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A review of age estimation methods in marine mammals with special reference to monodontids

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ABSTRACT

This paper presents a critical review of methods for estimating absolute or relative age in marine mammals. Absolute age is achieved by counting growth layer groups (GLGs) in hard structures such as teeth, ear plugs, baleen, bones and claws. Relative age can be obtained by methods such as aspartic acid racemisation, genetic telomeres, bone mineral density, fatty acid signatures and other methods. Each method is discussed in detail. Accuracy and precision, including inter-reader calibration and anomalies, as well as methods of validating GLG deposition rates are also addressed. Each section concludes with methods of age estimation applicable to monodontids, and suggestions on the focus of future age-estimation research.

INTRODUCTION

While methods to obtain age estimates from teeth for most marine mammal species have been available for some time, the use of those ages for research has been evolving. For example, in addition to traditional uses such as estimating age-specific vital rates, understanding the effects of contaminants on the health of marine mammals or accumulation of toxins, epizootic events, and epidemiology all require, to some degree, accurate age estimates of individuals. In addition, for some species many challenges remain, largely because age estimation from teeth is not a viable option. Addressing these challenges may require developing innovative approaches, often using emerging technologies. For many species, lack of direct validation or relatively high uncertainties in age estimates continues to create a necessity for further research.

Age estimates can be defined as 'absolute age' (also referred to as 'chronological age') when age is estimated according to calendar units, usually years for long-lived animals including all marine mammals, or

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'relative (or categorical) age' when the animal is assigned to an age class relative to other age classes (Laws 1952, Hohn 2009). Relative ages are generally categorised using the life-history status of the animal, for example, neonate/pup/calf, immature, and mature. While for most purposes absolute age is needed, the question of interest might be directly associated with relative age, *e.g.*, whether an individual is younger or older than the average age at sexual maturation.

Here we review methods for age estimation in marine mammals. Absolute and relative methods for estimating age are discussed, as well as methods for validation of deposition rates of tooth tissue and factors that might confound estimation of age. This is not meant as an exhaustive list of age estimation studies but rather a review of methods. We also suggest needs for future age-estimation research.

Applicable sections include a focus on monodontids. The narwhal (*Monodon monoceros*) and beluga whale (*Delphinapterus leucas*) comprise the only members of the Monodontidae family. Age estimation in monodontids has been particularly problematic and to date, no standard method has been agreed upon.

ACCURACY AND PRECISION IN ESTIMATED AGE

Accuracy refers to how close an age estimate is to true age while precision refers to the reproducibility of age estimates (Campana 2001). Care must be taken when using age estimation terminology; for example, age estimates are often referred to as 'accurate' when comparisons of multiple readings among and/or between readers are equal or similar when, in fact, it is a comparison of 'consistency'.

Accuracy and precision are affected by a number of factors. There is natural variation in the clarity, hence ease of interpretation, among and within species (see for example Christensen-Dalsgaard *et al*. 2010, Lockyer *et al*. 2010). Poorly prepared sections can influence interpretation and, thus, counts of growth layer groups (GLGs) (Hohn *et al*. 1989, Bjørge *et al*. 1995, Pinedo and Hohn 2000). Reader experience may affect counts in all but the most well-defined growth layers. While most studies strive for accuracy with high precision, ultimately, the levels of accuracy and precision required are also dependent on the research question, for example, an individual aged X±1 for investigating age-based life-history parameters is likely to be more influential to the results than an individual aged X±1 in an epidemiology study where knowledge of age class may be adequate.

Reader calibration

In earlier studies on age estimation, multiple age readings were not standard operating procedure (Stewart and Lavigne 1979). Since the 1980s, the vast majority of studies relying on ageing by counts of GLGs included multiple age readings conducted without reference to the biological data associated with each specimen or to prior readings. Although such repeat counts are conducted independently and systematically within working groups and institutes, the approach to replication of readings varies widely. For example, ages may be established as a function of one reading by two readers, two readings by two readers, or more readings or more readers. There is no universal optimal value for the number of replicates per specimen, but at least 3 is recommended. Mansfield (1991) found that 93% accuracy was achieved when three readings of known-age grey seals (*Halichoerus grypus*) were conducted, but when all five readings were considered, accuracy only improved to 94%. Five readings were required in over 50% of walruses (*Odobenus rosmarus*) to achieve three comparable age readings (Stewart and Stewart 2005).

When small inter-reader discrepancies in age are found, methods for attaining a 'final age' are not consistent. Final age estimates can be taken as identical readings when there are matches among multiple readings. Some working groups use the mean of multiple readings (*e.g.*, Johnston *et al*. 1987, Garlich-Miller *et al*. 1993, Frie *et al*. 2013, Brill *et al*. 2015) or the most frequently occurring reading (mode), while others may discuss the specimen until an age is agreed upon. Another approach is to remove outliers using maximum normalised residuals when, *e.g.*, fewer than three of five readings are the same (see Stewart and Lavigne 1979, Snedecor and Cochran 1980, Stewart and Stewart 2005). The overall goal is to obtain the best estimate possible, therefore when age estimates are ambiguous, age might be decided with the use of biological information, for example, body length of the individual (Lockyer *et al*. 2010). In general, with multiple readings obvious errors or outliers should be accounted for, such as through use of an appropriate central estimator.

Inter- and intra-reader variation has been discussed extensively in various publications (*e.g.*, Mansfield 1991, Bjørge *et al*. 1995, Evans *et al*. 2002, Christensen-Dalsgaard *et al*. 2010, Frie *et al*. 2011, 2013, Brill *et al*. 2015). The cyclic GLG pattern is not always distinct and anomalies such as accessory and marker lines might be counted as annual layers in younger individuals, while in older individuals, the GLGs become compact and difficult to differentiate (Fig. 1) (Grue and Jensen 1979, Hohn 1990, Mansfield 1991, Stewart *et al*. 1996, Evans *et al*. 2002, Christensen-Dalsgaard *et al*. 2010, Frie *et al*. 2011, 2013). Generally, however, GLG counts tend to be negatively biased because it is more likely for growth layers

to be lost, boundary layers of GLGs to not be detected, or compressed GLGs in old animals not be sufficiently visible than for significant supernumerary lines to be counted (Stewart and Stewart 2005).

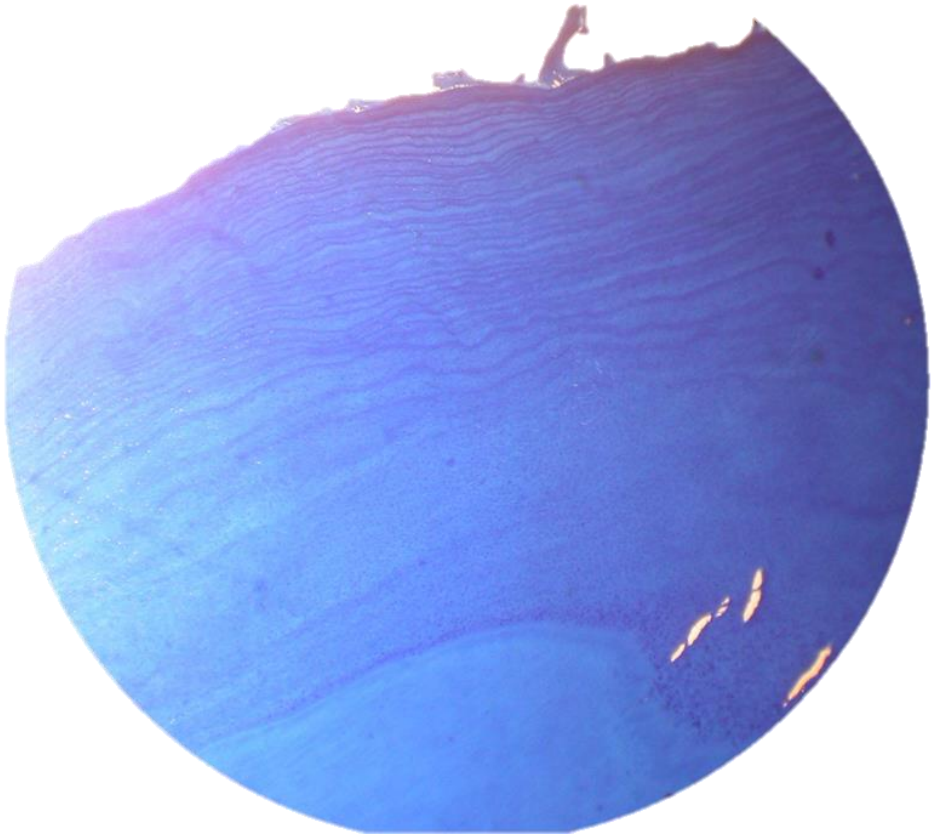

Fig. 1. Polar bear (*Ursus maritimus*) cementum in an animal aged 34 years, showing the compactness of later-forming GLGs.

Increased precision or within-reader consistency is generally positively correlated with experience (Campana 2001). However, both experienced and inexperienced readers showed significant levels of bias and imprecision in harp seal (*Pagophilus groenlandica*, formerly *Phoca groenlandicus*) age readings when age estimates were compared to the known age (Lawson *et al*. 1992, Frie *et al*. 2011). Similar studies with known-age grey seal teeth however, revealed no particular correlation of ageing error with reader experience and for all readers 89.3% of errors were within $\pm$1-2 years of known age (Frie *et al*. 2013).

Age reference collections are invaluable but rare and mainly occur for pinnipeds (*e.g.*, Frie *et al*. 2011, 2013). Known-aged animals are ideal for use in a reference collection. The majority of bottlenose dolphins (*Tursiops*

truncatus) in the Sarasota population, Florida, USA are known age (*e.g.*, Hohn *et al*. 1989) and could be used to establish a reference collection. When known-age samples are not available, which will be often, it is recommended to have an inter-laboratory reference collection for which age for a large number of specimens has been estimated by several experts. A species-specific publication of an ontogenetic series of photographs, with GLGs marked and containing a descriptive model for estimating age (*e.g.*, GLG appearance and size), is also helpful, such as those produced for bottlenose dolphins, harbour porpoises (*Phocoena phocoena*) and the Franciscana (*Pontoporia blainvillei*) (Hohn 1990, Hohn and Lockyer 1995, Pinedo and Hohn 2000).

Scoring specimens for section quality and GLG clarity following the criteria outlined in Stewart and Stewart (2005) can be valuable for analysis of potential biases that may influence readings and ultimately age estimation, but such scoring is rarely reported. Statistical models can be developed for age readings to account for biases due to reader experience, to differences between known-age and estimated-age and to between-reader errors, as well as for the accuracy of the estimated age in relation to the quality of the specimen (*e.g.*, Stewart and Stewart 2005, Frie *et al*. 2011, 2013, Brill *et al*. 2015). The importance of standardised counting methods with reference to known age animals is recognised.

The use of digital images instead of original specimens for age estimation is a relatively new approach to counting GLGs and may affect accuracy or precision. Accuracy of GLGs counts in digital images has been addressed by Evans *et al*. (2002), Christensen-Dalsgaard *et al*. (2010), Frie *et al*. (2011, 2013) and Lockyer *et al*. (2016) for known age animals of sperm whales (*Physeter macrocephalus*), polar bears (*Ursus maritimus*), harp seals, grey seals and beluga whales, respectively. In all cases, variation occurred between readers and between actual known age and GLGs counted. For example, Evans *et al*. (2002) found that counts derived from digital images were significantly higher than those derived from direct examination of teeth, although Frie *et al*. (2011) found no significant differences between digital images and original sections, which may reflect improvements in technology in digital imagery during the intervening years.

There are potential significant advantages to using digital images. For example, they can be easily shared without the same time, financial, and permit (*e.g.*, CITES) constraints applied to original specimens. Digital image characteristics, such as contrast, can be enhanced possibly increasing identification of GLGs, although it has not been determined to what extent enhancing images may influence GLG counts. Examination of ways to interpret the GLGs accurately in relation to actual age have been discussed,

and of how to deal with the problems that arise when age readings do not agree with actual age (*e.g.*, Frie *et al*. 2011).

ANOMALIES

In addition to GLGs, structures used for ageing also contain ultrastructural variation and anomalies, for example, marker lines, accessory lines, pulp stones, dental resorption and cemental disturbance (see Ichihara 1966, Myrick 1988, Lockyer 1993, 1995, Lockyer *et al.* 2010). Anomalies have been used to identify environmental variation, such as El Niño (Manzanilla 1989) and climate change (Boyd and Roberts 1993, Dellabianca *et al*. 2012), life history events (*e.g.*, Klevezal and Myrick 1984, Luque *et al*. 2009b, Medill *et al*. 2010) and stock structure (Lockyer 1995, 1999). In some cases, anomalies were positively correlated to age and occurred more frequently in females than males (Manzanilla 1989, Stewart and Stewart 2005, Luque *et al*. 2009b).

It is not fully understood what causes anomalies, but it is known that the physiological state of an individual is reflected in the morphology of the structure at the time of deposition (Klevezal 1996). Lockyer (1993) found that four out of five wild-caught short-finned pilot whales (*Globicephala macrorhynchus*) maintained in captivity showed a dentinal marker line within 9 months of capture, while the other whale had a pulp stone a few months after capture. It was proposed that the dental anomalies in these individuals were due to nutritional stress shortly after captivity. Nutritional stress due to environmental variation was also associated with a dental anomaly in dusky dolphins (*Lagenorhynchus obscurus*) when the dolphin's main prey item, anchoveta (*Engraulis ringens*), collapsed during the El Niño event in 1982-1983 in Peru (Manzanilla 1989).

Anomalies are not always useful in age estimation studies and may hinder GLG counts. Accessory lines in teeth and ear plugs may confuse the reading of actual GLGs and pulp stones obscure readings (Ichihara 1966, Roe 1967, Klevezal 1980, Hohn *et al*. 1989, Lockyer 1995, Evans *et al*. 2002, Stewart and Stewart, 2014, Garrigue *et al*. 2016) potentially leading to errors in age estimates. Dental anomalies such as accessory lines, pulp stones and dentinal resorption have been reported to be problematic for ageing monodontids (Sergeant 1973, Hay 1980, Perrin and Myrick 1980, Lockyer et al. 1999, Stewart and Stewart, 2014). It is recommended that, during age estimation studies, the occurrence of anomalies should be recorded and the extent, if any, to which they affect age estimates should be noted.

