INTRODUCTION: AGE ESTIMATION OF MARINE MAMMALS WITH A FOCUS ON MONODONTIDS

The Monodontidae is a family that exclusively inhabits the arctic regions and includes two genera, each with one species – *Delphinapterus leucas*, the beluga, and *Monodon monoceros*, the narwhal. Both species are classified as medium-sized toothed whales. The beluga, also known as the white whale, sea canary and melonhead, was first described in 1776 by Peter Simon Pallas (Mead and Brownell, 2005) The narwhal, also known as the sea unicorn, was first described by Linnaeus (Linnaeus, 1758).

The beluga has a near circumpolar distribution while the narwhal is found in the arctic seas around eastern Canada, Greenland, Norway and western Russia. The monodontids are similar in shape, with the narwhal longer and more streamlined than the beluga. The adult narwhal is greyish and flecked (Fig. 1); the adult beluga is all white (Fig. 2). Both differ from most other cetaceans by having an ontogenetic colour change from darker grey juveniles.

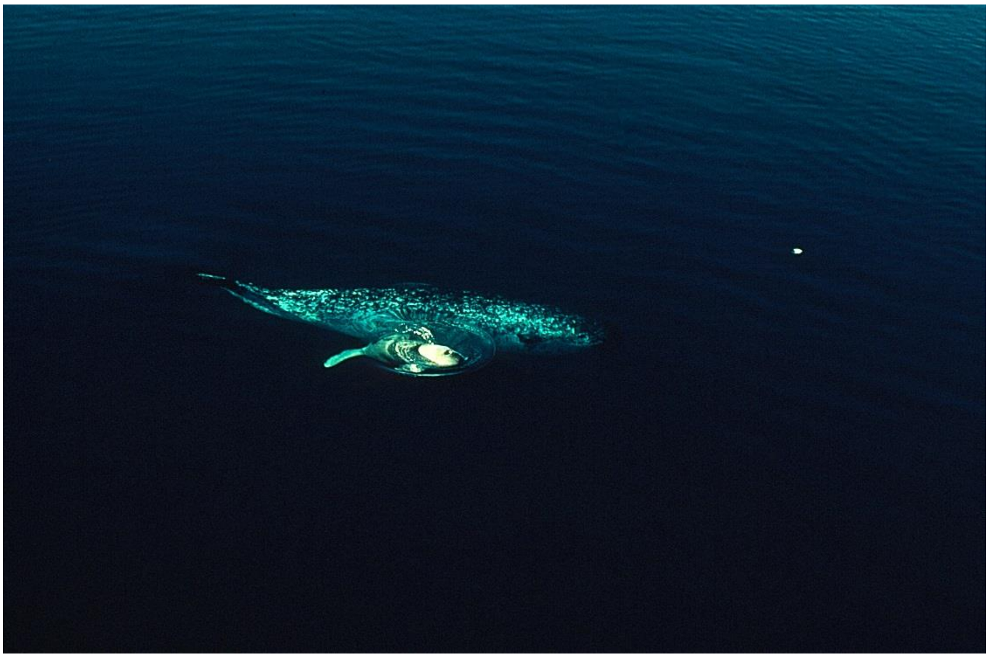

Fig. 1: Narwhal and calf. (Photo: R. E. A. Stewart)

The narwhal has embedded teeth that never erupt except for the canines. Male narwhals are characterised by the long straight helical tusk evolved from the upper left canine tooth while the right canine is also spiralled but is short and

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remains embedded in the upper jaw. The females have both canines embedded, although tusks have occasionally been observed in females and a very few narwhals bear twin tusks. In contrast, belugas have a full set of homodont teeth in both upper and lower jaws. One of the unusual characteristics of the belugas and narwhals compared to other cetaceans, is their ability to move the head in a manner more like other mammals because the cervical vertebrae are not fused as they are in most whales. This characteristic facilitates breathing in dense ice and feeding in shallows.

