



Two techniques of age estimation in cetaceans: GLGs in teeth and earplugs, and measuring the AAR rate in eye lens nucleus

Nynne H. Nielsen^{1*}, Gísli A. Víkingsson², Steen H. Hansen³, Susanne Ditlevsen⁴, Mads Peter Heide-Jørgensen¹

¹ Greenland Institute of Natural Resources, Box 570, DK-3900 Nuuk, Greenland

² Marine Research Institute, Skúlagata 4, IS-101 Reykjavík, Iceland

³ Department of Pharmacy, Faculty of Pharmaceutical Science, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen, Denmark

⁴ Laboratory of Applied Statistics, Department of Mathematical Sciences, University of Copenhagen, Universitetsparken 5, DK-2100, Copenhagen, Denmark

* Corresponding author: nel@ghsdk.dk

ABSTRACT

The ages of three species of cetaceans were estimated by counting the growth layer groups (GLG) and measuring the aspartic acid racemization rate (k_{Asp}) by what is referred to as the Aspartic Acid Racemization (AAR) technique. Data on k_{Asp} and the D/L ratio of aspartic acid at birth $[(D/L)_0]$ in North Atlantic common minke whales (*Balaenoptera acutorostrata*) are presented along with data on fin whales (*B. physalus*) and harbour porpoises (*Phocoena phocoena*) already published by Nielsen *et al.* (2012). The k_{Asp} specific for minke whales was $1.40 \times 10^{-3} \text{ yr}^{-1}$ (SE ± 0.00005) and the $(D/L)_0$ was 0.0194 (SE ± 0.0012). The correlation of GLG age and D/L ratio for all three species was highly significant; however, the correlation coefficient varied greatly (fin whales: $R^2 = 0.59$, $p < 0.0001$; minke whales: $R^2 = 0.96$, $p < 0.0001$; harbour porpoises: $R^2 = 0.36$, $p < 0.0001$). Asymptotic body length for all three species was estimated by a von Bertalanffy growth model on both the GLG and AAR techniques, and showed no difference.

INTRODUCTION

When it comes to management of wildlife populations, estimation of age is crucial in order to gain knowledge of their life history and population structure, and when managing harvested populations, knowledge of age composition is especially important for assessing sustainable quotas. In marine mammals, age estimation can be a delicate matter. Contrary to many terrestrial mammals, no obvious visual sign of age is present and up until today, a variety of different techniques have been used to estimate age in marine mammals, with variable success (Lockyer 1974, Watts and Gaskin 1989, Dietz *et al.* 1991, George *et al.* 1999, Olsen and Sunde 2002, Würsig and Jefferson 2004, Garde *et al.* 2010, Nielsen *et al.* 2012). Von Bertalanffy (1938, 1957) showed how length can indicate age up to the attainment of sexual maturity, after which this relationship ends. Chronological age estimation in cetaceans has traditionally been done by counting the growth layer groups (GLGs) deposited within a persistent tissue, primarily in earplugs, teeth, *bullae tympanica*

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(BT) or baleen (Lockyer 1972, Perrin and Myrick 1980, Christensen 1992). These GLGs are deposited over the years due to the seasonal (winter/summer) variation in food uptake and most studies support that one GLG represents one year (Hohn *et al.* 1989, Heide-Jørgensen *et al.* 1994, Hohn and Lockyer 1999). So far, the GLG technique has been used on different species, *e.g.* humpback whales (*Megaptera novaeangliae*; Chittleborough 1959, Gabriele *et al.* 2010), the southern fin whale (*Balaenoptera physalus*; Lockyer 1972), Antarctic minke whales (*B. bonaerensis*; Christensen 1992), North Atlantic minke whale (*B. acutorostrata*; Sigurjónsson 1980), narwhals (*Monodon monoceros*; Hay 1980), belugas (*Delphinapterus leucas*; Goren *et al.* 1987, Pleskach *et al.* 2016) and harbour porpoises (*Phocoena phocoena*; Ólafsdóttir *et al.* 2003); see also the review by Read *et al.* (this volume, in press). Counting the GLGs in hard tissues of cetaceans is a well-known technique, however due to natural wear and compression in the growth layers with time, it is inevitable that a temporal bias will appear escalating with age, making older individuals more difficult to age. More importantly, not all cetaceans form readable GLGs, *e.g.* in teeth or earplugs, or the patterns are too difficult to interpret (Lockyer 1974, Sigurjónsson 1980, George *et al.* 1999, Garde *et al.* 2007, 2012) leading to a cul-de-sac regarding life history of these species.

