Methods for the Preparation of Pacific Spiny Dogfish, *Squalus suckleyi*, Fin Spines and Vertebrae and an Overview of Age Determination

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Introduction

Age determination of elasmobranchs presents unique challenges compared to that of teleosts. Elasmobranchs do not have calcareous otoliths or scales, structures commonly used to age teleosts. Various techniques, including bomb-radiocarbon dating, histological staining, and X-radiography, have been applied to hard structures such as fin spines, vertebrae, and caudal thorns to age elasmobranchs (Cailliet and Goldman, 2004; Carlson and Goldman, 2006).

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doi: dx.doi.org/10.7755/MFR.78.1–2.1

ABSTRACT—The Pacific spiny dogfish, Squalus suckleyi, *is a small shark species commonly found in the North Pacific Ocean. Age determination for this species has historically been conducted by examination of the dorsal fin spine with little change in methodology since the 1930's. Despite extensive use, there are two major caveats associated with fin spines as age structures: 1) fin spines protrude from the body and are subject to damage, requiring estimation of annuli contained in missing portions of the fin spine and 2) there is a high degree of inter- and intra-reader variability due to difficulty in interpreting fin spine growth patterns. A new method was recently developed for* S. acanthias, *a North Atlantic Ocean congener of* S. suckleyi, *using histologically stained thin sections of vertebrae instead of dorsal fin spines for age estimation. Here, we apply this histological method to vertebrae of* S. suckleyi *and describe the historic methodology for dorsal fin spines. This document presents detailed procedures for both methods, including sample collection, sample preparation, and age estimation criteria for each structure.*

To be viable for an age determination study, a hard structure must form visible, annually-formed growth patterns. Typically, these growth patterns consist of alternating light and dark bands; each pair of growth bands is termed an "annulus," representing 1 year of growth. Note that this differs from the standard English definition of the word annulus, which is derived from the Latin word "anus" meaning "ring," not "annus" meaning "year" (Panfili et al., 2002).

In most elasmobranch studies, sagittally sectioned vertebrae are selected as the primary age structure (Cailliet and Goldman, 2004), although clarity of annuli within vertebrae is largely species-specific. For some species, vertebra growth patterns are not discernible and thus alternative structures such as the neural arch (McFarlane et al., 2002) or dorsal fin spine (Clarke and Irvine, 2006) must be used.

Squaliform sharks (dogfishes) are one of two orders of elasmobranchs possessing dorsal fin spines (Clark and Irvine, 2006). Pacific spiny dogfish, *Squalus suckleyi* (hereafter termed "spiny dogfish"), is a species of Squaliform shark found throughout the North Pacific Ocean, ranging from the Koreas and Japan through Russian and Alaskan waters to the North American west coast, reaching as far south as Baja California (Ebert et al., 2010). *Squalus suckleyi* was previously thought to be identical to its Atlantic counterpart, *S. acanthias*, but genetic, meristic, and morphological evaluation has proven them to be two distinct species (Ebert et al., 2010). Further, *S. suckleyi* differs from *S. acanthias* in several key life history attributes, including slower growth, larger maximum size, and later maturity. Much of the existing scientific literature from the North Pacific Ocean refers to spiny dogfish collected there as *S. acanthias,* and it is important to note that the species name is now considered incorrect in previous literature and should be considered *S. suckleyi.*

The dorsal fin spine method has been used to age *S. suckleyi* and *S. acanthias* since the 1930's. In this method, annuli on the enamel of the second dorsal fin spine are viewed and counted using reflected light and a dissecting microscope or with image analysis software. However, this may not be the best method to accurately describe age and growth of the species. Because the dorsal fin spine extends from the body into the environment, breakage and erosion of the fin spine often occurs over time. Thus, larger, older spiny dogfish tend to have fin spines with more wear than smaller, younger fish.

Ketchen (1975) developed an algorithm to estimate the number of missing annuli in the worn portion of the fin spine using the relationship between the enamel base diameter and the number of annuli counted on un-

worn fin spines. An alternative analytical method for estimating lost annuli was proposed by Cheng (2012); however, few laboratories have adopted this method to date.

Taylor et al. (2013) conducted a detailed examination of the Ketchen and Cheng analytical approaches and determined that both methods produced questionable age estimates for larger, older fish, and they further recommended an examination of new methods. Attempts to improve Ketchen's algorithm (McFarlane and King, 2009) have not addressed problems of error from other sources (e.g., natural variability, reader error); therefore, the historical method continues to be used (Tribuzio et al., 2010).

Fin spine-based age estimates of both *S. suckleyi* and *S. acanthias* have been validated using bomb-derived radiocarbon (Campana et al., 2006). Furthermore, annual periodicity of the fin spine banding pattern has been verified by oxytetracycline (OTC) injections and tag/recapture methods on *S. suckleyi* that were at liberty up to 21 yr (McFarlane and Beamish, 1987; McFarlane and King, 2009). However, the low precision of fin spine-based age estimates (CV = 19%; Rice et al., 2009; Tribuzio et al., 2010) is problematic, and systematic bias among age determination laboratories occurs despite age validation (Rice et al., 2009). Measurement errors among readers, potential systematic errors among laboratories, and process errors associated with estimating the number of worn annuli all combine to produce the low precision in age estimates and therefore growth parameters (Tribuzio et al., 2010). This error has ramifications for population modeling and biological reference points for fishery management (Tribuzio and Kruse, 2011).

To address these issues, a new method of age determination using vertebrae has been developed for *S. acanthias* in the northwest Atlantic Ocean (Bubley et al., 2012). This method employs histologically stained vertebra thin sections and presents two advantages over the old dorsal fin spine method. First, vertebrae do not wear or break over time as fin spines do, therefore reducing one source of variability and error in the age determination process (i.e., the need to use a modeled estimate for the number of missing annuli in worn fin spines). Furthermore, age estimates derived from vertebrae are far more precise than those from spines (Bubley et al., 2012).

Results are not presented in this paper as it is intended solely as a technical guide for the collection, laboratory processing, and interpretation of age structures. This paper is part of a larger spiny dogfish age determination project. While both methods are presented here to improve precision and document ageing criteria for use by other laboratories, it is important to note that at this time, the vertebra method does not appear to be appropriate for older spiny dogfish (Tibuzio et al., in press). Documenting age determination methods is imperative, as criteria used for identifying annuli can drift over time and among agencies. This paper provides a central reference for all laboratories involved in spiny dogfish age determination and will promote consistency between the two methods and among spiny dogfish age readers, in the hopes of improving inter- and intra-laboratory precision.

Sample Collection

Squalus suckleyi is a small shark with medium-brown to gray coloration dorsally and white ventrally; white spots are often present along the upper body. A prominent spine is present along the anterior edge of each dorsal fin. A complete description of identifying characteristics is available in Ebert et al. (2010). While there are at least 25 species of the genus *Squalus* occurring throughout the world, *S. suckleyi* is the only one occurring in the eastern North Pacific Ocean. However, the species can be confused with members of the Triakidae family (e.g., brown smoothhound, *Mustelus henlei*), which possess an anal fin and do not have dorsal fin spines. In the western North Pacific Ocean, *S. suckleyi* may overlap with other *Squalus* species near the far southwestern edge of its range, in particular, *S. japonicus*, *S. blainville,* and *S. brevirostris*. However, *S. suckleyi* is generally easy to distinguish from the other *Squalus* species because of the white spots along the sides and because the origin of the first dorsal fin spine is posterior to the rear free tips of the pectoral fins, whereas in most species of *Squalus* occurring in the western North Pacific Ocean the first dorsal fin and spine are located above the pectoral fins (Compagno, 1984).

