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

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Abstract

Telomere length (TL) is increasingly being 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, while terminal telomeres have been divided in shorter (Class II) telomeres and ultralong (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 ultralong 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 more quickly 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 TL may provide information on developmental history, while Class II TL provides information on telomere dynamics in adulthood. These findings have practical implications for telomere studies and raise the interesting question of what causes variation in TL dynamics between chromosomes within individuals and how this is related to development.

KEYWORDS
birds, microchromosomes, *Parus major*, qPCR, telomere distribution, TRF

1 | INTRODUCTION

Finding biomarkers of senescence is of wide interest in ecology and an increasingly used molecular marker is telomere length (TL) (e.g., Blackburn, Epel, & Lin, 2015; Haussmann & Heidinger, 2015). Telomeres are noncoding DNA repeats forming the end-caps of linear chromosomes, thereby 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, Richardson, & Richardson, 2017). TL generally shortens with age, and telomere shortening is accelerated by environmental challenges (Boonekamp, Mulder, Salomons, Dijkstra, & Verhulst, 2014; Reichert et al., 2014; Watson, Bolton, & Monaghan, 2015), reproductive effort (Bauch, Becker, & Verhulst, 2013), stress during adulthood (Hau et al., 2015) and disease (Asghar et al., 2015; Beirne, Delahay, Hares, & Young,

2014), and can differ between habitats (Angelier, Vleck, Holberton, & Marra, 2013; Stier et al., 2016). Finally, telomeres predict remaining lifespan or survival (e.g., Haussmann, Winkler, & Vleck, 2005; Bize, Criscuolo, Metcalfe, Nasir, & Monaghan, 2009; Salomons et al., 2009; Heidinger et al., 2012; Boonekamp, Simons, Hemerik, & Verhulst, 2013; Bauch, Becker, & Verhulst, 2014; Stier et al., 2016). Therefore, TL, and perhaps in particular telomere shortening, could be used as a 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, Krupkin, & Miller, 2000; Foote, Vleck, & Vleck, 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 ultralong 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 (Bauch et al., 2013; Salomons et al., 2009; Steenstrup et al., 2017). Delany et al. (2000) found no evidence for shortening of Class III telomeres in chickens, but their analyses were cross-sectional and data sets were small. Overall, little is known regarding the dynamics of ultralong 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, nondenaturing terminal restriction fragment (TRF) analysis is highly suitable (Haussmann & 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 ultralong 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 (ultralong) telomeres. We found great tits to have a broad TL distribution, ranging from 2.1 to >240 kb, and using multiple techniques (*Bal*31, mixture with seven restriction enzymes) we tested whether the long fragments in this distribution were telomeres rather than subtelomeric 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 (*Cyanistes caeruleus*) TL using quantitative PCR (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 ultralong telomeres in great tits can be attributed to subtelomeric 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.

2 | MATERIALS AND METHODS

2.1 | 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 nestboxes (Atema, Noordwijk, Boonekamp, & Verhulst, 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 data set and ranged from 0.8 to 7.6 years (mean ± *SE*: 2.24 ± 0.076). 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 3 weeks. Subsequently, samples were snap-frozen in 40% glycerol buffer and stored at −80°C.

2.2 | Telomere terminal restriction fragment analyses

We quantified TL in male great tits using TRF assays as described previously (Atema et al., 2015; Salomons et al., 2009) with some adjustments. Briefly, DNA from 4 µl of red blood cells was extracted in agarose plugs using the CHEF Mammalian Genomic DNA Plug kit (Bio-Rad Laboratories). Subsequently DNA from half a plug was digested overnight at 37°C with a mixture of the restriction enzymes *Hind*III (30 U), *Hinf*I (15 U) and *Msp*I (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. By cleaving appropriate sites the restriction enzymes digest specific DNA sequences, but they were selected in such a way as 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 0.5–10 kb; Molecular Weight Marker XV, Roche Diagnostics, range 2.4–48.5 kb; NEB MidRange PFG Marker I, New England Biolabs, range 15–242.5 kb) were separated on a 0.8% agarose gel by pulsed field

