Title page

The role of DNA integrity in opportunistic longitudinal telomere studies

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Running headline: *DNA integrity and relative telomere length*

Abstract

Determining both individual age and population age distribution is crucial for an array of ecological studies. This is generating growing interest in molecular age markers such as telomere length. Most studies exploring the relationship between age and telomere length have been cross-sectional, but such studies face problems with large individual variation and the selective loss paradox. Thus, interest is growing rapidly for turning to longitudinal studies. In this study, the rate of telomere loss was analyzed for the extraordinarily long-lived North Island brown kiwi, *Apteryx mantelli*. Relative telomere length (RTL) was quantified using qPCR from blood from four separate sampling occasions across 14 years were analyzed. Uniquely, the analysis of RTL was combined with high resolution analysis of genomic quality to get numerical values of DNA integrity. The analysis of RTL suggested a circa 5 % annual increase in *A. mantelli* telomere length. However, RTL was found to be highly correlative with DNA integrity, indicating that the perceived elongation of telomeres was a result of DNA quality differences between cohorts. Notably, the observed, positive correlation remained significant even when analyzing only samples classified as being of high DNA quality. Previous work has highlighted the potential impact of sample storage differences on RTL. However, to our knowledge, this is the first study to suggest that even small differences in DNA integrity between samples cohorts can impact the results of telomere studies. These findings are of great importance since longitudinal telomere studies of long-lived species tend to be “after the fact” utilizing already available samples for which handling and/or storage regimes might differ or be unknown. For such studies, we suggest that analysis of DNA quality with higher precision than traditional gel electrophoresis is needed to generate reliable results of telomere dynamics.

Key words

aging, DNA stability, sample storage, qPCR, relative telomere length, telomere attrition

Introduction

Telomeres are repetitive nucleotide sequences at the end of all linear, eukaryotic chromosomes (Allsopp et al., 1995). Due to the so-called end-copy-problem, the telomeric sequence shorten during each DNA replication, which gives telomere length the potential to act as a molecular clock indicating (biological) age (Olovnikov, 1996; Shay & Wright, 2000; Bize, Criscuolo, Metcalfe, Nasir & Monaghan, 2009; Smith, Wagner, Szép, Hoelzl & Molnár, 2016). This potential has brought a lot of attention to telomere research in ecology and conservation biology as age determination beyond broad categories such as "juvenile", "subadult" and "adult" is frequently hard, especially for long-lived species (Holmes & Martin, 2009; Wolfe, Ryder & Pyle, 2010; Töpfer, 2018). Meanwhile, determining age of individuals and/or age distribution within populations is highly desirable since it enables increased precision, for instance, in population modelling and conservation management evaluations (McCleery, Perrins, Sheldon & Charmantier, 2008; Wolfe et al., 2010; Martin, 2015). In addition, there is growing interest in looking at telomeres, and in particular at patterns of attrition (shortening) rate, in connection to several other aspects of evolution and ecology (Ringsby et al., 2015; Monaghan, Eisenberg, Harrington & Nussey, 2018; Tricola et al., 2018). A growing number of studies have connected accelerated telomere attrition to stress (Monaghan, 2014; Ibáñez-Álamo et al., 2018; Spurgin et al., 2018), for example. All these studies rely on accurate measurements of telomere length and/or telomere attrition rate. With the sharp increase in telomere quantification interest, the most frequently used method has shifted from Southern hybridization-based assays to quantitative real-time polymerase chain reaction (qPCR; Lai, Wright & Shay, 2018). The main motivators of this shift are the higher cost and complexity of the older methods, whereas qPCR is a relatively low-cost method that has the potential to be used as a relatively high throughput method with comparatively few and uncomplicated laboratory steps (Lai et al., 2018). Another benefit

with qPCR is that this method uses comparatively small amounts of template DNA (Lai et al., 2018). However, it does have the same requirement for high DNA quality (Fleige et al., 2006; Fernandez-Jimenez et al., 2011).

The relationship between age and telomere length has most frequently been explored through cross-sectional studies analyzing known-age individuals sampled at one point in time (see for example: Haussmann et al., 2003; Horn et al., 2011; Tricola et al., 2018; Wilbourn et al., 2018). Two problems potentially compromising the accuracy of such studies have been identified: (1) there tends to be large individual variation and/or low correlation between chronological (actual) age and telomere length for adult individuals (Haussmann et al. 2003; Horn et al. 2011; Dantzer & Fletcher 2015; Sudyka et al. 2016), and (2) the so-called selective loss paradox. The selective loss paradox (or the selective disappearance problem) implies that since shorter telomeres are associated with higher risk of mortality (Wilbourn et al., 2018) old individuals available for measuring risks being biased towards "long telomere individuals" while "short telomere individuals" to a larger extent have already disappeared from the population (Haussmann & Mauck 2008; Haussmann & Marchetto 2010; Dantzer & Fletcher 2015). These two problems have led to significant interest for moving from cross-sectional to longitudinal studies of telomere dynamics. Birds are comparatively well represented in these studies, likely for two key reasons. Firstly, this group constitutes a good study system for the evolution of telomere dynamics with species representing a wide spectrum of life-expectancies and sizes (Monaghan 2018). Secondly, the prevalence of long-term mark-recapture (banding) studies is higher among birds than other animal groups, which tend to render access to known age individuals and/or samples collected from the same individuals over time (Monaghan 2018). Previously collected samples from the same individuals over time constitute a very tempting resource to utilize for telomere studies. However, a major potential concern with such after-the-fact study scenarios is the limited

