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Harp seal ageing techniques—teeth, aspartic acid racemization, and telomere sequence analysis

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Lower jaws (containing the teeth), eyes, and skin samples were collected from harp seals (*Pagophilus groenlandicus*) in the southeastern Barents Sea for the purpose of comparing age estimates obtained by 3 different methods, the traditional technique of counting growth layer groups (GLGs) in teeth and 2 novel approaches, aspartic acid racemization (AAR) in eye lens nuclei and telomere sequence analyses as a proxy for telomere length. A significant correlation between age estimates obtained using GLGs and AAR was found, whereas no correlation was found between GLGs and telomere length. An AAR rate (k_{Asp}) of 0.00130/year ± 0.00005 *SE* and a D-enantiomer to L-enantiomer ratio at birth (D/L_0 value) of 0.01933 ± 0.00048 *SE* were estimated by regression of D/L ratios against GLG ages from 25 animals (12 selected teeth that had high readability and 13 known-aged animals). AAR could prove to be useful, particularly for ageing older animals in species such as harp seals where difficulties in counting GLGs tend to increase with age. Age estimation by telomere length did not show any correlation with GLG ages and is not recommended for harp seals.
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Key words: age determination, age estimation, amino acid racemization (AAR), aspartic acid, eye lens, growth layer group (GLG), *Pagophilus groenlandicus*, population management, telomere

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Reliable methods for estimating age of animals are fundamental for life-history and population-ecology studies. Management of many harvested species (e.g., many marine mammals and ungulate species) relies on age-specific reproductive rates and survival rates to calculate sustainable harvest levels (International Council for Exploration of the Seas 2004, 2008). Markers for age have been sought by examining age-related morphological changes in a variety of tissues such as teeth, claws, bones, baleen, blubber, and earplugs, and by examining molecular changes within tissues, including eye lenses, skin, blood, and blubber. Teeth have several advantages over other tissues because they do not remodel themselves like bones, nor abrade as quickly as claws or baleen (Hohn 2002). In addition, teeth are found in a wide range of marine mammals and are relatively easy to collect, store, and process for estimating age. Nevertheless, biases exist in ages derived from teeth (see below), and some species of marine mammals, such as baleen whales, have no teeth. For these reasons biologists continue to search for alternative methods for estimating the ages of marine mammals.

One alternative method is called the aspartic acid racemization (AAR) technique. The rationale behind this technique is that in metabolically inactive tissues, such as eye lens nuclei, the L-enantiomer (L) of aspartic acid converts to the D-enantiomer (D) over time, a process called racemization. This racemization occurs at a constant rate throughout the life of an individual. It is therefore theoretically possible to calculate the age of an animal when the racemization rate and the D/L ratio at birth $(D/L)_0$ are known (Bada et al. 1970; Bada and Protsch 1973; Wehmiller and Hare 1971). The

technique has been applied to age estimation of humans and other mammals (Masters et al. 1977; Othani et al. 1995; Yekkala et al. 2006), including a few whale species (Bada et al. 1980, 1983; Garde et al. 2007; George et al. 1999; Nerini 1983; Olsen and Sunde 2002). Although the technique has been used for more than 3 decades, it has yet to be validated for pinnipeds.

Another method for age estimation of animals investigated in recent years is based on measuring average telomere lengths (Haussmann and Mauck 2008; Haussmann and Vleck 2002; Vleck et al. 2003). Telomeres are the terminal nucleoprotein complexes of eukaryotic chromosomes (Blackburn 1991), which change their length throughout life in many species (Monaghan and Haussmann 2006). Telomeres consist of noncoding, highly conserved, repetitive DNA sequences and associated proteins that have a variety of functions including protecting the coding sequence of chromosomes from sequence loss during DNA replication (Blackburn 1991). In the absence of lengthening mechanisms, telomeres shorten each time a cell divides, and once a certain crucial length is reached the cell stops dividing or becomes unstable and dies (Hemann et al. 2001). Patterns of erosion of telomeres are thought to play a role in the ageing process (Monaghan and Haussmann 2006), although how this actually occurs in vivo is not well understood (Aubert and Lansdorp 2008), and it clearly does not follow uniform patterns in all tissues in the body (Goyns and Lavery 2000). If this method proves useful for reliable age estimation, live individuals could be aged from a simple tissue sample in a minimally invasive fashion (Nakagawa et al. 2004). However, the method has been reported to be inconsistent (Hall et al. 2004) or imprecise (Juola et al. 2006) for some species. Thus, it needs to be calibrated on a species-specific basis (Haussmann and Vleck 2002; Haussmann et al. 2003).

The aim of this study was to compare the 3 different age estimation techniques mentioned above: the traditional method of counting growth layer groups (GLGs) in teeth (Bowen et al. 1983; Frie et al. 2003; Lawson et al. 1992), AAR in eye-lens nuclei, and telomere sequence analysis, using harp seals (*Pagophilus groenlandicus*) as a model species.

Materials and Methods

Sampling of seals.—Lower jaws (with teeth), eyes, and skin samples were collected from harp seals sampled during commercial sealing operations in the southeastern Barents Sea (68°24′–70°12′N, 40°10′–45°35′E) on 9–24 April 2006. Lower jaws with teeth and eyes were stored frozen at −20°C until further processing in the laboratory. Skin samples were stored in 20% dimethylsulfoxide and 6 M NaCl until DNA extractions were performed.

A total of 113 seals were aged by counting GLGs in canine teeth. Eighty-eight of these also were aged based on AAR analysis; the other 25 seals were used to estimate the racemization rate and D/L_0 value (88 + 25 = 113). Samples from 92 seals with a matching GLG age were used for telomere analysis.