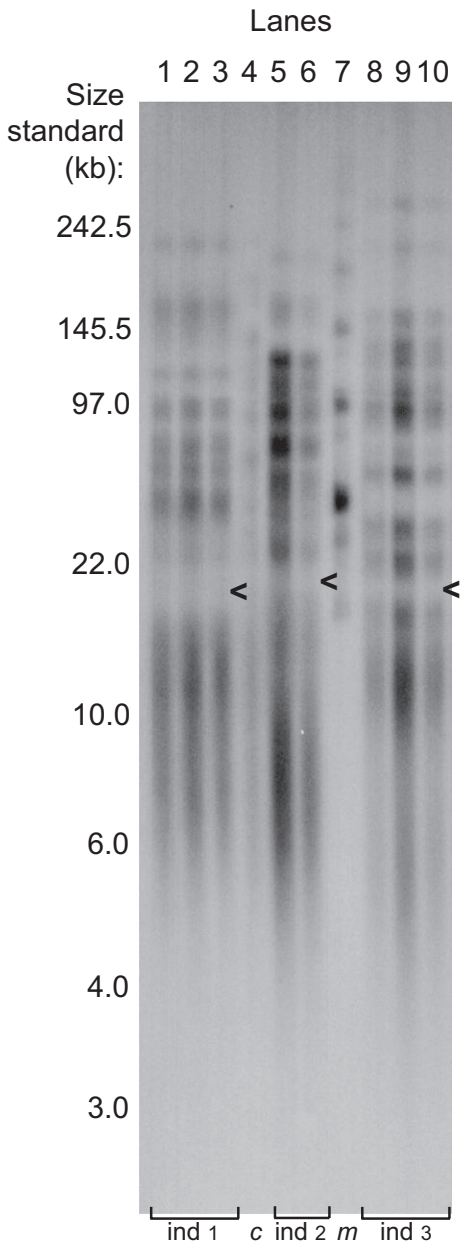

FIGURE 1 Subset of a nondenatured pulsed-field gel representing the variation in telomere distributions. Represented are repeated samples of three individuals, with the clusters of individuals in lanes 1–3, 5–6 and 8–10. Lane 4 includes a control sample and lane 7 includes the ultralong size standard 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

gel electrophoresis at 14°C for 22 hr (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), and the radioactive signal was visualized using a phosphor image (Cyclone Storage Phosphor System, PerkinElmer) (Figure 1).

We quantified the distribution of TLs based on densitometry using the open-source software IMAGEJ version 1.38× as described previously (Atema et al., 2015; Salomons et al., 2009). Our lower molecular weight limit was the point at which the optical density was lowest in the region of short telomeres (approximately 3 kb). As upper limit we set the point at which 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: (a) long telomeres, visible as the hypervariable banded pattern in the high-molecular-weight region (Class III, ~20–240 kb); and (b) short telomeres, which were visible on the gel as a continuous smear in the short-length region (Class II, ~3–20 kb). Like Delaney et al., we confirmed the end-chromosome location of the telomeres using *Bal*31 digestion (see below). Delany 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" (Figure 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 the results with those obtained with a fixed threshold at 20 kb.

2.3 | Testing for effects of subtelomeric regions

Great tit TLs were found 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 subtelomeric sites (Biscotti, Olmo, & Heslop-Harrison, 2015). Potentially, not all DNA other than telomeric repeats was digested by the three restriction enzymes we used, which would have left subtelomeric repeats intact. We therefore carried out three experiments to test the hypothesis that the long telomeres we measured can be attributed to subtelomeric repeats.

First, we applied the exonuclease *Bal*31, 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 *Bal*31 (0.1 U in 200 µl of reaction

buffer): 0, 20, 80 and 240 min. 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 electrophoresis, labelling with the oligonucleotide and visualization of the radioactive signal were performed as described above.

After digestion with *Bal*31 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 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) three times for 30 min and neutralizing it twice for 30 min 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 $(5'\text{-CCCTAA-}3')_4$ and the radioactive signal was visualized following the same protocol as used with the nondenatured 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 visualized with higher contrast, allowing for a more detailed comparison of different treatments.

