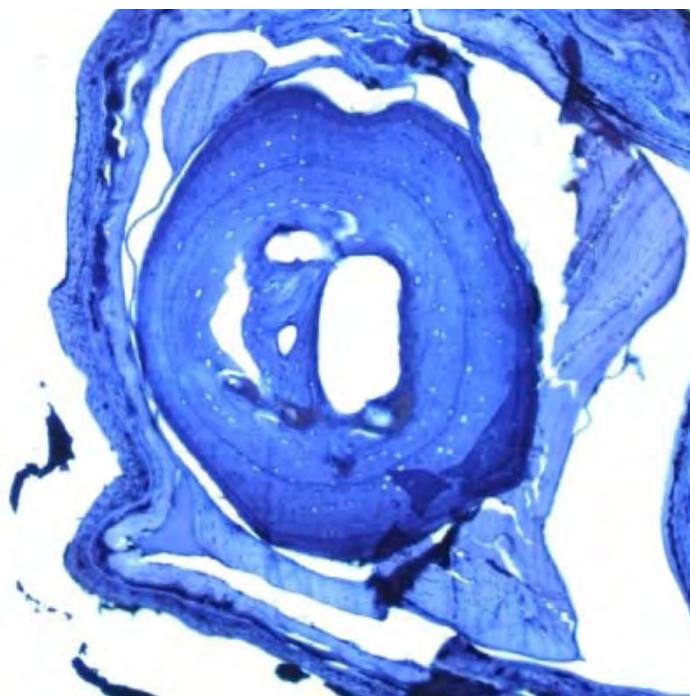


A Protocol for Aging Anurans Using Skeletochronology



Open-File Report 2008–1209

U.S. Department of the Interior
U.S. Geological Survey

Cover Photo: A stained cross-section taken from an anuran toe. All illustrations and photographs by Brome McCreary.

A Protocol for Aging Anurans Using Skeletochronology

By Brome McCreary, Christopher A. Pearl, and Michael J. Adams

Open-File Report 2008-1209

U.S. Department of the Interior
U.S. Geological Survey

U.S. Department of the Interior
DIRK KEMPTHORNE, Secretary

U.S. Geological Survey
Mark D. Myers, Director

U.S. Geological Survey, Reston, Virginia: 2008

For product and ordering information:
World Wide Web: <http://www.usgs.gov/pubprod>
Telephone: 1-888-ASK-USGS

For more information on the USGS—the Federal source for science about the Earth,
its natural and living resources, natural hazards, and the environment:
World Wide Web: <http://www.usgs.gov>
Telephone: 1-888-ASK-USGS

Suggested citation:
McCreary, Brome, Pearl, C.A., and Adams, M.J., 2008, A protocol for aging anurans using
skeletochronology: U.S. Geological Survey Open-File Report 2008-1209, 38 p.

Any use of trade, product, or firm names is for descriptive purposes only and does not imply
endorsement by the U.S. Government.

Although this report is in the public domain, permission must be secured from the individual
copyright owners to reproduce any copyrighted material contained within this report.

Contents

Abstract.....	1
Introduction	1
Fixation and Decalcification	2
Processing	5
Embedding	6
Facing and Sectioning	9
Preparations	9
Facing.....	11
Sectioning	12
Finishing.....	15
Staining.....	16
Mounting	17
Reading Sections.....	17
Procedure.....	17
General Section Viewing	18
LAG Spacing	19
Metamorphosis Line.....	19
Counting the Edge.....	19
Endosteal Bone and Resorption.....	20
Decreasing Error Caused by Resorption	20
Acknowledgements	21
References.....	22
Appendix 1. Selected Literature Review of Key Steps in Skeletochronology Procedure.....	25
Appendix 2. List of Materials	27
Appendix 3. Chemical Replacement Schedules	29
Appendix 4. Definitions for the Sample Datasheet.....	30
Appendix 5. Definitions for the Sectioning Datasheet.....	33
Appendix 6. Processing by Contractor	35
Appendix 7. Definitions for the Aging Datasheet.....	36

Figures

Figure 1. Foundation, lid, envelope, and labeled cassette containing envelope	2
Figure 2. Typical anuran fore-foot skeleton, phalange and cross sections with key features labeled	3
Figure 3. Toe placed in paper envelope	4
Figure 4. Foundation with a small section of grid removed	4
Figure 5. Forceps-warmer.....	6
Figure 6. Molds pre-warming on hot plate.....	7
Figure 7. Phalange gripped in curved forceps	8
Figure 8. Cold tray constructed from a Styrofoam shipping container and a glass baking dish	9
Figure 9. Paraffin blocks cooling in the cold tray, prior to facing or sectioning	10
Figure 10. Paraffin-block clamped in microtome chuck.....	10
Figure 11. Paraffin blocks	12
Figure 12. Close-up side view of the trimmed paraffin block	13
Figure 13. Ribbons of sections positioned on a slide	15
Figure 14. Slide baths set up for the staining process	16

Conversion Factors

Inch/Pound to SI

Multiply	By	To obtain
Length		
centimeter (cm)	0.3937	inch (in.)
millimeter (mm)	0.03937	inch (in.)
micrometer μm	0.00003937	inch (in.)
Mass		
gram (g)	0.03527	ounce, avoirdupois (oz)
Volume		
milliliter (ml)	.0211	pint

Temperature in degrees Celsius ($^{\circ}\text{C}$) may be converted to degrees Fahrenheit ($^{\circ}\text{F}$) as follows:
 $^{\circ}\text{F}=(1.8\times^{\circ}\text{C})+32$

A Protocol for Aging Anurans Using Skeletochronology

By Brome McCreary, Christopher A. Pearl, and Michael J. Adams

Abstract

Age distribution information can be an important part of understanding the biology of any population. Age estimates collected from the annual growth rings found in tooth and bone cross sections, often referred to as Lines of Arrested Growth (LAGs), have been used in the study of various animals. In this manual, we describe in detail all necessary steps required to obtain estimates of age from anuran bone cross sections via skeletochronological assessment. We include comprehensive descriptions of how to fix and decalcify toe specimens (phalanges), process a phalange prior to embedding, embed the phalange in paraffin, section the phalange using a microtome, stain and mount the cross sections of the phalange and read the LAGs to obtain age estimates.

Introduction

Determining the age distribution of a population of amphibians can be a valuable aspect of any research on that population. Although the most accurate method of determining age in an amphibian would be to track a marked individual through birth, growth and death, that is rarely the most practical and cost effective approach. That method also has little value when dealing with species that are difficult to recapture or historically preserved specimens, such as those in museum collections.

Skeletochronology is a widely used method of obtaining age estimates for some animals from the annual growth rings found in bones (appendix 1). A similar technique is the standard tool used to determine age in mammals through studying the rings in teeth cementum (Matson, 1981; Matson and others, 1993).

For amphibians in temperate climates, each year of growth during the warm season, and the subsequent slowing of growth during the cooler season, creates a repeated pattern in their bone. The lines created by a slowing of growth in the cool season (hibernation), commonly called Lines of Arrested Growth (LAGs), can be counted much like the growth rings in many trees. Because this growth pattern predominately corresponds to an annual cycle of increased and decreased activity, the LAG count can be taken as an estimation of age for that individual (Gibbons and McCarthy, 1983; Morrison and others, 2004).

It is now accepted that the middle portion of phalangeal bones can produce some of the best sections for age estimations (Gibbons and McCarthy, 1983; Rozenblut and Ogielska, 2005). This approach makes it possible for researchers to obtain information on age without sacrificing the animals in the study.

In this manual we attempt to describe in detail the steps required to produce transverse sections of anuran phalanges for estimating age through skeletochronology. This was written

initially as a manual for our laboratory at the Forest and Rangeland Ecosystem Science Center. Because there is some variation and flexibility in the procedures described herein (appendix 1), we hope that this document can serve as a protocol for those interested in using skeletochronology for aging anurans as well as a starting point for those who want to develop their own protocol to fit their individual needs and equipment.

We have included a list of the materials used for the procedures described in this manual (appendix 2) as well as a recommended changing schedule for some of the solutions (appendix 3). This manual assumes that anyone undertaking this type of work has sufficient knowledge in basic laboratory procedures and safety.

Fixation and Decalcification

1. Label a cassette (fig. 1), consisting of one foundation with a lid clipped to it, with an alcohol-proof pen or pencil and complete the Sample Datasheet (appendix 4). The label should be written on the slanted part of the foundation opposite the hinge.

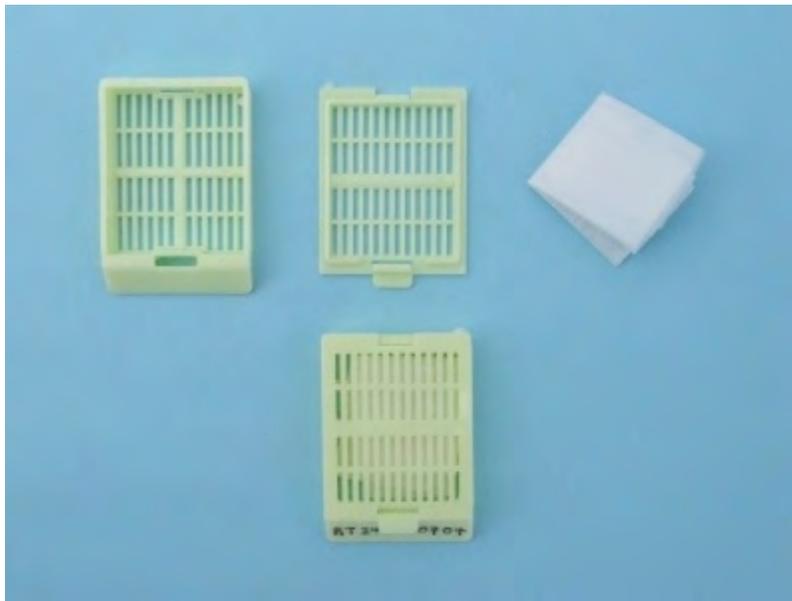


Figure 1. Foundation, lid, envelope, and labeled cassette containing envelope (from left to right, top to bottom).

