



# Inter-lab comparison of precision and recommended methods for age estimation of Florida manatee (*Trichechus manatus latirostris*) using growth layer groups in earbones

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## ABSTRACT

Manatees are routinely aged by counting Growth Layer Groups (GLGs) in periotic bones (earbones). Manatee carcasses recovered in Florida between 1974 and 2010 provided age-estimation material for three readers and formed the base for a retrospective analysis of aging precision (repeatability). All readers were in good agreement (high precision) with the greatest apparent source of variation being the result of earbone remodelling with increasing manatee age. Over the same period, methods of sample preparation and of determining a final age estimate changed. We examined the effects of altering methods on ease of reading GLGs and found no statistical differences. Accurate age estimates are an important component for effective management of the species and for better models of population trends and we summarize the currently recommended methods for estimating manatee ages using earbones.

## INTRODUCTION

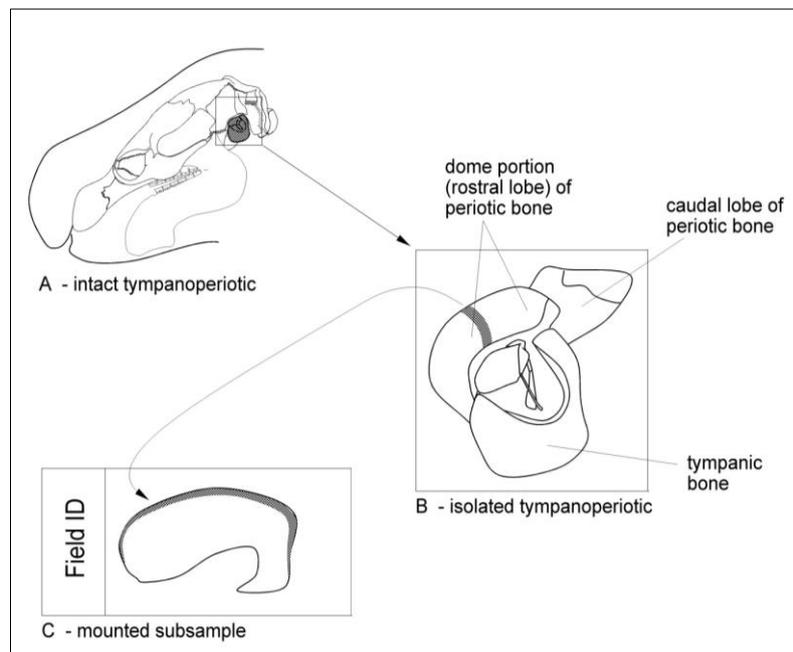
The Florida manatee (*Trichechus manatus latirostris*) is a subspecies of the West Indian manatee. It is a long-lived (maximum 50+ years) herbivorous marine mammal most commonly found in rivers and coastal bays of the Southeastern United States. Responsibility for manatee management is shared between the United States Fish and Wildlife Service (USFWS) and the Florida Fish and Wildlife Conservation Commission (FWCC) (Reynolds *et al.* 2007). The manatee is listed as ‘depleted’ under the Marine Mammal Protection Act, ‘endangered’ under the Endangered Species Act, and is a

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Convention on International Trade of Endangered Species of Wild Fauna and Flora (CITES) 'category I' species.

Scheffer (1950) determined the growth marks in skeletal tissue could be used to help estimate age in mammals. Teeth are the most commonly used structure (Hohn *et al.* 1989), in part because in many species of mammals the adult teeth store a lifelong record with no remodelling (Morris 1972). Growth layer group (GLG) counts in teeth have been used for age estimation in dugongs (*Dugong dugon*, Marsh 1980, 1995), cetaceans (Perrin and Myrick 1980, Pinedo and Hohn 2000), seals (Frie *et al.* 2011), and other mammals (Morris 1972). In contrast, tooth replacement is continuous in manatees and thus individual teeth are not present throughout life and another bony structure must be used for aging (Domning and Hayek 1984).

Marmontel (1993) refined a technique for aging manatees that used the dome portion of the periotic part of the earbone complex (tympanoperiotic) (Chapla *et al.* 2007) also known as the earbone (Fig. 1). The earbone increases in size throughout a manatee's lifetime by deposition of an outer yearly growth layer group (GLG) with some remodelling/restructuring beginning between years 10-15 (Marmontel *et al.* 1996). Each GLG consists of a broad zone (light stain) following an adhesion line (dark stain) (Marmontel *et al.* 1996).



**Fig. 1.** Manatee earbone: A) the tympanoperiotic complex (earbone) intact in the skull, left lateral view; B) the isolated earbone with subsample region of dome portion highlighted; C) the earbone slide and the subsample region in the orientation used to read GLGs under the microscope. (pers. communication, Rommel 2015)

Samples of manatee earbones were obtained from dead manatees that were found opportunistically in Florida and recovered by formal governmental programs instituted in 1974. The responsibility for salvage was transferred from the USFWS to the FWCC (formerly Florida Department of Natural Resources, FLDNR) in 1985. The Fish and Wildlife Research Institute (FWRI), located in St. Petersburg, Florida and part of the FWCC, maintains a mortality database, along with records pertaining to each necropsy. Current responsibility for the recovery and salvage of manatee carcasses in Florida lies with the Florida Fish and Wildlife's Marine Mammal Pathobiology Laboratory (MMPL). MMPL staff members, M. Bolen and K. Brill, used and modified Marmontel's methods for age estimates for reading earbones.

