be wise for researchers planning to carry out telomere length measurements in a new species to carry out pilot studies to ensure that telomeres are easily measurable, before investing a large amount of time and effort in collecting many blood or tissue samples.

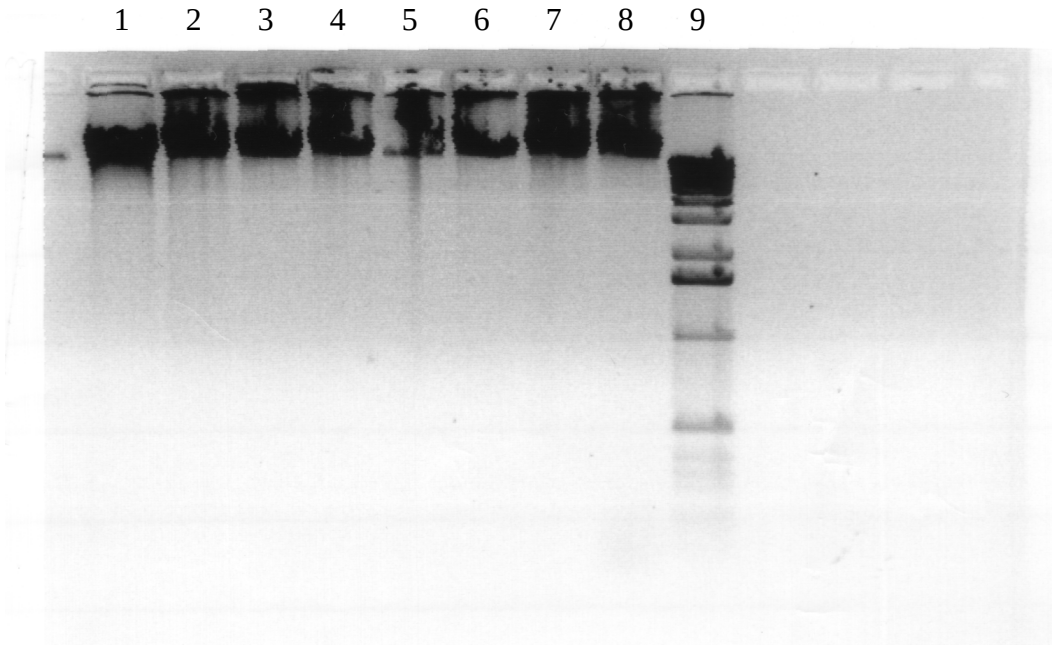


Figure 6.1: Genomic DNA from blue-footed boobies run on an electrophoresis gel. Lanes 1-8 contain boobie DNA, lane 9 contains a size marker lane. The boobie DNA consists of a single tight band, showing that the DNA is mostly intact, with little degradation.

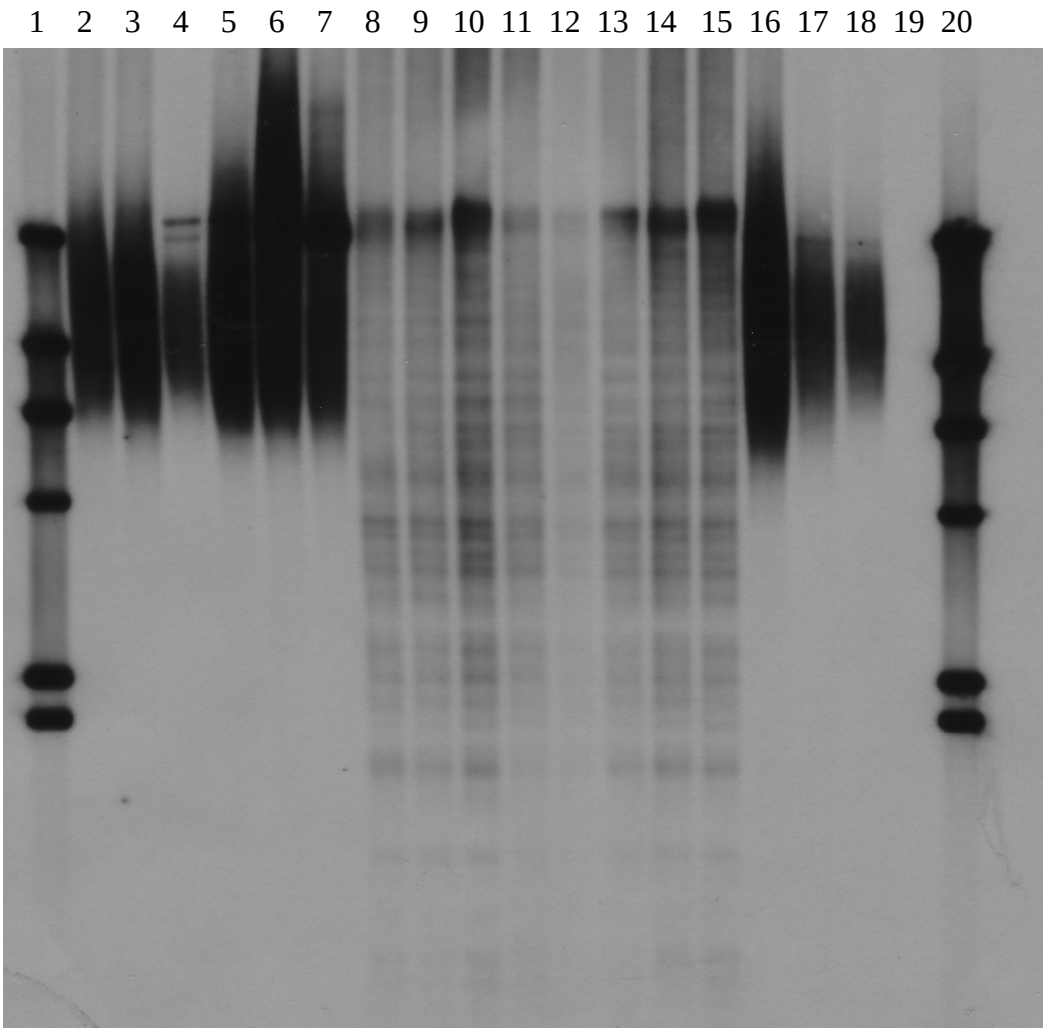


Figure 6.2: Representative blue-footed boobie TRF gel produced by constant-field gel electrophoresis, with DNA extracted by a chloroform-ethanol-precipitation method. Lanes 8-15 contain blue-footed boobie samples, with substantial banding present in the TRF smear. For comparison, lanes 2-7 and 16-18 contain TRF smears from European shag DNA; these smears are clear with no obvious banding. Lanes 1 and 20 contain markers, lane 19 is blank.

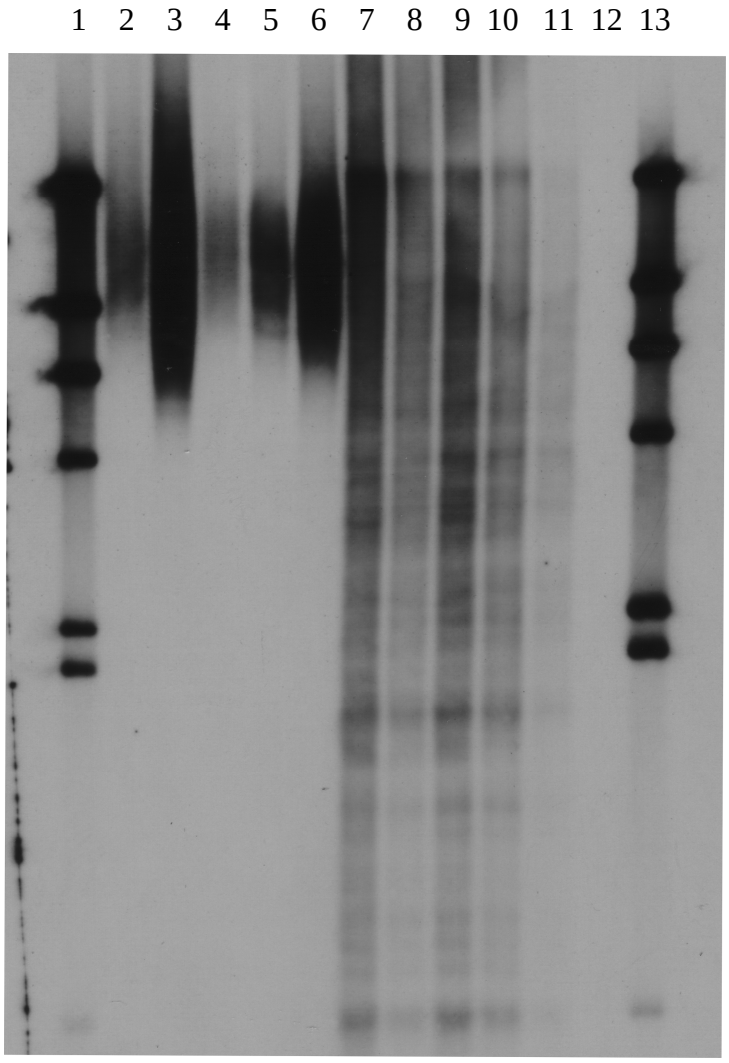


Figure 6.3: Representative blue-footed boobie TRF gel produced by constant-field gel electrophoresis, with DNA extracted using a DNeasy Blood and Tissue Kit. Lanes 7-11 contain blue-footed boobie samples, with substantial banding present in the TRF smear. For comparison, lanes 2-6 contain TRF smears from European shag DNA; these smears are clear with no obvious banding. Lanes 1 and 13 contain markers, lane 12 is blank.

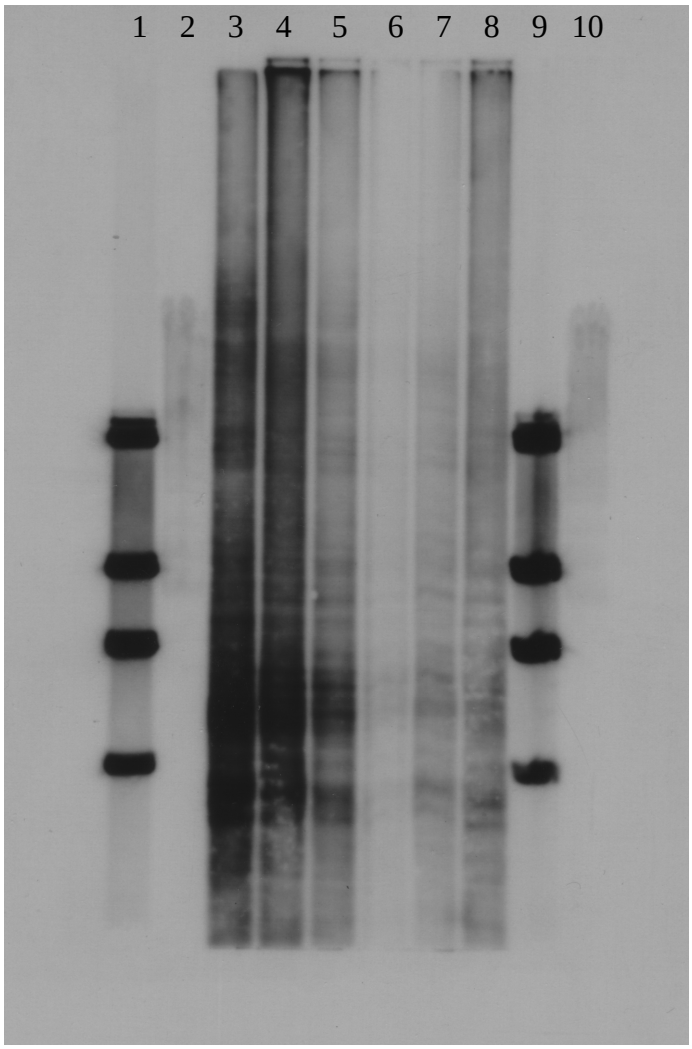


Figure 6.4: Representative blue-footed boobie TRF gel, produced by pulse-field gel electrophoresis. Lanes 1, 2, 9 and 10 contain markers. Lanes 3-8 contain boobie samples; substantial banding is present within the TRF smear.

