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Age Determination Studies
in Marine Turtles

Preliminary Report (I)

to

National Marine Fisheries Service
(Southeast Fisheries Center)

and

Fish and Wildlife Service
Endangered Species Program

(Region 2)

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I. Literature Review

Scientific papers relevant to the project have been reviewed, including those that deal with: age determination in reptiles, mammals and birds, utilizing bones, keratinous structures and eye lenses; osteology of vertebrates, especially turtles; techniques for marking bones; and techniques for examining Lammelar structures. Over 50 papers have been examined, and together with additional sources they will be listed in later reports to avoid redundancy.

II. Acquisition of Known-age Specimens

Any technique, to be credible, must be tested with known samples. The interpretation of age-related phenomena has to be made on the basis of studies of specimens of known age. Although the logic is simple, in practice this procedure is involved.

Known-age specimens are not common, especially with endangered animals that require elaborate husbandry. Hence, extensive enquiries have to be made, both nationally and internationally, to locate needed materials. This is a protracted process, and involves seemingly endless communications to locate just a potential source. Once a specimen has been located, it must be preserved, packed, and shipped. Instructions explaining these procedures are retarded by great distances between source and destination, and also by language problems.

As sea turtles are listed on CITES Appendix I and in the U.S. Endangered Species Act, it is necessary to obtain lengthy and elaborate documentation with official export and import permits. In some countries this takes months or years.

Notices have been publicized for acquiring species of known age and included in several international herpetological and zoological journals.

Known-age specimens that have been acquired, and addresses for more known-age specimens that have been located are listed in Tables 1 and 2. A great many critical specimens have been located, but from problems of communications, permits, and transportation they have not been acquired.

III. Preparation

Three hard tissues are being examined for age-related phenomena: bones, keratinous epidermal scales, and eye lenses. Each tissue type has distinct problems in preparation and in interpretation.

A. Bones

Virtually every bone that has been sectioned shows growth layers, but some bones have a larger proportion of compact bone and show more growth layers than do others. The most layers seem to occur in the humerus, that element under the most stress because of the powerful swimming movements. Some skull bones may also show large numbers of layers. Several methods of preparation are under trial.

1. Thin sectioning undecalcified sections

Sections can be cut directly from bone with a diamond bladed Isomet rotary saw, with a minimum thickness of about 70 μ . Wafers of about 120 μ thick show clearly defined growth layers, by several preparations:

- a. binocular microscope using reflected or transmitted light
- b. binocular microscope and transmitted polarized light
- c. microradiographs examined with transmitted light under binocular microscope.

After sectioning, wafers can be decalcified, in either 5% Formic Acid or EDTA, and further analyzed with:

- d. binocular microscope and transmitted light
- e. binocular microscope and transmitted polarized light
- f. stained (e.g. H & E) mounted, and examined with transmitted light under binocular microscope
- g. wafers of somewhat greater thickness (e.g. 250 μ) can be etched on one surface with formic acid, coated with gold-

platinum and examined under a Scanning Electron Microscope (SEM)

- h. wafers can also be examined with Scanning Laser Acoustical Microscope (SLAM). Pilot tests are presently being conducted and show promising results.

Of these techniques, microradiography, SEM, and SLAM clearly show that banding patterns relate to differences in mineral densities. The most countable bands are generally seen with decalcified, stained sections.

2. Decalcifying before thin sectioning

Samples of bone can be decalcified for conventional histologic preparation, and there are various agents for removing the hard mineral components, including:

- a. Formic acid and sodium citrate
- b. Formic acid and hydrochloric acid
- c. Nitric acid (diluted with either water or 95% alcohol)
- d. Trichloroacetic acid
- e. Ethyldiaminetetraacetate (EDTA)
- f. Commercial preparations (e.g. RDO).

Concentrations and treatment times can be varied.

Results to date show that a solution of formic acid and hydrochloric acid, recommended by one of the world's leading bone preparation laboratories at the Armed Forces Institute of Pathology (AFIP), works well. Other acids are either harsh on tissue or slow to decalcify. RDO shows promise, once decalcification times are worked out; this commercial preparation works fast and if it is not over used it leaves the material firm but decalcified.

Decalcified samples can be sectioned with a variety of techniques. Embedding in paraffin and treating as conventional histological specimens provides good results for histological work, and when thick sections are cut growth layers are clearly shown. Sections cut at 30 and 40 μ show better layers than those cut at 10 and 20 μ .

A table for sliding microtome with a freezing stage enables sections to be cut without embedding, thus saving time and materials. However, sectioning is more difficult. With further work it is hoped that the freezing microtome will provide a technique for rapid processing.

Decalcified thin sections are usually examined after staining, and Hematoxylin and Eosin (H & E) is the common stain. Other stains (e.g. Alizarin red) will be tried, but the results with H & E are good if the tissues are washed adequately to remove the acid.

