

## Examination of Layered Tissues of Odontocetes for Age Determination Using Polarized Light Microscopy

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### ABSTRACT

The polarized light microscope with a  $1/4 \lambda$  plate greatly improves resolution of the microstructure in layered hard tissues of dolphins by revealing changes in crystallographic alignment not visible using more conventional instruments. In adult specimens of *Stenella attenuata* the prenatal enamel contains up to 240 striae that are thought to represent daily records of prenatal growth. Prenatal dentine contains approximately 60 incremental layers, the significance of which is unknown at present. In *Lagenorhynchus*, *Delphinus*, *Tursiops* and *Stenella* spp. the neonatal 'line' consists of several incremental layers, suggesting that post-parturitional trauma may be a prolonged event. In these species the postnatal dentine contains 13 regularly-spaced accessory layers in the first five or six growth layer groups (GLGs). These accessory layers are thought to reflect lunar cycles, and each contains an average of 28 microlayers (lines of von Ebner) that may represent daily growth increments. Cemental GLGs show fewer accessory layers than those of dentine. GLGs in periosteal bone consist of accessory layers that may reflect lunar cyclicity. It is suspected that layering in dentinal, cemental, and periosteal tissues is in response to the same cyclic influences.

### INTRODUCTION

Investigators of dolphin life histories routinely use compound or dissecting microscopes to identify and count growth layer groups (GLGs) in thin sections of teeth and bones to determine relative ages. These instruments operate with plain light that is either transmitted through a section or reflected from the surface of a section to detect differential optical densities or topographic relief delineating GLG boundaries thought to represent uniform segments of time.

A prime cause of disagreement among investigators in counting layers has been the difficulty in deciding where to separate the layers. Also, there may be difficulty in distinguishing any regular layering at all in sections that have not undergone special preparation. The polarizing microscope assists in overcoming some of these problems by expressing structural and histological differences as highly contrasted patterns of light (i.e. as different colors or shades). It accentuates structure, not visible using more conventional instruments, that is of potential importance in studies of dental development, and it provides further bases for understanding and interpreting layering patterns for age determination.

This paper is an account of preliminary research conducted using the polarizing microscope in examination of the layered structure of hard tissues of dolphins as an aid to understanding more fully the macrostructures useful in estimating ages.

### BACKGROUND

The petrographic polarizing microscope was developed initially to determine the mineral composition of rocks by examination of their crystallography. It is a compound microscope with light-polarizing and phase-contrast capabilities that can be used to define differential optical and crystallographic features of translucent objects prepared in thin section (Moorehouse, 1959). The technique has been used widely in comparative dental histological studies (e.g. von Keil and von Nolting, 1968; Peyer, 1968).

Dental and periosteal tissues of dolphins and other vertebrates contain the mineral hydroxyapatite. Because growth is inconstant, layers of various optical densities are formed,

and crystal lattices of hydroxyapatite are deposited within the layers in orientations that tend to differ from increment to increment but which have a common alignment within a given growth increment. When layered tissue is examined with polarized light, each crystallographic alignment, and variations in the composition of the mineral phase and organic matrix are made visible.

### MATERIALS AND METHODS

#### 1. Ontogenetic series

Teeth and samples of periosteal bone, from the 'pan' region of the mandible and from the premaxillae at the base of the rostrum, were collected from approximately 50 female specimens of *Stenella attenuata* from the eastern tropical Pacific—the animals ranged from early fetal to old adult. Materials were prepared in undecalcified thin section ( $\cong 250 \mu\text{m}$ ) and arranged in order of body length to form developmental series. Each series was examined under polarized light in an effort to characterize developmental stages in the layering patterns and to inter-calibrate dentinal, cemental, and periosteal growth-layer-group (GLG) patterns.

#### 2. Known-age specimens

Thin sections ( $\cong 250 \mu\text{m}$ ) of undecalcified teeth from captive-born (known-age) individuals of *Tursiops truncatus* were examined by polarized light microscopy, without any advance knowledge of their ages, in an effort to identify reliable structures that may be useful in determining absolute ages.

#### 3. Tetracycline-marked specimens

Several teeth from specimens of *Tursiops truncatus*, *Lagenorhynchus obliquidens*, and *Delphinus delphis* containing tetracycline-marked tissues were examined by polarized light and under ultraviolet reflected light to intercalibrate postnatal dentinal and cemental layers. One of the known-age specimens was incidentally marked with tetracycline while *in utero*. The marked tissue was examined to identify the structural time of birth.

