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Telomere dynamics rather than age predict life expectancy in the wild

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Despite accumulating evidence from *in vitro* studies that cellular senescence is linked to telomere dynamics, how this relates to whole-organism senescence and longevity is poorly understood and controversial. Using data on telomere length in red blood cells and long-term survival from wild Alpine swifts of a range of ages, we report that the telomere length and the rate of telomere loss are predictive of life expectancy, and that slow erosion of relatively long telomeres is associated with the highest survival probabilities. Importantly, because telomere dynamics, rather than chronological age, predict life expectancy, our study provides good evidence for a mechanistic link between telomere erosion and reduced organism longevity under natural conditions, chronological age itself possibly not becoming a significant predictor until very old ages beyond those in our sample.

Keywords: ageing; Alpine swift; lifespan; longitudinal data; natural populations; telomere dynamics

1. INTRODUCTION

Telomeres are specialized nucleotide repeat sequences at the ends of eukaryotic chromosomes. They shorten at each cell division, in part because the normal DNA replication process does not fully copy the chromosome end (Blackburn 1991; Blasco 2007). In addition to the end replication problem (Blackburn 1991; Blasco 2007), there is good evidence that telomere erosion is accelerated by other factors, particularly oxidative stress (von Zglinicki 2002; Richter & von Zglinicki 2007). *In vitro* studies have shown that once a critical telomere length is reached, cells stop dividing and enter a state of replicative senescence, which may be followed by apoptosis (Blackburn 1991; Blasco 2007). Hence, it has been suggested that telomere length plays a crucial role in tissue functioning, and by extension, in the life expectancy of whole organisms (Blackburn 1991; Blasco 2007).

Studies in a range of organisms have shown that telomeres shorten with age in various somatic tissues (Allsopp *et al.* 1992; Jennings *et al.* 1999; Haussmann *et al.* 2003; Herbig *et al.* 2006; Jemielity *et al.* 2007), and individuals with relatively long telomeres have a greater life expectancy than those with short telomeres (Rudolph *et al.* 1999; Cawthon *et al.* 2003; Joeng *et al.* 2004; Haussmann *et al.* 2005; Pauliny *et al.* 2006). However, such an effect could be a consequence of either an initially long telomere length or a slow rate of telomere erosion. Comparison of the species-specific rates of telomere erosion calculated from the telomere lengths in different age categories suggests that telomere erosion rate might be most important, since species of birds and mammals with a low rate of telomere erosion have greater longevity (Haussmann *et al.* 2003). However, such cross-sectional studies have the disadvantage that differential survival of individuals with different initial telomere lengths can bias the estimation of erosion rates. A more direct test is whether the rate of telomere erosion within individuals is predictive of their future survival. Furthermore, the exposure and response to environmental factors that can influence telomere erosion might change under laboratory conditions. Therefore, we also need to know whether any relationships between telomere dynamics and life expectancy at the organismal level actually occur under natural conditions (Monaghan & Haussmann 2006).

Here, we make use of a long-term longitudinal study in a wild population of Alpine swifts (*Apus melba*) to investigate whether variation in the telomere length and the rate of telomere erosion in red blood cells (RBCs) are predictive of adult life expectancy. The Alpine swift is a 90 g insectivorous migratory bird that can live up to 26 years of age, with the median lifespan on reaching adulthood being 6 years ($n = 216$ individuals). Because adults return each year to breed in the same colony, often in the same nest, an annual census of breeders gives an accurate measure of individual survival rate (Bize *et al.* 2006). We show for the first time, to our knowledge, that both the telomere length and the rate of telomere erosion explain life expectancy in the wild, with slow erosion of relatively long telomeres being associated with the highest survival probabilities.

2. MATERIAL AND METHODS

(a) *Annual survival and life expectancy*

Data were obtained from a colony of approximately 100 breeding pairs in Bienne, Switzerland, where nestlings have

