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Ultra-long telomeres shorten with age in nestling great tits but are static in adults and mask attrition of short telomeres

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Ultra-long telomeres mask telomere shortening

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Abstract

Telomere length (TL) is increasingly used as a biomarker of senescence, but measuring telomeres remains a challenge. Within tissue samples, TL varies between cells and chromosomes. Class I telomeres are (presumably static) interstitial telomeric sequences, and terminal telomeres have been divided in shorter (Class II) telomeres and ultra-long (Class III) telomeres, and the presence of the latter varies strongly between species. Class II telomeres typically shorten with age, but little is known of Class III telomere dynamics. Using multiple experimental approaches, we show great tits to have ultra-long telomeres, and we investigated age effects on Class II and III telomeres using a longitudinal approach (our method excludes Class I telomeres). In adults, TL averaged over the whole distribution did not significantly change with age. However, more detailed analyses showed that Class II TL did shorten with age, and, as in other species, the longest Class II telomeres within individuals shortened faster with age. In contrast, Class III TL did not shorten with age within individual adults. Surprisingly, we found the opposite pattern in nestlings: Class III TL shortened significantly with age, while the age effect on Class II TL was close to zero. Thus, Class III telomere length may provide information on developmental history, while Class II telomere length provides information on telomere dynamics in adulthood. These findings have practical implications for telomere studies and raise the interesting question what causes variation in TL dynamics between chromosomes within individuals and how this is related to development.

Introduction

Finding biomarkers of senescence is of wide interest in ecology and an increasingly used molecular marker is telomere length (TL) (e.g. Blackburn *et al.* 2015; Haussmann & Heidinger 2015). Telomeres are non-coding DNA repeats forming the end-caps of linear chromosomes, hereby safeguarding chromosome integrity (Blackburn 1991). TL is to a large extent genetically determined, although heritability estimates vary widely between studies (range h^2 : 0 - 1; overviews in Atema *et al.* 2015; Dugdale *et al.* 2017). TL generally shortens with age, and telomere shortening is accelerated by environmental challenges (Boonekamp *et al.* 2014; Reichert *et al.* 2014; Watson *et al.* 2015), reproductive effort (Bauch *et al.* 2013), stress during adulthood (Hau *et al.* 2015), diseases (Beirne *et al.* 2014; Asghar *et al.* 2015) and can differ between habitats (Angelier *et al.* 2013; Stier *et al.* 2015a). Finally, telomeres predict remaining lifespan or survival (e.g. Haussmann *et al.* 2005; Bize *et al.* 2009;

Salomons *et al.* 2009; Heidinger *et al.* 2012; Boonekamp *et al.* 2013; Bauch *et al.* 2014; Stier *et al.* 2015a). Therefore TL, and perhaps in particular telomere shortening, could be used as biomarker of senescence and experienced life-stress.

Telomeric repeats are located at the ends of the chromosomes, but also at interstitial sites in the chromosome (Delany *et al.* 2000; Foote *et al.* 2013). It is unlikely that interstitial telomeres shorten, as terminal telomeres generally do, because this would involve double strand breaks. Hence, as a biomarker of senescence it is likely that only the terminal telomeres are of interest. Based on their length, two types of telomeres at the end of chromosomes have been described (Delany *et al.* 2000): shorter or Class II telomeres (8-40 kb) and ultra-long or Class III telomeres (up to 2.0 Mb). Class II telomeres are found to shorten with age in several species, such as humans, jackdaws and common terns (Salomons *et al.* 2009; Bauch *et al.* 2013; Steenstrup *et al.* 2017). Delany *et al.* (2000) found no evidence for shortening of Class III telomeres in chickens, but her analyses were cross-sectional and data sets were small. Overall, little is known of the dynamics of ultra-long telomeres.

Multiple techniques are available to measure TL and these techniques differ, among other things, in the information they provide, ranging from a point-estimate of TL in a sample (including interstitial sequences) to the length of single telomeres (Nussey *et al.* 2014). When the aim is to quantify the distribution of TLs at the ends of chromosomes, non-denaturing Terminal Restriction Fragment (TRF) analysis is highly suitable (Hausmann & Vleck 2002). This method yields an image of a smear which represents the frequency distribution of a range of TLs (Lansdorp *et al.* 1996). Because the DNA is not denatured in this approach, the probe binds to the single strand telomere overhang only, and hence interstitial telomeric sequences do not show up on the gel. Using this method, it was shown in jackdaws (Salomons *et al.* 2009) and common terns (Bauch *et al.* 2014) that within individuals the longer Class II telomeres shortened at a higher rate, and predicted survival and other fitness components best. However, these species have negligible ultra-long telomeres (Class III) and hence it is not known whether this finding extends to this Class of telomeres.

