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Age estimation of Antarctic minke whales *Balaenoptera bonaerensis* based on aspartic acid racemization technique

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Abstract   Counting of the growth layers in the earplugs is the most accepted technique for determining chronological age of Antarctic minke whales; however, unreadable growth layers form in some individuals, especially in young animals. In this study, aspartic acid racemization (AAR) technique was developed for estimating ages in this species with the aim of complementing the age estimated using earplugs. To validate the technique and to determine the specific coefficients for age estimation, the ratio of D and L-enantiomers of aspartic acid (Asp D/L) in lens of 18 whales and 20 fetuses were analyzed and compared with earplug-based age estimates. The equation for age estimation by AAR in this species was as follows: $\mathrm{Log}_e\{[1 + (\mathrm{Asp}\ D/L)_{\mathrm{act}}]/[1 - (\mathrm{Asp}\ D/L)_{\mathrm{act}}]\} = 2.30 \times 10^{-3} \times$ earplug age (year) + 0.0201 ($p < 0.001$, $r^2 = 0.918$). There is a strong correlation between the age estimates by AAR and earplugs. This study was successful in developing the AAR technique for the Antarctic minke whale, and the application of this technique can complement the age estimation of this species based on earplug readings, especially for young animals with unreadable earplugs.

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Introduction

Age is one of the most important life history parameters for assessment and management of marine living resources. In baleen whales, age has been estimated using a variety of methods such as eye lenses [1], baleen plates [2, 3], earplugs [4, 5] and tympanic bulla [6]. Counting of the growth layers deposited in the earplugs is the most accepted technique for determining chronological age of baleen whales [7]. Earplug-based age estimation has the advantages that it is time- and cost-efficient, and the technique can be used on available historical samples; however, unreadable growth layers form in some individuals [8] and species [9] of baleen whales. The aspartic acid racemization (AAR) technique for estimating ages is based on temporal changes of the ratio of D and L-enantiomers of aspartic acid (Asp D/L) in mammals [10, 11]. The technique is based on the principle that D-aspartic acid accumulates logarithmically with age, and the crystalline in the core of the lens has been conserved chemically since it is formed at the fetus stage and is metabolically inactive for all of the animal's life [10]. AAR is useful for age estimation in long-lived mammals because the rate of racemization process depends only on the age and temperature [10, 11].

Previous studies have reported on the application of the AAR technique to baleen whale species. Nerini [12] revealed a clear relationship between earplug-based ages and Asp D/L in fin whales. George et al. [9] applied AAR to estimate ages of bowhead whales, a species having unreadable earplugs. In that study, the rate of the racemization reaction of aspartic acid in eye lens (k_{Asp}) was extrapolated from human [10]

and fin whale [12], and the Asp D/L in eye lens at birth, $(D/L)_0$, was estimated from an average of eight bowhead whales aged 0 to about 2-years-old. Olsen and Sunde [13] applied AAR to estimate ages of common minke whales from the North Atlantic, and k_{Asp} was calculated from data in George et al. [9] as a function of body temperature. $(D/L)_0$ was also extrapolated from that study [9]. Rosa et al. [14] applied AAR for estimating the ages in bowhead whales. They calculated k_{Asp} from ovarian corpora counts, and estimated $(D/L)_0$ from corpora age assuming that the female age at sexual maturity was 20-years-old.

The studies mentioned above, excepting that of Nerini [12], were based on extrapolated k_{Asp} data from other studies as no age data were available for validation or comparison purposes. In addition, $(D/L)_0$ was also extrapolated or assumed in those studies because no sufficient fetus or neonate samples were available for such estimation. The earplug-based technique works well in the case of Antarctic minke whales *Balaenoptera bonaerensis* except with young animals where growth layers are difficult to read [8].

The aim of this study was to develop the AAR technique for age estimation in the Antarctic minke whale. Age information from the AAR will complement the age estimation based on earplug readings, especially for the more difficult young animals. There is plenty of biological information collected from Antarctic minke whales that makes assumptions and extrapolations of key parameters unnecessary. For example k_{Asp} and $(D/L)_0$ can be determined by direct comparison with earplug-based estimated ages, and $(D/L)_0$ can be estimated using available fetal samples from the Antarctic minke whales. Furthermore, the precision of the age estimation by the AAR ages of whales can be estimated.

