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Analysis of Growth Layers in the Teeth of *Tursiops truncatus* using Light Microscopy, Microradiography, and SEM

Aleta A. Hohn
Department of Zoology, University of Maryland, College Park, Maryland
and
Division of Mammals, Smithsonian Institution, Washington, D.C.

ABSTRACT

Preliminary results show microradiography and scanning electron microscopy (SEM) to be more accurate methods of accessing growth layer groups (GLGs) in the teeth of *Tursiops truncatus* than transmitted light microscopy. Microradiography shows the rhythmic deposition of mineral as alternating radiopaque and radiolucent layers. It improves the resolution of GLGs near the pulp cavity in older individuals, better than either SEM or light microscopy. SEM of etched sections show GLGs as ridges and grooves which are easily counted from the micrograph. SEM also shows GLGs to be composed of fine incremental layers of uniform size and number which may allow for more precise age determination. Accessory layers are usually hypomineralized layers within the hypermineralized layer of the GLG and are more readily distinguishable as such in SEM of etched sections and microradiographs than in thin sections viewed under transmitted light. The neonatal line is hypomineralized, appearing translucent under transmitted light, radiolucent in a microradiograph, and as a ridge in SEM.

INTRODUCTION

Conventional methods of counting growth layer groups (GLGs) in odontocete teeth, by use of undecalcified or decalcified and stained thin sections, have been used extensively in age determination (for example, Nishiwaki and Yagi, 1953; Sergeant, 1959, 1962, 1973; Klevezal' and Kleinenberg, 1967; Best, 1970, 1976; Christensen, 1973; Kasuya, 1972, 1976, 1977; Sergeant, Caldwell, and Caldwell, 1973) but do not always permit definition of all GLGs. Repeated counts on the same tooth by one person or several people sometimes give different results with the average or median count used as the age indicator (this workshop; Kasuya, Miyazaki, and Dawbin, 1974; Perrin, 1975; Kasuya, 1977). The development of a technique that will lead to better resolution of GLGs, and therefore more accurate age determination, would be useful. Preliminary work with microradiography and scanning electron microscopy (SEM) indicates their potential value in clarifying GLGs in the teeth of odontocetes.

Microradiography provides a non-destructive means of determining mineral density differences within hard tissues. It is particularly useful in resolving GLGs since GLGs consist of alternating poorly mineralized and more highly mineralized layers. It involves the use of very concentrated, low-voltage x-rays to expose a high resolution photographic plate, which increases sensitivity over standard x-rays, and gives sharply defined images. This permits the detection of local variation in mineral content in the tooth sections, since the x-rays are absorbed chiefly by the calcium and phosphate in the tooth (Trautz, 1967). Areas of greater mineral density are radiopaque and appear as light zones. Radiolucent or dark zones are the poorly mineralized areas that allow more x-rays to expose the plate.

An advantage of microradiography is that mineral density can be compared to optical density on the same thin section. This technique has been used by Sergeant (1962) and Nielsen (1972) to study odontocete teeth. Sergeant mentions that radiography confirmed his hypothesis that the opaque zones of teeth of *Globicephala melaena* were the better calcified dentine while the translucent zones were poorly calcified. In the teeth of *Phocoena phocoena*, Nielsen found that the radiopaque layers are equivalent to the light layers on thin sections when viewed with transmitted light. Because this result is contrary to Sergeant's (1962) findings, Nielsen cautions that optical density may not be dependent on mineral density.

A less ambiguous method of resolving GLGs may be by the removal of superficial calcium from a half-section of tooth, a procedure referred to as etching. The mineral is dissolved in an acid, usually formic, nitric, or hydrochloric, or removed by a chelating agent such as EDTA (see Boyde and Jones, 1974). After drying, the surface of the section has a topography of alternating ridges and grooves, each pair of which corresponds to a GLG, that results from the greater collapse of dentinal tissue in those layers which were initially hypercalcified.

