Preliminary estimation of the age of Antarctic minke whales based on aspartic acid racemization

GENTA YASUNAGA, TAKEHARU BANDO AND YOSHIHIRO FUJISE

The Institute of Cetacean Research, 4-5 Toyomi-cho, Chuo-ku, Tokyo 104-0055, Japan

Contact e-mail:yasunaga@cetacean.jp

ABSTRACT
To examine the approach of age estimation in Antarctic minke whales by ratio of aspartic acid enantiomers, the eye lenses of 18 whales and of 20 fetuses were measured. In the age estimation equation, the specific coefficient of hydrolysis effect in preparation and the constant conversion of aspartic enantiomers (*Kasp*) were estimated. We found that the D/L ratio of aspartic acid in the lenses of minke whales had increased by 0.0102 in the hydrolysis process (7 hr at 108°C), and estimated that the *Kasp* was 1.96×10-3 (/year) calculated from the earplug age and the age index with the D/L ratio of aspartic acid in minke whales. Consequently, formulae Age (year) = 511×Ln ((1+D/L)/(1-D/L)) -13.2 was obtained.

KEYWORDS: ANTARCTIC MINKE WHALE; AGE ESTIMATION; RACEMIZATION; ASPARTIC ACID

INTRODUCTION

Age composition is one of the life history parameters needed to assess the dynamics of whale populations. In the past, criteria such as body length, growth layers of the earplug (Purves, 1955; Zenitani and Kato, 2006), teeth (Scheffer and Myrick, 1980) and tympanic bulla (Christensen, 1995), and color of eye lenses (Nishiwaki, 1950), have been used to estimate physiological age in many species. For baleen whales, growth layers deposited in the earplug are the most useful criterion because they indicate chronological age. Earplug-age determination has become standard procedure in stock assessment and management decisions for baleen whales. Earplug-age determination has advantages in time- and cost-efficiencies and existing abundant historical data; however, there is age estimation bias among proficient readers and readable earplug whale species. Some species and individuals don’t have readable earplugs. Therefore, chemical age estimation is expected to gain support in the field as a reader is unnecessary.

The principles of age estimation with aspartic acid racemization had been elaborated on through comparison of the ratio of aspartic acid enantiomers and counting the number of growth layers in the teeth of tooth whales and pinnipeds study (Bade *et al.*, 1980; Bada, 1984). This method yielded the simple kinetic equation for the racemization reaction and the constant conversion of aspartic enantiomers (*Kasp*) which have to be determined through experiments used with animals which have available age data, and the hydrolysis effect should be further considered.

Nerini (1983) revealed the clear relationship between the earplug ages of fin whales which are easily determined by this method and their D/L ratios of aspartic acid in their eye lenses, whereas he ignored the hydrolysis effect. George *et al.* (1999) obtained the age estimation equation with aspartic enantiomers of bowhead whales from Alaska, whereas the *Kasp* were extrapolated by the previous studies of fin whales and humans. Auðunsson *et al.* (2013) obtained the age estimation equation with aspartic enantiomers of common minke whales from the North Atlantic Ocean, whereas the *Kasp* were extrapolated from the sample of Antarctic minke whales. Thus, certain age data, fetus samples and various biological data are needed to determine the specific values in the age estimation equation with aspartic enantiomers of baleen whales. In Antarctic minke whales taken from JAPRAII surveys, conclusive age data, fetus samples, reproductive status and other biological data are available for these estimations.

The objective of this report is to obtain the age estimation equation with aspartic acid enantiomers in the lenses of Antarctic minke whales. Then, we estimate the *Kasp* and correct for the effect of hydrolysis using the lens sample from Antarctic minke whales and their fetuses from the 2005/2006 and 2007/2008

JAPRAII surveys.

MATERIALS AND METHODS

Materials

Antarctic minke whales were collected from Area IV (70º-130ºE, Figure 1) in the 2005/2006 and 2007/2008 JAPRAII surveys. In the field, left eye lens samples of 18 females and 20 fetuses were stored in polyethylene bags at -80°C until analysis.