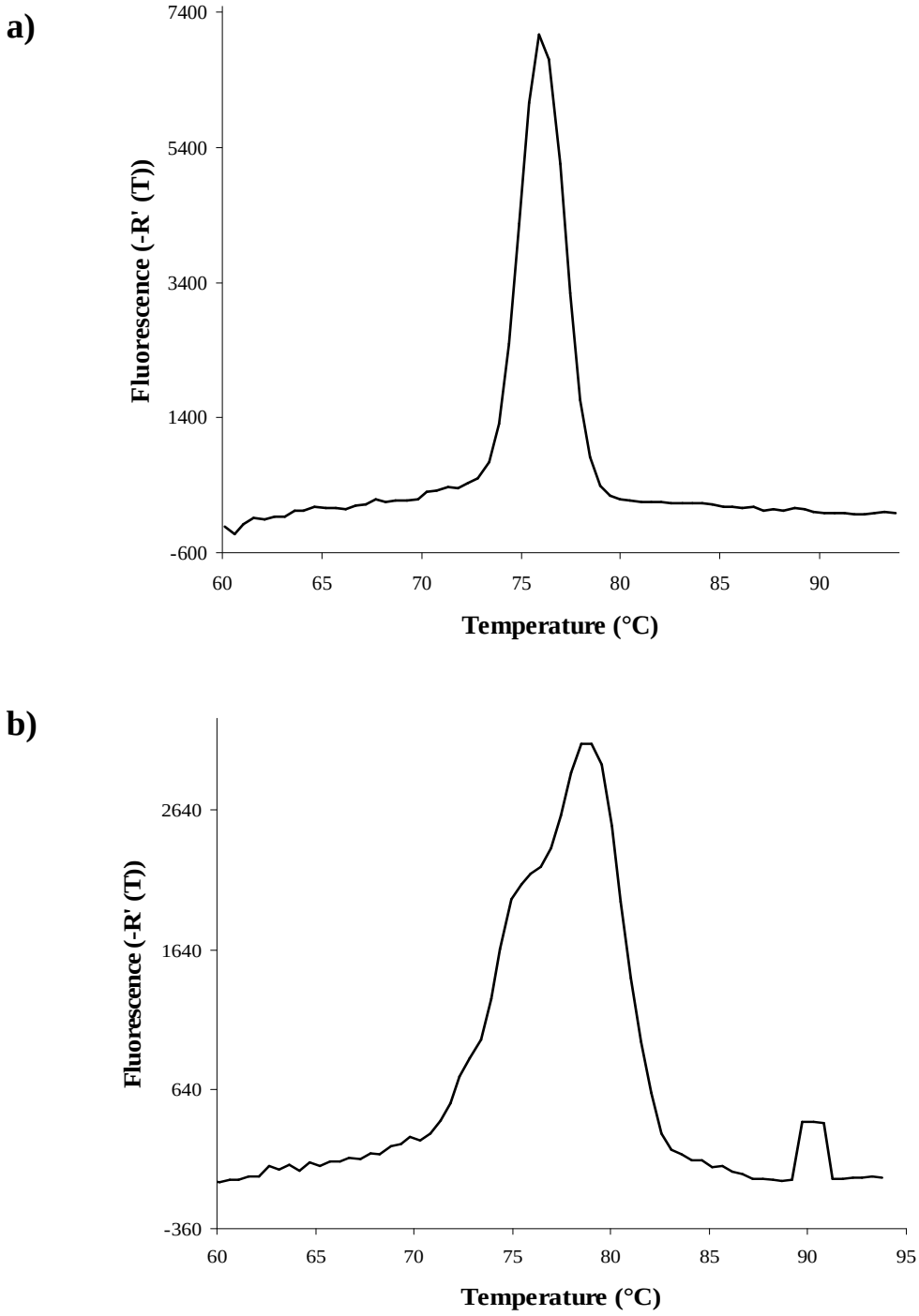


Figure 6.5: Representative dissociation curves for a) the GAPDH assay and b) the telomere assay. The curves show the amount of PCR product (measured in terms of fluorescence, y-axis) detected at different melting temperatures. If only one product is being amplified by the PCR assay, then fluorescence should peak at one temperature point, as in the GAPDH assay. In the telomere assay, although the curve has only one main peak, there is a long 'tail' to the left of the peak that suggests another product may have been amplified during the PCR reaction.

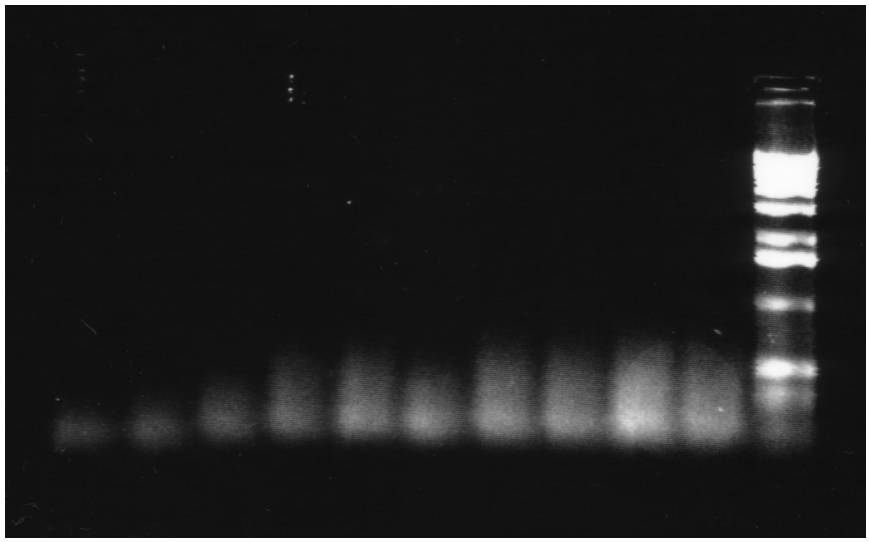


Figure 6.6: Gel electrophoresis of the product amplified during qPCR using telomere specific primers. Lanes 3 – 10 contain the amplification product from blue-footed boobie DNA. For comparison, lanes 1 - 2 show the amplification product from Alpine swift DNA (the same primers and reaction conditions were used for both species). Lane 11 contains a size marker. The product produced from swift DNA consists of one single tight band, as would be expected if only one product (i.e. telomeric DNA) was being amplified during the PCR. In contrast, the amplification product from boobie DNA, although consisting of one band, is much more diffuse. This suggests that, although only one product is being amplified during the boobie PCR, the size of that product is much less consistent than in the swift assay.

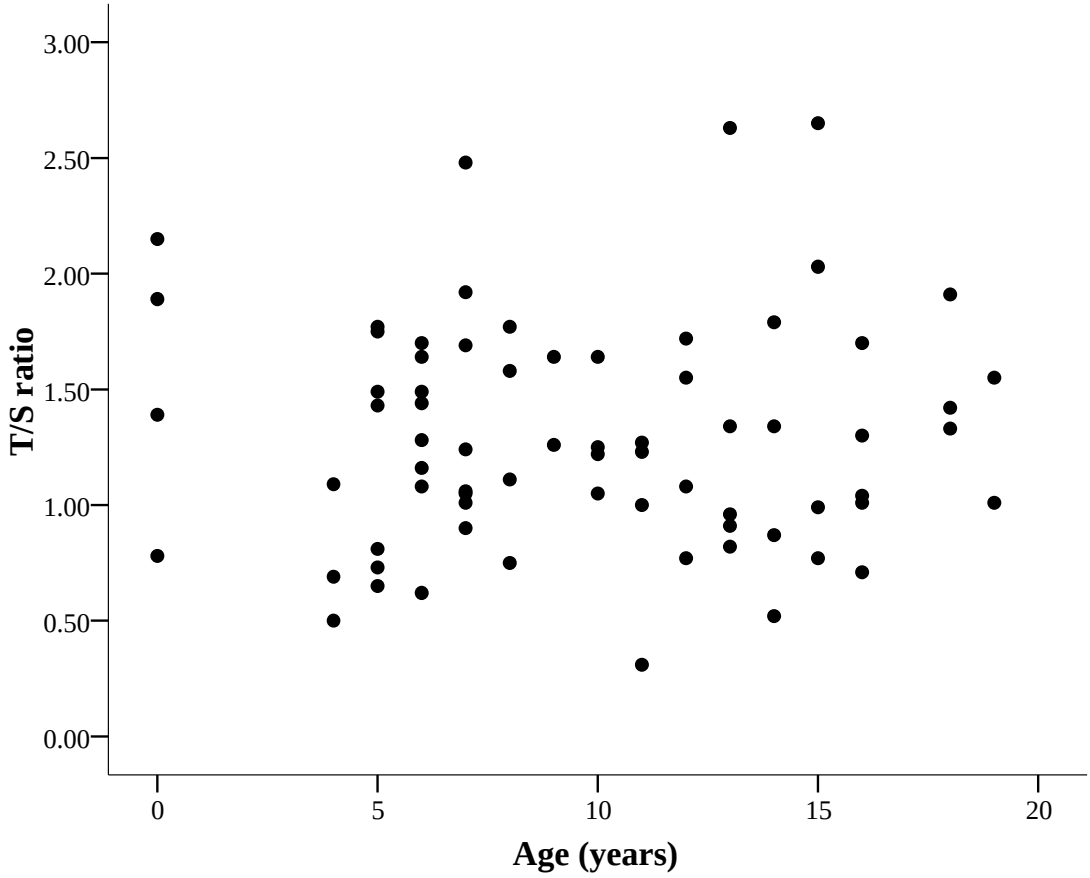


Figure 6.7: Age in relation to T/S ratio in the blue-footed boobie (n = 71). T/S ratio is a measure of relative telomere length; individuals with a low T/S ratio have relatively longer telomeres than individuals with a high T/S ratio.

Chapter 7: General discussion

Increasingly, telomeres are seen as more complicated than a 'mitotic clock', counting down at a constant rate towards cell senescence. Instead, the rate of telomere loss seems to be responsive to environmental conditions. Although telomeres do shorten with age in many species, the rate of loss is often not constant; in particular most telomere loss seems to occur in young individuals (e.g. Zeichner *et al.* 1999; Haussmann *et al.* 2003; Pauliny *et al.* 2006). The same pattern was demonstrated in the work on giant petrels *Macronectes* spp. in this thesis (chapter 5) with most telomere loss seen in young individuals. The significant amount of telomere loss seen in a very short period of time in the study of chicks of the lesser black-backed gull *Larus fuscus* (chapter 3) also supports the idea that most telomere loss is occurring in young individuals. This suggests that, if telomeres are a mitotic clock, the speed at which the clock ticks is not constant.

There are exceptions to this trend. For example some species show no telomere loss with age (sea urchins – Francis *et al.* 2006; *Drosophilia* – Walter *et al.* 2007). There are also species where telomere length apparently increases with age (e.g. Leach's storm petrel *Oceanodroma leucorhoai* – Haussmann *et al.* 2003; pine trees – Flanary & Kletetschka 2005). It would clearly be interesting to know what causes the difference in telomere dynamics between these species and those that do show telomere shortening with age. It should however be noted that the studies on these species were cross-sectional; it is possible that the pattern of telomere loss with age would be different if studied longitudinally. Indeed, Haussmann & Mauck (2008b) suggested that the apparent increase in telomere length with age in Leach's storm petrel was due to the differential survival of older individuals.

There are also species where no telomere shortening with age in adults is visible cross-sectionally (e.g. the European shag *Phalacrocorax aristotelis* and the wandering albatross *Diomedea exulans* – Hall *et al.* 2004; giant petrels – chapter 5). The difference between species such as these and species that do show telomere loss in adults might be related to lifespan. European shags, wandering albatross and giant petrels are long-lived species; perhaps these species invest more into maintaining telomere length as adults than shorter lived species which do show telomere loss in adults (e.g. zebra finches –

Haussmann *et al.* 2005). However this issue requires more investigation; in particular into whether no telomere loss in adults can be detected longitudinally in these species.

Telomere length is clearly not just a function of age; variation among same-age individuals has been seen in the vast majority of species studied. The avian species studied in this thesis were no exception; significant amounts of variation in telomere length among same-age individuals were seen in lesser black-backed gulls (chapter 3), northern and southern giant petrels (chapter 5) and blue-footed boobies *Sula nebouxii* (chapter 6). Given the almost universal nature of this pattern the question arises of what is causing this variation. It has been suggested that some of these differences are due to inbuilt differences between individuals. For example in humans some of the variation in telomere length seems to be due to parental inheritance (Nordfjäll *et al.* 2005; Njajou *et al.* 2007). However the extent to which telomere length is inherited has not been examined to any substantial extent in other species. There is no evidence for telomere length being paternally inherited in the species studied in this thesis, but none of the studies were specifically designed to examine this.

In this thesis, sex differences in telomere length were found in chicks of both the lesser black-backed gull (chapter 3) and the European shag (chapter 4). These sex differences were consistent for at least a few weeks during the chick period. A consistent sex difference was found across all ages in giant petrels (albeit with variation depending on the method used to analyse the TRF gels - chapter 5). It was notable that the direction of the sex difference differed between the gulls and shags (where males had longer telomeres than females) and the giant petrels (where females had longer telomeres than males). These sex differences are the first, to my knowledge, found in avian species. Sex differences have previously been found in humans (Benetos *et al.* 2001; Nawrot *et al.* 2004), rats (Cherif *et al.* 2003) and ants (Jemielity *et al.* 2007); in all these cases females had longer telomeres than males. What is needed now is an investigation of sex differences across a wider range of species to determine if there are general trends across taxa. It is possible that differences in telomere length between the sexes are linked to differences in life-histories between the sexes. For example, one might predict that in species where females are substantially larger than males (e.g. insects) they will have shorter telomeres than males, whereas in species where females are the smaller sex (e.g. humans) they will have longer telomeres than males. To a certain extent, the results obtained in this thesis fit this pattern. Giant petrels

are very sexually dimorphic by the standard of seabirds (Croxall 1982), with females smaller in size than males, while lesser black-back gulls show less (approx. 10%) difference in size between the sexes. Thus it makes sense that in the petrels females have longer telomeres than males, while this is not the case in gulls (although this hypothesis does not explain why males have longer telomeres than females in gulls). Conversely however, in the European shag females are substantially smaller than males, and yet they have shorter telomeres (at least in chicks).

Some of the variation in telomere length is probably inbuilt and due to factors such as sex and parental inheritance. However, given that most telomere loss seems to be occurring in young individuals it is logical to suggest that a lot of the variation in telomere length is linked to early life conditions. Very few studies have examined this however (e.g. Jennings *et al.* 1999; Hall *et al.* 2004). Work in this thesis on lesser black-back gull chicks (chapter 3) found some support for the idea of a relationship between early life conditions and telomere length. The pre-embryonic growth rate (as measured by hatchling size) was related to some of the variation in hatching telomere length. There was however no evidence of post-hatching growth rate affecting telomere length. There was a low sample size in this part of the study, but this finding might reflect a more general problem when trying to link growth and telomere length. A relationship between growth rate and telomere loss would probably only be expected if an increase in growth rate meant an increase in the rate of cell division; however, an increase in body size could be caused by an increase in cell size, which would confound attempts to link growth rate and telomere loss. More generally, there is a clear need for an experimental approach to studies of the cause of variation in telomere length. Given the number of factors that could potentially affect telomere length, identifying how important one factor is will always be difficult using a correlational approach. There is also the question of how important early variations in telomere length are. Are these early differences in telomere length maintained into adulthood? Can shorter telomeres in a young individual affect cell loss or the build up of senescent cells? If neither of these things were true then any early differences in telomere length would be unimportant, no matter the cause. There is a need for long-term longitudinal studies to investigate these questions, although there are obvious difficulties in conducting such studies.

