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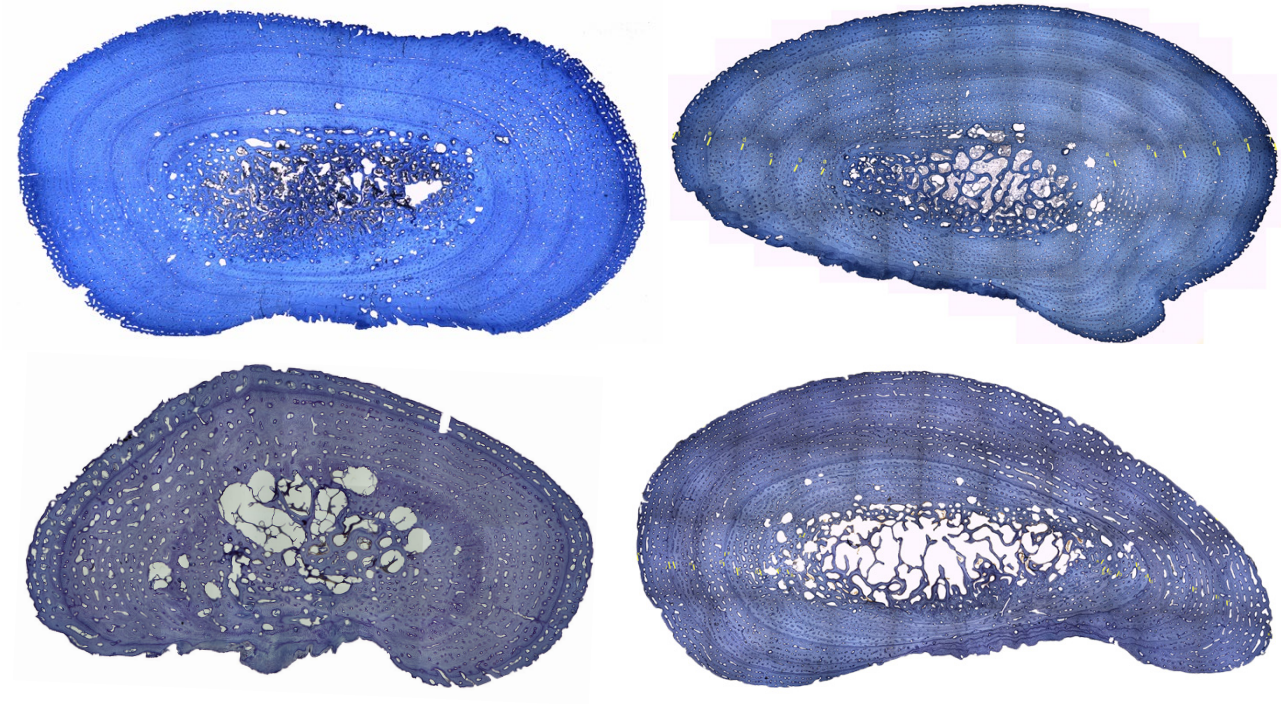
PROTOCOL FOR PROCESSING SEA TURTLE BONES FOR AGE ESTIMATION

LISA R. GOSHE
LARISA AVENS
MELISSA L. SNOVER
ALETA A. HOHN



U.S. DEPARTMENT OF COMMERCE
National Oceanic and Atmospheric Administration
National Marine Fisheries Service
Southeast Fisheries Science Center
Beaufort Laboratory

May 2020

Cover photos: Hematoxylin stained cross sections of sea turtle humerus bones. Species of turtles clockwise from top left: green *Chelonia mydas*, hawksbill *Eretmochelys imbricata*, loggerhead *Caretta caretta*, Kemp's ridley *Lepidochelys kempii*

Images by: Lisa Goshe, Shawn K. K. Murakawa, Cali Turner Tomaszewicz, Larisa Avens, and Matt Ramirez

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National Marine Fisheries Service
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Beaufort Laboratory
101 Pivers Island Rd
Beaufort, NC 28516

U.S. DEPARTMENT OF COMMERCE
Wilbur Ross, Secretary

NATIONAL OCEANIC AND ATMOSPHERIC ADMINISTRATION
Neil Jacobs, Under Secretary for Oceans and Atmosphere (Acting)

NATIONAL MARINE FISHERIES SERVICE
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May 2020

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Cover photographs by Lisa Goshe, Shawn K. K. Murakawa, Cali Turner Tomaszewicz, Larisa Avens, and Matt Ramirez

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Preface

Skeletochronology, or the histological preparation and analysis of skeletal growth marks in bones, has proven to be a useful tool in estimating the ages and growth rates of sea turtles. The ability to successfully carry out this technique can allow researchers to fill existing data gaps and detect temporal changes in vital rates. This report is intended to be a guide for reproducing the methods that have been refined over many years by National Marine Fisheries Southeast Fisheries Science Center researchers at the NOAA Beaufort Laboratory for the purpose of estimating the ages and growth rates of sea turtles. We describe each step in sufficient detail to guide histological processing of sea turtle bones so that the lines of arrested growth (LAGs) that delimit the outer edges of individual, annual skeletal growth marks may be visible in decalcified, stained bone cross sections. Bone histology is a technique that is not always straightforward and can present difficulties, which is why we include a troubleshooting section with a pictorial guide to common issues encountered and our suggestions for correcting course. The focus of this guide is on histological processing of juvenile and adult sea turtle humerus bones, as they are the primary structure typically used for age and growth analyses; however, brief methods for processing scleral ossicle bones and hatchling humeri are included in Appendices 4 and 5.

Introduction

Sea turtle age and growth research addresses the gaps in demographic data needed for modeling population trajectories of these protected species. Because sea turtles lack external structures suitable for estimating age, a method for age estimation of live sea turtles is not currently available. However, skeletochronology, or the analysis of growth marks deposited in bones or other calcified structures, has been reliably used to estimate ages and somatic growth rates of dead sea turtles for decades (reviewed by Avens and Snover 2013).

A growth mark consists of a wide zone of active growth followed by a line of arrested growth, referred to as a LAG, which is deposited during a period of slow or no growth (Castanet et al. 1993). The earliest growth marks are at the center of the bone, with the most recent growth occurring along the outer edge (Zug et al. 1986, Klevezal 1996). The humerus bone is most commonly used in sea turtle skeletochronology studies because it exhibits less resorption, or remodeling, of early growth marks than other bones when sections are taken near the narrowest part of the bone, distal to the deltopectoral muscle insertion scar (Zug et al. 1986). The high degree of resorption in the humeri of leatherback sea turtles *Dermochelys coriacea* prevents them from being useful for skeletochronological studies and, as a result, scleral ossicle bones from the eyes are examined instead (Zug and Parham 1996, Avens et al. 2009, 2020; Appendix 4).

Before the growth increments revealed using skeletochronology can be interpreted with confidence, it is necessary to determine: 1) how often LAGs are deposited, 2) how many LAGs may have been lost to bone remodeling, and if the intention is to estimate prior carapace lengths from LAG measurements, 3) the relation between bone growth and body growth. Greater detail on addressing these issues can be found in the review by Avens and Snover (2013).

The following is intended as a detailed guide for researchers seeking to replicate the methodology for applying skeletochronology to sea turtles. The laboratory protocol will be described with greater specificity than what is typically permitted in the methods section of published studies. Briefly, the histological process involves collecting humerus bones from dead sea turtles, cutting a cross section from the bone, fixing and decalcifying bone cross sections, then cutting thin sections that are stained in hematoxylin and mounted on slides in glycerin. Cover glass is secured in place over the sections with a sealing medium and digital images are collected so a calibrated, high-magnification composite image of each sample may be constructed, which is used for identifying and counting growth layers as well as measuring bone features. The detailed protocol, description of laboratory equipment and supplies, and troubleshooting suggestions for issues commonly encountered when carrying out the methods should allow for successful application of skeletochronology to sea turtles.

Bone Collection

Supplies needed:
Knife, nitrile gloves, ziptop plastic bags, permanent marker, waterproof paper, water-resistant string, hole-punch, calipers, freezer

From dead sea turtles, typically the left front flipper is removed at the joint between the humerus and the pectoral girdle using a knife, taking care to avoid cutting into the proximal head of the humerus bone (Fig. 1). The right flipper may also be collected for processing if the left humerus is not available. In addition to collecting flippers, it is important to record the species, date, straightline carapace length from the nuchal notch to the posterior tip (SCL, cm), stranding location, and turtle number by day. If tags are present, they should also be recorded. The flipper may be stored in a plastic bag that is labeled using permanent marker with the species, stranding date, turtle number by day, and carapace length. A 2" by 2" square of waterproof paper that has been hole-punched and has water-resistant string (e.g., masonry twine) attached should be labeled with the same information in permanent marker and included with the flipper. Flippers may be stored in a regular freezer until they are flensed, or the humeri are dissected out from the rest of the flipper.

Fig. 1 Dead loggerhead sea turtle (*Caretta caretta*) with the left front flipper removed at the joint and the proximal head of the humerus bone visible.

Flensing Humeri

Supplies needed:
Knife, kevlar glove, nitrile gloves, cutting board, knife sharpener, slow cooker or cooking pot, water, scrub brush, blunt forceps

Allow flippers to thaw if they are frozen. Using a knife, dissect away as much muscle from the humerus bone as possible without damaging the bone. Discard the remaining portion of the flipper from the elbow joint to the tip of the flipper. To protect hands from cuts during dissection, wear kevlar gloves in addition to nitrile gloves. Once the humerus has been removed, tie the string and waterproof label tightly around the center of the bone shaft and secure with several knots. Remove remaining tissue adhered to the flensed bones by placing them in water at a low simmer for approximately 6 hours until they are clean. A slow cooker works well for this step. Check the bones periodically to ensure they are removed from heat when the soft tissue begins to easily fall away from the bone, as smaller bones typically require less cooking time (~3 hours) than larger ones (~6 hours). Allowing bones to cook too long or at too high of a temperature can result in cracking and damage, in which case they will no longer be useful for histology.

Rinse bones with water to remove any remaining tissue and cartilage and use a scrub brush and/or forceps to assist with tissue removal where needed, then allow the bones to dry in the sun for approximately two weeks (Fig. 2). After this point, the humeri may be processed in the laboratory.

Fig. 2 Sea turtle humeri cleaned of tissue and drying in the sun prior to processing. Identification labels are tied around the bones.

Marking and Measuring Humeri Before Sectioning

Supplies needed:
Digital calipers, pencil, Humerus Bone Measurement sheet from Appendix 1

Histologically processing bones from start to finish requires a minimum of 6 days for the smallest bones and longer for larger bones, up to several weeks. We recommend processing bones in batches of 10 so that work can proceed at a comfortable pace; however, it is possible to process a greater number depending on the skill and number of technicians. We also recommend sorting bones by size, so that similarly-sized bones are processed in each batch.

Prior to cutting cross sections, bone measurements of interest may be taken using digital calipers. Analyses to date indicated that humerus diameter, also known as medial width, may be the most relevant of the many measurements that may be collected and should be prioritized (see Zug et al. 1986 for a full description of bone measurements collected prior to histological processing). Appendix 1 contains a datasheet for recording humerus bone measurements, as adapted from Zug et al. (1986).

To measure humerus diameter, first locate the deltopectoral crest and the muscle insertion scar (Fig. 3). Draw a line in pencil just distal to the muscle insertion scar on the ventral side of the bone (Fig. 4). Take care in drawing the orientation of this line, as this is where the cross section will be taken. The line should be horizontal and straight, not curving or at an angle, when the bone is viewed with proximal process up. When the bone is rotated 90 degrees, the line should be vertical and straight. Humerus diameter is essentially the diameter of the cross section and is measured along the pencil line that should now mark the distal end of the deltopectoral muscle insertion scar. We recommend drawing arrows pointing to the line from proximal and distal aspects to permit thin sections to be collected from the side of the cross section where humerus diameter was measured (Fig. 4).

Fig. 3 Ventral aspect of the left humerus bone from a green sea turtle (*Chelonia mydas*) with bone features and orientation labeled.

