Tetracycline Marking and the Possible Layering Rate of Bone in an Amazonian Manatee *(Trichechus inunguis)*[1]

Daryl P. Domning
Department of Anatomy, College of Medicine
Howard University, Washington, D.C.
and
Albert C. Myrick, Jr
National Oceanic and Atmospheric Administration, National Marine Fisheries Service
Southwest Fisheries Center, La Jolla, California

ABSTRACT

This paper reports the first successful use of tetracycline marking of layered bone in a manatee and suggests that this technique may be useful in estimating age for species of this family. A six-month-old male Amazonian manatee *(Trichechus inunguis)* was first injected with *Terramycin* on 23 August 1976 (2.2 mg/kg) and subsequently on 18 and 19 November 1977 (7.1 and 8.5 mg/kg, respectively). After the animal died, on 3 May 1978, a transverse thin section of one of its ribs was examined microscopically using plain and polarized transmitted light and ultraviolet reflected light. One fluorescent mark, probably representing the 1977 injections, was found in the periosteal bone between the first and second of three layers containing aligned Haversian systems. It is likely that, for this species, 2.2 mg/kg dosages are insufficient to mark bone, but dosages between 7.1 and 8.5 mg/kg are adequate. An average accumulation rate of one layer per year is suggested by our analysis of this specimen.

INTRODUCTION

In recent years the use of hard-tissue layering systems to determine age has become an important area of research in biological studies on sirenians. For dugongs the problem is simplified by the presence in these animals of tusk-like incisors that contain well-defined dentinal growth layer groups (Scheffer, 1970; Mitchell, 1976, 1978; Kasuya and Nishiwaki, 1978; Marsh, this volume). The manatees possess no incisors; their molariform teeth are rapidly and continuously replaced and growth layers do not accumulate (Odell, 1977). Age estimates for manatees have been based on body lengths (loc. cit.).

Since the histological study by Fawcett (1942) it has been known that certain bones of the manatee contain growth layers. His descriptive comments foreshadow the use of bone growth layers to determine age in manatees:

> The arrangement of periosteal bone [in the ribs of manatees], distinguished by slight differences of internal architecture, suggest that the osteogenic activity of the periosteum is periodic or seasonal. . . the most recently deposited bone is found. . .[on the outer curvature of the rib]. . . while older and older layers are encountered toward the inner edge. (p. 277)

Odell (1977) was unable to define growth lines in prepared histological sections of the manatee ribs that he examined. No other attempts at age estimation of manatees using growth layers have been reported.

Layering rates in hard tissues of sirenians are not known due to a lack of animals of known age (Marsh, this volume; Odell, 1977). Marsh (this volume) reports that

> There have been no successful field studies of specific individuals [of dugongs] over any time span. . . [and although they] have rarely been maintained in captivity [they] have never been bred in captivity.

Several manatees have been born in captivity (Odell, 1977). Apparently, none of these has been examined for growth layers.

Layering rates in marine mammals may be studied by use of *in vivo* administrations of tetracycline antibiotics which are incorporated into the hard tissue shortly after their introduction (Yagi, Nishiwaki and Nakajima, 1963; Best, 1976; Gurevich, Stewart and Cornell, this volume; Myrick, this volume). If given in dosages of sufficient strength, tetracycline labels the layer which is forming at the time the drug is introduced. The label may later be identified as a fluorescent band in the tissue when thin sections are viewed microscopically with reflected ultraviolet light.

The *average* layering rate may be calculated by dividing the number of layers that have accumulated since the marking event by the time elapsed between marking and death of the animal. By assuming that the layering rate is constant, one may estimate the animal's age by counting all layers and multiplying that value by the depositional rate.

This paper reports the first successful tetracycline-marking of skeletal tissues in an Amazonian manatee, indicates the adequate dosage required to produce a detectable mark in the tissues, and suggests the layering rate of periosteal bone. This technique should be of value in future studies of age and growth of sirenians.