Age estimation using teeth.—A canine tooth was extracted after boiling the lower jaw for approximately 45 min. A cross section was taken at the position of maximum circumference. Sections were photographed with a digital camera mounted on a binocular microscope (paying particular attention to contrast), and then read by a very experienced harp seal tooth-reader who has extensive training on known-age teeth.

Age estimation using AAR.—Eye lenses were dissected out, and lens layers surrounding the nucleus were removed by rolling the lens on paper. Any remaining layers were removed under a microscope. It is critically important to remove all lens layers from the nuclei, because any younger layers, blood, or other tissue will bias age estimates downward. Lens nuclei were cut in half and stored at −20°C. One half was used in the analysis and the other half was kept as a backup sample.

Hydrolysis of samples and high-performance liquid chromatography (HPLC) analysis were done using the procedures of Zhao and Bada (1995) and Garde et al. (2007). For hydrolysis, 1 ml of HCl was added to each glass tube containing half an eye-lens nucleus and then the tubes were placed in an oven at 100°C for 6 h. For data analysis an Agilent 1100 Series HPLC system (Agilent Technologies, Walbronn, Germany) was used. It consisted of a degasser G1379A, a capillary pump G1376A, an autosampler ALS G1313A, a column compartment G1316A, a fluorescent detector G1321A (excitation = 340 nm, emission = 450 nm), and a multi-wavelength detector MWD G1365B. The column was a Zorbax Eclipse XDB-C18, 4.6 × 150 mm, with particle size 3.5 μm. The HPLC system was connected to a Chemstation Software system, version A.08.03 (Agilent Technologies).

Ratios of D/L measured by HPLC were calibrated using the D/L standards 0.5/99.5, 1/99, 2/98, 5/95, 10/90, and 15/85, which were run at the beginning and end of each HPLC run. Measured D/L ratios from lens nuclei were recalculated using calibration equations (linear regression) for the D/L standards.

Ages were estimated using the equation

$$\text{age (years)} = \frac{\ln\left(\frac{1+\mathrm{D/L}}{1-\mathrm{D/L}}\right) - \ln\left[\frac{1+(\mathrm{D/L})_0}{1-(\mathrm{D/L})_0}\right]}{2k_{Asp}},$$

where D/L is the ratio between the D- and the L-form of aspartic acid, $(D/L)_0$ is the estimated D/L value at age 0, and k_{Asp} is the racemization rate for aspartic acid (George et al. 1999).

The racemization rate (k_{Asp}) and the $(D/L)_0$ value were estimated using 25 seals that had either a known age ($n = 13$; 12 were pups, 0 years old, sampled within a few weeks of their birth, and one was a 15-year-old animal, tagged as a pup in 1991 and collected in 2006) or were animals that had teeth that were deemed to be straightforward for ageing (high-readability teeth, spread across the range of ages in the complete sample). Individual values of $x = \ln[(1 + D/L)/(1 - D/L)]$ obtained by HPLC were regressed against GLG ages in this sample, where the slope of the regression line corresponds to twice the racemization rate ($2k_{Asp}$) and the intercept

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TABLE 1.—Terms and values used for estimation of seal ages and *SE*s.

Parameter description	Term	Calculation	Value
Slope of calibration line	$2k_{Asp}$	See Fig. 1	0.00260
Intercept of calibration line	$x_0 = \ln\{[1 + (D/L)_0]/[1 - (D/L)_0]\}$	See Fig. 1	0.03865
Average value of $x = [(1 + D/L)/[1 - D/L)]$	$\overline{x} = x_0 + 2k_{Asp} \times \overline{A}$ (where $\overline{A}$ = average age of the 25 seals for estimating $2k_{Asp}$ and $(D/L)_0$)	$0.03865 + 0.00260 \times 4.84$	0.05123
Slope of prediction line	$b = 1/2k_{Asp}$	1/0.00260	384.6
Variance of $\overline{x}$	$V(\overline{x}) = s_r^2/N$ (where $N = 25$)	0.0000160/25	6.40×10^{-7}
Variance of $2k_{Asp}$	$V(2k_{Asp}) = s_r^2 \Big/ \sum_{i=1^N} \left(A_i - \overline{A}\right)^2$ (where A = age)	0.0000160/1433	1.1×10^{-8}
Variance of b	$V_b = (2k_{Asp})^{-4} \times V(2k_{Asp})$	$0.00260^{-4} \times 1.1 \times 10^{-8}$	240.7
Variance of a new x	$V(x) = s^2/n$ (s^2 from nested ANOVA)	$1.354 \times 10^{-6}/3$	4.51×10^{-7}
V_x	$V(x) + V(\overline{x})$	$4.51 \times 10^{-7} + 6.40 \times 10^{-7}$	1.091×10^{-6}
Constant C	$C = b^2V_x + \overline{x}^2V_b + V_bV_x$	$384.6^2 \times 1.091 \times 10^{-6} + 0.05123^2 \times 240.7 + 240.7 \times 1.091 \times 10^{-6}$	0.79286
Variance of a new age estimate	$V(\hat{A}) = C + x^2V_b$	Example: $0.79286 + 0.08395^2 \times 248.3$	2.54
Standard error (*SE*) of age estimates	$SE(\hat{A}) = \sqrt{(C + x^2V_b)}$	$\sqrt{2.54}$	1.59

corresponds to twice the $(D/L)_0$ value. The $(D/L)_0$ term includes the part of the D-enantiomers present at birth plus the racemization that occurs when the samples are being hydrolyzed in HCl (Bada and Schroeder 1975). When calculating *SE*s the procedure of Garde et al. (2007) was followed (see Table 1 for terms and values used for calculations, including calculating *SE*s).

