Australian Marine Mammal Centre

Final Report

(subclause 9 and Schedule Item 5 of the Funding Agreement)

- **Title -** Investigative development of minimally invasive means to gather demographic information in Cetacea: A comprehensive comparative approach
- **Chief Investigator** – Mark Hindell and Glenn Dunshea
- **Organisation –** UTAS Innovation

Activity Period -

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1. Activity Summary

A clear summary of approximately 500 words outlining the work undertaken and any significant findings (for publication on the Department's web site)
Telomeres are nucleoprotein structures on the ends of linear chromosomes that have been shown to change with age in some animals. This project aimed to examine telomere dynamics in marine mammals with a view towards assessing the use of telomere change as an age marker for individual animals. There are various molecular biology methods for assessing telomere length, all of which revolve around detecting the specific repetitive DNA sequence of the telomere ($TTAGGG_n$ in vertebrates) and each with different requirements for starting material. It has previously been demonstrated that cetaceans' contain interstitial telomere sequence (ITS), which is DNA that has the same sequence as telomeric DNA but is not associated with the end of the chromosome (i.e. the telomere) and hence not bound by telomeric processes. We conducted experiments to assess the effect of this ITS sequence on traditional telomere measurements and found that cetacean ITS sequence can dramatically affect telomere length data gained by traditional telomere measurement protocols (i.e. denaturing terminal restriction fragment length assays) and these should probably be avoided. From these experiments we conclude that the best methodology for measurement of cetacean telomeres is a non-denaturing terminal restriction fragment length protocol, however we found this protocol prohibitive to apply to skin biopsy samples for a number of reasons mostly involving the quantity and quality of DNA that can be extracted from cetacean skin samples. A quantitative PCR (Q-PCR) technique was employed to look at relative changes in telomere amount within and between individuals of three species, the bottlenose dolphin (*Tursiops truncatus*), the southern right whale (*Eubalaena australis*) and the harp seal (*Phoca groenlandica*). While this technique is also effected by ITS and bound by certain assumptions, it can demonstrate relative changes. In bottlenose dolphins we found no significant change in telomere sequence amount between

individuals of different ages, however there were significant changes within individuals at different stages of development but these were not in a consistent direction. We found no relationship with telomere length and age in harp seals using both Q-PCR and terminal restriction fragment assays. In southern right whales we examined relative telomere sequence amounts between mothers and their calves and found that while there were generally significant differences between individuals in a cow-calf pair, the direction and magnitude of these differences were not consistent within and between cow-calf pairs and overall there were no differences between calves and cows.

This work has demonstrated that telomere correlates are likely not a good marker for age of individuals and demographic processes in populations of cetaceans and some pinnipeds. This is partly due to methodological constraints but our data also indicate considerable variation of telomere lengths and telomere dynamic processes within and between individuals. Within individuals telomere dynamic processes appear to differ in relation to developmental stage and between individuals telomere dynamic processes likely differ in relation to a number of intrinsic and extrinsic factors such as heredity and environmentally induced variation in growth and condition. Whilst correlations of telomere length and age are present in the literature, there is currently a poor understanding of processes that effect telomere dynamics and how these vary in space and time in wild animal populations.

This work has provided an advancement in the understanding of methodological considerations of telomere measurement in marine mammals and of comparative cellular aging processes in marine mammals and has proven valuable in guiding future research in this area which is on-going.

2. The Outcomes/Objectives

The degree to which the Activity has achieved the objectives

The degree to which this project has achieved the stated objectives will be explained by expanding upon each of the stated objectives in the original proposal. Since the project methodology changed in relation to which samples were used to gain data, this issue will initially be addressed in this section.

Assessment of telomere measurement methods for cetaceans and pinnipeds

Cetaceans are known to contain a portion of satellite DNA that has the same DNA sequence as vertebrate telomeric DNA sequence (Meyne et al., 1990). This sequence is known as interstitial telomere sequence (ITS) because it is not associated with the true telomere structure at the ends of chromosomes (i.e. is not true telomere), yet has the same repetitive DNA sequence as telomeric DNA. To measure telomeres there are many methods that have subtle differences, however all methods involve detecting the specific telomeric DNA sequence. The major distinguishing factor between molecular biology methods is whether DNA is denatured and thus the entire genome is exposed to the telomeric DNA probe when telomeres are detected, or whether DNA remains non-denatured and the telomere probe binds to the single stranded G-strand overhang at the ends of chromosomes only (ref). Although they are preferable, to use non-denaturing methods a very large amount of high quality DNA is required, which is was difficult to obtain from many samples in this project and subsequently these methods were unavailable to us. We attempted to employ these methods with DNA obtained from bottlenose dolphin blood since it is possible to extract large amounts of

DNA from mammalian whole blood; however we were unable to get a sufficiently strong signal autoradiographic signal to analyse the results.

