Comparison of two histological techniques for age determination in small cetaceans

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ABSTRACT

Age estimation in odontocetes is based on counts of growth layer groups (GLGs) deposited in recording structures such as teeth. There are several techniques for obtaining thin sections of odontocete teeth for microscopic examination. Generally, tooth sections are obtained using a freezing microtome or cryostat. However, some researchers prefer standard paraffin methods, obtaining thin sections using a "normal" or paraffin microtome. To date, very little information is available on the application of this method to dolphin teeth for age determination. The main aim of this study was to investigate whether the paraffin technique can be a viable alternative to the traditional cryostat technique for tooth preparation in small cetacean species. We considered whether estimated age would be affected by the effects of several factors related to methodology, namely preparation technique, staining method, section thickness, while controlling for effects of species, body length and sex. In addition, we analysed whether the staining method would affect the quality (readability) of sections in terms of contrast of the GLGs and the variability associated with age reading. Teeth from a total of 86 individual small cetaceans (representing seven species) were used in the study, although not all were prepared using both techniques since sufficient teeth were not available in all cases. Although the staining method had significant effects on the estimated age using both techniques, in general, the variability of GLG counts was small and appeared to be similar for both techniques. Using Mayer's haematoxylin stained sections of 8μm thickness, a good agreement (a high percentage of coincidence of age estimates) was obtained from both tooth-preparation techniques, although with a higher percentage of preparations classified as "good quality" for the paraffin technique. In addition, use of Mayer's haematoxylin provided the best contrast of the growth layers groups when using the paraffin technique. We conclude that the paraffin method represents a viable and cost-effective alternative to use of a cryostat or freezing microtome when preparing cetacean teeth for age determination.

Key words: age determination, small odontocete species, histological techniques.

INTRODUCTION

Age determination is a fundamental prerequisite for interpreting many aspects of the biology, ecology and physiology of marine mammals. The dynamics of a population cannot be determined without accurate information on of age composition, age at sexual maturity, age at first reproduction and natural longevity (Myrick *et al*. 1983, Hohn 2002). Furthermore, knowledge of age composition provides essential information for estimating fecundity or mortality rates (Hohn 2002).

Age estimation in odontocetes is based on counts of growth layers groups (GLGs) deposited in recording structures such as teeth (dentine and cementum) and bone tissue (Perrin and Myrick 1980, Hohn 2002). Teeth of most odontocetes are homodont and monophyodont (i.e. one set of permanent teeth that are all similar) with growth layers being deposited continuously throughout life (Lockyer 1995, Hohn 2002). Dentine constitutes most of the tooth and is the tissue most often used for estimating age in cetaceans. Cementum covers the tooth root and is also used to estimate ages of older animals in which dentinal age estimates are not possible. A growth layer group (GLG) is "a group of layers deposited parallel to the formative surface of a tissue that occur with cyclical and predictable repetition" and generally consists of a broad opaque layer and an adjacent narrow translucent layer when viewed under transmitted light, or as alternating stainable and unstainable layers in decalcified and stained sections (Perrin and Myrick 1980, Hohn 2002). The observed contrast is due to differences in content and distribution of the mineral component, resulting in differences in optical density and stainability, with the more transparent bands, except for the neonatal line, containing more mineral compounds (Klevezal 1996). The layers are thought to

be formed as a result of seasonal changes in the growth rate of the tooth, which are correlated to seasonal changes in the growth rate of the animal (Klevezal 1980). Thus, the broad layer is thought to record a period of rapid growth in spring-summer and the narrow layer forms in autumn-winter when growth is slower (Klevezal 1980, Myrick and Cornell 1990).

The annual deposition of growth layer groups have been verified in several cetacean species, including both captive and wild bottlenose dolphins (*Tursiops truncatus*) (Hohn 1980*b*, Hohn *et al*. 1989), short-finned pilot whales (*Globicephala macrorhynchus*) (Lockyer 1993), spinner dolphins (*Stenella longirostris*), common dolphins (*Delphinus delphis*) (Collet, 1981) and harbour porpoises (*Phocoena phocoena*) (Nielsen 1972), based on calibration studies (e.g. using tetracycline) (Gurevich *et al*. 1980, Myrick *et al*. 1984, Hohn *et al*. 1989, Myrick and Cornell 1990). Within taxonomic groups, (*e.g.,* the delphinids), the layering patterns of different species show many similarities (Hohn 1990, Lockyer 1995, Hohn 2002, Hohn and Pinedo 2000) and it is thought that calibrations obtained for one species can be applied to related species. Nevertheless, growth layer group (GLG) is a generic term and its equivalence to an annual growth increment needs to be determined in each instance of use (Perrin and Myrick 1980).

Counting of GLGs in teeth has been the most widely adopted method for age determination in odontocetes. Over the years, many preparation techniques have been developed (*see* Perrin and Myrick 1980), some of which have proved unsatisfactory in revealing GLGs (Perrin *et al*. 1977) or provide excellent resolution of GLGs but are too time consuming or expensive to be applied to large samples of teeth (e.g. Hohn 1980*b*). Hohn and Fernández (1999) suggested that the tooth preparation technique and the method used to section teeth could also introduce biases into the interpretation of age. The layering pattern in a tooth can be complex due to the presence of additional elements inside a layer, making it more difficult to identify a GLG (Hohn 1980*a*, Klevezal 1996).