Materials and methods

Biological sampling

Antarctic minke whales were sampled in the Antarctic sector corresponding to the Indo region, which correspond to the International Whaling Commission (IWC)'s management Area IV (70°–130°E), south of 62°S (Fig. 1). Whales were sampled during the austral summer seasons 2005/2006 and 2007/2008 in JARPAII (Japanese Whale Research Program under Special Permit in the Antarctic-Phase II). Whales were sampled through a pre-determined zigzag track-line designed to cover the whole research area (Area IV). In order to obtain biological samples representing the whole population in the research area, a random sampling method under the line transect sighting survey was adopted (see details in [15]). Whales were killed using penthrite grenade harpoon. The lethal sampling of whales under the JARPAII program was based on a permit issued by the Government of Japan in accordance with the provisions of Article VIII of the International Convention for the Regulation of Whaling (ICRW).

In the field, scientists collected the left eye ball from 20 fetuses and lens samples from 18 female Antarctic minke whales. The samples were stored in polyethylene bags at −80 °C until analysis.

Biological information

Table 1 shows information on sex, body weight, age, and measured and actual D/L ratios of aspartic acid, based on 20 fetus samples. The weight of the fetuses was measured
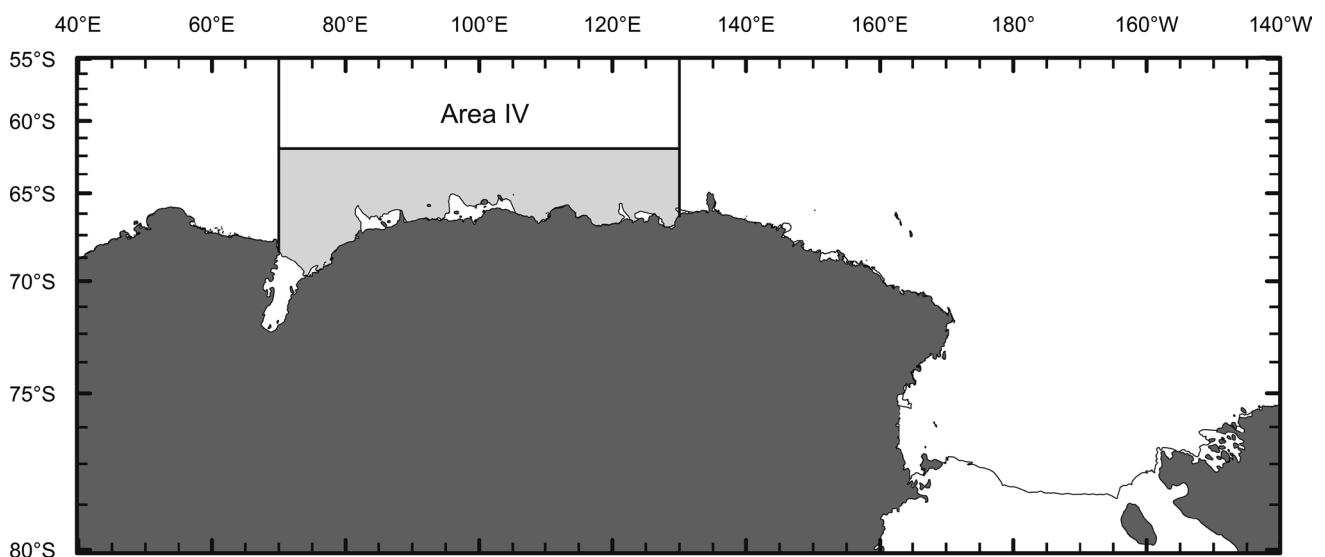

Fig. 1 Research area in the 2005/2006 and 2007/2008 austral summer seasons

Table 1 Biological information and the measured and actual *D/L* ratios of aspartic acid of 20 fetuses of Antarctic minke whale