2. Remove the toe from the container it was collected in. Check to verify that the toe was cut at or near the middle of the third toe bone, or phalange (fig. 2). The first phalange is the one with the claw and cannot be used for aging (Castanet and Smirina, 1990; Smirina, 1994). The second phalange is the best for aging analysis. Ideally the toe should be one of the outer toes from either front foot. Fore-foot phalanges are preferred because resorption (internal bone loss negatively affecting growth lines) may be less common in them (Hemelaar, 1985).

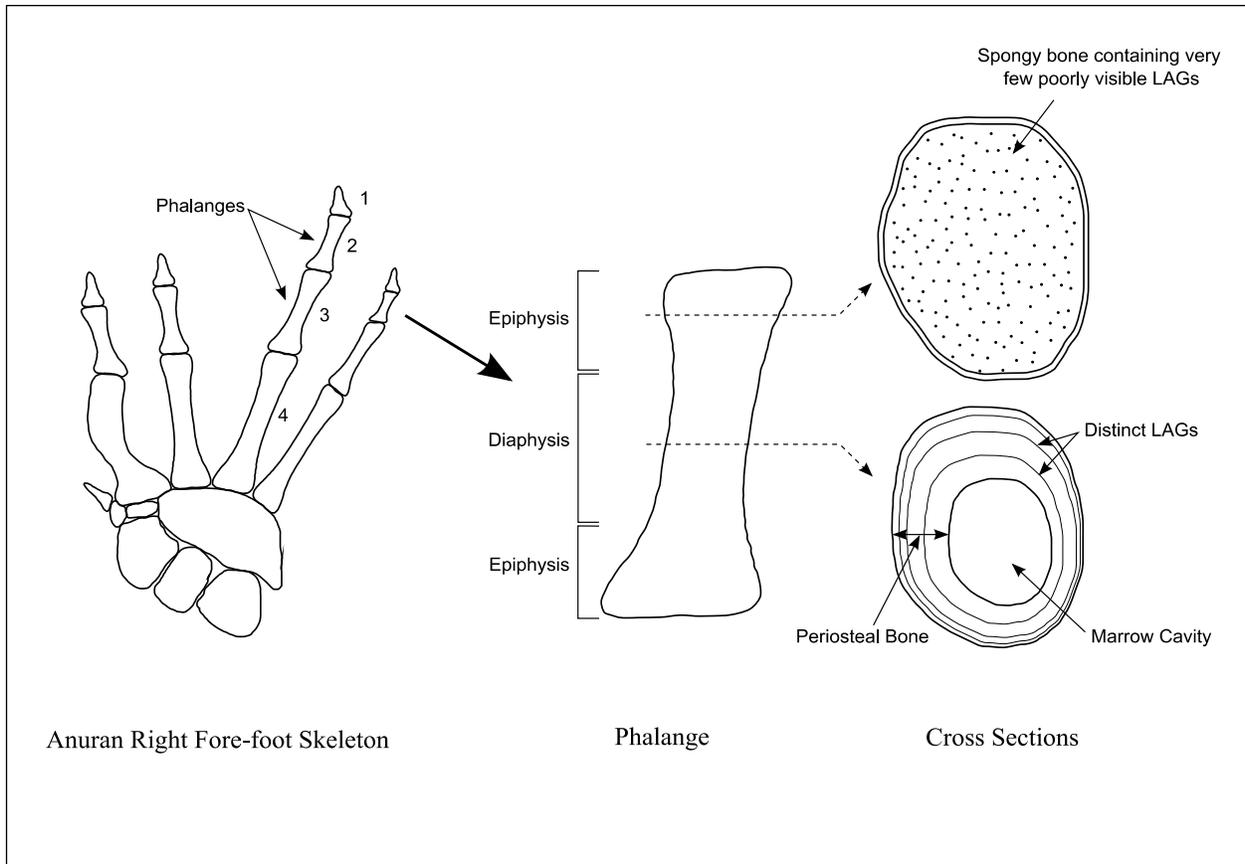


Figure 2. Typical anuran fore-foot skeleton, phalange and cross sections with key features labeled.

3. Place the clipped toe in a paper envelope made from a permanent end-paper (fig. 3). Fold up the envelope and place it inside the cassette, snapping the lid shut securely.
4. Place the loaded (with a toe) cassette in the fixing jar with 95 percent ethanol and seal. Fix for 24 hrs minimum. Multiple cassettes can be placed in a single fixing jar.
5. In the lab, remove a cassette from the fixing jar, carefully remove the toe from the paper envelope, and place the toe in a Petri dish. It might help to stick the toe to a small piece of double sided tape in the Petri dish to keep it secure (G. Matson, Matson's Laboratory, LLC, *written. commun.*). Under magnification (a stereoscope or dissecting scope works well), carefully separate the second phalange (counting from the tip of the toe) from any other phalanges. With one hand, use a pair of forceps to firmly grasp the second phalange; with the other hand use a scalpel to precisely cut right at the joints between the second phalange and the first and third phalanges. Removing the skin from the bone usually is not necessary as the shape of the bone and where each epiphysis (end of the phalange) meet should be readily discernable. To avoid losing the second phalange, it is important to maintain a firm hold on the second phalange during any trimming because sometimes trimmed-off pieces can be propelled out of the dish.



Figure 3. Toe placed in paper envelope.

6. Once trimmed, carefully measure the length of the second phalange to the nearest 0.25 mm using an optical micrometer in the stereoscope (Russell and others, 1996). Enter this value on the proper column on the Sectioning Datasheet (appendix 5). Be as accurate as possible when measuring phalanges. The second phalange has to be less than or equal to about 4.5 mm to fit vertically in the mold. If the second phalange is greater than 4.5 mm, a foundation with a small section of grid cut out of its center, creating a hole, will be needed (fig. 4).

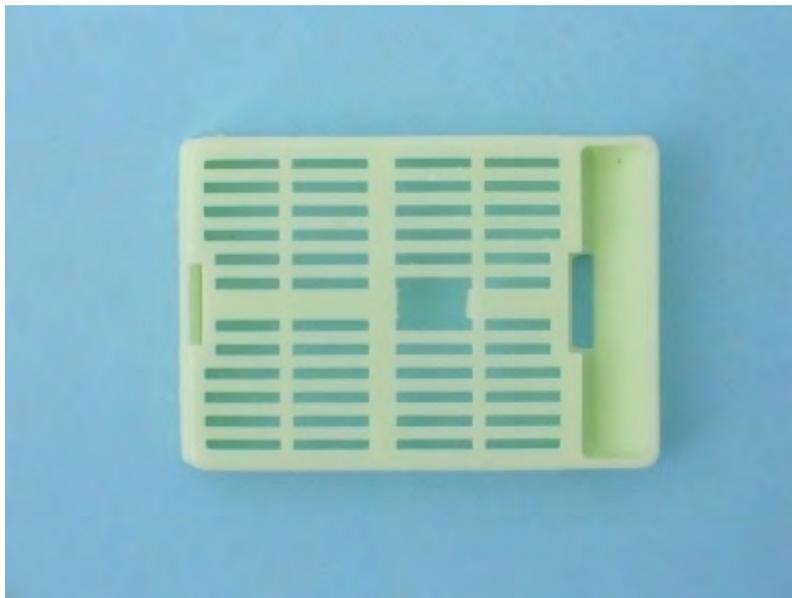


Figure 4. Foundation with a small section of grid removed.

7. Place the phalange back in its paper envelope (or make a new one if the original envelope was damaged) and refold it. Put the envelope back in its respective cassette and return the cassette to the fixing jar. If a separate vial or envelope was used to collect the toe, place any trimmed-off sections of the toe back in this vial for possible future use.
8. Repeat steps 4–6 with all fixed toes.
9. Remove loaded cassettes from the fixing jar and place them in jar of decalcifying solution for 8 hrs.
10. Move cassettes from decalcification solution to a beaker and rinse thoroughly in de-ionized water (DI-H₂O). The cassette can be placed in a large beaker in the sink and water can be left running slowly into the beaker, spilling out the top, overnight.
11. Return the cassettes to the 95 percent ethanol fixing jar until you are ready to process (dehydrate and clear) either in-house or by contracting out. A detailed example of the steps involved in contracting out the processing of phalanges can be found in appendix 6.

Processing

1. Remove the cassettes from the fixing jar and place them sequentially in the following solutions to dehydrate:

95% ethanol	1 hr
100% ethanol	3+ hrs (or overnight)
2. Remove cassettes from 100 percent ethanol solution and place them sequentially in the following solutions to clear:

Xylene substitute	1hr
Xylene substitute (fresh batch)	1hr

While phalanges are being cleared, preparations should be made for the infiltration process (soaking the toe bone in liquid paraffin) so that once clearing has been completed, the toes can go straight into paraffin immersion.

1. Place embedding paraffin in the wax dispenser (if not already full) and melt to liquid at 56–58°C. Be very careful to not overheat the paraffin. It may take several hours to a day to completely melt the paraffin, so it is important to allow for this time, prior to dispensing and using any paraffin, to assure a homogeneous solution.
2. Pre-warm the oven, to 56–58°C.
3. Label two clean oven-proof dishes or bowls “1” and “2” and place them in the oven to pre-warm them; these will be the infiltrating dishes (Gabe, 1976). The bowls or dishes used for infiltration should be of sufficient size to ensure that all the cassettes that are placed in them will be completely covered by molten paraffin.
4. Fill both infiltrating dishes with molten paraffin and return them to the oven. If the dishes already have paraffin in them, place them in the oven and allow the paraffin to liquefy.
5. Once cassettes have completed the clearing step and both infiltrating dishes have reached the appropriate temperature, remove the cassettes from the second clearing jar with a pair of forceps and immerse the cassettes in the paraffin in Infiltrating Dish 1. Let them sit for 2 hrs uncovered in the oven (if a vacuum oven is available, let it sit in that oven).

6. Transfer the cassettes to Infiltrating Dish 2, and let them sit uncovered on the oven for 2 hrs or overnight, if necessary.

While the phalanges are completing the last round of infiltration, make preparations for embedding the phalanges.

Embedding

1. Check the level of paraffin in the paraffin dispenser and fill it if needed.
2. Turn on the hot plate on the paraffin dispenser and allow it to warm up.
3. Turn on the forceps-warmer and place several pairs of forceps and probes, cleaned of wax, in the warmer (fig. 5). Use only warmed tools when working in the paraffin to decrease paraffin build-up.