The first steps in spiny dogfish collection are to sex and measure each fish. Sex can be determined externally; males have paired claspers attached to the pelvic fins. Special care may be needed when identifying sex of very small (immature) animals, as male claspers can be small. There are four commonly used length measurements for spiny dogfish (Tribuzio et al., 2009), measured from the tip of the snout to 1) the dorsal pre-caudal pit (pre-caudal length, PCL); 2) the deepest indentation of the tail fork (fork length, FL); 3) the dorsal tip of the tail in its natural position (natural total length, TL_{nat}); and 4) the dorsal tip of the tail with the upper lobe of the caudal fin depressed to align with the horizontal axis of the body (extended total length, TL_{ext}) (Fig. 1). However, PCL has the least amount of measurement error and is often the easiest to measure. This is the preferred length measurement for the species, but equations exist to convert among length measurements (Tribuzio et al., 2009; Tribuzio and Kruse, 2012).

Two age structures are collected: 1) the posterior dorsal fin spine (hereafter termed "spine" and 2) a section of the vertebral column. The posterior spine is preferred to the anterior spine because it is larger and generally subject to less wear. Vertebra samples can be collected at various positions along the vertebral column. The authors have found that vertebrae collected more anteriorly are generally larger, making them easier than posterior vertebrae to prepare and read. If time allows, it is preferable to collect vertebra samples ventral to the anterior dorsal fin;

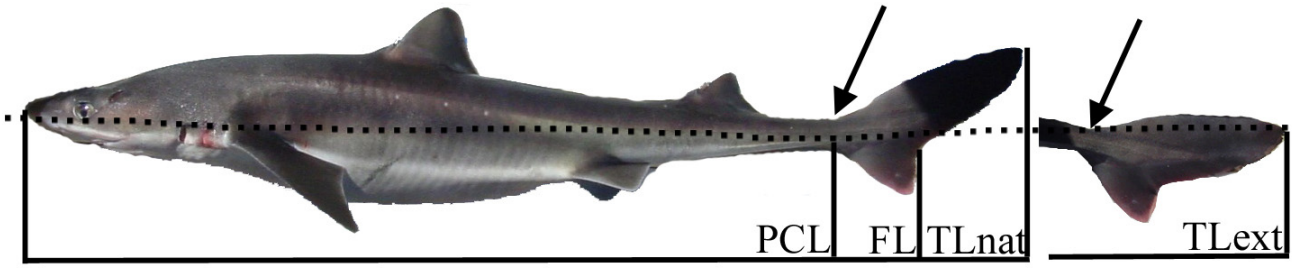

Figure 1.—External measurements for spiny dogfish. All measurements start at the snout and extend to either the pre-caudal length (PCL), fork length (FL) or total length (TL, both natural and extended). The dashed line shows the horizontal axis of the body for measuring TL extended). For this sampling plan only the PCL is used. The arrow points to the pre-caudal notch. From Tribuzio et al. (2009).

however, it is quickest to collect the vertebrae and posterior spine simultaneously (Fig. 2).

For collection, a vertical incision is made behind the posterior spine, slicing down (ventrally) through the vertebral column (Fig. 2A). Then an incision about 4 inches long is made horizontally towards the head, keeping the blade ventral to the vertebrae (Fig. 2B). The section is removed by cutting vertically to the dorsal side (Fig. 2C). Excess muscle tissue should be trimmed to make storage easier (Fig. 2D, 2E). Samples should be stored in individually labeled, single-specimen reclosable bags and frozen. Vertebra and spine samples from the same animal can be stored in the same bag. The same cutting and sample storage/preservation method can be used regardless of whether the vertebra samples are removed from the anterior or posterior portions of the vertebral column.

Specimen Preparation

Dorsal Fin Spines

Various techniques and tools can be used to prepare spines for age determination. The frozen spines and vertebrae need to be separated and the vertebrae retained for further processing as described in the next section; it is advisable to do this with the structures frozen or partially frozen to prevent tissue degradation of the vertebrae prior to processing.

Spines need to be heated to aid in tissue removal, taking care not to damage the spine enamel or base structure. Heating will create a strong odor and it is recommended to use a well-ventilated area or to process under a fume hood. Spines may be heated either using a microwave or hotplate. To microwave, spines should be placed, either individually or as batches if each is in an individually labeled microwave-safe bag, in a microwave-safe container filled with water. If spines are in individual bags, the bags should be punctured to allow water to flow through. Depending on the power of the microwave, the size of the container, and the number of spines, it should only require 2–6 min to adequately heat the spines. Alternatively, spines may be heated in large, water-filled beakers or in a divided basket within a large tray placed on a hotplate for several minutes. Tissue should easily scrape off the surface of the spine with gentle rubbing.

Tools that are helpful for cleaning the inner surfaces of spines include forceps, microprobes (i.e., dissecting needles), and dental picks. For cleaning the outer enamel, the best tool is a fingernail; however, a soft toothbrush (used gently) or a cloth can be useful. Metal tools should be used with caution on the outside of the spine because they may damage the enamel surface used for age determination.

All soft tissue needs to be removed from the exterior and interior of the spine. While working on a spine, it is helpful to keep the spine wet, otherwise the tissue becomes sticky and difficult to remove. The outside of the spine can be initially cleaned by carefully scraping away the tissue with a fingernail or other soft tool. Remaining tissue on the exterior of the spine can be carefully removed with forceps or another fine-tipped tool. The cartilage plug on the inside of the spine will generally slide out as one piece and can be pulled out with forceps or a dental pick. Once the plug is removed, any tissue remaining inside the spine can be scraped away without concern of damage to the interior of the spine.

Spines can be stored long term in individual paper coin envelopes. After cleaning, each spine should be allowed to air dry for at least 24 h to eliminate all moisture and potential sources of decay and to prevent the spine from sticking to the paper storage envelope. Spines may become brittle after many years of storage, so care is warranted when handling archived envelopes. Some laboratories use a barcode system to track samples; if this is the case, the barcode sticker can be placed inside the envelope just below the fold of the envelope's flap. Barcodes can still be scanned from this location, and if the adhesive weakens, the barcode label will not be lost.