Most researchers working with decalcified teeth have obtained sections at 15-20μm thicknesses by using a freezing microtome (*e.g.,* Perrin and Myrick 1980, Hohn and Lockyer 1995). This method is preferable when it is necessary to cut pieces of hard tissues more than 1cm thick. It is also possible to obtain thin sections of decalcified teeth using a cryostat which is a combination of a refrigerator with a microtome. This technique is more time-consuming than using a freezing microtome although it is extensively used for preparing dolphin teeth (Klevezal 1996). Other researchers ground sections to 20-30μm before decalcification (*e.g.,* Kasuya and Brownell 1979) and stained with haematoxylin for 30-40 min (*e.g.,* Scheffer and Myrick 1980, Myrick *et al*. 1983). The choice between the stained sections and ground sections is mostly determined by the growth layer patterns. Thus, the layers that are poorly revealed in ground sections may be distinct in stained sections or (more rarely) vice versa (Klevezal 1996). However, others prefer standard paraffin methods and prepare sections following standard microtechniques used with soft tissues. Thus, thin sections of decalcified teeth are obtained using a "normal" or paraffin microtome. Most of studies in which this technique has been applied refer to terrestrial mammals and very little information has been published on the application of this technique in dolphin teeth. For example, Slooten (1991), working on teeth of Hector´s dolphin (*Cephalorhynchus hectori*), suggested that it was possible to simplify the traditional procedure for preparing dolphin teeth by adopting standard procedures used on soft tissues, obtaining thin tooth sections (2-4μm thickness) on a standard microtome (which is more readily available than a freezing microtome or cryostat). Although the technique satisfactorily revealed the growth layers, no comparative analysis among different techniques using the same material was carried out, *e.g.,* to explore whether the resulting age estimates might differ between techniques and which technique might be generally most reliable.

In a more recent study, Duigan and Jones (2005) prepared Hector´s dolphin teeth according to Slooten`s (1991) protocol although they tried to obtain thicker tooth sections (at 10-20μm). Some technical problems were encountered with the sectioning equipment and, for several animals, tooth sections were not adequate to determine the age with accuracy. After wax embedding, some teeth became harder to section using the standard microtome (Duigan, pers. comm.). To date, Hector´s dolphin is the only species in which this technique has been applied successfully for preparing teeth and there is no published work about the use of wax embedding in other small cetacean species such as harbour porpoises or common dolphins.

The main aim of the present study was to investigate whether the paraffin technique can be applied as a viable alternative to the cryostat technique for preparing dolphin teeth for age determination. A comparative analysis among techniques was carried out. Furthermore, within each technique, particular investigations were carried out regarding four different staining methods (*i.e.,* Mayer's haematoxylin, Ehlrich's haematoxylin, Toluidine blue and Giemsa) and/or three section thicknesses (*i.e.,* 8μm, 16μm and 24μm) in order to see whether these factors contributed significantly in the estimation of age. We also analysed (a) the quality (readability) of sections in terms of the contrast of the GLGs using several staining methods (b) the variability associated with age readings. All these comparisons were made using duplicate sections from the same tooth or an extra tooth from the same animal. Teeth from several species of small cetaceans were used to investigate these questions and we therefore tested for differences between species (as well as effects of body length and sex). No teeth from known-age animals were available so, when possible, we applied the various methods to duplicate teeth from the same set of animals to facilitate comparisons.

MATERIALS AND METHODS

Samples for age determination

Teeth were acquired from small cetaceans stranded in two geographical areas of the northern Atlantic, Scotland (UK) and The Canary Islands (Spain). The material was collected by the local strandings schemes, coordinated by the Scottish Agricultural College (SAC) Veterinary Services in Inverness (Scotland) and by the Institute for Animal Health at the University of Las Palmas Gran Canaria (Spain), respectively. During the post mortem examinations, at least 4-5 teeth were removed from the middle of the lower jaw (Kuiken and Hartmann 1991) and fixed in 10% neutral buffered formalin (for about 24 hours) until processed.

For this study, teeth from 86 animals representing nine species were used although not all teeth were processed using both techniques since sufficient teeth were not available in all cases. Therefore, the material was selected according to availability (*see* Table 1 for details). For smaller species, a minimum of two teeth was required per individual (one per technique) since both techniques could not be applied to the same tooth. In harbour porpoises (*Phocoena phocoena*), because teeth were sectioned at two different orientations (*i.e.,* "porpoise" and "dolphin" cuts, respectively), four teeth were needed per individual (two teeth per technique). For those species with large teeth such as bottlenose dolphin (*Tursiops truncatus*) and pygmy sperm whale (*Kogia breviceps*), the same tooth was prepared by both techniques. Thus, the tooth was first cut along mid-longitudinal plane obtaining two halves and then each half was prepared by one of the two techniques. An extra tooth was also required when it was not possible to obtain good tooth sections.

Tooth-preparation techniques

Decalcification

After 24 hours in 10% formalin, teeth were gently rinsed in running water and placed in perforated plastic baskets for decalcification. Teeth were decalcified using a commercial rapid decalcifying agent RDO® (hydrochloric acid is the principal active ingredient) until they were flexible enough to section. The length of time required for decalcification varied between samples in relation to tooth size, age, and species, with longer times required for larger and older individuals. Teeth from small animals, including those from Atlantic spotted dolphin (S*tenella frontalis*), striped dolphin (*Stenella coerulealba*), common dolphin (*Delphinus delphis*), harbour porpoise (*Phocoena phocoena*) and Atlantic white-sided dolphin (*Lagenorhynchus acutus*) were decalcified whole, whereas larger teeth from bottlenose dolphin (*Tursiops truncatus*) and pygmy sperm whale (*Kogia breviceps*) take longer to be decalcified and these teeth were first cut along the mid-longitudinal plane using a *Buehler Isomet* low-speed diamond saw and the two halves then decalcified separately. Using RDO®, decalcification time ranged from 4-20 hours. Once teeth were rubbery in texture, they were rinsed in running tap water for several hours.