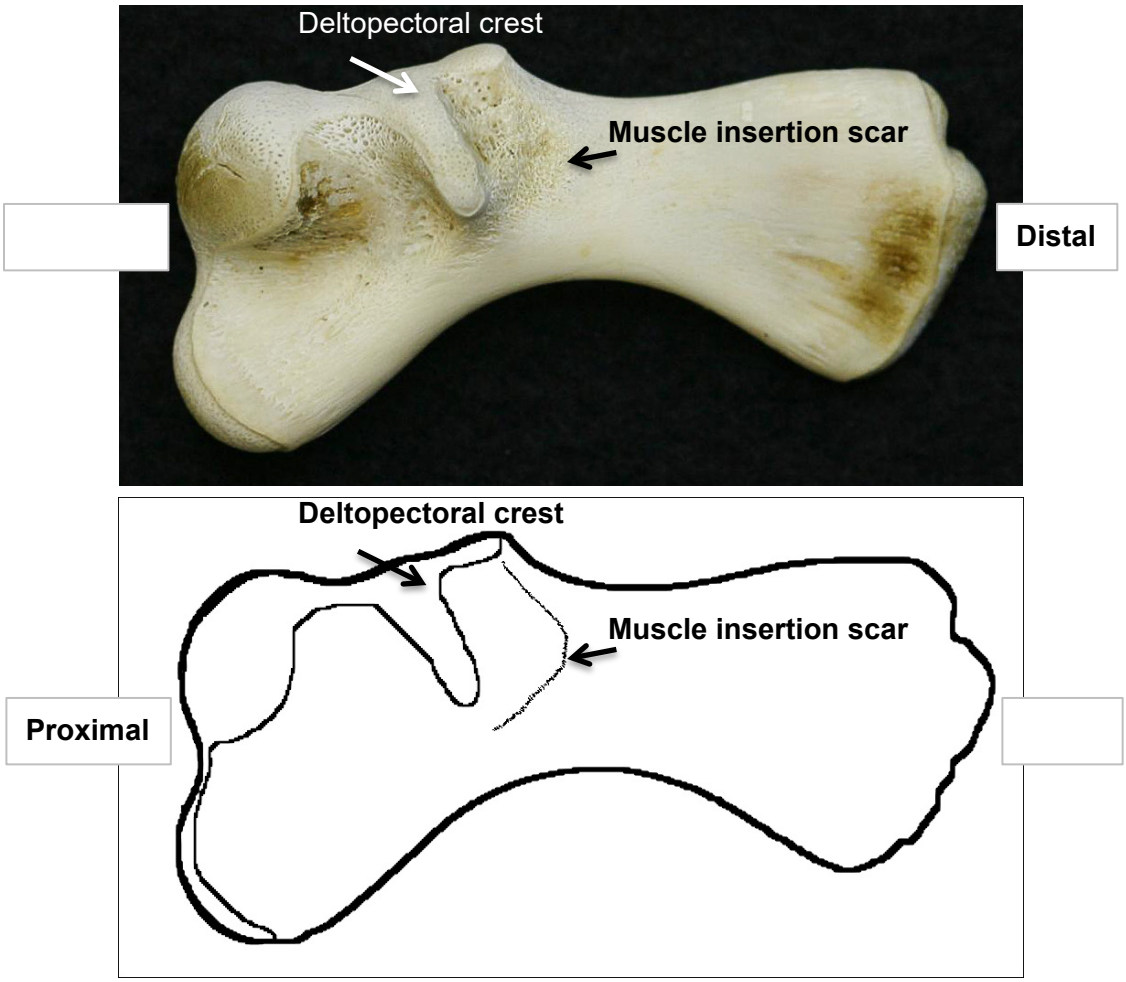
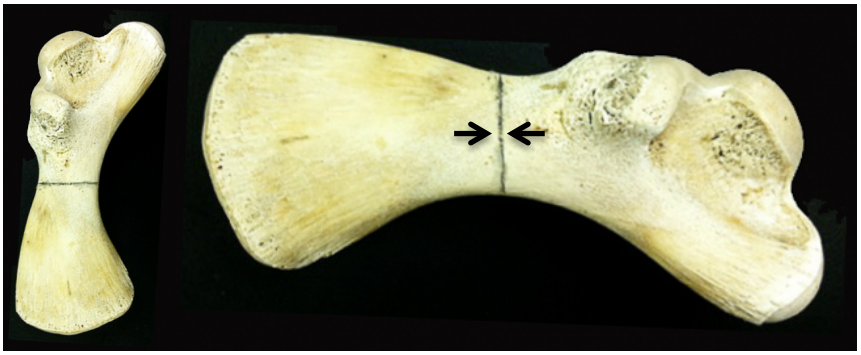


Fig. 4 Right humerus bone from green sea turtle (*Chelonia mydas*) with line drawn where the humerus diameter is measured and cross section will be taken. The line should be perpendicular to the long axis when the bone is rotated. Arrows should be drawn on the bone to identify the side of the cross section from which thin sections should be cut.



Cutting Cross Sections from Intact Bone

Supplies needed:
Digital calipers, scissors, pencil, low speed saw, 3", 4", and possibly 5" series 15 high concentration diamond blades, single saddle chuck, irregular specimen chuck, U-shaped bone chuck, small weights for IsoMet arm, hex wrench, water, dish soap, lab towel, small bowl, coping saw, waterproof paper, histo-cassettes, small bags if collecting additional cross sections, sealable bags for archiving remaining bone pieces, two 1x1" blocks of wood, label tape, small shop clamp

A low speed saw with diamond-embedded blades is typically used for cutting a cross section from the humerus bone. We use the IsoMet Low Speed Precision Cutter (Buehler, Lake Bluff, IL) with a bone chuck(s), a diamond wafering blade(s), and an arm attachment with weights (Fig. 5). Most bones can be sectioned with the single-saddle chuck and the 4" series 15 high concentration diamond blade. The 3" blade is used for the smallest of bones, to limit bone tissue loss when making the cross section cuts; the 5" is for the largest bones. To section, assemble the IsoMet saw so that the blade is secured between the two stabilizing plates, the blade speed is set between 5 and 6, one small weight is on the arm, and water with a few drops of dish-washing soap are in the lubrication basin below the blade. It is important that the blade is running though water when cutting, so make sure to move the basin into place and add water if necessary so that the bottom of the blade is submerged. A bowl and lab towel may be placed beside the IsoMet to catch water while the sections are being cut (Fig. 5).

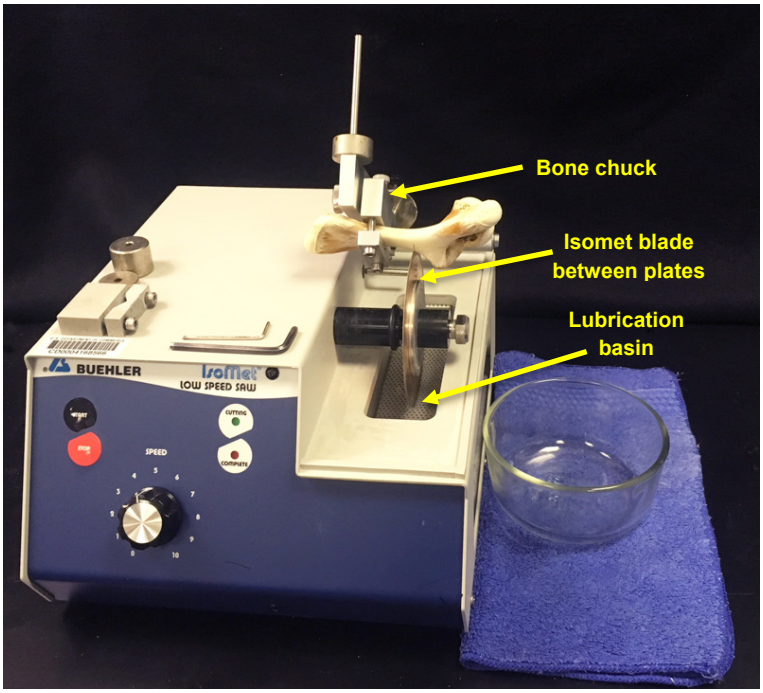
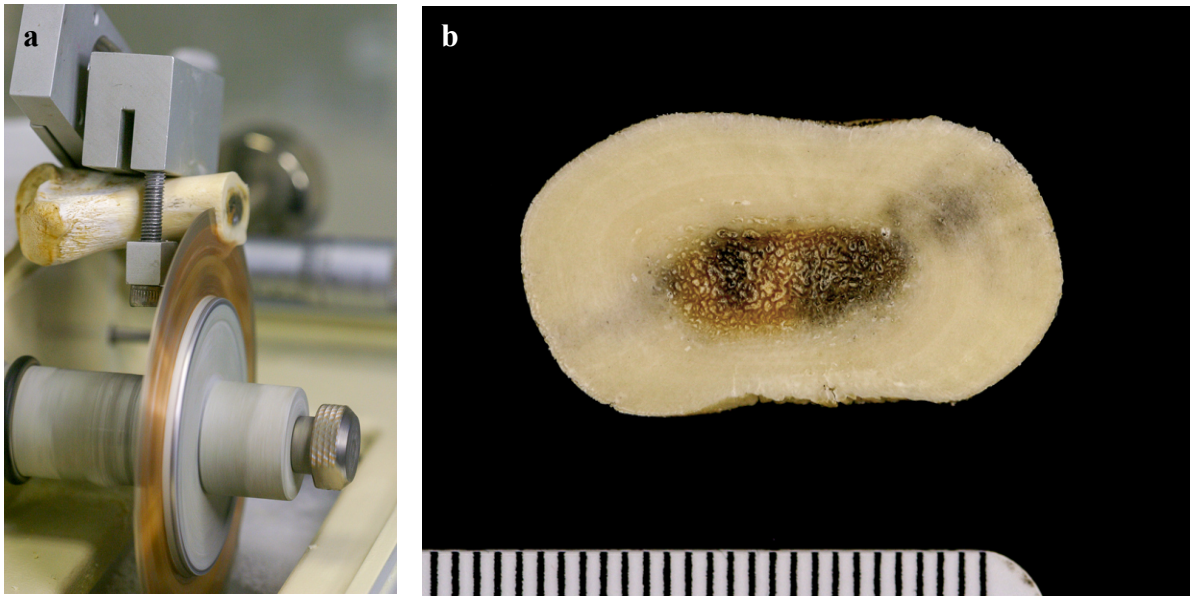


Fig. 5 IsoMet saw assembled with blade, water bath below the blade, and an extra bone chuck and weight. The humerus bone is properly oriented in the bone chuck, which is attached to the IsoMet arm.

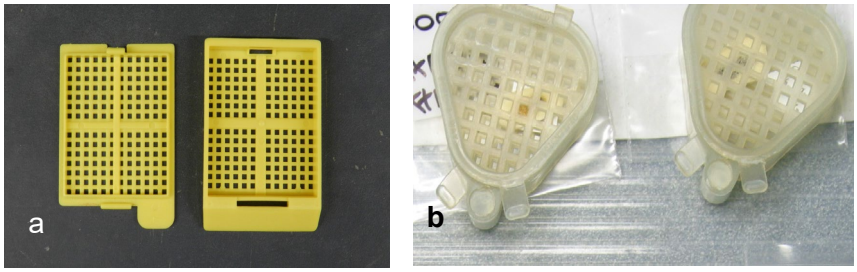
Place the bone in the single-saddle chuck so the ventral side is against the adjustable chuck bar and will be facing the blade once the chuck is tightened and attached to the arm. Identify the side of the chuck that will attach to the arm and position the bone so the distal process extends from that side. The chuck should be fastened so the bar is approximately 6 to 10 mm distal to the pencil line drawn on the bone. Check that the pencil line and chuck bar are parallel, then tighten the screws enough that the bone does not move easily from side to side but not so much that it is crushed (particularly for the smallest bones). Attach the chuck to the IsoMet arm and place one small weight on top of the arm. If two cross sections are needed, for example, one to be processed for aging and another for stable isotope or other analysis, adjust the arm so the blade will contact the bone 2 to 3 mm proximal to the pencil line. Start the IsoMet saw and gently lower the bone onto the rotating blade. The second cut is made on the pencil line, and the resulting cross section is typically reserved for stable isotope or other analysis as the position of the cut is slightly less optimal for aging than the next cut. Position the arm as to make a third cut to collect a 2 to 3 mm thick cross section for aging (Fig. 6a, b).

Fig. 6 (a) 2 to 3 mm sea turtle humerus cross section being cut using an IsoMet saw with a 4" diamond wafering blade; **(b)** 2 to 3 mm thick cross section of green sea turtle bone.



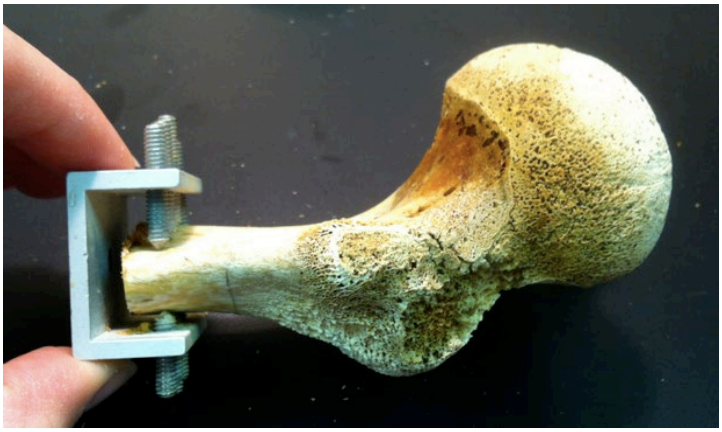
Create a 1 x 0.5 cm waterproof paper label using pencil for the aging section. Both the label and cross section may be placed in a plastic slotted or square mesh histo-cassette that has been labeled in pencil (Fig. 7a) or in a triangular plastic tissue capsule (e.g., Electron Microscopy Sciences, Hatfield, PA) (Fig. 7b). If a cross section was collected for stable isotope or other analysis to be performed at a later time, it may be allowed to dry and stored in a labeled small bag until processing. Once the remaining bone pieces are dry they may be sealed in plastic bags for archiving.

Fig. 7 a) Plastic slotted or square mesh histo-cassettes that may be used for fixation, decalcification, and staining; **b)** Alternative triangular plastic tissue capsule that may be used for fixation and decalcification of larger bones.