influence on how the available samples have been stored and handled over time. In particular, handling and storage might have differed among sample cohorts. In our case we had access to blood samples from brown kiwi (*Apteryx mantelli*) collected from the same individuals over 14 years in four discreet cohorts from four unrelated studies. Here we compare the relative telomere length (RTL) and DNA integrity of these samples to determine the annual telomere attrition rate for adult *A. mantelli* with the aim to develop a method for more accurate aging of these long-lived birds.

Optimal DNA for telomere analysis should be of high molecular weight. However, it is well documented that storage of tissue (including blood) as well as extracted DNA can affect rate and extent of DNA fragmentation and that high DNA concentration does not equal DNA of high integrity (Seutin, White & Boag, 1991; Freed & Cann, 2006; Shabihkhani et al., 2014; Rahikainen, Palo, de Leeuw, Budowle & Sajantila, 2016). Eastwood, Mulder, Verhulst and Peters (2018) as well as Reichert et al. (2017) found that sample handling and in particular sample storage media had a significant effect on relative telomere length measured using qPCR in sample from wandering albatross (*Diomedea exulans*), zebra finch (*Taeniopygia guttata*) and purple-crowned fairy-wren (*Malurus coronatus*). The authors of both these studies suggest that this is due to differences in DNA integrity. However, to our knowledge this is the first study ever to combine a longitudinal telomere length study using qPCR with direct quantification of DNA integrity.

Method

Samples

This study utilized brown kiwi (*A. mantelli*) samples from Ponui Island in the Hauraki Gulf along the eastern coast of New Zealand's North Island [36 55' S, 175 11' E]. Sample collection took place in 2004, 2006-2008, 2010 and 2017-2018, respectively, for four

separate studies. All samples were frozen within hours of collection and transferred to a -80ºC freezer within one week, but all samples have been subsequently thawed to take sub-samples for downstream analysis. The time the samples spent defrosted before being returned to long-term storage at -80ºC was unknown. Samples from 2004 were frozen in no media. Samples from 2006-2008 were stored in a combination of Queen lysis buffer (Seutin buffer) and 95% alcohol. In 2010, centrifuged red blood cells were stored in heparinized tubes and in 2017-2018 whole blood was stored in heparinized tubes. The sampling in 2004 was done in association with the establishment of a long-term study using radio transmitter fitted birds. Subsequent samples were collected from birds already part of the ongoing research program already fitted with radio transmitters. In total we analyzed the relative telomere length of 67 samples from 23 adult kiwi of unknown age. Each bird was represented by between two and four samples.

DNA Purification

DNA was extracted from 10 µl thawed whole *A. mantelli* blood using a High Pure PCR template preparation kit (Roche). Manufacturer's instructions were followed with the exception that elution was done in two rounds with 50µl of elution buffer each centrifugation round. The DNA extraction success and quality were validated using agarose gel electrophoresis (1.5-2% agarose in TAE buffer) and the concentration of DNA was measured using the Qubit dsDNA High Sensitivity assay (Life Technologies, CA, USA).

qPCR

For telomere amplification the generic (and consistently used) bird telomere primers tel1b (CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT) and tel2b (GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT; Criscuolo et al., 2009) were used. The single copy control or housekeeping gene used was GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) and the identified primer pair was a combination of an already

145 published reverse primer sequence (GAPDHR: CCATCAGCAGCAGCCTTCA; Criscuolo et
146 al., 2009) and a specifically developed forward primer (and GAPDHF_kiwi_n2:
147 CTTGCACAGCTGACACAATTTG). Primers were manufactured by Integrated DNA
148 Technologies (Ames, IA, USA). Primer efficiencies were first verified using standard PCR.

149 For telomere analysis 1ng of template DNA per reaction was amplified under the following
150 conditions: 1x Hot FIREPol EvaGreen qPCR Supermix (Solis, Biodyne, Tartu, Estonia),
151 250nM of each primer, made to a total volume of 20ul with nuclease free water. For GAPDH
152 analysis 4ng of template DNA per reaction was amplified under the following conditions: 1x
153 Hot FIREPol EvaGreen qPCR Supermix (Solis, Biodyne, Tartu, Estonia), 200nM of each
154 primer, made to a total volume of 20ul with nuclease free water. Amplification for both
155 primer sets occurred in a LightCycler 480 II (Roche, Basel, Switzerland) with the following
156 program: Pre-incubation at 95°C for 12 minutes, then 40 rounds of denaturation at 95°C for
157 15s, annealing at 60°C for 30s, and elongation at 72°C for 30s, followed by a melting curve
158 step. Samples were run as triplicates and each plate included a four set 2x dilution series and
159 a water control. The DNA amounts in the dilution series was 4, 2, 1 and 0.5 ng per reaction
160 on the telomere plates and 8, 4, 2 and 1 ng on the GAPDH plates, using DNA from one of the
161 samples. Telomere and GAPDH primers were run on separate plates to keep plate position
162 constant per sample (Eastwood et al., 2018). The longitudinal samples from the same
163 individual were also run on separate plates to keep plate position constant. However, to
164 determine variation in between runs, a subset of longitudinal samples was also run within the
165 sample plate.

