



Telomere length and age in pinnipeds: The endangered Australian sea lion as a case study

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ABSTRACT

Telomeres are the protective caps at the ends of all eukaryotic chromosomes. Because DNA replication of chromosome ends is incomplete, telomeres undergo sequence loss with each cell division resulting in the progressive shortening of their lengths. Telomere shortening with age is known from terrestrial mammals. We test

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whether this pattern is shared by marine mammals, by comparing telomere lengths between age classes in a pinniped species, the Australian sea lion (*Neophoca cinerea*). Telomere lengths were measured using a real-time quantitative polymerase chain reaction (PCR) method in specimens from three age classes: pup (<1.5 yr), juvenile (1.5–5 yr), and adult (>5 yr). Mean telomere lengths of the adults were significantly shorter than the juvenile and pup classes. However, we were unable to differentiate between pups and juveniles. These findings confirm that the Australian sea lion shares the general pattern of shortening telomere lengths with age as documented in terrestrial mammals. The application of telomere lengths as an age determinant requires considerable development to refine the scale of the age estimates derived, which will require the use of known-aged individuals. Nonetheless, measures of telomere lengths have the potential to become valuable tools in molecular ecology and forensics for assessing compliance in harvesting situations.

Key words: telomere length, pinniped, *Neophoca cinerea*, Australian sea lion, age class, management, forensics.

Telomeres are repeated noncoding nucleotide sequences that cap the terminal ends of all eukaryotic chromosomes (Blackburn 1990). They function to protect the coding ends of chromosomes by buffering against sequence loss as a result of faulty DNA replicative mechanisms and DNA damage events (Blackburn 1990). Telomere lengths may be maintained and, or extended by either the action of telomerase or by the mechanism of alternative lengthening of telomeres, which currently are only described in mammalian tumor cells (Reddel 2003). However, in most normal mammalian somatic cells, telomerase is not consistently expressed at levels to facilitate telomere maintenance, resulting in the progressive shortening of telomere lengths (Reddel 2003).

In general, mammals share a single pattern of telomere length attrition throughout their lifespan (Hastie *et al.* 1990, Allsopp *et al.* 1992). Moreover, this change in the length of telomeres is negatively correlated with age (Hastie *et al.* 1990, Vaziri *et al.* 1993). In humans, measurements of telomere lengths have been proposed as a forensic tool for determining the age of individuals (Tsuji *et al.* 2002, Lahert 2005). To date, there is an absence of published literature on patterns of telomere length change with age in marine mammal lineages.

The few studies available for pinnipeds suggest their telomeres would be subject to the same pattern of changes in length to those reported in terrestrial mammals. Many vertebrate species have intrachromosomal telomeric repeats varying in number and location throughout individual chromosomes (Meyne *et al.* 1990, Pagnozzi *et al.* 2000). However, in the pinniped species, the harbor seal (*Phoca vitulina*), telomeric sequences were located exclusively at the ends of chromosomes (Meyne *et al.* 1990). The absence of these intrachromosomal telomeric sequences in the harbor seal is encouraging, because their presence may confound measures of terminally located telomere lengths (Hausmann and Vleck 2002). In addition, undetectable levels of telomerase were found in the somatic tissues of Steller sea lion (*Eumetopias jubatus*) and the beluga whale (*Delphinapterus leucas*) (Elmore *et al.* 2008), which is indicative of subsequent change in telomere length. Furthermore, given the close phylogenetic relatedness between pinnipeds and terrestrial carnivores (Sato *et al.* 2006), for which relationships between telomere length and age exist (Nasir *et al.* 2001; Yazawa *et al.* 2001; Brümmendorf *et al.* 2002; McKevitt *et al.* 2002, 2003), it seems likely that the same pattern could exist in pinnipeds.

The further refinement of the telomere-based aging technique may provide a valuable tool for the noninvasive determination of the age structure of pinniped populations. Such a tool could have broad applications in wildlife biology and forensics. Telomere lengths may provide an immediately applicable forensic tool to identify broad age classes of beached animal carcasses (Tsuji *et al.* 2002). Moreover, telomeres may be used to forensically monitor the compliance of commercial pinniped hunters within demographically based harvest management systems, that is, the prohibition of targeting hooded and harp seal pups by the Canadian pinniped fishery,² where only trace samples are available. Forensic applications of telomere length determination are feasible with the recent development of a quantitative PCR (qPCR) assay (Alonso *et al.* 2004, Swango *et al.* 2006). The broader application of this technology to other marine mammal species would be equally valuable. For example, further modification of telomere-based aging in cetaceans has the potential to eliminate the necessity of lethal sampling practices of scientific whaling programs (Brownell *et al.* 2000, Nakagawa *et al.* 2004, Dennis 2006).

