Age determination methods in harbour seals *(Phoca vitulina)* with a review of methods applicable to carnivores

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ABSTRACT

The development of age determination methods in marine mammals is reviewed with particular reference to the use of teeth Growth Layer Groups (GLGs) formed in the dentine and cement of carnivores. Using this background, practices for sampling, tooth extraction and collection, storage and different methods of preparation of teeth as well as reading and counting GLGs are discussed and evaluated for the harbour seal *(Phoca vitulina)*. The paper includes comments on best practices for counting GLGs with new examples from known-age seals, and also a detailed examination of confounding factors in interpreting GLGs such as mineralization anomalies and the phenomena of accessory lines, "false annuli" and "paired laminae" which have not been discussed previously. The paper concludes with recommendations for undertaking age estimation in harbour seals from sampling through final GLG interpretation with special emphasis on standardization of methods with other researchers.

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INTRODUCTION

The ability to age individuals is critical in the investigation of the life history parameters and structure of any population. Knowing the age of individuals allows determination of mortality, longevity, and age specific-fecundity rates (Hewer, 1964). In turn, these age-specific vital rates allow the formulation of structured population models, where vital rates are age- or stage-specific, and such information is also crucial in contamination studies where loads can vary with age (Dietz *et al.* 1991). For the harbour seal *(Phoca vitulina),* knowing the age structure of the population is particularly important due to the age-specific susceptibility to phocine distemper virus (PDV) such as the epizootics in 1988 and 2002 (Härkönen *et al.* 2007), where half of the population along mainland Europe died (Härkönen *et al.* 2006).

This paper provides as a background, a general review of the methods used to age mammals using annual growth layers in teeth, highlighting how different approaches have been adopted to age individuals of both the same and different species. The paper then focuses on harbour seals with an evaluation of the best methods for this species considering which method is the most appropriate and accurate, and which factors may confound age estimates.

Background - the mammalian tooth

The teeth of all mammals are composed of both organic and mineral (predominantly calcium phosphate) material. The structure of any tooth is divided into three sections, the crown, the neck and the root. The crown is composed of the highly mineralized tissue of the enamel, which covers the dentine beneath. The neck of the tooth is the region immediately below the enamel, separating the crown from the root. The root is usually below the gum line and its surface is covered by cementum, with dentine underneath (Figs 1 and 2).

The pulp cavity is the central chamber of the tooth and is surrounded by dentine which arises from special cells, odontoblasts, situated at the pulp edge. During the life of an individual this pulp cavity contains sensory, connective, and nutritive tissues. Most of the structure of a tooth is dentine and, in general, 75% of the dentine's composition is mineral crystals in the form of hydroxyapatite (Langvatn, 1995). The

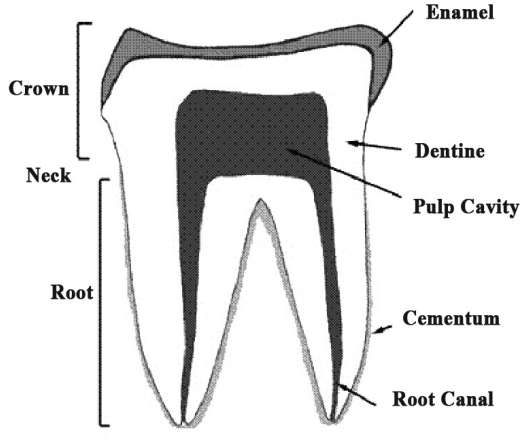

Fig. 1. *Diagrammatic representation of a mammalian tooth in buccal-lingual section.*

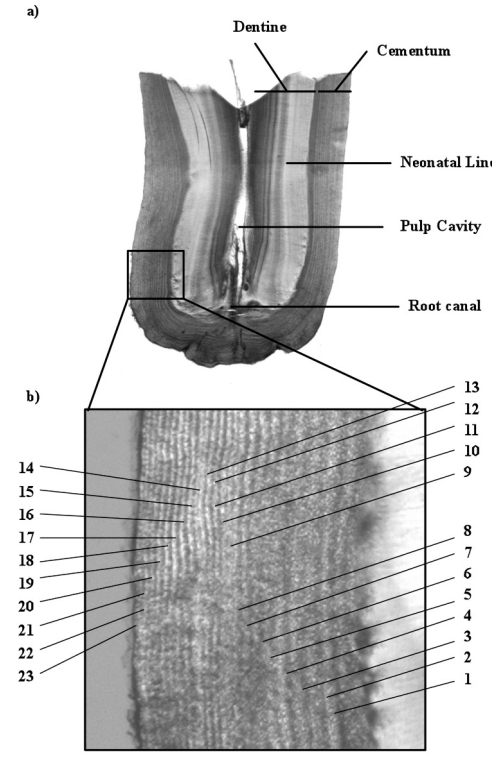

Fig. 2. *Example of a decalcified and stained buccal-lingual section from a ringed seal indicating, a) the main tooth structures and, b) how the Growth Layer Groups (GLGs) are counted. This individual was estimated to be 23 years of age at the time of its death.*

mineralization process takes place along the developing dentine front (Maas, 2002). The organic component of the dentine is small collagen fibrils, which are laid down both parallel and perpendicular to the developing dentine surface to provide tensile strength (Boyde, 1980, Maas, 2002). In many mammals the dentine continues to form throughout life with the deposition of incremental layers from the pulp edge.

The cementum which covers the dentine in the root of the tooth, has a similar composition to bone (Boyde, 1980) and is derived from the cementoblasts in the gum tissue adjacent to and surrounding the tooth. In general up to 20% of this tissue is organic material, including cementocytes, ground substance (containing proteoglycans) and both intrinsic and extrinsic collagen fibres (Maas, 2002). However, both between and within species differences in the percent by mass of carbon in cementum has been examined (Mackey, 2004). Cementum can be classified as cellular or acellular, dependant on both its fibre composition, and the relative proportions of cementocytes and ground substance in this composition. Cementocytes and ground substance are the primary constituents of cementum. Sharpey's fibres are well-mineralized extrinsic collagen fibre bundles that form the extrinsic fibre cement close to the alveolar bone. Towards the root of the tooth, mixed fibre cement contains both intrinsic collagen fibres, and Sharpey's fibres. In conjunction with the periodontal ligament, the cementum structure is responsible for tooth attachment (Lieberman 1993, Raspanti *et al.* 2000). Cementum is never remodelled, unlike dentine which can be resorbed but rebuilt with bone-like material. Its slow, but continuous deposition, in conjunction with the incremental growth layers, means that it provides an ideal long term recording structure (Klevezal 1980, Maas 2002, Raspanti *et al.* 2000).

