Government of South Australia

South Australian Museum

North Terrace
Adelaide SA 5000
ABN 39 808 959 302

Telephone +61 8 8207 7500
Facsimile +61 8 8207 7430
www.samuseum.sa.gov.au

AGE DETERMINATION OF MARINE MAMMALS USING TOOTH STRUCTURE

Marine Mammal Ageing Facility
South Australian Museum, South Australia

Workshop held 22–25 August 2007

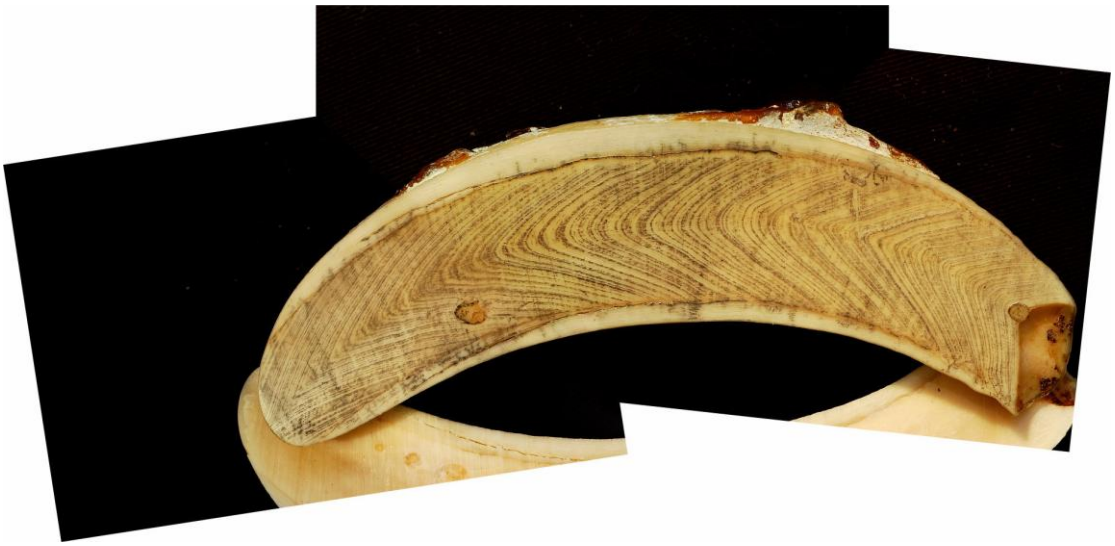
Handbook written and compiled by K. Evans, C. Kemper, J. McKenzie and R. McIntosh

Published by the South Australian Museum

AGE DETERMINATION OF MARINE MAMMALS USING TOOTH STRUCTURE

Marine Mammal Ageing Facility
South Australian Museum, Adelaide, South Australia

Workshop held 22–25 August 2007



Handbook written and compiled by K. Evans, C. Kemper, J. McKenzie and R. McIntosh

Published by the South Australian Museum

The content of this manual is based on a number of technical, circumstantial or otherwise specified assumptions and parameters. The user must make his or her own assessment of the suitability for its use of the information or material contained in or generated from the manual. To the extent permitted by law, the South Australian Museum excludes all liability to any party for expenses, losses, damages and costs arising directly or indirectly from using this manual.

The use of this manual is subject to the terms on which it was prepared by the South Australian Museum. In particular, the manual may be used only for the following purposes.

- it may be copied for distribution within the Client's organisation;
- the information in this manual may be used by the entity for which it was prepared ("the Client"), or by the Client's contractors and agents, for the Client's internal business operations (but not licensing to third parties);
- extracts of the manual distributed for these purposes must clearly note that the extract is part of a larger Report prepared by the South Australian Museum for the Commonwealth Department of Environment and Water Resources in 2008.

The manual must not be used as a means of endorsement without the prior written consent of the South Australian Museum.

The name, trade mark or logo of the South Australian Museum must not be used without the prior written consent of the South Australian Museum.

TABLE OF CONTENTS

INTRODUCTION

Determining the age of animals is essential to understanding the ecology and dynamics of populations. Knowing the age of individuals allows us to understand population demography, growth rates, population structure and age at sexual and physical maturity (Langvatn 1995). The structures used to determine age (*e.g.* teeth) can also yield information on general health, reproductive history and the influence of environmental factors on growth, health and reproduction (Lockyer 1995).

Methods of age estimation based on counts of growth layers in hard parts of marine mammals have been used in the study of age-related biology of marine mammals since the 1950s (Scheffer 1950; Laws 1952). For most species, the methods have involved the examination of incremental lines on the exterior or within the structure of teeth. However, for those species that do not grow teeth (*e.g.* baleen whales), or whose teeth are not suitable for aging (*e.g.* manatees because they lack tusks and have molars that are continually replaced throughout life) other structures such as bone, tympanic bullae and ear plugs have been used.

Regardless of which tissue is being used, four criteria must be met before age can be determined (Myrick *et al.* 1983).

(1) familiarity with the deposition and distribution of tissues being used to determine age;

(2) use of a reliable and effective system of preparing the tissue that gives clear resolution of growth layers;

(3) an ability to provide a detailed description of the structural pattern of growth layers as they appear in the tissue to ensure consistency of counts of layers;

(4) knowledge of how much time each layer or group of layers represents to ensure counts of growth layers provide an accurate assessment of age.

Of these criteria, the third and fourth are the hardest to consistently and robustly meet. Considerable effort has been put into defining and standardising the techniques associated with aging marine mammals (thereby enhancing the precision of age

estimates), and to calibrating growth layers across marine mammal species (thereby enhancing the accuracy of age estimates) as a result.

Attempts to standardise the definition and interpretation of growth layers as they relate to age determination were made during the International Whaling Commission's Workshop on Age Determination in Cetaceans and Sirenians (Perrin and Myrick 1980) and later in a similar workshop focused on age determination of the harbour porpoise (*Phocoena phocoena*, Bjørge *et al.* 1995). Numerous papers have also provided detailed definitions of growth layers in various tissues and species (see relevant literature section for a full list of references). However, no quantitative and objective method has yet been published to assist researchers. It is therefore left to an individual or laboratory to gain considerable experience in order to define growth layer structure and as a result, definitions of growth layers are the interpretation of the individual or the laboratory at which age estimates are being determined.

For some small cetaceans and a number of pinnipeds, calibration has been carried out using captive or wild 'known-age' animals (Goren *et al.* 1987; Hohn *et al.* 1989; Hohn 1990; Childerhouse *et al.* 2004; McKenzie *et al.* 2007). Tetracycline marking experiments have also enabled researchers to calibrate growth layers in captive animals (Best 1976; Brodie *et al.* 1990; Gurevich et al. 1980; Myrick 1988; Myrick and Cornell 1990) and more recently bomb radiocarbon dating has been used on belugas (Stewart *et al.* 2006). These studies have consistently concluded that growth layer groups are deposited annually. However, for some species, particularly large cetaceans, calibration is difficult because their size precludes them from being kept in captivity where their age can be monitored. Limited mark-recapture studies investigating the accumulation rate of growth layers and studies calibrating seasonal changes in the thickness of the most recently formed growth layer have been conducted (Ohsumi *et al*. 1963; International Whaling Commission 1967, 1971; Best 1970; Gambell 1977). Although far from conclusive, the general consensus of these studies is that growth layers in large cetaceans are also deposited on an annual basis for all tissues examined (International Whaling Commission 1967, 1971; Jonsgård 1969; Christensen 1995).

In the Australian region, few studies that involve estimating the age of marine mammals have been conducted, largely due to a lack of available expertise in the

techniques required. Researchers that have used growth layers to determine age have either undergone training in laboratories overseas, or have shipped samples to commercial facilities (also overseas) for preparation and analysis. Furthermore, they have been required to spend considerable time and expense ensuring that permitting conditions for the import and export of marine mammal specimens were met.

As a way of achieving better resources for researchers wanting to age marine mammals in Australia, funding was requested to establish an aging laboratory at a stable and specimen-based institution, namely the South Australian Museum. The development of such a facility would have the following benefits: it would (1) provide training facilities for researchers new to the field; (2) increase the pool of qualified readers for age estimation and validation; (3) provide a centralised facility for the preparation and analyses of samples for projects that did not have funds or personnel available for training; (4) provide a centralised laboratory, with high-quality equipment, for use by institutions that may not have the funds to purchase such equipment and (5) provide readily accessible and long-term storage of processed material for future reference. Such a facility would enable Australian researchers and government to provide more comprehensive assessments of the biology and ecology of marine mammals in Australian waters, thereby improving their management.

The funds granted by the Commonwealth also provided for a manual and workshop to train a small group of Australian researchers in the techniques involved in the preparation of cetacean and pinniped teeth for age determination.

The aims of this manual and the associated workshop were to:

(1) share the knowledge and expertise of scientists experienced in age determination with other marine mammal scientists in Australia and New Zealand;

(2) provide training under the supervision of scientists experienced in the techniques used to determine age in marine mammals using tooth structure;

(3) provide documentation detailing the techniques used in age determination;

(4) and in doing so, assess and improve the reliability of age determination in marine mammals throughout Australia and New Zealand.

Techniques for preparing marine mammal teeth for aging studies

Just as the type of tissue used for aging may vary both between and within species, the techniques used to prepare those tissues may also vary. For example, tissues may include bone, tympanic bullae, ear plugs and teeth (*e.g.* canine, post-canine, first mandibular, central mandibular). Tissues may be prepared using different methodologies but the basis is the same — a requirement to identify and determine the number of growth layers associated with growth and age.

Although this manual primarily details the two most commonly used methods for preparing teeth in pinnipeds and cetaceans: (1) decalcified and stained thin-sections of teeth and (2) acid-etched tooth halves, it is of use to mention the other tissues and preparation techniques. For further details on the use of these tissues and techniques, users of the manual are encouraged to seek more detail from primary sources.

Teeth

Choice of teeth

The tooth used for age determination is different depending on the species being studied more so than technique used, although preferences can also vary within a species.

Among odontocetes, there does not appear to be a preference for a particular tooth, with most literature recommending the teeth midway along the lower jaw. Exceptions to this include sperm whales, beaked whales (most species have only two teeth) and narwhals (one of the tusks is used). In sperm whales, either of the first mandibular (lower jaw) teeth is preferred, although if these are particularly worn, a straight un-erupted tooth from the upper jaw (maxilla) is recommended (Perrin and Myrick 1980).

For pinnipeds, either the canine or post-canine is most commonly used. Both have been used in a number of fur seal species and Weddell seals, and the canine has been used in elephant seals, crabeater seals, leopard seals, Ross seals, harp seals, and ringed seals (McCann 1993).

Preparation techniques

The most commonly used alternate technique to decalcification, thin-sectioning and staining (detailed in this manual) involves ground, polished sections of teeth. This may be carried out either on the whole tooth or on a longitudinal or transverse wafer (thick-section) of tooth. Longitudinal sections are more commonly used although transverse sections have been used for pinniped teeth. When preparing longitudinal sections, the labial (the side closest to the lips, also known as the buccal side) and lingual (the side closest to the tongue) sides of the tooth are sawn off using a diamond blade saw, leaving a wafer several millimetres thick containing the midline of the tooth. Both sides of the whole tooth or wafer are then ground alternately using either a whetstone or a lapping machine until the desired thickness (generally 30–80 µm) is achieved (see Perrin and Myrick (1980) for an overview of the thickness of sections for a number of odontocete species). A similar process is used for transverse sections (where the crown and tip of the tooth are removed), achieving a final thickness of 120–140 µm. Sections are viewed under a compound microscope, usually with polarised, ultraviolet and/or phase contrast facilities.

Bone

Although not commonly used in cetaceans and pinnipeds, bone is particularly useful for marine mammals such as manatees that do not grow tusks (unlike dugongs where the tusks can be used for aging) and whose teeth are rapidly and continuously replaced through life. Mandibles, ribs and the tympano-periotic complex (earbones) have been used in manatees and the tympanic bulla has been useful for aging baleen whales. Preparing bone for aging is similar to that for teeth *i.e.* either ground thin-sections (140–200 µm thick), decalcified and stained thin-sections or acid-etched ground sections (4.5–5.0 mm thick). Growth layer groups in the periosteal bone of thin-sections are then counted either under transmitted light, low-contrast polarised light, reflected ultraviolet light or using scanning electron microscopy.

Baleen whale ear plugs

The ear plugs of baleen whales have been used to estimate age since the 1950s when Laws and Purves (1956) found concentric laminations in the ‘wax’ plug that filled the proximal part of the external auditory canal and related these to the lengths of fin whales. The plug is largely composed of horny epithelial cells and fat cells that are laid down in alternating layers, a pair of which constitutes a growth layer.

Skin

More recently, there has been interest in using chromosome telomeres (a region of highly repetitive DNA at the end of chromosome) as a means of determining age (see Dennis 2006). Many in-vitro and in-vivo studies in mammals have demonstrated correlations between somatic cellular telomere length and life span. However, the relationship is not consistent between animals because telomeres shorten with age in some species, but not in others and some even lengthen with age. The relationship is likely to be species-specific and the technique has yet to be validated in marine mammals.

Obtaining teeth for aging studies

Before preparation can begin, a tooth must be obtained! This may either be extracted from a live animal (Figure 1, for example if determining the demography of a particular population and how this might change through time) or from the carcass of a dead animal (Figure 2, for example if age-related changes in the pollutant loading of a species from a particular region are being studied).

Live animals

This requires specific skills and should be conducted only by experienced personnel or under the supervision of a qualified person. In Australia, both State and Commonwealth permits may be required before pinnipeds or cetaceans can be handled and it would be a requirement to demonstrate adequate skill before the permits can be issued.

Animals must be adequately restrained and provided with pain management, the form of which will differ depending on the species (and animal care and use guidelines of

agencies involved). Sound knowledge of the root morphology and surrounding jaw structure of the species is required, including where the nerves are. For reference, we have provided a short overview of the techniques involved in the extraction of a single-rooted tooth (post-canine) taken from McIntosh (2007) and McKenzie *et al.* (2007). A more detailed description of the methods involved in tooth extraction can be found in Holstrom *et al.* (2004). This extraction method can also be used on dead animals.

Before extraction of the tooth can begin, the animal needs to be adequately restrained (usually using anaesthetic techniques). Once anaesthetised, the lower jaw of the animal is immobilised using a restraint board and the upper jaw is manually raised. This position is maintained using 30 cm of strong pliable nylon hose or webbing to enable access to either the lower left (if right-handed) or lower right (if left-handed) first post-canine (Figure 1). Before extraction, a preoperative nerve block is administrated using a local anaesthetic such as Lignocaine hydrochloride, to minimise the amount of general anaesthetic required during the procedure and to reduce post-operative pain. Care must be taken in the placement of nerve blocks to ensure the tongue is not desensitised, compromising the animal's ability to swallow.

To remove the tooth, the gum attached to the tooth is severed down to the jaw bone using a scalpel blade or dental elevator (3−4 mm). The tip of the dental elevator is then applied to the area between the root of the tooth and the bone surrounding the alveolus (the space in the bone within which the tooth root sits). The blade is moved into the periodontal ligament space using controlled force and then pushed down towards the root of the tooth (following the curve of the tooth), while slightly rotating the blade from side to side. This downward movement severs the periodontal ligament and jacks the tooth upward, while the rotation helps to stretch and loosen the ligaments on either side of the tooth. In general, the posterior periodontal ligament is severed first and then the anterior ligament is severed before repeating again on the posterior side. The dental elevator must be supported by the opposite hand at all times to prevent slipping while applying force (Figure 1). This procedure is repeated until the tooth either pops out of its own accord or becomes loose enough that it can be removed by gently twisting with dental forceps. The periodontal ligament on the buccal (cheek) and lingual (tongue) sides may require severing, however care must be

taken as the jaw bone at these locations may be thin in some animals and easily broken, and the elevator can easily slip.