Second, we used a mixture with seven restriction enzymes, instead of our standard three 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 *Hph*I (15 U), *Mnl*I (15 U), *Rsa*I (15 U) and *Hae*III (15 U) (New England Biolabs). Gel electrophoresis, labelling with the oligonucleotide and visualization of the radioactive signal were performed 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 (also sampled on Vlieland; N = 20) of which we also obtained TRF measurements of the same set of samples. 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 tits and blue tits (Atema, Van, & Verhulst, 2013). As a control "golden" sample we used a sample containing a mixture of DNA of four different great tit individuals. qPCR 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 tits and blue tits on the same plate. We calculated the Telomere/stable copy number gene ratio as (Pfaffl, 2001):

$$\frac{\text{Eff}_{\text{telo}} \wedge \Delta\text{Cq}_{\text{telo}}}{\text{Eff}_{\text{GAPDH}} \wedge \Delta\text{Cq}_{\text{GAPDH}}}$$

We calculated the mean amplification efficiency (Eff) and cycle quantification (Cq) values with the program LINREGPCR (version 12.13; Ruijter et al., 2009). ΔCq was calculated as the Cq values of the control minus the Cq value of the sample. Mean Eff_{telo} was 1.94 ± 0.014 and mean $\text{Eff}_{\text{GAPDH}}$ was 2.11 ± 0.0092, both close to the expected efficiency of 2.

2.4 | 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 data set in adults (two individuals sampled six times, four individuals sampled four times, 32 individuals sampled three times, 60 individuals sampled twice and seven individuals sampled once) and 24 nestlings (all sampled twice), 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 evidence for telomere elongation in a part of our data set, we tested whether telomere elongation was real or could be attributed to measurement error following Simons, Stulp, and Nakagawa (2014). Briefly, this method compares two estimates of variance due to measurement error using different assumptions. Error variance estimates under the first assumption, assuming TL increases and/or decreases, could be calculated using the residual sum of squares. Measurement error variance under the second assumption, that is telomeres do not elongate, is based on the difference in initial and last telomere measurement of individuals that showed an increase in TL. 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 ± standard error (*SE*).

3 | RESULTS

3.1 | 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 (Figure 2), and approximately seven times longer than telomeres of human adults (e.g., Verhulst, Aviv, Benetos, Berenson, & Kark, 2013). We restricted this comparison to species measured in our lab, to minimize methodological effects on TL.

3.2 | Ultralong telomeres or subtelomeric repeats?

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

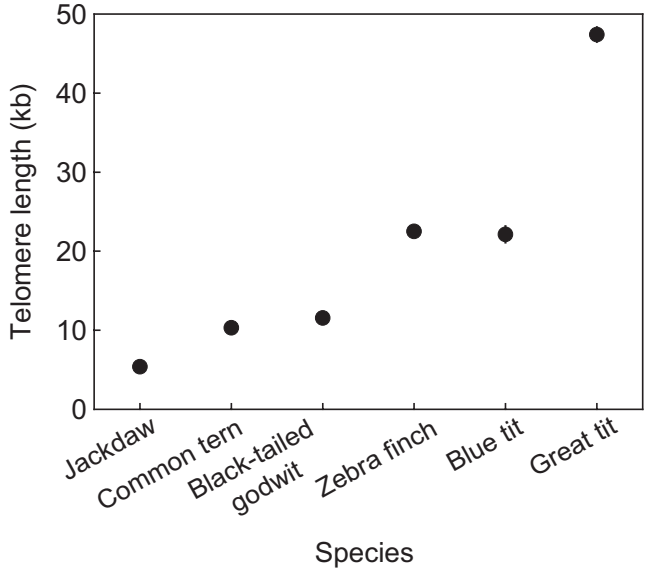

FIGURE 2 Population mean telomere length in adults of different bird species as measured in our lab with terminal restriction fragment analysis in jackdaw (Salomons et al., 2009), common tern (Bauch et al., 2014), black-tailed godwit (Atema, Mulder, Kentie, & Verhulst, 2011), zebra finch (Atema et al., 2015), and blue tit and great tit (this study). Error bars that are not visible are smaller than the marker

3.2.1 | *Bal*31

Over the gradient of digestion times, the signal in the larger weight region decreased and there was a shift in the entire distribution of telomeres towards the lower weight region in which the signal became more intense (Figure 3a). Mean TL estimated after 240 min of digestion with *Bal*31 in five 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 subtelomeric region, because otherwise the signal would have weakened more and shifted less.

3.2.2 | Seven restriction enzymes

We compared TL of four samples (two individuals, each sampled at two ages) with mixes containing three (used throughout our study) and seven 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 Figure 3b), suggesting that this extremely long telomeric repeat was confounded by subtelomeric repeats. The additional restriction enzymes on average caused a modest reduction of TL of 5.8%, which further indicates that the ultralong TL cannot be attributed to subtelomeric repeats in the flanking regions.