Between 1974 and 2010, 8,244 manatee carcasses were salvaged by either the USFWS or the FWCC. Approximately 20% of the salvaged carcasses were classed as perinatal (total length of  $\leq 150$  cm); in these cases, an earbone was not collected for aging. Thus 6,581 earbones were collected for aging; one earbone per manatee. Of these, 3621 earbones were aged using the earbone techniques outlined herein; 1,197 were read by Marmontel, 831 by Bolen, and 1,593 by Brill.

Age estimation of manatees is difficult and, as in all age determination processes, between-reader variation in estimation can be a concern. Here we provide the first comparative analysis of the methods used by the three readers who have generated the majority of manatee age estimates. The precision of the three readers was assessed using manatee earbones that were aged by two readers.

## **METHODS AND MATERIALS**

### **Samples**

Ideally, inter-lab comparisons would use common methods except for the variables being measured. Manatee material accumulates slowly and opportunistically. In most cases only one earbone has been collected for each manatee limiting inter-animal comparisons. In addition, there have been minor changes in specimen preparation and over time. Thus a rigorous experimental design was not possible due to these logistic constraints. Instead we offer a retrospective comparison. The 3,621 age estimates were prepared using variety of methods (Table 1) and the availability of readers overlapped. Some age estimates presented here have been previously published (e.g. Marmontel *et al.* 1996; Bolen 1997).

Earbone aging is a multi-step process. In general terms, samples were removed in the field or lab and stored temporarily before further processing in the lab. These periotic domes were subsampled, fixed and decalcified in

preparation for taking thin sections which were mounted on slides and read (GLGs counted).

There was variation in the details of these steps (Table 1). Briefly, all three researchers followed the collection protocol of Bonde *et al.* (1983) with minor changes to assure minimal damage. All earbones were washed with fresh water then stored in ETOH or formalin or glycerine for pre-storage. Brill subsectioned the earbone before placing the resultant subsamples in formalin. Later, the earbone was separated and the middle part of the dome was subsampled (Fig. 1B) and a section 2-4 mm thick removed. Bolen and Brill reduced the size of the subsample to 2-4 mm when possible. All labs fixed the subsample in 10% neutral buffered formalin at least overnight then decalcified the earbone subsamples in Rapid Decalcifer (RDO; Apex Engineering Products Corporation), constantly checking the subsamples. Earbones were fully decalcified when they were translucent, had a rubber-like consistency and bent without breaking. Therefore, decalcification times varied, not only between researchers, but also by specimen (Luque *et al.* 2009) and manatee age.

After the earbone subsample was decalcified and rinsed it was stored in 10% NBF (Table 1) prior to slide processing. Marmontel immediately took the earbone subsample for slide processing on a freezing microtome (AO Reichert Sliding Microtome Model 860) (Marmontel *et al.* 1990) instead of placing it back in formalin, or kept it refrigerated before sectioning. Bolen and Brill embedded subsamples in paraffin blocks before sectioning with a rotary microtome, which produced sections slightly thinner than the freezing microtome.

All researchers used a specific hematoxylin stain (Table 1). Bolen and Brill stained via an automatic staining processor (such as the Thermofisher Scientific Varistain Gemini ES). Brill tested different methods and stains prior to All Children's Hospital (ACH) to enhance the readability of the GLGs but none improved on H&E staining in distinguishing GLGs (Brill; unpublished data).

Usually there was one slide, bearing one section, produced for each manatee. Occasionally, two sections would be placed on one slide if the histologist had difficulties sectioning the sample. Readers selected the better section with the best reading area.

### **Reading**

All readers used a high resolution compound light microscope at 10-400 power to view each annual growth layer group, a GLG, defined as a broad lightly stained band followed by a narrow and intensely coloured line (Perrin

**Table 1.** Methods used by researchers in preparing manatee earbones for age determination.

	<b>Marmontel</b>	<b>Bolen</b>	<b>Brill</b>
<b>Removal</b>	Whole earbone	Whole earbone	Whole <2006; Partial earbone >2006
<b>Pre-Storage</b>	air dried <1989; ethyl alcohol >1989 and occasionally 10% neutral buffered formalin (NBF), 1:1 glycerol to water, or 1:1 glycerol to 70%	70% ethyl alcohol	70% ethyl alcohol <2009 10% NBF 2009-Present subsample prior to fixing in 10% NBF eliminating this step
<b>Subsampling/ Fixing</b>	cut to 4mm with rock saw rinsed ~6 h fixed in 10% NBF overnight	cut to 2-4mm with diamondblade saw rinsed 1 h fixed in 10% NBF for at least 24 h	cut to 2-4mm with diamondblade saw rinsed 3-8 h fixed in 10% NBF until decalcification
<b>Decalcification</b>	rinsed thoroughly RDO: 10-12 h	rinsed 1 h RDO: 4-17h	rinsed 1-3 h RDO: 24-72 h
<b>Fixing</b>	rinsed 3 h section immediately	rinsed 1 h 10% NBF	rinsed 3-8 hours 10% NBF $\geq$ 24 h
<b>Slide Processing</b>	freezing microtome section at 14 $\mu$ m standard glass slides	embed in paraffin rotary microtome section at 6-8 $\mu$ m positive glass slides	embed in paraffin. rotary microtome STAT decal (if needed) section at 3-4 $\mu$ m positive glass slides
<b>Staining</b>	Mayer's hematoxylin 30-60 min	Richard-Allan hematoxylin 2 h	Hematoxylin & Eosin 1 h
<b>Reading</b>	5 blind readings, 2 d to several mon. apart. Imaging: Black & white prints  Final Age: 3 identical or mean of 5  If medium or heavy remodelling, adjusted up for lost GLGs, recorded max and min	3 blind readings, 2 d to several mon. apart. Imaging: Sketched & described; some B&W prints  Final Age: determined on a 4 <sup>th</sup> reading, taking into account previous 3 blind readings.  If medium or heavy remodelling, adjusted up for lost GLGs, recorded maximum	3-6 blind readings, 2 d to several mon. apart. Imaging: Video camera & monitor; Digital pictures  Final Age: 3 identical or mean of 6  If heavy remodelling, final age = count or N/A not readable due to remodelling.

