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ESTIMATING AGE OF SPOTTED AND SPINNER DOLPHINS (Stenella attenuata AND Stenella longirostris) FROM TEETH

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U.S. DEPARTMENT OF COMMERCE
National Oceanic and Atmospheric Administration
National Marine Fisheries Service
Southwest Fisheries Center

NOAA Technical Memorandum NMFS

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NATIONAL OCEANIC AND ATMOSPHERIC ADMINISTRATION
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U.S. DEPARTMENT OF COMMERCE
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ABSTRACT

This paper is an account of preparation and examination techniques and criteria used to estimate age in decalcified and stained tooth thin sections from spinner and spotted dolphins. A dentinal growth layer group (GLG), composed of two thin light and two thicker dark-stained layers, is deposited annually. The GLG component layers are variably visible, but the "ideal" pattern and successive thinning of dentinal GLGs are used as a guide to determine GLG limits. Age-specific thicknesses of dentinal GLGs found in Hawaiian spinner dolphin teeth seem to be applicable to teeth of spotted dolphins and can be used as an aid in locating GLG boundaries. Cemental GLGs are composed of a dark-stained and a lightly stained layer and usually are deposited at a rate of one per year, but may be deposited every other year or two or three times per year. Two slightly different methods of counting dentinal GLGs are presented, along with guidelines for determining whether dentinal or cemental GLG counts provide the best estimate of age for a specimen.

INTRODUCTION

Age determination is a branch of research upon which dolphin population biology depends heavily. Without a clear and accurate understanding of age composition, age at sexual maturity, age at first reproduction and natural longevity, the dynamics of a population cannot be determined.

There are at least four requirements to determining age from growth layer groups (GLGs, terminology of Perrin and Myrick, 1980) in a dolphin tooth. First, one must be familiar with the timing of deposition and the distribution of dental tissues. Without knowledge of precisely where prenatal tissue ends and postnatal tissue begins, for instance, there can be no assurance that GLG counts are begun at the correct position in the tooth.

Second, a reliable and effective system of preparing teeth that gives clear resolution of GLGs is necessary. Over the years, many techniques have been developed (see Perrin and Myrick, 1980, Report of the Workshop). Some have proven unsatisfactory in resolving GLGs easily (e.g., Perrin et al.

1977), while others provide excellent resolution of GLGs but are too time consuming or prohibitively expensive for large samples of teeth (e.g., Hohn, 1980a).

Third, to count GLGs with consistency, one must define in detail the structural pattern of a GLG as it appears in prepared teeth for a given species. The definition of layers counted for determination of age is vague in much of the literature.

Finally, to relate GLG counts to age, the amount of time represented by a GLG should be demonstrated. With few exceptions (in which the results have been inconclusive, see references in Myrick et al. MS[1]), age-determination studies have been unable to establish the timing of GLG deposition, largely because of a lack of suitable material. Commonly, GLGs as counted in most studies have been interpreted as yearly increments despite the lack of experimental support.

Over the past several years the Marine Mammal Age Determination Project (SWFC, La Jolla, California) has conducted studies to improve techniques in preparation of thin sections of teeth for microscopic examination and to develop a real-time basis for estimating ages from dolphin teeth. This paper provides an account of preparation and examination techniques and the criteria developed to interpret age from the teeth of spotted and spinner dolphins, *Stenella attenuata* and *Stenella longirostris*. A study of seven captive Hawaiian spinner dolphins, *S. longirostris*, in which dental layers were calibrated using multiple tetracycline labels and periodic extractions of teeth (Myrick et al. MS[1]), forms the basis for definition of annual GLGs and age interpretation described below. No direct calibration of dental GLGs has yet been possible for spotted dolphins or for spinner dolphins from the wild, but the GLG pattern in such samples closely resembles that of the captive sample. We have assumed that the time represented in GLGs in the captive spinner dolphins is the same in those of the wild samples of both species.

DISTRIBUTION, DEPOSITION, AND UTILITY OF POSTNATAL DENTAL TISSUES

Dental tissues in all delphinids have a common basic pattern of distribution and deposition (Fig. 1). The tooth of a new-born dolphin consists of a thin, external, conical mantle of enamel, formed prenatally, covering a thicker, basally-tapered cone of prenatal dentine and part of a thin, inner, layered cone of neonatal dentine. These internally-nested cones surround a central pulp cavity.

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[1]Myrick, A. C. Jr., E. W. Shallenberger, I. Kang, and D. B. MacKay. MS Calibration of dental layers in seven captive Hawaiian spinner dolphins, *Stenella longirostris*, based on tetracycline labels.

Dentine

The neonatal cone, i.e. the "neonatal line" in thin sections, is the first in a series of internally-deposited, layered cones comprising the GLG of postnatal dentine, the number of which is dependent upon the length of the dolphin's life and deposition rate.

