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Toward calibrating dentinal layers in captive killer whales by use of tetracycline labels

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ABSTRACT

To establish a basis for accurate age estimation of killer whales, growth layer groups (GLGs) were examined in tetracycline-labelled and non-labelled teeth, prepared as decalcified and stained and undecalcified thin sections, from 13 animals with captive histories. Data concerning body length, tetracycline treatments and time in captivity were used in conjunction with labels and GLG patterns to identify annual rates of deposition. For this sample, the annual thickness of dentine deposited in the neck region of a tooth was never less than 700 μm and usually between 800 and 900 μm, even in adults. In this region, a standard measurement aids in identifying the extent of each annual GLG when accessory layers obscure its true boundary layers. Dentine was not readable after about the twentieth GLG, and cementum was usually poorly layered and very thin. Another age determination method should be sought for such cases if data for older age classes of a sample are desired.

INTRODUCTION

The method of age estimation of toothed cetaceans, based on counts of growth layer groups (GLGs, Perrin and Myrick 1981) of tissue in the teeth, was introduced 35 years ago (Nishiwaki and Yagi 1953). Since then GLG counting has become an important procedure when studying the age-related biology of odontocete populations (Scheffer and Myrick 1981; Kasuya 1976, 1977; Myrick *et al.* 1986).

The three most widely used techniques for tooth preparation and examination to estimate age are as follows:

1) Etched half-teeth – acid etching cut surfaces of longitudinally bisected teeth and "reading" dentinal GLGs in relief with reflected light, as a chevron-shaped series of valleys and ridges.
2) Untreated sections – sawing and polishing a thin longitudinal wafer from the center of a tooth and reading the GLG chevrons in transmitted light, as a series of darker and lighter layers.
3) Decalcified and stained (D&S) sections – cutting thin longitudinal sections from the central part of a decalcified whole tooth or central wafer with a freezing microtome, staining sections with hematoxylin and reading GLGs in cementum (along the external margins of the root) as well as in dentine (within the body of the tooth) in transmitted light, as a series of darker and lighter stained layers (See Perrin and Myrick 1981, pp. 3–48; Myrick *et al.* 1983).

Acid etching has been used chiefly for species in which teeth are large, such as sperm whales (Clark *et al.* 1968) and killer whales (Christensen 1982, 1984). Etching is a relatively simple method that produces a coarse relief thought to be adequate for identifying

GLGs in large teeth (Pierce and Kajimura 1981). D&S has been done most often on teeth of dolphins and porpoises because the teeth are small and GLG patterns are fine and difficult to see in acid-etched relief (Kasuya 1976, 1977; Perrin and Myrick 1981). D&S is a more complicated method than producing etched relief (Myrick *et al.* 1983).

The use of untreated sections has had limited success for age estimation (Perrin *et al.* 1976). Untreated sections have been used primarily to study coarse layering in large teeth, such as those of sperm whales (Klevezal and Tormosov 1971) and with ultraviolet light (UV) to examine teeth containing artificially introduced tetracycline labels (Nielsen 1972; Best 1976; Gurevich *et al.* 1981; Klevezal 1981; Myrick 1981; Myrick *et al.* 1984), in which fluorescent traces are lost if teeth are decalcified (Nielsen 1972).

A few published studies of killer whales have included age estimates based on GLG counts (Sergeant, in Caldwell and Brown 1964; Perrin and Myrick 1981; Christensen 1982, 1984). Of these, the latter three studies made counts on a substantial number of teeth and used etched half-teeth exclusively, except for the IWC workshop study (Perrin and Myrick 1981) which also employed one untreated and three D&S thin sections. In addition, the latter three studies indicated the nature of the counts and provided GLG definitions. To date, however, no study of killer whales has included known-age, minimum known-age and tetracycline-labelled specimens. Inclusion of such materials would permit calibration of tissue units with units of real time, as has been done for two other delphinid species, i.e. *Stenella longirostris* (Myrick *et al.* 1984) and *Tursiops truncatus* (Myrick, unpubl. data). Thus, as Christensen (1984, p. 256) stated, "[although] the etched-tooth technique provides clearly defined dentinal [GLGs in killer whale teeth], their interpretation with regard to [absolute age remains] provisional."