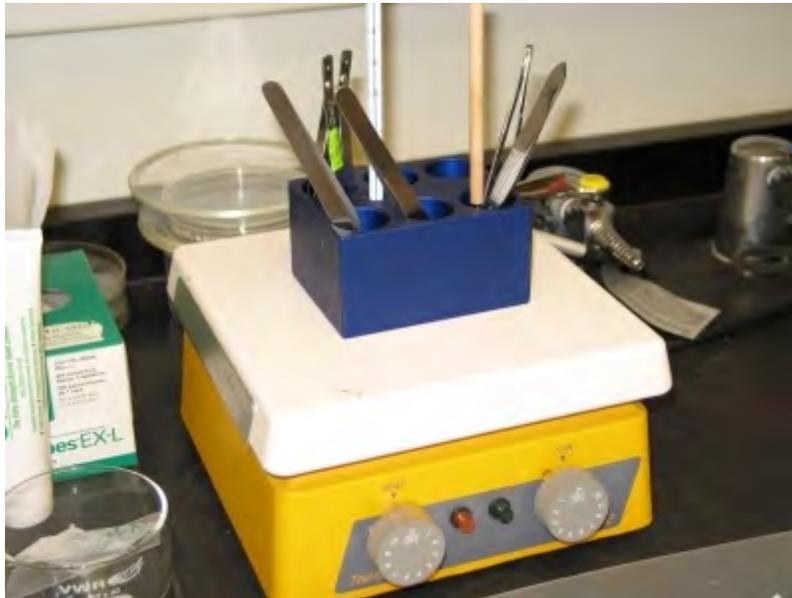


Figure 5. Forceps-warmer (made from block of aluminum) with holes for forceps and probes placed on hot plate. The alcohol thermometer visible vertically in rear helps monitor temperature of tools.

4. Carefully clean as many molds as will be needed (plus a few extras) with 95 percent ethanol and clean paper towels. Place several cleaned molds on the hot plate to pre-warm them (fig. 6).

It often helps to put some kind of barrier cream or lotion on your hands prior to working with the paraffin. This can help get the wax off of your hands later and keep your skin from drying and cracking.

Once several molds have dried, and the phalanges have completed their second infiltration step, embedding can begin.

1. Remove a cassette from Infiltrating Dish 2 with a pair of warmed forceps. Open and separate the two halves of the cassette (fig. 1), removing the paper envelope containing the phalange. Dispose of the cassette lid (the unlabeled part). Place the labeled part of the cassette (the foundation) on the hot plate.



Figure 6. Molds pre-warming on hot plate.

2. Open the envelope (it may be easier to tear it in half; just do not lose the bone). Remove the phalange and place it in the foundation on the hot plate. Dispose of the paper envelope.
3. In a warmed mold, add a very small amount of paraffin to just cover the bottom of the mold, leaving the mold on the hot plate. This should be about 1 millimeter of paraffin.
4. With a pair of warmed curved forceps, grasp the phalange. The forceps should grip the phalange perpendicular to the linear axis of the bone, near the end (fig. 7). Be very careful at this step. Grip the bone too hard and it can shoot off of the forceps and be lost; grip the bone too lightly and it could be dropped. Once you have a firm grasp on the phalange, move the mold off of the hot plate and onto a cool surface such as the bench-top. Quickly and carefully place one end of the phalange down in the paraffin in the mold and hold it there. The bone should be oriented vertically with one end pointing toward the ceiling. The paraffin should start to solidify from the bottom (nearest the cold surface) upwards. It should look like it is starting to whiten. When the phalange appears to be held in place by the hardening wax, but long before the wax completely hardens, carefully remove the forceps and set them back in the warmer. The forceps should detach from the phalange and not stick to it. Use a probe to further orient the bone, if necessary, being very careful to not let the paraffin completely harden.
5. Promptly (before the paraffin in the mold completely solidifies) place the foundation on top of the mold, oriented so the label is right-side up. Add liquid paraffin to the mold until it is full and paraffin has filled part of the foundation. It is very important that the paraffin in the bottom of the mold (holding the toe) is still semi-liquid when the rest of the paraffin is added. If the small amount of paraffin in the bottom of the mold is allowed to harden, a potential fracture zone could be formed, which could break and shear off later when performing sectioning.



Figure 7. Phalange gripped in curved forceps.

6. Allow the paraffin to solidify completely (at least 30-60 minutes at room temperature).
7. Carefully remove the mold from the wax block. The edge of a dull knife can come in handy to pry them apart. If the mold and the block do not separate, the paraffin has not set yet. Let them cool longer and try again. If the mold still will not come off, try chilling it in the refrigerator for 30 minutes or more.
8. If the mold pulls off part of the wax block, or if the block fractures in any way, the phalange should be re-embedded.
9. Once a block has been separated from its mold, check the label on the paraffin block to ensure it is still clear, place it in a clean plastic bag, and store it in the refrigerator at approximately 4°C. A block can be stored this way indefinitely. Multiple blocks can be stored in a single bag.

Manually embedding necessitates some rapid but smoothly performed actions and may require a little trial and error to perfect.

Facing and Sectioning

NOTE: This section is written based on the use of a Leica RM2235 manual rotary microtome. Some details of procedure may vary from one machine to another. Consult the user manual that came with the microtome for specific details of operation.

Preparations

1. Remove a cold plate or tray from the freezer and place it near the microtome. Alternately, a cold tray can be constructed from the bottom of a Styrofoam shipping container and a glass baking dish (fig. 8). The Styrofoam base can be filled with crushed ice or a re-usable ice pack from the freezer and the glass dish is then placed on top.

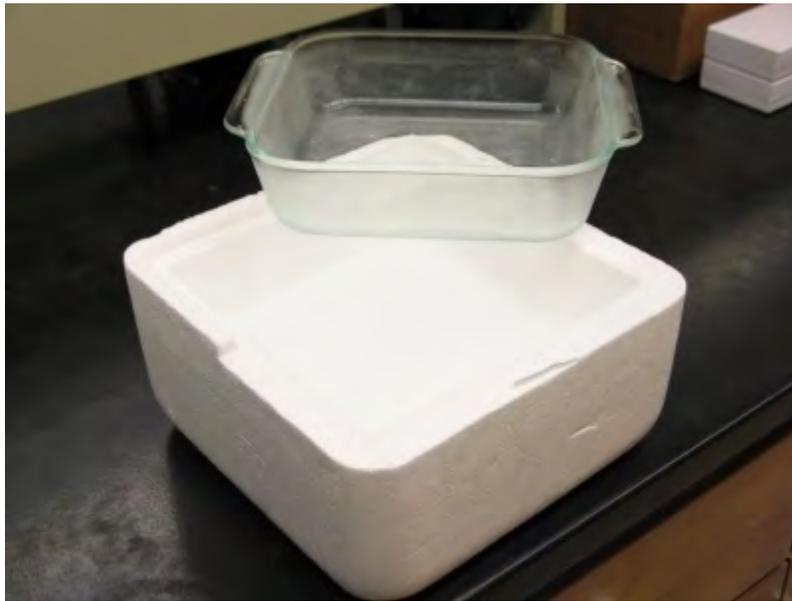


Figure 8. Cold tray constructed from a Styrofoam shipping container and a glass baking dish.

2. Wet the glass dish with a small amount of a 1 percent concentration of glycerol and DI-H₂O from a spray bottle.
3. Remove the paraffin blocks from the refrigerator and place them wax-face down on the cold tray (fig. 9).
4. Clean the water bath's glass dish with 95 percent ethanol.
5. Fill the water bath's dish to 80 percent of its height with DI-H₂O and warm the water to 39–40°C or 5–10 degrees below melting point of the paraffin being used.
6. If necessary, clean the rails of the microtome and all contact areas of wax buildup. Check the chuck and make sure it is also clean. Set and lock the universal cassette clamp in the uppermost position.
7. Set the knife angle to approximately 2.5 degrees. Check that the chuck, knife holder, and base are tight and solid.



Figure 9. Paraffin blocks cooling in the cold tray, prior to facing or sectioning.

8. Select a wax block from the cold tray and mount it in the universal cassette clamp. If necessary, complete any adjustments of the clamp by using the various setscrews on the object head (refer to user's manual that came with the microtome) so that orientation with respect to the blade is correct. Clamp blocks so their longest axis is horizontal and so that the label is on the right side when facing the machine (fig. 10).



Figure 10. Paraffin-block clamped in microtome chuck.

9. Check to make sure the block is clamped solidly in the chuck before proceeding.

10. Mount and fasten a blade (used but sharp is sufficient for this initial step) in the knife holder for trimming the block. Make sure the blade is seated properly in the holder, and is parallel to the upper edge of the pressure plate. If not, remove the blade and check for foreign objects in the blade channel.
11. Tighten the blade holding clamp.
12. Rotate the red knife guard handle upwards to cover the blade.

Facing

1. Release the brake lever for the hand wheel on the microtome.
2. Set the section thickness to 20 μm with the adjusting knob.
3. Release the lock on the hand wheel.
4. Rotate the hand wheel until the block is centered vertically in front of the blade. Check the space between the face of the block and the knife.
5. Adjust the knife holder base by loosening it and sliding it in or out (if necessary) on the rails until the blade is close to, but not touching, the face of the block.
6. While looking down from above directly over the block, rotate the course driving wheel to advance the block even closer to the blade, if necessary.
7. Rotate the red knife guard down into its resting position.
8. Turn the hand wheel until the blade just starts to cut into the block.
9. If the blade is not cutting the block, the block can be advanced more quickly by pressing all the way down on the trimming lever (to the 30 micron detent) while rotating the hand wheel, releasing the lever just when the blade begins to cut into the block.
10. Slowly continue cutting the block to check if the wax block is positioned correctly. If the knife cuts fairly evenly, continue cutting. If not, make the necessary fine adjustments.
11. Slowly face the block until you just reach the beginning end of the phalange's epiphysis and stop. The end of the bone will often not be right at the edge of the wax, but it also can be difficult to know exactly when it has been reached. The block may have to be trimmed a little, removed from the chuck (after locking the hand wheel and covering the blade), and examined under the stereoscope or dissecting scope to make sure the end of the bone has been reached. Alternatively, sections that have been cut can be examined under the compound microscope to see if the bone has been reached. Cut slowly, as it is important not to cut very far into the epiphysis before starting to count the number of sections being cut or any calculations concerning where to cut on the phalange will be inaccurate.
12. Once the epiphysis of the phalange has been reached, rotate the hand wheel until the block is at its uppermost position and engage the lock on the wheel.
13. Rotate the red knife guard up until it covers the blade.
14. Remove the block and return it to the cold tray.
15. Once the block has cooled for a few minutes, use a sharp single-edged razor-blade to carefully trim off the four sides of the block of wax to decrease the amount of wax around the phalange. Be careful not to cut off too much each time or the wax can crack. It is best to just shave off small curls of wax, as opposed to cutting off large chunks. Trim the block evenly around the embedded phalange and make every attempt to keep opposite sides of the block parallel to each other. Once trimmed, the block should have a smaller face

measuring about 5–8 mm square (fig. 11) that tapers back toward the plastic foundation. From the side, the block will look like a flat-topped pyramid with the phalange positioned in the center of the top of the pyramid (fig. 12).