Removed teeth are stored in 70% ethanol in individually labelled tubes. During and immediately following tooth removal some haemorrhaging may occur – this will generally stop after a short period or if not, may require a little pressure. Suturing of the gum is avoided (Figure 1) in order to prevent accidental entrapment of foreign material and infection. If the tooth breaks during extraction, all fragments must be removed to assist healing (broken teeth and fragments can still be thin-sectioned). Tooth removal generally takes between 1 and 3 min.

While extracting teeth from live odontocetes is not common (due to the difficulties the logistics of restraining animals and conducting extractions on an animal that is entirely aquatic), it has been done on a number of small captive odontocetes (see Ridgeway *et al.* 1975) and on some wild dolphins (Hohn *et al*. 1989). Again, extraction of a tooth from a live animal requires specific skills and should be conducted only by experienced personnel or under the supervision of a qualified person following the appropriate animal care and use guidelines of agencies involved.

Dead animals

The most common way of obtaining cleaned teeth from dead marine mammals is by macerating the whole skull. This process literally rots the flesh (at the same time the teeth fall out) and leaves a clean but sometimes greasy skull. Maceration is best carried out in water that is 25–35°C so it is usually necessary to gently heat the water, unless ambient temperatures are within this limit for several months. NEVER BOIL SKULLS OR TEETH! Boiling denatures and extracts collagen from the cementum and dentine in the tooth, damaging the cementum layers and the edge of the pulp cavity. In all situations (however see note on removal of teeth from dead pinnipeds below) it is better to macerate and gently clean any remaining soft tissue away from the tooth, making sure that that the external surface is not damaged. A dolphin skull will take about 2–3 months to macerate. If it is important to choose teeth at a certain position in the jaw the skull needs to be macerated for a week or two to allow the teeth to loosen, at which time they can be removed and their order recorded.

A

B

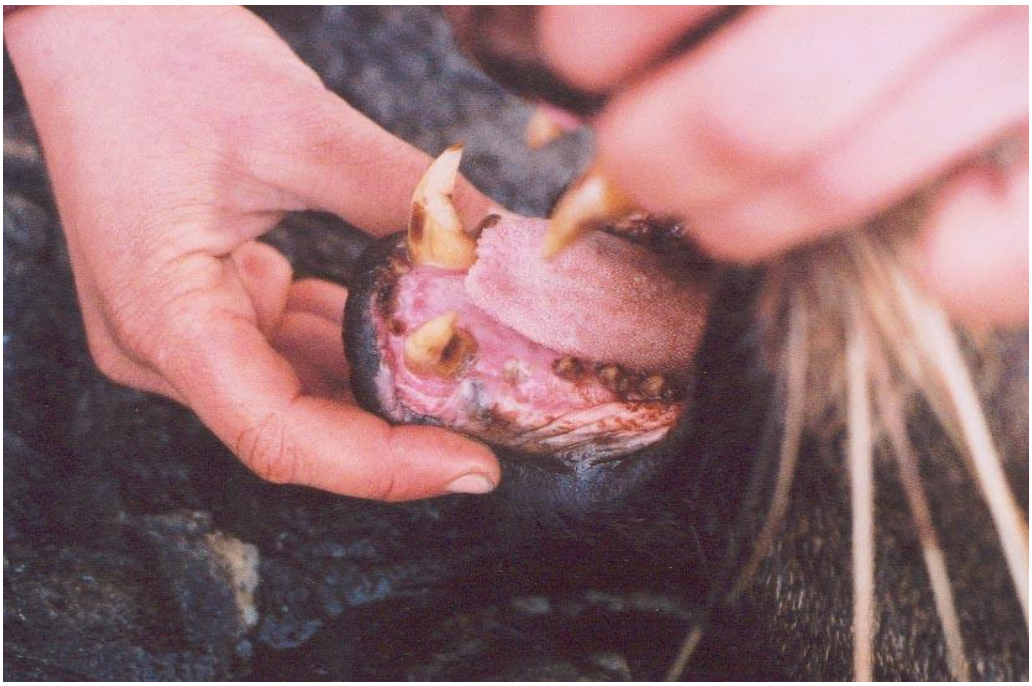
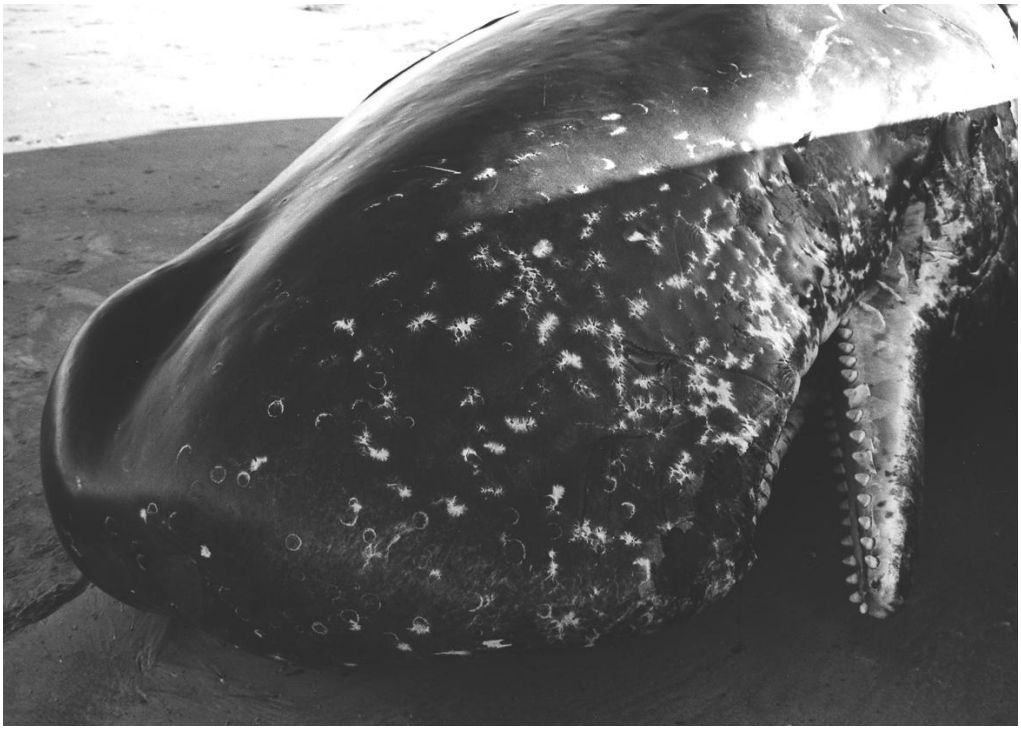


Figure 1. (A) Removal of a post-canine tooth from a New Zealand fur seal (*Arctocephalus forsteri*) mandible. (B) New Zealand fur seal jaw one week after removal of post-canine, illustrating rapid healing of gum.

A.



B.

Figure 2. Teeth in the lower jaw of a dead (A) sperm whale (*Physeter macrocephalus*) and (B) Indo-Pacific bottlenose dolphin (*Tursiops aduncus*). Note the wear on all but the very posterior teeth in the jaw of the bottlenose dolphin.

Removing cetacean teeth from a fresh carcass is not recommended, particularly for young animals because the base of the tooth is delicate and can easily be broken. If an individual tooth is required from a fresh carcass, similar procedures to those used on live animals need to be used. If there is no requirement to keep the skull intact or the whole skull is not required to be collected, cut a small section of the lower jaw from the animal and macerate it.

Removing pinniped teeth (or any other teeth where the cementum, opposed to the dentine, is to be used for aging) for thin-sectioning and staining should, where possible, be done on dead animals prior to maceration/cleaning. This ensures that the periodontal ligament tissue surrounding the cementum is not damaged. Macerating teeth in this situation is not advisable because it can damage the peripheral annulus, resulting in age being underestimated when counting growth layers in the cementum.

Storing teeth

Ideally, it is best to use teeth as soon as possible after removal from the animal, however, in many situations this is not possible (*e.g.* museum collections often store teeth for many years in various types of containers). For cleaned, dry teeth, optimal conditions for storage are 10–20° C and 40–70% relative humidity, without rapid changes in either temperature or humidity. Although teeth may be stored in a refrigerator, it is recommended that they not be frozen. Large teeth (*e.g.* sperm whales, killer whales and beaked whales) are sometimes stored in a solution of alcohol and glycerine so that environmental changes do not result in the teeth cracking. Fresh teeth removed with tissue still attached can be stored in 70% ethanol. It is important not to use chemicals such as formalin and strong degreasing agents to prepare teeth.

Before entering the laboratory

The importance of keeping a lab book for recording the various processes involved in preparing tissues for age determination cannot be emphasised enough. This will enable you to track the processes (*e.g.* acid-etching and decalcification times) and help develop guides relating to them for particular species. It will also be a record of

each specimen that can be later used to trouble-shoot problems (*e.g.* over- or under-decalcified teeth) and work out the best practices (*e.g.* temperature settings, thin-section thickness). In addition, a lab book can be used as a means of keeping track of the status of the equipment and consumables used. See Appendix C for an example decalcification and acid etching tracking tables.

TOOTH PREPARATION

Decalcified, stained, thin, longitudinal sections of pinnipeds, small to medium odontocetes and calf/juvenile large odontocetes

Teeth may need to be trimmed or wafered prior to decalcification to ensure that an even decalcification of the tooth is achieved and in doing so that partial over-decalcification or under-decalcification is avoided.

1. Trimming (all small to medium odontocetes): one side (either buccal or lingual) of the tooth is removed to produce unequal 'halves', the larger of which contains the centre of the tooth to be decalcified and sectioned.

2. Wafering (sub-adult and adult delphinids with large teeth): both sides are removed, leaving a 'wafer' that contains the centre of the tooth. As a rule of thumb, the teeth from bottlenose dolphins and pilot whales larger than 170 cm body length will require wafering.

If the teeth being prepared are particularly small (*e.g.* bottlenose dolphin calves, common dolphins), the whole tooth is decalcified. All otariid post-canines are decalcified whole or trimmed after partial decalcification to avoid over-decalcification of larger/older teeth.

If you are using a different saw from that of a low-speed rotary diamond saw (Isomet) the procedures used will be the same. However, if the saw uses coarser blades you may need to polish the cut surface of the tooth to reduce the number of saw marks on the cut surface of the tooth and to ensure an even surface for later thin-sectioning.

Trimming/wafering with low-speed rotary diamond saw (Isomet saw) – odontocetes only

Occupational Health and Safety

Wear safety glasses.

Keep hands away from saw.

Wear cotton gloves and do not touch melted thermoplastic cement...it will burn!

Use large forceps.

Equipment required

Low-speed rotary saw (Isomet) with slow water drip attachment or water reservoir
Diamond saw blade (4" or 5")
Small wooden blocks
Thermoplastic cement (quartz resin No. 70C) or hot glue gun
Bunsen burner (for thermoplastic cement)
Large forceps
Cotton gloves
Safety glasses
Tap water for rinsing
Plastic histology cassettes/small vials
Pencil/Permanent marking pen
Identification labels
Elastic bands

Note: cutting wafers of dolphin teeth on an isomet saw can take up to 30–40 min per tooth so be prepared to leave enough time to complete wafering. Note also that there are many ways in which teeth can be prepared for trimming/wafering – some labs embed teeth in resin blocks rather than attaching them to wooden blocks and varying materials can be used for attaching teeth to block for trimming/wafering (*e.g.* various glues). If you are using an alternative means of preparing teeth for trimming/wafering with an isomet, be aware of any future requirements of those parts of the teeth that you will not be using (*e.g.* stable isotopes, chemical analyses, genetics) and ensure that the materials you are embedding the teeth into or attaching the teeth to the wooden blocks with will not contaminate the teeth and

compromise those analyses. Also note that some resins and glues may react with the decalcifying agent used during decalcification so you may need to check on the stability of the resin/glue used to prevent this from occurring.

1. Choose 1–5 of the straightest, least-worn teeth (cleaned of flesh, preferably not cracked) from each individual, preferably derived from the middle of the lower jaw. If possible, prepare more than one tooth per individual to allow for back-ups in case of a poor-quality tooth, mistakes *etc*…

2. Most isomet saws have a holding stage that can either be used in a vice-like fashion to hold the tooth directly or hold a small wooden block (to which the tooth is adhered). If using a wooden block for fitting into the vice this needs to be large enough to hold the tooth but not so large that it cannot be held in the vice securely. In most cases the holding stage is aligned against the blade and the saw runs along a track so that the holding block automatically runs through the saw. Some, however, do not and you may need to manually feed the holding block through the saw. If the isomet saw does not have a holding stage you will need to prepare a block that can be used to manually feed each tooth through the saw. When preparing your block ensure that it is the appropriate size to (1) hold a tooth, (2) be fed through the saw (3) perform the actions without endangering any part of yourself.

3. Regardless of whether you are using a vice-like holding stage or a wooden block you will need to orient the tooth so that it is fed longitudinally through the saw and that either or both the buccal and lingual sides of the tooth can be trimmed. If using a vice, orient the tooth so that the convex surface of the tooth is downwards (and against the stage) and the concave surface of the tooth is facing upwards. Ensure that the tooth is aligned so that when cutting the saw blade is aligned as parallel to the middle of the tooth as possible. Lines can be drawn to each side of the midline with a pencil to assist with cutting in the right place. Lock the vice so that the tooth is held firmly and there is no chance that it will move as it is being trimmed.

4. If using a wooden block (either to be held by the vice or manually fed through the saw), teeth can be adhered to the wooden block using thermoplastic cement or hot glue. Cover the bench with a sheet of cardboard to keep it clean. Soften a small amount of thermoplastic cement by holding a stick of the cement using long forceps over a Bunsen burner flame. The cement will quickly become sticky and begin to melt, but will rapidly re-solidify once you remove it from the flame so you need to move quickly once you start to heat it. Once softened, place a sufficient amount of the cement on the block and before it hardens, press the tooth in the correct orientation (see point 3) into the cement. Add more cement around the sides of the tooth, making sure there is a strong bond with no gaps. Check that the tooth is secure and will not move as it is being trimmed. If it is not, either repeat the process again or secure the tooth by placing some more of the cement on either side of the tooth. Label the back of the block with the specimen number in pencil. Alternatively, hot glue guns have been used with great success for securing smaller teeth. Place a small amount of hot glue on the wooden block and lightly press the tooth into the glue. Hold in position for 30–50 seconds. If the bond is strong enough there is no need to add additional glue to the sides of the tooth. Most glues can be easily removed by peeling it off after cutting. Hot water can also assist to in removing the glue.

5. Before you start, ensure there is sufficient water in the tray of the saw so that the blade is just dripping as it rotates – never trim teeth using a dry blade. Put on your safety glasses. Start sawing at a medium speed and slowly work to a faster speed, being very careful not to cause too much friction heat. If trimming one side of the tooth slowly feed (either automatically or manually) the tooth through the blade so that either the buccal or lingual side is trimmed from the tooth, leaving two uneven halves, the thicker containing the longitudinal midline of the tooth. If the tooth requires wafering, trim both sides, leaving an approximately 3 mm thick wafer containing the centre of the tooth.

6. Place those parts of the tooth not required to one side and gently rinse the usable part of the tooth under tap water. Carefully remove as much of the

excess cement or glue from the tooth as you can. Place the trimmed tooth or wafer into a plastic histology cassette or if too large for the cassette a suitable container such as a small plastic vial, clearly labelling the container either with a pencil or permanent marking pen, or place a label inside. Wrap an elastic band around each container so that it won't accidentally open.

7. Use only quality, sharp diamond saw blades. Sharpening stones for blades are available and if using someone else's saw, be prepared to replace the blade if chipped before you begin.

8. Unused parts of teeth may be kept for genetic and isotope studies or the powder that collected in the water tray of the saw can be filtered and saved.

