GENERAL FISHERIES COMMISSION FOR THE MEDITERRANEAN

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STUDIES AND REVIEWS

AGE DETERMINATION OF ELASMOBRANCHS WITH SPECIAL REFERENCE TO MEDITERRANEAN SPECIES: A TECHNICAL MANUAL

STUDIES AND REVIEWS   **No. 94**

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AGE DETERMINATION OF ELASMOBRANCHS, WITH SPECIAL REFERENCE TO MEDITERRANEAN SPECIES: A TECHNICAL MANUAL

by

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FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS

Rome, 2013

PREPARATION OF THIS DOCUMENT

This document stems from the Workshop on age determination of elasmobranchs in the GFCM area, which was held at the Mediterranean Fisheries Research Production and Training Institute in Antalya (Turkey), from 8 to 12 October 2012, funded by the European Union and organized by the General Fisheries Commission for the Mediterranean (GFCM) of the Food and Agriculture Organization (FAO) of the United Nations. This hands-on workshop with an important training component was attended by participants from the GFCM member countries and from non-member countries. The purpose of this document is to provide a detailed and hands-on guide to successful and validated methods for ageing sharks, skates and rays of the Mediterranean.

ABSTRACT

Age information forms the basis for calculations of growth rate, mortality rate and productivity, ranking it among the most influential of biological variables. In the case of bony fishes, age determination methods based on annual growth marks are reasonably well established and are applied millions of times per year. However, age determination methods in elasmobranchs have lagged behind those applied to teleosts. In this manual, the best of the age determination methods for elasmobranchs have been brought together so as to provide a detailed and hands-on guide to successful and validated methods for ageing sharks, skates and rays. Particular emphasis is given to vertebral sections and image analysis techniques as applied to elasmobranch species from the Mediterranean Sea, and is suited to both novice and experienced age readers.

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FIGURES AND TABLES

1.   INTRODUCTION

Age information forms the basis for calculations of growth rate, mortality rate and productivity, ranking it among the most influential of biological variables. Calculations as simple as growth rate, or as complex as virtual population analysis, all require age data, since any rate calculation requires an age or elapsed time term. In the case of fishes, age can be estimated using radiochemical decay rates, tag-recapture studies and other methods, but it is usually periodic growth increments which are counted to estimate the age. Annual growth increments are found in otoliths, scales, fin rays and spines, vertebrae and bones, although it is the otolith which is applied over the broadest age range in many species (Campana 2001). In elasmobranchs, otoliths are absent and scales do not form discernable growth bands. Therefore, it is usually the vertebrae (or the fin spine, when present) which must be prepared for age determination.

Age determination in elasmobranchs is often more challenging than it is in teleosts, in part because of the absence of otoliths in the former, and in part because the number of teleosts that are aged annually is hundreds of times larger than is the case for elasmobranchs (Campana and Thorrold 2001). As a result, the implementation of modern age determination methods for elasmobranchs has tended to lag well behind that of teleosts. Hundreds of papers have been published on elasmobranch age, including methods for interpreting spines (Ketchen 1975; Irvine et al. 2006), whole vertebrae (MacNeil and Campana 2002), sectioned vertebrae (Natanson et al. 2002), neural arches (McFarlane et al. 2002), vertebral stains (Hoenig and Brown 1988), X-rays (Wintner 2000) and caudal thorns (Gallagher et al. 2006). Some of these methods, and a reasonably large proportion of the published applications, are now considered obsolete. A comprehensive summary of more modern ageing methods for elasmobranchs has not yet been published, although the paper by Cailliet and Goldman (2004) is the best attempt to date.

In this manual, I try to bring together the best of the ageing methods for elasmobranchs, drawing from the extensive literature on teleost ageing as well as the experience of our own laboratory (and that of others) with a long history of successful and validated elasmobranch age determinations. This manual is very much a technical manual, focused on hands-on techniques and relying heavily on modern image analysis methods. Many of the examples come from skates, rays and sharks in the Mediterranean Sea, since these were the target species in the *Training Workshop on Age Reading of Elasmobranchs in the GFCM Area* held in Antalya, Turkey in October 2012. This workshop was funded by the European Union and organized by the General Fisheries Commission for the Mediterranean (GFCM) of the Food and Agriculture Organization (FAO) of the United Nations. The meeting was attended by 20 participants from 9 Mediterranean countries and lasted one week.

2.   EMBEDDING AND SECTIONING OF VERTEBRAE

Vertebrae for age determination are usually removed from the elasmobranch from the cervical or thoracic regions, although the optimal location depends on the species. For many species, the area over the the posterior gill slit is often best. Excess tissue is cut away, then boiled or bleached to clean the vertebra, the neural and/or haemal arches and transverse processes are removed, and the vertebra is left to air dry before sectioning or embedding.

Although there are some young, fast-growing elasmobranch species for which the vertebrae can be aged intact (see chapter *Whole Vertebrae*), most vertebrae will be aged most accurately by first preparing a thin section through the centre of the centrum. Vertebral sections are particularly important to reveal growth bands near the distal growing edge in older elasmobranchs. Vertebrae with a centrum diameter of more than about 1 cm can often be sectioned directly with no embedding, but smaller vertebrae will need to be embedded in a rigid epoxy. Very small vertebrae (<1 mm diameter) can be mounted on a microscope slide with cyanoacrylate glue or epoxy and then sanded down to the midplane (McPhie and Campana 2009). It is also possible to use mass production methods to embed and section hundreds of vertebrae per day (McPhie and Campana 2009).
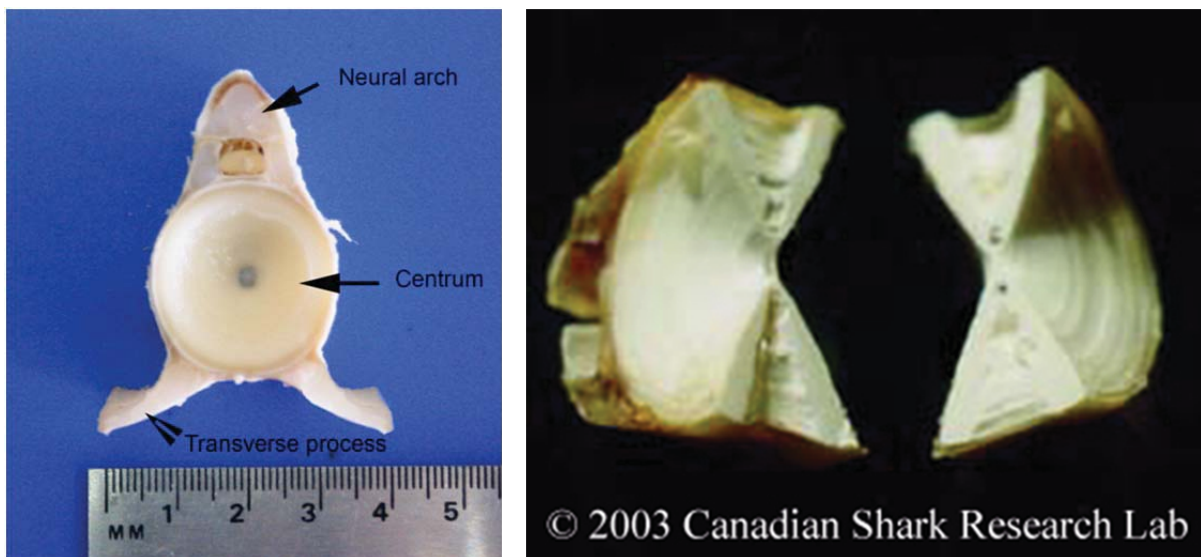
Figure 1. (Left) Intact vertebra and (Right) vertebra sectioned longitudinally to reveal the "bowtie" perspective. Ageing would use a thin longitudinal section rather than just cutting the vertebra in half.

2.1.   Embedding in epoxy

For precise sectioning with an Isomet saw, it is best to embed the vertebra in a hard epoxy. We use a two-stage liquid epoxy such as Araldite epoxy GY502 and hardener HY956 in a 5:1 weight ratio (available from Brenntag Canada Inc.). We also use Buehler Epo Thin resin (catalog # 20-8140-032) and Buehler Epo Thin hardener (catalog # 20-8142-016) (Buehler

catalog) in a 5:2 weight ratio. However, other epoxies and setting compounds, as long as they are transparent and very hard, will work.

To prepare the epoxy, add the epoxy and hardener together in a disposable plastic cup on a tared top-loading balance. 20 g Araldite epoxy and 4 g hardener will stay soft for about 10 mins, which is enough time to embed about 12 samples. Stir for a minimum of 3 mins. Unlike otoliths, sonification is not usually necessary. If there are only a few samples to embed, let the epoxy sit for 5-15 mins to set a little. All work should be done under a fume hood.

Many people use a 12-well silicone EPDM (silcone combined with a synthetic rubber) mold, 19 x 11 cm, but the number of wells and size are optional. Individual wells are roughly 4 x 2 x 0.5 cm and hold up to 4 ml of epoxy. It is sometimes helpful to prepare molds by first applying, with a small brush to each well, a light coat of release agent such as Frekote (Henkel/Loctite) and allow to dry. It is best to apply the release agent immediately following the last use of the mold. However, release agents are not necessary for all mold types.

Pour enough epoxy into the mold so that the vertebra is completely (but minimally) covered. Label a small piece of paper with the sample ID and stick the label into the narrow end of the epoxy pool. Push the vertebra into the epoxy, centrum face up, then use forceps to rotate the vertebra so that all faces are coated. Use a fine probe to remove all bubbles from around the sample, particularly those trapped at the center of the centrum. Push the vertebra to the bottom, making sure there are no bubbles. The vertebra may float up to the surface, but as long as it stays flat, that is not a problem. Allow to set 2-3 days before sectioning. Use acetone to remove spilled liquid epoxy.
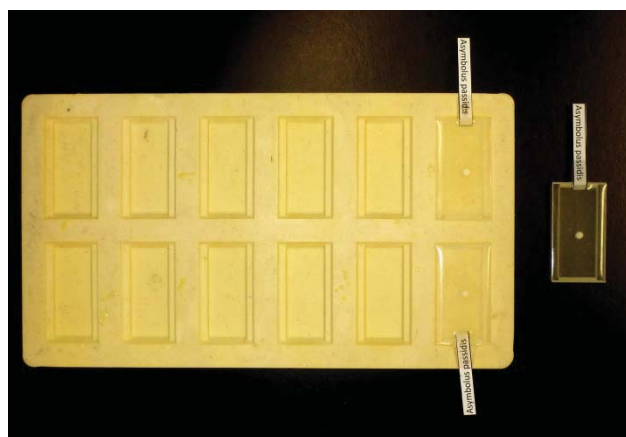
Figure 2. (Left) Mold for embedding multiple vertebrae at a time; (Right) example of embedded vertebra.

2.2. Sectioning

An Isomet low-speed diamond bladed saw is used for preparing vertebral sections, but any equivalent saw can be used. By fitting the saw with two blades separated by a spacer, a thin section can be prepared from an epoxy-embedded sample with a single cut in about 2 minutes. Single blades can also be used, but there can sometimes be more problems with the second cut breaking the section.

The epoxy-embedded sample is clamped perpendicular to the saw blade with a V-notch clamp, whereby the sample is positioned on the lower surface of the epoxy block. It is important to line the projected cut line as close to the centre of the vertebra as possible. It can be helpful to score the embedding medium with a straight edge directly over the area to be cut. The water tray for the saw is filled with 60% glycerin in water to a level where it just touches the tip of the blade. We use 4" blades between 3" flanges; with only 0.5" of free blade exposed, blade wobble is minimized. However, there must be enough free blade exposed to cut completely through the epoxy or sample. Where two blades are to be used, we separate them by a thin metal or plastic spacer with a diameter of 3" and a thickness appropriate to the section being prepared. Typically, a 800 μm spacer will be used for larger vertebrae, producing a 700-800 μm sample thin section. Sections of 500 μm thickness are more appropriate for small vertebrae (<7 mm in diameter). The above are only guidelines; different-sized saw blades and flanges can be used.

The saw can be run relatively quickly (which is still slowly) with up to 100 g of weight on top until the blade is almost through the epoxy. It is preferable to stop the saw just before one side of the epoxy block falls off. If the sections appear to be breaking while cutting, reduce the top weight. Remove the block from the clamp and use fingers or forceps to break off the section (which is probably attached to the block by very little). Rinse the thin section in 95% ethanol, then use a scalpel to cut off the broken block attachment point.

Figure 3. Preparing a section of a vertebra with an Isomet saw.

The section is then ready for imaging. Polishing is not usually necessary, but where done, should be to remove coarse scratches only. Sections can be stored dry, as is, or mounted onto a microscope slide with a mounting medium such as cyanocrylate glue (Krazy Glue), thermal-setting plastic (Crystalbond), or epoxy.

3.   IMAGING AND IMAGE ENHANCEMENT

Due to huge advances in digital imaging, direct microscopic examination of vertebral sections is seldom as effective nowadays as is the use of imaged and enhanced digital images. An exception might be where whole vertebrae or spines, with pronounced three-dimensional structure, makes focusing on a single plane difficult. However, with all vertebral sections, and even with many whole vertebrae and spines, imaging is usually the preferred approach. Imaging is usually done under a dissecting microscope fitted with a digital camera. However, sections which are too large for a microscope can also be photographed with a point-and-shoot digital camera mounted on a camera stand directly above the sample. The imaging camera can be either colour or greyscale, although colour images are seldom preferable. Cameras with a minimum greyscale resolution of 1280 x 1024 pixels are usually sufficient, but higher resolution may sometimes help. More important than resolution however, is the imaging depth of the camera. An 8-bit camera will provide some potential for image enhancement (as discussed later), but a 10-bit or higher camera will provide huge advances in image enhancement possibilities.

