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Utility of telomere length measurements for age determination of humpback whales

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ABSTRACT

This study examines the applicability of telomere length measurements by quantitative PCR as a tool for minimally invasive age determination of free-ranging cetaceans. We analysed telomere length in skin samples from 28 North Atlantic humpback whales (*Megaptera novaeangliae*), ranging from 0 to 26 years of age. The results suggested a significant correlation between telomere length and age in humpback whales. However, telomere length was highly variable among individuals of similar age, suggesting that telomere length measured by quantitative PCR is an imprecise determinant of age in humpback whales. The observed variation in individual telomere length was found to be a function of both experimental and biological variability, with the latter perhaps reflecting patterns of inheritance, resource allocation trade-offs, and stochasticity of the marine environment.

INTRODUCTION

The most commonly applied approaches for age determination of cetaceans are based on counting of growth layer groups (GLGs) in hard structures such as dentine, bone, baleen plates and ear plugs (Chittleborough 1965; Laws 1952, and this volume), as well as eye-nucleus aspartic acid racemisation (Garde *et al.* 2007; George *et al.* 1999; Nielsen *et al.* 2013; Olsen & Sunde 2002). However, with the exception of teeth, which can be collected in live-capture release projects of small cetaceans, these approaches typically require that the animal is dead. For free-ranging cetaceans, photo-ID records can be used to track identified individuals from their year of birth (Hammond *et al.* 1990; Katona & Whitehead 1981), but require substantial efforts in time and

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funding to establish a long-term photo record. Recently, two studies based on tissue biopsies from humpback whales have reported significant age-associations with blubber lipid profiles (Herman *et al.* 2009) and levels of cytosine methylation (Polanowski *et al.* 2014). Although very promising, the age-estimates of both methods were associated with 95% confidence intervals of up to 8-10 years, suggesting that alternative methods that are more accurate may still be of interest.

Here we investigate the utility of telomere length measurements in cetacean skin tissue samples. Telomeres are short tandem repeats situated at the end of chromosomes, where they provide a protective cap and ensured normal cell function (Blackburn 1991; Blackburn & Gall 1978). The interest in age determination by telomeres stems from the observation that telomere length declines over the lifespan in several animal species (Dunshea *et al.* 2011; Haussmann *et al.* 2003; Nakagawa *et al.* 2004; Vleck *et al.* 2003), including mammals (Allsopp *et al.* 1992; Garde *et al.* 2010; Hastie *et al.* 1990; Izzo *et al.* 2011; Pauli *et al.* 2011), birds (Haussmann & Mauck 2008b; Juola *et al.* 2006; Salomons *et al.* 2009), and reptiles (Hatase *et al.* 2008; Xu *et al.* 2009). If similar correlations can be found in cetaceans, telomere length estimates could provide a basis for genetic age determination of free-ranging individuals. Our assessment focuses on the humpback whale, for which life-history data and substantial sample collections are available from extensive long-term studies (Clapham 1992; Clapham 1996; Katona & Whitehead 1981; Robbins 2007).

MATERIALS AND METHODS

Study material

Samples and validating age data were obtained from a multi-decade study of North Atlantic humpback whales that summer in the Gulf of Maine (Center for Coastal Studies, Provincetown MA). Humpback whales are individually identified based on their natural markings (Katona & Whitehead 1981) and skin samples can be collected from free-ranging whales by biopsy sampling techniques (Palsbøll *et al.* 1991). For this study, we analysed samples from 28 individuals that had been catalogued in their first year of life and therefore of known age when sampled. The samples were stored at -20°C in a 10% DMSO solution to minimize potential effects of DNA degradation on telomere length measurements. Genomic DNA was extracted using the QIAGEN™ DNeasy Blood and Tissue kit according to the manufacturer's instructions (QIAGEN Inc.) and stored in TE buffer (10 mM Tris·Cl, 0.5 mM EDTA, pH 9.0) at -20°C. The DNA quality and concentration of each extraction was measured by gel-electrophoresis (Sambrook *et al.* 1989) and a Thermo Scientific NanoDrop 8000, respectively.