In order to assess how cetacean ITS would effect denaturing telomere protocols, we conducted a time series experiment with BAL-31, an exonuclease which degrades both 3' and 5' termini of duplex DNA without generating internal scissions. By analysing the DNA from each given period of BAL-31 digestion with normal denaturing terminal restriction fragment length measurements, it is possible to assess the effect of ITS on these style of telomere measurements. Put simply, by using the BAL-31 enzyme to eat away the ends of linear DNA molecules (i.e. the telomeres), it can be assessed what is left over (the ITS) and how this effects telomere measurements.

For this experiment we digested bottlenose dolphin DNA with BAL-31 and removed a portion from the digestion and stopped BAL-31 activity at 10 minute intervals from 0-60 minutes and left the final portion to digest for a further hour (2 hours digestion in total) before stopping BAL-31 activity. The amounts gathered at each time series interval were then halved and half was subject to a typical denaturing, terminal restriction fragment (TRF) length measurement protocol with the other half remaining as a BAL-31 control.

We found that the telomere signal from this experiment was digested after roughly 20 minutes of BAL-31 digestion and the signal present for the remainder of the time series was ITS, since it was immune to BAL-31 digestion and hence not situated on the ends of chromosomes (Fig. 1). ITS was present between the size range of approximately 160 base pairs to 9500 base pairs for these TRF assays (Fig. 1). Terminal restriction fragments (Telomeric DNA) were present up to approximately 23000 base pairs, however due to interference by ITS it is not possible to determine the lower end of the size distribution for telomeric DNA since it is impossible to distinguish telomeric DNA from ITS below roughly 9500 base pairs (Fig. 1). This has implications for telomere measurement using these techniques since telomere sequence is normally present over a wide range of sizes including below 9500 base pairs. Additionally, recent work has shown that the optimal area of image analysis for these types of telomere assays when attempting to establish telomere-age relationships is in the smaller size classes of the TRF size distribution (Haussmann and Mauck, 2007). Consequently, this style of telomeric assay (denaturing terminal restriction fragment analysis) is not suitable for measurement of cetacean telomeres because of the high interference from ITS DNA.

As well as indicating that these style of analyses are not suitable for cetacean DNA, this experiment also demonstrates that there is a considerable amount of ITS compared to true telomeric DNA and consequently ITS may affect other telomere measurement protocols that denature DNA such as Q-PCR assays. If ITS is variable between individuals it is likely to completely obscure any true telomeric signal gained by any denaturing assays. Further experiments on multiple bottlenose dolphin individuals (data not shown) indicate that the length of ITS restriction fragments is homogeneous and thus it appears that there is no variability between individuals arising from ITS length heterogeneity. However it has not been established whether the *amount* of the ITS repeat unit varies between individuals, which would also affect Q-PCR assays.

We therefore used Q-PCR methods for telomeric data collection from cetaceans under

the assumption that there is minimal inter-individual variation in cetacean ITS sequence.