A REVIEW OF GENERAL METHODS APPLICABLE TO CARNIVORES

Tooth selection for age determination

Carnivores (including the pinnipeds and fissipeds) have typical mammalian tooth formulae with incisors, canines, premolars (post-canines) and molars. The structural variation in teeth is largely related to method of feeding and whether or not biting, chewing or merely gripping is required. Most age research using mammalian teeth has been carried out using the canines, being the largest, and therefore easiest to study of the teeth (Dietz *et al.* 1991, Hewer 1960, Laws 1952). More recently however, the use of incisors and premolars (often referred to as post-canines) has developed. This has enabled the removal of teeth from live animals (Arnbom *et al.* 1992, Coy and Garshelis 1992), and has increased the ability for comparison of GLGs between teeth taken from the same individual over a period of time (Myrick *et al.* 1984). However, Bernt *et al.* (1996) used grey seal teeth to demonstrate that canine-derived estimates of age were more highly correlated with known age than incisor derived estimates, and they concluded that incisor-based estimates were less accurate than canine-based estimates, although they do provide a reasonable estimate of age for use in certain applications. The true accuracy of an individual's age estimate made using tooth GLGs can only be determined through the use of known age individuals. However, obtaining large samples of known age individuals by way of testing an experimental approach is not always possible, especially where populations of wild animals only exist (Watanabe *et al.* 1999). Ultimately, choice of tooth will depend on whether or not the animal is alive or dead, as choice may be restricted in live animals.

Collecting and sampling teeth

Carcases

In the field, skulls and/or mandibles are often collected from carcases. This enables a wide choice of teeth. The mandibles are then macerated in a heated tank where effectively the teeth loosen in the rotted jaw. The skulls/jaws can be tied up in nylon mesh (worn out ladies' nylon tights work well) and macerated at 40°C with a small amount of *Biotex* (enzyme detergent) for 2 weeks in a tank or merely identified with an attached tag. Generally it is considered harmful to subject the teeth to prolonged boiling; the process d enatures proteins and damages the cementum and dentine (Lockyer 1993, Perrin and Myrick

1980). They are then rinsed well afterwards. The teeth can then be removed relatively asily with dental retractors or in some stubborn cases with pliers by pulling and twisting. Cleaning the teeth with a tooth brush using dish washing detergent removes any remaining stickiness from the teeth. Individual teeth can also be removed from carcases using bolt cutters to cut small sections of jaw. Cleaning of any residual adhering tissue can be made with enzymes as outlined below.

Live animals
In live animals, extraction of incisors or post-canines requires the use of a scalpel/tooth chisel to separate the tooth from the surrounding gum tissue before pulling and twisting using dental elevators, and administration of either local anaesthetic during the procedure or *e.g.* isofluorane via a face mask to induce unconsciousness (Blundell and Pendleton 2008). However, this type of tooth removal can be difficult and may occasionally result in damage to the root.

For any specimens where gum tissue is still present, once the teeth are freed from the jaw, cleaning can be effected with the use of enzymes such as pepsin or trypsin in a water bath. A proprietory brand chemical, RMS Entkeimungsmittel - UN 201 (TEGEE-Chemie Bremen GmbH, Bergedorfer Str. 6-8, 28219 Bremen), used in a 5% solution and soaking the teeth overnight, produces clean specimens with hardly any residue attached. The teeth are thus more pleasant to handle and more importantly, the lack of external gum tissue makes it easier to identify GLGs at the growing edge in older animals. In general, enzyme use does not appear to affect the tooth tissue and the clarity of GLGs.

Long term storage of teeth
In previous studies, teeth have been stored in a variety of ways prior to ageing: frozen, dry, in ethanol or glycerol-ethanol mixture, or in neutral buffered formalin (Perrin and Myrick 1980). However, the method of storage has an observable effect on the quality of the tooth material. Storage dry may cause the teeth to crack, and formalin will decalcify the tooth over time and make the GLGs unreadable unless the solution is buffered and made neutral. The recommended method of storage for tooth material is in 70% ethanol (Perrin and Myrick 1980) which is also safer to handle than formalin. It is better to clean the teeth before storage. It is worth noting that certain preservatives may render the tooth or sections un-useable for subsequent chemical analysis.

Preparation of teeth
Once teeth have been extracted and cleaned, they need to be sectioned in order to expose the GLGs within the dentine and/or cement. Teeth can be sectioned in a buccal-lingual or transverse plane, depending on the species and whether dentine or cement is the tissue in focus. Simple untreated thin sections can be made with a circular diamond saw, and decalcified ultra-thin sections can be prepared for staining using a microtome. Details of these methods are provided later under Methods of Age Determination in Harbour Seals.

Estimating age from teeth
Incremental growth layers are present in both the dentine and cementum of carnivore teeth. Early studies using the teeth of fur seals *(Callorhinus ursinus)* and elephant seals *(Mirounga leonina)* showed that incremental ridges visible on the surface of the root were of an annual nature (Laws 1952, Scheffer 1950). Scheffer (1975) proposed that the annual growth layers could be correlated with seasonal changes in individual growth rates. Subsequently, Klevezal (1980) showed that annual layers in sectioned teeth could provide a record of yearly growth cycles in many species. Each of these annual layers is termed a growth layer group and each of these can be composed of several sub-layers or incremental growth layers with a specific pattern of broad and narrow bands associated with season, although growth is continuous.

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). The annual nature of these layers has been calibrated through investigations using tetracycline markers (Myrick *et al.* 1984, Lockyer 1993), individuals of known age (Bowen *et al.* 1983, Klevezal and Stewart 1994,

Harshyne *et al.* 1998) and repeated removal of teeth from individuals after known time intervals (Myrick *et al.* 1984, Lockyer 1993). Teeth have been used as indicators of age in several terrestrial (Johnston *et al.* 1987) and marine (Hohn 2002) species. In addition to ageing, growth layer characteristics and ultrastructural anomalies in tooth composition have been used to indicate life history events such as sexual maturation (Klevezal and Myrick 1984, Härkönen and Heide-Jørgensen 1990), environmental variation (Manzanilla 1989, Lockyer 1995), and stock structure (Lockyer 1999).