As a dolphin matures and dentine continues to accumulate at the wall of the pulp cavity, the pulp-cavity volume decreases gradually and successive GLGs become thinner. Typically, in regions of the late-formed dentine of older dolphins, the tissue shows increasingly irregular GLGs. Farther inward, the dentine may be convoluted and layering may be indiscernible. The pulp cavity tends to be exceedingly narrow or occluded. If a dolphin lives beyond the time that these conditions commence, the dentine may not be useful for estimating the dolphin's age accurately.

Cementum

Cementum is deposited postnatally; it is not known to occur in teeth of unborn dolphins. It is applied in concentric layers to the external surface of the root, below the "neck" of the tooth. Because its deposition is external and apparently unconfined, it is thought to form a continuous record representing a dolphin's entire life. Cementum, then, may be used to estimate maximum ages of older dolphins for which maximum dentinal age estimates are not possible.

PREPARATION OF THIN SECTIONS OF TEETH

Selection of Teeth

In dolphins, the largest, most uniformly layered teeth occur in the central regions of the maxillary and mandibular tooth rows (Hui, 1978). Dentinal layering may cease earlier in maxillary teeth than in teeth of the mandible (Gurevich et al. 1980); therefore, teeth from the center of the lower tooth row are recommended for use in age determination (Perrin and Myrick 1980, p. 40). The standard sampling procedure we use for spotted and spinner dolphins is to remove a four or five-cm-long section containing approximately eight to ten teeth from the middle of the dental series in the left mandibular ramus. Such sampling provides an adequate supply of teeth in case repeated preparations for a specimen become necessary. Any tooth from the mandibular section may be chosen for preparation. It is removed by breaking the alveolar bone away from the tooth. Utmost care is exercised to insure that the tooth is not chipped or otherwise damaged during removal.

Cleaning

Soft tissue is scraped from the tooth to be prepared with a scalpel or fingernail after the tissue has been made pliable by soaking in water. Care is taken to insure that the cementum is not damaged during soft-tissue removal.

Labels

Each tooth is accompanied by an identification label of pure, cotton fiber bond through every step of the preparation procedure. The specimen number is recorded in soft lead pencil.

Containers

Each tooth, with its label, is placed in a clear plastic, hinged container 2.8 cm on each side. To allow the free flow of fluids through the container, all sides are perforated with a teasing needle, or other fine metal probe, that has been heated. The holes must be small enough to prevent thin sections of teeth from being lost.

Decalcification

The teeth are decalcified in RDO[2], a commercial rapid decalcifying agent containing hydrochloric acid as the principal active ingredient. Approximately 10 spotted dolphin teeth, each in its own container, may be decalcified at one time in 500 ml of RDO. (Species in which teeth are larger, e.g., *Tursiops truncatus*, require a proportionately larger volume of RDO per tooth.) To increase circulation, containers are agitated in the solution every 1-3 hours. Decalcification times vary with the age of the animal, because the thickness in the tooth is a function of age, and with the species-specific size of tooth. For spotted dolphins, teeth of the youngest animals, such a neonates and calves, require only 1-3 hours decalcification. These from body-length classes 140-150, 150-160, 160-170, and 170-180 cm can require 3-5, 6-8, 9-13, and 11-13 hours respectively. Teeth of the oldest animals, 180-190+ cm in body length, require 11-15 hours for full decalcification. The 500-ml volume of decalcifier tends to lose strength after 20 hours of continuous use on 10 specimens at a time.

When decalcification is complete, the tooth is flexible and slightly translucent. A fully decalcified tooth may be pierced to the center without resistance with a fine, sharp needle, but this test disrupts the tissue of small teeth and is not recommended for routine use. Undue flexing of the tooth may damage internal tissues. Overdecalcification in RDO also causes damage and renders the tooth useless for age determination.

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[2]Reference to trade names does not imply endorsement by the National Marine Fisheries Service, NOAA.

Complete removal of acid from the teeth is necessary to prevent RDO from interfering with staining. After decalcification, the teeth are rinsed in running tap water by placing the containers in a large beaker under a faucet. The water flow causes the containers to tumble and optimizes circulation through the containers. Rinsing time is at least 3 hours.

Sectioning

Thin sections are cut with a sledge-type freezing microtome. A decalcified tooth is mounted on the cutting stage of the microtome, with the knife-blade set at about a 65° angle to the long axis of the tooth, and frozen with liquified CO^2. Serial longitudinal thin sections, 24 μm thick, are cut and discarded until the pulp cavity is revealed. Sectioning is continued until the entire extent of the pulp cavity is encompassed and thin sections cut subsequently fail to show the central cavity. This process yields a maximum number of near-center thin sections, which are returned to the container and rinsed in tap water to remove any residual RDO. The rest of the tooth is removed from the cutting stage and discarded.

Staining

The container of thin sections is immersed in Mayer's hematoxylin (see Appendix I for formula) for up to 30 minutes, until adequately stained. Excess stain is removed by a tap-water rinse. To enhance the contrast of the stained layers, the thin sections are "blued" in a 0.5% solution of ammonia water for 2-3 minutes. Prolonged exposure to the ammonia will reverse the stain. After blueing, the sections undergo a tap-water rinse for several minutes.