An alternative technique for age estimation in cetaceans is measuring the racemization rate of aspartic acid in the eye lens nucleus (Nerini 1983, Olsen and Sunde 2002, Garde *et al.* 2007, Nielsen *et al.* 2012, Rosa *et al.* 2012, Pleskach *et al.* 2016). The racemization of amino acids is based on the presence of biological proteins that are of two enantiomeric forms: the L-form which undergoes racemization to the D-form when the cells becomes metabolically inactive. The changing ratio of these two forms in tissues over time occurs at a constant rate until equilibrium is reached. After calibration, it is thus theoretically possible to estimate the biological age of an organism, once the rate of this racemization (k) and the D/L ratio of the amino acids at birth $[(D/L)_0]$ and at death, are known (Bada *et al.* 1980). However, in tissue with slow metabolism such as the eye lens the speed at which the L-form converts to the D-form increases with age (Masters *et al.* 1987). The rate of the amino acid racemization varies with the type of amino acid, but more important, as it is a chemical reaction it is also positively correlated with temperature. This results in an increasing k with increasing temperature.

Using k to estimate age was originally developed for dating old marine sediments (Bada 1970) and later it was applied to humans in the eye lens nucleus and, in forensic science, tooth enamel and dentine (Masters *et al.* 1977, 1978; for a review, see Meissner and Ritz-Timme 2010). Aspartic acid has the fastest racemization rate of the amino acids (Bada and Protosh 1973) making it suitable for estimating the age of living organisms. Measuring the aspartic acid racemization rate (k_{Asp}) was first applied to marine mammals by Jeffrey Bada and colleagues in the late 1970s (Bada *et al.* 1980), and to date, AAR has been a tool for estimating the age in three species of mysticetes — fin whales (Nerini 1983, Nielsen *et al.* 2012), bowhead whales (*Balaena mysticetus*; George *et al.* 1999, Rosa *et al.* 2004, 2012, Heide-Jørgensen *et al.* 2012), and North Atlantic minke whales (Olsen and Sunde 2002, Auðunsson *et al.* 2012) — and three species of odontocetes — narwhals (Garde *et al.* 2007), harbour porpoises (Nielsen *et al.* 2012) and belugas (Wetzel and

Reynolds 2015, Pleskach *et al.* 2016). It has been speculated that the k_{Asp} might be influenced by the longevity of the individual species of cetaceans (Nielsen *et al.* 2012), however, other factors are probably also effecting the k_{Asp} .

The aim of this paper is to discuss the usefulness of Aspartic Acid Racemization (AAR) as a tool for age estimation of fin whales, harbour porpoises and North Atlantic minke whales. Data on fin whales and harbour porpoises come from already published data by Nielsen *et al.* (2012) while new data for minke whales are presented. Readings of GLGs in the earplugs of Antarctic minke whales are used to estimate a general racemization rate for minke whales, thus, it is assumed they share general physiology. Age data from GLGs and the D/L ratio of aspartic acid for all three species are correlated in order to give an idea of the potential agreement between the two techniques.

MATERIAL AND METHODS

Sampling of fin whales and harbour porpoises are described in Nielsen *et al.* (2012), hence here is only a short review:

Fin whales

Eyeballs from 121 adult fin whales and 15 foetuses were collected during commercial catches in Iceland in 2009. Eye lenses were dissected out of the eyes and immediately stored at -18°C . Both left and right earplugs were collected from 98 of the whales and stored in 10% buffered formalin until further preparation. Earplugs were treated according to Lockyer (1974) prior to reading the GLGs. Two readers compared their counts and agreed on an age estimate for each individual whale. For computing a k , the D/L measurements were plotted against estimated age values based on GLG counts in 15 whales (range: 3 – 48 yr), and 15 foetuses all estimated to age 0.

Besides the 53 GLG aged fin whales in Nielsen *et al.* (2012), an additional 45 whales (of the 121 whales already age estimated by AAR) were aged by counting the GLGs in their earplugs (Iceland Marine Research Institute). This gave the opportunity to compare GLG ages and D/L ratios from a larger dataset than otherwise possible.

Harbour porpoises

The first author and employees of the Greenland Institute of Natural Resources collected the right eyeball and two teeth from the upper jaw from a total of 81 harbour porpoises. Two calves did not yet have erupted teeth so only their eyes were collected. All eyes and teeth were immediately stored at -18°C upon collection. The porpoises had been shot by Greenlandic hunters from the town of Maniitsoq three months earlier, and they had been stored at -18°C until the time of sampling. Teeth were prepared according to Kvam (1995) and a more detailed description can be found in Nielsen *et al.* (2012). The GLG readings were counted independently by two experienced readers on at least two occasions under a microscope. The count most frequently favoured was adopted but when there was disagreement the mean was taken if disagreement was within ± 1 yr. If it was larger than ± 1 yr, the count

by the more experienced reader was chosen. For computing a k , the D/L measurements were plotted against estimated age values based on GLG counts in 13 whales (range: 0.3 – 10 yr) and two calves estimated to age 0 based on length and the lack of teeth. The remaining 68 GLG aged harbour porpoises were then compared to the related measurements of D/L ratios of aspartic acid.