GLGs are visible in both the dentine and cementum, and in certain species either tissue can be used to accurately age individuals (Perrin and Myrick 1980). In many species of pinniped, including harbour seals, deposition of the dentine becomes disrupted due to apparent occlusion of the pulp cavity (Dietz *et al.* 1991, Hewer 1960, Norgaard and Heje-Larsen 1991, Stewart *et al.* 1996) and the number of GLGs within the dentine will not accurately represent an individual's age. Although dentinal GLGs may be more easily definable, only GLGs deposited in the cementum can be used to age individuals in several species, including the harbour seal as later discussed. It is important to note here that dentinal growth is centripetal while the cemental growth is centrifugal and thus not limited by lack of space in its development.

Methods of age estimation

The approach adopted in pinnipeds commonly varies in relation to the size of the tooth, but variation in the preparation of tooth sections and the use of specific stains also varies in relation to the equipment available, and has progressed through the years (*e.g.* Mansfield and Fisher 1960, Bowen *et al.* 1983, Dietz *et al.* 1991, Mansfield 1991, Lawson *et al.* 1992, Childerhouse *et al.* 2004). Sometimes more than one method is acceptably accurate, when choice of method may be guided by other considerations such as speed of preparation, cost, available equipment or precisely what data are expected from the teeth.

Despite the range of methods used in different ageing studies, there are only a few examples of direct comparisons between different experimental approaches for ageing individuals from the same or from different species (Hohn 1980, Smith *et al.* 1994, Stewart *et al.* 1996, Mackey 2004, Lastra-Luque 2008). One such study by Hohn (1980) compared the use of decalcified stained sections viewed under the light microscope, with the SEM (Scanning Electron Microscope) and microradiography in bottlenose dolphins. She found that in this species light microscopy (decalcified and stained sections) was less complicated in terms of preparation, but the technique was less accurate, and less reliable, than both the SEM, and microradiography. Microradiography was also shown to have increased sensitivity over the use of x-rays as the high-resolution photographic plate provided images that were more sharply defined. In this study the SEM was concluded to be the easiest method applied to count GLGs and accessory layers, due to the ease of reading the relief differences in the tooth surface. However, none of the individuals used in this study was of known age, so the accuracy of the methods could not be assessed. Smith *et al.* (1994) studying chemical ultrastructure of black bear cement using comparison of stained sections and SEM of untreated sections, found that the differentiation in dark- and light-staining was based on collagen density rather than calcification.

Counting the GLGs in either the dentine or cementum has previously been used to age phocids including harbour, grey and ringed seals (*e.g.* Mansfield and Fisher 1960, Bigg 1969, Hewer 1964, Dietz *et al.* 1991, Mansfield 1991, Stewart *et al.* 1996, Bernt *et al.* 1996). Pulp cavity occlusion means that the cementum should be used to more accurately age individuals, and in harbour seals effective occlusion occurs relatively early; estimates range from age four (Norgaard and Heje-Larsen 1991) to age ten (Dietz *et al.* 1991).

When ageing individuals using the GLGs in teeth, reading by more than one observer is often recommended. However, estimates of age have been shown to vary between readers with different levels of experience (Bernt *et al.* 1996, Lawson *et al.* 1992, Mackey 2004). As always, the issue of accuracy of age determination and precision remains. Age determination involves

different stages: selection of the most suitable method of tooth preparation; standardization of counting among readers for comparability of ages; validation of age from incremental layering using known-age or -history specimens; and, the final interpretation on the age to be used for analysis. At least with the first two stages, there is always room for error.

In the following section of the paper, we present and discuss the value of different methods for examining tooth GLGs in harbour seals, and conclude with the best techniques for use in this species.

METHODS OF AGE DETERMINATION IN HARBOUR SEALS

Historically, harbour seals have been aged from cementum GLGs using non-decalcified polished sections viewed using transmitted light (Mansfield and Fisher 1960) and polarized light (Boveng and Laidre 2001) from below, decalcified stained thin sections (Dietz *et al.* 1991, Norgaard and Heje-Larsen 1991), and x-rays (Norgaard and Heje-Larsen 1991). Mansfield and Fisher (1960) used layers in the cementum viewed using transmitted light to accurately age a harbour seal of known age in captivity. Despite ageing having been carried out in harbour seals through different experimental approaches the different approaches have not been formally compared. Therefore, uncertainty exists as to which approach is the most suitable. Three possible approaches to ageing harbour seals, based on methods used for carnivores, and the relative merits of each approach are detailed below.

Recently, Blundell and Pendleton (2008) investigated the correlation of ages derived from incisors, canines and post-canines from harbour seals, and also accuracy of age from incisors from known-age harbour seals with reference to morphometric data. Removal of post-canines in live animals is preferable for age accuracy, although removal of incisors is less invasive. However, morphometric data may be a reasonable substitute for tooth age in young animals older than one year when teeth cannot be removed. In general, tooth selection in harbour seals favours extraction of the canines or post-canines for accuracy although incisors should be taken from live individuals.

Comments on tooth preparation

The composition of cementum is not uniform along the tooth (Stewart *et al.* 1996), and so the cementum in the lower third of the tooth is generally favoured for harbour seals. The cutting plane and thickness of the section used in the preparation of tooth material can vary, dependant on methodological approach and tooth size (which determines how easy it is to cut a tooth in a certain plane). Transverse sections are often used with large or curved teeth, such as canines, as buccal-lingual sections (longitudinal through crown and root) may be off centre (Klevezal and Stewart 1994). Mansfield and Fisher (1960) believed that layers in the cementum of harbour seal canines were better resolved in transverse sections, but only a single tooth was used, which had no clear dentinal layers. Dietz *et al.* (1991) cut buccal-lingual sections from harbour seal canines in a plane parallel to the mid-longitudinal plane of the root-tip to provide a good grip on the freezing platform. No reference was made to the suitability of transverse or buccal-lingual sections for the readability of the teeth; the cutting plane was purely a practical consideration. Fiona Read tried cutting Dutch harbour seal teeth in different planes and found that the cementum tends to be thinner and harder to read in a buccal-lingual plane than a sagittal plane of the root end of the tooth. Sections made at different places down the tooth from the crown indicated that the thickest areas of cementum tend to be the root and are generally the best areas for counting.