MATERIALS AND METHODS

A male *Trichechus inunguis* calf, probably less than six months old and in severely malnourished condition (total curved-line length 93 cm, weight 9.1 kg), was given a single intramuscular injection of 20 mg of *Terramycin* (a tetra-

[1] Contribution No. 21 from the Department of Aquatic Mammal Biology, INPA, Manaus, Brazil.
A Portugese translation will appear in *Acta Amazônia*.

cycline antibiotic) (dosage 2.2 mg/kg) on 23 August 1976. It was maintained thereafter on a diet of milk formula and aquatic plants. By 16 November 1977 it had reached a curved-line length of 125 cm and a weight of 35.2 kg. Single intramuscular injections of 250 and 300 mg of *Terramycin* were given on 18 and 19 November, respectively (dosages 7.1 and 8.5 mg/kg). The animal died on 3 May 1978, at a length of about 128 cm and still weighing about 35 kg; the lack of further growth was attributed to termination of the milk diet. The frozen carcass of the animal used in this study is preserved at the Instituto Nacional de Pesquisas da Amazônia (INPA), Departamento Peixe-Boi, Manaus, Brazil (catalog number INPA-PB 11).

A left rib from the middle thorax (chord length 15.5 cm) was cleaned by maceration and cut transversely at the midpoint of its length, and a thin section (anteroposterior diameter 15 mm, mediolateral diameter 10 mm, thickness ≅ 75 μm) was cut with a diamond saw (Fig. 1). The section was mounted in *Permount* and viewed at 50 to 200 X in reflected ultraviolet light, using a *Zeiss* fluorescent vertical illuminator with a filter reflector No. 48-77-05 combination and in plain and polarized transmitted light. Plain and polarized transmitted-light examinations of several thin sections taken from ribs and mandibles of adult and young adult manatees from Florida were made for histological comparison.

RESULTS

Histology

The histological arrangement of the bone in the thin section examined by one of us (Myrick) corresponds to that described by Fawcett (1942) for thin sections of manatee ribs. The section consists of a stratified series of concentric zones of densified bone that arch outwards from the pleural edge. According to Fawcett (1942) these zones result from different proportions and arrangements of primary (fetal) trabeculae that have become filled gradually by secondary bone, forming a dense central core. They are invested by abundant nutritive channels, but contain progressively fewer Haversian systems toward the outer curvature.

Toward the outer curvature of the section the original fetal trabecular architecture of primary spongiosum is easily recognizable (see Figs. 1 and 2); the inter-trabecular spaces are large and the walls have not been thickened appreciably by deposition of secondary bone. The more proximal trabeculae are thickened, with spaces that are

Fig. 1. Composite photograph of transverse thin section of rib from 2.2 year old male *Trichechus inunguis*, viewed in low-contrast crossed polarized light (×12). Abbreviations: N = probable location of neonatal line separating periosteal bone from spongiosum. T = approximate level of periosteal tissue in which fluorescent mark from *Terramycin* injection may be seen under ultraviolet light.