Telomere length by quantitative PCR

Approaches to telomere length measurement can be classified as either hybridization or PCR based (Nakagawa 2004). The quantitative PCR (qPCR) method for telomere length estimation builds on the principle that telomere repeats can be amplified by specific PCR primers and relative telomere length (RTL) determined by scaling the amount of telomere repeat to the amount of a simultaneously amplified single-copy reference gene (Cawthon 2002; Cawthon 2009). Here, telomere lengths were measured using a modification of Cawthon's (2009) multiplex qPCR method. Each qPCR reaction was conducted in a total volume of 25 µl consisting of 50% ABsolute™ QPCR SYBR© Green Mix Plus ROX vial (Thermo Fisher Scientific, Inc.), 800 nM of each primer, and 20 ng of template DNA. Telomere primers and reference gene primers (albumin) were as described by Cawthon (2009) with the exception that we used 500 nM and 1300 nM of each telomere and reference gene primer, respectively. Amplifications were performed in a QIAGEN Rotor-Gene Q qPCR cycler with the following conditions: 95°C for 15 min, 2 cycles of 94°C for 15 s and 49°C for 15 s, 40 cycles of 94°C for 15 s, 62°C for 10 s, 74°C for 15 s with signal acquisition, 84°C for 10 s, 88°C for 15 s with signal acquisition, and a dissociation curve ramping from 72°C to 95°C, rising by 0.5°C in steps of 30 s. Each qPCR batch (i.e. 96-well qPCR plate) contained three replicates of each sample of genomic DNA from humpback whales of known age, a total of three no template controls (NTCs), and serial dilution series of standard DNA, also in triplicate, used to construct standard curves. The applied method has been subject to extensive quality control and was found to be the best performing of several different qPCR assays for estimation of humpback whale telomere length (Olsen *et al.* 2012). Briefly, this assay has high and constant reaction amplification efficiencies (E: 98-104%), low inter-batch standard deviation (SD) of Cq values for the telomere (average SD = 0.116) and reference gene reactions (average SD = 0.196), and relatively low coefficient of variation (CV) of RTL estimates (average CV = 8.8%; range: 1.3-29.6%).

Data analysis

The raw data were baseline corrected in LinRegPCR version 12.16 (Ramakers *et al.* 2003; Ruijter *et al.* 2009; Tuomi *et al.* 2010) using the "strict" baseline correction option to adjust for background fluorescence noise, a common or window of linearity (W-o-L), and a fixed fluorescence threshold line for determination of Cq values. Manual adjustments of the baseline and/or W-o-L were made in a few cases where visual inspection suggested that these were wrongly set by the program. Relative telomere length was estimated as the ratio between the observed amount of telomere and reference gene averaged for triplicate reactions and calibrated by the standard curve to account for any variation between batches (Pfaffl 2001).

The correlation between RTL and age was determined using linear regression, statistical significance tested using ANOVA, and the uncertainty of the population mean determined by the 95% confidence intervals (CI) of the regression line. Moreover, to account for uncertainty in individual telomere length estimates we calculated the 95% prediction interval (PI) (Crawley 2013), which reflects the degree of accuracy that can be expected in age estimates based on observed telomere lengths and the relationship between telomere length and age presented here.

The applicability of telomeres for age determination largely depends on the experimental and biological variability associated with individual telomere length estimates. Experimental variability is defined as the variance in RTL estimates due to, for example, pipetting errors and slight variations in amplification efficiency. Biological variability is the variation in RTL estimates among individuals of similar age caused by individual life-history, inheritance and environment as discussed below. The contribution of experimental and biological variability to variability in RTL estimates was determined by estimating the coefficient of variation (CV) among triplicate RTL estimates (experimental variability), the CV of RTL estimates averaged across individuals (overall variability), and the difference between these two (biological variability). For this analysis, we grouped telomere length estimates obtained from individuals assigned to the following categories; <1-4 yrs (n=7), 5–9 yrs (n=7), 10–14 yrs (n=6), 15–19 yrs (n=4), and 20–26 years (n=4).