To gain an understanding of telomere dynamics of the different TL classes, we investigated age dependent patterns in subsets of telomeres in free-living adult and nestling great tits (*Parus major*). Following Delany *et al.* (2000) we divided the distribution into Class II (short) telomeres and Class III (ultra-long) telomeres. We found great tits to have a broad TL distribution, ranging from 2.1 till >240kb, and using multiple techniques (Bal 31, mixture with 7 restriction enzymes) we tested

whether the long fragments in this distribution were telomeres rather than sub-telomeric regions that by chance were not removed by our cocktail of restriction enzymes. In a further test of the latter hypothesis we measured great tit and blue tit TL using qPCR and the TRF method, and compared whether the relative species difference in TL was method dependent, with method dependence being an indication that the ultra-long telomeres in great tits can be attributed to sub-telomeric regions. To illustrate that great tits have unusually long telomeres we compared the TL we found in great tits with other species previously measured in our lab.

Materials and methods

Study species and blood sampling

We sampled a population of great tits on Vlieland (53.178 N, 5.038 E), an island in the Dutch Wadden Sea, in the years 2011-2015 and 2017 (nestlings only). During the breeding season, adults were caught with spring traps while feeding 8-10 day old nestlings and in winter while roosting in nest boxes (Atema *et al.* 2016). Individuals were identified by their ring number; 70% of the captured birds used in this study had been ringed as nestling. Individuals captured for the first time were ringed and their age (yearling or older) was estimated based on the colour of the wing coverts (Svensson 1992). Including these birds with age estimated based on plumage, age was known for 101 out of the 105 adults in our dataset and ranged from 0.8 - 7.6 (mean \pm s.e.: 2.24 ± 0.076) years. Baseline samples of nestlings were collected when they were 4 days old, and follow-up samples were taken when they were 15 days old (N = 19).

Blood samples were taken from the brachial vein and stored in 2% EDTA at 4-7 °C for up to three weeks. Subsequently, samples were snap-frozen in 40% glycerol buffer and stored at -80 °C.

Telomere terminal restriction fragment analyses

We quantified TL in male great tits using terminal restriction fragment (TRF) assays as described previously (Salomons *et al.* 2009; Atema *et al.* 2015) with some adjustments. In short, DNA from 4 μ l of red blood cells was extracted in agarose plugs using the CHEF Mammalian Genomic DNA Plug kit (Bio-Rad Laboratories, Inc., USA). Subsequently DNA from half a plug was digested overnight at 37 °C with a mixture of the restriction enzymes *HindIII* (30 U), *HinfI* (15 U) and *MspI* (30 U) in NEB2

buffer. These three restriction enzymes have proven to be sufficient, in that additional restriction enzymes did not result in markedly shorter telomeres in other species tested in our lab. The restriction enzymes by cleaving appropriate sites digest specific DNA sequences, but were selected in such a way to leave the telomere sequence intact. The intact terminal (telomere) restriction fragments are the targets for the succeeding steps in the protocol, namely pulse field gel electrophoresis and hybridization with the radioactive marker.

The restricted DNA and the ^{32}P end-labelled size standards (1 kb DNA ladder, New England Biolabs, range 0.5-10kb; Molecular Weight Marker XV, Roche Diagnostics, Basel, Switzerland, range 2.4-48.5 kb; NEB MidRange PFG Marker I, New England Biolabs, range 15-242.5 kb) were separated through a 0.8% agarose gel by pulsed field gel electrophoresis at 14 °C for 22 h (4.8 V/cm, initial switch time 1 s, final switch time 25 s). Gels were dried (gel dryer model 538, Bio-Rad Laboratories) and hybridized overnight at 37 °C with ^{32}P -labelled oligonucleotide (5'-CCCTAA-3')₄, which bound to the single-stranded overhang of the telomeres. Gels were exposed overnight to a phosphor screen (PerkinElmer Inc., Waltham, USA), and the radioactive signal was visualized using a phosphor image (Cyclone™ Storage Phosphor System, PerkinElmer) (Fig. 1).