Sample no.	Sex	Body weight (kg)	Fetus age	$(D/L)_{meas}$ (days)	$(D/L)_{act}$
07/08-AM363F	M	49.4	227	0.0279	0.0117
07/08-AM380F	M	19.0	185	0.0283	0.0122
07/08-AM383F	F	64.1	241	0.0289	0.0130
07/08-AM392F	M	29.9	203	0.0277	0.0116
07/08-AM393F	M	19.2	186	0.0276	0.0114
07/08-AM461F	M	59.3	236	0.0287	0.0127
07/08-AM474F	M	35.8	211	0.0276	0.0114
07/08-AM475F	F	63.2	240	0.0280	0.0119
07/08-AM481F	M	21.9	191	0.0280	0.0119
07/08-AM502F	M	78.5	252	0.0287	0.0127
07/08-AM504F	M	14.0	174	0.0276	0.0114
07/08-AM516F	F	22.1	191	0.0285	0.0124
07/08-AM523F	M	108.0	272	0.0284	0.0123
07/08-AM527F	M	34.6	210	0.0286	0.0126
07/08-AM531F	M	100.0	267	0.0284	0.0123
07/08-AM533F	F	194.5	315	0.0297	0.0138
07/08-AM542F	F	98.0	266	0.0288	0.0127
07/08-AM550F	F	58.8	236	0.0287	0.0127
05/06-AM588F	M	49.0	226	0.0284	0.0123
05/06-AM820F	F	53.6	231	0.0271	0.0108

onboard the research-base vessel, using a marine scale (M1100 PL2260, Marel Co. Ltd.) equipped with motion compensation devices (precision <1%). To convert the fetal weight (*W*: g) into fetal age (*t*: day), the following equation was used [16]:

$$t = W^{1/3}/0.24 + 74. \tag{1}$$

Table 2 shows information on sex, age (year) estimated from earplugs [4, 5] and measured and actual *D/L* ratios of aspartic acid based on 18 female samples.

Preparation of samples

Lenses were rinsed first with phosphate-buffered saline. Then their outermost layers were removed with mucus, and the cores were dissected out using a surgical knife.

Sample preparation and the high performance liquid chromatography analysis (HPLC) were done mainly by following the procedures by Fujii et al. [17]. Then, 10 mg of core samples were homogenized with 1 ml of tris-buffer (200 mM Tris, 150 mM NaCl, pH 8.0) using an ultrasonic homogenizer. The homogenate was centrifuged at 15,000×*g* for 15 min at 4 °C, and 100 μl of the supernatant was then desalted with acetone and air-dried. They were hydrolyzed in the gas-phase HCl (6N-HCl) for 7 h at 108 °C (Pico Tag Work Stations, Waters, Tokyo). The hydrolysates were evaporated under reduced pressure.

Table 2 Biological information and the measured and actual *D/L* ratios of aspartic acid of 18 female Antarctic minke whales

Sample no.	Earplug age	$(D/L)_{meas}$	$(D/L)_{act}$	$\mathrm{Log_e}\,[(1 + D/L)/(1 - D/L)]$
05/06-AM348	14	0.0371	0.0223	0.0446
05/06-AM349	21	0.0463	0.0328	0.0656
05/06-AM352	9	0.0370	0.0222	0.0444
05/06-AM361	26	0.0555	0.0432	0.0865
05/06-AM372	40	0.0552	0.0430	0.0860
05/06-AM382	7	0.0328	0.0173	0.0347
05/06-AM386	8	0.0342	0.0190	0.0380
05/06-AM398	7	0.0320	0.0164	0.0329
05/06-AM488	4	0.0295	0.0136	0.0272
05/06-AM498	4	0.0308	0.0151	0.0302
05/06-AM517	3	0.0269	0.0106	0.0212
05/06-AM539	1	0.0285	0.0124	0.0249
05/06-AM565	2	0.0328	0.0174	0.0348
05/06-AM592	4	0.0324	0.0169	0.0337
05/06-AM603	6	0.0328	0.0173	0.0346
05/06-AM615	7	0.0322	0.0167	0.0333
05/06-AM630	5	0.0309	0.0152	0.0305
05/06-AM634	3	0.0286	0.0125	0.0250