Fig. 2: Beluga and calf. (Photo: R. E. A. Stewart)

Both species occur in the arctic zone of the North Atlantic region covered by the NAMMCO Agreement of 1992. NAMMCO's objective is to provide advice "on the conservation status, sustainable removals and responsible hunting methods of marine mammals" (https://nammco.no/, accessed 23 June 2018) and both belugas and narwhals have been hunted throughout their range and are still a valuable food resource - especially highly prized as mattak - for Inuit in Greenland (a member nation) and in Canada. Beluga whales occur off the arctic coast of Norway and both species occur around Svalbard, but hunting is prohibited by Norway. NAMMCO recognises that stocks are migratory and are the shared responsibility of other nations such as Canada and Russia. NAMMCO has a scientific joint working group with the Canada-Greenland Joint Commission on Narwhal and Beluga (JCNB) that is concerned with management of the stocks within the shared waters. The 4th volume in the NAMMCO Scientific Publications series, *Belugas in the North Atlantic and the Russian Arctic*, published in 2002, addressed beluga biology, life history, distribution, stock identity, status and catches and is still an authoritative source of information on this arctic circumpolar species (http://septentrio.uit.no/index.php/NAMMCOSP/issue/view/245). NAMMCO recently convened a Global Review of Monodontids workshop where more than 20 stocks of belugas and 13 stocks of narwhals were reviewed, and knowledge gaps as well as global and regional issues related to the conservation of belugas and narwhals were identified and discussed. A number of recommendations for research and cooperation was also presented. The report is available at: (https://nammco.no/wp-content/uploads/2018/05/report-global-review-of-monodontids-nammco-2018_after-erratum-060518_with-appendices_2.pdf).

The topic of age estimation, important for all exploited species, is of concern to NAMMCO, as age estimates are important for determining catch composition and stock demography, both, in turn, essential for efficient management. A common problem for all species in all areas is that of obtaining accurate age estimates, which has been particularly difficult in monodontids. Teeth have been successfully used for many odontocete species for estimating age from growth layer groups (GLG) in the dentine and / or the cementum. Teeth are available for belugas and have been studied extensively, however, pragmatically not for narwhals, although their unerupted and embedded teeth have been investigated. Relative means of ageing have been used, such as body colouration or overall size in length. More recently for narwhals, investigation of Aspartic Acid Racemisation (AAR) in eye lenses has provided a potential means of estimating age. Calibration of the AAR method to provide actual age requires known ages but GLGs in sectioned tusks have provided independent data on ages. Recognizing that age-estimation research on other mammals can inform monodontid ageing, NAMMCO commissioned a review of ageing methods and convened two international workshops. This volume is the product of those three sponsored events.

The volume opens with the review of age estimation in marine mammals by Read *et al* (Paper 1). The paper addresses the need for accurate and precise age estimation, describes the similarities and differences in teeth across marine mammal taxa, and lists, explains, and evaluates various methods tried and tested in species ranging from fissipeds, pinnipeds, odontocetes, balaenopterids to sirenians, with reference to terrestrial mammals when relevant. The potential application of new techniques, *e.g.*, genetic telomere length and fatty acid profiles, is discussed, and their use in the future is noted should these techniques be perfected to provide greater age accuracy. The techniques found most suitable in different species are elaborated on. For monodontids, the use of GLGs in teeth of belugas and use of AAR in the nucleus of the eye lens in narwhal are seen as the most useful techniques presently, despite underestimation of age associated with tooth crown wear (and loss of GLGs) in belugas, and the need for careful validation and calibration of AAR in narwhals, where presently age estimates are imprecise relative to GLG counts.

The first international workshop was a two-day meeting immediately before the Society for Marine Mammalogy biennial conference in Tampa, Florida, US, 26-27 November 2011. Workshop participants discussed a number of issues related to marine mammal age estimation and made clear that most issues concerning monodontid age estimation are not unique. Hohn *et al* (Paper 2) include extended abstracts of the presentations, many illustrated, detailing a diversity of methods and tissues (many recent or novel) to reveal biological records of age in many species of marine mammals. During the workshop, the usefulness of different biological tissues was assessed, along with preferred tissues in species as diverse as manatees (periotic ear bones) and baleen whales (ear plugs). In addition, aspects of accuracy and precision of the GLG counts and other methods were considered, as well as their interpretation. The use of chemical markers in hard tissues, such as atomic bomb isotopes and tetracycline antibiotics, were discussed in providing validation for life-history events and deposition rates of growth layers. The merits of relatively novel techniques such as analyses of genetic telomere lengths, stable isotopes and fatty acid ratios, and AAR for estimating age in species where traditional methods have failed, have been reported in some species, and may be or have already been shown to be promising in narwhals and belugas. Some of these methods are treated more fully in subsequent papers. A valuable tabulation of methods that may be applicable in monodontids is provided in the report. One of the main conclusions from the workshop was that teeth are still the preferred tissue for ageing in belugas, and that an annual deposition rate of tooth GLGs was to be the accepted standard for belugas. Regarding narwhals, AAR was deemed to be promising.