RESULTS

Relative telomere lengths were highly variable among humpback whales, but nevertheless showed a decline over time (R^2 = 24.4%; F = 8.41; P = 0.008) (Fig. 1). In the humpback whales studied here, the relationship between telomere length and age was described by:

$$RTL = -0.0394*(Age) + 2.0222 \pm \varepsilon \qquad \text{Equation 1}$$

where ε is the variation in RTL estimates for individuals of similar age as defined by upper and lower boundaries of the 95% prediction intervals (here ε = 1.1442). To determine the age of unknown individuals based on their RTL we rearrange Equation 1 to:

$$Age = ((RTL – (2.0222 \pm 1.1442)) / -0.0394 \qquad \text{Equation 2}$$

From this, it is clear that individual age estimates are associated with a large degree of uncertainty, amounting to approximately ±28 years for intermediate RTL values.

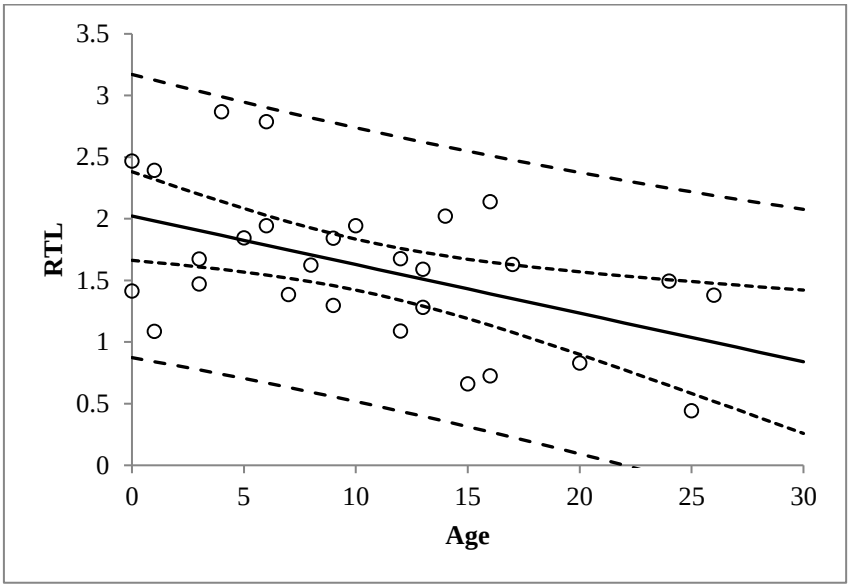

Fig. 1. The observed relationship between relative telomere length (RTL) and chronological age in 28 humpback whale samples. Solid line=trend line; inner stippled lines=boundaries of the 95% CI; outer stippled lines=boundaries of the 95% PI.

