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Age estimation of Antarctic minke whales based on aspartic acid racemization: technical development

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ABSTRACT

At present, examination of earplugs is the only practicable means to obtain age data at the annual scale in baleen whales. It is the only method providing age data accurate enough for population-level analyses such as the Statistical Catch-at-Age Analysis (SCAA) of Antarctic minke whales. Because there are a number of whales sampled with unreadable earplugs, the feasibility of using other techniques for age determination is being investigated by scientists at the Institute of Cetacean Research with the aim of determining the age of those samples where earplugs does not work. One of those techniques is based on enantiomers of aspartic acid in eye lens, the aspartic acid racemization (AAR) technique. This paper presents a brief review of the technical development of the AAR technique at the Institute of Cetacean Research, and the preliminary results of using this technique for age determination of Antarctic minke whales.

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 determined using a variety of methods such as examination of baleen plates (Nishiwaki, 1951; Zenitani and Kato, 2010), earplugs (Lockyer, 1984) and tympanic bulla (Christensen, 1995). Counting of the growth layers deposited in the earplugs is the most accepted technique for determining chronological age of baleen whales (Lockyer, 1984). Earplug-based age determination 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 baleen whales (Maeda *et al.*, 2013; George *et al.*, 1999). In such cases alternative methods of age determination are required.

Indices of chronological age of whales have been developed using molecular approaches. For example length of telomeres, nucleoprotein caps flanking DNA (Olsen *et al.*, 2012), and methylation levels at the C5 position of cytosine residues adjacent to guanidine residues (Polanowski *et al.*, 2014) were examined in relation to age using skin of humpback whales (*Megaptera novaeangliae*) in the Gulf of Maine. The feasibility of the DNA methylation technique to determine age in Antarctic minke whales (*Balaenoptera bonaerensis*) has been evaluated by scientists at the Institute of Cetacean Research (ICR) (Goto and Inoue, 2018). Currently, these epigenetic approaches face challenges such as measurement of indices error and whale specific biological processes, which makes it difficult to interpret the age of whales.

Regarding biochemical approaches, the Advanced Glycation End (AGE) products was proposed as one of the possible factors underlying the functioning of the biological clock for mammals (Severin *et al.*, 2013), however no relationship was observed between AGE levels and age in bowhead whales (Rosa, 2006). Another technique is based on enantiomers of aspartic acid in eye lens, the aspartic acid racemization (AAR) technique, which is being developed at the ICR.

This paper presents a brief review of the technical development of the AAR technique at the ICR, and shows preliminary results of using this technique for age determination of Antarctic minke whale.

PRINCIPLE OF THE AGE ESTIMATION BY THE ASPARTIC ACID RACEMIZATION TECHNIQUE

The AAR technique for determining age is based on temporal changes of the ratio of D and L-enantiomers of aspartic acid in mammals (Helfman and Bada, 1975) (Figure 1). 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 (Masters

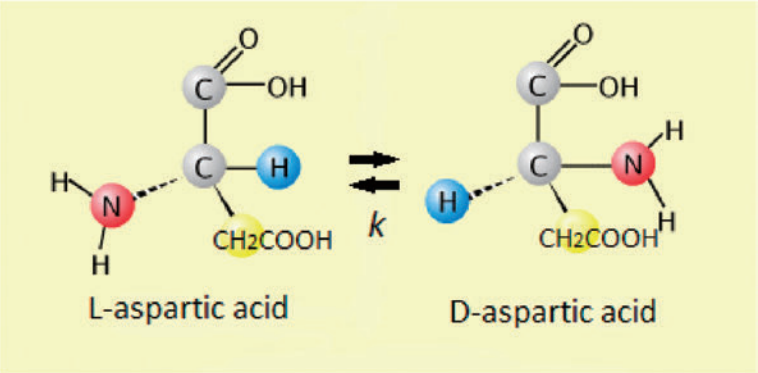

Figure 1. Scheme of racemization of aspartic acid.

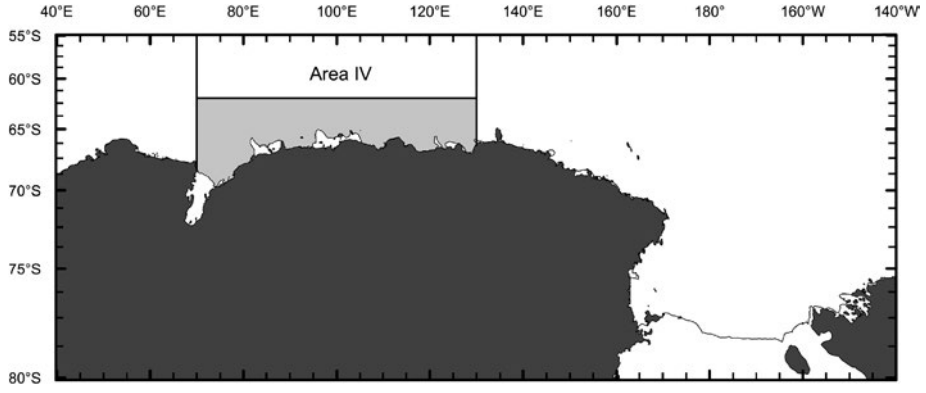

Figure 2. Research area of JARPAII in the 2005/2006 and 2007/2008 austral summer seasons.

et al., 1977).