The second workshop, the report of which is Paper 3, by Lockyer *et al*, held in Beaufort, North Carolina, US, 5-9 December 2011, focussed on practical aspects of age estimation in beluga. Participants estimated age for a number of specimens from a variety of geographic areas and prepared using several methods. The emphasis was on variation among readers with hands-on lab work regarding tooth preparation and identification of GLGs. Details of the processes used to compare sections, images, and GLG interpretation were described. The participants generated guidelines for best practices in beluga age estimation based on the collective experience of the group. The report is, effectively, a manual that describes the complete processes of preparation of, examination and counting of GLGs in beluga teeth, that are considered to be "best practices". Participants agreed that counting GLGs using well prepared, thin, untreated sections was the preferred method because there was more agreement among readers compared to other methods, and concomitantly, these sections are simpler to prepare than decalcified thin stained sections. The value of using high-resolution digital images was also assessed relative to examining actual tooth sections and found to be potentially an excellent method for exchange of materials without requiring international export/import permit applications. These images also provide an archive that can be annotated to document readings and facilitate discussion among readers.

An important part of Paper 3 describes a comparative study on common dolphins which statistically quantified and analysed counting differences among readers and found that precision of experienced readers was good, and higher than reported for other odontocetes. Information on storage, preparation, examination and GLG interpretation of teeth for ageing is tabulated (Table 2) in the paper for easy access.

Stewart and Stewart (Paper 4) provide an in-depth ontogenetic study of age estimation from beluga teeth, examining the biology behind the GLG counts and tooth development related to age estimation. The widely accepted method of estimating ages of beluga is to count dentinal GLGs in median, longitudinal sections of a tooth. It is essential to understand how these growth layers form and to consider developmental factors that can confound their enumeration to be able to provide meaningful age estimates. Information is provided, with illustration, on key factors: evaluating the presence and occlusal wear of foetal dentine; interpreting early-formed diagnostic features such as the neonatal line; assessing the last-formed growth layer adjacent to the pulp cavity; identifying the presence of nodes at the dentine-cementum interface to assist in counting GLGs; and recognizing pulp stones and accessory lines in the dentine which may hinder and mislead the age estimation process. Particular attention is given to the impact on ageing when there is significant wear at the tooth crown and if the cut along the centre longitudinally is oblique or off centre, both of which can result in a minimum age.

This paper is especially valuable for researchers studying age in belugas because it gives detailed examples with illustrations about how to count and interpret GLGs and associated micro-structures in the dentine.

There has long been a controversy regarding the deposition rate of growth layers in teeth of belugas (one or two GLGs per year), which has important implications in assessing population parameters. Campana and Stewart (Paper 5) review the technique of bomb dating for age validation and quality control of age estimates of monodontids and other marine mammals. The basis and application of bomb radiocarbon to marine mammal age validation are discussed, highlighting its value for providing unambiguous estimates of age for belugas and other long-lived animals which form GLGs. Bomb radiocarbon is particularly useful for marine mammals, given that the age of an individual animal can be determined to within ±1-3 years, so long as it was alive during the 1960s (during the time of atomic tests). Ongoing studies involving ageing require careful monitoring to ensure that age interpretations remain consistent across ages and through time. In addition, quality-control protocols using reference collections of ageing material are recommended.

Matthews and Ferguson (Paper 6) investigate the validation of dentine deposition rates in beluga whales by interspecies cross dating of temporal trends in stable isotope ratios of carbon ($\delta^{13}C$) in teeth. The theory behind this technique is based on the fact that isotopic time series from sequentially sampled GLGs in marine mammal teeth can be combined to build chronologies allowing assessment of isotopic variation in marine ecosystems. Synchronous recording of baseline isotopic variation across dentinal GLGs of species with temporal and spatial overlap in foraging offers a unique opportunity for validation of marine mammal age estimation. It calibrates GLG deposition rates in one species against another whose GLG deposition has been independently determined. In this study, trends in $\delta^{13}C$ across dentinal GLGs of three eastern Canadian Arctic (ECA) beluga populations through the 1960s-2000s were compared with a $\delta^{13}C$ time series measured across dentinal GLGs of ECA/Northwest Atlantic killer whales (*Orcinus orca*) from 1944-1999. Confirmed annual GLG deposition in killer whales was used as a means to assess beluga GLG deposition. It was shown that linear $\delta^{13}C$ declines across chronologies of both species were statistically indistinguishable when based on annual GLG deposition in beluga whales, but differed when based on biannual deposition. The authors propose that $\delta^{13}C$ declines reflect the oceanic ^{13}C Suess effect. This study provides additional support for annual GLG deposition in beluga whales.