We quantified the distribution of TLs based on densitometry using the open-source software IMAGEJ v. 1.38x as described previously (Salomons *et al.* 2009; Atema *et al.* 2015). Our lower molecular weight limit was the point where the optical density was lowest in the region of short telomeres (approximately 3 kb). As upper limit we set the point where the optical density dropped to the background density in the region of long telomeres, which was maximally 240 kb (approximately the limit of our molecular size standards). In rare cases telomeres extended somewhat beyond this upper limit, which we could not quantify.

For each sample we calculated mean TL, as well as the TL of every 10th percentile (range 10-90%). Our preliminary results suggested that the short and long telomeres are two different traits reflecting Class II and III telomeres as described by Delany *et al.* (2000). Hence, we continued the analyses of age dependent patterns by dividing the distribution of telomeres into two regions according the approach taken by Delany *et al.* (2000). We used their description to distinguish between the two Classes of telomeres: (i) long telomeres, visible as the hypervariable banded pattern in the high molecular weight region (Class III, approximately 20-240 kb) and (ii) short telomeres, which were visible on the gel as a continuous smear in the short length region (Class II, approximately

3-20 kb). Like Delaney et al, we confirmed the end-chromosome location of the telomeres using Bal31 digestion (see below). Delaney et al (2000) suggested that the transition of Class II to Class III telomeres was at 30-40 kb in chicken, without defining a clear border. Based on the optical density plots we defined a border between the end of the short “smear” and the beginning of the long “bands” for each sample individually (range 9-32 kb). This border was visible on the images of the gels as a small gap with a lower optical density between the Class II “smear” and Class III “bands” (Fig. 1). We identified the border by first inspecting the gel image to pinpoint the range in which the border was located, after which we defined the border as the point in that range with the lowest optical density values. However, because there is a slight subjective element in the visually scored threshold we verified results with results obtained with a fixed threshold at 20 kb.

Testing for effects of sub-telomeric regions

Great tit TLs turned out to be extremely long compared with other bird species (see below). A substantial part of the genome consists of repetitive DNA, amongst which are repeats of sequences at sub-telomeric sites (Biscotti et al. 2015). Potentially, not all DNA other than telomeric repeats was digested by the 3 restriction enzymes we used which would have left sub-telomeric repeats intact. We therefore carried out three experiments to test the hypothesis that the long telomeres we measured can be attributed to sub-telomeric repeats.

First, we applied the exonuclease *Bal31*, an enzyme that preferentially digests double-stranded DNA ends when DNA is intact. DNA was extracted in agarose plugs as described above that we subsequently cut in four equal-sized pieces. These pieces were subjected to different digestion times with *Bal31* (0.1 U in 200 µl of reaction buffer): 0, 20, 80 and 240 minutes. To stop the reaction, plugs were transferred to a tube on ice containing 20 mM Tris, 50 mM EDTA (pH 8.0) buffer. Subsequently plugs were immediately washed three times (10 min each) in EDTA buffer, three times in Tris buffer and placed in 150 µl restriction enzyme reaction buffer for 60 min. Finally, DNA was restricted using the mix with three restriction enzymes and gel-electrophoreses, labelling with the oligonucleotide, and visualization of the radioactive signal were done as described above.

After digestion with *Bal31* there is no single-stranded overhang to label with the telomeric probe and hence DNA had to be denatured for this assay. To this end we followed the procedure as described by Foote et al. (2013). Denaturing and subsequent labelling of telomeric sequence will

visualize all three Classes of telomeres. The protocol involved denaturing the gels with a buffer (1.5 M NaCl, 0.5 M NaOH) during three times 30 minutes and neutralizing it during two times 30 minutes with a buffer (0.5 M Tris-HCL pH 8.0, 1.5 M NaCl) at room temperature. Gels were hybridized with the ^{32}P -labelled oligonucleotide (5'-CCCTAA-3')₄ and the radioactive signal was visualized following the same protocol as used with the non-denatured gels. Because labelling now occurred along the full length of telomeric repeats, differences in the banding pattern in the long region of the telomere distribution were visualised with higher contrast, allowing for a more detailed comparison of different treatments.

Second, we used a mixture with 7 restriction enzymes, instead of our standard 3 restriction enzymes, to increase the likelihood that all DNA other than telomeric sequence was digested. The restriction enzymes we added to our standard set were *HphI* (15 U), *MnII* (15 U), *RsaI* (15 U) and *HaeIII* (15 U) (New England Biolabs, Inc., Beverly MS, USA). Gel-electrophoreses, labelling with the oligonucleotide, and visualization of the radioactive signal were done as described above, including denaturation after the normal labelling protocol.