Decalcification

Decalcification of both otariid and odontocete teeth requires the same methodology. Teeth can either be decalcified in histology cassettes or if the trimmed tooth or whole tooth is too large for histological cassettes they can be decalcified in clear plastic vials which have had several small holes drilled into the sides and bottom. A small drill bit can be used to make approx. 20 holes in the sides and bottom of the vial. Ensure that there are enough holes in the vial to adequately mix the decalcification fluid around the tooth and that any rough bits remaining from the drilling process are cleaned off.

Occupational Health and Safety

Use RDO only under a fume hood.
Make sure used RDO is clearly labelled and stored under a fume hood.
Wear gloves when handling RDO.
Filter paper should go in a biological waste bin and waste RDO into a waste chemical bin.

Equipment required

Plastic histology cassettes or vials with small holes
Pencil
Permanent marking pen
Identification labels
Elastic bands
Large glass jar or beaker
Tap water for rinsing
Plastic tube extender for tap
Decalcifying agent (RDO)

- Large glass funnel
- Filter paper to fit funnel
- Wide mouth glass jar with screw lid (1–2 L)
- Heavy gloves
- Fume cupboard
- Timer with alarm
- Long forceps
- Tap water for rinsing
- Storage jars for decalcified teeth
- Distilled water or 99.5% glycerine
- Blue medical padded sheets to protect benches

Note: see Appendix A for a guide to decalcification times for a range of odonotocete and pinniped species and Appendix C for an example table for recording decalcification times. The decalcification times for otariid teeth can be decreased by trimming the buccal and lingual sides of the tooth (and the crown if the tooth is particularly large) with a scalpel or razor blade when the tooth is partially decalcified. Take care when doing so that the periodontal ligament and the anterior or posterior edges of the tooth are not dislodged or damaged.

1. Place tooth specimens into individual cassettes/vials and clearly label the container either with a pencil or permanent marking pen and place an identification label inside the container. If using cassettes, wrap an elastic band around each cassette so that it won't accidentally open and empty its contents

2. If teeth have been stored in ethanol they will need to be rinsed in tap water prior to decalcification. Place containers in a large glass jar or beaker and using a plastic tube extender or hose (ensures that water is directed to the bottom of jar and that there is adequate mixing) on a cold-water tap rinse the teeth for a minimum of 2 h.

3. In the meantime, under a fume hood filter the decalcification agent (RDO) using a large glass funnel and filter paper into a wide-mouth glass jar with a screw-on lid (needs to be able to hold at least 1 L). Filtering removes the dark precipitate in the RDO. Don't shake the RDO before filtering. Only do 1–3 pours per piece of filter paper as it may tear, remixing the precipitate into the solution. It is best to use two jars, filtering small amounts into one jar and

transferring this into a second jar every so often. It will take about 1 h to filter 1 L of RDO.

4. Note that once opened RDO will be effective for approximately 4 d but can be used multiple times during this period.

5. If decalcifying a number of teeth, sort the teeth into species and size groups because decalcification times will vary depending on these factors. Place groups of teeth into separate jars containing RDO, no more than about 20 teeth per 2 L of RDO. Screw the lids on and agitate each jar to ensure circulation of the RDO through the containers holding the teeth specimens. Agitate the jar again every 1–2 h to ensure proper circulation of the RDO around the tooth specimens.

6. If the decalcification time of the species/teeth is unknown check the teeth every half hour for smaller teeth (*e.g. Delphinus* and thin wafers) and every 1 h for larger teeth (use a timer with an alarm to keep track of time). Once decalcification times have been established the teeth can be left for longer if appropriate (see Appendix A for approximate decalcification times for a range of species). When checking the tooth specimens remove the containers from the RDO using long forceps or tongs, and place them into a large glass jar or beaker. Using a plastic tube extender or hose, rinse the containers under running water for 5 min. Once rinsed, remove each tooth from its container and check the state of the tooth specimen using your bare hands (do this individually so you don't mix up teeth and cassettes), taking care not to damage the tooth, especially with fingernails. When fully decalcified, the tooth should be pliable (rubbery) throughout its whole length and be reasonably translucent when held up to the light. Spots of opaque material and rigidity within the tooth indicate that it is not fully decalcified. Care must be taken not to over-decalcify, which will result in damage to the growth layers in the dentine or cementum. To avoid over decalcification of cementum layers in pinniped teeth, stop the process and trim the buccal and lingual sides of the tooth using a scalpel or razor blade while the dentine is still a bit hard.

7. If the tooth specimens are not fully decalcified, replace the teeth into their containers, securing the cassettes with an elastic band. Return the containers into the RDO and repeat step 6. Reduce the time in RDO to 30 min as decalcification nears completion.

8. Once fully decalcified, place the containers into a large glass jar or beaker. Using a plastic tube extender or hose on a cold-water tap, rinse the teeth for at least 3 h (preferably overnight) to ensure that all the RDO is removed. Take care that the tap is on enough to ensure adequate rinsing but not enough that the containers float out of the jar/beaker.

9. If the decalcified tooth specimens are not to be sectioned and stained immediately, they can be stored in distilled water for a few days, making sure that the water is replaced with fresh distilled water each day. If the decalcified tooth specimens are to be stored indeterminately they can be stored in 99.5% glycerine. To use glycerine stored-teeth/wafers/tooth halves, just place back in baskets/vials and rinse in running tap water for 2–3 d.

10. If it becomes necessary to stop the process at any time before the tooth specimens are fully decalcified, they need to be rinsed under running water for a minimum of 6 h (*e.g*. overnight) so that decalcification process is fully stopped.

Thin-sectioning, staining and mounting onto slides

Thin sectioning, staining and mounting are usually carried out in sequence. If you do not plan to stain/mount your thin sections immediately after sectioning/staining, and want to store sections for only a short amount of time (a few days at most) ensure that you have pre-prepared small storage jars containing distilled water and an identification label into which the baskets containing the thin sections can be placed prior to staining/mounting. Again, replace the distilled water each day and if sections need to be stored indeterminately place them in 99.5% glycerine. To use glycerine stored sections, rinse in tap water for 30 min prior to staining/mounting.

Note: some pre-preparation is required for both the stain and the slides prior to staining and mounting of the sections so ensure that you leave adequate time for these

when planning your laboratory time. Once made up, the haematoxylin stain must be left overnight to prove, so make up a batch of the stain the day before you plan to stain your tooth sections (see 'Preparation of stain' section for details on preparing this stain). Try to avoid pre-made versions of haematoxylin stain because they are generally not as good as freshly made. The slides onto which the stained sections are to be mounted need to be coated in a 5% agarose/gelatine solution and so need to be pre-prepared prior to the slide mounting session (see 'Preparing slides' section for preparing agarose/gelatine solution and coating slides). Coating the slides holds the sections on slides more securely therefore preventing sections from falling off the slide.

Thin-sectioning

Occupational Health and Safety

Do not operate the machine if tired or under the influence of alcohol.

Operators are required to be trained in both OHS&W and the techniques involved in thin-sectioning before they use the cryostat or slide microtome.

Operators should read this manual (thin-sections procedures) before using the machine.

Operators should read the manual relating to the machine that is being used before commencing thin-sectioning.

Keep hands away from the knife at all times.

Do not try to catch a falling knife.

Always lock the turning handle after cutting sections *i.e.* before removing sections from blade.

Cover the knife-edge with the guard and close the chamber window when not sitting at the machine.

Operate the machine with the chamber light on.

Take the knife blade out of the stage when the machine is not in use *e.g.* overnight, lunch break.

Move the blade stage to one side and the specimen cylinder to the rear when changing or manipulating the specimen/chuck.

When cleaning the blade, use a small paintbrush or low-lint wipes and a motion working away from the blade edge.

When cleaning out the dish of waste sections, make sure the specimen cylinder is towards the rear and the blade is to one side.

Avoid contact between skin and very cold parts of the machine as this may result in fingers becoming stuck to the cold metal.

Equipment required

- Cryostat machine/slide microtome with freezing attachment
- Low profile new/clean unchipped microtome blades /sliding (sledge) microtome knife
- OCT embedding compound
- Fine-hair paintbrush (camel hair is best)
- Petri dishes (2–3 medium)
- Distilled water
- 70% ethanol
- Low-lint wipes
- Soft forceps (*e.g.* entomological forceps)
- Plastic histology cassettes (deep)
- Pencil
- Permanent marking pen
- Identification labels
- Elastic bands
- Nylon stocking pieces or small bags sewn with ball-point needle
- Large glass jar/beaker
- Tap water for rinsing
- Paper towel
- Storage jars
- Distilled water or glycerine

Note: Thin-sectioning may be carried out using either a cryostat or a freezing slide (sledge) microtome. If you are unsure of the difference between a cryostat and a sliding microtome a cryostat is a climate controlled chamber containing a semi-automated microtome for sectioning frozen tissue. A sliding microtome is a bench-top unenclosed microtome that is used to cut organic tissue into thin sections to which a freezing stage can be attached. The specimen being sectioned is made to slide on a track and the operation of the microtome is fully manual (Figure 3). Although the end result is the same, the methodology for operating the two is slightly different. For trouble shooting related problems associated with thin-sectioning refer to Appendix B.

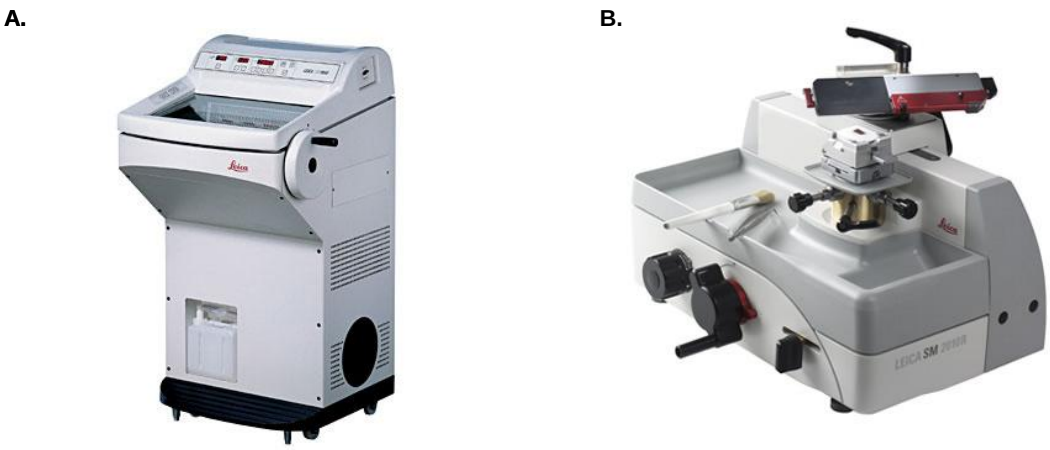


Figure 3. Examples of (A) a cryostat and (B) a sliding (sledge) microtome.

Also note that from the time the thin-sections are cut, any associated equipment (*e. g.* forceps, Petri dishes) need to be rinsed with distilled/deionised water to prevent contamination.

1. If decalcified specimens have been stored in glycerine, rinse in running tap water for 2–3 d.

2. Set up 2–3 Petri dishes with distilled water for collecting sections in a position where they can be easily reached (*e.g.* on a bench nearby or on top of the cutting machine). Label each dish with the animal number and sequence of groups of sections.

3. If using a cryostat, turn on the machine and allow chamber temperature to lower to operating temperature. Generally this is around –5° C but may differ depending on the machine (the dual temperature cryostat at the SA Museum is set at –3 to –8° C for the chamber and –10° C for the stage). Place the blade in the blade holder and secure (remembering that you would have removed it after the last use of the cryostat) ensuring that an unused edge is exposed. Set the angle of the blade (it is set at 2º out of a scale of 0–10 on the SA Museum cryostat). Always allow the blade to come to operating temperature before sectioning. This may take as long as 20 min. Cool the mounting discs on the shelf in the cryostat. Set the cryostat to section at a thickness of 20–40 µm,

depending on species and possibly with condition of tooth (*i.e.* fresh or from a museum specimen) and be prepared to experiment with practice teeth). *Arctocephalus forsteri* and *Neophoca cinerea* are sectioned at 20 µm, most delphinids are sectioned at 25 µm, *Globicephala melas* is sectioned at 28 µm, *Physeter macrocephalus* is sectioned at 40 µm.

4. If using a freezing slide (sledge) microtome, turn on the CO_2. Set the microtome to section at a thickness of 20–40µm (as above, this will vary depending on species and possibly with condition of tooth).

5. Remove decalcified specimen from its container and let dry for a few seconds on paper towel. Before mounting the tooth onto the cryostat disc or the freezing slide (sledge) microtome stage, ensure that the blade head is moved to a position well away from the cryostat stage clamp or the freezing slide (sledge) microtome stage and take extreme care to avoid the blade. Place enough embedding compound (OCT) onto the disc or stage to allow the tooth to be embedded (usually the size of an Australian 10 cent piece). Avoid the formation of bubbles in the medium. Either allow the OCT to become somewhat opaque (*i.e*. the bottom layer is frozen) in the cryostat or if using a freezing slide (sledge) microtome feather the CO_2 to the same point before placing the tooth in position. Make sure the tooth is as level as possible and that the orientation of cutting will be from crown to root tip. Because it will be difficult to see the tooth once fully embedded, when using a cryostat always orient the tooth in the same position relative to the mark on the disc. Allow the OCT and specimen to freeze on the shelf of the cryostat or open the CO_2 valve to freeze the tooth onto the microtome stage. If the tooth is not level, when using a cryostat remove the mounting disc and reposition with your finger or a flat object as the OCT thaws. When using a freezing slide (sledge) microtome close the CO_2 valve and allow the OCT to thaw, after which the tooth can be repositioned. Replace the cryostat disc onto the shelf of the cryostat and allow to re-freeze or for the freezing slide (sledge) microtome, re-open the CO_2 valve and allow the tooth to re-freeze onto the stage.

6. Once the specimen is satisfactorily frozen in place, add additional OCT around tooth to enclose tip and root, building up the sides with OCT. If a large specimen is being cut, cover the entire specimen and allow this to freeze.

7. Be careful not to over-freeze the specimen as this will result in scrape marks being left on the section by the blade. Under-freezing will result in the specimen falling off the mount. Keep in mind that you may have to alter the chamber temperature on the cryostat depending on room temperature when operating with the window open.

8. If using a cryostat, align disc so that it is parallel to the blade. Ensure that the disc is tightly secured (but do not over tighten) and will not move if blade hits an under-decalcified spot in the centre of the tooth.

9. Start sectioning. There will be several layers of OCT to shave off before getting to the decalcified tooth. If you are using a freezing slide (sledge) microtome that has a collection tray, stop when you reach the tooth and discard the sections out of it. This ensures that if a tooth section falls into the tray it can be easily retrieved. Collect the thin-sections produced by the freezing slide (sledge) microtome as you section through the tooth with a fine-haired paintbrush and place these in a Petri dish with distilled water (the OCT will dissolve off). Place those sections away from the tooth centre and those from the centre into different Petri dishes. If using a cryostat, you can operate with or without the roll/safety plate in position and collect the sections from the blade with a fine-haired paintbrush. In both situations take care not to freeze the brush to the blade and avoid touching the mounted tooth with the brush (particularly if the tooth has partially unfrozen because there is a chance you will dislodge it). Use a smooth steady motion when sectioning, particularly at the centre of the tooth and just each side of it. If you are using a cryostat, with experience, it should be possible to collect ‘ribbons’ of sections. If generating ribbons of sections, remove the ribbon before it becomes too long or it will drop off the blade. Sort the sections as you go into those from the midline and those just off centre into separate Petri dishes making sure that it is clear which Petri dish contains each type of section. The midline of the tooth is the most important part for aging and includes the centre of the crown,

the pulp cavity and the centre of the root. In teeth that still have a pulp cavity, this will include the widest portion of the tooth cavity. If ice or tissue builds up on the blade remove using a low-lint wipe and ethanol, wiping in an upward direction and taking extreme care of the blade. Remove all tissue from blade and machine between samples and reposition the blade to use a fresh section of the blade (to ensure that the sharpest part of the blade is used each time). Once you have finished sectioning, if your microtome has a fixed stage allow the OCT to thaw from the remaining part of the tooth and remove it from the microtome stage. If your microtome has a removable stage, take this off and place in water to dissolve the OCT from the tooth. If using a cryostat, move the blade head well away from the stage clamp before removing the disc. Wipe down any remaining OCT from the stage or disc with a paper towel or cloth.