Telomere length measurements.—Biopsy tissue blocks from 92 seals were stored in 20% salt-saturated dimethylsulfoxide solution until analysis. Muscle and blubber tissue were trimmed away from the skin, and hair was removed with a scalpel. DNA was extracted from each skin sample multiple times using a standard proteinase K–phenol–chloroform procedure. The multiple DNA extracts for each seal were pooled, ethanol precipitated, and re-eluted in a small volume of preserving solution. DNA quantity and quality was checked by agarose gel electrophoresis. Some samples displayed poor DNA yields or DNA degradation and were omitted from further analysis ($n = 16$). Most remaining samples also contained some degraded DNA, and some samples ($n = 30$) had a band of high-weight DNA and large amount of degraded DNA. The high-weight DNA was purified by cutting it from gels and using the Gelase protocol according to manufacturer's instructions (Epicentre Biotechnologies, Madison, Wisconsin). This procedure removed most of the degraded DNA from the samples.

For quantitative polymerase chain reaction (PCR) assays DNA was diluted to 1–5 ng/μl, and 2 μl of 10 mg/ml Rnase A was added to each well and incubated at room temperature for 15 min. To reduce variation between runs *Escherichia coli* DNA was added to all samples to achieve a final concentration of 5 ng/μl (R. Cawthon, pers. comm.).

Telomere assays were performed using quantitative PCR as described in Cawthon (2002), including modifications by O'Callaghan et al. (2008), for absolute telomere sequence quantization. This assay measures telomere sequence in a given amount of sample, using vertebrate telomere sequence ($TTAGGG_n$)–specific primers. The signal is normalized against the number of nuclear genomes present in the same amount of sample as indicated by amplification of a single copy nuclear locus. Telomere sequence specific primers, reagents, and reaction conditions used in this study followed those of O'Callaghan et al. (2008), except that reactions were performed in a 15-μl total volume with 5 μl of template DNA. In this study a pinniped-specific and a cetacean-specific single copy nuclear assay for the BM1 locus were used (Morin et al. 2006). Conditions for the BM1 assay were 1× SensiMix SYBR Plus master mix (Quantace, London, United Kingdom), 150 nM each of forward and reverse primers with 5 μl of template DNA in a 15-μl total volume. Thermocycling for the BM1 assay was optimal at 95°C for 10 min, 40 cycles of 95°C for 10 s, and 60°C for 75 s. Standards used for absolute quantization of the telomere sequence were serially diluted HPLC-purified oligonucleotides (Geneworks, Australia) of $TTAGGG_{14}$, and standards for the single-copy nuclear locus were a 52-base pair (bp)–long oligomer spanning both primer regions of the target BM1 sequence (Morin et al. 2006; O'Callaghan et al. 2008). Quantitative PCR reaction runs were set up using the Corbett Robotics liquid handling robot system (Qiagen, Copenhagen, Denmark). The fluorescence baseline to select the threshold cycle for subsequent standard curve fit and sample quantization was set the same for all PCR runs at $10^{-1.5}$ (units).

Samples were assayed in duplicate in 2 separate runs for the BM1 locus and 1 or 2 separate runs for the telomere sequence PCR to examine assay consistency. If samples displayed an intrarun coefficient of variation ($[SD/\overline{X} \times 100)$ of $\geq 20\%$ for a BM1 or a telomere PCR, the result was discarded because this was interpreted as PCR inhibition resulting from the quality of the DNA extract. The geometric mean of each duplicate was used as the final value per run, because quantitative PCR is an exponential process. Thus, 2 replicate measures of telomere sequence per genome existed for most samples, based on 2 averaged values of each BM1 gene and telomere sequence. The geometric mean of the BM1 assay was divided by 2, because there are 2 copies per genome. Then it was normalized to the geometric mean of 1 telomere sequence amount per sample to yield a value for telomere sequence per

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genome. This process was repeated for the 2nd group of assays. The telomere sequence per genome presented is the average of the 2 replicate values.

Terminal restriction fragment assays also were performed on a subset of samples ($n = 25$) to provide confidence that the values obtained from the quantitative PCR assays were comparable. Here ≈5 µg of DNA from each individual was digested overnight at 37°C with 20 units of *Hae*III and *Rsa* I (New England Biolabs, Ipswich, Massachusetts). The following day 10 µl of Rnase A was added to each sample, and the sample was incubated at room temperature for 15 min. DNA was quantified with the Picofluor system (Turner Biosystems, Sunnyvale, California) and Picogreen dye (Invitrogen, Mulgrave, Victoria, Australia). Then 3–4 µg of DNA was loaded into a 0.5% 1 × TAE agarose gel, which was run at 55 V for 20 h with recirculating 1 × TAE buffer kept at a constant 4°C. Gels were dried for 8 min per side at room temperature, and DNA was denatured by soaking them for 5 min in denaturing solution (0.5 M NaOH, 1.5 M NaCl), followed by two 5-min soaks in neutralizing solution (1 M Tris HCl, 1.5 M NaCl). Dried, denatured gels were prehybridized for 1 h in hybridization solution (0.5 M Na_2HPO_4, 7% sodium dodecyl sulfate, 1 mM ethylenediaminetetraacetic acid, 1% bovine serum albumin) and hybridized overnight with fresh hybridization solution to a ^{32}P-labeled $TTAGGG_4$ oligonucleotide (Geneworks, Thebarton, South Australia, Australia). The following morning gels were washed at high stringency (final wash 0.25 × saline-sodium citrate, 1 h, 37°C), and exposed to Kodak BioMax MS film (Sigma-Aldrich, Sydney, Australia) with an intensifying screen for autoradiograph acquisition. Autoradiographs were digitized on an Epson 2450 PHOTO flatbed scanner (Epson, Nagano, Japan) at maximum resolution and analyzed with image J software according to Haussman and Mauck (2008). This was necessary for finding the optimal analysis window across the length range of the telomeric smear by searching for telomere intersection points among old and young individuals. No clear telomere intersection points were found between the youngest and oldest individuals, so densitometry was performed across most of the telomeric smear (29–3 kilobases [kb]), using the formula $L = \sum(OD_i \times L_i)/\sum(OD_i)$, where OD_i is optical density output at position i, L_i is DNA size at position i, and L is terminal restriction fragment length. DNA under 3 kb was not analyzed, because an approximately 1,700-bp band was present on the hybridized gels, which was interpreted to be an interstitial (i.e., nonterminal) telomeric sequence. Two samples were run in duplicate within gels, 1 sample was run in duplicate between gels, and a sample was run singly on either gel to determine intra- and intergel coefficients of variation. We used 14 samples with a terminal restriction fragment estimate of average telomere length and a quantitative PCR estimate of telomere sequence content per genome. A strong relationship existed between both measures (linear regression: $F_{1,12} = 24.61$, $P < 0.0001$, $R^2 = 0.65$), suggesting the methods were comparable.