Two international meetings of cetacean biologists have indicated that: 1) data are needed as a basis for age determination of killer whales (Perrin and Myrick 1981; IWC 1982); 2) improvement is needed in preparation and examination techniques to age killer whales using the D&S method (Perrin and Myrick 1981); and, in general, 3) known-age, known minimum-age, and tetracycline-labelled (captive) specimens should be studied "for calibration of dentinal layer groups and for cross-calibration of dentinal [and] cemental... layering" (Perrin and Myrick 1981, pp. 39–40). Using teeth and records from all available specimens known to us with captive histories, we have responded to the research recommendations concerning killer whale age determination as suggested above. We here account the methods and results of a study that was conducted to calibrate dental layers in killer whales *(Orcinus orca)*.

MATERIALS AND METHODS

The Sample

We used a single tooth from each of 13 killer whales, 12 dead and one live, captured from the wild and held in captivity at U.S. oceanaria for various periods. Ten of the 13 animals had been treated with tetracycline for physical ailments during their captive lives. The teeth were studied in conjunction with data on capture dates, length at capture, therapeutic treatment dates and dates of death (the date of tooth extraction for the live animal) from medical records of varying completeness (Table 1).

Labels

Using reflected UV light, we examined untreated sections from all individuals to identify fluorescent labels corresponding to recorded, as well as unrecorded, tetracycline treatments. By turning the transmitted plain light source on and off while the reflected UV remained on, we were able to locate precisely the positions of the labels within the layering patterns. Following Myrick *et al.* (1984), we photographed labelled regions of untreated sections in UV and plain light separately and in UV and plain light simultaneously to estab-

TABLE 1

Background data and age estimates of 13 captive killer whales used to calibrate GLGs in the teeth (Origin: A *= North Atlantic,* P *= northeastern Pacific.* * *= Estimated age at capture based on body length).*

		Capture						*Estimates of total age (y) based on:*		
No.	*Sex*	*Orgin*	*Date*	*Length cm*	*Age* (y)*	*Extraction or Death*	*Time Captive (y)*	*Capture length and captive time*	*Capture length, captive time, labels and GLGs*	*Captive time and GLGs with and without labels*
001	m	P	11 Mar 67	277	1	23 Sep 78	11.5		12.5	
002	m	P	29 Apr 68	401	?5	5 Dec 70	2.7	7+	10.5	
003	f	P	27 Dec 69	290	1.3	4 Aug 71	1.7			3.0
004	f	P	27 Aug 71	379	4	15 Jun 75	3.8	7.8	6.0	
005	m	P	12 Dec 69	412	?5	20 May 72	2.4		7.4	8+
006	f	P	26 Aug 71	434	5+	28 Sep 77	6.1	12.0		15.0
007	m	P	12 Mar 72	354	3+	1 Dec 74	2.7	6.0		7+
008	f	P	Mar 73	488	<7	22 Oct 77	4.6	12+		19+
009	m	A	12 Oct 77	350	3	2 Aug 81	3.8		7+	
010	f	A	Oct 78	287	1+	21 Mar 82	3.5			5.0
011	f	?	65			29 Aug 71	6.0			12.0
012	m	P	Oct 68	427	5+	Oct 82	14.0			20+
013	f	P	1 Mar 70	335	3	2 Nov 72	2.7		6.0	

lish the positions of labels within GLG patterns (Figs. 1A and 1B).

To make the connection between layers and time, dentinal thicknesses were measured in a standard position in the neck region of each tooth (Fig. 1B). Measurements were made of dentine accumulated between the neonatal line and labels, between each label and between labels and the pulp-cavity margin. Each measurement was made at least three times and averaged. We then matched labels to recorded tetracycline treatments by interpreting distance between labels as corresponding to time between treatment dates, thickness of labels as duration of treatment, and brightness as strength of dose. Each measurement (in μm) was divided by the time elapsed between dates to yield the annual depositional thickness of dentine. Then, measurements from each specimen were totalled and divided by total captive time to get a mean annual depositional rate (Table 2). To mitigate the effects of local variation and measuring error, all values were rounded to the nearest 50 μm. Rounding made the values easy to deal with and, considering the large sizes of the GLGs, made little difference in absolute values.