10. It is advisable to check thin-sections under a dissecting microscope for scrape marks (associated with a blunt or damaged blade or over-freezing) and other problems early in the sectioning process (see Appendix B for trouble-shooting).

11. When first learning how to thin-section you may like to keep more sections than you might with more experience. This will allow you to build up a ‘library’ of sections from which mistakes can be highlighted and others also learning can refer to.

12. Using a pair of soft, smooth forceps, remove the sorted sections from the Petri dishes, place into the container you will be using for staining (cassette or vial) and clearly label the outside with a pencil or permanent marking pen. Do not crowd the container with too many sections or they will overlap during the staining process and the teeth will be unevenly stained. For large teeth place 1–2 sections in each basket/vial; for smaller teeth 3–20 sections (depending on size) in each basket/vial. Wrap the container in a small square or bag of stocking (try and cover with only one layer to allow liquid to circulate) securing this with an elastic band or small clip (this stops small sections sliding through the holes in the container). Do not use any metal product to

close the stocking pieces because this will react with the stain and form a precipate.

13. Place the containers in large glass jar or beaker and using a plastic tube extender or hose on a cold-water tap rinse under running tap water for 5 min to remove any remaining OCT. Clean the Petri dishes and replace the distilled water. Repeat steps 2–12 for additional teeth.

14. Once the thin-sections have been rinsed, remove them from the jar/beaker and let the containers drain on paper towel (to avoid excess water diluting the stain). If you are not staining the tooth sections immediately, they can be stored in distilled water for a few days, making sure that the water is replaced with fresh distilled water each day. If the sections are to be stored indeterminately they can be stored in 99.5% glycerine. To use glycerine stored tooth sections, just place back in baskets/vials and rinse in running tap water for 30 min.

Preparing stain

Occupational Health and Safety

Wear surgical gloves and safety glasses.
Do not inhale or touch toxic chemicals.

Equipment required

Surgical gloves (latex)
Safety glasses
Haematoxylin powder
Sodium iodate powder (GR for analysis)
Aluminium potassium sulphate (12 hydrate crystal, $AlK(SO_4)_2.12H_2O$)
Filter paper
Electronic balance
Powder spatula
Glass conical flask with stopper
Distilled water
Magnetic stirrer
Warming plate
Label for flask

Note: Preparing haemotoxylin stain takes about 30 min. Batches of stain must be made up in exactly the amounts given – doubling the amounts in an effort to make up

a larger quantity will not work. If you require more than the 500 ml in this recipe, make up the stain in separate amounts and combine. As a very rough guide, 1 L of stain will be sufficient for 150 baskets of thin-sections. The stain, once proved, will last approximately one week and needs to be discarded once a glaze forms on the surface or within a week (which ever occurs first). Keeping the stain in the refrigerator while it is not being used will lengthen its life.

1. Measure the following chemicals accurately (particularly the sodium iodate) onto filter paper on an electronic balance using a powder spatula or teaspoon.

   0.5 g haematoxylin
   0.1 g sodium iodate
   25.0 g aluminium potassium sulphate

2. Dissolve the haematoxylin in 500 ml distilled water in a large, stoppered glass conical flask by stirring (use a magnetic stirrer) and using gentle heat (hot plate on low ~37º C). You are aiming for the liquid to be an orange/brown colour.

3. Add the sodium iodate, and when it is dissolved (the liquid will now be red), add the aluminium potassium sulphate. Stir 3 min to allow it to completely dissolve. The liquid should be a dark purple colour.

4. Allow the stain to cool to room temperature and test the colour of the stain on filter paper. The stain should be purple in colour when wet – if it is a red-orange colour, too much sodium iodate may have been added.

5. Clearly label the stain with the date and contents and store (stoppered) in the fridge overnight to prove. Allow the stain to return to room temperature before using.

Staining thin-sections

Occupational Health and Safety

Wear safety glasses and gloves.

Equipment required

Surgical (latex) gloves
Safety glasses
Deep glass dish
Prepared haematoxylin stain

Timer with alarm	Dissecting microscope
Large glass jars or beakers (x2)	Paper towel
Tap water for rinsing	2% ammonia solution (100 ml 25% NH_4 aqueous solution + 1250 ml distilled water)
Soft forceps (entomological forceps)	Storage jars
Petri dishes (2–3 medium)	Distilled water
Distilled water	Dissecting microscope

Note: Using the histology cassettes for staining can result in uneven staining even when the mixture is agitated. It may be necessary to place tooth sections into small plastic vials with holes for staining in this case.

1. Pour the haemotoxylin stain into a deep glass dish or jar (deep enough to completely submerge the containers in which the tooth sections have been placed).

2. Group the sections according to species and size groups if you are staining a variety of tooth sections and place the containers containing the tooth sections into the dish. Gently agitate the containers so that the sections mix with stain. Staining times will vary depending on species and section thickness so check the sections every 5 min (use a timer with an alarm). Once you have established staining times for your specimens, the initial stain period may be increased (staining time for *A. forsteri* is 20–25 min, *N. cinerea* is 25 min, most odontocetes is 25–40 min). Agitate the containers frequently and use forceps to submerge any containers that may float. New stain will work more quickly than stain that is old or has already been used.

3. Remove the containers using forceps after 5 min (or longer once staining times are determined) and let them drain for a few seconds over the stain bath. Quickly place the containers in a glass jar/beaker and using a plastic tube extender or hose on a cold-water tap, rinse in running water until the water runs clear.

4. To check if the sections are adequately stained, open each container one at a time and using soft forceps remove the sections from the container placing the sections into a Petri dish of distilled water. Check the sections for the darkness of stain under a dissecting microscope – sections will be stained adequately when they are a dark violet colour and/or GLGs clearly visible. If the sections are too pale, replace the sections into their container and re-wrap the container with stocking/pantyhose. If checking a number of teeth at the same time, place the containers into the glass jar or beaker of water until you have finished checking all the sections.

5. Drain water from containers requiring further staining and repeat steps 1–4 until the sections are stained adequately.

6. Once the sections are stained adequately, using a plastic tube extender or hose on a cold-water tap, rinse the wrapped containers until it runs clear. 'Blue' the sections (and 'fix' the stain) by placing the containers in glass jar/beaker containing 2% solution of ammonia made up with distilled water for 30 sec to 1 min (use a timer with an alarm). Agitate the containers to ensure adequate mixing in the ammonia solution. If an oily slick or bits appear in the ammonia solution, replace the solution. Once 'blued' remove the containers with forceps, place in glass jar/beaker and using a plastic tube extender or hose on a cold-water tap rinse in running water for 30 min. Be sure not to leave the sections in ammonia for too long as the ammonia solution will continue to lighten the stain and will result in sections that are too pale.

7. Once rinsed, open one container at a time in a Petri dish partly filled with distilled water. The sections should float out but may need to be gently pulled out with soft forceps. Choose the best sections for mounting onto slides using a dissecting microscope to check for the best stained specimens closest to the midline of the tooth. Discard the sections unsuitable for mounting.

8. If you are not planning to immediately mount the sections return the best sections to the container, secure with stocking and place in glass jar containing distilled water. These can be stored in distilled water for a few days, making sure that the water is replaced with fresh distilled water each day. If the

stained sections are to be stored indeterminately they can be stored in 99.5% glycerine. In order to use the glycerine stored stained sections, just place back in baskets/vials and rinse in running tap water for 30 min.

Preparing slides

Slides can either be coated with an agar or a gelatine solution – either is suitable so it will depend on what is available to you. When choosing the slides to be used for mounting of sections (the choice is endless!) make sure they are suitable for the size of your specimens and always ensure that your slide cover size matches your slide size. It is preferable to use slides with a frosted end for easy labelling of each slide.

Occupational Health and Safety

Wear surgical gloves.

Equipment required

Agar, coarse powder/gelatine, type A
Distilled water
Glass beaker
Powder spatula
Magnetic stirrer
Warming plate/Microwave oven
Glass slides with frosted edge for labelling
Surgical gloves (latex)
Slide storage boxes
Low-lint wipes

Note: slides are usually cleaned and coated in advance and stored in a slide box away from dust. Preparing the mixture takes approx. 30 min and mixing the agar (if preparing agar coated slides) takes 20 min at high heat. The solutions must be used immediately after they are made.

1. Clean the slides you intend to use for mounting with 70% ethanol using a low-lint wipe or something similar.

2. To make up an agar solution, mix together 95 ml of room-temperature, distilled water and 5 g agar powder in small glass beaker heated on a hot plate (high setting) or a microwave at a low setting. When completely mixed (*i.e.* not gritty) the mixture should be clear. If you require a larger amount of

solution made up remember to keep the proportion of agar to distilled water the same.

3. To make up a gelatine solution, mix together 95 ml of distilled water heated to 60°C in a small glass beaker on a hot plate with 5 g gelatine. A few crystals of thymol can be added to the solution as a preservative. When completely mixed the mixture should be clear. Must be used within 1–2 h of being made.

4. The solution (either agar or gelatine) must be kept warm (40–50° C) when being used to coat the slides so it is advisable to keep the beaker/jar containing the solution on a hot plate at low–medium heat.

5. Apply a thin coat (smear) of agar or gelatine solution by dipping the short edge of a slide into the mixture and drawing it across the surface of second slide. If your beaker is deep enough to dip each slide in completely simply dip the slide into the beaker and then drain the excess agar/gelatine solution onto paper tissue. Try to avoid streaks as these interfere with reading the teeth.

6. Allow the coated slides to air dry at least 30 min (preferably longer and overnight if possible) before using. Slides can also be dried in an incubator at 37° C

7. Discard the agar/gelatine solution after use.

Mounting thin-sections onto slides

Occupational Health and Safety

Wear surgical gloves and safety glasses when handling chemicals.
The cover slip mounting medium must be used under a fume hood.

Equipment required

- Surgical (latex) gloves
- Safety glasses
- Petri dishes (2–3)
- Pre-prepared agarose/gelatine coated glass slides
- Cover slip mounting medium
- Glass cover slips
- Soft forceps (entomological forceps)
- Pencil/permanent marking pen
- Air-drying rack
- 70% ethanol

Low-lint wipes	99.5% glycerine
Plastic disposable pipettes/glass rod	Slide box
Warming plate	
Storage vials	

Note: there are many mounting mediums available on the market (*e.g.* DPX, DEPEX, Clear-Mount, Cytoseal) and your choice will depend largely on what is available to you. When purchasing a mounting medium make sure that it is compatible with haemotoxylin stain (all of the examples given above are).

1. Float the sections you have chosen as your most suitable for mounting from their container into a Petri dish with small amount of distilled water, lining the sections up in the orientation you want them to be in on the slide. Place the long edge of a prepared and labelled (pencil or permanent marking pen) slide into the Petri dish and briefly submerge slide to moisten the agar/gelatine. Lift the slide up so that it is on a slight angle and so the long edge is still in the water. Using soft forceps pull each section up onto the slide, positioning all sections in the same orientation. When placing the sections onto the slide keep in mind the size of your cover slip and the area of the slide it will cover. The slide can be re-wet if the sections are not placed on the slide properly (*i.e.* if they are creased, folded *etc…*) the first time, but try to avoid this as the agarose/gelatine becomes messy. The number of sections able to be fit on each slide will depend on the size of the sections.

2. Once you are happy with the placement of sections, remove the slide from the Petri dish and place the slide on a rack to air dry in dust free environment (at least 2 h, preferably overnight). Label the slide either with a pencil or a permanent marker. Rinse the Petri dish and fill with fresh distilled water for the next slide.

3. Once dry, transfer the slides to a fume cabinet where coverslips will be applied. Clean the coverslips with 70% ethanol using a low-lint wipe. Place a line of mounting medium down the centre of the slide using either disposable

pipette or a glass, bulbed dip-stick, ensuring that all sections have some mounting medium on them. Be careful not to use too much as it is likely to introduce bubbles under the coverslip and these are difficult to remove once dry. Place the cover slip into the medium at one end of the slide and carefully place over the section making sure you avoid getting air bubbles underneath.

4. Place the slide onto a warming plate on low setting (if the warming plate is too high you will generate bubbles under the cover slip) to promote even spreading of the mounting medium. Remove the slide once the mounting medium is evenly spread and place onto a rack to air dry in a dust-free environment at least overnight until completely dry. Depending on the mounting medium used and the temperature of the room this may take several days.

5. Once dry, any excess mounting medium can be removed with a one-sided razor blade. Be careful not to chip the cover slip or introduce air underneath it (Figure 4).

6. Any stained sections remaining can be stored in glass screw-top vials containing 99.5% glycerine and an identification label. Before mounting, the stored sections will need to be placed back into a histology cassette or perforated vial, wrapped in stocking/pantyhose and secured with an elastic band. Rinse in running tap water for 30 min.

A.

B.

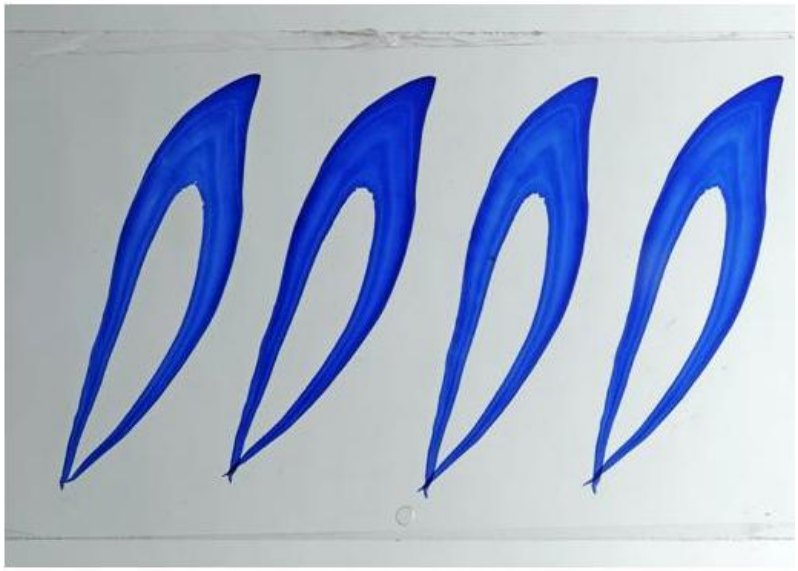


Figure 4. Finished slides ready for aging. (A) Adult female New Zealand fur seal (*A. forsteri*) post-canine sections. Note the undecalcified spot at the centre of the tooth (darker purple spot), which resulted in damage to some sections during sectioning. (B) Juvenile Indo-Pacific bottlenose dolphin (*Tursiops aduncus*) tooth sections estimated at 2 y.

Acid etching – large pinniped canines, adult/sub-adult large odontocetes

Halving with a diamond saw

Cutting large teeth in half for acid etching is done using the same principles as that for smaller teeth; the major difference lie with the size – everything is bigger!

Teeth being prepared for acid etching are in most cases halved using a low-speed diamond saw or a band saw. If using a band saw the cut is somewhat coarser than that of a diamond saw and requires more polishing afterwards. As a result, teeth cut with a band saw must be cut slightly further off centre to accommodate for the more extensive polishing.

If using a diamond saw ensure that good quality, sharp diamond saw blades are used. Sharpening stones for blades are available and if using someone else's saw, be prepared to replace the blade if the blade is chipped – do not use a chipped blade as it will gouge the surface of the tooth.

Occupational Health and Safety

Wear safety glasses.
Keep hands away from saw.
Wear cotton gloves and do not touch melted thermoplastic cement….it will burn!
Use large forceps.
Follow guidelines for the machine being operated.

