Age estimation for bowhead whales, *Balaena mysticetus,* using aspartic acid racemization with enhanced hydrolysis and derivatization procedures

D.L. WETZEL[1], J.E. REYNOLDS, III[1], P. MERCURIO[1,2], G.H. GIVENS[3], E.L. PULSTER[1] AND J.C. GEORGE[4]

Contact e-mail: dana@mote.org

ABSTRACT:

Important information for determining effective conservation strategies for bowhead whales (*Balaena mysticetus*) includes accurate and precise determination of the ages of individual whales. Attempts to develop reliable methods to determine age for this species have included baleen carbon cycling analysis, aspartic acid racemization measurements, and assessments of corpora accumulation. Each of these methods has limitations affecting its utility. The objectives of this work were to identify the deficiencies and limitations of the existing methods for aspartic acid racemization measurement and to improve the effectiveness of such procedures to obtain consistent, reproducible results; this has been problematic to date. Using D/L ratios of aspartic acid in bowhead whale lens nuclei obtained from optimized analytical methods, we estimate whale ages employing a previously published aspartic acid racemization rate. The improved methods significantly increase the precision of D/L measurements. The resulting age estimates are also more precise than estimates generated from corpora count data. The enhanced techniques for measuring D/L may serve equally well for other hard-to-age species including other mysticetes, birds, and even homeothermic ectotherms.

KEY WORDS: BOWHEAD WHALE, AGE DETERMINATION, ASPARTIC ACID RACEMIZATION

INTRODUCTION

Conservation of great whales and other marine mammals can be hindered by a lack of knowledge regarding the health of individuals, population demography and the extent to which threats are under control (Reynolds et al. 2005). Long-term survival of bowhead whales (*Balaena mysticetus*) and other ice-adapted species will be particularly influenced by direct effects of climate change and resultant changes in human activities. Ice-free seas will foster expanded commercial fishing and shipping, and oil and gas production, among other changes (Arctic Climate Impact Assessment 2004), which could affect life history parameters, carrying capacity and even the sustainability of these species (Reeves et al. 2012).

Models to evaluate impacts of threats on marine mammals, including the great whales, must include data on factors such as age-specific reproductive performance and survival rate, population size and trend, and animal health (e.g., Runge et al. 2004). An important component of evaluating the effects of environmental change and consequent human activities on bowhead whales involves precise and accurate estimation of ages of individual whales.

For most marine mammals, age determination is done by counting growth layer groups (GLGs) in teeth (Hohn 2009). Certain marine mammals, however, either lack teeth altogether (e.g., the members of the Suborder Mysticeti [baleen whales; Order Cetacea]) or replace teeth at an uncertain rate (manatees [Family Trichechidae; Order Sirenia]) making GLG counts inappropriate (Hohn 2009). Bowhead whales are mysticetes, so an alternative to counting GLGs must be used to estimate age. Attempts to develop reliable age estimation methods for this species have used baleen carbon cycling (Lubetkin et al., 2008; 2012), amino acid racemization (AAR) (George et al. 1999; Rosa et al. 2013), and corpora counting (Olsen and Sunde, 2002; George et al., 2011). However, there are shortcomings associated with each of the techniques used to date.

The accuracy and precision of the corpora counting method are low for young animals, and are not applicable for males. Moreover, resulting age estimates can have high standard errors (e.g., Olsen and Sunde, 2002; George et al. 1999; 2011). To appropriately apply corpora aging techniques, it is necessary to know life history and other parameters including age of sexual maturity, age of onset of senescence (or even whether there

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[1] Mote Marine Laboratory, 1600 Ken Thompson Parkway, Sarasota, Florida 34236.
[2] Current Address: National Research Centre for Environmental Toxicology (Entox), The University of Queensland, Queensland Health Precinct, 39 Kessels Road, Coopers Plains, Qld 4108, Australia.
[3] Department of Statistics, Colorado State University, Fort Collins, CO 80523.
[4] Department of Wildlife Management, North Slope Borough, Barrow, AK 99734.

is senescence), ovulation rate (and potential changes thereof) and whether corpora albicantia persist through the life of the animal (Olsen and Sunde, 2002).