A total of 72 samples was analyzed with the telomere Q-PCR assay. Three samples failed to amplify. Thirteen samples were discarded because the intrarun coefficient of variation (*CV*) was >20% for the single copy, or the telomere PCR, or both. A further 4 samples were discarded because *CV*s for the average of the 2 final values of telomere sequence per genome were >20%. We had 7 samples with only a single (albeit precise within each assay) estimate of telomere sequence per genome; these were retained. This left 52 samples with a relatively precise measure of telomere sequence per genome.

Aspartic acid racemization statistics.—The precision of the HPLC system for D/L measurements was tested using 9 randomly chosen seals, each of which was analyzed 3 times (a, b, and c runs). One seal was analyzed twice (× 3 runs). The $x = \ln[(1 + D/L)/(1 - D/L)]$ values were compared using a nested analysis of variance (ANOVA). Homogeneity of variance was confirmed via an Fmax test. A paired *t*-test showed no within-individual difference between the $x = \ln[(1 + D/L)/(1 - D/L)]$ values obtained by the HPLC. Thus, the average value was used when calculating the age of the 9 seals.

Growth layer group and AAR comparisons.—Growth layer group age estimates and AAR age estimates were evaluated in linear regression models using *F*-statistics and Akaike's information criterion (AIC). Residuals were plotted to ensure that linearity and homoscedasticity were met. Positive values indicate overestimation by the AAR technique (compared to GLG ages), and negative values indicate underestimation. Precision was estimated as the residual *SE* of the observed values around the regression line for age groups 0–7, 8–11, and 12–25 years. Regression models were run in R (R Development Core Team 2008) using the MASS library. Following recommendations by Sakamoto et al. (1986), terms were retained in the final model only if their omission caused an increase in AIC of more than 1.5.

Results

Analysis of teeth.—A total of 113 seals were aged by counting of GLGs in the dentine of a canine tooth (Table 2); a majority of the teeth (60%) were deemed high readability. Low-readability teeth were predominantly (75%) >20 years of age. No occluded pulp cavities were found in this study.

Age estimates using AAR.—Twenty-five of the seals were used to estimate the racemization rate of aspartic acid and the $(D/L)_0$ value, and therefore had only the D/L value determined and not an age estimate. These seals included 12 animals that had been sampled within a few weeks of their birth and were therefore 0 years old. Another 12 seals with GLG ages from 1 to 28 years were chosen based on an assessment of their high readability. The last seal (number 115) had been tagged as a pup in 1991 and was 15 years old when it was collected in 2006. This was the only older individual of known age in this sample. The known GLG ages were correlated to individual values of $\ln[(1 + D/L)/(1 - D/L)]$ ($r^2 = 0.96$). The derived value of 0.03865 ± 0.00095 *SE* corresponds to a $(D/L)_0$ value of 0.01933, and the $2k_{Asp}$ value of 0.0026 ± 0.0001 *SE* corresponds to a racemization rate of 0.0013 (Fig. 1). The D/L

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Table 2.—Summary data for 88 harp seals (animal identification number and sex [M = male; F = female]) aged via aspartic acid racemization (AAR) and growth layer groups (GLGs) in the teeth.