16. Return the block to the cold tray.

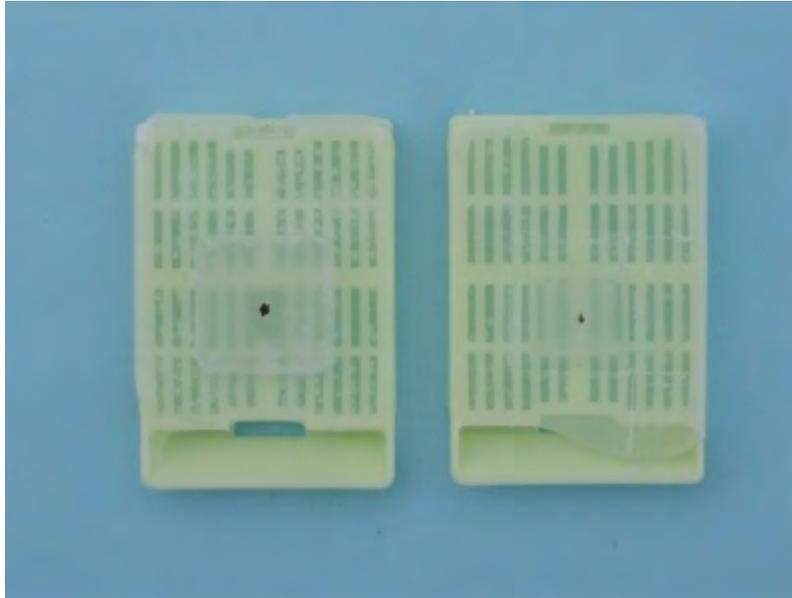


Figure 11. Paraffin blocks. Block on the left is untrimmed as it comes out of the mold; block on the right has been trimmed. Dark object in center of block is the end of the phalange.

Sectioning

Throughout the sectioning process it is very important to keep track of the number and thickness of all sections cut. This is the only way to know where sections are being cut from on the length of the bone. It is best to use a counter to tabulate cuts. If the thickness of the cut sections changes at any point during sectioning, start a new tally for the new thickness.

1. Complete the necessary calculations on the Sectioning Datasheet (Appendix 5) to determine the proper location on the phalange from which to save sections for aging. The datasheet is currently set up on the assumption that sections will be saved from the middle of the diaphysis (mid-section of phalange, fig. 2), which is considered to yield the best sections for accurate aging (Gibbons and McCarthy, 1983; Rozenblut and Ogeilska, 2005). However, it is not difficult to locate other regions of the phalange to sample from using the same calculation method.

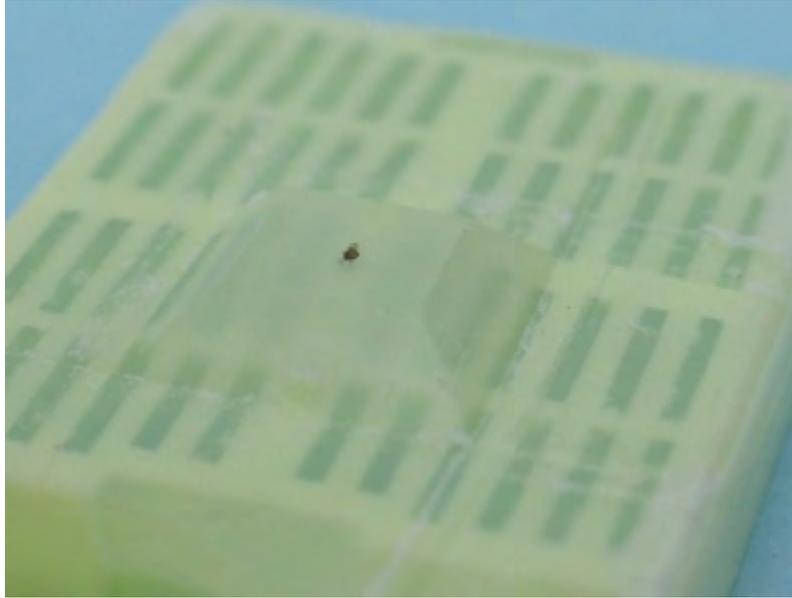


Figure 12. Close-up side view of the trimmed paraffin block.

2. Remove the used blade from the knife holder and install a new or sharpened one.
3. Make sure the blade is seated correctly, and is parallel to the upper edge of the pressure plate. If not, remove the blade and check for foreign objects in the blade channel. If sectioning is begun by using one end of the blade, the life of the blade edge can be maximized.
4. Tighten the blade holding clamp.
5. Rotate the red knife guard handle upwards to cover the blade.
6. After the embedded phalange has been kept cool for about 10 minutes, place it in the microtome chuck.
7. If this is the same block that was just faced, almost no adjustments should be necessary. Cutting sections can proceed.
8. If several blocks have been faced, it may be necessary to repeat steps 5 and 6 detailed in the section on “Facing” (above) to bring the block closer to the blade.
9. If a region from which sections to be saved for aging has been reached, set the section thickness to 10 μm with the adjusting knob. If additional trimming is still needed to reach the desired region of the phalange, the thickness can be set to 20 μm to speed things up.
10. Rotate the red knife guard down into its resting position.
11. Release the lock on the hand wheel.
12. Rotate the hand wheel to cut a continuous wax ribbon of sections. Make sure to use a counter to keep track of how many sections are being cut, and write that number down, along with the section thickness, on the datasheet when sectioning stops or pauses. It is very important to not lose track of this information.

13. Although it may not always be necessary, sectioning can be greatly enhanced by wetting the surface of the wax with the 1 percent concentration glycerol solution either with the spray bottle or a soaked Kimwipe[®].
14. If cutting sections becomes difficult, adjusting cutting speed can help. Return the block to the cold tray as often as possible during sectioning, as the temperature of the block makes a big difference in the quality of the sections cut. If streaks begin to appear in sections, the blade could be dirty and may need cleaning (a cotton swab soaked in xylene (or a substitute) may be used to gently wipe the front edge of the blade, always upwards, to remove built-up wax. If you continue to get streaks or splits in sections, the blade needs to be changed (or moved over to a new, unused area). If a sectioning problem persists, look for a section on trouble-shooting in the user's manual that came with the microtome, or refer to a general histology text (Sheehan and Hrapchak, 1973; Gabe, 1976; Presnell and Schreibman, 1997).
15. When sections look good and a region of the bone has been reached from which sections are to be saved, grasp the leading edge of the ribbon of sections with a pair of clean (non-warmed) forceps in the left hand and gently lift up to feed it off of the blade (while continuing to cut sections). Cut until there is a strip of 8–10 sections together. Stop cutting. Gently peel the ribbon off of the blade (a second pair of forceps or a probe often helps) and move it from the microtome to the water bath. The ribbon of sections should smooth out as they float on the surface of the warm water.
16. Take a charged slide and copy the label code exactly as it is written from the cassette foundation to the slide with an alcohol-proof pen or pencil
17. Holding the labeled slide flat, move it under a quality ribbon of sections floating in the bath and gently lift up. The ribbon of sections should be positioned parallel to the long axis of the slide and be covering no more than half of the available space on the slide. In this way, at least 16–20 sections can be placed on each slide, in two rows side-by-side running the length of the slide (fig. 13). Once the first ribbon of sections is on the slide, it can be difficult to get the second ribbon onto the same slide without the first floating off in the water. Once the first ribbon has been placed on one half of the slide, the tip of a probe can be carefully run down the edge of the wax ribbon on the inside of the strip of sections (the side that will touch the second ribbon of sections near the middle of the slide). This tends to help “glue” that edge of the first ribbon of sections to the slide. Then when the slide is re-dipped in the water bath to pick up a second ribbon, the first ribbon will be less likely to float off. The two ribbons of sections can sometimes be overlapped a little so that they are closer to the center-line of the slide. A probe can be used to carefully “cut” off any attached sections or wax that does not fit on the slide. Positioning sections on slides can be challenging and will take practice to perfect.

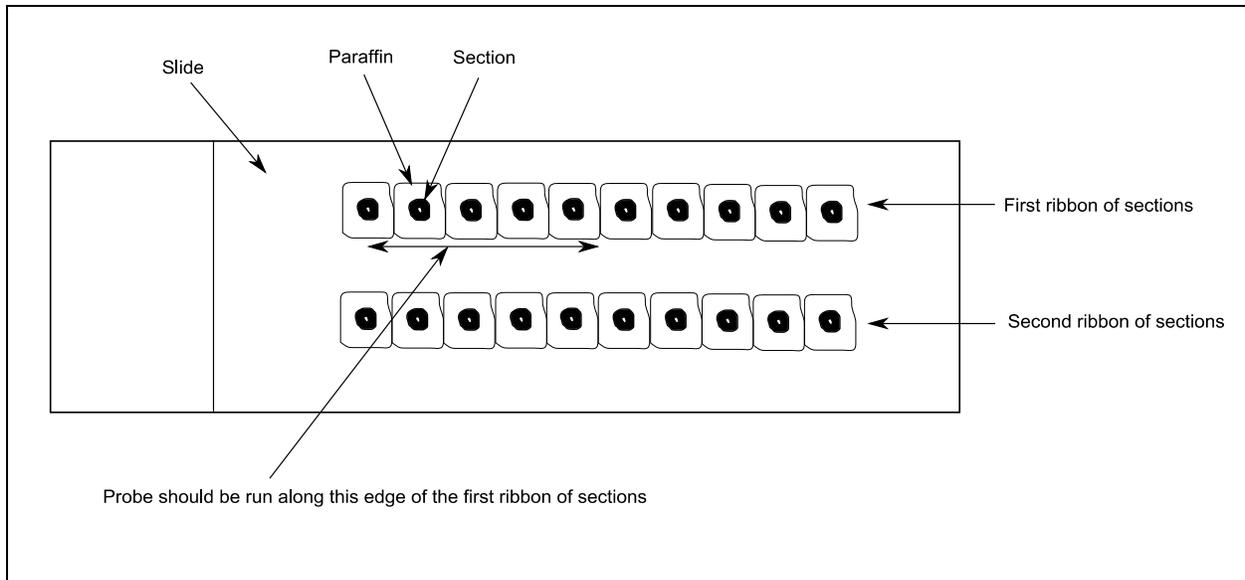


Figure 13. Ribbons of sections positioned on a slide.