and Myrick 1980, Hohn 2002). All readers also used photographs, videos, and image analysis programs to assist in reading.

All readers first read the slides with no reference to biological data or previous readings (blind replicates) eliminating those possible biases in the reading. Slides from many animals were examined during each aging session, making it difficult to remember the previous age estimate for a given animal. Making multiple readings of the same sections increases accuracy and consistency (Pinedo and Hohn 2000). After the final reading when the aging is completed, a final age estimate was derived (“age class (AC)” in Marmontel *et al.* 1996 terminology).

While all readers waited two days to several months between readings, the number of blind readings and generation of a final age estimate varied among readers (Table 1). Marmontel and Brill both read sections in multiple (five and six respectively) blind readings. Both took three identical readings to be the final age estimate. If there were not three identical readings Marmontel used the mean of all five readings and Brill used the mean of all six readings. Bolen read the slide four times and used the first three blind readings to inform the fourth count, which was taken as the final age estimate.

Remodelling increases with age making the earbones of older animals particularly difficult to age. Sections are routinely assessed by all readers as having no, little, medium or heavy remodelling (numerically 0-3). Marmontel *et al.* (1996) outlined a protocol for dealing with resorption, also followed by Bolen. With moderate to severe remodelling, GLGs were followed and matched on the other side of any discontinuity and the number of completely missing lines estimated on the basis of relative GLG width. The first three to six GLGs are typically wider than the more recent GLGs (Marmontel *et al.* 1996) although these GLGS also become progressively thinner.

The three readers differed in the manner in which they dealt with moderate to severe restructuring. When remodelling was not so severe as to obliterate all the wider GLGs, Marmontel used the count of GLGs (minimum age) and an estimate of missing lines to produce a maximum age at each reading, and took the mid-point of each as the age estimate from a reading. Three identical or the average of five such estimates was the final age estimate for the animal, designated as ‘approximate age class.’ When restructuring was an issue, Bolen used the microscope micrometer to measure the span of GLGs present then applied that rate of deposition to the resorbed area to estimate the number of lines lost. Brill attempted to maximize each count by searching the whole section and following partial lines. This maximum count was recorded for each reading with no adjustment for missing lines and the final age was three identical counts or the mean of 5-6 counts. When remodelling was severe, she

recorded that the estimate was a minimum or concluded no age estimate (N/A) was determined.

For severely resorbed earbones, Marmontel recorded three types of final reading (minimum, maximum and mid-point) and we opted, *a priori*, to use the maximum final estimate to compare to Brill's final counts for between reader analyses. In the end, there were only three such maximum values in the final data set. Similarly, we compared Bolen's final estimates, adjusted for lost lines, to Brill's final counts.

### Data Analysis

Precision, defined as repeatability of GLG counts, was assessed by two or more readers reading the same earbone section on the same slide without reference to the other readers' results. Sections prepared by Marmontel were read by Bolen and Brill; those prepared by Bolen read by Brill. None of Brill's preparations was read by the other two.

Agreement between readers was assessed using linear regression analysis of independent readings from the same sections by each member of the same pair of readers. Perfect agreement would be a regression with a 0-intercept and slope of 1. Deviations from this theoretical fit indicate overall between-reader differences that are constant (intercept  $\neq 0$ ) or vary (slope  $\neq 1$ ) with manatee age. The degree of remodelling associated with each earbone section was included in graphic representations but not in the regression analysis. We also calculated the concordance correlation coefficient (Lin 1989, 2000; Murie and Parkyn 2002) using the on-line calculator at <https://www.niwa.co.nz/node/104318/concordance> for discontinuous (quanti-tray methods) data.