The information in Papers 4-6 answers NAMMCO's key questions about beluga age estimation. Counting GLGs in teeth is appropriate for age estimation in beluga and guidelines for best practices in age estimation have been developed. How to interpret what is seen at various stages of tooth development has been documented. The use of 1 GLG/year to interpret age from those counts is irrefutable.

Further information about ageing using tooth and other hard tissue GLGs in other, diverse, species is presented in four additional papers. Murphy *et al* (Paper 7) examine deposition of GLGs in dentine tissue of captive common dolphins *Delphinus delphis*, and its continuation throughout life. Estimation of age in common dolphins (*Delphinus* spp.) is primarily based on counting GLGs in the dentine of thin, decalcified and stained sections of teeth. While an annual incremental deposition rate was validated for *Delphinus* spp. more than 30 years ago using tetracycline antibiotic time-marking, it was not known whether the pulp cavity becomes occluded in older individuals or if GLGs continue to be deposited in dentine tissue. This factor is important as pulp cavity occlusion may lead to underestimation of age in old animals. The targets of this study were two short-beaked common dolphins which were captured together in Hawkes Bay, North Island, New Zealand and classified as juveniles based on physical appearance. They were held in captivity for 31 and 33 years. The teeth were processed in two specialist laboratories, using several different techniques. An age estimated for one of the dolphins was in line with that anticipated based on estimated age at capture and period in captivity, thus allowing estimation of maximum age. The other animal unfortunately had a mineralisation anomaly that interfered with age estimation.

Lockyer and Braulik (Paper 8) examine teeth of highly endangered, geographically isolated, freshwater platanistid species: the South Asian river dolphins (*Platanista gangetica minor* and *P. g. gangetica*). The teeth of *P. gangetica* vary greatly in size and shape both with age and along the jaw of an individual, with longer and thinner teeth situated forward in the jaw and teeth in older animals broader and flatter with associated changes in the root. Thus the teeth are significantly different from most dolphin species, which are homodont. The authors detail methods for preparing optimal sections which rendered both dentinal and cemental GLG readable. Cemental GLGs were generally easier to interpret. Ages ranged from newborn / young of year (with no laminae or only a neonatal line present) to a maximum age of 30 GLGs. There is currently no validation available for GLG deposition rate, but it is likely annual. This study highlighted the problems that may be encountered when examining irregular-shaped teeth. The paper includes an illustrated reference of a chronological age series.

Brill *et al*. (Paper 9) examine the technique of age estimation in Florida manatees (*Trichechus manatus latirostris*) using GLGs in periotic (ear) bones, with the focus on precision (repeatability of counts among readers). Manatees are long-lived (>50 yr) and although they bear teeth, the teeth constantly grow, move forward in the jaw and are replaced over time. Thus, they are not useful for age estimation. Manatee carcasses recovered in Florida between 1974 and 2010 provided material for a retrospective analysis of ageing precision (repeatability). All three readers were in good agreement (high precision) with the greatest apparent source of variation being the result of earbone remodelling with increasing manatee age (>15 yr). Remodelling is often the result of resorption, and subsequent bone regrowth. Over the same period of carcass recovery, methods of sample preparation and of determining a final age estimate changed. However, on analysis, the effects of altering methods on ease of reading GLGs yielded no statistical differences in age estimates. The authors describe the currently recommended methods for estimating manatee ages using earbones, from sample collection protocol, preparation and histological treatment, to reading and interpreting GLGs, and how to interpolate age when remodelling occurs in the tissue (illustrated) and obscures the GLGs. The method of using GLGs in periotic earbones could potentially be an interesting technique to try in monodontids, with initial trials on belugas where GLG comparison with teeth is feasible. The paper underlines the importance of standardising age reading in labs, and in training and testing for precision over time. This issue is important for all species where GLGs are being counted, in order to ensure consistency in ageing and comparability of age counts in population studies.

Micro-Computed Tomography (Micro-CT) was used by Loch *et al.* (Paper 10) for dental studies in modern and fossil odontocetes. The GLGs recorded in dentine have demonstrated application for ageing studies, but also have the potential to elucidate life-history phenomena such as metabolic or physiological events. Micro-CT is a non-invasive and non-destructive technique that allows three-dimensional study of mineralized tissues, such as human teeth, and their physical properties. This property alone has potential value in examining tooth specimens from all species where the teeth have become fragile, are from rare species, or for various reasons the teeth must not be cut or destroyed. Micro-CT has been proven to be a useful tool for resolving the internal morphology of fossil and extant teeth of cetaceans before they are sectioned for other morphological analyses. However, some methodological refinements are still necessary to allow better resolution of dentine for potential application in non-destructive age determination studies.