18. Drain off excess water by placing the slide with sections upright against a clean object on a clean paper towel for a minute or two.
19. Place the slide in a holder and move it into the oven at 60°C for 1 hr to dry. In the oven, position the slides so that they are resting on their sides on a paper towel. This can help the wax melt off the slides and get wicked up by the paper.
20. The next day the slides can be stained.
21. An open Kimwipe[®] can be used to skim excess wax/sections from the surface of the water bath.

Finishing

1. When all sectioning has been completed for a block, raise the block to the uppermost position by rotating the hand wheel.
2. Engage the brake lever and the lock on the hand wheel.
3. Cover the blade edge with the guard.
4. Unclamp the block from the chuck and place it in the cold tray or return it to a bag in the refrigerator.
5. Turn course driving wheel until the chuck is in its farthest rearward position.
6. A new block can now be clamped in the chuck, or the microtome can be cleaned.
7. If all sectioning has been completed for the day, loosen the clamp and remove the blade from the knife holder and dispose of it or store it for future sharpening.
8. Using a brush and/or rag, brush all loose wax debris from the chuck, knife holder, and base into the waste tray. It may be necessary to loosen the base and remove it to facilitate thorough cleaning.
9. Empty the waste tray and cover the machine.
10. If further cleaning is necessary, refer to the proper section in the microtome manual.

11. Turn off the power to the water bath.
12. Return the cold tray or plate to the freezer.

Staining

1. In the fume hood, fill all of the slide baths (fig. 14) with the solutions listed below.
2. Place slides to be stained in a slide rack.
3. To remove the paraffin, dip the rack of slides sequentially into the following baths:

Xylene substitute	10 min
Xylene substitute(fresh batch)	10 min

Agitating the slides a little by dipping them up and down every 3–5 minutes can help clean them of wax (K. Fischer, Oregon State University Veterinary Diagnostics Laboratory, *oral. commun.*). After each wash, check the slides to see how the cleaning is progressing. Almost all the wax should come off. If wax is still seen on slides, put them back in the wash. Depending on how thick the wax is, the slides may need to be washed in xylene for 30-45 minutes. If there is a small lump or two of wax that just won't come off the slide, a cotton swab or paper towel dipped in xylene (or a substitute) can be used to rub the wax off, but be very careful not to touch any sections.



Figure 14. Slide baths set up for the staining process.

4. Clear the slides to remove the xylene substitute by dipping the slides sequentially into the following baths:

100% ethanol	2 min
95% ethanol	2 min
70% ethanol	2 min
5. Wash the slides in:

De-ionized water	2 min
------------------	-------

6. Stain the sections by placing the slides in:

Harris' Hematoxylin	8 min
---------------------	-------
7. Rinse off excess stain by washing the slides in:

De-ionized water	2 min
------------------	-------
8. Check the quality of the stain by viewing a few of the sections under a microscope.
9. If the stain is too light, re-stain the sections in:

Harris' Hematoxylin	8 min
---------------------	-------
10. Rinse the slides in de-ionized water again.
11. Re-check the quality of the stain under the microscope.
12. When the stain looks good, dehydrate the sections by dipping the slides sequentially in:

70% ethanol	2 min
95% ethanol	2 min
100% ethanol	2 min
13. Clear the sections to remove the alcohol by dipping the slides in:

Xylene substitute	3–5 min
-------------------	---------
14. Keep all sections moist with xylene substitute before mounting.

Mounting

1. Remove a slide from the rack and place it on a clean paper towel in the fume hood. When mounting cover slips, always use a clean paper towel under the slide.
2. Place a narrow line of mountant adhesive (make sure it is miscible with the xylene or substitute being used in the staining process) down the middle of the slide, staying 8–10mm away from the ends of the slide, and add a cover slip (22×50 mm). Too much mountant can create a big mess, and it is easy to put too much on. Practice.
3. Use a probe to push any air bubbles toward the edges of and out from under the cover slip.
4. Once the slide looks good, it should sit for 30–45 minutes before it is viewed. After about 24 hrs the slide should be thoroughly dry.
5. Once dry, excess glue can be removed from the slide with a razor blade.
6. Check the slide's label for clarity, and then place it in a slide box for storage.
7. After closing the mountant bottle, it helps to wipe off any glue that has accumulated around the tip, especially if it will not be used for more than several minutes. This glue will harden if left on the tip and can make future use of the bottle more difficult.
8. After mounting each slide, clean the cover slip forceps and the probe of any mountant, using xylene substitute if necessary.

Reading Sections

Procedure

1. Select a slide from the storage box.
2. Write the ID Code of the slide on the Aging Datasheet (appendix 7).

3. Place the slide on the stage of the compound microscope.
4. Starting at 10x, adjust the focus and the position of the first section until the specimen is clearly visible.
5. Switch to the 20x or 40x ocular and count the Lines of Arrested Growth (LAG) in the section.
6. View all sections and compare counts to come to a final count for the specimen.
7. Enter the final count into the No. LAGs field for the specimen on the datasheet, as well as the high and low estimates.
8. In the Notes field, make any notes about evidence of partial or reabsorbed, indistinct, double or confusing LAGs. Note the quality of the sections and any other information that could be relevant to the accuracy of the LAG count.
9. If necessary, measure the diameter of the LAGs (see section on “Decreasing Error due to Resorption” below).
10. Replace the slide in the storage box and select the next slide.

General Section Viewing

If a particular section is difficult to read, do not hesitate to move on to another one on the slide. Not all sections will be easy to read; that is why more than one section should be saved. Think of the sections as rough copies of the same thing and understand that some will be better (easier to read) than others. When a slide is first put under the microscope, give all the sections a cursory look to identify which ones might be better and then start with those, working from the good sections down to those that do not appear to be as good quality.

Make sure to review all sections for an individual (given ID Code) before settling on a count of LAGs. All sections may be on one slide, or spread over two or more slides. Check the storage box for all slides from a particular phalange. Even bad sections can yield good information.

In addition to recording the best estimate of the total number of LAGs present, note the high and low estimated count of LAGs, if applicable.

The number of LAGs for each ID Code should be counted independently by two or more people, or at least twice by the same person but on different occasions (Sagor and others, 1998). Once completed, the two readers can compare their results. If the readers cannot come to a consensus about what the final count should be and the counts differ by more than 1 LAG, a decision needs to be made whether or not to throw out those estimates (Castanet and others, 1996). It should be noted that as much as 26 percent of a sample has been off by ± 1 yr or greater between readers in past studies (Marnell, 1997; Sagor and others, 1998; Miaud and others, 1999).

Here are a few tricks that might help when interpreting sections on slides.

- Try adjusting the focus on each section, as LAGs can sometimes actually be clearer when the section is viewed just slightly out of focus.
- Adjusting the light level and contrast while viewing a section can also help with reading.
- Flipping the slide over and viewing the section from the other side may also help.
- Switching back and forth between magnifications can also help with interpreting sections, especially those with many LAGs.

- Occasionally, all the sections from an individual will simply not be interpretable. This may be due one or more variables. In those cases, not much can be done to get counts for the sections from that individual, and that individual must be rejected from the study. It is not unusual to have to reject 9–15 of the individuals in a sample (Marnell, 1997; Marnell, 1998; Miaud and others, 1999; Olgun and others, 2001; Cvetkovic and others, 2005).
- Make sure to take regular breaks to rest and stretch every few hours at least.

LAG Spacing

It is important to guard against undercounting LAGs. A LAG may be indistinct or incomplete, especially near the endosteal bone in specimens 3–4 yrs-old or older. Most periosteal bone development (bone growth) occurs during the second growing season. During the third and fourth years, around the point of sexual maturation, growth slows down, causing the LAGs for those years to be closer together at the outer edge of the bone (Gibbons and McCarthy, 1983; Leclair and Castanet, 1987; Miaud and others, 1999; Rozenblut and Ogielska, 2005). In older specimens, later LAGs may be very thin and compressed into the edge of the periosteal bone (Leclair and Castanet, 1987; Castanet and Smirina, 1990; Sagor and others, 1998; Miaud and others, 1999). Accuracy of aging decreases when many indistinct LAGs occur on the edge of the bone, so it is important to pay close attention in these situations.

Watch for inconsistent changes in the growth pattern or spacing between LAGs in a given section. The spacing between LAGs should decrease at a steady rate as you go from the center of the cross section toward the edge, reflecting the slowing of bone growth as the animal ages (Castanet and Smirina, 1990). If you notice what appears to be unusual spacing between two or three LAGs that seems inconsistent with the prevailing spacing on that section (either too wide or too narrow), this could indicate a false or double LAG (close spacing) or a missing LAG (wide spacing) (Castanet and Smirina, 1990; Sagor and others, 1998). Make sure to note any evidence of these unusual spacing changes in the Notes section of the datasheet.

Metamorphosis Line

Be aware that if the individual the phalange was collected from was a young-of-the-year (YOY; individual that transformed in the year the bone was collected) it will not have any LAGs yet since it has not experienced hibernation. However the phalange possibly will show a faint line from the slowing of bone growth caused by metamorphic climax. Often called a metamorphosis line, this line can look like a LAG and forms within the one-year growth bone (Hemelaar, 1985; Castanet and Smirina, 1990; Rozenblut and Ogielska, 2005).

A metamorphosis line will most likely be absent in phalanges from individuals in their second year (one hibernation) due to resorption of that inner periosteal bone by endosteal bone growth (see below).