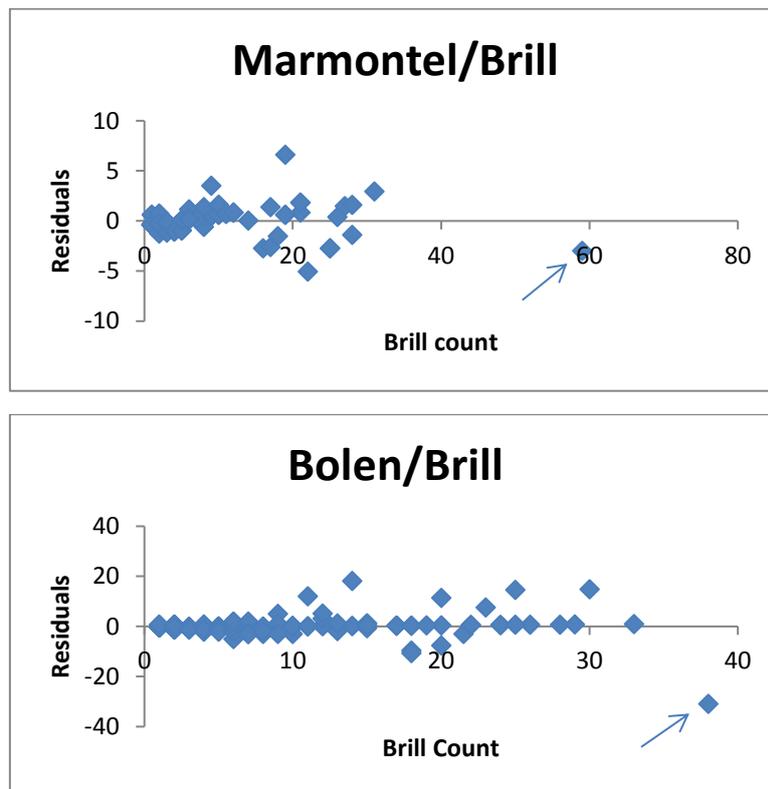
Finally, to statistically assess any changes in precision resulting from changes in methods we examined data from one reader (Brill) reading sections prepared by all three observers. Each section was read three to five times and we compared the frequency with which the final age was obtained in 3, 4, or 5 readings to see if changes in methods changed readability ( $\chi^2$ ).

## RESULTS

There were 250 manatees aged by the two pairs of readers (Ma-Br  $n = 110$ ; Bo-Br  $n = 140$ ). For both pairs of readers, examination of residuals from preliminary regressions indicated there was a statistical outlier in each data set (Fig. 2). These outliers were excluded from subsequent analyses.

For Marmontel and Brill, the regression was not significantly different than the hypothetical model of slope = 1, (Ma/Br  $t_{\text{slope}} = -5.36$ ,  $p = 0.36$ ) but nearly

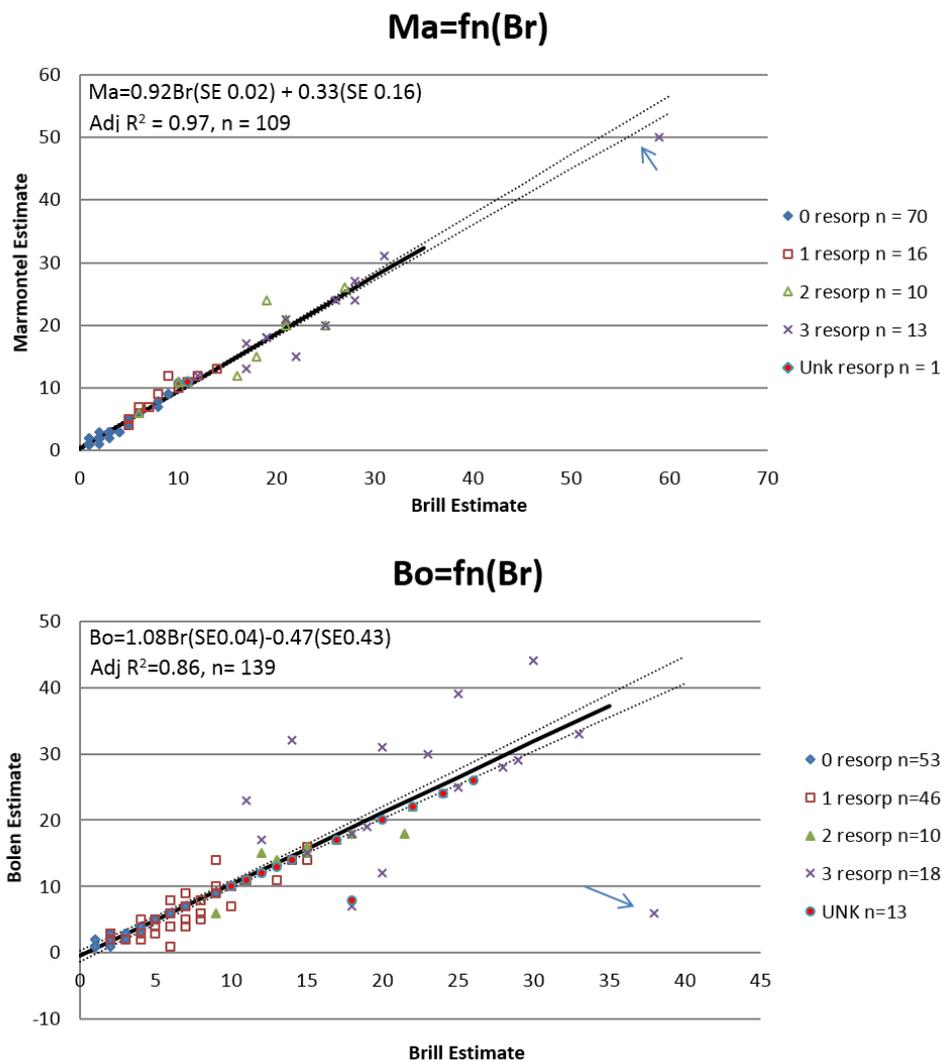
significantly different from an intercept of 0 ( $t_{\text{intercept}} = 1.97$ ,  $p = 0.051$ ; Fig. 3). The Bolen-Brill regression was not significantly different from a slope of 1 ( $t_{\text{slope}} = 2.09$ ,  $p = 0.28$ ) and intercept = 0 ( $t_{\text{intercept}} = -1.09$ ,  $p = 0.28$ ; Fig. 3). Adjusted  $R^2$  values were high for both regressions although there was more variation in the Bolen-Brill dataset, especially for earbones with severe remodelling. Concordance analysis indicates “almost perfect agreement” for discontinuous data for both Marmontel-Brill (Sample concordance correlation coefficient ( $\rho_c$ ) = 0.98 95% CI = 0.97-0.99) and Bolen-Brill ( $\rho_c$  = 0.92 95% CI = 0.89-0.94).



