Calibrating Dental Layers in Captive Bottlenose Dolphins from Serial Tetracycline Labels and Tooth Extractions

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INTRODUCTION

Scientists studying marine mammal populations have become increasingly dependent on age estimates from tooth layer counts to assess the population dynamics (Scheffer and Myrick, 1981). Such dependence has underscored the importance of understanding the patterns and rates of tooth layer deposition.

Cetacean teeth have complex patterns of layers, or growth layer groups (GLGs, terminology of Perrin and Myrick, 1981), which are similar to each other in detail. The GLG has been assumed to represent deposition during a 1-year period or some other constant unit of time (Sergeant, 1959). However, neither GLGs nor the variably prominent finer layers that they contain can be interpreted with certainty until GLG patterns are calibrated with units of absolute time.

Rough calibrations of GLGs with absolute time can be achieved by using layers known to have been deposited between two dates (Myrick, 1981a; Myrick *et al.*, 1984; Hohn, Chapter 33, this volume). However, calibration based only on two dates (usually birth and death) gives only a mean annual rate that provides no information on whether the GLG pattern and formation period are dependent on endogenous or exogenous factors. If rates are factor-dependent, then GLGs cannot be used with confidence to estimate ages unless the nature and timing of the factors are known.

Three techniques have been used to calibrate GLGs with two dates. In one, layer patterns were examined in single teeth from a few captive-born (known-age) bottlenose dolphins, *Tursiops truncatus* (Sergeant, 1959; Sergeant *et al.*, 1973; Hui, 1978). In another, a single lead acetate or tetracycline label was introduced into the layering pattern, and tooth layers deposited after the label

The Bottlenose Dolphin.

were examined (*Stenella coeruleoalba*, Nishiwaki and Yagi, 1953; *Phocoena phocoena*, Nielsen, 1972; *Lagenorhynchus obscurus*, Best, 1976; and *Delphinus delphis*, Gurevich *et al.*, 1981). In the third, two teeth from a bottlenose dolphin were extracted a few years apart, and the difference in the number of GLGs was noted (Hui, 1978).

The successful uses of these techniques have led to recommendations calling for more sophisticated studies that include combinations of the following: (1) monitoring captive-born animals, whose ages are exactly known, together with wild captives of the same species to compare GLG patterns; (2) introducing tetracycline labels weeks or months apart over several years to mark segments of time in tissue that could be studied alone or with other segments; (3) studying the timing of unique or repeated physical events to determine any association with changes in GLG patterns during a monitored experimental period; and (4) using serial extractions to compare tissue deposition between teeth from one animal as well as between teeth of different animals to detect similarities and differences produced over the same period (Myrick, 1981a,b; Myrick *et al.*, 1984).

In addition, after it was realized that multiple tetracycline labels may be inadvertently produced in teeth of captive dolphins through repeated therapeutic treatments of the animals, Myrick (1981a) suggested studying layering rates retrospectively by matching labels to treatment dates from the medical records maintained by oceanaria. Myrick *et al.* (1984) conducted such a study of seven Hawaiian spinner dolphins (*Stenella longirostris*) (four dead, three living), held at Sea Life Park in Hawaii, that had been treated intermittently with tetracycline for up to 8 years. To ensure that at least some labels could be documented if therapeutic records and labels could not be matched, experimental injections of tetracycline were given and three tooth extractions were made during a 1-year period for each of the three live animals.

The Hawaiian spinner dolphin study showed annual dentinal GLGs to be of age-specific thickness, which varied only slighty from tooth to tooth in each individual and between animals of the same age. Annually deposited dentine revealed that a GLG consists of two light layers, each followed by a dark layer. In addition to the coarse GLG pattern, about 13 pairs of finer layers were visible in annual GLGs, each pair consisting of a dark layer and a light layer. Where fine layers were especially distinct and tetracycline labels closely spaced, label dates showed that a pair of fine layers was formed each month. No differences were noted between the GLG patterns formed while the animals lived in the wild and the patterns formed in captivity. This led to the conclusion that neither the captive environment nor the natural environment influenced dental layering patterns in the specimens studied (Myrick *et al.*, 1984).

In 1979, as part of a continuing effort to examine GLG patterns and deposition rates in delphinids, we began a 3.5-year project to calibrate tooth layers in bottlenose dolphins. Our two objectives were to use multiple tetracycline injections and tooth extractions in captive bottlenose dolphins to monitor rates of tooth tissue deposition, in relation to measurable factors that might affect deposition, and to calibrate GLGs with absolute time. Here we describe the protocol and results of the project.

METHODS AND MATERIALS

The Sample

We used data from 12 bottlenose dolphins (three females and nine males), hereafter called "animals" or "dolphins," maintained for public display at the three parks operated by Sea World, Inc., in San Diego, California (SWSD), Orlando, Florida (SWO), and Aurora (near Cleveland), Ohio (SWA). The dolphins ranged from captive-born yearlings to wild animals that had been held in captivity for up to 9 years (Table 1). All but one of the wild animals were collected from coastal waters off Florida; the other was captured in the Pacific Ocean off California. One of the three females (animal M) died 1 year and 3 months after the project began.

Tetracycline Labeling and Tooth Extractions

Treatments, begun in the spring of 1979, were of four types: tooth extraction and tetracycline labeling, tetracycline labeling only, extraction only, and

Table 1
Background Data and Age Estimates of 12 Captive Bottlenose Dolphins Used in Dental Layer Calibration Project

Animal	Sex	Origin[a]	Date of Birth or Capture	Capture Length/Age or Age By GLG Inspection (Years)[b]	Preproject Time in Captivity (Years)	Age at End of Project (Years)
A	M	cb:P/F	5-78		1.1	4.5
B	M	cb:F/F	5-78		1.1	4.5
C	M	cb:F/P	4-78		1.2	4.6
D	F	wc:F/F	9-77	195 cm/2	1.8	7.2
E	M	wc:F/F	1-77	201 cm/2	2.3	8.0
F	M	wc:F/F	10-75	2	3.7	8.5
I	F	wc:F/F	6-70	1–2*	9.0	14.0
J	M	wc:F/F	12-72	3–4*	6.4	13.0
M	F	wc:F/F	2-79	196 cm/2	0.3	3.0[c]
P	M	wc:P/P	2-78	3*	1.3	7.0
Controls						
K	M	wc:F/F	12-72	1–2	6.4	11.0
L	M	wc:F/F	11-72	4	6.5	14.0

[a] cb, Captive born; wc, wild captured; P/F or F/P, hybrid; origin of mother/sire; P/P, Pacific purebred; F/F, Florida coastal purebred.
[b] An asterisk indicates that age was estimated based on GLG counts relative to label introduced at capture.
[c] Animal M died in the second year of the project.

handling (i.e., sham procedures) only. Biomycin, a tetracycline, was the labeling agent used; it was injected into the dorsal musculature slightly off the midline of the body and anterior to the dorsal fin, at a dosage of 20–30 mg/kg body weight. Teeth were extracted from the middle of the row of either lower jaw using anesthetization and extraction methods similar to those described by Ridgway *et al.* (1975) and used successfully on Hawaiian spinner dolphins by Myrick *et al.* (1984).

Each animal had one tooth extracted and received one injection at the beginning of the study. All except the two animals (K and L) that were used as controls received tetracycline injections approximately every 3 months and had one or (occasionally) two teeth extracted each year for the next 3.5 years. All intact teeth were permanently labeled each time tetracycline was injected. A label introduced after the first tooth was extracted appeared in the second and third teeth and so on, but not in the first tooth (Table 2). In sham procedures, dolphins were lifted out of the water, weighed, measured, and returned to the pool. Sham procedures were done at random in lieu of real treatment or extraction to determine whether merely removing an animal from the water and weighing and measuring it affected its tooth-layering patterns.

The two control animals were not experimentally treated after the initial treatment and extraction at the start of the project. A second tooth was extracted from them only at the termination of the experiment (fall 1982) when a final extraction was carried out on all animals involved in the project.

Dolphin Health and Environment

We recorded average weekly changes in water temperature and salinity, and the average daily amount and species of food consumed by each animal, to identify any effects of these factors on dental-layering pattern or deposition rate (water

Table 2
Extraction and Labeling Schedule for Animal F as an Example of How Serial Data Were Generated for Analysis

Date or Period	Tooth Extracted	Labels Present	Labels Introduced
6-5-79	F1	None	A
9-7-79			B
9-11-79 to 10-9-79 daily			C (therapeutic)
11-1-79 to 11-4-79 daily			D (therapeutic)
12-3-79			E
3-10-80			F
5-28-80	F2	A through F	G
9-5-80			H
12-4-80	F3	A through H	I
12-10-80			J (therapeutic)
3-5-81			K
6-9-81			L
9-8-81			M
12-14-81	F4	A through M	N
1-15-82			O (therapeutic)
3-5-82			P (therapeutic)
6-4-82			Q
9-7-82			R
1-11-82	F5[a]	A through R	None

[a] Last tooth extracted; end of project.

temperatures and salinities were artificially controlled at Ohio and Florida parks). In addition, daily records were made of behavior, including type of activity (e.g., free feeding, training, show), periods of illness and treatment, and within-park transfers from one tank or pool to another. Finally, because SWA is 11° latitude farther north than SWSD and 14° latitude farther north than SWO, we tracked transfer of dolphins between parks (interpark transport) to try to detect possible effects of long-distance transport or changes in latitude on dental-layering patterns.