Biological information

The weight of the fetuses was measured by the marine scale (M11000, Marel Co. Ltd.). To convert the fetal weight (g) into fetal age (t: day) we used the equation of Kato and Miyashita (1991):

$$t=W^{1/3}/0.24+74 \qquad (1)$$

Table 1 shows the number of fetus samples used in the chemical analysis divided by sex, body weight and fetus age (day). The whale body length was measured from the jaw to the tail notch by researchers onboard the vessels. Ages of the whales were determined by counting the growth layers on the core of the earplugs (Purves, 1955; Zenitani and Kato, 2006).

Table 2 shows the number of whale samples used in the chemical analysis divided by sex, body length and earplug ages (year).

Preparation of samples

The lens samples were stripped externally to avoid contamination and homogenized with Tris-buffer (200mM Tris, 150mM NaCl, pH 8.0) using ultrasonic disruptors. The homogenate was centrifuged at 1,5000 × g for 15 min. at 4°C, and it was then desalted with acetone and air-dried. The sample re-dissolved in water was then set up within a glass tube for hydrolysis and freeze-dried. The purified samples were lyophilized in tubes and were hydrolyzed in the gas-phase 6N-HCl for 7 hr at 108°C (Pico Tag Work Stations, Waters, Tokyo). The hydrolysates were evaporated under reduced pressure.

For validation of the effect on hydrolysis, 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.

Apparatus and quantitative analysis

The hydrolysates were dissolved and incubated in 0.1N-HCl and Borate buffer (0.1M, pH10.4), and were incubated with *o*-phthalaldehyde (OPA) and n-*tert*-butyloxycarbonyl-L-cysteine (Boc-L-Cys) to form diastereoisomers. The D/L ratio of aspartic acid was determined using RP-HPCL (Alliance ® HPLC systems e2696, Waters) with a Nova-Pak ODS column (3.9mm ×300mm, Waters) using fluorescence detection (344 nm excitation wavelength and 433 emission wave length). Elution was carried out with a simple isocratic adsorption of 3% acetonitril+3% tetrahydrofuran /0.1M acetate buffer pH6.0 in 45 min at a flow rate of 0.8 ml/min, at 23°C. Sensitivity of intensity for both enantiomers of aspartic acid was corrected by 100ppm DL-Aspartic acid (minimum 99% TLC, SIGMA-ALDRICH) solution.

The precision and accuracy and linearity of the D/L ratio were assessed with the 3 mixtures of D- and L-aspartic acid standards, which were 1.5ppm: 100ppm, 3.0ppm: 100ppm, 5.0ppm. Good reproducibility and linearity in the D/L ratio range from 1.5 and 5.0 were obtained (Table 3).

Method of age estimation

To estimate ages by ratio of enantiomers of aspartic acid, we used the formula of Bada *et al.* (1980):

$$2Kasp \cdot t = \mathrm{Ln}((1+D/L)/(1-D/L)) - \mathrm{Ln}(1+D/L)/(1-D/L))_{t=0} \qquad (2)$$

where *Kasp* is the rate constant of conversion between L and D enantiomers, t is the age of the whale, D/L is the ratio of the enantiomers in the lens, and “t=0” is the ratio at birth.

Statistics

The correlations between ratio of aspartic acid enantiomers, and hydrolysis time and fetal age were assessed by the Pearson correlation test (Zar, 1999). The Kasp and the constant terms at birth were assessed by linear regression analysis (Zar, 1999). To estimate for the error of the predicted value of the constant term at birth, we used the equation of Devore and Berk (2012):

$$SE(age)=\delta^2\left[\frac{1}{n}+\frac{(x^*-\bar{x})^2}{S_{xx}}\right] \quad (3)$$

where x* is the predicted x value, SE(age) is the predicted value used by the regression equation, n is the number of x data, Sxx is the variance of x×n, and δ is the standard error in the regression equation. A *p* value of less than 0.05 was used as a criterion on statistical. These statistical analyses were executed by PASW Statics 17.0 for Windows (SPSS Co. Ltd.).