In four specimens (Nos. 005, 006, 007, 011), treatments had been administered on or shortly after the date of capture. This gave a complete dentinal record of time in captivity from the first label (date of capture) to the pulp-cavity margin (date of death or tooth extraction, Fig. 1C). The number of specimens with a label introduced at capture was expanded from four to six (by adding Nos. 001 and 003) after study of other specimens had shown that, although the earliest labels in the latter two specimens lacked dates, the label distances and positions relative to other structures and labels corresponded in time to the capture dates. (It is not unusual to find poor records of medications given during the first days of captivity to protect newly caught animals during their adjustment – Myrick *et al.* 1984).

GLG Patterns

To obtain the best resolution of layering patterns, D&S sections were prepared from

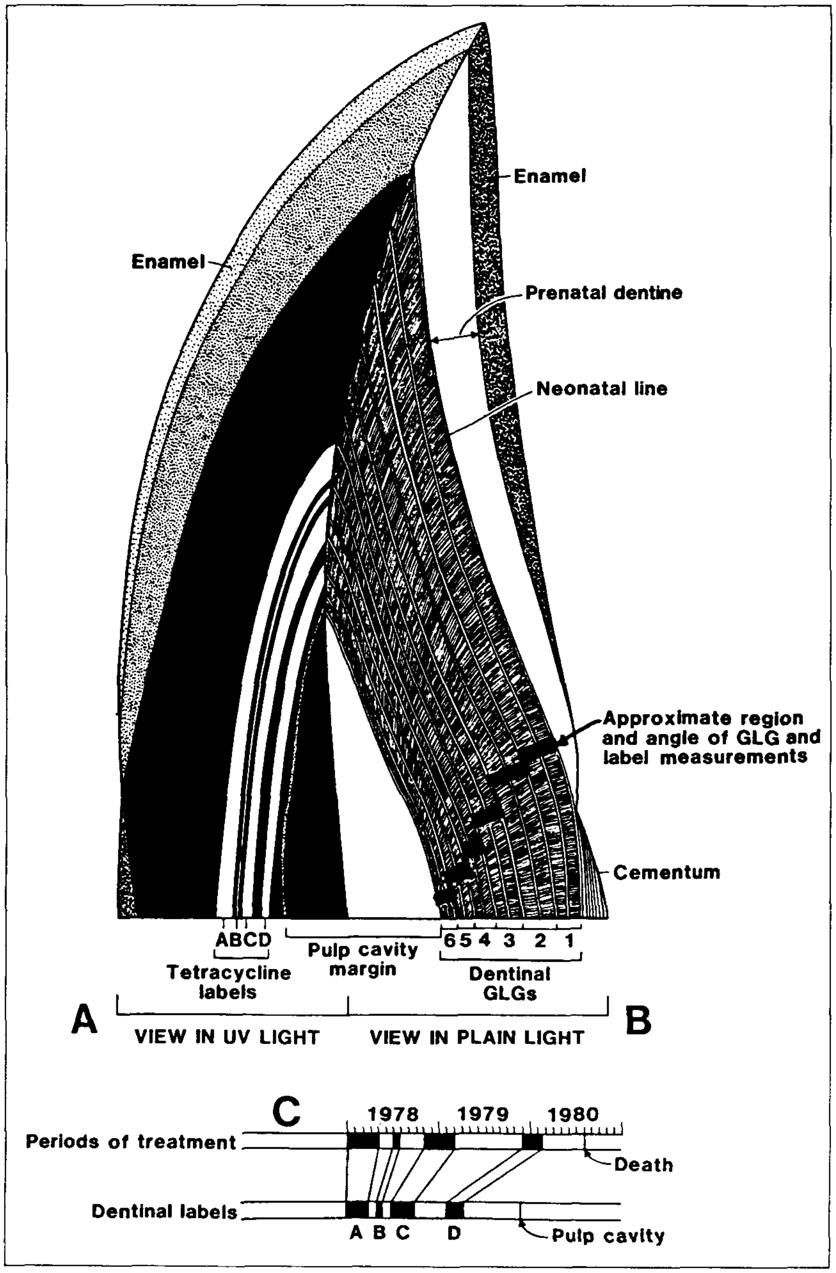

Fig. 1. Diagram of tooth thin section from hypothetical captive delphinid (other than killer whale) showing A) tetracycline labels under UV reflected light (left) and B) dentinal GLG patterns under plain transmitted light and standard positions where labels and GLG thicknesses are measured (right). The map C) illustrates the method of identifying labels in a tooth section for which tetracycline treatment dates are known, by comparing relative thickness and spacing of labels with intervals and durations of treatments. (Modified from Myrick *et al.* 1984).

remaining parts of the teeth from which untreated sections were made. This facilitated the comparison of gross GLG patterns, structures and labels seen in untreated sections with GLGs defined in D&S sections.

The basic D&S procedure consisted of making 2-mm-thick wafers from near the center of a tooth with a small saw, decalcifying the wafers in a rapid bone decalcifier, cutting the wafers to fit the microtome stage and then dividing the wafers into 30-μm-thin long sections. Rinses and staining followed procedures by Myrick *et al.* (1983). The stained thin-section pieces were reassembled on glass slides in pure glycerol to prevent shrinkage. A full account of the D&S preparation method for killer whale teeth will be reported elsewhere (Yochem, in prep.).