3.2.3 | 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; Figure 4). This further indicates that the ultralong telomeres as quantified by the TRF assay represented telomeric repeats rather than subtelomeric regions.

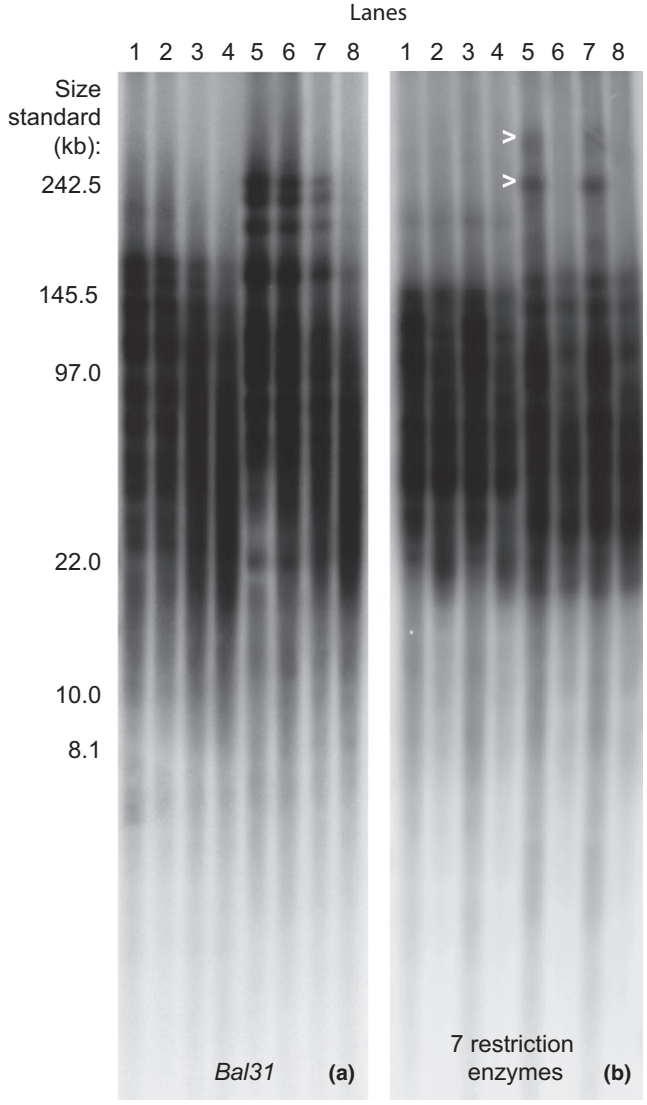

FIGURE 3 Tests to verify that we quantified exclusively end-telomeric repeats. (a) The telomere distribution as visible after digestion of double-stranded DNA ends with *Bal*31. DNA was digested over a gradient of time (0, 20, 80 and 240 s) as shown in two individuals (lanes 1–4 and 5–8). Note further 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 three (odd lanes) or seven (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

3.3 | 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; $F_{1,218.8} = 0.41$, $p = 0.52$; Figure 5a). TL at the 10th percentile was 6.0 ± 0.12 kb, whereas TL at the 90th percentile was 134.8 ± 4.6 kb (Supporting Information 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 (50th–90th percentiles, Supporting Information 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 evidence for the expected telomere shortening with age (Supporting Information Table S1). In subsequent analyses we therefore analysed the ultralong 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; $F_{1,147.8} = 7.63$, $p = 0.0065$, Supporting Information Figure 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, Figure S2).

3.4 | 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 data set, we found little evidence for an age effecton TL in the Class III telomeres (slope: −73.6 ± 428.1 bp/year; $F_{1,241} = 0.029$, $p = 0.86$; Figure 5b).

We quantified TL at the 10th–90th percentiles 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 (Figure 6), confirming the result found for average Class III TL.