Change in the deposition rate of GLGs, for example, the transition zone, where widely-spaced GLGs become narrower, has been found to indicate attainment of sexual maturity in several species including grey seals and bottlenose dolphins (Hohn 1980, Mansfield 1991) as well as some species of

baleen whales (Lockyer 1972, Lockyer 1974, Masaki 1979, Kato and Sakuramoto 1991). Calving and lactating events have been identified in teeth of captive bottlenose dolphins (Myrick 1991), as well as in free-living spinner (*Stenella longirostris*) and spotted dolphins (*Stenella attenuata*) (Klevezal and Myrick 1984). Furthermore, characteristics of the growth layers, such as bipartite or tripartite sub-annual incremental layers, can make readings misleading (Myrick and Cornell 1990).

ABSOLUTE AGE ESTIMATES

Owen (1845) first documented the presence of 'concentric layers' in marine mammal teeth. Two marine mammalogists contemporaneously pioneered age estimation in pinnipeds using these concentric layers in teeth (Scheffer 1950, Laws 1952) and that work was followed shortly by identification of growth layers in teeth from odontocetes (Laws 1953, Nishiwaki and Yagi 1953, Nishiwaki 1958). Most work since has focused on expanding the methods to other species, on refining methods, and on validation for ensuring that age estimates are accurate and precise (Morris 1972, Fancy 1980, Johnston *et al*. 1987, Hohn 2009). Several techniques have been established and are widely used for ageing marine mammals, some more accurate than others. Due to the vast differences in the biology of marine mammals, however, no 'one size fits all' method exists. Teeth are the primary structure used for age estimation, although other structures used historically or currently include bones, tusks, claws, ear plugs, and baleen plates. Obtaining accurate and precise age estimates requires knowledge of the best tissue available for each species, the deposition rate of growth layers within that tissue, the best practices for preparing the tissue and counting growth layers, and the advantages and limitations of each method (see also Avens and Snover 2013). We will address each aspect.

Absolute age estimates are primarily obtained from counts of growth layer groups (GLGs) in teeth or bones. A GLG has been defined as a "semi-repeating pattern of adjacent groups of incremental growth layers within the dentine, cementum, or bone which is defined as a countable unit" (Perrin and Myrick 1980). Although the definition of GLG is of structures and not time, generally the implied time frame of interest is annual (Hohn 2009). An encompassing definition was needed, however, because annual incremental layers in turn contain sub-annual incremental layers. All incremental layers reflect the physiology of the individual at the time of deposition, thus, they serve as recording structures (Lockyer 1993, Klevezal 1996). Sub-annual incremental layers serve as "marker lines" (Lockyer 1995) if they can be associated with, for example, a life-history event (*e.g.*, Bengtson 1988). Alternatively, and generically, they are referred to as accessory layers, which can interfere with the identification and counting of annual layers and, thus,

influence both the accuracy and precision of age estimates (see 'Anomalies' section).

Teeth

Teeth have proven to be invaluable for age estimation in most species of marine and terrestrial mammals. Dental tissues are highly mineralized and deposition patterns remain largely unaltered throughout life and post-mortem (Medill *et al*. 2010 and references therein). Most cetacean and pinniped species have transitioned from diphyodont to functional monophyodont dentition (Stewart and Stewart 1987, Kubota *et al*. 2000), resulting in permanent teeth representing the full postnatal record for an individual, except in the case of tooth wear. Teeth are readily collected during necropsies and also are one of the few biological samples taken during organised hunts (Lee and Taylor 1994, Frie *et al*. 2011). Teeth are relatively easy to store and handle. Depending on the species, teeth may even be taken from live animals (*e.g*., Garshelis 1984, Hohn *et al*. 1989, Arnbom *et al*. 1992) and, for most species, at least one duplicate sample can be taken for future studies or replication. For some species, the annual deposition rate has been validated (*e.g*., Hohn *et al*. 1989 and Figs 2a and b). Fortunately, patterns of annual layer formation in hard tissues seem to be similar for mammals of different taxa and ecological groups (Klevezal 1980), suggesting that it is reasonable to apply patterns of growth-layer deposition determined from species where validation is available to similar taxa for which there has been no validation (Hohn 1990). For all these reasons, teeth are the 'gold-standard' structure for age estimation.

Within a tooth there are four primary macrostructural components: dentine, cementum, enamel, and the pulp cavity. Dentine is covered by enamel in the crown of the tooth and by cementum in the root. The pulp cavity is the central chamber of the tooth and, due to the deposition of dentine at the edge of the pulp cavity, it fills in with age. Cementum also is deposited throughout the life of the animal, with the most recently deposited layers on the external surface. Thus, GLGs can be found in dentine and cementum. For a more detailed description of the structure of mammalian teeth, see Boyde (1980) and Lockyer *et al*. (2010).

In mammals, dentition is heterodont or homodont. Odontocetes (toothed whales), with few exceptions (*e.g.*, Amazon River dolphins (*Inia geoffrensis*), and narwhals), are homodont, meaning that all their teeth have the same morphology. In homodont teeth, age estimation is primarily from dentine; the cementum is relatively thin. Exceptions include Mesoplodon species and some river dolphins, for which cementum is the primary tissue. In some species, GLGs can be counted in both tissues (*e.g*., beluga whales). Most other marine mammals, including polar bears, sea otters (*Enhydra lutris*) and

the pinnipeds, have heterodont teeth, generally including incisors, canines, premolars, and molars. Age estimation in these species is primarily from GLGs in cement. An advantage of homodont teeth for age estimation is that the dentinal layering pattern is essentially the same among teeth within an individual. While the number of cemental layers would be the same among heterodont teeth, the clarity of those layers and of GLGs in dentine can vary among teeth.

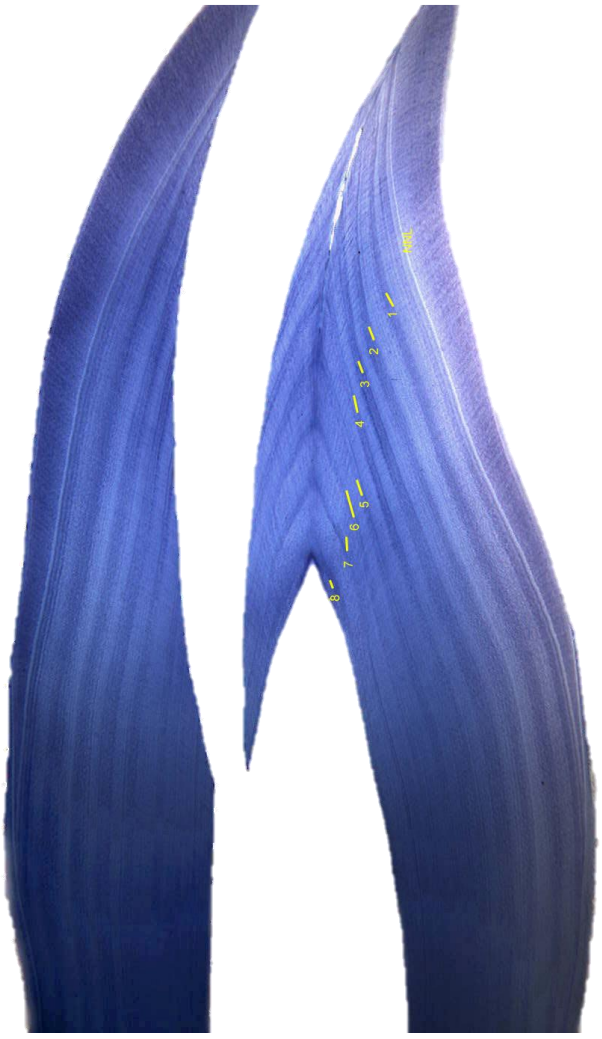

Fig. 2a. Dentinal growth layers identified in stained section of bottlenose dolphin (*Tursiops Truncatus*) age 8 years. NNL is the neonatal line.

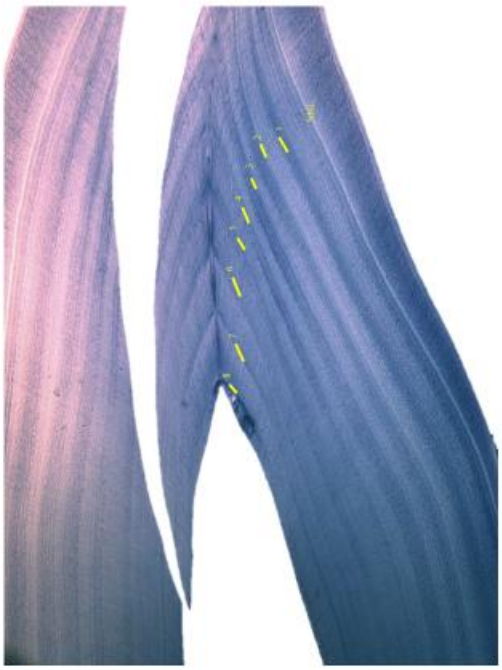

Fig. 2b. Dentinal growth layers identified in stained section of bottlenose dolphin (*Tursiops Truncatus*) age 16 years. NNL is the neonatal line.

Manatees (*Trichechus manatus*) and dugongs (*Dugong dugong*), unique among marine mammals and as a legacy of the common evolutionary ancestry with elephants (genus Loxodonta) (Domning 2009), have continuous replacement of molariform cheek teeth by means of posterior to anterior migration (Domning and Hayek 1984, Lanyon and Sanson 2005). As a result, their cheek teeth are not suitable for estimating age of an individual beyond a minimum age, which is the age of the tooth.

Tusks are another form of tooth found in some species. Dugong tusks (upper posterior incisors) are their only permanent teeth, although generally they erupt only in males. These tusks have GLGs that can be used for age estimation in both males and females, although wear can result in only a minimum age in quite old animals (Mitchell 1978, Marsh 1980, 2009). In walruses, the tusks are elongated upper canines with continuous growth and wear in older animals, erupting in both sexes and also containing GLGs (Stewart and Stewart 2005). Male narwhals have tusks, which are also elongated upper canines, generally erupting only on the left side. Beaked whales of the genus *Mesoplodon* (family Ziphiidae) have two tooth-like tusks half-way along the lower jaw that only erupt in males. Non-erupted teeth do not develop (Dalebout *et al*. 2008).

Narwhals are heterodonts. In both sexes, the upper canines develop into tusks.

In females they usually do not erupt. Generally in males, the left upper canine develops into an elongated, spiralled tusk while the right tusk does not erupt. Occasionally, the one or two tusks erupt in females and males, and some males may be tuskless (Bada *et al*. 1983, Perrin and Myrick 1980, Neve 1995).

A further characteristic of teeth is that growth in length of a tooth is either finite or continuous, which further affects the ability to obtain age estimates. For most marine mammals, tooth growth is limited, that is, teeth reach a maximum length, usually at a relatively young age. In these species, wear may occur but generally it does not extend below the gum. As a result, the neonatal line (reference point for birth) remains present and visible and a full GLG count is possible. The delphinids, phocoenids, and most pinnipeds are included in this category. In species that have continuous tooth growth, the tooth continues to erupt such that the neonatal line eventually extends above the gum. Only a minimum age can be determined once the crown becomes worn to the extent that the neonatal line and first GLG(s) are no longer present, such for dugongs (male tusk wear), sperm whales, and belugas resulting in an underestimation of age (Lockyer *et al.* 1999). There is currently no means to correct for the number of GLGs worn away (Hohn and Lockyer 1999). Brodie *et al*. (2013) suggested that the changing angles of GLG formation within the dentine of beluga teeth might be extrapolated in worn teeth to estimate the number of missing GLGs (see Fig. 2 in Hohn *et al*. 2016) and this should be investigated further.

Because of the variability among taxa in size and shape of teeth, as well as preferred tissue (dentine or cement) for age estimation, for each taxon it is important to determine the appropriate tissue and optimal tooth for counting GLGs. For odontocete species with homodont dentition, tooth selection should not influence estimated age, although Hui (1980) found the pulp cavity in the small posterior teeth in bottlenose dolphins to be open longer than in more anterior teeth. To standardise methods, Perrin and Myrick (1980) and Kuiken and Hartman (1991) recommended taking teeth from the mid-section of the left mandible, although no significant differences between teeth taken from the left or right side of the mandible were noted in sperm whales (Evans *et al*. 2002). Hohn *et al*. (1989) found that the dentinal layering pattern in bottlenose dolphin teeth are like a 'fingerprint' and they were able to identify a previously aged individual that was found badly decomposed using the pattern in the dentine. Although not formally investigated, a GLG layering pattern has also been observed in a replicate tooth of other species including harbour porpoise, short-beaked common dolphins (*Delphinus delphis*) and harbour seals (*Phoca vitulina*) (F. Read, pers. obs). Brodie et al. (2013) found that longitudinal sections of beluga teeth exhibit a general pattern of GLG deposition which appears to be consistent between populations. However,

some species-specific variation exists, *e.g.*, teeth from the middle of the mandible are generally the largest and least worn in beluga whales (Heide-Jørgensen *et al*. 1994) so they offer the best opportunity for obtaining the maximum count of GLGs (Lockyer *et al*. 2016).

In species with heterodont dentition, some differences have been documented in GLG counts or characteristics among teeth (*e.g.*, Bernt *et al*. 1996, Stewart and Stewart 2005, Medill *et al*. 2009). In ringed seals (*Pusa hispida*), GLGs counts from the first post-canine were more accurate than those from the second incisor (Chambellant and Ferguson 2009), affecting which tooth is best to pull from live animals. Bernt *et al*. (1996) found that age estimates from canines were more accurate and less variable than those from incisors in stained sections of teeth from grey seals. In contrast, in harbour seals, Blundell and Pendleton (2008) found high correlations between GLG counts in canine, post-canine, and incisor teeth. In walrus, Stewart and Stewart (2005) found GLGs in canines to have higher clarity than the first and second post-canines, especially for males, resulting in higher GLG counts. In general, however, they determined that the first three post-canines would be suitable alternatives for the canine in males while only the first post-canine was a suitable replacement for canines in females, indicating that a sex effect (due to differences in tooth size) might also be a factor to consider. Stewart and Stewart (2005) found that if one type of tooth was difficult to read, the others were also difficult to read. Medill *et al*. (2009), with an interest in using changes in GLG growth in cement to reconstruct life history, found differences in GLG widths in different teeth and different aspects of the same teeth. It is not always possible to choose the optimal tooth for ageing, particularly when teeth are extracted from live animals (Chambellant and Ferguson 2009, Lockyer *et al*. 2010), so it is important to know if inter-tooth differences exist in the number of readable GLGs, the magnitude of any differences, and whether the differences are predictable and, thus, correctable.