Fixation of bone

It is maintained by experts at AFIP that bone must always be fixed in formalin before being sectioned. However, useable results have been obtained without fixation, and it is necessary to test the quality of results with and without fixation. If the lack of fixation in formalin does not lower the quality of the results, the collecting, preserving and preparing of materials will be greatly facilitated. Salt (NaCl) is more easily obtained in field situations, less toxic, and seems to be an adequate preservative. However, trials are being run on the advantages of salt over formalin.

B. Keratinous epidermal scales

The most common method of determining the age of a testudine is by counting "rings" on its scales. Rarely has the technique been "standardized" with known-aged animals, and rings are normally referred to as "annuli" despite the fact that their annual nature has not been established.

This technique is not useable in sea turtles, for rings on scales are usually inconspicuous and few, if present at all. A young, rapidly-growing turtle shows conspicuous growth marks on its shell, but after the first year (in captivity) these are usually incomplete and hard to see. Evidently as an adaptation to reduce drag, the older layers of shell are abraded or sloughed, leaving a smooth outer surface.

The exception is Eretmochelys imbricata, the hawksbill, in which some individuals show non-concentric growth marks. These can occasionally be on the exterior of the carapace and/or plastron scales, but an unknown number of outer layers are not present, and if the animal is adult, some recent rings are so tightly spaced that they are not countable.

Thin cross section (e.g. 100 to 200 μ) of thick pieces of keratin have been cut on a diamond bladed Isomet rotary saw, and these show distinct layering. Thick keratin is difficult to soften and to cut on a microtome, however.

In general, keratinous scales appear to hold little promise as indicators of age, except in Eretmochelys imbricata. The material proves to be difficult to prepare by standard histology, and of limited value as a record of age.

3. Eye lenses

The weight of dried eye lenses increases with age in some vertebrates and this measure can be used as an estimator of age. However, lens weight is not a useful age determiner for many animals, especially birds. Little work has been done on testudines.

Lenses are removed from preserved heads, stored in 10% buffered formalin, cleaned, dried to a constant weight, and weighed. To date, lenses are still being removed. Cleaning, drying and weighing will be done latter.

Because growth rates are extremely variable in sea turtles, lens

weight is not expected to be an accurate predictor of age.

Layering in eye lenses is occasionally apparent, and this will be investigated.

IV. Results

The project is still in an experimental/developmental phase, so known age material has not been systematically examined. Once techniques are developed, detailed studies of this material will be made and reported on.

Table 1. Known-age specimens that have been acquired.

Species	Locality	Age (Years)	Number	Material acquired	Source
<u>Chelonia mydas</u>	Cayman Turtle Farm	1.2	6	limb bones	J. Wood
<u>Chelonia mydas</u>	Cayman Turtle Farm	1.5	4	limb bones	J. Wood
<u>Chelonia mydas</u>	Cayman Turtle Farm	2.5	4	limb bones	J. Wood
<u>Chelonia mydas</u>	Cayman Turtle Farm	3.5	2	limb bones	J. Wood
<u>Lepidochelys kempi</u>	Galveston, Texas	0.75	1	entire	J. Leong
<u>Caretta caretta</u>	Florida	20.0	1	part of skeleton (remainder to be acquired)	R. Wilton
<u>Caretta caretta</u>	Florida	3.0	1	entire	R. Wilton
<u>Caretta caretta</u>	Zurich, Switzerland	10.0	1	entire	R. Honegger
<u>Caretta caretta</u>	Florida	1.2	1	entire	E. Phillips
<u>Dermochelys coriacea</u>	Florida (Mass.)	0.6	1	entire	E. Phillips

Table 2. Contacts that have been developed for known-aged specimens.

Species	Locality	Age (years)	Number	Material available	Source	Problems
<u>Chelonia mydas</u>	Bonin Islands, Japan	1-3	?	Skeleton	Y. Kurata	Permits & shipping
<u>Chelonia mydas</u>	Bonin Islands, Japan	6	1?	Skeleton	Y. Kurata	Permits & shipping
<u>Chelonia mydas</u>	Bonin Islands, Japan	4 or 5	1?	Skeleton	Y. Kurata	Permits & shipping
<u>Chelonia mydas</u>	Phuket, Thailand	8	2	Entire	H. Chansang	Permits
<u>Chelonia mydas</u>	Florida, U.S.A.	?	Several	?Entire	Christianson	Permits
<u>Caretta caretta</u>	U.S.A.	8	1	?Entire	J.K. Langamer	No response
<u>Caretta caretta</u>	Florida, U.S.A.	10	1	Skeleton	F. Beng	No response
<u>Lepidochelys olivacea</u>	Mexico	3	Several	Entire	Christianson	Permits
<u>Dermochelys coriacea</u>	Ceylon	2	1	?	Mrs. Ratnapala	No response
<u>Chelonia depressa</u>	Yorke Island, Australia	?	1	Skeleton	D. Mosby	No response