Harp seal no.	Sex	D/L (cor.)[a]	AAR age (years)	± *SE*	GLG age (years)
18	F	0.03029	8.4	± 1.3	10
19	F	0.02159	1.7	± 1.1	2
20	M	0.04838	22.4	± 1.7	16
21	M	0.03238	10.1	± 1.3	7
22	M	0.04711	21.4	± 1.7	16
23	F	0.01946	0.1	± 1.1	1
24	M	0.01807	−1.0	± 1.1	2
28	M	0.02370	3.4	± 1.2	5
35A	M	0.05614	28.4	± 2.0	22
37	F	0.02289	2.7	± 1.1	1
39	F	0.02543	4.7	± 1.2	5
40	M	0.05114	24.5	± 1.8	22
41	F	0.05489	27.4	± 1.9	25
44		0.02229	2.3	± 1.1	4
46	M	0.02476	4.2	± 1.2	5
47	M	0.02323	3.0	± 1.1	5
49	M	0.02744	6.3	± 1.2	8
50	M	0.04848	22.5	± 1.7	16
51	F	0.05402	26.7	± 1.9	18
54	F	0.02497	4.3	± 1.2	5
55	M	0.02761	6.4	± 1.2	7
57	M	0.02516	4.5	± 1.2	6
58	M	0.03597	12.8	± 1.4	12
59	F	0.05222	25.3	± 1.9	15
60	F	0.05225	25.4	± 1.9	20
61	M	0.05432	27.0	± 1.9	16
63	M	0.03771	14.2	± 1.5	13
64	F	0.05367	26.5	± 1.9	19
65	M	0.05444	27.0	± 1.9	19
66	M	0.05554	27.9	± 1.9	19
67	F	0.05748	29.4	± 2.0	23
68	M	0.03270	10.3	± 1.4	12
70	M	0.02859	7.1	± 1.3	9
71	F	0.06195	32.8	± 2.1	25
72	F	0.01844	−0.7	± 1.1	1
73	M	0.04309	18.3	± 1.6	18
74	M	0.04650	20.9	± 1.7	18
75	M	0.02028	0.7	± 1.1	3
76	M	0.04569	20.3	± 1.7	14
79	F	0.05778	29.6	± 2.0	14
80	M	0.02843	7.0	± 1.3	8
81	F	0.02418	3.7	± 1.2	6
84	F	0.05760	29.5	± 2.0	24
85	M	0.03380	11.1	± 1.4	11
86	M	0.05069	24.2	± 1.8	20
87	M	0.04587	20.4	± 1.7	18
88	F	0.02519	4.5	± 1.2	6
91	F	0.02418	3.7	± 1.2	6
92	M	0.04195	17.4	± 1.6	25
94	F	0.02264	2.5	± 1.1	5
95	M	0.05134	24.7	± 1.8	12
96	F	0.04971	23.4	± 1.8	16
97	M	0.02000	0.5	± 1.1	2
98	F?	0.04835	22.4	± 1.7	20
99	M	0.03810	14.5	± 1.5	13
100	M	0.04679	21.2	± 1.7	20
101	M	0.03188	9.7	± 1.3	9
104	M	0.04243	17.8	± 1.6	21
105	F	0.01899	−0.3	± 1.1	1
106	M	0.02068	1.0	± 1.1	2
107	M	0.02351	3.2	± 1.2	2
108	F	0.05358	26.4	± 1.9	18
109	M	0.03242	10.1	± 1.3	11
111	M	0.04416	19.1	± 1.6	18
112	M	0.03424	11.5	± 1.4	14
113	M	0.02172	1.8	± 1.1	3
114	M	0.03387	11.2	± 1.4	14
116	M	0.05297	25.9	± 1.9	18
117	M	0.02542	4.7	± 1.2	7
118	F	0.02396	3.6	± 1.2	6
119	M	0.04874	22.7	± 1.8	17
120	M	0.02797	6.7	± 1.2	9
121	F	0.04934	23.1	± 1.8	16
123	F	0.03351	10.9	± 1.4	11
124	M	0.03122	9.2	± 1.3	9
125	F	0.04508	19.8	± 1.7	15
126	M	0.02518	4.5	± 1.2	7
127	M	0.02266	2.6	± 1.1	5
128	M	0.04796	22.1	± 1.7	16
138	F	0.02792	6.6	± 1.2	8
139	M	0.03240	10.1	± 1.3	10
140	M	0.04604	20.6	± 1.7	18
141	F	0.03053	8.6	± 1.3	9
142	M	0.03158	9.4	± 1.3	8
143	M	0.04957	23.3	± 1.8	24
144	M	0.04636	20.8	± 1.7	17
145	F	0.02322	3.0	± 1.1	6
146	M	0.03081	8.8	± 1.3	7

[a] D/L (cor.) refers to corrected (according to regression equations) D/L values.

values for the twelve 0-year-old seals used to estimate the $(D/L)_0$ value (Fig. 1) ranged from 0.01831 to 0.02388 (7 individuals had a value of 0.01982). The estimated racemization rate and $(D/L)_0$ value specific to harp seals were used subsequently to estimate the ages of the remaining 88 harp seals (Table 2; 1 animal was disregarded because of confusion regarding its identification number).

Among the subset of samples that were run multiple times (Table 3), we found no significant difference between a and b runs ($t_9 = 0.88$, $P = 0.40$), a and c runs ($t_9 = 0.86$, $P = 0.41$), or b and c runs ($t_9 = 0.99$, $P = 0.35$). Exclusion of the 2nd value for animal 85 did not affect the results of these tests. Average D/L values from the 3 runs were used when calculating the ages of these 9 seals. The residual variance (s_r^2) of the line estimating the $2k_{Asp}$ and the $(D/L)_0$ value (Fig. 1) was 1.60×10^{-5}. Hence, the variance of $\overline{X}$ was 6.40×10^{-7}. The variation of the racemization rate was 1.1×10^{-8}, and V_b was 240.7. For seals analyzed multiple times a within-individual variance (s^2) of 1.354×10^{-6} was obtained using a nested ANOVA (Table 3). The variance of the new x was 4.51×10^{-7} and V_x was 1.091×10^{-6} (also see Garde et al. 2007).

