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Comparative Study of Dentine Staining Techniques to Estimate Age in the Chilean Dolphin, *Cephalorhynchus eutropia* (Gray, 1846)

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Abstract

Three staining methods were used to assess their potential for resolution of growth layer groups (GLGs) and identification of accessory layers in dentine for age determination of the Chilean dolphin, *Cephalorhynchus eutropia*. Tooth sections from 10 dolphins were stained with Giemsa stain, Harris' hematoxylin and Mayer's hematoxylin. The general GLG pattern in the Chilean dolphin teeth is similar to that described for other delphinids. Since the three methods revealed the same number of GLGs, it is possible to use any of these techniques to better discern and count GLGs in Chilean dolphin teeth.

Introduction

Estimating age of marine mammals from growth layer groups (GLGs, see Perrin & Myrick, 1980) is a basic tool for study of the population dynamics of these species, and since discovery of the technique a number of different methods have been developed (Scheffer & Myrick, 1980). The most often used techniques for counting GLGs in odontocete teeth require undecalcified or decalcified and stained sections. These techniques have been extensively used in age determination by several authors (Nishiwaki & Yago, 1953, 1954; Klevezal & Kleinenberg, 1967; Christensen, 1973; Sergeant, 1959; Sergeant *et al.*, 1973; Best, 1976; Kasuya, 1977; Hohn, 1980; Lockyer *et al.*, 1981). However, some of these techniques do not allow a clear resolution of the GLGs, especially the last GLGs in old animals (Myrick *et al.*, 1983).

Within the genus *Cephalorhynchus*, Lockyer *et al.* (1981, 1988) studied age and growth in the Commerson's dolphin, *C. commersonii*, using decalcified sections stained with Ehrlich's hematoxylin. Little is known however on life history parameters of the other species of the genus, including the Chilean dolphin *Cephalorhynchus eutropia*, a coastal endemic species from Chilean waters (Aguayo, 1975; Oporto, 1988). The aim of this work was to compare three different staining techniques, assessing their potential in the resolution and identification of GLGs in decalcified, stained thin-sectioned teeth of Chilean dolphins.

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Table 1. Data on specimens of *Cephalorhynchus eutropia* used in this study

Number	Date	Sex	Length (cm)	Weight (kg)
JAO 034	20JUN87	F	147.5	45.0
JAO 035	20JUN87	M	147.5	45.0
JAO 037	20JUN87	M	143.0	40.9
JAO 039	20JUN87	M	151.0	51.0
JAO 040	20JUN87	F	142.0	42.6
JAO 044	16DEC88	M	147.0	48.0
JAO 045	16DEC88	M	157.0	52.0
JAO 068	27DEC89	M	131.0	30.0
JAO 078	19MAR90	F	144.5	38.5
JAO 079	19MAR90	M	128.0	36.0

Material and Methods

Teeth from 10 Chilean dolphins, three females and seven males, were analyzed. The dolphins were incidentally caught by local fishermen off Queule (39°22'S; 73°13'W), between 1987 and 1990 (Table 1). Six teeth from the mid-left mandibular ramus were taken from each specimen. They were cleaned, examined, measured externally and stored in 70% ethanol. A sample of four teeth chosen from each series was decalcified with RDO, a rapid commercial bone decalcifier (See Myrick *et al.*, 1983). Decalcification time varied from 2–15 hours, depending directly on the length of the specimen (i.e., teeth from smaller animals required less time). After decalcification, the teeth were cut longitudinally with a freezing microtome. As no previous information was available on ageing in the Chilean dolphin, we experimented with several section thicknesses (8, 10, 16, 20, 30 and 40 μm). On-center sections were stained using the following techniques.