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been ringed each year since 1968 and adults have been the subject of an individual-based study since 2000 (Bize et al. 2006). Capture–recapture analyses have demonstrated that, in this study colony, the probability of recapturing breeders while they are sitting on eggs or hatchlings is virtually 1.0 (Bize et al. 2006). Moreover, because we have never observed breeding dispersal from Bienne to neighbouring colonies, we can confidently conclude that breeders which do not return to the colony from one year to the next are dead (Bize et al. 2006). To investigate the relationship between age, telomere length and life expectancy, we collected blood in 2001 from 98 adult breeders that had been ringed as nestlings, and hence were of known age (mean $\pm$ s.e. $= 6.9 \pm 0.4$ years; range: 2–19 years), and we followed their survival over the next 6 years until 2007. For each individual, we used its last year of capture to calculate its life expectancy between 2001 and 2007. That is, an individual that was last captured in 2001 was given a life expectancy of 0, in 2002 a life expectancy of 1, and so on. To investigate whether the rate of telomere erosion is a better predictor of future survival than the telomere length, in 2006, we re-sampled 22 individuals for which we had measures of their average RBC telomere length, in 2001. We analysed adult annual survival between 2006 and 2007, giving a score of 1 to individuals recaptured in 2007 and of 0 to individuals not recaptured in 2007.

(b) Blood sampling and genomic DNA extraction

We measured telomeres from whole-blood samples, since small blood samples can be collected without negative effects, blood is a highly mitotic tissue, and RBCs are nucleated in birds. Blood samples were collected from the foot of the birds, stored in EDTA buffer and preserved at $-20°C$ before analyses. Genomic DNA was extracted from whole-blood samples by using DNeasy Blood & Tissue Kit (Qiagen) and by following the manufacturer's protocol. The ratio of red : white blood cells is approximately 200 : 1 in birds (Sturkie 1986), and thus the telomere length measured from whole-blood samples provides measurements of the telomere length in the RBCs (hereafter referred as RBC telomere length).

(c) Telomere measurements

Telomere length was quantified using a recently described quantitative PCR assay developed to measure relative telomere length in humans (Cawthon 2002), which we have adjusted to measure relative telomere length in birds (Criscuolo et al. in press). Briefly, we measured relative telomere length by determining the ratio (T/S) of telomere repeat copy number (T) to single control gene copy number (S) in focal samples when compared with a reference sample. We used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a single control gene. Forward and reverse telomeric primers were 5′CGGTTTGTTTGGGTTT GGGTTTGGGTTTGGGTTTGGGTT-3′ (Tel1b) and 5′-GGCTTGCCTTACCCTTACCCTTACCCTTACCC TTACCCT-3′ (Tel2b), respectively, and forward and reverse primers for the GAPDH gene (GenBank accession no. AF255390) were 5-AACCAGCCAAGTACGATGACAT-3′ (GAPDH-F) and 5′-CCATCAGCAGCAGCCTTCA-3′ (GAPDH-R), respectively. Telomere and GAPDH real-time amplifications were carried out on two different plates. Each reaction for the telomere (or GAPDH) plates was performed using 20 ng of DNA with sets of primers Tel1b/Tel2b (or GAPDH-F/GAPDH-R), each used at a concentration

of 200 nM/200 nM, in a final volume of 25 μl containing 12.5 μl of Brilliant SYBR Green QPCR Master Mix (Stratagene). PCR conditions for the telomere portion of the assay were 10 min at 95°C followed by 30 cycles of 1 min at 56°C and 1 min at 95°C, while conditions for the GAPDH portion of the assay were 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 an Mx3000P QPCR System (Stratagene). To test the efficiency of each PCR reaction (accepted efficiency range $100 \pm 15\%$), a standard curve was produced in every plate by serially diluting one sample (40, 20, 10 and 5 ng of DNA per well) and by running it in triplicate. To be able to compare measurements among plates, one individual was used as a reference and run in triplicate on every plate. The threshold C_t of this reference sample was then calculated for each plate; 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 other samples were run in duplicate on the plates, and mean values per plate were used to calculate relative T/S ratios of target individual relative to the reference individual using the formula $2^{\Delta\Delta C_t}$, where $\Delta\Delta C_t = (C_t^{\text{telomere}} - C_t^{\text{GAPDH}})_{\text{reference}} - (C_t^{\text{telomere}} - C_t^{\text{GAPDH}})_{\text{target}}$ (introduction to quantitative PCR: methods and application guide by Stratagene 2007). Quantitative PCR was performed a minimum of two times (i.e. two telomere and two GAPDH plates) for each sample, and mean T/S ratios were used in the statistical analyses. Mean intraplate coefficient of variation was 1.17 per cent for the C_t values of GAPDH assays and 2.37 per cent for the C_t values of telomere assays (Criscuolo et al. in press), and interplate coefficient of variation was 17.09 per cent for ΔC_t values of the reference sample.