Counting the Edge

It is important to decide whether the very outermost edge of the periosteal bone will be counted as a LAG or not. Knowing the date when the phalange was collected (removed from the amphibian) can be helpful in making this decision. If the phalange was collected in spring, the last LAG (from the previous winter's hibernation period) will be very close to the edge of the bone, compared to a phalange collected later in the year or in autumn. Because the amphibian probably just came out of hibernation, very little if any new periosteal bone has had a chance to grow between the last LAG and the edge of the bone (Kusano and others, 1995; Sagor and

others, 1998; Khonsue and others, 2002). This last LAG's proximity to the edge of the bone can make it difficult to discern, so it may be necessary to count the edge of the bone as a LAG in those individuals collected soon after hibernation (Redmer, 1994; Khonsue and others, 2002; Eaton and others, 2005).

Endosteal Bone and Resorption

If endosteal bone is present in the cross section, it is important to identify its presence and prevalence. The hematoxylinophilic zone between the endosteal and periosteal bone should not be accidentally included in any LAG counts. LAGs may occasionally be visible in the endosteal bone and should not be confused with those in the periosteal bone (Smirina, 1994; Rozenblut and Ogielska, 2005). Endosteal bone usually will be visible in phalanges older than 2 yrs and will appear as a lighter area of bone between the marrow cavity and the periosteal bone, separated from the periosteal bone by a line of resorption often with visible gaps (Rozenblut and Ogielska, 2005). Periosteal bone will stain darker and the periosteal LAGs should be much darker than those in the endosteal. There may be as many LAGs in the endosteal bone as in the periosteal bone, but generally they will be fewer and less distinct (Redmer, 1994).

The growth of endosteal bone replaces the inner edge of the periosteal bone and therefore can eliminate all or, more often, part of inner (older) LAGs (Hemelaar, 1985; Castanet and Smirina, 1990; Morrison and others, 2004). Although its function is not well understood, this process, often called remodeling or resorption, may serve to lighten the skeleton and thus aid in amphibian buoyancy (Leclair, 1990).

In the northern leopard frog, endosteal bone growth has been determined to be slight or nonexistent in 1-yr-old frogs (Leclair and Castanet, 1987). Partial resorption by endosteal bone of the first LAG was detected in a few 2-yr-old frogs and was more developed in 4 yr-old frogs. The first LAG was completely removed in 17 percent of those frogs sampled older than 1 year of age.

After first hibernating, part of the first LAG may be resorbed, but usually it will be untouched, (Rozenblut and Ogielska, 2005). After second hibernation, part of the first LAG can be missing due to resorption, whereas after third or fourth hibernation ("3" or "4" year-old frogs), all of the first LAG may be missing. The second LAG is usually not resorbed completely (only 0.1 percent of sample in Rozenblut and Ogielska, 2005).

If present, resorption appears to occur mostly prior to reaching sexual maturity (2–4yr-old) and decreases after that age (Hemelaar, 1985; Cvetkovic and others, 2005). Resorption is rare or nonexistent in some populations (Morrison and others, 2004; Eaton and others, 2005; Meddeb and others, 2007).

In the common toad, the rate of resorption has been determined to be higher in phalanges from females (Hemelaar, 1985; Cvetkovic and others, 2005) and in the rear phalanges of males (Hemelaar, 1985)

Decreasing Error Caused by Resorption

Two basic methods can be used to decrease the error in LAG counts due to the effect of periosteal bone resorption by endosteal bone growth.

The most precise method is to use known aged animals within each population/sample to determine the rate of resorption (Smirina, 1994; Tejedo and others, 1997; Sagor and others, 1998; Eden and others, 2007). By comparing the number and completeness of LAGs between individuals of known but different age, one can generally confidently determine the rate and severity of any resorption.

When individuals of known age are not available, comparisons of bone size can be made between individuals of different age (Hemelaar, 1985; Smirina, 1994). By measuring the size of the marrow cavity and outer edge of a bone collected from a young-of-the-year individual just prior to or just after its first hibernation, and comparing that value to the size of the marrow cavity and suspected first LAG of an older individual, one can deduce if LAGs may be missing due to resorption. Ideally comparisons should only be made between the same phalange bone from each of the individuals being examined (Sagor and others, 1998; Sinsch and others, 2001).

Because phalange cross sections are typically roughly oval, calculations to obtain a mean diameter must be made by taking the square root of the product of the maximum diameter and the diameter taken at a measurement perpendicular to the maximum (Hemelaar, 1985; Sagor and others, 1998). A more detailed description of this technique is available in Hemelaar, 1985.

Acknowledgements

The authors would like to thank Kay Fischer at the Oregon State University Veterinary Diagnostics Laboratory (Corvallis, Oregon) for sharing her histological knowledge during the development of this protocol. This project would have been considerably more difficult without her willingness to take time out of her busy work schedule to answer our questions and share her experiences. We also would like to thank Gary Matson (Matson's Laboratory, LLC, Milltown, Montana), for his encouragement and contributions to the field of skeletochronology. We thank Dr. Brian Eaton, (Alberta Research Council, Canada), Janice Engle (U.S. Fish and Wildlife Service, Sacramento, California), Mizra D. Kusrini (Departemen Konservasi Sumberdaya Hutan and Ekowisata, Fakultas Kehutanan, Indonesia), Carla Piantoni (Smithsonian Institution, Washington, D.C.), Mike Redmer (U.S. Fish and Wildlife Service, Chicago, Illinois), Dr. Ulrich Sinsch (University of Koblenz-Landau, Germany) for sharing their experiences regarding the details of specific techniques. We express our gratitude to the two individuals who contributed to the peer review of this document.

References

- Acker, P.M., Kruse, K.C., and Krehbiel, E.B., 1986, Aging *Bufo americanus* by skeletochronology: *Journal of Herpetology*, v. 20, p. 570–574.
- Ash, A.N., Bruce, R.C., Castanet, J., and Francillon-Vieillot, H., 2003, Population parameters of *Plethodon metcalfi* on a 10-year-old clearcut and in nearby forest in the southern Blue Ridge Mountains: *Journal of Herpetology*, v. 37, p. 445–452.
- Bastien, H., and Leclair, R., Jr., 1992, Aging wood frogs (*Rana sylvatica*) by skeletochronology: *Journal of Herpetology*, v. 26, p. 222–225.
- Bruce, R.C., Castanet, J., and Francillon-Vieillot, H., 2002, Skeletochronological analysis of variation in age structure, body size, and life history in three species of desmognathine salamanders: *Herpetologica*, v.58, p.181–193.
- Castanet, J., and Smirina, E., 1990 Introduction to the skeletochronological method in amphibians and reptiles: *Annales des Sciences Naturelles, Zoologie*, v. 11, p. 191–196.
- Castanet, J., Francillon-Vieillot, H., and Bruce, R.C., 1996, Age estimation in desmognathine salamanders assessed by skeletochronology: *Herpetologica*, v. 52, no. 2, p. 160–171.
- Cvetkovic, D., Tomasevic, N., Aleksic, I., and Crnobrnja-Isailovic, J., 2005, Assessment of age and intersexual size differences in *Bufo bufo*: *Archives of Biological Sciences*, [Belgrade], v. 57, p. 157–162.
- Driscoll, D.A., 1999, Skeletochronological assessment of age structure and population stability for two threatened frog species: *Australian Journal of Ecology*, v. 24, no. 2, p. 182–189.
- Eaton, B.R., Paszkowski, C.A., Kristensen, K., and Hiltz, M., 2005, Life-history variation among populations of Canadian Toads in Alberta, Canada: *Canadian Journal of Zoology*, v.83, p. 1421–1430.
- Eden, C.J., Whiteman, H.H., Duobinis-Gray, L., and Wissinger, S.A., 2007, Accuracy assessment of skeletochronology in the Arizona tiger salamander (*Ambystoma tigrinum nebulosum*): *Copeia*, v. 2, p. 471–477.
- Erismis, U.C., Kaya, U., and Arikan, H., 2002, Observations on the histomorphological structure of some long bones of the Water frog (*Rana bedriagae*) from the Izmir area: *Turkish Journal of Zoology*, v. 26, p. 213–216.
- Gabe, M., 1976, *Histological Techniques*: New York, Springer-Verlag, 1106 p.
- Gibbons, M.M., and McCarthy, T.K., 1983, Age determination of frogs and toads (Amphibia, Anura) from Northwestern Europe: *Zoologica Scripta*, v. 12, no. 2, p. 145–151.
- Hasumi, M., and Watanabe, Y., 2007, An efficient method for skeletochronology: *Herpetological Review*, v. 38, no. 4, p. 404–406.
- Hemelaar, A., 1985, An improved method to estimate the number of year rings resorbed in phalanges of *Bufo bufo* (L.) and its application to populations from different latitudes and altitudes: *Amphibia-Reptilia*, v. 6, p. 323–341.
- Hemelaar, A.S.M, and van Gelder, J.J., 1980, Annual growth rings in phalanges of *Bufo bufo* (Anura, Amphibia) from the Netherlands and their use for age determination: *Netherlands Journal of Zoology*, v. 30, no. 1, p. 129–135.
- Khonsue, W., Matsui, M., and Misawa, Y., 2002, Age determination of Daruma pond frog, *Rana porosa brevipoda* from Japan towards its conservation (Amphibia: Anura): *Amphibia-Reptilia*, v. 23, no. 3, p. 259–268.