#### 4. Preparation of samples

All teeth were sectioned mid-longitudinally using a *Buehler Isomet* saw equipped with two diamond-embedded blades separated by a 200- $\mu\text{m}$  shim to produce a wafer 200  $\mu\text{m}$  to 300  $\mu\text{m}$  thick. Bone samples were sectioned transversely using the same equipment. Thin sections were mounted on glass slides with *Permount* and covered with glass coverslips.

#### 5. Method of examination

All sections were examined at between 57 x and 1200 x using a *Zeiss* photomicroscope No. 472190 equipped with a rotatable polarizer and analyzer, a rotary stage, petrographic objectives, and a  $1/4 \lambda$  (first order red) quartz plate. A fluorescent vertical illuminator with a filter-reflector No. 44-77-05 combination was used for examination of the tetracycline-marked specimens in ultraviolet light.

### RESULTS AND DISCUSSION

#### 1. Enamel

**A. Ontogenetic development of layers.** Enamel is deposited prenatally. Polarized light microscopy of this tissue in *Stenella attenuata* reveals up to 240 growth lamellae lying parallel to each other, arranged in an offlapping pattern downward from near the apex and oriented subnormal to the prismatic structure (Fig. 1). Analyses of the ontogenetic series indicate that these layers represent growth increments that first appear in teeth of fetuses at body lengths of between 25 cm and 28 cm. There is an increase in the num-

ber of layers with increasing body length. At 50 cm the enamel contains approximately 40 layers, at 70 cm there are 150 layers, and in teeth of full-term fetuses (approximately 82.5 cm) and postnatal animals including old adults (up to 220 cm) up to 240 enamel layers have been counted. This suggests that incremental growth in enamel probably does not continue after parturition.

#### B. Significance and possible applications of enamel layering.

Perrin, Coe and Zweifel (1976) concluded that the average gestation period for *S. attenuata* is approximately 11.5 months (or 345 days). If the layers in the enamel represent daily growth, as much as 240 days (or 8 months) of fetal development could be accounted for. The unrecorded 3.5 months would represent the period from conception to the stage at which the tooth germ is developed immediately prior to layer formation.

If examination of larger samples substantiate these tentative findings, enamel layers may be used in conjunction with labelling of fetal hard-tissues in captive, pregnant females and with embryological data from terrestrial mammals to estimate more precisely the fetal growth stages and gestation periods of delphinid species.

#### 2. Prenatal dentine

**A. Ontogenetic development of layers.** Prenatal dentine has been relatively ignored except as a 'landmark' tissue internal to which GLG counts are begun. It has been described as unlayered or granular in appearance, but polarized-light

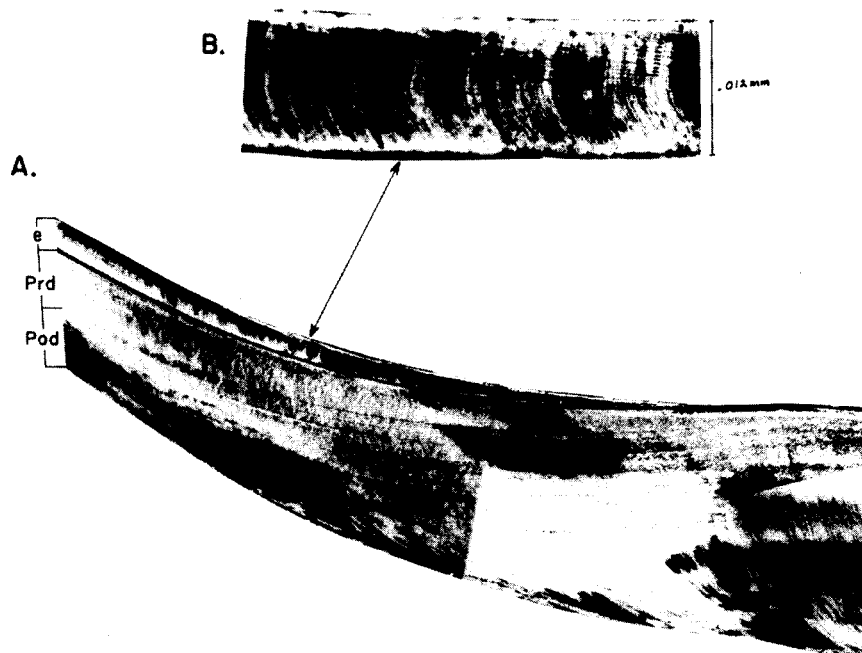


Fig. 1. Daily (?) layering in enamel in a tooth of a female of *Stenella attenuata* (TCF 060).