Vertebrae

To prepare spiny dogfish vertebrae for age determination, they must be individually dissected, thin-sectioned, stained, and mounted on glass slides. Histological staining methods have been adapted from Bubley et al. (2012). Supplies needed to prepare

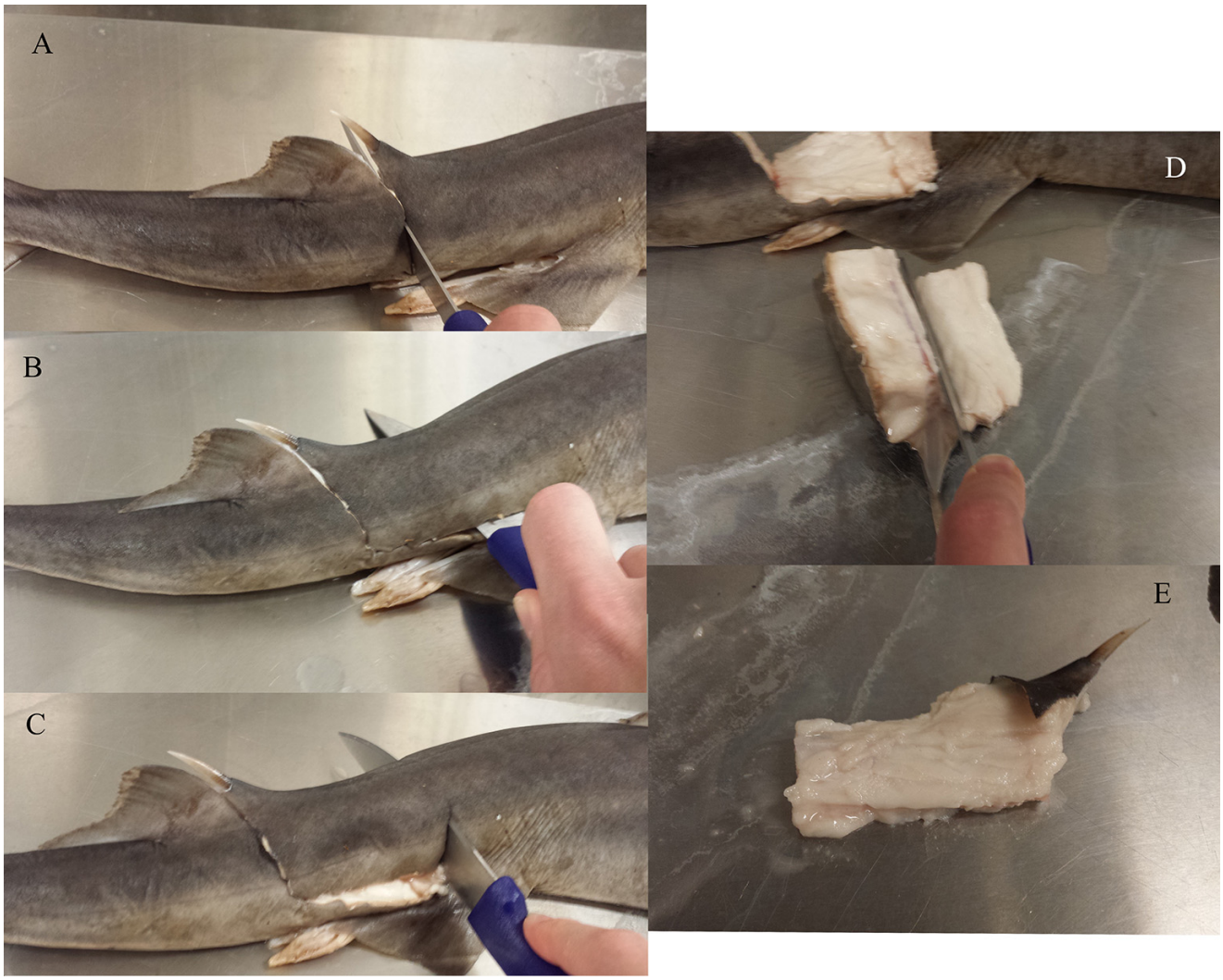

Figure 2.—Dorsal fin spine and vertebrae dissection. A) A downward incision is made posterior to the dorsal fin spine, followed by B) a horizontal incision ventral to the vertebral column, and finally C) an upward incision is made to simultaneously remove the spine and a portion of vertebral column. D) Excess tissue is removed from the spine and vertebrae to produce E) a single sample ready to be frozen until further processing in the laboratory is possible.

vertebrae are listed in Table 1. Personal protective equipment such as eye goggles, latex or nitrile gloves, and laboratory coats should be worn when handling chemicals. A chemical fume hood should be used where indicated, and refer to material safety data sheets (MSDS) for proper handling, storage, and disposal of all chemicals.

Dissection

Vertebral column sections must be at least partially thawed before excess soft tissue can be scraped away. Once thawed, a scalpel or sharp knife is used to cut excess tissue from around the vertebral column section. The axial processes (neural and hemal arches) may be left attached to the vertebrae to simplify cleaning, and any remaining soft tissue can then be scraped off with the back of a knife.

Individual centra are separated from the vertebral column using a scalpel. The axial processes and remaining soft tissue can then be trimmed from each centrum. Care should be taken when removing excess tissue from each centrum; fine forceps can be used to remove stubborn tissue. It is often not possible to remove all the tissue; however, this will not impact age determination. Several vertebra centra per animal can then be stored in individual vials containing 70% ethanol to await sectioning and staining. If desired, the remainder of the vertebral column may be stored frozen.

Thin-sectioning

Silicone molds (suggested size: 64 × 70 × 12 mm) are used to encase

rows of vertebra centra in resin for sectioning multiple specimens simultaneously. A brand of resin that works well for this purpose is Polytranspar Artificial Water[1], a clear casting resin that requires a catalyst to harden (available at taxidermy supply businesses). A new batch of resin must be mixed for each use because it starts to harden immediately once the catalyst has been added. Wax cups pre-marked in 2 oz increments are quite helpful for mixing the resin as they can be disposed of once the unused catalyzed resin hardens.

First, working in a fume hood, a batch of resin is mixed and a thin layer is poured into the mold such that it just barely covers the bottom of the mold. The mold should be left undisturbed for about 40 min to allow the resin to harden until tacky. A line should be drawn in the resin, using a probe and a ruler as a guide, for aligning multiple vertebra centra. Either the anterior or the posterior end of each centrum is then pressed into the resin with the focus on the guideline, to result in a longitudinal section. Space should be left at the top of each block to place a label with the specimen numbers for each row. Each block can accommodate 2–3 rows of vertebrae, with 4–7 vertebrae per row.

Once aligned, the vertebrae need to dry in place (about 30 min) so they are held firm in the bottom layer of resin. This prevents the vertebrae from shifting as the top layer of resin is poured. A new batch of resin is then poured over the centra so that they are fully covered while striving to keep block thickness to a minimum. Excess resin can cause difficulties when sectioning. The resin should be allowed to dry a minimum of 2 days in the fume hood.

After the resin is fully cured, vertebrae are sectioned with a high-speed saw such as an IsoMet 5000 (Buehler-ITW, Lake Bluff, IL). Optimal section thickness for spiny dogfish vertebrae is 0.4 mm, although some variation is acceptable as long as stain penetration and annulus clarity are not affected. We recommend checking the thickness of the distal ends of each section with calipers to verify that the saw is cutting accurately. Thin sections can be stored in 70% ethanol indefinitely. Excess resin should be trimmed away from the vertebra sections using a sharp scalpel prior to staining.

[1]Mention of trade names or commercial firms is for identification purposes only and does not imply endorsement by the National Marine Fisheries Service, NOAA.

Table 1.—Supplies used for sectioning, staining, and mounting vertebra centra.