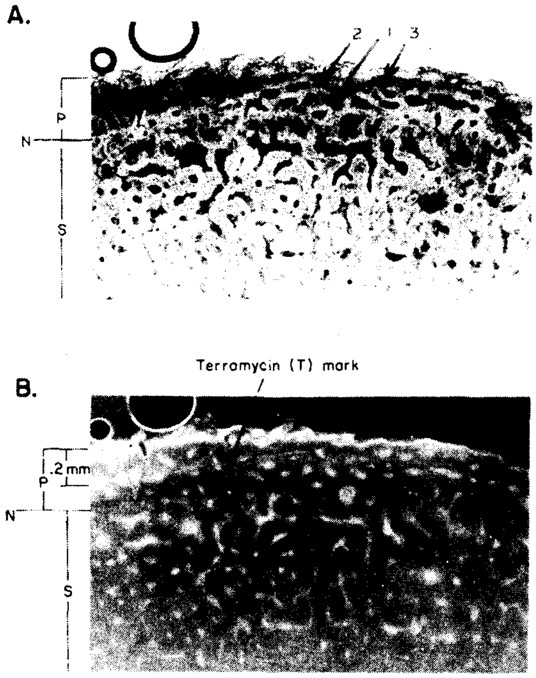

Fig. 2. Transverse thin section of rib as shown in Fig. 1, showing histology near the external margin (x50).
A. Low-contrast polarized light photograph showing three layers (numbered) of aligned Haversian systems in periosteal bone (P). N indicates neonatal line separating periosteal bone from spongiosum (S).
B. Section as shown in A using reflected ultraviolet light. *Terramycin* (T) is represented by fluorescent band (arrow). N indicates well-defined dark region representing neonatal line.

almost entirely filled. Apparently, the deposition of secondary bone commences in the pleural region and progresses outwardly toward the external surface (Fawcett, 1942).

The boundary between the periosteal bone and the fetal core of primary spongiosum is recognized by a sharp change in orientation and shape of the trabeculae. The trabeculae of the core tend to be columnar and radiate outwards from the pleural region in an irregular pattern. The neonatal boundary, i.e. where deposition of periosteal bone begins, truncates the fetal trabeculae with a thickened, continuous envelope of bone containing abundant, flattened osteocytes whose long axes lie parallel to the external curvature of the rib.

External to the neonatal boundary, the bone contains two, possibly three layers of Haversian systems that tend to be open and flattened somewhat, with long axes that are arranged end-to-end, parallel to the external curvature. The bone surrounding the Haversian systems forms a series of more or less continuous walls that accentuate the stratified pattern, but no cemental (adhesion) lines, as described in Fawcett's study, were found in the periosteal bone in the section.

Structures marked by tetracycline

Ultraviolet light produced fluorescence as follows (see Figs. 2 and 3).

(1) Fluorescent rings in the walls of open trabeculae internal to the periosteal (i.e. neonatal) boundary.

(2) Fluorescent rings in the walls and fluorescence of the canaliculi of the Haversian systems comprising the first and second layers, but not the third layer (Figs. 3A, B).

(3) A single, continuous, somewhat irregular fluorescent band situated between the first and second layers of Haversian systems, most closely associated with the second layer, approximately 0.2 mm internal to and parallel with the outer curvature of the bone (Figs. 2B, 3B).

DISCUSSION

Osteogenic activity indicated by fluorescent labels

(1) ***Deposition of secondary bone*** – As indicated by the fluorescent labels, the peripheral systems immediately internal to the periosteal boundary were active when the bone was labelled. This agrees with the interpretation (Fawcett, 1942) that the inter-trabecular spaces of the fetal spongiosum become filled with secondary bone after the fetal framework is formed.

(2) ***Activity and deposition of periosteal bone*** – Fluorescence in the walls and canaliculi of the Haversian systems in the first and second layers indicate that osteoblastic systems in both layers were active at the time tetracycline was introduced. Intimate association of the fluorescent band with the Haversian systems of the second layer suggests that this layer was forming at the time the bone was being labelled. The absence of fluorescence in Haversian systems comprising the third, outermost layer shows that this layer was formed after the tetracycline was incorporated into the system.

(3) ***Adequate dosage required to label bone*** – Three *in vivo* injections of *Terramycin* were given; one (2.2 mg/kg) on 23 August 1976 and the others (7.1 and 8.5 mg/kg) on 18 and 19 November 1977, respectively. Because only one fluorescent band was visible in the section and the 1977 dosages were stronger, we interpret the 18–19 November administration dates to be represented by the band. Apparently, the injection given in 1976 was of inadequate strength to produce a visible mark. We conclude that a dosage of between 7.1 and 8.5 mg/kg is sufficient to label the bone.

