A New Simple Method for Age Determination of Harbour Porpoises (*Phocoena phocoena*)

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Abstract

Age determination of marine mammals is important for understanding the impact of anthropogenic disturbances as well as for population management. Toothed whales are usually age-determined by counting annually formed layers in their teeth. This includes a time-consuming sequence of preparations, usually involving chemical treatment. This study tested a quicker and simpler method for age determination of harbour porpoises (Phocoena phocoena), originally developed for age determination of foxes and other terrestrial carnivores. The tooth was ground with finegrained sandpaper, and the age lines were directly read using a binocular microscope. To evaluate the usability of the grinding method for harbour porpoises, three tests were used: (1) the number of growth layer groups (GLGs) in teeth from 66 harbour porpoises by the grinding method were compared by two readers; (2) GLGs in teeth from six harbour porpoises prepared by the grinding method and by the decalcification method were compared in a blinded set-up with two readers; and (3) the GLGs in teeth from two individuals with known ages prepared by both the grinding method and the decalcification method, respectively, were compared. A Bland-Altman plot showed high agreement between the determined age of individuals by the two different methods. The average age difference was -0.56 years, and the 95% confidence interval for the average difference was [-4.3, 3.2] years. The grinding method is therefore considered to be a valid alternative and quicker method for age determination of harbour porpoises.

Key Words: age methodology, grinding teeth, counting growth layers, toothed whale, mortality, demography

Introduction

Individual age determination is essential to understand population dynamics and to adequately address conservation measures for threatened species by investigating, for example, age at mortality, age-related diseases, survivorship, and demography (Fontaine et al., 2014; Jacobson et al., 2020; Rouby et al., 2021; Betty et al., 2022).

The harbour porpoise (*Phocoena phocoena*) is one of the most abundant coastal cetacean species in the Northern Hemisphere (Elliser & Hall, 2021). It is considered to be of "least concern" according to the International Union for Conservation of Nature's Red List, even though some populations are threatened (Carlén et al., 2018). Harbour porpoises are exposed to a number of human activities such as bycatch, disturbance, habitat loss, and chemical and noise pollution (Wisniewska et al., 2018; Siebert et al., 2020; Gallagher et al., 2021; Larsen et al., 2021). To understand these impacts at a population level, and to develop the best management strategies, information on the population size, habitat use (Hammond et al., 2013; Carlén et al., 2018), age structure (Lockyer & Kinze, 1995; Siebert et al., 2006), reproduction status (Kesselring et al., 2017), and health status (Siebert et al., 2001, 2020, 2022) is required.

In addition to several international treaties and obligations protecting the harbour porpoises in Danish waters, there are national objectives for the Danish National Contingency Plan concerning

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stranding of marine mammals. These include participation in the monitoring of seal and cetacean health, deaths and population status through registration, and the sampling and necropsy of stranded animals (Jensen et al., 2012). The National Contingency Plan is coordinated by the Danish Environmental Protection Agency in close cooperation with the Danish Nature Agency, the Fisheries and Maritime Museum, the Nature History Museum of Denmark, Department of Ecoscience, Aarhus University, Aalborg University, and Centre for Diagnostics DTU. Since 1991, the National Contingency Plan has facilitated the collection of dead, stranded harbour porpoises for necropsy (Thøstesen, 2021). Each year, up to 25 harbour porpoises are collected.

The age of harbour porpoises and other odontocetes can be determined by counting annual growth layer groups (GLGs) in their teeth (Lockyer & Kinze, 1995). Dentine is deposited and gradually fills the pulp cavity (centripetal growth; Grue & Jensen, 1979); therefore, a wide pulp cavity is only observed in young animals. In the teeth of harbour porpoises, dentine growth layers are visible as dark lines or "arches" around the pulp, usually forming annually between January and September (Kinze & Sørensen, 1984). It is usually feasible to determine age with an interval of 1 y per age category, even though dark and light dentinal layers may be difficult to interpret. One of the most common methods to count GLGs is described by Lockyer (1995). This method requires several days of preparation and decalcification before it is possible to determine the number of growth layers (Lockyer, 1995).

Herein, the simpler grinding method is tested for age determination in harbour porpoises. The grinding method is faster and does not use any chemicals. The tooth is ground down to the midline through crown and root after which the GLGs are counted in the dentine. The grinding method has previously been used to determine the age of mammals such as red foxes (Vulpes vulpes) and raccoon dogs (Nyctereutes procyonoides) (Roulichová & Andera, 2007; Pagh et al., 2018, 2020), but it has not previously been tested on harbour porpoises or other cetaceans. In this article, we compare the feasibility of the grinding method with Lockyer's (1995) decalcification method on wild animals with unknown age and on animals with known age held in captivity.