Laboratory and quantitative procedures

The Asp *D/L* was determined using HPCL (Alliance® HPLC systems e2696, Waters) with a Nova-Pak ODS column

(3.9 mm × 300 mm, Waters) using fluorescence detection (344 nm excitation wavelength and 443 nm emission wave length). Elution was carried out with an isocratic adsorption of 3% acetonitrile + 3% tetrahydrofuran/0.1 M acetate buffer pH 6.0 at a flow rate of 0.8 ml/min and column temperature of 23 °C. Then, 70 μl of borate buffer (0.1 M, pH 10.4), 5 μl of *n-tert*-butyloxycarbonyl-L-cysteine and 5 μl of *o*-phthalaldehyde were successively added to 5 μl of the hydrolysate dissolved in 0.1 N-HCl. The measured Asp *D/L*, $(D/L)_{\text{meas}}$, was calibrated against real ratios in the standard solutions, and the calibrated ratio was represented as $(D/L)_{\text{calib}}$. Precision, linearity and accuracy were assessed using the standard solutions of aspartic acid (Asp *D/L* 0.010, 0.020, 0.030, 0.040, 0.050, 0.060, 0.070 and 0.075).

Correction for hydrolysis effect

For validation of the hydrolysis effect, the purified fetus samples, 07/08-AM533F, were hydrolyzed in the gas-phase 6N-HCl for 7, 14, 21, 28 and 35 h at 108 °C, respectively.

Method of age estimation and statistical procedure

The formula of Bada et al. [10] was used:

$$2k_{\text{Asp}}t = \text{Log}_e[(1 + D/L)/(1 - D/L)] - \text{Ln}\{[1 + (D/L)_0]/[1 - (D/L)_0]\},$$
(2)

where *t* (year) is the age of the whale. Standard error (SE) and 95% confidence interval for the AAR-based age estimates were calculated following the procedures of Rosa et al. [14]. First, 2,000 pairs of $(D/L)_0$ and k_{Asp} were generated from a bivariate normal distribution with mean values of the actual estimates and covariance matrix derived from the regression. Second, 2,000 replicates of $(D/L)_{\text{act}}$ for each whale were generated from normal distribution with mean whales' $(D/L)_{\text{act}}$ and their standard deviations (SDs). Then 2,000 replicate values of age calculated from the replicate pairs of $(D/L)_0$ and k_{Asp} provided averages and the SDs of age.

The correlations between $(D/L)_{\text{meas}}$ and the real ratios in the standard solutions, $(D/L)_{\text{meas}}$ and hydrolysis time, and $(D/L)_{\text{act}}$ and fetal age in fetuses were assessed by the Pearson correlation test. Differences of $(D/L)_{\text{meas}}$ analyses among measurement days were assessed by one-way ANOVA. The k_{Asp} and the $(D/L)_0$ were assessed by simple linear regression analysis. The SEs of AAR ages were performed by bootstrap simulation. A *p* value of 0.05 was the significant level of the test. Pearson correlation test, one-way ANOVA and simple linear regression analysis were executed by PASW Statics 17.0 for Windows (SPSS Co. Ltd.). Bootstrap simulation was executed by R environment version 3.2.2 [18].

Results

Linearity, calibration and precision of the Asp *D/L* analysis

To evaluate linearity of the Asp *D/L* analysis, *D/L* mixtures of aspartic acid (0.010, 0.020, 0.030, 0.040, 0.050, 0.060 and 0.070) were measured three times each.

Table 3 shows the relationship between measurements and real ratios of Asp *D/L* of the standard solutions. The $(D/L)_{\text{meas}}$ correlated with real Asp *D/L* (Pearson correlation test; $p < 0.05$, $r^2 = 1.000$). The data yielded the following linear regression equation:

$$(D/L)_{\text{calib}} = 1.142 \times (D/L)_{\text{meas}} + 0.00119 \quad (p < 0.05, \ r^2 = 1.000).$$
(3)

To evaluate the precision of $(D/L)_{\text{calibs}}$, *D/L* mixtures of aspartic acid (0.025, 0.050 and 0.075) were measured ten, ten and nine times, respectively. Their precision of $(D/L)_{\text{calibs}}$ were averaged 0.0143 (SD = 0.00022, CV = 0.0156), 0.0331 (SD = 0.00025, CV = 0.00759) and 0.0526 (SD = 0.00055, CV = 0.0105), respectively. These results showed a high precision of $(D/L)_{\text{calib}}$ analyses in this study.