166 ## Longitudinal RTL comparison

167 To calculate relative telomere length, we used the formula developed by Eastwood *et al.*
168 (2018). This formula combines the threshold cycle (C_t), the individual qPCR efficiency (E)
169 and a "goldstar value" for both the telomere and the GAPDH run of the individual sample

(Equation 1). We used C_t as well as the individual qPCR efficiency values calculated using LinRegPCR (Academic Medical Center, Amsterdam, NL). The "goldstar value" was extracted by reading the "ideal" C_t value for the DNA concentration used (1ng and 4ng per reaction, respectively) as indicated by the slope of the plate-specific efficiency curve based on the plate-specific dilution series. This slope was generated on the LightCyclers own software.

$$RTL = \frac{E_{Tel}^{(Goldstar\ value\ ¿¿\ Tel - C_{t_{Tel}})}}{E_{Control}^{(Goldstar\ value ¿¿ Control - C_{tControl})}} ¿$$

Equation 1. Calculation of relative telomere length (RTL) based on threshold cycle (C_t), the individual qPCR efficiency (E) and a "goldstar value" extracted by reading the "ideal" C_t value for the DNA concentration used as indicated by the slope of the plate-specific efficiency curve, and comparing this for telomeres (Tel) and GAPDH (control) for each sample (Eastwood et al., 2018).

Statistics

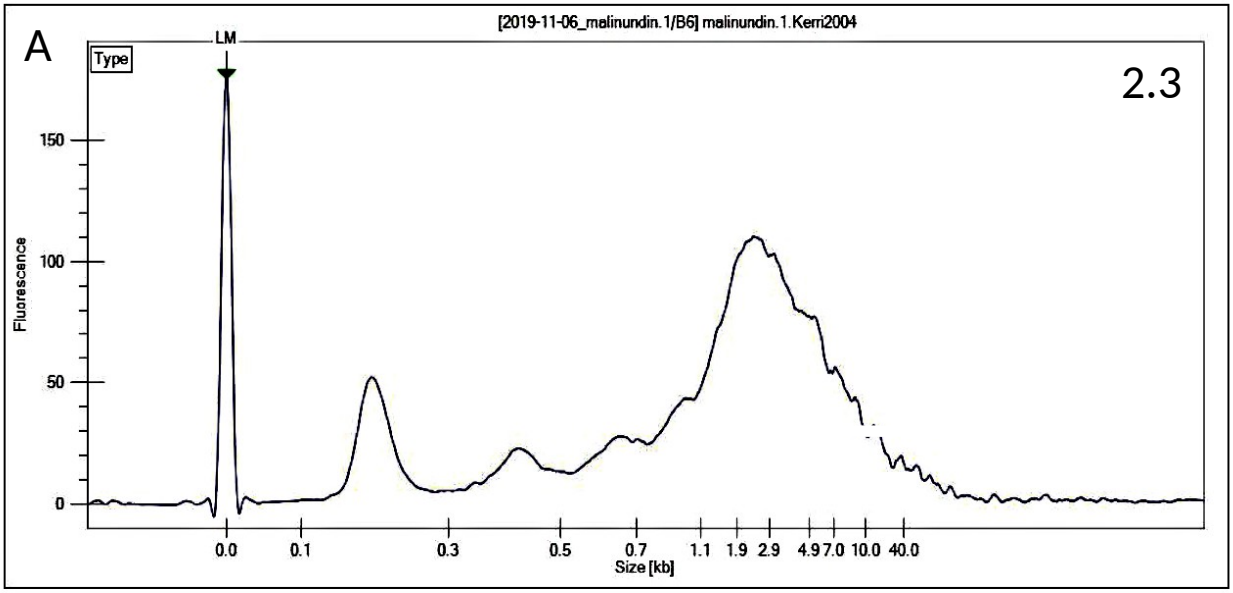
Statistical analyses were conducted in R (R core team version 3.6.2). Correlation analyses were used to evaluate the relationship between relative telomere length and genomic quality score, and between each factor and sample age. Regression analyses were used to determine the average annual change in *A. mantelli* telomere length. ANOVA was used to compare the four sample cohorts with respect to individual qPCR efficiency, relative telomere length and genomic quality score.

DNA Quality Analysis

A subset of 20 samples were analyzed using the Perkin Elmer LabChip® GX Touch HT (Perkin Elmer, Waltham, MA, USA) fragment analyzer using the Hi Sensitivity LabChip at Massey Genome Service (Massey University, Palmerston North, New Zealand) to obtain a

193 quantification of DNA integrity known as genomic quality score (GQS, developed by Massey
194 Genome Service). GQS is a score from 0 to 5 based on a fragment size distribution where “5”
195 corresponds to intact genomic DNA and “0” to “highly degraded” genomic DNA (Fig. 1).