We test whether the mammalian pattern of telomeric sequence loss is shared by a pinniped species. We assess telomere length change at the population level in the endangered Australian sea lion, *Neophoca cinerea*, by comparing mean telomere lengths between broad age classes from a single breeding colony using a qPCR-based assay.

METHODS

Sample Collection and DNA Extraction

Biopsy samples were collected opportunistically from Australian sea lions at the Olive Island breeding colony (South Australia) in April 2009. Animals from both sexes were captured while ashore on the island and assigned to one of three broad life history stages: pup (<1.5 yr), juvenile (1.5–5 yr), and adult (>5 yr). However, as animal ages were not determined from counts of growth rings in the animal's teeth, these are approximate age ranges. Sex was determined by the presence of external reproductive morphology and age was determined using size, pelage coloration, and gross morphology (McIntosh 2007). Due to logistical limitations, biopsy samples from adult males were not obtained.

Animals were restrained for a short period of time using a purpose-built V-net and tissue samples were obtained by removing a small (5 mm²) biopsy from one of the hind flippers. All procedures were approved by the University of Adelaide Animal Ethics Committee (S-008-2006) and conducted under a South Australian Department for Environment and Heritage permit (A-24684). Flipper biopsies were stored in ethanol prior to DNA extraction. DNA was isolated following prescribed methods (Genra Systems, Inc., Minneapolis, MN) and DNA quality was assessed through gel electrophoresis.

Primer Optimization

PCR conditions for primer pairs were optimized using standard PCR techniques and gel electrophoresis. Forward and reverse telomeric primers used to amplify the

²Canadian Department of Fisheries and Oceans. 2009. Overview of the Atlantic seal hunt 2006–2010. Unpublished; available at <http://www.dfo-mpo.gc.ca/fm-gp/seal-phoque/reports-rapports/mgtplan-plangest0610/mgtplan-plangest0610-eng.htm> (accessed 1 September 2009).

84 bp telomeric amplicon (which comprised 14 tandem repeats of the telomeric unit: TTAGGG) were 5' CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT 3' and 5' GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT 3', respectively (Callicott and Womack 2006). The interphotoreceptor retinoid binding protein (*IRBP*) has been shown to be a single-copy gene in mammals (Borst *et al.* 1989, Sato *et al.* 2003) and an 81 bp amplicon was used to determine the relative quantity of the telomeric repeat sequence (Cawthon 2002). Sequences of the forward and reverse *IRBP* primers designed for this study were: G1781F—5' CAC TCA CCA ACC TCA CAC AAG A 3' and G1782R—5' CCA CAT TGC CCT CCA GAA C 3', respectively.

Annealing temperatures were optimized by running thermal gradient cycles. Each PCR was carried out in a volume of 15 μ L with a final concentration of 1 \times GENEAMP PCR Gold buffer, 2 mM MgCl₂, 200 μ M of each dNTP, 0.2 μ M of each primer, 0.5 U of AMPLITAQ Gold DNA polymerase (Applied Biosystems, Foster City, CA), and approximately 100-ng genomic DNA. All standard PCR assays were performed on a Gradient Palm-Cycler (Corbett Life Science, Mortlake, NSW, Australia). Amplifications comprised an initial denaturation step of 94°C for 9 min, followed by 34 cycles of: 94°C denaturation for 45 s, an annealing gradient from 52°C to 64°C for 45 s, and a 72°C extension for 60 s; with a final 72°C extension for 6 min. The optimal annealing temperatures for the telomere and *IRBP* primer pairs were 62°C and 55°C, respectively.