Common minke whales

A total of 303 eye lenses from North Atlantic common minke whales (body length between 4.9 – 9.7m) was collected by scientific personnel from the Icelandic Marine Research Institute during both commercial (in the years 2006, 2008 - 2011) and scientific whaling (from 2003 - 2007) in Iceland. As soon as the whales were onboard (within one hour *post-mortem*) the eyes were dissected out of the skull and stored at -20°C and later eye nucleus was dissected from the eye in the laboratory. Eye lenses and earplugs from 17 Antarctic minke whales (*B. bonaerensis*; body length between 4.9 – 10.2m) from a scientific catch under the Japanese JARPA survey in 2001/2002 were included to calculate the racemization rate of aspartic acid (k_{Asp}), and 13 fetuses from Iceland were used to calculate the ratio of D- and L amino acid at age 0 [(D/L)₀]. A total of 263 eye lenses were age estimated by AAR. Due to the difficulties in reading the GLGs in North Atlantic common minke whales, Antarctic minke whales were used for determining a general k_{Asp} for minke whales. It is hereby assumed that these two species are similar in their physiology and thereby share the same k_{Asp} . The Antarctic minke whales were read three times each by one reader and the individual GLG age was estimated on the background of the mean age of these three readings of each animal. The minke whales in this study were also a part of a large study on age estimation by Auðunsson *et al.* (2012).

Preparation of eye lenses for hydrolysis and High-Performance Liquid Chromatography (HPLC)

Prior to analysis, all eye lenses were kept frozen (-18°C) and when analysing, each sample was kept on a cooler brick to prevent fast thawing. In order to get to the nucleus of the eye lens, the lens was rolled on paper removing the outer cell layers. The remaining layers were subsequently removed under a stereo microscope for removal of layers not visible to the naked eye, and to ensure only the nucleus was left behind. Great care was taken not to pollute the nucleus with younger layers or blood and all tools involved were washed in Extran (2%) and wiped off with ethanol (96%) before preparation of a new lens. Half of the lens nucleus was stored as backup and the other half hydrolysed for later analyses in the high-performance liquid chromatography (HPLC) system (see Nielsen *et al.* 2012 for technical description). To each sample was added 1 mL 6 M HCL and hydrolysed for 6 h at 100°C (Garde *et al.* 2007) to release the amino acids from the proteins. After hydrolysis the samples were cooled at room temperature for 30 min. and kept frozen until HPLC analysis. The HPLC system separates the D- and L- aspartic acid enantiomers and a computer connected to the HPLC integrates the peak areas, providing the ratio between D and L.

Racemization rate (k_{Asp}), AAR age estimation and D/L ratio at age 0 [(D/L)₀]

The calculations were made as in Nielsen *et al.* (2012). A regression of $X = \ln[(1+D/L)/(1-D/L)]$ on A (Age) was used to estimate the k_{Asp} . It was based on

animals where reliable age estimates were assumed (30 fin whales, 17 harbour porpoises and 30 minke whales). The ages were estimated by GLG counts (15 fin whales, 15 harbour porpoises and 17 Antarctic minke whales) or given age 0 yr (15 fin whales and 13 minke whale foetuses), or estimated to age 0.1 yr based on their length (two harbour porpoise calves). The equation for the line is as follows (modified from Masters *et al.* 1977):

$$1) \quad \ln\left(\frac{1+(D/L)}{1-(D/L)}\right) = 2k_{\text{Asp}} A + B$$

where $2k_{\text{Asp}}$ is estimated as the slope of this regression line, A is the age of the animal and B the intercept, equalling twice the $(D/L)_0$ value. The $(D/L)_0$ term represents the part of the D-form present at birth plus the racemization that happens when samples are being hydrolysed (Bada and Schroeder 1975).

Age estimation of the 106 fin whales, 68 harbour porpoises and 253 minke whales was done as in Ohtani and Yamamoto (2010). The equation is derived from the same data as above by weighted linear regression of A (Age) on X :

$$\begin{aligned} 2) \quad A &= 353.85X - 19.06 \text{ (fin whales)} \\ A &= 128.56X - 4.90 \text{ (harbour porpoises)} \\ A &= 311.59X - 11.16 \text{ (minke whales)} \end{aligned}$$

where A and X are as above.

The approach of regressing X on age as done in 1) is the obvious choice if the main interest is to estimate the k_{Asp} (Bada *et al.* 1983, Nerini 1983), but it is not suitable to predict age. If the age were to be predicted directly from the estimated k_{Asp} , this corresponds to a prediction of age from an inverse regression, which is biased.

