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An Evaluation of Histological Techniques Used in Skeletochronological Age Estimation of Sea Turtles

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ABSTRACT. – **Skeletochronological analysis was used to compare stained and unstained cross sections of humeri from Kemp's ridley (*Lepidochelys kempii*) and loggerhead (*Caretta caretta*) sea turtles to determine if the 2 histological techniques yielded an equal number of visible lines of arrested growth (LAGs). Stained sections viewed at high magnification under a compound microscope revealed the presence of closely spaced and splitting LAGs, resulting in a greater number of individual LAG counts for these sections when compared to unstained and stained sections viewed at a lower magnification under a dissecting microscope. Prior studies have shown that some of these closely spaced LAGs are annual, and therefore the inability to detect such marks could result in a downward bias in age estimates.**

Over the past several decades, skeletochronological analyses of growth marks in sea turtle bones have provided age and growth data necessary for accurate parameterization of population models and predicting the effects of management decisions (Heppell et al. 2003, 2005; National Marine Fisheries Service and US Fish and Wildlife Service 2008). The value of the age estimates for these models is contingent on meeting the assumptions that the methods used by various studies to obtain age data yield comparable results and that those results are accurate and unbiased. A review of the sea turtle skeletochronology literature reveals that when conducting these analyses, researchers have used different histological techniques for bone preparation. Five skeletochronological studies of sea turtles were conducted using unstained cross sections of the humerus bone (Zug et al. 1995, 1997, 2006; Zug and Glor 1998; Coles et al. 2001), which means that subsequent to sectioning, the bone was simply immersed in an ethanol:glycerin solution while being viewed under a microscope for analysis. By contrast, 11 used decalcified and stained sections of skeletal structures (Zug et al. 1986; Klinger and Musick 1992, 1995; Zug and Parham 1996; Bjorndal et al. 1998, 2003; Snover and Hohn 2004; Avens and Goshe 2007; Snover et al. 2007a, 2007b; Avens et al. 2009), one used a combination of stained and unstained sections (Parham and Zug 1998), and one did not specify which histological technique was used (Zug et al. 2002). Parham and Zug (1998) called for a comparison of stained and unstained sections to determine if the staining of sections is necessary for both small and large turtles. In addition, studies in other species have shown that preparation technique affects the ability to detect growth marks and obtain accurate age estimates (Stewart et al. 1996; Hohn and Fernandez 1999). However, to date such an assessment has yet to be conducted for sea turtles. Given that there may be differences in the extent to which each technique accentuates growth marks within bone sections, it is important to evaluate the 2 techniques to determine their relative effectiveness.

Previous studies have validated the deposition rate of growth marks in Kemp's ridley (*Lepidochelys kempii*) (Snover and Hohn 2004) and loggerhead (*Caretta caretta*) sea turtle bones (Klinger and Musick 1992; Coles et al. 2001; Snover and Hohn 2004). Although the goal of this study is not to estimate age, the validation studies are important to determine which growth structures in bone must be visible to obtain an accurate age estimate. In this study, we therefore address the question of whether the 2 most common histological techniques result in an equal number of visible lines of arrested growth (LAGs), which delimit the outer edges of skeletal growth marks. We assess the number of LAGs visible in unstained and stained sections taken from the same turtles and also determine whether LAG counts are affected by the type of microscope used for analysis, as well as turtle size.

Methods. — Humeri used in this study were obtained from 20 Kemp's ridleys ranging from 30.3 to 48.7 cm straight carapace length (SCL) and 10 loggerheads ranging from 61.2 to 69.2 cm SCL that stranded dead along the Atlantic coast of the southeastern United States (Virginia to Texas) from 2000 to 2005. SCL was measured from the nuchal notch to the posterior tip of the carapace by an observer at the time of stranding. For those turtles for which SCL was not reported, the following regression equations, as established by Snover et al. (2007b) for Kemp's ridleys and Snover (2002) for loggerheads, were used to convert curved carapace length (CCL) to SCL:

Kemp's ridleys: $SCL = 0.957 \times CCL - 0.696$
Loggerheads: $SCL = 0.923 \times CCL + 0.189$