One might also consider using baleen length to estimate age. However, baleen is continuously worn down as bowheads grow older and wear rates need to be estimated to apply the technique, hence accurate age estimates can only be made for young whales. In contrast, there is significant information available in baleen carbon cycling analysis (Lubetkin et al, 2008; 2011). However, this technique has significant age range limitations and can only reliably be used for young whales.

Measuring body length is also not an effective method for estimating age. The correlation between body length and whale age is poor, as we will see below, and the relationship is sex-specific.

Aspartic acid racemization of the lens nucleus is an alternative method used for calculating ages of bowhead whales. Whereas the baleen cycling and corpora count estimation methods are fundamentally based on biological processes of an individual whale, AAR is based on physical chemistry of the rate of a chemical reaction. During gestation, aspartic acid is laid down in the nucleus of the lens where no metabolic activity occurs that would convert the L form of aspartic acid to the D form (Bada et al., 1980). Thus, in absence of metabolism, the conversion of L to D aspartic acid takes place only due to racemization over time. The kinetics of the rate of reaction constants of aspartic acid enantiomers can be ascertained using the Arrhenius equation which accounts for the effect of a change of temperature on the rate constant and, therefore, on the rate of the reaction. Whereas the mechanics of the mathematical treatment for the Arrhenius equation are clear and simple to apply, the data generation needed to calculate the reaction rate, K_{asp}, can be compromised by analytical error. Although the use of AAR has shown considerable promise and led to several published analyses specifically for bowhead whales, a number of limitations and problems have been encountered when using this approach for ageing whales. The most serious of these problems seems to be with reproducibility of the D/L ratio data as a consequence of both analytical chemistry protocols and sample/standard instability.

In light of such problems, a primary focus of our efforts has been to examine heretofore untested assumptions with regard to the efficacy of AAR approaches that have been used in previous studies of bowhead ages, starting with Bada et al. (1980). Wetzel et al. (2007) performed such testing and modified methods used by George et al. (1999) and Rosa et al. (2004, 2013) to provide bowhead age estimates that are significantly more precise than those of the earlier studies. This paper describes further refinement of methods to promote improved precision for age determination of bowhead whales, which has implications for enhanced ability to determine age of individuals for other taxa including other mysticete species.

When this approach is applied to a large number of bowhead whales of various body sizes, sampled during different harvests, managers will have data that will improve their ability to assess both fundamental life history parameters and the relationships of threats to the ages of affected whales, as well as detect changes in such relationships over time. Having an accurate aging technique for both sexes will allow scientists and managers to understand whether particular demographic groups are most vulnerable to ship strikes and entanglements; monitor changes in life history and other parameters; assess changes over time in the extent to which fishing, shipping and other threats (e.g., contaminants) affect particular age groups; and inform effective mitigation actions *before* consequences of threats become critical.

MATERIALS AND LAB METHODS

Sample acquisition
For approximately three decades, scientists with the North Slope Borough, Department of Wildlife Management (NSB-DWM), have worked closely with Alaska Native hunters to examine bowhead whales taken during subsistence hunts. During this period, a large number of eyes from freshly-dead whales have been preserved (frozen, intact) along with other tissue samples and basic biological data for each whale. For this study we selected lenses from 67 bowhead whales, ranging from fetuses to very large adults. We analyzed the D/L ratios for each of the 67 lenses and then used lens material from two whales to begin a pilot artificial aging study to evaluate the efficacy of using artificial aging to estimate K_{asp} and, ultimately, to use such methods to estimate age.