Standard curves and linear regression equations.—Linear regression was run on theoretical D/L standards versus measured D/L standards for each of the HPLC runs. The 113 samples were run in 4 different HPLC runs; the coefficient of determination was $r^2 = 0.999$ for each run. Equations with a slope closest to 1 (range: 1.0719–1.3243)

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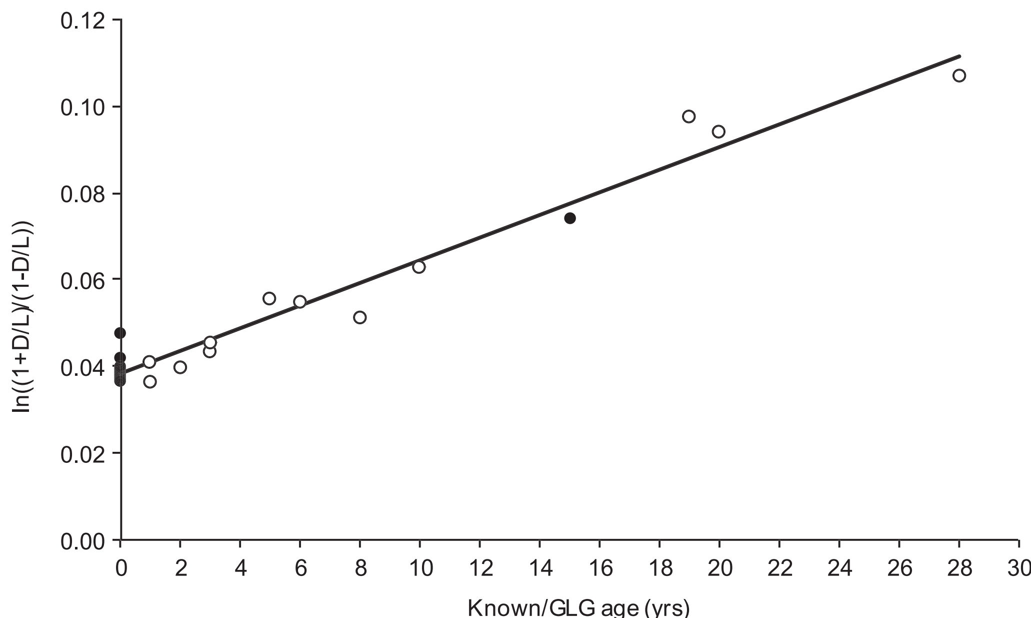

Fig. 1.—Regression line of D/L ratios against known or growth layer group (GLG)–estimated ages in years (yrs) for 25 harp seals. The intercept is 2 times the $(D/L)_0$ value (0.01933). The slope corresponds to $2k_{Asp}$. Solid symbols are known-age animals, and open symbols are selected high-readability teeth used for counting GLGs. See Table 1 and text for explanations of abbreviations, symbols, and y-axis label.

from each run were used to recalculate D/L ratios for the samples.

Comparison of age estimates using GLG and AAR.—The oldest individual, estimated by AAR, was 33 years of age compared to 25 years of age for GLG analysis. GLG versus AAR ages showed a strong relationship ($r^2 = 0.86$; intercept -2.09, slope = 1.32, residual $SE = 3.86$; $F_{1,85} = 514.33$, $P < 0.0001$; Fig. 2), but variance around the total-sample regression line was not homogeneous; it increased with increasing age and was skewed. A linear regression run for only young animals (1–11 years) was significant ($y = -1.42 + 1.08x$; $F_{1,42} = 259.62$, $P < 0.0001$, $r^2 = 0.86$), suggesting that AAR ages are somewhat lower than GLG ages for young animals. For GLG age classes 1–7 years (which are assumed to reflect true age) direct estimation of bias in a linear model showed a constant negative bias of -1.13 years with a residual SE of 1.38 years for AAR ages. A similar analysis for GLG age classes 8–11 years showed similar results, with a slightly lower associated SE than for younger seals (intercept = -0.57; residual $SE = 1.13$). For GLG age classes 12–25 years AAR ages were on average higher than GLG ages. For older animals a significant linear relationship was found for both the total data set ($y = 8.45 + 0.78x$; $F_{1,41} = 16.74$, $P < 0.001$, $r^2 = 0.29$) and a high readability subset ($y = 5.32 + 0.8x$; $F_{1,14} = 5.82$, $P < 0.05$, $r^2 = 0.29$), although coefficients of determination were <30% in both analyses (Fig. 2). The average age difference between methods was estimated to be 4.37 years (residual $SE = 4.55$) for the older animals in total and 2.06 years (residual $SE = 4.80$) for the high-readability data set (Table 4).

Telomeres—quantitative PCR assays.—Table 5 shows summary statistics for intra- and interassay precision for the telomere analyses. The telomere sequence per genome varied from 11.9 to 278.3 million base pairs (megabase pairs [Mbp]), approximately 2–3 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 3.72×10^5 to 8.7×10^6 bp of telomere sequence per chromosome (where *P. groenlandicus* diploid number [2n] = 32). We found no relationship between telomere sequence content and GLG age ($F_{1,50} = 0.12$, $P = 0.73$).

Terminal restriction fragment assays.—Terminal restriction fragment estimates of average telomere length were obtained for 23 seals, 22 of which had accompanying GLG age estimates. The telomere signal from 2 seals was not analyzed because of lack of a defined signal. *CV*s for terminal restriction fragment assays were 0.3% intragel (mean range: 0.16–0.41%) and 2.9% intergel (mean range: 1–4.8%) for the 17–3 kb analysis, and were similar for the 29–3 kb analysis. We found no relationship between telomere length (as determined by terminal restriction fragment analysis) and GLG age (17–3 kb analysis: $F_{1,20} = 0.19$, $P = 0.67$; 29–3 kb analysis: $F_{1,20} = 0.41$, $P = 0.53$).

Discussion

Traditional age estimation of harp seals based on enumeration of dentinal annuli is thought to be reasonably accurate, although it is subject to errors due to reader bias and increasing difficulty in discerning very thinly spaced GLGs in older animals (Bowen et al. 1983; Lawson et al. 1992; Scheffer and Myrick 1980; Stewart et al. 1996). Additionally, dentine is deposited into the pulp cavity, which eventually becomes occluded, thus preventing further accumulation of GLGs in old individuals. However, complete occlusion of the pulp cavity is not thought to occur until an age of 25–30 years

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Table 3.—Nested ANOVA used to compare D/L values for 9 harp seals that were run 3 times each; seal ID 85 was run 6 times (2 × 3). Nested ANOVA, average of a, b, c: 1.35×10^{-6}.