Electing the use of either dentine or cementum as the primary ageing tissue could have critical implications. Dentinal growth is centripetal, progressively occluding the pulp cavity, while cemental growth is centrifugal with different constraints in its development (Lockyer *et al*. 2010). Generally, cementum in delphinids and phocoenids is not well developed and has inconspicuous layers so dentine is used for age estimation (Lockyer *et al*. 1981, Myrick *et al*. 1983, Hohn 2009). Cementum normally provides better clarity of GLGs for carnivores such as pinnipeds, sea otters and polar bears because the pulp cavity often becomes occluded (*e.g.*, McLaren 1958a,b, Hewer 1964, Dietz *et al*. 1991, Garlich-Miller *et al*. 1993, Stewart *et al*. 1996, Calvert and Ramsay 1998, Amano *et al*. 2000, Lockyer *et al*. 2010) or dentine deposition is too irregular to resolve GLGs, especially last-deposited small layers (Myrick *et al*. 1983, Bjørge *et al*. 1995, Hohn 2009).

Childerhouse *et al*. (2004) examined the lower first canine tooth from known-age New Zealand sea lions (*Phocarctos hookeri*) and reported that GLG counts in cementum were more accurate than those in dentine. Cementum is also used to age most species of beaked whales (Perrin and Myrick 1980) and the Franciscana (Pinedo and Hohn 2000), and is equally as valuable as dentine in South Asian river dolphin (*Platanista gangetica*) (Lockyer and Braulik, 2014). For pinnipeds, the use of dentine for young animals and cementum for older animals has been suggested by several authors (*e.g.*, Smith 1973, Bowen *et al*. 1983, Murphy *et al*. 2012). Stewart *et al*. (1996) found a good correlation between the two tissues in ringed seal up to the age of about 10 years. In general, and for consistency, dentine should be used for species in which the pulp cavity does not become occluded early in life and cementum for species in which it does. In the best cases, both tissues contain countable GLGs and the counts from each tissue can help with overall accuracy and precision of age estimates.

Attempts to estimate age in narwhals have been made using GLGs in stained and untreated sections of the mandibular embedded (unerupted) tusks (Hay 1980, 1984, Neve 1995, Garde *et al.* 2012, Stewart 2017). Cementum in the embedded tusk was not found to be useful for ageing narwhals because it is extremely thin and not evenly distributed (Hay 1980). In contrast, Neve (1995) found that although ageing is often confounded by compression of the GLGs, in the embedded tusk cementum was readable and the results correlated with dentine and mandibular GLG readings. However, only 3 of 96 animals with more than 20 GLGs were readable, and overall 47% of specimens could not be aged accurately from GLG counts (Neve 1995).

The use of dentine for age estimation in narwhals is also restricted because at the age of sexual maturation (around 9 GLGs) the dentine in embedded tusks becomes occluded so only a minimum age can be estimated (Hay 1980, Neve 1995, Garde *et al*. 2012, Stewart 2017). Prior to dentinal occlusion, mandibular, dentinal and cemental GLGs were found to be deposited at the same rate. After occlusion, mandibles consistently show more GLGs than dentine (Hay 1980) and cement (Neve 1995). Acid etching of the embedded tusk made the neonatal line invisible in many specimens, and the high frequency of accessory lines (up to 6-9 per GLG) were found to confound true GLGs (Hay 1980, Perrin and Myrick 1980). More recently, Garde *et al.* (2012) successfully used bisected and acid-etched tusks to count GLGs and estimate age. The results were very promising, but because of the valuable nature of the tusks and the difficulties in preparing them for age estimation, only a few have so far been examined.

For a detailed explanation of extraction, cleaning and storage of tooth samples for age estimation studies see Lockyer *et al*. (2010). After extraction, two

methods have traditionally dominated for preparing teeth for age estimation: 1) "thick" sections not otherwise treated, that is, not decalcified or stained, and 2) decalcified and stained thin sections. The methods will be further referred to as 'untreated' and 'stained', respectively. Early studies used untreated sections but more recently the use of untreated or stained sections is seen as species- or taxa- specific, with one technique better for some than others. While these two methods significantly overlap in the initial and ultimate stages, the end products are quite different and the resulting age estimates may differ. Thus, in general, when different teeth and/or methods have been used, 'pooling data' should be avoided unless results have been calibrated and demonstrated to be equivalent.

The main advantage of using untreated sections is that they are cost-effective and time-efficient relative to stained sections, facilitating processing of large sample sizes. Probably the most valuable use is for counts of cemental GLGs from transverse sections of some pinniped teeth: for example, hooded seals (*Cystophora cristata*; Born 1982) and harp seals (Fisher 1954, Bowen *et al*. 1983, Frie *et al*. 2011) and grey seals (Frie *et al*. 2013) (Fig. 3). Untreated sections may also be more practical for counting GLGs in dentine or cement in longitudinal sections of large odontocete teeth, such as sperm whales or killer whales (*Orcinus orca*) and beluga whales (Stewart 2012, Stewart and Stewart 2014). Untreated sections are needed for mineral chemistry (Evans *et al*. 1995) or other studies when the mineral must remain in the tooth (*e.g.*, Manzanilla 1989).

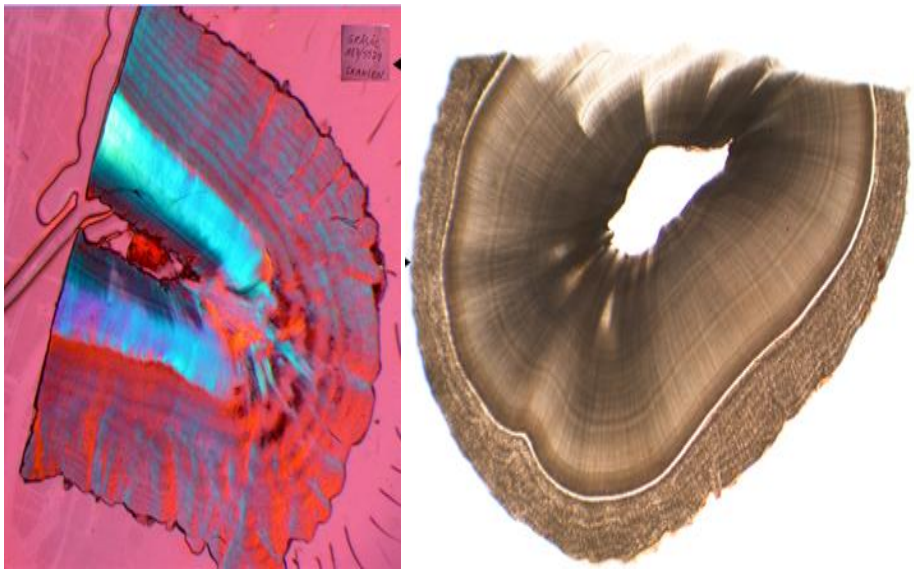

Fig. 3. Grey seal (*Halichoerus grypus*) untreated tooth sections, sections in the longitudinal plane and viewed under polarised light (left) and in the transverse plane viewed under plain light (right).

Untreated sections range in thickness from about 150 µm (grey seals, Mansfield 1991 and harp seals, Frie *et al*. 2011) to 350 µm (walruses, Stewart and Stewart 2005). A thick section from a tooth is relatively easily obtained by grinding or sawing the whole tooth to produce a section containing the desired part. The type of saw depends on the size of the sample. A low-speed saw with a circular diamond-embedded blade (*e.g.*, Buehler Isomet) can be used for most teeth, although for very large teeth, such as adult sperm whales, use of a saw with a larger blade, *e.g.*, hack saw or a slow-rotating band saw is needed. With the Isomet saw, two parallel blades can be used simultaneously to produce a 'wafer' of the desired thickness with a single cut. The preferred type of cut (longitudinal for odontocetes, or transverse for some pinnipeds) and the thickness of the section depend on the species. The initial cut should be slightly off centre, allowing for the kerf (blade thickness), to ensure the resulting section reveals the medial surface.

With use of the low speed saw, untreated sections are cut after attaching the tooth to a platform that will fit a chuck that comes with the saw. Simple, inexpensive methods, such as a hot glue gun, can be used to attach the tooth to the platform, *e.g.*, a small block of wood (see Appendix 3 in Lockyer *et al*. 2016). Polyester resin embedding has been explored (Wainwright and Walker 1988) although not widely adopted, possibly because it takes more time than other simpler methods, may alter the teeth, and may complicate future chemical analysis (Keklikoglu and Akinci 2013). There is potential value for this method for species with straight teeth, or for species with curved teeth (such as harbour seals) when cementum is the desired tissue and therefore maintaining the crown as part of the section is not important. Embedding of the tooth is recommended when dry, fragile teeth *e.g.*, museum specimens, that would otherwise fragment, are sectioned.

Manual polishing or grinding of a whole tooth may be required when a saw blade would remove too much tissue from teeth 2-3 mm in diameter. Polishing of the cut surface of a section may be required if the initial section is too thick or the cut surface is rough, such as might occur when the sectioning is done with a band saw; diamond-embedded blades used with the low-speed saw cause minimal scratching of the exposed surface. Teeth and/or sections are polished with grit-paper or sandstones to the appropriate thickness. Sydney and Monteiro-Filho (2011) embedded estuarine dolphin (*Sotalia guianensis*) teeth in resin, ground the resin and tooth down until the central point of the tooth was reached, and then acid etched (see below) the specimen to count the GLGs.

Untreated sections may be mounted permanently or stored in a vial, wet or dry, and temporarily placed on a slide for reading. Resin-embedded sections

may be left in the resin or removed and then handled as other untreated sections.

Untreated sections are generally viewed with a dissecting (stereo) microscope. Enhancements employed in attempts to aid GLG clarity or resolution include polarized light which may increase contrast between adjacent GLGs due to birefringence of the tooth mineral (Figs 3 and 4). Application of polarized light is easy and inexpensive, essentially just requiring polarizing filters used in conjunction with the microscope.

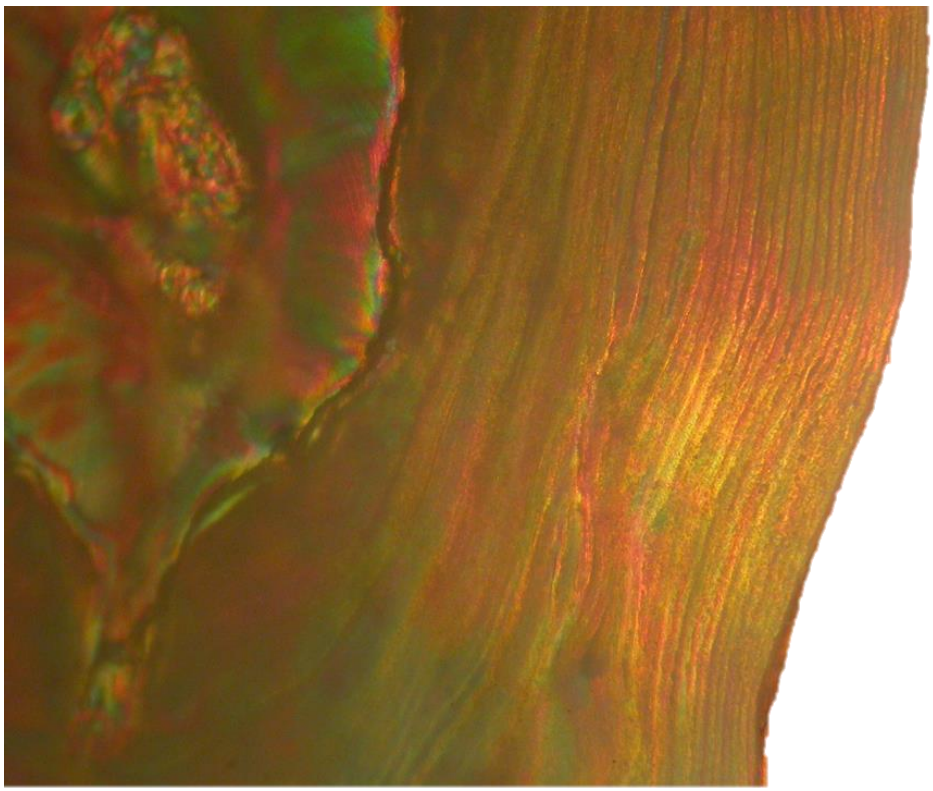

Fig. 4. Walrus (*Odobenus rosmarus*) cementum in untreated tooth section viewed under polarised transmitted light.

For untreated thin sections or half sections, acid etching has also been used to increase the clarity of GLGs. It relies on the differential demineralization of GLG tissue (Hohn 1980). The cut surface of the tooth is exposed to 5-15 % formic (or other) acid to remove the superficial calcium. After rinsing and drying (usually some hours), alternating grooves and ridges representing the GLGs are exposed. The tooth surface can be rubbed with a soft lead pencil to emphasise the etched surface. Acid etching is a simple and relatively inexpensive method that requires minimal equipment.