There are several key steps to imaging vertebral sections:

1) **Always image wet!** Sections with a dried surface are invariably more difficult to interpret than are sections coated with, or preferably, immersed in a thin layer of ethanol or water. Oil can also be used for immersion, although oil tends to irreversibly clear a sample after some weeks.

2) **Use diffuse lighting from a double-armed fibre optic light source for optimal illumination**. Lighting should not be so bright as to "wash out" parts of a sample; that is, if parts of the sample become pure white in the digital image, no enhancement will be possible for that part of the image. Lighting coming in from either side at about 30 degrees from the horizontal is often best, but different combinations should be explored. Always be sure to eliminate surface glare. In some cases, transmitted light will work best, but only if the section is sufficiently thin. There are relatively few instances where transmitted light will out-perform reflected light, but to some extent, this is a personal preference.

3) **Image the section from both sides.** It can be challenging to section a small vertebra so that the centre is perfectly sandwiched between the twin saw blades. Usually however, one side or the other provides a better view of the growth bands. By imaging both sides, it is easier to decide which view of the section should be used for age determination.

4) **If at all possible, include a ruler scale in the image during photography**. This will make any subsequent measurements much easier.

5) **If at all possible, use the same microscope magnification for all images of the same species**. This will make it easier to interpret the age of young elasmobranchs, since the many false checks that are present in young, fast-growing fish can be confusing if enlarged to the same size as that of an older individual.

The product of the imaging will be a digital image, usually in .tif or .jpg format, which can then be digitally enhanced on the computer screen prior to age determination.

Growth bands in vertebrae are seldom as clear as those in otoliths or bivalves. For that reason, dyes and stains were often used in the past to enhance the vertebral growth bands. However, virtually all modern examinations of vertebrae and other ageing structures now take advantage of computer-assisted microscopic imaging techniques, or image analysis. Image analysis systems allow for image enhancement, manipulation, storage and quantification with an accuracy and speed that cannot be matched with the eyes or a microscope alone. Perhaps more importantly, simple and fast image enhancement techniques can produce images of vertebrae which are as clear, or more often clearer, than any stain or dye.
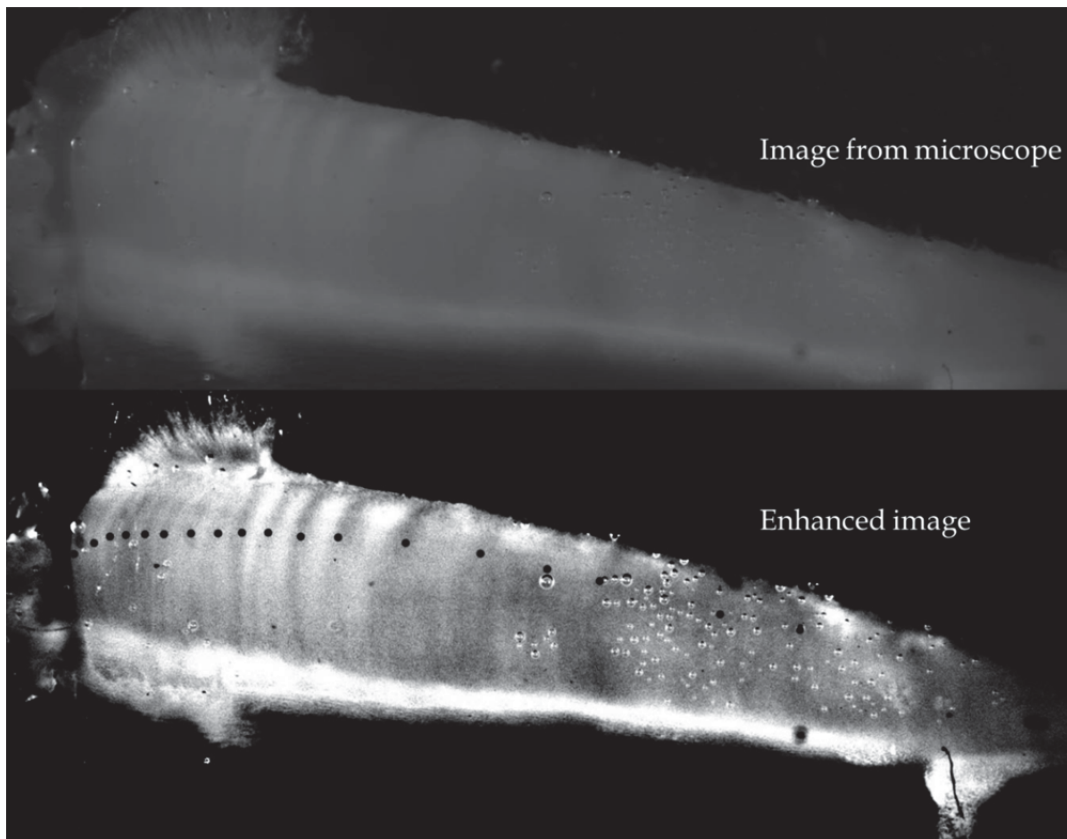
Figure 4. An example of a portion of a vertebral section as originally captured by the camera (Top) and after 30 seconds of enhancement in Adobe Photoshop (Bottom).

Most image analysis systems allow for image enhancement at the time of image capture. However, we have found that image capture with the camera or image analysis software, followed by image enhancement using Adobe Photoshop provides the greatest flexibility and power. What follows are a few easy steps that can be applied to any image to enhance its clarity.

Colour does not usually help, and may hinder, age determination from images. If the camera is of high resolution (> 3000 x 2000 pixels) or if it has a 10-bit or higher colour depth, colour will not degrade image resolution. However, if the camera is of lower resolution, or
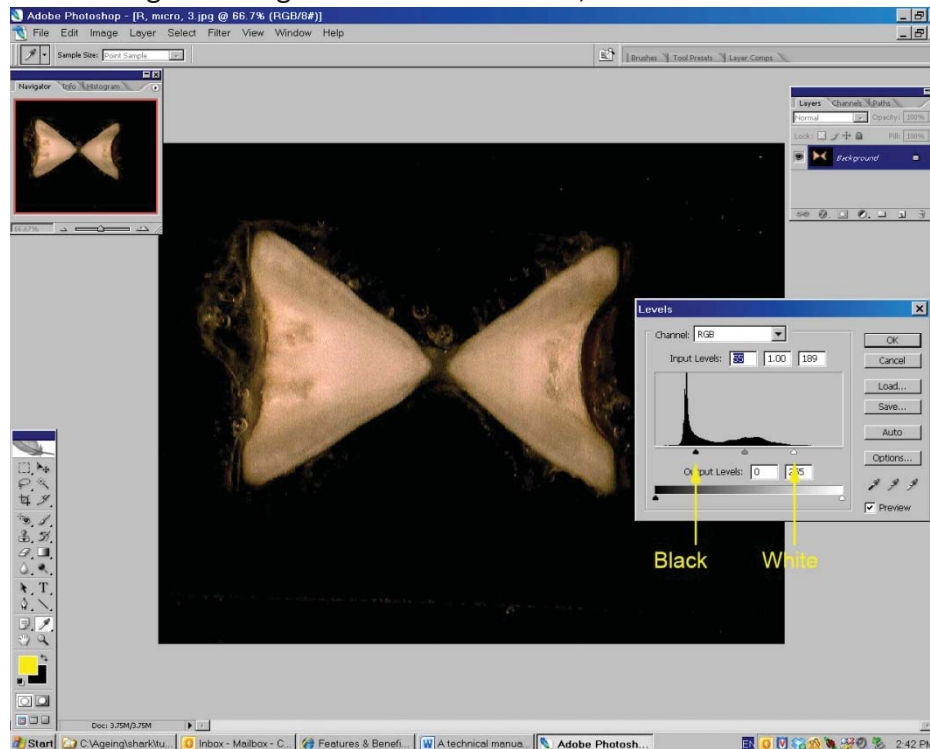
particularly if it is an 8-bit camera, a grey-scale image will provide greater enhancement capabilities. It is best if the camera is set to grey-scale before the image is captured. If it is not however, use Photoshop to change to grey-scale

Figure 5. Photoshop screen for adjusting grey levels. The black and white set points (arrowed) are moved as described below.

To change an image from colour to grayscale:
- Image-Mode-Grayscale

To show the windows that you'll need for image enhancement:
- Window-Navigator (to move around and zoom in on an image)

There are two key steps that are almost always helpful in enhancing an image. The first is the adjustment of grey levels, which effectively spreads the light levels in the image across the entire possible range (256 grey levels in an 8-bit camera; 1024 grey levels in a 10-bit camera). Adjusting grey levels is not the same as adjusting contrast; the former is much more powerful.

To adjust the grey levels:
- Image-Adjustment-Levels
- Move the white setpoint arrow to the left until it is at the right-most base of the histogram, or just before any point on the vertebra (in an area which might be examined for ageing) becomes pure white
- Move black setpoint arrow to the right as much as possible, but not to the point where the edge of the corpus calcareum (see Fig. 6) begins to disappear
- Move central arrow to point where image is pleasing to eye; usually it is safe to leave it where it is

- Try unchecking the Preview box on the Levels popup window to ensure that you haven't lost any crucial detail; then recheck to turn back on
- select OK

The second step of most (but not all) enhancements is to apply an Unsharp Mask. The unsharp mask is perhaps the most powerful edge enhancement filter available for general use. Its use may not be necessary if the image is sufficiently clear after the grey level adjustment. But if it's not, the Unsharp Mask can be helpful.

To apply the Unsharp Mask:
- Filter-Sharpen-Unsharp Mask
- Leave Threshold between 0 and 2
- Move Radius to between 10-30
- Move Amount to 100-300%, or until it suits taste
- Try unchecking the Preview box on the Unsharp Mask popup window to ensure that you haven't lost any crucial detail; then recheck to turn back on

Layers in Photoshop are like transparent plastic overlays for your image; you can annotate or mark them anyway you want, without affecting the underlying image. They are ideal for comparing age interpretations with another reader, since you can mark annuli on a layer, and then hide it so that another reader can mark the same image on their own layer. Both layers can subsequently be unhidden to compare the growth band annotations.

To add a layer which can be annotated for training or quality control:
- Window-Layers (to see the layers that you'll use for annotation)
- Layer-New layer; give it any title or your name (or none at all)
- Insure that layer is selected (will appear highlighted in layers window); if the layer is not highlighted, the annotation will not take place on the layer
- To annotate the growth bands, select a brush tool on the tool popup window; change the size using Windows-Brushes, or click on the brush tool at top left; a brush hardness of 100% is best
- To change the brush colour, double-click on the colour panels in the left-hand menu bar; note that a coloured brush cannot be used on a grayscale image, unless the image is first changed to RGB mode
- Use the brush to mark the growth bands; use Text tool (T) to add text to layer
- To hide a layer during testing, click on the eye in the layer popup window to make it disappear; click on the eye box again to make it reappear
- Layers are collapsed (not kept separate) if the final image file is saved as a .jpg or other format. To save the ability to hide or show a layer, one must save the image files as a Photoshop (.psd) file.

In most cases, the above steps will enhance the image as much as can be done, allowing for the number of grey levels and resolution of the raw image. In some cases however, more aggressive steps might need to be taken. Try these steps:

Isolate your sample from the background and enhance:

- Click on the Magic Wand tool on the floating tool bar; click on the background to select it; the selection doesn't need to be perfect, just enough to exclude most of the background and none of the vertebra
- Select-Inverse to reverse the selection and select the vertebra
- Image-Adjustment-Auto Levels

If you're not satisfied with the Auto Levels, Edit-Undo to undo the auto levels. Then, use the Lasso tool from the floating toolbar to draw a rough shape within the bounds of your vertebra. Make sure you don't include any of the background, but it doesn't need to include more than half of your vertebra. Then:

- Image-Adjustments-Equalize; on the following popup window, select either one of the options for where it is applied

The Equalize adjustment is the most aggressive enhancement possible. It's usually too strong. But if no growth bands are visible with it on your within-vertebra selection, there's nothing there.

Measurements are best made at the time of imaging with the calibrated measurement tools available in most imaging software. Measurements from the image can also be made in Photoshop using the Ruler tool (access by right clicking the Eyedropper tool). However, Photoshop measurements are made in terms of pixels, not SI units. To convert pixel measurements to more useful units, one would need to measure something else in the same image that is of known dimensions (eg- a ruler scale that lay beside the sample when being photographed).

4. INTERPRETATION OF VERTEBRAL SECTIONS

Age interpretation of vertebral sections is an acquired skill. Although some people seem to have more aptitude with it than others, it is definitely a skill that improves with practice and experience. As a rule of thumb, a novice elasmobranch age reader should plan to age at least 100-200 vertebral sections before even beginning to use the age data in any application.