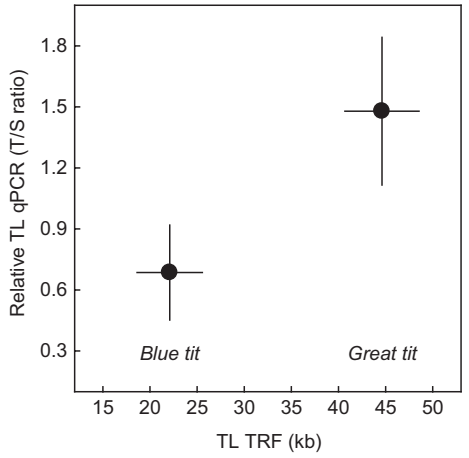

FIGURE 4 Mean telomere lengths (TL) (±*SE*) quantified with terminal restriction fragment (TRF) assays (*x*-axis) and relative qPCR (*y*-axis) in blue tits and great tits.

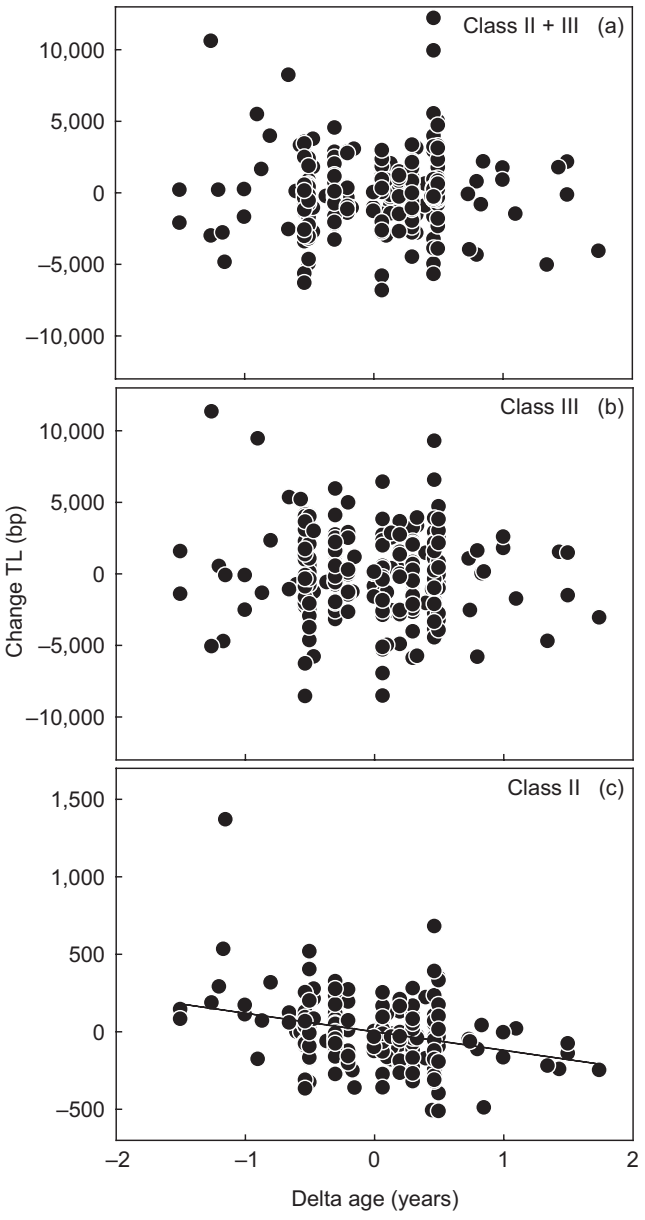

FIGURE 5 Change of telomere length (TL) (bp) in the full (a), long Class III (b) and short Class II (c) telomere distribution plotted against change in age (years). TL did not significantly decline with age in the complete sample and Class III ($p > 0.50$), while Class II telomeres shortened significantly with age. In the analysis, individual identity was included as a random effect, and, to let the figure reflect the analysis, the data on both axes were expressed as deviations from the individual mean age and TL calculated over the different samples per individual. Note substantially smaller scale on the *y*-axis of panel (c).