Tooth Preparation and Examination

Preparation and examination of teeth followed the methods described by Myrick *et al.* (1983). Each tooth was prepared in untreated mid-longitudinal thin section between approximately 100 and 150 μm thick. In a few cases, decalcified and hematoxylin stained thin sections, about 40 μm thick, were prepared from the remaining fragments (leftovers) to enhance resolution of layering patterns. Microscopic examination was in plain and polarized transmitted light to determine layering structure and in UV reflected light to locate tetracycline labels, visible as fluorescent lines (Fig. 1A,B).

The best sections of the series of teeth extracted from each animal were photographed in plain and UV light, and their interstructural (e.g., between the neonatal line and the pulp-cavity margin) and interlabel distances were measured. Distance measurements (in micrometers) were taken in a step-down fashion toward the pulp cavity from the neck region of the tooth near the base of and perpendicular to the neonatal line (Fig. 1B; see

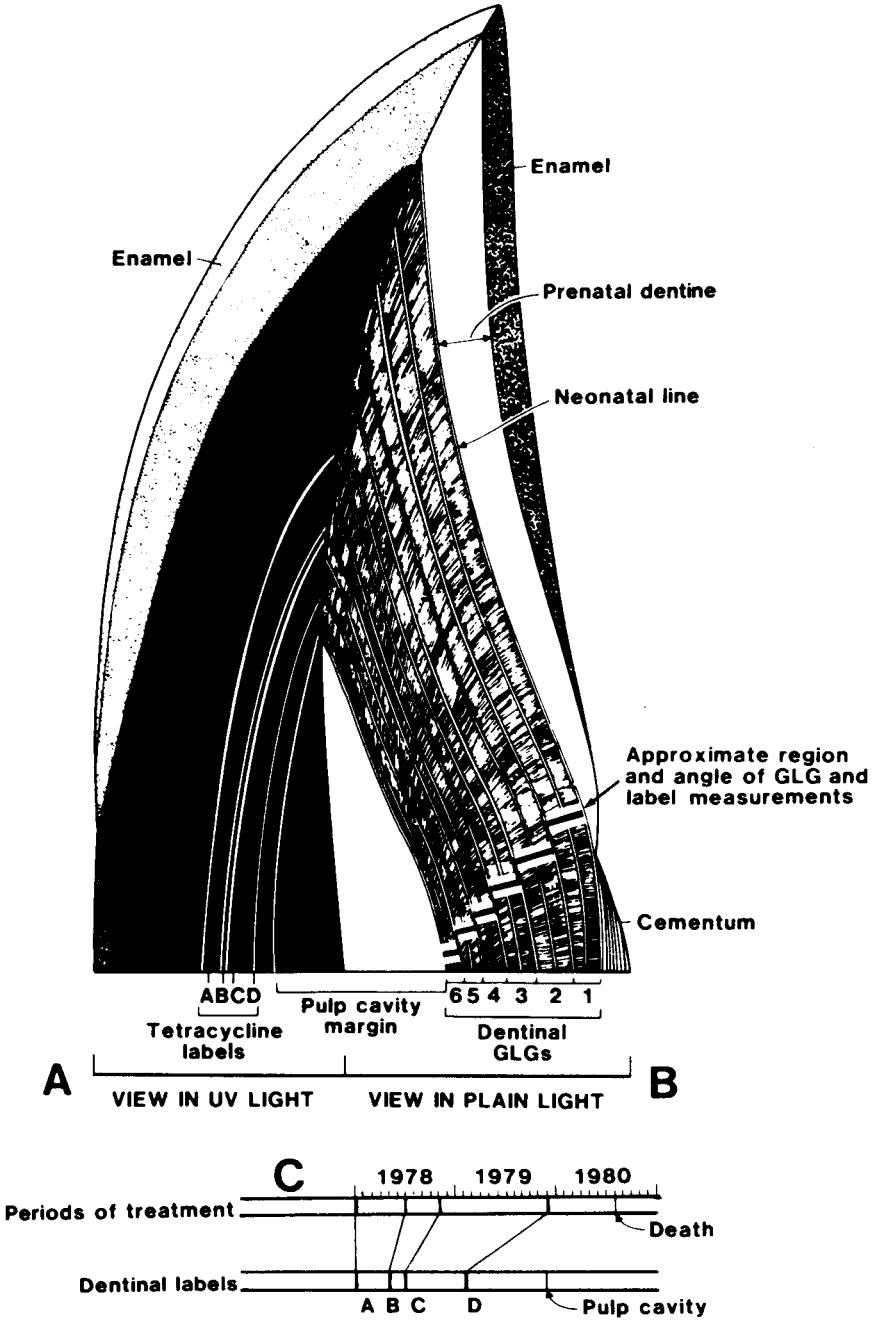


Figure 1 Diagram of tooth thin section from hypothetical project dolphin showing tetracycline labels, A–D under ultraviolet light (A, left-hand side) and dentinal growth layer group (GLG) layering patterns under plain light and standard positions in tooth where labels and GLGs are measured (B, right-hand side). (C) Method of identifying labels introduced into tooth and determining elapsed time between labels by comparing relative spacing of labels. (Modified from Myrick *et al.*, 1984.)

also Myrick *et al.*, 1984). (In this region the layers are usually the least distorted, neither highly expanded as in the apex, nor strongly narrowed as in the root base.)

The mean of three to five series of measurements was used for final values of GLG thickness (Table 3). Each series of thickness measurements was taken at a different time to minimize measurer bias. At least one full series of measurements was taken from the last tooth pulled from an animal because it contained the complete continuous record of the project period. Because the full 3.5-year monitored record had not been completed in teeth extracted before the end of the project, measurements taken from such teeth were augmented by measurements from teeth that were extracted later in the project, including the last tooth extracted. This helped to ensure, as well as possible, that GLG and interlabel distances in most of the teeth extracted from each animal were represented in calculating final mean values.

Data Analysis

Label locations on the UV micrographs were marked on clear plastic overlays, which were then aligned on the corresponding plain light micrographs to determine the location of the labels among the layers. Label positions for each animal were transcribed onto a calibration chart as a row of vertical lines, each separated from others and tooth landmarks (such as the neonatal line) on a scale representing 10-μm intervals. Above this row of label distances, the treatment and extraction data were transcribed as a row of lines scaled in months, representing time of treatment. The two data rows completed for each animal were used to identify corresponding project treatments and labels, designated alphabetically in the order that they were introduced (Fig. 1C).

Time-Calibrated Dentinal Patterns

To define annual or subannual depositional segments within the dentinal patterns, distance measurements between labels spanning a year or half-years were added together. Because the animals were often treated on different days or months, spaces between labels in the teeth represented various lengths of time. These different time-calibrated segments provided many opportunities to examine layering patterns in the continuum within 3.5 years of monitored deposition. Repeating patterns of yearly deposition could be identified, annual GLGs could be defined, and time represented by GLG component (fine) layers could be investigated.

Monthly Depositional Rates

There were enough data points from most of the dolphins to calculate average monthly depositional rates by dividing segment distance (in micrometers) by elapsed time of segment deposition. Although all twelve project animals were useful in determining annual GLGs, only eight of the twelve animals were useful in studying monthly rates. This was because the two controls did not have enough labels, and in one other animal (J) distances between labels were so short that measurements lacked precision. For the fourth animal (M, which died after 1 year and 3 months), the period was too short to yield enough data. For each of the eight measurable animals, average monthly depositional rates were compared with season, water temperature, and salinity data, and with periods of illness, behavior and feeding, and interpark transport.

Calibration of Preproject Layers

Because nine of the animals were captured at about 2 years old or older, the earliest formed layers were accumulated before capture and it was not possible to calibrate those GLGs directly. We characterized the patterns of initial GLGs exhibited in the three captive-born animals (A, B, and C), which, by the end of the project, had four and a half completely documented GLGs. However, we were reluctant to use these patterns before interpreting initial annual GLGs in the remaining animals for three reasons: (1) two of the captive-born dolphins were hybrids (mother and sire were from different populations), and it was not known whether hybridization affected dentinal-layer patterns; (2) all three had always been in captivity,

Table 3
Number and Mean Thickness (μm) of Completed Annual Growth Layer Groups in Dentine of 12 Captive Bottlenose Dolphins[a]