Every species has its own characteristic growth pattern. Even experienced agers will interpret 20 or more images to develop a "search image" for that species before beginning the recording of the age data. Fish length should <u>not</u> be available to the age reader while ageing; it tends to guide the age reader into expected ages, which is not desirable. However, knowledge of the month or season of collection is useful, since it can aid in interpretation of the growing margin.
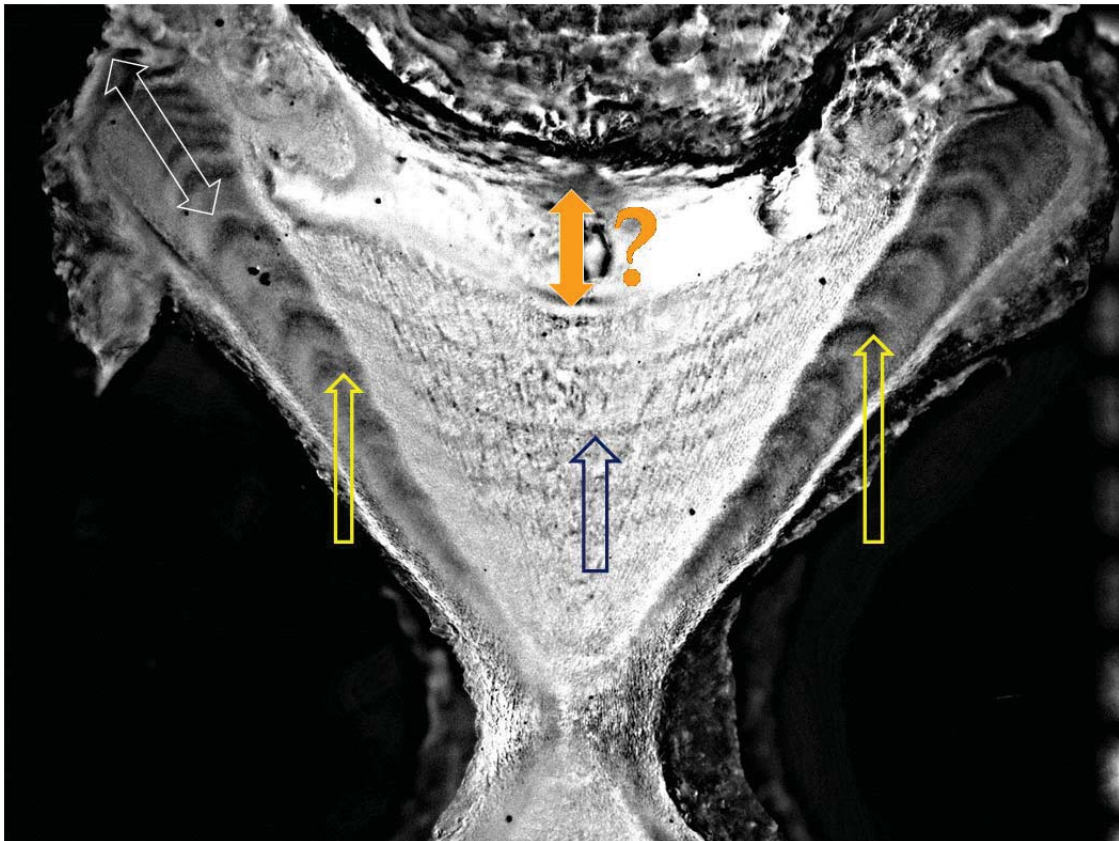

Figure 6. Age interpretations should be made along the corpus calcareum region (yellow arrows and blue arrow in upper left), not the centrum (orange arrow). However, the innermost growth bands in the centrum (blue arrow) can be useful in interpreting the first few annuli.

Many environmental and physiological factors can produce growth marks; the trick is to identify the annual growth marks. Here are some tips for interpreting vertebral sections:

- Age along the corpus calcareum region (near the lateral edge) of the section, particularly in older elasmobranchs. Growth bands may also be visible in the centrum, but use them only for the innermost few annuli; they become increasingly unreliable as age increases.
- Seek out clear or unambiguous areas to develop a "search image" for the section, then use the appearance of the growth bands in this region to help you interpret the remainder of the section (eg- the upper part of the corpus calcareum above the yellow arrow on the left side of Fig 6)
- Continuity in contrast and spacing of the growth band is the rule, not the exception. Use that principle as a guide to differentiating annuli from false checks. True annuli will tend to be somewhat similar in appearance and width to those around them. Very narrow (<50% of the width of the adjacent bands) or faint growth bands usually typify false checks, not annuli, since elasmobranch annual growth will seldom differ that much from year to year
- Higher magnification will almost always reveal more growth markings. If possible, keep the image magnification constant between images, and zoomed out enough to see the entire half of the "bowtie" section. However, zooming in on narrow annuli formed after sexual maturity is often necessary
- In some species, the first few (innermost) annuli tend to be split into multiple false checks, making the interpretation of the annulus challenging. In contrast, annuli usually get narrower and more regular after sexual maturity, making them easier to interpret
- Each intact section contains four possible growth axes along the corpus calcareum. Where possible, each growth axis should be aged. However, use the growth axes that gives you the most confidence in assigning a final age
- During the training stage, access to reliable, annotated images can be invaluable in developing a search image. Having just a single section from a known-age individual can make a huge difference in age interpretation accuracy.
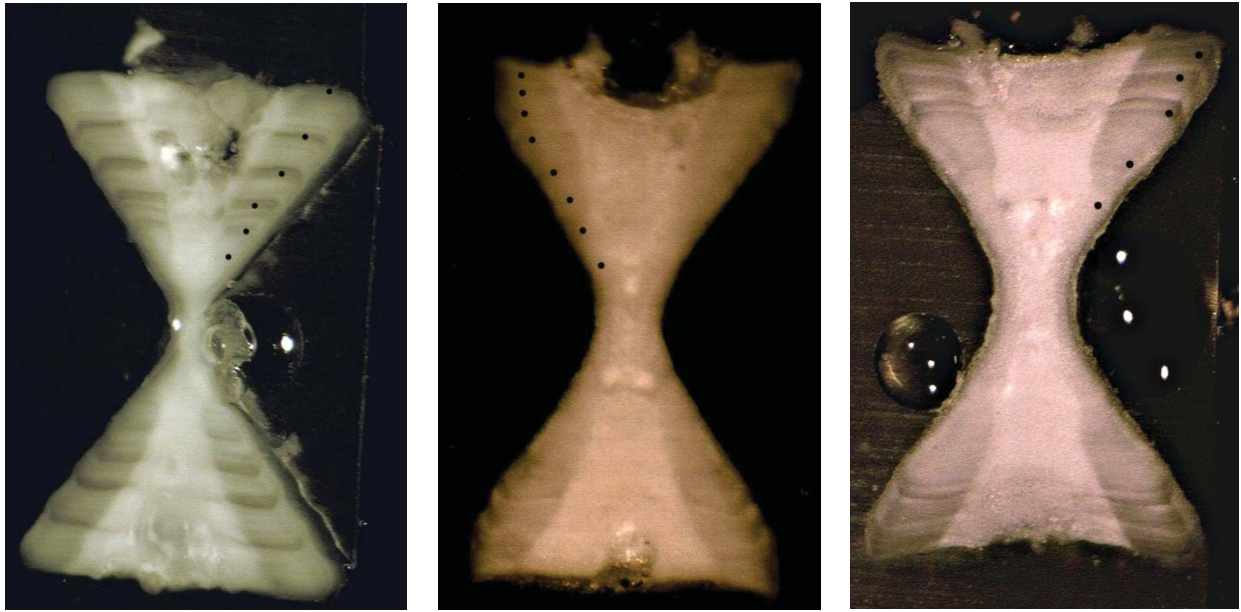
Fig. 7. Enhanced vertebral images of *Dasyatis pastinaca*, annotated to show annual growth bands.
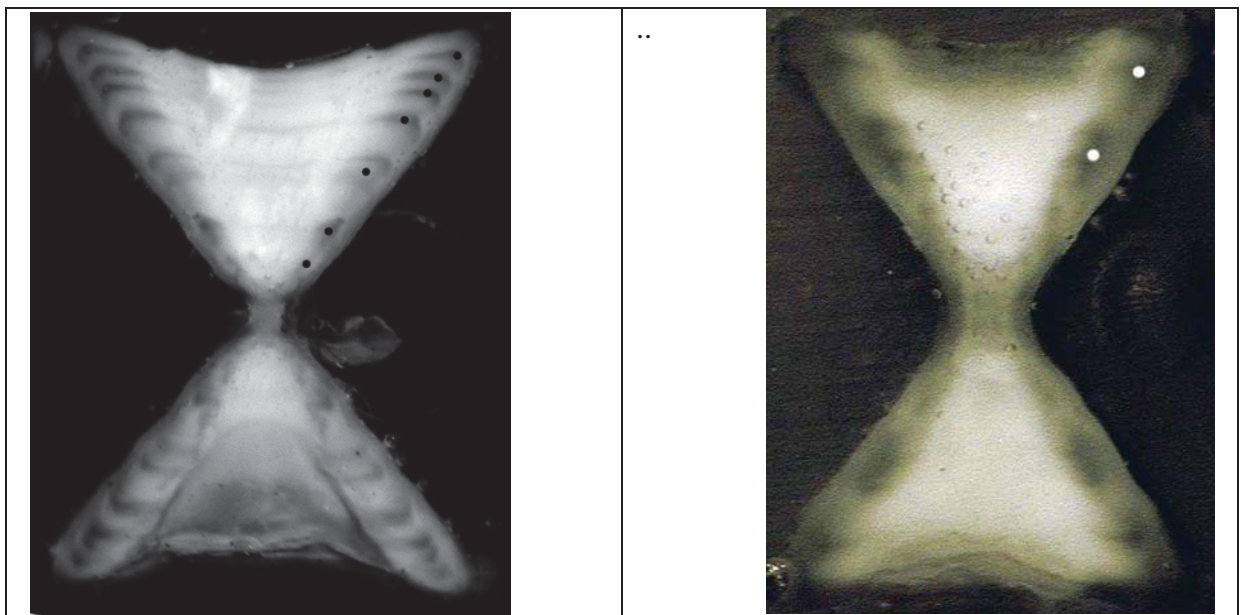
Fig. 8. Enhanced vertebral images of *Leucoraja ocellata*, annotated to show annual growth bands.
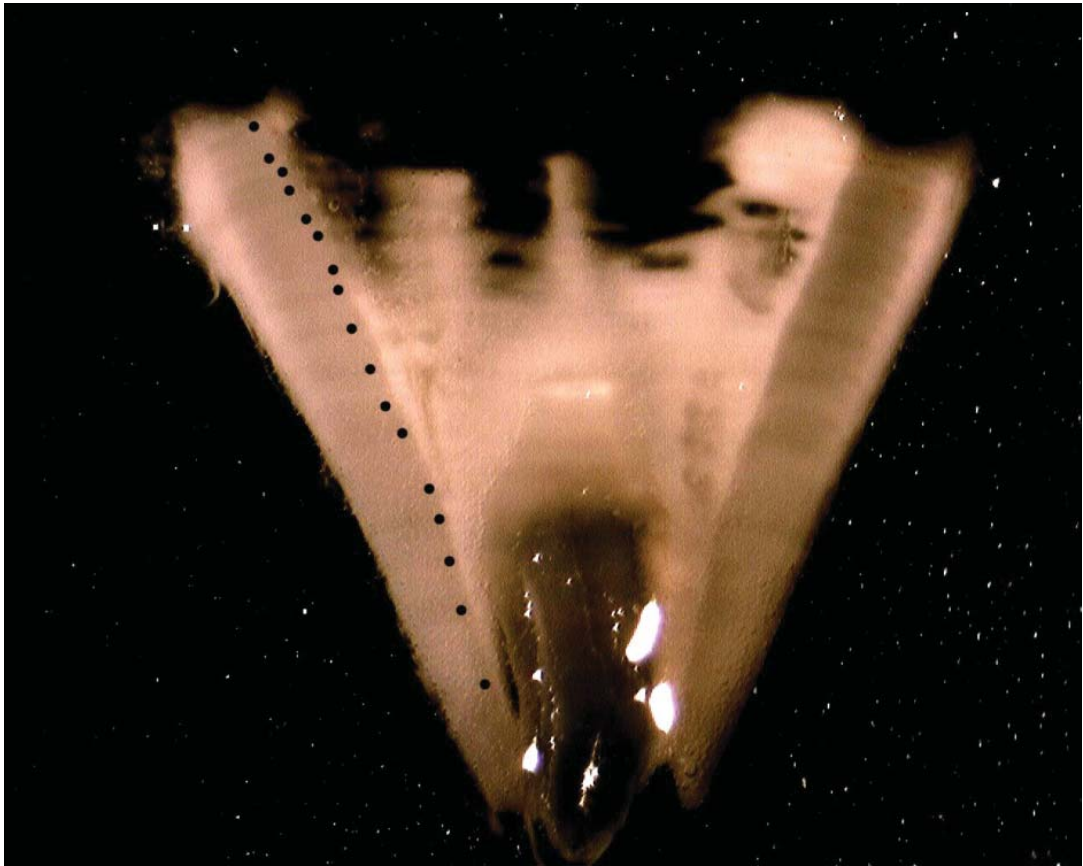
Fig. 9. Enhanced vertebral images of *Lamna nasus*, annotated to show annual growth bands.
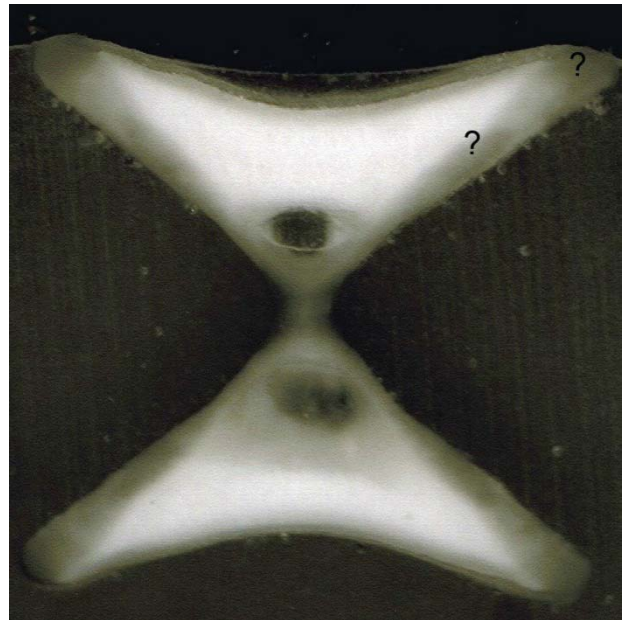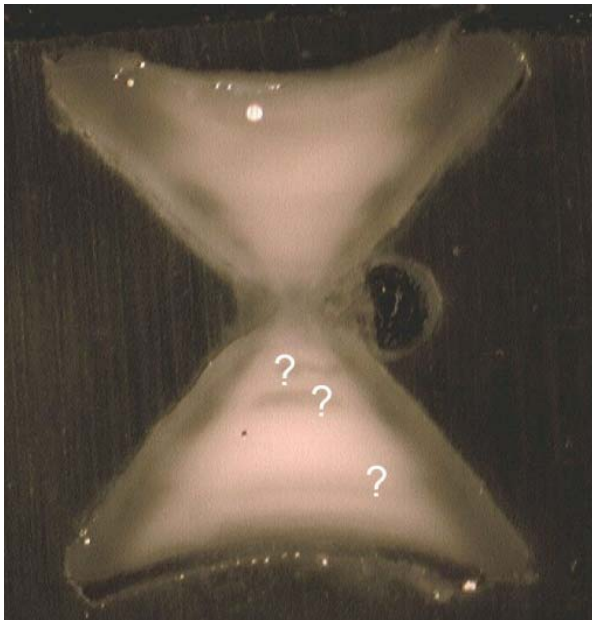
Fig. 10. Enhanced vertebral images of *Mustellus mustellus*, annotated to show what may (or may not be) annual growth bands
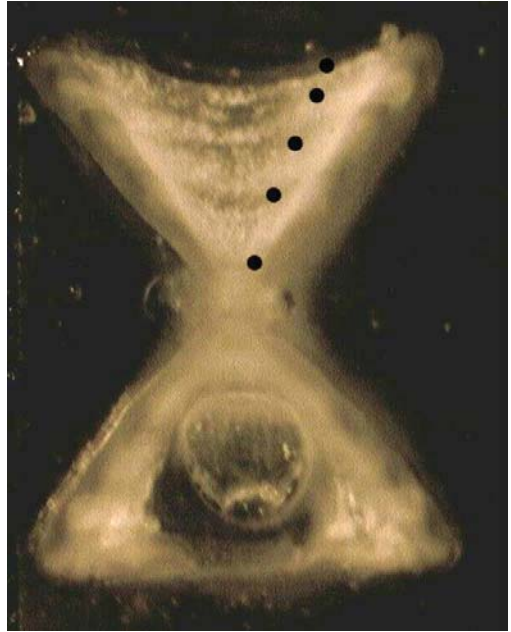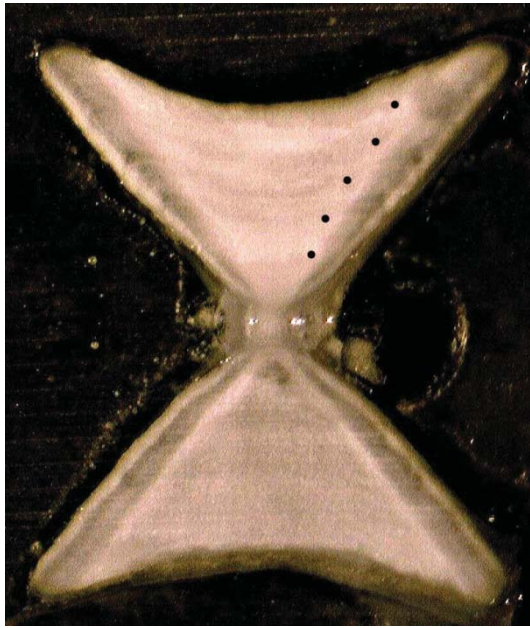
Fig. 11. Enhanced vertebral images of *Raja clavata*, annotated to show annual growth bands.
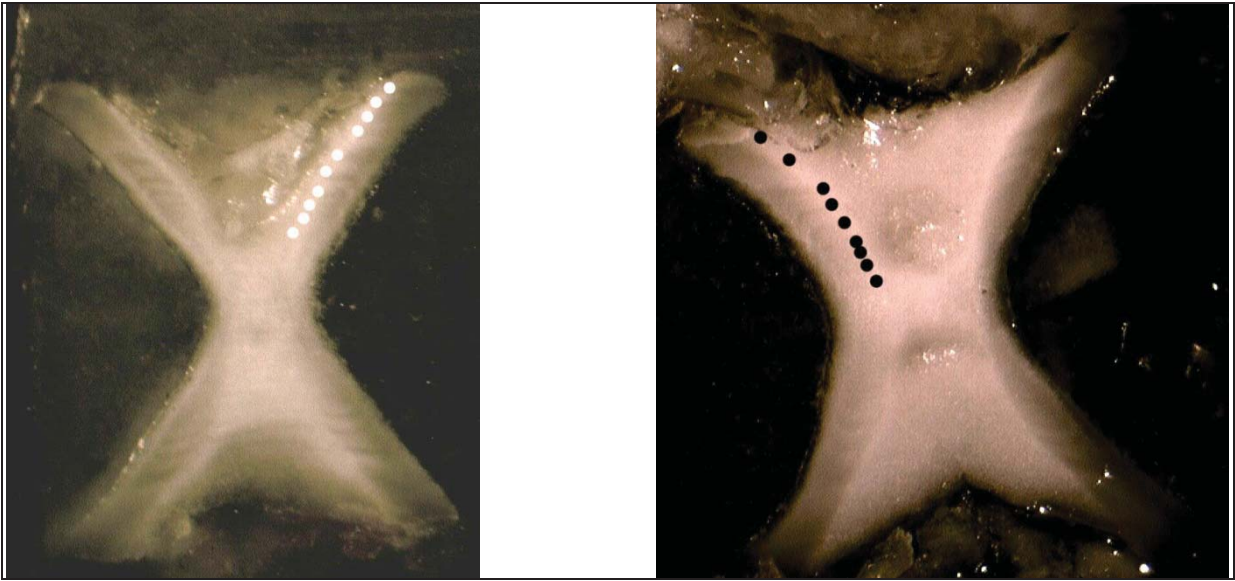
Fig. 12. Enhanced vertebral images of *Raja asterias*, annotated to show annual growth bands.
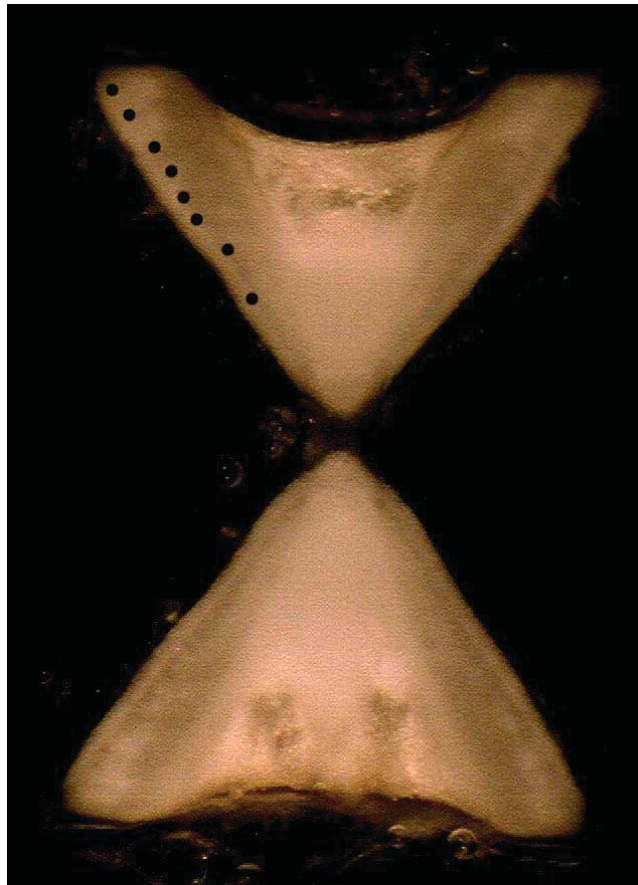
Fig. 13. Enhanced vertebral images of *Raja miraletus*,
annotated to show annual growth bands.
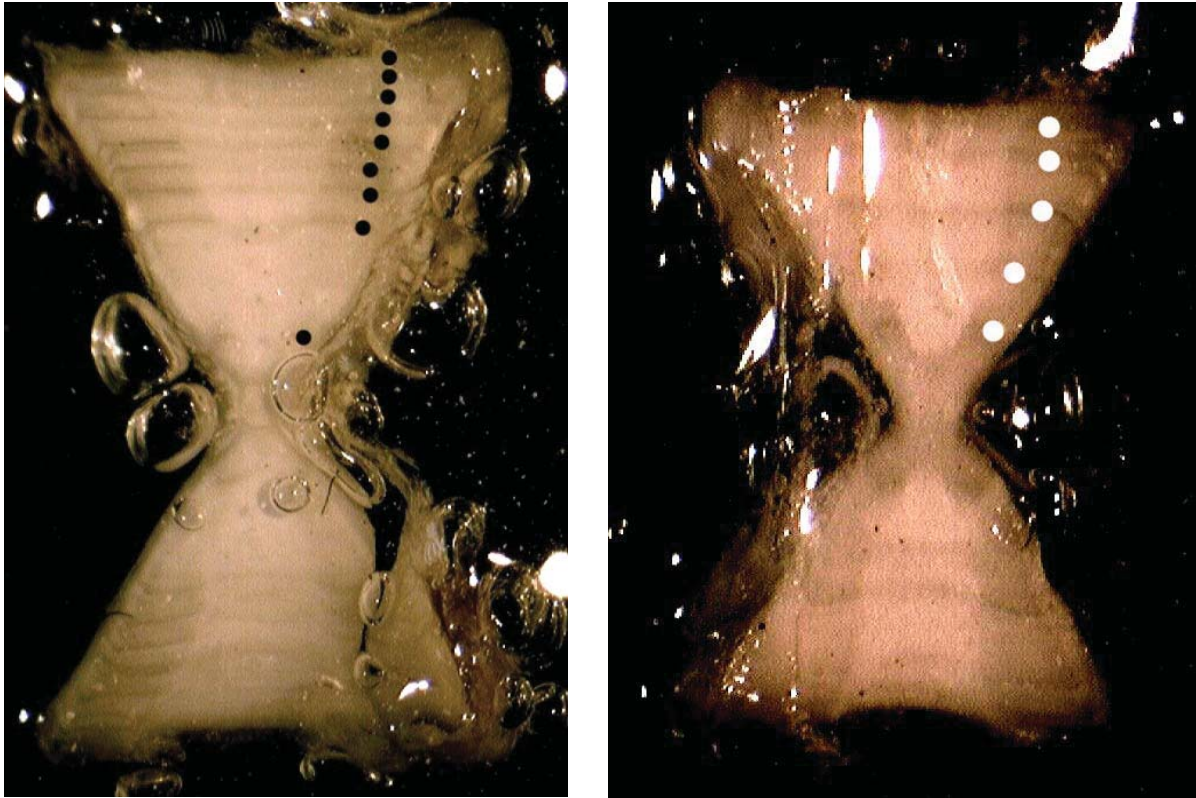
Fig. 14. Enhanced vertebral images of *Rhinoptera marginata*, annotated to show annual growth bands

5. DETERMINING THE LOCATION OF THE FIRST GROWTH BAND

Accurate identification of the first, or innermost, growth band is an important component of any age determination study; without a correctly defined starting point, age determinations will be consistently wrong by a constant amount. For example, uncertainty over the identification of the first vertebral annulus in porbeagle shark (*Lamna nasus*) in two independent studies resulted in size at age estimates which consistently differed by one year (Francis & Stevens 2000; Natanson et al. 2002).

As a general rule, the first (innermost) prominent growth band that is visible is also the growth band formed during, or at the end of, the first year of life. In some species however, a "birth band" is also visible, with a presumed time of formation at or before birth (Fig. 15). Birth bands are not counted when assigning an age. Birth bands often appear differently than the subsequent annual growth bands, in that the birth bands may either be sharply delineated and very narrow, or very vague and diffuse. If a birth band is visible, the subsequent (more distal) prominent growth band that is visible is usually the first annulus.
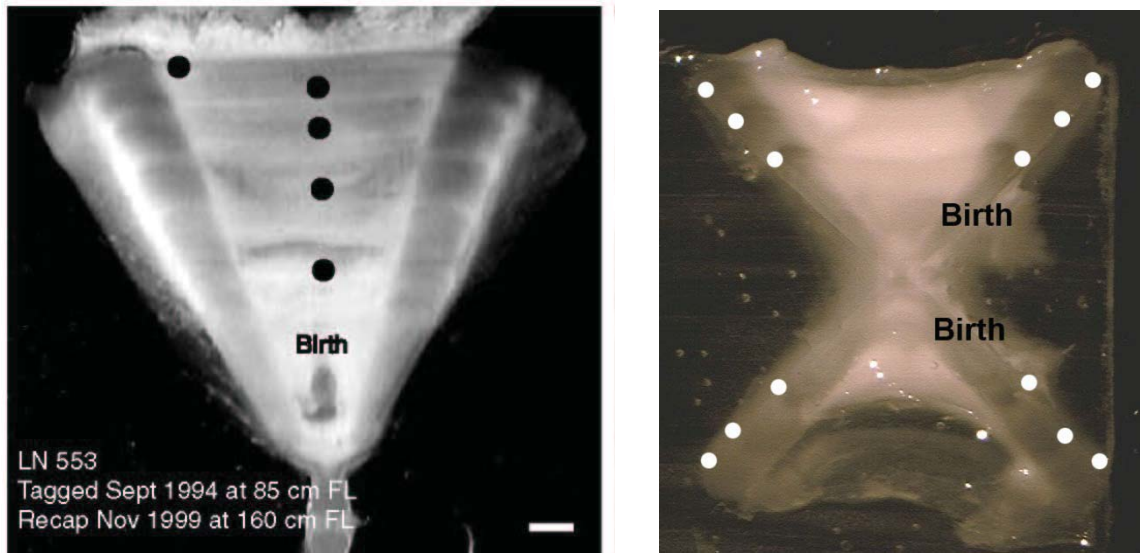
Figure 15.: Presumed birth bands in a 5-yr old *Lamna nasus* (left image) and in a 3-yr old *Raja clavata* (right image), with the annual growth bands being indicated by filled symbols.