Sectioning supplies	Staining supplies
Wax cups marked in 2 oz increments	Histology tissue cassettes
Tongue depressors	Large beakers
Strips of paper ~ 1 cm wide (to label blocks)	Graduated cylinders
Dried, cleaned vertebrae	Containers to store reagents
Silicone molds	Funnel
Polyester resin	Glass slides
Forceps	Cover slips
Probe	Slide boxes
Paper towels	Hot plate
Scalpel	Orbital shaker or stir plate with bars
Scissors	Stopwatch
Precision saw with diamond blade	Small and large forceps
Dissecting microscope	Scalpels
Ruler	Flammable chemical disposal container
Calipers	Dissecting microscope
	Plastic tub
	Trays
	Paper towels
	Distilled water
	Modified Harris hematoxylin
	Gelatin
	Glycerol
	Listerine
	Hydrochloric acid (12 M)
	Ethanol (100%)
	RDO Rapid Decalcifier

Solution Preparation

Several of the solutions used to stain and mount specimens can be prepared in advance: acid-alcohol, a series of concentrated glycerin solutions, and Kaiser glycerin jelly. Acid-alcohol, used to destain specimens, is prepared using 65% distilled water, 35% ethanol, and 12 drops of 12M hydrochloric acid per 300 ml water/ethanol mixture. Acid alcohol should not be reused and should be disposed of according to local and federal regulations.

A series of increasingly concentrated glycerin solutions (25, 50, 75, and 100%) is used to hydrate stained thin sections to prepare them for mounting. Distilled water is added to 100% glycerin to make the glycerin solutions (25, 50, and 75%). Each glycerin solution can be stored in a separate container and reused, although the weaker solutions (25 and 50%) are more prone to mold formation and must therefore be changed more frequently.

Kaiser glycerin jelly is used to adhere stained specimens to glass slides and affix coverslips. It is made using 40 ml distilled water, 7 g Knox gelatin, 50 ml glycerol, and 10 ml Listerine antiseptic mouthwash (any flavor which has thymol as an active ingredient). The water and glycerol are mixed together, then sprinkled with the gelatin, and allowed to sit for 5 min. The solution is then melted at low heat using a hot plate or double boiler (gelatin's melting point is 40°C). After the gelatin has dissolved, the solution is removed from the heat and Listerine added, stirring slowly to prevent formation of bubbles. The Kaiser glycerin jelly solution is then allowed to cool until it is completely solid prior to use.

Staining

Vertebra thin sections are placed in histology tissue cassettes labeled with specimen numbers. Multi-chamber cassettes may be used to prepare many specimens simultaneously if desired.

Cassettes are placed in a large beaker on an orbital shaker and fully immersed in RDO Rapid Decalcifier (Apex Engineering Products Corp.). A partially full bottle of water or Erlenmeyer flask may be nested within

the beaker to keep the cassettes submerged. Sectioned vertebrae should be soaked in RDO until softened (flexible) and yellowed. It is recommended to check sections after 5–10 min and re-immerse in RDO if not adequately decalcified. In general, larger vertebra sections require longer soak times, but a total soak time of roughly 15 min is adequate for most specimens. Soaking too long will cause the sections to decalcify to the point where annuli are not visible after staining. Once the specimens are sufficiently decalcified, cassettes are removed from the RDO and placed in a beaker under running water for 1 h. RDO can be reused until precipitates form. Following decalcification, cassettes need to be soaked in 100% distilled water for at least five minutes, although the process can be stopped here overnight.

The following seven steps need to be conducted sequentially, i.e., no overnight breaks. Solutions are placed in a beaker on an orbital shaker to ensure constant movement.

1) Soak the cassettes in modified Harris hematoxylin (Richard-Allen or Thermo Fisher Scientific) for about 8 min. Total soak time may vary depending on size and thickness of vertebra sections, and it is recommended to do some test samples to determine the best soak time to achieve optimal staining. Fresh hematoxylin should be used because this chemical loses potency after expiration. Precipitates may sometimes form within open containers of hematoxylin and can be filtered out using conical paper coffee filters.
2) Rinse the cassettes in a laboratory sink by soaking in multiple water baths until the water runs clear. The staining level should be checked for adequacy by examining sections under a dissecting microscope. Staining should be uniform and dark, and annuli should be visible although contrast between light and dark bands may be low. If additional staining is required, Steps 1 and 2 can be repeated until the desired level of staining is achieved.
3) De-stain the specimens with acid alcohol solution for about 4 min to make the banding pattern more visible and improve contrast between light and dark bands.
4) Place the cassettes in a tub filled with water in the sink and rinse using agitation for about 1 min. Again, check sections under the microscope to ensure de-staining is adequate. A pattern of alternating light and dark bands, purple or red in color, should be evident. If necessary, Steps 3 and 4 can be repeated, although the time spent in acid alcohol may be adjusted based on staining strength.
5) Rinse the cassettes in the sink under running water for 10 min.
6) Soak the cassettes in distilled water for 2 min.
7) Soak the cassettes in gradually increasing concentrations of glycerin: 10 min each in 25, 50, 75, and 100% glycerin. Vertebrae can be left in 100% glycerin overnight if there is insufficient time for slide-mounting.

Specimen Mounting

To mount specimens, a stained vertebra thin section and a small (pea-sized) amount of Kaiser glycerin jelly is placed next to each other on a labeled glass slide. The slide is then placed on a hot plate set to the lowest temperature that will melt the jelly in a period of 30–60 sec. The temperature should be set as low as possible to prevent formation of air bubbles, which can occlude specimens.

Once the jelly is melted, a coverslip is placed over the vertebra. It is best to hold the cover slip at roughly a 30° angle to the slide, touching the jelly, and then slightly drag it towards the specimen, which will cause the jelly to melt around and underneath the specimen when the cover slip is placed on top (i.e., there is no need to move the vertebra on top of the melted jelly).

The slide is then allowed to cool completely. Excess jelly that leaks from under the cover slip should be removed using a razor blade so the slides won't stick together during storage.

Age Determination

Dorsal Fin Spines

As described, accurate age estimation of spiny dogfish spines is difficult because the spines are external to the body and are often broken or worn, resulting in "missing" annuli, the number of which must be estimated using regression methods based on morphometric relationships. Several different measurements taken on each spine (Fig. 3) are used to calculate the number of potentially missing annuli. Measurements can be taken using either calipers or image analysis software, but the method should be consistent for all specimens in a given study.

In the Ketchen (1975) approach, the two key measurements used in estimating lost annuli are the enamel base diameter (EBD) and the diameter at the last readable point (LRP, also called the "no-wear point" and the diameter at this point is LRD) (Fig. 3). For the Cheng (2012) approach, the mid-point measurement (MID) is used in addition to the EBD and LRD (Fig. 3). The MID is somewhat subjective, based on the reader's interpretation of the spine, and represents the location along the spine where the reader feels that the readability of the spine degrades and confidence in the interpretation of annuli decreases. Three additional measurements, total length (TL), stem length (SL), and spine base diameter (SBD) (Fig. 3) can be used for other applications such as determining relationships with animal size; however, they are not necessary for estimating the number of missing annuli.

Further descriptions of the spine reference its orientation in situ (Fig. 4). The enamel on the tip (dorsal edge) is typically the most worn portion of the spine. Wear progresses ventrally from the anterior to the posterior edge of the spine. It is important to accurately identify the LRP, which is the most dorsal point of enamel on the anterior edge (Fig. 5A). The LRD is measured on the enamel between the anterior

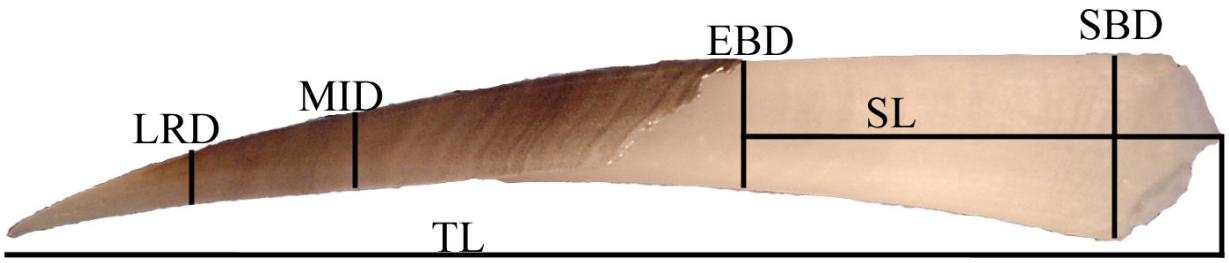

Figure 3.—Measurements taken for each fin spine: LRD=last readable point diameter; MID=mid-point diameter; EBD=enamel base diameter; SBD=spine base diameter; SL=stem length; TL=total length.