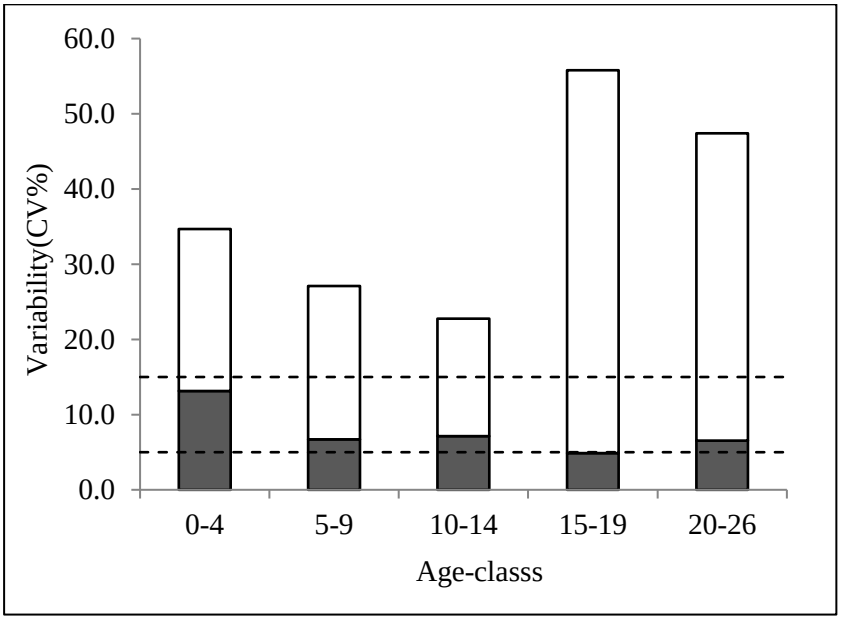

Fig. 2. Variability in average RTLs estimated each age-classes divided into experimental variability (average CV of individual RTL estimates; dark grey) and biological variability (the difference between overall variability in RTL and experimental variability; white). The stippled lines mark the range of RTL CVs reported in recent applications of the multiplex qPCR telomere assay (Aviv *et al.* 2011; Cawthon 2009; Kim *et al.* 2011).

The total variability in the average RTL determined for each age category ranged between a CV of 23% to 56% (Fig. 2). Of this, the experimental variability was relatively constant across age-classes ranging from 5% to 15%, whereas biological variability ranged from 15% and up to 50% with highest values for the youngest and oldest age-categories.

DISCUSSION

Interest in using telomeres as markers for chronological age in non-model species arose about a decade ago (Monaghan & Haussmann 2006; Nakagawa *et al.* 2004; Vleck *et al.* 2003). However, to date the vast majority of published telomere studies focus on birds and reptiles (Bize *et al.* 2009; Hall *et al.* 2004; Haussmann *et al.* 2003; Juola *et al.* 2006; Pauliny *et al.* 2006; Ujvari & Madsen 2009) and there is a striking paucity of studies on mammals other than humans (Garde *et al.* 2010; Izzo *et al.* 2011; Pauli *et al.* 2011).

Here, we applied the qPCR approach to telomere length estimation (Cawthon 2002; Cawthon 2009) to investigate the prospect of using telomere length as a proxy for chronological age in a cetacean species in which individuals have been studied for nearly four decades. We found that, for the described sampling scheme and experimental techniques, telomere lengths in humpback whales only weakly correlated with age and were highly variable among individual whales (Fig. 1). Dunshea and co-authors (2011) recently suggested that the large biological variation in telomere lengths among individuals of similar age implies that exact telomere-based age determination may be unachievable for most vertebrates. In our study, telomere length did decrease over time. However the observed levels of variability strongly indicate that telomere length is insufficiently precise to serve as an indicator of chronological age in humpback whales. Recently, two studies on humpback whales have reported significant age-associations with blubber lipid profiles (Herman *et al.* 2009) and levels of cytosine methylation (Polanowski *et al.* 2014). The 95% confidence intervals of these methods were less than 10 years, suggesting that they are more appropriate for age determination of humpback whales than the highly variable telomere length estimates. In the following we highlight some of the sampling, experimental and biological factors causing this variability.

Sampling issues

The observed individual variation and rate of telomere shortening implies low precision of age estimates. In particular, this may be an issue in cases like ours where the initial samples used to establish the relationship between telomere length and age does not cover the entire lifespan of the study species. For most large whale species, the availability of validated samples from known age individuals is limited by the effort and duration of photo-

identification studies. In the Gulf of Maine, humpback whales have been studied since the 1970s and our study included individuals with known ages up to 26 years. However, humpback whales may live for 48 years or perhaps up to 100 years (Chittleborough 1965; Gabriele *et al.* 2010), implying that skin samples with validated age data are currently available for only 25-50% of the potential lifespan of humpback whales. Human telomere studies indicate that telomere shortening is not linear, but occurs rapidly during maturity, more slowly over adulthood, and faster again late in life (Allsopp *et al.* 1992; Aubert & Lansdorp 2008; Coviello-McLaughlin & Prowse 1997; Hayflick 2003; Lee *et al.* 2002). Hence, the temporal coverage of available known-age samples may be too limited to adequately describe and apply the relationship between telomere length and age in humpback whales and other long-lived cetaceans.