The remaining parts of several half-teeth were also prepared using the etching technique to compare the GLGs in relief to those seen in D&S section. Results of that comparison have been summarized earlier and will be described fully in a forthcoming report (Yochem, in prep.).

Calibration

In calibrating dentinal layers, our primary objective was to define the killer whale annual GLG by imposing the time data upon the layering patterns and vice versa. To do this, we first identified repeating layering patterns (pattern GLGs) in D&S sections. Then using direct measurements from natural markers and superimposed photographs of tooth tissues, we located the same regions in untreated sections, enveloped by series of dated labels (Fig. 2). From the elapsed time between labels represented in the untreated section, we then determined the time interval of the pattern of GLGs identified in D&S sections. Finally, we compared results from all study specimens with adequate labels to define the annual GLG, both visually and in terms of dentinal thickness.

Our GLG definition was tested by using it on the specimens to estimate their ages. The counts of annual GLGs were used to determine, in the blind, the ages of specimens for which total or minimum age estimates were already available. These earlier estimates had been derived from age based on length at capture plus total time in captivity. As a basis for age-at-length estimates, we adopted Bigg's (1982) interpretation that a body-length of 9 feet (274 cm) represents a one-year old, and that length increases by about 1.2 feet (35.6 cm) per year to a length of 16 feet (488 cm) (Table 1).

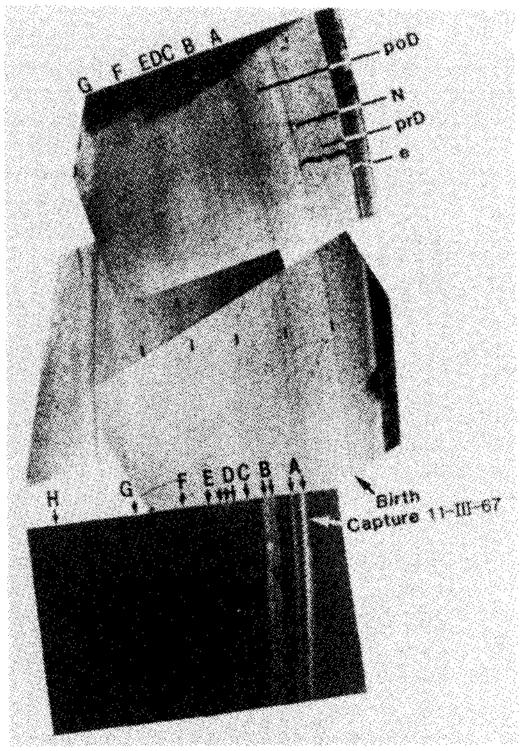

Fig. 2. Matched UV and plain-light photographs of an untreated tooth section of killer whale No. 001, showing the location of tetracycline labels (lettered arrows). The right-hand label in group A was introduced at capture and is separated from the neonatal line (N) by one GLG. GLGs are about 900 μm thick and are shown here bounded by vertical bars. The left-hand label of group B, label C and label F were introduced 1.0, 0.6 and 1.9 years apart, respectively. Dates of other labels were not identified (see Table 2). e = enamel, PoD = postnatal dentine, PrD = prenatal dentine.