Sectioning

The sectioning equipment differed between the two techniques. The cryostat technique was adapted from Hohn and Lockyer (1995). Teeth from a total of 86 individuals were prepared using this technique and after being rinsed in running tap water, they were mounted

on freezing blocks with O.C.T® (Sakura Finetek) compound and sectioned longitudinally using a freezing (-10°C) microtome (TE Electronic Cryostat). For 67 of these 86 individuals, sufficient teeth were available to prepare sections of 8μm thickness while for only 30 of these 86 individuals, teeth were sectioned at three different thicknesses (*i.e.,* 8μm, 16μm or 24μm). Since harbour porpoise teeth are spatulate in shape, two teeth were used for each individual and sectioned in two different orientations, one parallel to the mandible (porpoise cut) and one perpendicular (dolphin cut). Both cuts were made to ensure the optimum sections were obtained. The other delphinid species used had cone-shaped teeth and sections were cut in the bucco-lingual plane (dolphin cut).

The wax embedding or (paraffin technique) was adapted from Slooten (1991). In her study, once decalcification was complete, about one-third was cut off each tooth longitudinally to hasten the process of reaching the pulp cavity during sectioning. Then, teeth were placed in separate plastic cassettes and put through a standard tissue processing and wax embedding process, as used for soft tissues. Teeth were sectioned at 2-4μm using a standard microtome. In our study, decalcified teeth from 67 individuals were prepared using the paraffin technique. For 61 of these individuals, sufficient teeth were available to prepare stained sections using three different staining methods (further details are given below). The protocol was modified at several stages as follows: decalcified teeth were rinsed in running tap water and bisected longitudinally using a microtome blade. The cut surface was placed face down into a plastic histology cassette for embedding in (paraffin) wax. The cassettes were processed in an automated machine for embedding the tissue in paraffin wax (*Leica TP1050*®). During the process of (paraffin) wax embedding, tissues were dehydrated through increasing concentrations of alcohol then xylene and molten paraffin (*see* below for detail). This took place on an automated machine (*Leica TP1050* ®). One of the technical problems that we found using the (paraffin) wax embedding procedure was that dolphin teeth were

hardened and it was therefore difficult to obtain tooth sections using the standard microtome. Therefore, several trials were carried out to optimise the length of time that the teeth remained in ethanol, alcohol and xylene to avoid excessive dehydration that consequently hardens the tissue. The optimum programme lasted around 3.5 hours (as compared to 24 hours for soft tissues), as follows:

1. 50% ethanol 30 min
2. 96% ethanol 30 min 20ºC
3. Absolute alcohol (100%) 15 min at 20ºC
4. Absolute alcohol (100%) 15 min at 20ºC
5. Xylene 30 min at 20ºC
6. Xylene 30 min at 20ºC
7. Molten wax (60ºC) 30 min
8. Molten wax (60ºC) 30 min

Finally the teeth were embedded in molten paraffin wax (60ºC) using a blocking machine (*Sakura Tissue- Tek TEC*). They were then sectioned at 8µm thickness by using a standard paraffin microtome (*Leica*®) with stainless steel disposable microtome blade. The effect of section thickness was not investigated in the paraffin technique because the sectioning equipment available did not allow preparation of thicker sections of high quality. Before staining, it is necessary to remove the paraffin wax from tissues, allowing the stains to penetrate. Sections were oven-dried for 15 min at 100ºC and the paraffin wax was then removed by passing the sections through the following stages:

1. Xylene 2 min
2. Xylene 2min
3. Alcohol 100% 2min
4. Alcohol 100% 2min
5. Alcohol 70% 2min
6. Distilled water 2 min
7. Distilled water 2 min
8. Distilled water 2 min
9. Distilled water 2min

Staining and mounting

For both techniques, multiple sections were obtained and only those most central and complete, which included the crown and the maximum area of pulp cavity, were selected and then stained using four different stains which are commonly used for revealing the growth layers groups (GLGs) present in dentine and/or cementum: Mayer's haematoxylin (*e.g.,* Thomas 1977, Myrick *et al*. 1983, Hohn and Lockyer 1995), Ehlrich's haematoxylin (*e.g.,* Klevezal and Kleinenberg 1967, Korytin 1984), Toluidine blue (*e.g.,* Thomas 1977, Graf and Wandeler 1982, Allen and Melfi 1985) and Giemsa (*e.g.,* Stone *et al*. 1975, Matson 1981, 1993, Molina and Oporto 1993). Mayer's haematoxylin and Ehlrich´s haematoxylin stained sections were "blued" in a weak ammonia solution and rinsed in distilled water, whereas Toluidine blue and Giemsa stained sections were placed directly in distilled water before mounting.

Stained sections were mounted onto microscope slides pre-coated with a 5% gelatine solution in order to prevent curling. Once the sections were fully dried on a warm hot plate, permanent slides were prepared using DPX-mountant.

Age determination

Stained tooth sections were examined under a binocular microscope (×10-50 magnification). The age was estimated by counting the growth layer groups, (GLGs) (Perrin and Myrick 1980) in the dentine. If annual growth layers could be easily distinguished in the cementum, these were also counted. We assumed that the GLGs represent one year's individual growth (although for the purpose of comparing techniques this assumption was not strictly necessary). Ages of teeth showing less than one full GLG were estimated to the nearest 0.5 GLG (6 months).

The reading of the GLGs was done "blind" with no reference to biological data, to avoid any possible biases in the estimation. Tooth sections were read three times by two independent readers. Finally the estimated age was achieved by consensus between the two readers. If no consensus was reached, the tooth was excluded from subsequent analysis.

Data analysis

Quality of tooth preparations

We defined quality of the tooth preparation based on how clearly the annual growth layers groups (GLGs) could be identified as countable units. Thus, tooth preparations were categorized as follows: good-quality (when layers were distinct, and easily counted), satisfactory (layers could be distinguished but there was some uncertainty about how many GLGs were present) and poor-quality (a reasonable estimate of the number of GLGs was impossible).