Third, we measured telomeres using qPCR in great tits (N = 20), to be compared with blue tits (*Cyanistes caeruleus*, also sampled on Vlieland; N = 20) of which we also obtained TRF measurements of the same set of samples. The mean TL measured with TRF is half as long in blue tits compared to great tits (see below). Finding a similar ratio with qPCR, which we can safely assume to measure exclusively telomeric repeats, would further support our results found with TRF. As a control gene we used GAPDH, amplified with primers we previously designed for great- and blue tits (Atema *et al.* 2013). As a control “golden” sample we used a sample containing a mixture of DNA of four different great tit individuals. The qPCR reaction was executed as previously described (Atema *et al.* 2013), with small adjustments. DNA samples were diluted to a concentration of 0.83 ng/μl, each reaction contained 5 ng of DNA. The reaction for telomere (500 nM for forward and reverse primer) and GAPDH (100 nM for forward and reverse primer) was done on separate plates, with DNA of great- and blue tits on the same plate. We calculated the T/S ratio as:

$$\frac{\text{Eff}_{\text{telo}}^{\Delta\text{Cq}_{\text{telo}}}}{\text{Eff}_{\text{GAPDH}}^{\Delta\text{Cq}_{\text{GAPDH}}}} \quad (\text{Pfaffl 2001})$$

We calculated the mean amplification efficiency (Eff) and cycle quantification (Cq) values with the programme LinRegPCR (version 12.13; Ruijter *et al.* 2009). ΔCq was calculated as the Cq values of the control subtracted by the Cq value of the sample. Mean Eff_{telo} was 1.94 ± 0.014 and mean Eff_{GAPDH} was 2.11 ± 0.0092 , both close to the expected efficiency of 2.

Statistics

We tested for effects of age on TL (Class II, Class III and Class II + Class III) using linear mixed models on a longitudinal dataset in adults (2 individuals sampled 6 times, 4 individuals sampled 4 times, 32 individuals sampled 3 times, 60 individuals sampled 2 times and 7 individuals sampled once) and 24 nestlings (all sampled 2 times), including individual identity and gel identity as random effects. As we found shortening with age in Class II TL, we also tested for effects of season in this set.

Given that we found hints for telomere elongation in a part of our dataset, we tested whether telomere elongation was real or could be attributed to measurement error following Simons *et al.* (2014). Briefly, this method compares two estimates of variance due to measurement error using different assumptions. Error variance estimates under the first assumption, assuming telomere length increases and/or decreases, could be calculated using residual sum of squares. Measurement error variance under the second assumption, telomeres do not elongate, is based on the difference in initial and last telomere measurement of individuals that showed an increase in telomere length. The hypothesis that telomeres show no elongation can be tested with a variance ratio test between the two estimated error variances. In further analysis, using the upper confidence limit of the error variance under the first assumption, individuals that are likely to show true telomere elongation could be identified.

Analyses were done in JMP 7.0 and R 3.0.2 (R Development Core Team 2008). Unless mentioned otherwise, estimates are presented as mean \pm standard error (s.e.).

Results

Full telomere distribution

The distribution of TLs included extremely long telomeres, exceeding 240 kb in some cases. Mean TL was 47.1 ± 2.0 kb (N = 251), which is more than twice as long as found previously in other bird species analysed in our lab (Fig. 2), and approximately seven times longer than telomeres of human adults (e.g. Verhulst *et al.* 2013). We restricted this comparison to species measured in our lab, to minimize methodological effects on TL.

Ultra-long telomeres or sub-telomeric repeats?

We ran three experiments to test whether we quantified exclusively telomeric repeats.

Bal31: Over the gradient of digestion times, the signal in the larger weight region reduced and there was a shift in the entire distribution of telomeres towards the lower weight region in which the signal became more intense (Fig. 3a). The mean TL estimated after 240 minutes of digestion with *Bal31* in 5 different samples was reduced by 32% compared to the start of the digestion procedure. This supports the hypothesis that the long restriction fragments consist of telomeric sequences, as opposed to being largely composed of a sub-telomeric region, because otherwise the signal would have weakened more and shifted less.