Our final objective was to compare GLG counts from patterns in the cementum with those made in dentine (in D&S sections) to evaluate the possiblity of using cementum reliably in age estimation.

A special problem emerged in analyzing the labels for specimen No. 001 (Tables 1 and 2, Fig. 2). This whale had been captured as a one-year old (9 feet 1 inch or 277 cm) and held in captivity for 11.5 years. During that time many tetracycline treatments were given, but only five treatment dates were preserved in its medical records. By studying dentinal de-

TABLE 2

Life history events and tetracycline treatments accounting for spacing between structures and labels in killer whale teeth used to define annual depositional rates (see Materials and Methods). (NNL = *neonatal line, formed at birth;* PC = *pulp-cavity margin or label at pulp-cavity margin, indicating end of tooth record);* A: *North Atlantic;* B: *northeastern Pacific;* d: *day(s);* y: *year(s);* * *indicates age estimated from body length at capture).*

Spec. No.	Event	Date* from length at capture	Treatment period dose	Structure or Label: With data	Structure or Label: No data but used	Structure or Label: No data not used	Elapsed time (y)	Distance (μm)	Rate (μm/y)
001	Birth	~ Mar 66*		NNL					
	Capture	11 Mar 67	unknown		A		~1.0*	900	900
		27 Mar 68	unknown	B			1.0	800	800
		17 Nov 68	13d – unknown	C			0.6	500	850
		unknown				D			
		unknown				E			
		28 Oct 70	5d – unknown	F			1.9	1600	850
		unknown				G			
		unknown				H			
		21 Apr 76	8d – 75g/d	I			5.5	5000	900
		13 Sep 78	3d – 30g/d	(PC)					
	Death	23 Sep 78		PC			2.4	2000	850
		total time captive					11.5		
		total age and average rate					12.5*		850
002	Birth			NNL					
	Capture	29 Apr 68	unknown		A		7.5	7000	
		5 Jan 70	80d – 20g/d	B			1.7	1600	950
		unknown				C			
	Death	25 Dec 70		PC			1.0	850	850
		total time captive and average rate					2.7		900
		total age					10.7		
003	Birth	Jul 69*		NNL					
	Capture	27 Dec 69							
		5 Jan 70	unknown – 20g/d	A			1.3*	1100	850
		22 Apr 70	unknown – 20g/d	B			0.3	250	850
	Death	4 Jul 71		PC			1.3	1250	950
		total time captive					1.7		
		total age and average rate					3.0*		850
004	Birth			NNL					
	Capture	27 Aug 71	unknown		A		2.3	2100	900
			unknown			B			
			unknown			C			
	Death	15 Jun 75		PC			3.8	3200	850
		total time captive and average rate					3.8		850
		total age					~6.0		
005	Birth			NNL					
	Capture	12 Dec 69							
		6 Jan 70		A			5.5*	5150	950
	Death	20 May 72		PC			2.4	2000	850
		total time captive and average rate					2.4		850
		total age					~8.0*		
006	Birth			NNL					
	Capture	26 Aug 71							
		18 Jun 72	12d – unknown	A			0.8	8100	
		7 Jul 72	10d – 38g/d	A					

Table 2 (continued)