Method 1:
Light microscopy on untreated teeth

Examination of tooth sections under the light microscope allows the relative opacity and translucency of the tissues of the dentine and cementum to be assessed. Tooth sections can be examined using transmitted light from below with a polarizing filter, with no prior decalcification nor staining.

Sectioning untreated teeth
This method has been used on harbour seal canines (Mansfield and Fisher 1960, Boveng and Laidre 2001, Mackey 2004). With smaller teeth (such as incisors) it may be more difficult to use. Teeth should be sectioned using a low speed circular saw such as Buehler Isomet (from Buehler Ltd, Illinois, USA) with a diamond wafering blade.

- Sections used should be relatively thick, approximately 80-100 μm, although Ilka Hasselmeier reports that 40-50 μm can be satisfactory. Sections can be buccal-lingual (or facial-lingual for incisors) or transverse. Transverse sections are easier to cut because there are more opportunities to section along the tooth, but may provide less options of seeing well defined GLGs within the cementum.
- The teeth sections should be examined while wet under a light microscope with a polarizing filter.
- As the plane of the GLGs is not constant through the tooth cementum these relatively thick sections allow the depth of focus to be varied through the tooth, and the best age estimate is gained by rereading the tooth at several regions within the root.

This method provides a very quick solution and excellent results in grey seals but when applied to harbour seals, GLGs appear less well defined (Mackey 2004) than the alternatives detailed below (see Figs 3a, 4a and 5a).

Method 2:
Light microscopy on decalcified and stained teeth
Decalcified and stained tooth sections are examined under the light microscope using transmitted light from below.

Decalcification and staining of teeth
Decalcification of the whole tooth is generally recommended for the study of small marine mammal teeth (Perrin and Myrick 1980). In early studies, nitric acid solution was commonly used, with decalcification being a protracted procedure lasting several days or weeks (Dietz *et al.* 1991, Norgaard and Heje-Larsen 1991). More recently the development of commercially prepared mixtures of acids, such as RDO (Apex Engineering Products Corporation, Illinois, USA), has substantially reduced decalcification times to a matter of hours, although this time is dependent on the density and size of the tooth (Lockyer 1993, Lockyer 1995, Hohn and Fernandez 1999).

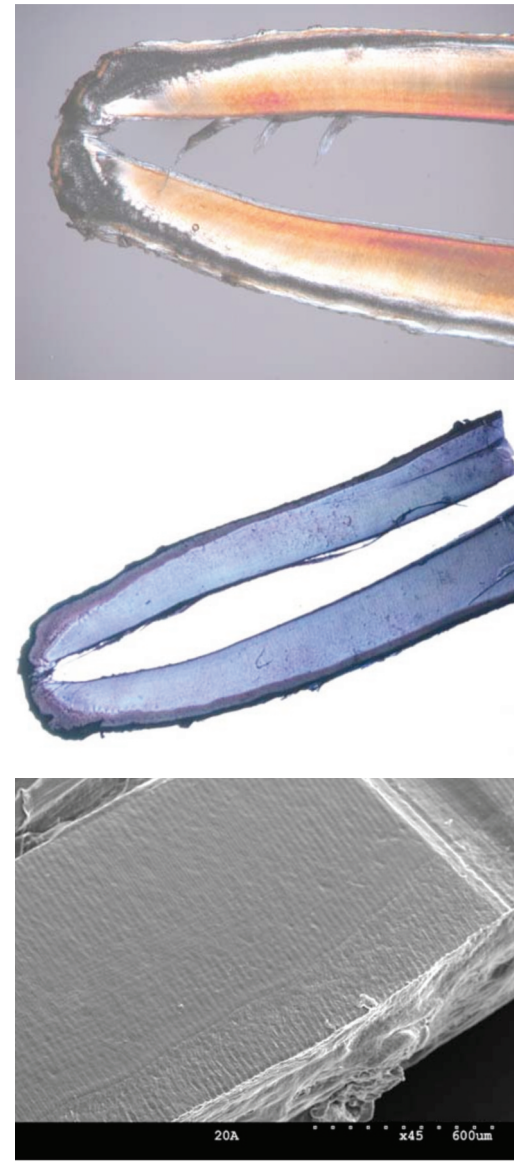

Fig. 3. *Buccal-lingual sections of a canine tooth from a harbour seal less than one year old viewed a) with transmitted polarized light b) decalcified and stained, and c) under the SEM. The large pulp cavity is visible in a) and b) which is not yet occluded. Growth lines are also visible within the dentine in each image.*

The recommended thickness for decalcified sections of tooth is dependent on whether the dentine or cementum is to be examined. A thickness of 12-14 µm is recommended for the examination of the cementum. Sections of teeth can be examined either stained or unstained. When examining the cementum to determine the age of an individual it is also important to remember that not all of the cementum surface may be forming at once. This means that relatively high levels of magnification need to be used to determine layer distribution (Boyde 1980). Cementum is very similar in growth and structure to bone. Dietz *et al.* (1991) were the first to validate the examination of cementum GLGs using Toluidine Blue-stained decalcified sections of harbour seal teeth.