196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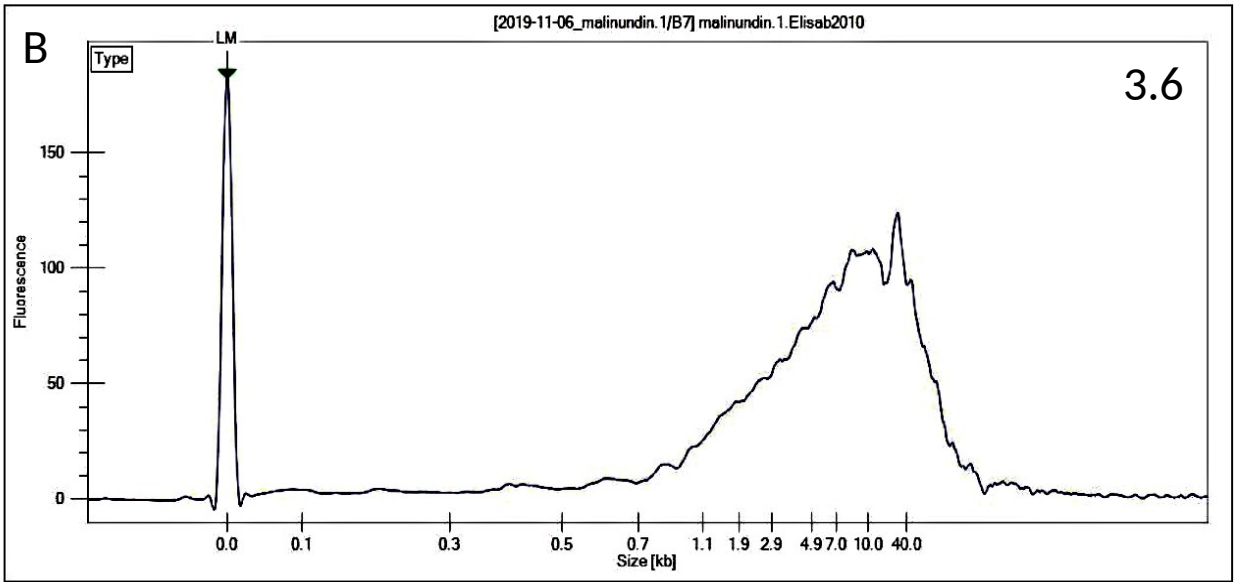
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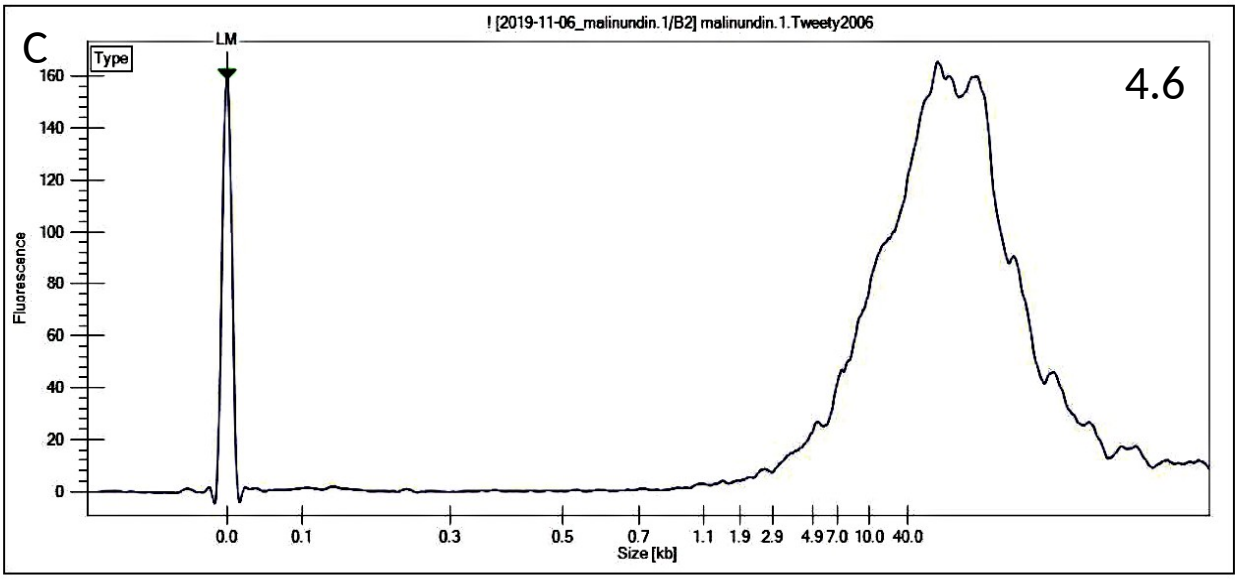
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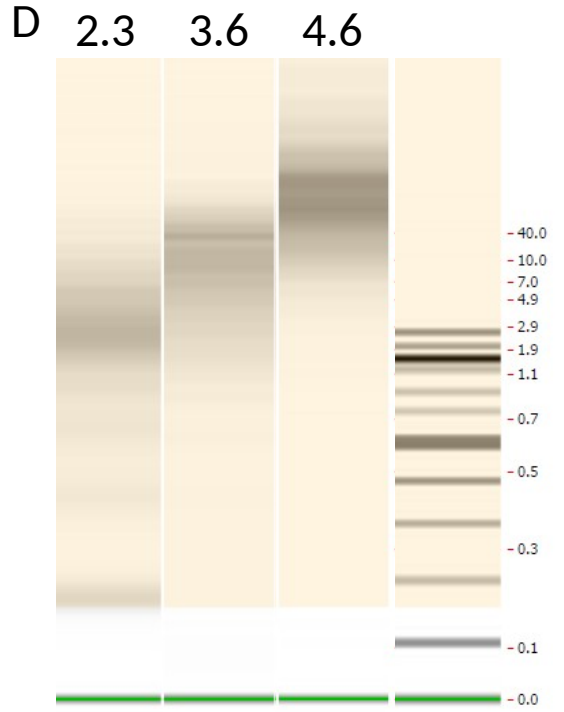