Not all bones will fit in the single-saddle chuck; an alternative is the irregular specimen chuck (Fig. 8). The distal end of the largest bones will need to be removed before these bones will fit in the irregular specimen chuck. A coping saw can be used to make a cut about an inch distal to where the pencil line was drawn marking where the cross section will be taken. Securing the bone in a vise helps for safety and ensuring the desired cut is obtained. The irregular chuck has individual screws that may be tightened to hold the cut end of the bone in place (Fig. 8). As with the single-saddle chuck, visualize how the blade will pass through the bone and align the pencil line and edge of the chuck so they are parallel, then secure the chuck to the IsoMet arm so the deltopectoral crest is oriented downward, facing the water bath, and process as described above. The 4” blade will not pass completely through the largest bones, which makes the 5” blade necessary for cross sectioning some samples.

Fig. 8 Large sea turtle humerus bone showing the distal process has been removed so it may be secured in the irregular specimen chuck for cross sections to be collected using an IsoMet saw.



When an additional cross section is collected for stable isotope or other analysis, this section and the aging section are notched using the IsoMet for post-processing alignment so that the skeletochronology image (see **Reading Sections**) can be used to guide skeletal growth increment-specific stable isotope sampling. To create notches, use label tape may be to hold each cross section pair together, then place between two 1 x 1” blocks of wood so the ventral side of the sections extends below the blocks. It is helpful to tape the blocks before securing them in the

U-shaped bone chuck (Fig. 9). Place a small shop clamp may be placed mid-way on the blocks so the bone sections do not move (Fig. 9). Attach the 3" blade to the IsoMet saw, add water to the basin so the blade is submerged, and remove any weights from on top of the arm. Check the orientation of the sections before tightening the chuck to the IsoMet arm, making sure that the blade will make contact with both sections once the arm is lowered. Position the arm so one notch is made midway on the ventral side. Start the blade and lower the sections onto the blade for just four or five seconds, then lift and adjust the arm so the second notch is made lateral to first. Check that the blade went into both sections before removing them from the chuck. It is fastest to simply remove the clamp and sections from the chuck, leaving the chuck secured to the IsoMet arm and carefully placing the next sections in to be notched during batch processing.

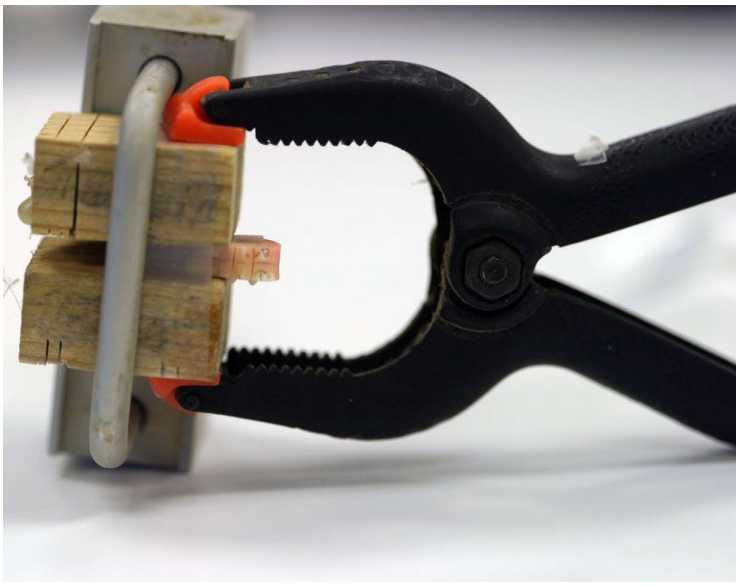


Fig. 9 Set-up for using an IsoMet low speed saw to create notches in the ventral side of sea turtle bone cross sections intended for aging and stable isotope analysis.

Fixation and Decalcification

Supplies needed:
2 liter glass or plastic jar with lid, fixative and/or decalcifier (e.g., Cal-Ex II (fixative + decalcifier) or 10% neutral buffered formalin (fixative) and RDO (dilute hydrochloric acid decalcifier), labeling tape, marker, nitrile gloves, ventilation hood, goggles, long forceps, chemical spill kit, laboratory notebook, empty liter size wide mouth jars, lab towels

Once placed into plastic histo-cassettes or triangular plastic tissue capsules with labels, bone cross-sections can then be fixed and decalcified. Various acids or combinations of acid can be and have been used to decalcify bone. Initially, we used RDO (dilute hydrochloric acid decalcifier; Apex Engineering Products Corporation, Aurora, IL, USA) and found that some of the bone sections became macerated before the entire bone was fully decalcified. Using a weaker decalcifying agent (Cal Ex II) has produced better results, albeit decalcification time is longer. Cal-Ex II provides both fixation and decalcification using just one chemical, avoids the

potentially hazardous mixing of formalin with hydrochloric acid (see below), and produces a more predictably decalcified section that is not overdecalcified. However, RDO or other rapid decalcifiers may still be used if necessary.

When using Cal-Ex II (Fisher Chemical), one liter of the chemical should first be transferred to a wide mouth glass or plastic 2-liter jar with a tight fitting lid. Label the jar with the contents and date. Retain the empty bottle for waste Cal-Ex II. Working in a ventilation hood wearing personal protective equipment such as nitrile gloves and goggles, use long forceps to submerge the 10 to 12 cassettes containing similarly sized bone cross sections in the Cal-Ex II and allow them to soak for the amount of time required for decalcification (Table 1). Record in a laboratory notebook (Appendix 2) the date sections were placed in Cal-Ex II and when they should be removed. Once decalcified, use long forceps to remove the sections in a ventilation hood, tapping them well to remove excess Cal-Ex II, and place them in a jar of tap water. Thoroughly rinse the samples in tap water and allow them to soak overnight.

Table 1. Recommended soak time in Cal-Ex II for approximately 2 mm thick humerus bone cross sections from Kemp's ridley (*Lepidochelys kempii*), loggerhead (*Caretta caretta*), green turtle (*Chelonia mydas*), and hawksbill (*Eretmochelys imbricata*) sea turtles from the Atlantic coast of the United States. Straightline carapace length = SCL.

SCL	Days in Cal-Ex II*
hatchlings-20 cm	1
20-25 cm	2
25-35 cm	3
35-45 cm	4
45-60 cm	5
60-70 cm	6
70-80 cm	7
80-90 cm	8
90+ cm	9 to 14

*soak time varies depending on freshness of Cal-Ex II

The same liter of Cal-Ex II can be used to decalcify about 40 cross sections from 20 to 35 cm SCL turtles, but only 10 to 15 sections from ≥ 80 cm SCL turtles. When the product begins to turn yellowish in color and/or if sections do not completely decalcify after soaking, the Cal-Ex II needs to be replaced.

To use RDO, sections in cassettes are submerged in 10% neutral buffered formalin for 2 to 3 hours, or longer. Cassettes are then removed and rinsed well in tap water. **Do not combine formalin and RDO; hydrochloric acid (active ingredient of RDO) and formaldehyde vapors have been reported to form a potent carcinogen, bischloromethyl ether.** Dry the cassettes and place

in RDO. Decalcification may take 7 to 48 hours (Tables 2, 3); larger bones will take longer; smaller bones may take less time. Check bones periodically for decalcification. Wearing gloves, examine their flexibility and color (the color will turn dark yellow-orange as the sections decalcify). Return sections to RDO if they are not ready. Once decalcified, soak the sections in cassettes in tap water overnight to remove as much of the decalcifying agent as possible.

Table 2. Recommended soak time in RDO for 2-3 mm thick humerus bone cross sections from loggerheads (*Caretta caretta*) and green turtles (*Chelonia mydas*) from the Pacific coast of Baja, California, Mexico. Curved carapace length = CCL.

CCL	Mean time in RDO Loggerheads	CCL	Mean time in RDO Greens
45-55 cm	8 hrs	50-54 cm	12 hrs
55-59 cm	9 hrs	55-59 cm	18 hrs
60-69 cm	14 hrs	60-65 cm	15 hrs
70-79 cm	17 hrs	73 cm	17 hrs
80-85 cm	20 hrs	85 cm	23 ¼ hrs
85-88 cm	22 hrs	95 cm	37 hrs

Table 3. Recommended soak time in RDO for 2-3 mm thick humerus bone cross sections from green turtles (*Chelonia mydas*) from the Atlantic coast of the United States. Straight carapace length = SCL.

SCL	Mean time in RDO
20-30 cm	4.25-5 hrs
30-40 cm	6.5-8 hrs
40-50 cm	7-9 hrs
50-60 cm	7-9 hrs
60-70 cm	9-10 hrs
70-80 cm	10-16 hrs
80-90 cm	13-17 hrs
90-100 cm	17-19 hrs
100-102 cm	20 hrs

Record in a laboratory notebook the date sections were placed in RDO and the number of hours required for decalcification. From this point, the laboratory steps are the same as using Cal-Ex II, except that sections decalcified using RDO will not be placed into Cal-Ex II (or RDO) after thin sectioning (see **Thin Sectioning**). RDO can be filtered to improve clarity, but this is not necessary, and it needs to be replaced infrequently when inadequate decalcification is observed.

Thin Sectioning

Supplies needed:
Embedding medium for frozen-tissue sectioning, dissecting microscope with bottom transmitted light source, small paint brush, probe, forceps, paper towels, 2 small glass petri dishes filled with tap water, 1 small beaker of tap water, 1 small beaker with 50% glycerin 50% water mixture, 1 large jar/beaker to hold cassettes in tap water, 10 vials with glycerin, fine marker, pencil, nitrile gloves, labeling tape, microtome or cryostat, microtome blades, Cal-Ex II, long forceps

Either a freezing stage microtome or a cryostat may be used to thin-section the 2 to 3 mm decalcified cross section. The SEFSC has used a Leica SM2000-R microtome with a small freezing stage (Fig. 10) along with the associated Physitemp controller and pump and tank unit (not pictured) for controlling freezing temperature. A cryostat has also worked well for quick collection of high quality thin sections of uniform thickness with minimal scratches from the blade (Fig. 11).

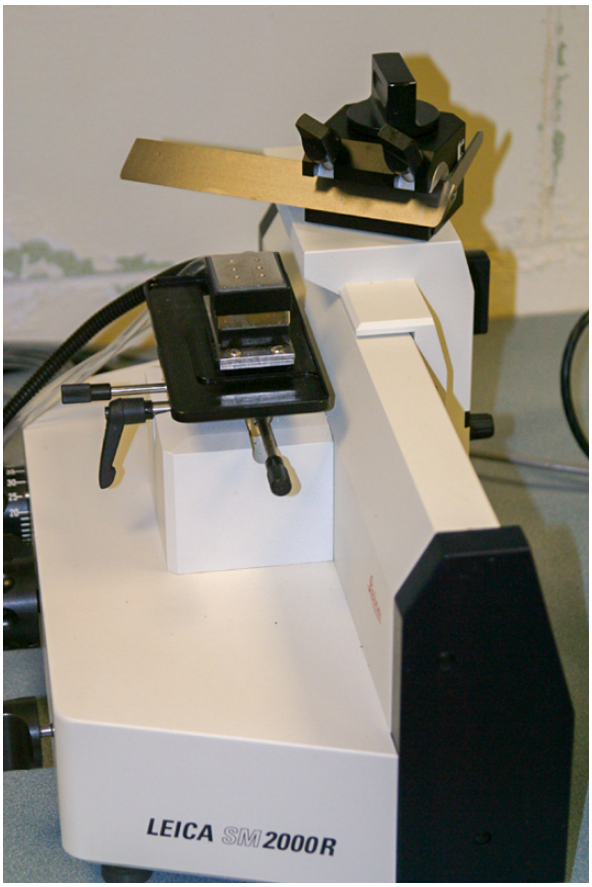


Fig. 10 Microtome with small freezing stage and blade attached.

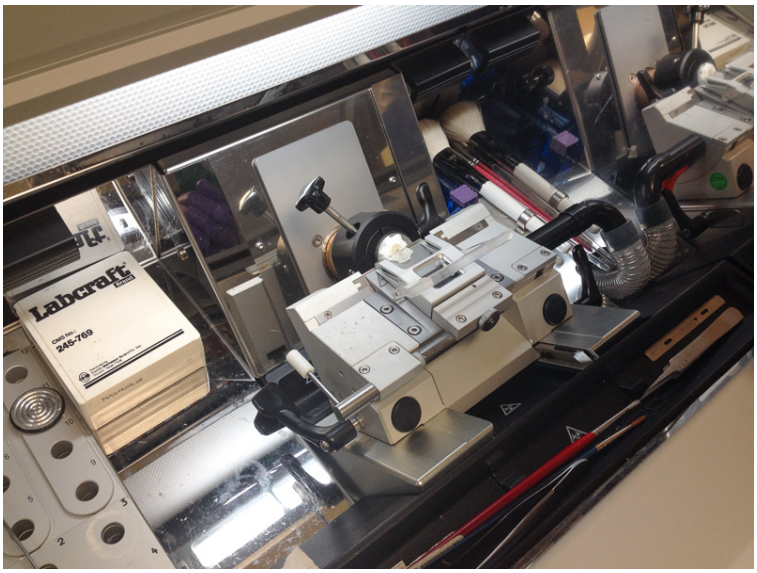


Fig. 11 Cryostat being used to collect 25 µm thin sections from a decalcified Kemp's ridley (*Lepidochelys kempii*) humerus bone cross section.