**Fig. 2.** Residual plots for each pair of readers with outliers identified (arrow).

Brill read sections prepared by Marmontel ( $n = 97$ ), Bolen ( $n = 122$ ) and herself ( $n = 63$ ). For age-distribution analysis, samples were grouped 1-5, 6-10, ... 40+ based on Brill’s final age estimates. However, to have sufficient numbers expected in each cell, the older animals were further pooled with the final bin limits being 0, 5, 10, 15, 20, and 25+. The age distributions of the three samples were significantly different ( $\chi^2 = 44.5$ , 8 d.f.,  $P < 0.005$ ,  $n = 282$ ), with far fewer animals  $< 5$  yr final age available to Brill ( $n = 9$ , 14.3%) than the other two readers (59, 60.1% and 59, 48.4%). When these younger animals were removed, the age distributions of the three data sets were not

significantly different ( $X^2 = 11.3$ , 8 d.f.,  $P > 0.05$ ,  $n = 155$ ). In this reduced data set, there were no significant differences in the proportion of samples requiring 3, 4, or 5 readings to attain a final age by Brill ( $X^2 = 0.7$ , 4 d.f.,  $P \gg 0.05$ ,  $n = 155$ ) based on preparation method. Using the full data set despite difference in age distributions, and recognizing that over 60% of the  $\leq 5$  year old group required only three readings, there was still no significant difference among preparation methods, albeit close to significance ( $X^2 = 9.9$  ( $X^2_{0.05} = 9.5$ ), 4 d.f.,  $n = 282$ ).



**Fig. 3.** Regression results comparing age estimates from Marmontel and Brill (upper panel) and Bolen and Brill (lower panel). The fitted line (solid) does not include outliers (arrow) but the 95% confidence intervals (dotted line) were extended to overlap outliers.

## DISCUSSION

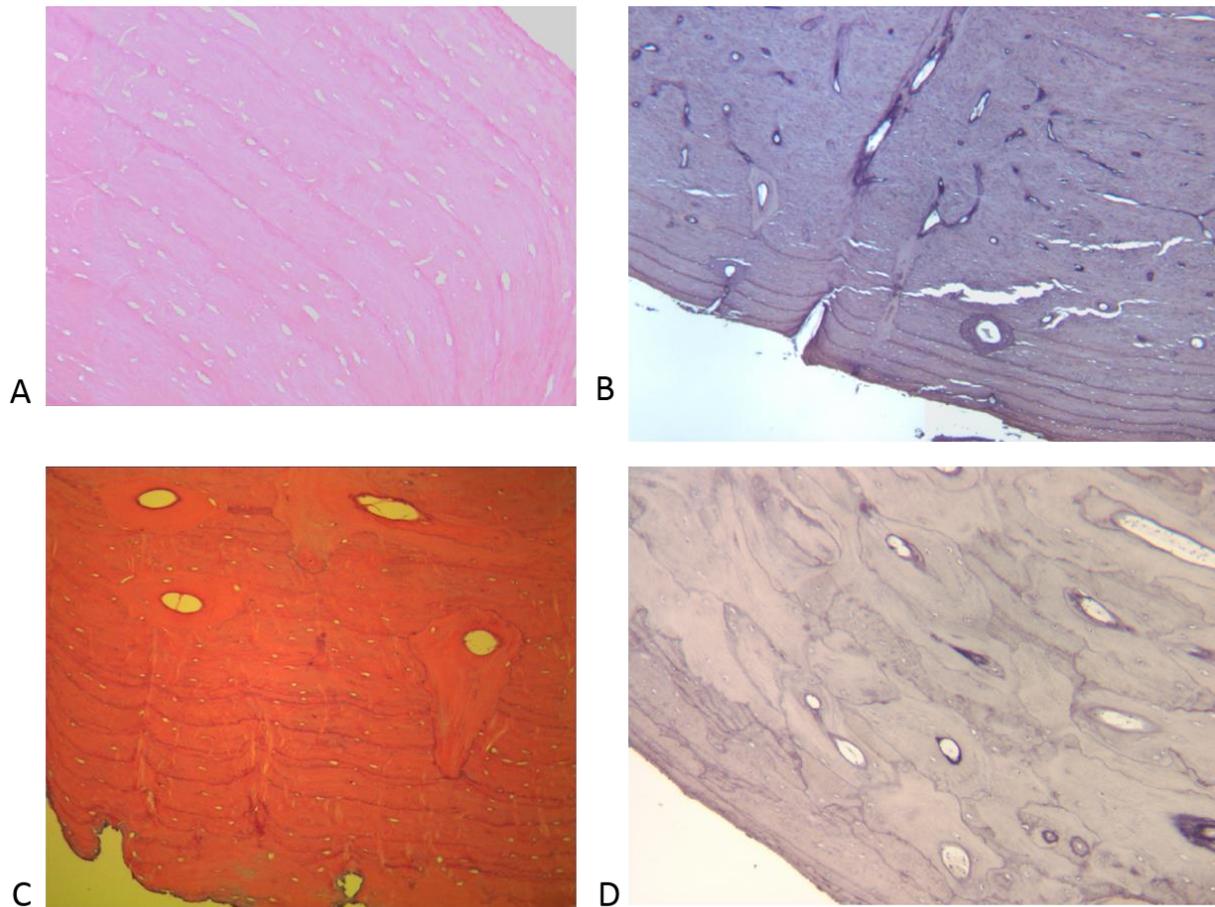
Our results show that precision in age estimation of these three readers was excellent. Experienced age-readers tend to have fewer discrepancies than novice readers (Kitakado and Punt 2007). Novices therefore often require considerable training to close this gap. Marmontel trained Bolen who in turn trained Brill and the value of that direct training probably contributed to the good agreement among the readers.