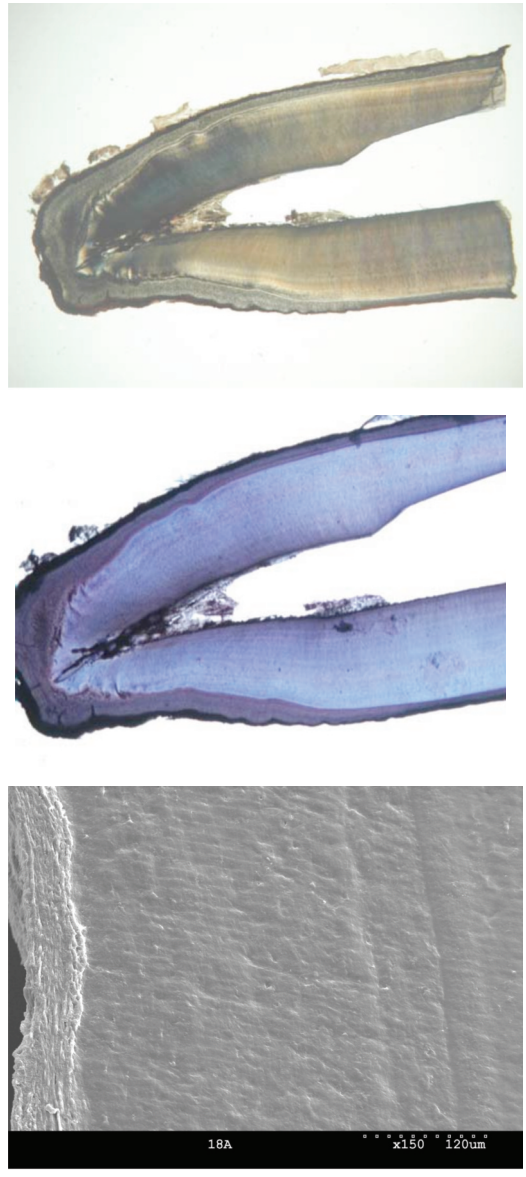

Fig. 4. *Buccal-lingual sections of canine tooth from a harbour seal estimated to be 2 years old viewed a) with transmitted polarized light b) decalcified and stained, and c) under the SEM. The pulp cavity is visible in a) and b) which is not yet occluded.*

This method has been used successfully on harbour seal incisors, canines, premolars and molar teeth (Dietz *et al.* 1991, Härkönen and Heide-Jørgensen 1990, Mackey 2004). A Buehler Isomet low speed saw is used to cut off the crown of the sample so only part of the tooth is decalcified (*e.g.* lower third including the root). This shortens decalcification time, and also trims the outside of the tooth when sectioning in "longitudinal" plane. This also eliminates the problems with teeth that have a pronounced curve at the tip. A wafer of about 2.5-3 mm thickness is adequate for decalcification.

- Prior to decalcification, fresh teeth should be fixed in buffered 10% formalin for a minimum of 12 hours, and then rinsed well in water. This stage is often by-passed, but integrity of the tooth tissue and subsequent sectioning are better with fixation. Teeth previously stored in alcohol need to be re-hydrated in water for a few hours before treatment.
- The teeth, once wafered, can then be decalcified using a commercial preparation of acids, RDO (see above) for between 2 and 12 hours. Decalcification times depend on the relative density of the tooth and thickness of the wafer. Whether decalcification is complete can be determined by bending the tooth material gently to assess pliability. The teeth should then be rinsed well in running water, and stored in water overnight to remove excess acid.
- Sections should be made of between 12 and 14 µm, using a freezing microtome at a temperature of –10°C to avoid excessive tearing of the tooth surface. Teeth can be cut in either transverse or buccal-lingual (facial-lingual for incisors) section; although authors are not in agreement as to whether transverse or "longitudinal" sections are better for reading GLGs. However, in general we all agree that

buccal-lingual sections provide a larger area for examination in canines and post-canines and would be our choice.

Following Dietz *et al.* (1991) sections are collected and rinsed in distilled water and then stained free-floating in 1% Toluidine Blue solution for between 20 and 40 minutes. Time in stain will vary among teeth, so sections should be checked at regular times to avoid over- staining. However, Christina Lockyer has found and recommends that for sections already floated onto and mounted on 5% gelatine-coated slides, a stain concentration of 0.3% Toluidine Blue with zinc chloride in a solution containing 1% sodium bicarbonate is best for cement with staining taking only 15-20 seconds. A low concentration of 0.2% Toluidine Blue is also recommended by Gurr (1963) with times of 20-30 seconds. With a lower concentration it is easier to control the extent of staining. Over-staining can be reversed for either technique if sections are left in running water for some hours.

- If the free-floating approach to staining is adopted each stained section should be examined to determine the best (least scoring from the cutting blade, or clearest GLGs) before mounting. After removal from Toluidine Blue the sections – whether free-floating or already mounted on slides – are placed in running water to remove excess stain. The best sections can be floated in water onto slides coated in 5% gelatine solution (to act as an adhesive to the slide and prevent the sections from curling or shrinking as they dry). Slides should be left to dry (about 15-30 min) before mounting the cover slip with DPX (a xylene-based mounting medium). This stage should be done in a fume cupboard. The slides need to be left overnight to firm but will require some days to fully harden.
- Slides should be examined under the light microscope (x40 and x100) with plain transmitted light (Figs 3b, 4b and 5b). Individuals are aged by counting the number of GLGs in the cementum. Each tooth should be read on at least two independent occasions, information on the date of the death of the animal or live extraction allows accurate ageing to within months – after the initial counting.

Compared to using just polarized light, decalcification and staining makes reading cementum GLGs in harbour seals more consistent and easier (Mackey 2004).

Fig. 5. *Buccal-lingual sections of canine tooth from a harbour seal known to be 11 years old when it died viewed a) with transmitted polarized light b) decalcified and stained, and c) under the SEM. The pulp is occluded in this individual.*

Method 3:
Scanning Electron Microscope (SEM)
The use of the SEM has aided in detailed studies of the processes associated with tooth growth, and age determination. Etching or demineralization is used to dissolve calcium phosphate in the tooth below the surface of a cut and polished section (Boyde 1980). The difference in density and degree of mineralization within each GLG leads to visible ridges and grooves that can easily be counted (*e.g.* Hohn 1980). Teeth can be viewed and aged in buccal-lingual and transverse sections with the scanning electron microscope. The approach has been successfully carried out using canine teeth from harbour seals (Mackey 2004).

Preparation for SEM
- First the teeth are sectioned using a Buehler Isomet low speed saw with a diamond wafering blade. The sections need to be a minimum of approximately 2-3 mm thick for ease of manipulation.
- The sections are etched in 5% formic acid at room temperature (20-25°C). In harbour seals etching times between 15 and 25 minutes appear to provide the best GLG clarity (Mackey 2004).
- On removal from formic acid each sample is rinsed thoroughly in distilled water to remove any excess.
- Samples are then air-dried and mounted on carbon tabs on aluminium specimen stubs.
- The samples are plated with gold using a sputter coater (*e.g.* Bal-Tec SD005) for 60 seconds at a 30 μm current.
- A scanning electron microscope (*e.g.* Hitachi S-4300) is then used to examine the mounted samples.
- Micrographs should be taken of the cementum at relatively low magnifications (x25-x90) at 5 kV. The exact magnification and working distance reflects the relative size of the tooth sections.
- The sections should be viewed, and micrographs taken at an angle of 40° to provide good relief. Examples are shown in Figs 3c, 4c and 5c.