(4) ***Possible layering rate in periosteal bone*** – If, as we presume, the animal was (at most) six months old when the first injection was given (August 1976), it would have been about 2.2 years old when it died in May 1978. The total thickness of periosteal bone, containing two and part of a third layers of Haversian systems, would then represent 2.2 years of accumulation. Division of layers by that elapsed time gives an *average* layering rate of approximately one layer per year for the specimen (see Figs. 3B, 3C). However, the animal was young and in poor health and the layers in its rib used in the calculation of layering rate are not separated by adhesion lines observed in peripheral periosteal layers in ribs of adults. No ontogenetic series of rib sections of young and mature animals was available for detailed histological comparison of the early layers. This precludes a confident blanket statement regarding layering rates in periosteal bone of manatees in general.

(5) ***Periosteal layers in other specimens***
Ribs – Haversian systems were not discernible in large numbers in thin sections of periosteal bone from other

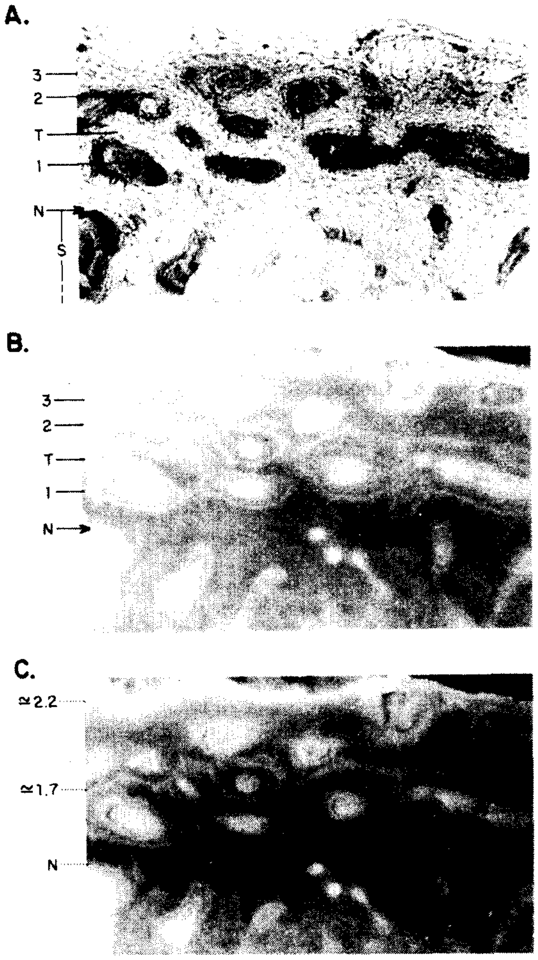

Fig. 3.A. Highly magnified (x200) view in low-contrast polarized light of section shown in Fig. 2. Two and part of a third layer(s) of aligned Haversian systems are shown (numbered). B. Section as in A viewed with reflected ultraviolet light showing fluorescent labels ringing Haversian systems in layers one and two but not in those of the third. Brightness at external margin (top) is autofluorescence which is not of same quality or colour as that produced by the *Terramycin*. (See Fig. 2 for abbreviations.)
C. Section as in B showing time brackets applied to layers used to obtain estimate of layering rates. N indicates time of birth. ≃1.7 indicates accumulation after 1.7 years when the second and third injections of *Terramycin* were administered. ≃2.2 indicates approximate age in years when animal died.

specimens *(T. manatus)* examined by one of us (Myrick). In these specimens the layers contained only flattened osteocytes aligned parallel to the bones' external curvatures. Nevertheless, the boundary (earliest) layer in each section truncated the columnar arrangement of the spongiosum as was observed in the section of marked manatee rib. An ontogenetic series of ribs from neonates through young adults (not available to us) might help in determining whether or not the presence of Haversian systems in the boundary layers is characteristic of the formation of early periosteal bone in manatees. It would also be useful in defining the absorption rate (if any) of periosteal layers in adult specimens.