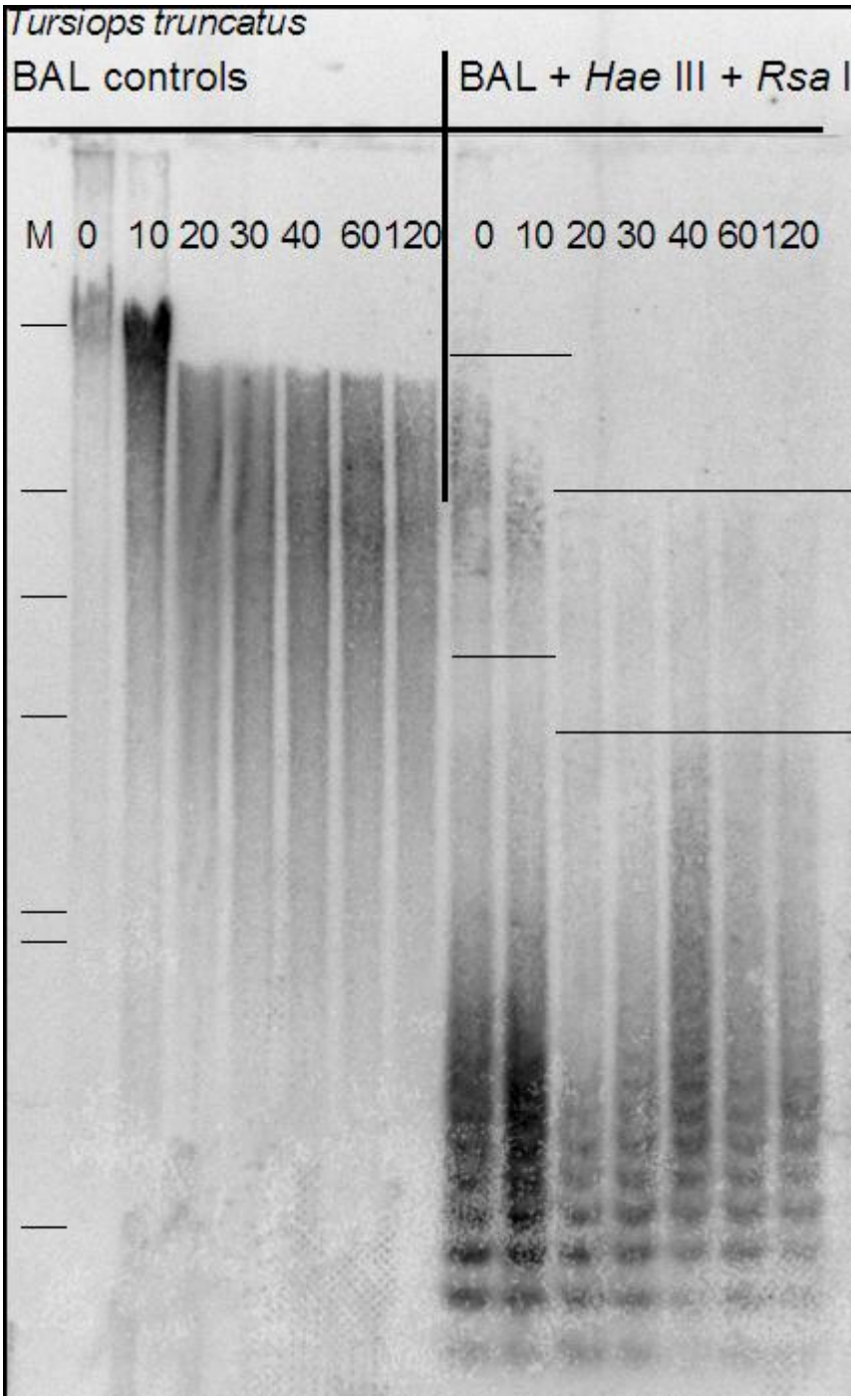

Figure 1. Results of BAL-31 time series experiment to determine the extent of interstitial telomere sequence interference for denaturing terminal restriction fragment assays. Top of image indicates whether sets of lanes are BAL controls or TRF assays performed on time series BAL-31 digested DNA (i.e. left side = BAL controls, right side = TRF assays. M indicates a molecular size marker was run in this lane and numbers indicate the time of BAL-31 digestion for each lane in minutes. Size of marker from top = 23, 9.4, 6.5, 4.3, 2, 0.5 kilo-base pairs. Note the presence of telomeric DNA between 23000 and 9500 base pairs that disappears after 20 minutes of BAL-31 digestion and the resistance to BAL-31 digestion of DNA below approximately 9500 base pairs indicating an interstitial location.

Objective 1: Gain an understanding of age-based molecular processes in marine mammals by examining how telomeres change during the course of an animal's

lifespan

We were able to meet this objective in three study species to date; two cetacean species and one pinniped species. Of the five original study species outlined in the proposal (*Tursiops truncatus*; *Phocoena phocoena*; *Balaena mysticetus*; *Eubalaena australis; Globicephala melaena*) we were only able to use two (*Tursiops truncatus* and *Eubalaena australis*) because, despite considerable laboratory effort, we could not isolate large amounts of high weight DNA from *Balaena mysticetus* and *Phocoena phocoena* samples. This was due to the fact that these samples were historical samples that in one case (*Balaena mysticetus*) were not stored in buffer at all and were defrosted when they were shipped and in the second case may have been stored in buffer for excessive periods. In any case sufficient quantities and quality DNA could not be gained from these samples which is most likely related to the samples age and storage conditions. The third of the original study species excluded (*Globicephala melaena*) was not included because supporting age data was not made available in the timelines of the project. The major findings to date from each species for which data were collected are summarised below:

Tursiops truncatus

Given the variable quality of DNA that was extracted from frozen *T. truncatus* skin, we used samples of *T. truncatus* blood that were available from the Sarasota population for this component of the project as large amounts of good quality DNA could be extracted from these samples. We used a Q-PCR methodology to determine telomere sequence amount per genome for these samples. Currently there are 16 samples for which we have generated telomere data, 8 of which are samples from four individuals that were repeat sampled at different stages throughout their life. For cross sectional analysis of telomere data between individuals, the older samples of each of the duplicate samples from each individual are excluded leaving 12 samples. There was considerable variation in telomere sequence amount between individuals compared to previously published values for other species of mammals and no significant relationship between age and telomere length in these samples ($F_{(1,10)}$= 1.84, P = 0.21; Fig 2).

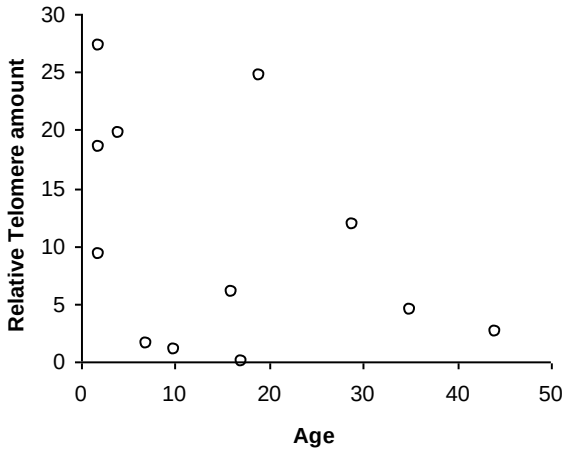

Figure 2. The relationship between telomere sequence content and age in Sarasota bay bottlenose dolphins as estimated with a Q-PCR telomere assay.