7 Restriction enzymes: We compared TL of 4 samples (2 individuals, each sampled at 2 ages) with mixes containing 3 (used throughout our study) and 7 restriction enzymes. The only notable difference from visual inspection of the distribution was the disappearance of a band outside our measurement range (i.e. >240 kb, indicated in Fig. 3b), suggesting that this extremely long telomeric repeat was confounded by sub-telomeric repeats. The additional restriction enzymes on average caused a modest reduction of TL of 5.8%, which further indicates that the ultra-long TL cannot be attributed to sub-telomeric repeats in the flanking regions.

qPCR: We compared TLs quantified with TRF and qPCR in the same samples. For both techniques TLs were significantly longer in great tits compared to blue tits (TRF: t-ratio = -19.23, $P < 0.0001$; qPCR: t-ratio = -8.22, $P < 0.0001$). More importantly, the species difference in TL was almost the same, independent of technique (ratio blue tit:great tit TRF = 1:2.02; qPCR 1:2.16; Fig. 4). This

finding further indicates that the ultra-long telomeres as quantified by the TRF assay represented telomeric repeats rather than sub-telomeric regions.

Full telomere distribution: effect of age

There was no significant effect of age on the mean TL calculated over the full distribution (slope: 213.5 ± 335.1 ; $F_{1,218.8} = 0.41$, $P = 0.52$; Fig. 5a). TL at the 10th percentile was 6.0 ± 0.12 kb, whereas TL at the 90th percentile was 134.8 ± 4.6 kb (Table S1). We found no significant effect of age on TL at any of the nine percentiles (P : 0.31-0.88). Individual identity (random effect) in the mean TL of the full distribution explained 51.2% of the total variation.

In contrast to what could be expected based on our findings in other bird species, the data suggested that on average telomeres in the long region of the distribution (percentile 50-90, Table S1) elongated with age. We tested whether there was statistically significant elongation using the approach proposed by Simons et al. (2014) but could not reject the null hypothesis that Class III telomeres did not elongate ($F_{16,37} = 1.46$, $P = 0.17$). We therefore assume the apparent telomere lengthening can be attributed to measurement error, which is larger at the higher end of the telomere distribution due to the logarithmic nature of DNA on gels. In the range representing short telomeres (10-30%) we found hints for the expected telomere shortening with age (Table S1). In subsequent analyses we therefore analysed the ultra-long Class III and short Class II telomere distributions separately. The individual border between Class II and III telomeres correlated with age (slope: -211.1 ± 76.4 bp; $F_{1,147.8} = 7.63$, $P = 0.0065$, Fig. S1), indicating that the assignment was biologically relevant. Verification of this border with a fixed threshold at 20 kb showed the same results (see supporting information S3, Fig. S2).

Class III telomeres: effect of age

The mean TL of the Class III telomeres was 83.6 ± 2.0 kb. Individual variation in the mean TL in this region of the distribution of TRF assays accounted for 73.3% of the total variation. Despite the longitudinal character of our dataset, we found little evidence for an age effect on TL in the Class III telomeres (slope: -73.6 bp/yr ± 428.1 ; $F_{1,241} = 0.029$, $P = 0.86$; Fig. 5b).

We quantified TL at percentiles 10-90% within the Class III TL distribution. Although the estimates of the change of TL with age were negative up to the 80th percentile, we found no significant changes of TL with age across the distribution of Class III telomeres (Fig. 6), confirming the result found for average Class III TL.

Class II telomeres: effect of age

The mean TL of the Class II telomeres was 8.7 ± 0.2 kb. Individual identity (random effect) in mean Class II TL accounted for 80.5% of the total variation (Fig. 7). Class II TL shortened significantly with age (slope: -111.2 ± 27.1 bp/year; $F_{1,155.1} = 16.78$, $P < 0.0001$; Fig. 5c). Verification of this result with a fixed threshold at 20 kb instead of an individually set border yielded the same result (see supporting information S3, Fig. S3). There was no significant difference between winter TL and breeding TL (all years combined) in our dataset; the least square means corrected for bird ID and age are respectively 8.40 ± 0.15 and 8.45 ± 0.14 bp (season as factor: $P = 0.55$).

When quantifying age effects on TL at percentiles 10-90% within the Class II TL distribution we found significant shortening of TL with age at all percentiles (all $P \leq 0.0092$). More importantly, because in agreement with our findings in other species, higher percentiles within the Class II TL distribution lost more base pairs per year than the lower percentiles (Fig. 6). Verification of this result with a fixed threshold at 20 kb instead of an individually set border yielded the same result (see supporting information S3, Fig. S4).