Since the quantitative PCR assay measures both terminal and interstitial telomeric repeats (Nakagawa et al. 2004) and birds can have large amount of interstitial telomeric repeats (Delany et al. 2003), we validated our relative measure of the telomere length (T/S ratio) against absolute measures of the telomere length values determined by assessment of terminal restriction fragments using the conventional Southern blot technique. As found in humans (Cawthon 2002), mice (Callicott & Womack 2006) and birds (Criscuolo et al. in press), measures of the telomere length using those two different approaches correlate well (for the Alpine swift the Pearson's correlation is $r = 0.76$, $n = 15$, $p = 0.001$; Criscuolo et al. in press). Therefore, because interstitial telomeric repeats do not vary with age and show low variation between individuals within the same species (Delany et al. 2003), the use of a quantitative PCR assay is an appropriate method for intraspecific investigations of variation in relative telomere length and erosion rate (Cawthon et al. 2003; Epel et al. 2004; Kotrschal et al. 2007; Criscuolo et al. in press).

(d) Statistical analyses

Two pairs of individuals were siblings born from the same nest and, to minimize pseudoreplication, one individual chosen at random per pair of siblings was kept in the final analyses. Because parents were not systematically captured before 2000, no information is available on possible kinship of individuals born in different years. All the analyses were performed using the statistical package JMP v. 7.0 (SAS Institute Inc.). We used a Cox regression to analyse adult survival between 2001 and 2007, where the chronological age and the telomere length were entered as two

explanatory variables; we controlled for the fact that some individuals were still alive in 2007 by entering a censor group in the analysis. We analysed within-individual change in the telomere length between 2001 and 2006 using an ANOVA where change in the telomere length was the dependent variable, and telomere length in 2001 and the chronological age were entered as two explanatory variables. We analysed adult annual survival between 2006 and 2007 (coded 1 or 0) using a logistic regression where telomere length in 2001, within-individual change in telomere length between 2001 and 2006 and the chronological age were entered as three explanatory variables. Analyses were conducted with a full model and interactions with p-values greater than $p = 0.10$ were dropped from the final analyses in order to produce minimum adequate models. Although telomere dynamics are often reported to differ between the sexes (Benetos *et al.* 2001; Cherif *et al.* 2003; Nawrot *et al.* 2004), we found no difference between male and female Alpine swifts in average telomere length (Student's t-test: $t = 0.16$, $p = 0.87$), life expectancy ($\chi^2 = 0.14$, $p = 0.71$) or in the rate of telomere erosion between 2001 and 2006 ($F_{1,19} = 0.07$, $p = 0.79$, controlling for the effect of initial telomere length). Sex was therefore dropped from the final analyses in order to produce minimum adequate models.

3. RESULTS AND DISCUSSION

We found that survival between 2001 and 2007 was related to RBC telomere length measured in 2001 (Cox regression: $\chi^2 = 5.90$, $p = 0.015$; figure 1) but not to the chronological age at that time ($\chi^2 = 0.05$, $p = 0.83$), which was only weakly related to telomere length (figure 2). When used as a single explanatory variable, in either a linear or a quadratic relationship, age did not explain variation in survival (all p-values > 0.52). This finding fits with previous studies showing that mortality is often catastrophic in long-lived bird species, with individuals maintaining a high level of physical fitness until shortly before the acute failure that results in death (Ricklefs 2008). Thus, individuals with short telomeres in 2001 had a shorter life expectancy than individuals with long telomeres (figure 1), irrespective of whether they were young or old adults when first sampled in 2001. Subsequent survival has been linked to blood cell telomere length in a narrow age range of individuals, either early (Haussmann *et al.* 2005; Pauliny *et al.* 2006) or very late in life (Cawthon *et al.* 2003; Njajou *et al.* 2007). Our results in the Alpine swift demonstrate that the telomere length until late adult age predicts subsequent survival, and that age in itself is not the key variable.