Equipment required

Low-speed diamond saw or band saw
Diamond saw blade (size will depend on saw)
Slow water-drip attachment or water reservoir
Wooden blocks
Thermoplastic cement (quartz resin No. 70C) or hot glue gun
Bunsen burner (for thermoplastic cement)
Large forceps
Cotton gloves
Safety glasses
Matches/lighter

Sandpaper for polishing (grades from coarse to superfine)
Tap water for rinsing
Pencil/Permanent ink (technical or Rotring) pen
Air-drying rack
Paper towel

Note: There are many ways in which teeth can be prepared for halving. Some labs embed teeth in resin blocks rather than attaching them to wooden blocks and varying materials can be used for attaching teeth to block for trimming/wafering (*e.g.* various glues). If you are using an alternative means of preparing teeth, be aware of any future requirements of those parts of the teeth that you will not be using (*e.g.* stable isotopes, chemical analyses, genetics) and ensure that the materials you are embedding the teeth into or attaching the teeth to the wooden blocks with will not contaminate the teeth and compromise those analyses. If you want to compare the external growth ridges on pinniped teeth to the internal GLG, use a glue which can be easily removed from the tooth after cutting (*e.g.* Bosch hot glue). Also be aware that some resins and glues may react with either the formic acid solution or acetone used during the acid etching process and should be avoided.

1. For cetaceans, choose one of the front teeth from the lower jaw or the straightest tooth (cleaned of flesh) from each individual. For pinnipeds the upper canine is straighter and less twisted than the lower canine and therefore easier to cut following the midline.

2. The saw you are using may have a holding stage that can either be used in a vice-like fashion to hold the tooth directly or to hold a small wooden block (to which the tooth is adhered). If using a wooden block for fitting into the vice this needs to be large enough to hold the tooth but not so large that it cannot be held in the vice securely. In most cases the holding stage is aligned against the blade and the saw runs along a track so that the holding block automatically runs through the saw. Some do not and you may need to manually feed the holding block through the saw. If the saw does not have a holding stage you will need to prepare a block that can be used to manually feed each tooth through the saw. When preparing your block ensure that it is the appropriate

size to (1) hold a tooth, (2) be fed through the saw (3) perform the actions without endangering any part of yourself.

3. You will need to orient the tooth so that it is fed longitudinally through the saw root tip first and that either or both the buccal and lingual sides of the tooth can be trimmed. If using a vice, orient the tooth so that convex surface of the tooth is downwards (against the stage) and the concave surface of the tooth is facing upwards. Ensure that the tooth is aligned so that when cutting the saw blade is aligned as parallel to the middle of the tooth as possible. Lines can be drawn on each side of the midline with a pencil to assist with cutting in the right place. If the tooth is twisted, position the tooth so the blade is aligned as parallel to the midline of the lower half of the tooth and root tip (this ensures that the most recent dentine layers are exposed). Lock the vice so that the tooth is held firmly and there is no chance that it will move as it is being trimmed.

4. If using a wooden block (either to be held by the vice or manually fed through the saw), teeth can be adhered to the wooden block using thermoplastic cement or glue. If using thermoplastic cement, cover the bench with a sheet of cardboard to keep it clean. Soften a small amount of thermoplastic cement by holding a stick of the cement using long forceps over a Bunsen burner flame. The cement will quickly become sticky and begin to melt, but will rapidly re-solidify once you remove it from the flame so you need to move quickly once you start to heat it. Once softened, place a sufficient amount of the cement on the block and before it hardens, press the tooth in the correct orientation (see point 3) into the cement. Add more cement around the sides of the tooth, making sure there is a strong bond with no gaps. Check that the tooth is secure and will not move as it is being trimmed. If it is not, either repeat the process again or secure the tooth by placing some more of the cement on either side of the tooth. Label the back of the block with the specimen number in pencil. An alternative way of securing small or medium teeth (*e.g.* pinniped canines and dolphin teeth) is to use a hot glue gun. Place a small amount of hot glue (*e.g.* about the size of an Australian 10 cent coin) on the wooden block and lightly press the tooth into the glue. Hold in position for 30–50 sec. If the bond is strong enough there is no need to add additional glue to the sides of the tooth.

However, run a bead of glue along any visible cracks to prevent the tooth breaking when cut.

5. Fix the mounted tooth to the saw. Before you start, ensure there is sufficient water in the tray of the saw so that the blade is just dripping as it rotates – never trim teeth using a dry blade. Slowly feed it through the blade (either automatically or manually) so that one side is trimmed off, leaving two 'halves', one slightly thicker than the other and containing the longitudinal midline of the tooth. Label each side of the tooth with a permanent-ink (technical) marking pen. The 'half' not being used for aging can be stored dry in a stable environment (low humidity, cool temperature) and may be of use for other analyses.

6. Polish the 'half' of the tooth containing the longitudinal midline of the tooth with progressively finer sandpaper grades until all saw marks are removed from the cut surface and it has become very smooth. The grade of sandpaper to start with will depend on the extent of the saw marks on the tooth. However, the grade for the final polish needs to be in the order of 320–400 for large teeth such as whales and 800–1200 for smaller teeth such as pinniped canines. Wetting the sandpaper slightly will assist in polishing in the final stages. Ensure the surface that the sandpaper is placed on is smooth and even (can use a piece of marble slab or many geology departments may have a lap-polishing machine available).

7. Once the tooth 'half' is sufficiently polished (the midline of the tooth should now be exposed), it can be rinsed under tap water to remove any excess sanding residue and air dried on a rack.

Acid etching

Occupational Health and Safety

Wear heavy gloves and safety glasses when using chemicals.
Work in a fume hood.

Equipment required

Fume hood
Heavy gloves

Safety glasses
Deep, large glass dishes (2) with lids (*e.g*. Pyrex casserole dish) for large whale teeth or glass petri dishes for pinniped teeth.
Formic acid (AR grade)
Acetone (AR grade)
Distilled water
Large forceps/tongs (2)
Tap water for rinsing
Paper towel
Soft-leaded pencil (No. 1)/graphite transfer paper
Permanent marking pen/ink technical (Rotring) pen
Air-drying rack
Magnifying lamp/dissecting microscope

Note: see Appendix C for example tables for recording acid etching times.

1. Make up a solution of 15% formic acid using distilled water, large enough to fill a large glass dish to a depth of at least 1 cm for large teeth or enough to almost cover smaller teeth.

2. Place the tooth 'half' cut surface down into the dish making sure that the acid solution covers the complete cut surface and comes approximately 1 cm up both sides of the tooth. Agitate the tooth to ensure that any air bubbles trapped between the bottom of the dish and the cut surface are dissipated.

3. If the etching time is unknown, check the tooth every 30 min for large teeth (*e.g. P. macrocephalus*) and after 10–15 min for smaller teeth (*e.g.* pinniped canines). Use a timer with an alarm to ensure that teeth are not left in the acid for too long. Once etching times have been established, the tooth can be left for longer if appropriate before the first check (*e.g. P. macrocephalus* teeth can be left for 3 h, *A. forsteri* and *A. pusillus doriferus* canines for 10 min before the first check). As the acid is chemically reacting with the surface of the tooth and essentially eroding the surface of the tooth, it is important to regularly agitate the dish every few minutes to dispel any bubbles produced by the process as they can create an unevenly etched surface.

4. After 10–30 min (depending on species) remove the tooth from the dish using large forceps or tongs and rinse under tap water for 2–3 min.

5. While the tooth is rinsing, fill a second glass dish under the fume cupboard with a similar amount of acetone to that of the acid solution.

6. Pat the tooth dry with paper towel and place into the acetone bath for 3 min (use a timer with an alarm). Agitate the tooth to ensure that any air bubbles trapped between the bottom of the dish and the cut surface of the tooth are dissipated.

7. Remove the tooth from the dish using large forceps or tongs and rinse under tap water for 2–3 min. Pat dry with a paper towel and air-dry on a rack. The tooth needs to completely dry before it can be checked as the growth layer groups (GLGs) will not be easily distinguished until it is.

8. Once dry, rub the etched surface with a soft leaded pencil (No. 1) or graphite transfer paper to emphasise the relief of the etched surface. Check the state of the acid etching under a magnifying lamp or dissecting microscope. When fully etched, the GLGs should be clear and distinguishable from any accessory layers (Figures 5 and 6).

9. If not fully etched, repeat steps 2–8 until complete, reducing the time period between checks to 30 min for large teeth (*e.g. P. macrocephalus*) or 3–5 min for small teeth (*e.g.* pinniped canines). Keep in mind that tooth density can also affect etching time more than actual size.

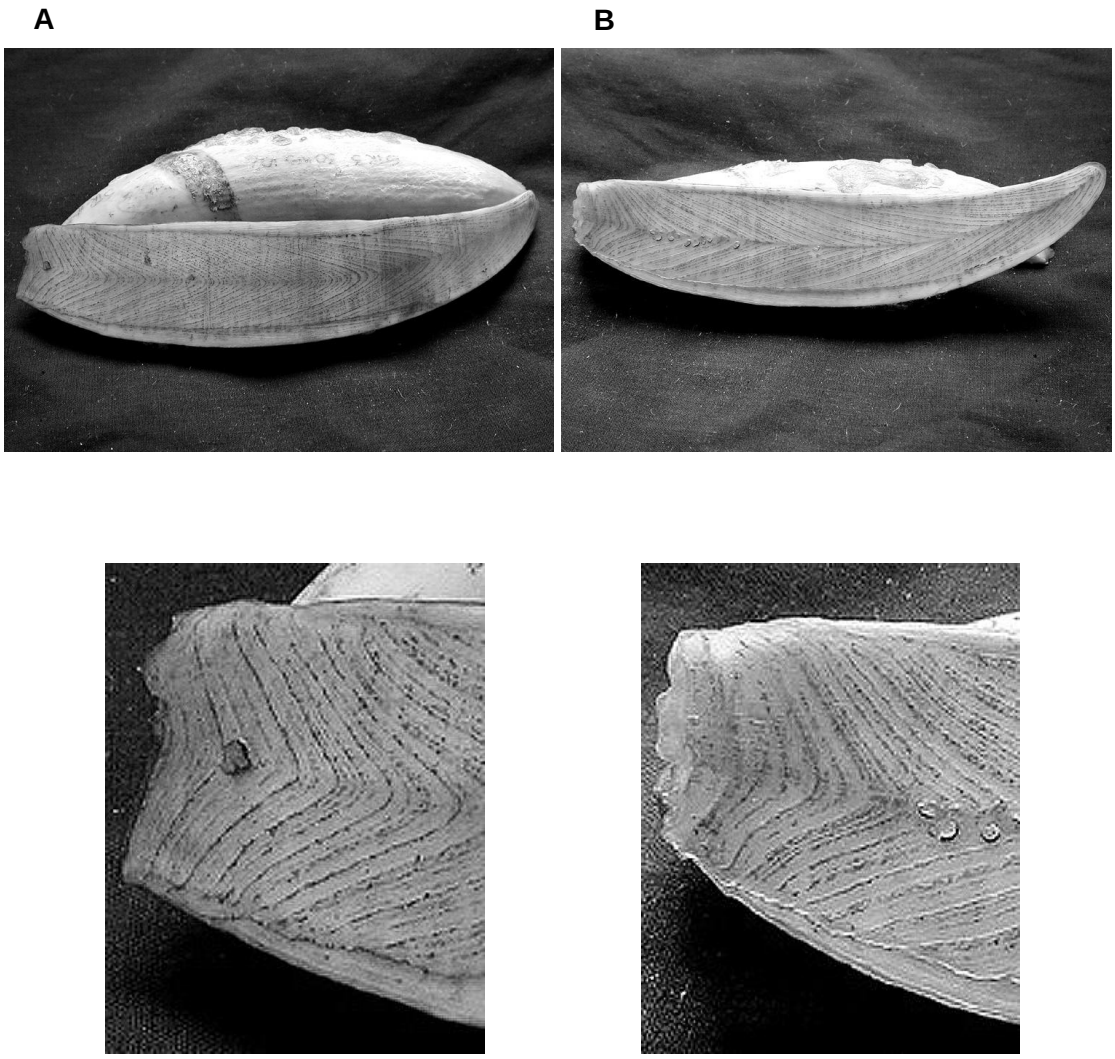


Figure 5. Longitudinal, acid-etched sperm whale tooth with detail of etched GLGs (bottom panel). (A) Tooth properly etched and ready for aging. (B) Tooth under-etched and requiring further etching.

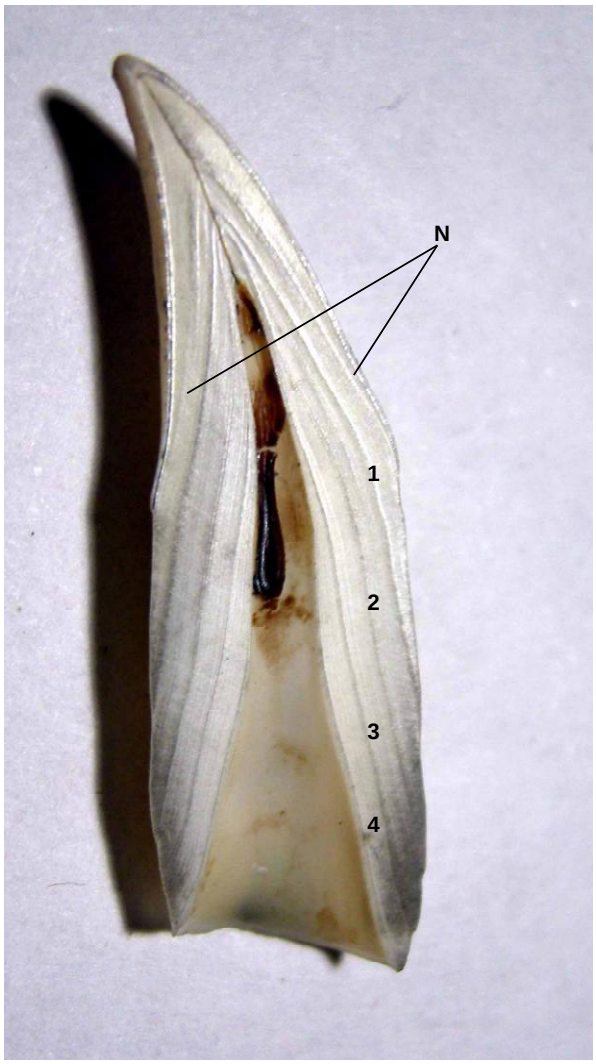


Figure 6. Longitudinal, acid-etched upper canine tooth section of a female northern fur seal (*Callorhinus ursinus*), estimated to be 4 y. Four annual narrow dark bands (dominant ridges) can be clearly seen. N = neonatal line.

AGING TEETH

Tooth morphology

The first step in aging your prepared specimen is familiarising yourself with the anatomy of the tooth so that you can identify the key features (Figure 7) involved in determining age.

The portion of the tooth that lies above the gum line is called the crown and is covered by a thin layer of enamel that protects the underlying dentine. If preparing teeth using decalcification, the acids used will dissolve the enamel. The part of the tooth that lies below the gum line is the root. A root or pulp cavity may be present depending on the age of the specimen. The root is bonded to the bone of the jaw by the periodontal membrane. The outermost surface of the root is covered by a relatively thin layer of cementum, which runs along either side of the tooth. In decalcified, stained thin-sections, the cementum contains a series of alternating darkly and lightly stained layers orientated sub-parallel to the root cavity or tooth midline (Figures 7, 8 and 9). Within the dentine (the inner portion of the tooth below the enamel), the prenatal dentine occurs as a darkly stained relatively unlayered chevron at the apex of the tooth (Figures 4 and 8). This will taper down either side of the tooth to varying degrees depending on the animal's age and species. Adjacent and internal to the prenatal dentine is the neonatal line, a thin unstained line. The remainder of the dentine (postnatal dentine) forms a series of internally nested elongate chevrons of decreasing thickness with alternating dark and light layers. In younger animals, the root or pulp cavity will be open and the most recent dentine growth layers will be adjacent to this area.