A low-speed isomet saw (Buehler, Lake Bluff, IL, USA) was used to cut 2 consecutive cross sections at a standardized location for all humeri, just distal to the deltopectoral muscle insertion scar (Zug et al. 1986): 1) a 1–3-mm-thick cross section to be histologically processed and stained, followed by 2) a 0.6–0.8-mm cross section that was not decalcified or stained (hereafter referred to as

unstained). The 1–3-mm cross sections for staining were processed following the methods of Snover and Hohn (2004). They were fixed in 10% neutral buffered formalin (Fisher Scientific, Fairlawn, NJ), rinsed with tap water, then allowed to soak in RDO (Apex Engineering Products Corp., Aurora, IL), a commercial decalcifier, for 6–36 h, depending on the size of the bone. After allowing sections to soak in water overnight, 25-µm-thick sections were taken using a freezing stage microtome (Leica Microsystems, Inc., Bannockburn, IL) and stained using Ehrlich's hematoxylin diluted 1:1 with water filtered by reverse osmosis (Klevezal 1996). Stained sections were mounted on slides in 100% glycerin under cover glass sealed in place with a high-viscosity mounting medium (Cytoseal 280, Thermo Scientific, Waltham, MA), which allowed the sections to be viewed and archived. Unstained sections were stored dry until viewing in a solution of 4:6 glycerin and ethanol, according to the methods of Parham and Zug (1998).

The stained sections were viewed using transmitted light on both a Nikon SMZ-U dissecting microscope (Nikon Inc., Melville, NY) and an Olympus BX41 trinocular compound microscope (Olympus America Inc., Melville, NY). Unstained cross sections were viewed using transmitted and reflected light in a solution of 4:6 glycerin and ethanol on the dissecting microscope only, as it was not possible to view sections in solution using the compound microscope. Both types of sections were viewed at up to ×7.5 magnification on the dissecting microscope; the compound microscope allowed stained sections to be viewed at up to ×20 magnification. Sequential images of the stained sections were taken at ×4 magnification on the compound microscope using an Olympus Colorcube-12 Color CCD digital camera with Olympus Microsuite image analysis software (Olympus America) and stitched using Adobe Photoshop (Adobe Systems Inc., San Jose, CA) to obtain high-resolution composite digital images. Digital images of the stained and unstained sections were taken on the dissecting microscope at ×0.75–×1.5 magnification, as the lack of a movable stage did not permit the precision of movement required to obtain sequential images at higher magnifications.

The number of individual LAGs in stained and unstained sections was counted by 3 independent observers (LRG, LA, and JB), after which a consensus among observers was reached to yield a final LAG count for each section. Consensus reads of the stained sections were conducted using the composite digital images taken on the compound microscope. All other digital images were used only to mark LAGs once consensus was reached. We compared the number of LAGs detected in 1) stained and unstained sections viewed under a dissecting microscope and 2) stained sections viewed under a dissecting vs. compound microscope. Additionally, we attempted to determine whether turtle size influenced LAG visibility by analyzing these comparison-count data for a subset of small (30–40 cm SCL)

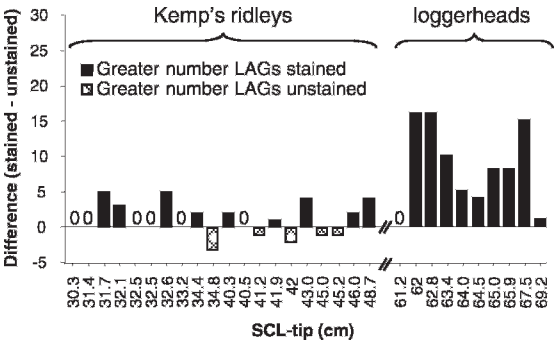

Figure 1. Comparison between stained and unstained bone sections using a dissection microscope. A significantly greater number of individual LAGs was visible in Kemp's ridley and loggerhead stained sections than in unstained sections when the dissecting microscope was used to view both (Kemp's ridleys $p < 0.05$, $n = 20$; loggerheads $p = 0.0025$, $n = 10$).

Kemp's ridleys separately. The Wilcoxon paired-sample test (Zar 1996) was used for all LAG count comparisons.