Nestlings

The mean TL of the full distribution in nestlings was longer than in adults (day 4: 60.8 ± 0.3 kb; day 15: 58.8 ± 0.3 kb; Fig. 8a), indicating considerable telomere shortening between fledging age and adulthood. Class II TL changed surprisingly little over this period (difference: $0.32 \text{ bp} \pm 29.9$; $F_{1,23} = 0.0001$, $P = 0.99$; Fig. 8b), whereas Class III TL became substantially shorter (difference: $-559.7 \text{ bp} \pm 187.6$; $F_{1,46} = 8.90$, $P = 0.0046$; Fig. 8c). Given these results it was somewhat unexpected that the average of the full TL distribution did not shorten significantly in these 11 days (difference: $-178.7 \text{ bp} \pm 133.0$; $F_{1,46} = 1.81$, $P = 0.19$; Fig. 7a).

Discussion

We were interested in telomere dynamics of great tits, a short-lived bird species with extremely long telomeres. Following Delany *et al.* (2000), we separated the telomeric distribution in two different Classes (II and III). In adults, Class III telomeres did not shorten with age, and, because Class III telomeres constitute a large proportion of the full distribution, this explains there to be no effect of age on TL calculated over Class II and III telomeres combined. That Class III telomeres did not shorten with age in adults was unexpected, because within individual birds and humans the longer Class II telomeres lose more base pairs with age than the shorter telomeres (Kimura *et al.* 2007; Salomons *et al.* 2009; Bauch *et al.* 2014). In theory, the finding that Class III telomeres do not shorten with age in adults could be due to insufficient statistical power, and we can of course not exclude the possibility that a small age effect would be detectable in a much larger data set. However, we anticipate that such an (as yet undetected) effect would be small, given that (i) the precision of our measurements was high, (ii) due to our method, we included only terminal telomeres in our measurements, and (iii) we had a large set of longitudinal measurements that yields substantially more statistical power than cross-sectional data, due to large TL variation between individuals.

In contrast to Class III telomeres, Class II TL did shorten with age in adults. The observed rate of telomere shortening of Class II telomeres was 111 bp/year, which falls well within the expected range (Dantzer & Fletcher 2015). Moreover, we found the higher telomere loss rates at the longer percentiles of the Class II telomere distribution, as previously shown using the same approach in jackdaws (Salomons *et al.* 2009) and common terns (Bauch *et al.* 2014). Because of the similar dynamics as found in other species, we suggest that Class II telomeres act as indicators of biological age, which can be further tested in relation to for instance survival. The distribution of Class II telomeres in great tits overlaps with the telomere distribution in other species with few Class III telomeres such as jackdaws and common terns. It is interesting to note therefore that great tit Class II telomere dynamics resembles the dynamics of these same species, whereas Class III telomeres do not.

The border to distinguish between Class II and III telomeres was not clearly defined by Delany *et al.* (2000), and we were fortunate that it was relatively straightforward to distinguish Class II and Class III telomeres in great tits by the gap in optical density distribution (Fig. 1). Our interpretation of the gel data is supported by our finding that the border value consistently varied between individuals

and decreased with age, and our separation of the two Classes is further supported by the finding that age impacts Class II and Class III telomeres differently. However, some subjectivity may be unavoidable, and it is important therefore that when we estimated Class II and III TL using a fixed border at 20 kb, the same pattern emerged, with the telomeres <20 kb shortening with age, while no such effect was found in telomeres >20 kb. This approach is therefore an alternative for species in which there is no clear gap in the TL distribution, although care must be taken to ensure that the setting of the fixed border is done independently of the results, e.g. by using different data sets to set the border and for testing hypotheses.

While Class III TL was inert in adults, and Class II TL declined with age, we found the opposite pattern in nestlings. Apparently, telomere dynamics differs not only with age, but in addition there is a striking difference in the age effect between different parts of the TL distribution. We attribute the lack of significant telomere shortening in nestlings to the modest sample size in combination with the high variance in the dynamics of the Class III telomeres. At what age the Class II telomeres start shortening significantly, and when the shortening of Class III telomeres becomes undetectable we cannot say exactly, but it will be sometime between fledging (in May) and December when we first recapture the birds. When indeed the Class III telomeres are predominantly at the ends of micro-chromosomes, the complex telomere dynamics could be due to a difference between early life and adulthood in the function of micro-chromosomes, perhaps related to development, but this remains to be tested. In any case, the complex telomere dynamics we observed is in agreement with other recent findings in the same species using qPCR based TL measurements, which measures all telomeric repeats (i.e. Classes I, II and III pooled; Nussey *et al.* 2014), and hence best resembles our measurement of the complete distribution. Given that the complete distribution is dominated by the Class III telomeres, which shortened with age in nestlings but not in adults, we expect qPCR based TL estimates to decline with age in nestlings but not in adults. The available data are in agreement with this prediction (nestlings: Stier *et al.* 2015b; adults: Salmón *et al.* 2017). A consequence of these findings is that the different parts of the telomere distribution contain different information when one is aiming to use TL as biomarker. While Class II TL dynamics may be informative on factors affecting telomeres in adulthood, the Class III telomeres may provide information on factors affecting telomeres prior to adulthood.