The following describes using a microtome but the process is similar if using a cryostat. At a work station beside the microtome, set up (Fig. 12):

- a small glass petri dish of tap water
- small beaker of tap water
- small beaker containing a mixture of about 10 ml water and 10 ml glycerin
- small paint brush
- forceps
- probe
- fine-tip marker
- paper towels
- pencil
- one-liter jar of tap water, and
- an equal number of small plastic or glass glycerin filled vials as cross sections to be thin sectioned

A dissecting microscope with a transmitted light source below the stage and a second small petri dish of tap water should be situated nearby. The microscope set-up is helpful for viewing sections to confirm that they are complete and do not exhibit scratch marks from under-decalcification or nicks in the blade. Secure the microtome blade in the holder on the microtome and set the sectioning thickness at 25 microns. Check that the microtome stage is level using a round bubble level or, for newer microtomes, rotating the leveling arms until they click in place. Then squeeze an embedding medium for frozen-tissue sectioning onto the stage in the shape of a diamond, roughly the size of the bone cross section. We use O.C.T. Compound by Tissue-Tek. Dry the decalcified bone section and using forceps press it into the embedding medium so it is flat against the stage with the arrow drawn on the bone pointing up. More embedding medium may be applied to completely surround the bone edges. Turn the freezing stage temperature controller to the lowest temperature or -40°C and wait several minutes for the section and embedding medium to freeze completely (Fig. 13a, b).

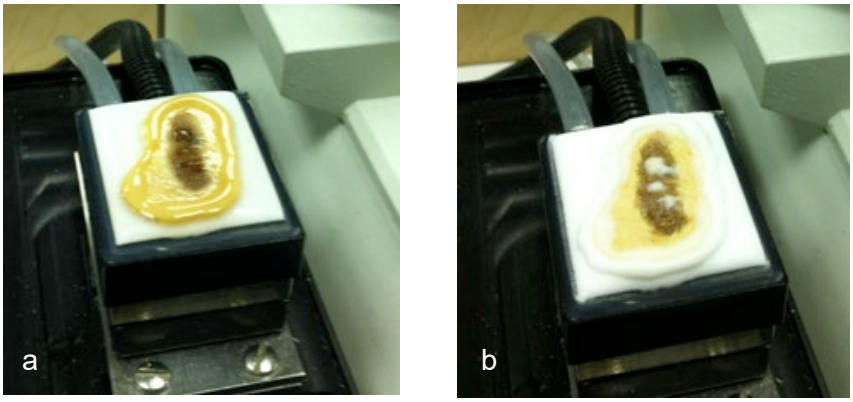


Fig. 13 a) Decalcified bone cross section beginning to freeze in Tissue Tek O.C.T. compound on a freezing stage microtome. **b)** Completely frozen decalcified bone section on freezing stage.

The outside of a screened histo-cassette may be labeled in pencil with the specimen ID, species, stranding date, turtle number by day, SCL, and any tag numbers. The waterproof label containing the specimen ID information may be retained and placed in the histo-cassette. The temperature may then be increased to approximately -10°C for sectioning. Manually adjust the blade so it is above but very close to the top of the cross section. Wearing examination gloves (e.g., nitrile, latex), rub the top of the cross section with a fingertip to warm it slightly, and then begin sectioning by drawing the blade over the top of the cross section. It may take several passes before the blade makes contact with the top of the bone, which is normal. If using a cryostat, the section may be frozen to a block, which is moveable instead of the blade. If using a microtome, moisten the small paint brush and use it to pick-up and remove incomplete section debris from the blade until a complete section is acquired. Cryostats typically have suction to remove and collect debris in a trap. Water must not come into contact with a cryostat, so use a dry brush, probe, or forceps to carefully remove thin sections from the blade. It is important to remove each thin section from the blade using the brush before allowing the blade to pass back over the frozen cross section. Taking one section at a time, place it in the petri dish of water under the dissecting microscope and check for complete sections without evidence of scratches from the blade. (Fig. 14).

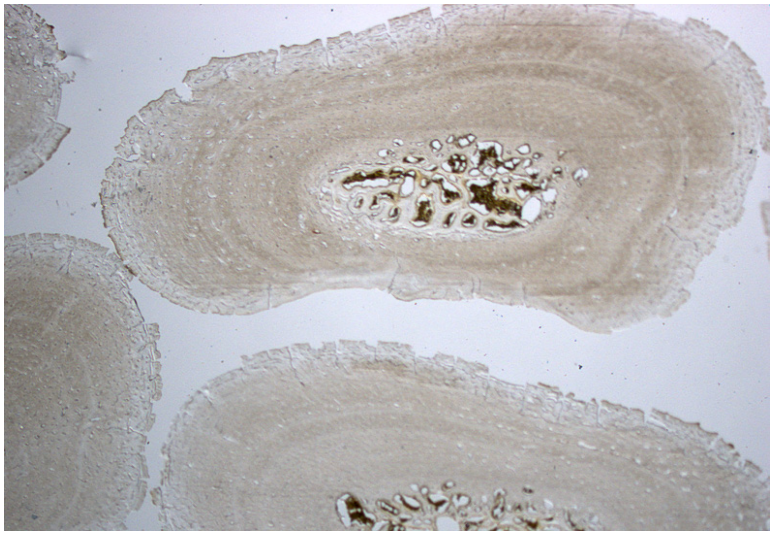


Fig. 14 Dissecting microscope view of 25µm thick decalcified green sea turtle (*Chelonia mydas*) bone cross sections

If scratches are detected on the sections (**see Troubleshooting**), you may attempt to remedy the problem by moving the blade slightly to move any damaged areas of the blade away from the surface of the cross section. Collect 8 to 10 thin sections of good quality, placing them in the center of a histo-cassette to keep them away from the seam between the lid and body of the cassette, as the sections may be damaged if the lid closes on them. Then, use the thaw function on the temperature controller to release the mounting medium and using forceps, gently lift the frozen section off the freezing stage and place it in the small beaker of water to remove the mounting medium. This residual section should be retained in case re-sectioning is necessary. It

may be stored in a labeled vial of 100% glycerin after moving it from water to a mixture of 50% water, 50% glycerin, where it should soak for 15 to 20 minutes. The cassette of thin sections should be submerged in a one-liter jar of tap water while the remaining cross sections are thin sectioned. Once thin sectioning is complete, remove the histo-cassettes from water, dry them off, and place them into Cal-Ex II to soak overnight. Remove the cassettes from Cal-Ex II the next day, rinse well, and allow them to soak in tap water overnight before attempting to stain. Thin sections collected from bone cross-sections decalcified using RDO may simply be soaked in tap water overnight prior to staining; no additional re-immersion in RDO is needed.

Staining

Supplies needed:
Stain ingredients (see Ehrlich's Hematoxylin recipe), distilled or reverse osmosis water, Erlenmeyer flask, stirring magnet, stir plate, weighing boats, scale, lab scoop/spatula to transfer dry chemicals, bulb and 5 ml graduated pipette, 50 ml graduated cylinder, stir plate, glass staining box, orbital shaking plate, 10 extra histo-cassettes, pencil, long forceps, short forceps, timer, nitrile gloves, dissecting microscope with bottom transmitted light, lab towels, probe, 2 small glass petri dishes, at least 2 1 L jars of tap water, 2 large petri dishes, 1 L jar of 50% glycerin, 50% distilled water solution, 1 L jar of 100% glycerin

At least one week prior to staining, make a batch of Ehrlich's hematoxylin (Table 4). Following this recipe makes about 150 ml of Erhlich's hematoxylin that should be stored so that it is exposed to natural light, which will darken the stain. Preparing stain one week in advance of beginning to stain decalcified bone sections yields optimal results. Undiluted, this stain keeps for about 30 days. It has been suggested that sodium iodate is not necessary if the stain is allowed to age naturally with exposure to sunlight for 3 months, however this has not been attempted by the SEFSC. Sodium citrate ($Na_3C_6H_5O_7 \bullet 2H_2O$) has also been suggested as a substitute for sodium iodate although this also has not been tested by the SEFSC.

Table 4. Erhlich's Hematoxylin stain recipe

Ehrlich's Hematoxylin

Hematoxylin crystals	1.0 g
95% Ethanol	50.0 mL
Aluminum potassium sulfate ($AlK(SO_4)_2 \bullet 12H_2O$)	1.5 g
Distilled water	50.0 mL
Glycerin ($C_3H_8O_3$)	50.0 mL
Glacial acetic acid (CH_3COOH)	5.0 mL
Sodium iodate ($NaIO_3$)	0.075 g

To make stain, transfer the dry chemicals to weighing boats on a tared laboratory balance using a lab scoop to acquire the exact weight required. Place an Erlenmeyer flask containing a stirring magnet on a stir plate and then dissolve the hematoxylin in the ethanol by turning the plate on so that the magnet spins at low speed. Once the hematoxylin dissolves, add the remaining ingredients. Applying low heat may facilitate dissolution of the hematoxylin crystals. Glacial acetic acid is best measured using a bulb and graduated pipette. The stain should begin to turn purple with the addition of sodium iodate. Cover the top of the flask and allow the stain to stir for at least 2 hours.

Prior to staining, dilute 50 ml of hematoxylin with 50 ml of distilled water or water filtered by reverse osmosis (RO) in a glass staining box and allow it to mix on an orbital shaking plate. As the stain is mixing, prepare one cassette of sections to test the stain. First, remove all sections from the cassette using small forceps and/or a dissecting probe and place them into a petri dish of tap water (Fig. 15). This helps prevent them from sticking together when staining. Label a second cassette with the turtle ID information and place half of the sections in each cassette.

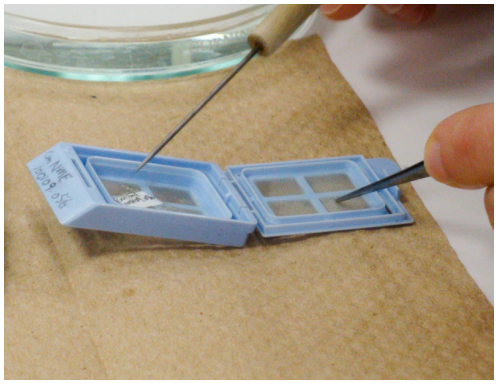


Fig. 15 Transferring unstained thin sections to a petri dish of water so that they will unfold and be less likely to stick together prior to dividing them between two cassettes for staining.

Using forceps, place cassettes in the diluted stain sitting on top of the orbital shaking plate, shaking the cassette and tapping it to remove air bubbles. Turn the shaking plate on and agitate for 8 minutes. Next, use forceps to remove the cassette and immediately submerge it in a nearby jar of tap water to rinse off the excess stain. Remove the cassette from water, carefully open it and use small forceps and/or a dissecting probe to transfer a few of the sections to a small petri dish of water to examine using a dissecting microscope with transmitted light from below (Fig. 16).

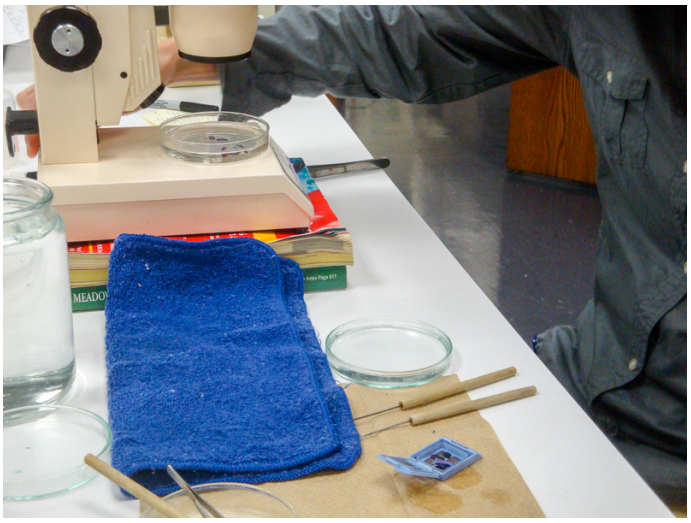


Fig. 16 Viewing stained humerus thin sections in water using a dissecting microscope with transmitted light.

Sections should be examined for clarity of growth layers, including differentiation of diffuse growth marks, without overstaining the surrounding bone tissue. The length of time that subsequent cassettes are left in stain may be increased or decreased depending on what is necessary to achieve optimal results. If more time in stain is required, place the sections back in the cassette, ensuring they will not be compressed in the seam of the closing lid. Use a lab towel to dry the outside of the cassette and place it back in stain for an additional amount of time, starting with 1 to 2 minutes before checking it again. Once you are satisfied with the results, immediately submerge the cassette containing the stained thin sections in a jar of tap water and allow it to soak for 20 minutes (Fig. 17). This turns the sections blue without the need for a counterstain. After testing the first cassette, four or more cassettes may be stained simultaneously following the same process.

Fig. 17 Submerging a histo-cassette of stained thin sections in tap water so they will turn blue.

After the sections have soaked in tap water for 20 minutes, remove them from water and dry them well. Next, place the cassettes into a solution of 50% glycerin:50% distilled or reverse osmosis (RO) water to soak for 20 minutes (Fig. 18). Using long forceps, remove the cassettes and place them on a large petri dish set at an angle so excess solution may drain. Once they have drained for about 10 minutes, cassettes should be transferred to a jar of 100% glycerin, where they may remain until they are mounted on slides. Gradually increasing the glycerin concentration to 100% prevents dehydration from being too sudden, which can damages the section tissue architecture causing them to "wrinkle" permanently and preventing them from laying flat when mounted on slides later in the process. Draining as much of the 50% glycerin as possible from the sections and cassettes reduces dilution of the 100% glycerin when the cassettes are transferred. Used stain may be reserved and used again without further dilution. Once the sections begin to stain too dark too quickly, the stain should be replaced with fresh, newly diluted stain.