There may be some elasmobranch species in which the birth band is associated with a change in angle of the corpus calcareum; that is, the edge of the sectioned vertebra appears to curve slightly at the birth band, becoming relatively straight afterwards. Although some species seem to show this property (Cailliet and Goldman 2004), it does not appear to be present in most species.

There is no definitive method to confirm the identity of the first growth band in an individual elasmobranch which is more than 1-yr old. However, the easiest method to determine the approximate location of the first growth band is to collect young-of-the-year individuals in the season in which the first band is being formed, prepare a vertebral section in the same manner as would be done with an adult, and then to measure the distance from the "waist" of the section (the central part of the bowtie) to the distal edge of the corpus calcareum (Fig. 16). This distance corresponds to the radius of the first growth band. If this same measurement is repeated on a number of young-of-the-year individuals, the mean measurement then becomes the expected mean radius of the first growth band. It is then possible to superimpose this measurement on a vertebral section of an older individual, beginning at the waist of the section, to determine the expected location of the first growth band. Clearly, this approach cannot adjust for individual variability in the location of the first growth band in the older elasmobranchs, but it can be extremely helpful in identifying the most likely first growth band.

In principle, identification and validation of the first growth increment can also proceed using any of the available age validation methods. These methods are described later (and in Campana (2001) in more detail).

1) Measure radius of vertebral section in elasmobranch at end of first year of life.

2) Overlay measurement on section from older elasmobranch to identify likely first annulus.

X mm

X mm
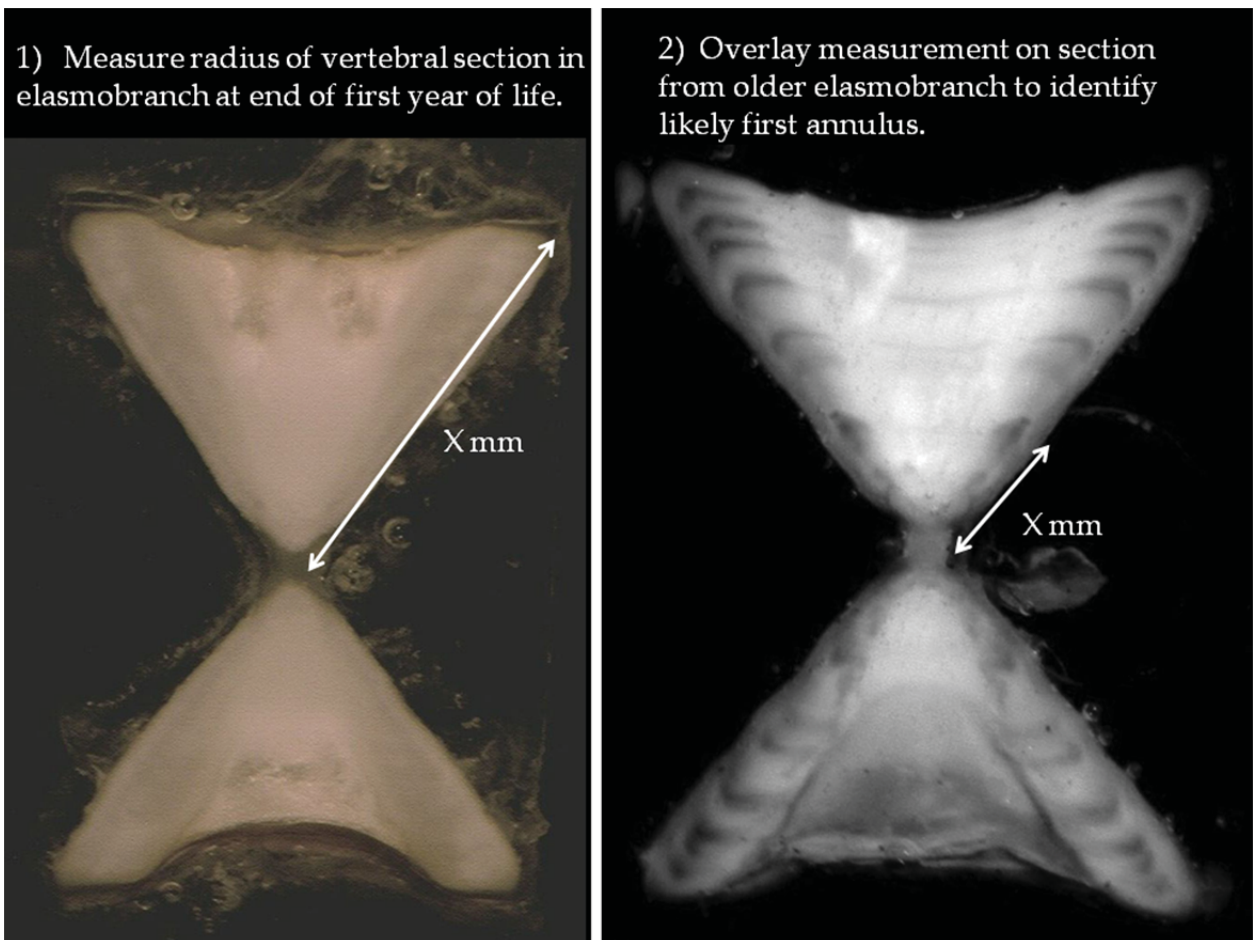
Figure 16. A simple approach to identifying the most likely location for the first growth band.

6.   Whole Vertebrae

There are relatively few species for which examination of unsectioned vertebrae will provide an accurate age. Unlike otoliths, vertebrae grow in direct proportion to the growth of the body, so that vertebral growth becomes very small in sexually mature, older elasmobranchs. Such limited growth is usually detectable in vertebral sections, but is much more difficult (or impossible) in intact vertebrae. Nevertheless, intact vertebrae can often be used to determine age in young, fast-growing species and individuals such as blue shark (*Prionace glauca*) (MacNeil and Campana 2002).

Whole vertebrae for ageing must be first cleaned of all adhering tissue, then dried. Growth bands of large species can often discerned with the naked eye, but age determination is best done under a dissecting microscope at low magnification or from digital images. Digital images can be captured using a standard digital camera mounted on a camera stand. Proper lighting is very important; oblique lighting from twin arms of a fibre optic light source are best, oriented low and from each side to maximize contrast. If digital images are being

captured, it is important to ensure that the growth axes being examined are all in the same focal plane, or alternatively, that multiple images at different focal planes be captured.
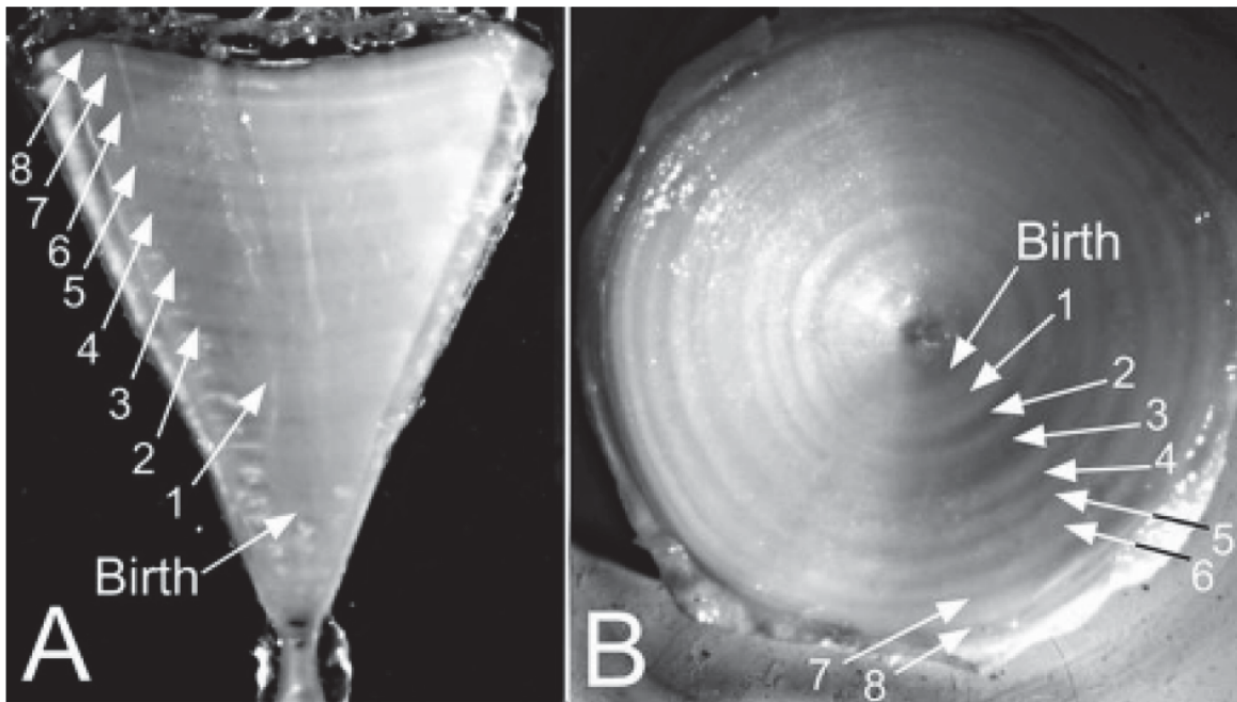
Figure 17. Comparison of annual growth bands visible in a single *Prionace glauca* vertebra after sectioning (A) and as viewed whole (B). Annual growth bands are indicated.

7. SPINES

7.1. Imaging and Interpretation

Elasmobranchs with fin spines sometimes show distinct growth bands. These growth bands have been validated to form annually in species such as spiny dogfish (*Squalus acanthias*) (Campana et al. 2006). In dogfish such as *Squalus acanthias*, the second dorsal spine is generally used for age determination, since it tends to be less worn.

Fin spines are generally too large to image under a dissecting microscope, but the growth bands can sometimes be counted visually under the microscope. The best approach is to capture a digital image of the spine with a standard digital camera, preferably mounted on a camera stand, and then to enhance the image prior to age determination. Growth bands are usually most clear if the spine is held with the convex side of the curve facing upwards, but offset slightly from vertical. As was the case with whole vertebrae, proper lighting is important, and is best provided from a fibre optic light source, although at relatively low levels.
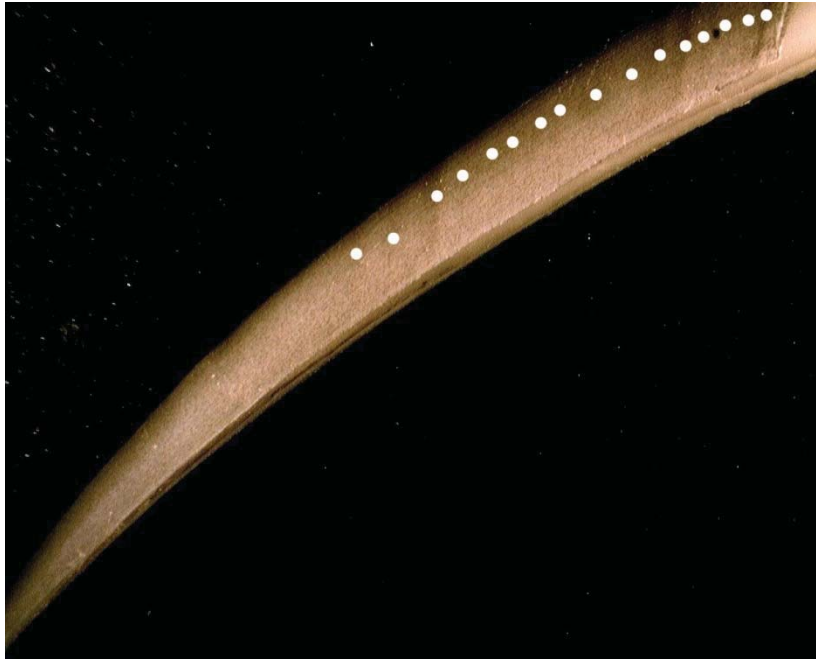
Figure 18. Spine of *Squalus blainvillei*, annotated to show (faint) growth bands.

7.2.   Adjustment for Worn Spines

Fin spines grow conically from the base, implying that the tip of the spine is the oldest part of the spine. As a result, and combined with other sources of wear throughout the life, the spine enamel often becomes so eroded that any growth bands that were present become lost.   Without correcting for the eroded growth bands, any age determined from the remaining growth bands will underestimate true age.

Figure 19. Fin spine of *Squalus acanthias* indicating annual growth bands as well as the anterior region in which growth bands have been lost due to erosion.

Estimation of the number of growth bands lost through erosion is a two-step process. First, the relationship between number of growth bands and basal diameter of the spine needs to be determined from unworn spines. Unworn spines will usually only be seen in relatively young elasmobranchs, but that is acceptable since it is usually the growth bands formed in the young ages that are worn away.