	GLG Number														
Animal	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
M	900*	500*	343												
	(41)	(44)	(69)												
	4	4	5												
A	858	536	458	461	( )										
	(38)	(56)	(19)	(11)											
	4	3	3	3											
B	881	500	348	375	( )										
	(22)	(56)	(38)	(21)											
	4	4	4	4											
C	471	327	298	281	( )										
	(13)	(15)	(5)	(13)											
	5	5	5	5											
D	825*	500*	370*	340*	234	239	190	( )							
	(35)	(0)	(28)	(14)	(11)	(18)	(10)								
	3	3	3	3	5	5	5								
P	505*	375*	350*	339*	352	350	337	( )							
	(10)	(50)	(0)	(14)	(22)	(35)	(32)								
	4	4	4	4	4	3	3								
E	875*	500*	425*	347*	300*	303	215	211	( )						
	(29)	(0)	(25)	(42)	(35)	(16)	(11)	(14)							
	4	3	5	4	5	5	5	4							
F	925?	500?	450?	375?	300?	195	291	191	( )						
	(29)	(0)	(0)	(29)	(0)	(31)	(41)	(53)							
	4	3	3	4	3	5	5	5							
I	813*	465*	440*	360*	300*	280*	225*	195*	180*	185*	170*	185	245	237	( )
	(35)	(21)	(10)	(10)	(0)	(0)	(15)	(15)	(0)	(25)	(65)	(39)	(66)	(32)	
	3	3	3	3	3	3	3	3	3	3	3	3	3	3	
J	890*	463*	450*	407*	353*	293*	230*	220*	207*	187*	167	140	143		
	(17)	(12)	(0)	(23)	(25)	(12)	(10)	(20)	(12)	(12)	(29)	(10)	(12)		
	3	3	3	3	3	3	3	3	3	3	3	3	3		
Controls															
K	817?	520?	450?	373?	320?	240?	237?	182?	160	137	(117)	( )			
	(58)	(27)	(0)	(58)	(35)	(30)	(35)	(17)	(10)	(15)	(6)				
	3	3	3	3	3	3	3	3	3	3	3				
L	820?	483?	450?	417?	407?	367?	283?	263?	243?	170?	170?	150	130	125	
	(27)	(29)	(0)	(58)	(51)	(29)	(29)	(47)	(60)	(17)	(17)	(0)	(0)	(0)	
	3	3	3	3	3	3	3	3	3	3	3	3	3	3	
Florida coast averages (A, B, D, E, F, I, J, K, L, M) (up to 14 GLGs)															
	862	497	418	384	316	274	239	210	198	170	156	158	173	181	
	(40)	(23)	(46)	(39)	(54)	(55)	(36)	(30)	(36)	(23)	(26)	(24)	(63)	(80)	
Pacific coast averages (C, P) (7 GLGs in one of two)															
	488	351	324	310	352	350	337								
	(24)	(34)	(37)	(41)	(0)	(0)	(0)								

[a] ?, Estimated annual GLG by inspection plus time in captivity; *, estimated annual using some documentation; empty parentheses indicate additional partial GLG present; values are means calculated from three to five measurements of GLG thickness; parenthetical values are rounded standard deviations of mean values; third row of values for each specimen is number of measurements used to calculate the mean.

and the effects of captivity were not known; and (3) the dentinal thicknesses of the first three GLGs in one (animal C) of the two hybrids was obviously different from those of the other two captive-born animals (A and B).

To try to calibrate preproject and precapture GLGs of the wild animals, we used estimated age at capture based on capture body length, or on increasing body lengths during the project, plus time in captivity to get a total age in years. (We knew that each animal must have been at least 1 year old when captured and thus the preproject age would have had to be at least 1 year plus total time in captivity.) We then looked for the same number of repeating layer groups as our age estimate. If our GLG count was one or two different than the age in years estimated for a young animal based on body length, we concluded that the body-length age was incorrect and that the GLGs were annual units. For a hypothetical example, based on a capture body length of about 225 cm, an animal estimated to be 3 years old is held captive for 1 year before its first label is introduced. Its teeth should have approximately four repeating GLG units between the neonatal line and the first project label. If only three GLGs occur, we would have to consider the capture-length age estimate of 3 years at 225 cm too high.

In some cases, we tried to augment the calibration of preproject layers by also using certain undocumented labels to define annual preproject GLGs. Here, we assumed that the label closest to the neonatal line and formed before the project began had been introduced at about the time of capture. We hoped to determine whether the number of GLGs formed prior to and after the "capture label" corresponded to age (in years) based on length at capture and time in captivity, respectively (Table 1).

When we decided that the first preproject label in a tooth was introduced at or near the time of capture (as was done in three of five animals), we used the label with the other, better documented labels from project treatments to calibrate all dentine, by inspection, behind and in front of the label. (In a case where the undocumented label was close to the first project label and the animal was captive for several years, we regarded the undocumented label as not a capture label, and calibration by this method was not attempted.) After preproject GLGs were defined in wild specimens by this method, we compared their thicknesses and patterns with those of the three captive-born animals to determine what similarities and differences existed. Adding the three captive-born dolphins to the animals with useful presumed capture labels brought to six the number of animals for which the initial GLGs were defined. We defined initial GLGs in the remaining six animals by inspection, estimated age at length, and time in captivity.

RESULTS AND DISCUSSION

Tetracycline Labels

Preproject Labels

Examinations of sections of the extracted teeth from captive-born dolphin C revealed a fluorescent label in the postnatal dentine approximately 25 μm from the neonatal line in the direction of the pulp cavity (Fig. 2A, C). This male calf was about 3 weeks old and nursing when its mother became sick and was placed on a 10-day treatment of 5 g of tetracycline daily. The closeness of the label to the neonatal line in animal C's teeth is evidence that tetracycline was transferred from mother to calf through the milk during the 10 days of treatment. It is also compatible with the popular assumption that the neonatal line is formed near the time of birth (Nishiwaki and Yagi, 1953; Myrick, 1981b). The calf was never given tetracycline before the project and could not have ingested medicated fish intended for other dolphins sharing the pool because he could not yet eat solid food.

In addition to the label produced in C through nursing, labels introduced before the start of the project were found in the teeth of five of the wild project animals, dolphins I, J, K, L, and P. There were no medical records of tetracycline treatment to account for these labels. Nevertheless, the earliest formed label in each of the three other noncontrol animals (I, J, and P) was used as though it had been introduced at or about the time of the animal's capture (Table 1). This was done to see if estimates of age at capture plus time in captivity in