Although etching seems to be a good technique for enhancing GLGs in sperm whale teeth (Bow and Purday, 1966), it has not frequently been applied to small odontocetes, probably because of difficulty in counting individual ridges and grooves. One method to facilitate counting is by use of the SEM. The advantage of the SEM is that it produces a three dimensional image of the surface of the tooth, allowing for more accurate interpretation of the GLGs.

The purpose of this study is to compare the techniques of transmitted light microscopy, microradiography and SEM for examination of *Tursiops* teeth for the clear resolution of GLGs, identification of accessory layers, and correlation of mineral density with optical density for the layers.

METHODS

Two teeth were taken from each of 15 *Tursiops truncatus* females which had stranded on the east coast of the United States, and which are in the collection of the National Museum of Natural History. Generally the teeth were removed directly from the skulls, most of which had been boiled clean before addition to the collection. When possible, central mandibular teeth were extracted. If the teeth

had already been removed from the jaws, large relatively straight teeth were chosen.

All teeth were sectioned with a *Buehler Isomet* 11-1180 low speed saw. Longitudinal half-sections were cut by gluing the tooth on a microscope slide with a cellulose nitrate base adhesive and cutting medially. For thin sections, the cut surface of one half-section was glued down on a slide and all but 150 μm cut off, leaving the thin section adhering to the slide. Sections thicker than 150 μm were sanded down using 320 and 400 grit sandpaper. To prepare thick cross sections the tooth was placed in a clamp-like chuck accessory of the saw and cut in the zone of most recent growth as determined from the longitudinal thin section. Another cut was made farther down the tooth to make the sections about 0.25 cm thick for use in the SEM.

Preparation for microradiography involved removal of the longitudinal thin sections from the slide and then cleaning it in an ultrasonic cleaner to remove loose surface particles. One section from a tooth of each animal was microradiographed, following the method of Ortner and Yong (1975), for one and one-half hours at 11 kv. GLGs were counted from the microradiograph using a dissecting microscope.

The cross sections prepared for SEM were soaked in 5% formic acid for three hours, rinsed in water, then sonically cleaned. Each section was air dried, mounted on an SEM stub, plated with gold-palladium, and viewed with a *Cambridge Stereoscan* Mk IIA or S4-10 or AMR 1000A SEM. Micrographs were taken at a 45° tilt-angle under low magnification (15–21X) at 10 kv for large sections and 20 kv for smaller sections, including at least one-half of the surface of the tooth from the center to the periphery. Details were viewed under higher magnification at 20 kv.

RESULTS

GLGs

GLGs are the most prominent layered components within the tooth (see workshop glossary for definitions), within which there are finer layers. Using light microscopy with undecalcified thin sections, each GLG is composed of a translucent and an opaque layer (Fig. 1). In etched sections, each GLG consists of a ridge and an adjacent groove (Fig. 2). In microradiographs, each GLG consists of one radiopaque and one radiolucent layer, indicating that each GLG comprises one hypermineralized layer and one hypomineralized layer (Fig. 3). Cross counts of GLGs using the three methods gave the same whole-year counts for most of the specimens. Discrepancies occurred in some cases where the microradiograph resolved the beginning of a new layer, resulting in a slightly higher count (Table 1), and where it made visible last-formed layers in older animals in which the pulp cavity had been occluded (Figs. 1, 2, and 3). In the latter case, the additional GLGs followed the periodicity and intensity of the preceding GLGs.

Figs. 1–3. Comparison of GLGs in the same tooth by each of the techniques used.