For the paraffin technique we examined the quality of tooth preparations stained using Mayer's haematoxylin, Ehlrich's haematoxylin and Toluidine blue. The staining method which gave the highest percentage of good quality preparations was selected for examining the difference in quality between paraffin and cryostat techniques. Poor-quality preparations were excluded from further analyses, i.e. teeth from two pygmy sperm whales, one dwarf sperm whale (*Kogia simus*) and three bottlenose dolphins.

From the literature, for the cryostat technique, we may expect that staining does not affect preparation quality.

Variability in counting of GLGs

For the cryostat technique, we assessed the degree of variability associated with counts of GLGs for each particular staining method. Thus, we treated age estimates from each section thicknesses (*i.e.,* 8, 16 and 24μm) as individual observations and calculated the standard deviations values (SD) between them for each of the four stains (*i.e.,* Mayer's haematoxylin, Ehlrich's haematoxylin, Toluidine blue and Giemsa). The overall variability for each particular stain is expressed using the frequency distribution of SD values.

To assess the variability between the two techniques, we treated estimates from each stain (*i.e.,* Mayer's haematoxylin, Ehlrich's haematoxylin and Toluidine blue) as observations and calculated the SD between them for the two techniques.

Statistical analysis: the effects of several factors on the estimation of age within each technique

Linear mixed modelling techniques (Pinheiro and Bates 2000, West *et al.* 2006, Zuur *et al.* 2007) were used to model estimated age in relation to several explanatory variables, namely staining method, section thickness (μm), species, body length (cm), sex, and individual. Due to the nested structure of the data, species and dolphin identity (nested in species) were used as random effects.

An initial analysis indicated violation of homogeneity, and therefore we allowed for heterogeneous residual variance structures (Pinheiro and Bates 2000, West *et al.* 2006, Zuur *et al.* 2007). Therefore, the following model was applied on the data obtained by the paraffin technique:

$$Age_{ijk} = \alpha + Length_{ijk} \times Sex_{ijk} \times Stain_{ijk} + a_i + b_{ij} + \varepsilon_{ijk}$$

Age_{ijk} is the age obtained by stain k for specimen j of species i, where k = 1, …,3, i = 1, …,6, and j takes any value between 3 and 25. The notation above means that Age is modelled as a function of length, sex and stain using the main terms, two-way interactions and the three-way interaction. The terms a_i and b_i are random effects representing the between species variation and between-specimen variation within a species. Both are assumed to be normally

distributed with mean 0 and variances σ_a^2 and σ_b^2 respectively. The term ε_{ijk} is the unexplained noise (or within specimen variation), and is assumed to be normally distributed with mean 0 and variance given by:

$$\sigma^2 \times |Length_{ijk}|^{2\delta}$$

This variance structure allows for the modelling of heterogeneous residuals using a power function; *see* Pinheiro and Bates (2000) for the mathematical details. To avoid numerical problems, length was expressed in metres in the variance structure.

A similar model was applied on data obtained by the cryostat technique, in this case also including the effect of section thickness, and also for the comparison of data obtained by both main techniques.

The model selection followed the step-down approach described in West *et al.* 2006. All analysis were done in R (R Development Core Team, 2006) using the nlme library (Pinheiro *et al.* 2006).

RESULTS

Quality of tooth preparation

Using the paraffin technique, the quality of tooth-preparations stained using the three staining methods, was examined for 61 individuals including several small cetacean species: harbour porpoises (*Phocoena phocoena*), common (*Delphinus delphis*), striped (*Stenella coeruleoalba*), Atlantic spotted (*Stenella frontalis*) and bottlenose dolphins (*Tursiops*

truncatus). Results indicated that the percentage of tooth preparations falling into each quality category varied substantially between stains (*see* Fig. 1). Thus, using Mayer's haematoxylin, 70% of tooth preparations were considered "good quality", 21% "satisfactory" and 8% "poor quality". Considerably lower proportions of good quality sections were obtained using Toluidine blue or Ehlrich's haematoxylin (51% and 31% of tooth preparations respectively). Giemsa stained sections were considered to be of the poorest quality by both readers and were not included in any further comparative analysis since GLGs could not be distinguished.

We examined the quality of tooth preparations processed by both techniques in 67 animals (all species combined). Based on Mayer's haematoxylin stained thin (8μm) sections, the percentage of preparations considered of "good quality" was higher for the paraffin technique (37%) than for the cryostat technique (10%) although the percentage of "satisfactory" preparations was the same (46%) for both techniques (Fig. 2). Note that accessory lines were identified within the first two growth layers of some teeth and may sometimes have been counted as boundary layers leading to a wrong interpretation of age.

Variability in counting of GLGs

Using the cryostat technique, the frequency distribution of the standard deviation values, (note: each individual SD value expressing variation in estimated ages from different section thicknesses for a single tooth), was similar for the four staining methods. For most individual animals, differences between age estimates obtained using different section thicknesses were small, as indicated by standard deviation values that range from 0 to 1.5 (Fig. 3). The absence of any high SD values suggested that variability of GLG counts was slightly lower for Mayer's haematoxylin than other staining methods.

The frequency distributions of the standard deviation values expressing variation in estimated ages from different stains (namely Mayer's haematoxylin, Ehlrich's haematoxylin and Toluidine blue) were similar for both techniques. For most of individuals, differences between age estimates were small, as was indicated by standard deviation values that range from 0 to 1.5 (Fig. 4). The variability of GLG counts thus appeared to be low and similar for both techniques.

Paraffin technique

The optimal model for estimated age using, the paraffin data, contained the main terms stain and body length, and the interaction between them. Estimated parameters are given in Table 2. Using a likelihood ratio test, a model with, and a model without the interaction term were compared, and the results indicated that the interaction term was significant ($L = 25.420$, $df = 2$, $P < 0.001$).