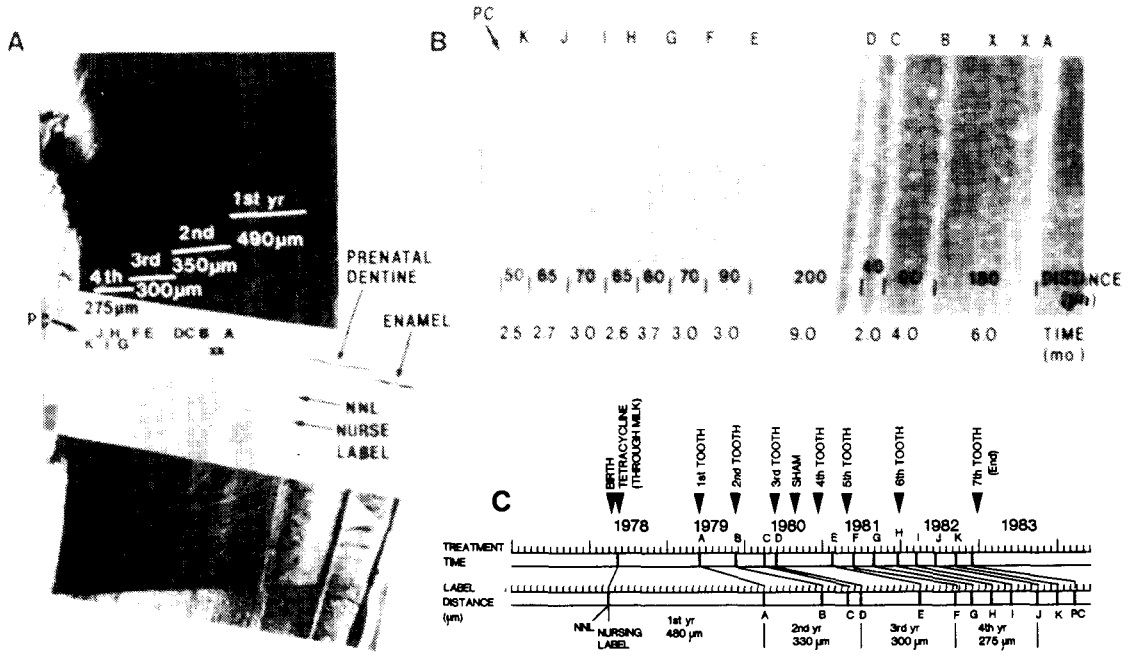


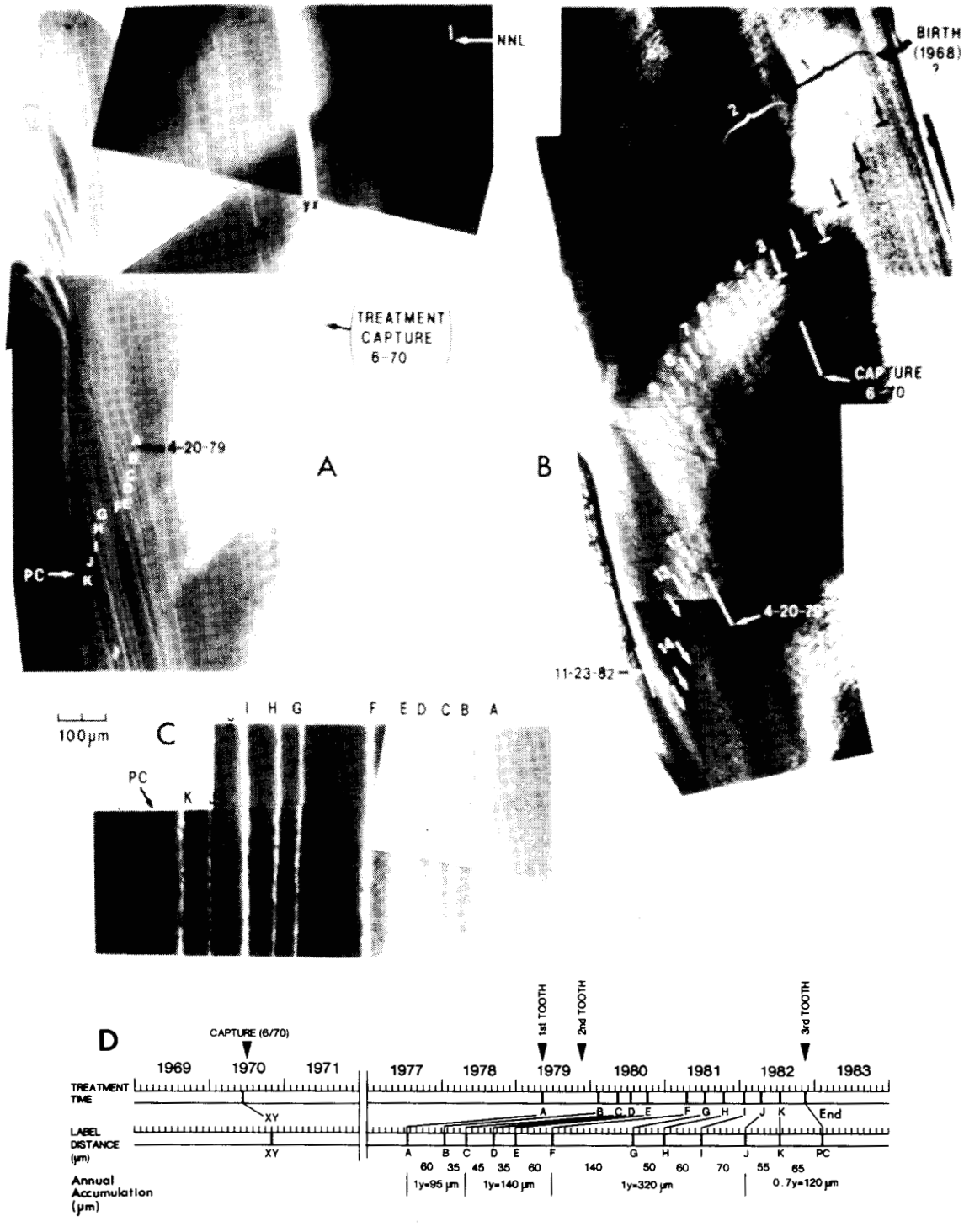
Figure 2 Dentine calibration of animal C. (A) Swatch of UV micrograph with tetracycline labels (lettered) superimposed on light micrograph of dentinal pattern (GLGs marked and measured); ×39. Nurse label internal to the NNL reflects introduction of tetracycline imparted by the mother through milk at 3 weeks after birth. Distance between the NNL and label A is the thickness of dentine deposited in the first 1.1 years. Faint labels between A and B and marked with X are without records. (B) Magnified (×150) UV micrograph of labels showing distances (micrometers) between labels (lettered) and time elapsed between labels (months). (C) Calibration chart showing labeling treatments and tooth extractions, label identifications, and annual dentinal thicknesses. (Abbreviations in this and other figures: PC, pulp-cavity margin; mo, months; yr, year; End, termination of project; 1st TOOTH, 2nd TOOTH, etc., tooth extractions; NNL, neonatal line formed at birth.)

years matched the number of GLGs defined in thickness and pattern that were counted in front of and behind the label.

Preproject labels were not used to estimate age at capture for the two control animals (K and L). This decision was made for animal K because its tooth layers lacked clear patterns in regions critical to interpretation of the labels. The two preproject labels in the teeth of L were too close to the project label (<300 μm), given the animal's 6.5 years in captivity, to have been introduced at capture.

For animal I, a pair of bright, closely spaced labels (X and Y, Fig. 3) occurred about two GLGs inward from the neonatal line (formed at birth) and nine GLGs external to the first project label introduced 20 April 1979. If the XY label is taken as having been introduced at or near capture, June 1970, the project would have begun approximately nine annual GLGs later, which is what the layering pattern showed. Accordingly, animal I would have been about 2 years old at capture, and birth would have been in 1968 (Fig. 3B, D).

For animal J, the presumed capture label was separated from the first project label (A, produced 11 April 1979) by six GLGs, which if formed annually would be compatible with our interpretation that the undocumented label was introduced at capture (December 1972) (Table 1). For animal P, we estimated that the preproject label (X, Fig. 4) was introduced at or about the time of capture for the following reasons. First, P was captured in February 1978 (Table 1), only slightly more than a year before the project began. Second, label X was slightly more than one GLG external to the first


NNL
TREATMENT
CAPTURE
6-70
4-20-79
PC
A
BIRTH
(1968)
?
CAPTURE
6-70
4-20-79
11-23-82
B
100μm
C
I H G F E D C B A
PC
K
D
CAPTURE (6/70)
1st TOOTH
2nd TOOTH
3rd TOOTH
1969
1970
1971
1977
1978
1979
1980
1981
1982
1983
TREATMENT TIME
XY
A B C D E F G H I J K End
LABEL DISTANCE (μm)
XY
A B C D E F G H I J K PC
60 35 45 35 60 140 50 60 70 55 65
Annual Accumulation (μm)
1y=95 μm
1y=140 μm
1y=320 μm
0.7y=120 μm

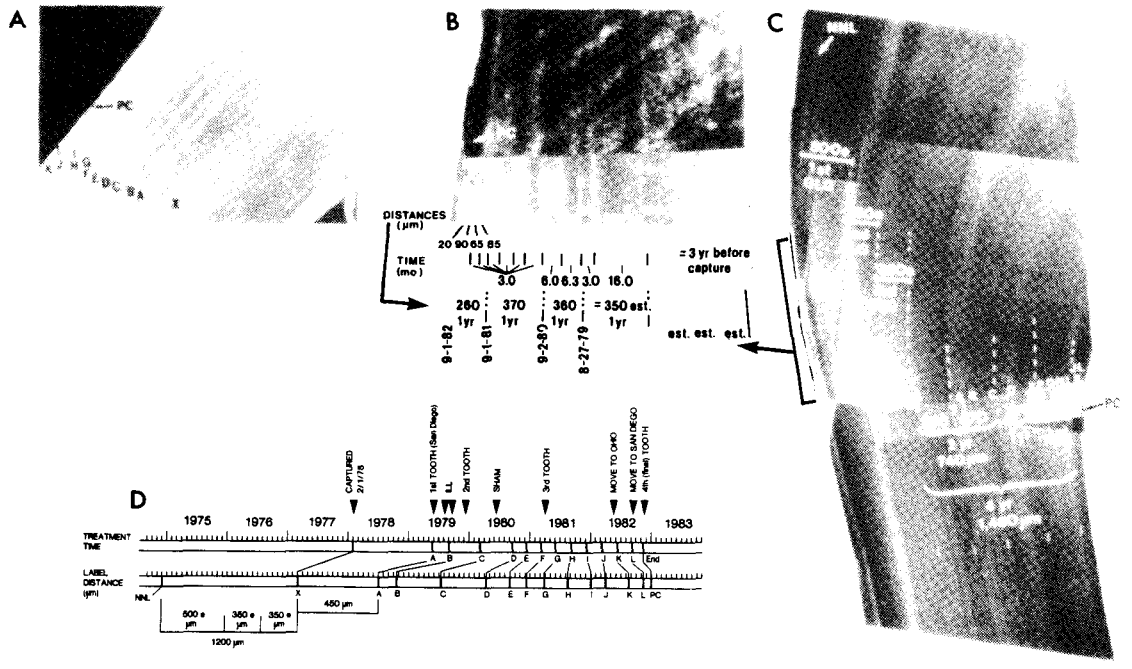


Figure 4 Dentine calibration of animal P augmented by a capture label. (A) UV micrograph showing all labels including one, X, introduced before the beginning of project, probably at about capture. (B) Light micrograph with swatch of UV micrograph showing positions, distances (in micrometers), and time (in months), and estimated annual depositional thicknesses. (C) Position of labeled portion of dentine and total presumed annual GLGs (marked by dashed lines) in last tooth extracted. Based on project body length increases, P was about 3 years old when captured. (D) Calibrated labels and estimated annual GLG thicknesses deposited before the project label (label A). (Original magnification of A and B, ×150; C, ×39. e, Limits of annual GLGs estimated by inspection and consideration of preproject labels and background data; see Fig. 2 for other abbreviations.)

project label (A). Third, the distance of label X from label A was about the same as or slightly greater than the distance between successive project labels representing almost the same amount of elapsed time. Finally, four GLGs occurred between the neonatal line and the first project label (A), and the body length of P, when label A was introduced, was 268 cm (length continued to increase through the project period to 284 cm). The length at the beginning of the project indicates that of a juvenile of about 4 years old (Table 1).

Thus, there is support, from the three specimens in which the earliest preproject label was assumed to have been introduced at capture, that GLGs defined in this study represent annual

Figure 3 Dentine calibration of animal I. (A and B) UV and light micrographs, respectively, of final extracted tooth (pulled 11-23-82). Three preproject labels (X, Y, Z) are contained in the dentine. Labels XY may have been introduced at or about the time of capture considering the number of apparent GLGs (i.e., double dark and light layer pairs) between the XY labels and the neonatal line and between the labels XY and A, introduced at the beginning of the project, 4-20-79, for this animal. This estimate would be compatible with the capture date of June 1970 given by park records. Numbered paired arrows indicate paired dark layers in the second GLG; triple arrows point out three dark layers in GLG number one. (C and D) The gap between labels F and G is unexpected and represents a break in the rather regular deposition rate. Label X is a faint label without a record. (See Fig. 2 for other abbreviations.)

units. Nonetheless, whether the labels we used actually represented treatments at or near capture is not directly proved.