Methods

Data Collection

In total, the age of 66 harbour porpoises was determined with the grinding method. Out of these, 58 were Danish stranded porpoises, which had never been aged before, and six were stranded porpoises from Germany, which had previously been aged according to the Lockyer (1995) method. Two porpoises held in human care with known ages were used as reference for the aging method.

The stranded Danish harbour porpoises were collected between April 1993 and March 2021 from the Danish coastline of Jutland (n = 56)and Zealand (n = 2). They were stored in a -20°C freezer and macerated in hot water. Teeth were contained in small acid-free bags at the Fisheries and Maritime Museum (Esbjerg, Denmark). Six porpoises collected from the German Baltic and North Sea coasts by the Institute for Terrestrial and Aquatic Wildlife Research (ITAW), University of Veterinary Medicine Hannover, Germany, had previously been age-determined using the decalcification method by Lockyer (1995). The teeth from the German samples were preserved in water, cleaned, and then stored in 70% alcohol. After arrival, they were dried for 6 d before grinding. Teeth from the two porpoises held in human care with known ages of 5.5 and 13 to 14 y were made available from Fjord&Bælt (Kerteminde, Denmark). The younger animal ("Frigg") was born in the facility, and the older one ("Sif") arrived at about 6 mo of age. Sif, measuring 106 cm in length, was bycaught in a pond net and brought to Fjord&Bælt in late July. According to growth curves derived from animals of known age (Stepien et al., 2023), Sif was less than 1 y old at this time. The age of both porpoises had previously been determined with Lockyer's (1995) method. The teeth from Frigg were stored similarly to the other samples from Germany. The teeth from Sif were retrieved from a deep-frozen cranium 1 mo before being analyzed.

The Decalcification Method

The most common method for age determination of harbour porpoises is to count annual tooth GLGs as described by Lockyer (1995). When using this method, the teeth are first cleaned; decalcified in a rapid decalcifying agent (RDO) for 2 to 8 h, depending on size and likely age; and then rinsed in running water for several hours. Then, the teeth are fixed in 10% buffered formalin before they are sectioned with a carbon dioxide-freezing microtome at 20 to 25 µm thickness and then stained in haematoxylin for 15 min. Sections are then "blued" in weak ammonia solution, rinsed in distilled water, and dehydrated in 70% alcohol before floating onto 5% gelatincoated slides. Lastly, all sections, once dried, are immediately mounted using Protex under a glass coverslip and then placed for several days on a slide warmer to harden before examination with a binocular microscope (Lockyer, 1995).

The Grinding Method

Three to five teeth were extracted from each animal. Most teeth had been stored individually in small acid-free bags for many years in a museum collection. Dried teeth were ground with 800-corn sandpaper on a modified stone polishing machine down to the midline through the crown and root while restraining the tooth with a pincer or preferably a surgical scissor with clamp due to the small size of the harbour porpoise teeth. The teeth were ground vertically on the flat side, from the root towards the crown. If necessary for identifying all GLGs, teeth were further gently ground with 1,200-corn sandpaper. Two teeth from all individuals were ground for testing reproducibility between teeth. The grinding process was performed with a grinding machine (originally for jewelry stones).

For a trained laboratory technician, the process to prepare a tooth for reading GLGs will take less than a minute. The ground teeth were examined with a binocular microscope (most commonly using 25× magnification), photographed, and measured with a microscope camera (5 mp Wi-Fi; Frederiksen Scientific, Ølgod, Denmark) using Micro Capture, Version 6.20, software. A Starlite Inspection Light source was used during the examinations. High-magnification images of the GLGs were acquired using an iPhone 8 with a smartphone adaptor for binocular microscope. Age was determined by counting the number of GLGs. When inspecting the grinded tooth through a binocular microscope, the optimal location for counting dentine growth layers is usually along the side of the pulp in the mid-area of the tooth or in the crown above the pulp. For example, if two dark dentinal lines are read (beside the neonatal line which is deposited at birth and represents time zero), then the age of this animal is estimated to be 2 y old. Furthermore, to test a supplemental age determination method, the width of the pulp cavity was measured.