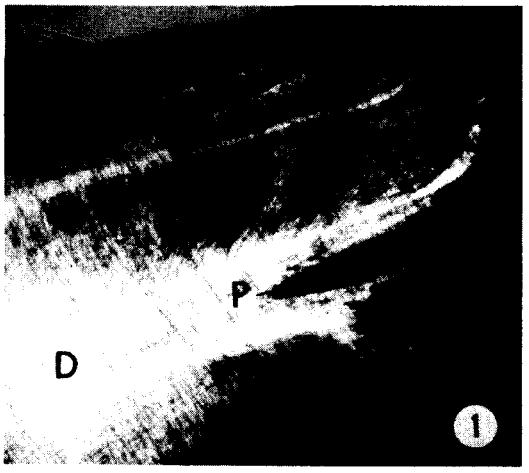

Fig. 1. Ground longitudinal section (150 μm) under polarized transmitted light. GLGs visible only on the sides of the tooth and near the base close to the pulp cavity. Bars demarcate GLGs. Original magnification: x17. P – pulp cavity, C – cement, D – dentine.

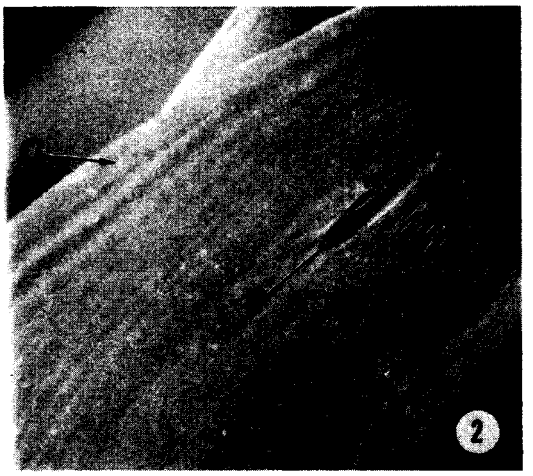

Fig. 2. Scanning electron micrograph of etched longitudinal half-section. GLGs more clearly resolved than in thin section. Original mag.: x15.

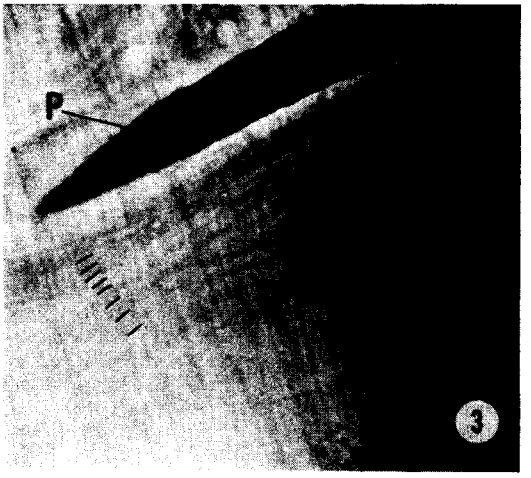

Fig. 3. Microradiograph of ground thin section. More GLGs appear surrounding the pulp cavity than are resolved either by light microscopy or SEM. Original mag.: x25.

Table 1
Examples of discrepancies in dentinal GLG counts in teeth of *Tursiops* compared by method of examination. Light microscopy counts were made using transmitted polarized light. TL – total length of the animal

USNM No.	TL (cm)	Number of GLGs counted		
		Light microscopy	SEM	Micro-radiography
504590	155	1	1	1¼
504583	185	3	3	3¼
395670	–	12	12½	12½
504559*	252	14	14	18

*Pulp cavity occluded.

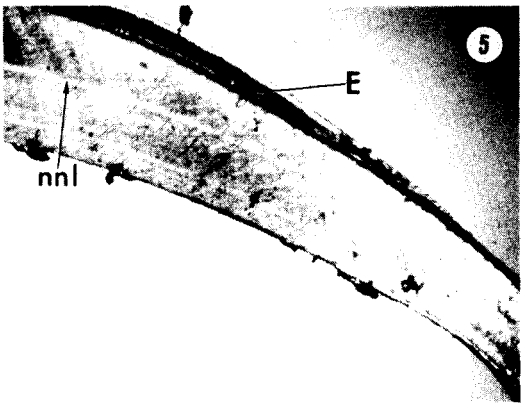

Fig. 5. Ground thin section from the tooth of a young animal viewed under polarized transmitted light. The many accessory layers prevent accurate distinction of the beginning of a new GLG. The neonatal line appears translucent. Original mag.: x30. E – enamel, nnl – neonatal line.