When examining repeat samples for changes within individuals we found significant changes within 3 of 4 individuals (paired samples T-test on duplicate measures at each time period), however the direction of changes was not consistent between individuals (Fig. 3). While two individuals lost telomere sequence with age, one individual didn't appear to lose telomere sequence over time and another appeared to gain telomere sequence over time (Fig. 3). This could be related to developmental stage or environmental influences but at the moment sample sizes are too small to examine these issues. Although this data appears counter-intuitive given mechanisms of telomere loss, it does indicate considerable variability between individuals in both relative telomere sequence content and in longitudinal telomere regulation processes. In light of both of these sources of variability, it is not surprising that we have not detected a significant cross sectional relationship between telomere length and age (Fig. 2).

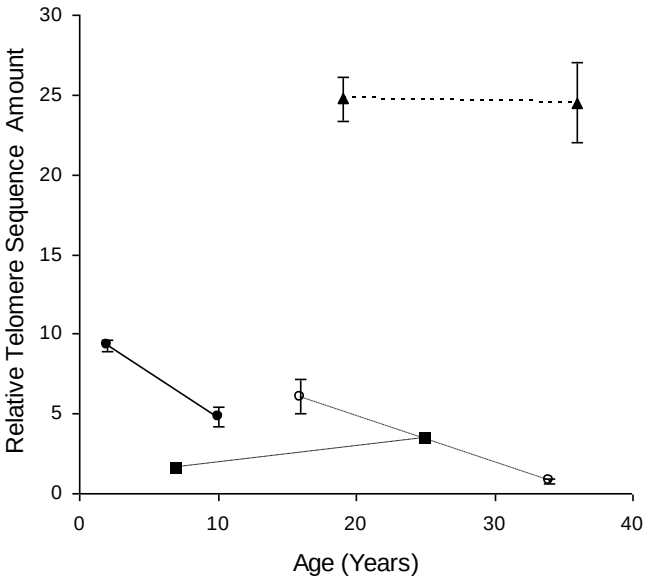

Figure 3. Telomere dynamics within four individuals with samples taken at different ages. Whisker bars represent standard deviations from multiple measurements: Filled circles = GDRW 113, a significant decline in telomere amount (T = 22.7, P = 0.03); Filled squares = GDRW 10, a significant increase in telomere amount (T = -45.8, P = 0.01); Blank circles = GDRW 36, a significant decrease in telomere amount (needs log transforming); Filled triangles = GDRW 90, no significant change in telomere amount (T = 0.27, P = 0.83). Telomere sequence amount (x-axis) is given in million base pairs.

There are still samples available from this population as well as additional repeat measures from the above samples. However given the variability within and between individuals in telomere sequence amount and regulation processes it is unlikely that a strong predictive relationship between telomere length and age will be found in this species.

Eubalaena australis

The southern right whale portion of this project is in collaboration with C. Scott

Baker and Nathalie Patenuade of the University of Auckland. An annual field season has been conducted since 2006 biopsying southern right whales of the Auckland Islands and over 500 contemporary samples have been collected. These contemporary samples will be individually matched to samples collected in 1995-1998 by Patenaude et al by genotyping microsatellite loci. This analysis is still on-going however preliminary results from the 2006 sample set indicate no matches between the Patenuade sample set and the 2006 samples. Good quality DNA has been extracted from the 2006 and 2007 samples and provided to the Baker laboratory for genotyping.

To gather an initial idea of southern right whale telomere dynamics we examined differences between cows and their calves for 12 cow-calf pair and also variation between calf telomere amounts, since all calf samples are from young of the year and thus they are roughly the same age. For this analysis we also used the Q-PCR technique. If telomere sequence declines with age in this species it would be predicted that on average calves would have more telomere sequence than adults. Thus far we have successfully gathered data from 10 cows and 7 calves consisting of six cow-calf pairs and found no significant difference in telomere sequence amount between calves and adults (stats) and no consistent relationship as to whether calves have more telomeres than cows within each cow-calf pair (Fig. 4).