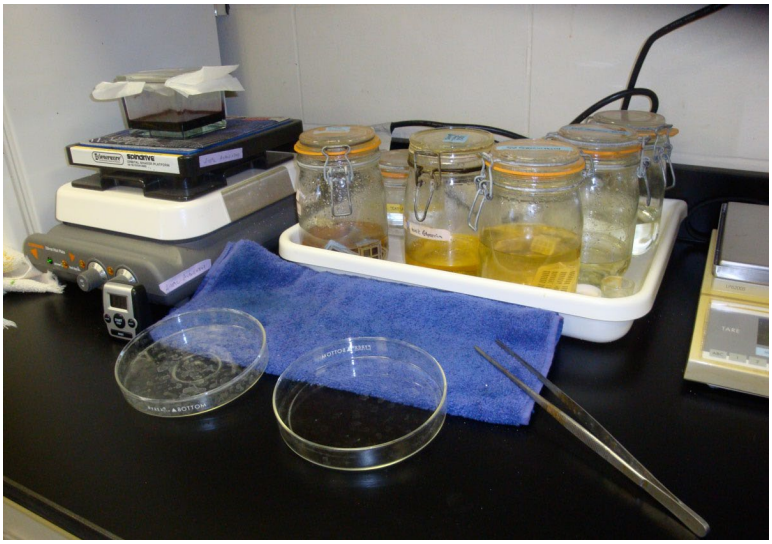


Fig. 18 Set-up for transferring stained sections in cassettes from 50% glycerin to 100% glycerin with petri dishes for draining.

Mounting to Slides

Supplies needed:
Long forceps, large petri dish, small petri dish, dissecting microscope with a bottom transmitted light, a small or large petri dish of 100% glycerin, fine forceps, probes, standard glass slides with frosted ends that may be written on, 22 x 30 mm and 22 x 40 mm cover glass, a fine tip marker, paper towels, lint-free wipes for cleaning excess glycerin, a slide tray, the labeled vials of glycerin containing the cross sections left over from thin sectioning

Set up a workstation with a dissecting microscope that has transmitted light, a small or large petri dish of 100% glycerin, forceps, probes, standard glass slides with frosted ends for labeling, 22 x 30 mm and 22 x 40 mm cover slips, a fine tip marker, paper towels, lint-free wipes

for cleaning excess glycerin, a slide tray, high viscosity mounting medium, such as Cytoseal 280, and the labeled vials of 100% glycerin containing the cross sections left over from thin sectioning. Using long forceps, transfer the cassettes from 100% glycerin to a large petri dish to drain. Find two cassettes with matching IDs and place them in a small empty petri dish. At the workstation, use the probe and fine forceps to pry open both cassettes, to avoid getting glycerin on your fingers and thus on the slide and cover slip. Use the probe or forceps to carefully transfer all of the stained thin sections to the glass petri dish containing 100% glycerin (Fig. 19).

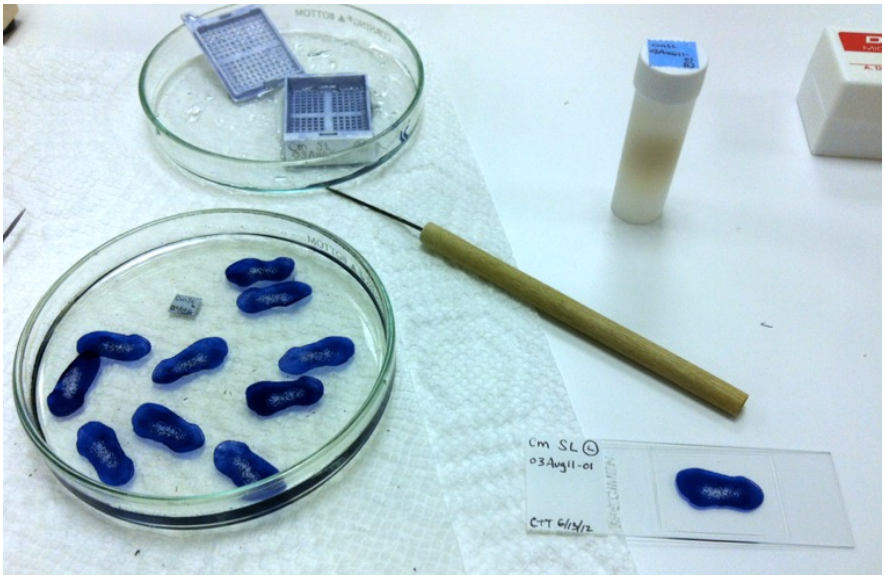


Fig. 19 Stained thin sections in petri dish of 100% glycerin, with histo-cassette used in staining, storage vial, and thin section mounted on a slide with cover slip applied.

Arrange the sections in rows to facilitate comparison and evaluation of the best section when viewing them with transmitted light using the dissecting microscope. Optimal section characteristics include one that is not missing any of the edge, is not torn or showing scratches, and has good definition of the growth layers including those that appear as diffuse marks and not just distinct lines. Compare the sections to see if an annulus (diffuse first year mark) is visible on any. In making your final selection, check that the lines of arrested growth (LAGs) are visible on each of the lateral aspects of the section as this may allow LAG diameter measurements later during analysis. Once you have chosen the best section, lift it from the glycerin using the probe, allow excess glycerin to drip off, and place the section on a clean microscope slide. Examine the section using the dissecting microscope with transmitted light and remove air bubbles, debris, and dust using a dissecting probe. It is not necessary to add any additional glycerin to the slide before applying a cover slip, but a probe can be used to spread the existing glycerin beyond the edges of the section to minimize introduction of bubbles as the cover slip is applied. Choose a cover slip that is large enough to cover the section, keeping in mind that room must be allowed to run a bead of medium along the edges of the cover glass to seal it and the glycerin in place. At an angle, slowly lower the cover slip onto the section, using a fingernail to apply enough pressure to keep air bubbles from forming as the glass covers the stained thin section. If

remounting is necessary, gently slide the cover slip off of the slide, put the thin section back into 100% glycerin, and either wash and clean the original slide of glycerin or select a new slide. Once a section has been successfully mounted onto a slide, record the specimen ID and date the slide was prepared on the frosted end of the slide and use a lint-free paper wipe to slowly wipe excess glycerin away from the surface of the slide near the edges of the cover glass. Place the slide on a slide tray and transfer the remaining stained sections and the waterproof label to the vial containing the decalcified cross section in glycerin and place it in storage. Once the batch of stained thin sections have been mounted on slides with cover slips and cleaned of excess glycerin, images may be collected prior to sealing the slides. It is important that the surfaces in contact with mounting medium are completely cleaned of glycerin or there will be gaps and the glycerin will leak. The glycerin in the petri dish should be covered and reserved for next time.

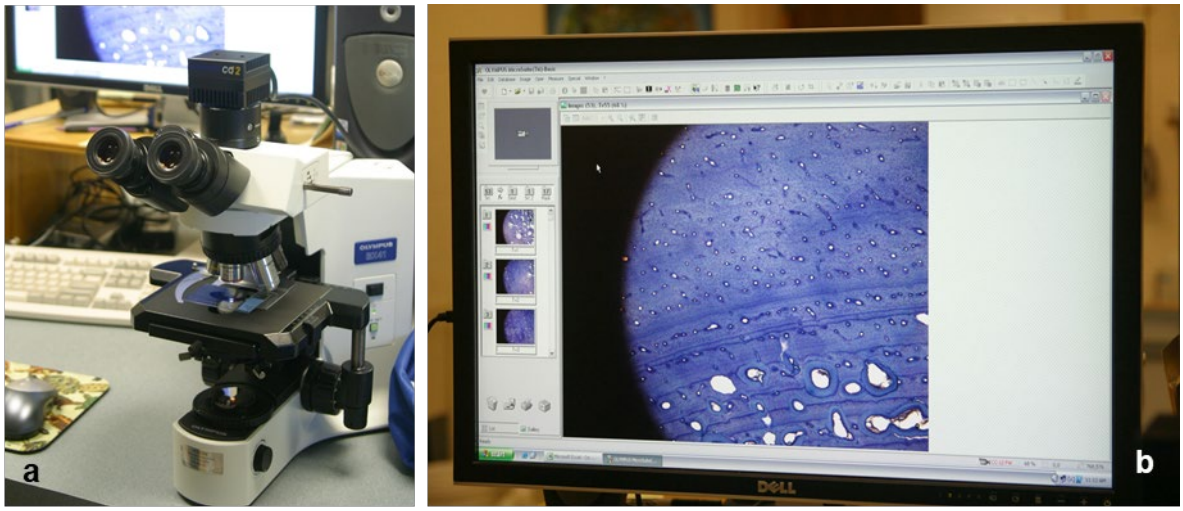
Imaging

Supplies needed:
Compound microscope with movable stage, high-resolution digital camera tethered to computer, computer, 4× objective, image analysis software, calibration slide for setting the scale bar

Stained sections are typically imaged on the same day they were mounted on slides to avoid fading. We also capture images before applying a seal around the cover slip to prevent inadvertently applying sealing medium over the section, which would obscure the view of the entire stained thin section.

Individual images can be captured using a compound microscope with a movable stage and a high-resolution digital camera tethered to a computer with image analysis software installed. We recommend creating a new folder for each individual bone section, giving it a name that corresponds with the individual ID from which the bone was collected, such as LkAKB120401_01. With the microscope set at 4x magnification, the light source on, and the image analysis software ready to collect still images, place a slide on the stage (Fig. 20a, b). Set the camera on live feed and adjust the white balance and light.

Fig. 20 a) Compound microscope with mounted slide on the movable stage and tethered digital camera. **b)** Computer monitor showing the digital camera live feed of 4× magnification sequential, partial images being collected of stained thin sections.



Starting with the upper left quadrant of the stained section, capture a still image and save it in the folder for that individual. Saving each sequential image in the folder as 1, 2, 3 and so on makes for an efficient workflow both when collecting images and later when stitching them together to form a composite image. With the live view turned on, adjust the stage so you are collecting images with 40% overlap with the previous image captured. We recommend collecting images starting from left to right, then down and working from right to left, continuing this pattern of collection until the entire stained section has been captured. We also recommend adding a calibrated scale bar to the final image collected, to provide calibration for the final, composite image (see **Stitching mosaics**).

Sealing Slides

Supplies needed:
High viscosity permanent mounting medium, nitrile gloves, fume hood, slide storage box
Wearing gloves and working in a fume hood, slowly apply a steady bead of permanent mounting medium so it slightly overlaps the cover glass and seals the cover to the glass slide (Fig. 21). As long as sealing medium is not covering or obscuring the stained section, you may re-image the slide if necessary. When all of the slides have been sealed, allow them to dry in the hood overnight. Once the mounting medium has dried, the slides may be stored in a slide box.

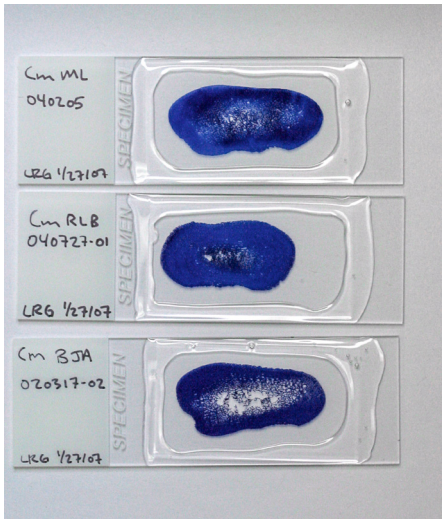


Fig. 21 Stained green sea turtle (*Chelonia mydas*) thin sections mounted on slides in 100% glycerin with mounting medium applied to seal the cover glass to the slide.

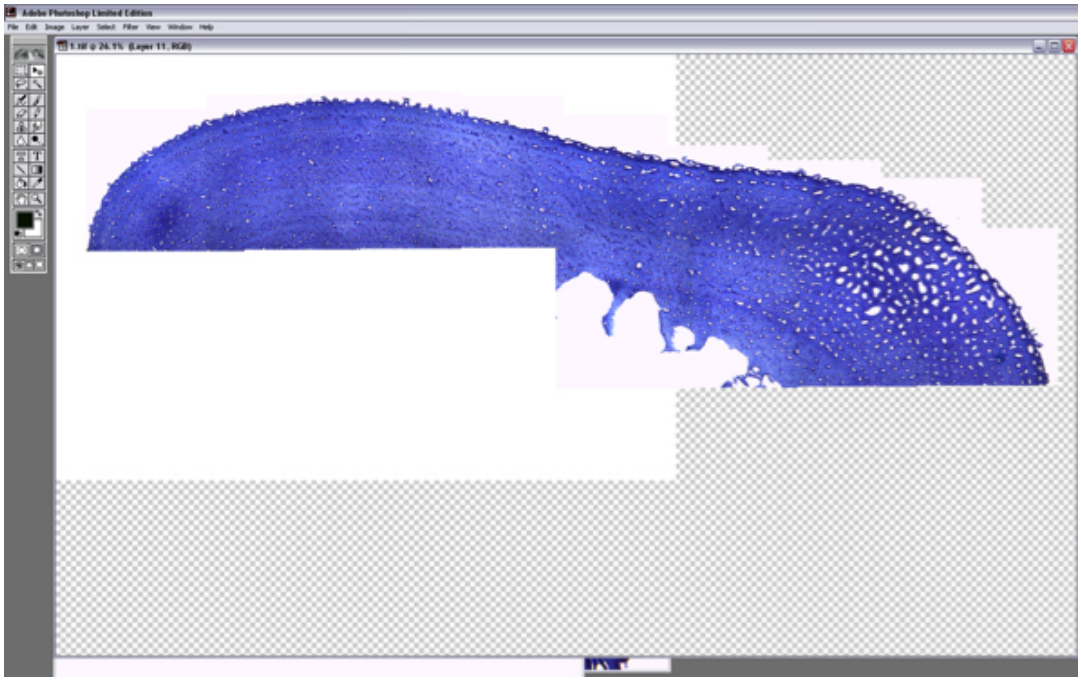
Stitching Mosaics

Supplies needed:
Computer, imaging software

SEFSC researchers manually stitch the partial, sequential images of each stained section together to form a composite image using imaging software, such as Adobe Photoshop. To carry out this approach, first navigate to the folder of images you would like to stitch together and open image 1 in the imaging software program. Using the marquee tool, select the image, crop it, and then resize the canvas, choosing a size that should approximate that of the entire stitched section. Move the image to the upper left quadrant by selecting it with the marquee tool and then using the move tool. Open image 2, select the portion of the image that you want, then copy and paste it onto what was image 1. Use the move tool to move image 2 to match up with image 1, zooming in to place it accurately. The arrow keys on the keyboard are useful in making fine scale movements to match the image up exactly. Continue this process (Fig. 22) until all of the images have been stitched together, forming one composite image of the stained section. For the best file stability and resolution, the composite image should be named and saved as a tagged image file (.tif).