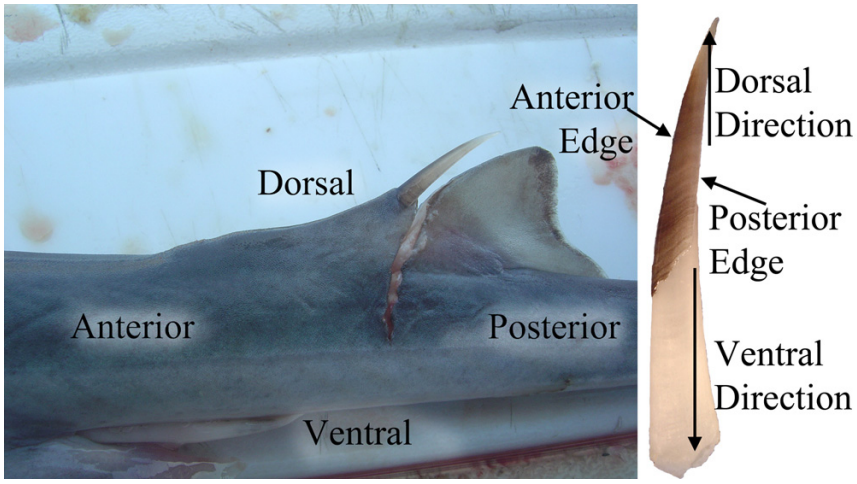

Figure 4.—Fin spine orientation.

and posterior edge (Fig. 5B). In general, a relatively larger LRD indicates a greater degree of wear.

If the LRP is improperly identified, over- or underestimation of the number of worn annuli can occur. For example, if the location of the annulus just ventral to the worn enamel is assumed to be the LRP, then the information in the area between that annulus and the worn edge of the enamel is lost, resulting in a potential overestimate of the number of worn annuli. Conversely, if annuli are evident dorsal to the worn edge of the enamel, as in both spines in Figure 5, it is inappropriate to identify the LRP dorsal to the worn edge of the enamel. This is because it is not possible to accurately measure the spine diameter at locations lacking enamel. Thus, measuring the diameter at this location would result in an underestimation of the true diameter at that point and a potential underestimation of the number of worn annuli.

Annuli are laid down as the spine grows in length. The oldest annuli are at the dorsal tip and those most recently formed are at the ventral edge of the enamel. The enamel gland deposits enamel as the spine grows from the base. In periods of slower growth annuli can be close together and sometimes appear to be stacked upon one another (Fig. 6). Growth patterns on the enamel do not appear to match those on dentine or the base cones (white structure) of the spine and the deposition mechanisms are different (Irvine et al., 2006). Thus, it is not possible to use the base cones or dentine to verify when annuli appear stacked on top of each other.

To estimate spine age, the dark bands are counted. For some spines, this is a relatively easy task (e.g., Fig. 5), but for others it can be much more challenging. In addition to being stacked upon each other (Fig. 6), annuli can appear compressed and almost indistinguishable in portions of the spine (Fig. 7). Annuli can also be very faint or diffuse, especially closer to the tip (Fig. 8A). For smaller animals, it appears that there are multiple "false annuli," or "checks," at the base of the spine. These fade with age and are less noticeable in older animals. This is likely caused by feeding pulses occurring in years of faster growth. While spiny dogfish are generalist feeders, they are also opportunistic and have been documented gorging when seasonal food sources are abundant (Tribuzio, 2010). For larger animals, the dark bands near the base are more distinct and are more likely to be true annuli. It is also possible to find spines with a bold white band at the enamel base (Fig. 8). This is the area where new annuli form but the enamel has not been laid down yet. If a dark area appears ventral to this white band, it is not counted in the age estimate.

There are a number of methods that have been used to make identifying annuli easier. Newer ventral portions of the enamel tend to have ridges associated with the dark bands (the older ridges, located more dorsally, are generally more worn), and dynamically adjusting the light source at different angles can be helpful in seeing the ridges. Only the most prominent ridges should be counted, as the smaller ridges between them are likely false