Not all marine mammals have teeth or permanent hard structures that can reliably be used for age investigation, and development of new ageing methods not relying on hard structures may be applicable to narwhal.

Maeda *et al* (Paper 11) describe a successful new technique for extraction of ear plugs in dead minke whales (*Balaenoptera acutorostrata*). These ear plugs are usually small and fragile, and introducing liquid gelatin into the surrounding ear canal provides a supportive medium when set (by freezing), so that extraction minimises breakage and possible destruction of the ear plug. While this technique is not directly applicable to monodontids, the use of gelatin as a support medium, *e.g.* in examination of old, fragile and cracked teeth during processing, is potentially valuable. The method of extraction may also be valuable for removing ear plugs in other dead stranded balaenopterids. In addition, the authors reported on the use of alizarin red S stain in histological sections of ear plugs to highlight calcium cycles and improve the readability and identification of GLGs. Traditionally, GLGs have been identified mainly on the annual lipid density changes.

Tange-Olsen *et al.* (Paper 12) describe the utility of a technique of measuring telomere length using quantitative PCR for age estimation in humpback whales (*Megaptera novaeangliae*). The technique is minimally invasive and could be used on living free-ranging cetaceans. Skin samples from 28 living North Atlantic humpback whales, ranging from 0 to 26 years of age, were examined. The results suggested a significant correlation between telomere length and age in humpback whales. However, a major drawback was that telomere length was highly variable among individuals of similar age, suggesting that telomere length measured by quantitative PCR is an imprecise determinant of age in humpback whales. The observed variation in individual telomere length was found to be a function of both experimental and biological variability, with the latter perhaps reflecting patterns of inheritance, resource allocation trade-offs, and stochasticity of the marine environment. The technique has been used with some success in other species, but is still experimental, and is not likely to be a useful tool in monodontids at this stage.

Paper 13 by Nielsen *et al*. examines different methods of age estimation, comparing (and thus partly validating) age from GLGs in teeth of relatively short-lived harbour porpoises (*Phocoena phocoena*) and GLGs in ear plugs of long-lived fin whales (*Balaenoptera physalus*) and common minke whales with ages estimated from AAR rate (k_{Asp}) – known as the AAR technique using eye lens nucleus. The correlation of GLG age and D/L enantiomer ratio for all three species was highly significant, but varied greatly by species, and was strongest in minke whales and weakest in harbour porpoises. The study nevertheless demonstrated the potentially reliable use of AAR technique in age estimation in some species, particularly longer-lived ones, albeit the need for increased precision.

Garde *et al*, (Paper 14) examine the accuracy of age estimation using the AAR technique, with reference to 25 different mammalian species, using eye lens nucleus from a sample size of 124 animals. D/L enantiomer ratios for these samples were obtained using the AAR technique. The animals were either of known age or had the age estimated by other methods. The sample size from a single species of known age, the pygmy goat (*Capra hircus*), was also large enough to investigate the accuracy of ages obtained using the AAR technique. The $2k_{Asp}$ values from 12 species, four estimated in this study and another eight published, were used to examine the effect of core body temperature on the rate of racemization. A positive relationship between AAR and temperature was found but results also suggested that other factors are involved in the racemization process in living animals. Based on their results the authors emphasize that non-species-specific racemization rates should be used with care in AAR age estimation studies, and that the period of postnatal growth of the eye lens be considered when estimating species-specific D/L_0 values and ages of young individuals.

At this stage, it is not possible to identify a best practice for age estimation in narwhal. Now that it has been established that the GLG deposition rate in beluga teeth is annual, it is likely that GLGs in narwhal tusks also form annually. Belugas and narwhals may have a greater longevity than originally thought and recent studies indicate that ages exceeding well over 70 years may not be unusual in these species.

This volume 10 - *Age estimation of marine mammals with a focus on monodontids* – represents an authoritative collection of reviews and articles on historical approaches to ageing, development of new methods sometimes in concert with established methods, and innovation research for and application of techniques to increase the range of species for which accurate and precise age estimates can be obtained. The papers in this volume have mainly arisen from the output of two workshops addressing the question and validity of age estimation methods in general and also specifically addressing the beluga tooth preparation, GLG interpretation, and accuracy and precision. It is hoped that this volume will become a standard reference work for learning how to age monodontids, but, even more, provide inspiration and motivation for tackling ageing in diverse species not previously examined, and for developing or refining innovative techniques in species already under scrutiny.

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