Correction for hydrolysis effect

The $(D/L)_{\text{calibs}}$ in lens of the fetus hydrolyzed for 7, 14, 21, 28 and 35 h at 108 °C were 0.02853, 0.03958, 0.04865, 0.05937 and 0.06943, respectively. The $(D/L)_{\text{calib}}$ correlated with the hydrolysis times (Pearson correlation test; $p < 0.05$, $r^2 = 1.000$) (Fig. 2), and yielded the following linear regression equation:

$$(D/L)_{\text{calib}} = 0.00145 \times \text{hydrolysis time (h)} + 0.0186, \quad (4)$$

$(p < 0.05, \ r^2 = 0.999).$

The initial $(D/L)_{\text{calib}}$ in the lenses of whales was 0.0186 (SD = 0.00112) calculated in Eq. (4).

Table 3 Relationship between the measurements and the real ratios of the standard solutions of aspartic acid

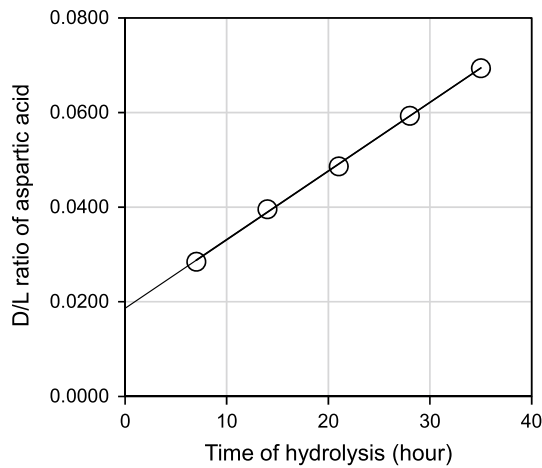| Asp *D/L*	Measurements of Asp *D/L*		
	1	2	3
0.010	0.00797	0.00800	0.00810
0.020	0.0163	0.0160	0.0158
0.030	0.0254	0.0255	0.0255
0.040	0.0339	0.0336	0.0340
0.050	0.0427	0.0430	0.0428
0.060	0.0518	0.0516	0.0513
0.070	0.0602	0.0603	0.0603

Fig. 2 Relationship between time of hydrolysis in sample preparation (h) and D/L ratios of aspartic acid in lens of fetus of Antarctic minke whales

Therefore, to correct for the hydrolysis effect, $(D/L)_{act}$ 0.0186 was subtracted from the $(D/L)_{calib}$.

Aspartic D/L ratio at birth of whales

Table 1 shows the $(D/L)_{act}$ in the lenses of fetuses of whales, and Fig. 3 shows the relationships between the $(D/L)_{act}$ and fetal ages (day). The $(D/L)_{act}$ correlated with fetal ages (Pearson correlation test $p < 0.05$, $r^2 = 0.394$). The data yielded the following linear regression equation:

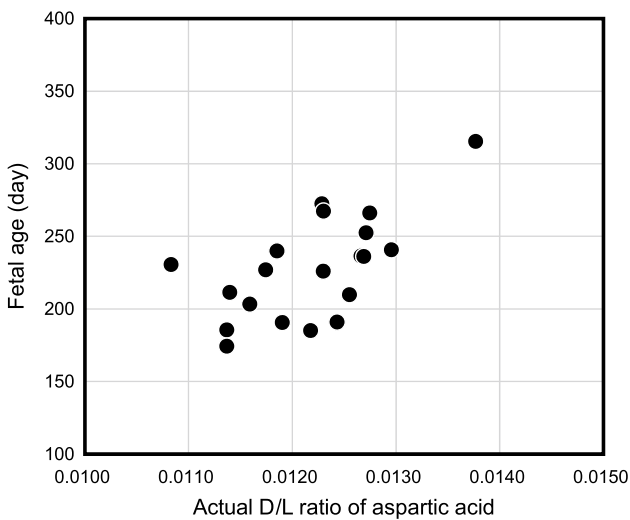$$(D/L)_{act} = 0.0000122 \times \text{fetal age (day)} + 0.00941, \quad (5)$$

$p < 0.05$, $r^2 = 0.400$.