Acid etching has been used on teeth from several cetacean species including various delphinids, beluga whales, Cuvier's beaked whales (*Ziphius cavirostris*), sperm whales (Gambell and Grzegorzewska 1967, Hui 1980, Pierce and Kajimura 1980, Myrick *et al*. 1988, Evans and Robertson 2001, Evans *et al*. 2002, Best *et al*. 2010, Sydney and Monteiro-Filho 2011), and pinnipeds (Pierce and Kajimura unpublished from Pierce and Kajimura 1980). Acid etching did not improve readability in longitudinal sections of beluga teeth (Perrin and Myrick 1980, Pierce and Kajimura 1980). These studies all concluded that acid etching works for most large cetacean species but that for small species, for example harbour porpoises and most delphinids, and young animals, the resolution is insufficient and stained sections are better (Pierce and Kajimura 1980, Evans and Robertson 2001). A challenge of acid etching is resolving the small GLGs deposited in dentine of older animals and without which age would otherwise be underestimated (Hohn 1980). No rigorous comparative study of counts from etched sections relative to stained or untreated sections has been published. For a young killer whale (Fig. 5) clarity of an etched and a stained section were similar but, contrary to Best *et al*. (2010), age estimation from acid etching was not possible for an older killer whale aged >20 years (F. Read, pers. obs.). Reflected light is generally used for reading GLGs in etched teeth.

Fig. 5. A comparison of GLG clarity from an acid etched (left) and stained, decalcified section (right) of a killer whale (*Orcinus orca*) aged 9 years.

Etched sections have also been viewed via scanning electron microscopy (SEM) to create high-resolution images. SEM produces a 3-dimensional image of the tooth surface, allowing high clarity, and, therefore, more

accurate interpretation of the GLGs (Hohn 1980). There is also a reduced effect of dental anomalies, for example, accessory lines, confounding GLG readings. This method has been used to age bottlenose dolphins (Hohn 1980) and harbour, grey and ringed seals (Mackey 2004). Both researchers found that readability is equal or superior to that of the untreated sections. Additional information, for example, for tooth composition, can be obtained on non-plated samples by using mass spectroscopy (Mackey 2004). SEM was not found to be useful for ageing belugas because, although GLGs were visible, they were not countable from the photomicrographs (Goren *et al*. 1987). The method is more complicated, expensive and time consuming than other methods (Hohn 1980, Mackey 2004).

Microradiography (MRG) also takes advantage of the mineralization in the tooth and produces a high-resolution image of a thin section (150µm) on specialised photographic film. The main advantage of MRG is its sensitivity to spatial variation in mineral densities, which allows for identification of GLGs, and resulting non-destructive images can then be examined with transmitted light microscopy (Hohn 1980). In bottlenose dolphin teeth, MRG resulted in higher resolution of the older, smaller GLGs around the pulp cavity relative to SEM and untreated sections (Hohn 1980). Additionally, the process of cyclic mineralization and the mechanisms influencing it can be determined and may be related to other aspects of the animal's biology, such as life-history events. MRG requires high precision in section thickness and x-ray time, and the film is relatively expensive, thus, the method is not considered feasible for ageing a large number of specimens (Hohn 1980).

Despite the relative ease of preparation of untreated sections, there has been a shift away from their use for most species. In comparative studies, age from untreated teeth from ringed seals (Stewart *et al.* 1996) and bottlenose dolphins (Hohn and Fernandez 1999) were substantially underestimated in older individuals relative to stained sections. Thus, the primary disadvantage of untreated sections is inherent bias in age estimates. Unless already tested for a species (or taxonomic group), it would be necessary to compare counts from untreated and stained sections to ensure results are equivalent.

Due to the limitation of using untreated teeth for many species, use of stained sections has become prevalent. Lockyer (1993) and Lockyer *et al.* (2010) provided detailed reviews of methods for preparing thin stained sections. Here we provide a summary of methods. For many species, stained sections highlight GLGs better than untreated sections, which is particularly important for accurate counting of the small, last-deposited GLGs in dentine (Hohn and Fernandez 1999) and increasing the clarity of cemental layers (Stewart *et al*. 1996). The disadvantages of stained sections are that the process requires additional equipment, is more complicated, and takes longer. Nonetheless, if

accuracy and precision are sacrificed by using a simpler, faster method, choose the method that provides the most robust age estimates.

The fundamental steps of preparing stained sections are the same. Variation may occur depending on which tissue is of interest (dentine or cementum), what equipment is available, and researchers' preferences, for example the type of stain. The initial stage of the preparation requires decalcifying (after formalin fixation). Small teeth, such as incisors or molars or teeth from small odontocetes, for example striped dolphins (*Stenella coeruleolba*), can be decalcified whole (Perrin and Myrick 1980). For large teeth, it is recommended to first prepare a thick, central section (wafer) of the tooth 2.5–3 mm thick encompassing the centre of the tooth (Hohn *et al*. 1989) then decalcify the wafer. Otherwise, the outer tissue will become over-decalcified before the centre has fully decalcified.

Various decalcifying agents have been used (for a generic review of the advantages and disadvantages of various decalcifying agents, see Sheehan and Hrapchak 1980). In earlier studies, nitric acid solution was frequently used for decalcification, with the process lasting from several days to weeks (Grue and Jensen 1979, Dietz *et al*. 1991, Norgaard and Heje-Larsen 1991, Slooten 1991). Formic acid, although a decalcifying agent, is more commonly used for acid etching (*e.g.*, Evans *et al*. 2002, Sydney and Monteiro-Filho 2011). In more recent years, the development of commercially prepared mixtures of acids, such as RDO (Apex Engineering Products Corporation, Illinois, USA), has significantly lowered decalcification times to hours. Murphy *et al*. (2014) investigated the use of 4 different decalcifying agents, and found that Formical-4™ (Decal Chemical Corporation, New York, USA) provided the best sections for age estimates of two captive short-beaked common dolphins known to be over 30 years old. However, decalcification took 4-6 weeks. Regardless of the agent used, decalcification time is dependent on the density and size of the tooth (or wafer) and the size of the pulp cavity (Lockyer 1993, 1995, Hohn and Fernandez 1999, Lockyer *et al*. 2010). Additonally acid age, temperature, and the degree of pH-neutralisation due to re-use all impact the decalcification time (Johnston *et al*. 1987).

During the decalcification process, gentle mechanical agitation of the solution is recommended. The end point can be determined physically, by the pliability of the specimen, or chemically, by an ammonium oxalate turbidity test which detects the presence of calcium ions (Yalman *et al*. 1959). For larger teeth, radiography can be used to determine if a tooth is fully decalcified. If a tooth is not fully decalcified, it is possible to re-submerge it in acid after beginning sectioning provided the sectioning has not progressed too close to the medial line of the tooth.

The primary equipment used for thin sectioning is a freezing-stage microtome, which can be a sledge microtome with a freezing stage or a portable radial microtome that clamps to a table top. Both are referred to as 'freezing microtomes'. The stage with the sample is cooled by electrical means or a gas to between -10 and -15°C, depending on the size of the sample, while the knife and body of the microtome remain at room temperature. In these models, the stage is fixed and the blade is mobile. An alternative device for sectioning frozen samples is a cryostat, which is a microtome in a freezing cabinet wherein the entire chamber and internal equipment, including the knife, is maintained at below-freezing temperature. In many models the temperature of the cabinet, the blade and the freezing stage can all be controlled separately. In the cryostat, the type of microtome is usually a rotary model, where the blade is fixed and the freezing stage is mobile. The two platforms are quite different, and it is important to be clear about which device was used, which is not always the case in the literature.

Use of the cryostat is more time-consuming than for the freezing microtome (Klevezal 1996). Both techniques work equally well for small teeth, but in the cryostat the small size of the stage and blade limits use to small teeth. A large stage can be used to section larger teeth with a cryostat, but even with experience using a cryostat for sectioning teeth, it is very difficult to get good sections from large teeth, *i.e.* the most central sections and uniform thickness (F. Read, pers. obs.), perhaps because of the number of temperature variables to control and optimise (blade, stage and cabinet) in commercial cryostats. Therefore, for larger specimens, such as bottlenose dolphins and pilot whales (*Globicephala spp.*), it is advised to use a sledge-type freezing microtome which can accommodate a significantly bigger platform for the specimen and has a more robust blade, and more control over the orientation of the sample for sectioning. On the whole, use of a cryostat is more complicated than that of a freezing microtome, and the cost of the former, at outlay, is significantly more than that of the freezing microtome.

After thin-sectioning, the desired sections are stained, customarily with Mayer's haematoxylin (see below), and mounted on glass slides. The section thickness found most effective for resolving GLGs in dentine and cement is commonly 10-25 µm for cementum studies and 20-25 µm for dentine. GLGs in dentine generally are read using a binocular or compound microscope at magnifications from 10x to 100x, depending on the size of the tooth, and up to 150x for cement (*e.g.*, Franciscana dolphin, Pinedo and Hohn 2000).

Paraffin embedding for thin sectioning has also been explored. Slooten (1991) used a rotary microtome to obtain 2-4 µm thin sections from Hector's dolphins (*Cephalorhynchus hectori*) teeth embedded in paraffin. Stewart *et al.* (1996) mentioned the possibility of using paraffin embedded samples for

neonatal ringed seals to prevent damage of delicate teeth in the cryostat. Luque *et al*. (2009a) found no significant differences in GLG counts between sections cut from paraffin blocks on a rotary microtome and those cut using a cryostat. While age could successfully be estimated using paraffin embedding for larger teeth such as bottlenose dolphin teeth and pygmy sperm whale (*Kogia breviceps*), the method worked best with small teeth, for example, harbour porpoise. It did not work well for the dwarf sperm whale (*Kogia sima*).

Advantages of paraffin embedding are that the block can easily be stored for further sectioning if necessary (Slooten 1991) and embedding helps sections remain intact after cutting rather than disintegrating. Disadvantages include that (1) as with the freezing microtome and cryostat, the equipment is expensive, (2) the method requires some knowledge and experience of histological techniques, (3) the additional chemicals required are expensive, (4) the process is more time-consuming than using a freezing microtome (Luque *et al*. 2009a), (5) paraffin-embedded samples are restricted to the size of the histological cassette that holds the sample in the microtome, and (6) a number of studies have found that GLGs are better resolved in the thicker sections produced using the freezing microtome than the thin sections cut using a rotary microtome. It can also be difficult to 'set' the tooth at the correct angle in the paraffin to avoid off-centre sections, thus rendering the final product sub-optimal.

The orientation of the section also could have critical implications. To obtain the most accurate age estimate from dentine, it is important to produce mid-longitudinal (central) cuts which include the crown and the maximum area of pulp cavity, generally in the buccal-lingual plane (transverse to the jaw). At least in harbour porpoise and the South Asian river dolphin, GLGs are equally clear in sagittal sections (parallel to the jaw) (Fig. 6). The objective of section orientation is to ensure that all of the GLGs are visible along the pulp cavity. For a few species of pinnipeds, transverse sections from the midpoint (maximum circumference) of the tooth have been commonly used (e.g., Bowen *et al.* 1983), although Stewart *et al.* (1996) found that for ringed seals stained longitudinal sections provided higher GLG counts and more agreement between readers. They also found that, below the age of 10, there was little agreement between paired cementum-dentine readings although the overall difference was not statistically significant. After that age cemental GLG counts became increasingly greater than dentinal counts. A similar study would be warranted for other species. With regard to cemental GLGs, it is critical that all of the GLGs are visible. In as much as cementum thickness and appearance are not equal along the length of the tooth and the thickest area tends to be at the root, counting at or near the root has been recommended (Stewart *et al.* 1996, Lockyer *et al.* 2010). However, other regions higher

along the sides below the gum line may have greater clarity (as in polar bears and harbour seals – C. Lockyer and F. Read, pers. comm.)

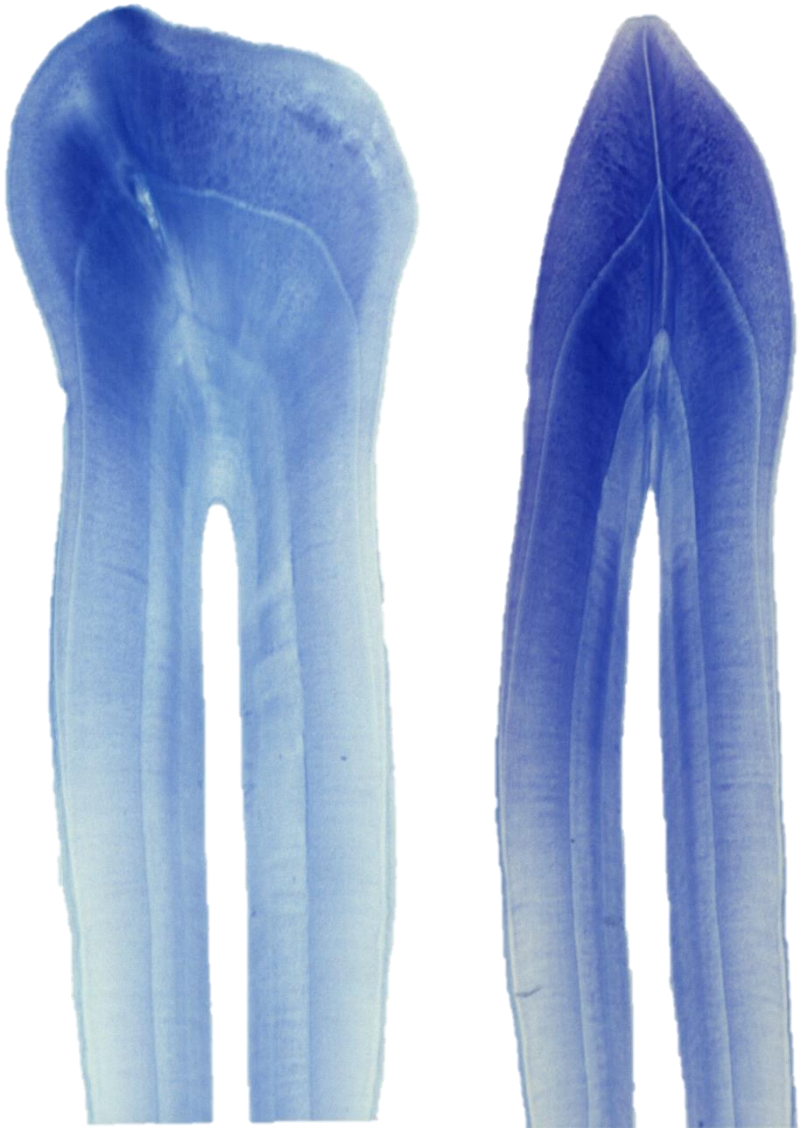

Fig. 6. Harbour porpoise (*Phocoena phocoena*) teeth from the same animal cut at two planes; sagittal plane (left) and buccal-lingual plane (right).