Advances in automated stitching software programs may speed the stitching process and should be tested to determine if they are able to produce results that are as accurate as those yielded by hand stitching using the described methods. Using the shortcuts in Photoshop as part of your workflow should decrease the amount of time required to hand stitch composite images (Appendix 3).

Fig. 22 Stitching individual, partial images of a stained sea turtle humerus cross section into a composite image using imaging software.



Reading Sections

Supplies needed:
Computer, imaging software

To identify and count growth layers, open the mosaic image in the imaging software and begin visually tracking a LAG completely around the cross section. Most of the time it will not be possible to track LAGs along the dorsal side because the layers are more compressed. Once a LAG has been tracked across the bone to both lateral sides mark each LAG in a bright color, like yellow, on both the right and left sides (in Photoshop, use the line tool) where LAG diameters will be measured. Continue this process of tracking and marking LAGs until all of them have been marked. Assign each LAG a letter (e.g., using the text tool) to mark both sides of each tracked LAG.

SEFSC researchers typically marks LAGs keeping in mind that LAG diameter must be measurable for the innermost LAG marked. Many more LAGs may be visible interior to this mark but if their diameters are not measureable because of the loss of tissue due to bone remodeling, or resorption, these LAGs would not be marked. Instead, correction factors may be used in estimating the mean number of LAGs lost to resorption (see Avens et al. 2017). The exception to this is when the annulus is visible but not measurable, in which case it should be marked because the presence of an annulus allows the turtle to be directly aged without using a

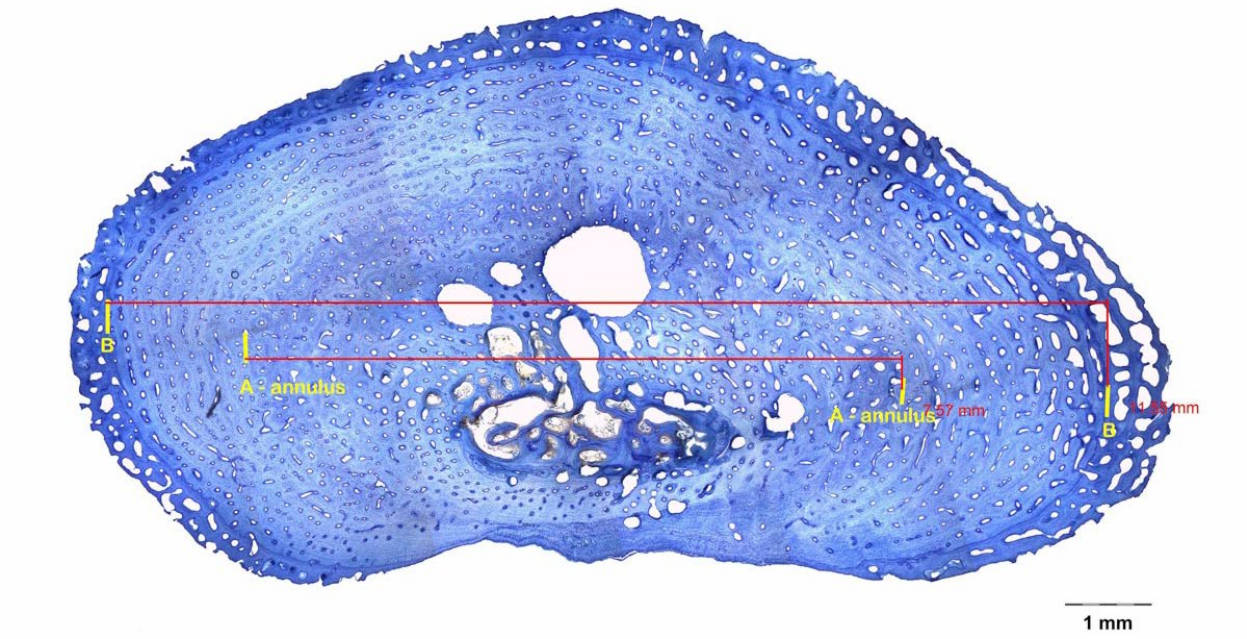
correction factor to estimate lost growth marks. Once the innermost LAG is identified, all LAGs exterior to it are marked and labeled for counting regardless of whether or not they are measurable. It is important to inspect the outer edge of the section, as sometimes LAGs form close to it. When finished with marking and counting, name the file with the marked LAGs so it ends with the initials of the person who conducted the read and save it as a new jpg so the original unmarked mosaic is not overwritten.

Measuring LAG Diameters

Supplies needed:
Computer, image analysis software, spreadsheet software

It is usually possible to acquire measurements using the image analysis software that was used to collect the individual images to be stitched together. We recommend using the image analysis software for measurements instead of other programs, as not to introduce discrepancies or errors that would translate into errors in correcting for resorbed LAGs and/or in back-calculating carapace lengths. LAG diameters are measured on a horizontal axis from each lateral side at the point where the LAG diameter would be the widest (Fig. 23). Record the measurements in a spreadsheet program for later analyses, which are beyond the scope of this document. For example, see Avens et al. (2017) for details on how SEFSC researchers have used these measurements to estimate carapace lengths, somatic growth rates, and estimate numbers of LAGs lost in the resorption core.

Fig. 23 Stitched composite image of a stained Kemp's ridley sea turtle (*Lepidochelys kempii*) humerus cross section with horizontal lines depicting where line of arrested growth (LAG) diameters marked and labeled for measurement.



Troubleshooting

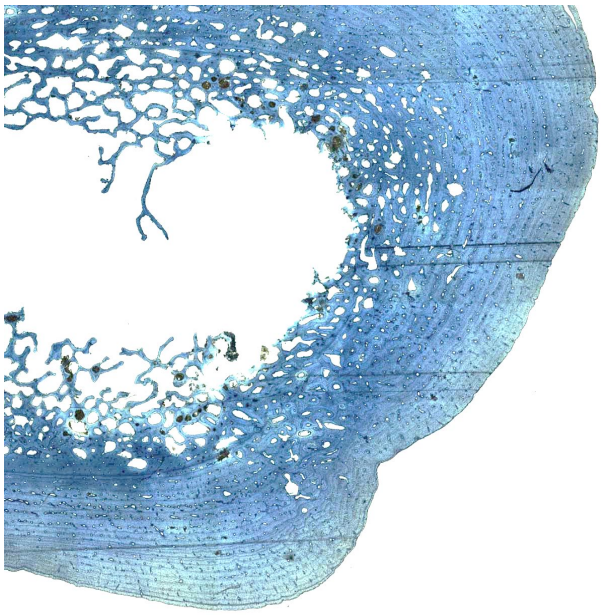
There are times during processing when issues arise, impeding researchers from obtaining the best possible cross sections. Because this guide is meant to be comprehensive in allowing researchers to repeat these methods in the future, we will describe common problems that we have encountered during different stages in the process and how to correct them.

Cutting Cross Sections from Intact Bone

Edges of bones crumble when cutting on low-speed saw: The bones likely boiled at too high of a temperature or spent too long boiling during the process of removing all tissue after flensing. There is no way to correct for this, other than processing the other humerus bone from that individual turtle (if both were collected and the other humerus did not sustain similar damage during boiling).

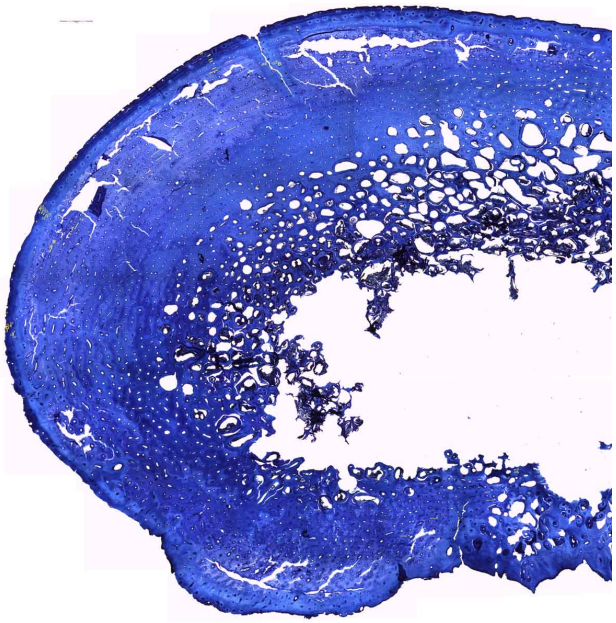
Thin Sectioning

Scratches appear on thin sections: The knife may be damaged. Shift the position of the microtome knife in the holder so that a different part of the knife blade cuts through the section. If possible, have the microtome knife sharpened or, if using disposable blades, replace it with a new blade. It is possible to check the edge of the blade for damage using a dissection microscope. If scratches continue to appear in the same place and/or the sound of the blade scraping the section during the cut is heard, it is possible that the section might be under-decalcified.



If this is the case, place the cross section back in decalcifier and attempt thin sectioning at a later time. If scratches still appear and the issue is not resolved by moving the knife, it is possible that the decalcifier is not performing effectively and should be replaced.

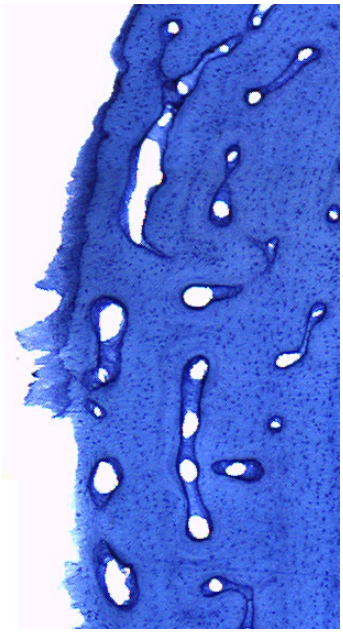
Sections curl when placed in water: The section was likely too cold when thin sectioning. Wearing nitrile gloves, warm the frozen cross section with the tip of a finger for 1 to 2 seconds before thin sectioning. Check that the temperature is set at approximately -10°C. If curling persists and is accompanied by thin sections turning brown (instead of transparent), the sections are likely to be under-decalcified and warming it will not help. In this case, stop sectioning and return the thick section to decalcifier until it is fully decalcified.



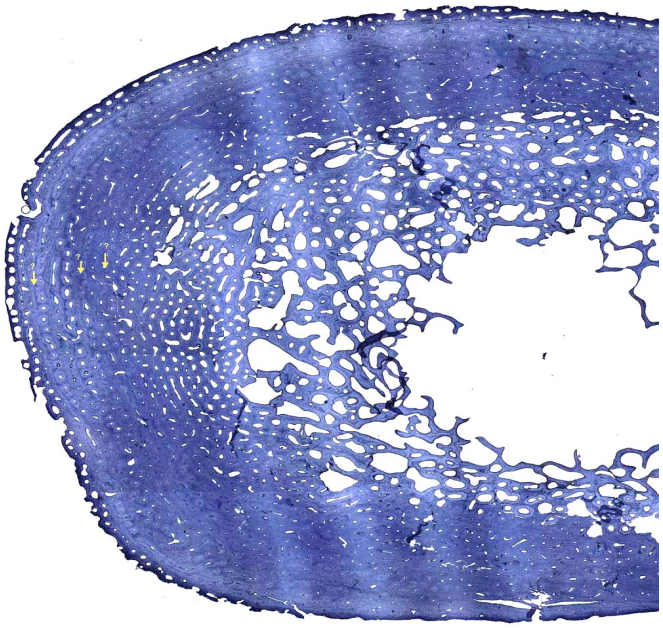
Sections are uniformly brownish: It may also sound crunchy or louder than normal when drawing the blade across the section. The section is under-decalcified. Stop sectioning and return it to decalcifier.

Sections have brown patches or parts of the section tear out when sectioning: The section is over-decalcified. When staining, this appears as missing patches of bone or weak areas that are torn. This is more commonly seen when using RDO than with Cal Ex II. There is no remedy other than to process the humerus from the other flipper.