Results. — Stained vs. Unstained, Dissecting Microscope. — When both groups of samples were read on the dissecting microscope, a significantly greater number of individual LAGs was visible in the stained sections when compared to the unstained sections for both the Kemp's ridleys (Fig. 1; $p < 0.05$, Wilcoxon paired-sample test, $n = 20$, mean absolute difference = 1.8) and loggerheads (Fig. 1; $p = 0.0025$, Wilcoxon paired-sample test, $n = 10$, mean absolute difference = 8.3).

Stained Sections: Dissecting vs. Compound Microscope. — As the stained sections were examined on the dissecting and compound microscopes, it was possible to compare the number of individual LAGs visible in those sections to determine if the type of microscope affected LAG detection. For the Kemp's ridley stained sections, a greater number of individual LAGs was visible using the compound microscope (Fig. 2; $p < 0.0005$, Wilcoxon paired-sample test, $n = 20$, mean absolute difference = 5.3). There was no significant difference between

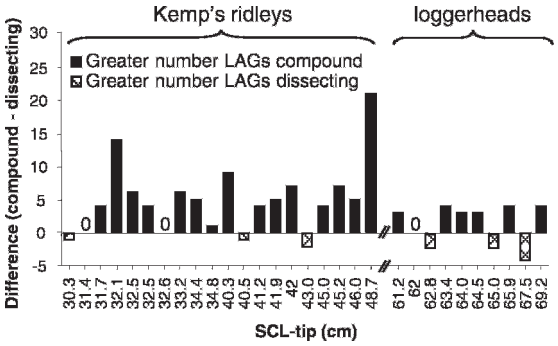

Figure 2. Comparison between stained bone sections viewed under a dissecting vs. a compound microscope. In stained Kemp's ridley sections, a significantly greater number of individual LAGs was visible when viewed on the compound microscope than when viewed on the dissecting microscope ($p < 0.0005$, $n = 20$). There was no significant difference in the number of individual LAGs visible in the loggerhead stained sections using either microscope ($p > 0.10$, $n = 10$).