Ehlrich´s haematoxylin and Toluidine blue stains were different from the baseline (Mayer's haematoxylin), and the age-length relationship differed between staining methods.

Cryostat technique

Similar results were obtained for the cryostat technique data. Thus, the optimal model contained the main terms stain and body length, and the interaction between them (*see* Table 3 for the estimated parameters). Using a likelihood ratio test, a model with, and a model

without the interaction term were compared, and the results indicated that the interaction term was significant (L = 10.079, df = 3, $P = 0.0179$).

Ehlrich´s haematoxylin and Giemsa stains were different from the baseline (Mayer's haematoxylin), giving higher ages (as indicated by positive coefficients) and the apparent age–length relationship differed between stains, as indicated by the significant interaction terms. There was no effect of section thickness.

Comparison of both techniques

The optimal model for the comparison of both techniques data contained only body length as the main term. There was no difference between techniques. Estimated parameters are given in Table 4. There was more variation in the age readings of larger animals.

DISCUSSION

Quality of tooth preparations

Results from the paraffin technique showed that the best contrast of growth layers groups was obtained using Mayer's haematoxylin which gave the highest percentage of tooth preparations classified as good quality. Use of Ehlrich's haematoxylin did not improve and even decreased the contrast of the growth layers in comparison to Mayer's haematoxylin and Toluidine blue. Giemsa was the staining method that was least successful in terms of revealing growth layer patterns in comparison to the other staining methods. The Giemsa-

stained preparations were considered the "poorest" quality by both readers and were not included in the comparative analysis since GLGs could not be identified. In contrast, Giemsa-stained sections obtained using the cryostat technique showed a reliable contrast of the growth layers.

This suggests that the effectiveness of Giemsa in revealing dentinal growth layers might depend on the procedure for preparing teeth before staining. For example, using the paraffin technique it is necessary to remove the paraffin wax from sections to allow the stains to penetrate into the decalcified tissue. Sections are oven-dried for 15 min at 100ºC and the paraffin wax is then removed by passing the sections through several stages of xylene and decreasing grades of alcohol (*see* material and methods above). It is possible that the procedure for removing paraffin affects the penetration of Giemsa stain into the tissue, giving different interpretations of growth layers and therefore differences in estimated ages. Giemsa stain has been used for age determination from cementum layers Matson (1981, 1993) and has been recommended by some other authors (*e.g.,* Stone *et al.* 1975). However, no published information is available about the application of this method for revealing dentinal growth layers or even its application on dolphin teeth. Results of this study indicated that further experimental work is required, for example, to investigate what are the main components of the dentine and cementum tissues (*i.e.,* the organic and inorganic material) for which Giemsa stain has more affinity.

We also examined the quality of tooth preparations processed by both techniques based on Mayer's haematoxylin-stained thin sections. Results showed that the percentage of preparations considered of "good quality" was higher for the paraffin technique than for the cryostat technique. This might suggest that the standard microtome is rather better for obtaining thin sections than the freezing microtome, particularly working with small teeth. Nevertheless, most of preparations showed an acceptable (satisfactory) layering pattern

regardless of technique. This indicated that the paraffin technique can be applied as a viable tooth preparation technique, being especially successful for preparing small teeth (*e.g.,* harbour porpoises (*Phocoena phocoena*), for which thin sections are recommended to ensure multiple replicates from the same tooth close to the pulp cavity. In addition, the paraffin technique was successfully applied to a reasonably wide range of small cetacean species including those with small teeth such as spotted dolphin (S*tenella frontalis*), common dolphin (*Delphinus delphis*) and Atlantic white-sided dolphin (*Lagenorhynchus acutus*), and other species with larger teeth including bottlenose dolphin (*Tursiops truncates*) and pygmy sperm whale (*Kogia breviceps*).

Variability in counting of GLGs

Results indicated that, using the cryostat technique, differences between estimates from different section thicknesses were small and similar for the four staining methods. However, the variability of GLG counts was lower for Mayer's haematoxylin than other staining methods. As was mentioned above, regarding the quality of tooth preparations when using the paraffin technique, the highest percentage of tooth preparations catalogued of good quality was obtained using Mayer's haematoxylin. This might indicate that Mayer's haematoxylin provides a reliable contrast of the growth layers regardless of the prior preparation technique.

Using both techniques, differences between estimates from three staining methods (*i.e.,* Mayer's haematoxylin, Ehlrich's haematoxylin and Toluidine blue) were also small. The variability in GLG counts appeared to be similar for both techniques. Again, regardless of the staining method, the paraffin technique can be considered a reliable tooth preparation technique.

The effect of stain on estimated ages

Regarding staining methods, results showed that the stain contributed significantly to the estimated ages using either the paraffin or the cryostat technique.

Other aspects of the procedure for preparing teeth (*e.g.,* decalcification, sectioning and mounting) can affect the ease of distinguishing growth layers. For example, some researchers (*e.g.,* Graf and Wandeler 1982, Allen and Melfin 1985) have preferred Toluidine blue to haematoxylins but they suggested that it is necessary either to mount sections in synthetic resins or to study a preparation just after it has been made because the stain (*i.e.,* Toluidine blue) disappears in glycerine. Korytin (1984) tested four haematoxylins for staining teeth and bone tissue. He concluded that the best contrast of layers was obtained by using Krutsai haematoxylin with an increased amount of haematoxylin powder and decreased amount of HCL. Thomas (1977) tried 20 different stains, besides Ehrlich's and Harris's haematoxylins, for staining cementum layers in carnivores and ungulates. He concluded that the order of preference of the stains in revealing cementum layers can be different for different species. Therefore, other factors such as the recipe for the stain, time of staining, age of the stain as well as inter and intra-specific differences in the layering pattern may also affect the visualisation of growth layer groups and therefore the interpretation of age.