Various stains have also been tested. Luque *et al*. (2009a) tested four stains previously reported in the literature and recommended Mayer's haematoxylin overall. A commonly used modification of Mayer's haematoxylin that eliminates the need for aging of the stain is provided in Myrick *et al*. (1983). Lockyer *et al*. (2010) reported that GLGs in the cementum of harbour seal tooth sections are more prominent with Toluidine Blue while haematoxylins

such as Mayer's or Ehrlich's were better for dentine. Murphy *et al*. (2014) suggested that Toluidine Blue may have a better affinity for tissues decalcified using organic acids and chelating agents. Lockyer (1993, 1995) preferred Ehrlich's acid haematoxylin as it is more durable and does not fade so rapidly over time. Lockyer and Read prefer Toluidine Blue stain (pers. comm.) for examining cemental GLGs (as in polar bear (Fig. 7), ringed seal, harbour seal and most other carnivore species). It is advisable to store stained sections in the dark, as light will accelerate fading.

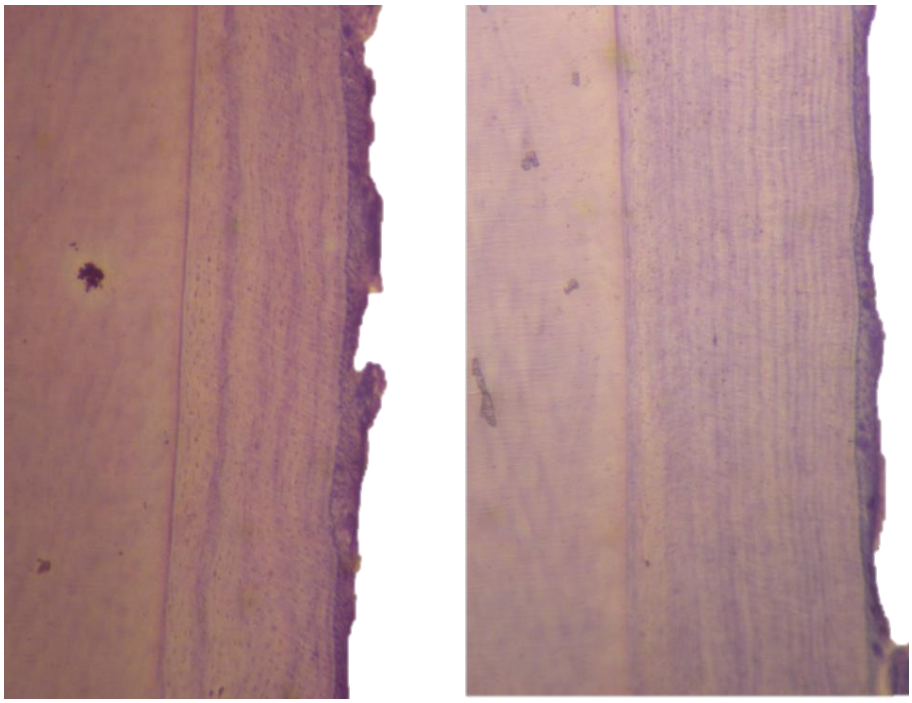

Fig. 7. Polar bear (*Ursus maritimus*) cementum in animals of differing ages (5 GLGs, left and 17 GLGs, right), sections stained with toluidine blue.

For sections requiring longer immersion (many minutes) in stain (*e.g.*, haematoxylin types), sections are frequently left free-floating in a cassette or small container (for larger teeth) with a means of agitating the liquid. With rapid stains, such as Toluidine blue which requires only a few seconds, sections are generally pre-mounted on gelatine-coated slides before staining.

In addition to the foregoing, age estimation of belugas using thick (150-200 µm) untreated longitudinal sections of teeth were recommended for reading dentine while thin stained sections were recommended for cementum examination (Perrin and Myrick 1980). GLG counts from cementum and dentine agree approximately, although dentine counts can be higher (Sergeant 1973).

In many species, cementum is often difficult to read due to compression of and less well defined GLGs relative to dentine (Lockyer *et al*. 1999, Stewart *et al*. 2006). Sergeant (1973) reported that female belugas generally had thinner dentinal and cemental GLGs than males making age estimation more difficult. Details for methods of collection, preparation and age estimation have been compiled by Stewart (2012) and Stewart and Stewart (2014). In addition, the report of the Beaufort Workshop on Age Estimation in Belugas (NAMMCO 2013 (summary), Lockyer *et al*. 2016) gives specific methods and reading techniques.

Claws

Historically, claws have been used for age estimation in several pinniped species, for example, Baikal seals (*Pusa sibirica*, formerly *Phoca sibirica*) (Pastukhov 1969), ringed seals (McLaren 1958a) and bearded seals (*Erignathus barbatus*) (McLaren 1958b, Benjaminsen 1973). In Benjaminsen's (1973) study, claws were removed and sectioned longitudinally to 500-800 µm and GLGs read with the unaided eye and compared to GLGs from a canine tooth. Results showed that GLGs in claws and canine teeth agreed until the age of eight; older GLGs had worn away in claws. The advantage of using claws is that the method is quick, easy, and inexpensive. The disadvantage is that the use of claws for age estimation is only applicable to young animals due to wearing away of the GLGs distally. The method is no longer used for age estimation in marine mammals.

Baleen

Scoresby (1820) first suggested using baleen for age estimation although the technique of counting baleen GLGs was applied only many years later (Ruud 1940). Similar to claws, also of epidermal origin and composition, baleen wears down relatively rapidly at the distal end and is constantly replaced from the root so GLG counts are only applicable for young individuals. In fin whales (*Balaenoptera physalus*), after 3 years of age the neonatal mark on the baleen plate begins to wear off (Ichihara 1966). Chittleborough (1959) found that GLG counts were not accurate age estimators in humpback whales (*Megaptera novaeangliae*) over 5 years old, while Rice and Wolman (1971) found no more than 4 GLGs in grey whale (*Eschrichtius robustus*) baleen. The use of this method is severely limited given that its application is to species that do not become sexually mature until later than 5 years of age and are long-lived (for example, minke whales (*Balaenoptera acutorostrata*; Lockyer, 1984), bowhead whales (Lubetkin *et al*. 2008), humpback whales (Clapham 1992), fin whales (Lockyer 1972) and sei whales (*Balaenoptera borealis*; Lockyer 1974). Relative age can be estimated from baleen length in bowhead whales (*Balaena mysticetus*) under 2 years old (Lubetkin and Zeh 2006).

Ear plugs

Purves (1955) discovered alternating laminations of dark and light areas in the core of fin whale ear plugs and suggested that they might be useful for estimating age. Ear plugs have subsequently been used for other species of baleen whales including southern hemisphere blue whale (*Balaenoptera musculus*), humpback, minke and sei whales (Ohsumi 1979, Lockyer 1974, Lockyer 1984, Gabriele *et al*. 2010). The advantage of using ear plugs is that the GLGs persist throughout life and are not affected by resorption and wear (Lockyer 1984, Gabriele *et al*. 2010). Ohsumi (1979) reported ages of 110 and 114 years old for a southern hemisphere blue whale and fin whale, respectively.

To expose the GLGs, ear plugs are bisected longitudinally using a traditional cut-throat style razor blade (or similar style large disposable blade in holder) to pare away tissue, and the new surface is polished with a wet sandstone (Chittleborough 1959, Lockyer 1974, Ohsumi 1979, Lockyer 1984). Lockyer (1974) found that exposing the cut ear plugs of sei whales to a weak solution of hydrogen peroxide increased the number of samples classified as 'satisfactorily readable' from 50 to 93% by lightening the pigmentation. GLGs are often poorly defined in young individuals. Accessory lines may confuse readings (Ichihara 1966, Roe 1967) and counting accessory lines might inflate age estimates, especially in immature animals. Sometimes the most recently formed GLGs become compressed in older individuals making complete identification difficult, so that ages of old animals might, on occasion, be underestimated (Chittleborough 1959, Lockyer 1974). Age at sexual maturation can also be determined from the transition phase when widely spaced GLGs become narrower (Lockyer 1972, Lockyer 1974, Masaki 1979, Kato and Sakuramoto 1991; Fig. 8).

Ear plugs are challenging to collect and the method is restricted to a few species of whales (Lockyer 1984). For example, minke whales have soft small ear plugs that are often damaged during the extraction stage, preventing their use for age estimation. Maeda *et al*. (2013) found that extracting minke whale ear plugs is more successful if gelatine solution is injected into the outer ear canal surrounding the ear plug and cooled with gas to solidify it before extraction. This method allowed the complete ear plug to be extracted and readability was found to be improved.

Ear bones

The auditory bulla ("ear bone") in cetaceans comprises the periotic and tympanic bones, both of which are exceptionally dense, compact bone (Ketten 1992, Ketten *et al*. 1992) that contain GLGs in the outer periosteal, compact bone (Klevezal and Mitchell 1971). Because there is little to no remodelling

(Buffrénil *et al*. 2004), these bones have more potential that most other bones for absolute age estimation. Preparation is as untreated or stained sections using methods similar to those previously described for teeth, although most early studies used untreated sections. Calibration of deposition rates has been conducted by comparing GLGs counts in ear bones to counts in ear plugs with various results. For example, GLG counts in bullae were lower than counts from ear plugs in older animals (fin whales, Konrádsson and Sigurjónsson 1989), while in other studies counts were similar (minke whales, Christensen 1981, Sigurjónsson 1989). It should be noted that known age is not generally possible in baleen whales, so that calibration using a standard known age is not feasible. Use of the ear bone for age estimation of harbour porpoises was inconclusive (Perrin and Myrick 1980). Given that GLGs in bullae are compressed, similar to the small, late-deposited GLGs in dolphin teeth, additional studies using stained sections seem warranted.

Fig. 8. Fin whale *(Balaenoptera physalus*) ear plug aged to be >52 years showing the neonatal line, GLGs becoming more compacted with age and a transition of GLGs at ca. 14 years. Photo courtesy of Sverrir Daniel Halldórsson.

The periotic dome of the tympano-periotic complex has been used for age estimation in manatees (Marmontel *et al*. 1996, Brill *et al*. 2015) (see Fig. 4 in Brill *et al*. 2015). Following the same method as stained tooth sections, stained sections of the periotic bone provided higher GLG counts in comparison with teeth, mandibles and ribs due to slower resorption rates. The authors validated the method with known-aged animals and concluded that GLGs in the periotic bone are also useful for identifying life-history and

environmental events (Marmontel *et al*. 1996).

The advantages of using ear bones are that they are found in species without teeth, the GLGs have a life-long persistence or, at least, a low rate of remodelling or absorption relative to other bones, and they are relatively easy to collect from dead animals and store. However, as with cementum in teeth, GLGs are not consistent throughout the ear bone tissue, requiring that the best location for a species be identified. Also, particularly with the need to evaluate GLGs in stained sections, laboratory equipment similar to that used for preparing stained teeth will be required.

Other bones

The use of growth layers in non-tympano-periotic bones for age estimation has been investigated in several species (Laws 1960). GLGs were not observed in histological sections of manatee ribs (Odell 1977), but were observed in those of dugongs (Domning and Myrick 1980, Marsh 1980). GLGs have been detected in the mandible of manatees (Domning and Myrick 1980), common dolphins (*Delphinus* spp.) (Kleinenberg and Klevezal 1962, Gurevich *et al*. 1980), harbour porpoise (Buffrénil 1982), sperm whales (Nishiwaki *et al.* 1961) and walruses (Petersen and Born 1982). In contrast, no clear GLGs were observed in the mandibles of sei and fin whales (Klevezal and Mitchell 1971). In the mandibular bone of narwhals substantial resorption of the tissue occurs, and the neonatal line is often lost. In older females, there is a balance of deposition and resorption in the mandible, and so age estimation using mandibles is more accurate in males than females (Hay 1980). Neve (1995) found that 41% of specimens could not be aged accurately with mandibular GLGs. Lockyer (unpubl. data) found multiple GLGs in stained mandibular sections from an elderly harbour porpoise whose teeth had fallen out. Brodie (1969) also reported on mandibular layering and age estimation in belugas.

Bone is lost due to remodelling and resorption, resulting in loss of the GLGs, and most bones do not meet the requirement for ageing of having negative allometry, *i.e.*, growing more slowly than the skeleton as a whole (Klevezal 1996). Thus, most studies have found significantly fewer GLGs than in other hard tissues, *e.g.*, teeth, in the same animal, especially in older animals (Nishiwaki *et al*. l961, Kleinenberg and Klevezal 1962, Petersen and Born 1982, Garlich-Miller *et al*. 1993). Therefore, the use of most bones to obtain absolute age estimates is unsatisfactory when age can be obtained using other tissue or methods.

Myrick (1980, p. 111) stated that ‘cemental, dentinal, and periosteal layering probably is influenced by the same physiological mechanism. This may eventually permit use of all three systems interchangeably in estimating ages

of old animals.' Interchangeability in methods is ideal but validation is required to prevent potential biases and uncertainties to be incorporated into data analysis, and if possible, avoided. Most work on age estimation using bones (with the exception of ear bones) is out-dated and advances in age estimation methods have left these methods obsolete for most species.

RELATIVE (CATEGORICAL) AGE METHODS

Despite the progress made with methods to estimate absolute age, there are situations in which it is not possible to obtain the necessary tissues for ageing and for many species these methodologies do not work well. In such cases, other, often innovative and novel, techniques have to be developed. Many of these methods do not produce a chronological (absolute) age but rather a relative age. Nonetheless, when using these methods the goal is to produce relative age estimates close to the absolute age without the limitation of estimating an age for only the youngest animals or having to use broad categories such as neonate/pup/calf, immature, and mature.