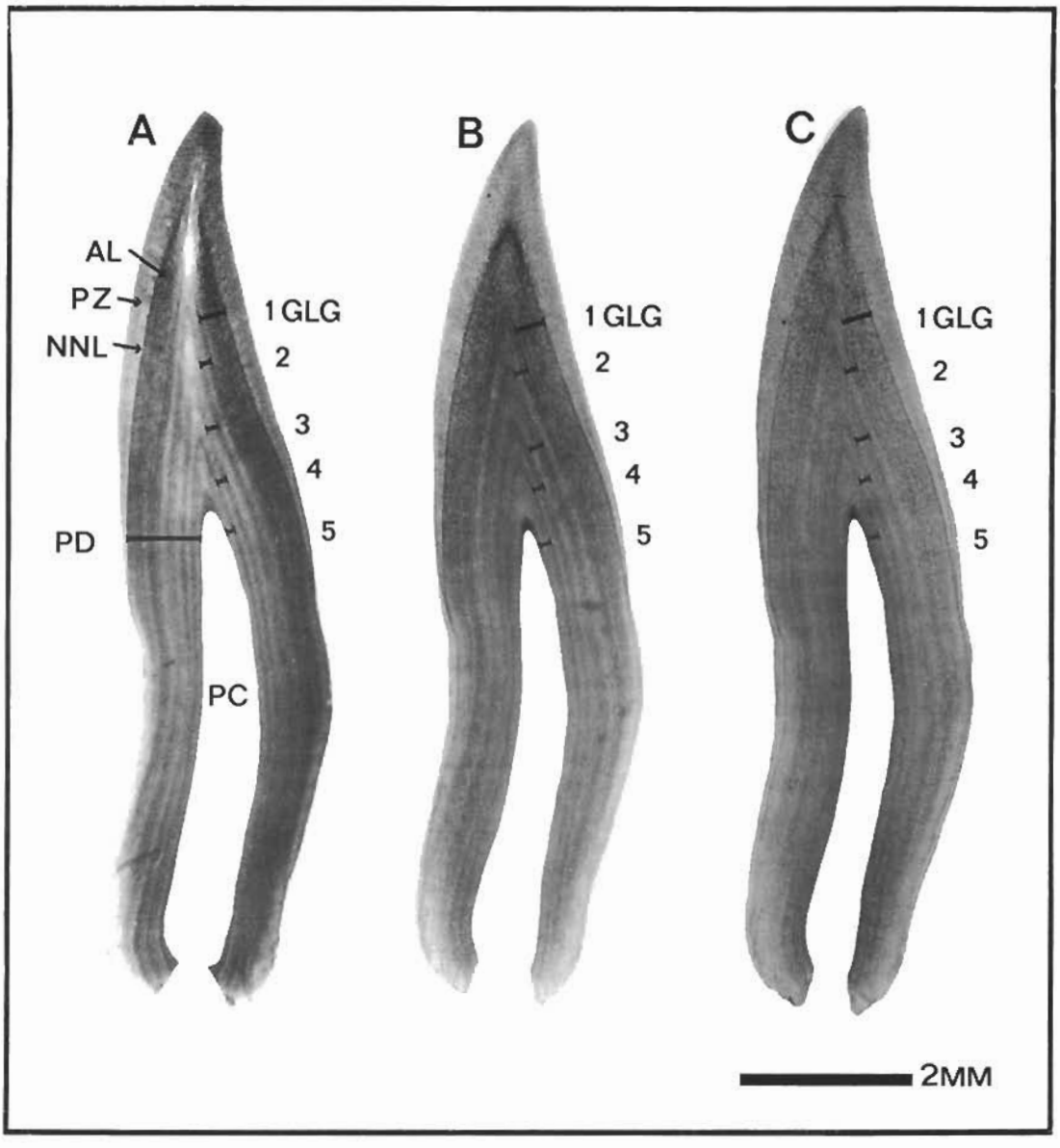

Figure 1. Longitudinal tooth section of Chilean dolphin (JAO 044) stained with three different methods, showing five Growth Layer Groups (GLGs). A = Giemsa stain; B = Harris' hematoxylin; C = Mayer's hematoxylin; NNL = Neonatal Line; PC = Pulp cavity; PZ = Prenatal Zone; PD = Postnatal dentine; AL = Accessory Layer.

a) Geimsa stain. For this method, each section was immersed during 60 min in a staining solution of 40% buffered phosphate at 6.8 pH. Excess of stain was removed with distilled water for 10 min. To dehydrate and fix the section onto slide, it was necessary to cover it with a coverslip and immersed liquid nitrogen at −85°C for 5 sec. The coverslip was removed with a razor blade and the section mounted in Permount.

b) Harris' hematoxylin. Here, sections were stained during 100 mins and excess stain removed with 1% sodium borate (1 min) to increase the contrast between layers. Sections were then rinsed with tap water for 10 min and placed in 100% glycerin to help retain the stain of the preparations. Permount was used for mounting.

c) Mayer's hematoxylin (recommended by Myrick *et al.*, 1983). For the Chilean dolphin, the best results in

Table 2. Comparison of three staining techniques applied to teeth of Chilean dolphins for age determination. G = Giemsa stain; H = Harris' hematoxylin; M = Mayer's hematoxylin.