Using the paraffin technique, Ehlrich´s haematoxylin and Toluidine blue stains were different from the baseline (*i.e.,* Mayer´s haematoxylin) giving higher ages (as indicated by positive coefficients). Similar results were obtained from the cryostat technique data, in which Ehlrich´s haematoxylin and Giemsa stains were different from Mayer´s haematoxylin. Results of this study that concerns the quality of stained tooth sections suggest that, Ehlrich´s haematoxylin provides a less reliable layering pattern in comparison to other stains.

Furthermore, in both techniques, the apparent age–length relationship differs between stains, as indicated by the significant interaction terms showing that the estimate age readings were larger for larger animals. Overall, the age was probably underestimated for older adults in which pulp cavity was almost closed. Bryden (1989) pointed out that one of the problems encountered in age determination by counting growth layers in cetacean teeth, particularly those from small cetacean species (*e.g.,* harbour porpoises) is that teeth are small and dentinal deposition may occlude the pulp cavity before the animal dies, giving only minimum age estimates for older adults. When the tooth grows, dentinal growth layers are deposited inwards until the pulp cavity is occluded. Thus, layers become narrower and it is more difficult to identify the boundary of the most recently deposited growth layers, potentially leading to incorrect interpretation of age. Thus an underestimation of age is expected for older adults due to the occlusion of the pulp cavity regardless of the tooth preparation technique used.

The effect of section thicknesses

Using the cryostat technique, teeth were sectioned at 8, 16 and 24μm thicknesses. Results showed that section thicknesses did not contribute significantly on the estimation of age. Using the paraffin technique, teeth were sectioned at 8μm thickness. Particularly when working with small dolphin teeth, this can be considered as an advantage since this guarantee a suitable set of central sections close to the pulp cavity, which is recommended in order to ensure a reliable interpretation of the layering pattern and an accurate estimated age. The ability to obtain thin sections might provide an additional advantage for those cases in which

tooth sample sizes are small. Thus, guarantee a higher number of multiple sections from the same tooth.

In cetacean species with larger teeth (*e.g.,* bottlenose dolphins), thicker tooth sections are recommended (Perrin and Myrick 1980). However, using the wax embedding procedure, it is very difficult to obtain thick sections using the standard or paraffin microtome because the process of embedding hardens the tissue. In a recent study, Duigan and Jones (2005) found some technical problems with sectioning when they tried to obtain sections at 10-20μm thicknesses from Hector´s dolphin (*Cephalorhynchus hectori*) teeth. The tooth sections were of poor quality and age was not estimated with certainty. In our study, we solved some of the technical problems that we found with sectioning, although we were not able to obtain sections thicker than 8μm. However, results showed that thin sections provided a reliable estimated age for all studied species, even for those with larger teeth.

Accessory layers were identified within the first two GLGs and for some animals they may have been counted as boundary layers. "Accessory lines" (or layers) are considered "false" incremental layers that occur within GLGs and which can be confused with the boundary of the annual layer leading to incorrect interpretations of age (Klevezal 1980, Hohn *et al.* 1989). It has been suggested that these incremental layers represent monthly records (Hohn 1980*b*, Klevezal 1980, Myrick *et al.* 1984). Therefore, to identify them is essential for a reliable age determination (Hohn 1990).

According to Hohn (1990), a misinterpretation of annual boundaries between GLGs seems to be most problematic in the first two annual growth layers, affecting the age estimation at an early age. Therefore, determination of some life history parameters (*e.g.,* age at weaning or sexual maturation) would be particularly difficult in species with a relative short life-span (*e.g.,* harbour porpoise).

Finally, we investigated whether the technique (*i.e.,* paraffin or cryostat) had a significant effect on the estimated ages. The absence of significant differences between estimated ages from both techniques indicated that the paraffin technique can be applied as a tooth preparation technique alternative to the cryostat technique.

As no teeth from known age animals were available, we were not able to show which technique was more efficient in obtaining accurate counts. Ideally similar experimental work is required using teeth from known age animals to explore whether estimated age corresponds to the real age.

Concluding remarks

We conclude that the paraffin technique represents a viable and cost-effective alternative to use of a cryostat or freezing microtome when preparing cetacean teeth for age determination.

However:

- The effect of the staining method on the estimated age should be taken into account in both techniques.
- Using the paraffin technique, Ehlrich´s haematoxylin and Toluidine blue stains are different from the baseline (Mayer's haematoxylin), and the apparent age-length relationship differs between staining methods. However, the variability of GLG counts is small and appears to be similar for the three staining methods.
- Using the cryostat technique, the variability of GLG counts is lower for Mayer's haematoxylin than other staining methods. Moreover, Ehlrich´s haematoxylin and

Giemsa stains are different from the baseline (Mayer's haematoxylin), giving higher ages and the apparent age–length relationship also differs between stains.

- We recommend use of thin sections, particularly in small cetacean species and when the availability of tooth samples is limited.
- Regarding staining methods, when using the paraffin technique, we recommend use the Mayer's haematoxylin, which gave the highest percentage of preparations classified as a "good quality".
- The paraffin technique requires less specialist technical experience and is more readily available than the cryostat technique.

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FIGURE LEGENDS

Figure 1 Frequency distribution for quality of tooth preparations (n=61) using the paraffin technique and three different staining methods (MH: Mayer's haematoxylin, EH: Ehlrich's haematoxylin and TB: Toluidine blue). Tooth preparations were categorized using a three grade scale as follow: poor quality, satisfactory quality and good quality.