Eye lens removal and analysis of extracts
The methods employed for acquisition of lens nuclei were generally similar to those described by George et al. (1999), Olson and Sunde (2002), and Rosa et al. (2004, 2013). Prior to the analyses, no information about whale size and sex was known– i.e., the samples were run 'blind'. For each eye, the lens nucleus was removed and trimmed. One half of the nucleus was retained frozen in a clean glass vial, and the other half was hydrolysed.
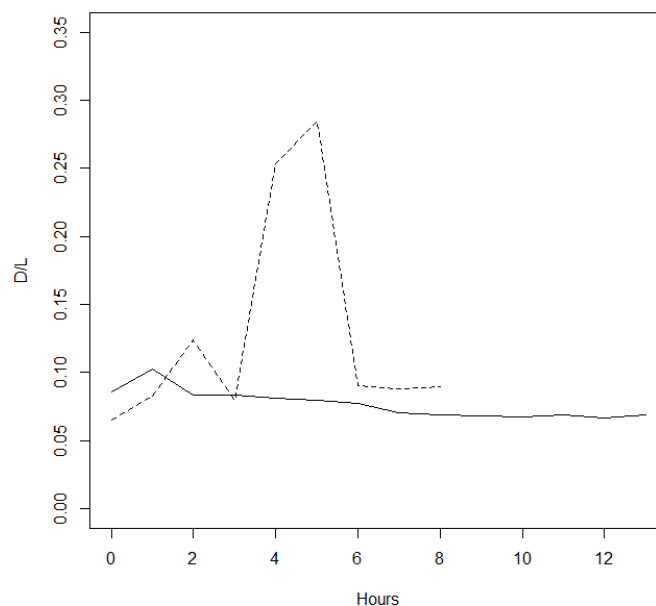
Figure 1: D/L ratio versus time for hydrolysis at two hydrolysis temperatures, 80ºC (solid) and 100ºC (dashed).

Time (min)	% A (methanol)	% B (acetonitrile)	% C (N-acetyl-L-cysteine)
5	5.0	0.0	95.0
25	23.0	0.4	76.6
30	0.0	60.0	40.0
40	5.0	0.0	95.0

Table 1: Liquid phase program used for separation of D- and L-Asp enantiomers.

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Sample extracts were hydrolyzed and derivatized using methods which were modified from previous studies (George et al. 1999; Olson and Sunde 2002; Rosa et al. 2013). Analyses for D and L isomers of aspartic acids were done in triplicate on a HyperClone reverse phase C18 column (120A, 250 x 4mm, 5micron; Phenomenex, Torrance, CA, USA) using a high performance liquid chromatograph (HPLC; Agilent Technologies, Santa Clara, CA, USA), equipped with an autosampler and scanning fluorescence detector (λ_{ex}=230nm; λ_{em}=445nm). The HPLC flow rate was 1.5 ml/min, the column temperature was set to 30ºC and methanol (A), acetonitrile (B) and N-acetyl-L-cysteine (C) were used as eluants with the gradient program in Table 1.

Hydrolysis

George et al. (1999) and others have followed the methodological lead of J.L. Bada, a pioneer in the application of amino acid racemization to age estimation for large whales (e.g., Bada et al. 1980). These scientists typically conducted hydrolysis of bowhead lens samples in 6M HCl at 100ºC for 6 hours. In contrast, our study tested a range of temperatures and durations for hydrolysis to find the point at which hydrolysis is optimized for the species in question (in this case, the bowhead whale). That optimum occurred using 6M HCl at 80ºC for 8 hours. Specifically, our study found that hydrolysis produces stable results after 8 hours, but more variable outcomes over shorter time periods. These results are illustrated in Figure 1. Neither 100ºC nor 80ºC is a temperature at which aspartic acid structure is affected in a manner that affects D/L ratios (Goodfriend 1997; Goodfriend and Meyer 1991).

Standards. Under most chemical analysis scenarios, the normal quality assurance and quality control information regarding chemical standard calibration is rather rote and unexceptional. However, in many previous studies (e.g., Olsen and Sunde 2002; Rosa et al. 2013) researchers have found that responses in calibration curves of the D and L isomers of aspartic acid were notably atypical. In almost all previous whale AAR research, day-to-day instrument responses were reported as inconsistent, totally absent, impaired or just "touchy", to the point where, in one study, multiple models for standard responses needed to be developed using robust regression and then model selection techniques were applies (e.g. George et al. 1999).

Because of this, we paid particular attention to designing a robust calibration curve comparison, bracketing each set of three lens samples with a standard and running all analyses in triplicate. All standards in this study were corrected for cross contamination as there were residual D isomers in the L isomer aspartic acid standard and vice versa. This cross contamination is expected and is due to the fact that the chemical reaction (racemization) is constant. The standards used for the calibration curve consisted of seven different ratio mixtures of D to L isomers of aspartic acid which were analyzed in triplicate for each set of 10 lens sample analyses. For each standard calibration curve analysis, we required the regression coefficient of determination (R^2) to be at least 0.99 or a new standard calibration mixture was made and analyzed until our acceptable R^2 value was achieved.