Choice of method for telomere length estimation

Despite its conceptual simplicity, we found the qPCR method for telomere length estimation difficult to optimize and subject to considerable experimental variability. As a result of the methods' high sensitivity even slight variations in qPCR cycling temperatures and pipetting volumes translated into considerable variation in the resulting RTL estimates (Olsen *et al.* 2012). When the method was optimized and subjected to extensive quality control, experimental factors still accounted for average CVs ranging between 5 and 13% (Fig. 2). That said, similar levels of variability was reported by Cawthon (2009) and in recent telomere studies (Aviv *et al.* 2011; Barrett *et al.* 2013; Kim *et al.* 2011), indicating that although a CV>10% might be considered high, such levels of experimental uncertainty is intrinsic to most qPCR approaches to telomere length estimation. In comparison, Aviv and co-authors (2011) reported that telomere length estimates obtained by the telomere restriction fragment (TRF) method was associated with a CV<2%. The TRF method has been widely used for telomere measurement in birds, reptiles and smaller mammals (Haussmann & Mauck 2008a). It is based on hybridizing a radioactive labeled oligonucleotide probe to telomere restriction fragments obtained by digesting genomic DNA with *Hinf*1 or other short-sequence recognition site restriction enzymes. Telomere restriction fragments may differ among cells and chromosomes and consequently TRF assays produce an autoradiographic smear which may be analyzed with some element of subjectivity (Haussmann & Mauck 2008a; Nakagawa *et al.* 2004). Moreover, previous attempts to measure cetacean telomere length using TRF were unsuccessful (Dunshea *et al.* 2011). The most recently devised technique for telomere length measurement is the dot blot method (Kimura & Aviv 2011). In this method, relative telomere length is estimated as the ratio between the amount of telomere in a sample (measured by hybridization to a DIG labelled telomeric probe) and the amount of DNA (measured as the fluorescence produced by a SYBR Dx DNA Blot Stain). Although yet to be

tested under a wider range of settings, the reported simplicity and rapidity of the technique as well as its modest requirement on DNA quality and amount seems promising.

A limitation to both qPCR, TRT and dot blot approaches, is that they measure genome-wide telomere length, including interstitial telomere repeats if they exist (Dunshea *et al.* 2011). Interstitial telomeres are telomere-like TTAGGG sequences that randomly occur in the genome in the form of e.g. satellite DNA or transposable elements (Lin & Yan 2008). Such interstitial telomere sequences could cause variation in individual RTL estimates if these sequences are more frequent in some individual than others. A recent study in birds suggests that inter- and intra-individual differences in interstitial telomere signal may add noise to RTL estimates (Foote *et al.* 2013). Cetaceans are known to harbour telomere-like satellite DNA (Arnason *et al.* 1988; Arnason & Widegren 1984), but the degree of individual differences in the length of these satellites and their influence on the observed variation in individual telomere lengths is unknown.

In addition to the approaches described above, several other approaches allow for telomere length measurements (reviewed in Nussey *et al.* 2014), however we will not discuss these in detail as they typically require fresh blood samples, substantial effort to establish in the technique in the lab and are associated with moderate to high handling time per sample, and thus seem inappropriate for telomere measurements in free-ranging cetaceans.