The reverse approach of regressing age on X as done in 2) reduces the analysis to a simple linear regression allowing the use of standard tools for prediction and variance estimation. Moreover, a linear regression assumes that covariates are measured without noise, and the ages used to derive the prediction equation are measured with a large uncertainty, whereas the measurement errors on D/L ratios are small. When estimating the AAR age, whales of known-age are needed as a fix point to the linear regression. There are no North Atlantic common minke whales with known age, hence GLG aged Antarctic minke whales ($n = 33$) were used to provide an approximate age which again was used to estimate an AAR based age. Due to this circularity in age estimation, correlation of age within the two techniques is not possible, thus data on raw D/L content of aspartic acid in the North Atlantic common minke whales were compared with all the GLG ages in order to compare the two techniques.

Growth and age

For all three species, body lengths were plotted against AAR and GLG age, respectively. The whales were divided into males and females and the growth curve was fitted to the data using a von Bertalanffy model (von Bertalanffy 1938; George *et al.* 1999):

$$3) \quad \text{Length} = L_{\max}(1-b^{-(k(t-t_0))})$$

where L_{\max} is the asymptotic body length at which growth is zero, t is age and b and k are model constants.

RESULTS AND DISCUSSION

Racemization rate (k_{Asp}), D/L ratio at birth [(D/L)₀] and comparison of AAR and GLG

The k_{Asp} for the minke whales were estimated by regression values of $\ln((1+D/L)/(1-D/L))$ against ages of 30 minke whales (13 North Atlantic common minke whale fetuses and 17 <2 yr GLG aged Antarctic minke whales, Fig. 1). This corresponds to a k_{Asp} of $1.40 \times 10^{-3} \text{ yr}^{-1}$ (SE ± 0.00005) specific to minke whales and a (D/L)₀ value of 0.0194 (SE ± 0.0012). k_{Asp} and (D/L)₀ of all three species are shown in Table 1. The k_{Asp} for fin- and minke whales are in agreement with other values obtained for *e.g.* bowhead whales ($0.977 \times 10^{-3} \text{ yr}^{-1}$; Rosa *et al.* 2012), narwhals ($1.145 \times 10^{-3} \text{ yr}^{-1}$; Garde *et al.* 2012), and in particular, fin whales ($1.160 \times 10^{-3} \text{ yr}^{-1}$; Nerini 1983) which are in close agreement with our data for fin whales. Auðunsson *et al.* (2012) found a k_{Asp} of $1.147 \times 10^{-3} \text{ yr}^{-1}$ for minke whales which is in agreement with this study although it lies closer to the k_{Asp} for narwhals found by Garde *et al.* (2012). Rosa *et al.* (2012) estimated a k_{Asp} of minke whales based on data from Olsen and Sunde (2002), who use the k_{Asp} of fin whales (Nerini 1983) to calculate the age of minke whales. They estimate a minke whale k_{Asp} of $1.051 \times 10^{-3} \text{ yr}^{-1}$ which is a somewhat lower rate than obtained in both this and the Auðunsson *et al.* (2012) study, thus indicating the need for a specific species rate rather than reusing the rate and D/L₀ obtained for other species.

The k_{Asp} values for harbour porpoises lies somewhat higher than that for other cetaceans and are among the highest k_{Asp} measured in mammals. Pleskach *et al.* (2016) found a k_{Asp} of $3.48 \times 10^{-3} \text{ yr}^{-1}$ in belugas, and their findings contradict the speculations that species longevity has a positive influence on k_{asp} . In fact, Pleskach *et al.* (2016) advocate for being very cautious when comparing k_{asp} values across species, due to the impact of different laboratories protocols potentially influencing the k_{asp} .