The main benefit of using the SEM to age individuals is the clarity of the GLGs due to the differential relief after etching the surface of the tooth. In general, Tero Härkönen and Beth Mackey found harbour seal tooth sections prepared in this way to be of equal readability to decalcified and stained sections. Using the SEM also enables additional information to be gained on tooth composition and life history by using mass spectroscopy for example (Mackey 2004).

Other methods
Alternatively, the use of microradiography provides a non-invasive technique (in terms of preservation of the sample material) to determine the mineral density of the teeth. The x-rays are absorbed by calcium and phosphate in teeth; high mineral density provides a radio-opaque zone, and poorly mineralized areas are radiolucent (Hohn 1980). Norgaard and Heje-Larsen (1991) applied radiography to study the lower canines of harbour seals from the Wadden Sea. By examining the relative thickness of the dentine and cementum combined, younger seals (up to age class 3) could be assigned to a stage class. The method was used as a tool to distinguish juveniles from older individuals, but direct estimates of age could not be made.

Summary of methods
Our overall recommendation is for the use of decalcified stained thin sections of cementum for age determination in harbour seals. The extra labour and increased cost involved in additional preparation steps over the use of untreated sections is outweighed by the improved clarity of the GLGs and the display of other structural details in the tooth tissue. However, the actual choice of method may also be influenced by the number of tooth samples requiring processing and the time available. We therefore summarize the methods discussed with their various benefits and disadvantages so that a researcher can make an informed decision regarding which method will suit their situation best (Table 1. below).

HARBOUR SEALS - APPRAISAL OF METHODS USED, AGE VALIDATION AND NEW INVESTIGATIONS

Harbour seals appear more difficult to age than other species of phocid seal (Mackey 2004). In common with other species, older individuals appear more difficult to age (Mackey 2004). Also Dietz *et al.* (1991) highlighted the difficulty in reading the ages from growth layers in harbour seals between the ages of 2 and 10, since the deviation among readers increased in this span. There also appear to be differences in the relative clarity of the GLGs between populations of harbour seals. For example, Tero Härkönen and Beth Mackey found that teeth taken from animals of Swedish origin (Skagerrak) had much clearer GLGs, and were subsequently easier to age than teeth from Scottish populations (Moray Firth). We believe that differences in seasonality, food availability, or differences in pollutant burdens may be important underlying causes for these differences.

In choosing the most appropriate method for ageing in any species it is important to weigh the costs of the experimental approach. Preparation of samples for the SEM, and decalcified and stained sections for viewing with the light microscope, both take longer than the preparation of samples for viewing under polarized light. Therefore, a trade off may be made between the most appropriate method and the associated costs.

However, where no strong case can be made for a particular method being more accurate, decisions on the method adopted may purely come down to time and money (refer to Table 1 and the associated discussion earlier).

Interpretation of GLGs and example age series including known-age animals

Ultimately, interpretation of GLGs requires validation from known-age animals (Figs 5 and 6) where GLGs can be directly correlated with known age in years and in order to establish accuracy. Based on this GLG interpretation, there should be standardization among readers. When using cementum as the age medium, it is important to bear in mind its formation and growth characteristics. New cement forms from the gum so that the most recently formed layers are always on the outside of the tooth with the first year growth adjacent to the dentine. Usually the junction between cement and dentine is clear so that identification of the first GLG is easier once this is noted. Normally, the first GLG is very broad with a narrowing of GLG bands with advancing age (Fig. 6b), especially after GLG 2-3 with more densely packed GLGs after 3-4. This may be associated with sexual matura-

Table 1. Summary of cost and benefits of different tooth preparation methods in harbour seal.

Method	Relative cost	Time required	Precision	Accuracy
Untreated sections (ca 100 μm) and polarized light	Low	Short	Low	Low/Medium – where tested
Decalcified stained thin sections (ca 12 μm)	Medium	Long	High	Medium/High – where tested
SEM	High	Medium	High	Medium/High – where test
Microradiography	Medium	Short	Low	Unknown/Low

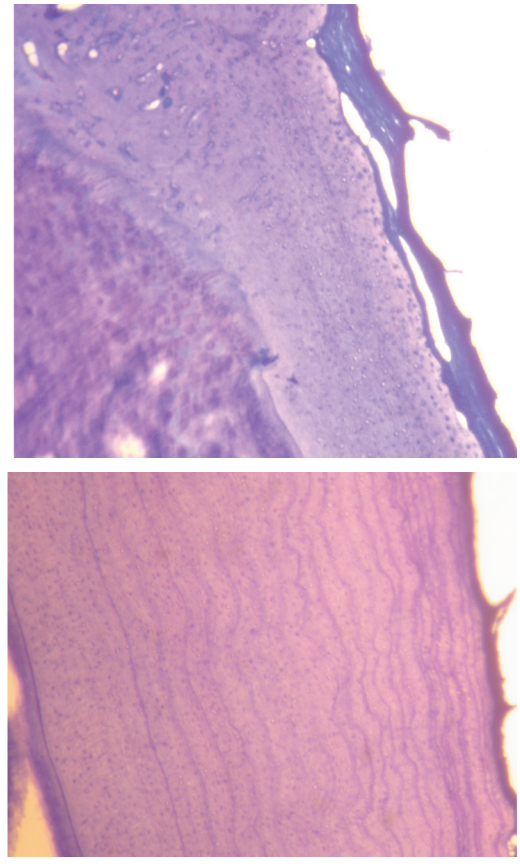

Fig. 6. *Cemental layers in known-age harbour seals from Inner Danish Waters: a) Seal no 245 born July 1985 with 2 GLGs in the cementum; died in third year of life ; b) Seal no 2 born June 1970 with 21 GLGs in the cementum; died in twenty second year of life. Stain used is Toluidine Blue. Specimens courtesy of Rune Dietz, National Environmental Research Institute, Roskilde, Denmark. Photographs by Christina Lockyer.*

tion and a general slowing of body growth. However, this pattern is not always the case and irregularities in GLG width as well as accessory lines within GLGs are common and can cause errors in ageing. Cement – as in all species - can also appear porous in young animals, especially in the root, and other anomalies may render GLG interpretation difficult. This will be addressed below under mineralization anomalies, especially with regard to the presence of "paired" and "false annuli".