- Kumbar, S.M., and Pancharatna, K., 2001, Determination of age, longevity and age at reproduction of the frog *Microhyla ornata* by skeletochronology: *Journal of Bioscience*, v. 26, no. 2, p. 265–270.
- Kusano, T., Fukuyama, K., and Miyashita, N., 1995, Age determination of the stream frog, *Rana sakuraii*, by skeletochronology: *Journal of Herpetology*, v. 29, no. 4, p. 625–628.
- Kusrini, M.D., and Alford, R.A., 2006, The application of skeletochronology to estimate ages of three species of frogs in West Java, Indonesia: *Herpetological Review*, v. 37, no. 4, p. 423–425.
- Leclair, R., Jr., 1990, Relationships between relative mass of the skeleton, endosteal resorption, habitat and precision of age determination in ranid amphibians: *Annales des Sciences Naturelles, Zoologie, Paris*, v. 11, p. 205–208.
- Leclair, R., Jr., and Castanet, J., 1987, A skeletochronological assessment of age and growth in the frog *Rana pipiens* Schreber (Amphibia, Anura) from Southwestern Quebec: *Copeia*, v. 2, p. 361–369.
- Leskovar, C., Oromi, N., Sanuy, D., and Sinsch, U., 2006, Demographic life history traits of reproductive natterjack toads (*Bufo calamita*) vary between northern and southern latitudes: *Amphibia-Reptilia*, v. 27, no. 3, p. 365–375.
- Lu, X., Li, B., and Liang, J.J., 2006, Comparative demography of a temperate anuran, *Rana chensinensis*, along a relatively fine elevational gradient: *Canadian Journal of Zoology*, v. 84, p. 1789–1795.
- Marnell, F., 1997, The use of phalanges for age determination in the smooth newt, *Triturus vulgaris* L.: *Herpetological Journal*, v. 7, p. 28–30.
- Marnell, F., 1998, A skeletochronological investigation of the population biology of smooth newts *Triturus vulgaris* L. at a pond in Dublin, Ireland: *Biology and Environment, Proceedings of the Royal Irish Academy*, v. 98B, no. 1, p. 31–36.
- Matson, G.M., 1981, Workbook for cementum analysis: Milltown, Mont., Matson's Laboratory, 30 p., accessed January 30, 2008, at: <http://www.matsonslab.com/>
- Matson, G.M., van Daele, L., Goodwin, E., Aumiller, L., Reynolds, H., and Hristienko, H., 1993, A laboratory manual for cementum age determination of Alaska brown bear PM1 teeth: Milltown, Mont., Alaska Department of Fish and Game and Matson's Laboratory, 52 p.
- Measey, G.J., 2001, Growth and ageing of feral *Xenopus laevis* (Daudin) in South Wales, U.K.: *Journal of Zoology [London]*, v. 254, p. 547–555.
- Meddeb, C., Nouira, S., Cheniti, T.L., Walsh, P.T., and Downie, J.R., 2007, Age structure and growth in two Tunisian populations of green water frogs *Rana saharica*—a skeletochronological approach: *Herpetological Journal*, v. 17, p. 54–57.
- Miaud, C., Guyétant, R., and Elmberg, J., 1999, Variations in life-history traits in the common frog *Rana temporaria* (Amphibia: Anura): A literature review and new data from the French Alps: *Journal of Zoology [London]*, v. 249, p. 61–73.
- Morrison, C., Hero, J.M., and Browning, J., 2004, Altitudinal variation in the age at maturity, longevity, and reproductive lifespan of anurans in subtropical Queensland: *Herpetologica*, v. 60, no. 1, p. 34–44.
- Olgun, K., Miaud, C., and Gautier, P., 2001, Age, growth, and survivorship in the viviparous salamander *Mertensiella luschani* from southwestern Turkey: *Canadian Journal of Zoology*, v. 79, p. 1559–1567.
- Patón, D., Juarranz, A., Sequeros, E., Pérez-Campo, R., López-Torres, M., and de Quiroga, G.B., 1991, Seasonal age and sex structure of *Rana perezi* assessed by skeletochronology: *Journal of Herpetology*, v. 25, no. 4, p. 389–394.

- Piantoni, C., Ibarquengoytía, N.R., and Cussac, V.E., 2006, Growth and age of the southernmost distributed gecko of the world (*Homonota darwini*) studied by skeletochronology: *Amphibia-Reptilia*, v. 27, no. 3, p. 393–400.
- Presnell, J.K., and Schreibman, M.P., 1997, *Humason's Animal Tissue Techniques* (5th ed.): Baltimore, Md., The John Hopkins University Press, 572 p.
- Redmer, M., 1994, Relationships of demography and reproductive characteristics of three species of *Rana* (Amphibia: Anura: Ranidae) in southern Illinois: Carbondale, Ill., Southern Illinois University, M.S. dissertation, 93 p.
- Rossell, C.R., Jr., and Sheehan, J.L., 1998, Comparison of histological staining procedures for skeletochronological studies: *Herpetological Review*, v. 29, p. 95.
- Rozenblut, B., and Ogielska, M., 2005, Development and growth of long bones in European water frogs (Amphibia: Anura: Ranidae), with remarks on age determination: *Journal of Morphology*, v. 265, p. 304–317.
- Russell, A.P., Powell, G.L., and Hall, D.R., 1996, Growth and age of Alberta long-toed salamanders (*Ambystoma macrodactylum krausei*)—A comparison of two methods of estimation: *Canadian Journal of Zoology*, v. 74, no. 3, p. 397–412.
- Sagor, E.S., Ouellet, M., Barten, E., and Green, D.M., 1998, Skeletochronology and geographic variation in age structure in the wood frog, *Rana sylvatica*: *Journal of Herpetology*, v. 32, no. 4, p. 469–474.
- Sheehan, D.C., and Hrapchak, B.B., 1973, *Theory and Practice of Histotechnology*: Saint Louis, Ill., The C.V. Mosby Company, 218 p.
- Sinsch, U., Oromi, N., and Sanuy, D., 2007, Growth marks in natterjack toad (*Bufo calamita*) bones—histological correlates of hibernation and aestivation periods: *Herpetological Journal*, v. 17, no. 2, p. 129–137.
- Sinsch, U., Di Tada, I.E., and Martino, A.L., 2001, Longevity, demography and sex-specific growth of the Pampa de Achala toad, *Bufo achalensis* CEI, 1972: *Studies on Neotropical Fauna and Environment*, v. 36, no. 2, p. 95–104.
- Smirina, E.M., 1994, Age determination and longevity in amphibians: *Gerontology*, v. 40, p. 133–146.
- Tejedo, M., Reques, R., and Esteban, M., 1997, Actual and osteochronological estimated age of Natterjack toad (*Bufo calamita*): *Herpetological Journal*, v. 7, p. 81–82.
- Tessa, G., Guarino, F.M., Giacomini, C., Mattioli, F., and Andreone, F., 2007, Longevity and body size in three populations of *Dyscophys antiongilli* (Microhylidae, Dyscophinae), the tomato frog from north-eastern Madagascar: *Acta Herpetologica*, v. 2, p. 139–146.

Appendix 1. Selected Literature Review of Key Steps in Skeletochronology Procedure

Source ¹	Fixative	Decalcifier (type/duration)	Section ²	Stain (type ³ /duration)
Acker and others, 1986(a)	1-2% trypsin	RDO/2 h	15 µm/r	Delafield;s hema./30-35 m
Ash and others, 2003(u)	70% ethanol	3% nitric acid/6-8 h	15 µm/f	Ehrlich's hema./7 m
Bastien and Leclair, 1992(a)	ns ⁴	3%nitric acid/6 h	16 µm/f	Ehrlich's hema./ ns
Bruce and others, 2002(u)	(see Castanet and others, 1996)			
Castanet and others, 1996(u)	10%formalin/70% ethanol	3% nitric acid/1-2 h	15 & 20 µm/f	Ehrlich's hema./15 & 20 m
Driscoll, 1999(a)	Bouins Fluid	Bouins Fluid/overnight	7 µm/r	Ehrlich's hema./15 m
Eaton and others, 2005(a)	70% ethanol	RDO Rapid Decal/3 h	8 µm/r	Harris' hema./3-10 m
Eden and others, 2007(u)	70% ethanol	3% nitric acid/ns	20 µm/f	Harris' hema./ ns
Erismis and others, 2002	70% alcohol	5% nitric acid/4 h	10-12 µm/ns	ns/ns
Gibbons and McCarthy, 1983(a)	10% formol	Dilute formol-nitric acid/1-2 d	10-15 µm/ ns	Ehrlich's hema./ ns
Hasumi and Watanabe, 2007	10% formalin	5% formic or nitric acid/24 h+	8-16 µm/r	Carazzi's hema. or Jordet Dip-Quick/ns
Hemelaar, 1985(a)	(see Hemelaar and van Gelder, 1980)			
Hemelaar and van Gelder, 1980(a)	70% ethanol	5% formic acid/ ≥1 h	20 µm/f	Delafield's hema./30 m
Khonsue and others, 2002(a)	10% formalin	5% nitric acid/60-90 m	20-22 µm/f	Hayer's acid hemalum hema./30 m
Kumbar and Pancharatna, 2001(a)	10% formalin	5% nitric acid/ns	8 µm /r	Harris hema./ns
Kusano and others, 1995(a)	10% formalin	5% nitric acid/2-3 h	20 µm/f	Delafield;s hema./30 m
Kusrini and Alford, 2006(a)	4% formalin	10% formic acid/~24 h	10 µm/r	Mayer;s hema./ ns
Leclair, 1990(a)	(see Leclair and Castanet, 1987)			
Leclair and Castanet, 1987(a)	70% alcohol	3% Nitric acid/9 h	20 µm/f	Ehrlich's hema./25 m
Leskovar and others, 2006(a)	ns	ns	10-12 µm/r	Cresylviolet/ 5-10 m
Lu and others, 2006(a)	ns	ns	ns/r	Ehrlich's hema./ ns
Marnell, 1997(u)	70% alcohol	Rapid Decal/1 h	10-12 µm/r	Harris' hema./5 m