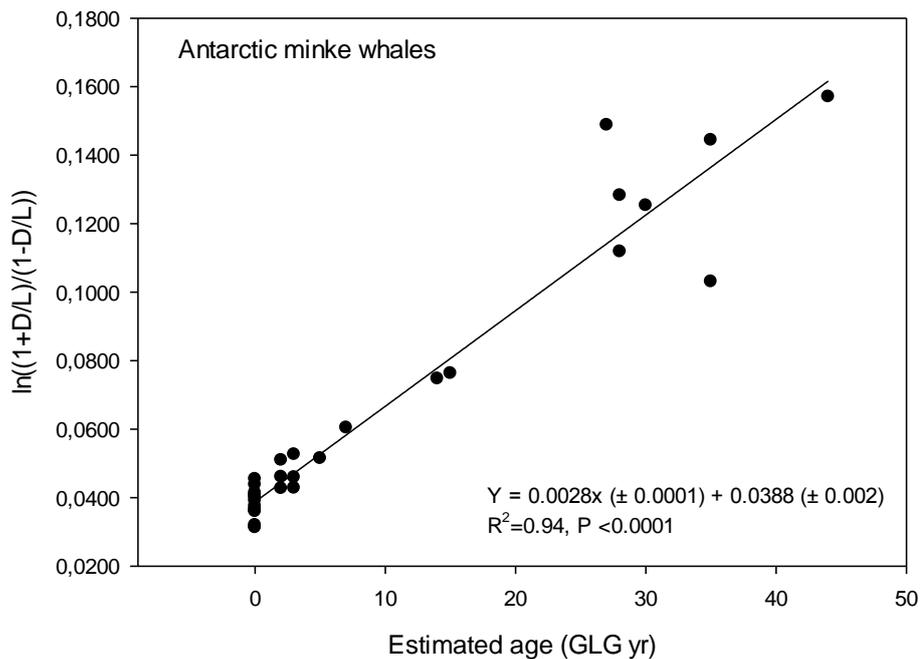


Fig. 1. Regression line of D/L ratios against GLG readings from 17 Antarctic minke whales and 13 features (0 yr.). The slope of the regression line corresponds to twice the racemization rate ($2k_{Asp}$) and the intercept corresponds to twice the (D/L_0) .

As mentioned above, k_{Asp} is highly influenced by temperature thus it is obvious to conclude that the species specific variation of k_{Asp} is due to the individual body temperature, also pointed out by Rosa *et al.* (2012). The body temperature of the three species in this study is presented in Table 1. There is no indication of a correlation between k_{Asp} and body temperature within the three species in this study. However, the low sample size is probably not large enough to establish this. Furthermore, this will, in particular, not explain the very high k_{Asp} in harbour porpoises. Pleskach *et al.* (2016) suggests several sources of variation influencing k_{Asp} values, and therefore that direct interspecies comparison should not be made.

A simple linear regression of the two techniques is shown in Fig. 2a-c. A Pearson product-moment correlation coefficient was used to assess the relationship of the estimated GLG age and the D/L ratio of aspartic acid content. There was a highly significant correlation for all three species, however, the correlation coefficient varied greatly (fin whales: $R^2 = 0.59, P <0.0001$; minke whales: $R^2=0.96, P <0.0001$; harbour porpoises: $R^2=0.36, P <0.0001$). The small age span is partly to blame for the poor ability to explain the large variability in the porpoises, and the opposite is seen for the minke whales, where a small sample size gives rise to a very low variability.

Asymptotic body length (L_{max}) of females and males from all three species was estimated from both AAR and GLG age (eq. 3). There was no difference in the outcome of L_{max} between AAR and GLG for female (AAR: 19.89m, GLG: 19.88) and male (AAR: 18.57m, GLG: 18.52m) fin whales, female (AAR: 1.48m, GLG: 1.47m) and male (AAR: 1.34m, GLG: 1.32m) harbour porpoises (Fig. 3). No data

on sex were available for the GLG aged Antarctic minke whales thus only L_{\max} for female (AAR: 8.48m) and male (AAR: 7.86m) AAR aged minke whales is presented here (Fig. 4). All three species showed sexual dimorphism with females displaying a larger L_{\max} than males, a finding that is also demonstrated in other studies (George *et al.* 1999, Ólafsdóttir *et al.* 2004; see also a general review on the subject in Ralls and Mesnick 2008).

Table 1. k_{Asp} , D/L_0 and body temperature for the three species of cetaceans presented in this paper.

Species	k_{Asp}	$(D/L)_0$	Body temperature (°C)
<i>Fin whale</i>	$1.15 \times 10^{-3} \text{ yr}^{-1}$ (SE \pm 0.00005)	0.028 (SE \pm 0.0012)	36.1 (Brodie and Paasche 1985)
<i>Minke whale</i>	$1.40 \times 10^{-3} \text{ yr}^{-1}$ (SE \pm 0.00015)	0.019 (SE \pm 0.00045)	34.7 (Folkow and Blix 1992)
<i>Harbour porpoise</i>	$3.10 \times 10^{-3} \text{ yr}^{-1}$ (SE \pm 0.0004)	0.023 (SE \pm 0.0018)	36.1 (Desportes <i>et al.</i> 2003)

A general look at pros and cons of the AAR and GLG age estimation techniques

Fig. 1 shows the variation of the D/L content (between 0.03126 – 0.04541) in minke whale fetuses with age \approx 0 yr. A variation in the D/L content among fetuses and young animals is also seen for other species (Garde *et al.* 2012; Nielsen *et al.* 2012; Rosa *et al.* 2012), and this variation is most likely due to an individual biological variation in the amount of D and L aspartic acid. Hence it is suggested that a more cautious approach is taken when aging young individuals with the AAR technique, and a combination of AAR and GLG might be the solution where the GLG age is estimated for the young individuals and the AAR is used for older animals. Age estimation from AAR is a costly technique that needs to be carried out in a laboratory with expertise in HPLC. The GLG technique is less expensive, however the teeth and earplugs need correct preparation and experienced readers to interpret the GLGs. AAR is time-consuming with few animals in regard to preparation and analysing the samples, however, with a high number of animals the AAR technique is by far favoured when the HPLC machine is already prepared for it.