Thus, the $(D/L)_0$, which is the $(D/L)_{act}$ in lens of the Antarctic minke whale at birth, was estimated from this equation and 330 days of the gestational period as assumed for balaenopterid species by [19]. The estimate was 0.0134 (SE = 3.78×10^{-4}; 95% confidential interval 0.0121–0.0147).

Slope of the age estimation equation

Table 2 shows the ratio of $(D/L)_{act}$ in the lenses of Antarctic minke whales corrected by the hydrolysis effect and the age index which is "$\text{Log}_e\{[1 + (D/L)_{act}]/[1 - (D/L)_{act}]\}$". Figure 4 shows the relationships between the age indexes and earplug ages in Antarctic minke whales.

The single outlier at 40-years-old for which Cook's distance exceeded 2 was eliminated in the first regression analysis. Then, the two equations of age estimation are shown below [Eqs. (6) and (7)].

In Eq. (6) linear regression analysis were re-performed to determine k_{Asp} in Eq. (2) substituting $(D/L)_0 = 0.0134$ using dataset of the $(D/L)_{act}$ and the earplug ages in the 17 whales (Fig. 4) as follows:

$$\begin{aligned}\text{Log}_e\{[1 + (D/L)_{act}]/[1 - (D/L)_{act}]\} \\ = 1.79 \times 10^{-3} \times \text{earplug age (year)} + 0.0268,\end{aligned} \quad (6)$$

$\therefore \quad p < 0.001, \quad r^2 = 0.890, \quad k_{Asp} = 8.94 \times 10^{-4},$

$\text{SE}(2k_{Asp}) = 1.52 \times 10^{-4}.$

In Eq. (7) linear regression analysis was performed to determine k_{Asp} and $(D/L)_0$ in Eq. (2) using dataset of the

Fig. 3 Relationship between fetal ages (days) and ratios of aspartic enantiomers (D/L) in fetuses of Antarctic minke whales
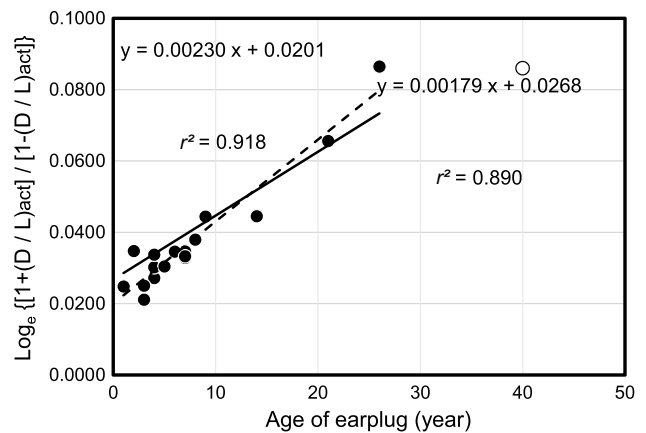

Fig. 4 Relationship between age indexes, $\text{Log}_e[1 + (D/L)_{act}]/[1 - (D/L)_{act}]$, and ages of earplugs of Antarctic minke whales: *open circle* was excluded from simple linear regression analysis as an outlier, and a *solid* and *broken line* is calculated by Eqs. (5) and (6), respectively

$(D/L)_{act}$ and the earplug ages in the 17 whales (Fig. 4) as follows:

$$\text{Log}_e\{[1 + (D/L)_{act}]/[1 - (D/L)_{act}]\}$$
$$= 2.30 \times 10^{-3} \times \text{earplug age (year)} + 0.0201, \quad (7)$$

$$\therefore \quad p < 0.001, \quad r^2 = 0.918, \quad k_{Asp} = 1.15 \times 10^{-3},$$

$$\text{SE}(2k_{Asp}) = 1.71 \times 10^{-4}, \quad \text{SE (intercept)} = 1.72 \times 10^{-3}.$$

Squared correlation coefficient of the Eq. (7) was higher than that of the Eq. (6), and the SE of $2k_{Asp}$ and intercept of the Eq. (7) were lower than those of the Eq. (6). Therefore Eq. (7) is more precise and accurate to estimate ages of whales than Eq. (6).