A. Half of a tooth section viewed with polarized light and showing layering in the enamel (arrow) (57x).

B. Highly magnified section of enamel as shown in A showing layers as indicated by marks. Because of the oblique (offlapping) arrangements of layers many transects at various intervals from apex to neck may be necessary to obtain full counts. A total of 223 enamel layers was seen in the tooth.

Abbreviations: e. enamel, n. neonatal line, prd. prenatal dentine, pod. postnatal dentine.

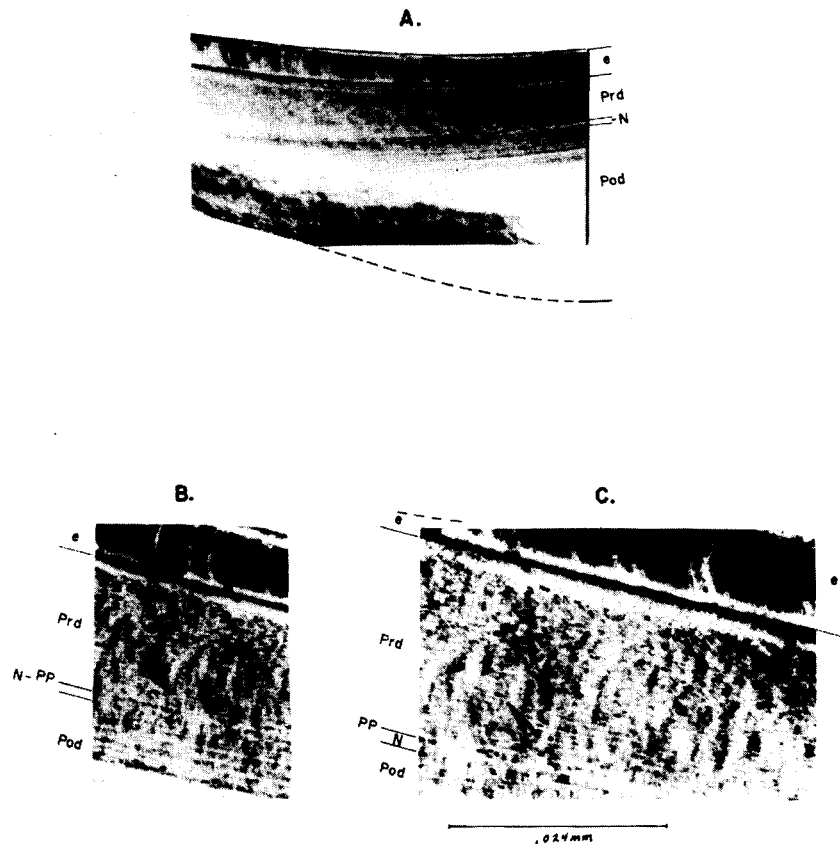


Fig. 2. Layering in prenatal dentine and neonatal line in *Stenella attenuata* (TCF 060).  
 A. Thin section of half of tooth as viewed with crossed polarizers and phase contrasted light showing prenatal dentine and neonatal line with layers not readily apparent (57x).  
 B. Magnified view (200x) of prenatal dentine as in A showing layering in prenatal dentine and neonatal line.  
 C. Highly magnified view (250x) of same section as in B with 60 counted layers in the prenatal dentine and 5 layers visible in the neonatal layer. Major layers often bifurcate in prenatal dentine (arrows). Note that layering is difficult to resolve in the earliest deposited tissue near the enamel. Abbreviations: PP, structural point of parturition, N neonatal line.

microscopy reveals that layers are fairly well developed in prenatal dentine (Fig. 2).

Layers first appear at the same stage of fetal development as do layers in enamel, i.e. at 25 cm to 28 cm. Unlike enamel layering however, prenatal layers seem to accumulate at a considerably lower rate. At 50-cm body lengths the teeth show only 25 prenatal dentinal layers, at 70 cm approximately 45 layers are present, and in the teeth of full-term fetuses and postnatal animals only 55 to 60 layers have been counted.