Any curvilinear equation can be fitted to the band count – basal diameter data; exponential equations are often used, but as long as the fitted curve represents the data well, any equation can be used (Ketchen 1975; Campana et al. 2009). It is important to note that the relationship between band count and basal diameter in unworn spines will vary with the growth rate of the population; published equations from one population <u>cannot</u> be applied to a different population unless their growth rate is similar.

$$Age = 0.594 \, e^{\,0.696 \, Diameter}$$
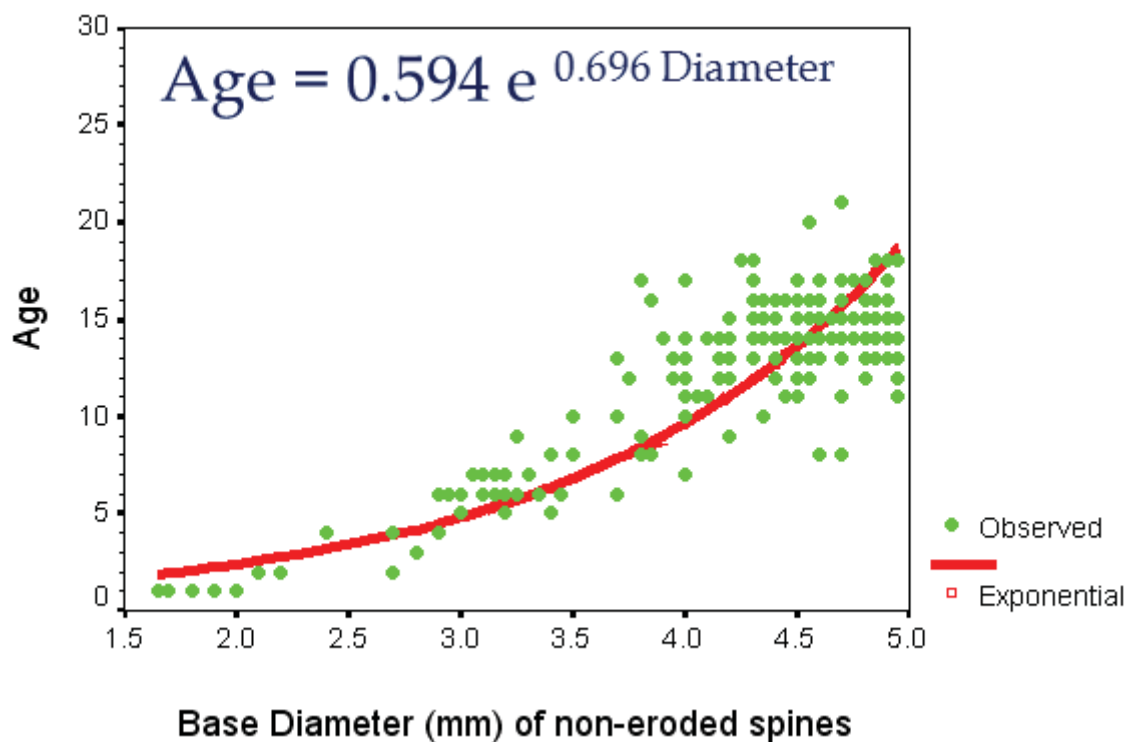
Figure 20. Example of relationship between the number of growth bands in unworn spines and the diameter of the spine base.
The resulting equation is used to estimate the number of missing growth bands in worn spines. Note that the fitted equation differs among species and populations.

Once the relationship between band count and basal diameter has been determined in unworn spines, the same relationship can be used to estimate the number of missing growth bands in worn spines. The spine diameter in the worn spine is measured at the most distal point at which growth bands are still visible. This measurement is then inserted into the equation calculated from the unworn spines to estimate the number of missing growth bands. That number can then be added to the observed number of growth bands in the worn spine to derive the estimated age of the elasmobranch with the worn spine.

8. AGE VALIDATION METHODS FOR ENSURING ACCURACY

Consistency does not imply accuracy! There are many high-profile examples in the scientific literature of age readers with years of experience, and capable of extremely consistent replicate age readings, being wrong (Campana 2001). Consistency implies excellent precision, and although precision is good, and is often the sign of a good age reader, it is all too possible to be consistently wrong. Accuracy on the other hand implies that the reader is providing the correct age (on average), even if they are not particularly precise. Assessing accuracy usually requires some objective, independent means of determining the age of the organism. The process of confirming that accuracy is called age validation.

A variety of methods exist through which age interpretations can be validated. Although the distinction has often been blurred in the literature, methods can be classified as either validating absolute age, validating the periodicity of growth increment formation, or of corroborating (but not validating) an existing set of age estimates. A complete review of age validation methods is available from Campana (2001).

Age validation methods suitable for elasmobranchs can be ranked in descending order of rigor as per the following:

- Release of known age and marked fish into wild
- Bomb radiocarbon
- Mark-recapture of chemically-tagged wild fish
- Ageing of discrete length modes
- Marginal increment analysis
- Rearing in captivity

Release of known age and marked fish into the wild is probably the most rigorous of the age validation methods for many species, since the absolute age of the recaptured fish is known without error. Since the released fish are generally less than 1 yr old, recaptured fish will have spent the majority of their lives in natural surroundings. Fish can be marked either externally with tags, or injected with calcium-binding chemicals so as to leave a permanent mark on the bony structures used for ageing. This approach is not well suited to long-lived species, since recapture rates of old fish tend to be minimal. Nor can this method be used on species which cannot be reared in captivity prior to release. However, there is a variation on this method which makes it more widely available at the expense of relatively minor assumptions. This variation involves the tagging of young fish where age can reliably be approximated by size. This approach was used by Natanson *et al.* (2002) in their study of porbeagle shark (*Lamna nasus*), whereby sharks estimated to be young of the year at the time of tagging were subsequently recaptured up to 6 years later. Although there was the potential for a ±1 year margin of error around the age estimate at the time of tagging, that margin was too small to change the conclusion that vertebral growth marks were formed annually after tagging.
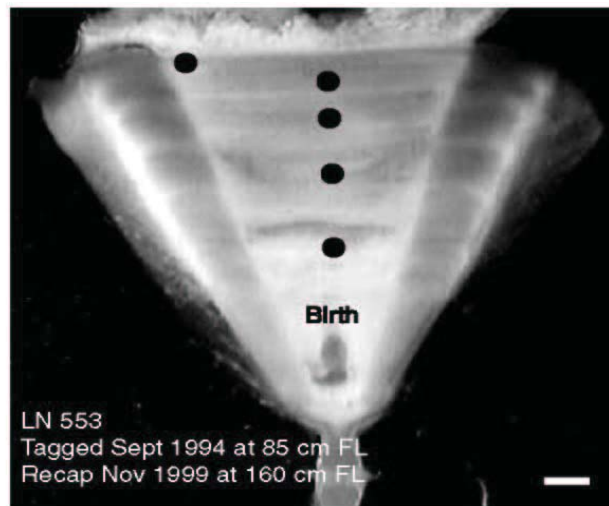
Figure 21. Vertebral section of a known-age but wild porbeagle shark.

Bomb radiocarbon derived from nuclear testing provides one of the best age validation approaches available for long-lived elasmobranchs (Campana et al. 2002, 2006; Francis et al. 2007; McPhie and Campana 2009). The onset of nuclear testing in the late 1950s resulted in an abrupt increase in atmospheric ^{14}C, which was soon incorporated into corals, bivalves, teleosts, elasmobranchs and other organisms that were growing at the time. Thus the period is analogous to a large-scale chemical tagging experiment, wherein all growth bands deposited before 1958 contain relatively little ^{14}C and all those hatched after 1968 contain elevated levels. Elasmobranchs born in the transition period contain intermediate levels. As a result, the interpretation of the ^{14}C chronology in growth bands isolated from vertebral sections or spines is relatively simple; the growth band ^{14}C chronology spanning the 1960s should match other published ^{14}C chronologies for the region (vertebral chronologies tend to be lagged by a couple of years relative to carbonate chronologies such as those from otoliths) as long as the annular age assignments (= year-class) are correct. Any under-ageing would phase shift the growth band ^{14}C chronology towards more recent years, while over-ageing would phase shift it towards earlier years. Sample contamination with material of more recent origin can only increase the Δ^{14}C value, not decrease it. Thus the growth band Δ^{14}C value sets a minimum age to the sample, and the years 1958-1965 become the most sensitive years for Δ^{14}C-based ageing. For elasmobranchs born during this time period, bomb radiocarbon can be used to confirm the accuracy of more traditional ageing approaches with an accuracy of at least ± 1-3 yr; the discriminatory power of samples born before or after this period is more than an order of magnitude lower. Since the ^{14}C signal recorded in deepsea and freshwater environments can be different from that of surface marine waters (deepsea=delayed; fresh water=advanced), reference ^{14}C chronologies appropriate to the environment experienced during the period of growth band formation must be used. However, the radiocarbon chronologies are not species-specific, which means that reference chronologies from one area and species can often be applied to another area and species. Clearly, this approach is not well suited to studies of short-lived (<

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5 yr) species, in instances where the presumed birth dates do not span the 1960s, or in environments where appropriate reference chronologies are not available. On the other hand, the low radioactive decay rate of ^{14}C implies that both archived and recent collections are appropriate for assay. For example, the innermost growth band isolated from a 50-yr old shark collected in 2010 would be just as ideal for assay as that of the innermost growth band of a 10-yr old shark collected in 1970 and stored since that time. Bomb radiocarbon assays of elasmobranchs have an advantage over those of teleosts, in that individual growth bands can often be removed from any location in the growth sequence through micromilling, and need not be restricted to the innermost growth band. In addition, radiocarbon chronologies can be developed from assays of multiple growth bands from a single individual (eg- Passerotti et al. 2010). Although this method is expensive ($500-$1000 per sample, not including the cost of growth band isolation), relatively few samples are required for age validation.
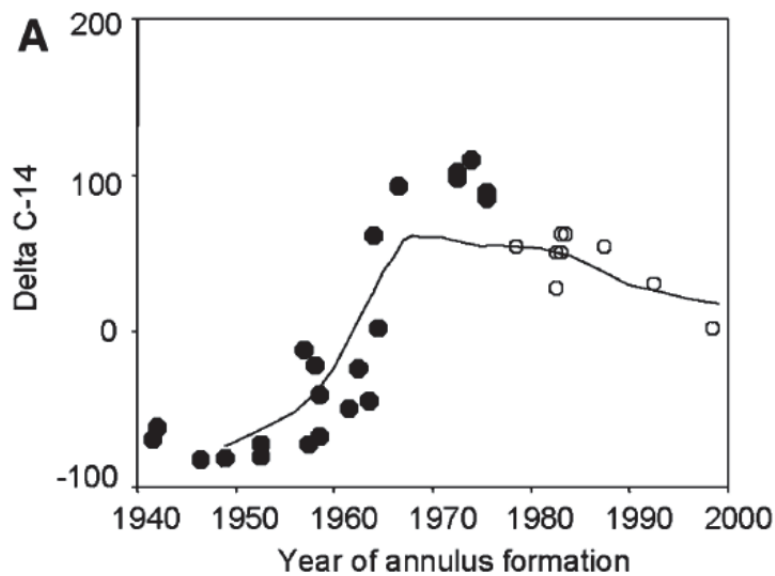
Figure 22. Radiocarbon (delta C-14) from individual growth bands of spiny dogfish spines from the northwest (solid symbols) and northeast Atlantic (open symbols) compared to the carbonate reference chronology for the northwest Atlantic.

Mark-recapture of chemically-tagged wild fish is one of the best methods available for validating the periodicity of growth band formation in elasmobranchs. The method is based on rapid incorporation of calcium-binding chemicals such as oxytetracyline, alizarin, calcein or strontium, injected at the time of tagging, into the vertebrae and spines (Campana 1999; Natanson et al. 2002). The result is a permanent mark, visible under fluorescent light (except strontium), in the growth band being formed at the time of tagging. The number of growth bands formed distal to the chemical mark is then compared to the time at liberty after tagging. This approach has been used to validate annual growth band formation in a wide range of elasmobranch species, in both vertebrae (Brown & Gruber 1988; Natanson et al. 2002) and spines (Beamish & McFarlane, 1985). A major advantage of this approach is

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that the growth bands being validated are formed while the fish is growing in a natural environment. Experiments in which fish are chemically-tagged and then reared in the laboratory or an outside enclosure are less optimal, although they are logistically easier to carry out. A disadvantage of the chemical tagging approach is that the number of increments formed after tagging is often low, resulting in a potentially large relative error if one of the increments (such as that at the growing edge) is misinterpreted. For example, misinterpretation of a single growth zone in a fish at liberty 2 years would result in a 50% error, whereas the same misinterpretation in a fish at liberty 10 years would only produce a 10% error. For this reason, fish tagged at a young age and recaptured at an old age provide the most robust validation results. Notwithstanding the caveat that this method only validates growth band formation for the size/age of fish tagged, this is a powerful method, and one of the two (along with bomb radiocarbon) readily applied to adult wild elasmobranchs.
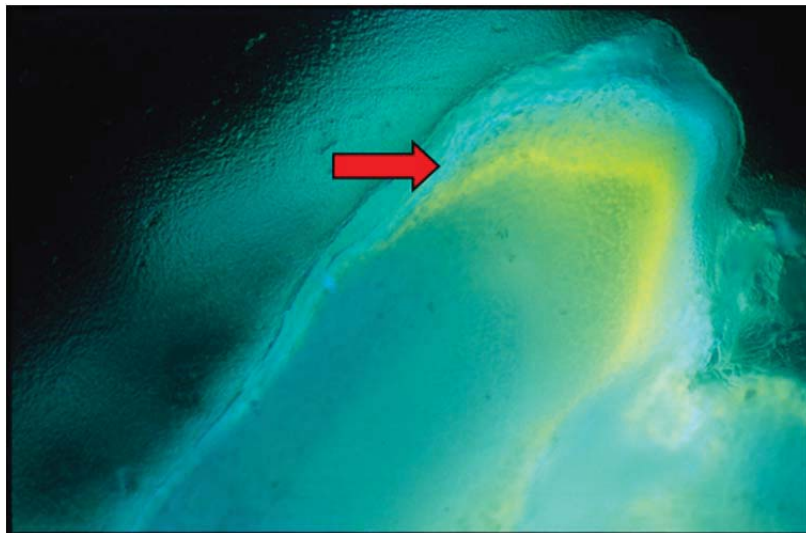
Figure 23. Chemical mark (visible under fluorescence) produced by tetracycline (OTC) injected at the time of tagging, and visible in a sectioned skate vertebra after one year at liberty (from McPhie 2007).