Figure 2 Frequency distribution for quality of Mayer's haematoxylin stained preparations using the paraffin and the cryostat techniques (n=67). Tooth preparations were categorized using a three grade scale as follow: poor quality, satisfactory quality and good quality.

Figure 3 Frequency distribution of values for standard deviation (expressing variation in estimated ages between the three section thicknesses) for the four staining methods (i.e. MH: Mayer's haematoxylin, EH: Ehlrich's haematoxylin, TB: Toluidine blue, G: Giemsa) (n=30 in each case).

Figure 4 Frequency distribution of values for standard deviation (expressing variation in estimated ages between three staining methods) for the two techniques (n=34).

Figure 1

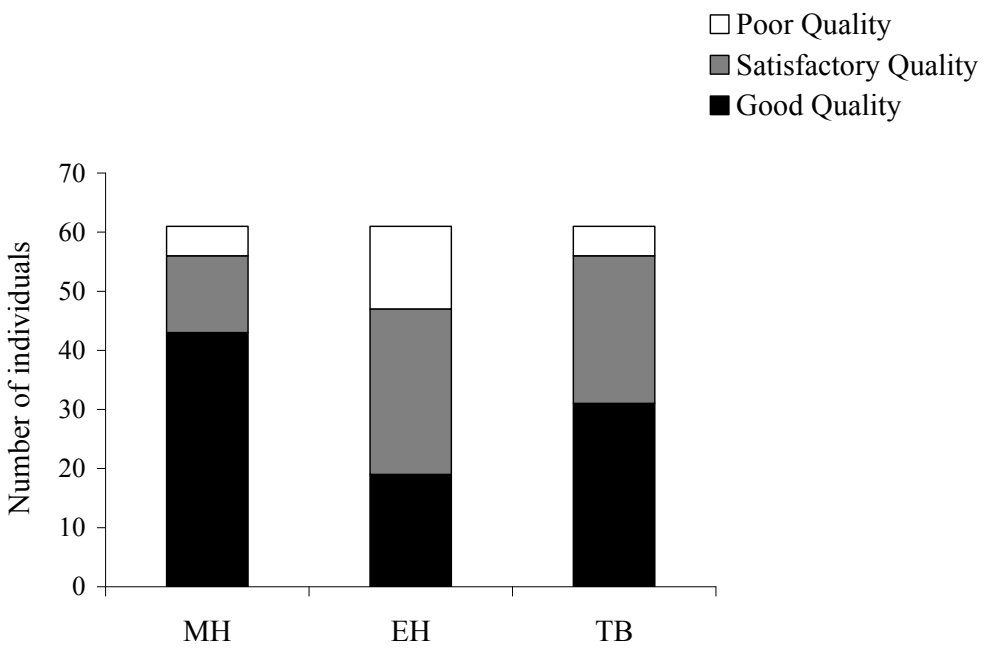

Figure 2

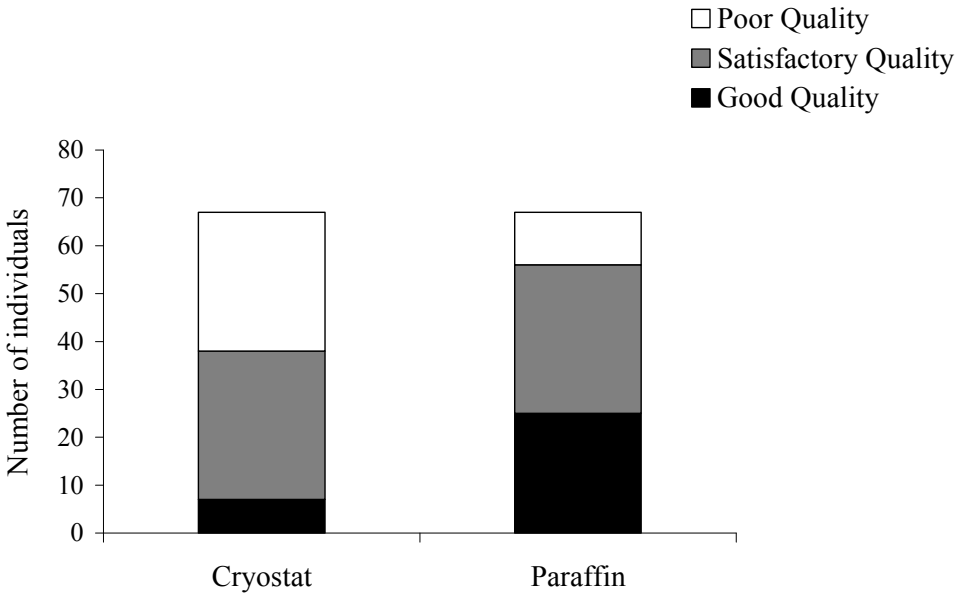

Figure 3

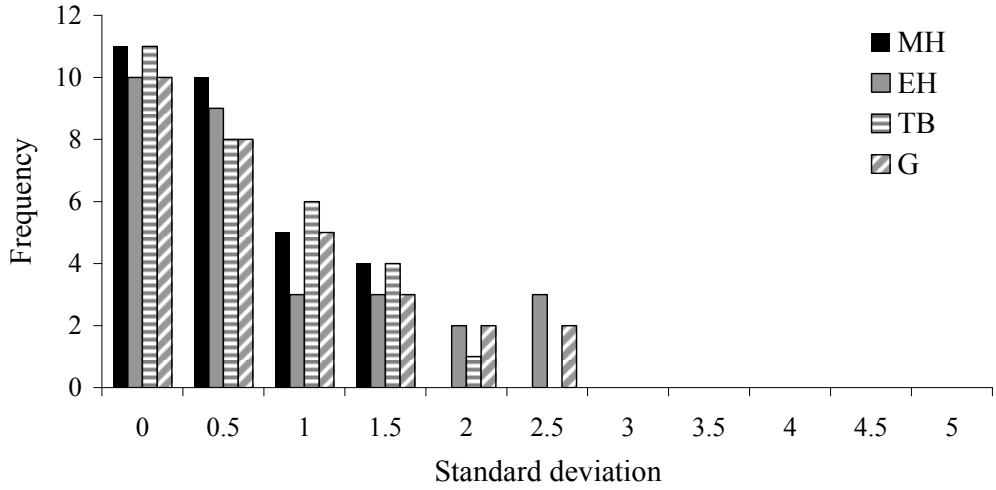

Figure 4

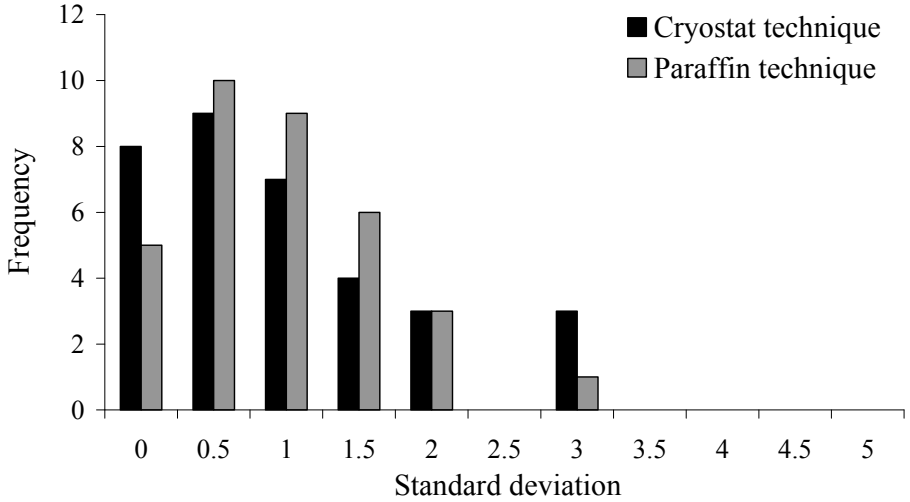

Table 1. Summary of total number of teeth processed, by species and geographical area (M = male, F = female).

Species	Code	Scotland	Canary Islands	Total
Harbour porpoise (*Phocoena phocoena*)	Pp	20 M 16 F	-	36
Common dolphin (*Delphinus delphis*)	Dd	3 M	4 F	7
Atlantic white-side dolphin (*Lagenorhynchus acutus*)	Lac	3 M, 3 F	-	6
Striped dolphin (*Stenella coeruleoalba*)	Sc	1 M	8 M, 1 F	10
White-beaked dolphin (*Lagenorhynchus albirostris)*	Lal	5 M	-	5
Atlantic spotted dolphin (*Stenella frontalis*)	Sf	-	3 M, 6 F	9
Bottlenose dolphin (*Tursiops truncatus*)	Tt	-	5 M, 5 F	10
Pygmy sperm whale (*Kogia breviceps*)	Kb	-	2 M	2
Dwarf sperm whale (*Kogia simus*)	Ks	-	1 M	1
TOTAL		51	35	86

Table 2. Estimated parameters for the paraffin data. The term stain was fitted as a nominal variable and length as a continuous variable. The random effect a_i representing the between species variation is N $(0,6.70^2)$ and the random effect b_{ij}, representing the between animal variation in the same species is N$(0, 3.05^2)$. The estimated values for σ and δ are 0.23 and 2.21, respectively.