Figure 1. DNA fragment size distribution as visualized in two ways by the Perkin Elmer LabChip GX Touch HT fragment analyzer. Results for three representative samples from our study, exemplifying partially degraded DNA (2004 sample; Genome Quality Score (GQS) 2.3; panel A and D far left), good quality DNA (2010 sample; GQS 3.6; B and D middle) and essentially intact DNA (2006 sample; GQS 4.6; C and D right; maximum GQS is 5).

Results

Based on the overall dataset of 67 samples from 23 birds, our results suggest that relative telomere length (RTL) increase with about 5% per year in adult *A. mantelli* both based on regression analyses for all samples combined (4.7 % annual increase, $R^2 = 0.25$; Supplementary Fig. 1), and on calculating the average of all individual rates of change (5.5% annual increase STD 5.52; Fig. 2).

However, there was a significant cohort effect for RTL ($f = 20.44$, $df = 81$, p-value < 0.001; Fig. 3 B) with samples from the 2004 and 2010 having shorter telomeres than those from 2006-2008 and 2017-2018. Control experiments with longitudinal samples run within plates

supported that this was a true cohort effect and not caused by inter-plate difference (Supplementary Fig. 2). In addition, there was no cohort effect, in terms of individual qPCR efficiency ($f = 0.67$, $df = 82$, p-value = 0.57; Fig. 3 C), indicating consistency of the qPCR process.

Focusing in the subset of samples for which both genomic quality score (GQS) and relative telomere length (RTL) were examined, there was a significant difference between sample cohorts for the GQS with 2006-208 samples having higher and 2004 samples lower DNA integrity than other cohorts ($f = 15.36$, $df = 23$, p-value < 0.001; Fig. 3A). The differences in GQS and RTL did not correlate with sample age *per se*. Instead there was a significant positive correlation between RTL and GQS ($t = 4.26$, $df = 25$, p-value < 0.001, $R^2 = 0.42$, adjusted $R^2 = 0.40$; Fig. 4). This correlation remained significant even after excluding the three samples of lowest DNA quality to focus only on samples with an GQS higher than three (which is an accepted cut off for a level of quality "good enough NGS library prep and sequencing"; X. Lin pers. comm.; $t = 4.12$, $df = 22$, p-value < 0.001). In addition, pairwise comparisons of longitudinal samples showed that increasing RTL between samples was, in all cases except one, associated with higher GQS for the new samples while lower GQS for the newer sample was associated with decreasing or, in one case, unchanged RTL (Fig. 5).