RESULTS

Effect for the acid hydrolysis

Examining the racemization effect on hydrolysis intensity, the purified lens samples of fetal Antarctic minke whales, 07/08-AM533F, were hydrolysed several times. The D/L ratios of the samples hydrolyzed for 7, 14, 21, 28 and 35 h at 108°C were 0.0285, 0.0396, 0.0486, 0.0594 and 0.0694, respectively (Fig. 2). The D/Lasp ratio correlated with fetal age (Pearson correlation test; r = 1.000, $p < 0.001$). The data yielded the following linear regression equation:

Hydrolysis time (hr) = 689×D/Lasp-12.8 ($p<0.001$, $r^2=0.999$) (4)

Aspartic D/L ratio in whales

Table 1 shows the ratio of D/Lasp ratios in the lenses of fetuses of Antarctic minke whales, and figure 3 shows the relationships between the D/Lasp ratios and fetal ages (day). The D/Lasp ratio correlated with fetal ages (Pearson correlation test; r = 0.628, P < 0.01). The data yielded the following linear regression equation:

Fetal age (day) = 39400×D/Lasp-768 ($p<0.01$, $r^2=0.394$) (5)

Table 4 shows the ratio of D/Lasp ratios in the lenses of Antarctic minke whales.

DISCUSSION

Effect for the acid hydrolysis

Bada (1984) suggested that the age estimation equation with aspartic acid enantiomers is needed to account for the fact that the initial D/L ratio in the system under investigation is not exactly 0 mainly because some slight racemization takes place during the protein hydrolysis step. We compared this with the measurement of the D/L ratio affected by the hydrolysis step and the estimation of the D/L ratio at birth by the D/L ratio in the lenses of fetuses and their fetal ages. The correlation coefficient of the D/L ratios and the hydrolysis times were obviously higher than that of the D/L ratios and fetal ages; therefore, the age estimation equation in our system is corrected by the hydrolysis bias. The initial D/L ratio in the lenses of whales was 0.0102 calculated in equation (5). After this, 0.0102, the hydrolysis effect, was subtracted from the crude D/Lasp ratios in whales.

Determination of the *Kasp*

Table 4 shows the ratio of D/Lasp ratios in the lenses of fetuses of Antarctic minke whales corrected by the hydrolysis effect and the age index which is "Ln ((1+D/L)/(1-D/L))". Fig. 4 shows the relationships between the age index in Antarctic minke whales and their earplug ages.

The single outlier at 40 years old for which Cook's distance exceeded 2 was eliminated in the first time regression analysis. Then, the age estimation equation is given to:

Age (year) = 511×Ln ((1+D/L)/(1-D/L)) -13.2 ($r^2=0.923$) (6)

∴ 2*Kasp*=1.96×10-3 (/year)

Estimated age used in equation (6) and their standard deviation used in equation (3) in the aspartic acid enantiomers of the lenses of minke whales are shown in Table 4, and the relationships between the estimation age and the earplug age are shown in fig. 4.

The value of 2*Kasp* in this study is lower than that which was found for fin whales (2.21×10^{-3} in Nerini, 1983), Antarctic minke whales (2.94×10^{-3} in Auðunsson *et al.*, 2013) and humans (2.50×10^{-3} in Masters *et al.*, 1997). The values were inconsistent among the previous studies and this study. Bada (1984) supported that the 2*Kasp* is mainly affected by the temperature of the lens during its life-span and the hydrolysis process during preparation, and recommended that the hydrolysis effect be corrected by the Ln

((1+D/L)/(1-D/L)) at birth. We suggest direct correction of the hydrolysis effect in the system, because estimating the hydrolysis effect is more precise while estimating the age index value at birth is difficult.