Number	Resolution of GLGs(*)			Resolution of accessory layers			Number of GLG		
	G	H	M	G	H	M	G	H	M
JAO 034	V	V	V	V	V	S	5.0	5.0	5.0
JAO 035	E	S	V	V	S	S	4.5	4.5	5.0
JAO 037	V	V	S	V	V	S	4.5	4.5	4.5
JAO 039	E	E	E	V	V	V	5.0	5.0	5.0
JAO 040	V	V	V	S	V	V	6.0	6.0	6.0
JAO 044	E	E	E	E	E	V	5.0	5.0	5.0
JAO 045	V	S	S	S	P	P	7.0	7.0	7.0
JAO 068	E	E	E	V	V	V	1.5	1.5	1.5
JAO 078	E	V	V	V	V	V	1.5	1.5	1.5
JAO 079	E	V	V	V	V	V	1.5	1.5	1.5

(*)E = excellent; V = good; P = poor.

this technique were achieved after 120 min in the stain. To obtain an appropriate contrast, 0.1% ammonium solution was used for 5 sec, and the section was then mounted in Permount.

Sections were examined under transmitted light with a dissecting microscope at the lowest magnification (10–50 ×). The readings were made by a single observer (DMM). The deposition rate of GLGs is unknown for *C. eutropia*, since the date specimens of known age are lacking. To identify GLG deposition in dentine, the protocol described by Hohn *et al.* (1989) was utilized. A GLG was defined as having a light layer followed by a dark layer for an annual dentine deposition group.

Results and Discussion

Description of GLGs

Sections 20 μm thick demonstrated the best dentinal GLG resolution. A similar optimum thickness applies for sections of *Stenella* spp. (Myrick *et al.*, 1983), and *C. commersonii* (Lockyer *et al.*, 1988). Identification of GLGs in sections 16, 30 and 40 μm thick was difficult, while sections 8 and 10 μm thick did not show dentinal GLGs at all.

The general GLG pattern in the Chilean dolphin teeth is similar to that described for other delphinids (Hohn, 1980; Gurevich *et al.*, 1980; Myrick *et al.*, 1983; Lockyer *et al.*, 1981, 1988). The prenatal dentine is a homogeneous, unlayered or poorly layered area much different from the postnatal dentine. The neonatal line (NNL) is a narrow clear layer, usually followed by a narrow intense dark layer. The first GLG is approximately the same width as the prenatal zone at a point in the prenatal zone about one third the distance from the apex of the tooth to the base of the prenatal zone on the buccal side of the tooth (Figure 1).

Sometimes the boundary layer for the first GLG is readily apparent only in the crown or in the root of the tooth as a sudden change in color resulting from a difference in stainability of the dentine in the first and second GLGs. A similar feature has been observed in sections of bottlenose dolphin teeth (Hohn *et al.*, 1989). The second GLG is about the same width as the first GLG. The boundary layer between the second and third GLGs is usually more darkly stained and hence easier to identify. As the animal gets older, the pulp cavity fills up and the GLGs start to diminish in width. However, there were no difficulties in identifying all GLGs, since old animals were not present in the sample.

Accessory layers in dentine make difficult the identification of the first two GLGs, because their accessory layers appear irregular and there is no clear boundary layer between the two. Although distribution and optical density of the accessory layers vary for each species, those observed in the Chilean dolphin present an optical density similar to that described for bottlenose dolphins by Hohn (1980). In studying teeth of Commerson's dolphins for age determination, Lockyer *et al.* (1981, 1988) did not describe the presence of accessory layers, thus a comparison with the allied Chilean dolphin is at present not feasible. The use of the model proposed by Hohn *et al.* (1989) for those samples for which it was not possible to calibrate deposition time of the GLG, has allowed us to distinguish, with some confidence, the patterns of growth layer groups in the Chilean dolphin, especially when the reader lacks experience.

Comparison of the techniques

The advantage of the Giemsa staining technique is that less preparation time is needed and, at the same

time, it provides a direct contrast of the layers. It should be pointed out, however, that all three techniques showed a clear identification of the accessory layers present between the NNL and the first GLG. The three methods used revealed the same estimated number of GLG for all specimens of *C. eutropia* analyzed in this study (Table 2) Therefore, it is possible to use any of these techniques to reveal and count the GLGs in the Chilean dolphin teeth.

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