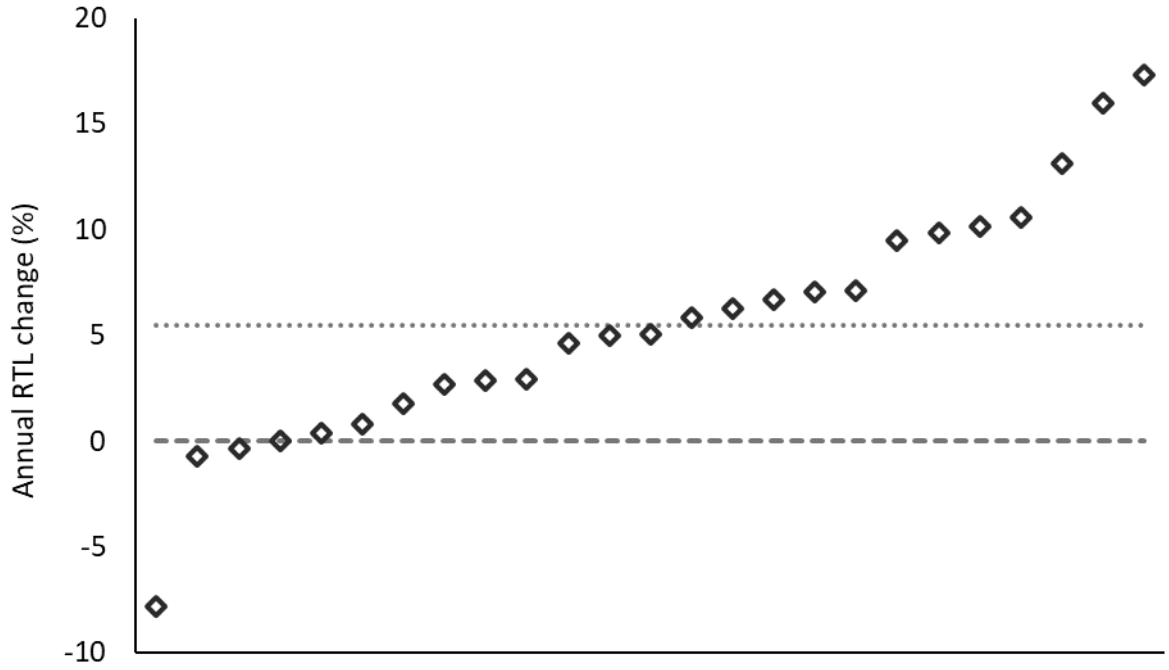
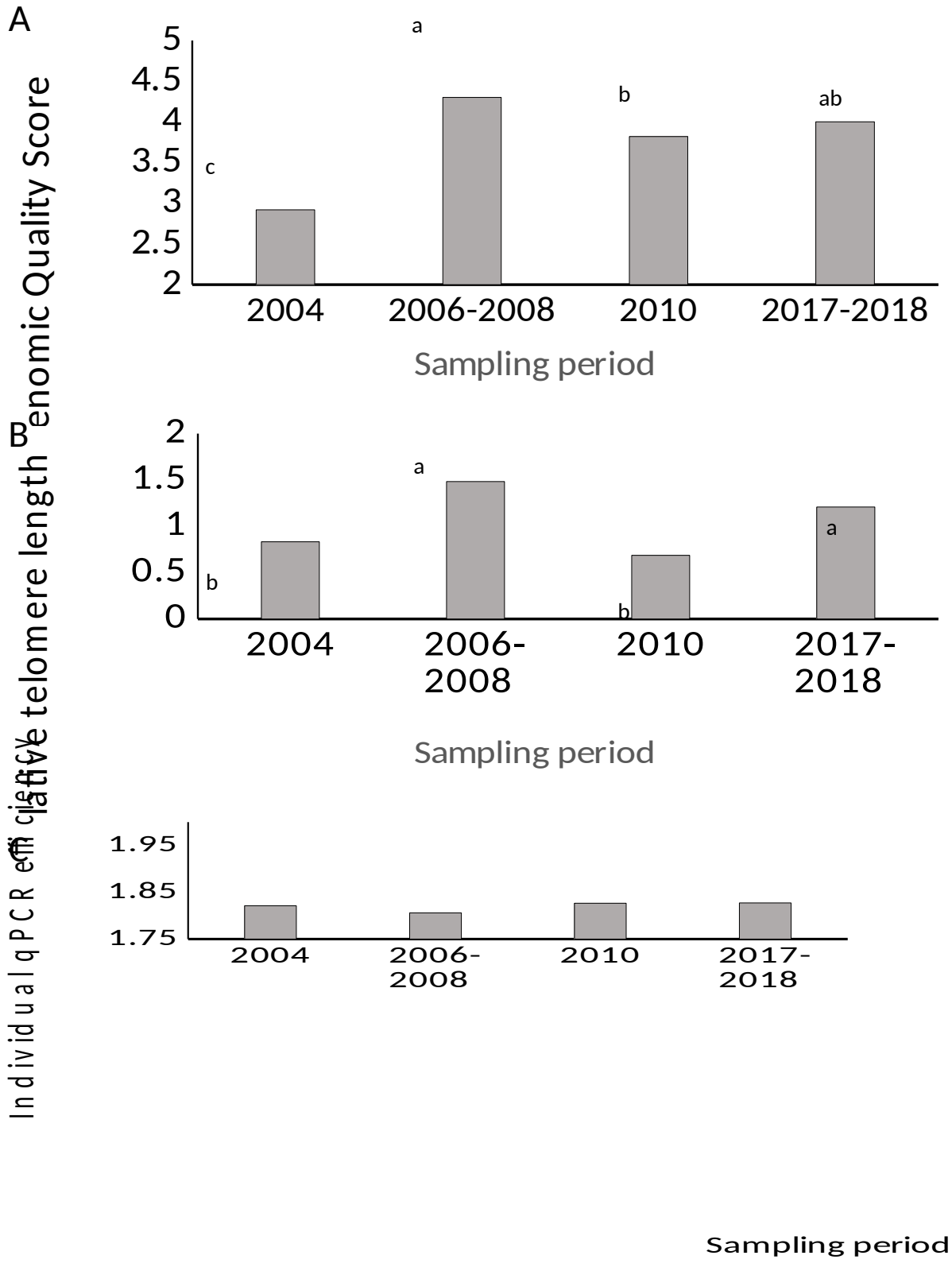