Glycerin exchange

Stained thin sections retain their color best when mounted in pure glycerin. Thin sections and label are first transferred from the container to a petri dish containing 1:1 solution of glycerin and distilled water to soak for 5-10 minutes. Then they are placed in a petri dish of 100% glycerin to complete the exchange of glycerin for water in the thin sections.

Selection of thin sections

The petri dish is placed on the stage of a dissecting microscope and the thin sections to be mounted are selected. Ideally, those selected should be mid-longitudinal. Each section should have a sharply-pointed apex. The dentinal matrix should be light blue, with contrasting darkly-stained and lighter stained layers appearing as sharply-pointed chevrons along the mid-line. Changing the focal depth by adjustments of the focusing knob of the microscope should produce only slight relative displacement of layers and pulp cavity margins. The cementum should reveal more deeply-stained thin layers against a light blue background. Thin sections without a pulp cavity, those stained too dark or too light, or cut off mid-center are not used.

Mounting

Several thin sections are placed in 100% glycerin on a slide. Wrinkles are smoothed out with a small blunt instrument so that each specimen lies flat. Excess glycerin is drained from beneath the cover slip, and cover-slip margins are cleaned with ethanol and sealed to the slide with Permount[2]. Specimen numbers and coded preparation information are recorded in waterproof ink on the frosted glass surface of the slide. The finished slides are placed on racks to dry.

Appearance of the mounted thin section

The thin mantle of enamel originally covering the apex of the tooth is absent, having been dissolved completely during decalcification. The prenatal dentine occurs as a darkly-stained, relatively unlayered chevron, thickest at the apex, with two tapered legs pointed basally. Adjacent and internal to the prenatal dentine, the neonatal line appears as a thin, distinctly unstained chevron. The remainder of the postnatal dentine occurs as a series of internally-nested, elongate chevrons of decreasing thickness with alternating darkly-stained and lightly-stained layers (Fig. 2A).

Cementum occurs along both sides and at the base of the root external to the dentine. Its pattern consists of alternating darkly-stained and lightly-stained layers oriented subparallel to the root and off-lapped basally (Fig. 2B).

DEFINING DENTINAL GLGs

GLG appearance

Ideally, each of our GLGs, (which in these species we have calibrated as representing one year-- see above), consists of an external, thin, lightly-stained or unstained boundary layer (beginning with the neonatal line), followed in succession pulpwards by a thick, dark-stained layer, another thin, lightly-stained mid-GLG layer (usually less distinct than the boundary layer), and a second thick, darkly-stained layer. A GLG is considered to be complete when it contains all four layers (Fig. 3).

Commonly, however, the four components are variably visible. In most regions of the first several GLGs, the two thick darkly-stained components may be light near the mid-GLG layers and darkest at the margins near the boundary layers. This gives the GLG an appearance of containing only the light boundary layer and one broad layer that is stained slightly darker. Examination of the GLGs through their entire longitudinal extent, especially in the apical region where components may be followed basally, often permits identification of all or some of the layers. Usually, at least the boundary layer or the contrasting dark margin of an adjacent darker-stained layer is recognizable. Such landmarks are most reliable in delimiting a GLG. Despite the problem that all GLG components are not equally apparent, we have found that the "ideal" pattern and the progressive thinning of the GLGs pulpwards are important aids in defining GLGs. For adjacent GLGs, the later-formed GLG should be the same thickness or thinner than the earlier-formed GLG.

The eighth to sixteenth GLGs are almost uniform in thickness, less variable in component pattern, and easier to distinguish. Usually, their boundary layers or strong, dark adjacent margins are sharply demarcated.

Age-specific thickness of GLGs

Studies of tetracycline-labeled teeth of seven Hawaiian spinner dolphins, S. longirostris, have shown that each GLG (as defined above) has a rather specific thickness and that the thickness of a given GLG varies little between teeth of the same individual or between teeth of different individuals (Myrick et al. MS[1]). GLG-thickness measurements were made perpendicular to the long axis of the teeth, from the base of the neonatal line, inwardly and downwardly at a 20 to 40° angle to the wall of the pulp cavity. Measurements made on the labeled Hawaiian spinner teeth yielded the following thicknesses for GLGs:

	$\bar{x}$		S	N*		$\bar{x}$		S	N*
First GLG	240.0	μm	0	7	Ninth GLG	61.3	μm	2.5	4
Second "	238.6	"	3.8	7	Tenth "	58.1	"	2.4	4
Third "	171.0	"	11.0	7	Eleventh "	55.0	"	5.0	3
Fourth "	145.0	"	10.5	6	Twelfth "	65.0	"	-	1
Fifth "	119.6	"	8.4	6	Thirteenth "	55.0	"	-	1
Sixth "	95.4	"	16.5	6	Fourteenth "	40.0	"	-	1
Seventh "	87.0	"	5.6	6	Fifteenth "	40.0	"	-	1
Eighth "	71.3	"	10.3	4					

*Sample sizes of Hawaiian spinner dolphins used.

Age-specific measurements of GLG thickness have been shown to have some constancy in S. longirostris (Perrin et al. 1977), Pontoporia blainvillei; (Kasuya and Brownell, 1979), and Tursiops truncatus (Hohn, 1980b), although neither labeled nor known-age specimens were studied.