Given that there is significant variation in telomere length among individuals, the next obvious question is what effect this variation has. One would expect telomere length to be linked to senescence, but there is little actual evidence for this *in vivo*; telomere length has been linked to life expectancy in humans (e.g. Cawthon *et al.* 2003) and Alpine swifts (Bize *et al.* in prep.). However, there is increasing evidence that telomere length is linked (directly or indirectly) to other measures of individual quality. For example, telomere length has been linked to short term survival in tree swallows (Haussmann *et al.* 2005) and *C.elegans* (Joeng *et al.* 2004). The work in this thesis on southern giant petrels *Macronectes giganteus* (chapter 5) seems to provide further support for this idea; telomere length was linked to the survival of adult individuals in the 8 years after they were initially sampled. This relationship was independent of age and so was obviously not linked to cell senescence (the same is true in tree swallows and *C.elegans*). Whether telomeres are directly linked to survival, through a mechanism other than cell-senescence, or if telomere length just correlates with individual quality is unknown. However, the idea that telomere length might provide a measure of an individuals quality is clearly an exciting one. More study is required to identify both how widespread this effect is and if telomere length is linked to other measures of individual quality such as reproductive success.

In recent years, the range of taxa whose telomere dynamics have been studied has gradually expanded, from initial work on human cells *in vitro*, to work *in vivo*, first on humans and lab animals, and now on a variety of wild taxa. Although the questions they seek to answer might be different, work done on cell culture by molecular biologists is highly applicable to the work being done by behavioural ecologists working on wild animals (and *vice versa*). This is equally true of work conducted by behavioural ecologists working on different taxa; work done on avian telomere dynamics should not just be of interest to other avian researchers. Indeed these points are more relevant to research on telomeres than they are to other areas of biology, because of how conserved the basic structure and function of telomeres is across taxa.

This conservation of the structure and function of telomeres should theoretically make the application of methods to measure telomeres easily applicable across taxa. Problems could still arise however. For example, there is the question of how best to measure telomeres in a new species; several different methods are available to measure telomeres, but as the work on blue-footed boobies in this thesis (chapter 6) showed, these methods

might not necessarily work with every new species they are applied to. There is also the question of the extent to which study design needs to be adapted to different species. One such example was presented in this thesis; 10 days was enough time to see a significant longitudinal telomere loss in lesser black-back gull chicks (chapter 3), but 13 days was not enough to see a similar loss in chicks of the European shag (chapter 4).

Other factors, not considered in this thesis, will also be important considerations in future studies. For example, telomerase was generally not considered in 'traditional' studies of human telomere dynamics, as it is not expressed in human somatic cells. However, this is not the case in many non-human species. In the few avian species studied, telomerase is expressed in many somatic tissues and, furthermore, the levels of expression vary with age (Haussmann *et al.* 2004, 2007). Thus, there are important questions to answer in relation to telomerase in avian species; could an increase in telomerase levels in adults be partly responsible for the slower rate of telomere loss in adults seen in some species? Similarly, do levels of telomerase expression vary between same age individuals? If so, this could be responsible for some of the variation in telomere length we see. The ideal future approach would be to measure telomerase levels at the same time as telomere lengths are measured. However this would present problems; for example it is not known if telomerase levels are constant within an individual at all times of the year, or even at all times of the day.

There is also the question of the type of cell used in telomere studies. Throughout this thesis, red blood cells (rbcs) have been the cell type examined, as is the case in the vast majority of studies done on wild taxa (or white blood cells in taxa that do not have nucleated rbcs). The obvious benefit of using blood cells is that they can be easily collected with the minimum of interference to the study individuals. However, in terms of senescence, rbcs are unlikely to be important; when we use rbcs in telomere studies we assume that what is happening in rbc telomeres is representative of what is happening to the telomeres of more important tissues. However telomere lengths vary among different tissues in humans (e.g. Ishii *et al.* 2006; Nakamura *et al.* 2007) and rats (Jennings *et al.* 1999; Cherif *et al.* 2003) and both telomere lengths and telomerase levels vary in birds (Haussmann *et al.* 2004, 2007). Ideally we would always take samples from a variety of tissues, but this is not always possible in studies on wild populations, especially in longitudinal studies. Even in studies of humans, non-blood cell tissue samples tend to

come from deceased individuals, giving the risk of a biased sample. The considerable amount of cell culture work done on different human cell types at least allows some estimate to be made of how their telomere dynamics differ. Similar cell culture work on non-human species could give us an equal insight into how telomere dynamics in blood cells relate to telomere dynamics in other tissues.

An increase in our knowledge of the mechanisms affecting telomere loss would also benefit studies of telomere dynamics. For example, it is generally assumed that higher levels of oxidative stress result in higher rates of telomere loss, but this is primarily based on work done *in vitro* (von Zglinicki 2002; Richter & von Zglinicki 2007). If we could experimentally show that individuals with a higher rate of telomere loss also have higher levels of oxidative stress *in vivo*, then this would further increase the evidence for this hypothesis. Again though, whether this is practical or not will depend on the species studied and the conditions in which samples are being collected.

Clearly there is huge potential in the study of telomeres. Behavioural ecologists were initially attracted to telomeres as a potential way of measuring the age structure of wild populations (e.g. Juola *et al.* 2006). The realisation that telomeres do not decrease at a constant rate over time removed this hope, but opened up many more avenues of research. It now seems possible that telomeres might provide both a way of measuring the long-term costs of early life-conditions and a way to measure the quality of an individual. More work is needed to investigate to what extent this is true across taxa. In particular an experimental approach needs to be applied to these questions, especially experiments involving longitudinal analysis of telomere length. As discussed above, the effects of telomerase and cell type needs to be considered more in studies of telomere dynamics, as do the proximate mechanisms affecting telomere loss. In addition, there still needs to be an increase in the variety of taxa whose telomere dynamics are studied, to include taxa with more varied life-histories. A huge amount of what we know about how telomeres still comes from studies on humans; we know very little about the pattern of telomere loss, the expression of telomerase and the variation among tissue types and individuals in non-human species. Filling in these gaps in our knowledge can only be beneficial to our understanding of life-history strategies.

Appendix: Real-time quantitative PCR assay for measurement of avian telomeres

This appendix contains a copy of work recently accepted for publication in the Journal of Avian Biology. Although I was not one of the lead authors on this study, I contributed to the work it reports on; it is thus included as additional support for this thesis.

FRANÇOIS CRISCUOLO[*‡], PIERRE BIZE[*], LUBNA NASIR[†], NEIL B. METCALFE, CHRIS G. FOOTE*, KATE GRIFFITHS[*], ELIZABETH A. GAULT[†] AND PAT MONAGHAN*

* Ornithology Group, Division of Environmental and Evolutionary Biology, Institute of Biomedical and Life Sciences, Graham Kerr Building, University of Glasgow, Glasgow G12 8QQ, UK; and [†]Institute of Comparative Medicine, University of Glasgow, Faculty of Veterinary Medicine, Bearsden Road, Glasgow G61 1QH, UK

Abstract

We present the application of a real-time quantitative PCR assay, previously developed to measure relative telomere length in humans and mice, to two bird species, the zebra finch (*Taeniopygia guttata*) and the Alpine swift (*Apus melba*). This technique is based on the PCR amplification of telomeric $(TTAGGG)_n$ sequences using specific oligonucleotide primers. Relative telomere length is expressed as the ratio (T/S) of telomere repeat copy number (T) to control single gene copy number (S). This method is particularly useful for comparisons of individuals within species, or where the same individuals are followed longitudinally. We used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a single control gene. In both species, we validated our PCR measurements of relative telomere length against absolute measurements of telomere length determined by the conventional method of quantifying Telomere terminal Restriction Fragment (TRF) lengths using both the traditional Southern blot analysis (alpine swifts) and in gel hybridization (zebra finches). As found in humans and mice, telomere lengths in the same sample measured by TRF and PCR were well correlated in both the Alpine swift ($r = 0.76$, $P = 0.001$) and the zebra finch ($r = 0.82$, $P < 0.001$). Hence, this PCR assay for measurement of bird telomeres, which is fast and requires only small amounts of genomic DNA, should open new avenues in the study of environmental factors influencing variation in telomere length, and how this variation translates into variation in cellular and whole organism senescence.

Introduction

Telomeres are specialized nucleotide repeat sequences at the end of eukaryotic chromosomes that play a crucial role in preventing chromosome degradation and fusion (Blackburn 2001). In vertebrates, telomeres are composed of numerous tandem repeats of $(TTAGGG)_n$ sequences bound by shelterin-telosome proteins (Blasco 2007). Telomeres shorten at each division of most somatic cell types because of the inability of normal DNA polymerase to produce a complete new lagging strand (5'-end-replication problem; Watson 1972; Blackburn 2001). The rate of telomere shortening can be further accelerated by the increase in single-strand breaks due to oxidative stress (von Zglinicki 2002). *In vitro* studies have demonstrated that telomere shortening puts a finite limit on cell replication, and evidence is accumulating from *in vivo* studies that relatively short telomere length is associated with reduced whole organism lifespan (Monaghan and Haussmann 2006).

Telomere lengths are most commonly measured using the Telomere (terminal) Restriction Fragment method (TRF), where average lengths of TRFs are measured by Southern blot or in-gel hybridization with a specific phosphorescent or radioactive oligonucleotide probe, after digestion of the DNA by a cocktail of restriction enzymes (Nakagawa et al. 2004, Haussmann and Mauck 2008). This relatively easy method has the advantage of providing highly repeatable absolute measures of telomere lengths (intra- and inter-gel co-efficients of variation in birds usually less than 2%, e.g. see Haussmann et al. 2003), thus allowing straightforward inter- and intra-specific comparisons of telomere lengths (e.g. Haussmann et al. 2003, Hall et al. 2004). However, the TRF method suffers from several major drawbacks (see also Baird 2005, Aviv et al. 2006): (i) it relies on autoradiographic smears that are difficult to interpret (but see Haussmann and Mauck 2008 for recent methodological advances); (ii) depending on which restriction enzyme is used, TRF measures may include noise from the sub-telomeric fragments close to the nearest restriction site (Nakagawa et al. 2004); (iii) it requires a large amount of DNA (around 5-10 µg); and (iv) it is a very time consuming method, *c*. 4 days being required to process 26 samples, and much longer when pulse field electrophoresis and in gel hybridisation are required (Haussmann and Mauck 2008). These last two restrictions severely limit the applicability of the TRF method in ecological and evolutionary studies where telomere lengths must be obtained from small amounts of DNA (e.g. from blood samples of small

passerines, a volume often less than 100 µl) and where sample sizes should ideally be large.

Both of these problems can be avoided by the specific amplification of the telomere sequence by the polymerase chain reaction (PCR)-based method (Cawthon 2002). One of the major methodological problems of the PCR method was the auto-dimerisation of primers designed to hybridize to the TTAGGG and CCCTAA DNA repeats, a major methodological hurdle recently overcome for human telomeres (Cawthon 2002). The use of the real-time quantitative PCR method (hereafter referred to as qPCR), which is relatively rapid (2 days for 96 samples, so almost over seven times as many samples being done per unit time compared to the standard TRF approach), has recently enabled researchers to process large sample sizes, bringing to the fore evidence on the heritability of telomere length (Nordfjäll et al. 2005) and the impact of stress on telomere dynamics (Epel et al. 2004). However, as with the TRF method, qPCR measurement of telomere sequences also carries methodological difficulties. Firstly, telomere length is expressed as the ratio (T/S) of telomere repeat copy number (T) to a control single gene copy number (S). Hence, the qPCR method measures relative telomere lengths and not absolute telomere lengths (Cawthon 2002), (though absolute telomere lengths can be generated when interstitial repeats are not included/not high, see Callaghan et al. 2008). The choice of the control gene (S) is therefore important: it should not vary in copy number between individuals, nor within individuals over time, to ensure that variation in T/S ratios is only due to variation in telomere size (T). Secondly, qPCR primers amplify all $(TTAGGG)_n$ sequences in the genome, including interstitial sequences that are not part of the telomeres; this needs to be taken into account in those animals that have abundant interstitial $(TTAGGG)_n$ sequences (including many birds: Venkatesan and Price 1998, Nanda et al. 2002). However, since the interstitial repeat levels do not change with age, and vary little among individuals of the same species (Delany et al. 2003), the qPCR method is very useful for studies where known individuals are sampled repeatedly, and where comparisons are made amongst individuals of the same species. To validate the qPCR assay for measurement of telomeres, it is important to check that T/S ratios are correlated with TRF measurements. This could potentially allow calibration of the T/S ratios for the species in question, and enable estimation of absolute measures of telomere length (for a given interstitial telomeric length) that could be compared with previous studies.

Here we show that the qPCR method can be used to measure telomere lengths in birds, and validate our qPCR measurements of telomere lengths against conventional TRF measurements. We performed the analyses in two bird species from different Orders, the zebra finch (*Taeniopygia guttata*, from the Passeriformes) and the Alpine swift (*Apus melba,* from the Apodiformes). Because of the possibility of relatively high levels of interstitial repeats in some birds, including zebra finches, it has been suggested that it is best to use the in gel hybridization method for measuring TRFs (Haussmann and Mauck 2008); this avoids de-naturing the DNA, and this the telomeric probe binds only to the telomere, and not to the interstitial repeats. We therefore examined the correlation between the qPCR method using both in-gel hybridization (zebra finches) and the standard southern blot method (alpine swifts).