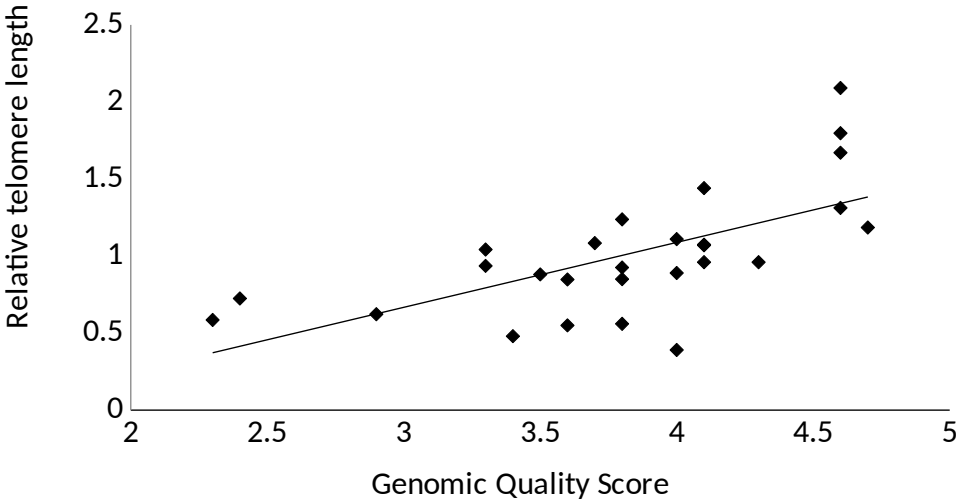
Figure 2. Annual change in relative telomere length (RTL) for each of the 23 analyzed *A. mantelli* graphed in order of rate of telomere change from fastest loss (far left) to greatest annual increase (far right). Three samples fall below the zero-change line (darker dotted line) indicating annual telomere shortening. Light grey dotted line indicates the average annual change across all sampled birds: 5.5% yearly increase.



236 **Figure 3.** A cohort effect was evident for genomic quality score (A) as well as relative

237 telomere length (B) but not for individual qPCR efficiency (C) was found. Bars indicate

238 average, error bars indicate standard deviation, numbers indicate sample size and different
239 lowercase letters above bars indicate significant difference.



241 **Figure 4.** Relationship between relative telomere length (RTL), quantified by qPCR, and genomic
242 quality score (GQS), quantified by the Perkin Elmer LabChip GX Touch HT fragment analyzer.
243 Mathematical relationship: $y = 0.42x - 0.60$; $R^2 = 0.42$. A higher GQS indicates higher DNA integrity
244 with a GQS of 5 implying no detectable degradation.

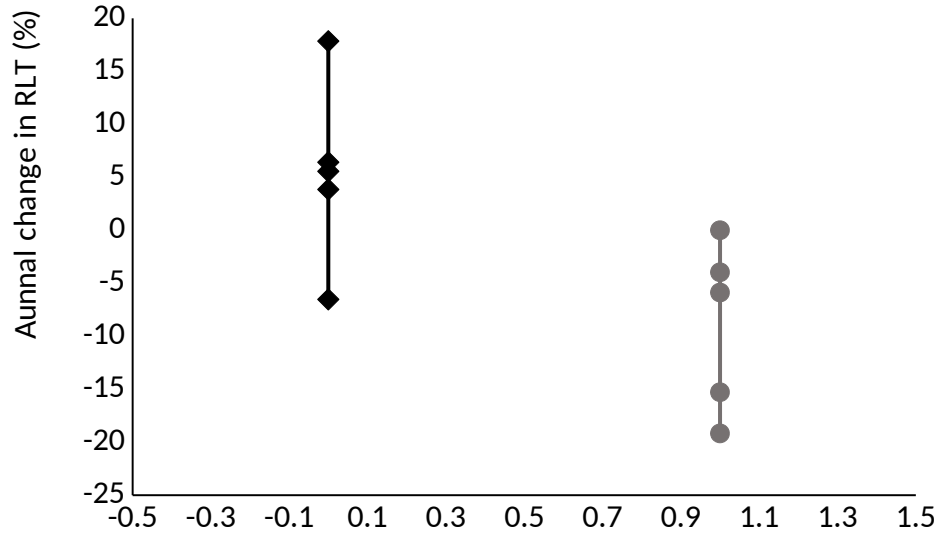


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Figure 5. Annual change (percentage) of relative telomere length (RTL) between pairwise longitudinal samples. Each point indicates one pairwise comparison (N = 10). Comparisons are grouped by whether the newer sample had a higher (left) or lower (right) genomic quality score (GQS) and are connected for clarity. Negative y-axis values indicate telomere attrition (shortening) and dotted line highlights the transition from attrition to increasing telomere length over time.

Discussion

In this paper we show for the first time that even a small amount of DNA decay affects relative telomere length (RTL) measurements enough to impact the overall results of after-the-fact longitudinal telomere studies. Our longitudinal study of *A. mantelli* telomeres suggested a 5% annual increase in RTL. However, we found (1) a strong positive correlation between genomic quality score (GQS) and RTL and (2) that increasing RTL between samples from the same bird, in all cases except one, was associated with a higher GQS of the newer sample. Most noticeably, this association between GQS and RTL was significant even then only considering samples with GQS suggesting "high quality genomic DNA" (a GQS above 3). Thus, we suggest that the quantified annual telomere growth in *A. mantelli*, is equally or more likely to represent a quantification of DNA integrity rather than a cell division (and thus age) related telomere attrition. Further, we suggest that these detrimental differences in DNA integrity between sample cohorts is more likely to be related to storage and handling conditions than to time between blood sampling and laboratory analyses *per se,* which is consistent with previous findings (Reichert et al., 2017; Eastwood et al., 2018).