Constancy of GLG thickness for spotted and spinner dolphins

GLGs in wild northern offshore spotted dolphins appear to have the same component-layer pattern and thicknesses as those in the Hawaiian spinner dolphins studied by Myrick et al. (MS[1]). As shown in Figure 4, when the dentine from one of the captive Hawaiian spinner dolphins is compared with that of a wild spotter dolphin the resemblance between the two patterns is striking. When the GLG measurements determined from the captive sample were applied to the dentinal pattern of spotted dolphins, they corresponded approximately with the boundary layers of each of the first 15 GLGs. By continuing to apply the same last measurement on subsequent GLGs, boundary layers for up to the eighteenth GLG could be identified in cases where layering remained undistorted. Despite the problem that experimental work on time calibration of dentinal layering has been possible only for the captive Hawaiian spinner dolphins, until known-age or labeled spotted dolphins become available, we assume that a GLG represents the same amount of time in spinner and spotted dolphins.

Extrapolating in this way provides a method for definition of GLGs in spotted dolphins. Nevertheless, because some variation occurred in GLG thickness between individual spinner dolphins studied and because the degree of individual variation is unknown for spotted dolphins, the series of measurements is recommended only as an aid in locating GLG boundaries.

Methods used to count dentinal GLGs in spotted dolphins

To identify and count dentinal GLGs in the teeth of spotted dolphins, two slightly different methods have been used (by Hohn(AAH) and Myrick(ACM)).

a. Method 1(AAH). The whole tooth section is first inspected to determine the general pattern of layer groups. Then the layer groups are examined to define GLGs. Use is made of the "ideal" GLG pattern of component layers, depending heavily on the position and spacing of the dark margins of the darker-stained layers and on the progressive narrowing of layer groups pulpwards. The first two GLGs are identified using the prenatal dentine as a guide. Each of these GLGs is approximately the thickness of the prenatal dentine measured at a point about two-thirds of the distance down from the apex to the basal end of the prenatal wedge. All GLGs are examined throughout their longitudinal extent before making final interpretations.

The chief advantages of this method are that: 1) it does not require an external aid that may affect interpretations if applied rigorously, 2) no single region is used principally to locate boundaries, and 3) GLG distortion has a minimal effect on final interpretations. However, locating boundary- and other component-layers may be difficult in regions with faint dentinal layers and some GLGs may be overlooked, resulting in an understimate of age. The relative order of magnitude of decreasing GLG thicknesses is evaluated without the aid of quantified reference points. In addition, the method is not extrapolated from real-age studies of related species.

b. Method 2(ACM). The whole tooth section is inspected to characterize the pattern of layer groups. Although not applied rigorously, the series of GLG measurements derived from the study of Hawaiian spinner dolphins is used as an aid in locating GLG boundaries. The segment of dentine enclosed by each successive measurement is inspected for GLG-component layers throughout its longitudinal extent and in relation to the general layering pattern of the whole tooth. Adjustments to the "standard" thicknesses are made on the basis of the general dentinal pattern and specific layer-component patterns observed relative to the "ideal" pattern. The first measurement inward of 240 μm from the prenatal edge of the neonatal line, taken at a point near the base of the prenatal dentinal wedge, gives the approximate distance of the external, thin, lightly-stained boundary layer of the second GLG. A 240-μm measurement from the adjusted boundary layer gives the approximate distance to the external boundary of the third GLG. Successive GLG boundaries are located using the appropriate standard thicknesses as a guide.

The advantages of the method are that: 1) progressive thinning of GLGs can be quantified, aiding decisions about the limits of successive GLGs, 2) it provides a means of locating GLGs in faintly stained regions with relative ease, 3) it is extrapolated from GLG thicknesses in a related species with a similar dentinal pattern for which real time was determined. Nevertheless, in applying the method rigorously to define GLGs, identification of component layers may tend to be forced by anticipation of the pattern. This is especially true in cases where layers are variably visible or compressed and indistinct. In addition, GLG thicknesses are not uniform from apex to base. Natural regional distortion in a tooth or imposed distortion from off-center sections affect GLG thickness, causing variability to be high.

Differences between the methods

Method 1 uses dark-stained boundary layers. Method 2 uses light boundary layers. Method 1 uses no external aid, but does use the prenatal thickness to estimate the limits of each of the first two GLGs. Method 2 uses the measurements as a guide. Method 1 was developed without extrapolation from real-time documentation of GLGs. Method 2 is aided by real-time documentation of GLG thickness in teeth of similar pattern representing a related species.

Similarities between the methods

Despite the differences, both methods are basically similar. GLGs are defined with reference to the same "ideal" pattern and progressive GLG thinning. Both methods require inspection of the whole tooth to identify general layer group patterns. Both rely on position and spacing of boundary layers. Both methods have resulted in the determination that the first two GLGs are of similar thicknesses. Finally, both evaluate subsequent layer groups subjectively after the regular layering pattern ceases.