Standard error of age estimated by AAR

Ages estimated from Eq. (7) and $(D/L)_{act}$ in each whale, including their SEs and 95% confidence intervals estimated by bootstrap simulation, are shown in Table 4. Figure 5 shows the relationships between ages estimated from earplugs, and ages (including SEs and 95% confidence intervals), estimated by the AAR. The range of SEs of the AAR-based age estimates was 0.9 and 2.9 years, and they were within the 95% confidence interval, except for one case.
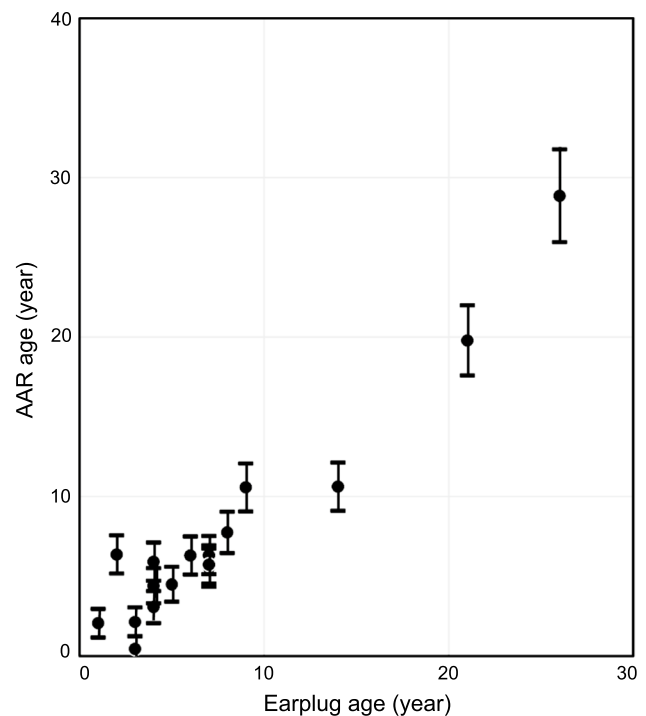

Fig. 5 Relationship between ages estimated by AAR and earplugs (averages and SEs estimated by bootstrap simulation) in Antarctic minke whales

Table 4 Age estimated from earplugs and by the AAR in Antarctic minke whales

Sample no.	Age estimated from earplugs	Age estimated by the AAR[a]		
		Age	SE	95% CI
05/06-AM348	14	10.6	1.5	8.1 to 14.2
05/06-AM349	21	19.8	2.2	16.1 to 25.0
05/06-AM352	9	10.6	1.5	8.2 to 14.2
05/06-AM361	26	28.9	2.9	24.2 to 35.7
05/06-AM382	7	6.3	1.2	4.4 to 9.1
05/06-AM386	8	7.8	1.3	5.6 to 10.8
05/06-AM398	7	5.6	1.2	3.7 to 8.2
05/06-AM488	4	3.1	1.0	1.4 to 5.3
05/06-AM498	4	4.4	1.1	2.6 to 6.8
05/06-AM517	3	0.5	0.8	−0.9 to 2.3
05/06-AM539	1	2.1	0.9	0.5 to 4.1
05/06-AM565	2	6.4	1.2	4.4 to 9.1
05/06-AM592	4	5.9	1.2	4.0 to 8.6
05/06-AM603	6	6.3	1.2	4.3 to 9.1
05/06-AM615	7	5.8	1.2	3.8 to 8.5
05/06-AM630	5	4.5	1.1	2.8 to 7.0
05/06-AM634	3	2.2	0.9	0.7 to 4.2

[a] Ages estimated by the AAR were derived from Eq. (7), and their SEs and 95% CIs were estimated from bootstrap simulation

Discussion

Analytical linearity, precision and calibration of Asp *D/L* analysis

One of the important aspects of Asp *D/L* analysis using the HPLC system is the validation concerning linearity, calibration and precision using the standard solutions of enantiomer mixtures of aspartic acid. In this study such evaluation was conducted, and the $(D/L)_{meas}$ showed excellent linearity in the range of 0.010 and 0.070 of Asp *D/L* in the standard solutions, and their precision (CV) was under 0.0156. The $(D/L)_{meas}$ was corrected to $(D/L)_{act}$ by Eq. (4).