To our knowledge, our research group is the first ever to analyze *Apteryx* telomeres. The results presented here do not necessarily rule out annual telomere increase in *A. mantelli* only that the quality of our samples rendered it impossible to accurately quantify this. Interestingly, results from studies of certain species, such as Leach's storm petrel (*Oceanodroma leucorhoa;* Haussmann et al., 2003; Tricola et al., 2018), Eurasian

oystercatcher (*Haematopus ostralegus*; Tricola et al., 2018) and Edible dormouse (*Glis glis*; Hoelzl et al., 2016), and of certain individuals within other species such as Blue tits (*Cyanistes caeruleus*; Sudyka, Arct, Drobniak, Gustafsson & Cichoń, 2019), Seychelles warbler (*Acrocephalus sechellensis*; Spurgin et al., 2018; Wood & Young, 2019), European storm petrel (*Hydrobates pelagicus*; Watson, Bolton & Monaghan, 2015), Alpine swift (*Tachymarptis melba*; Bize et al., 2009), and Zebra finch (*Taeniopygia guttata*; Heidinger et al., 2012) indicate elongating telomeres over time. Such results are often dismissed as measuring errors or the result of selective loss that skews data (see introduction; Bateson & Nettle, 2017; Tricola et al., 2018). However, some authors have found convincing statistical support for that increasing telomere length should not be dismissed so quickly, but rather that lengthening can be expected to occur in a small proportion of the population (Bateson & Nettle, 2017; Spurgin et al., 2018). Along similar lines, several studies of other long-lived bird species, such as Leach's storm petrel (Haussmann et al., 2003; Tricola et al., 2018), thick-billed murre (*Uria lomvia;* Young et al., 2013), kakapo (*Strigops habroptilus;* Horn et al., 2011), European shag (*Phalacrocorax aristotelis*) and wandering albatross (Hall et al., 2004) have not found support for shortening telomeres with increasing age, in particular among adult individuals, however there are exceptions such as great frigatebird (*Fregata minor*; Juola, Haussmann, Dearborn & Vleck, 2006). Lastly, there are ways through which telomeres can elongate, the most common mechanism in animals is through the activity of the enzyme telomerase (Greider & Blackburn, 1985), but other mechanisms also occur even in vertebrates (see for instance Foley et al., 2018). Thus based on these studies and the long lifespan of *A. mantelli* there is a theoretical potential that our results indicating a yearly increase in telomere length is be correct, but we withhold that further study based on longitudinal samples of equally high DNA quality is needed to definitively determine this.

Conclusion

Our results are consistent with previous studies (Reichert et al., 2017; Eastwood et al., 2018), However, to our knowledge, this is the first study to suggest that differences in DNA decay between sample cohorts (due to different storage and handling regimes) can be big enough to directly influence the calculated annual change in telomere length. In fact, we suggest that qPCR measurements of telomeres might under these conditions act more as a measure of DNA integrity than of true relative telomere length. Thus, there is reason to believe that RTL measurements comparing samples that have been stored and handled in different ways is not a reliable resource for longitudinal studies of telomere dynamics. Our results can have wide implications since we have observed an significant increase in telomere studies using qPCR over the last few years and telomere length and attrition rate is discussed more and more frequently as a tool for everything from evaluating habitat quality, population health, and age distribution to answering big questions about the evolution of cancer prevention mechanisms. Thus, incorrect results caused by un-accounted for sample decay can have severe consequences, not the least for populations in need of conservation management.

We do recognize the potential and value in analyzing telomere length using qPCR, and do not suggest against using this method. However, we recommend that there is a need to make sure samples are handled and stored in a consistent way, and if this is not possible, at least that the studies include analyses of DNA integrity beyond standard gel electrophoresis. Our results suggest that ensuring comparable intactness/quality between samples compared is very important and that this likely has a bigger impact on the results than striving for highest possible quality *per se*. Noticeable is that in this study we were not able to detect the degradation differences through comparing qPCR efficiency.

Lastly, we suggest that more research is needed into exactly what elements of storage and handling affect DNA quality in a way that impact RTL measurement. We suggest that this would be performed through an extension of the work of Eastwood et al. (2018) by intentionally exposing aliquots of the same samples to different settings after which GQS and RTL be analyzed and compared. This would enable the identification of an ideal protocol for accurate telomere quantification using qPCR and judging by the growing interest in these types of studies, such a protocol is urgently needed.

Authors contribution

MU and IC conceived the ideas; MU and KG designed methodology; MU collected and analyzed the data; MU led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Acknowledgement

Sample collection for this research was conducted with permission from the Massey University Animals Ethics Committee, Department of Conservation as well as the traditional owners and the current dwellers of the sample site. Funders making this enable this research were: Bernard Sabrair and Elka Gouzer-Waechter through the Massey Foundation, the JS Watson trust, the Julie Alley Bursary, and the Kiwi Recovery Group. In addition, this research was made possible by numerous sample collectors over the years and by skilled and by Anja, Briana and Trish who assisted in the lab.

Data Availability statement

There is no data to be archived linked to this manuscript.

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