CEMENTUM

In general, each cemental GLG consists of two components: a darkly stained layer and a lightly stained layer. The problem with such a simple definition is that although the number of cemental GLGs often may equal the number of dentinal GLGs counted, they are not always equal. There is no regular thickness for a cemental GLG as there is for a dentinal GLG. In several regions along the tooth, cementum may contain GLGs in multiples, i.e. with two or three times the number of GLGs observed in the dentine. In other regions it may be thin, with no distinct layering or with only half the number of GLGs observed in the dentine. Myrick et al. (MS)[1] have made similar observations in Hawaiian spinner dolphin teeth using tetracycline labels to calibrate cementum and dentine.

SUGGESTED PROTOCOL FOR DETERMINING AGE FROM GLGs

The finding that cemental GLGs may occur in multiples or halves and the problems with pulp-cavity occlusion or layer indistinctness in dentine point up the hazards of using either tissue independently for age determination. Before analyzing the layering pattern of cementum, we recommend that one should first evaluate the dentine and condition of the pulp cavity. In teeth with clear, regularly-formed dentinal GLGs and with an open pulp cavity, dentinal GLG counts should be relied upon for estimating age. The cemental count may be used to check the count from dentine; it should be the same as, or half, or exact multiples of the dentinal count. In other cases, cementum should be relied upon for the best estimation of age, with the best dentinal count used to evaluate whether the cemental GLGs were deposited as multiples or halves in the cementum.

Specifically, we recommend the following protocol:

1. If the pulp cavity is open:

   a. and regularly layered dentine is present throughout the tooth, the dentinal count should be used as the best indicator of age; the cemental count should be used to check the dentinal count. If the cemental count is half, two times, or three times the dentinal count, assume that the cementum has been deposited as halves or multiples of the dentinal GLGs.

   b. and irregularly layered dentine is present, preventing a complete count of dentinal GLGs, count the dentinal GLGs as fully as possible, then count the cemental GLGs. Use the number of cemental GLGs as the age if it is less than or more than an exact multiple of the dentinal count. If it is equal to a multiple of the dentinal count, use the dentinal count as the age. If the maximum cemental count is much less than, but greater than half of the dentinal count, assume that cemental GLGs are halves and double the number to estimate age. If the cementum is unlayered or indistinctly layered, the only age estimate available is a minimum age as indicated by the dentine.

2. If pulp cavity is narrow or almost occluded:

   a. and regularly layered dentine occurs throughout the section, use the same procedure as in 1a. An exception is in cases where the cemental count is more than the dentinal count but less than a multiple or more than half of the dentinal count, then use the same procedure as in 1b.

   b. and irregular dentine is present, use the same procedure as in 1b.

3. If pulp cavity is closed or is filled with irregularly layered dentine, use the same procedure as 1b.

Since all of the criteria for final determinations of age rest chiefly on the accuracy of the dentinal counts, it is essential that careful evaluation of the pulp-cavity condition and scrupulous definition of dentinal GLGs be carried out. A tooth in which the pulp cavity is interpreted incorrectly as being closed and in which the dentinal GLGs are miscounted, may lead one to use the count of cemental GLG multiples as the estimated age.