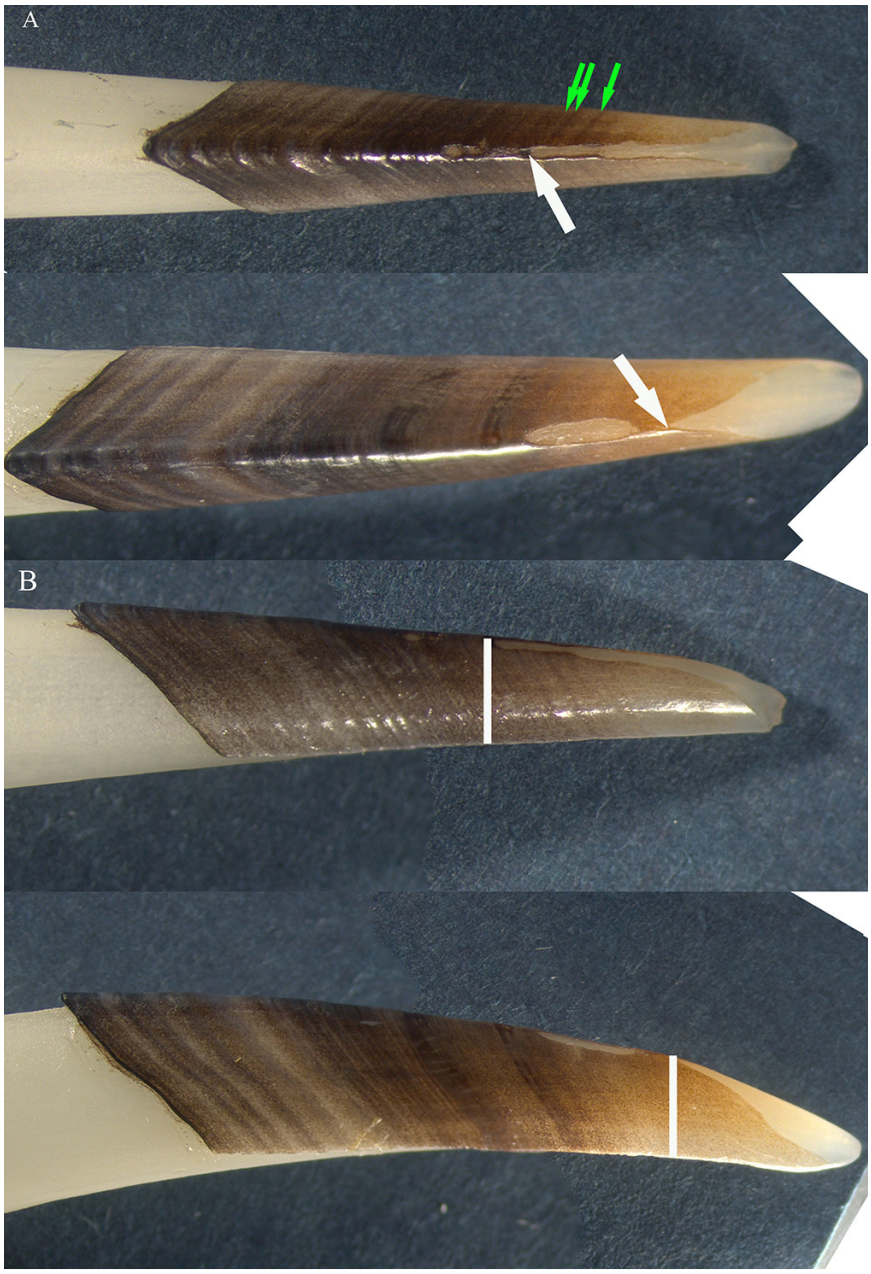

Figure 5.—A) Identification of the Last Readable Point (LRP) on worn fin spines from two different spiny dogfish. White arrows point to the LRP on the anterior edge of the enamel. The three small green arrows on the top fin spine highlight dark bands that should not be counted by the age reader because they are dorsal to the LRP. B) Side view, with white lines showing measurements of the fin spine diameter corresponding to the LRP.

annuli. A thin layer of mineral oil is also helpful to increase light reflection. When viewing the dorsal portion of the spine, where the enamel is thinner, shining reflected light on the spine from directly above can make it easier to see the color differences between dark bands while providing more contrast.

Additionally, spines can be viewed either dorsally or laterally to assist with identifying annuli and aid in following annuli around the spine. True annuli typically encircle the spine, whereas false annuli are often incomplete. Displaying spines on a monitor and either zooming out or standing back from the screen may aid in the identification of prominent dark bands. Applying color filters to high-resolution images of spines using image software such as Adobe Photoshop can also be helpful (Fig. 9). There are unlimited combinations of potential image transformations, and different transformations may be used for examining different portions of the spine. Transmitted light or polarizing filters have also been found to be useful when examining small or embryonic spines.

Interpretation of annuli can vary substantially between readers. To address this imprecision, a reference set of photographs of known-age specimens has been assembled, the ages of which were validated using bomb-radiocarbon dating (Campana et al., 2006). These specimens range in difficulty of interpretation from “easy” (Fig. 8A), where annuli are consistently spaced and relatively easy to identify, to “hard” (Fig. 8B), where annuli are more closely spaced and difficult to discern from one another on certain locations of the spine. These high resolution images and a set of calibration spines, for which multiple readers have come to an agreed age, are stored as part of the permanent collection of the NMFS Alaska Fisheries Science Center’s Age and Growth Program and are available for future calibrations between readers and laboratories. Such reference sets are invaluable for evaluating accuracy of age estimates and

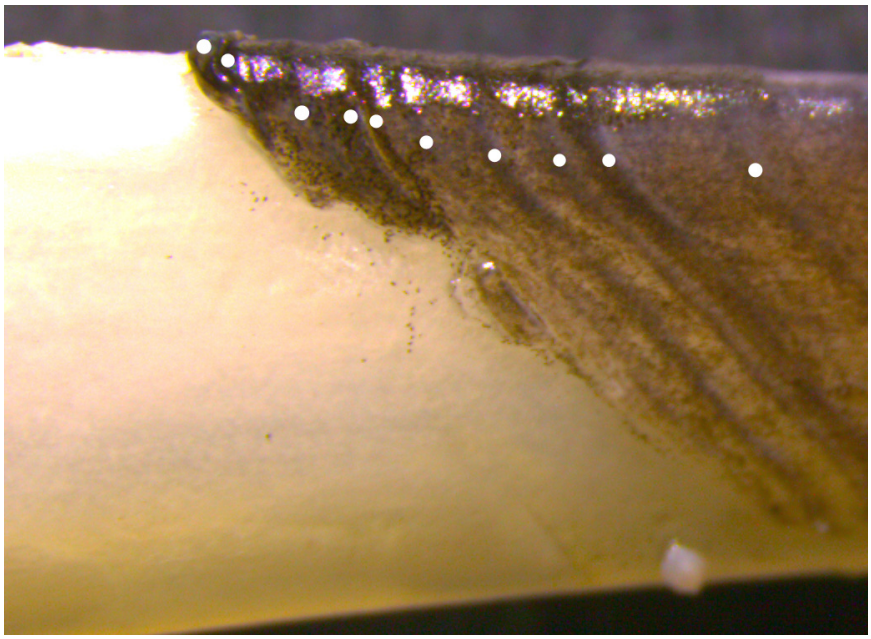

Figure 6.—Magnified image of spine enamel showing annuli stacked on top of one another. White dots identify ridges corresponding to annuli.

periodically testing inter-reader and inter-laboratory precision.

Vertebrae

Stained spiny dogfish vertebra thin sections are typically viewed with transmitted light, and fish age is estimated by counting the dark purple bands (Fig. 10). As with viewing spines, a number of filters either attached to the microscope or applied in imaging software can enhance contrast between light and dark bands. The morphological features of sectioned vertebrae are shown in Figure 11. Annuli are most easily seen on the corpus calcareum and radiate outwards from the focus. In many shark species, annuli can be followed through the intermedialia to the other edge of the corpus calcareum, but the intermedialia are absent or inconsistent in the *Squalus* species examined to date.

Vertebra measurements, if desired, should be taken along a single transect. The vertebra radius is taken from the focus (center) to the distal (outer) edge of the vertebra along the corpus calcareum (Fig. 11). Measurements are important for determining periodicity (i.e., marginal increment analysis), confirming location of the birthmark, and correlations between growth and annulus deposition.

Vertebrae accrete material around the focus as the shark grows. The annuli located closest to the focus are the earliest deposited, while those located furthest are the most recently deposited (Fig. 12). The growth pattern of an individual changes as it gets older, with the annuli closer to the focus being broader and more diffuse than the more distal (outer) annuli, which are thinner with sharper edges. The distance between annuli also decreases with increasing distance from the focus, such that annuli can become quite compressed in appearance near the distal edge of the vertebra (Fig. 12).

To determine age, first the location of the birthmark (age-0) must be identified. The birthmark usually corresponds with an angle change at the interface of the corpus calcareum and intermedialia (Goldman, 2004) (Fig. 12). The birthmark is excluded from the annulus count, and the following dark band is the first one counted in the age estimate. The birthmark's location may be confirmed by measuring vertebra radii of full-term embryos obtained from gravid females and comparing the average to measurements from the focus to the presumed birthmark on adult specimens.