**B. Significance of prenatal dentinal layering.** The significance of layering in this tissue is not readily apparent. If prenatal dentinal growth occurs concomitantly with that of enamel (i.e. for a presumed period of approximately 240 days), then prenatal dentinal layers would be deposited at an average rate of one every four days. Such a rate would not seem to be correlated to any known exogenous cycle. On the other hand, distinguishing layers in this tissue is difficult, especially in the region of earliest deposition

(Figs. 2B–C). It is possible that either some of the ‘growth pulses’ governing layering in enamel are not of sufficient intensity to produce visible layers in the prenatal dentine, or that another technique is needed to detect existing, but indistinct layers.

**3. Neonatal line**

The neonatal ‘line’ has been thought to demarcate the point of parturition—recording post parturitional trauma of the neonate as it is confronted with the external environment and a new mode of feeding. Polarized-light microscopy at 200 X shows that this ‘line’ is composed of an alternating series of three or more pairs of opaque and translucent layers bounded on either side by a rather bright translucent layer (Figs. 2B–C).

**4. Postnatal dentine**

**A. Composition of GLGs.** Microscopic examination (at 57 X) of postnatal dentine using plain transmitted or reflected light reveals repeating or semi-repeating patterns

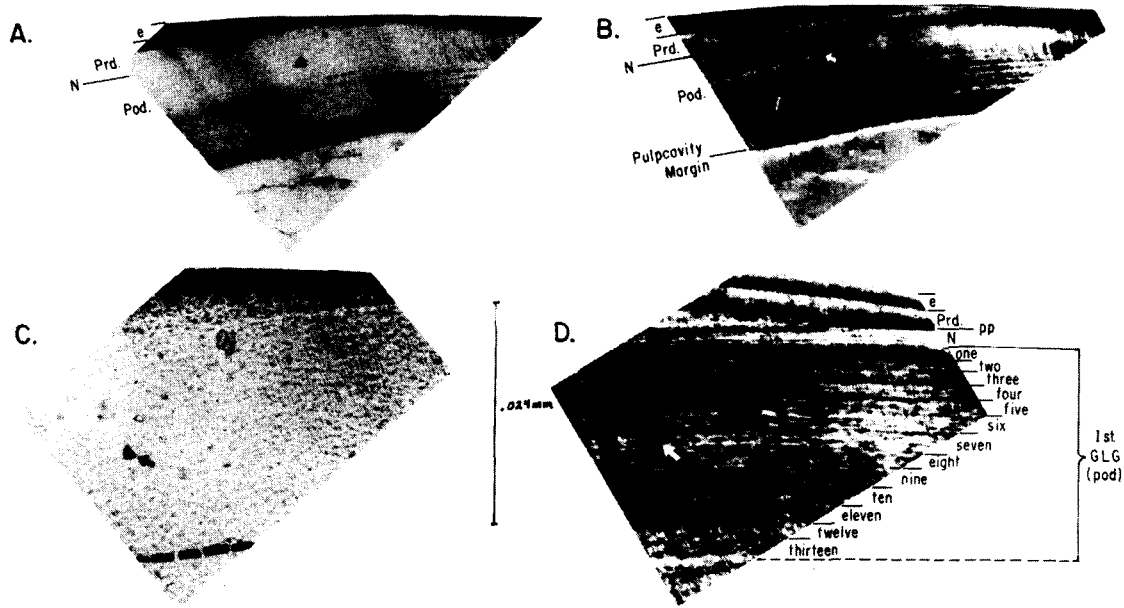


Fig. 3. Lunar layering in postnatal GLGs of a tooth of a specimen of *Stenella attenuata*.

A. Plain-light view of one-half of tooth section showing postnatal dentine. Dashed line indicates boundary between first and second growth layer groups. Triangle (an artifact) used as point of reference (57x).

B. Section as in A, viewed with crossed polarizers and phase-contrasted light. Note presence of multiple layers brought out by this technique.

C. Plain-light view of section as in Fig. A (250x). Arrow indicates triangle reference point.

D. Section as in C viewed with crossed polarizers and phase-contrasted light. Note layered components of neonatal line and 13 counted accessory layers within first GLG (bracketed). At this power the more intense aggregations of microlayers (lines of von Ebner) become apparent. Abbreviation: GLG = growth layer groups.

of accessory layers that may be used to define GLGs. Each accessory layer consists of a pair of components—a translucent layer and an opaque layer, which, in turn, are composed of fine dark and light incremental layers. With ordinary light microscopy, accessory layers are seen to occur in varying intensities, and commonly the accessory layers that mark the boundaries of GLGs are no more distinct than some of the more strongly developed accessory layers within the GLGs. Hence problems often arise in defining GLGs that introduce considerable subjective error into age estimates.