Spec. No.	Event	Date* from length at capture	Treatment period dose	Structure or Label: With data	Structure or Label: No data but used	Structure or Label: No data not used	Elapsed time (y)	Distance (μm)	Rate (μm/y)
		12 Sep 77	1d – 30mg/d	B	(PC)				
		15 Sep 77	7d – various	B	(PC)				
	Death	28 Sep 77		PC			6.1	5000	800
		total time captive and average rate					6.1		800
		total age					15.0		
007	Birth			NNL					
	Capture	12 Mar 72							
		21 Apr 72	4d – 60g/d	A			0.1		
		25 Mar 74	4d – 10g/d	B			1.9	1700	900
		21 Sep 74	3d – 20g/d	(PC)					
	Death	21 Dec 74		PC			0.7	650	900
		total time captive and average rate					2.7		900
		total age					7.0		
008	Birth			NNL					
	Capture	Mar 73							
	Death	22 Oct 77		PC					
		total time captive					4.6		
		total age					19+		
009	Birth			NNL					
	Capture	12 Oct 77					3.0*		
		23 Jun 80	24d – 20g/d	A			5.7	4950	850
		22 Jul 81	6d – 40g/d	B	(PC)				
	Death	2 Aug 81		PC			1.1	1000	900
		total time captive					3.8		
		total age					7+		
010	Birth	summer 77*		NNL					
	Capture	Oct 78					1+*		
		27 May 79	3mo – 10g/d	A			0.6		
		20 Mar 82	1d – 20g/d	B	(PC)				
	Tooth Extraction								
		21 Mar 82		PC			2.8	2400	850
		total time captive					3.4		
		total age					5.0*		
011	Birth			NNL					
	Capture	?-65							
		20 Dec 65	8mo – various	A					
		7 Jan 67	19d – unknown	B			1.0	850	850
		3 Jul 67	17d – —	C			0.5	400	800
		30 Aug 67	19d – —	C					
		10 Oct 67	27d – —	C					
		24 Feb 68	7d – —	D			0.6	1000	1650
		3 Mar 68	4d – —	D					
		30 Mar 68	14d – —	D					
		18 Aug 68	30d – —	E			0.4	350	850
		27 Sep 68	31d – —	E					
		31 Dec 68	48d – —	E					
		24 Jul 70	9d – —	F			1.9	1550	800
	Death	29 Aug 71		PC			1.1	900	800
		total time captive and average rate					5.7		900
		total age					12.0		

Table 2 (continued)

Spec. No.	Event	Date* from length at capture	Treatment period dose	Structure or Label: With data	No data but used	No data not used	Elapsed time (y)	Distance (μm)	Rate (μm/y)
012	Birth			NNL					
	Capture	Oct 68							
	Death	Oct 82		PC					
		total time captive					14.0		
		total age					20+		
013	Birth			NNL					
	Capture	1 Mar 70							
	Death	2 Nov 72		PC					
		total time captive					2.7		
		total age					5.7		

position rates of the other specimens and assuming that No. 001 had a similar rate and pattern, we were able to predict where the dated treatments should occur in the layered dentine. In this way it was possible to sort out the dated from the non-dated labels. As it turned out by this assumption, the preserved dates corresponded in time with the dentinal spacing of labels. Moreover, using the distance between label A and the neonatal line on one side and the distance between label A and the second label group (group B) on the other, we determined that the most external label in label A group (Fig. 2) was introduced at about the time the yearling was captured. The dentinal pattern deposited in its first year (in the wild) could then be defined.

RESULTS

Dentinal GLG Pattern

In D&S thin section, the GLG pattern in our sample seemed deceptively simple. Each GLG consisted of a broad, lightly stained layer containing a varying number of thin dark-stained (accessory) layers. The thin layers near the middle bisected the broad layer subequally, while those at the sides separated it from other broad layers contiguous with it (Fig. 3, lower left region). However, in many areas of the sections, the more centrally positioned accessory layers resembled boundary layers so closely that we could not decide whether the GLG to be counted was double-layered or single-layered (Fig. 3, upper right region). It was only through calibration of dentine that the composition of the annual GLG was defined.

Direct measurements of GLGs in D&S sections of all specimens showed that usually single layered GLGs were about half the value (about 450 μm) of the double layered GLGs, which were typically between 800 and 900 μm thick. Repeated measurements of all GLGs in tooth neck regions indicated that although values fell below 800 in some of the last-formed double layered GLGs of the three oldest specimens (Nos. 006, 008, 012 – Table 2), none was less than 700 μm thick.