Absolute qPCR

Absolute qPCR reactions for the separate telomere and *IRBP* assays were carried out in a volume of 10 μ L with a final concentration of 1 \times EXPRESS SYBR GreenER qPCR master mix (Invitrogen, Carlsbad, CA), 500 nM ROX reference dye (Invitrogen), 200 mM each of the forward and reverse primers, and approximately 100-ng genomic DNA. All qPCR assays were performed in the Rotor-Gene 6000 automated thermocycler (Corbett Life Science). Amplifications comprised an initial denaturation step of 95°C for 5 min, followed by 40 cycles of: 95°C denaturation for 10 s and a 62°C and 55°C (for the telomeric and *IRBP* assays, respectively) annealing step for 20 s; followed by an extension and data acquisition step at 72°C for 15 s. At the completion of the reactions, melt curves, increasing from 55°C to 95°C, were run to assess the formation of primer-dimer products.

A standard curve for the *IRBP* assays was calculated by serially diluting known concentrations of the *IRBP* amplification product generated by standard PCR modified by running it for 80 cycles to produce large amounts of the *IRBP* target sequence. *IRBP* PCR products were cleaned using a vacuum plate clean-up procedure and their concentration determined by electrophoresing a specified volume against a ladder of a range of molecular weights (MassRuler Low Range DNA Ladder: Fermentas International Inc., Burlington, ON, Canada). The telomeric standard curve was calculated using serial dilutions of known concentrations of a synthesized telomeric oligomer (GeneWorks, Adelaide, SA, Australia) following O'Callaghan *et al.* (2008). Standard curves and reaction efficiencies for the telomeric repeat and the *IRBP* assays were calculated using the Rotor-Gene 6000 analytical software (Corbett Life Science). The resultant efficiencies of the telomere and *IRBP* reactions were 100%, indicating specific primer binding and the exponential amplification of the target region with each cycle.

All telomeric and *IRBP* assays were run in triplicate. In order to convert the resulting cycle threshold (C_T) values into telomere lengths, the number of telomeric repeats, and *IRBP* copies per reaction were calculated following O'Callaghan *et al.* (2008). Telomeric repeat numbers were then divided by the numbers of *IRBP* copies per diploid genome, as there are two copies per genome, to give a final telomere length in kb per diploid genome (O'Callaghan *et al.* 2008).

Statistical Analysis

The variability and precision of the repeated measurements of telomere length were analyzed using the coefficient of variation and index of precision (Chang 1982), as well as the index of average percentage error (IAPE) (Beamish and Fournier 1981). The data set violated the assumptions of homogeneity of variance; hence, the nonparametric Kruskal-Wallis (KW) test of independent samples was used to compare mean telomere lengths between sexes and among age classes. Statistical analyses were performed using SPSS 15.0 (SPSS, Inc., Chicago, IL). To test the discriminatory power of measurements of telomere length, a canonical principal components analysis (CAP: Anderson and Willis 2003) was performed using Primer version 6.0 (Clarke and Gorley 2005). Telomere lengths were $\log(x + 1)$ transformed prior to calculating the Euclidean distances between measurements to obtain a resemblance matrix.

RESULTS

In total, telomere lengths were measured in 86 individual Australian sea lions. Repeated measurements of telomere length had a mean coefficient of variation and measure of precision of 47.16% and 33.35%, respectively. The mean IAPE for measurements was 28.19%. Total telomere lengths ranged from 16.49 to 379.85 kb per diploid genome. In general, the pups showed the greatest range in telomere lengths of the three age classes examined (Table 1, Fig. 1).

Mean total telomere lengths exhibited a general decline with increasing age (Fig. 1). When the data were pooled across all age classes for each sex, no significant difference in the mean total telomere lengths was identified (KW: $\chi^2 = 2.040$, $P = 0.153$). Similarly, there was no significant difference between the sexes within both the pup and juvenile classes ($P > 0.05$). As there was no significant difference in mean total telomere length between the sexes, the data were pooled across sexes for each age class. No significant variation among age classes was identified (KW: $\chi^2 = 4.920$, $P = 0.058$). Further analysis determined that the adult age class had significantly smaller mean total telomere lengths than the pups (KW: $\chi^2 = 3.658$, $P = 0.046$) and the juveniles (KW: $\chi^2 = 4.343$, $P = 0.037$). However, there was no

Table 1. Summary of telomere length measurements of the Australian sea lion, *Neophoca cinerea* (in kb per diploid genome).