Masters *et al.* (1977) and Bada *et al.* (1980) proposed that the racemization of amino acids follows a first-order reversible rate law, where the racemization equation is:

$$2k_{\mathrm{Asp}} \cdot t = \mathrm{Log_e}\left[(1+D/L)/(1-D/L)\right] - \mathrm{Ln}\left\{\left[1+(D/L)_0\right]/\left[1-(D/L)_0\right]\right\}. \quad (1)$$

A linear regression model is constructed using this expression, where D/L is the ratio of D- and L-aspartic acids, t is any given time during racemization, and the logarithmic term at t=0 describes the amount of D-aspartic acid formed at birth.

RESEARCH ON ASPARTIC ACID RACEMIZATION AT THE INSTITUTE OF CETACEAN RESEARCH

At the ICR, the feasibility of using the AAR technique to determine the age of Antarctic minke whales has been investigated (see details in Yasunaga *et al.*, 2017). The JARPA and JARPAII research programs in the Antarctic have provided sufficient biological information on this species that makes assumptions and extrapolations of key parameters used in this technique unnecessary. For example k_{Asp} and $(D/L)_0$ in Eq. (1) can be determined by direct comparison with earplug-based estimated ages, and $(D/L)_0$ can be estimated using available foetal samples.

Samples

Antarctic minke whales used in this study were sampled in the Indo region of the Antarctic, which corresponds to the International Whaling Commission's (IWC)'s management Area IV (70°–130°E), south of 62°S (Figure 2). Whales were sampled during the austral summer seasons 2005/2006 and 2007/2008 by JARPAII surveys.

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

Preparation of lens of Antarctic minke whale

Lenses of all samples (foetuses and females) were rinsed first with phosphate-buffered saline (Figure 3). Then their outermost layers were removed with mucus, and the cores were dissected out using a surgical knife. Approximately 10 mg of core samples were homogenized with 1 ml of tris-buffer (200mM Tris, 150mM 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) (Figure 4). The hydrolysates were evaporated

Figure 3. Lens of Antarctic minke whale rinsed with phosphate buffer.

Figure 4. Hydrolysis of lens of Antarctic minke whale using Pico Tag Work Stations (Waters, Tokyo).

under reduced pressure.

Laboratory 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) (Figure 5). Elution was carried out with an isocratic adsorption of 3% acetonitrile +3% tetrahydrofuran/0.1M 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 (0.1M, 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* was calibrated against real ratios in the standard solutions and then it was corrected for hydrolysis effect under our laboratory conditions, which was represented as $(D/L)_{act}$.

Figure 5. HPCL system (Alliance® HPLC systems e2696, Waters) used for fluorescence detection.

Results

Table 1 shows $(D/L)_{act}$ and the age index calculated for the 18 female Antarctic minke whales. The $(D/L)_0$ which is one of two specific coefficients of AAR, was determined using lenses of foetuses at various developing stages because their $(D/L)_{act}$ can be approximated to those at birth. However, precision of the $(D/L)_0$, which is estimated using foetuses was unsatisfactory ($(D/L)_0$=0.0134; SE=3.78×10^{-4}; 95% confidential interval 0.0121–0.0147).

Taken together, the slope of the age estimation equation was examined for two cases. Figure 6 shows the relationships between the age indexes and earplug ages. The single outlier at 40-years-old for which Cook's distance exceeded 2 was eliminated in the first regression analysis. The two equations (cases) of age estimation are shown below as Eq. (2) and Eq. (3). In Eq. (1) linear regression analysis were re-performed to determine k_{Asp} substituting $(D/L)_0$=0.0134 using dataset of the $(D/L)_{act}$ and the earplug ages in the 17 whales as follows:

$$\begin{aligned} &\mathrm{Log_e}\left\{\left[1+(D/L)_{act}\right]/\left[1-(D/L)_{act}\right]\right\} \\ &=1.79\times10^{-3}\times\text{earplug age (year)}+0.0268, \\ &\therefore p<0.001,\ r^2=0.890,\ k_{Asp}=8.94\times10^{-4}, \\ &\mathrm{SE}\ (2k_{Asp})=1.52\times10^{-4}. \end{aligned} \quad (2)$$

In Eq. (3) linear regression analysis was performed to determine k_{Asp} and $(D/L)_0$ in Eq. (1) using dataset of the $(D/L)_{act}$ and the earplug ages in the 17 whales as follows:

Table 1
The actual D/L ratios of aspartic acid in lens and AAR ages of Antarctic minke whales.