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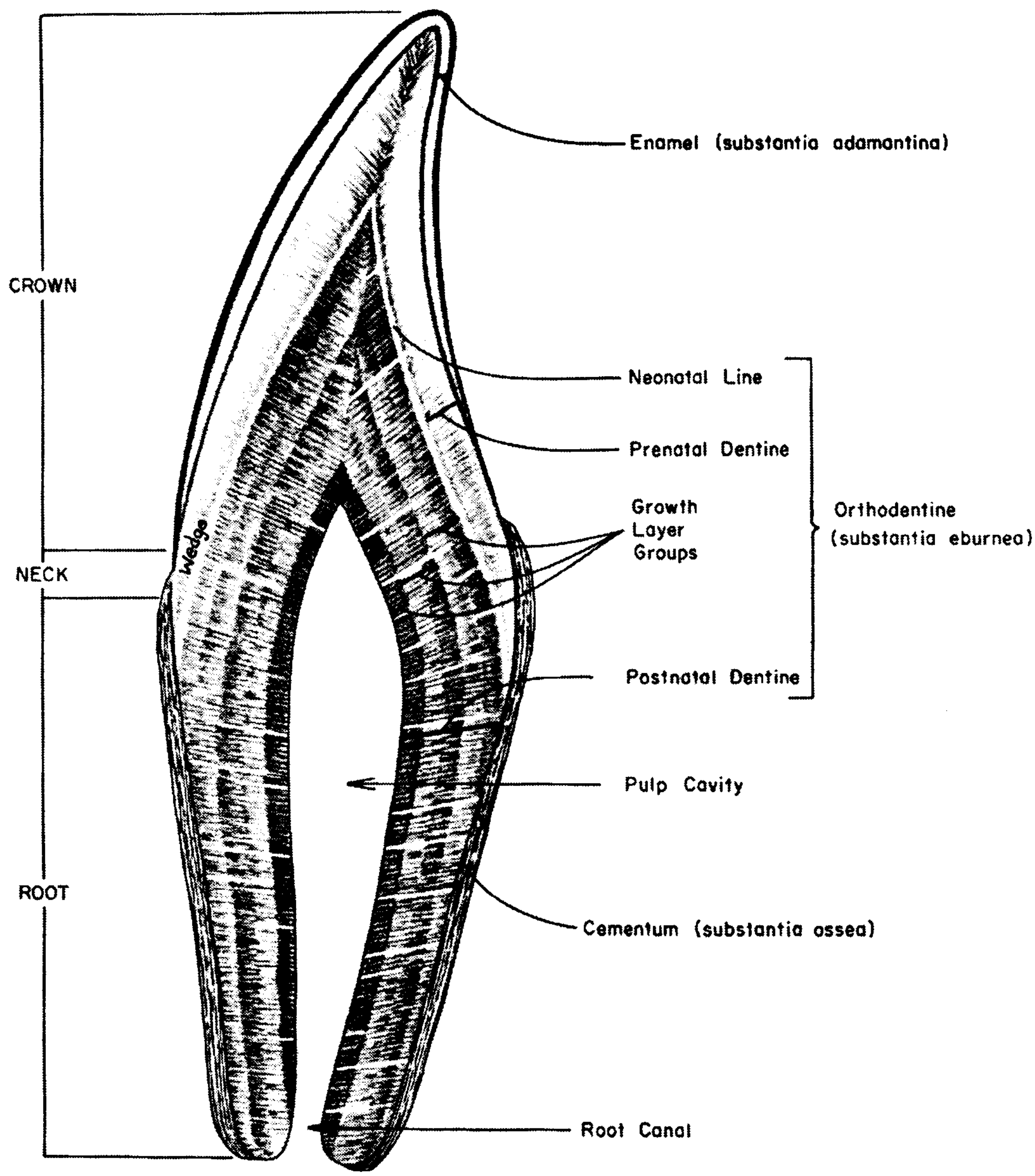

Fig. 1. Line drawing of dolphin tooth in thin section showing distribution of tissues and structural anatomy (from Perrin and Myrick, 1980).