In acid-etched teeth, the enamel may still be present if not naturally worn due to tooth wear. The prenatal dentine and a number of the outer dentinal layers may also be missing as a result of wear. The general appearance of the remainder of the tooth is the same as that described above, although instead of alternate darkly and lightly stained layers, the cementum and dentine will comprise alternate ridges and grooves (Figure 5).

There are often mineralisation anomalies in the dentine. These may comprise pulp stones (Figures 5 and 8) or occlusions. Large pulp stones can bend growth layers, or may obscure that part of the growth layer situated in the area of the pulp stone. In most cases, regardless of pulp stone size, growth layers can still be identified in the dentine. Occlusions may obscure growth layers by disrupting lamina formation to the extent that they are no longer clearly defined. This may not affect the precision of counts of growth layers, since the same number of laminae actually defined within and outside the mineralisation interference area can be identified. However, such events have implications for the accuracy of age estimates, especially in older animals, in which both the incidence and the number of mineralization anomalies are higher. If possible, it is recommended that several teeth from the same specimen be prepared to maximize the chances of a specimen clear of occlusions and pulp stones.

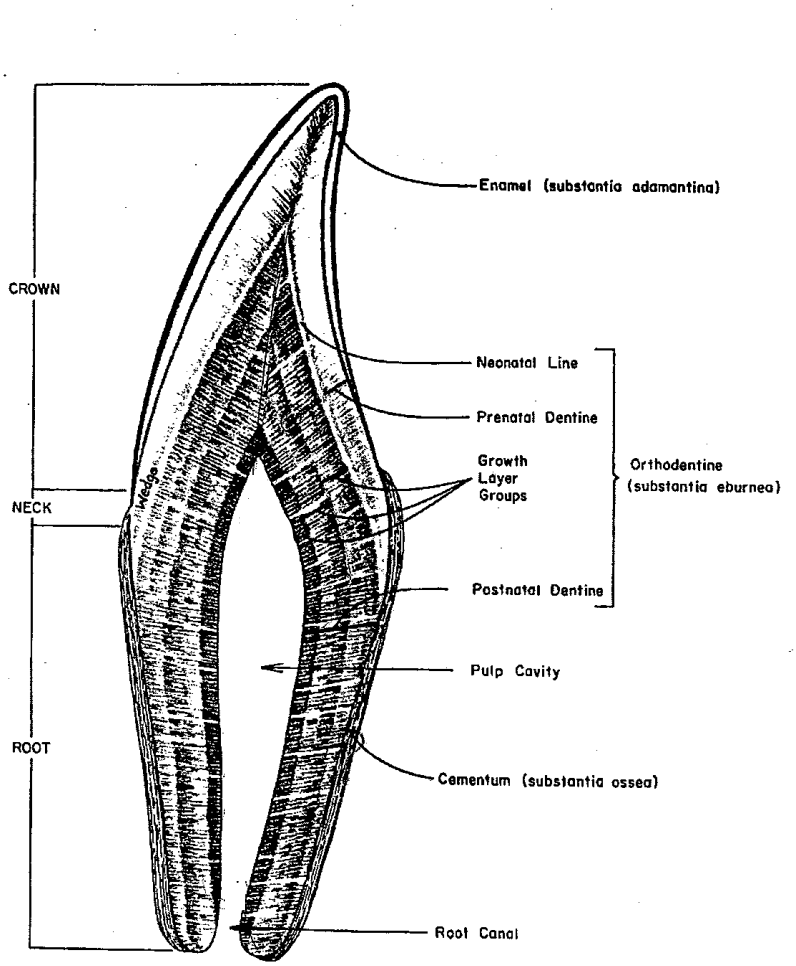


Figure 7. Generalised longitudinal section of a dolphin tooth. Taken from Perrin and Myrick (1980).

Definition of growth layer groups

Growth layer groups as defined in the report of the workshop on age determination of toothed whales and sirenians (Perrin and Myrick 1980) are: "groups of incremental growth layers which may be recognised by virtue of a cyclic repetition, generally occurring at constant or regularly changing relative spacing. Such a cyclic repetition of incremental growth layers must involve at least one change, *i.e.* between translucent and opaque, dark and light, ridge and groove, more stained, less stained but may involve more than one change". They are further defined as "a repeating or semi-repeating pattern of adjacent groups of incremental growth layers within the dentine (or cementum) which is defined as a countable unit involving a change….from a ridge to groove" in the case of etched teeth and "intensely stained to lightly stained" in the case of stained, thin-sectioned teeth".

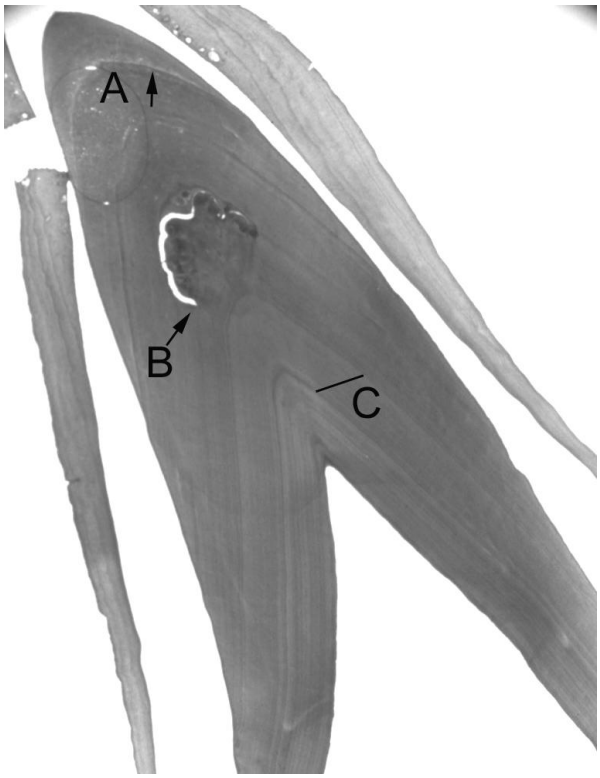


Figure 8. Decalcified, thin-sectioned and stained juvenile sperm whale tooth estimated at 5 y. The neonatal line (A), a pulp stone (B) and growth layers groups (C) can be clearly identified. Note also that growth layer groups in the cementum are also clearly identifiable.

Definitions of GLGs may vary depending on the species and the clarity of the growth layers in the tooth being examined. Growth layer groups in *Stenella* spp. were described by Myrick *et al.* (1983) as consisting of a thin, lightly-stained boundary layer, a thicker darkly-stained layer, another thin lightly-stained mid-GLG layer and a second thick darkly-stained layer. Most of the time, however, all four layers were not distinct and the GLG appeared to contain only a light boundary layer and a thick darkly stained layer. In the same species Perrin and Myrick (1980) described GLGs as consisting of a thick translucent layer, a bright, thin translucent layer and a thick translucent layer which tends to become darker towards the internal edge. The GLGs of *D. delphis* have been described as alternating structures stained with varying intensity, with the boundary layers of each defined by a region of highest intensity in the stain (Perrin and Myrick 1980). Growth layer groups in the dentine of *T. truncatus* have been described as a thick layer of intermediate to slightly opaque optical density with fine substructure followed by a slightly thinner layer containing an alternate sequence of two to five opaque and translucent layers. Each GLG is bordered by thin opaque margins (Perrin and Myrick 1980). The GLGs of bottlenose whales (*Hyperoodon* spp.) have been described as consisting of a wide layer of intermediate density, followed by a thin translucent layer and a thin opaque layer. Those of *Globicephala* spp., *Pseudorca crassidens*, *Grampus griseus* and *P. phocoena* are described as consisting of a regular series of thick opaque and thin translucent layers (Perrin and Myrick 1980) and those of young *P. macrocephalus* are also best broadly described in this fashion (Figure 8). The GLGs in the cementum of the pinnipeds *A. forsteri* and *N. cinerea* are described as a layer including two distinct incremental zones; a wide poorly-stained zone containing lightly stained accessory laminae and a narrow deeply stained zone (McKenzie *et al.* 2007; Figure 9). However, the first GLG may not always be distinct from the dentine-cementum junction and in some regions of the tooth may appear as a single thickened dark layer. Two narrow, dark zones may also occur within the same annual GLGs, being clearest in the region of the root tip.

Growth layers groups in acid-etched teeth have most commonly been described as alternating ridges and grooves (Figures 5 and 6) that correspond to the dark and light laminae seen in decalcified stained thin-sections (Perrin and Myrick 1980; Lockyer 1980).

Growth layer groups often contain a number of narrow layers of contrasting density, termed accessory layers. In general, these are distinguished by their smaller thickness, their position and their irregular occurrence in relation to the more regular occurrence of GLGs. It is not entirely clear what these accessory layers represent, but it has been suggested that they may be associated with such events as feeding patterns (McCann 1993), lunar cycles (Myrick *et al.* 1984) nursing bouts (Baker 1991) or periods of arrested annual growth (Klevezal' 1980).

In reality, the decision as to what constitutes a GLG is made on the basis of an inspection of the overall pattern of layering within the tooth, keeping in mind what has previously been defined as a GLG in other teeth examined (*i.e.* past experience). Definitions of GLGs depend on the interpretation of the individual or the laboratory at which age estimates are being determined and are therefore, qualitative and subjective. It is important to become familiar with the level of variation in the appearance of GLGs in different regions of the cementum or dentine and among different specimens.

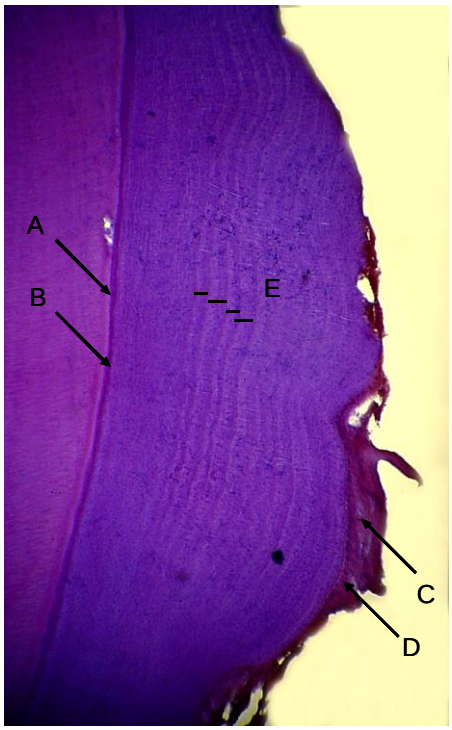


Figure 9. Decalcified, stained, thin-section of a New Zealand fur seal (*A. forsteri*) post-canine, estimated at 18 y (in comparison to 17.6 y based on capture date). (A) dentine-cementum junction, (B) first annulus, (C) periodontal ligament tissue, (D) new cementum forming and (E) GLGs.

Dentine vs *cementum*

Whether you count growth layers in the cementum or the dentine of a tooth will depend on the species, the preparation method used and the age of your specimen. There is no hard and fast rule for using either and it usually a choice of which yields the clearest GLGs.

In general, GLGs are usually identified in the dentine of odontocete teeth after either acid etching or decalcification, thin-sectioning and staining. This is largely because growth layers are often clearer in the dentine and more easily readable. The cementum tends to be quite thin and poorly layered in a number of odontocete species (many of the delphinids *e.g. Orcinus orca*, *Globicephala* spp., *Grampus griseus*). The growth layers in the cementum of a number of beaked whales and larger delphinids (*e.g. Pseudorca crassidens*) overlap towards the root of the tooth. As a result, the full length of the root is needed for examination if GLGs are to be read from the cementum.

In most pinniped teeth, growth layers in the cementum are identified for aging after decalcification, thin-sectioning and staining. This is because growth layers in the dentine are, in general, not easily distinguished and frequently contain accessory lines. Also, the pulp cavity closes with age, resulting in the underestimation in older individuals if dentine is used. Complete teeth are required because the most recent cementum layer forms at the root and may not extend up the full length of the root. In those pinnipeds where the tooth is acid-etched (*e.g.* canines), the dentine is generally used because the cementum layer is extremely narrow.

Reading teeth

Decalcified, stained thin-sections are usually examined under a compound transmitted light microscope at magnifications of 40–100X depending on the size of the tooth and whether the dentine or the cementum is being examined. When identifying each GLG, trace it along its length from the point of examination to ensure that it is not compressed (fused). This also avoids counting double layers as one (McKenzie *et al.* 2007). Note that in *A. forsteri* post-canines, the first annual layer in the cementum is

not always distinct from the cementum-dentine junction along the length of the tooth, and may appear as a thickened cementum-dentine junction. Also, the most recent layer of growth may not be visible in all regions or may be partially formed or damaged, particularly if the periodontal ligament is not intact.

Acid-etched teeth are usually examined under a magnifying lamp but if the specimen is small, a dissecting microscope may be required. Reflected light directed across the surface of the tooth helps to highlight the ridges and grooves.

Note that in all teeth, the GLGs will become more compressed as the individual ages. This is because the pulp cavity fills in as more layers of dentine are deposited. Once the cavity is closed, the most recently deposited dentinal layers become compacted near the root of the tooth and are subsequently hard to discern. Care must be taken interpreting these.

In most situations, prepared teeth are read blind, that is, without prior knowledge of any of the associated biological data (sex, length *etc…*) of the individual, and in random order so as not to identify particular individuals. This is done to reduce conscious or unconscious bias produced by perceptions of how old the individual 'should' be. Such bias can result in under-estimation of biological variation and may result in the production of invalid demographic measurements such as growth curves, life tables, measures of survival *etc*… It is best to cover the true specimen number with a temporary numerical or alphabetical label to avoid making connections with particular individuals. Age estimates can be cross-referenced back to the original identification of the animal after reading is complete. Where ages are being verified against known-age specimens and/or the identification and definition of GLGs is being established, it is acceptable to have prior knowledge of biological data when reading the teeth.

Given that in most situations an animal will not be able to be aged accurately (this can only occur in known-age animals that have had markers introduced to their skeletal structure at regular intervals throughout their lives or their birth date is known), it is important that the precision of the estimates, that is, the repeatability of counts of GLGs within a specimen, is maximised. Being able to consistently achieve the same

estimate in an individual (not only by yourself but also by other readers) demonstrates that the age you have assigned the individual is as robust as is possible.

To produce a statistically robust measure of precision, it is recommended that the GLGs in each tooth should be counted at least three and preferably five times. Cross validation of age estimates with at least one other reader experienced in age estimation (and particularly with the species you are aging) should be carried out when ever possible. Quantitative tools for assessing precision of age estimates can be found in Beamish and Fournier (1981), Chang (1982), Reilly *et al.* (1983), Campana *et al.* (1995) and Campana (2001) and their use is encouraged.

Assigning the final age estimate to your specimen from multiple readings can be simple in the case that all readings are the same. However, it is more likely that at least a proportion of your readings will not be the same and a statistically valid decision will need to be made to achieve a final estimate of age.

Tools for identifying growth layers and maximising precision of age estimates

Given that definitions of GLGs are largely subjective assessments made by the reader, concerted efforts have been made by numerous researchers to standardise interpretations of GLGs and thereby assessments of age (Perrin and Myrick 1980; Merrick *et al.* 1983; Evans and Robertson 2001; Evans *et al*. 2002). Through the use of high resolution photographs of acid-etched teeth (Figure 5) and decalcified, stained thin-sections (Figure 8), Evans *et al*. (2002) were able to reduce overall variation in estimates of age. This was thought to be due to greater clarity of and contrast in growth structures resulting in less confusion in interpreting between GLGs and accessory layers, thereby reducing the incidence of differences in the definition of accessory layers and GLGs. Photographs were also considered easier to read as a result of greater ease in the sequential identification of GLGs.