Derivatization

Previous studies (e.g., George et al., 1999; Rosa et al., 2004) conducted derivatizations in which the amino acid extract being assessed was diluted 1:1000 with distilled water, and a subsample of the dilution was placed in a centrifuge with 10ul of OPA-NAC (ortho-phthaldialdehyde and N-acetyl-L-cysteine). This mixture was shaken for 20 seconds, and centrifuged for 15 minutes, at which time 475 ul of 0.05 M sodium acetate buffer was added. Finally, 200 ul of this was analyzed by HPLC using methanol and sodium acetate for mobile phase at 1ml/min.

In contrast to the multi-step process described above, our study followed a different approach (e.g., Kaufman and Manley 1998). We used OPA-IBLC (ortho-phthaldialdehyde and N-isobutyryl-l-cysteine) instead of OPA-NAC; our amino acid extract was rehydrated with 0.01M HCL and sodium azide (antibacterial) at 0.04ml/mg lens; and our extract was placed on the HPLC where derivatization was performed in a single step *within the autosampler syringe*. Conducting the derivatization in a single step within the syringe eliminates the possibility of inconsistencies or errors being introduced at each step of more traditional processes and the chemical stability problems that have been observed with previous methods.

Artificial aging studies

The assessment of possible age-related changes in the racemization rate in lenses of a particular species can be done through artificial aging studies (Goodfriend 1997; Goodfriend and Meyer 1991). Fundamentally, heating of the lens extracts mimics the aging process, but at a faster reaction rate.

Following heating of the hydrolysed extracts over a range of lengths of time and at three incubation temperatures, D/L ratios of the extracts are measured. The standard analysis (Goodfriend 1997; Goodfriend and Meyer 1991) applies the Arrhenius equation to these data to assess how the aspartic acid racemization rate (i.e., K_{asp}) changes with age.

To conduct an artificial aging experiment for bowheads, lens samples were heated to three temperatures: 80ºC, 100ºC, and 120ºC, for a total of 50 samples analyzed in triplicate. After incubation of the extracts for different time periods at each temperature, the D/L ratio of aspartic acid was measured, as described above.

STATISTICAL METHODS

Age estimation

Estimates of $(D/L)_i$ for the *i*th whale and $(D/L)_0$ are used to estimate age according to the equation

$$Age_i = \frac{\log\left\{(1+(D/L)_i)/(1-(D/L)_i)\right\} - \log\left\{(1+(D/L)_0)/(1-(D/L)_0)\right\}}{2K_{asp}}$$

(George et al., 1999). The $(D/L)_0$ value was estimated using an inverse variance weighted average of five values. The first value is 0.0250 (s.e. 0.0013) from Rosa et al. (2013). This is estimated from a regression model using D/L data mostly for young whales of known ages (using corpora counts, baleen growth increments and fetus data). The remaining values are means of triplicated D/L measurements for four fetuses included in the present dataset. These values ranged from 0.0256 to 0.0292 with standard errors ranging from 0.0001 to 0.0005. For the *i*th whale, the observed data value of $(D/L)_i$ is taken to be the average of our three replicated measurements.

Variance estimation used a hybrid parametric and non-parametric bootstrap approach (Davison and Hinkley, 1997). The variance and 95% confidence interval were estimated separately for each whale. Also separately, for each whale, we re-sampled the three independent D/L measurements uniformly with replacement. Within each bootstrap iteration we also employed parametric re-sampling of $(D/L)_0$ and K_{asp}. The approximate correlation between the estimates of $(D/L)_0$ and K_{asp} is 4e-9, so this was ignored during re-sampling. Together, these bootstrap sampled quantities were used to generate one bootstrap pseudo-estimate of Age_i. We used 10,000 bootstrap replications for each whale. Confidence intervals were generated using the percentile method.

Using these age estimates and other estimates from 161 whales based on aspartic acid racemization, corpora counts, and baleen aging methods (Lubetkin et al., 2008; George et al., 2011; International Whaling Commission, unpublished), we fit the two-stage von Bertalanffy II (1938) model to estimate sex-specific growth curves. This is the same approach used by Lubetkin et al. (2012) except that we did not include growth spurts.