CONCLUSION

The present study reported the preliminary age estimation of aspartic acid enantiomers in the lenses of Antarctic minke whales. We proposed a new approach to correct for the bias of the hydrolysis step during preparation. In the future, this estimation method needs to be extended to some whales and individuals which don't have readable earplugs.

ACKNOWLEDGMENTS

We would like to thank L.A. Pastene of the Institute of Cetacean Research for helpful suggestions and criticism of the manuscript. N. Fujii of the Research Reactor Institute of Kyoto University has been incredibly tolerant and supportive in the racemization analysis.

REFERENCES

Auðunsson, G.A., Nielsen, N.H., Víkingsson, G.A., Halldórsson, S.D., Gunnlaugsson, þ., Elvarsson, B.þ., Kato, H. and Hansen, S.H. 2013. Age estimation of common minke whales (*Balaenoptera acutorostrata*) in Icelandic waters by aspartic acid racemization (AAR),-AAR and earplug readings of Antarctic minke whales (*B. bonaerensis*) used as a reference. Paper SC/F13/SP15 presented the Icelandic a review panel of experts in a workshop, Reykjavik, February 2013 (unpublished). 17pp.

Bada, J.L., Brown, S. and Masters, P.M. 1980. Age determination of marine mammals based on aspartic acid racemization in the teeth and lens nucleus. *Rep. Int. Whal. Commn. Spec*. (Special Issue 3), 113-8.

Bada, J.L. 1984. In vivo racemization in mammalian proteins. *Methods Enzymol.* 106:98-115.

Christensen, I. 1995. Interpretation of growth layers in periosteal zone of tympanic bulla from minke whales *Balaenoptera acutorostrata*. In A.S. Brix, L. Walløe and Ø. Ulltang (eds.). *Whales, seals fish and man.* Elsevier Science, Amsterdam, pp413-23.

Devore, J.L. and Berk, K.N. 2012. *Modern mathematical statistics with applications* second edition, Springer, New York, NY, 845pp.

George, J.C., Bada, J., Zeh, H., Scott, L., Brown, S.E., O'Hara, T. and Suydam, R. 1999. Age and growth estimates of bowhead whales (*Balaena mysticetus*) via aspartic acid racemization. *Can. J. Zool.* 77:571-80.

Kato, H. and Miyashita, T. 1991. Migration strategy of southern minke whales in relation to reproductive cycle estimated from foetal lengths. *Rep. Int. Whal. Commn.* 41:363-69.

Masters, P.M., Bada, J.L. and Zigler J.S. Jr. 1977. Aspartic acid racemization in the human lens during ageing and in cataract formation. *Nature* 268:71-3.

Nerini, M.K. 1983. Age determination of fin whales (*Balaenoptera physalus*) based upon aspartic acid racemization in the lens nucleus. *Rep. Int. Whaling Comm.* 34:447-8.

Nishiwaki, M. 1950. Determination of the age of Antarctic blue and fin whales by the colour changes in crystalline lens. *Sci. Rep. Whal. Res. Inst.* 4:115-61.

Purves, P.E. 1955. The wax plug in the external auditory meatus of the Mysticeti. *Discoverty Rep.*, 27: 293-302.

Scheffer, V.B. and Myrick, A.C. Jr. 1980. A review of studies to 1970 of growth layers in the teeth of marine mammals. *Rep. Int. Whal. Commn.* (Special Issue 3), 51-64.

Zar, J.H. 1999. *Biostatistical analysis*, 4th ed., Prentice Hall, New Jersey, USA, 663pp.

Zenitani, R. and Kato, H. 2006. Temporal trend of age at sexual maturity of Antarctic minke whales based on transition phase in earplugs obtained under JARPA surveys from 1987/88-2004/05. In: Report of the intersessional workshop to review data and results from special permit research on Minke Whales in the Antarctic. Tokyo. 4-8 December 2006. SC/D06/J15. 9pp.