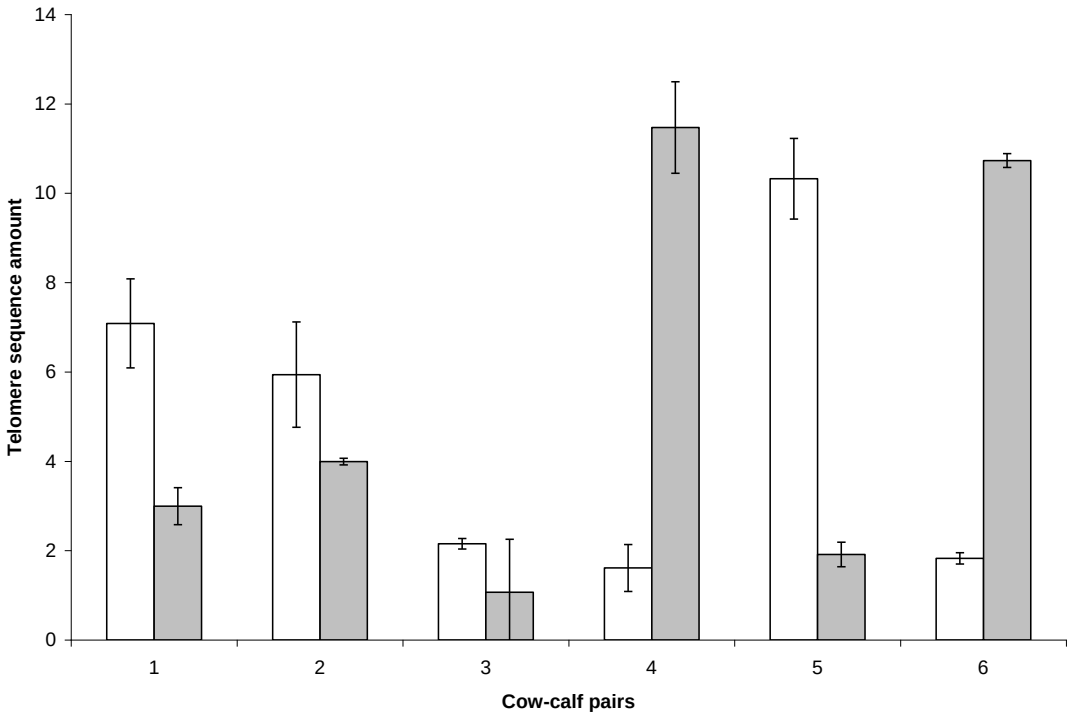

Figure 4. Comparison between telomere lengths of cows and calves in six cow-calf pairs of southern right whales. Blank bars represent calves and grey bars represent cow values. Whiskers are standard deviation from replicate measurements. Telomere sequence amount (x-axis) is given in million base pairs

Phoca groenlandica

In collaboration with Christian Lydersen of the Norwegian polar institute, we were able to obtain 92 samples of *Phoca groenlandica* skin from individuals whose age had been estimated with both sectioned tooth growth layer group tooth (tooth GLG) count techniques and aspartic acid racemization (AAR) techniques. We were able to

extract predominately good quality DNA (although there was some degradation present in most samples) from 76 samples; 30 of these had good quality DNA further isolated from extracts by cutting the high weight DNA portion from gels and purifying it.

We were able to perform telomere measurement using Q-PCR methods for most of these samples and using denaturing terminal restriction fragment analysis for a subset of samples. We were able to use denaturing terminal restriction fragment length analysis for these samples since they appeared to have little interstitial telomere sequence which would otherwise confound this type of telomere measurement (see above).

Seventy five samples were analysed with the telomere Q-PCR assay. For the harp seal samples we excluded samples from which values where either the single copy, telomere PCR or the telomere to single copy ratio had a coefficient of variation (CV) of $\geq 20\%$, as these samples were assumed to be affected by PCR inhibitors. Three samples failed to amplify with either or both the single copy or telomere PCR. Of the 72 samples assayed that amplified successfully, data from 14 samples was discarded as the inter-assay CV was >20% for the single copy and/or the telomere PCR. There were seven samples with only a single (albeit precise within each assay) estimate of telomere sequence per genome and these were retained. A further 4 samples were discarded because CV's for the average of the two final values of telomere sequence per genome were >20%. This left 54 samples with a relatively precise measure of telomere sequence per genome and three of these were discarded as there were no accompanying age estimates. Table 1 shows summary statistics for intra and inter-assay precision prior to and following filtering out imprecise assays.

Table 1. Summary statistics for precision of telomere sequence per genome quantitative PCR assays as indicated by coefficient of variation (CV= Standard Deviation/Mean*100) calculations. Filtered final ratio values are those where samples with inter-run CV of >20% are excluded.

Assay	*n*	*CV Range*	*CV Mean*	*CV Median*	*CV St. Dev.*
Within BM1	142	0.1 - 22.9	6.2	4.3	5.2
Between BM1	71	0.4 – 38.4	7.8	4.8	8
Within Telomere	119	0 - 85.2	9	4.7	14
Between Telomere	45	0.1-26.5	7.3	6.6	5.5
Final ratio values	51	0.1-32	7.9	5.3	6.9
Filtered final ratio values	47	0.1-16.1	6.2	4.5	4.5

The amount of telomere sequence per genome varied from 11.9 -278.3 million base pairs (mega base pairs; Mbp); approximately 2 orders of magnitude greater than the amount of telomere sequence in the human genome (O'Callaghan et al., 2008). This equates to an average of $9.28 \times 10^4 – 2.17 \times 10^6$ bop of telomere sequence per chromosome (where *Phoca groenlandica* 2n = 32). There was no relationship between telomere sequence content and minimum tooth age ($F_{(1,49)} = 0.71$, $P = 0.41$; Fig. 5). Sex had no effect on this outcome.