Seal ID	Run a: ln[(1 + D/L)/(1 − D/L)]	Run b: ln[(1 + D/L)/(1 − D/L)]	Run c: ln[(1 + D/L)/(1 − D/L)]	Average of run a ,b, c	Nested ANOVA (a)	Nested ANOVA (b)	Nested ANOVA (c)
120	0.05395	0.05814	0.05577	0.05595	4.43946×10^{-7}	5.29698×10^{-7}	3.78355×10^{-9}
128	0.09558	0.09369	0.09870	0.09599	1.872×10^{-8}	5.87348×10^{-7}	8.15783×10^{-7}
139	0.06696	0.06266	0.06483	0.06482	5.11334×10^{-7}	5.16228×10^{-7}	1.16534×10^{-11}
28	0.04817	0.04806	0.04601	0.04741	6.40487×10^{-8}	4.64947×10^{-8}	2.19684×10^{-7}
30	0.03783	0.03882	0.03874	0.03846	4.47588×10^{-8}	1.44357×10^{-8}	8.35651×10^{-9}
31	0.04068	0.04012	0.04060	0.04047	4.92263×10^{-9}	1.29882×10^{-8}	1.91881×10^{-9}
46	0.04927	0.04992	0.04937	0.04952	6.92086×10^{-9}	1.78018×10^{-8}	2.52323×10^{-9}
92	0.08415	0.08416	0.08353	0.08395	4.66024×10^{-9}	5.07256×10^{-9}	1.94569×10^{-8}
85	0.06620	0.06735	0.06628	0.06661	1.85664×10^{-8}	6.06601×10^{-8}	1.21075×10^{-8}
85	0.06835	0.06929	0.06829	0.06864	9.25081×10^{-9}	4.59332×10^{-8}	1.39569×10^{-8}
					1.12713×10^{-6}	1.83666×10^{-6}	1.09758×10^{-6}

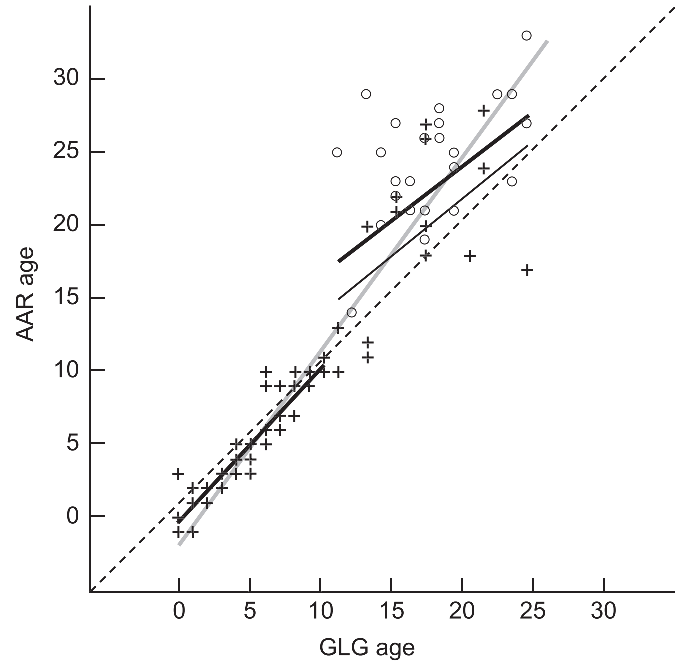

Fig. 2.—Aspartic acid racemization (AAR) ages plotted against growth layer group (GLG) ages. Linear regression expressions including regression lines are shown for age groups 1–11 and 12–25 years (thick black lines). High-readability data are plotted with black crosses, and low-readability data are plotted with gray circles. For older seals a separate regression line is shown for high-readability data (thin black line). Additionally the 1:1 relationship (dashed line) and the entire age-span regression (light gray line) are plotted for comparison.

(Bowen et al. 1983). No instances of complete occlusion of the pulp cavity were seen among harp seals in this study, but our maximum GLG estimate was 25 years.

In the present study the AAR technique showed potential as an alternative to GLG age estimation of harp seals; the relationship was strong between ages derived using the 2 methods, although variance increased among older animals. For GLG age classes 1–7 years we observed a consistent negative bias of −1.37 years for the AAR technique. For older animals AAR ages were generally higher than GLG ages, but the modeled difference between the methods for older seals was large (4.37 years). AAR age estimates suggest that the present study included many seals older than 20 years and that these seals had a much higher incidence of low GLG readability compared with younger age classes. Removing low-readability specimens resulted in halving of the estimated difference between the 2 methods.

Ageing errors in seals will necessarily affect estimates of age at sexual maturity and estimates of longevity and senescence-related parameters. However, correct age estimation of 1- to 7-year-old seals is particularly important in studies of age at maturity and somatic growth rates. A better basis for relative weighting of GLG ages and AAR ages might be obtained in the future if sufficient numbers of known-aged seals (based on pup tagging) become available for study.

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TABLE 4.—Results from linear modeling of differences between growth layer groups (GLGs) ages and aspartic acid racemization (AAR) ages for 3 age groups. Residual *SE*s (RES.SE) and Akaike's information criteria (AIC) are shown for the full model (GLG age term included), the constant-only model (C-model), and the 0-model (C = 0). The terms included in the most-parsimonious model based on AIC selection are indicated in the last column, and the estimated parameters for this model are shown in boldface type in the table. For the oldest GLG age class, estimates are shown both for the total data set, AAR(TOT), and for a high-readability subset (of aged teeth—GLGs), AAR(HR).