Radiography

Several researchers had used x-rays in for age estimation, but the attempts were not successful and only mentioned in passing. Norgaard and Heje-Larsen (1991) used radiographs (x-rays) of the lower canines of harbour seals from the Wadden Sea to determine age. They examined the relative thickness of dentine and cementum on x-rays to distinguish juveniles from adults and assign young seals to age classes but age could not be determined. Marsh *et al*. (1980) noted earlier use of x-ray images of dugong tusks and found that a section 1 mm thick worked best. They concluded that more 'trial and error' studies would be needed to get the settings right, but that x-rays were too expensive to consider as a viable option for tusks.

The degree of fusion of ephiphysial plates or the hyoid complex as revealed by means of x-ray examination has been used in the past to estimate the age of stranded cetaceans based on bone development (*e.g.*, Ogden *et al*. 1981, Calzada *et al*. 1997, DiGiancamillo *et al*. 1998, Galatius *et al*. 2006). As with radiography of harbour seal teeth, the degree of fusion only allowed age to be ascribed to a general age class. The advantages of this method may be for identification of immaturity or maturity; overall the technique is not recommended for age estimation given the current state of knowledge and availability of other, better, techniques.

Bone mineral density

Bone mineral density (BMD) is generally used clinically to test for bone pathologies (*e.g.*, Sweeny *et al*. 2005). It is measured using a dual-energy x-ray absorption device, and the method has been applied to several species

including rodents, cats (*Felis catus*), dogs (*Canis lupus familiaris*), non-human primates, and horses (*Equus ferus caballus*) (Grier *et al*. 1996). Several studies have evaluated whether BMD has value for estimating age.

Significant correlation between body length, age from GLG counts in teeth, and BMD in flippers was found in 15 striped dolphins (Guglielmini *et al*. 2002) using multiple regression analysis and in bottlenose dolphins (Butti *et al*. 2007) using a linear model. Guglielmini *et al*. (2002) suggested that the model could predict the age of an individual if both length and BMD were known and there is a tight correlation between age and length, while Butti *et al*. (2007) concluded that BMD values are reliable indicators of age when using a predictive model based on body length only. However, Azevdeo *et al*. (2015) found that in the Guiana dolphin (*Sotalia guianensis*) BMD is only reliable up to the age when the peak in bone density is reached.

The main advantages of BMD for age estimation are that the method is non-invasive, rapid and relatively inexpensive. The flipper is an easy structure to take from stranded animals but the method could be useful for live animals, badly decomposed carcasses, or species possessing only a few teeth, for example, members of the Family Ziphiidae (Guglielmini *et al*. 2002). However, the method does require specialised equipment and species-specific age-at-length reference data where age and length are tightly correlated. Other factors such as sex would also need to be considered. More relevant is that the resulting age estimates from BMD for an individual are imprecise (see Butti *et al*. 2007), rendering this method suitable for relative but not absolute age. This imprecision may result from both individual variation in BMD and the poor predictive power of length as an indicator of age for animals older than juveniles as noted by Azevedo *et al*. (2015). Furthermore, BMD may be influenced by other factors, *e.g.*, overall health of an individual and/or ingested pollutant such as cadmium.

Genetic telomeres

Nakagawa *et al*. (2004) noted that "telomeres are short, tandem repeated sequences of DNA found at the ends of eukaryotic chromosomes that function in stabilizing chromosomal end integrity". Telomeres shorten as a result of cell division and shortening is associated with greater longevity in birds and mammals (Haussmann *et al*. 2003). Thus, telomere length may serve as a molecular clock from which the age of an individual may be estimated (Haussmann and Vleck 2002, Vleck *et al*. 2003, Haussmann and Mauck 2008). Telomere length also provided a rough estimate of age and was suggested to be of value for human (*Homo sapiens*) health (Lahnert 2005) and forensic age estimation when no other morphological information was available (Tsuji *et al*. 2002). Telomere length was generally associated with longevity in birds, but estimated ages are sufficiently imprecise as to not be

predictive (Hall *et al*. 2004, Juola *et al*. 2006). Bize *et al*. 2009) found that telomere length was associated with increased life expectancy but only weakly related to chronological age.

If successful, telomere length for age estimation studies would have many advantages. The method would be valuable for species for which absolute age data are hard to obtain, it requires small sample of easily obtainable tissue, such as skin, including from remote biopsies, does not require lethal sampling, and is readily repeatable (Brownell *et al*. 2000, Nakagawa *et al*. 2004, Dennis 2006, Izzo *et al*. 2011). Telomere length assays would provide a direct and cost-effective approach to obtain immediate knowledge of age and population age structure without long-term longitudinal studies (Nakagawa *et al*. 2004).

In marine mammals, Garde *et al*. (2010) found no relationship between harp seal telomere length and dentinal GLG age and concluded that telomere length is not suitable as a tool for age estimation in harp seals. The use of telomere length for age estimation in the Australian sea lion (*Neophoca cinerea*) was investigated by Izzo *et al*. (2011). No known-age animals were used in the study, so the animals were categorised into the following classes: pups, juveniles and adults, and males and females. The results showed that only broad age classes could be discriminated, because although mean telomere lengths of adults were significantly shorter than those of juveniles and pups, no differences were observed between juveniles and pups. It was concluded that the application of telomere length as a method for age estimation in pinnipeds requires considerable development to refine the scale of the age estimates derived, with the use of known-age animals (Izzo *et al*. 2011). Olsen *et al*. (2014) examined the use of telomere length measurements using quantitative PCR of free-ranging cetaceans. They analysed telomere length from skin samples of 28 North Atlantic humpback whales of known age, ranging from 0 to 26 years of age. Whilst they found a significant correlation between age and telomere length telomere length varied substantially in individuals of similar age. This suggests that the use of quantitative PCR to measure telomere length is imprecise for estimating age of humpback whales. The variations found in individual telomere length were due to both experimental (*e.g.*, pipetting errors, slight variations in amplification efficiency) and biological variability, with the latter perhaps reflecting patterns of inheritance, resource allocation trade-offs, and stochasticity of the marine environment. Nonetheless, various authors (*e.g.*, Monaghan 2010, Dunshea *et al*. 2011) have described the restricted use of telomeres, including the limitations for age estimation. However, Dunshea *et al*. (2011) and Olsen *et al*. (2014) concluded that telomeres should not be used for age estimation.

Amino Acid Racemisation (AAR)

Amino acids exist in two isomeric configurations, called L- and D-enantiomers. The L-enantiomers are unstable and over time racemise to form D-entantiomers (Helfman and Bada 1975, 1976). The rate of racemisation is a function of the specific amino acid, the ambient pH, and the ambient and body temperature (Bada *et al*. 1974). Because the half-life of racemisation ranges from a few thousand to millions of years, the amino acid with the shortest half-life, aspartic acid, is generally chosen for ageing (Bada and Schroeder 1975, Bada *et al*. 1980). The method also requires the use of a metabolically stable tissue, such as the proteins in the nucleus of the eye and in teeth (Bada *et al*. 1980, George *et al*. 1999). Within the context of those constraints, the process occurs at a constant rate through time making it possible to use the D/L ratio to calculate the relative age of an animal (Masters *et al*. 1977).

Age estimation by AAR has been applied to humans and other mammals, including harp seals and several cetacean species (Masters *et al*. 1977, Bada *et al*. 1980, 1983, Nerini 1983, George *et al*. 1999, Olsen and Sunde 2002, Garde *et al*. 2007, 2010, Rosa *et al*. 2012, Nielsen *et al*. 2013, 2017, Pleskach *et al*. 2016, Garde *et al*. 2018). Bada *et al*. (1980) found that racemisation in the teeth of marine mammals is more useful for age estimation in species with larger teeth due to the ability to isolate a large enough sample from within GLGs. The use of the eye lens provides an alternative; correlations between dentinal GLGs counts and AAR in the eye lens nucleus were found in shorter lived species such as the spotted dolphin (Bada *et al*. 1980), harp seal (Garde *et al*. 2010) and harbour porpoise (Nielsen *et al*. 2013, 2017). AAR analysis has now been shown to be successful using the lens nucleus from the eye (Master *et al*. 1977, 1978), and is useful in species for which teeth are not available for ageing. The use of teeth for AAR analyses has largely become obsolete, because age can usually be directly estimated from tooth GLGs.

In narwhals, age estimates obtained from GLG counts from tusks were lower than those obtained from AAR (Bada *et al*. 1983). However, the result likely was influenced by the use of the D/L ratio from human teeth (Helfman and Bada 1976), and comparability was not known. Errors in the D/L ratio measurement account for most of the variability in the age estimates of younger animals. Overestimation of the $(D/L)_0$ value (the D/L value at age 0) results in underestimation of age and vice versa (George *et al*. 1999, Rosa *et al*. 2012).

Garde *et a*l. (2010) noted that "Bada *et al*. (1980) suggested that racemisation rates in eye lens nuclei were similar within mammal species". However, Garde *et al*. (2010) found that applying the rate from one species to another can result in significant errors in estimated age. For example, by applying the

narwhal racemisation rate to calculate harp seals ages, there was an overestimation of seal age, which was caused by a slightly lower racemisation rate in narwhals than in harp seals (Garde *et al*. 2007). Furthermore, some species, such as bowhead whales, naturally have higher asparagine residues than other mammals, potentially resulting in overestimates of age (George *et al*. 1999). For all species, near-term foetuses or postpartum individuals and the use of known-age animals are required to establish a baseline for the D/L ratio estimates and racemisation rates, respectively (Olsen and Sunde 2002, Garde *et al*. 2007, Garde *et al*. 2018). Furthermore, Garde *et al*. (2018) found that continued growth of the eye lens postnatally has significant implications for estimation of species-specific D/L_0 values and the accuracy of age estimation using the AAR technique in young marine mammals.

The use of AAR has potential for determining longevity in long-lived animals for which other methods either cannot be applied or have severe limitations. AAR is the only technique that has provided direct data on ages of mature bowhead whales and the maximum age obtained by AAR was 211 (± 35) years (George *et al*. 1999).

For shorter lived species, Olsen and Sunde (2002) found AAR age at sexual maturation of minke whales was consistent with other studies. However, they concluded that precision of the method is low, most likely because it used the racemisation rate from another species and no replicate samples from the same individual. Lubetkin *et al*. (2008) found that age estimates using stable isotopes in baleen GLGs were more accurate than AAR for animals in their teens or younger, due to the large standard errors in AAR (George *et al*. 1999, Rosa *et al*. 2012).

The use of AAR for age estimation has been described as 'touchy and complicated' (George *et al*. 1999, Olsen and Sunde 2002) although once the method is 'up-and-running' it is relatively straightforward. When possible, all specimens should be analysed in a single series to reduce error. AAR age estimates using the nucleus of the eye lens will be over-estimated if the animal has a brunnescent group IV cataract (Masters *et al*. 1977) and under-estimated if tissue or blood contaminates the sample because the D/L ratio may be significantly lowered (George *et al*. 1999). Furthermore, lens samples can only be collected from very fresh dead animals, which is why most work to date has focused on animals that were hunted or recently deceased captive animals. Statistically significant differences have been found in paired eye samples from the same individual of minke and bowhead whales (Olsen and Sunde 2002, Rosa *et al*. 2012), however these differences were biologically insignificant considering the lifespan of the whales (Olsen and Sunde 2002). Regression models can be applied to the data to reduce the standard errors for individual ages (Olsen and Sunde 2002) and species-specific data on body

temperature are required (Garde *et al*. 2018). A primary limitation, even with all of the above considerations taken into account, is the one in common with use of telomere, that is, AAR values are highly variable among individuals of the same age.

AAR is the first technique that has provided data on longevity of narwhals (Garde *et al*. 2007). Based on eye lens samples from 75 harvested narwhals, maximum ages were calculated as 84 (± 9) and 115 (± 10) years for males and females, respectively. These estimates are consistent with modern beluga estimates, *i.e.*, a similar and similar sized species (*e.g.*, Stewart *et al*. 2006). Previous studies had significantly lower maximum ages (30–50 GLGs) from mandibles and teeth (Hay 1980, Neve 1995). Garde *et al*. (2007) found around 20% of the sampled whales to be older than 50 years.

Results of AAR on the tusk combined with age estimated from GLGs in the tusk found 0.7-0.8 (±0.15) GLG deposition annually (Bada *et al*. 1983). However, Garde *et al*. (2012) were able to estimate a species-specific racemization rate for narwhals by regressing aspartic acid D/L ratios in eye lens nuclei against growth layer groups in tusks (n=11), assuming annual GLG deposition. The tusks varied in length (34 -255 cm) and age (5-70 GLGs). They proposed that this species-specific racemization rate and $(D/L)_0$ value could be used in future AAR age estimation studies of narwhals with increased accuracy.

Fatty acid signatures

The use of fatty acids (FA) from blubber to estimate age was suggested when Koopman *et al*. (1996, 2003) identified very short-chain fatty acids in the blubber that increased with body length and age of belugas and approximate known-age harbour porpoise. Herman *et al*. (2008) found that a linear combination concentration of two fatty acids resulted in a good correlation between FA and age for known-age killer whales ranging from 0 to 60 years old. The precision was greater than 7 years (SD=3.82 yr), there were no trends in residuals with age. Herman *et al*. (2009) predicted the age of known-age humpback whales with >95% confidence with a precision of a little more than 10 years. Further, from duplicate biopsies taken from two humpback whales more than 2 years apart, Herman *et al*. (2009) reported that the ages predicted were in "reasonable" agreement the time between the samplings. Marcoux *et al*. (2015) found that FAs that correlated with GLGs in teeth allowed age to be estimated with a precision of around 7-10 years in beluga from three distinct populations in Canada.

No single model is likely to be adequate to estimate age as a result of blubber FA compositions for all cetacean species; the FA-age relationships will be taxon- or species-specific. FAs most correlated with age in North Pacific

killer whales were shorter-chain monounsaturated and iso- and anteiso-branched chain FAs (Herman *et al*. 2008). In contrast, humpback whale age was most correlated with long-chain monounsaturated and multi-methyl branched-ch ain FAs (Herman *et al*. 2009). Herman *et al*. (2009) also found that no single FA can accurately predict the age of unidentifed individuals within a study; this method still requires further development.