Methods

Study species and blood collection

The zebra finch is a 18 g passerine bird that can live up to 5 years in the wild (Zann 1996) and slightly longer in captivity, while the Alpine swift is a 100g bird that can live up to 26 years (Bize et al. 2006). Samples were collected in 2006 from a captive zebra finch colony at the University of Glasgow, UK, and from a wild Alpine swift colony in Bienne, Switzerland.

In the zebra finch, 100µl of blood was collected from the brachial vein into heparin capillaries and centrifuged for 5 min at 6000 RPM to separate red blood cells (RBC) from the plasma. RBC were stored in Eppendorf tubes at -80°C prior to analysis. We used a similar procedure for the Alpine swift with the only difference being that RBC were stored at -20°C. We maximized the range of telomere lengths in both datasets by blood sampling both young and older individuals. This also allowed us to check that the reference gene copy number used for the qPCR did not change with age, which, for the method to be effective, it should not. In the zebra finch, we sampled 13 young (< 60 days) and 13 older birds (5-6 years), and in the Alpine swift we sampled 6 young (< 50 days) and 9 older birds (8-19 years). We also checked whether the relationship between telomere length measured by the qPCR and TRF held within the age classes.

TRF assay

TRF assay was conducted following the method previously described in several papers (Haussmann and Vleck 2002, Haussmann et al. 2003, Haussmann and Mauck. 2008). In the zebra finch, we extracted genomic DNA from fresh blood in an agarose plug as follows. Immediately after blood sampling, ice cold 2% EDTA pH7.7 was added into the Eppendorf tubes to give equal volumes of whole blood to buffer. Samples were spun at 3500 rpm at 4°C for 4 min after which the plasma/EDTA supernatant was removed, and the red blood cells (RBC) were stored on ice. 5µl of RBC were used in a 100µl volume of cell suspension buffer/agarose solution to form an agar plug, giving a final agarose concentration of 0.8%. Plug formation and DNA extraction were carried out according to the manufacturer's protocol (CHEF Genomic DNA Plug Kits BIO-RAD 170-3591). In the Alpine swift, genomic DNA was extracted from previously frozen RBC using DNeasy Blood and Tissue Kit (Qiagen) and following the manufacturer's protocol.

For both species, genomic DNA was digested overnight at 37°C using three restriction endonucleases: *Hinf* I (15U), *Hind*III and *Msp*I (30 U of each, New England Biolabs). The restricted DNA and Kb DNA size standards (CHEF DNA size standard 8-48 kb, BIO-RAD cat. 170-3707, DNA Molecular weight marker II, Digoxigenin labelled 0.12-23.1 kbp Roche cat. 11 218 590 910) were electrophoresed through non-denaturing agarose (0.8%) using pulsed field gel electrophoresis (CHEF-DR®II Pulsed Field Electrophoresis Systems) for 24 hours, using the following conditions: voltage: 3.5v/cm, initial switch time: 0.5 seconds, final switch time: 7 seconds. On completion of pulsed field gel electrophoresis of Alpine swift DNA the gel was dried using a gel drier for 25 minutes at 80°C and subsequently hybridized overnight at 37°C with the ^{32}P γ-ATP labelled telomeric probe $(C_3TA_2)_4$ for the zebra finches. Signals were visualized by phosphor imaging. The alpine swift DNA was transferred to a nylon membrane by the downward capillary transfer method of Southern blotting, during which process the DNA is denatured by soaking the gel in 0.4 N NaOH for 15 min (Southern blotting by alkaline transfer, Koetsier et al. 1993). After DNA transfer, the membrane was hybridized overnight with the same oligonucleotide probe as used for the zebra finch. After scanning the image, TRF smears of different sizes were measured using Totallab software. Using each band of the CHEF DNA ladder, a mean TRF length per lane was then calculated as mean TRF length = $\sum (OD_i)/ \sum (OD_i/L_i)$ where OD_i is total radioactivity above background in interval i and L_i

is the average length of i ladder base pairs. The window for telomere length analysis (lower limit: 10 kb; upper limit: 48 kb; fig. A.1) was defined to avoid extrapolation of data points at high molecular weights (over the range given by the ladder, see fig. A.1b) that may greatly influence telomere measurements (Pauliny et al. 2006, Haussmann and Mauck 2008). TRF measurements were carried out blindly with respect to qPCR values. Due to practical and time constraints, and because TRF measurements are highly repeatable in our own and other studies (e.g. Jeanclos et al. 2000, Hall 2004, Haussmann and Mauck 2008), we ran each sample singly in the TRF analyses.

Quantitative PCR assay

Telomere qPCR was performed as described by Cawthon (2002) with the following modifications. In both species, genomic DNA was extracted from previously frozen RBC using DNeasy Blood and Tissue Kit (Qiagen) and following the manufacturer's protocol. The control single copy gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified using the primers GAPDH-F (5'-AACCAGCCAAGTACGATGACAT-3') and GAPDH-R (5'-CCATCAGCAGCAGCCTTCA-3'). These primers are specific to the zebra finch GAPDH (Genbank Accession No: AF255390) but also amplify the Alpine swift GAPDH gene. Telomere primers were: Tel1b (5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3') and tel2b (5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'). qPCR for both GAPDH and telomeres was performed using 20 ng of DNA per reaction. The telomere primers tel1b / tel2b were used at a concentration of 100 nM and 200 nM for zebra finch and Alpine swift respectively, and primers GAPDH-F / GAPDH-R at 200 nM in both cases, for a final volume of 25 µl containing 12.5 µl of Brilliant®SYBR®Green QPCR Master Mix (Stratagene). Telomere and GAPDH real time amplification were performed on two different plates. Telomere PCR conditions were 10 min at 95°C followed by 30 cycles of 1 min at 56°C and 1 min at 95°C. GAPDH PCR started with 10 min at 95°C followed by 40 cycles of 1 min at 60°C and 1 min at 95°C. PCRs were performed in a Mx3000P® QPCR System (Stratagene). Each 96-well (finch and swift) plates included serial dilutions (40ng, 20ng, 10ng, 5 ng of DNA per well), run in triplicate, of DNA from the same reference bird for each species, which was used to generate a reference curve to control for the amplifying efficiency of the qPCR (amplification efficiencies for GAPDH and telomere amplification were 100-105% in zebra finches and 100-110% in alpine

swifts; accepted range 100 ± 15%, Stratagene) and to set up the threshold C_t value. The C_t of a DNA sample is the fractional number of PCR cycles to which the sample must be subjected in order to accumulate enough products to cross a set threshold of magnitude of fluorescent signal. All samples other than the reference were run in duplicate. Amplicon size for the GAPDH was measured after migration of PCR products on agarose gel, and was around 50 bp, which was the size expected by the primers' design (fig. A.2). Telomere length is expressed relative to the internal single gene control (GAPDH) measured on the same sample of DNA. Using the standard curves of Tel and GAPDH, dilution factors of standards corresponding to the telomere (T) and the single gene control (S) amounts in each sample were calculated to obtain a relative T/S ratio, which reflects the length differences in telomeric DNA relative to the constant GAPDH amplicon and is calculated following the formula: telomere length = $2^{(-\Delta Ct)}$ where $\Delta C_t = C_t^{telomere} - C_t^{Gapdh}$. Standardisation of T/S measurements to an internal control, often termed 'the golden sample' is the usual method of accounting for intra and inter qPCR assay variability (Cawthon 2002), and this procedure has been followed in the presentation of results here. All qPCR measurements were carried out blindly with respect to TRF values for the same bird.

Statistical analyses

We used Pearson correlation analysis to establish the relationship between telomere measurements obtained by TRF and qPCR methods. In order to check that these relationships held within young and older birds, and still retain a reasonable sample size for each age class, we transformed the data for each species age class to standard normal distributions using the relevant age specific mean for adults and older birds. We then examined the relationship between the TRF telomere measurement and the qPCR measurement for the both the combined young and the combined old birds for each species.

Results

Mean telomere length measured by TRF method ranged from 20 to 30.4 kb in captive zebra finches and from 13.7 to 21.5 kb in wild Alpine swifts (fig. A.1).

In both species, C_t^{Gapdh} and $C_t^{telomere}$ values obtained by qPCR varied little for the same sample, the average co-efficient of variation being around 1-2% (see table A.1), and hence we used mean C_t values to calculate the T/S ratio. We found a strong correlation between the relative measures of telomere length obtained by qPCR (i.e. the T/S ratio) and absolute measurements of telomere length obtained by the TRF method in both the zebra finch ($r = 0.82$, $P < 0.001$, $n = 26$) and the Alpine swift ($r = 0.76$, $P = 0.001$, $n = 15$) (fig. A.3). When the normalised data for the young birds and the old birds of both species were combined, there was a significant relationship between the two measures in both cases (young birds: $r = 0.66$, $P = 0.002$, $n = 19$; old birds: $r = 0.65$, $P = 0.001$, $n = 22$). There was no relationship between C_t values of our single copy gene (C_t^{Gapdh}) and age in either the zebra finch ($r = 0.12$, $P = 0.56$, $n = 26$) or the Alpine swift ($r = 0.10$, $P = 0.71$, $n = 15$), indicating that gene copy number does not change with age in either species.

Discussion

It is important to remember that qPCR amplification is not restricted to the end-telomeric sequence, but is extended to non-telomereic, i.e. interstitial and centromeric, $(TTAGGG)_n$ sequences (Venkatesan and Price 1998). The avian genome shows a high density of non-telomeric sequences linked to the important proportion of microchromosomes (Nanda et al. 2002). When in-gel hybridization is used, as was the case here for the zebra finch, the TRF estimates do not include the non-telomeric sequences. Where the Southern blot method is used, as was the case here for the Alpine swifts, the TRF measurements will include the non-telomeric repeats. Many of these interstitial repeats are likely to be small sequences, and may often be below the detection limit of the hybridization method on which the TRF is based, or below the lower measurement window on the TRF smear (usually 10 kb). Very short sequences are included in the qPCR measurement, since the lower detection limit is around 78 base pairs (fig. A.2; see Cawthon 2002). However, for most bird species, we simply do not know the pattern of occurrence of interstitial repeats of the telomeric sequence. Furthermore, ultra-long telomeres have also been found in some birds (Delany et al. 2003), and these are also likely to be included in the qPCR measurement but not in the TRF measurement; the magnitude of the effect of these ultra-long telomeres on the TRF telomere measurement is unclear and likely to vary amongst species, and amongst age classes, but again we generally do not know the pattern in most bird species. This would require an *in situ* hybridization approach, at present likely to be beyond the scope of most

ecological studies (Nakagawa et al. 2004). These considerations make it very important to examine the correlation between the qPCR method of telomere estimation and the TRF measurement. Our study shows that the correlation is good, whatever method in used for the TRF measurement, and that the correlation is significant even when only young birds are considered. This demonstrates that telomere size evaluation by qPCR is a reliable method for estimating telomere length in birds, as has been found in mammals (Cawthon 2002, Callicot and Womack 2006). The strength of the correlations obtained for both the zebra finch and the alpine swift in this study are similar whether the more traditional Southern blot approach for TRF measurement is used, (alpine swifts) or the in gel hybridization (zebra finches) (fig. 2). The latter suggests that the amount of interstitial sequences varies little between individuals, and that most of the variation between individuals is a consequence of variation in the end telomere length. A similar result was obtained by Callicot and Womack (2006) using Southern Blotting and qPCR in their study of mice, which also have extensive interstitial $(TTAGGG)_n$ sequences. The correlation coefficients between the two methods in the present study (R^2 = 0.58 and 0.67) are similar to those previously reported in humans (R^2 = 0.68, Cawthon 2002) and mice (R^2 = 0.66, Callicott and Womack 2006). Our results also suggest that variable detection of the ultra-long telomeres does not cause a problem, perhaps because they are not present in sufficient quantities. However, inclusion of these longer telomeres in the TRF analysis might improve the correlations between the two methods further. In future, the sequencing of the whole genome of species like the zebra finch will, concomitantly with the development of a detailed method of analysis of telomere length (like single telomere length analysis, STELA, Baird et al. 2003), enable us to increase the sensitivity of the qPCR approach for telomere length measurement in birds.

The qPCR method necessitates the use of a reference gene that does not change with age, thereby avoiding the possibility of misleading results (Aviv et al. 2006). Although GAPDH may not be a suitable internal control for RNA analyses in ageing studies (Lowe et al. 2000), we found no change in GAPDH gene amplification with age in either of our study species, thereby confirming that GAPDH can be used as a reference for qPCR determination of telomere length in these species. However, while the qPCR method appears to be highly suitable for intra- or inter-individual comparisons in telomere sizes within a species, it will need more methodological development for inter-specific studies. The necessity for each species to share the same single copy gene sequence (Nakagawa et

al. 2004), and the need to optimize the primer concentrations for telomeres and the control gene, which are likely to be different for each species (see Methods), makes it unlikely that inter-specific comparisons of telomere size by qPCR can be done on the same plate using the same PCR conditions. In addition, while it is possible to convert the relative measure of telomere length obtained by qPCR to an absolute telomere length (Thomas et al. 2008, Callaghan et al. 2008), in birds this needs to be adjusted to take account of the level of interstitial repeats of the telomeric sequence. While this can be done by *in situ* hybridization, this would need to be done for each species since there is interspecific variation amongst birds (Nanda et al. 2002). The qPCR technique is however very well suited to analyses of intra-specific variation and intra-individual changes in telomere length (since interstitial sequences will remain constant over time, so that any changes in the C_t measurement are due to telomere attrition). The speed and relative simplicity of the assay will thus make it far easier to process the number of samples that are needed for full investigation of the environmental factors that cause changes in telomere dynamics and the rate of cellular senescence.