Artificial aging studies
The standard statistical analysis for our artificial aging study would follow the procedures developed by Goodfriend (1997), estimating K_{asp} for the three temperatures and then using the Arrhenius equation $K_{asp} = A \exp\{-E_a / (RT)\}$ to estimate the relationship between K_{asp} and temperature. Here, K_{asp} is the rate constant for the reaction from the L form to the D form of aspartic acid, R is the universal gas constant, and T is the temperature of the living whale in degrees Kelvin. The parameters A and E_a are estimable from the heating experiment data via linear regression based on the log transform of the above model. The results may be extrapolated to estimate K_{asp} for lens temperatures appropriate for bowheads. However, our results indicated that this standard analysis would not be appropriate for the bowhead data obtained from our heating experiment for reasons discussed further below.

RESULTS AND DISCUSSION

Age estimation
Table 2 provides estimated ages, bootstrap standard errors and bootstrap 95% confidence intervals for the whales analysed in this study. Negative age estimates are sensible because the D/L values include measurement uncertainty; such estimates should be interpreted as 'very young'. The most striking aspect of these results is that there is evidence that some bowhead lifetimes may extend nearly 200 years or beyond. This is consistent with previous findings from other researchers. Excluding negative age estimates, 80% of the coefficients of variation for these estimates are within 0.01 of 0.16, indicating a very consistent degree of uncertainty on the log scale.

The ages of eleven of the whales we analysed here have previously been estimated by other researchers, using a variety of techniques including corpora counting and baleen isotope cycle analysis. Table 3 compares our estimates to these previous results. Our aspartic acid racemization estimates are consistent with the baleen cycle estimates from Lubetkin et al. (2008). However, the corpora counting age estimates of George et al. (2011) appear to be generally higher than the estimates from other methods.

Figure 2 shows a plot of the age estimates for the whales we analysed and 172 additional whales previously aged by other researchers. Each whale is represented by a dot (the point estimate) and a horizontal bar (spanning the 95% confidence interval). Red bars correspond to females, and males are represented by blue. The whales aged in our study are shown with heavier lines than for the whale ages from other researchers. The black lines in Figure 2 show the fitted sex-specific von Bertalanffy II (1938) growth curves, with female whales being larger than males of the same age.

Artificial aging experiment
Applying the Goodfriend (1997) heating experiment method to the bowhead data yields an estimated relationship between K_{asp} and temperature. Lens temperature for living bowhead whales is unknown, however Sformo et al. (unpublished report, 2011) attempted to measure the temperature of lenses from a few very freshly dead bowhead whales. Although those measurements occurred some time after death, and the data should be considered quite preliminary, the mean temperature was 11.3°C (SE ± 1.9°C), which is considerably lower than the core body temperature of 33.8° (George, 2009). Sformo et al. suggested that (a) lens temperature may be higher if peripheral cooling after death occurs, or (b) lower lens temperatures are possible if extrapolations are made from cornea temperatures.

Using the estimated relationship between K_{asp} and temperature, if we set K_{asp}=0.000977, which is the estimate of Rosa et al. (2013), our analysis yields age estimates that resemble the estimates of those authors, but correspond to a lens temperature that is considerably lower than the conventional wisdom or the range suggested by the scant empirical data of Sformo et al. (unpublished report, 2011). Conversely, if we set lens temperature to be similar to the estimate of Sformo et al. and solve for K_{asp}, the resulting age estimates are surprisingly low.

We have not presented the estimated parameters of the model or ages here. There are two key reasons why.

5

First, due in part to the increased precision of our D/L measurements, we have discovered that the heating experiment yields data indicating a statistically significant nonlinear relationship between duration of heating exposure and D/L. This nonlinearity is most pronounced for the 80º data, and diminishes as temperature increases. Such a relationship was unexpected and prevents application of the Goodfriend (1997) method unless the nonlinearity is ignored.

Second, it is important to recognize that application of the estimated Arrhenius equation to bowheads requires an enormous extrapolation from temperatures around 100º to perhaps 10º or less. The modest estimation uncertainty in the vicinity of tested temperatures is greatly magnified by this extrapolation, even assuming that the linear regression model is still appropriate at such extreme untested temperatures.

Thus, it is apparent that the artificial heating data contain a strong statistical signal, but that further work must be done to develop an appropriate analysis approach akin to that of Goodfriend (1997) for simpler data. Such research is ongoing.