Table 1. Biological information and ratios of aspartic enantiomers of fetuses of Antarctic minke whales

Sample No.	Sex	Body weight (kg)	Foetal age (day)	Ratio of aspartic enantiomers (D/L)
07/08-AM363F	M	49.4	227	0.0254
07/08-AM380F	M	19.0	185	0.0248
07/08-AM383F	F	64.1	241	0.0260
07/08-AM392F	M	29.9	203	0.0256
07/08-AM393F	M	19.2	186	0.0240
07/08-AM461F	M	59.3	236	0.0254
07/08-AM474F	M	35.8	211	0.0246
07/08-AM475F	F	63.2	240	0.0252
07/08-AM481F	M	21.9	191	0.0255
07/08-AM502F	M	78.5	252	0.0254
07/08-AM504F	M	14.0	174	0.0243
07/08-AM516F	F	22.1	191	0.0254
07/08-AM523F	M	108.0	272	0.0252
07/08-AM527F	M	34.6	210	0.0254
07/08-AM531F	M	100.0	267	0.0255
07/08-AM533F	F	194.5	315	0.0264
07/08-AM542F	F	98.0	266	0.0256
07/08-AM550F	F	58.8	236	0.0248
05/06-AM588F	M	49.0	226	0.0255
05/06-AM820F	F	53.6	231	0.0259

Table 2. Biological information of Antarctic minke whales

Sample No.	Sex	Body length (m)	Earplug age (year)
05/06-AM348	F	9.56	14
05/06-AM349	F	9.28	21
05/06-AM352	F	8.64	9
05/06-AM361	F	8.84	26
05/06-AM372	F	9.02	40
05/06-AM382	F	7.82	7
05/06-AM386	F	7.84	8
05/06-AM398	F	7.96	7
05/06-AM488	F	6.61	4
05/06-AM498	F	7.14	4
05/06-AM517	F	5.82	3
05/06-AM539	F	5.31	1
05/06-AM565	F	5.44	2
05/06-AM592	F	7.43	4
05/06-AM603	F	7.23	6
05/06-AM615	F	7.03	7
05/06-AM630	F	7.32	5
05/06-AM634	F	6.21	3

Table 3. Precision of the D/L ratio of mixtures of D- and L- aspartic acid standards

Reagent	n	Age index*		
		Average	SD	error (%)
Asp-L 100ppm D1.5ppm	10	1.43	0.02	1.3
Asp-L 100ppm D3.0ppm	10	2.99	0.02	0.8
Asp-L 100ppm D5.0ppm	9	4.75	0.05	1.1

Table 4. The ratio of aspartic acid enantiomers, age index and estimation age of Antarctic minke whales

Sample No.	Ratio of aspartic enantiomers (D/L)		Age index**	Estimation age (year)	
	Actual value	Correction value*		Age	SE
05/06-AM348	0.0333	0.0231	0.0463	10.4	2.0
05/06-AM349	0.0416	0.0314	0.0629	18.9	1.9
05/06-AM352	0.0333	0.0231	0.0462	10.4	2.0
05/06-AM361	0.0498	0.0396	0.0793	27.3	1.7
05/06-AM372	0.0496	0.0394	0.0789		
05/06-AM382	0.0295	0.0193	0.0385	6.5	2.1
05/06-AM386	0.0307	0.0205	0.0411	7.8	2.1
05/06-AM398	0.0287	0.0185	0.0371	5.8	2.1
05/06-AM488	0.0265	0.0163	0.0326	3.5	2.1
05/06-AM498	0.0277	0.0175	0.0350	4.7	2.1
05/06-AM517	0.0241	0.0139	0.0279	1.0	2.2
05/06-AM539	0.0256	0.0154	0.0308	2.5	2.2
05/06-AM565	0.0295	0.0193	0.0386	6.5	2.1
05/06-AM592	0.0291	0.0189	0.0378	6.1	2.1
05/06-AM603	0.0294	0.0192	0.0385	6.5	2.1
05/06-AM615	0.0289	0.0187	0.0375	5.9	2.1
05/06-AM630	0.0278	0.0176	0.0352	4.8	2.1
05/06-AM634	0.0257	0.0155	0.0309	2.6	2.2