Incremental growth layers

Incremental growth layers are layers which occur parallel to the formative surface of the dentine (see workshop glossary) and compose GLGs. In cross sections of teeth, incremental growth layers are arranged concentrically around the pulp cavity (Figs. 4 and 7). They are most prominent in SEM micrographs where they appear as fine ridges and grooves within the ridges and grooves of the GLGs. In microradiographs and thin sections viewed with light microscopy, incremental growth layers appear as adjacent fine radiolucent and radiopaque layers or translucent and opaque layers, respectively, but are not as clear as in the SEM micrographs. The incremental growth layers averaged 10 to 13 per GLG and 2.0 μm to 3.5 μm in width measured from SEM micrographs, with those in the most recently formed GLGs sometimes smaller than those in the first formed GLGs. Although they were in all of the teeth, they were often not apparent in old animals in the first-formed GLGs nor in the compressed, last-formed GLGs. In sections which showed incremental growth layers in the last-formed GLG, there was a constant number in the teeth of animals that died during the same time of year.

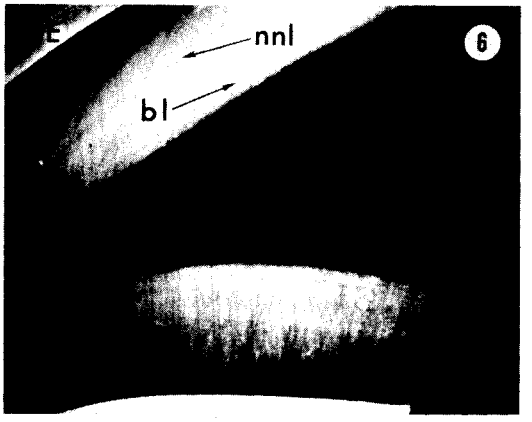

Fig. 6. Microradiograph of the ground thin section in Fig. 5. One radiolucent line stands out and is considered the GLG boundary layer. Original mag.: x30. bl – boundary layer.

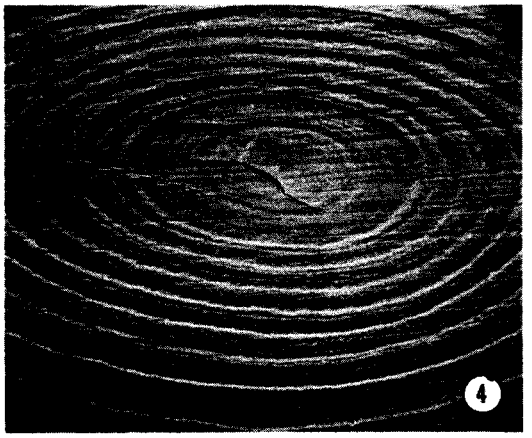

Fig. 4. SEM of etched cross section showing the alternating ridges and grooves. Also faintly visible are some incremental growth layers which compose the GLGs. Arrows indicate GLGs. Original mag.: x50.

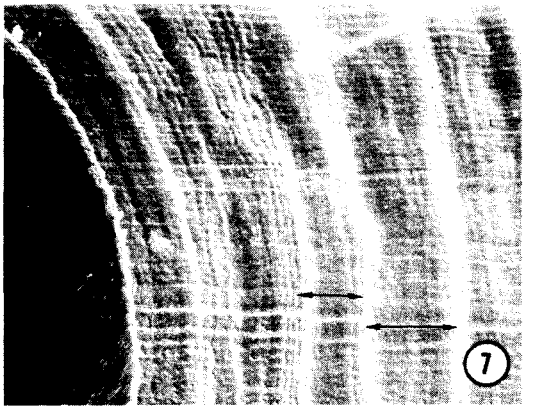

Fig. 7. SEM micrograph of an etched cross-section showing the incremental layers, arranged concentrically around the pulp cavity, which compose the GLGs. The lines running from left to right are saw marks; dentinal tubules are not visible. The arrows denote GLGs. Original mag.: x180.