Nonetheless, there were some unexplained outliers and variation. The extreme outliers in each dataset were few; 1 or 0.9% in the Ma-Br comparison and 1 (0.7%) in the Bo-Br data. Possible sources of such discrepancies include recording or transcription errors, misidentification of the animal or slide, or perhaps a particularly difficult earbone to read. While we cannot retroactively determine the source of these errors, we consider them to be too infrequent to undermine the methods or overall level of precision.

Variation appeared to increase after about age 20 (Ma/Br) or 10 (Bo/Br) and greatest source of variation may be associated with increasing degrees of earbone remodelling (Figs. 2 and 3). Evidence of remodelling is seen in the interruption of the GLGs as the animals get older. In this study relative remodelling rates were scored from 0-3 with 0 showing no evidence of remodelling (i.e. no remodelling of GLGs) and 3 showing the most (i.e. GLGs nearly completely resorbed) at each reading. There was no variation in remodelling scores among replicate readings by an individual or between readers examining the same sections (Brill; unpublished data) so evaluating the degree of remodelling appears highly repeatable.

The remodelling score helps the reader assess the possibility of underestimation of the final age estimate by an unknown amount. No remodelling (Fig. 4A) meant that the reader was able to read the earbone without a problem. Light remodelling (Fig. 4B) displays a low number of Haversian systems and widely scattered secondary osteons. The earbone can be read to obtain a final age estimate, since typically the light remodelling does not interfere with the reading. Moderate remodelling (Fig. 4C) may interfere with obtaining an accurate GLG count due to the more frequent but sporadic small aggregations of Haversian systems. In earbones with heavy remodelling (Fig. 4D), early-formed lines are hard or impossible to read.

Marmontel addressed this difficulty by examining the relative widths of existing GLGs and estimating how many lines may have been lost to remodelling. She added half the difference between the count and maximum adjusted count to the count, generating the final age reading, then used the mean to generate the final age estimate. This technique makes use of the clear



**Fig. 4.** Four levels of remodelling in manatee earbones are documented with each reading to help assess accuracy and precision of the reading. A) No remodelling (score 0) is represented (original magnification 40x) with GLG lines roughly parallel and showing no Haversian systems to interfere with reading. B) Light remodelling (score 1) has a low number of Haversian systems and widely scattered secondary osteons (photo original magnification 10x). C) Moderate remodelling (score 2) is the start of possible age-assigning difficulties and there are more sporadic small aggregations of Haversian systems (photo original magnification 10x). D) In heavy remodelling (score 3), the Haversian systems are abundant and make the GLGs very hard to read (photo original magnification 10x).

differences in early-formed GLGs and cannot be used if the area is entirely restructured. What was a little surprising, because Brill made no adjustment for lost GLGs, was the absence of greater variation in the Ma/Br dataset. Marmontel may have been extremely conservative in augmenting counts with estimates or her process of using mid-points for each reading and an average for the final age may have dampened the effects. Brill's efforts to maximize each count may have favoured congruence.

Methodical differences may have contributed to the greater variation in final estimates between Bolen and Brill for severely resorbed earbones. Brill trained on 200-300 previously read sections while time constraints limited Bolen to about 20 (Bolen 1997). A regression of age estimates derived from earbones with little or no remodelling (scores 0-1, not shown) explained more of the variation (Adjusted  $R^2 = 0.88$ ) in the Bo/Br dataset than when all earbones were used (Adjusted  $R^2 = 0.77$ , Fig. 3) but not as much as the Ma/Br regression.

The difference in training may have contributed to more variation in Bo/Br data than the Ma/Br data, but does not explain the bias among severely restructured sections in which Bolen counts generally were higher than Brill counts (Fig. 2). Bolen estimated the number of lines lost based on the distance spanned by apparent lines and the distance without countable lines. This technique makes some untested assumptions and could lead to an over estimate of the number of lines lost. Bolen took the first three readings into account when determining the final age on a fourth reading. A review of a subset of her original data indicated reading 4, the final age estimate, tended to reflect the highest of the previous counts in restructured earbones. Bolen's adjustment for lost lines coupled with the use of the maximal age estimate likely contributed to the positive bias compared to Brill's final estimates based on the mean.