* (hydrolysis effect correction value): Actual value-0.102
** (Age index by Bada 1980): LN(1+D/L)/(1-(D/L))

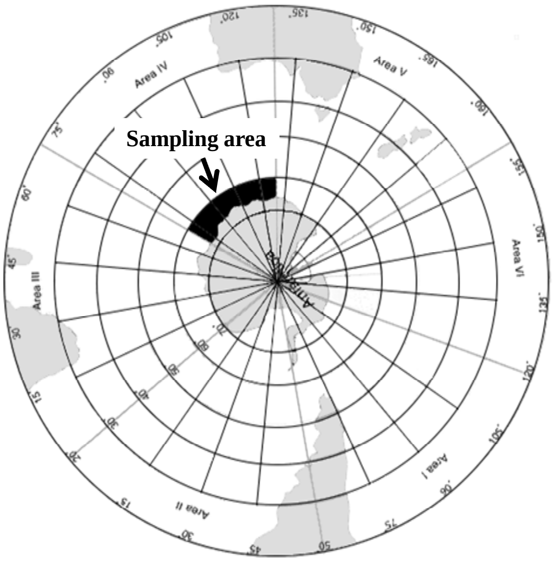

Figure 1. Sampling area of Antarctic minke whales in 2005/2006 and 2007/2008 JAPRAII research cruises

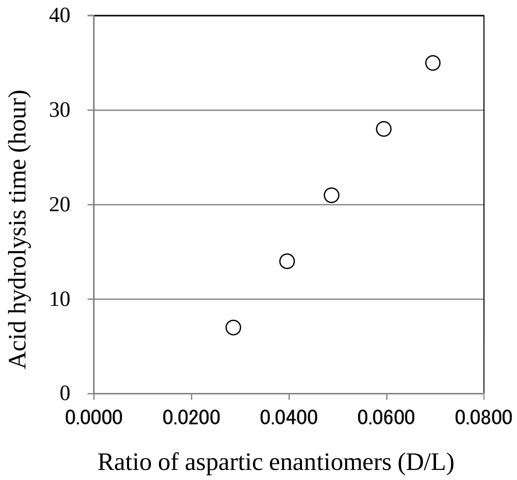

Figure 2. Relationship between acid hydrolysis times and ratio of aspartic enantiomers (D/L) in lenses of fetuses of Antarctic minke whales

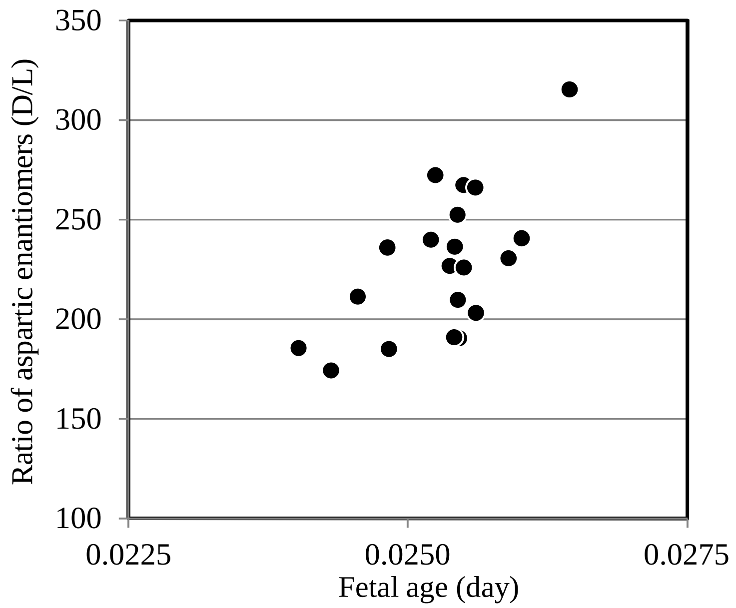

Figure 3. Relationship between fetal age and ratio of aspartic enantiomers (D/L) in fetuses of Antarctic minke whales