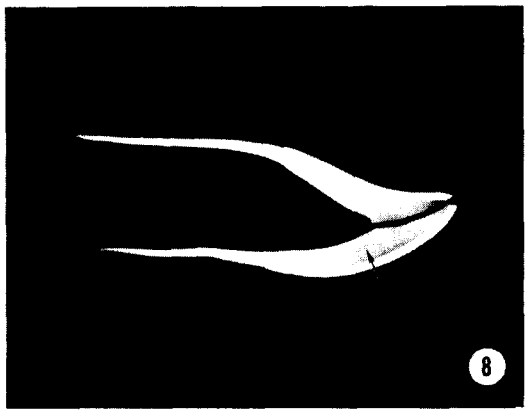

Fig. 8. Microradiograph of ground thin section. The enamel is strongly radiopaque (light), the neonatal line radiolucent. Slight differences in thickness of the section prevented the x-rays from exposing the plate at the root end of the tooth, so it appears highly mineralized (radiopaque). The arrow indicates the neonatal line. Original mag.: x7.

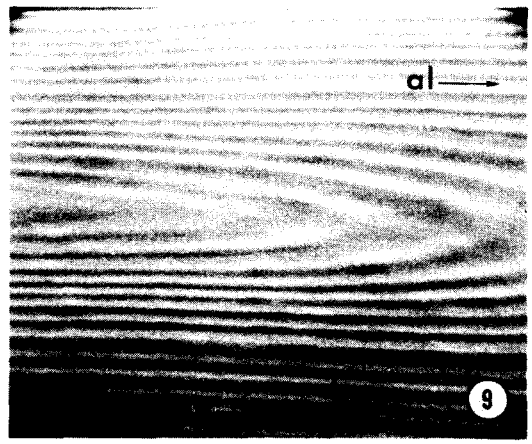

Fig. 9. SEM micrograph showing an accessory layer as an additional ridge between two GLG ridges.

Accessory layers

Sometimes an incremental layer appears particularly prominent within the GLG because it is relatively more hypo- or hypermineralized than the GLG layer in which it is located (Fig. 9). For purposes of this study, these prominent layers are referred to as accessory layers, defined as irregularly occurring, nonrhythmic layers that disrupt the expected mineralization pattern within a GLG and complicate the problem of counting GLGs. They seem to occupy the same relative position in GLGs in the teeth of a given individual, but vary in number and position among teeth of various specimens. Accessory layers occur most commonly near boundaries of GLG layers.

Comparison of techniques for counting GLGs

Scanning electron micrographs of etched sections is the easiest method by which to count GLGs, because the contrast in topographic relief between the layers of a GLG is generally great enough to easily distinguish adjacent GLGs (Fig. 4). Although incremental layers and accessory layers are apparent using SEM, their effect in confusing the counting of GLGs is diminished by etching. In microradiographs, the first-formed GLGs are not as clearly delineated, because the contrast between adjacent layers of the GLG is less than in etched sections. In undecalcified thin sections examined with light microscopy, the accessory layers were sometimes so conspicuous near the boundary layer of GLGs that divisions between GLGs were obscured. This problem occurred most often within the first four or five GLGs. When the boundaries of the GLGs were not definable, the number of GLGs could only be estimated.