GLG Calibration

Mean annual rates of dentinal deposition for 10 of the 13 specimens in which they could be calculated from labels are presented in the right-hand column of Table 2. These averages fell within the same range of values (800–950 μm) as the individual annual thickness in specimens that have numerous labels separated by about one year (Nos. 001, 002, 003, 011). An exception to this rate was found only in specimen No. 011. Here, a rate of 1650 μm/year was calculated for a thickness of 1000 μm deposited over five months. We were unable to explain this apparent anomaly. We concluded, nevertheless, that the data in Table 2 overwhelmingly supported the use of the double-layered GLG greater than 700 μm thick (in the neck region) as a mean annual dentinal unit.

Ages from Calibrated GLGs

If this definition of an annual GLG is accurate for all killer whales in our sample, then a count of annual GLGs in the specimens should agree with their estimated ages from capture length/age and time in captivity. The estimates based on capture length and time in captivity are compared to those from GLG counts independent of age-at-length data in Table 1.

In five of the 12 specimens with capture length (Nos. 002, 004, 006, 007, 008), there was a difference of > 1.0 to 7.0 years between age estimated from length plus captive time and estimates made from GLG counts. All five were more than 11.5 feet (350.5 cm) long at capture; two of them were more than 14 feet (488 and 434 cm) long. Only in the case of No. 004 was the GLG age estimate less than the age/length estimate (6.0 years vs. 7.8 years).

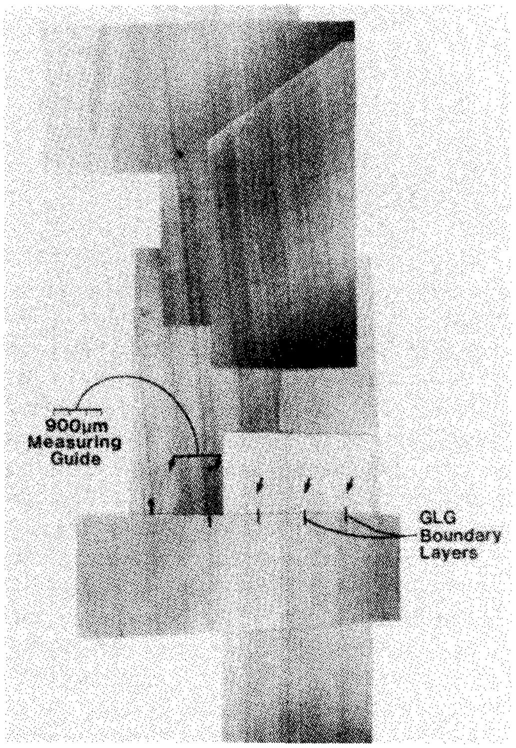

Fig. 3. D&S section of killer whale tooth showing annual GLGs in the region of the tooth neck. Each GLG is characterized by a broad, lightly stained layer, bounded and bisected by a variable series of closely-spaced, thin, dark stained layers. Note, especially in the upper right-hand area, how boundary layers become confused. Because the GLGs are usually between 800 and 900 μm thick, a standard measured scale helps in identifying the extent of annual deposition.

For two reasons we concluded that the GLG estimates were closer to the actual age than the body-length plus captivity age estimates. First, in the other seven specimens, almost all smaller and easier to age at capture than the five, the two estimates were within a year of each other. Second, GLG thickness varied little from year to year. Thus, total thickness of dentine deposited between birth and capture could be divided by about 900 μm to get an approximate age at capture (Table 2). When this could be done (i.e. when a physical capture point could be identified in the tooth) for four of the other five specimens (Nos. 002, 004, 006, 007), age at capture plus time in captivity was within a year of the age estimated from GLG counts (Tables 1 and 2).

The maximum age estimated from dentinal GLG counts for one specimen was about 21 years; the next oldest specimen was a little more than 19. In both specimens, additional but irregular and unreadable dentine occurred near the pulp cavity. Because it had no structural resemblance to the GLG, we found no basis for increasing the age estimates. Neither specimen had age-at-capture estimates or tetracycline labels. Thus, it is possible that both of these specimens were somewhat older than the age estimates given.