	Value	Std.Error	DF	t-value	P-value
(Intercept)	-23.1723	3.8571	114	-6.0076	0
factor (Stain: Ehlrich´s haematoxylin)	1.8687	0.4030	114	4.6368	0
factor (Stain: Toluidine blue)	1.1414	0.4030	114	2.8320	0.0055
Length	0.1737	0.0155	51	11.1356	0
factor (Stain: Ehlrich´s haematoxylin):Length	-0.0178	0.0032	114	-5.5483	0
factor (Stain: Toluidine blue): Length	-0.0097	0.0032	114	-3.0366	0.0030

Table 3. Estimated parameters for the cryostat data. The term stain was fitted as a nominal variable and length as a continuous variable. The random effect a_i representing the between species variation is N $(0,6.29^2)$ and the random effect b_{ij}, representing the between animal variation in the same species is N $(0, 3.73^2)$. The estimated values for σ and δ are 0.096 and 3.798.

	Value	Std.Error	DF	t-value	P-value
(Intercept)	-17.0723	4.3406	313	-3.9331	0.0001
factor(Stain: Ehlrich´s haematoxylin)	1.0183	0.3486	313	2.9211	0.0037
factor(Stain: Toluidine blue)	0.5585	0.3486	313	1.6020	0.1102
factor(Stain)4(Giemsa)	0.6814	0.3486	313	1.9548	0.0515
Length	0.1362	0.0204	20	6.6573	0
factor(Stain Ehlrich´s haematoxylin):Length	-0.0088	0.0028	313	-3.1268	0.0019
factor(Stain: Toluidine):Length	-0.0049	0.0028	313	-1.7328	0.0841
factor(Stain: Giemsa):Length	-0.0059	0.0028	313	-2.0911	0.0373

Table 4. Estimated parameters for the comparison. The term technique was fitted as a nominal variable and length as a continuous variable. The random effect a_i representing the between species variation is N $(0,4.708^2)$ and the random effect b_{ij}, representing the between animal variation in the same species is N $(0, 2.295^2)$. The estimated values for σ and δ are 0.156 and 3.822.

	Value	Std.Error	DF	t-value	P-value
(Intercept)	-23.9111	2.9073	65	-8.2243	0
Length	0.1737	0.0128	56	13.5622	0