Sample No.	Sex	Earplug Age	$(D/L)_{act}$	$Log_e[(1+D/L)/(1-D/L)]$	Age estimated by the AAR*		
					Age	SE	95% CI
05/06-AM348	F	14	0.0223	0.0446	10.6	1.5	8.1–14.2
05/06-AM349	F	21	0.0328	0.0656	19.8	2.2	16.1–25.0
05/06-AM352	F	9	0.0222	0.0444	10.6	1.5	8.2–14.2
05/06-AM361	F	26	0.0432	0.0865	28.9	2.9	24.2–35.7
05/06-AM372	F	40	0.043	0.086			
05/06-AM382	F	7	0.0173	0.0347	6.3	1.2	4.4–9.1
05/06-AM386	F	8	0.019	0.038	7.8	1.3	5.6–10.8
05/06-AM398	F	7	0.0164	0.0329	5.6	1.2	3.7–8.2
05/06-AM488	F	4	0.0136	0.0272	3.1	1	1.4–5.3
05/06-AM498	F	4	0.0151	0.0302	4.4	1.1	2.6–6.8
05/06-AM517	F	3	0.0106	0.0212	0.5	0.8	−0.9–2.3
05/06-AM539	F	1	0.0124	0.0249	2.1	0.9	0.5–4.1
05/06-AM565	F	2	0.0174	0.0348	6.4	1.2	4.4–9.1
05/06-AM592	F	4	0.0169	0.0337	5.9	1.2	4.0–8.6
05/06-AM603	F	6	0.0173	0.0346	6.3	1.2	4.3–9.1
05/06-AM615	F	7	0.0167	0.0333	5.8	1.2	3.8–8.5
05/06-AM630	F	5	0.0152	0.0305	4.5	1.1	2.8–7.0
05/06-AM634	F	3	0.0125	0.025	2.2	0.9	0.7–4.2

* Ages estimated by the AAR were derived from Eq. (3)

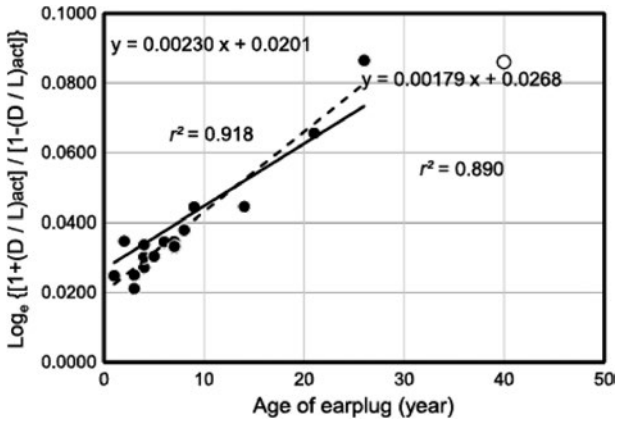

Figure 6. Relationship between age indexes, $Log_e\{[1+(D/L)_{act}]/[1-(D/L)_{act}]\}$, and ages of earplugs of Antarctic minke whales: open circle was excluded from simple linear regression analysis as an outlier, and a solid and broken line is calculated by Eq. (2) and Eq. (3), respectively.

$$\begin{aligned}&Log_e\left\{\left[1+(D/L)_{act}\right]/\left[1-(D/L)_{act}\right]\right\}\\&=2.30\times10^{-3}\times\text{earplug age (year)}+0.0201,\\&\therefore p<0.001,\ r^2=0.918,\ k_{Asp}=1.15\times10^{-3},\\&\text{SE}\,(2k_{Asp})=1.71\times10^{-4},\ \text{SE (intercept)}=1.72\times10^{-3}.\end{aligned}\quad (3)$$

Squared correlation coefficient of the Eq. (3) was higher than that of the Eq. (2), and the SE of $2k_{Asp}$ and intercept of the Eq. (3) were lower than those of the Eq. (2). Therefore, Eq. (3) is considered more precise and accurate to estimate ages of whales than Eq. (2).

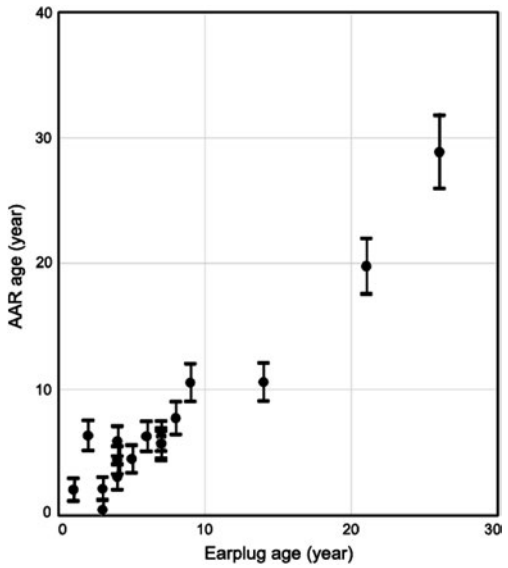

Figure 7. Relationship between ages estimated by AAR and earplugs (averages and SEs estimated by bootstrap simulation) in Antarctic minke whales.

Finally, the standard errors were calculated for the ages estimated by AAR. Ages estimated from Eq. (3) and $(D/L)_{act}$ in each whale, including their SEs and 95% confidence intervals estimated by bootstrap simulation, are shown in Table 1. Figure 7 shows the relationships be-

tween 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.

CONCLUSIONS

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

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