CONCLUSIONS

Considerable effort has gone into development and application of methods to age bowhead whales. Several approaches have emerged, including analysis of AAR of the nucleus of the eye lens. Whereas earlier studies using AAR have provided valuable insights into bowhead longevity and life history, the methods described here present an opportunity to further improve such analyses. The novel methodological changes described herein provide D/L data that are remarkably consistent. The specific modifications developed by this study include (a) species specific hydrolysis time and temperature that optimizes the preparation of the lens aspartic acid for further analysis; (b) stable and consistent calibration curves to eliminate the need for modelling standards; (c) use of single step, within-syringe derivatization; and (d) investigation of artificial aging experiments that may shed light on questions such as constancy of K_{asp} with age, lens temperature, and ultimately age estimates using data based on physical chemistry rather than more plastic features. In concert with the K_{asp} estimate of Rosa et al. (2013), the data presented here provide age estimates for 64 whales not previously aged, and the results are strongly consistent with previous studies that support the hypothesis of extreme longevity for some bowhead whales. Further research with AAR, other aging techniques and, particularly, artificial aging experiments offers promising avenues for continuing to improve our understanding of bowhead life history.

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Whale	Age	2.5%	97.5%	SE	Whale	Age	2.5%	97.5%	SE
02B17	7.5	5.7	10.2	1.2	07B9F	-2.1	-3.0	-1.6	0.4
02B2	52.0	39.4	71.9	8.3	07G3	39.9	30.2	55.1	6.5
02B21	12.6	9.4	17.4	2.0	07G4	29.3	22.2	40.6	4.8
02B22	1.7	1.2	2.4	0.3	07S1	9.5	7.1	13.3	1.6
02B3	106.3	80.6	146.0	16.9	07S2	7.5	5.6	10.4	1.2
02B5	4.8	3.6	6.6	0.8	07S3	17.7	13.3	24.2	2.8
03B6	19.4	14.6	26.7	3.2	07S4	34.8	26.3	47.7	5.5
03B9	68.3	51.6	93.5	11.0	08B14	27.4	20.6	37.8	4.4
04B4	22.2	16.8	30.4	3.5	08S3	187.7	141.9	258.0	29.8
04B5	80.1	60.4	111.0	13.0	09KK1	-3.1	-4.3	-2.4	0.5
04B8	24.0	18.3	32.9	3.9	10B15	20.1	15.2	27.6	3.2
04B9	18.6	14.1	25.7	3.0	11B3	56.1	42.3	77.8	9.1
04G2	4.4	3.3	6.0	0.7	11B4	2.5	1.3	4.1	0.7
04KK1	123.3	93.2	169.3	19.9	11B5	37.2	28.2	51.3	6.0
05B11	17.5	13.3	24.2	2.9	11B6	71.3	53.8	98.1	11.3
05B12	28.2	21.3	38.8	4.6	11B7	157.3	118.8	214.8	25.0
05B21	7.2	5.3	10.1	1.2	12B15	-0.6	-1.2	-0.1	0.3
05B25	14.8	11.2	20.3	2.4	12S2	23.3	17.7	32.2	3.7
05B8	1.3	0.8	2.0	0.3	12S2F	-1.6	-2.3	-1.0	0.3
05B8	0.3	0.1	0.6	0.1	12S3	1.2	0.6	2.0	0.4
05S5	47.4	36.1	65.7	7.6	81WW2	73.2	55.6	100.1	11.5
05S7	81.5	61.6	112.7	13.0	96B5	121.4	91.8	166.6	19.5
06B10c	-5.1	-6.9	-3.9	0.8	97B10	58.1	44.1	79.6	9.2
06B18	54.5	41.3	75.1	8.8	97B12	67.4	50.9	93.1	10.9
06B6	28.0	21.2	38.6	4.5	97B5	5.0	3.8	6.9	0.8
07B10	37.9	28.7	52.2	6.1	97B7	13.1	10.0	18.1	2.1
07B11	78.1	58.7	108.1	12.6	97B8	18.6	14.0	25.5	3.0
07B12	32.3	24.4	44.6	5.2	98B20	16.2	12.2	22.5	2.6
07B13	88.6	66.9	122.2	14.3	98B21	48.4	36.4	66.5	7.8
07B16	28.4	21.5	39.0	4.5	98B4	22.1	16.8	30.5	3.6
07B8	87.6	66.3	119.9	14.0	98B5	95.3	72.0	130.9	15.1
07B9	31.5	23.8	43.1	4.9	98WW2	21.7	16.5	29.7	3.4