While there was good agreement between Bolen and Brill until the earbones were severely restructured, using early readings to inform the final reading which becomes the age estimate is hard to replicate and should be avoided. Also, it is clear that all three readers were counting the same things in the same way but that dealing with lost lines is fraught with greater uncertainty. When earbones have heavy remodelling it is often useful to prepare multiple slides. It may also be useful to develop a numerical model for Bolen's method, quantifying lines/mm that can be seen and projecting that rate of deposition to the obscured area.

In cetaceans, methods of preparation affect age estimates and each species should have an established preparation technique and counting method (Perrin and Myrick 1980). Past studies on manatees not only indicate that the

periosteal bone rostral lobe is the most consistent of all the other bones viewed but also that layers are produced annually (Marmontel *et al.* 1990, 1996). Our results indicate that manatee age estimation using earbones is robust to minor changes in preparation protocols. Although not apparent statistically, these modifications have improved ease and efficiency of sample preparation. Here we outline the currently recommended method for manatee aging using earbones, as it has evolved from Marmontel (Marmontel *et al.* 1996) to Bolen to the most recent preparation technique by Brill informed by our analysis.

### **Sample Preparation**

Many factors affect the production of good sections for microscopic examination. Critical steps include fixation, decalcification, embedding, sectioning, and mounting. For decalcification to be effective the earbones must be well-fixed; cutting the earbone into a smaller subsample allows for the formalin to fix the earbone more efficiently and thoroughly (<http://www.statlab.com/technical-procedures/histology/decalcification>).

Fixing the entire earbone takes time and it is still questionable whether the thicker, bigger subsample would allow proper penetration of the formalin. Subsampling immediately does not allow the bone to dehydrate and allows more rapid perfusion by the preservative. At some necropsy sites, this step may not be possible due to limited resources, in which case a whole earbone should be maintained in fluid and fixed as soon as possible.

We recommend that initial storage be in 10% neutral buffered formalin unless it is a known tetracycline animal, in which case 70% ETOH is a suitable replacement. Some earbones initially preserved in alcohol were more brittle than those stored in buffered formalin and crumbled (Brill and Carney, unpublished data) because they dehydrate. However, when a subsample is not dry enough during the histological paraffin processing, the paraffin blocks are filled with water droplets which makes for difficult microtomy. There is a fine line between being too dry and too wet. Using formalin throughout the steps process allows for more control of fixing the earbone correctly and produces a good final result.

The decalcification process needs to be monitored closely as each specimen reacts differently, and neither over- or under-decalcification is conducive to good sectioning or staining. All formalin needs to be thoroughly rinsed with water from the subsample before decalcification. A rapid decalcifier is used over a regular decalcifying agent because it is faster. Manatee bone is denser than the bone of most other mammals and takes approximately twice as long to decalcify. If necessary, a decalcification process can also be slowed down by adding water thus diluting the rapid decalcifier solution. Slower, longer decalcification with diluted RDO facilitated smooth sectioning, made

mounting easier, and produced better staining results (clearer GLGs) than did full-strength RDO (Brill and Carney, unpublished data). However, the subsample can also be degraded and discoloured by decalcifying with RDO too long (>72 hours depending on the condition of the specimen). Transferring back and forth between alcohol, formalin, and decalcifying agent affects the integrity of bone by making it too hard, which in turn compromises sectioning and staining.

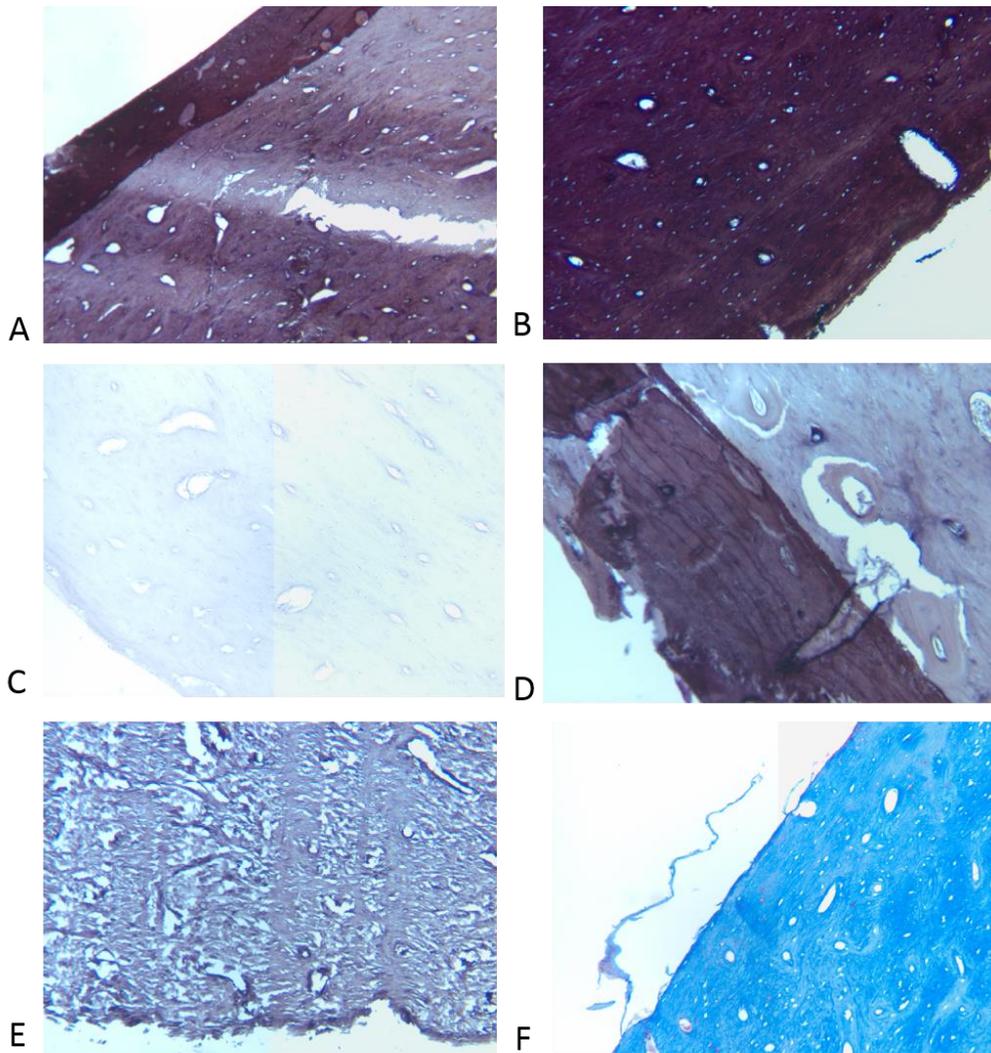
Occasionally, a subsample is not properly decalcified, especially if it is more than 4 mm thick. Improper decalcification may result from inadequately fixing the earbone thoroughly with formalin immediately upon collection, leaving the subsection in formalin for too long or too short a time, leaving it in RDO for too long or not long enough, or using old RDO. The subsample could pop out of the paraffin block, chip, cause microtome banding of the section (Fig. 5A), and the section could stain poorly if the sample is too hard (Fig. 5B & C). When minerals are removed from the improperly fixed specimen through decalcifying, the remaining bone will be extremely soft and will not section.