The age estimates from AAR can be influenced by a range of conditions. First and most important is the laboratory protocol. If the lens nucleus gets contaminated with younger cells (*e.g.* from blood or outer lens layer cells) the D/L_0 ratio will not be as significant from the D/L ratio at death and thus point to an

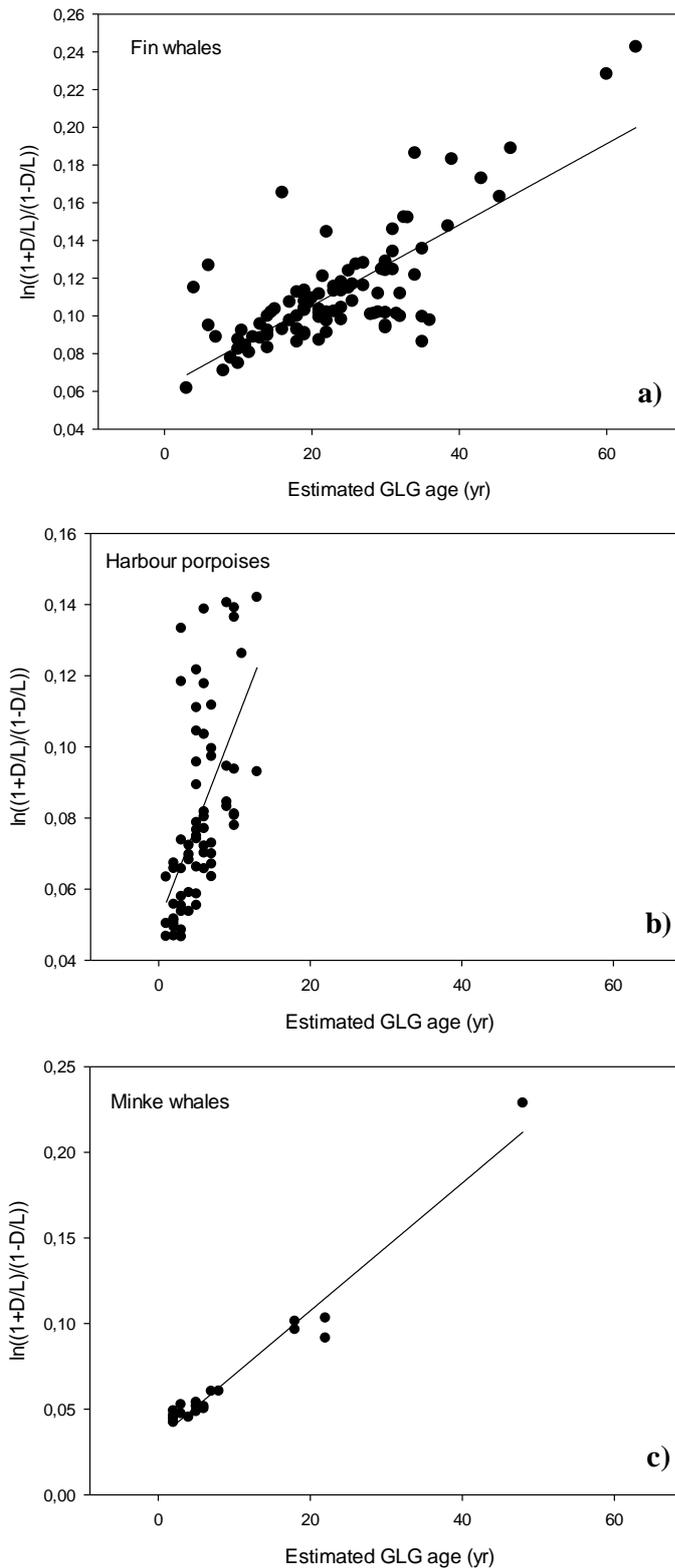


Fig. 2a-c. Linear regression between GLG ages and D/L content in fin whales [$y=0.0021 (\pm 0.0002)x + 0.062 (\pm 0.004)$, $n=98$, $R^2 = 0.59$, $P < 0.0001$], harbour porpoises [$y=0.0006 (\pm 0.0009)x + 0.051 (\pm 0.006)$, $n=66$, $R^2 = 0.36$, $P < 0.0001$] and minke whales [$y=0.004 (\pm 0.0002)x + 0.033 (\pm 0.003)$, $n=19$, $R^2 = 0.96$, $P < 0.0001$].