Class	Sex	<i>n</i>	Range	Minimum	Maximum	Mean	SE
Pup	Female	20	339.11	20.03	359.14	179.60	28.32
	Male	20	347.13	32.72	379.85	183.44	24.88
Juvenile	Female	10	310.44	16.49	326.93	169.05	32.79
	Male	15	248.82	71.23	320.04	164.45	18.08
Adult	Female	21	215.40	25.44	240.84	116.01	13.89

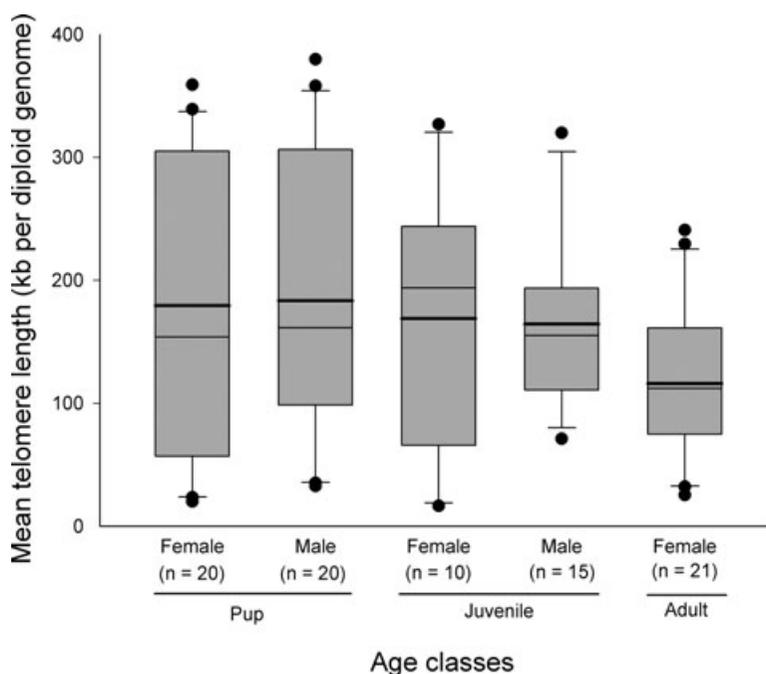


Figure 1. Comparisons of mean telomere lengths between age classes separated by sex, of the Australia sea lion, *Neophoca cinerea* (sample sizes [n] in parenthesis). The boxes represent the ranges of telomere lengths measured. The mean and median telomere lengths are shown by the thick and thin horizontal lines in box, respectively. The tails are \pm the standard errors of the mean. (●) denote outlying values.

significant difference between the pup and juvenile age classes (KW: $\chi^2 = 0.059$, $P = 0.408$). In order to avoid the difficulties associated with differentiating between large pups and small juveniles (McIntosh 2007), these animals were pooled as immature animals ($n = 65$) and compared with the mature adult females ($n = 21$). A significant difference between the two groups was found (KW: $\chi^2 = 4.913$, $P = 0.027$); hence, the discriminatory ability of telomere length was tested for these maturity groups. The combined CAP assignment score for both immature and mature animals was 58.14%. The cross-validated scores for the correct assignment of samples into the immature and mature groups were 55.58% and 66.67%, respectively.

DISCUSSION

We identified a general decline in telomere length with age in the Australian sea lion. Specifically, the mean total telomere length of adults was significantly shorter than for pups and juveniles. Furthermore, mean total telomere lengths in mature animals were significantly shorter than for immature animals (both pups and juveniles). These findings fit the generalized mammalian pattern of telomere length attrition with age. Mean total telomere length measurements presented here are within the same size range as those reported in humans and had comparable variance in telomere lengths within age classes to other species of mammals (O'Callaghan

et al. 2008, Thomas *et al.* 2008). This study marks the first such reported finding for a pinniped species.

The adult age class comprised sexually mature females that were identified as such because they were observed suckling pups. The average age at which a female Australian sea lion reaches sexual maturity is 5 yr (Ling *et al.* 2006, McIntosh 2007) and they may continue to breed until 26 yr (McIntosh 2007). The age of the adults that we sampled ranged widely based on body length and tooth condition (McIntosh 2007). Despite this range in age among the adults, the variance of telomere lengths was smaller in adults compared with pups and juveniles.