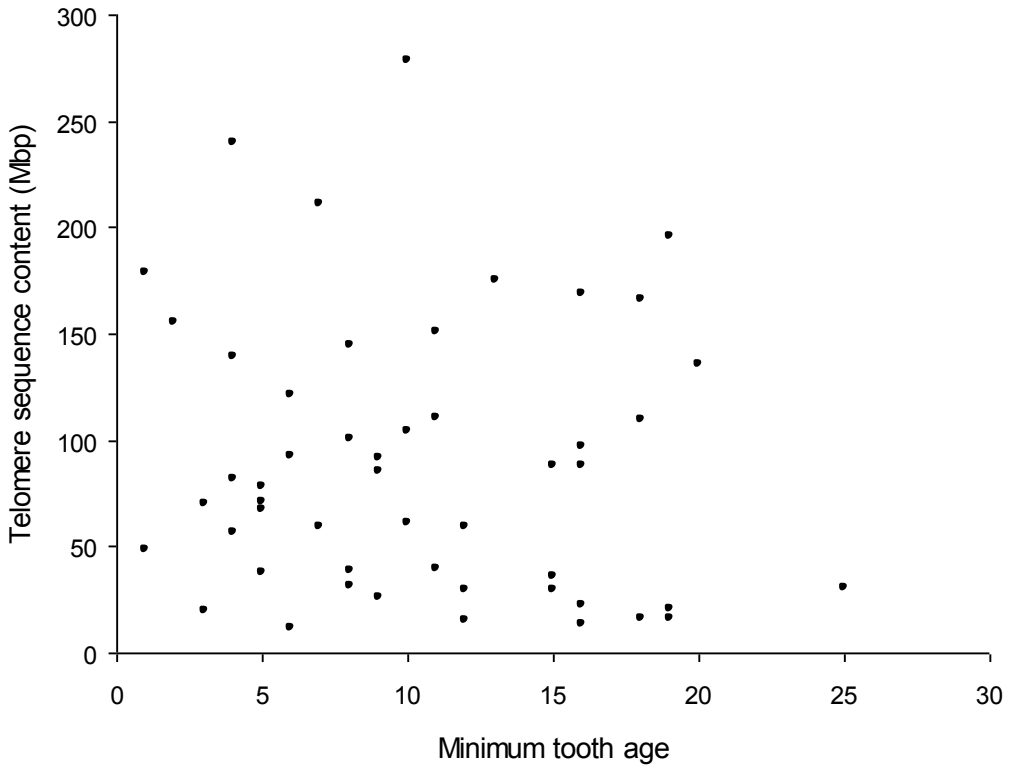

Figure 5. Scatterplot of telomere sequence content and minimum tooth age for *Phoca groenlandica*.

TRF assays

TRF estimates of average telomere length were gained for 23 individuals, 22 of which had accompanying tooth age estimates. Telomere signal from two individuals was not analysed because of lack of a defined signal. Three other lanes representing replicate measures of individuals were also not analysed because of interference in the radioactive signal from contamination that produced blotches on the autoradiograph within the lanes that could not be avoided in the analysis window (n = 2) or saturation of the signal (n=1). Coefficients of variation for TRF assays were 0.3% (mean; range=0.16-0.41%) intra-gel and 2.9% (mean; range=1- 4.8%) inter-gel for the 17-3 kbp analysis and similar for the 29-3 kbp analysis. There was no relationship between telomere length as determined by TRF analysis and age (17-3kbp analysis: ($F_{(1,20)}$ = 0.58, P = 0.45; 29-3kbp analysis: $F_{(1,20)}$ = 0.67, P = 0.42 (Fig. 6)). There were two influential points in this analysis with high leverage, both from individuals 1 year of age with a short average telomere length (Fig. 6). If these points are excluded there is a significant relationship between telomere length and age (17-3kbp analysis: $F_{(1,18)}$ = 7.66, P = <0.05, r^2=0.26; 29-3kbp analysis: $F_{(1,18)}$ = 7.77, P = <0.05, r^2=0.26), however there was no valid reason to exclude these data points; their DNA quality appeared similar to other samples assayed and a strong autoradiographic signal was detected. The effect of sex on the relationship of average TRF length with age was not assessed due to small sample sizes; sex data was available for 19 animals with TRF data, only 6 of which were male.