Polarized light microscopy of the tissue enhances resolution of accessory layers already optically apparent using more conventional microscopes and makes visible other accessory layers that cannot otherwise be detected easily. At 200X the first (i.e. externalmost) GLG is shown to consist of 13 accessory layers excluding the one represented by the neonatal layer (Figs. 3D and 4). Although younger GLGs are deposited as increasingly thinner, more compressed layering sequences, the first three or four GLGs usually show 13 accessory layers also (Figs. 3 and 4). Younger GLGs usually are too thin to permit delineation of all incremental components, but in large, well layered teeth 13 accessory layers can be detected in the fifth and (rarely) sixth GLGs.

**B. Possible significance of accessory layers.** There are 13 lunar months (synodic cycles) in a year. It is possible that, as in many other marine organisms, the physiologies of odontocetes are or were influenced either directly or indirectly by lunar periodicity and that this periodicity is

reflected in the layering patterns of hard tissues. If accessory layers correspond with lunar cyclicity, they could be used as countable units to determine age. To test this, accessory layers in the teeth of two known-age specimens of *Tursiops truncatus* ('Pinger' and 'Moe B') were counted without advance knowledge of their true ages. Teeth of both specimens contained between 42 and 45 accessory layers. 'Pinger' (SWFC 0007) was born in captivity at the Naval Ocean Systems Center, San Diego in November 1970 and died in February 1974 (Hui, 1978). 'Moe B' (SWFC 0008) was born at Sea World in San Diego on 14 April 1975 and died on 4 July 1978. Both animals were 3 years, 3 months old (or 43 lunar months old). The close agreement between the number of accessory layers counted and the age of the specimens in lunar months strengthens the hypothesis that accessory layers, when optimally viewed, may be used to estimate ages for young specimens.

**C. Lines of von Ebner: daily(?) records.** At high magnifications (800 to 1200X), polarized-light microscopy of postnatal dentine reveals a system of minute incremental growth layers (so-called 'lines of von Ebner') within the accessory layers (Figs. 3D and 5). Often these structures are difficult to see, but in accessory layers where they are most distinctive, I have counted an average of 28 such microlayers. Because they are contained within accessory layers that I interpret to represent 28-day lunar cycles, I suggest that the lines of von Ebner in dolphin teeth may reflect daily growth.

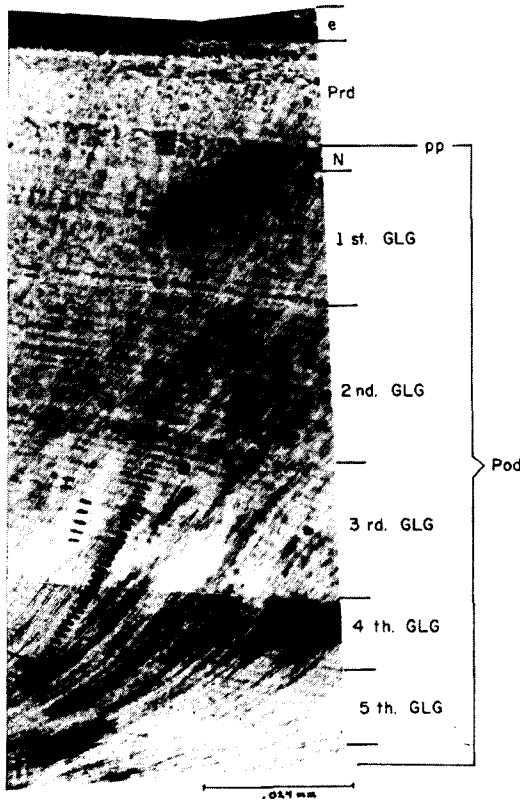


Fig. 4. Partial tooth section of a female specimen of *Stenella attenuata* (TCF 060) viewed with cross polarizers and phase-contrasted light. Section shows 13 regularly spaced accessory layers in each of the first five GLGs (each GLG defined by translucent boundary layers) (200x). Regular spacing and number of accessory layers indicated lunar cyclicity (see text).