To be an effective tool for ageing animals, the empirical models obtained from animals of known-age need to be independent of diet, so that minimal changes in diet in the future will not indriectly impact the estimated ages (Herman *et al*. 2009). Herman *et al*. (2008, 2009) found that FA-age relationships most correlated with age were also largely independent of diet and sex. Marcoux *et al*. (2015) found that half of the FAs that correlated with age were from dietary sources in beluga, which might reflect age-related changes in diet. Nonetheless, when analysing FA composition, samples taken from animals with abnormal fatty acid and/or lipid class compositions need to be eliminated from analysis. Multi-dimensional scaling or other appropriate analyses of fatty acid composition data along with lipid class analyses should be used as pre-screening tools to flag samples that exhibit abnormal lipid and/or FA profile as uncertain based on fatty acid-age models (Herman *et al*. 2009).

The main advantage of the use of FA signatures for age estimation is that samples can be collected relatively easily, including remote biopsy, facilitating ageing a large number of live animals. However, at present, results are not likely to be comparable between laboratories, so each laboratory needs to derive its own set of empirical FA-age relationships from animals of known-age (Herman *et al*. 2009), precision is relatively low and transformation of fatty acids may occur in dead animals. The use of fatty acids in age estimation holds promise and additional effort towards increasing precision of age estimates is encouraged.

Ovarian corpora

Counts of *corpora albicantia* (CA) and *corpora lutea* (CL) in the ovaries of mature females have correlated with age (from counts of GLGs in earplugs) in several mysticete species including humpback whales (Chittleborough 1959), fin whales (Laws 1961, Lockyer 1972), minke whales (Masaki 1979, Kato *et al*. 1984), grey whales (Rice and Wolman 1971, Zimushko and Ivashin 1980), and sei whales (Lockyer 1974). Ages estimated from *corpora* counts allowing for age at first ovulation, were found to be the same as ages estimated from AAR in bowhead whales (Rosa *et al*. 2012). However, correlation of *corpora* accumulation to estimate age does not appear to be valid for odontocetes, sirenians, and pinnipeds. Accessory CLs occur in beluga whales (Brodie 1972), which confounds using the number of *corpora*

as an estimate of age.

The relationship between age and the number of CAs in short-beaked common dolphins was very weak (Dabin *et al*. 2008). For spotted dolphins, Myrick *et al*. (1986) found a significant slope but a low correlation for number of corpora at age starting at the average age at sexual maturation. In manatees and pinnipeds, CAs are resorbed within a few months to a couple of years, (*e.g.*, Bester 1995, Marmontel *et al*. 1996, Odendaal *et al*. 2002). In narwhals, combining results from *corpora albicantia* counts with dental and mandibular GLGs and AAR did not show a strong relationship (Hay 1984, Garde *et al*. 2015). In belugas, *corpora albicantia* steadily accumulated until around 40 years of age (Burns and Seaman 1986, Suydam 2009). No specific studies using *corpora* as relative age have been reported for monodontids.

The main advantage of using corpora counts for age estimation is that these counts are relatively easy to obtain with good accuracy as long as both ovaries are analysed. Disadvantages include that the use of ovarian *corpora* is only valid for mature females and can only be applied to dead animals. Use of *corpora* requires knowledge of age at sexual maturation and ovulation rate, does not account for individual variation in life-history parameters (Nerini 1983), and, if reproductive senescence occurs, the method underestimates age (George *et al*. 2011). As a sole method of ageing, *corpora* are no longer in use to estimate age.

Colour change with maturity

Numerous species undergo skin or pelage colouration changes with age. For some species of pinnipeds, pups pass through species-specific, easily identifiable stages over the lactation period and the first several weeks post weaning. For example, harp seal pups are classified into 7 age categories (Stewart and Lavigne 1980, Kovacs and Lavigne 1985) and grey seal pups into 5 age categories (Kovacs and Lavigne 1986, Meyers *et al*. 1997). However, Stewart and Lavigne (1980) and Kovacs and Lavigne (1985) reported slightly different age estimations for harp seal pups based on pelage colour. After the initial moult, age can only be estimated to age class from pelage colouration (*e.g.*, McConkey et al. 2002).

Belugas and narwhals undergo colour changes with age. At birth, belugas are pinkish grey and become darker grey or brownish during their early years (Caron and Smith 1990), gradually turning from grey to white as they mature (Hazard 1988). Burns and Seaman (1986) found that all white belugas are sexually mature, but some animals may be sexually mature before they turn white. In belugas colour change has been documented in Alaska (US), Greenland and Russia (Heide-Jørgensen and Lockyer 2001, Suydam 2009). Colour change is highly variable among individuals, and so its use as a

diagnostic for estimated age is unreliable (Heide-Jørgensen and Lockyer 2001).

At birth narwhals are blotchy grey, becoming solid grey or black after weaning then gradually more mottled grey with age. Old individuals may be almost completely white with some dark spots on their dorsal surface (Hay 1984, Hay and Mansfield 1989). Colour change relative to age has not been documented. In general, colour may only be used as a very relative age indicator.

Stable isotope ratios

Patterns of stable isotope ratios of carbon in baleen plates thought to reflect dietary changes, were used to age bowhead whales (Lubetkin *et al*. 2008 and references therein). The method identified annual cycles for individuals of around 10-13.5 m in length and < 20 years of age; nonetheless, it was concluded that incremental counts of baleen GLGs were more accurate (and less laborious and costly) for age estimation in young animals, and relative *corpora* counts and AAR were more effective for older females (Lubetkin *et al*. 2008). Matthews and Ferguson (2014) investigated "synchronous recording of baseline isotopic variation across dentinal GLGs of species with temporal and spatial overlap in foraging". They determined that this technique "offers a unique opportunity for validation of marine mammal age estimation procedures through calibration of GLG deposition rates in one species against another whose GLG deposition has been independently determined". Linear $\delta^{13}C$ isotopic declines across chronologies of both beluga and killer whales were statistically indistinguishable when based on annual GLG deposition, but differed when based on biannual deposition. This method could be of use in validating deposition rates yet is unlikely to be an important method for estimating age due to the time and specialized equipment requirements.

Radiometry

Kastelle *et al*. (2003) applied radiometry on the tympanic bullae of mysticetes (5 grey whales and 2 bowhead whales) from neonate to full-grown size, by investigating the disequilibrium (ratio) between radionuclide ^{210}Pb and ^{226}Ra to predict age. The method depends on the accumulation over time of the naturally occurring radionuclides ^{226}Ra in the bone tissue and subsequent retention of its progeny ^{210}Pb. The results were not conclusive, but this method might be explored with other species.

Eye lens weight

The eye lens continues to grow throughout adult life and a number of studies across various taxa of mammals identified a correlation between dry weight of the lens and age, *e.g.*, in cottontail rabbits (*Sylvilagus floridanus*; Lord

1959), kangaroos (*Macropus* spp.; McLeod *et al*. 2006) and humans (Augusteyn 2007). Age was also estimated using the eye lens weight for northern fur seals (*Callorhinus ursinus*) for which known-age seals could be aged to the nearest year through age 2 years (Bauer *et al*. 1964). In harp seals, lens weight correlated well with GLG counts (0 to 25 and 1 to 25) but had low regression coefficients and little predictive precision; lens weight did not correlate with age in the first month of life (Stewart 1983, Stewart and Lavigne 1980). For accurate age estimates from eye lens weight, a large sample size is required covering the whole age range from foetal to adult life, and lenses need to be from individuals with healthy eyes. Storage may affect the results, for example, formalin fixing reduces water content (Augusteyn 2007), and may also alter the chemical balance and thence the weight of the lens. Further, similar to use of telomeres, AAR, and other methods, lack of precision is likely to hinder the overall value of the age estimates. This method is essentially obsolete for estimating age in marine mammals.

Age-related colour changes in the eye

Nishiwaki (1950) noted that the colour of blue and fin whale's eyes appeared to change with body size. This led to absorption of light by the eye lens to be investigated and a linear relationship was found between absorption of light in relation to body length and *corpora* count. No age validation exists for this method and it is unlikely to become a common method for ageing.

Morphometric-based age estimates

If morphometry closely and precisely correlates with age, then it would provide a mechanism for quickly estimating approximate age for an individual. In their study of harbour seals, including a subset of known-age animals, Blundell and Pendleton (2008) developed predictive equations regressing mass, length, and girth against the known age or estimated age from GLGs in teeth. While morphometrics and age were correlated, predicted age estimates were only within one year for seals ≤ 3-yr old while seals > 3-yr old had larger discrepancies. In general, the use of morphometric-based age estimates is not likely to be sufficiently precise to replace age estimates except for very young animals.

VALIDATION TECHNIQUES

An essential assumption of age estimation is that the estimates are accurate. The deposition rate of the structures must be validated to ensure that the GLGs counted represent the period assumed, *e.g.*, a year. For growth layers in hard tissue, the term 'age validation' is often used misleadingly in that the frequency of GLG deposition is validated rather than the age of the individual (Campana 2001). In addition, GLG deposition of immature animals seldom resembles that of mature animals and the youngest and oldest age groups,

which are often the most difficult to age accurately, are most influential in estimates of growth, mortality or longevity (Campana 2001). Therefore, validating the periodicity of GLG formation and absolute age should be conducted for all age groups (Beamish and McFarlane 1983, Campana 2001).

Age-validation studies in marine mammals have been conducted by: 1) analysis of GLGs of known-age or known-history animals; 2) chemical time-marking of GLGs; 3) comparison of GLGs in samples taken at known intervals (multiple extractions; Hohn 2009); and 4) artefacts (George and Bockstoce 2008).

Known age or known history

The most common method for validating the deposition rate and characteristics of growth layers is to use individuals identified as newly-born calves or pups and sampled in subsequent years (Hohn *et al*. 1989). Individuals may be 'known approximate-age' if their birth date is unknown but an approximate age or minimum age can be estimated at first capture/sighting. Age validation using known-age animals generally requires sightings data from long-term studies which can be expensive and time-consuming, so known-age animals are relatively rare. Nonetheless, known-age individuals have been used to validate annual GLG deposition for seals (*e.g.*, Scheffer 1950, Laws 1952, Bowen *et al*. 1983, Oosthuizen 1997), sea otters (Bodkin *et al*. 1997), bottlenose dolphins (Hohn *et al*. 1989), and humpback whales (Gabriele *et al*. 2010). Animals for which the minimum age was known can be useful if the minimum age is relatively old, for example, Gabriele *et al*. (2010) combined 27 years of sightings data (the same size as other adult whales when first sighted) and GLGs (around 44 GLGS) in the ear plug to estimate the minimum age of a humpback whale. This also validated the annual deposition rate of ear plug layers. Multiple extractions of age-estimation samples from individuals after known time intervals can also provide known minimum-aged specimens and for known-age animals, provide calibration of GLG deposition between the two extractions (*e.g.*, Sergeant *et al*. 1973, Hui 1980, Myrick *et al*. 1984, Hohn *et al*. 1989, Lockyer 1993).

Validating GLGs with known-age animals is relatively easier for marine carnivores (*e.g.*, seals, sea otters and polar bears) as their amphibious lifestyle makes them easier to catch, mark, and recapture at their haul-out site or den. Known-age cetacean and sirenian studies require other methods; photo-identification is now a commonly used method to identify and follow several species (*e.g.*, bottlenose dolphins (*e.g.*, Cheney *et al*. 2014), manatees (*e.g.*, Langtimm *et al*. 2004), killer whales (*e.g.*, Young *et al*. 2011) and humpback whales (*e.g.*, Mizroch *et al*. 2004)), so the number of known-aged animals for a wider variety of species, should become increasingly available. Photo-id is

being conducted using nicks and notches on the dorsal ridge of narwhals (Auger-Méthé et al. 2010) so in the future, known-age animals should be available. The only data on known-age wild beluga comes from long term investigations of St Lawrence belugas in Canada using photo-identification and subsequent recovery of known stranded animals which indicated that the tooth GLGs agreed with the known ages of these animals thus supporting an annual deposition (Michaud 2011).

Bio-markers for time-marking

A biological marker, or bio-marker, is a substance that is used as an indicator of a biological state. For age estimation, bio-markers are generally calciphilic chemicals that bind permanently to actively mineralizing tissue (Frost 1968) and usually fluoresce when the specimen is viewed in ultraviolet illumination (Fig. 9). Bio-markers can be injected intramuscularly or ingested by an animal to place a time-specific mark in mineralizing tissue. Bio-markers applied specifically for validating GLGs in marine mammals are administered annually, ideally on the animal's birth date, but may also be administered daily, weekly or monthly. Good records of bio-marker administration are required for accurate results so that the resulting mark in teeth or bone can be evaluated relative to deposition of GLGs.

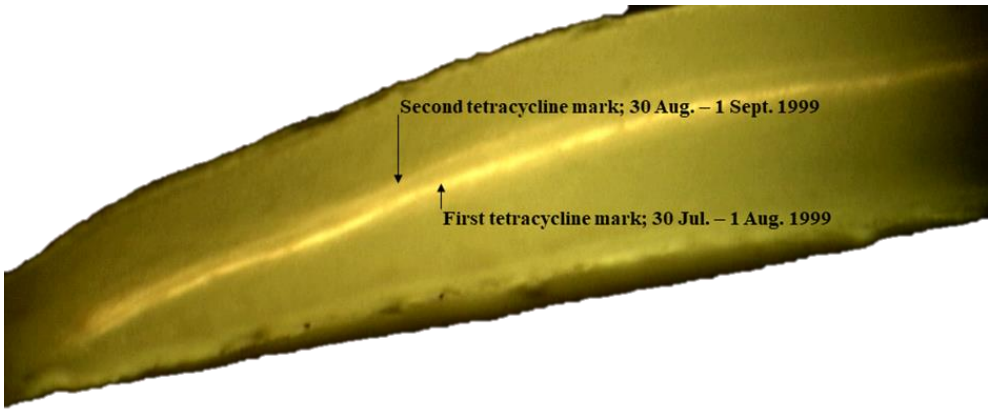

Fig. 9. Tetracycline marking in untreated harbour porpoise (*Phocoena phocoena*) dentine showing fluorescence under reflected UV light.