Testing the Reliability of the Grinding Method

Two teeth from each harbour porpoise were prepared for testing reproducibility of age determination. The lines of the two teeth from the same animal were read by Reader A (the first author of this article who had just finished her master study about age determination of harbour porpoises) multiple times to test intra-observer reproducibility in age determination. There was at least 1 d between readings to avoid fatigue and to ensure the reader could not remember the result of the previous reading (double-blind procedure). The results of the readings of the two teeth were investigated with linear regression and Analysis of Variance (ANOVA). To assess variation between observers, the ages determined from all 66 harbour porpoises were compared using linear regression between two readers (Readers A and B). Reader A was inexperienced in reading GLGs prior to the study, whereas Reader B (SP) had previous experience aging foxes, raccoon dogs, and American mink (*Neogale vison*) (Pagh et al., 2018, 2020, 2021). To assess the accuracy of each reader, the teeth from each of the 66 harbour porpoises were examined five times by Reader A and twice by Reader B. The first and fifth estimated ages by Reader A were compared using linear regression.

In addition, the age of eight harbour porpoises previously aged by Lockyer's (1995) method, including the two individuals with known ages, were compared to the estimated age of the individuals aged by the grinding method. A Bland– Altman plot, also known as the Tukey meandifference plot, was used to test the agreement between the two methods for animals with estimated ages between 1 and 13.5 y.

Measuring Pulp Cavity

To correlate pulp width with age, the maximum tooth width and maximum pulp cavity width (usually found in the middle of the tooth) were measured using *Micro Capture*, Version 6.20. The average pulp size was calculated in percent by dividing maximum pulp cavity width with the maximum tooth width.

Results

Testing the Grinding Method with Multiple Readings and Two Readers

The average difference between the first and last readings by Reader A of the same tooth was 1.3 y. There was a significant correlation between the first and the last determined age of the individuals ($R^2 = 0.93$, paired *t* test, n = 66, p < 0.001), with very few outliers (Figure 1). The correlation between the first age determination (AD1) and the fifth age determination (AD5) showed a trend of underestimation of GLGs at AD1 compared to AD5, especially for animals older than 6 y (Figure 1).

There was a high correlation between readings by Readers A and B ($R^2 = 0.93$), even though some outliers were found (Figure 2). The estimates of Reader A are from the fifth reading and from the second reading of Reader B due to the likelihood of these readings being the most correct ones after gaining experience in correctly finding the GLGs (see the Supplemental Appendices; the supplemental appendices for this article are available in the "Supplemental Material" section of the Aquatic Mammals website).



Figure 1. The estimated ages of the first and fifth readings by the inexperienced Reader A of 66 harbour porpoises. Data points have been jittered slightly vertically to display points with overlapping values. Solid line indicates a one-to-one relationship between the first and fifth age determination. Stippled line is a linear regression (equation $y = 1.1 \times + 0.06$).



Figure 2. Estimated ages (years) for 66 harbour porpoises read by Readers A and B. Solid line indicates a one-to-one relationship between Readers A and B. Stippled line is a linear regression (equation $y = 0.87 \times + 0.32$).

Comparing the Methods of Grinding and Decalcification (Lockyer, 1995)

There was high agreement between the age estimates of the eight individuals using Lockyer's



Figure 3. Eight harbour porpoises' ages estimated by two different methods: New and Traditional (Lockyer, 1995). The solid line indicates a one-to-one relationship between the two methods. The stippled line is a linear regression (equation $y = 1.25 \times -0.51$). Data points with identical values were jittered slightly for visualization.

(1995) method and the grinding method (R^2 =0.80; Figure 3). The grinding method seemingly provided fewer visible lines than the decalcification method so that the age was sometimes underestimated (Figure 3).

The Bland–Altman plot indicated low variation and similar deviations for all age groups between the two methods (Figure 4). The average difference was -0.56 y in the range from 1 to 13.5 y, and the 95% confidence interval for the average difference was [-4.3, 3.2].

Methods Tested on Harbour Porpoises with Known Ages

Two harbour porpoises with known ages of 5.5 and 13.5 y were analyzed. The number of GLGs visible in the teeth of these individuals treated by the Lockyer (1995) method showed a match with age for both individuals, while the GLGs using the grinding method showed three less visual lines for the older individual when analyzed both by Readers A and B (Table 1).

Pulp Cavity Size Changes with Age

As in other carnivores, the pulp size in harbour porpoise teeth decreases in size relative to the age of the animal. A fitted exponential function of estimated age (years) was fitted to pulp size (%) with $R^2 = 0.93$ (Figure 5).



Figure 4. Bland–Altman plot of the difference in age determination between the grinding and decalcification methods with the two methods as a function of their mean

 Table 1. True age of two harbour porpoises (*Phocoena phocoena*) housed at Fjord&Bælt, with three different age estimations by two different methods. The grinding method was measured by two different readers (A and B). All ages are in years.