Intentional Project Labels

In all specimens, all intentionally introduced tetracycline labels were identified in the dentine (e.g., Figs. 2, 3, 4, and 5) but not in the cementum. Apparently the tetracycline doses (20–30 mg/kg body weight) used to label dentine were too weak to produce distinct cemental labels. Because of this, we concentrated mainly on calibrating dentinal layers, and only when strong therapeutic concentrations of tetracycline had been used were labels in dentine and cementum compared.

Therapeutic Project Labels

Labels other than those scheduled in the project were inadvertently introduced when tetracycline was used medicinally during ill health of some of the animals. These therapeutic treatments occurred in the teeth as exceptionally bright, wide labels (e.g., Fig. 5B). We used them as additional data points and to demarcate tissue formed during sickness and recovery.

Unidentified Project Labels

Dolphins A, C, and I each had undocumented labels, introduced during the project, that were much fainter and more irregularly spaced than

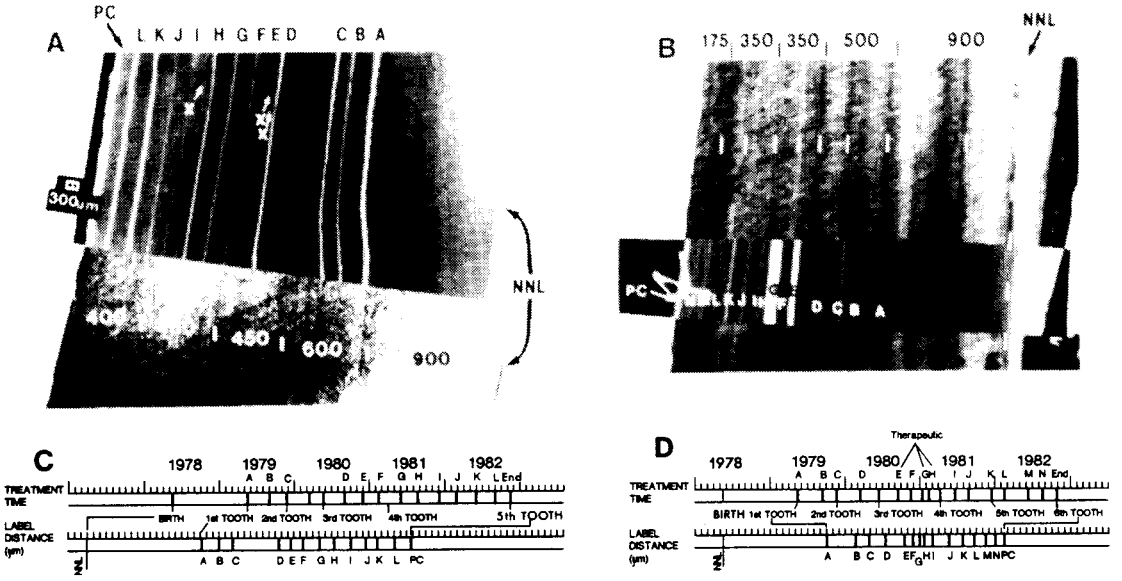


Figure 5 Dentinal calibration for animals A and B. (A and B) UV micrographs with tetracycline labels (lettered) superimposed on GLG patterns (measurements) of the final tooth extracted from animals A and B, respectively. (B) White vertical bars indicate the tripartite nature of first (900-m-thick) GLG and bipartite nature of subsequent GLGs. Faint labels indicated by X's in A are without dates. (C and D) Calibration charts for animals A and B with dated tetracycline labelings and tooth extractions connected with labels, lettered in order of introduction. The substantial gap between labels C and D in micrograph A reflects protracted period, shown in chart C, during which no tetracycline was administered to animal A. Chart D for animal B indicates therapeutic treatments of tetracycline as evidenced by thick, bright labels E, G, and H shown in micrograph B. (Original magnification of photos, ×39. See Fig. 2 for abbreviations.)

other labels (Figs. 2A, 3C, and 5A). Because all intentional labels were accounted for in these specimens, we ignored the faint labels. Presumably the labels were formed when the animals ingested fish medicated with tetracycline, intended for other dolphins sharing a common tank. A similar case was reported by Myrick *et al.* (1984) in their study of tetracycline-labeled Hawaiian spinner dolphins.

Dentinal Growth Layer Groups

Untreated Sections

In transmitted light, well-prepared untreated sections of teeth from all project animals had a repeating pattern of layers comprising a GLG similar to those described by Myrick *et al.* (1984) for Hawaiian spinner dolphins. Except for the first-formed GLG, which exhibited three pairs of layers when resolution was adequate, a GLG consisted of two light layers each followed by a darker layer. The more external light layer formed the outer boundary of the GLG, and the second light layer usually was found about midway through the GLG (i.e., "mid-GLG layer," Myrick *et al.*, 1984). Each new GLG began with a light boundary layer formed at the internal margin of the second dark layer of the previous GLG; the dark layer usually highlighted the light boundary layer (Figs. 3B and 5B).

Decalcified and Stained Sections

Because leftovers from the sides of a central untreated section were used to produce decalcified and stained thin sections, most were noticeably off-center, and few were of sufficiently high quality for detailed GLG thickness comparison with their untreated counterparts. The best specimens showed GLGs with thin, dark-stained boundary and mid-GLG layers and a more lightly stained background (Fig. 8A).

The first GLG consisted of three pairs, instead of two pairs, of thin, dark-stained layers and thick, light-stained layers. The two-pair pattern was common in the dentine formed after the first GLG. However, in some cases, the GLG boundary layer was indistinct because one or more thin, dark-stained accessory layers occurred nearby [e.g., note the boundary layer(s) between GLGs 4 and 5 in Fig. 8A].

Calibration of Growth Layer Groups

Age-Specific Growth Layer Group Thickness

Mean thicknesses of GLGs in the 12 animals are presented in Table 3 along with rounded standard deviations. In comparisons of thickness between GLGs, annual dentine deposition had decreased with increased age in all 12 of the project dolphins. Additionally, serial measurements showed that each annual dentinal GLG was deposited at a more or less predetermined thickness according to the age of the animal.

The age-specific GLG thickness pattern (especially for the first three GLGs) was similar in all wild animals from the same geographic area whether the layers were formed in captivity or in the wild. All wild dolphins had already formed at least two GLGs before capture, and the teeth of two captive-born dolphins exhibited GLG thicknesses similar to the Florida specimens. The other captive-born dolphin (animal C) had GLG thicknesses resembling those of the wild animal (P) from California.

Population-Specific Patterns

Comparisons of mean age-specific dentinal thicknesses for the 12 individuals shown in Table 3 indicate the possibility that two depositional patterns exist: one for Florida-coast animals and one for California-coast animals. All wild Florida-coast animals deposited more than 800 μm of dentine the first year, more than 450 μm the second year, and usually more than 350 μm (418 μm average) the third year. This was also true of the captive-born Florida purebred (animal B) and the captive-born hybrid of a Florida male and a Pacific-coast female (animal A) (refer to Table 1). The one wild animal from California (P) apparently deposited about 500, 375, and 350 μm of dentine in his first, second, and third years, respectively. The captive-born hybrid (C) sired by a purebred California

male shows a dentinal-depositional pattern (i.e., 471, 327, and 298 μm) much nearer that of animal P than those from Florida animals (Table 3 and Fig. 6).

The Pacific-coast sample (n = 2, including animal C) was too small for conclusive results. Nonetheless, it is at least interesting that a thickness pattern so different from the other 10 project animals was found in the wild specimen from California and the one captive-born hybrid male sired by a wild California animal. Figure 6 includes mean values and standard deviations for GLGs one and two. In these GLGs, the two specimens have values more than three standard deviations below mean values of the Florida-coast animals. This indicates that the probability of these two patterns being part of the same population is very low; for the first GLG the probability that the two patterns are from the same population is less than 1.0%.

Monthly Layers

Labels were spaced closely enough and fine dentinal layering was clear enough in several specimens (especially in B, D, and F) to investigate the timing of what have been thought to be monthly layers (e.g., Laws, 1962; Kasuya, 1977; Myrick, 1979) or lunar monthly layers (Myrick, 1981b; Myrick *et al.*, 1984). In the second extracted tooth of D, labels A and B had been introduced 3 months apart, and 3 additional months were represented between label B and the pulp-cavity margin (Fig. 7). Almost four full layers occurred between labels A and B, and there were three layers between label B and the edge of the pulp cavity. In the third extracted tooth of animal D, 12 months were represented between labels A and D. Thirteen pairs of dark–light layers were visible within that segment. In the sixth (last) tooth taken from D, further deposition of monthly layers was identified by comparing layers with elapsed time between labels on the calibration chart (Figs. 7 and 8). In other animals, tooth sections with well-developed fine layering show similar results. This supports calibration observations made earlier by Myrick *et al.* (1984) that fine dentinal layers in dolphins are deposited with lunar (or at least monthly) regularity. Thus, monthly layers have now been verified for time in specimens of two species, Hawaiian spinner dolphins and bottlenose dolphins.