Correlation between optical and mineral density

Optical density corresponds to mineral density for each layer of the GLG when comparing the three methods (Table 2). Sections viewed with transmitted light show the neonatal line as translucent with the prenatal zone and the immediately postnatal incremental layer of the first GLG appearing opaque, and the second layer of the GLG appearing translucent. In etched sections, the neonatal line appears as a ridge, and the prenatal and immediately postnatal incremental layer are grooves. In the microradiographs, the neonatal line is radiolucent (relatively hypomineralized), the prenatal and postnatal incremental layers are radiopaque (relatively hypermineralized), with the second incremental layer of the GLG radiolucent. This cyclic deposition of mineral, i.e. adjacent hypermineralized and hypomineralized layers, continues throughout the postnatal dentine.

Table 2

Correlation of GLG components with relative mineral density in the teeth of 15 specimens of *Tursiops* using transmitted light microscopy, SEM, and microradiography

Method of examination			
Transmitted light	Micro-radiography	SEM	Relative mineral density
opaque	radiopaque	groove	greater
translucent	radiolucent	ridge	less

DISCUSSION

GLGs

Each of the preparations should permit adequate age estimates to the nearest whole GLG, but microradiography seems to be the best of the three techniques for accurately identifying the nature, i.e. mineral density, and extent of growth of the newly forming GLG in teeth which were still accumulating dentine when the animals died. This information, collected over at least a year for a species, will show the cycle of mineralization, helping to explain the mechanisms influencing mineralization and its relationship to life history attributes of the animals. Previous work, e.g. on *Tursiops truncatus* (Sergeant, 1959), *Globicephala melaena* (Sergeant, 1962), *Hyperoodon ampullatus* (Christensen, 1973), *Delphinapterus leucas* (Sergeant, 1973), and *Berardius bairdii* (Kasuya, 1977), has shown the consistency of the stainability or optical density of the last GLG component with the season of death for each species.

In some cases, more GLGs may be deposited, in terms of mineralization cycles, than may be determined using con-

ventional light microscopy. Because of its sensitivity to differential mineral density, microradiography may be used to detect these GLGs and extend the maximum GLG count.

Incremental growth layers

Early work in dental histology demonstrated the existence of incremental lines in dentine that express the constant rhythmic changes in the degree of mineralization of the matrix (Schour and Steadman, 1935; Schour and Hoffman, 1939a; Schour and Massler, 1940). According to Schour and Hoffman (1939a), two adjacent lines, one more and one less mineralized, measured 16 μm in all species examined. These lines were later interpreted as incremental lines of von Ebner. More recently, smaller incremental lines (von Ebner lines), e.g. 5 μm, have been described that represent daily increments of growth of the tooth (Krauss and Jordan, 1965; Miani and Miani, 1972; Newman and Poole, 1974; and Yilmaz, Newman, and Poole, 1977). However, neither incremental growth of teeth on other than a daily rhythm nor the annual mineralization pattern of mammal teeth have been well demonstrated.

Sub-annual incremental layers, where GLGs in at least *Tursiops* teeth are considered to be annual (see Sergeant, 1959; Klevezal' and Kleinenberg, 1967; Sergeant *et al*, 1973; this workshop–known-age dolphins), have been described in some marine mammals. Kasuya (1977) found the 'long cycles' (GLGs) in *Berardius* teeth to contain many (11.0–13.4) 'short cycles', and Kasuya and Nishiwaki (1978) found that coarse layers (GLGs) in *Dugong* tusks contain 10 to 15 fine growth layers (see also Scheffer, 1970). They interpreted the fine growth layers or short cycles as representing the lunar cycle or an endogenous rhythm of about one month manifested in the growth of the tooth. Myrick (this volume) noted incremental layers (his accessory lines) of the same periodicity in the teeth of different species of dolphin, as well as von Ebner lines which are interpreted as representing a daily growth pattern. The rhythmicity and periodicity of the incremental lines or layers, i.e. 10 to 13 per GLG, in this study, also suggests the incremental layers in the North Atlantic Ocean *Tursiops* to be incremental growth of the tooth similar to that described by Kasuya (1977), Kasuya and Nishiwaki (1978) and Myrick (this volume). In this case, the incremental layers appear to be the result of a finer mineralization pattern within the more gross annual mineralization cycle of the GLGs. This is evident in the three-dimensional pattern of the incremental layers in etched sections, where calcium has been removed, and in microradiographs.