References

Aviv, A., Valdes, A.M. and Spector, T.D. 2006. Human telomere biology: pitfalls of moving from the laboratory to epidemiology. - Int. J. Epidemiol. 35: 1424-1429.

Baird, D.M. 2005. New developments in telomere length analysis. - Exp. Gerontol. 40: 363-368.

Baird, D.M., Rowson, J., Wynford-Thomas, D. and Kipling, D. 2003. Extensive allelic variation and ultrashort telomeres in senescent human cells. – Nat. Genet. 33: 203-207.

Bize, P., Gasparini, J., Klopfenstein, A., Altwegg, R. and Roulin, A. 2006. Melanin-based coloration is a nondirectional selected sex-specific signal of offspring development in the alpine swift. - Evolution 60: 2370-2380.

Blackburn, E.H. 2001. Switching and signaling at the telomere. - Cell 106: 661-673.

Blasco, M. 2007. Telomere length, stem cells and aging. – Nat. Chem. Biol. 3: 640-649.

Callaghan, N.J., Dhillon, V.S., Thomas, P. and Fenech, M. 2008. A quantitative real-time PCR method for absolute telomere length. – BioTechniques 44: 807-809.

Callicott, R.J. and Womack, J.E. 2006. Real-time PCR assay for measurement of mouse telomeres. - Comparative Med. 56: 17-22.

Cawthon R.M. 2002. Telomere measurement by quantitative PCR. - Nucleic Acids Res. 30: E47.

Delany, M.E., Daniels, L.M., Swanberg, S.E. and Taylor, H.A. 2003. Telomeres in the chicken: genome stability and chromosome ends. - Poultry Sci. 82: 917-926.

Epel E.S., Blackburn, E.H., Lin, J., Dhabhar, F.S., Adler, N.E., Morrow, J.D. and Cawthon, R.M. 2004. Accelerated telomere shortening in response to life stress. – Proc. Natl. Acad. Sci. USA 101: 17312-17315.

Hall, M.E. 2004. Senescence and reproductive performance in the European shag (*Phalacrocorax aristotelis).* PhD Thesis, University of Glasgow, UK.

Hall, M.E., Nasir, L., Daunt, F., Gault, E.A., Croxall, J.P., Wanless, S. and Monaghan, P. 2004. Telomere loss in relation to age and early environment in long-lived birds. - Proc. R. Soc. B271: 1571-1576.

Haussmann, M.F. and Mauck, R.A. 2008. New strategies for telomere-based age estimation. - Mol. Ecol. Res. 8: 264-274.

Haussmann, M.F., Winkler, D.W., O'Reilly, K.M., Huntington, C.E., Nisbet, I.C.T. and Vleck, C.M. 2003. Telomeres shorten more slowly in long-lived birds and mammals than in short-lived ones. – Proc. R. Soc. B270: 1387-1392.

Haussmann, M.F. and Vleck, C.M. 2002. Telomere length provides a new technique for aging animals. - Oecologia 130: 325-328.

Jeanclos, E., Schork, N.J., Kyvik, K.O., Kimura, M., Skurnick, J.H. and Aviv, A. 2000. Telomere length inversely correlates with pulse pressure and is highly familial. Hypertension 36: 195-200.

Koetsier, P.A., Schorr, J. and Doerfler, W. 1993. A rapid optimized protocol for downward alkaline Southern blotting of DNA. - BioTechniques 15: 260-262.

Lessells, C.M. and Boag, P.T. 1987. Unrepeatable repeatabilities: a common mistake. - Auk. 104: 116-121.

Lowe, D.A., Degens, H., Chen, K.D. and Always, S.E. 2000. Glyceraldehyde-3-phosphate dehydrogenase varies with age in glycolytic muscles of rats. – J. Gerontol.: Biol. Sci. 55A: B160-164.

Monaghan, P. and Haussmann, M.F. 2006. Do telomere dynamics link lifestyle and lifespan? - Trends Ecol. Evol. 21: 47-53.

Nakagawa, S., Gemmell, N.J. and Burke, T. 2004. Measuring vertebrate telomeres: applications and limitations. – Mol. Ecol. 13: 2523-2533.

Nanda, I., Schrama, D., Feichtinger, W., Haaf, T., Schartl, M. and Schmid, M. 2002. Distribution of telomeric $(TTAGGG)_n$ sequences in avian chromosomes. - Chromosoma 111: 215-227.

Nordfjäll, K., Larefalk, A., Lindgren, P., Holmberg, D. and Roos, G. 2005. Telomere length and heredity: indications of paternal inheritance. - Proc. Natl. Acad. Sci. USA 102: 16374-16378.

Pauliny, A., Wagner, R.H., Augustin, J., Szep, T. and Blomqvist D. 2006. Age-independent telomere length predicts fitness in two bird species. Mol. Ecol. 15: 1681-1687.

Thomas, P., Callaghan, N.J.O. and Fenech, M. 2008. Telomere length in white blood cells, buccal cells and brain tissue in and its variation with ageing and Alzheimer's disease. Mech. Ageing Dev. 129: 183-190.

Venkatesan, R.N. and Price, C. 1998. Telomerase expression in chickens: constitutive activity in somatic tissues and down-regulation in culture. - Proc. Natl. Acad. Sci. USA 95: 14763-14768.

Von Zglinicki, T. 2002. Oxidative stress shortens telomeres. - Trends Ecol. Evol. 27: 339-344.

Watson, J.D. 1972. Origin of concatameric T4 DNA. - Nature New Biology 239: 197-201.

Zann, R.A. 1996. The zebra finch: a synthesis of field and laboratory studies. Oxford University Press, Oxford.

Acknowledgments

We are grateful to John Laurie, Graham Law, Christine Gould, Alister Kirk, San Kim and Dorothy Armstrong for help with bird husbandry and the Natural Environment Research Council for funding (NE/C004353/1 to PM, LN and NBM). The authors would also like to thank Dr. Monika Mihm for help with qPCR. PB was funded by a fellowship from the Swiss National Research Foundation (PPOOA-109009).

Figure legends

Figure A.1:

Southern blot hybridized with ^{32}P γ-ATP labelled telomeric probe $(C_3TA_2)_4$ in zebra finches (A) and Alpine swifts (B). The size standard is a 48kb ladder ranging from 8 to 48 kb (second standard on the other end of the gel not shown). Pulse field electrophoresis was used to resolve the length of telomere fragments larger than the usual upper limit of 23 kb. The mean TRF length was calculated within the outlined window, with reference to the size of the standard (shown in kilobases), using TotalLab TL100 software. Lane 5 in the zebra finch autoradiographic smear failed.

Figure A.2:

Agarose gel electrophoresis of qPCR products obtained on 6 different zebra finch samples, both for GAPDH and telomere gene amplification. qPCR amplicons were separated in 3% agarose gel run in standard TBE buffer (100 V), and visualized by ethidium bromide staining. Amplification of GAPDH sequence (lanes 1-6, right handside) led to a product size of around 50 pb, which was expected by the alignment of primers on zebra finch GAPDH gene sequence (not shown). For telomere qPCR products, a smear with greatest intensity around the lowest predicted size (78 pb) was observed (lanes 7-12 on the left hand side). The qPCR amplification of this shortest product is expected to be proportional to the total telomere length in each sample.

Figure A.3:

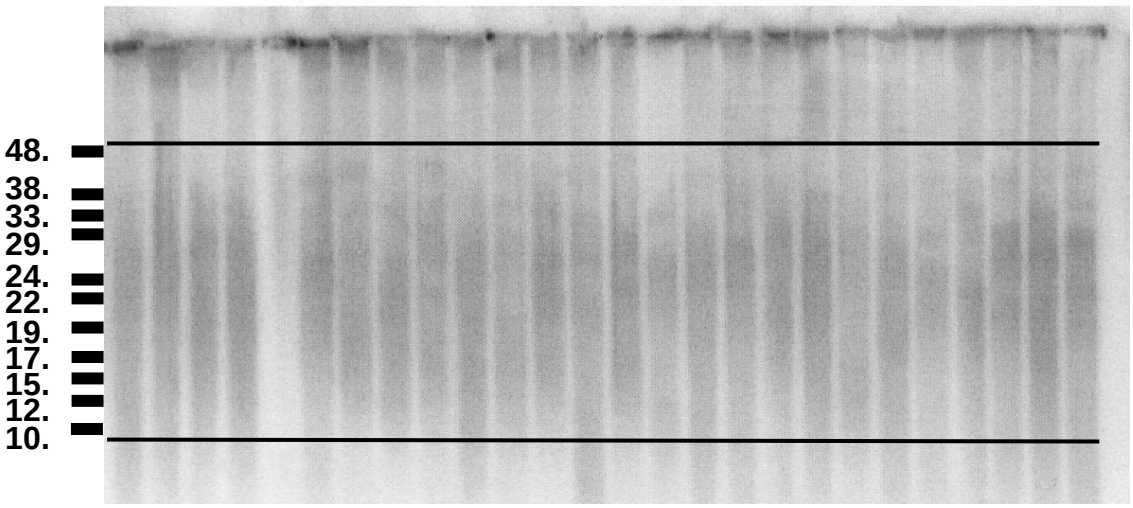
Relationship between relative telomere length obtained by quantitative PCR and mean TRF lengths determined by Southern blot analysis in zebra finches (A) and Alpine swifts (B). T/S ratios have a value $0 > x > 2.5$ because they are relative to the mean ratio (15.08 in zebra finches and 14.43 in Alpine swifts) observed among the samples The linear regression line best fitting the data are shown for each species. The equations were $y = 4.33x + 20.13$, $R^2 = 0.67$, $p<0.0001$ and $y = 7.19x + 9.63$, $R^2 = 0.55$, $p<0.0016$ for the zebra finch and Alpine swift, respectively.

Table A.1 Intra-plate correlation (Pearson correlation co-efficients) for the duplicate measurements of the telomere assay and for the duplicate GAPDH assay from red blood cell DNA samples for 26 zebra finches and 15 Alpine swifts using qPCR. The mean intra-plate coefficient of variation, calculated for each sample as (100*SD/mean value), is also given (± SE) for each species.

			Coefficient of correlation (r)	Coefficient of variation
Zebra finch		C_t^{GAPDH}	0.78	0.95 ± 0.18%
		$C_t^{telomere}$	0.83	2.00 ± 0.34%
Alpine swift		C_t^{GAPDH}	0.84	1.17 ± 0.21%
		$C_t^{telomere}$	0.92	2.37 ± 0.47%