Other variants on this theme could include the use of imaging software for use on dissecting and compound microscopes, allowing a larger view of the tooth or tooth sections. If considering using imaging software or photographs, care must be taken to ensure that the image is at high enough resolution and the complete image is clearly in

focus. If sections of the tooth or tooth section are not properly in focus (this may occur particularly in acid-etched teeth as a result of curvature of the tooth) or of an adequate resolution, any identification of GLGs and subsequent age estimation will be compromised. The process may involve taking several photographs of the tooth/tooth section and then stitching them together using appropriate imaging software (Figure 10). Contrast or colour levels for digital images may also be adjusted to enhance GLGs, but care must be taken to ensure the details of less distinct GLGs are not lost or additional lines introduced. Similarly, the light setting and use of colour filters (typically blue) on microscopes can be used to enhance the appearance of GLGs in decalcified, stained thin-sections, particularly when sections have been under or over stained.

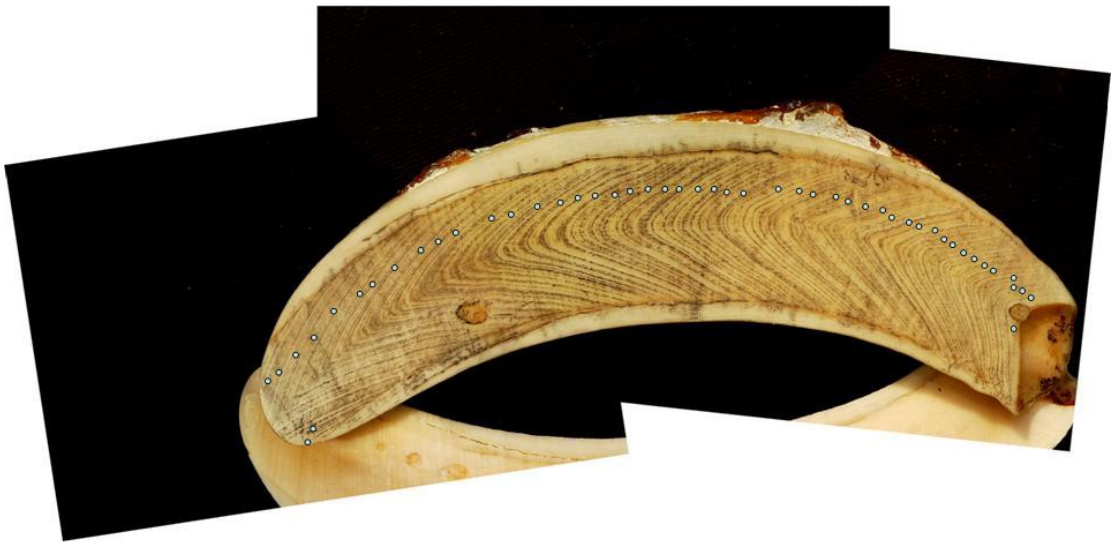


Figure 10. Composite photograph of an acid-etched sperm whale tooth in which the GLGs have been marked.

ACKNOWLEDGEMENTS

The authors would like to thank those who taught us all of the techniques that we are now passing on to others. Kelly Robertson, Jessica Lipsky and Susan Chivers of the Southwest Fisheries Science Center, NOAA and Peter Boveng of the National Marine Mammal Laboratory, NOAA are thanked for their time and generosity in sharing their skills. Funding was made available from the Department of Environment, Water Heritage and Arts, Canberra under the Australian Centre for Applied Marine Mammal Science (ACAMMS). The authors are grateful to the South Australian Museum and volunteers for support in running the workshop and producing the manual, particularly Amy Stump, Sue Gibbs and Ikuko Tomo. Further input into the manual was provided by Elena Trentin and Mauro Talamonti.

LITERATURE CITED

Baker JD. 1991. Trends in female northern fur seal, *Callorhinus ursinus* feeding cycles indicated by nursing lines in juvenile male teeth. MSc. Thesis, University of Washington, Seattle, Washington.

Beamish RJ and Fournier DA. 1981. A method for comparing the precision of a set of age determinations. Canadian Journal of Fisheries and Aquatic Sciences 48: 1007–1014.

Best PB. 1970. The sperm whale (*Physeter catadon*) off the west coast of South Africa. 5. Age, growth and mortality. Investigational Reports of the Division of Sea Fisheries of South Africa 79: 1–27.

Best PB. 1976. Tetracycline marking and the rate of growth layer formation in the teeth of a dolphin (*Lagenorhynchus obscurus*). South African Journal of Science 72: 216–218.

Brodie PF, Geraci JW and St. Aubin DJ. 1990. Dynamics of tooth growth in beluga whales, *Delphinapterus leucas*, and effectiveness of tetracycline as a marker for age determination. Pages 141–148 in Smith TG, St Aubin DJ and Geraci JR (eds.). Advances in Research on the Beluga Whale, *Delphinapterus leucas*.

Canadian Bulletin of Fisheries and Aquatic Science 224, Department of Fisheries and Oceans, Ottawa.

Bjørge A, Hohn AA, Kvam T, Lockyer C, Schweder T and Aarefjord H. 1995. Report of the harbour porpoise age determination workshop, Oslo, 21–23 May 1990. Report of the International Whaling Commission (special issue) 16: 477-496.

Campana SE. 2001. Accuracy, precision and quality control in age determination, including a review of the use and abuse of age validation methods. Journal of Fish Biology 59: 197–242.

Campana SE, Annand MC, McMillan JI. 1995. Graphical and statistical methods for determining the consistency of age determinations. Transactions of the American Fisheries Society 124: 131–138.

Chang WYB. 1982. A statistical method for evaluating the reproducibility of age determination. Canadian Journal of Fisheries and Aquatic Science 39: 1208–1210.

Childerhouse S, Dickie G, Hessel G. 2004. Ageing live New Zealand sea lions (*Phocarctos hookeri*) using the first post-canine tooth. Wildlife Research 31: 177–181.

Christensen I. 1995. Interpretation of growth layers in the periosteal zone of *tympanic bulla* from minke whales *Balaenoptera acutorostrata*. Pages 413–423 in Blix AS, Walløe L and Ulltang Ø (eds). Whales, seals, fish and man. Elsevier Science, BV.

Dennis C. 2006. Conservation at a distance: a gentle way to age. Nature 442: 507-508.

Evans K, Hindell MA, Robertson K, Lockyer C and Rice D. 2002. Factors affecting the precision of age determination in sperm whales, *Physeter macrocephalus*. Journal of Cetacean Research and Management 4(2): 193–201.

Evans K and Robertson K. 2001. A note on the preparation of sperm whale teeth (*Physeter macrocephalus*) for age determination. Journal of Cetacean Research and Management 3(1): 101–107.

Gambell R. 1977. Dentinal layer formation in sperm whale teeth. Pages 583–590 in Angel M (ed.). A Voyage of Discovery. Pergamon Press, Oxford.

Goren AD, Brodie PF, Spotte S, Ray GC, Kaufman HW, Gwinnett AJ, Sciubba JJ and Buck JD. 1987. Growth layer groups (GLGs) in the teeth of adult belukha whale (*Delphinapterus leucas*) of known age: evidence of two annual layers. Marine Mammal Science 3(1): 14–21.

Gurevich VS, Stewart BS and Cornell LH. 1980. The use of tetracycline in age determination of common dolphins, *Delphinus delphis*. Report of the International Whaling Commission (special issue 3): 165–169.

Hohn AA, Scott MD, Wells RS, Sweeney JC and Irvine AB. 1989. Growth layers in teeth from known-age, free-ranging bottlenose dolphins. Marine Mammal Science 5(4): 315–342.

Hohn AA. 1990. Reading between the lines: analysis of age estimation in dolphins. Pages 575–584 in Leatherwood S and Reeves RR (eds). The Bottlenose Dolphin. Academic Press, San Diego.

Holstrom SE, Eisner ER and Frost Fitch P. 2004. Veterinary dental techniques for the small animal practitioner. WB Saunders/Elsevier Science, BV.

International Whaling Commission. 1967. Report of the Scientific Committee. Appendix E. Sperm whale sub-committee meeting report. Report of the International Whaling Commission 17: 120–127.

International Whaling Commission. 1971. Annex D. Report of the special meeting on sperm whale biology and stock assessments. Report of the International Whaling Commission 21: 42–50.

Jonsgård Å. 1969. Age determination of marine mammals. Page 1-30 in Andersen HT (ed.) The biology of marine mammals. Academic Press, New York.

Klevezal' GA. 1980. Layers in the hard tissues of mammals as a record of growth rhythms of individuals. Report to the International Whaling Commission (special issue 3): 89–94.

Langvatn R. 1995. Age determination of mammals – some aspects of biochemistry and physiological mechanisms relating to deposition of incremental lines in dental tissues. Report of the International Whaling Commission (special issue 16): 499–510.

Laws RM. 1952. A new method for age determination for mammals. Nature 169: 972.

Laws RM and Purves PE. 1956. The ear plug of the Mysticeti as an indication of age with special reference to the North Atlantic fin whale. The Norwegian Whaling Gazette 8: 413–425.

Lockyer C. 1980. Age determination studies on *Physeter macrocephalus*. Report of the International Whaling Commission (special issue) 3: 216.

Lockyer C. 1993. A report on patterns of deposition of dentine and cement in teeth of pilot whales, genus *Globicephala*. Reports of the International Whaling Commission (special issue) 14: 137–161.

Lockyer C. 1995. A review of factors involved in zonation in odontocete teeth, and an investigation of the likely impact of environmental factors and major life events on harbour porpoise tooth structure. Report of the International Whaling Commission (special issue) 16: 511–529.

McCann TS. 1993. Age determination. Pages 199-227 in Laws RM (ed.) Antarctic seals, research methods and techniques. University Press, Cambridge.

McIntosh R. 2007. Life history and population demographics of the Australian sea lion. PhD Thesis, Zoology Department, La Trobe University, Melbourne, Australia.

McKenzie J, Page B, Shaughnessy PD and Hindell M. 2007. Age and reproductive maturity of New Zealand fur seals (*Arctocephalus forsteri*) in southern Australia. Journal of Mammalogy 88(3): 639–648.

Myrick AC. 1988. Is tissue resorption and replacement in permanent teeth of mammals caused by stress-induced hypocalcaemia? Pages 379–389 in Davidovitch Z (ed.). The Biological Mechanisms of Tooth Eruption and Root Resorption. EBSCO Media, Birmingham.

Myrick AC and Cornell LH. 1990. Calibrating dental layers in captive bottlenose dolphins from serial tetracycline labels and tooth extractions. Pages 587–607 in Leatherwood S and Reeves RR (eds). The Bottlenose Dolphin. Academic Press, San Diego.

Myrick AC, Hohn AA, Sloan PA, Kimura M and Stanley DD. 1983. Estimating age of spotted and spinner dolphins (*Stenella attenuata* and *Stenella longirostris*) from teeth. NOAA Technical Memorandum. NOAA-TM-NMFS-SWFC-30.

Myrick AC, Shallenberger EW, Kang I and MacKay DB. 1984. Calibration of dental layers in seven captive Hawaiian spinner dolphins, *Stenella longirostris*, based on tetracycline labelling. Fisheries Bulletin 82(1): 207–225.

Ohsumi S, Kasuya T, and Nishiwaki M. 1963. Accumulation rate of dentinal growth layers in the maxillary tooth of the sperm whale. Scientific Reports of the Whales Research Institute 17: 15–35.

Perrin WF and Myrick AC (eds). 1980. Report of the International Whaling Commission (Special Issue 3). Age determination of toothed whales and

sirenians. International Whaling Commission, Cambridge, UK.

Reilly SB, Hohn AA and Myrick AC. 1983. Precision of age determination of northern offshore spotted dolphins. NOAA Technical Memorandum, NOAA-TM-NMFS-SWFC-35. Southwest Fisheries Center, National Marine Fisheries Service, California.

Ridgeway SH, Green RF and Sweeney JC. 1975. Mandibular anesthesia and tooth extraction in the bottlenose dolphin. Journal of Wildlife Diseases 11: 415–418.

Scheffer VB. 1950. Growth layers on the teeth of Pinnipedia as an indication of age. Science 112: 309–311.

Stewart REA, Campana SE, Jones CM and Stewart BE. 2006. Bomb radiocarbon dating calibrates beluga (*Delphinapterus leucas*) age estimates. Canadian Journal of Zoology 84: 1840–1852.

APPENDICES

Appendix A: Decalcification guide

Note: this information should be used only as an indication of times taken to decalcify teeth. The actual time will depend on the (i) species, (ii) tooth used, (iii) age (size) of animal/size of tooth and (iv) age/strength of RDO used. Good alternate guides on this method have been published in Evans and Roberston (2001), Lockyer (1993), McIntosh (2007), McKenzie *et al.* (2007), Myrick *et al.* (1983).

Otariids

Arctocephalus forsteri: 5–12 h

Arctocephalus pusillus doriferus: 6–8 h for young animals, up to 48 h for older animals

Neophoca cinerea: 5–35 h (mean = 17.6 ± s.d. 6.12, n = 228)

Phocarctos hookeri: 5% nitric acid for 24 h, rinsed and trimmed, 1 part formic acid: 9 parts 10% formalin for 48–65 h (as per Childerhouse *et al.* 2004. Note use of nitric acid, formic acid and formalin rather than RDO).

Odontocetes—whole teeth

Delphinus delphis

Total length 120–50 cm: 2 h

Total length 150–200 cm: 6–8 h

Total length >200 cm: 8–15 h

Lissodelphis peronnii

Total length 214 cm: 7 h

Sousa chinensis

Total length <190 cm: 4 h

Total length 190–210 cm: 16 h

Total length >210 cm: 16–19 h

Stenella longirostris

Total length <160cm: 2–3 h

Total length 160–190cm: 4–6 h

Total length >190cm: 6–8 h

Stenella attenuata

Total length <120cm: 2 h

Total length 120–150cm: 4 h

Total length 150–170cm: 6–8 h

Total length 170–200cm: 8–11 h

Tursiops truncatus

Total length <150 cm: 3–4 h

Total length 150–200 cm: 6–10 h

Total length 200–250 cm: 6–17 h

Total length >250 cm: 17–30 h

Mesoplodon hectori

Total length 334 cm: 3 h

Phocoena phocoena

Total length <120 cm: 1–2 h

Total length 120–150 cm: 2–4 h

Total length >150 cm: 4–6 h

Phocoena dioptrica

Total length 119 cm: 2 h

Kogia sima

Total length 214 cm: 7 h

Physeter macrocephalus

Total length 400–500 cm: 4–12 h

Total length 500–650 cm: 12–24 h

Total length 650–750 cm: 22–30 h

Odontocetes—wafers (approx. 3 mm)

Globicephala melas

Total length 250–350cm: 2–6 h

Total length >350cm: 4–12 h

Tursiops truncatus

Total length 170–250 cm: approx. 6 h

Total length >250: 6–14 h

Tursiops aduncus

Total length <140: <2.5 h

Total length 140–170: 2.5–5 h

Total length >170: 5–15 h

Appendix B: Trouble-shooting when cutting thin-sections

Some of these comments were taken from the cryostat manual and apply to other types of tissue but the advice may be pertinent to cutting marine mammal teeth.