One remedy is to take a second subsample from the original earbone and begin again. Performing the entire decalcification process again on the first sample is not successful because the earbone becomes too soft after it has been put back in formalin. If needed, the histotechnologist at most can perform a surface STAT decal (a type/brand of rapid decalcifer) of the paraffin block subsamples for five minutes right before sectioning to help obtain a smoother sectioning if the bone is too hard. These salvaged thin sections are sometimes too thin and fold and tear easily during mounting (Fig. 5D & E).

We have found that sectioning at 3-4  $\mu\text{m}$  limits the likelihood of edges folding over and allows a good level of staining. Mounting on positively charged glass slides improves adhesion.

### **Interpreting Annual Lines**

Factors other than specimen preparation that cause difficulty in reading earbones include remodelling, bifurcated lines, and faint traces of lines (accessory lines). Remodelling affects how final age estimates are derived. Trying to estimate the numbers of lines that have been lost due to remodelling undermines the repeatability of final age estimates. It is best to read minimally remodelled areas, so a reader can continue reading uninterrupted GLGs. We recommend that final ages always be presented as the best count of GLGs seen. Adjustments to 'best age' can be provided additionally with details of how they were derived. The end users of the data then have all the information they need to assess which estimate is most appropriate for their application.



**Fig. 5.** Errors in processing result in flawed sections. A) Microtome banding from a damaged knife shown on the earbone due to specimen being too hard (photo original magnification 10x). B) Staining time was too long resulting in a specimen that is too dark (photo original magnification 10x). C) Poor fixation, poor decalcification and too brief a staining time result in an under-stained section (photo original magnification 40x). D) Edges of the earbone folded because the section is too thick (photo original magnification 10x). E) Torn sections can result from poor initial processing or poor sectioning technique or both (photo original magnification 40x). F) An alternate stain (trichrome stain) tried but the GLGs showed up poorly (photo original magnification 10x).

“Bifurcated lines” occur when two lines come together or when a line splits in two. A novice reader may read this as two GLGs. Bifurcated lines need to be followed throughout the specimen. At the opposite outer points of the earbone section, the layers also tend to run closer to the edge and sometimes disappear. Therefore, the best area to read is in between those two points.

Finally, extensive training and practice is recommended until the reader becomes comfortable dealing with the various factors that affect GLG formation and interpretation. Having new readers age already-aged specimens and to have their specimen ages double-checked by a second or third experienced reader will also increase the accuracy of a new reader’s readings and provide a useful training tool to refine the new reader’s technique and skills.

### **Future Research**

The study reported here was a *post hoc* attempt to synthesize what we know about precision in ageing manatees. While our results strongly indicate concurrence among readers, a future study which includes multiple readers examining the same sections prepared by a single method would be useful.

The remodelling of the earbone clearly limits our ability to determine ages of very old manatees. Research should be directed at establishing a numeric algorithm for estimating the number of GLGs lost based on the spacing of early-formed GLGs. Counts could then be adjusted on a repeatable manner and final age estimates incorporate the error associated with conversion equation. It is unlikely even then that the technique can be used if the area is entirely restructured.

Methods of preparing samples and interpreting GLGs continue to evolve; new approaches such aspartic acid racemization may be explored. But new methods must be calibrated against predecessors to ensure there is no loss in precision. Additional research requirements include the examination of more known-age wild animals, especially some of advanced age for further calibration of age versus GLGs and investigation of GLG spacing relating to growth and/or life traumas.

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