Fig. 5 demonstrates 3 different preparation methods of a known age animal's tooth where all methods provided an accurate age. Fig. 6a shows the broad cemental GLGs of a juvenile, with a substantial development of part of the third GLG in a decalcified and stained thin section. Fig. 6b demonstrates in a decalcified thin stained section, the narrowing of the adult GLGs and the appearance of confusing double laminae towards the outer edge. The calibration of age from known age animals is particularly valuable for deciding how to interpret such double lines and whether to count them as one GLG (as here) or individually.

As noted earlier, the cement does not form evenly all along the tooth below the gum, and can also be thicker in one plane or along one side than the other. We often found a difference of one GLG in different sites on the tooth. It is therefore important to check the cemental GLGs along and around the entire tooth edge before assigning an age.

In the initial stages of counting it is best to do the work without reference to biological information on the animal, and also to undertake independent readings at least twice by either the same reader with a time interval between the reading events, or two experienced readers. In the event of disagreement on the age among readers or even the same reader, additional readings may be necessary, with discussion about where disagreement occurs. However, in finalising an age estimate for an individual, it can then be useful to refer to other biological data after the GLG counting has been done to improve accuracy. This is especially valuable for age estimation when, for various reasons, readers disagree and/or the GLG count is uncertain because of presence of accessory lines and there are various interpretations. In adopting a final age, it should be recognized that uncertainty of +1 in a young animal may be more significant than uncertainty of +2 in an old animal, and the degree of accuracy required also depends on what use the age data will be put to.

As a final comment, it is less important to employ several readers than a single reader with experience in a standardized counting method. Indeed all readers should refresh their technique before a reading programme by first examining a reference set of teeth for which ages are agreed.

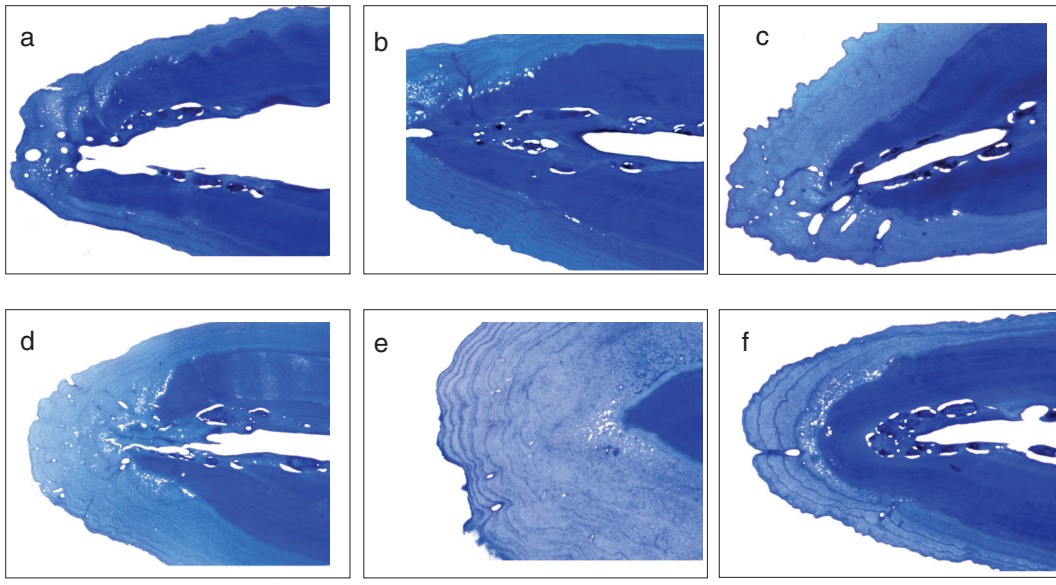

Fig. 7. *Examples of different mineralization anomalies in harbour seal teeth. Stain used is Toluidine Blue. a) Seal K0774: pulp stones and cemental disturbance; b) Seal K0818: pulp stones; c) Seal K0908: "wobbly" cement on outside of tooth ; d) Seal K0965: cementum and dentine border hard to see at the pulp cavity opening; e) Seal K0968: paired laminae in older GLGs; f) Seal K0938: pulp stones and dentinal resorption.*

This can also be undertaken as an exercise periodically to maintain standards, and it is recommended to compile a reference set of teeth representing various ages for this purpose. Furthermore, we propose that it would be a valuable exercise to examine old material - especially from known age animals if feasible - using different readers, and even to try new techniques on older material to compare with former preparation methods. Perhaps the most important point is that apart from striving for accuracy, all readers should establish a reading standard and interpret GLGs in a comparable manner. The value of a standard reference set of material is thus imperative for maintaining ageing standards of accuracy and precision.

Mineralization Anomalies in Teeth

Annual GLGs also contain daily layers, lunar layers, accessory layers and other features formed during the year (Lockyer 1995; Klevezal 1996). Deposition patterns in GLGs are also thought to indicate general health, sex, reproductive history, attainment of sexual maturity, periods of physiological or environmental stress (Klevezal and Myrick 1984, Lockyer 1995) and even suckling lines (Bengtson, 1988).

Disturbances in the regular rate of deposition of cementum and dentine are termed mineralization anomalies and 5 different types have been defined (Myrick 1988, Lockyer 1993, 1995). Here we investigate the occurrence of such anomalies in harbour seal teeth based on previously identified characteristics and also ones we have noted:

- *Pulp stones* – discrete nodules containing concentric rings in the dentine but may also remain free in the pulp. (Lockyer 1993) (Figs 7a and b)
- *Marker lines* – found in both dentine and cementum. Discrete laminae that are regular but noticeably different in appearance from the boundary layers of GLG *e.g.* deeply staining or non-staining (Lockyer 1993).
- *Cemental disturbance* – any anomalous appearance of the usual laminated cemental tissue (Figs 7a and 7c, including mineralization interference and resorption (Myrick 1988). Cemental resorption may progress into dentine (Fig. 7d) and irregularly around the root due to excessive or unequal forces on the tooth (Armitage 1976).
- *Mineralisation interference* – irregularities in the lamina formation, generally found in the pulp cavity edge, resulting in wavy lines,

Table 2. The presence of anomalies recorded in 206 harbour seal teeth.