Biological factors causing individual variation

The observation that the correlation between telomere length and age is not strong in humpback whales, even when experimental bias is minimized, reflects the importance of biological factors causing individual variability in telomere dynamics. Telomeres shorten during cell replication due to the directionality of DNA polymerase, and by oxidative damage brought about by the uncontrolled oxidation of DNA by reactive oxygen species (ROS) (Harley *et al.* 1990; Richter & Zglinicki 2007; Watson 1972). ROS's can be introduced by exogenous sources, but the majority are generated endogenously as an inevitable by-product of aerobic metabolism (Balaban *et al.* 2005; Beckman & Ames 1998). Telomeres may be synthesized de novo by the enzyme telomerase (Greider & Blackburn 1985; Masutomi *et al.* 2003) and oxidative damage reduced via neutralization of ROS by antioxidant enzymes (Ames *et al.* 1993; von Zglinicki 2002). Telomere shortening, protection and elongation occur at different degrees in different individuals and give rise to observed differences in telomere dynamics. A portion of the individual variation in telomere length is likely to be determined by inheritance. Heritability of telomere length can be substantial and differs

among species, study population or age and lifespan of parents (De Meyer *et al.* 2007; Njajou *et al.* 2007; Nordfjall *et al.* 2005; Slagboom *et al.* 1994; Unryn *et al.* 2005). Individuals are also subjected to different levels of oxidative stress by their environment, which can lead to stochastic variation in telomere lengths. Hall *et al*. (2004) found that the environmental conditions experienced early in life correlated with the loss of telomere repeats in two species of long-lived birds, and it is well known that contaminants can increase oxidative stress (Li *et al.* 2003; Risom *et al.* 2005).

Synthesizing telomeres and protecting them from oxidative damage may be costly and hence associated with trade-offs between allocating internal resources to maintaining telomeres versus growth and reproduction (Speakman *et al.* 2002; Williams 1966). Individuals may differ in their energetic requirements and how effectively they utilize available and stored energy resources, and individuals are subject to natural selection resulting in diverse life history strategies which emerges as differential telomere lengths and rates of shortening. Self-maintenance (i.e. avoiding loss of telomere repeats) to delay senescence and onset of age-related diseases in long-lived species is facilitated by constant expression of telomerase and/or a highly efficient antioxidant system (Harman 1956; Haussmann *et al.* 2007; Haussmann *et al.* 2003; Ogburn *et al.* 2001). In addition, cetaceans and other marine mammals often experience periodic inaccessibility to oxygen, which likely result in substantial variations in the oxygen levels of most tissues (Filho *et al.* 2002; Schreer & Kovacs 1997). When surfacing to breathe, oxygen restricted organs and tissues are subject to a dramatic oxygenation and high rates of ROS formation (Halliwell & Gutteridge 2007). Animal species that routinely are subjected to aerobic/anoxic transitions due to diving or extracellular-freezing, lack of environmental oxygen, and hibernation, may minimize oxidative damage by maintaining high antioxidant activity (Storey 1996). Indeed, high antioxidant activity has been observed in dwarf minke whales (*Balaenoptera acutorostrata*), two species of dolphins (*Stenella clymene* and *Pontoporia blainvillei*), and the ringed seal (*Pusa hispida*) (Filho *et al.* 2002; Vazquez-Medina *et al.* 2006; Vázquez-Medina *et al.* 2007). The general operation of an efficient antioxidant system, as observed in other birds and mammals, as well as specific adaptations to the marine environment, could be responsible for the observed rate of telomere shortening observed in humpback whales.

In summary, although our study suggests a significant correlation between telomere length and age in humpback whales, the large variation in telomere length among individuals of similar age imply that telomere length measured by quantitative PCR is a poor determinant of age in humpback whales. Assuming that this individual variation to a large extent results from the stochasticity of biological processes, it may be beneficial to focus future

efforts on fine-tuning existing age-determination alternatives, such as blubber lipid profiles (Herman *et al.* 2009) and levels of cytosine methylation (Polanowski *et al.* 2014).

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