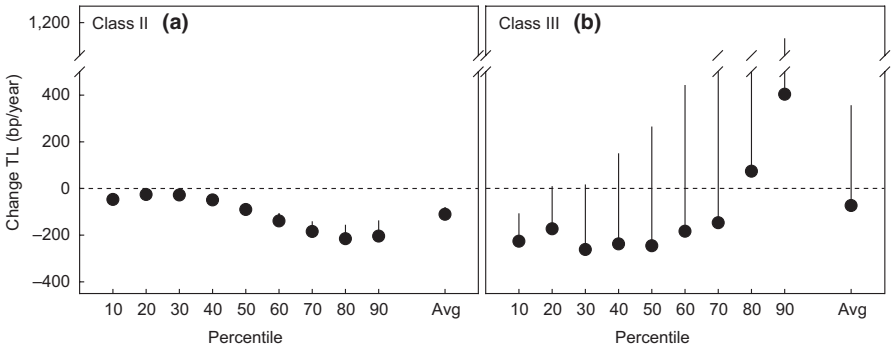

FIGURE 6 Telomere shortening rate (bp year^{-1}; mean ± *SE*) at different telomere length (TL) percentiles as estimated with the mixed model. (a) Class II TL significantly shortened across all percentiles (*p*: >0.0001–0.0092). (b) No significant shortening across the Class III TL percentiles (10th percentile $p = 0.055$; other percentiles *p*: 0.34–0.92). Note the increase in *SE* 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 (Supporting Information 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

3.5 | 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 (Figure 7). Class II TL shortened significantly with age (slope: −111.2 ± 27.1 bp/year; $F_{1,155.1} = 16.78$, $p < 0.0001$; Figure 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, Figure S3). There was no significant difference between winter TL and breeding TL (all years combined) in our data set; 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 the 10th–90th percentiles within the Class II TL distribution we found significant shortening of TL with age at all percentiles (all $p \leq 0.0092$). More importantly, and 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 (Figure 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, Figure S4).

3.6 | 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; Figure 8a), indicating considerable telomere shortening between fledging age and adulthood. Class II TL changed surprisingly little over this period (difference: 0.32 ± 29.9 bp; $F_{1,23} = 0.0001$, $p = 0.99$; Figure 8b), whereas Class III TL became substantially shorter (difference: −559.7 ± 187.6 bp; $F_{1,46} = 8.90$, $p = 0.0046$; Figure 8c). Given these results it was somewhat unexpected that the average of the full TL distribution did not shorten significantly over these 11 days (difference: −178.7 ± 133.0 bp; $F_{1,46} = 1.81$, $p = 0.19$; Figure 7a).

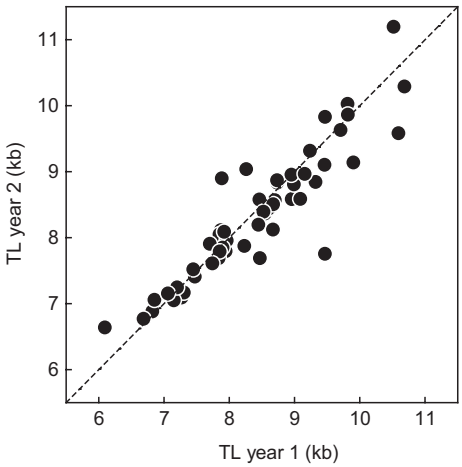

FIGURE 7 Class II telomere length (TL) in year 2 plotted against TL in year 1 ($N = 53$ individuals). The dotted line shows where subsequent measurements are equal ($y = x$). All dots which fall below that line are individuals in which TL was shorter when recaptured 1 year later. For graphical purposes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

4 | 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 into 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 why there is 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 (Bauch et al., 2014; Kimura et al., 2007; Salomons et al., 2009). In theory, the finding that Class III telomeres do not shorten with age in adults could