Cemental GLGs

Cementum of killer whales that we examined was very thin and poorly layered in D&S sections (Fig. 4). Where tetracycline labels could be detected in the cementum, no consistent correspondence could be found of GLGs before and after the cemental labels with GLGs between labels in the dentine.

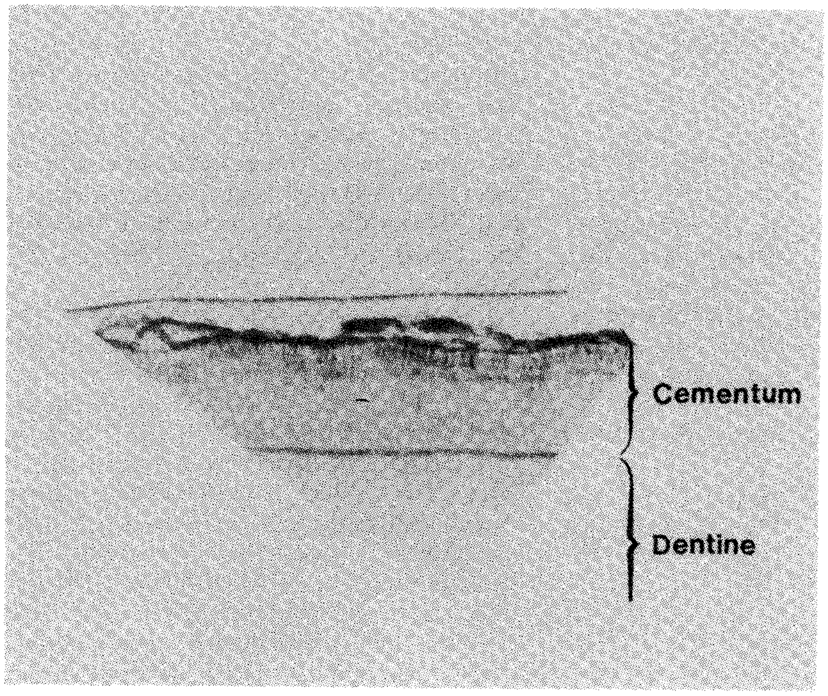

Fig. 4. D&S thin section of cementum of No. 001, a 12.5-year-old killer whale, showing GLGs. Cementum was very thin and poorly layered in the samples examined.

DISCUSSION AND CONCLUSIONS

Our study has shown that, with proper records and specimens, tetracyline can be used to calibrate killer whale dentine. The sample we used indicated consistently that in D&S thin section an annual GLG is made up of a broad, lightly stained layer bounded and bisected by a variable number of thin, dark-stained accessory layers. Usually, this annual unit, measured in the region of the tooth neck, was between 800 and 900 μm thick. However, it was never less than 700 μm thick, even in mature animals. We suggest that where accessory layers blur GLG boundaries (Fig. 3) a standard measure could be used to identify the annual unit. Although a 900-μm measurement was used as a guide in this paper, we think that any scale capable of delineating clearly between 450 μm and 700 μm would be useful.

The finding that GLG thickness tended to be uniform throughout the dentine (Table 2) is a dramatic departure from the pattern established for other delphinid species, as depicted in Figure 1B. For non-killer whales, each GLG tends to be thinner than that deposited in the previous year (Best 1976; Gurevich *et al.* 1981; Myrick *et al.* 1983, 1984). The difference in depositional patterns may be connected with functional difference between the biting teeth of killer whales and the prey-manipulation teeth of most smaller delphinids. Whatever the case, it should be kept in mind that we could not test the hypothesis that uniformly thick GLGs occur in animals older than 20 or so years of age. Teeth from animals more than 20 years of age should be studied to resolve this question.

Because of the difficulty in reading cemental GLGs and in linking them in some consistant manner to dentinal GLGs, we view as unreliable the method of using cemental layers alone for age determination.

Our sample yielded two specimens which, based on the dentinal GLG counts, were estimated to be approximately 20 years old. Additional but disordered dentine had been accumulated in these specimens subsequent to the formation of the GLGs, possibly indicating older ages. Thus, new methods for estimating age in very old individuals should be explored.

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