5. Cementum

**A. Resolution of GLGs.** In the teeth of most species of small delphinids, cemental GLGs are difficult to define without special preparations because of the extreme thinness and poor layering of this tissue. Typically, cementocytes are stratified, but their parallel alignments do not correspond necessarily to cemental layering where it is distinguishable.

Compared to ordinary light, polarized-light microscopic examination provides somewhat better resolution of cemental layers because of the color contrast produced. Nevertheless, only decalcification and staining procedures (Kasuya, 1977) seem to enhance cemental layering adequately.

**B. Accessory layers.** When visible under polarized light, GLGs in cement exhibit fewer accessory layers than the 13 found in early dentinal GLGs. Tooth sections of captive specimens of *T. truncatus* clinically treated with tetracycline show fluorescent markers in the dental tissue when examined microscopically with reflected ultraviolet light (Figs. 6A, B, C and D). These markers extend down the dentinal layers, wrap around the bases of the teeth, and are continuous with markers within the cemental tissue (Figs. 6C and D). This indicates that the same mechanism which governs dentinal layering also influences cemental layering, but it is not known why fewer accessory layers are found in the cemental GLGs. The problem may be one of preparation and examination techniques (see Kasuya, 1977).

6. Periosteal bone

Polarized-light microscopy helps in delineating GLGs in transverse sections of periosteal bone from the 'pan' region of the mandible (Fig. 7) and from the premaxilla near the base of the rostrum (Fig. 8). A small ontogenetic series of bone samples compared with teeth from the same individuals indicates that periosteal GLGs seem to accumulate at the same rate as dentinal GLGs. In old specimens, some resorption of the early periosteal GLGs takes place, but relicts of these resorbed layers remain discernible with the polarizing microscope (Fig. 8).

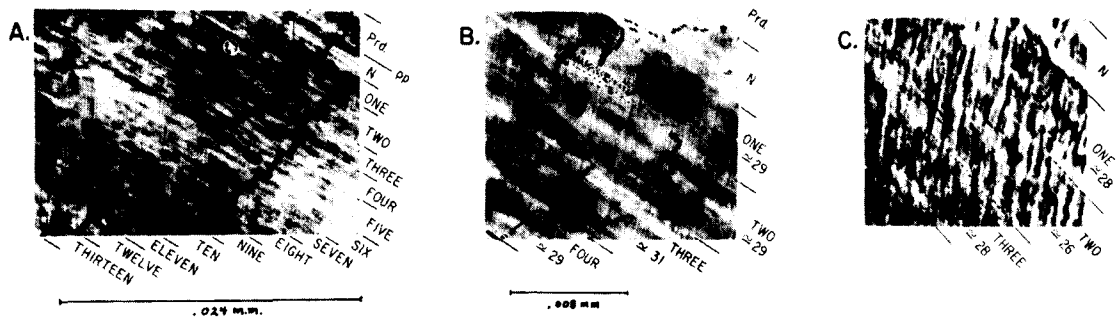


Fig. 5. Daily layering in postnatal GLGs in *Stenella attenuata*.  
 A. Magnified view (250x) of layers as shown in Fig. 3D showing 13 (bracketed) lunar layers within the first growth layer group.  
 B. Magnified view (800x) of inset in 5A, showing system of lines of von Ebner in first four accessory layers. Counts average 29 lines per accessory layer. This suggests that daily growth is represented. Circled arrow points to small triangle in second accessory layer used as point of reference.  
 C. Lines of von Ebner in another region of same tooth showing greater resolution produced by changing angle of section relative to polarizers. An average of 27 or 28 lines may be seen in first three accessory layers (800x).