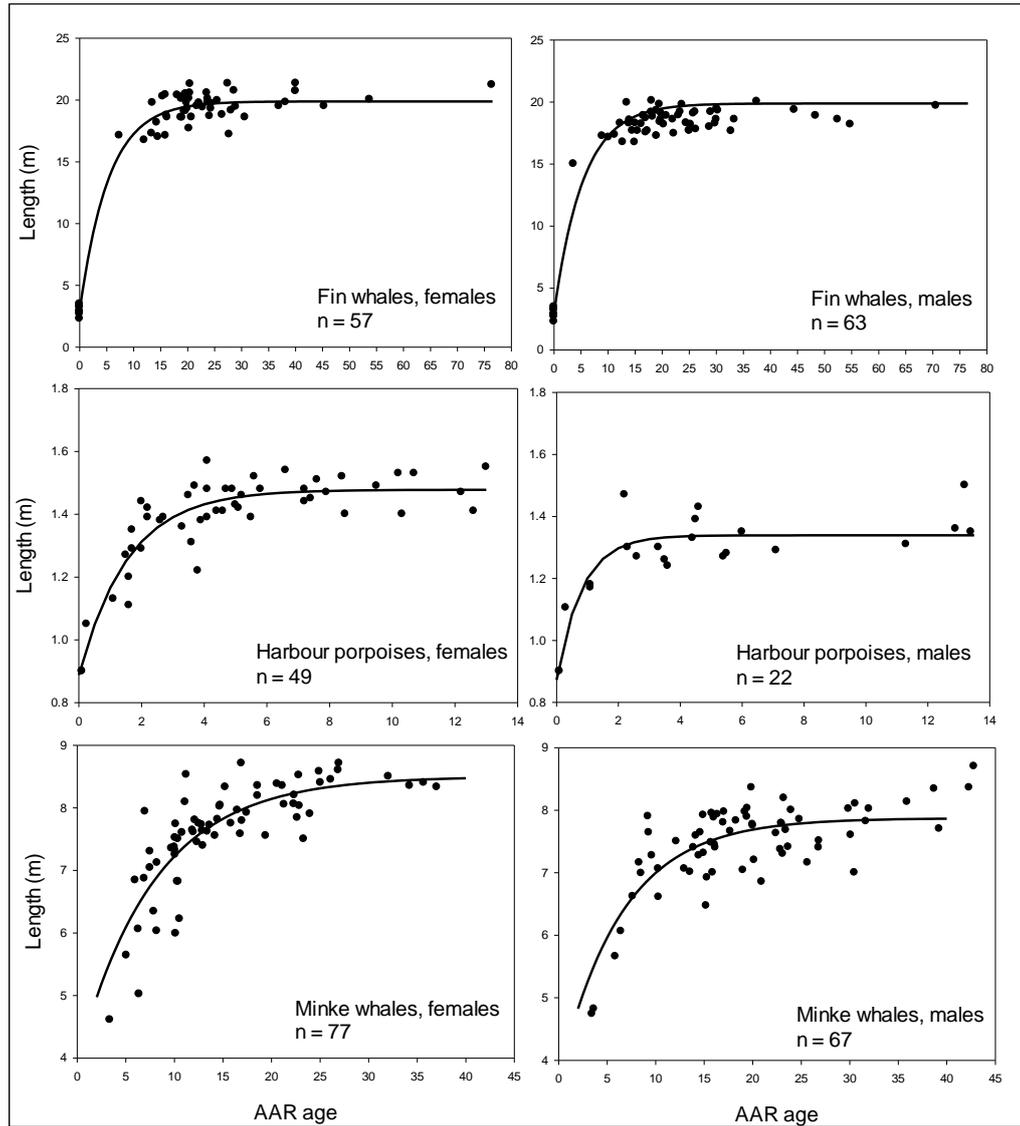


Fig. 3. Growth in female and male fin- and minke whales and harbour porpoise for AAR age. The line is mean asymptotic growth in length (m).