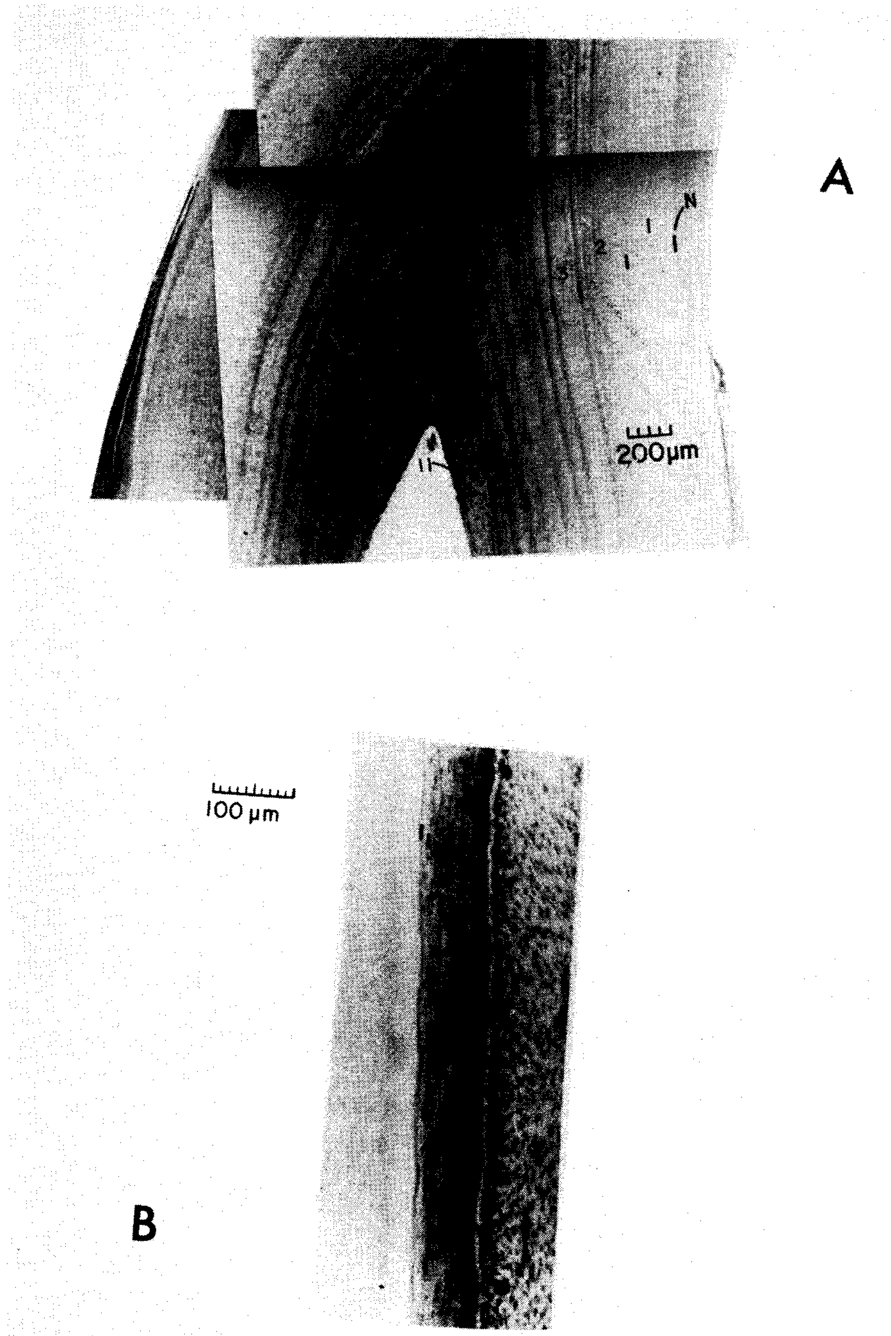

Fig. 2. Decalcified and stained thin section of tooth from a female northern offshore spotted dolphin (specimen no. SWM 0001) viewed in plain transmitted light (composite photograph).
A. Dentine showing 11 GLGs. (39X) N=neonatal line.
B. Cementum showing 11 GLGs. (150X)

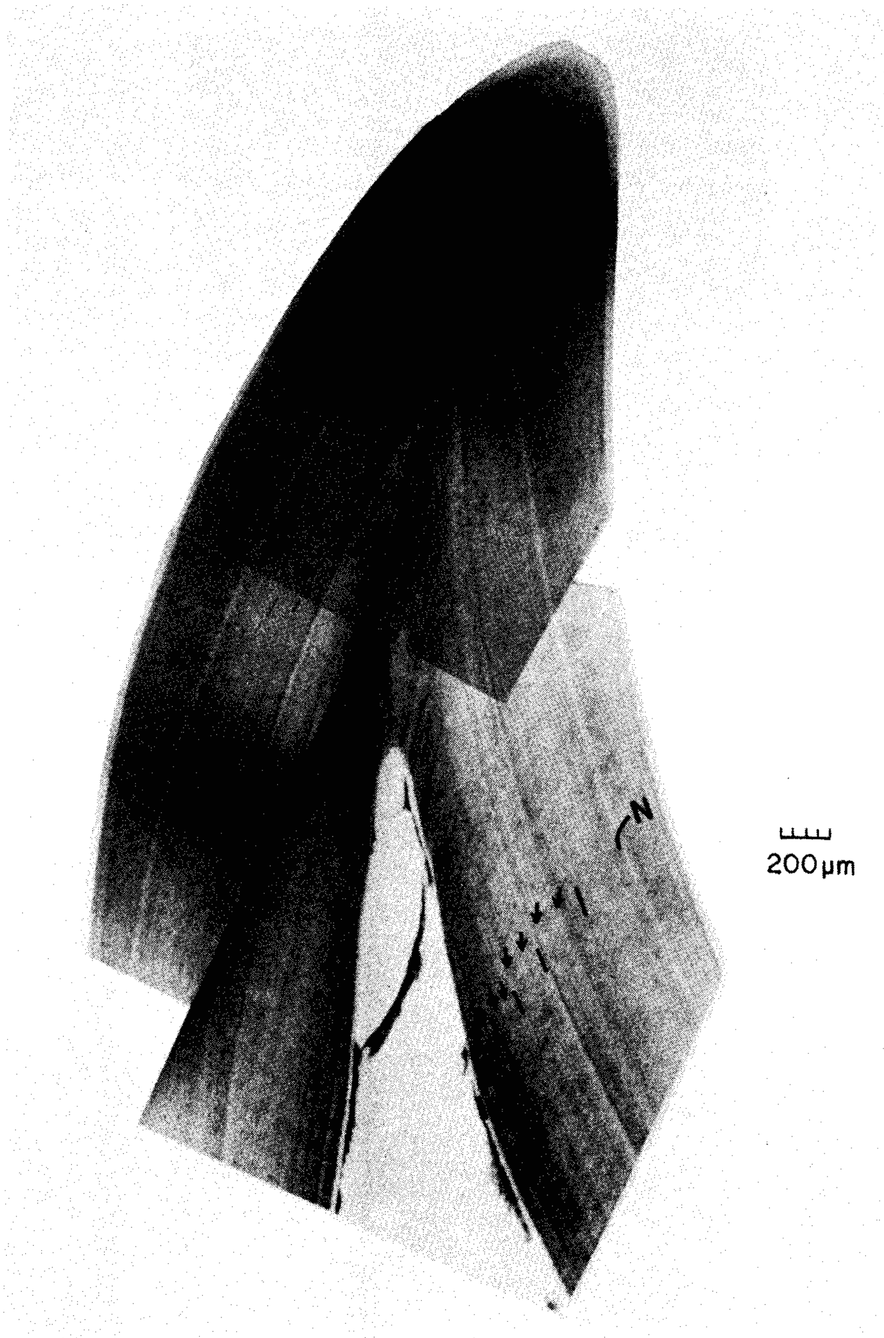

Fig. 3. Decalcified and stained thin section of tooth from a six-year-old female northern offshore spotted dolphin (specimen no. SER 0059) showing six dentinal GLGs (composite photograph). Each GLG contains two dark-stained layers (arrows) (plain transmitted light, 39X).