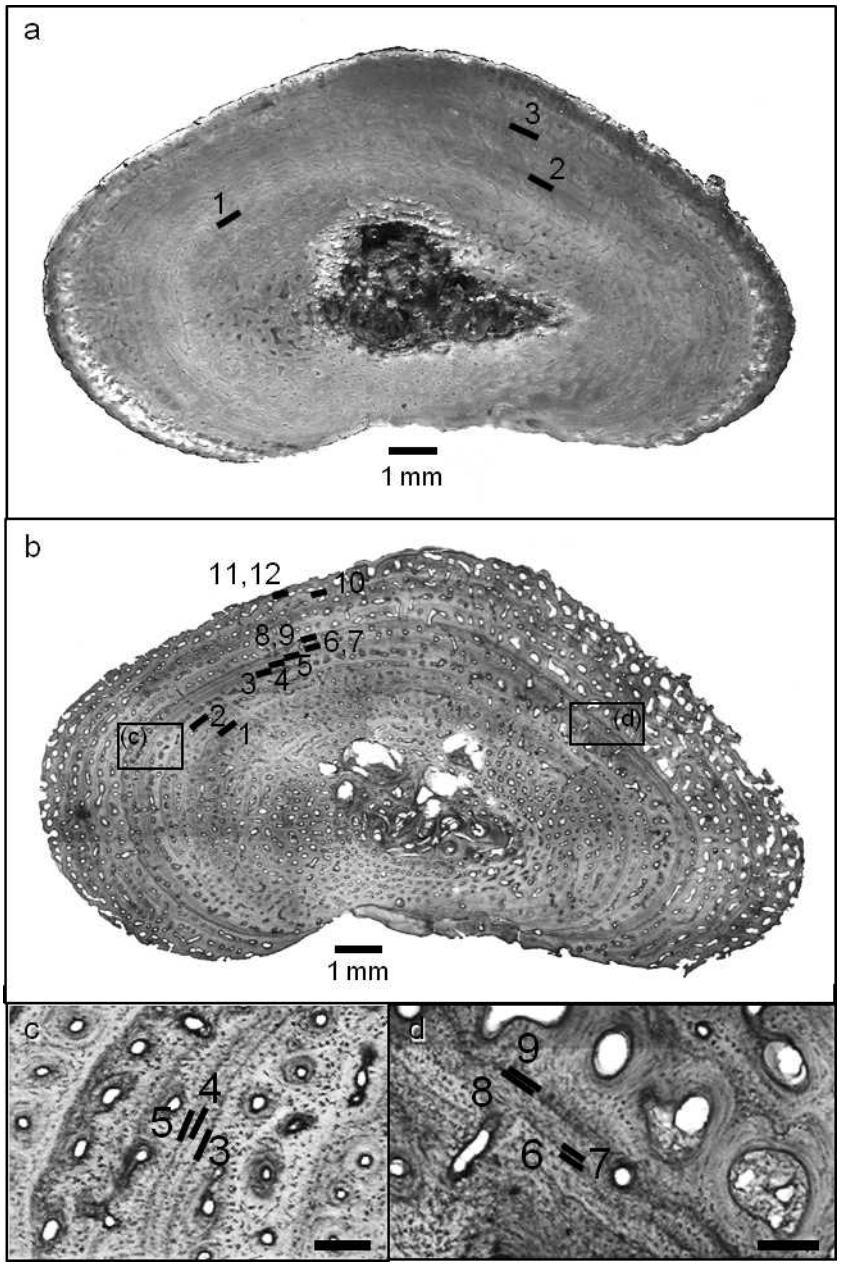

Figure 3. Images of stained and unstained bone sections from a Kemp's ridley sea turtle (ID: Lk JVK 001122-03; SCL: 31.7 cm). a) Unstained section showing 3 LAGs and b) stained section showing 12 LAGs. c) and d) Closely spaced individual LAGs are discernible on the stained section under high magnification. LAGs are denoted by black lines. Horizontal black bars in c) and d) represent 0.25 mm.

the 2 microscopes in the number of individual LAGs visible in the loggerhead stained sections (Fig. 2; $p > 0.10$, Wilcoxon paired-sample test, $n = 10$, mean absolute difference = 2.9).

Small Individuals: Stained vs. Unstained. — We tested whether the difference in count results between species might be due to the small size of Kemp's ridley bones. Ten Kemp's ridleys between 30.3 and 34.8 cm SCL comprised the smallest individuals in this study. When the dissecting microscope was used to read both the stained and unstained sections, there was no significant difference in the number of individual LAGs visible ($p > 0.20$, Wilcoxon paired-sample test, $n = 10$, mean absolute difference = 1.8). However, when the stained sections were viewed on the compound microscope, a significantly greater number of individual LAGs was visible compared to the unstained sections viewed on the dissecting microscope ($p = 0.01$, Wilcoxon paired-sample test, $n = 10$, mean absolute difference = 5.7).

Discussion. — During our analyses, LAGs appeared as dark lines in both the unstained (Fig. 3a) and stained sections (Fig. 3b) but were less distinct in unstained sections. Additional fine-scale histological features were visible in the stained sections that were not visible in

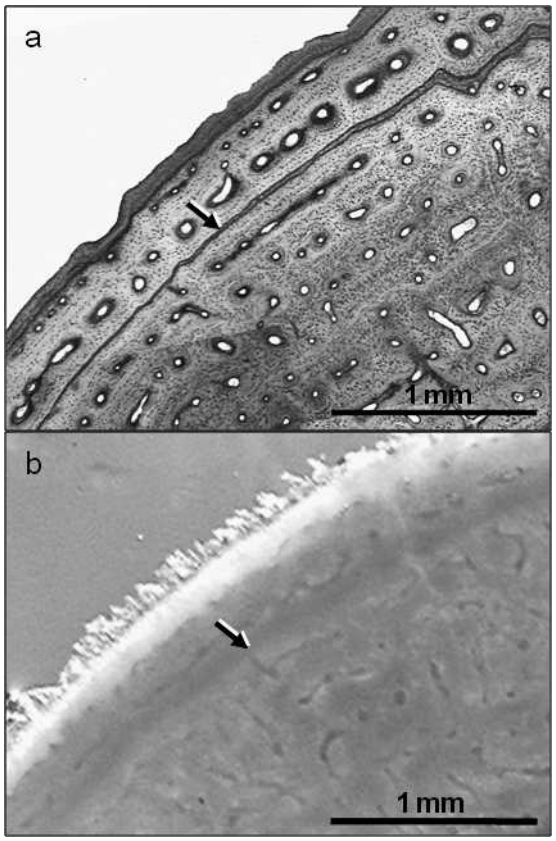

Figure 4. Partial image of stained and unstained bone cross sections from a Kemp's ridley sea turtle (ID: Lk DMB 041111-01; SCL: 32.1 cm). Arrows denote splitting LAG a) identified in stained section and b) not identified as a splitting LAG in unstained section taken from the same turtle.