Table 2: Age estimates, 95% confidence interval limits, and standard errors.
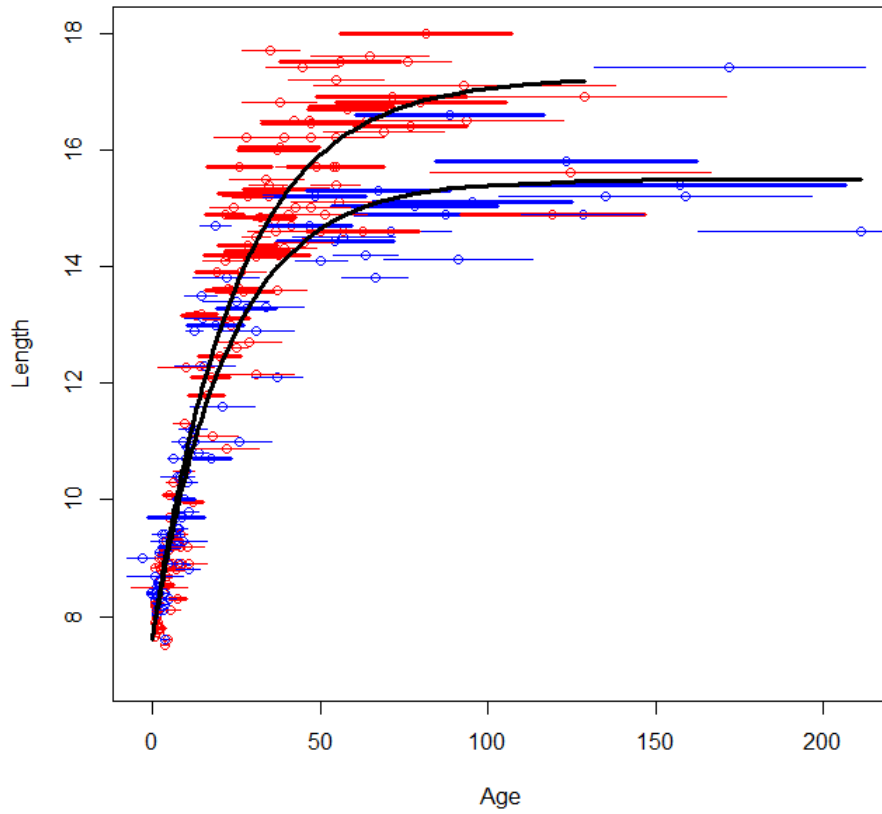
Figure 2: Fitted von Bertalanffy growth curves. Each whale is represented by a dot (the point estimate) and a horizontal bar (spanning the 95% confidence interval). Red bars correspond to females, and males are represented by blue.

Whale	Age	s.e.	Method	Citation
02B17	7.5	1.2	AAR	Here
	6.8	1.1	Corpora	DAA
	8.25	Na	Baleen	L08
02B2	52.0	8.3	AAR	Here
	79	18	Corpora	G11
	65.9	12.0	Corpora	DAA
02B21	12.6	2.0	AAR	Here
	11.7	2.3	Baleen	L08
02B3	106.3	16.9	AAR	Here
	139	38	Corpora	G11
	114.1	23.5	Corpora	DAA
03B9	68.3	11.0	AAR	Here
	102	26	Corpora	G11
	85.0	15.9	Corpora	DAA
04B8	24.0	3.9	AAR	Here
	31	6	Corpora	G11
04B9	18.6	3.0	AAR	Here
	43	8	Corpora	G11
05B12	28.2	4.6	AAR	Here
	38	7	Corpora	G11
96B5	121.4	19.5	AAR	Here
	125	38	Corpora	G11
	114.1	23.5	Corpora	DAA
97B10	58.1	9.2	AAR	Here
	65	14	Corpora	G11
	55.2	9.3	Corpora	DAA
97B8	18.6	3.0	AAR	Here
	31	6	Corpora	G11
	27.5	5.1	Corpora	DAA

Table 3: Comparison of age estimates from various studies. L08 refers to Lubetkin et al. (2008). G11 refers to George et al. (2011). DAA refers to data available from the International Whaling Commission under its Data Availability Agreement (see www.iwc.int).