Progression of discrete length modes sampled for age structures has seldom been applied rigorously, but it is a reasonably robust approach for validating the interpretation of annuli in young fish. By monitoring the progression of discrete length modes across months within a year, it is relatively straight forward to determine if the modes correspond to age classes (Natanson *et al.* 2002). In instances where the length modes are well separated, can be tracked throughout the year, are not confounded by size-selective mortality, migration or multiple recruitment pulses within a year, and the mode corresponding to the young-of-the-year can be unequivocally identified, absolute age is confirmed. Examination of the ageing structures sampled from those same modes can then be used to test the validity of the presumed annual growth bands as age indicators. This approach is not equivalent to that which is more commonly applied, in which discrete length modes observed in a single sample are each assumed to correspond to an age class. While the latter approach provides corroboration for an age interpretation, there is no independent evidence that the length

modes represent age classes; thus strictly speaking, an approach that does not track modal progression through the year does not validate either absolute age or annulus periodicity.
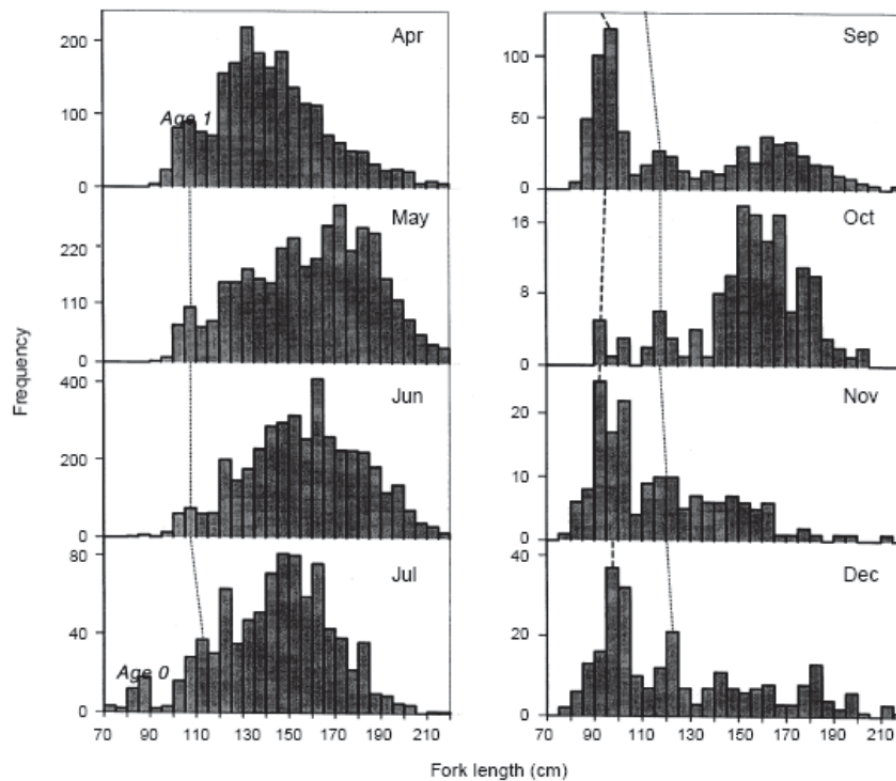
Figure 24. Monthly progression of Age 0 and Age 1 length modes in a well-sampled porbeagle fishery. Since the Age 0 sharks could be clearly identified, their length progression to Age 1 is confirmed. Interpretation of the vertebrae from those two length modes could then be used to validate Ages 0 and 1, at least on average.

Marginal increment analysis (MIA) is the most commonly used, and the most likely to be abused, of the age validation methods. The underlying premise as a method for validating growth band periodicity is sound: if a growth band is formed on a yearly cycle, the average state of completion of the outermost growth band should display a yearly sinusoidal cycle when plotted against season (Fig. 25). The popularity of this method can be attributed to its modest sampling requirements and low cost. However, in many ways, this is one of the most difficult validation methods to carry out properly, due to the technical difficulties associated with viewing a partial increment affected by variable light refraction through an edge which becomes increasingly thin as the margin is approached, as well as light reflection off the curved surface of the edge. The absence of an objective means of interpreting the data further complicates the situation.
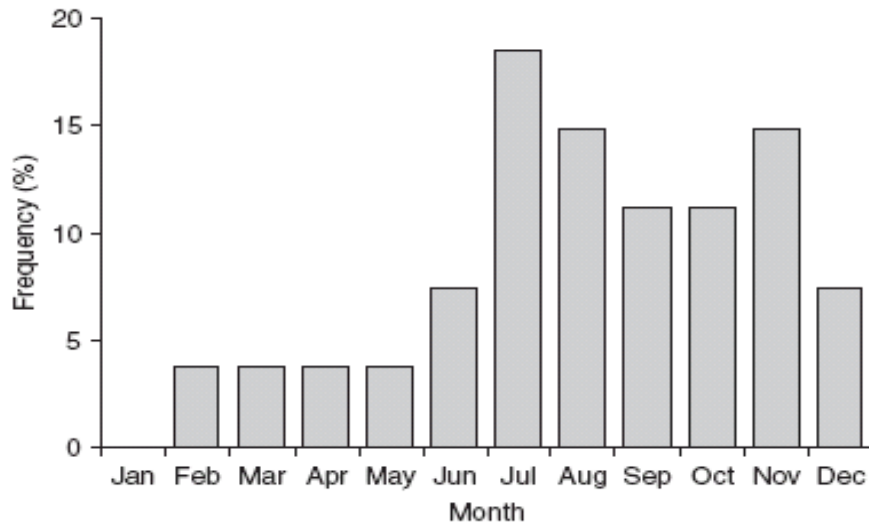
Figure 25. Proportion of sharks in a sample that display an opaque zone at the growing edge of the vertebral section versus the month of collection.
An annual cycle should be evident if the growth bands are formed once per year.

Marginal increment analysis is sometimes differentiated from edge analysis, but when used as a validation method, has similar properties. The marginal increment is usually calculated as a proportional state of completion, ranging from near zero (an increment is just beginning to form) to one (a complete increment has formed) as well as all values in between. When plotted as a function of month or season, the mean marginal increment should describe a sinusoidal cycle with a frequency of one year in true annuli. Edge analysis does not assign a state of completion to the marginal increment, but rather records its presence as either an opaque or translucent zone. It is the change in relative frequency of each edge zone which is plotted across months or seasons, but as with MIA, the cycle frequency should equal one year in true annuli. In both MIA and edge analysis, a yearly cycle of formation can be difficult to distinguish from other frequencies, contributing to their poor performance as validation methods. A proper statistical method for confirming the presence of an annual pattern in marginal increment data was presented by Okamura and Semba (2009).

There are several reasons why MIA may provide misleading results. Prominent among these is the fact that the marginal increment is most easily discerned in young, fast-growing fish, a life history stage where the marginal increment may accurately confirm the formation of annual increments. The problem arises when the 'validation results' are later applied to older fish, contrary to the assumptions of all age validation methods. Many studies have reported age validation based on MIA of young fish, but noted that the same ageing structure and/or approach provided incorrect ages in older fish (Campana, 2001).

Marginal increment analysis is valid if done with sufficient rigor, but must meet four requirements: 1) samples must be completely randomized before examination, with no indication to the examiner when the sample was collected; 2) a minimum of two complete

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cycles needs to be examined, in accordance with accepted methods for detecting cycles; 3) the results must be interpreted objectively/statistically (i.e. Okamura and Semba 2009), extending well beyond the "looks like a cycle to me" interpretation that is so commonly used; and 4) the MIA should be restricted to only a few age groups at a time, ideally only one. In particular, old fish should be analysed separately from young fish.

Captive rearing is not a reliable means of validating annual growth band formation, but can provide some information if no better method is possible. Laboratory environments are seldom able to mimic natural environments, due to their artificial photoperiods, temperature cycles, feeding schedules and limited space for diurnal vertical migrations. Since annulus formation is strongly influenced by the environment, an artificial environment is likely to produce artificial annuli. Mesocosms, ocean pens and outside enclosures provide improved and more natural rearing environments for validation studies than do indoor locations. Thus, outdoor rearing could be expected to produce growth bands which are quasi-natural in appearance and frequency, although growth rates would be unlikely to reflect those of natural conditions, resulting in unnatural growth band width.

9. METHODS FOR AGE CORROBORATION

Methods for age corroboration are not equivalent to those for age validation, since corroboratory methods support or are correlated with a particular method of ageing, but are not directly or logically linked. As a result, it is entirely possible to have an age corroboration method which reinforces an incorrect age interpretation. Nevertheless, a well designed corroboratory study can provide valuable support for a proper age validation study in confirming the accuracy of an age estimate or method. The following briefly summarizes the major age corroboration methods that are currently available, some of which have previously been called (incorrectly) age validation methods.

Tag-recapture analysis, along with length frequency analysis, is a member of a suite of methods which provides growth rate estimates which can be compared with those derived from annulus counts. The growth comparison is by inference, since none of the recaptured fish are of known age. Nonetheless, if sufficient tag returns are available, and particularly if the capture and release sizes were carefully measured, the resulting growth rate estimate is an important check on the accuracy of the age determination method. The traditional method of Gulland & Holt (1959) uses a graph of annualized growth rate after tagging plotted against average length between tagging and recapture to calculate the von Bertalanffy growth parameters, L_∞ and K. This method has been widely used, but assumes von Bertalanffy growth, no measurement error and no seasonal variability in growth rate. A more rigorous approach is the GROTAG analysis of Francis (1988), which uses maximum likelihood methods to estimate growth rate, growth variability and measurement error at two lengths. The approach properly differentiates between growth at length and growth at

age, and produces reliable growth estimates, but at the cost of considerable recapture data (e.g. Natanson *et al.*, 2002).
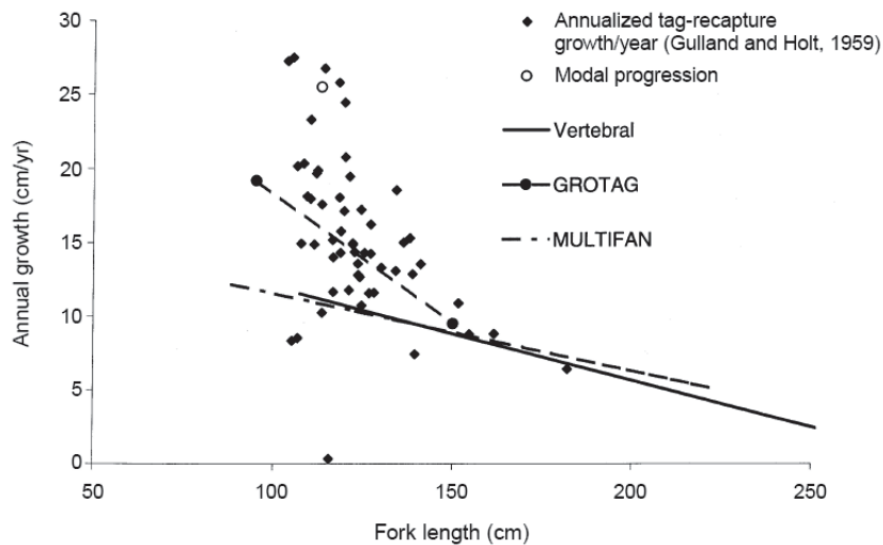

Figure 26. Use of tag-recapture data in both Gulland and Holt (1959) and GROTAG method to estimate growth rate at length.
Those estimates are then compared with those predicted of accurate ageing data.

Length frequency analysis subsumes a variety of different length-based methods, all of which produce estimates of growth rate. The corroboration occurs when the resulting growth estimate is compared to that of the age determination method. Monitoring of the progression of length frequency modes through time is one of the most basic of the length frequency analyses which is possible, and can be a reliable form of age corroboration in young, fast-growing fish. If monitoring occurs throughout the year, the results can be used to verify the annual frequency of the length modes, even if the corresponding age structures are not sampled. The subsequent comparisons of length at age or growth rate between length- and age-based methods must then be considered reasonably robust. Substantially less robust is the simple observation of the position of length modes in a single sample; in this approach, there is no confirmation that the modes actually correspond to any age classes, let alone the identity of that age class. At a more advanced level, methods such as Multifan fit a von Bertalanffy growth curve to multiple length frequency samples using maximum likelihood estimation (Fournier *et al.* 1990). In instances where monthly length samples are available throughout the year, this method is a valuable approach for integrating multiple samples to produce estimates of growth rate (Francis & Francis, 1992). However, here as with the other length-based methods, the approach is most suited for young, fast-growing fish where the length modes for each age group are easily distinguished; Multifan will use the well-defined length modes of the younger fish to fit a growth model to all fish, even if the length frequency of the older fish is nonsensical. Size-selective migration into or out of the study area is not an allowable assumption of this, or any other length-based method.
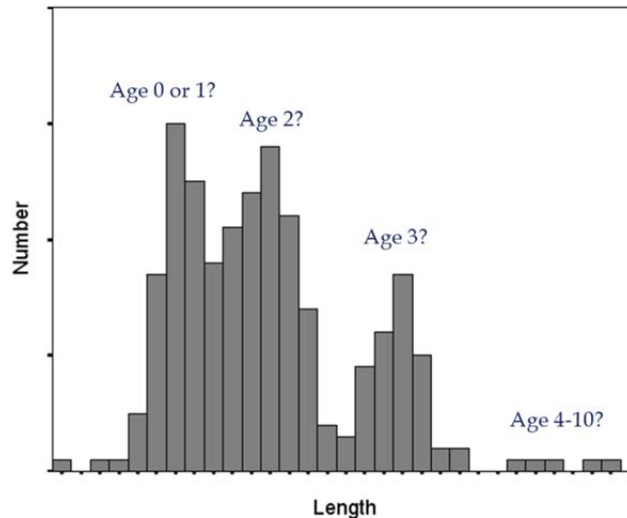
Figure 27. Length frequency modes can provide guidance on age determination, particularly when comparing the assumed lengths of the first two age groups, but age assignments are assumed, not known.