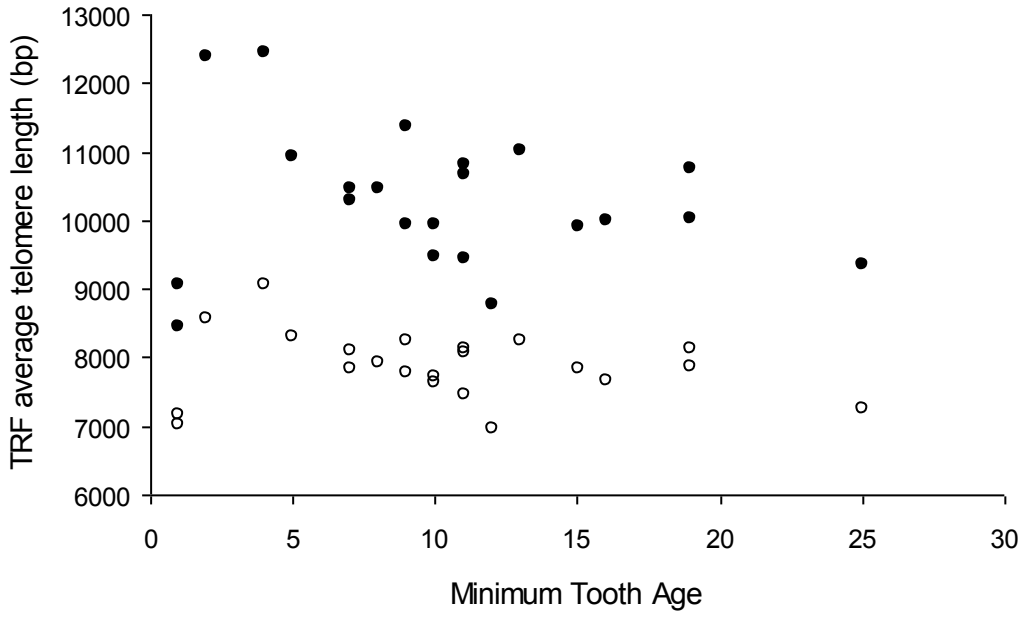

Figure 6. Scatterplot of average TRF length and minimum tooth age for *Phoca groenlandica*. Filled circles are values from analysis where the telomeric smear is averaged over 29-3kbp, blank circles are values are from analysis conducted over 17-3kbp.

Quantitative PCR and TRF methods compared

There were 14 samples with a TRF estimate of average telomere length and a quantitative PCR estimate of telomere sequence content per genome. There was a strong relationship between both measures ($F_{(1,12)} = 24.61$, $P = <0.0001$, $r^2=0.65$; Fig 7.) suggesting the methods were comparable.

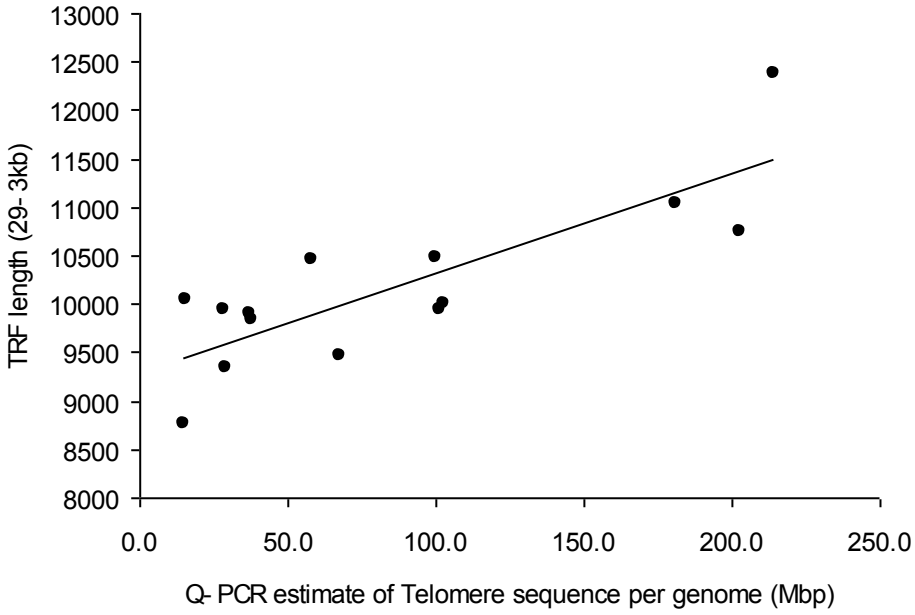

Figure 7. Relationship between telomere sequence content per genome and telomere length as determined by TRF analysis. This relationship is significant ($F_{(1,12)} = 24.61$, $P = <0.0001$, $r^2=0.65$)

Objective 1 Summary:
Although there are some methodological assumptions that must be made to use the methods employed to meet this objective, this objective is essentially met since some data (particularly from the same or closely related individuals) is directly comparable regardless of these assumptions. We have been able to demonstrate that telomere sequence content is extremely variable between individuals and that different telomere dynamic patterns can be seen between individuals. We have also shown that although there appear to be differences within some species between adults and calves, the differences are not consistent in regards to direction and magnitude of difference. All these conclusions demonstrate how telomeres change throughout the course of an animal's life span and how that varies widely between individuals.