Figure A.1

a



b

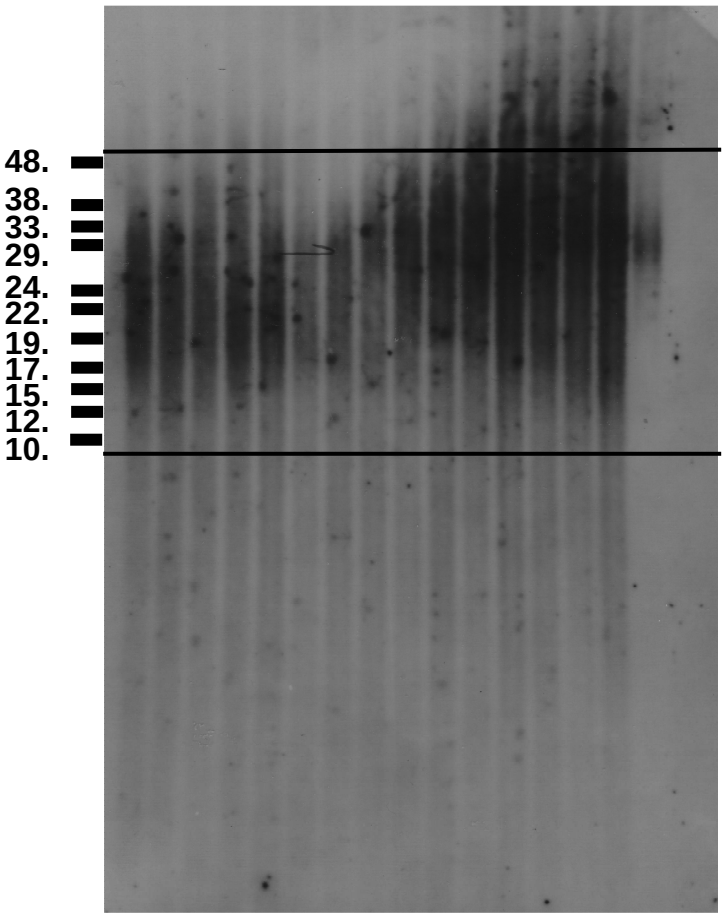


Figure A.2

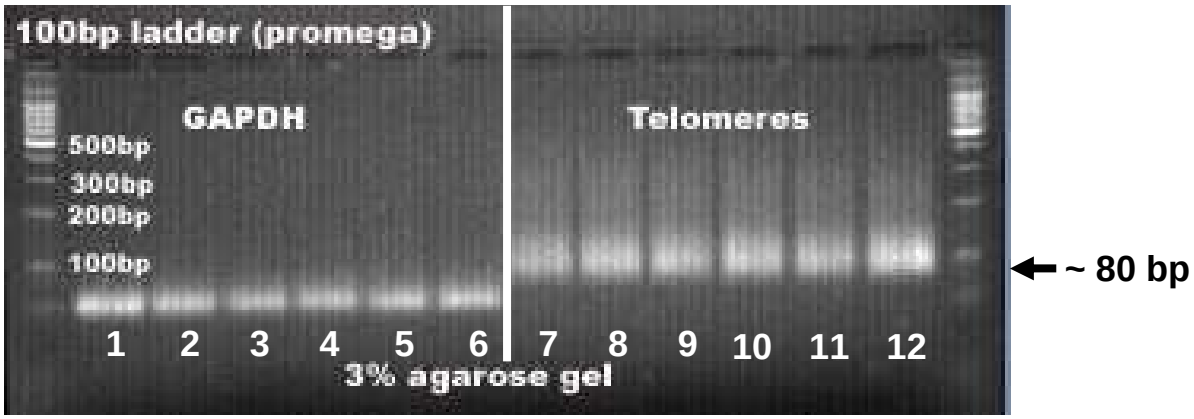
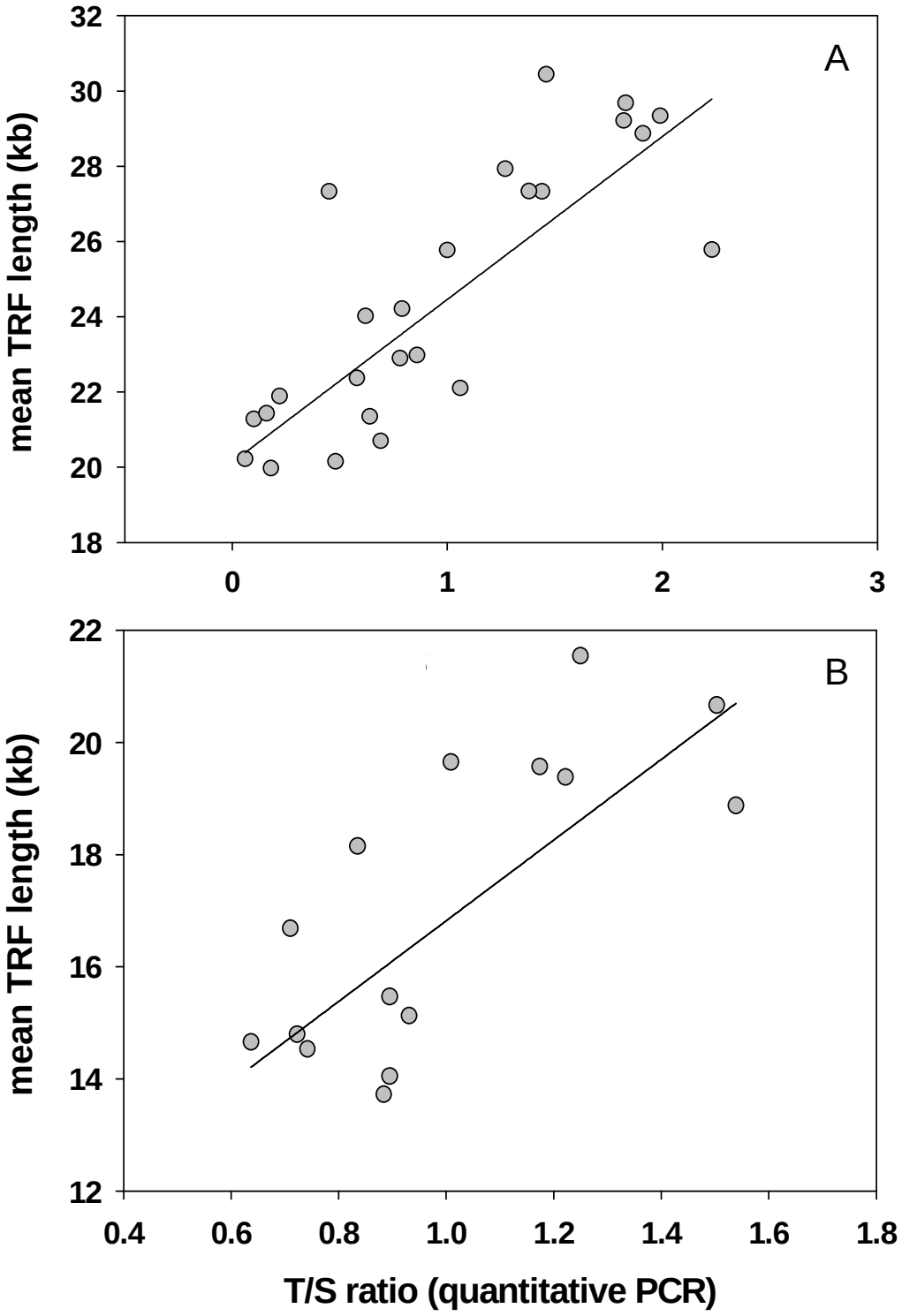

100bp ladder (promega)
GAPDH
Telomeres
500bp
300bp
200bp
100bp
1 2 3 4 5 6 7 8 9 10 11 12
3% agarose gel
~ 80 bp

Figure A.3


A
B
mean TRF length (kb)
mean TRF length (kb)
T/S ratio (quantitative PCR)
32
30
28
26
24
22
20
18
0
1
2
3
22
20
18
16
14
12
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8

References

Allsopp RC, Vaziri H, Patterson C, *et al.* (1992) Telomere length predicts replicative capacity of human fibroblasts. *Proceedings of the National Academy of Sciences, USA,* **89,** 10114-10118.

Andrew T, Aviv A, Falchi M, *et al.* (2006) Mapping genetic loci that determine leukocyte telomere length in a large sample of unselected female sibling pairs. *The American Journal of Human Genetics,* **78,** 480-486.

Arendt JD (1997) Adaptive intrinsic growth rates: An integration across taxa. *The Quarterly Review of Biology,* **72,** 149-177.

Arendt JD (2003) Reduced burst speed is a cost of rapid growth in anuran tadpoles: problems of autocorrelation and inferences about growth rates. *Functional Ecology,* **17,** 328-334.

Baerlocher GM, Rice K, Vulto I & Lansdorp PM (2007) Longitudinal data on telomere length in leukocytes from newborn baboons support a marked drop in stem cell turnover around 1 year of age. *Aging Cell,* **6,** 121-123.

Baird DM (2006) Telomeres. *Experimental Gerontology,* **41,** 1223-1227.

Baird DM, Britt-Compton B, Rowson J & Amso NN (2006) Telomere instability in the male germline. *Human Molecular Genetics,* **15,** 45-51.

Baird DM, Rowson J, Wynford-Thomas D & Kipling D (2003) Extensive allelic variation and ultrashort telomeres in senescent human cells. *Nature Genetics,* **33,** 203-207.

Barja G, Cadenas S, Rojas C, Perez-Campo R & Lopez-Torres M (1994) Low mitochondrial free radical production per unit O 2 consumption can explain the simultaneous presence of high longetivity and high aerobic metabolic rate in birds. *Free Radical Reseach,* **21,** 317-328.

Barja G & Herrero A (2000) Oxidative damage to mitochondrial DNA is inversely correlated to maximum lifespan in the heart and brain of mammals. *FASEB Journal,* **14,** 312-318.

Barker DJP (2006) Chronic disease in humans originates in the intra-uterine environment. *Animal Reproduction Science,* **94,** 343-354.

Barker DJP, Osmond C, Forsén TJ, Kajantie E & Eriksson JG (2005) Trajectories of growth among children who have coronary events as adults. *The New England Journal of Medicine,* **353,** 1802-1809.

Bateson P (2003) The promise of behavioural biology. *Animal Behaviour,* **65,** 11-17.

Baur JA, Zou Y, Shay JW & Wright WA (2001) Telomere position effect in human cells. *Science,* **292,** 2075-2077.

Bechter OE, Zou Y, Walker W, Wright WE & Shay JW (2004) Telomeric recombination in mismatch repair deficient human colon cancer cells after telomerase inhibition. *Cancer Research,* **64,** 3444-3451.

Benetos A, Okuda K, Lajemi M, *et al.* (2001) Telomere length as an indicator of biological aging: the gender effect and relation with pulse pressure and pulse wave velocity. *Hypertension,* **37,** 381-385.

Bianchi A, Smith S, Chong L, Elias P & de Lange T (1997) TRF1 is a dimer and bends telomeric DNA. *Embo Journal,* **16,** 1785-1794.

Bianchi A, Stansel RM, Fairall L, Griffith JD, Rhodes D & de Lange T (1999) TRF1 binds a bipartite telomeric site with extreme spatial flexibility. *Embo Journal,* **18,** 5735-5744.

Bischoff C, Graakjaer J, Andersen-Ranberg K, Vaupel J, Bohr VA, Kolvraa S & Christensen K (2006) No association between telomere length and survival among the elderly and oldest old. *Epidemiology,* **17,** 190-194.

Bize P, Criscuolo F, Metcalfe NB, Nasir L & Monaghan P Telomere dynamics rather than age predict life expectancy in the wild. *In prep.*

Blackburn EH (2005) Telomeres and telomerase: their mechanisms of action and the effects of altering their functions. *FEBS Letters,* **579,** 859-862.

Blount JD, Surai PF, Nager RG, Houston DC, Moller AP, Trewby ML & Kennedy MW (2002) Carotenoids and egg quality in the lesser black-backed gull *Larus fuscus*: a supplemental feeding study of maternal effects. *Proceedings of the Royal Society of London. Series B, Biological Sciences,* **269,** 29-36.

Bodnar AG, Ouellette M, Frolkis M, *et al.* (1998) Extension of Life-Span by Introduction of Telomerase into Normal Human Cells. *Science,* **279,** 349-352.

Bolton M (1991) Determinants of chick survival of in the lesser black-backed gull: relative contributions of egg size and parental quality. *Journal of Animal Ecology,* **60,** 949-960.

Bolton M, Houston DC & Monaghan P (1992) Nutritional constraints on egg formation in the lesser black-backed gull: An experimental study. *Journal of Animal Ecology,* **61,** 521-532.

Bourne WJP & Warham J (1966) Geographical variation in the giant petrels of the genus *Macronectes*. *Ardea,* **54,** 45-67.

Britt-Compton B, Rowson J, Locke M, Mackenzie I, Kipling D & Baird DM (2006) Structural stability and chromosome-specific telomere length is governed by cis-acting determinants in humans. *Human Molecular Genetics,* **15,** 725-733.

Brümmendorf TH, Mak J, Sabo KM, Baerlocher GM, Dietz K, Abkowitz JL & Lansdorp PM (2002) Longitudinal studies of telomere length in feline blood cells: Implications for hematopoietic stem cell turnover in vivo. *Experimental Hematology,* **30,** 1147-1152.

Bryan TM, Englezou A, Gupta J, Bacchetti S & Reddel RR (1995) Telomere elongation in immortal human cells without detectable telomerase activity. *Embo Journal,* **14,** 4240-4248.

Burnham KP & Anderson DR (2002) *Model selection and multimodel inference: A practical information-theoretic approach,* 2nd Ed., Springer, New-York

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

Campisi J & d'Adda di Fagagna F (2007) Cellular senescence: when bad things happen to good cells. *Nature Reviews: Molecular Cell Biology,* **8,** 729-740.

Campisi J, Kim S, Lim C & Rubio M (2001) Cellular senescence, cancer and ageing: the telomere connection. *Experimental Gerontology,* **36,** 1619-1637.

Capper R, Britt-Compton B, Tankimanova M, *et al.* (2007) The nature of telomere fusion and a definition of the critical telomere length in human cells. *Genes and Development,* **21,** 2495-2508.

Cawthon RM (2002) Telomere measurement by quantitative PCR. *Nucleic Acids Research,* **30,** e47-e47.

Cawthon RM, Smith KR, O'Brien E, Sivatchenko A & Kerber RA (2003) Association between telomere length in blood and mortality in people aged 60 years or older. *The Lancet,* **361,** 393-395.

Celli G & De Lange T (2005) DNA processing not required for ATM-mediated telomere damage response after TRF2 deletion. *Nature Cell Biology,* **7,** 712-718.

Chan SRWL & Blackburn EH (2004) Telomeres and telomerase. *Proceedings of the Royal Society of London. Series B, Biological Sciences,* **359,** 109-121.

Chang B-D, Watanabe K, Broude EV, Fang J, Poole JC, Kalinichenko TV & Roninson IB (2000) Effects of p21Waf1/Cip1/Sdi1 on cellular gene expression: Implications for carcinogenesis, senescence, and age-related diseases. *Proceedings of the National Academy of Sciences, USA,* **97,** 4291-4296.

Chang E & Harley CB (1995) Telomere length and replicative aging in human vascular tissues. *Proceedings of the National Academy of Sciences, USA,* **92,** 11190-11194.

Cherif H, Tarry JL, Ozanne SE & Hales CN (2003) Ageing and telomeres: a study into organ- and gender-specific telomere shortening. *Nucleic Acids Research,* **31,** 1576-1583.

Cimino-Reale G, Pascale E, Battiloro E, Starace G, Verna R & D'Ambrosio E (2001) The length of telomeric G-rich strand 3'-overhang measured by oligonucleotide ligation assay. *Nucleic Acids Research,* **29,** e35.

Copello S, Quintana F & Somoza G (2006) Sex determination and sexual size-dimorphism in Southern Giant-Petrels (*Macronectes giganteus*) from Patagonia, Argentina. *Emu,* **106,** 141-146.

Court R, Chapman L, Fairall L & Rhodes D (2005) How the human telomeric proteins TRF1 and TRF2 recognize telomeric DNA: A view from high-resolution crystal structures. *Embo Reports,* **6,** 39-45.

Crawley MJ (2002) *Statistical computing: an introduction to data analysis using S-plus,* Chichester, Hoboken, NJ

Criscuolo F, Bize P, Nasir L, *et al.* (2008) Real time quantitative PCR assay for measurements of bird telomeres. *Journal of Avian Biology,* **In Press**.

Cristofalo VJ, Lorenzini A, Allen RG, Torres C & Tresini M (2004) Replicative senescence: a critical review. *Mechanisms of Ageing and Development,* **125,** 827-848.