Problem	**Cause**	**Remedy**
Frost on chamber walls and microtome of cryostat	Cryostat is exposed to air currents (open windows and doors, air conditioning)	Change place of installation for the cryostat
	Frost built up by breathing into the cryochamber	Wear mouth protection
Cryostat sections curled	Static electricity/air currents	Remove cause
	Specimen not cold enough	Select lower temperature
	Large area of specimen	Increase section thickness
	Anti-roll plate poorly positioned	Reposition anti-roll plate
	Anti-roll plate poorly aligned with knife edge	Align correctly
	Incorrect knife angle	Set correct angle
	Blunt knife	Use different part of knife or replace knife
Cryostat sections curled despite correct temperature and correctly aligned anti-roll plate	Dirt on knife and/or anti-roll plate	Clean with dry cloth or brush
	Top edge of anti-roll plate damaged	Replace anti-roll plate
	Blunt knife	Use different part of knife or replace the knife
Sections do not uncurl in distilled water	Tooth wafer may have been over-decalcified	Open with paintbrush, check for over-decalcification. Use alternative tooth for aging
Sections curl on anti-roll plate of cryostat	Anti-roll plate does not protrude far enough beyond the knife edge	Re-adjust correctly

Problem	Cause	Remedy
Scraping noise during sectioning	Anti-roll plate of cryostat protrudes too far beyond the knife edge and is scraping against the specimen	Re-adjust correctly
	Specimen over-frozen	Warm the specimen slightly
	Knife damaged	Replace the knife
Tooth dislodges from OCT during sectioning	Specimen insufficiently frozen onto specimen disc	Re-freeze specimen onto disc
	Specimen disc not clamped tightly	Check disc clamping
	Knife not clamped tightly enough	Check knife clamping
	Specimen has been sectioned too thickly and has detached from disc	Re-freeze specimen onto disc
	Blunt knife	Use different part of the knife or replace knife
	Knife profile inappropriate for specimen cut.	Use knife with different profile
	Incorrect knife angle	Change angle
Condensation on anti-roll plate of cryostat or knife during cleaning	Brush, forceps and/or cloth too warm	Store all tools on storage shelf in the cryochamber of the cryostat or cool tools before use on freezing slide (sledge) microtome
Anti-roll plate on cryostat damaged after adjustment	Anti-roll plate too high above the knife edge	Replace anti-roll plate
	The adjustment was carried out in the direction of the knife	Be more careful next time!
Sections are variable in thickness	Temperature incorrect for the tissue being cut.	Select correct temperature
	Knife profile inappropriate for the specimen cut	Use knife with different profile
	Ice build-up behind the knife	Remove ice
	Hand wheel/slide speed not uniform	Adapt speed

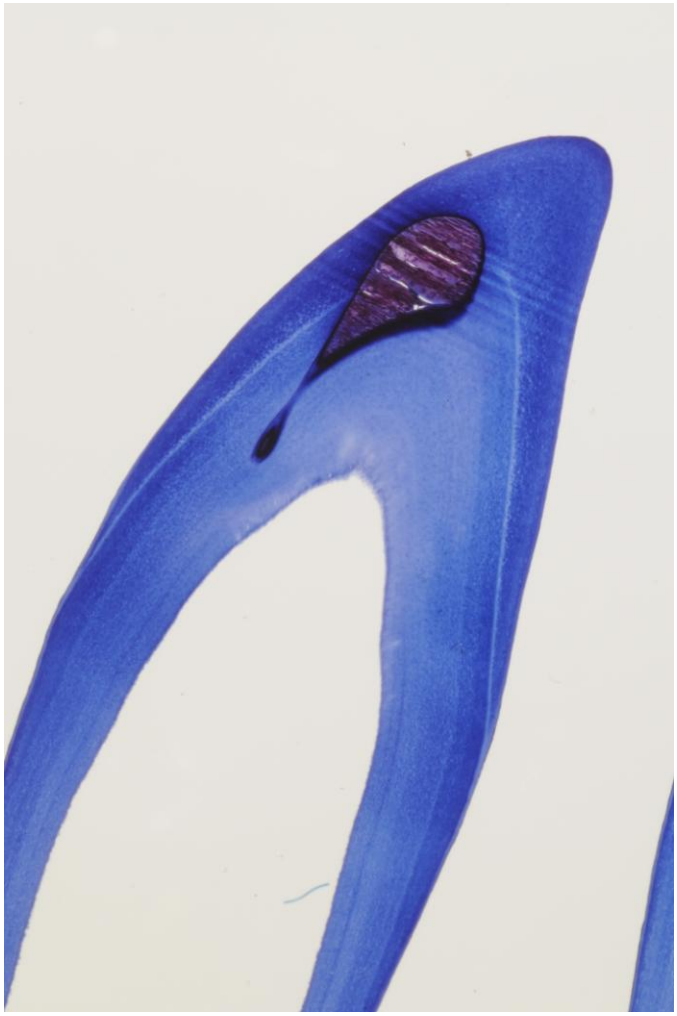
Problem	Cause	Remedy
	Knife not clamped tightly enough	Check knife clamping
	Specimen disc not clamped tightly enough	Check disc clamping
	Cryocompound (OCT) applied to cold specimen disc: specimen detached from disc after freezing	Apply cryocompound to warm disc, mount specimen and freeze
	Blunt knife	Use different part of the knife edge or replace knife
	Inappropriate section thickness.	Select correct section thickness.
	Incorrect knife angle	Set correct angle
	Microtome not properly dry (*e.g.* after cleaning)	Dry microtome thoroughly
	Specimen too dry	Prepare new specimen
Tissue sticks or breaks up on the anti-roll plate of the cryostat	Anti-roll plate too warm or incorrectly positioned	Cool down anti-roll plate, or reposition correctly
	Fat on the corner or edge of the anti-roll plate	Remove fat from anti-roll plate
	Anti-roll plate not correctly fixed.	Fix correctly
	Rust on the knife	Remove rust
Flattened sections curl up when anti-roll plate of cryostat is lifted	Anti-roll plate too warm.	Cool down anti-roll plate.
Sections tear	Temperature too low for type of tissue being cut	Increase temperature and wait
	Dirt, dust, frost or rust on the knife/blunt knife.	Remove cause/use different part of the knife edge or replace knife
	Top edge of the anti-roll plate on the cryostat is	Replace anti-roll plate

Problem	Cause	Remedy
	damaged Hard particles in the tissue being cut Dirt on back of knife	 Choose another piece of tissue or tooth Clean
Inconsistent or insufficient specimen feed on the cryostat microtome	Microtome not entirely dry when refrigeration turned on. The result is ice build-up in the microtome feed system. Microtome defective	Remove microtome and dry thoroughly Call technical services
Cyrostat inoperational	Mains plugs not properly connected Defective fuses or automatic fuse has triggered	Check if properly connected Replace fuses, or switch automatic fuse back on. If not possible, call technical services
Specimen disc cannot be removed from the stage in the cryostat	Moisture on the underside causes the specimen to freeze to the freezing shelf or specimen head	Apply concentrated alcohol to the contact point
In sufficient refrigeration of the cryochamber or the freezing microtome stage	Stopper not placed in cryostat drain hole. Cooling system or electronic drive of cryostat defective CO_2 not properly turned on CO_2 valve or hose not properly connected/defective CO_2 cylinder empty	Replace the stopper Call technical services Turn on properly Re-connect valve or hose properly/replace valve or hose Replace with full CO_2 cylinder
Sliding window of cryostat collects condensation	Air humidity and room temperature too high	Comply with the requirements for the installation site
Insufficient refrigeration of the specimen cooling system of the cryostat	Cooling system or electronic drive defective	Call technical services
Cryostat lamp does not work	Lamp defective Switch defective	Check lamp and replace it if necessary Call technical service

Appendix C: Example table for recording decalcification or acid-etching times.

Specimen	Date	Time In	Time out	Time (h:min)	Cumulative time (h:min)	Comments
M21314	22 August 2007	16:30	18:35	2:05	2:05	overnight rinse
	23 August 2007	8:45	10:45	2:00	4:05	
	23 August 2007	11:00	11:30	0:30	4:35	
	23 August 2007	11:55	12:30	0:35	**5:10**	**finished**
M11099	22 August 2007	16:30	18:35	2:05	2:05	overnight rinse
	23 August 2007	8:45	10:45	2:00	4:05	
	23 August 2007	11:00	11:30	0:30	4:35	
	23 August 2007	11:55	12:30	0:35	5:10	
	23 August 2007	12:45	13:45	1:00	6:10	
	23 August 2007	13:55	14:20	0:25	**6:35**	**finished**

Appendix D: Example of under-decalcification in part of a young bottlenose dolphin tooth.



Appendix E: Equipment suppliers (as of 2008)

Most laboratory consumables can be sourced from your local laboratory supplier. The following are specialised items.

Cryostats

Leica Microsystems (Leica also supplies slide (sledge) microtomes)

(see your local Leica distributor for contact details)

CM 1900 (has chamber and block freezing)

CM 1850 (has chamber freezing)

Cost: approx AU$37,000

Decalcifying solution (RDO)

Available from RYDLYME International Pty Ltd, 76 McCoy Street, Myaree, WA.

Phone: (08) 9333 0777

Cost: AU$75 per 4L bottle (ex. GST)

Lakeside Thermoplastic Cement (No. 70C)

Available from Hugh Courtright and Co., 26200 South Whiting Way, Monee, IL 60449, USA.

Email info@rite-tape.com

Cost: US$65 per carton of 12 sticks

Tissue Freezing-Compound 125 ml (N 14020108926)

Sourced from Leica (see your local Leica distributor for contact details)

Cost: AU$21 per bottle

Alternate sources: ProSciTech, see your local laboratory supplier

Appendix F: additional relevant literature

Anas RE. 1970. Accuracy in assigning ages to fur seals. Journal of Wildlife Management 34: 844–52.

Arnbom TA, Lunn NJ, Boyd IL and Barton T. 1992. Aging live Antarctic fur seals and southern elephant seals. Marine Mammal Science 8: 37–43.

Bell CM. 1997. Growth of the southern elephant seal *Mirounga leonina* (Linnaeus 1758) at Macquarie Island. MSc thesis. University of Tasmania.

Bernt KE, Hammill MO and Kovacs KM. 1996. Age estimation in grey seals (*Halichoerus grypsus*) using incisors. Marine Mammal Science 12: 476–482

Bow JM and Purday C. 1966. A method of preparing sperm whale teeth for age determination. Nature 210: 437–438.

Bowen WD, Sergeant DE and Øritsland T. 1983. Validation of age estimation in the harp seal, *Phoca groenlandica*, using dentinal annuli. Canadian Journal of Fisheries and Aquatic Science 40: 1430–1441.

Boyd IL and Roberts JP. 1993. Tooth growth in male Antarctic fur seals (*Artocephalus gazella*) from South Georgia: an indicator of long-term growth history. Journal of Zoology, London 229: 177–190.

Cope JM and Punt AE. 2007. Admitting ageing error when fitting growth curves: an example using the von Bertalanffy growth function with random effects. Canadian Journal of Fisheries and Aquatic Sciences 64: 205–218.

Dickie GS and Dawson SM. 2003. Age, growth, and reproduction in New Zealand fur seals. Marine Mammal Science 19: 173–185.

Donovan GP. 1985. A brief review of aging techniques for toothed cetaceans within the context of marine parks. Proceedings of the Symposium on Endangered Marine Animals and Marine Parks 1: 84–92.

Donovan GP, Breiwick J and Bannister JL. 1982. A note on comparative readings of sperm whale teeth. Reports of the International Whaling Commission 32: 251–252.

Doubleday WG and Bowen WD. 1980. Inconsistencies in reading the age of harp seal (*Pagophilus groenlandicus*) teeth, their consequences and a means of reducing resulting biases. NAFO SCR Doc. 80/XI/160. Northwest Atlantic Fisheries Organisation.

Grue H and Jensen B. 1979. Review of the formation of incremental lines in tooth cementum of terrestrial mammals. Danish Review of Game Biology 11(3): 1–48.

Hohn AA. 1980. Analysis of growth layers in the teeth of *Tursiops truncatus*, using light microscopy, microradiography, and SEM. Report of the International Whaling Commission (special issue) 3: 155–160.

Hohn AA and Fernandez S. 1999. Biases in dolphin age structure due to age estimation technique. Marine Mammal Science 15(4): 1124–1132.

Hui CA. 1980. Variability of dentin deposits in *Tursiops truncatus*. Canadian Journal of Fisheries and Aquatic Science 37: 712–716.

Kvam T. 1995. Procedures and techniques applied by NINA for cutting, staining, mounting and ageing porpoise teeth. Report of the International Whaling Commission (special issue) 16: 545–552.

Laws RM. 1956. Determination of the age of the larger whales (Mysticeti). Polar Record 8(58).

Lockyer C, Smellie CG, Goodall RNP and Cameron IS. 1981. Examination of teeth of Commerson's dolphin *Cephalorhynchus commersonii* for age determination. Journal of Zoology, London 195: 123–131.

Marmontel M, O'Shea TJ, Kochman HI and Humphrey SR. 1996. Age determination in manatees using growth-layer-group counts in bone. Marine Mammal Science 12: 54–88.

Marriott RJ and Mapstone BD. 2006. Consequences of inappropriate criteria for accepting age estimates from otoliths, with a case study for a long-lived tropical reef fish. Canadian Journal of Fisheries and Aquatic Science 63: 2259–2274.

Mattlin RH. 1978. Population biology, thermoregulation and site preference of the New Zealand fur seal, *Arctocephalus forsteri* (Lesson, 1928), on the Open Bay Islands, New Zealand. PhD thesis, University of Canterbury, New Zealand.

Mikhalev YA. 1982. Subjective and objective assessments of the laminations in sperm whale teeth. Report of the International Whaling Commission 32: 227–233.

Mitchell J. 1978. Incremental growth layers in the dentin of dugong incisors (*Dugong dugon* (Muller)) and their application to age determination. Zoological Journal of the Linnean Society 62(4): 317–348.

Myrick AC. 1991. Some new and potential uses of dentinal layers in studying delphinid populations. Pages 251-279 in Pryor K and Norris KS (eds.). Dolphin societies: discoveries and puzzles. University of California Press, Oxford.

Myrick AC, Yochem PK and Cornell LH. 1988. Toward calibrating dentinal layers in captive killer whales by use of tetracycline labels. Rit Fiskideildar 11: 285–296.

Nishiwaki M, Hibiya T and Ohsumi S. 1958. Age study of sperm whale based on reading of tooth laminations. Scientific Report of the Whales Research Institute 13: 135–154+Pl I–II.

Ohsumi S. 1977. Age determination and growth of Cetacea. Honyu-Kagaku 34(3): 54–63.

Oosthuizen WH. 1997. Description and validation of an efficient method of estimating age of Cape fur seals using ground tooth sections. Marine Mammal Science 13: 683–693.

Oosthuizen WH, and Bester MN. 1997. Comparison of age determination techniques for known-age Cape fur seals. South African Journal of Zoology 32: 106–111.

Payne MR. 1978. Population size and age determination in the Antarctic fur seal, *Arctocephalus gazella*. Mammalian Review 8: 67–73.

Pierce KV and Kajimura H. 1980. Acid etching and highlighting for defining growth layers in cetacean teeth. Report of the International Whaling Commission (special issue 3): 99–104.

Pinedo MC and Hohn AA. 2000. Growth layer patterns in the teeth from the franciscana, *Pontoporia blainvillei*: developing a model for precision in age determination. Marine Mammal Science 16: 1–27.

Scheffer VB and Myrick AC. 1980. A review of studies to 1970 of growth layers in the teeth of marine mammals. Report of the International Whaling Commission (special issue) 3: 51–64.

Stewart REA, Stewart BE, Stirling I and Street E. 1996. Counts of growth layer groups in cementum and dentine in ringed seals (*Phoca hispida*). Marine Mammal Science 12: 383–401.

Stirling, I. 1972. Observations on the Australian sea lion, *Neophoca cinerea* (Péron). Australian Journal of Zoology 20: 271–279.

Troy SK, Mattlin R, Shaughnessy PD and Davie PS. 1999. Morphology, age and survival of adult male New Zealand fur seals, *Arctocephalus forsteri*, in South Australia. Wildlife Research 26: 21–34.

Van Utrecht WL. 1981. Comparison of accumulation patterns in layered denitinal tissue of some Odontoceti and corresponding patterns in baleen plates and ear plugs of Balaenopteridae. Beaufortia 31(6): 111–122.