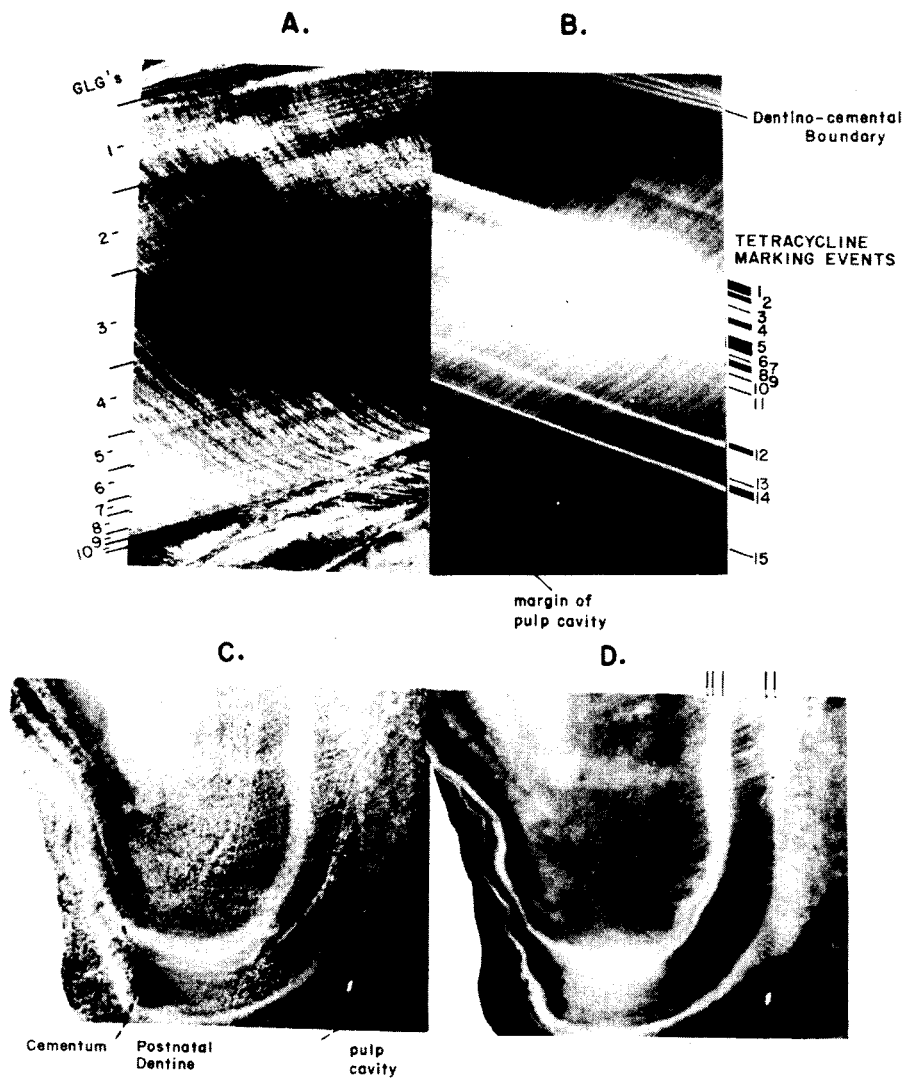


Fig. 6. Tetracycline-marked partial tooth sections of *Tursiops truncatus* (#AB718) showing fluorescent bands in relation to layers in postnatal dentine and cementum.

A. Dentinal tissue viewed using polarized light, showing GLG's.

B. Mirrored view of dentinal tissue as shown in A viewed under ultraviolet light showing fluorescent bands (150x).

C. Base of tooth viewed using combination of reflected ultraviolet light and transmitted plain light showing relationship of fluorescent markers to layers in cementum and dentine (150x).

D. Base of tooth viewed under ultraviolet light showing continuity of fluorescent markers in dentine and cementum (150x).