The analysis of the change in RBC telomere length of the 22 individuals sampled in 2001 and 2006 shows that, although the majority of birds showed an erosion in the RBC telomere length (paired t-test comparing lengths in 2001 and 2006: $t_{21} = 5.43$, $p < 0.001$), there was marked variation in the degree of change in the RBC telomere length over this 5 year period, with some individuals showing little telomere erosion and surprisingly two individuals exhibiting an increase in the RBC telomere length with age (figure 3). Variation among individuals in the change in RBC telomere length was independent of their chronological age (ANOVA: $F_{1,18} = 1.82$, $p = 0.19$). It was, however, related to the RBC telomere length in 2001 (hereafter termed 'initial telomere length';
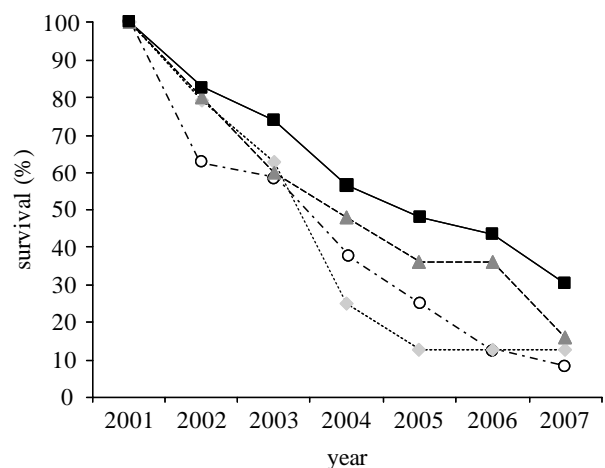
Figure 1. Survival curves for adult Alpine swifts in relationship to the telomere length. Results are based on 96 birds sampled as breeding adults in 2001 and then followed to 2007; data are divided into quartiles on the basis of average telomere length in 2001. Squares, 4th quartile—long telomeres; triangles, 3rd quartile; diamonds, 2nd quartile; circles, 1st quartile—short telomeres.
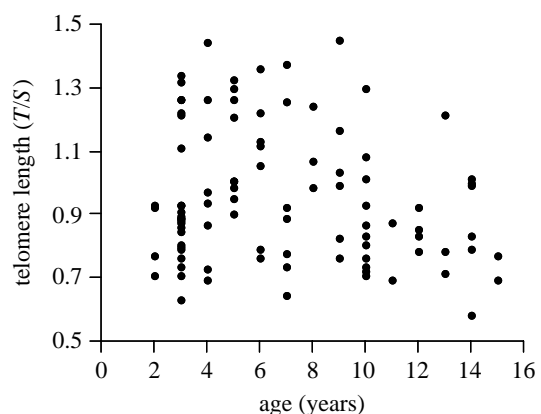
Figure 2. Age-related variations in the telomere length in 96 adult Alpine swifts. There was a weak quadratic relationship between telomere length and age at the time of sampling (age: $F_{1,93} = 0.11$, $p = 0.74$; age squared: $F_{1,93} = 5.53$, $p = 0.021$; $r^2 = 0.09$).

$F_{1,18} = 25.63$, $p < 0.001$). As has been found in other studies (e.g. Hall *et al.* 2004), longer telomeres were associated with faster attrition rates. There was also a suggestion that longer telomeres show faster attrition rates in old than young birds, but this was not quite significant (chronological age by initial telomere length interaction: $F_{1,18} = 3.92$, $p = 0.063$). Most importantly, after controlling for the initial telomere length, we found that an individual's average RBC telomere attrition rate, as measured over the 5 years preceding 2006, differed significantly between those adults that survived to 2007 and those that did not ($F_{1,19} = 19.05$, $p = 0.007$). Individuals with a slower RBC telomere erosion for a given initial telomere length were more likely to survive to the next year (figure 4).

We then used a logistic regression to assess the relative importance of age, telomere length and rate of telomere erosion in predicting life expectancy. Both erosion rate ($\chi^2 = 8.51$, $p = 0.0035$) and RBC telomere length ($\chi^2 = 7.90$, $p = 0.0049$) were highly significant, predictors of survival to the next year, while age was not significant, whether it was considered together with the two measures
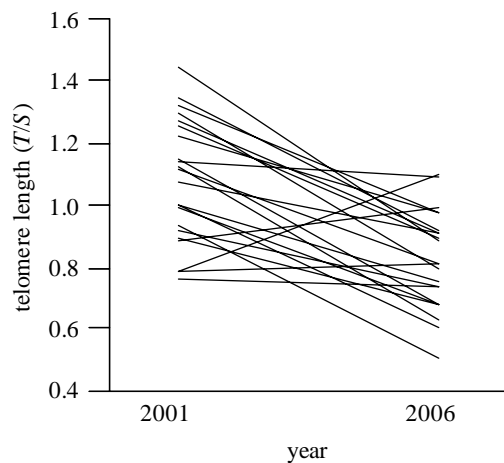
Figure 3. Change in the telomere length over a 5 year period in 22 adult Alpine swifts. Lines link measurement of the same individual sampled in 2001 and 2006.
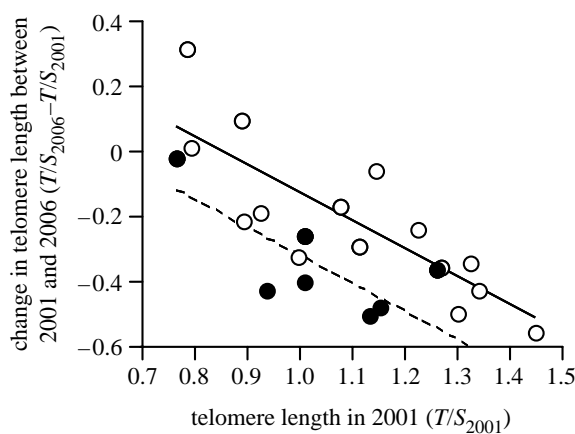