Croxall JP (1982) Sexual dimorphism in Snow Petrels *Pagodroma nivea*. *Notornis,* **29,** 171-180.

d'Adda di Fagagna F, Reaper PM, Clay-Farrace L, *et al.* (2003) A DNA damage checkpoint response in telomere-initiated senescence. *Nature,* **426,** 194-198.

Daunt F, Monaghan P, Wanless S, Harris MP & Griffiths R (2001) Sons and daughters: age specific differences in parental rearing capacities. *Functional Ecology,* **15,** 211-216.

de Lange T (2005) Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes and Development,* **19,** 2100-2110.

Dimri GP, Lee X, Basile G, *et al.* (1995) A Biomarker that Identifies Senescent Human Cells in Culture and in Aging Skin in vivo. *Proceedings of the National Academy of Sciences, USA,* **92,** 9363-9367.

Dunham MA, Neumann AA, Fasching CL & Reddel RR (2000) Telomere maintenance by recombination in human cells. *Nature Genetics,* **26,** 447-450.

Epel ES, Blackburn EH, Lin J, Dhabhar FS, Adler NE, Morrow JD & Cawthon RM (2004) Accelerated telomere shortening in response to life stress. *Proceedings of the National Academy of Sciences, USA,* **101,** 17312-17315.

Epel ES, Lin J, Wilhelm FH, *et al.* (2006) Cell aging in relation to stress arousal and cardiovascular disease risk factors. *Psychoneuroendocrinology,* **31,** 277-287.

Finkel T & Holbrook NJ (2000) Oxidants, oxidative stress and the biology of ageing. *Nature,* **408,** 239-247.

Flanary BE & Kletetschka G (2005) Analysis of telomere length and telomerase activity in tree species of various life-spans, and with age in the bristlecone pine *Pinus longaeva*. *Biogerontology,* **6,** 101-111.

Fraga CG, Shigenaga MK, Park J, Degan P & Ames BN (1990) Oxidative damage to DNA during aging: 8-Hydroxy-2'-Deoxyguanosine in rat organ DNA and urine. *Proceedings of the National Academy of Sciences, USA,* **87,** 4533-4537.

Francis N, Gregg T, Owen R, Ebert T & Bodnar A (2006) Lack of age-associated telomere shortening in long-and short-lived species of sea urchins. *FEBS Letters,* **580,** 4713-4717.

Frenck RW, Blackburn EH & Shannon KM (1998) The rate of telomere sequence loss in human leukocytes varies with age. *Proceedings of the National Academy of Sciences, USA,* **95,** 5607-5610.

Funk WD, Wang CK, Shelton DN, Harley CB, Pagon GD & Hoeffler WK (2000) Telomerase expression restores dermal integrity to *in vitro*-aged fibroblasts in a reconstituted skin model. *Experimental Cell Research,* **258,** 270-278.

Furumoto K, Inoue E, Nagao N, Hiyama E & Miwa N (1998) Age-dependent telomere shortening is slowed down by enrichment of intracellular vitamin C via suppression of oxidative stress. *Life Sciences,* **63,** 935-948.

Gluckman PD & Hanson MA (2006) *Developmental origins of health and disease,* Cambridge University Press, Cambridge

González-Solís J (2004) Sexual size dimorphism in northern giant petrels: ecological correlates and scaling. *Oikos,* **105,** 247-254.

Gonzalez-Solis J, Croxall JP & Wood AG (2000) Sexual dimorphism and sexual segregation in foraging strategies of northern giant petrels, *Macronectes halli*, during incubation. *Oikos,* **90,** 390-398.

Gorbunova V & Seluanov A (2008) Coevolution of telomerase activity and body mass in mammals: From mice to beavers. *Mechanisms of Ageing and Development,* **In Press,** Corrected Proof.

Gottschling DE, Aparicio OM, Billington BL & Zakian VA (1990) Position effect at S. cerevisiae telomeres: Reversible repression of Pol II transcription. *Cell,* **63,** 751-762.

Graakjaer J, Bischoff C, Korsholm L, *et al.* (2003) The pattern of chromosome-specific variations in telomere length in humans is determined by inherited, telomere-near, factors and is maintained throughout life. *Mechanisms of Ageing and Development,* **124,** 629-640.

Graakjaer J, Der-Sarkissian H, Schmitz A, Bayer J, Thomas G, Kolvraa S & Londoño-Vallejo J-A (2006) Allele-specific relative telomere lengths are inherited. *Human Genetics,* **119,** 351-351.

Greider C & Blackburn EH (1985) Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts. *Cell,* **43,** 403-413.

Griffith JD, Comeau L, Rosenfield S, Stansel RM, Bianchi A, Moss H & de Lange T (1999) Mammalian Telomeres End in a Large Duplex Loop. *Cell,* **97,** 503-514.

Griffiths R (1992) Sex-biased mortality in the lesser backed gull *Larus fuscus* during the nestling stage. *Ibis,* **134,** 237-244.

Griffiths R, Double MC, Orr K & Dawson RJG (1998) A DNA test to sex most birds. *Molecular Ecology,* **7,** 1071-1075.

Hall ME, Nasir L, Daunt F, Gault EA, Croxall JP, Wanless S & Monaghan P (2004) Telomere loss in relation to age and early environment in long-lived birds. *Proceedings of the Royal Society of London. Series B, Biological Sciences,* **271,** 1571-1576.

Harley CB, Futcher AB & Greider C (1990) Telomeres shorten during aging of human fibroblasts. *Nature,* **345,** 458-460.

Haussmann MF & Mauck RA (2008a) New strategies for telomere-based age estimation. *Molecular Ecology Resources,* **8,** 264-274.

Haussmann MF & Mauck RA (2008b) Telomeres and Longevity: Testing an Evolutionary Hypothesis. *Molecular Biology and Evolution,* **25,** 220-228.

Haussmann MF, Winkler DW, Huntington CH, Nisbet ICT & Vleck CM (2004) Telomerase expression is differentially regulated in birds of differing life span. *Annals of the New York Academy of Sciences,* **1019,** 186-190.

Haussmann MF, Winkler DW, Huntington CH, Nisbet ICT & Vleck CM (2007) Telomerase activity is maintained throughout the lifespan of long-lived birds. *Experimental Gerontology,* **42,** 610-618.

Haussmann MF, Winkler DW, O'Reilly KM, Huntington CH, Nisbet ICT & Vleck CM (2003) Telomeres shorten more slowly in long-lived birds and mammals than in short-lived ones. *Proceedings of the Royal Society of London. Series B, Biological Sciences,* **270,** 1387-1392.

Haussmann MF, Winkler DW & Vleck CM (2005) Longer telomeres associated with higher survival in birds. *Biology Letters,* **1,** 212-214.

Hayflick L (1965) The limited in vitro lifetime of human diploid cell strains. *Experimental Cell Research,* **37,** 636.

Hayflick L (2003) Living forever and dying in the attempt. *Experimental Gerontology,* **38,** 1231-1241.

Hemann MT & Greider CW (2000) Wild-derived inbred mouse strains have short telomeres. *Nucleic Acids Research,* **28,** 4474-4478.

Hemann MT, Strong MA, Hao L & Greider C (2001) The shortest telomere, not average telomere length, is critical for cell stability and telomere viability. *Cell,* **107,** 67-77.

Henderson E (1995) Telomere DNA structure. IN Blackburn EH. & Greider C (Eds.) *Telomeres.* Cold Spring Harbor Laboratory Press.

Herbig U, Ferreira M, Condel L, Carey D & Sedivy JM (2006) Cellular senescence in aging primates. *Science,* **311,** 1257.

Herbig U, Jobling WA, Chen BPC, Chen DJ & Sedivy JM (2004) Telomere shortening triggers senescence of human cells through a pathway involving ATM, p53, and p21CIP1, but not p16INK4a. *Molecular Cell,* **14,** 501-513.

Hockemeyer D, Sfeir AJ, Shay JW, Wright WE & de Lange T (2005) POT1 protects telomeres from a transient DNA damage response and determines how human chromosomes end. *Embo Journal,* **20,** 2667-2678.

Holmes DJ & Austad SN (1995) The evolution of avian senescence patterns: Implications for understanding primary aging processes. *American Zoologist,* **35,** 307-317.

Holmes DJ, Flückiger R & Austad SN (2001) Comparative biology of ageing in birds: an update. *Experimental Gerontology,* **36,** 869-883.

Huffmann KE, Levene SD, Tesmer VM, Shay JW & Woodring EW (2000) Telomere shortening is proportional to the size of the G-rich telomeric 3'-overhang. *Journal of Biological Chemistry,* **275,** 19719-19722.

Hunter S (1984) Movements of giant petrels *Macronectes* spp. ringed at South Georgia. *Ring Migration,* **5,** 105-112.

Hunter S (1987) Species and sexual isolating mechanisms in sibling species of giant petrels *Macronectes*. *Polar Biology,* **7,** 295-301.

Ishii A, Nakamura K, Kishimoto H, *et al.* (2006) Telomere shortening with aging in the human pancreas. *Experimental Gerontology,* **41,** 882-886.

Jemielity S, Kimura M, Parker KM, Parker JD, Cao X, Aviv A & Keller L (2007) Short telomeres in short-lived males: what are the molecular and evolutionary causes? *Aging Cell,* **6,** 225-233.

Jennings BJ, Ozanne SE, Dorling MW & Hales CN (1999) Early growth determines longevity in male rats and may be related to telomere shortening in the kidney. *FEBS Letters,* **448,** 4-8.

Jennings BJ, Ozanne SE & Hales CN (2000) Nutrition, oxidative damage, telomere shortening, and cellular senescence: individual or connected agents of ageing? *Molecular genetics and metabolism,* **71,** 32-42.

Joeng KS, Song EJ, Lee KJ & Lee J (2004) Long lifespan in worms with long telomeric DNA. *Nature Genetics,* **36,** 607-611.

Johnson JB & Omland KS (2004) Model selection in ecology and evolution. *Trends in Ecology & Evolution,* **19,** 101-108.

Juola FA, Haussmann MF, Dearborn DC & Vleck CM (2006) Telomere shortening in a long-lived marine bird: cross-sectional analysis and test of an aging tool. *The Auk,* **123,** 775-783.

Kakuo S, Asaoka K & Ide T (1999) Human is a unique species among primates in terms of telomere length. *Biochemical and Biophysical Research Communications,* **263,** 308-314.

Kanai N, Fujii T, Saito K & Tokoyama T (1994) Rapid and simple method for preparation of genomic DNA from easily obtainable clotted blood. *Journal of Clinical Pathology,* **47,** 1043-1044.

Kim NW, Piatyszek MA, Prowse KR, *et al.* (1994) Specific association of human telomerase activity with immortal cells and cancer. *Science,* **266,** 2011-2015.

Kotrschal A, Ilmonen P & Penn DJ (2007) Stress impacts telomere dynamics. *Biology Letters,* **3,** 128-130.

Kruk PA, Rampino NJ & Bohr VA (1995) DNA damage and repair in telomeres: relation to aging. *Proceedings of the National Academy of Sciences, USA,* **92,** 258-262.

Ku HH & Sohal RS (1993) Comparison of mitochondrial pro-oxidant generation and anti-oxidant defenses between rat and pigeon: possible basis of variation in longevity and metabolic potential. *Mechanisms of Ageing and Development,* **72,** 67-76.

Kurz DJ, Decary S, Hong Y, Trivier E, Akhmedov A & Erusalimsky JD (2004) Chronic oxidative stress compromises telomere integrity and accelerates the onset of senescence in human endothelial cells. *Journal of Cell Science,* **117,** 2426.

Lessells CM & Boag PT (1987) Unrepeatable repeatabilities: a common mistake. *The Auk,* **104,** 116-121.

Li B, Oestreich S & de Lange T (2000) Identification of human Rap1: Implications for telomere evolution. *Cell,* **101,** 471-483.

Liu D, O'Connor MS, Qin J & Songyang Z (2004a) Telosome, a mammalian telomere-associated complex formed by multiple telomeric proteins. *Journal of Biological Chemistry,* **279,** 51338-51342.

Liu D, Safari A, O'Connor MS, Chan DW, Laegeler A, Qin J & Songyang Z (2004b) PTOP interacts with POT1 and regulates its localization to telomeres. *Nature Cell Biology,* **6,** 673-680.

Loayza D & De Lange T (2003) POT1 as a terminal transducer of TRF1 telomere length control. *Nature,* **424,** 1013-1018.

Loayza D, Parsons H, Donigian JR, Hoke K & de Lange T (2004) DNA binding features of human POT1: A nonamer 5'-TAGGGTTAG-3' minimal binding site, sequence specificity, and internal binding to multimeric sites. *Journal of Biological Chemistry,* **279,** 13241-13248.

Louis EJ & Vershinin AV (2005) Chromosome ends: different sequences may provide conserved functions. *BioEssays,* **27,** 685-697.

Makarov VL, Hirose Y & Langmore JP (1997) Long G tails at both ends of human chromosomes suggest a C strand degradation mechanism for telomere shortening. *Cell,* **88,** 657-666.

Mantell LL & Greider C (1994) Telomerase activity in germline and embryonic cells of *Xenopus*. *Embo Journal,* **13,** 3211-3217.

Martens UM, Chavez EA, Poon SSS, Schmoor C & Lansdorp PM (2000) Accumulation of short telomeres in human fibroblasts prior to replicative senescence. *Experimental Cell Research,* **256,** 291-299.

Martin-Ruiz C, Gussekloo J, van Heemst D & Von Zglinicki T (2005) Telomere length in white blood cells is not associated with morbidity or mortality in the oldest old: a population based study. *Aging Cell,* **4,** 287-290.