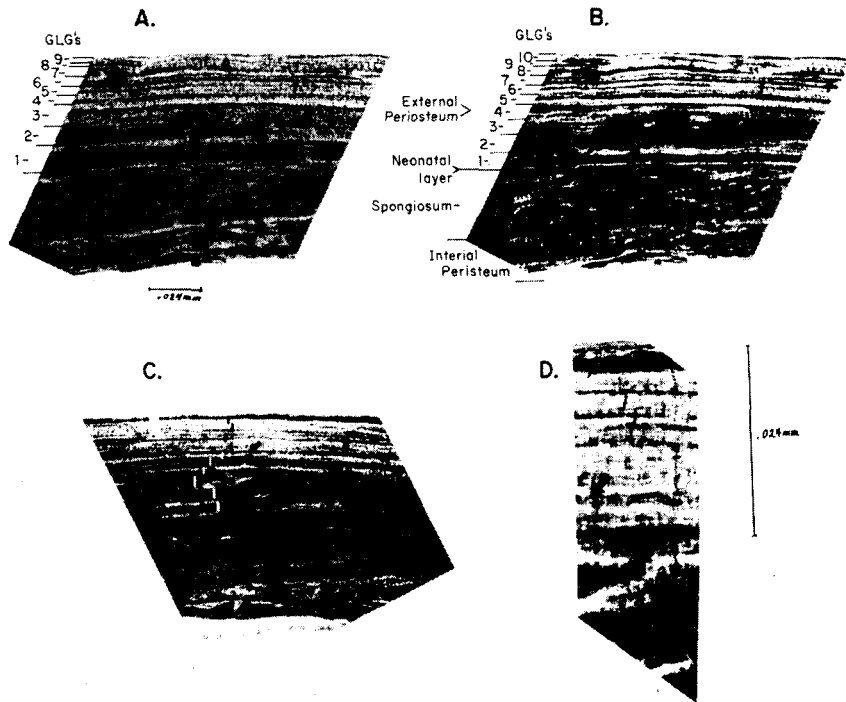


Fig. 7. Periosteal layering in mandible of *Stenella attenuata* female (TCF 060).  
 A. Transverse section of mandible from the "pan" region viewed in plain light (57x).  
 B. Section as in A showing strong contrast between GLGs produced by polarized and phase-contrasted light.  
 C. Another section of same bone viewed with polarized light. GLGs are marked. Arrow indicates point of reference between fifth and sixth GLG (57x).  
 D. Magnified (150x) area of section as in C showing 13 accessory layers per GLG.

The technique shows also that each periosteal GLG is composed of 13 accessory layers—most apparent in early layers (Fig. 7D). As in the dentinal GLGs the accessory layers within later GLGs in the bone become compressed. The presence of lunar-cycle layers in bone suggests that dentinal, cemental, and periosteal layering is influenced by a common mechanism. If this proves to be true, eventually all three layering systems may be used interchangeably to obtain maximum age estimates of very old individuals in which dentinal layering has stopped because of pulp-cavity occlusion.

**SUMMARY**

Polarized-light microscopy gives high resolution and color contrast to the ultrastructure in layered hard tissues of delphinids. Visual access to the ultrastructure is useful in gaining a more complete understanding of the factors influencing layering and layering rates. With the use of this method to examine ontogenetic series (in *S. attenuata*), and known-age and marked tissues of other delphinids — the following tentative conclusions were reached:

(1) Enamel layers occur that may represent daily growth records. Because enamel is developed prenatally, the layers may be used in concert with other reproductive

and embryological data to estimate gestation periods and fetal age-growth stages in dolphins.

- (2) The neonatal line is a complex layer, the initial component of which may mark the point of parturition. The presence of its other components may indicate that post-parturitional trauma is a prolonged event.
- (3) The prenatal dentine has a layered pattern that commences consonantly with enamel layering, but its layering rate may be governed by different physiological factors.
- (4) Each of the first five or six postnatal dentinal and periosteal GLGs are composed of 13 accessory layers that seem to correspond to lunar months. If this is true, the age of young animals may be estimated more accurately than in the past.
- (5) Lines of von Ebner in postnatal dentine of delphinids may represent daily growth.
- (6) Cemental, dentinal, and periosteal layering probably is influenced by the same physiological mechanism. This may eventually permit use of all three systems interchangeably in estimating ages of old animals.

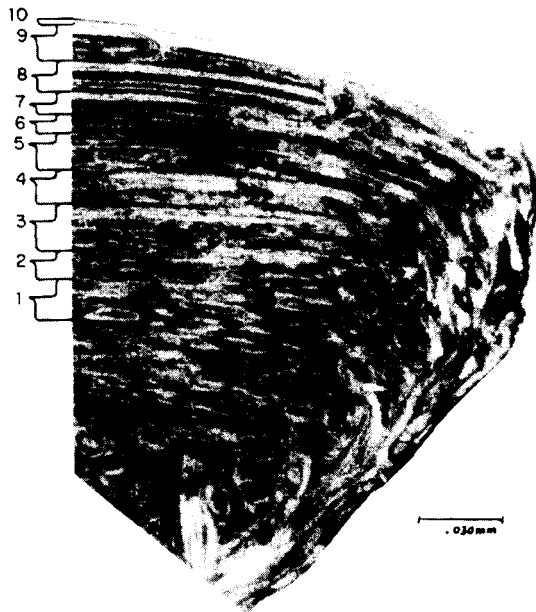


Fig. 8. Periosteal layering in premaxilla of *Stenella attenuata* female (TCF 060).

A. Transverse section of premaxillary bone taken near base of rostrum and viewed with polarized light, showing GLGs. The first GLG shows incipient resorption as indicated by presence of secondary Haversian systems (arrows) (80 $\times$ ).

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