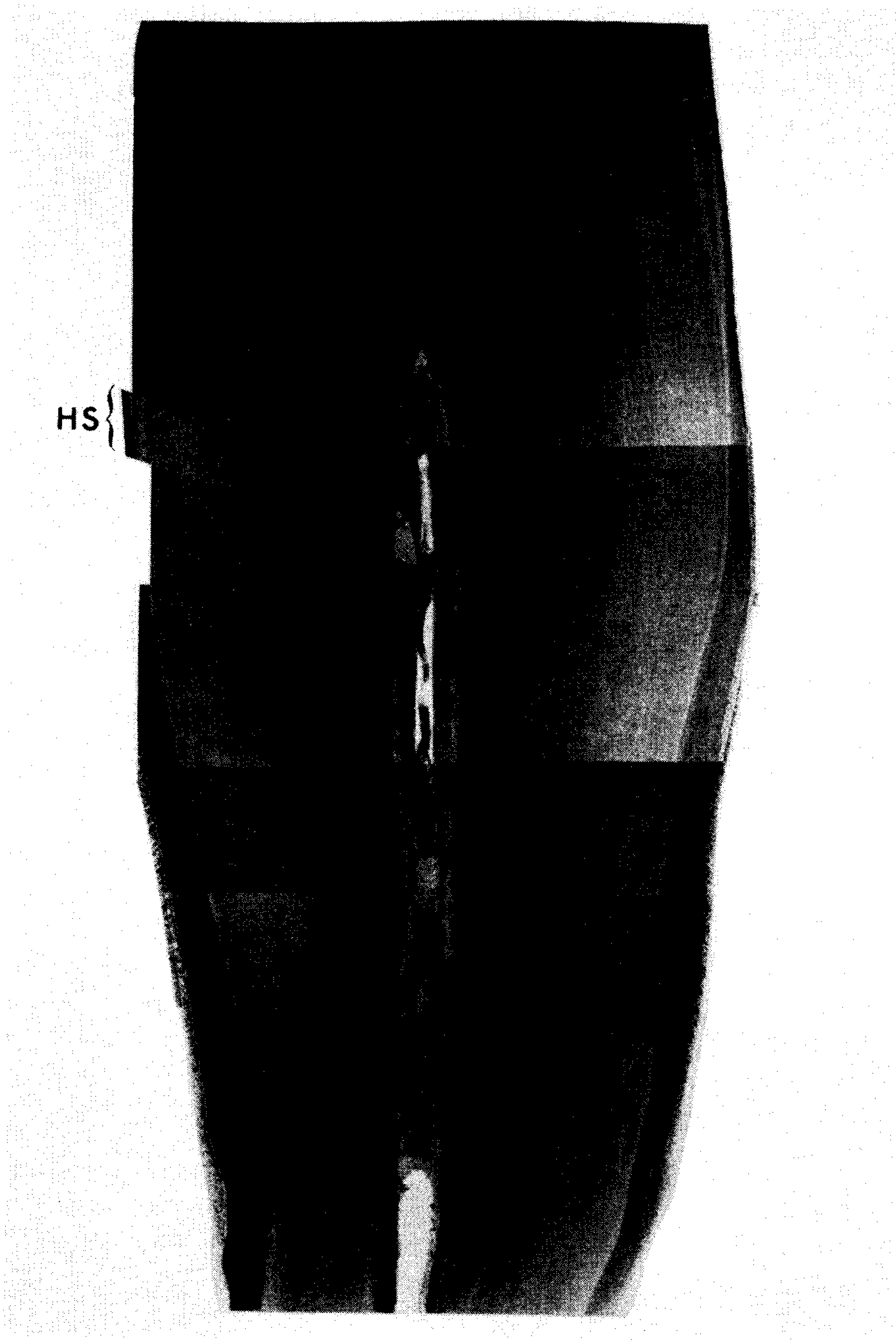

Fig. 4. Swatch from thin section of tooth of a captive Hawaiian spinner dolphin (specimen no. SWFC 104) imposed upon left "shoulder"(HS) of thin section from a northern offshore spotted dolphin (specimen no. WHB 0025) taken from the wild, showing almost identical dentinal layering patterns (composite photograph).

APPENDIX 1. Formula for Mayer's hematoxylin (Modified by Helen Grue)

Hematoxylin	1.0 gm
Distilled water	1000.0 cc
Sodium iodate	0.1 gm
Aluminum potassium sulfate (alum)	50.0 gm

Dissolve hematoxylin in water, using gentle heat if necessary. Add the sodium iodate, then the alum. Stir until the alum is dissolved. Store stain in the refrigerator to retard overripening. Stain may be used immediately after preparation.

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29 Report of eastern tropical Pacific research vessel marine mammal survey, May 15-August 3, 1982.
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