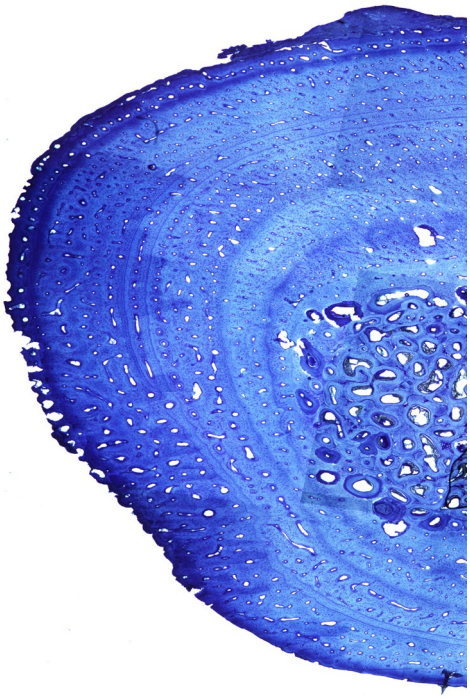
Crunchy sound when thin sectioning: The sections are likely under-decalcified and should be returned to decalcifier. Also, confirm that the thickness of sections is not greater than 25µm.



Lip at the edge of thin section: Sometimes a 'lip' or additional amount of bone is present on the thin sections and visible when examined under a dissecting microscope. The section is likely too warm when thin sectioning and the edge has ripped. Refreeze, or decrease the temperature of the freezing stage or cryostat before taking additional sections, then return it to the sectioning temperature, warm it slightly with a fingertip, and section as normal. An alternative solution is to take a single, slightly thicker section (60µm), then reset the thickness to resume sectioning at 25µm. You may also thaw, reset, and refreeze the section before starting again, or rotate the frozen section so the blade contacts the opposite end of the section first.



Dark and light bands appear on thin sections: This is most obvious after staining but with careful inspection it can be seen after thin sectioning. The section is likely under-decalcified. Return the section to decalcifier, allow to soak in tap water overnight, then attempt re-sectioning.

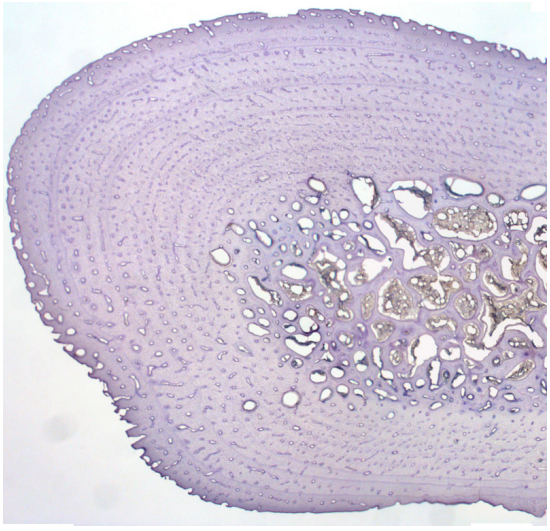


Blade begins to take sections that appear thicker than settings indicate and/or cuts at an angle into the thick section: The section may be becoming too warm and is thawing. Thaw the section, then reset and refreeze it. Also, stop sectioning even if the blade has passed part-way through the section, in order to prevent damage to that section. Another option is to rotate the section to begin cutting from its opposite end.

Section detaches from freezing stage when knife makes contact: The temperature of the section is likely too cold and should be increased from that at which it was initially frozen, to about -10° C. Also, the knife may not have been adjusted to pass just above the section on the first approach. Inadvertently attempting to pass the knife through the section at a thickness much greater than 25µm can cause the section to dislodge from the stage.

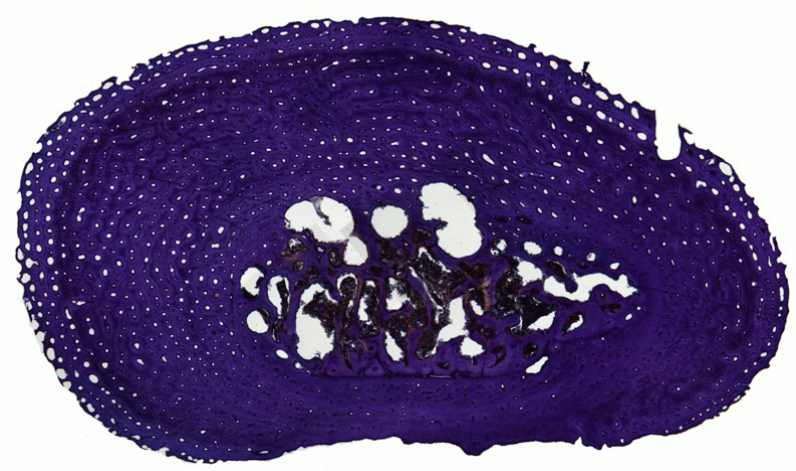
Staining

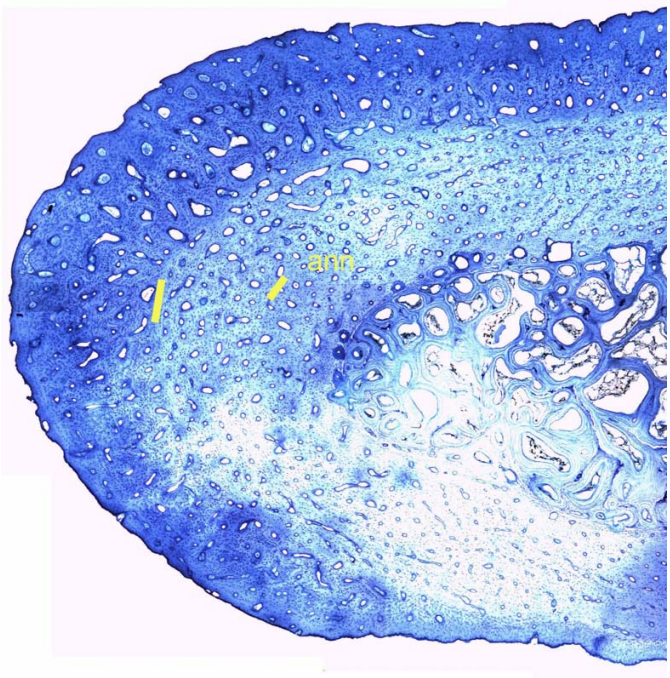
Sections stain too light: Increase the amount of time in stain. Check that the correct chemicals and quantities of chemicals were added to the stain when preparing it and ensure that it was diluted prior to staining sections. Avoid using deionized water in the stain recipe or for dilution. Allow the stain to age for a longer period of time while exposed to natural light.



Sections stain too dark: Reduce the amount of time in stain. Or, if the diluted stain has been used to stain over 20 cassettes it is likely time to replace it with freshly diluted stain. Check the age of the undiluted stain; you may need to make fresh stain if it is older than 1 month. Check that the stain was diluted 1:1 with distilled or reverse osmosis water and is not 100% undiluted stain.

Sections appear uniformly dark blue and/or burnt. Start by checking that the sections were not under- or over-decalcified. Decrease the amount of time in stain. Check that the stain was prepared properly. Check that the stain is not greater than 1 month old and that it has been used to stain no more than 20 cassettes. Check that the section thickness is not greater than 25µm.





Portions of section stain very light or not at all: Section is likely under-decalcified. The reserved decalcified section stored in glycerin may be returned to decalcifier and re-sectioned after first stepping it down from 100% glycerin to 50% glycerin for 15 minutes, then to tap water prior to returning it to decalcifier.

Mounting to Slides

Stained sections are wavy and do not lay flat on slides: Make sure to allow sections to soak at least 20 minutes in 50% glycerin before moving them into 100% glycerin. Check that the sections were fully decalcified and increase the amount of time spent in decalcifier if necessary.

Leatherbacks and Hatchlings

The laboratory methods are slightly different for histologically processing ossicles (e.g., for leatherback sea turtle (*Dermochelys coriacea*) age estimation; Appendix 4) and hatchling humeri for all sea turtle species (Appendix 5). Brief descriptions of the processing methods for these structures are included in the appendices. For processing scleral ossicle bones, the main differences from the methods described for processing humeri are that fixation and decalcification is achieved with 10% neutral buffered formalin and RDO, and they stain best with Modified Mayer's Hematoxylin instead of Ehrlich's Hematoxylin. Hatchling humeri are tedious to process, but it is possible following the guide in Appendix 5.

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Appendix 1

Humerus Bone Measurement Sheet

Measured by: _____ Turtle ID# _____

Date measured: _____

Right or left humerus: _____

Carapace length

curved: _____

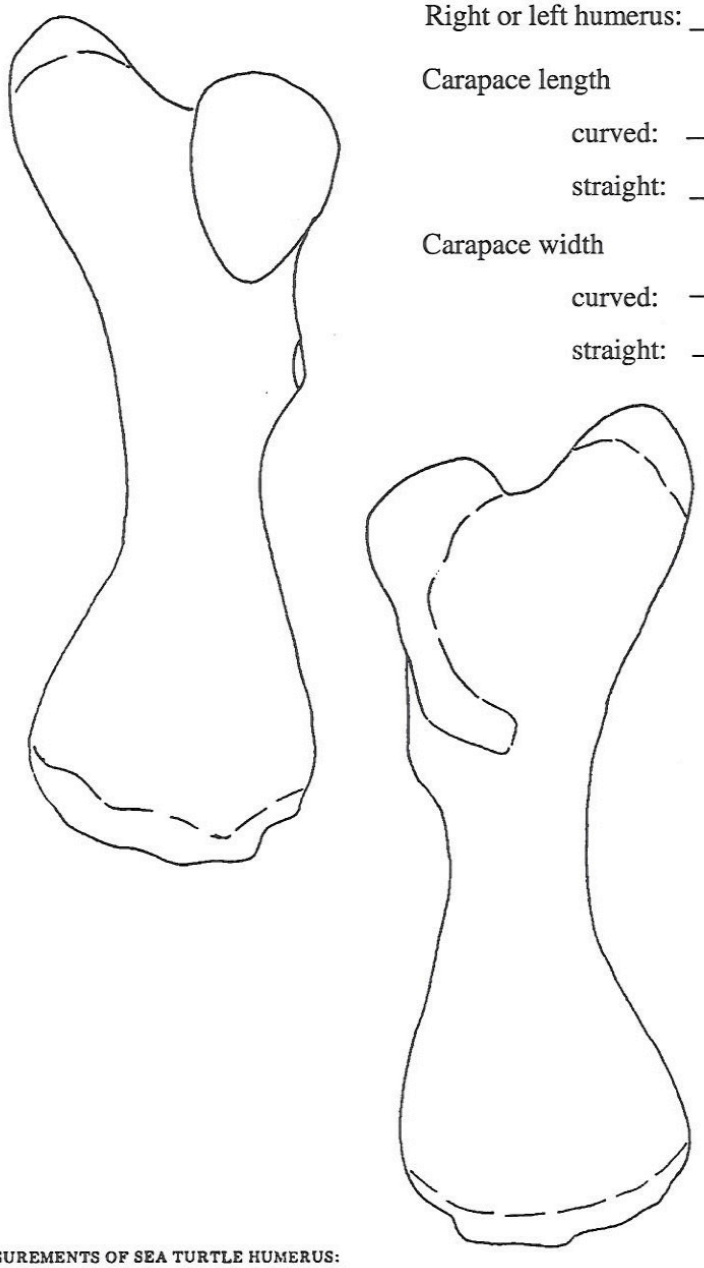
straight: _____

Carapace width

curved: _____

straight: _____

Sex: _____



ML: _____

LL: _____

PW: _____

DPPW: _____

MW: _____

DW: _____

T: _____

Weight: _____

MEASUREMENTS OF SEA TURTLE HUMERUS:
All Straight Line Distances in MM.

SI-848 9-16-80
Reptiles/Amphibians
Dept of Vertebrate Zoology
National Museum of Natural History

Appendix 2

Example Lab Notebook Page

Turtle ID	Left or Right Humerus	Isomet Date	Date in CalEx II	Date out CalEx II	Date thin sectioned	Date out CalEx II & in water	Date stained, mounted, and imaged	Notes

Appendix 3

Shortcuts in Photoshop

Open: ctrl o

Marquee tool: m

Crop (Image-crop)

Canvas size (Image-canvas size)

Zoom out/in: ctrl -, ctrl +

Zoom out to see entire image: ctrl 0

Move tool: v

Hand tool: h

Line tool: u

Text tool: t

Appendix 4

How to Process Sea Turtle Ossicles

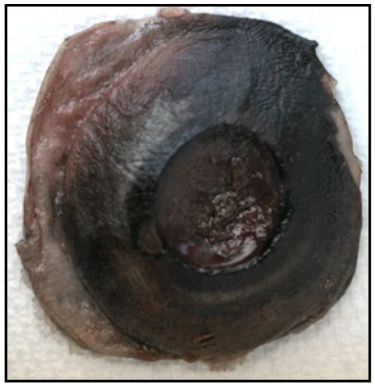
Dissecting and Cleaning

Dissect ossicles one by one out of the eyeball using a scalpel blade.