Interpreting annuli on vertebrae, as with spines, is essentially counting the number of dark bands deposited. In some cases, determining what constitutes an annulus can be challenging,

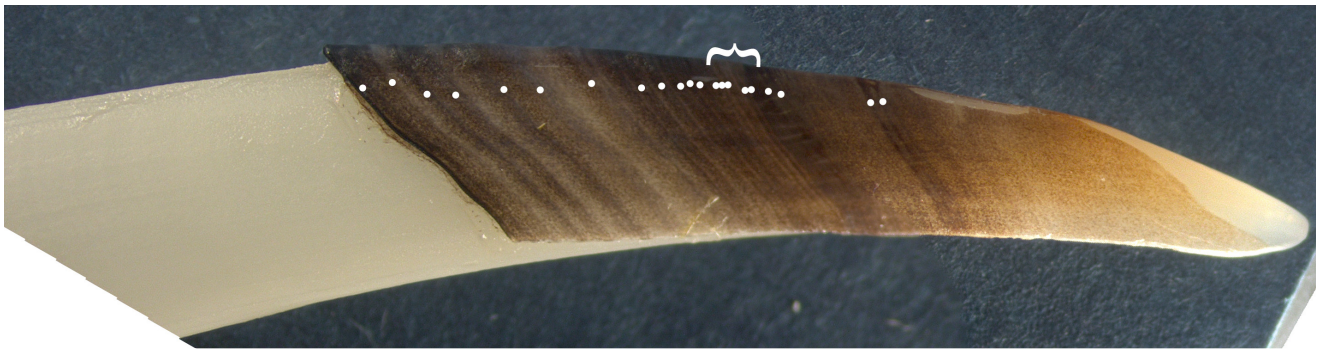

Figure 7.—Spine showing area with annuli very closely spaced or compressed. White dots identify selected individual annuli.

Figure 8.—Fin spines that are A) easy and B) difficult to interpret, with ages validated by bomb radiocarbon dating. Dots represent annuli. Images courtesy of the Canadian Shark Research Laboratory, Bedford Institute of Oceanography, Nova Scotia, Canada.

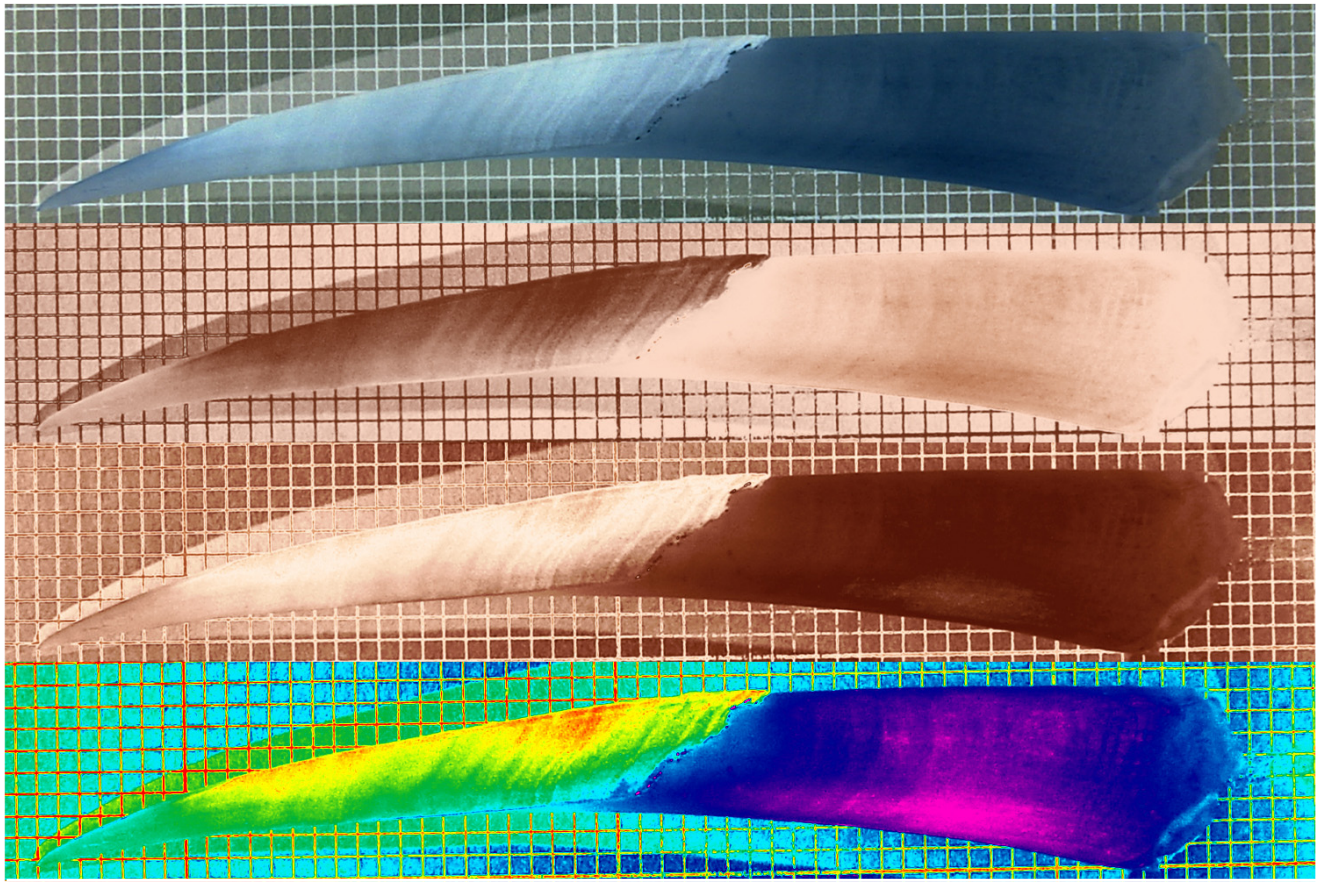

Figure 9.—Image analysis. Examples of four possible image transformations (performed using Adobe Photoshop Elements). From the top: inverted color, inverted then copper gradient, copper gradient only, rainbow gradient. Backgrounds are 1 mm x 1 mm grids.

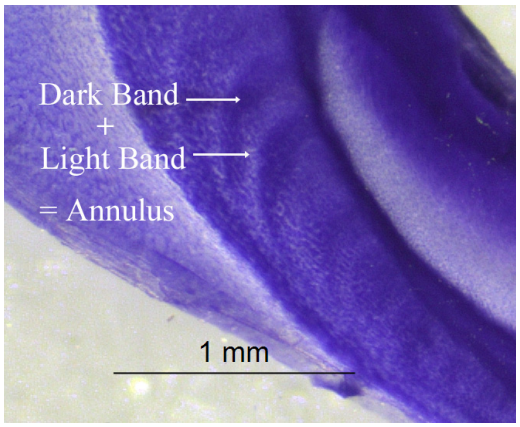

Figure 10.—Identifying bands and annuli on vertebrae.

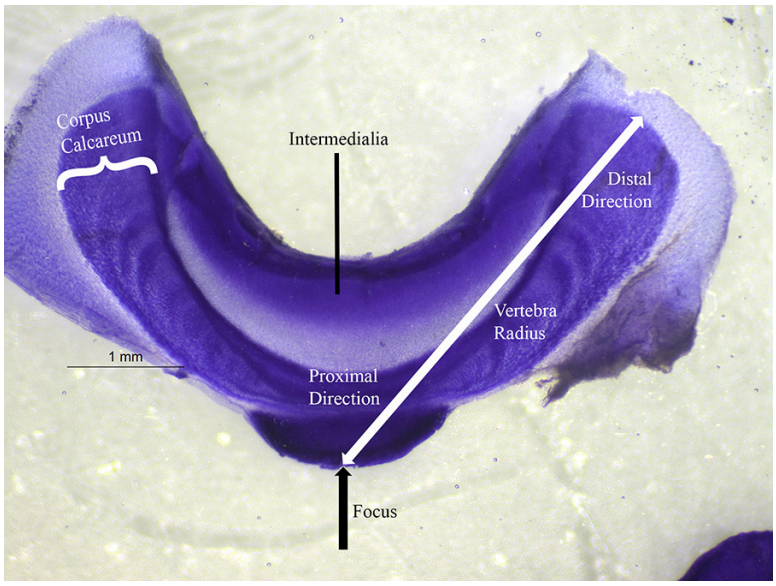

Figure 11.—Vertebra anatomy, with orientation and vertebra radius measurement shown.