Marnell, 1998(u)	(see Marnell, 1997)			
Matson's Lab Manual (unpub)	10% formalin	Hydrochloric acid/48 h	14 µm/r	Harris' hema./10 m
Measey, 2001(a)	10% formalin	2.5% nitric acid/10 h	15 µm/r	Harris' hema./ ns
Meddeb and others, 2007(a)	20% formalin	5% nitric acid/48 h	20 µm/f	Ehrlich's hema./1.5 h
Miaud and others, 1999(a)	ns	ns	15 µm/ ns	Ehrlich's hema./ ns
Morrison and others, 2004(a)	FAACC/70% alcohol	10% nitric acid/24-48 h	10 µm/r	Ehrlich's hema./ ns
Olgun and others, 2001(u)	ns	3% nitric acid/4 h	14-16 µm/f	Ehrlich's hema./ ns
Patón and others, 1991(a)	ns	3% nitric acid/75-90 m	15-25 µm/f	Ehrlich's hema./45-60 m
Piantoni and others, 2006(l)	ns	5% nitric acid/5-17 h	13 µm/r	Masson's Trichromic/ ns
Redmer, 1994(a)	10% formalin/70% ethanol	Kristensen's formic acid/6-10 h	10 µm/r	Shandon instant regressive hema./ 15m
Rossell and Sheehan, 1998	10% formalin/70% ethanol	35 nitic acid/ns	5 µm/ns	Mayer's hema./15 m or Dip-Quick Stain
Rozenblut and Ogielska, 2005(a)	4% formalin/80% ethanol	1:1 10% formic acid and 4% formalin/2-4 h	12 µm/f	0.05% cresyl violet acetate/1-5 m
Russell and others, 1996(u)	10% formalin	3% nitric acid/5 d	9 µm/r	Gill's No. 2 hema./20 m
Sagor and others, 1998(a)	10% formalin	3% nitric acid/3 h	16-20 µm/f	Ehrlich's hema./22 m
Sinsch and others, 2007(a)	70% ethanol	ns	10 µm/r	0.05% cresyl violet acetate/1-5 m
Sinsch and others, 2001(a)	Bouin's solution	5% formic acid/1 h	10 µm/r	0.05% cresyl violet acetate/1-5 m
Tejedo and others, 1997(a)	70% ethanol	3% nitric acid/5 h	15 µm/f	Ehrlich's hema./15 m
Tessa and others, 2007	90% ethanol	5% nitric acid/2 h	12 µm/f	Ehrlich's hema./10 m

¹ a=anuran, u=urodel, l=lacertilia

² f=freeze microtome, r=rotary microtome (paraffin)

³ hema.=hematoxylin

⁴ ns= not specified

Appendix 2. List of Materials

Collection and Decalcification

1. Sharp clippers
2. Cassettes (for example, Yellow LabStorage Uni-Capsette™)
3. Permanent (hair-styling) end-papers (for example, Sally Beauty™ Jumbo End Wraps)
4. Alcohol-proof pen (for example, Statlab StatMark®) or pencil
5. Petri dishes
6. Stereoscope with optical micrometer
7. Forceps
8. Scalpel and spare blades
9. 95% ethanol in sealable jar
10. Decalcification solution (for example, Fisher Scientific Cal-Ex® II) in sealable jar
11. Beaker, 200-400 ml

Processing

1. 95% ethanol in sealable jar
2. 100% ethanol in sealable jar
3. Xylene or a substitute (for example, Thermo Scientific Shandon xylene substitute), two washes, in sealable jars
4. Embedding media (for example, Paraplast® Plus), two soaks, in oven proof dishes or jars
5. Paraffin dispenser with hot plate (for example, Leica EG1120)
6. Warming oven with precise temperature control
7. Forceps

Embedding

1. Forceps warmer with thermometer
2. Disposable base molds, 15 mm x 15 mm x 5 mm
3. Embedding media
4. Forceps, curved tip, 2-3 pair
5. Probes, 2-3 pair
6. 95% ethanol in squirt bottle
7. Paper towels
8. Barrier cream
9. Plastic bags-sealable

Facing and Sectioning

1. Microtome blades
2. Rotary microtome (for example, Leica RM2235)
3. Positively-charged slides (for example, Superfrost[®] plus), white
4. Forceps, two pair
5. Probes, two pair
6. Single edged razor blades
7. Spray bottle containing 1% concentration of glycerol in di-H₂O (diluted Downy[®] fabric softener also works)
8. Cold plate or tray (can be made with a glass dish fitted into an ice-filled Styrofoam dish)
9. Water bath with heater (for example, Leica HI1215)
10. Counter
11. Medium sized brush
12. Alcohol-proof pen or pencil
13. Slide grippers (for example, Peel-A-Way[®])
14. Kimwipes[®]

Staining and Mounting

1. Fume hood
2. Slide baths and racks
3. Xylene or a substitute
4. 70% ethanol
5. 95% ethanol
6. 100% ethanol
7. De-ionized water
8. Hematoxylin (for example, Harris')
9. Cover glass, 22 mm x 50 mm
10. Mountant (for example, Shandon Xylene Substitute mountant)
11. Probes
12. Slide storage boxes

Reading Sections

1. Compound microscope (with camera mounted and linked to a monitor, and computer with image software)

Appendix 3. Chemical Replacement Schedules

Collection and Decalcification

95% ethanol: Change every ~200 specimens

Decalcification solution: Change every 150 specimens

Processing

(if done in-house)

95% ethanol: Change every ~750 specimens

100% ethanol: Change every ~750 specimens

Xylene (or substitute): Change every ~750 specimens

Paraffin: Change every ~750 specimens

Staining and Mounting

Xylene (or substitute): Change every ~300 slides

Ethanol: Change all ethanol every ~300 slides

Hematoxylin: After ~300 slides, it should be filtered and changed with a mixture of 1:1 used/filtered and new.

Appendix 4. Definitions for the Sample Datasheet

ID Code: The code for this specimen. This is the same code that should be written on the cassette. There are various ways to build codes. The only real restrictions are that the code should be unique from all other samples and it must be short enough fit in the label space provided on the cassette.

Here is one way to build a unique ID code:

1. First, species identifier

RP=RAPR (Oregon spotted frog)
RL=RALU (Columbia spotted frog)
RS=RACAS (Cascades frog)
RA=RAAU (Red-legged frog)
RT=RACAT (Bullfrog)
BB=BUBO (Western toad)

When necessary, subspecies or regional population distinctions may be denoted by adding a third letter to the code. Just make sure abbreviations are clearly defined and used consistently.

2. Second, date

04APR07

3. Third, within day species number

If five RAAU are caught at the first site of the day, number them 1 through 5. If 7 RACAS are caught later in the day at another site, number them 1 through 7. If an additional 3 RAAU are caught at that second site, number them 6 through 8.

Examples:

RS13MAY0712 = The 12th Cascades frog sampled on May 13, 2007

RT03SEP0606 = The 6th Bullfrog sampled on September 3, 2006

Date Col: The date that the sample was collected.

Location: Location where the sample was collected. Coordinates are best but simple site name, refuge name, and (or) state can work.

Species: The species code of the frog or toad from which the sample was collected.

Use these codes:

RAPR (Oregon spotted frog)
RALU (Columbia spotted frog)
RACAS (Cascades frog)
RAAU (Red-legged frog)
RACAT (Bullfrog)
BUBO (Western toad)

Remember, additional letters may be added to the code to denote subspecies distinctions.

Sex: The gender of the frog or toad the specimen was collected from. Please do not guess.

Use these codes:

M = male
F = female

U = unknown

Stage: The best assessment of the developmental stage of the frog or toad. Most of the time all toes will be collected only from adults. However, it may be informative, depending on the objectives of the study and the population, to collect toes from juvenile or metamorphic individuals to establish when LAGs first form, resorption rates, etc.

Use these codes:

M = metamorph or a frog or toad that still has indications it metamorphosed recently, or is in the process.

J = juvenile or young of the year (transformed within the year captured)

A = any individual that looks to have transformed prior to the year in which it was captured.

SUL: The snout-to-urostyle length, measured in millimeters of the frog or toad. This is often measured with a flexible ruler over the dorsal side of the slightly flattened anuran from the tip of the snout to the base of the urostyle (long bone of fused vertebrae at the base of vertebral column ending just above the vent). Alternatively, snout-to-vent length may be recorded. There is some variation in measuring lengths of amphibians. Just make sure that whatever method of measurement that is chosen is standardized and maintained consistently throughout the study.

Mass: Measure the mass in grams of the frog or toad.

Notes: If known, record which toe was clipped. Also note if toes were counted from the inside or outside. For example RF4 would be the Right, Front outside (4_{in}) toe, if counting from the inside out looking down at the frog or toad from above.

Appendix 5. Definitions for the Sectioning Datasheet

ID Code: This is the code for this specimen. This code should be on the cassette front and match the ID code on the Collection Datasheet.

Second Phalange Length: Using the stereoscope (or dissecting scope) and the ocular micrometer, measure the length of the second phalange as precisely as possible, in millimeters. Multiply this value by 1,000 to get the length in micrometers or microns.

Cut Calculations:

No. μm : Multiply the length of the phalange by 0.49. This will indicate the location of the beginning of the region of the central diaphysis from which sections will be saved.

At 20 μm : Divide the number above (No. μm) by 20 to get the number of 20- μm thick cuts that need to be made to get to the location of interest.

No. cuts: Enter the number of 10 μm cuts that were made during the process of cutting sections for aging. The location on the phalange where cutting was stopped can be determined from this number..

Notes: Notes about how sectioning went, problems, quality of sections, etc.

ID Code	Second Phalange Length			Cut Calculations		Notes
	mm	μm	No. μm (49%)	At 20 μm = (number of cuts)	No. cuts (at 10 μm)	
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						
13						
14						
15						
16						
17						
18						
19						
20						
21						
22						
23						
24						
25						

Appendix 6. Processing by Contractor

NOTE: This is written based on the use of a local contracted histology lab. It serves only as an example.

Take a maximum of 50 cassettes to the front office of Oregon State University's Veterinary Diagnostics Lab (College of Veterinary Medicine, Room 134, Magruder Hall, 30th and Washington Way) in a sealed labeled container of 95 percent ethanol. Route the samples to the histology technician.

Also give the laboratory a glass container (oven proof) to put the cassettes in after they come out of the processor.

It takes about 24 hrs for the samples to be processed and be ready for pick-up.

When cassettes are picked up, make sure all billing paperwork is current at the front office.

When the infiltrated cassettes have been picked up and returned to the lab, place the container holding the cassettes in the oven at 58°C to re-melt the paraffin. Make all preparations to embed the phalanges.

Appendix 7. Definitions for the Aging Datasheet

ID Code: This is the code for this specimen. This code should be on the slide and should match the ID code on the Collection and Sectioning Datasheets.

No. LAGs: The number of Lines of Arrested Growth (LAG) counted from the section(s). Record the high and low estimates of LAGs as well.

Notes: Any notes about the sections, quality of staining, problems reading, evidence of resorbed LAGs, etc.

ID Code

No. LAGs

Notes

1		
2		
3		
4		
5		
6		
7		
8		
9		
10		
11		
12		
13		
14		
15		
16		
17		
18		
19		
20		
21		
22		
23		
24		
25		

This page left intentionally blank