Bio-markers for age estimation can be used to 1) monitor GLG patterns of captive animals for comparisons with wild conspecifics; 2) calibrate GLG deposition rate; and 3) study the timing of physical events related to GLG patterns (Myrick and Cornell 1990). To date, most studies of bio-markers have been based on captive animals, although the method is also possible for free-living animals, if they can be caught, administered a bio-marker and

released (Hohn 2009) or a naturally occurring biomarker is found.

The primary bio-marker used for marine mammals is the antibiotic tetracycline, although use of lead acetate has also been reported. Tetracycline has been used successfully to calibrate annual GLG deposition and deposition patterns in several marine mammals including common, bottlenose, and spinner dolphins, killer and pilot whales, and Amazonian manatees (*Trichechus inungis*) (Yagi *et al*. 1963, Best 1976, Domning and Myrick 1980, Gurevich *et al*. 1980, Myrick *et al*. 1983, 1988, Myrick and Cornell 1990, Lockyer 1993). Tetracycline marks were observed only in teeth taken from the mid-lower jaw in common dolphins (Gurevich *et al*. 1980) and were not observed in common dolphins and killer whales marked at an old age when the GLG deposition rate is low (Gurevich *et al*. 1980, Myrick *et al*. 1988). Thus, one drawback to use of bio-markers for teeth and bone is the decrease in tissue deposition rates with age which limits incorporation of the chemical.

Tetracycline initially was administered for clinical treatment rather than for calibrating dentinal deposition rates. However, incidental tetracycline administration has often been insufficient for later detection in teeth, *e.g.*, in spinner dolphins, pilot whales, and beluga whales (Myrick *et al*. 1984, Lockyer 1993, Lockyer *et al*. 2007). Tetracycline can also be administered to nursing calves through treatment of the lactating mother (Myrick *et al*. 1984). Marked tissues lose their biomarkers if stored in formalin or are decalcified (Nielsen 1972, Johnston *et al*. 1987).

Tetracycline marking of some of the captive belugas has been inconclusive with respect to deposition rate, perhaps because the animals were of known history but not of known total age (Lockyer *et al*. 2007, Hohn and Lockyer 2011, Brodie *et al*. 2013). Instances when an annual deposition rate of GLGs was verified hinged on rare examples of multiple time markings, and the time elapsed between tetracycline markings or up until the time of death (Fig. 2 in Lockyer *et al*. 2007).

The atmospheric testing of atomic bombs in the 1950s and 1960s resulted in a rapid and well-documented increase in bomb radiocarbon (^{14}C) in the world's oceans (Druffel and Linick 1978, Kalish 1995). That ^{14}C is incorporated into tissues and remains static throughout the life of the structure, means that ^{14}C can be used as a bio-marker to validate age for individuals alive at the time of testing. It has been used for a range of species, from trees (Worbes and Junk 1989) to fish, bivalves, and corals (Kalish 1993, Weidman and Jones 1993, Campana 1997), and beluga whales. Mass spectrometry is used to identify the isotopic signatures of ^{14}C. The method is best applied to samples that span pre- and post-bomb eras and that are large

enough to permit micro-milling of individual GLGs (see also Campana and Stewart 2014). It requires high technology equipment and is expensive, but an ageing method needs to be validated only once.

The results of studies on radio isotopes present in GLGs from atomic bomb fallout clearly indicated that in GLGs formed annually in beluga (Stewart *et al*. 2006, Campana and Stewart 2014), despite criticisms of the method (see Brodie *et al*. 2013). Stewart *et al*. (2006) found that beluga whale teeth recorded and preserved a bomb radiocarbon pulse in GLGs formed during the 1960s. Analysis of teeth collected from 1865 to the late 1950s showed the pre-bomb ^{14}C signal was relatively constant and significantly lower than tooth material deposited after bomb testing (Fig. 3 in Stewart *et al*. 2006). Results from using this approach disproved the 2 GLG/year hypothesis, and showed longevity to be 77 and 79 years old for male and female belugas respectively (Stewart *et al*. 2006), far longer than previously anticipated, but more in line with large odontocetes such as pilot, and killer whales and narwhals.

Captive studies

With the increasing success of captive breeding programmes, many marine mammals (*e.g.*, seals, sea lions, bottlenose dolphins, walruses, polar bears, and killer and beluga whales) are now born in captivity providing potential access to known-age specimens. Studies with captive animals can complement field studies on the same species or provide opportunities for species for which field studies are not feasible for providing information to validate GLG deposition. Use of captive animals assumes that captive and free-living animals have the same GLG deposition rate on the basis that the deposition rate is a cyclical, endogenous process. No change in the pattern of overall pattern of GLG deposition was recorded in bottlenose and spinner dolphins and manatees after short-term periods of stress (Myrick *et al*. 1984, Hohn 1990, Marmontel *et al*. 1996). GLG counts corresponded to age in captive bottlenose dolphins, pilot whales, and harbour seals (Sergeant 1959, Mansfield and Fisher 1960, Lockyer 1993). Furthermore, the first calibration of dentinal GLGs in captive bottlenose dolphins by Sergeant (1959) was later confirmed by wild bottlenose dolphins of known age and life history (Hohn *et al*. 1989).

In captivity, controlled diet and day-night rhythm, as well as constant water temperature and salinity, might be argued to affect the pattern of GLGs. Lockyer (1993) noted in captive pilot whales marker lines and anomalies in the dentine that appeared to be correlated with stress due to illness, reproductive events, and also dietary changes on first coming into captivity. However, age estimates for captive harp seals are probably not reliable. A harp seal caught as a pup showed no GLGs after 9 years in captivity, and a female caught after she had attained the 'harp' markings, and was therefore

likely over 6 years old, showed 9 GLGs after 8 years in captivity (Stewart 1983).

Clark *et al.* (2000) stated that "in spite of the likely influences of genetics, differences in nutrition, parasitic infestations, energetics, prey availability and various other environmental conditions between captive animals and wild animals", captive animals provide a control. Most captive-animal studies to validate age estimation use animals that were removed from the wild and assigned an "age" at the time of capture using body length (and skin colour for beluga). Combining age-at-capture and time in captivity gives the animal a known-minimum age at the time of tooth extraction or death (*e.g.*, Sergeant 1959, Lockyer 1993). The benefit of using captive animals is that health status, medical history, and life-history events are generally well documented. Multiple extractions over time allow inter-specific differences in GLG patterns as well as in different teeth of the same individual, or different individuals over the same period to be studied (*e.g.*, Myrick and Cornell 1990, Lockyer 1993).

No consistent evidence exists that captivity, *per se*, alters GLG deposition and overall growth rates for belugas (Goren *et al*. 1987, Lockyer *et al*. 2007), although GLGs from captive belugas appear less defined and harder to read by virtue of the presence of accessory laminae, than wild animals (Heide-Jørgensen *et al*. 1994, Lockyer *et al*. 2007). No GLGs were visible in the tooth of a beluga that had lived in captivity for 13 years (Brodie 1982). It is important to note however, that most captive belugas that have been studied for age validation, have originated from the same geographical region *e.g.*, Churchill, Canada. In assessing readability of GLGs, it is important to compare tooth structure from both free-living and captive animals originating from the same geographical region.

With the success of captive breeding programmes of belugas and cooperation from aquaria (*e.g.*, tetracycline marking and records of individual life history events) and photo-identification projects (*e.g.*, Upper Cook Inlet, Alaska and the St. Lawrence Estuary, Canada) the availability of known-age belugas will increase and potentially allow for age validation of tooth GLGs. Brodie *et al*. (2013) discussed the data presented therein in relation to the question of annual or biannual GLG deposition rates with reference to conclusions made by Luque *et al*. (2007).

Validation using captive animals

Although growth and reproduction of free-ranging belugas have been studied throughout most of the species' range (*e.g.*, Kleinenberg *et al*. 1964, Brodie 1971, Sergeant 1973, Ognetev 1981, Burns and Seaman 1986, Braham 1984, Doidge 1990, Heide-Jørgensen and Teilmann 1994, Stewart 1994, Vos 2003,

Suydam 2009), few studies on narwhal life history parameters have been conducted. This is most likely because reliable age estimation methods have not been developed (Hay 1980). Estimated asymptotic body length varies considerably among studies on narwhals by Hay (1984), Neve (1995) and Garde *et al*. (2007). The differences are probably due to the methods of age estimation rather than temporal or spatial differences.

It has been particularly important for the assessment and management of monodontids to validate the deposition rate of dental GLGs (Waugh *et al*. 2018). Sergeant (1959) initially suggested that 2 GLGs were deposited annually (age = GLG/2) in belugas by analogy with sperm whales (Gambell and Grzegorzewska 1967). Later studies revealed sperm whales deposited GLG/1 per year (*e.g.*, IWC 1969, Best 1970) and despite Sergeant (1981) noting that there was not *a priori* reason for beluga to be unique the GLG/2 interpretation remained. In samples from captive animals, Brodie *et al*. (2013) recently supported the hypothesis of GLG/2. One hypothesis is that several GLGs are deposited annually during the juvenile growth phase and GLG/1 is deposited once sexual maturity is reached (Lockyer *et al*. 2007, Luque *et al*. 2007). However, this does not fit well with 1 *versus* 2 GLG/year. Such a pattern, if it existed, would make the average 1+ where + is very small in a long life. For example, if GLG/2 were deposited from 0-10 years a 60-year-old would have 70 lines for an average of 1.2, not nearly 2/year.

Studies of belugas that were removed from the wild, with a contemporary estimate of age based on size or colour, and that have remained in captivity have been not been in agreement with respect to the rate of GLG deposition (*e.g.*, Brodie 1971, 1982, Goren *et al*. 1987, Heide-Jørgensen *et al*. 1994, Hohn and Lockyer 1999, Lockyer *et al*. 2007, Luque *et al*. 2007, Brodie *et al*. 2013). However, there is no explanation as to why beluga should be an exception to the rule of GLG/1 as seen in other mammals (*e.g.*, Klevezal and Kleinenberg 1967, Sergeant 1973, 1981, Grue and Jensen 1979). The deposition of GLGs in belugas is also unlikely to vary between populations (Sergeant 1973). See Lockyer *et al*. (2007) for a thorough overview of age estimation, reader comparability and age validation of ten belugas live-captured near Churchill, Canada and maintained in captivity for 4-30 years.

To date, no narwhals have been kept in captivity and no known-age animals have been used for age estimation studies, so no calibration and validation studies have been conducted. In narwhal, Hay (1980) reported that based on length frequency distributions, it looked like 3 layers were deposited annually in the first 2 or 3 years, and then annual deposition of a single GLG once sexual maturity is attained. However, the validity of these results was suspect (Hay 1984). Neve (1995) assumed 2 GLGs were deposited per year.

The main conclusion of the Workshop on age estimation in Monodontids, 26-27 November 2011, Tampa, Florida, after reviewing the evidence, was that one GLG formed annually. This has also recently been determined by Waugh et al. (2018) for beluga. All previously generated data should be revised when the GLG deposition rate is determined because all life history parameters will potentially have up to a two-fold difference and the consequences of age-estimation differences on population studies may be considerable (Stewart *et al*. 2006). Management plans should account for actual age. Stewart *et al*. (2006) found that exploited populations of beluga whales recover more slowly when comparing models using age based on GLG/1 rather than GLG/2.

Artefacts

George and Bockstoce (2008) used artefacts from whaling to determine minimum age, longevity and movements of bowhead whales. During the nineteenth century, not all bowhead whales struck by whalers died and some whaling weaponry became embedded in the surviving whale. Each whaler's iron has the manufacturer's mark and a private mark allowing identification of the origin of the whaler. Whaling technology changed significantly in the late 1880s when commercial whaling stations were established and mostly this new technology was only used soon after manufacture in the nineteenth century (George and Bockstoce 2008). Therefore, combining the period of manufacture of whaling gear and dates when marked gear was last used allows estimation of the period when the gear was embedded in the whale. In 1980 and 2007, bomb lance fragments dating back to between 1879 and 1885 were found embedded in two bowhead whales, with a large degree of healing around the wounds (George and Bockstoce 2008). These artefacts support the results of age estimation studies by AAR which found that bowhead whales can live in excess of 100 years (George *et al*. 1999, Rosa *et al*. 2012). This method is potentially applicable to other hunted species with long lives.

CONCLUSION

This review of methods for estimating age in marine mammals demonstrates the complexity of the issue. The degree of accuracy required for the application of ages depends in part on the application. Relative ages may suffice in some cases, chronological age may be required in others. But since broader age categories can be formed by collapsing detailed data and the converse is not true, we concentrate on obtaining chronological age, usually by counting GLGs in hard structures. The tissue type and preparation and viewing methods that produce the most accurate and precise age estimates are species specific. Teeth appear to be the most suitable tissue for age estimation in belugas, despite crown wear, and GLG readability problems.

The objective is to provide precise and accurate age estimates. Standardization of methods improves precision and allows direct comparison between studies although different techniques may be calibrated against each other. Efforts should be made to quantify bias and precision in all methods. Validation of accuracy should be done for each species and sometimes, for each population. Age estimation work on belugas should follow the guidelines outlined in Lockyer *et al*. (2007), Stewart and Stewart (2014) and the report of the Beaufort Workshop on Age Estimation (NAMMCO 2013 (summary), Lockyer *et al*. 2016). These include the provision of details on section quality and GLG clarity, and recording structural anomalies. Guidelines need to be developed for narwhals.

Several methods have recently been applied to marine mammal ageing. The use of techniques such as telomere length, bomb radiocarbon isotopes, fatty acid analysis, and aspartic acid racemisation (AAR) for ageing monodontids should be continued or explored. Methods such as telomere length and fatty acid analysis can be utilised in living animals from biopsies. More effort should be invested into interpreting GLGs (Lockyer *et al*. 2007) in general and comparing GLG structure between captive and wild belugas in particular. Aspartic acid racemisation (AAR) appears to be a possible option for ageing narwhals but results have a high level of uncertainty and a better method is needed. As for tusks, this method depends on availability of dead animals and tusks are often not available from hunters.

Ultimately, it should be remembered that age data for all individuals is not feasible, even in well-documented populations, and the length of research has still not covered the potential longevity of many species (Herman *et al*. 2009).

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