Effect of hydrolysis

Another important aspect of this kind of study is the preparation process, especially hydrolysis, which could affect measurement values [20, 21]. Different conditions of hydrolysis would make inter-laboratory comparisons of k_{Asp} and $(D/L)_0$ difficult. The hydrolysis process for amino acid analysis induces racemization of amino acids, and the degree of racemization is affected by experimental conditions such as processing time, temperature, phase of acid, and additive agent [22]. Therefore, the degree of racemization must be determined by each laboratory in order to allow

inter-laboratory comparison of the results, and this was done in the present study.

A decrease in the degree of racemization through the hydrolysis process contributes to improve the accuracy and precision of AAR age. This study adopted gas-phase hydrolysis, because the degree of racemization in gas-phase hydrolysis was lower than that in the liquid-phase [22] which is the common method for whale age estimation using AAR.

Estimation of $(D/L)_0$ and k_{Asp}

$(D/L)_0$ determined directly from data of fetus or neonate contributes to age estimation by AAR with higher precision. Previous studies inferred the $(D/L)_0$ from a few data of juveniles [9] or data of whales in a wide range of age [12, 14]. The present study attempted to determine $(D/L)_0$ based on a larger number of fetus's lens samples; however, the $(D/L)_0$ were not determined with a high degree of precision because of the hydrolysis effects.

It should be noted that only female samples were used as postnatal animals. This is potentially useful because ovulation rate in common minke whales is well documented, and it is possible to use the post-sexual maturity age as an independent validation of the age estimate [13]. On the other hand, the racemization rate of aspartic acid is only dependent of body temperature of whales, it is whereas unaffected by metabolism [13]. Then the method developed in this study should be also applicable to males.

A single outlier at a 40-years-old animal (earplug age) was eliminated based on Cook's score. Another reason for this was the possibility that the accelerated aspartic acid racemization rate originated from cataracts in lens of old mammals such as human [23] and rat [24]. The amount of changes of Asp D/L in lens of younger animals was higher than older animals, because the Asp D/L exponential decays in lens of mammals, suggesting that k_{Asp} is more precisely estimated for young animals. Therefore, the k_{Asp} and the intercept for age estimation were calculated from 17 whales.

The k_{Asp} in Antarctic minke whale was estimated to be 1.15×10^{-3}/year (SE = 1.71×10^{-4}/year) from Eq. (7). This value was similar to 1.16 in fin whales [12], 1.18×10^{-3}/year in bowhead [9] and 1.10×10^{-3} in common minke whale from the Atlantic [13], whereas it was slightly higher than 0.977×10^{-3} in bowhead whales [14], suggesting the k_{Asp} in the present study was appropriate.

Standard error of the estimation of age from AAR

In the present study the SEs of the age estimates from AAR were in the range of 0.8–2.9 for age estimates in the range 0.5–29.2 years old. These SEs were lower than those of common minke whale from the Atlantic (SE 4.5–8.77 for 0–30 years old, [13]) and bowhead whales (SE 6–7 for −6 to 20 years old, [9]; and SE 2−5.5 for −6.5 to 29 years old [14]). Kitakado et al. [25] reported that the CV estimates for age estimation in relatively young Antarctic minke whales based on reading growth layers from earplugs among the five readers were in the range of 0.0041–0.0155. In animals under the age of three, the AAR ages differed from the earplug ages. The reason for this is that the amount of Asp D yielded just after birth would be extremely lower than that produced by the hydrolysis process. This tendency had been reported in previous studies [9, 14].

This study was successful in developing the AAR technique for Antarctic minke whale with a few issues including possible bias due to cataract, requiring further consideration in the future. The application of this technique can complement the age estimation of this species based on earplug reading, especially for young animals with unreadable earplugs.

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