TL typically varies between species (Haussmann *et al.* 2003; Lorenzini *et al.* 2009) and we found great tits to have very long telomeres compared to humans and other bird species. Long sub-telomeric regions, not digested by our standard set of three restriction enzymes, could potentially explain this finding. However, the combined results of three different experiments make this explanation unlikely, and we therefore conclude that great tits have long TLs compared to other bird species. TL measured in our lab using the same technique in samples from great tits in Wytham woods (UK) showed similar long telomeric patterns as found in our Vlieland population (Mulder, Bouwhuis *et al.* unpublished observations), suggesting that ultra-long telomeres are a general feature of great tits. In mammals, TL variation was in part explained by body size, with small mammal species having longer telomeres (Lorenzini *et al.* 2009). In the small set of species for which we could compare TL (Fig. 2), there is also a trend that smaller and shorter-lived species have longer telomeres, but clearly a much larger species set is required to analyse interspecific variation in avian TL.

Ultra-long telomeres were previously found in wild Iberian shrews (Zhdanova *et al.* 2010), chickens, several raptor species and cranes (Delany *et al.* 2000). Why Class III telomeres are numerous in some species and not in others is not fully resolved. Nanda *et al.* (2002) using FISH on 16 bird species, found micro-chromosomes to display a large number of telomere sequences, and bird species can have many micro-chromosomes. Furthermore, Delany *et al.* (2000) found that Class III telomeres were more numerous in species with more micro-chromosomes. Great tits were estimated to have 40 different chromosomes ($2n = 80$) of which about half were classified as micro-chromosomes (Nanda *et al.* 2011; van Oers *et al.* 2014). Thus the large number of micro-chromosomes might, at least partly, explain the high prevalence of ultra-long telomeres in great tits. It is worth noting that this explanation contrasts with the positive correlation between chromosome size and telomere length observed in the human genome (Wise *et al.* 2009). On the other hand, the smallest human chromosome (#21) is larger than all great tit micro-chromosomes (Santure *et al.* 2013), and different processes may act on different size ranges.

We would have concluded that adult TL was independent of age when we had relied on a technique that yields a single estimate to characterize telomere length, such as qPCR or dotblot (Nussey *et al.* 2014). Hence, it is possible that other studies that found TL to be independent of age would have reached a different conclusion when a more informative technique had been employed.

For instance no effect of age on TL estimated with qPCR was found in leatherback turtles (Plot *et al.* 2012), which could be due to the presence of Class III telomeres, given that turtles also have micro-chromosomes (Ellegren 2013). It has previously been recommended to investigate the TL distribution with a suitable technique such as TRF before investing in techniques that yield only a single TL estimate (e.g. Nussey *et al.* 2014), and our findings illustrate the value of this recommendation. The few species for which we have comparable telomere length estimates suggest that this may more often be necessary in small and/or short-lived species than in larger and/or longer-lived species.

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Data accessibility

The data on which this paper is based are available through Dryad (doi:10.5061/dryad.kv319sc) in the following three files:

The file “qPCRdata.xlsx” contains telomere length data measured using two different techniques, TRF (in bp) and qPCR, for great tits and blue tits (i.e. the data used for figure 4 and associated analyses).

The file TLadults.xlsx contains the information on the telomere measurements (TRF in bp) in adults with age in years. The file contains three sheets: (1) measurements of the complete telomere range, (2) measurements of the ‘smear’ at the lower end of the distribution only, (3) measurements of the telomere range above the ‘smear’.

The file TLchicks contains the information on the telomere measurements (TRF in bp) in nestlings with age in days. The file contains three sheets: (1) measurements of the complete telomere range, (2) measurements of the ‘smear’ at the lower end of the distribution only, (3) measurements of the telomere range above the ‘smear’.

Author contributions

EA, AJN and SV designed the study, EA and AJN collected samples, EA and EM analysed samples, EA performed statistics and drafted the manuscript with SV, all authors commented on the manuscript.

Supporting information

Additional supporting information could be found in the online version of this article.