Accessory layers

If the mineralization cycles, both annual and sub-annual, seen in dolphin teeth are the result of extrinsic environmental factors, then irregular changes in the expected deposition pattern may be a visible manifestation of a change in environmental parameters. Similarly, if mineralization is intrinsically controlled, appropriate changes within the animal's system should appear in an actively growing tooth. Accessory layers may be a result of either of these types of fluctuations. If so, they may be useful in distinguishing stocks of animals, and careful notation of these changes may aid in explaining mechanisms responsible for the mineralization patterns seen in dolphin teeth; therefore, it is important to distinguish accessory layers as such. But, in regards to a practical application of odontocete teeth for age determination, a method is needed that permits clear resolution of individual GLGs without the sometimes overwhelming interference of accessory layers. Of the three methods in this study, SEM best serves this purpose.

Correlation between optical and mineral density

The determination of the mineral density of each layer of a GLG, especially the last-formed layer, may be important for resolving the basis of formation of GLGs in odontocete teeth. However, there has been disagreement about the interpretation of optical density and mineral density in some of the species examined (Sergeant, 1962; Klevezal' and Kleinenberg, 1976; Nielsen, 1972; and Kasuya, 1976). In response to these discrepancies, Nielsen (1972; this volume) feels that no species-wide generalization can be made to correlate optical to mineral density.

In this study, the correlation of mineral density to optical density in the teeth of *Tursiops* agrees with Sergeant's (1962) results with *G. melaena*. In agreement with many previous studies (*Stenella coeruleoalba*, Nishiwaki and Yagi, 1953; *T. truncatus*, Sergeant, 1959; *G. melaena*, Sergeant, 1962; *B. bairdii*, Kasuya, 1977; and *Phocoena phocoena*, Gaskin and Blair, 1977) the neonatal line is translucent (or unstainable) and the immediately postnatal layer is opaque (transmitted light). Although the event causing the formation of the neonatal line does not occur again, the neonatal line is hypomineralized (Irving and Weinmann, 1948) and is generally easily identifiable. Therefore, it can be used as a landmark for identification of mineral densities of the following layers. In other words, if the neonatal line appears translucent, and we know it to be hypomineralized by microradiography, then each translucent layer within subsequent GLGs should be hypomineralized. This is the case in all of the *Tursiops* teeth examined.

Evaluation of techniques

Of the three techniques, SEM is the easiest method by which to count GLGs. While the method is procedurally more complicated than preparation of thin sections, the results justify the slight additional preparation time, especially for a large number of specimens. Microradiography, valuable as a source for information on mineral density, requires exceptional precision in thickness of sections and timing of the x-ray to achieve worthwhile results and would be too demanding for age determination of a large number of specimens. The conventional method of light microscopy, although less complicated in terms of tooth preparation, seems to be least reliable for accurate counting of GLGs.

Although each of the methods permits adequate estimates of GLGs for age determination, additional information about the deposition and mineralization of dentine is available from microradiography and SEM. Such information helps to better define GLGs, as well as to ascertain what processes influence layering in odontocete teeth.

ACKNOWLEDGEMENTS

I am grateful to D. Ortner and D. Yong, Department of Anthropology, U.S. National Museum of Natural History (NMNH), Smithsonian Institution, for the suggestion and use of their microradiograph. C. Potter and G. Morgan, NMNH, critically reviewed the manuscript; J. Bittner, NMNH, helped in preparation of figures. M. Jacque Mann, SEM Lab, NMNH, prepared most of the SEM micrographs. A. Myrick, National Marine Fisheries Service, encouraged me during the early stages of this research. I am especially indebted to J.G. Mead, NMNH, whose advice and support enabled me to complete this work.

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