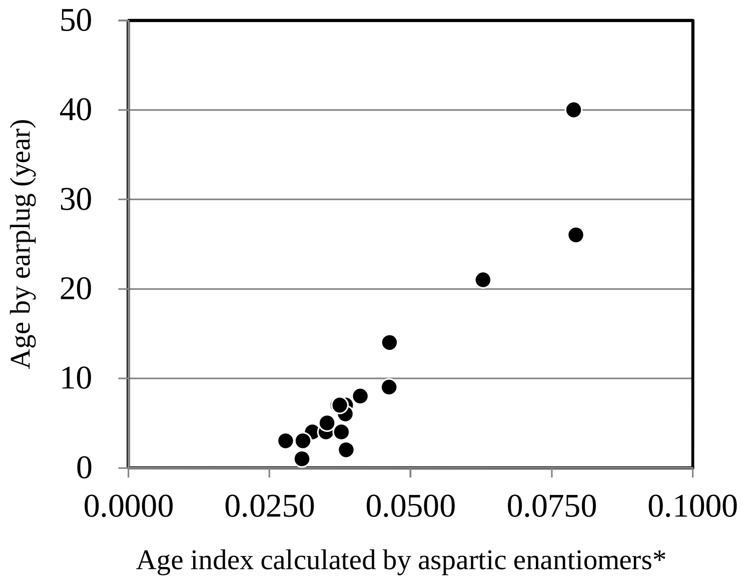

Figure 4. Relationship between age index calculated by aspartic enantiomers in lenses of Antarctic minke whales and age determined by earplug

*: Age index=Ln ((1+D/L)/(1-D/L))

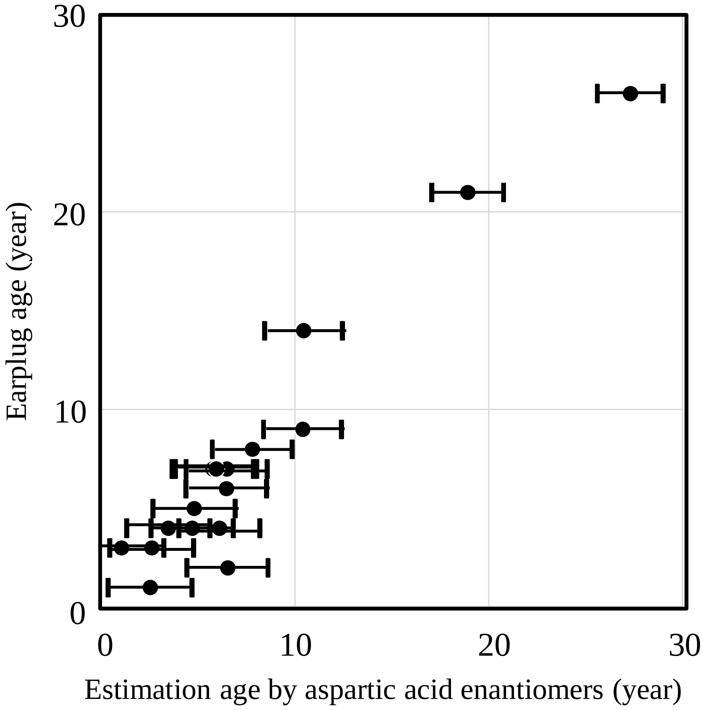

Figure 4. Relationship between earplug age and estimation age by aspartic acid enantiomers in lenses of Antarctic minke whales