10. METHODS WHICH ARE NEITHER AGE VALIDATION NOR AGE CORROBORATION

There are three methods which are sometimes reported in the literature as confirming the accuracy of the age determinations, but they actually do no such thing, although the may serve some other purpose:

- *Back-calculated lengths* are useful for inferring the diameter of the first annulus and providing a continuous sequence of lengths for a growth curve. However, the similarity of back-calculated lengths across several ageing structures in no way validates or corroborates any age interpretation; it merely shows consistency in the interpretation of the sequence of growth increments, independent of whether or not the interpretation is correct.

- *Comparison of multiple ageing structures within each fish* will often show great consistency, reflecting the fact that the growth of all structures within a given fish tends to be influenced by the same environmental and physiological factors in the same manner. That is, whatever forms a check in one structure will almost certainly form it in other structures, even if it is a false or stress check. While structure comparisons are very useful during the selection of a preferred ageing method, they provide no information on ageing accuracy.

- *Comparison among or within age readers* is an excellent method to estimate ageing precision or consistency. However, the comparisons say nothing about ageing accuracy. When age readers are wrong, they tend to be consistently wrong.

11.   STATISTICAL ANALYSIS OF AGE DATA

Age data are most commonly used for developing growth curves, and as input into age-structured population models. Before that can be done however, a few simple steps need to be undertaken to assess the quality of the age data. These steps are: 1) descriptive statistics of the age and length variables to insure that there are no inputting or data errors; 2) an age bias plot to check for bias; and 3) calculation of a precision index to assess consistency or reproducibility.

The descriptive statistics used for checking for data input errors can be as simple as producing separate frequency histograms of the age and length variables to insure that all of the measurements fall within reasonable bounds. A scatterplot of length versus age can also be useful in identifying obvious errors, but it is important here that the data not be "pruned" to eliminate observations that don't fit prior expectations of growth. Rather, the intent is to identify data input or recording errors.

The age bias plot is the primary tool for assessing bias; that is, a systematic difference between two age readers or ageing methods. It is the ideal tool for detecting under- or over-ageing of one ager relative to another, even if the ageing error is restricted to the youngest or oldest fish.
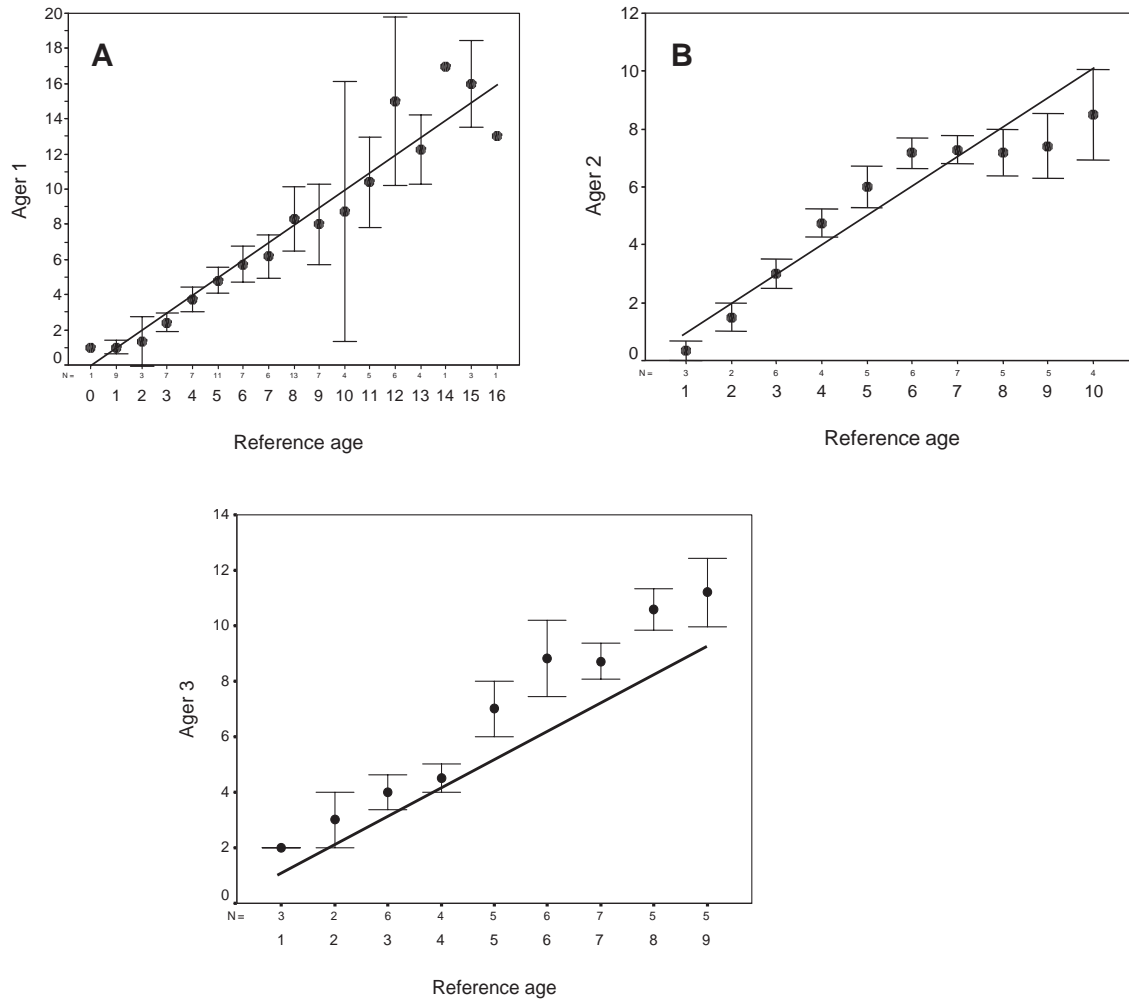
Figure 28. Examples of age bias plots where no bias is present (A) and where bias is present (B and C).
In (B), Ager 2 has over-aged ages 4-6 but under-aged ages 8-10. In (C), Ager 3 has consistently counted 1-2 extra growth
bands compared to the Reference age. Each error bar represents the 95% confidence interval about the mean age
assigned all samples of a given age by a second age reader (Reference). The 1 : 1 equivalence (solid line) is also indicated.
Numbers plotted below symbols are the sample size at each age.

Ideally, age bias plots are prepared where the age reader compares against a known-age reference collection. When this is done, the age bias plot becomes a check on the accuracy of the age reader. Where this is not possible, the most reliable set of ages is used as the reference age on the X axis. However, only relative accuracy is being assessed at this point, since neither set of age readings is known to be correct. When two age readers are being compared, or when the comparison is between two ageing structures or methods, the age bias plot can only a systematic difference eg- in Fig 28b above, Ager 2 is under-ageing relative to the reference age, but it could be that Ager 2 is correct and the reference age is over-ageing.

It is important to note that the interpretation of the age bias plot is in terms of overall patterns, not individual points. The intent is to detect consistent deviations from the 1:1 line, not to examine the deviation of any one age from the line. So for example, in Fig 28a, there are some points that lie above the 1:1 line and some points that lie below it; some mean values are statistically different from the 1:1 line (as indicated by the extent of the

error bar), and others that are not. But there is no overall pattern, and therefore no bias. In Fig 28b, there is a trend; several continuous ages (ages 4-6) are about 1 yr above the line, and ages 8 and above are all increasingly below the line. The easiest type of age bias to deal with is shown in Fig 28c, where Ager 3 has consistently counted 1-2 extra growth bands relative to the reference age. This type of bias usually occurs where one reader is counting the edge (or a first annulus) and the other ager is not. A brief comparison of annotated images is usually sufficient to get rid of this type of bias.

Ageing precision refers to the the reproducibility or consistency of repeated age determinations on a given structure, whether or not those age readings are accurate. It is not unusual for inaccurate age readings to be highly reproducible (in other words, precisely wrong). Therefore, precision cannot be used as a proxy for accuracy. Nevertheless, a measure of precision is a valuable means of assessing the relative ease of determining the age of a particular structure, of assessing the reproducibility of an individual's age determinations, or of comparing the skill level of one ager relative to that of others.

There are two widely used and statistically sound measures of ageing precision: average percent error (APE) and coefficient of variation (CV). Although percent agreement is the traditional index of ageing precision, it varies widely both among species and among ages within a species. For example, 90% agreement to within one year between two age readers would be poor precision if there were only 3 year classes in the fishery. On the other hand, 90% agreement to within one year would constitute excellent precision for spiny dogfish *Squalus acanthias* L., given its 60-yr longevity.

The coefficient of variation (CV), expressed as the ratio of the standard deviation over the mean, is the most widely used measure of precision, and can be written as:

$$CV_j = 100\% \times \frac{\sqrt{\sum_{i=1}^{R} \frac{(X_{ij} - X_j)^2}{R-1}}}{X_j}$$

where CV_j is the age precision estimate for the jth fish. The CV is calculated across all age readings for each elasmobranch, and is usually averaged across fish to produce a mean CV.

The average percent error (APE), is defined as:

$$APE_j = 100\% \times \frac{1}{R} \sum_{i=1}^{R} \frac{|X_{ij} - X_j|}{X_j}$$

where X_{ij} is the ith age determination of the jth fish, X_j is the mean age estimate of the jth fish, and R is the number of times each fish is aged. When averaged across many fish, it becomes an index of average percent error.

Both CV and APE are easily calculated using a spreadsheet, as shown in the following example:

Reader 1	Reader 2	Reader 3	SD	Mean	CV (%)	APE (%)	
9	8	9	0.6	8.7	6.7	5.1	
8	5	6	1.5	6.3	24.1	17.5	
12	14	13	1.0	13.0	7.7	5.1	
					12.8	9.3	Mean

CV and APE are mathematically related, with CV being about 40% higher than APE for any given set of ageing data. It is important to note that all measures of precision will be artificially inflated by any bias which exists between agers, implying that bias should be dealt with before calculating precision. There is no single value of precision that can be used as a target level for ageing studies, but a CV of 5% is often used for otolith studies. CV values of more than 10% are common in studies reporting shark ages based on vertebrae.

12. REFERENCE COLLECTIONS AND QUALITY CONTROL OF AGE READINGS

There are four steps leading to the development and continued success of an ageing program:

1. development of an ageing method
2. age validation
3. preparation of a reference collection
4. quality control monitoring

Quality control monitoring monitors ageing consistency through time, under the working assumption that the method is accurate (an assumption that was presumably tested and confirmed in the age validation stage). As noted by Campana (2001), the monitoring process ensures 1) that the age interpretations of individual age readers do not 'drift' through time,

introducing bias relative to earlier determinations; and 2) that the age interpretations by different readers are comparable. Such a protocol monitors both relative accuracy and precision at regular intervals, and is completely analogous to quality control protocols in a manufacturing process. Integral to the quality control process is the reference collection and the two statistical monitoring tools discussed previously: age bias plots and the CV.

Reference collections of ageing structures are important elements of an ongoing ageing program. Ideally, a reference collection is a collection of prepared ageing structures, of known or consensus-derived ages, representative of all factors which might reasonably be expected to influence the appearance or relative size of the growth bands. Ideally, a list of such factors might include all combinations of age, sex, season, and source of collection, spanning the entire length range, a representative sample of the geographic range, and several collection years. The primary role of the reference collection is to monitor ageing consistency over both the short and long term, as well as among age readers. The collection is particularly important for testing for long-term drift, something that cannot be detected through simple re-ageing of samples from the previous year, or through use of a secondary age reader. A second role of the reference collection is for training purposes; a representative subsample of the collection can be imaged and annotated, thus simplifying the training of new age readers and insuring consistency in the type of structures which are interpreted as growth increments. It is important to note that ideal reference collections are rare. It is far more important to have something – anything – than to have nothing. A collection of 200 vertebrae or other ageing structures is good, and can be added to through time, but again, a few dozen vertebral images is better than nothing.

Once assembled, the reference collection can be sent out for ageing as part of an exchange program, either physically or in the form of digital images. The preparation of digital images insures long-term availability, facilitates exchanges with other laboratories, and simplifies the training of new age readers. The use of annotated 'layers' (*sensu* Photoshop) which can be toggled off and on, allows the image to be interpreted with or without the annotation.

For quality control monitoring, a subsample of the reference collection is intermixed with a subsample of recent samples and then aged without the age reader knowing which is which. An age bias graph comparing test versus reference ages for the reference structures would confirm long-term ageing consistency, while a separate age bias graph comparing test versus original ages for the production subsample would insure consistency between the most recent production run and the QC test. If both tests indicate lack of bias, the same ageing criteria must have been used for both reference and production samples. The CV of original and new ages provides the measure of ageing precision. The combination of the new vs original age bias graph and CV is sufficient to detect almost all sources of ageing error.

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