GLG (years)	Method	*n*	Full model	RES.SE	AIC	C-model (0-model)	RES (SE)	AIC	Selected model
1–7	AAR	30	−1.15 − 0.01 × age	1.41	22.4	**−1.13** (0.00)	**1.38** (2.03)	**20.4** (42.3)	**C**
8–11	AAR	14	−1.83 + 0.14 × age	1.05	3.1	**−0.57** (0.00)	**1.02** (1.13)	**1.4** (3.6)	**C**
12–25	AAR(TOT)	43	8.35 − 0.21 × age	4.53	131.8	**4.37** (0.00)	**4.55** (6.27)	**131.2** (157.9)	**C**
	AAR(HR)	16	5.32 − 0.19 × age	5.00	52.8	**2.06 (0.00)**	**4.80 (5.08)**	**51.13 (52.0)**	**0**

When using the AAR technique to obtain age estimates for mammals it is crucially important to estimate a specific $(D/L)_0$ value for the species investigated. This is important because the $(D/L)_0$ value differs among species, a feature explained by differences in protein composition of the eye lens nucleus (Bada et al. 1980). The present study is the 1st large-scale study performed on a pinniped species. A $(D/L)_0$ value of 0.01933 was determined for harp seals. Studies on whales have shown $(D/L)_0$ values ranging from 0.027 to 0.0291 (Garde et al. 2007; George et al. 1999; Olsen and Sunde 2002). These values are fairly similar, especially when compared to the human $(D/L)_0$ value of 0.056, and might reflect a similarity in $(D/L)_0$ values among cetaceans. The value for the harp seal found in this study therefore might reflect values for other pinniped species; but definitive conclusions require that more species be studied.

Overestimation of the $(D/L)_0$ value results in underestimation of age and vice versa, especially affecting age estimates of young individuals (George et al. 1999). The wide range of D/L values for newborns suggests intraspecific variation in this parameter for this species, which might explain some of the observed underestimation in harp seal age class 1–7 years in this study. Additional samples from newborn harp seals likely will improve the estimate for the $(D/L)_0$ value for this species.

Ideally, ageing methods would be tested against a large sample of known-aged animals, but among pinnipeds large-scale tagging programs and subsequent collections of tissues from known-aged specimens currently are relatively rare. In this study we calculated a $2k_{Asp}$ value of 0.00260, specific for harp seals, based on 13 seals of known age (twelve 0-year-olds and 1 tagged 15-year-old) and 12 seals with GLG age estimates that were thought to be quite precise. A $2k_{Asp}$ of 0.0023 and a D/L_0 value of 0.01980 were produced using only known-age animals. Using these values, instead of those in Fig. 1, for estimating the age of the remaining seals resulted in age differences ranging from 0.01 to 3.87 years. A racemization rate of 0.0023 is closer to the rates found for whales, and the slightly higher $(D/L)_0$ value also would mean less underestimation of seals in age class 1–7 years. However, the known-age sample in our study included data points for only twelve 0-year-olds and 1 for the 15-year-old. Collection of eyes from seals of known age that have been tagged as pups or born in captivity might provide a more-precise racemization rate, which could potentially be used in studies of other pinniped species.

Bada et al. (1980) suggested that racemization rates in eye lens nuclei were similar within mammal species. However, studies of whales, humans, and the present one on seals show considerable differences among species. Although rates might seem similar (harp seals, 0.00260 [present study]; narwhals, 0.00209 [Garde et al. 2007]; fin whales, 0.002209 [Nerini 1983]; and humans, 0.0025 [Masters et al. 1977]) using a rate from another species can result in errors in age of several years. For example, using the narwhal rate to calculate harp seal ages results in overestimation of seal ages by up to 8 years.

Racemization rates are temperature dependent (Bada et al. 1983; Bada and Schroeder 1975), and differences in rates could be due to different internal body temperatures or different environmental temperatures experienced by different species. Eye lens temperature of the harp seal is not known, but core temperature is approximately 36°C (Kvadsheim and Folkow 1997). We assume that the innermost part of the eye-lens nucleus has a uniform temperature among individuals, but small difference in temperatures among individuals potentially could introduce significant variance to this kind of study.

Using telomere sequence content per genome as a proxy for telomere length, we found no relationship between telomere length and age of harp seals. Two possible explanations for these results are that either age does not influence telomere dynamics in harp seals, or an influence exists, but the DNA quality of the samples in this study, or the methods used, were not sufficient to detect it. That a strong relationship was found between different telomere measurement techniques and

TABLE 5.—Summary statistics for precision of telomere sequence per genome quantitative polymerase chain reaction assays as indicated by coefficient of variation ($CV = SD/\bar{X} \times 100$) calculations. Filtered final ratio values are those where samples with interrun *CV* of >20% are excluded.

Assay	*n*	*CV* range	*CV* $\bar{X}$	*CV* median	*CV SD*
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

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values from both techniques showed no relationship with GLG age suggests that telomere length is not influenced by chronological age in this species. This is not the first study to report a lack of relationship between age and telomere length in a wild animal (Bize et al. 2009; Hall et al. 2004; Hatase et al. 2008; Pauliny et al. 2005) and reinforce the taxon-specific nature of these relationships.

To summarize, this study has shown that AAR produces results that are similar to counting of GLGs in teeth for harp seals. The former technique might produce more-accurate results for older age classes, although this suggestion needs to be verified using samples of seals of known age in additional studies. Additional calibration using known-age seals (including newborn seals) for estimation of an even more-precise racemization rate and $(D/L)_0$ value also are desirable. No relationship was found between telomere lengths and tooth-ageing in this species. Telomere length does not appear to be a useful tool for age estimation in harp seals, at least from material collected under normal field-sampling conditions.

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