Animal names	True age	Lockyer (1995) method	Grinding method (Reader A)	Grinding method (Reader B)
Frigg	5.5	5.0	5.5	5.0
Sif	13.5	13.5	9.0	9.0

Young individuals with an estimated age of 0 to 1 y had a wide pulp cavity that fills up approximately 90% of their tooth. An older animal at an estimated age of 12 to 13 y had a pulp cavity that only fills up 3% of their tooth (Figure 5).

In harbour porpoise teeth, GLGs usually annually form a paired dark thin line and adjacent lighter thicker lines. The older the animal, the less pulp cavity remains (Figures 5 & 6). For example, if two dark dentinal lines are read (besides the neonatal line which is deposited at birth), then the age of this animal is estimated to be 2 y old (Figure 6).



Figure 5. Average pulp size and the estimated age for all 66 collected harbour porpoises (*Phocoena phocoena*). The dotted line is an exponential function (y = 109.47e-0.285x) fitted to the data.



Figure 6. Longitudinal sections of a tooth, through crown and root, from harbour porpoises with the following estimated ages: (A) 8 y (A) and (B) 2 y.

Discussion

This study suggests that the grinding method is a reliable and methodologically simpler way of assessing the age of harbour porpoises. Young individuals are easier to age. However, as age increases beyond 6 y, the number of GLG lines may be more difficult to detect.

The observer's experience in the process increases the number of GLGs that can be discerned (Figure 1). Still, there was a high agreement between the first and fifth readings of the individuals (Figure 1). Also, the co-author who was more experienced in reading teeth from terrestrial mammals (Reader B) detected more lines than the less experienced reader (Reader A; Figure 2), indicating that training is fundamental for accurate age determination. It requires practice to detect all the correct number of lines, and the estimates possibly become more precise with more practice.

Comparison Between the Decalcification Method and the Grinding Method

The high agreement between the age determined by the decalcification and the grinding methods indicates that the new simpler grinding method can be a valid alternative for age determination. As the ages of six out of the eight harbour porpoises were not known, it is not possible to decide which of the two methods was the most exact.

The readability of the GLGs varied between teeth. For unknown reasons, the harbour porpoises sent from Germany showed less visible growth lines than harbour porpoises from the Danish samples. This might be due to differences in the preparation or storing conditions. This could also be due to regional differences in porpoise teeth (Lockyer, 1999).

The high correlations between the two methods were supplemented with the Bland–Altman plot, showing good agreement between the two methods for both young and adult animals.

The Two Methods Tested on Individuals of Known Ages

For unknown reasons, only nine clear lines could be seen with the grinding method using the teeth of the 13.5-y-old animal Sif, while the true age of the younger animal Frigg (5.5 y) was correctly interpreted by the grinding method. The age of the two individuals had previously been accurately determined by the decalcification method. The teeth from Sif illustrate that some teeth may be difficult to read using the grinding method. Hindrances to reading GLGs can occur in samples (e.g., crown wear, split or overground teeth), which can present difficulties in spotting the correct number of lines by the grinding method. This emphasizes the importance of collecting at least three to five teeth from each individual to get the best possible sample for age determination. Currently, there are only a handful of harbour porpoises of known age kept in captivity, so the prospects are very slim for obtaining a larger dataset of teeth from porpoises of different ages for studying the precision of aging techniques.

Pulp Size Correlation with Age

Besides GLGs, the size of the pulp cavity also gives information about the age of an individual. On average, the pulp size of individuals less than 1 y old is around 85 to 90%. The pulp size decreases to 53 to 61% for 3-y-old individuals, and it is almost completely closed (less than 7%) in individuals older than 6 y (Figure 5). As the size of the pulp is highly negatively correlated with the number of GLGs, it is a good indicator for age. The annual change in relative pulp cavity size decreased with age; therefore, the pulp size is a less precise indicator of age than GLGs. Two teeth with approximately the same estimated age can appear very different from each other, also in regard to the size of the pulp cavity, meaning that the pace at which the pulp cavity fills up may vary among individuals. But even so, pulp size can be used as an easy way to separate juveniles from adults.

Perspectives of the Grinding Method

We found the grinding method usable for age determination of harbour porpoises. The method requires few materials, and the entire process takes less than 5 min from obtaining the tooth to age determination. One of the few limitations of the grinding method is that it requires practice and experience in identifying the correct lines and counting the correct number of GLGs in the teeth as it is known from the decalcification method and other age determination methods (Evans et al., 2002). Also, old, damaged, and worn teeth seem to be slightly more difficult to read for GLGs with the grinding method.

Note: The supplemental appendices for this article are available in the "Supplemental Material" section of the *Aquatic Mammals* website: https://www.aquatic mammalsjournal.org/index.php?option=com_conte nt&view=article&id=10&Itemid=147.

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