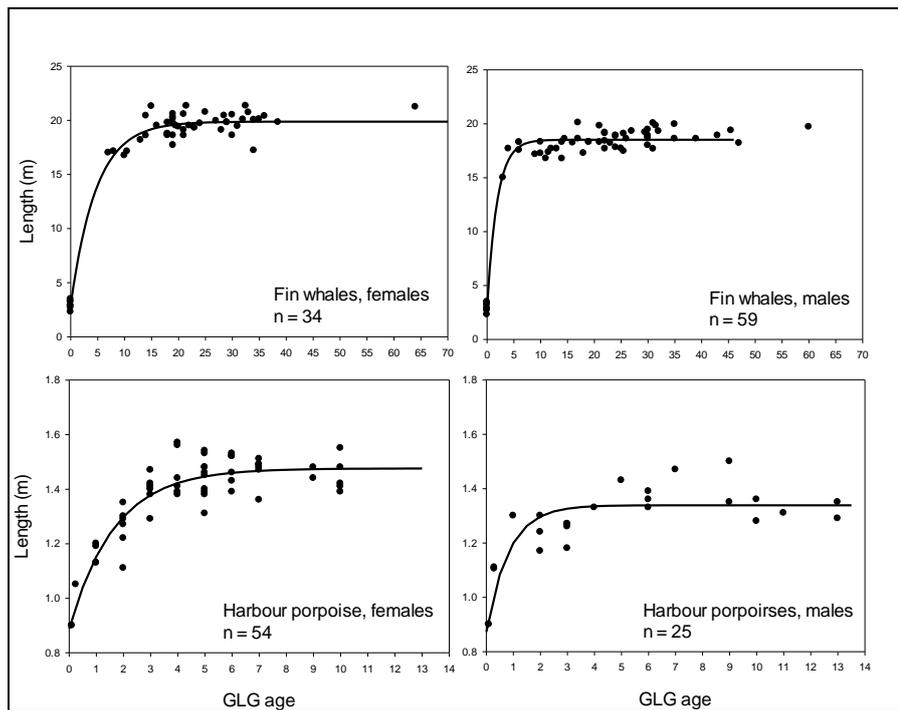


Fig. 4. Growth in female and male fin whales and harbour porpoises for GLG age. No data on sex for the GLG aged Antarctic minke whales were available thus no data on the asymptotic growth within this group are presented. The line is mean asymptotic growth in length (m).

underestimation of age. Another source of bias using AAR is the presence of a cataract in the eye nucleus, but only few studies on cetaceans have addressed this matter. Cataract is a condition in the mammalian eye lens that appears to accompany the latter stages of lifespan in all mammals studied (*e.g.* Williams *et al.* 2004; Fernandes *et al.* 2003 and Wolf *et al.* 2000). Studies on humans have shown that cataract of the type brunnescent group IV is especially of major concern (Masters *et al.* 1977). It decreases protein activity inducing a fourfold amount of the D-aspartate and thus over-estimating the age. Few cetaceans have been studied with respect to cataract, but so far it has not been located in bowhead whales (n=50) (Qian 1993), fin whale (n=1) and narwhals (n=3) (Nielsen *et al.* 2012). Obviously, a larger sample size is needed in order to clarify the extent of cataract in cetaceans, but if the already studied cetaceans set the standard it is indeed a very interesting finding.

Studies on GLG readings in harp seals (*Pagophilus groenlandicus*) indicate a tendency towards greater disagreement in the older animals (>8 yr.) probably due to a more complex layering structure with age (Frei *et al.* 2011). In order to correctly interpret the layers a high degree of experience is needed. Reading the GLGs in either earplugs or teeth requires training, using criteria rarely based on known-age or known-history animals of the target species, so the degree of accuracy is uncertain. Readings should therefore at least be made consistent among readers using reference collections for training to avoid potential random biases in the

readings from subjective interpretation. For a full review and discussion on the problems of accuracy and precision in age estimation from GLGs, see Read *et al.* (this volume, in press).

In conclusion, calibration of both techniques of age estimation with known-age cetaceans is desirable, but is also very difficult to obtain. Known-age individuals from few species of mammals have provided validation of the GLG technique *e.g.* bottlenose dolphins (*Tursiops truncatus*; Hohn *et al.* 1989) and European harbour seals (*Phoca vitulina*; Dietz *et al.* 1991), and AAR has likewise only been applied to very few mammals with known-age, *e.g.* humans (Ohtani *et al.* 1995) and pygmy goats (*Capra hircus*; Garde *et al.* 2010). All four studies mentioned above showed a high correlation between actual age and the estimated ages even though validation of the GLG and AAR at species level would be preferable. So far only a few cetaceans have been born in captivity and lived long enough to provide a basis for such calibration and similarly few if any cetaceans have been tagged at birth and recaptured as adults. Some cetaceans such as the North Atlantic common minke whales have proven unsuited for age estimation by counting the GLGs (Christensen 1992), thus an alternative method is needed, such as AAR. However, even though using the AAR technique shows promising results for estimating the age in minke whales and other cetaceans which cannot be aged by other techniques (*e.g.* bowhead whales (Rosa *et al.* 2012) and narwhals (Garde *et al.* 2007)), it is still a technique that needs more studies in order to clarify the relationship between the individual D/L measurements, body temperature and age.

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