S1. Relation TL full distribution and age

Table S1 Estimates (s.e.), F ratio and P-value from mixed models testing for effects of age on TL across the percentiles in the full distribution.

S2. Relation individual border and age

Figure S1 The assigned individual border in year 2 plotted against the border in year 1 for the same individuals (N = 53). The dotted line shows where the assigned borders are equal ($y=x$). All dots which fall below that line are borders which were shorter in year 2 than in the first year. Shown data points are the selection of all first two samples of all individuals taken one year apart, but in the analyses we included all samples.

S3. Comparing individual border with 20 kb border

Figure S2 The correlation between Class II TLs estimated with a fixed upper border of 20 kb and Class II TLs estimated with an individual upper border set for each sample.

Figure S3 Class II TL (kb) plotted against age (years).

Figure S4 Telomere shortening rate of different Class II TL percentiles and the mean TL as estimated with the mixed model (bp/year).

Figure legends

Figure 1 Subset of a non-denatured pulsed-field gel representing the variation in telomere distributions. Represented are repeated samples of three individuals, with the clusters of individuals in lane 1-3, 5-6, 8-10. Lane 4 includes a control sample and lane 7 includes the ultra-long size standard NEB MidRange PFG Marker I. The border between Class II and III telomeres is indicated with black arrows per sample cluster. Note the consistent individual variation in telomere distribution pattern.

Figure 2 Population mean TLs in adults of different bird species as measured in our lab with TRF analysis in jackdaw (Salomons *et al.* 2009), common tern (Bauch *et al.* 2014), black-tailed godwit (Atema *et al.* 2011), zebra finch (Atema *et al.* 2015), blue- and great tit (this study). Error bars that are not visible are smaller than the marker.

Figure 3 Tests to verify that we quantified exclusively end-telomeric repeats. (a) The telomere distribution as visible after digestion of double-stranded DNA ends with *Bal* 31. DNA was digested over a gradient of time (0, 20, 80 and 240 seconds) as shown in two individuals (lanes 1-4 and 5-8). Note further that, against expectations, no Class I (interstitial repeat) telomeres are visible on the gel. This may be due to their signal being too weak to be picked up in the presence of the dominant signal of the Class III telomeres. (b) The telomere distribution as visible after restriction with 3 (odd lanes) or 7 (even lanes) restriction enzymes. The test was run in two individuals (lanes 1-4 and 5-8) and in each individual at two different ages (age 1: lanes 1, 2 and 5, 6; age 2: lanes 3, 4 and 7, 8). The difference caused by the additional restriction enzymes, situated in the extremely large region of the distribution, is depicted with white arrows.

Figure 4 Mean TLs (\pm s.e.) quantified with TRF assays (x-axis) and relative qPCR (y-axis) in blue- and great tits.

Figure 5 Change of TL (bp) in the full (a), long Class III (b) and short Class II (c) telomere distribution plotted against delta age (years). TL did not significantly decline with age in the complete sample and Class III ($P > 0.50$), while Class II telomeres shortened significantly with age. In the analysis, individual

identity was included as random effect, and, to let the figure reflect the analysis, the data on both axis were expressed as deviations from the individual mean age and telomere length calculated over the different samples per individual. Note substantially smaller scale on y-axis of panel (c).

Figure 6 Telomere shortening rate (bp/year \pm s.e.) at different TL percentiles as estimated with the mixed model. (a) Class II TL significantly shortened across all percentiles (P: >0.0001-0.0092). (b) No significant shortening across the Class III TL percentiles (10th percentile P = 0.055; other percentiles P: 0.34-0.92). Note increase in s.e. with percentile, which is due to the logarithmic nature of the gels. Furthermore, the rate of shortening in Class II telomeres was higher compared to the shortening rates in the 10th-30th percentiles in the analyses of the full distribution (table S1). This is due to the fact that the 30th percentile of the full distribution in some cases will include Class III telomeres, causing underestimation of telomere shortening.

Figure 7 Class II TL in year 2 plotted against TL in year 1 (N = 53 individuals). The dotted line shows where subsequent measurements are equal (y=x). All dots which fall below that line are individuals in which TL was shorter when recaptured 1 year later. For graphical purpose we only plotted individuals with two samples with 1 year interval; in the analyses we included all measurements.

Figure 8 Full TL distribution (a), short Class II TL (b) and long Class III TL (c) at day 15 plotted against TL at day 4 (N = 19 individuals). The dotted line shows where subsequent measurements are equal (y=x). All dots which fall below that line are individuals in which TL was shorter when resampled 11 days later.