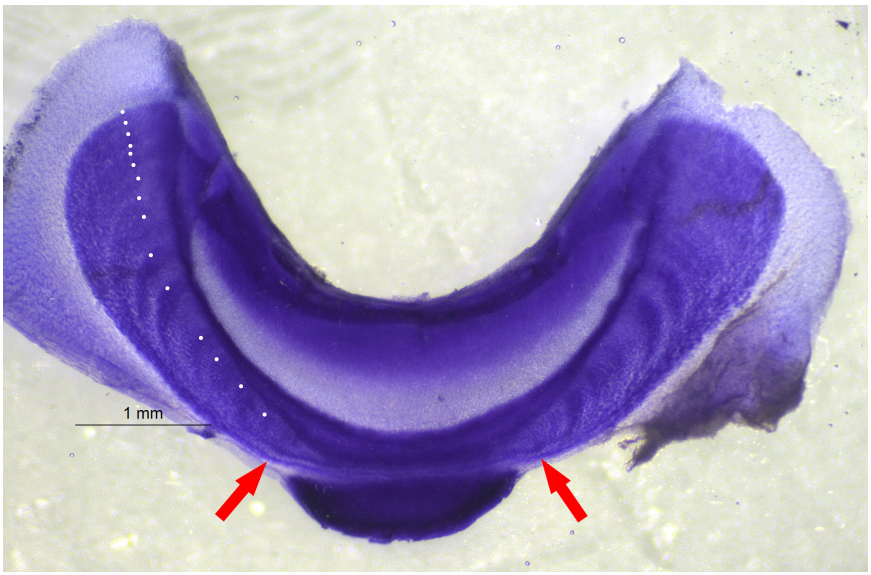

Figure 12.—Sectioned and stained vertebra. Each dark band is marked with a white dot. The birthmarks are noted with red arrows. Note that annuli become more compressed with increased distance from the focus.

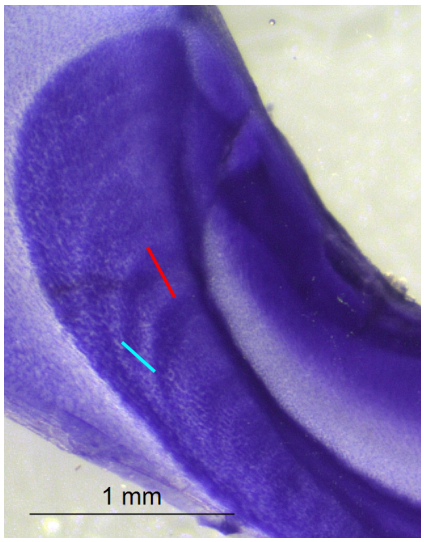

Figure 13.—Annulus width on vertebra (indicated by colored lines). Note the change in distance between annuli from the edge of the corpus calcareum nearest (red arrow) and furthest from the intermedialia (blue arrow).

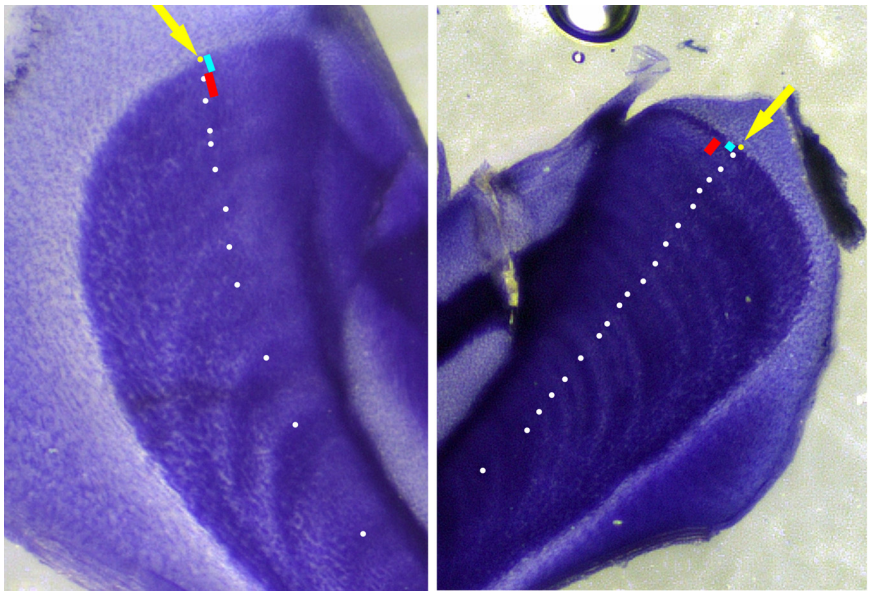

Figure 14.—Two different vertebrae with dark bands and marginal increment ratio (MIR) measurements identified. The blue line represents the edge width (EW) and the red line represents the last annulus width (LAW). The yellow arrow and dot mark the edge of the centrum.

while others are relatively easy to differentiate. By examining vertebrae using some of the following techniques, the reader can have more reproducible age estimates. If time allows, examining more than one vertebra per animal may also aid in age estimation.

Several criteria can assist with annulus identification. Annuli tend to be clearer and less compressed near the inner edge of the corpus calcareum (adjacent to the intermedialia) than on the outer edge (Fig. 13). However, the outer edges of the corpus calcareum can occasionally provide more evidence as there can be a slight notch corresponding to each dark band at the outer edge.

One must take into account the location on the vertebra in relation to the focus when determining whether a dark band is a single diffuse annulus or actually consists of multiple annuli, remembering that broad, diffuse annuli tend to be located nearest the focus. With increasing distance from the focus, annuli tend to become more condensed, requiring higher magnification. However, the vertebra may look somewhat granulated under higher magnification. It is sometimes easier to distinguish annuli near the focus by reducing magnification when viewing that portion of the vertebra.

The vertebra technique is relatively new for spiny dogfish and must be verified (or validated). Unlike spines, known-age vertebra samples are unavailable. The marginal increment ratio (MIR) is one method that is easily implemented and can be used to verify the annual periodicity of growth patterns. To compute the MIR, the reader measures the edge width (EW) and the width of the last fully formed annulus (LAW) (Fig. 14). The ratio of EW (blue line in Fig. 14) to LAW (red line in Fig. 14) is then averaged by month of collection to determine if the pattern of deposition is annual.

Similar to spines, a set of specimens aged by multiple laboratories and for which a consensus age was reached have been archived in a calibration collection, stored as part of the permanent collection of the NMFS Alaska Fisheries Science Center's Age and Growth Program.

Acknowledgments

The authors would like to give special thanks to Cal Blood, Sandy Rosenfeld, and Patrick McDonald, who helped with the development of this paper and participated in method development and inter-laboratory studies. This project was funded by the North Pacific Research Board (www.nprb.org), project number 1106 and is publication number 463. The findings and conclusions in the paper are those of the authors and do not necessarily represent the views of the National Marine Fisheries Service, NOAA.

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