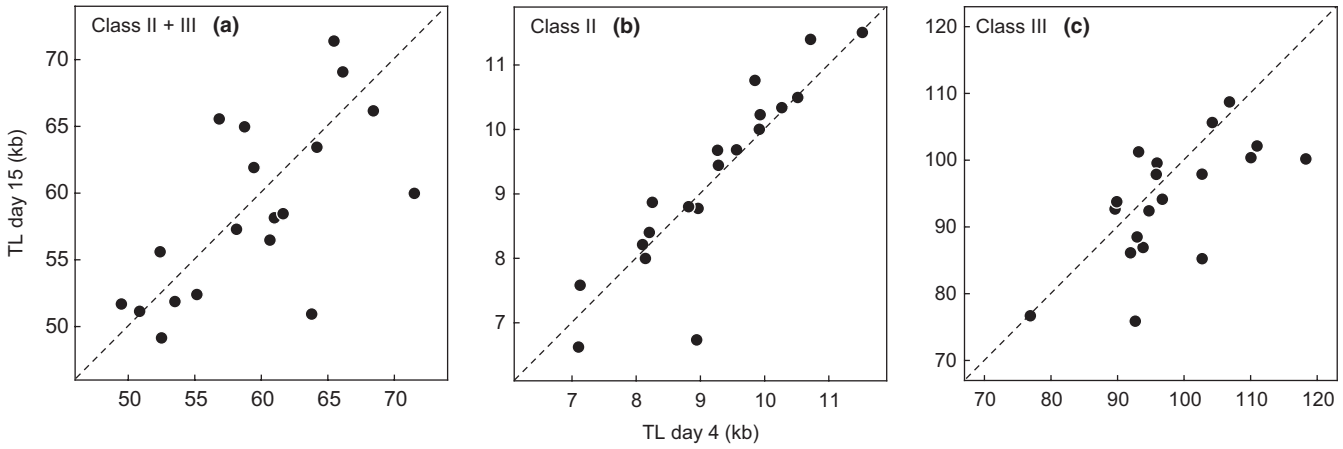

FIGURE 8 Full telomere length (TL) distribution (a), short Class II TL (b) and long Class III TL (c) at day 15 plotted against TL at day 4 (N = 19 individuals). The dotted line shows where subsequent measurements are equal ($y = x$). All dots which fall below that line are individuals in which TL was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

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 (a) the precision of our measurements was high; (b) due to our method, we included only terminal telomeres in our measurements; and (c) 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 example, 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 based on the gap in optical density distribution (Figure 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 setting of the fixed border is done independently of the results, such as by using different data sets to set the border and for testing hypotheses.

While Class III TL was stable 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. We cannot say exactly at what age the Class II telomeres start shortening significantly, and when the shortening of Class III telomeres becomes undetectable, but it will be sometime between fledging (in May) and December when we first recapture the birds. When the Class III telomeres are indeed predominantly at the ends of microchromosomes, the complex telomere dynamics could be due to a difference between early life and adulthood in the function of microchromosomes, perhaps related to development, but this remains to be tested. Regardless, 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, Massemin, Zahn, Tissier, & Criscuolo, 2015; adults: Salmón, Nilsson, Watson, Bensch, & Isaksson, 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 a biomarker. While Class II TL dynamics may be informative regarding

factors affecting telomeres in adulthood, the Class III telomeres may provide information on the 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 subtelomeric 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 (M. Mulder *et al*., unpublished observations), suggesting that ultralong 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 (Figure 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.

Ultralong telomeres were previously found in wild Iberian shrews (Zhdanova, Minina, Karamysheva, Rubtsov, & Londono-Vallejo, 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 fluorescence in situ hybridization on 16 bird species, found microchromosomes to display a large number of telomere sequences, and bird species can have many microchromosomes. Furthermore, Delany et al. (2000) found that Class III telomeres were more numerous in species with more microchromosomes. Great tits were estimated to have 40 different chromosomes ($2n = 80$) of which about half were classified as microchromosomes (Nanda, Benisch, Fetting, Haaf, & Schmid, 2011; van Oers et al., 2014). Thus, the large number of microchromosomes might, at least partly, explain the high prevalence of ultralong telomeres in great tits. Note that this explanation contrasts with the positive correlation between chromosome size and TL observed in the human genome (Wise et al., 2009). On the other hand, the smallest human chromosome (no. 21) is larger than all great tit microchromosomes (Santure et al., 2013), and different processes may act on different size ranges.

We would have concluded that adult TL was independent of age if relying on a technique that yields a single estimate to characterize TL, 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 if a more informative technique had been used. For instance, no effect of age on TL estimated with qPCR was found in leatherback turtles (Plot, Criscuolo, Zahn, & Georges, 2012), which could be due to the presence of Class III telomeres, given that turtles also have microchromosomes (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 TL 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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AUTHOR CONTRIBUTIONS

E.A., A.J.N. and S.V. designed the study, E.A. and A.J.N. collected samples, E.A. and E.M. analysed samples, E.A. performed statistics and drafted the manuscript with S.V., and all authors commented on the manuscript.

DATA ACCESSIBILITY

The data on which this paper is based are available through Dryad (https://doi.org/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: (i) measurements of the complete telomere range, (ii) measurements of the "smear" at the lower end of the distribution only, (iii) 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: (a) measurements of the complete telomere range, (b) measurements of the "smear" at the lower end of the distribution only, and (c) measurements of the telomere range above the "smear".

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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