Martin-Ruiz C, Saretzki G, Petrie J, *et al.* (2004) Stochastic variation in telomere shortening rate causes heterogeneity of human fibroblast replicative life span. *The Journal of Biological Chemistry,* **279,** 17826-17833.

McClintock B (1938) The fusion of broken ends of sister half-chromatids following chromatid breakage at meiotic anaphase. *Res. Bull. Mo. Agric. Esp. Stn,* **290,** 1-48.

McClintock B (1942) The stability of broken ends of chromosomes following nuclear fusion. *Proceedings of the National Academy of Sciences, USA,* **28,** 458-463.

Metcalfe NB & Monaghan P (2001) Compensation for a bad start: grow now, pay later? *Trends in Ecology & Evolution,* **16,** 254-260.

Metcalfe NB & Monaghan P (2003) Growth versus lifespan: perspectives from evolutionary ecology. *Experimental Gerontology,* **38,** 935-940.

Meyne J, Ratliff RL & Moyzis RK (1989) Conservation of the Human Telomere Sequence (TTAGGG)n among Vertebrates. *Proceedings of the National Academy of Sciences, USA,* **86,** 7049-7053.

Monaghan P (2008) Early growth conditions, phenotypic development and environmental change. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences,* **363,** 1635-1645.

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

Muller HJ (1938) The remaking of chromosomes. *Collect. Net.,* **13,** 182-198.

Murnane JP, Sabatier L, Marder BA & Morgan WF (1994) Telomere dynamics in an immortal human cell line. *Embo Journal,* **13,** 4953-4962.

Nager RG, Monaghan P, Griffiths R, Houston DC & Dawson R (1999) Experimental demonstration that offspring sex ratio varies with maternal condition. *Proceedings of the National Academy of Sciences, USA,* **96,** 570-573.

Nager RG, Monaghan P & Houston DC (2000a) Within-clutch trade-offs between the number and quality of eggs: experimental manipulations in gulls. *Ecology,* **81,** 1339-1350.

Nager RG, Monaghan P, Houston DC & Genovart M (2000b) Parental condition, brood sex ratio and differential young survival: an experimental study in gulls (*Larus fuscus*). *Behavioural Ecology and Sociobiology,* **48,** 452-457.

Nakagawa S, Gemmel NJ & Burke T (2004) Measuring vertebrate telomeres: applications and limitations. *Molecular Ecology*, **13,** 2523-2533.

Nakamura K, Takubo K, Izumiyama-Shimomura N, *et al.* (2007) Telomeric DNA length in cerebral gray and white matter is associated with longevity in individuals aged 70 years or older. *Experimental Gerontology,* **42,** 944-950.

Nasir L, Devlin P, Mckevitt T, Rutteman G & Argyle DJ (2001) Telomere lengths and telomerase activity in dog tissues: A potential model system to study human telomere and telomerase biology. *Neoplasia,* **3,** 351-359.

Nawrot TS, Staessen JA, Gardner JP & Aviv A (2004) Telomere length and possible link to X chromosome. *The Lancet,* **363,** 507-510.

Nelson JB (1978) The Sulidae: gannets and boobies. Oxford University Press, Oxford.

Njajou OT, Cawthon RM, Damcott CM, *et al.* (2007) Telomere length is paternally inherited and is associated with parental lifespan. *Proceedings of the National Academy of Sciences, USA,* **104,** 12135-12139.

Nordfjäll K, Larefalk Å, Lindgren P, Holmberg D & Roos G (2005) Telomere length and heredity: Indications of paternal inheritance. *Proceedings of the National Academy of Sciences, USA,* **102,** 16374-16378.

Nosek J, Kosa P & Tomaska L (2006) On the origins of telomeres: a glimpse at the pre-telomerase world. *BioEssays*, **28,** 182-190.

Ogburn CE, Austad SN, Holmes DJ, Kiklevich JV, Gollahon K, Rabinovitch PS & Martin GM (1998) Cultured renal epithelial cells from birds and mice: enhanced resistance of avian cells to oxidative stress and DNA damage. *Journal of Gerontology,* **A53,** B287-B292.

Olovnikov AM (1973) A theory of marginotomy: the incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the problem. *Journal of Theoretical Biology,* **41,** 181-190.

Packer L & Fuehr K (1977) Low oxygen concentration extends the lifespan of cultured human diploid cells. *Nature*, **267,** 423-425.

Pamplona R, Prat J, Cadenas S, Rojas C, P,rez-Campo R, Lop,z-Torres M & Barja G (1996) Low fatty acid unsaturation protects against lipid peroxidation in liver mitochondrial cells from long-lived species: the pigeon and human case. *Mechanisms of Ageing and Development,* **86,** 53-66.

Parrinello S, Coppe J-P, Krtolica A & Campisi J (2005) Stromal-epithelial interactions in aging and cancer: senescent fibroblasts alter epithelial cell differentiation. *Journal of Cell Science,* **118,** 485-496.

Pauliny A, Wagner RH, Augustin J, Szép T & Blomqvist D (2006) Age-independent telomere length predicts fitness in two bird species. *Molecular Ecology,* **15,** 1681-1687.

Petersen S, Saretzki G & von Zglinicki T (1998) Preferential accumulation of single-stranded regions in telomeres of human fibroblasts. *Experimental Cell Research,* **239,** 152-160.

Price JS, Waters JG, Darrah C, Pennington C, Edwards DR, Donell ST & Clark IM (2002) The role of chondrocyte senescence in osteoarthritis. *Aging Cell,* **1,** 57-65.

Prowse KR & Greider C (1995) Developmental and tissue-specific regulation of mouse telomerase and telomere length. *Proceedings of the National Academy of Sciences, USA,* **92,** 4818-4822.

Rahman R, Forsyth NR & Cui W (2008) Telomeric 3'-overhang length is associated with the size of telomeres. *Experimental Gerontology,* **43,** 258-265.

Raices M, Maruyama H, Dillin A & Karlseder J (2005) Uncoupling of longevity and telomere length in *C.elegans*. *PLOS Genetics,* **1,** 295-301.

Richter T & von Zglinicki T (2007) A continuous correlation between oxidative stress and telomere shortening in fibroblasts. *Experimental Gerontology*, **42,** 1039-1042.

Rohme D (1981) Evidence for a Relationship between Longevity of Mammalian Species and Life Spans of Normal Fibroblasts in vitro and Erythrocytes in vivo. *Proceedings of the National Academy of Sciences, USA,* **78,** 5009-5013.

Rollo CD (2002) Growth negatively impacts the lifespan of animals. *Evolution and Development,* **4,** 56-61.

Royle N (2000) Overproduction in the lesser black-backed gull - can marginal chicks overcome the initial handicap of hatching asynchrony? *Journal of Avian Biology,* **31,** 335-344.

Royle N, Surai PF & Hartley IR (2001) Maternally derived androgens and antioxidants in bird eggs: complementary but opposing effects? *Behavioural Ecology,* **12,** 381-385.

Rubin H (2002) Promises and problems in relating cellular senescence in vitro to ageing in vivo. *Archives of Gerontology and Geriatrics,* **34,** 275-286.

Rufer N, Dragowska W, Thornbury G, Roosnek E & Landsdorp PM (1998) Telomere length dynamics in human lymphocyte sub-populations measured by flow cytometry. *Nature Biotechnology,* **16,** 743-747.

Saretzki G, Sitte N, Merkel U, Wurm RE & Von Zglinicki T (1999) Telomere shortening triggers a p53-dependant cell cycle arrest via accumulation of G-rich single stranded DNA fragments. *Oncogene,* **18,** 5148-5158.

Schaetzlein S, Lucas-Hahn A, Lemme E, *et al.* (2004) Telomere length is reset during early mammalian embryogenesis. *Proceedings of the National Academy of Sciences, USA,* **101,** 8034-8038.

Sedivy J, Shippen DE & Shakirov EV (2003) Surprise ending. *Nature Genetics,* **33,** 114-116.

Seluanov A, Chen Z, Hine C, *et al.* (2007) Telomerase activity coevolves with body mass not lifespan. *Aging Cell,* **6,** 45-52.

Serra V, von Zglinicki T, Lorenzi M & Saretzki G (2003) Extracellular superoxide dismutase is a major antioxidant in human fibroblasts and slows telomere shortening. *The Journal of Biological Chemistry,* **278,** 6824-6830.

Sfeir AJ, Chai W, Shay JW & Wright WE (2005) Telomere-end processing the terminal nucleotides of human chromosomes. *Molecular Cell,* **18,** 131-138.

Shay JW & Bacchetti S (1997) A survey of telomerase activity in human cancers. *European Journal of Cancer,* **33,** 787-791.

Shelton DN, Chang E, Whittier PS, Choi D & Funk WD (1999) Microarray analysis of replicative senescence. *Current Biology,* **9,** 939-945.

Simon NM, Smoller JW, McNamara KL, *et al.* (2006) Telomere Shortening and Mood Disorders: Preliminary Support for a Chronic Stress Model of Accelerated Aging. *Biological Psychiatry,* **60,** 432-435.

Smith JR & Hayflick L (1974) Variation in the life-span of clones derived from human diploid cell strains. *Journal of Cell Biology,* **62,** 48-53.

Sohal RS, Svensson I & Brunk U (1990) Hydrogen peroxide production by liver mitochondria in different species. *Mechanisms of Ageing and Development,* **53,** 209-215.

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology,* **98,** 503-517.

Stansel RM, de Lange T & Griffith JD (2001) T-loop assembly in vitro involves binding of TRF2 near the 3' telomeric overhang. *Embo Journal,* **20,** E5532-E5540.

Starck JM & Ricklefs RE (1998) *Avian growth and development*, Oxford University Press, Oxford

Starr JM, McGurn B, Harris SE, Whalley LJ, Deary IJ & Shiels PG (2007) Association between telomere length and heart disease in a narrow age cohort of older people. *Experimental Gerontology,* **42,** 571-573.

Takai H, Smogorzewska A & de Lange T (2003) DNA Damage Foci at Dysfunctional Telomeres. *Current Biology,* **13,** 1549-1556.

Tarry-Adkins JL, Ozanne SE, Norden A, Cherif H & Hales CN (2006) Lower antioxidant capacity and elevated p53 and p21 may be a link between gender disparity in renal telomere shortening, albuminuria, and longevity. *American Journal of Physiology - Renal Physiology,* **290,** F509-F516.

Unryn BM, Cook LS & Riabowol KT (2005) Paternal age is positively linked to telomere length of children. *Aging Cell,* **4,** 97-101.

Valdes AM, Andrew T, Gardner JP*, et al.* (2005) Obesity, cigarette smoking, and telomere length in women. *The Lancet,* **366,** 662-664.

Vasile E, Tomita Y, Brown LF, Kocher O & Dvorak HF (2001) Differential expression of thymosin {beta}-10 by early passage and senescent vascular endothelium is modulated by VPF/VEGF: evidence for senescent endothelial cells in vivo at sites of atherosclerosis. *FASEB Journal,* **15,** 458-466.

Venkatesan RN & Price C (1998) Telomerase expression in chickens: constitutive activity in somatic tissues and down-regulation in culture. *Proceedings of the National Academy of Sciences, USA,* **95,** 14763-14768.

Verboven N, Monaghan P, Evans DM, Schwabl H, Evans N, Whitelaw C & Nager RG (2003) Maternal condition, yolk androgens and offspring performance: a supplemental feeding experiment in the lesser black-backed gull (*Larus fuscus*). *Proceedings of the Royal Society of London. Series B, Biological Sciences,* **270,** 2223-2232.

Vleck CM, Haussmann MF & Vleck D (2003) The natural history of telomeres: tools for ageing animals and exploring the ageing process. *Experimental Gerontology,* **38,** 791-795.

von Zglinicki T (2002) Oxidative stress shortens telomeres. *TRENDS in Biochemical Sciences,* **27,** 339-344.

von Zglinicki T (2003) Replicative senescence and the art of counting. *Experimental Gerontology,* **38,** 1259-1264.

von Zglinicki T, Pilger R & Sitte N (2000) Accumulation of single-strand breaks is the major cause of telomere shortening in human fibroblasts. *Free Radical Biology and Medicine,* **28,** 64-74.

von Zglinicki T & Schewe C (1998) Mitochondrial water loss and ageing of cells. *Cell Biochemistry Function,* **13,** 181-187.

Walter MF, Biessmann MR, Benitez C, Török T, Mason JM & Biessmann H (2007) Effects of telomere length in *Drosophila melanogaster* on life span, fecundity, and fertility. *Chromosoma,* **116,** 41-51.

Watson JD (1972) Origin of concatameric T4 DNA. *Nature New Biology,* **239,** 197-201.

Wright WA, Tesmer VM, Huffmann KE, Levene SD & Shay JW (1997) Normal human chromosomes have long G-rich telomeric overhangs at one end. *Genes and Development,* **11,** 2801-2809.

Ye JZ-S, Donigian JR, van Overbeek M, *et al.* (2004) TIN2 binds TRF1 and TRF2 simultaneously and stabilizes the TRF2 complex on telomeres. *Journal of Biological Chemistry,* **279,** 47264-47271.

Zeichner SL, Palumbo P, Feng Y, *et al.* (1999) Rapid Telomere Shortening in Children. *Blood,* **93,** 2824-2830.

Zijlmans JM, Martens UM, Poon SSS, Raap AK, Tanke HJ, Ward RK & Lansdorp PM (1997) Telomeres in the mouse have large inter-chromosomal variations in the number of T2AG3 repeats. *Proceedings of the National Academy of Sciences, USA,* **94,** 7423-7428.

Zou Y, Sfeir A, Gryaznov SM, Shay JW & Wright WE (2004) Does a sentinel or a subset of short telomeres determine replicative senescence? *Molecular Biology of the Cell,* **15,** 3709-3718.