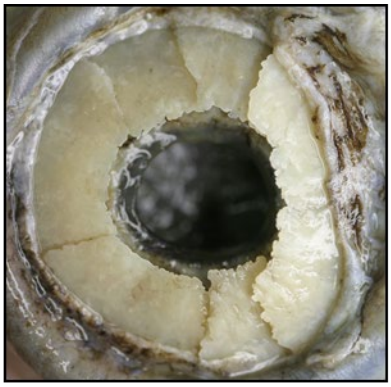
Clean individual ossicles in a petri dish of tap water by gently scraping tissue away using probes. Do not use bleach in cleaning tissue from the ossicles.

Dry each ossicle with a paper towel and place on a black background, maintaining the order they were in.

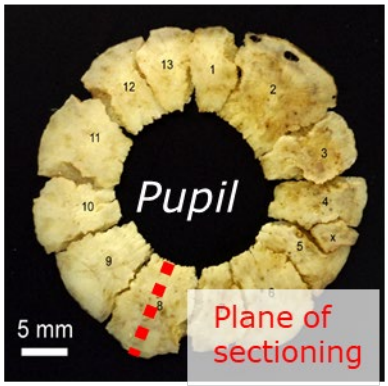
Photograph entire ring of separated ossicles and transfer files to computer.



Outer half of leatherback eyeball with scleral ossicle bone ring intact.



Tissue dissected away from ring of scleral ossicle bones



Cleaned, separated, and numbered scleral ossicles with the plane of cross-sectioning shown by the dashed line

Fixation and Decalcification

Assign a number to each ossicle.

Label a histo-cassette and make a small label with waterproof paper for inside the cassette.

Place ossicles in individually numbered cassettes.

Place cassettes in 10% neutral-buffered formalin for 2-3 hours.

Remove and rinse well with tap water.

Dry the cassettes, then place them in RDO for approximately 2 hours. Large ossicles (leatherback, some loggerhead) may take 2.5 to 3 hours. Hatchling ossicles need only 5 to 10 minutes in RDO.

Remove and soak in tap water overnight prior to thin sectioning.

Thin Sectioning

Ensure stage is level.

Place blade on microtome. Ensure settings are correct (blade is at a 0º angle and set to take 25µm thick cross-sections). A cryostat may also be used instead.

Place a small dot or line of embedding medium for frozen-tissue sectioning (we use Tissue-Tek O.C.T.) onto the freezing stage, large enough for the ossicle.

Dry the ossicle and use forceps to hold it on the stage in the tissue-tek, orienting it so that the outside edge of the ossicle is facing the knife.

Turn stage on to the coldest possible temperature to freeze (typically -40°C).

Hold the ossicle in place with forceps until it is frozen.

Surround the ossicle with embedding medium for frozen-tissue sectioning, one layer at a time until it is surrounded with the compound.

Label a vial filled with 100% glycerin.

When completely frozen, adjust the blade and increase the temperature to -10°C for sectioning.

Begin taking thin sections.

Use a small paintbrush to collect the sections from the blade.

View the sections in a petri dish of water on a dissecting microscope with a transmitted light. Check for complete sections, rips, and tears. The goal is to acquire sections from the middle of the ossicle.

Separate good sections from bad sections in the petri dish.

Once finished sectioning, turn the microtome to defrost and take the ossicle off the stage, placing it in a beaker of water.

Place the remaining portion of the ossicle into 50% glycerin for 15 minutes, followed by 100% glycerin, then into the properly labeled vial.

Collect the good 25µm thin sections and place them in the labeled histo-cassettes that have small mesh screen windows so the ossicles will not slip through when staining.

Place histo-cassettes into a jar of tap water to soak overnight prior to staining, changing the water periodically if possible.

Staining

Modified Mayer's Hematoxylin works best for staining ossicles. The day before you plan to stain, make the stain using the following recipe:

Modified Mayer's Hematoxylin Recipe*
(1/4 batch)
*Modified by Helen Grue

0.25 g Hematoxylin
250 mL Distilled or Reverse Osmosis filtered water
0.025 g Sodium Iodate
12.5 g Aluminum Potassium Sulfate

1. Dissolve the hematoxylin in water in a flask on a stirring hot plate, using low heat if necessary.
2. Add the sodium iodate, followed by the aluminum potassium sulfate.
3. Stir until the aluminum potassium sulfate is dissolved.

Store the stain in a refrigerator to prevent over-ripening.

The stain must be refrigerated, but does not need to be diluted as Ehrlich's does and it will keep for about a month.

When ready to stain, remove stain from refrigerator, pour into staining box and allow it to come to room temperature. Return the unused portion to the refrigerator.

Test 1 histo-cassette of sections by placing it in the stain.

Agitate for 12 to 18 minutes.

Remove the cassette from stain, place it in a jar of tap water, and view sections in a petri dish of tap water using a transmitted light dissecting microscope.

Look for growth layers and good color.

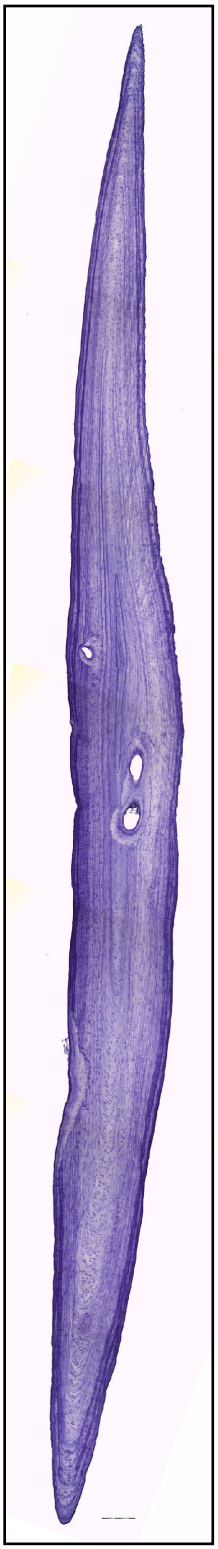
If the test sections look good, stain the remaining sections in the same manner (up to 4 at a time).

After staining, soak the sections in histo-cassettes in a jar of tap water for 20 minutes to blue the sections.

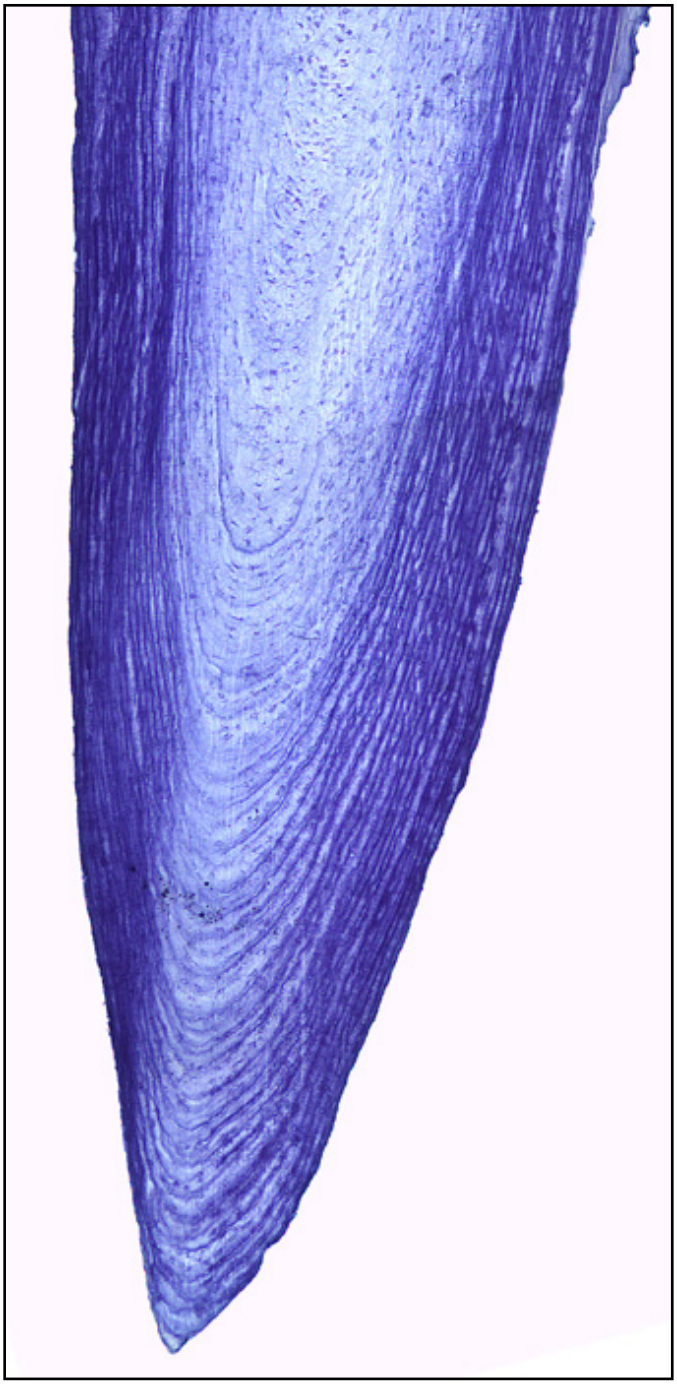
Dry the cassettes well and transfer them to a solution of 50% glycerin, 50% distilled water to soak for 20 minutes.

Drain the cassettes on a petri dish.

Transfer them to 100% glycerin for at least 20 minutes.



Stained leatherback scleral ossicle thin section. The core mark (hatch mark; see Avens et al. 2009) is visible in the center and a small amount of resorption is present.



Wide tip of decalcified and stained leatherback scleral ossicle thin section where growth marks are counted.

Mounting Slides

Remove cassettes from 100% glycerin and allow them to drain on a petri dish.

Place stained ossicle sections in a small petri dish of 100% glycerin and view them using a dissecting microscope under transmitted light.

Choose 3 to 5 of the best complete sections with the least amount of resorption, layers that show up well, and minimal scratches.

Place the chosen sections onto a slide labeled with a fine point marker.

Add a few drops of glycerin, using a probe to spread the glycerin around the tops of the sections.

Place the slide on the stage of a dissecting microscope to make sure the sections are flat and to remove air bubbles and debris.

Choose a cover slip to fit.

Carefully and slowly place the cover slip at an angle, starting from the top rather than from the side as for the humerus sections.

Check the mounted slide using the microscope.

Clean any excess glycerin from the slide if necessary.

Place the extra sections in the labeled vial (containing the extra portion of ossicle left over from thin sectioning) and store away from sunlight.

Collect sequential images of the ossicles using a compound microscope, digital camera and computer with image analysis software.

Seal the slides with a bead of high viscosity mounting medium such as Cytoseal 280, making sure to cover both the cover slip and the slide for a good seal. Take care as to prevent covering the ossicle sections when sealing the slides.

Allow slides to dry overnight.

Appendix 5

How to Process Hatchling Humeri

Dissecting and Cleaning

Dissect the left humerus using a scalpel blade. Remove as much tissue as possible using probes, working in a petri dish of tap water.

Fixation and Decalcification

Place the entire humerus in a labeled histo-cassette and soak in decalcifier for 1 day in Cal Ex II or 2 to 3 hours in 10% neutral buffered formalin, followed by a water rinse and 2 to 3 hours in RDO. Do not attempt to cut them prior to decalcifying whole. Allow the bones to soak in water overnight prior to thin sectioning to remove as much of the decalcifying agent as possible.

Thin Sectioning

You may carefully cut the proximal and distal ends off with a scalpel when you are ready to thin section. The remaining piece is easy to lose at this point so you might place a small amount of mounting medium for frozen sections (we use Tissue Tek O.C.T.) on the bone prior to using the scalpel to cut them so the remaining bone will stick to the forceps, making it easier to pick up.

Thin sections may be acquired using a microtome with a freezing stage or a cryostat.

Secure the microtome blade in the holder on the microtome and set the sectioning thickness to 25 μm.

Check that the microtome stage is level, then squeeze a small amount of mounting medium for frozen sections onto the stage.

Dry the decalcified bone section and using forceps, hold it on the stage so it is flat. More mounting medium for frozen sections may be applied to completely surround the bone edges. Turn the freezing stage temperature controller to the lowest temperature, or -40°C, and wait several minutes for the section to freeze completely.

Increase the temperature to -10°C, check the position of the blade, warm the top slightly with a gloved finger, and begin thin sectioning.

Use a small paintbrush to remove thin sections from the blade before drawing it back over the top of the frozen section.

View the thin sections in tap water in a petri dish using a dissecting microscope and transmitted light.

Once about 10 sections have been collected from the narrowest portion of bone shaft, you may either stain them or proceed directly to collecting images, as they are typically needed only for humerus diameter measurements.

Staining

Staining is optional.

If staining is desired, small mesh screened histo-cassettes work best for containing the tiny thin sections.

Proceed following the methods described earlier for processing humeri.

Less time in Ehrlich's hematoxylin is required than the standard 8 minutes (typically 3 to 4 minutes).

Mounting Slides

Transfer cassettes of thin sections (stained or not) to a solution of 50% glycerin 50% water to soak for 20 minutes.

Drain the cassettes well, then transfer them to 100% glycerin for 20 minutes.

Drain on a petri dish, then use a probe to transfer a thin section to a slide.

Spread glycerin around the thin section.

Cover the slide with cover glass by applying it at an angle and slowly pressing it down.

Imaging

Collect an image at 4× magnification using a compound microscope with a digital camera tethered to a computer with image analysis software.

Save the image with a scale bar in it.

Humerus diameter may be measured later using the saved image files.