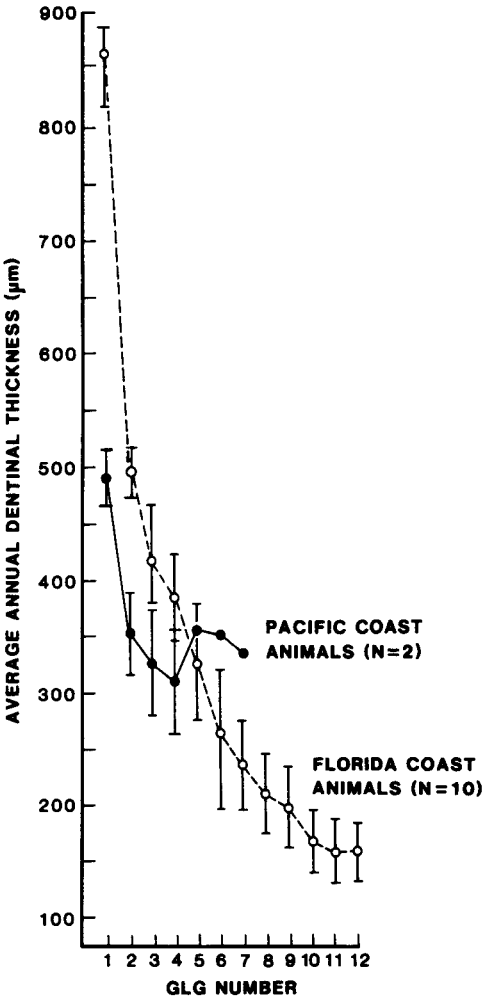


Figure 6 Average dentinal GLG thickness in Pacific-coast and Florida-coast bottlenose dolphins. Vertical lines represent one standard deviation from mean values.

Monthly Rates of Deposition

Enough between-label measurements were available to plot mean monthly accumulated thicknesses (in micrometers) of dentine during the

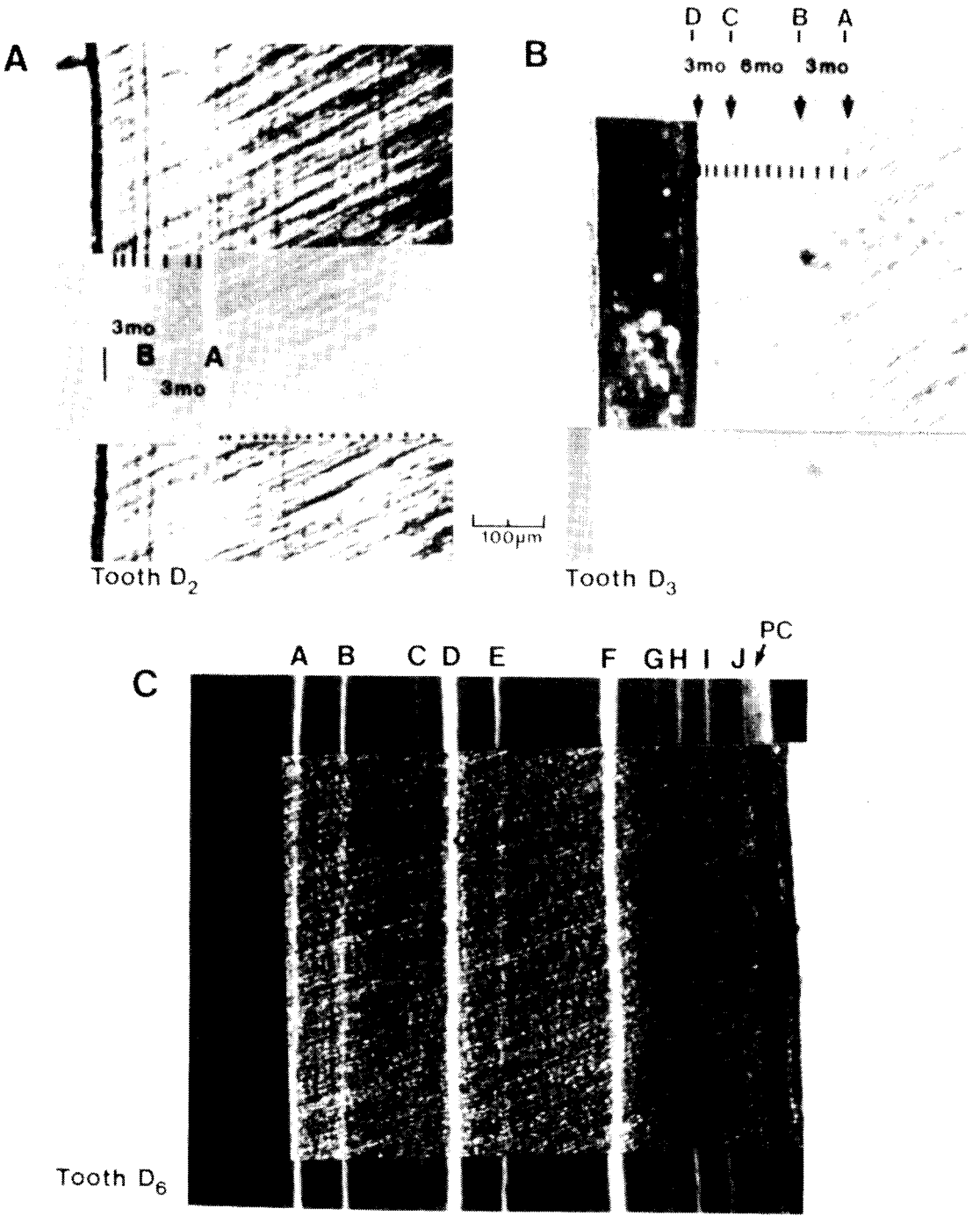


Figure 7 Accumulation of labeled dentine in animal D. (A and B) UV micrograph swatches on light micrographs of dentine in second and third tooth extracted, respectively. The accumulating labels, dentine, and elapsed time are indicated. Drawn marks suggest numbers of fine layers between labels. (C) Swatch of light micrograph on UV micrograph of dentine in the last tooth extracted from animal D. (Original magnification, ×150.)

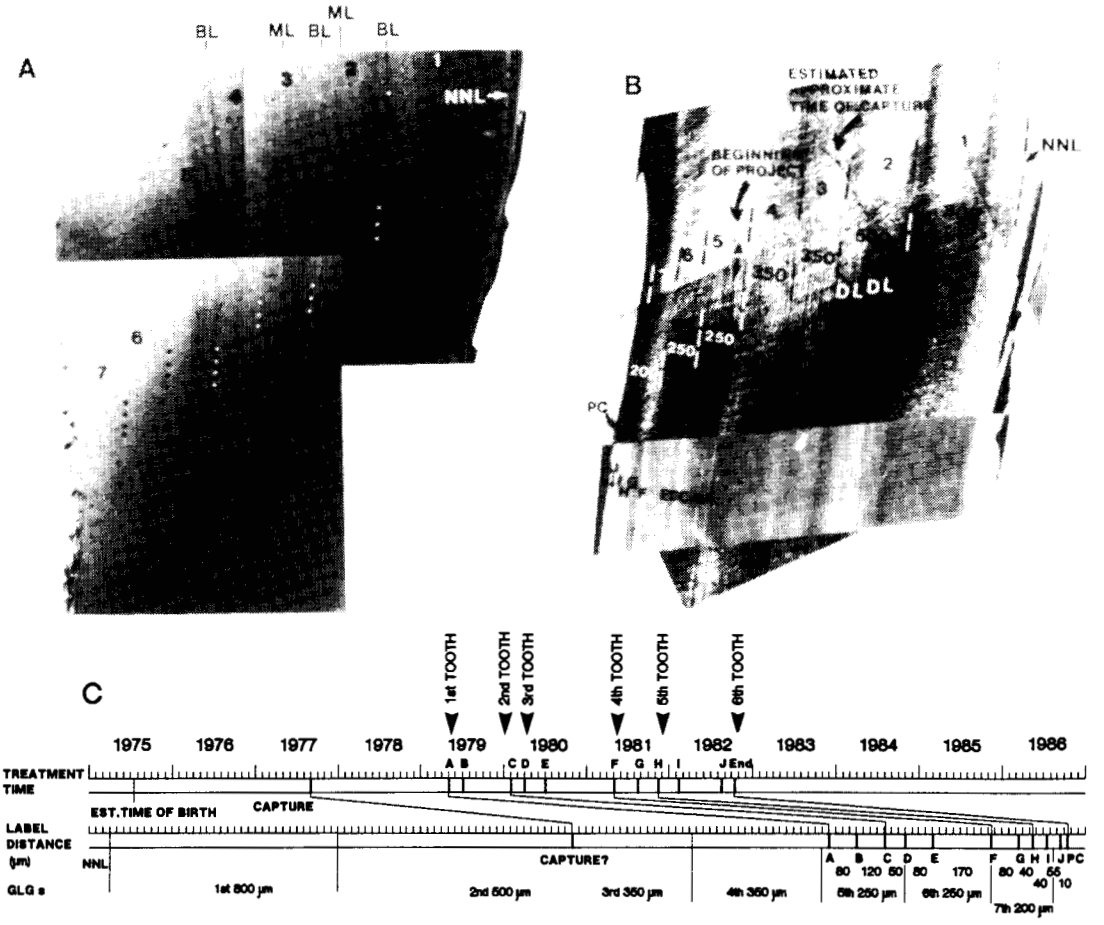


Figure 8 Calibration of GLGs in the last extracted tooth of animal D. (A) Decalcified and stained section showing GLG patterns featuring a single thin dark boundary layer (BL) (dotted lines) and a single thin dark mid-GLG (ML) layer. The boundary between the apparent fourth and apparent fifth GLG is indistinct due to the occurrence of several thin, dark-stained layers. (B) Untreated section with UV swatch showing labels (lettered). GLGs (bounded by dashed lines) are numbered and show thicknesses in micrometers. (C) Calibration chart showing birth and capture dates (estimated from capture body length and time in captivity) and annual GLG thicknesses based on estimated and monitored dentine deposition. (Original magnification of A and B, ×39. DLDL indicates dark–light–dark–light layer pattern in second GLG; GLG number one has a tripartite pattern; see Fig. 2 for other abbreviations.)