unstained sections, such as closely spaced double LAGs and splitting LAGs, which are LAGs that appear to be single but can occasionally be resolved into 2 or more individual LAGs when tracking the LAG around the entire circumference of the section (reviewed by Castanet et al. 1993) (Figs. 3c, d, 4a, b). Although growth marks are typically described as consisting of 1 wide zone followed by 1 dark line, or LAG (Castanet et al. 1993), anomalous LAGs are not uncommon. Double and split LAGs have been observed in the bones of amphibians and reptiles (for review, see Castanet et al. 1993; Snover and Hohn 2004). As bone growth is related to somatic growth (Snover et al. 2007a), if a turtle experienced zero growth in a given year, as Braun-McNeill et al. (2008) measured in loggerheads, true annual LAGs would be spaced very closely together, appearing as a double or split LAG. Closely spaced LAGs are difficult to distinguish as individual marks, particularly if the sections are unstained, as well as when stained sections are viewed at lower magnification, as was the case for the small Kemp's ridley humeri analyzed using the dissecting microscope. In addition, splitting LAGs that are often resolved as several LAGs (reviewed by Castanet et al. 1993) can be missed on unstained sections or at lower magnification (Fig. 4a, b). In both cases there is the danger of underestimating age if the appropriate analytical technique is not applied. Comparison of the number of observed LAGs for loggerhead stained sections viewed under the dissecting microscope at low magnification and the compound microscope at higher magnification yielded no significant difference. However, this result may be a function of small sample size, as the mean absolute difference for the loggerhead comparison was 2.9, greater than the absolute difference of 1.8 for the stained vs. unstained Kemp's ridley comparison involving a greater number of humeri, which did produce a significant result.

It is important to mention that the number of individual LAGs does not necessarily correspond with age estimates, as not all individual LAGs are annual marks. LAG deposition can also be caused by interruptions in growth related to seasonal cycles as well as noncyclical events such as hatching, physiological stress, and irregular climatic conditions (Klevezal 1996). For example, more than 1 LAG is deposited each year in species of newts in which growth is interrupted by aestivation and hibernation in the same year (Jakob et al. 2002; Olgun et al. 2005). Supplemental LAGs that do not demarcate annual cycles have been documented in sea turtles (Snover and Hohn 2004). However, regardless of the ultimate interpretation of the marks being analyzed, the results of this study indicate that fine-scale LAG characteristics were most visible, and thus available for analysis, when sections were stained and viewed under high magnification. Although occasionally a greater number of LAGs was initially found for unstained sections (see Fig. 1), post hoc side-by-side comparison with stained sections revealed that this discrepancy resulted from erroneous interpretation of shading as LAGs in the unstained sections. The persistence of the use of 2 different histological techniques in sea turtle skeletochronological analyses is likely related to the cost, time, and labor associated with such studies. Unstained sections require less time, processing, and equipment compared to that needed when staining sections. Whereas it is possible to examine unstained sections for growth marks immediately after taking a cross section, a number of days of processing are required before stained sections can be read. Use of a simpler technique is, of course, reasonable as long as the results are accurate; however, the results of the current analyses indicate that this might not always be the case when using unstained sections.

Conclusions. — On the basis of the results of this study, we recommend that stained sections be used when obtaining age and growth data for sea turtles through skeletochronological analysis. We further recommend that growth marks in these stained sections be examined using a high-magnification microscope. Although we did not find a significant difference in LAG counts between techniques for the smallest Kemp's ridleys in our sample when viewed at low magnification under a dissecting microscope, we extend these recommendations to turtles of all sizes and

species, as methodological consistency is essential for allowing comparisons among samples and studies.

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