	Immature		Mature		Total		
	Female	Male	Female	Male	Females	Males	Total
AL (n=11)	0.5%	0.5%	2.4%	1.9%	2.9%	2.4%	**5.3%**
CD (n=110)	10.7%	16.5%	14.6%	11.6%	25.3%	28.1%	**53.4%**
DR (n=32)	0.5%	3.4%	7.8%	3.9%	8.3%	7.3%	**15.6%**
PS (n=136)	8.3%	16.1%	24.1%	17.5%	32.4%	33.6%	**66.0%**

KEY:
AL= accessory lines
CD= cemental disturbance
DR= dentine resorption
PS=pulp stones

swirl patterns and asymmetry which disrupt usual patterns (Lockyer 1993).

- *Dentinal resorption* – actual erosion and frequent repair of existing regular laminated dentinal tissue, resulting in an amorphous and/or globular appearance. Frequently with holes, cutting across and into regular tissue (Myrick 1988) (Fig. 7f).

In addition we include the following:
Accessory lines or false annuli – additional layers within a GLG which may stain light or dark observed in both dentine and cementum. They may follow the true pattern of the GLGs, be 'patchy' or continuous and may even form a "double GLG" pattern called paired annuli with the GLGs (Fig. 7e). Fig. 6b demonstrates a good example of what could be interpreted as "paired annuli" in the outer part of the cement.

It is still not fully understood what causes these anomalies to occur. For harbour seals, cemental disturbance and accessory lines appear to be the only anomalies that may affect age estimates, due to the fact that we count GLGs in the cementum.

Age was estimated for 206 harbour seals that died during the 2002 PDV epizootic in the Netherlands and the presence and type of anomalies recorded. The preparation method used was decalcified thin-sectioning (14 µm) and staining with Toluidine Blue. Maturity was based on the analysis of reproductive organs (Read *et al.* 2007). Ages ranged from 0-21 years. Overall, 81.2% (n=168) of the seals had at least one type of anomaly in their teeth (Table 2). Neither marker lines nor mineralization interference were recorded in any of these teeth. When accessory lines were recorded, all the animals were over 4 years of age and with the exception of one immature male, all the animals also had cemental disturbance. Seals of one year had only cemental disturbance as an anomaly. All seals with dentine resorption were over 3 years of age and had pulp stones in their teeth. Additionally, over half of the animals with dentinal resorption also had cemental disturbance. Pulp stones were the most commonly seen anomaly in 66% (n=136) of the seals and were always recorded in seals over 2 years old. When we exclude all newborns of the year and 1 year olds, 93.2% of all the seals have anomalies (n=151), mainly pulp stones and cemental disturbance. The animals without any anomalies in this sub-set (n=11) were all between 2 and 4 years old.

In the present study, accessory lines were not very common among the harbour seal teeth analysed. However, along with bears (Ursus spp.), harbour seals have the most accessory lines in their cementum and these can be very problematic for ageing. Coy and Garshelis (1992) found that only 3% of 146 male black bears (Ursus americanus) had no accessory lines. Generally, accessory lines are easily identified as anomalies. However, sometimes accessory lines are continuous and follow the same pattern as the GLGs making it hard to

distinguish actual GLGs from the accessory lines. Additionally, they may form "paired annuli" with the actual GLG to give the appearance of double lines and therefore double the age. Paired annuli in female black and polar bears are thought to indicate cub rearing (lactation) and were only observed in GLGs after sexual maturity (Coy and Garshelis 1992, Kirkegaard *et al.* 2005). Therefore, paired annuli are counted as 2 years in females and one year (and one "false annuli") in males. However, this does not appear to be the case in harbour seals, most likely due to the considerably shorter lactation period (3-4 weeks compared to that of up to 2 years in bears) therefore, for both sexes, paired annuli in harbour seals are counted as "one year and one false annuli" (refer also to discussion earlier of the GLG interpretation of known age seal in Fig. 6b).

The best way to overcome the problem of accessory lines is to try to determine a pattern in the cementum layers and ensure that it is possible to follow the GLG boundary line along the section. With experience, detecting accessory lines becomes easier.

CONCLUSIONS

Tooth selection in harbour seals depends in part on whether the animal is alive or dead and accessibility. Teeth are best removed from carcase jaws after some maceration to ease extraction. Lengthy boiling is not recommended because denaturation of the tooth protein may result in destruction of the GLG contrast. However, incisors, canines and post-canines (premolars) can all be used with less age accuracy reported in incisors which are often taken in live animals. Teeth are best stored in fluid to avoid drying and cracking, and 70% alcohol is suitable. Tooth cementum is a suitable material for estimating age of harbour seals. All round, consistently good results are obtained by decalcifying tooth wafers cut in the buccal-lingual – canines and post-canines and facial-lingual – incisors (longitudinal) planes, sectioning at 12 µm and staining with Toluidine Blue. Use of a microscope with plain transmitted light is best for examining the sections and counting GLGs. We recommend this method. Most other methods may be useable under certain circumstances *e.g.* equipment unavailability, and other stains *e.g.* Haematoxylin may be substituted. However, results will not be as consistently satisfactory as the recommended method.

The most important factor in reading the tooth sections is that the reader(s) are familiar with the standard method of counting and interpreting GLGs for the species. New readers and indeed experienced readers can standardize their counts by periodically referring to a standard collection of tooth sections for which there are agreed ages. In this respect it is more valuable to have a single experienced reader than a number of lesser experienced readers. Counts are normally done on at least 2 separate occasions and initially performed without reference to biological data that could bias results. However, reference to biological data can be useful when counts are ambiguous and it can be seen that "paired laminae" and indeed other types of mineralization anomalies may be confounding age estimates.

Our final recommendation is that it is a constructive practice to exchange a few samples and photographs of teeth with other workers from time to time in order to maintain standards. In addition, each ageing laboratory should hold a reference set of tooth sections – to include some known age specimens if possible – that can be used for standardization each year by both experienced and new GLG readers in order to maintain standards. Furthermore, the publication of photographs of an ontogenetic series of ages for harbour seals – in a similar manner as done for harbour porpoises (Hohn and Lockyer, 1995) - would form the basis of a universal reference for ageing.

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