3.5-year project for eight of the twelve dolphins. Rates of deposition were computed for each animal by dividing thickness by the elapsed time in tenths of months. Because of age-specifc depositional patterns, younger individuals accumulated considerably more dentine per month than others. For this reason, and because of the possibility of population-specific thickness patterns, average monthly deposition by each animal during the monitored period was plotted on the same graph. The abscissa is scaled in 10-μm intervals, with absolute values omitted, and the period of the project, from spring 1979 to fall 1982, is represented on the ordinate with vertical lines set at successive January firsts (Fig. 9).

A common pattern in dentine depositional rates was discerned to some extent in all specimens: increased rates in spring and summer with de-

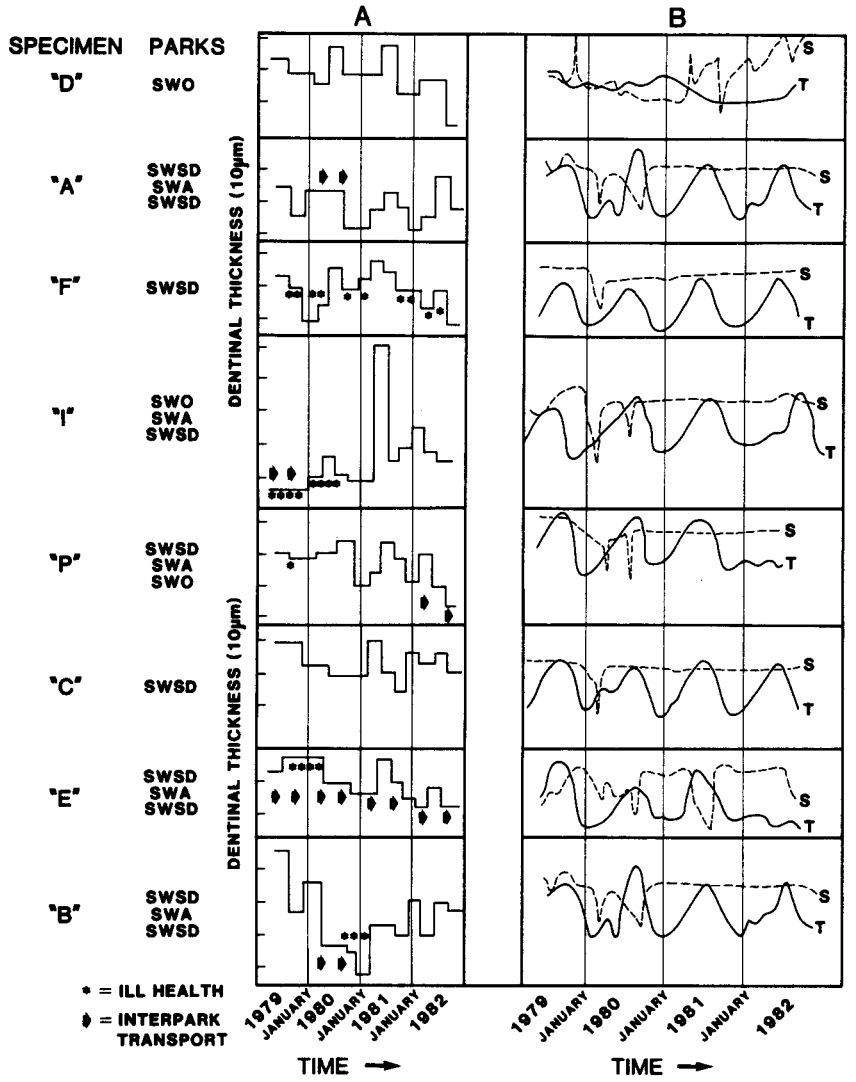


Figure 9 Monthly depositional rates and external environmental factors. (A) Relative average monthly rates of dentine deposition in eight bottlenose dolphins over a 3.5-year period, with plots of weekly measurements of water temperature (T) and salinity (S) (smoothed by eye) shown for each. (B) Vertical lines indicate January first of years shown. Rates tend to increase in individuals in spring–summer months and to drop in fall–winter months, although considerable wandering from this pattern is apparent. Depositional rate changes roughly correspond to but are not preceded by fluctuations in water temperature. They are not correlated with changes in salinity, nor with transport between Sea World parks (SWO, Florida; SWA, Ohio; SWSD, California). Arrows represent times of interpark transport, and asterisks indicate periods of ill health.

creased rates in fall and winter (Fig. 9A). This pattern, typified in plots for dolphins D and F, showed distinct and synchronized peaks and dips in mean rate over the entire 3.5-year period. The resemblance of relative changes in rates between these two animals was especially remarkable considering that D remained at SWO (Florida), where temperature and salinity are manipulated, and F remained at SWSD (California), where these two parameters are subject to ambient fluctuation.

Despite the striking similarities among many of the animals in depositional-rate cycles, however, there was noticeable variation in timing (e.g., B and E) and amplitude (e.g., I and P) within and between individuals. To test whether the spring–summer rate increase and fall–winter decrease amounted to a significant pattern, we compared synchronized drops and rises in rates among the eight animals for the 3.5-year period. For an animal, in each time segment (spring–summer or fall–winter) there can be only one of three possible outcomes: a peak matching a spring–summer segment, a drop matching a fall–winter segment, or something else that disrupts the ideal model of synchronized rate changes. If there is a common seasonal pattern that animals follow, there should be little difference in the frequency distribution of synchronized rate changes among animals.

A contingency table was set up with the rows representing animals and columns representing the three possible outcomes. Calculations gave a χ^2 value of 13.174 with $(8 - 1)(3 - 1) = 14$ df ($p = .5$). This indicates that the frequency distribution of the three outcomes is independent of the animals, and we conclude that the animals follow the synchronized cyclic model. Out of a potential 32 peaks and 32 dips in an ideal model of eight eight-season rate changes, the sample showed a 40% probability of peak (26 out of 64), a 40% probability of dip (again, 26 out of 64), and 12 out of 64 or a 20% probability of neither a synchronized dip nor peak.

Factors Possibly Affecting Dentinal Deposition

Exogenous Factors

The cyclic depositional rates of D illustrate how parameters were evaluated as possible influencing factors. Animal D had one of the most regular, cyclelike depositional patterns, yet experienced the most uniform environment. She was not transported between parks during the project, was never ill, and was not subjected to much or sudden fluctuation in water temperature (although water salinities temporarily fluctuated sharply several times). She usually ate no less than about 7.5–13 kg (20–35 pounds) of fish daily. She was not trained and did not participate in shows in the first year of the project. In the second year, training and show activity was frequent but did not affect her daily fish consumption; nor was it reflected by any noticeable difference in layering patterns or depositional rates in her teeth. In addition, D was kept at a lower latitude (Orlando, Florida) than any other animal in the project, yet she had one of the most distinctly cyclic changes in rate. This is contrary to what one might expect if day length were an important *zeitgeber* for changes in dentinal-deposition rates.

For all individuals, with the important exception of D, there were regular seasonal changes in water temperature which corresponded roughly with depositional rate changes in dentine, but none of the changes in water temperature or other physical parameters that we monitored consistently preceded changes in depositional rate (Figs. 9A, B). Thus, they do not seem to be causally related. In some cases, when water temperature had risen or had begun to rise, dentinal rate remained unchanged (e.g., P and C in 1980) or dropped (e.g., B and E in 1980). In other cases, when water temperature had started to decrease or had already reached a nadir, dentinal-deposition rate rose (e.g., E and B in winter 1979) or remained unchanged (e.g., C and E in winter 1980).

Perhaps more importantly, at various times depositional rates changed while water temperature (and other parameters) did not change. The rates of D in all years and of P and E from fall of 1981 to fall of 1982 followed a pattern of fall–winter decrease and spring–summer increase even though water temperature remained fairly constant. For D, mean water temperature was 16.4°C, with gradual changes between 13° and 20°C; for P and E water temperatures for 1982 fluctuated slowly between 14° and 18°C (Fig. 9B).

Changes in types of food given had no detectable effect on depositional rate. For example, A was fed fish of only one species in 1979, fish of

mixed species in 1980, and fish and squid in 1981–1982. None of these changes were reflected in A's near-perfect cyclicity of winter decreases and summer increases in dentinal deposition.