Objective 2: Relate telomere dynamics information to the different life-spans and growth schedules of the study species in a phylogenetic context

It is difficult to directly compare data between species since pinnipeds don't appear to have ITS (judging from electrophoretic patterns from harp seal TRF analyses), or if they do, it is of a very different structure from that of cetaceans. It is also unknown how cetacean ITS varies between species. Both of these facets effect how the Q-PCR assays employed should be compared between species and thus it appears that this objective cannot currently be met. However, there is a commonality between study species in that none of them appear to have relationships between telomere sequence content and age.

Objective 3: Synthesize the information gained from those aims above to assess the applicability of telomere based methods for estimating the age of live cetaceans

This objective is effectively met. Since there is no consistent relationship between telomere length and age in the current study species, it appears unlikely that telomere-based age estimation methods will be applicable to either cetaceans or pinnipeds, at least given the limitations of the methodologies used in this study.

Objective 4: Synthesize the information gained from those aims above to add data to relationships that may aid in estimating longevities in species where they are unknown

This objective cannot effectively be met. Since there is no relationship between telomere length and age in the current study species, there is no data we can add to existing relationship examining telomere rates of change and longevity relationships. Additionally, since these data are from Q-PCR techniques and other existing data are from other methods (mostly denaturing terminal restriction fragment length methods), it is unclear how data from this study relate to those of previous studies.

3. Appropriateness

The appropriateness of the approaches used in the development and implementation of the Activity
The techniques used in this study are all that were available to us given the condition of the samples and DNA that could be attained from them. It was highly appropriate to examine the effect of cetacean interstitial telomere sequence on conventional terminal restriction fragment analyses despite the fact that the results were unfavourable. There are reasons as to why the data gained may not be representative of true telomere dynamics that mostly revolve around interference of the assays from interstitial telomere sequence (ITS). This would be a large and completely confounding effect if the ITS was highly variable between individuals. However despite this being the case, this effect would not occur in the repeat samples from the same individuals (from bottlenose dolphins: Fig. 3). These data clearly indicate different telomere dynamic processes within individuals. This level of variability in telomere regulatory processes within individuals tends to support the lack of relationship between individuals, as even if all individuals started at the same telomere point, this regulation variability would have a confounding effect on the formation of any strong telomere-age relationship. It is clear from the current literature that there is a wide variability in telomere length at birth, and this in combination with variable longitudinal telomere regulation processes strongly suggests that strong predictive telomere length – age relationships are likely not to exist for many species, particularly long-lived ones since the slower rate of change in long lived species (Haussmann et al., 2003) makes changes difficult to detect in spite of the above issues.

4. Effectiveness

The degree to which the Activity has effectively met its stated objectives
This activity has effectively met its stated objectives to the best of the limitations set by the sample condition and methodological constraints. There are clear indications as to the ability of telomere dynamics to be an accurate predictor of age that comes from comparisons within individuals, between individuals and between species. All of the data generated from the project suggest that these tools will not be useful for age estimation in the taxa studied and this project has also highlight issues which should be considered when conducting similar work on other species. Since the main objective of this project was to examine the ability of telomeres to provide demographic information for marine mammal individuals and populations and this considers not just empirical relationships but practical matters (such as sample collection and age issues and laboratory methodologies), these objectives have been met and thus the project has been effective.

5. Financial Account of the Activity (refer to subclause 9.6, and Schedule Item 5.10 of the Funding Agreement)

Nearly all funding was spent on wages for a research assistant. Dr Bruce Deagle was initially the research assistant for this project for a short period at the beginning and after this Dr Ben Maynard was employed for the remainder of the duration. There were three separate additional items bought with the funding apart from wages, two of which were laboratory consumables misallocated internally to this account (items ICMS20080228 and ICMS20080328 – see below). These two items will be written out of the account and allocated appropriately in the coming month (for further details contact Barry Rumbold; the School of Zoology Finance Officer). The third item was a maxtor hard drive for data backup and storage for Glenn J Dunshea.

References:

Haussmann, M. F. and R. A. Mauck (2007). New strategies for telomere-based age estimation. *Molecular Ecology Notes* **8**(2): 264-274.

Haussmann, M. F., D. W. Winkler, K. M. O'Reilly, C. E. Huntington, I. C. T. Nisbet and C. M. Vleck (2003). Telomeres shorten more slowly in long-lived birds and mammals than in short-lived ones. *Proceedings of the Royal Society of London. B Series* **270**(1522): 1387-1392.

Meyne, J., R. J. Baker, H. H. Hobart, T. C. Hsu, O. A. Ryder, O. G. Ward, J. E. Wiley, D. H. Wurster-Hill, T. L. Yates and R. K. Moyzis (1990). Distribution of non-telomeric sites of the (TTAGGG)n telomeric sequence in vertebrate chromosomes. *Chromosoma* **99**: 3-10.

O'Callaghan, N. J., V. S. Dhillon, P. Thomas and M. Fenech (2008). A quantitative real-time PCR method for absolute telomere length. *Biotechniques* **44**: 807-809.