The pup age class consisted of animals with less than 1 yr difference in ages, but had much larger variance in telomere length. The variance of telomere lengths reported in pups may be the result of: (1) rapid somatic growth of pups in the first year of life (Bell *et al.* 1997, McIntosh 2007), (2) differences in the length of telomeres at birth (Monaghan and Haussmann 2006), or (3) marked variance in the rate of change in telomere length among individuals (Bize *et al.* 2009). The observed variance is somewhat characteristic of the heterogeneous nature of telomeres among individuals in a typical mammalian population (Takubo *et al.* 2002, Monaghan and Haussmann 2006).

A similar situation may exist in the juvenile age class, with the added complication of a greater range of ages (1.5–5 yr) compared with pups (0–1.5 yr). These results indicate that there is a greater variance in the rate of decline in telomere lengths in younger age classes compared with older age classes. As animals become older, the rate of decline in telomere length reduces and tends to plateau, thus compressing the overall telomere lengths in older age classes.

When Australian sea lions were separated by their state of sexual maturity (*i.e.*, 0–5 yr and 5+ yr), mature animals had significantly shorter mean total telomere lengths. Overall, significant telomere length reduction may occur upon or before the onset of sexual maturity. This is the general pattern of telomere length loss also seen in humans, with children to the age of 5 yr undergoing the most rapid phase of telomere length change (Frenck *et al.* 1998, Rufer *et al.* 1999, Zeichner *et al.* 1999). The long-term measurement of telomere lengths of individuals within the population, that is, longitudinal sampling, is needed to discern the causes and consequences of the variability of telomere lengths between individuals observed in Australian sea lions. The cross-sectional design of this study may mask different patterns of telomere length change among individuals within the population (Bize *et al.* 2009); hence, resulting in the large degree of variation in telomere lengths seen here among individuals. Longitudinal sampling may be viable in the Australian sea lion given that individuals are routinely tagged (Higgins 1993, Gales *et al.* 1997) and display a measure of philopatry (Campbell *et al.* 2008), allowing for repeated tissue sampling on an annual basis.

The sex of the animals sampled did not influence telomere lengths in immature Australian sea lions. However, differences in telomere length between sexes have been reported previously in other mammalian species, with females generally possessing longer telomeres than males (Coviello-McLaughlin and Prowse 1997, Jeanclos *et al.* 2000, Cherif *et al.* 2003). A similar situation would be expected among Australian sea lions, given that telomere length is inversely proportional to somatic growth and that adult males are markedly larger than adult females (Ling 1992, McIntosh 2007). However, due to logistical limitations, we were unable to obtain samples from adult males to compare their telomere lengths with adult females. Therefore, future studies may benefit from the inclusion of adult males.

It has been suggested that a measure of an individual's telomere lengths provides a potential indicator of the long-term survival and fitness of an individual (Hall *et al.* 2004, Pauliny *et al.* 2006, Bize *et al.* 2009). Longer telomeres are associated with extended individual longevity (Jemielity *et al.* 2007) and an extended reproductive lifespan (Keefe and Liu 2009). Natural selection theory predicts that young Australian sea lions with relatively short telomeres would be disadvantaged and are eliminated from the population (Hausmann *et al.* 2005, Pauliny *et al.* 2006, Bize *et al.* 2009) and variance in telomere length should gradually decrease over time in a population or species. This potentially also explains why the adult females had less variability in the telomere length measurements.

These findings confirm that Australian sea lions share the same general pattern of declining telomere length with age as reported for other terrestrial mammals such as dogs (Nasir *et al.* 2001, Yazawa *et al.* 2001, McKevitt *et al.* 2002), cats (Brümmendorf *et al.* 2002, McKevitt *et al.* 2003), and humans (Hastie *et al.* 1990). We have shown that telomere lengths can be used to discriminate between broad age classes, specifically between pups and adult females, juveniles and adult females, or in a more collective sense between immature and mature animals. However, low measurement repeatability as well as the low degree to which individuals can be assigned to relatively broad groups based on telomere lengths (*i.e.*, immature *vs.* mature animals), currently limits the applicability of this tool for estimating age.

The application of telomere lengths as an age determinate requires considerable development to refine the scale of the age estimates derived, which will require the use of known-aged individuals. Nonetheless, telomeres have the potential to become valuable tools in molecular forensic ecology, providing a tool for assessing compliance in demographically based harvest management systems in exploited pinniped populations,² the importance of which cannot be understated, given the growing social and political pressure regarding the harvest of marine mammal populations.

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