Depositional rate changes and layering patterns typified by A, D, and F were unaffected by interpark transport. This is illustrated in A in 1980 and P in 1982. It is shown especially well in E. Dentinal accumulation for E was normal in 1981 and 1982 despite semiannual airplane transport of this dolphin (shown as arrows in Fig. 9A) between California and Ohio in all years during the project.

Endogenous Factors

A rare accident permitted us to examine possible effects of short-term stress in measurably altering layering patterns. A project animal leaped out of its tank and apparently was out of the water on the ground for hours before it was discovered. It was returned to its pool, presumably somewhat stressed. Using tetracycline labels to pinpoint the region of dentine formed at the time the dolphin was out of the tank, we were unable to detect any unusual lines or layers that might have reflected a physiological response to that incident.

Periods of sickness, however, did appear to affect depositional rate. The rate plot for F (Fig. 9A) is accompanied by several clusters of asterisks symbolizing bouts of sickness, usually indicated by inappetence. Distribution of these symbols corresponds with diminution of amplitude in changes of rate. For example, the drop in the rate in January 1980–1981, common in other dolphins plotted, is shallow in F, and the expected rise in spring–summer of 1982, common in most of the other animals, is equivocal in F. Conversely, the peaks in spring–summer 1980 and 1981, when F was not sick, are present in the plot. Similarly, P did not exhibit a decrease in depositional rate during fall–winter 1979, when he had a poor appetite and was receiving medication. However, his depositional pattern cycled normally during other years.

Animal E was not well during fall–winter of 1979. Beginning at about the same time, his dentinal-deposition rates departed from the presumed common pattern, which was not reestablished until winter 1980–1981. For I, symbols for interpark transport are concurrent with those of ill health in 1979, and the condition apparently was not normalized until mid 1980. During the period of sickness, there was very little change in the slow dentinal-deposition rate. The common pattern appeared in I in the spring of 1981 in what could be considered a compensatory acceleration of the rate. In fact, however, we have no data that provide a satisfactory explanation of this sudden escalation in rate. It is possible that this spurt in deposition represents variation in one tooth. It was documented in only the last tooth that was pulled.

For C, we have no data to account for either the lack of a peak in mid 1980 or an early increase in rate in winter of 1982. He remained in California during the entire period and was never ill or medicated. Salinity decreased sharply in early 1980 in C's tank, but similar sudden drops in salinity for other animals on various occasions (e.g., A in 1980 and E in 1981) did not result in disruptions of normal depositional cycles. If ill health was a factor in the apparent distortion of C's pattern, it was not detected by our observers.

Depositional rates of B differ markedly from those of A, although the two dolphins were subjected to almost identical conditions during the project (Fig. 9A, B). Part of this difference may be artifactual; the broad peak in the first half of 1980 for A is due in part to a mean rate computed using one measurement over 9 months during which no labels were introduced (Fig. 5A, C). However, comparisons of thicknesses of the second, third, and fourth GLGs for the two animals show that annual deposition was indeed less in B (Table 2). Animal B had a decreased appetite shortly before returning to San Diego from Ohio in 1980, and his record for the next 6 months is a picture of intermittently low food consumption accompanied by therapeutic treatments to help correct the condition (Figs. 5D and 9A). He was fully recovered by spring 1981, but his dentinal-deposition rate pattern remained largely unsynchronized relative to that exhibited in the other animals for the remaining 17 months of the study.

Cementum

Although cemental GLGs, each consisting of a light layer and a dark layer, were visible and countable, calibration of cementum was not possi-

ble in this study. The labels that we tried to introduce experimentally did not show up distinctly, apparently because our dosages of tetracycline were too weak for detection in cementum. We also were unable to match dentinal GLG counts with those of cementum using therapeutic labels. Often the cementum was too thin, or had fewer GLGs than the dentine, or showed the therapeutic labels in the wrong relative position. For example, in animal I the *XY* label, visible in about the third annual dentinal GLG (Fig. 3), was identified in about the eighth or ninth cemental GLG (Fig. 10). Cemental GLG counts, therefore, may be difficult to use as reliable indicators of age for this species.

CONCLUSIONS

Results of this long-term monitoring and marking study suggest that bottlenose dolphins deposit a specific annual thickness of dentine at a given age in a predetermined pattern which is largely under endogenous control. In healthy animals of our sample, the pattern of GLG deposition was generally unaffected by conditions of captivity, including changes in water temperature or salinity, physical activity, at least some kinds of short-term stress, type of food consumed, and possibly even differences in latitude.

Rates of dentinal deposition were fairly cyclic, tending to increase in the spring and summer and to decrease in the fall and winter. They seem to have altered somewhat when the animals were ill and for some period after the illnesses. Apparently, they were not strongly entrained by any external *zeitgeber* that we were able to detect. However, the following observations suggest that some entrainment cue or complex of cues exists: (1) there was a rough correspondence of depositional rate changes with regular changes in water temperature, with a number of important exceptions, (2) to some extent rate peak and depression varied in time and in amplitude in an animal and

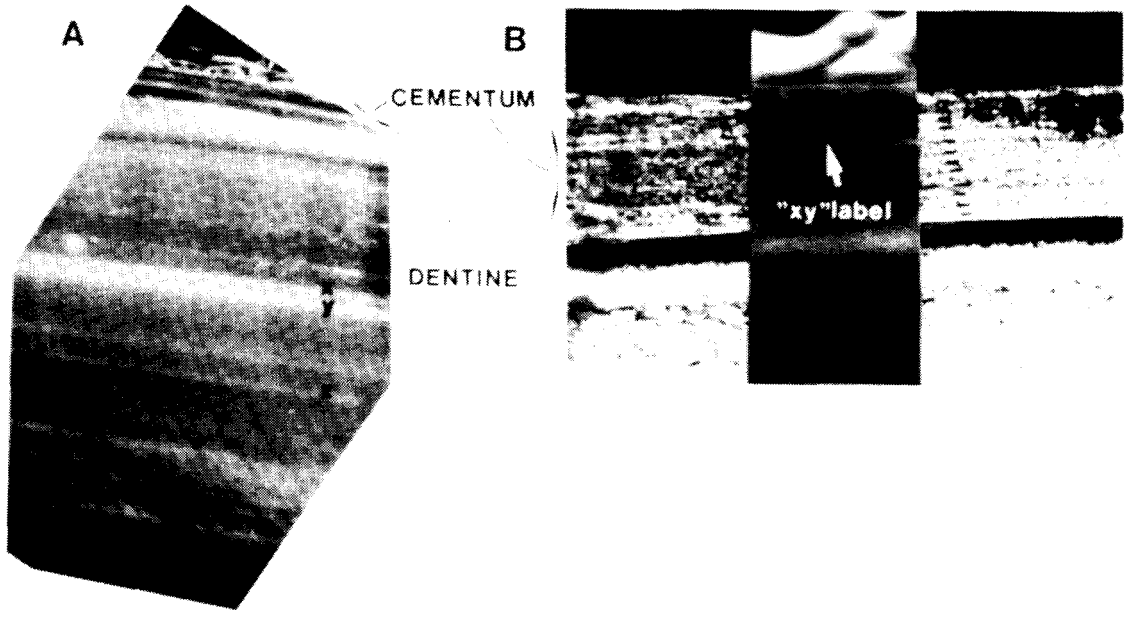


Figure 10 Unreliability of cemental layers for estimating age. (A) UV micrograph of area of tooth tissue including cementum from animal I (more detail and calibration are shown in Fig. 3). Preproject labels *X* and *Y* were presumably introduced at or near capture, when I was only a few years old. The labels occur in approximately the third dentinal GLG (×39). (B) Light micrograph of cementum from I with swatch of UV micrograph showing what is interpreted to be the *XY* label in approximately the eighth or ninth cemental GLG (×150). This example suggests that cemental GLG deposition is not necessarily correlated with that of dentine.

between animals, and (3) in some specimens, after being unsynchronized for a time, deposition cycles usually became resynchronized. The *zeitgeber* was not identified in this study.

Although the sample from the California coast consisted of only two animals including one captive-born hybrid, comparisons with the sample from Florida suggest that the age-specific annual thickness of dentine may be manifested as two population-specific patterns: one for Atlantic animals and one for Pacific animals. Studies using labels in additional captive animals from the Pacific should help to decide this question.

In untreated thin sections viewed in plain light, the annual GLG consists of two light layers each followed by a darker layer. In decalcified and stained thin sections, in plain transmitted light, each annual GLG tends to have one or more dark-stained layers near its boundary and one or more near its midpoint. The dark-stained mid-GLG layer(s) bisects the otherwise lightly stained GLG. All GLGs tend to be bipartite except the first GLG formed, which tends to be tripartite. Fine layers within the GLGs are probably formed monthly.

We were not able to calibrate cemental layers with dentinal layers in this study. Tetracycline labels could not be used to match GLGs in dentine and cementum. This suggests that cemental layers may have no simple relationship to annual GLGs deposited in dentine. In our opinion, cemental GLGs alone should not be relied on for estimating ages of bottlenose dolphins.

A tetracycline label immediately internal to the neonatal line in one captive-born animal was coincident with tetracycline treatment of its mother. This supports the suggestion by Myrick *et al.* (1984) that it may be possible to treat nursing calves in ill health with certain drugs that combine with constituents of milk by medicating the lactating mothers. The label's closeness to the neonatal line is also corroborating evidence that the neonatal line forms at or near the time of birth.

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