Figure 4. Change in the telomere length between 2001 and 2006 in relationship to the telomere length in 2001, plotted separately for adult Alpine swifts that did (open circles and solid line; $n=15$ individuals) and did not survive (filled circles and dashed line; $n=7$) between 2006 and 2007. A negative value for the change in telomere length indicates that the telomeres became shorter.

of telomere dynamics ($\chi^2=1.35$, $p=0.25$) or as a single explanatory variable ($\chi^2=0.08$, $p=0.78$). Thus, for a given RBC telomere length, the rate of erosion had an independent and important effect on survival probability; a slow erosion rate coupled to a long telomere length gave the highest probability of survival. The removal from the logistic regression analysis of the two individuals showing an elongation in the telomere length does not affect the interpretation of our results (erosion rate: $\chi^2=5.72$, $p=0.017$; RBC telomere length: $\chi^2=7.45$, $p=0.0063$). There was no difference between the sexes in life expectancy, RBC telomere length or telomere erosion rate (all p-values > 0.70; see §2).

The finding that future life expectancy differs between adults with different rates of the RBC telomere erosion, independent of telomere length, supports the suggestion that we need to look beyond simple telomere length to understand the relationships between telomeres and life expectancy (Blackburn 2000). There are a number of non-mutually exclusive mechanisms that can produce a link between telomere dynamics and life expectancy (Monaghan & Haussmann 2006; Blasco 2007; Verdun & Karlseder 2007; Kappei & Londoño-Va 2008;

Shawi & Autexier 2008). For example, some individuals may be more resistant to oxidative stress than others, and thereby able to mitigate the deleterious effects of oxidative stress on telomere dynamics and, in turn, to live longer. Because oxidative stress can damage many physiological and molecular pathways besides telomeres (Beckman & Ames 1998; Finkel & Holbrook 2000), the relative importance of telomere homeostasis in determining life expectancy remains to be tackled in detail. A recent study in this Alpine swift population has showed that males with high resistance to oxidative stress have a higher annual survival than those with low resistance to oxidative stress (Bize et al. 2008), and thus investigation of the links between oxidative stress, telomere erosion and organism lifespan is likely to be worthwhile. Individuals could of course also differ in telomere restoration through the activation of the enzyme telomerase (Verdun & Karlseder 2007; Shawi & Autexier 2008). The recent finding that two long-lived bird species, common terns (*Sterna hirundo*) and Leach's storm petrels (*Oceanodroma leucorhoa*), show high levels of bone marrow telomerase activity throughout life (Haussmann et al. 2007) suggests that such differences could occur, and the observation that telomere length increased within two individual swifts supports this possibility (figure 3). Clearly, more work is required to find out why some individuals show an apparent increase in the telomere length with age.

That telomere length in RBCs, which is linked to life expectancy, supports the suggestion that this is indicative of tissue functioning in other parts of the body (Takubo et al. 2002). Our results show that individuals with, on average, relatively long telomeres and slow rates of telomere erosion in RBCs have a higher life expectancy, with their chronological age probably not becoming a significant predictor until they are very old. However, because the Alpine swift can live to more than 20 years of age, while in the present study, individuals were between 2 and 19 years of age, the relative importance of age and telomere length in predicting life expectancy in very old individuals remains to be established. This study also demonstrates that the telomere length cannot be assumed to act as a clock, providing a measure of chronological age. Elucidating the importance of genetic (Njajou et al. 2007; Kappei & Londoño-Va 2008) and environmental (Epel et al. 2004; Kotrschal et al. 2007) factors on telomere dynamics is likely to play an important part in developing our understanding of the life-history trade-offs that shape the evolution of lifespan and determine individual longevity.

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