Mandibles – Although layers were fairly well definable in some of the rib sections examined, they do not always occur with equal clarity or in adjacent areas of the same rib (see Fawcett, 1942; Marsh, this volume). On the other hand, transverse thin sections taken from the posterior regions of mandibular rami of two Florida manatees were found (by Myrick) to show layering very clearly and continuously. These layers appear to be more easily distinguishable than the periosteal layers in ribs. Odell (1977) suggested that investigation of the layers in flipper bones of the manatee might prove useful in making age estimates, but we now believe that periosteal layering in the mandible will give superior results (see also Mitchell, 1978).

SUMMARY

The study suggests the following:

(1) Tetracycline antibiotics may be used safely to label periosteal bone of manatees.

(2) The dosage sufficient to produce a detectable mark under ultraviolet light is between 7.1 and 8.5 mg/kg of body weight.

(3) Periosteal bone may be deposited at a rate of one layer per year in manatees, but data are inconclusive.

(4) In the rib of the manatee, the boundary (i.e. neonatal line) between the fetal spongiosum and the periosteal (postnatal) compactum is demarcated by a truncation of the columnar trabeculae by tissues arranged parallel to the external curvature of the bone.

We make the following recommendations:

(1) Age determination studies of manatees should include investigations of the layering patterns of periosteal bone in the posterior region of the mandible which seems to be superior to ribs in discernibility of layers.

(2) When possible, future studies should include construction of an ontogenetic series of bone samples ordered by carcass body length and separated by sex. Samples would provide information on relative age, ontogenetic development of layers, and deposition and resorption patterns.

(3) When feasible, live animals should be sexed, measured, tagged, and injected with tetracycline in anticipation of future recovery of their carcasses.

ACKNOWLEDGEMENTS

This study is part of an ongoing INPA research program on the biology of the Amazonian manatee. This program is supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) of Brazil, and by grants from the Vancouver Public Aquarium, British Columbia, Canada, and the Fauna Preservation Society of Great Britain. We thank C. W. Asling, R. Best, D. Magor, J. Mead and W. Perrin for their assistance. K. Raymond prepared the figures for this paper. H. Marsh, J. Mitchell and W. Perrin reviewed the manuscript. D. Odell supplied samples of manatee bones for comparisons.

REFERENCES

Best, P.B. 1976. Tetracycline marking and the rate of growth layer formation in the teeth of a dolphin *(Lagenorhynchus obscurus). S. Afr. J. Sci.* 72: 216–18.

Fawcett, D.W. 1942. Amedullary bones of the Florida manatee. *Amer. J. Anat.* 71(2): 271–309.

Gurevich, V.S., Stewart, B.S. and Cornell, L.H. 1980. The use of tetracycline in age determination of common dolphins, *Delphinus delphis,* (published in this volume).

Kasuya, T. and Nishiwaki, M. 1978. On the age characteristics and anatomy of the tusk of *Dugong dugon. Sci. Rep. Whales Res. Inst., Tokyo* 30: 301–11.

Marsh, H. 1980. Age determination of the dugong (*Dugong dugon* (Müller)) in northern Australia and its biological implications, (published in this volume).

Mitchell, J. 1976. Age determination in the dugong *Dugong dugon* (Müller). *Biol. Conserv.* 9: 25–8.

Mitchell, J. 1978. Incremental growth layers in the dentine of dugong incisors (*Dugong dugon* (Müller)) and their application to age determination. *Zool. J. Linn. Soc.* 62: 317–48.

Myrick, A.C., Jr. 1980. Examining layered tissues for age determination of odontocetes using polarized light microscopy, (published in this volume).

Odell, D.K. 1977. Age determination and biology of the manatee. (Report to Director, U.S. Fish Wildl. Serv. Contract No. 14-16-0008-930, 123 pp., unpublished.)

Scheffer, V.B. 1970. Growth layers in a dugong tooth. *J. Mamm.* 51(1): 187–90.

Yagi, T., Nishiwaki, M. and Nakajima, M. 1963. A preliminary study on the method of time marking with lead salt and tetracycline on the teeth of fur seal. *Sci. Rep. Whales Res. Inst., Tokyo* 17: 191–5.