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Proceedings of the Workshop on Aging and Sexing American Eel

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Edited by
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Atlantic States Marine Fisheries Commission

Convened by:
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November 30-December 1, 2000
Laurel, Maryland

Preface

This document was prepared in cooperation with the Atlantic States Marine Fisheries Commission's American Eel Management Board, Technical Committee, Plan Development Team, Stock Assessment Subcommittee and the Advisory Panel.

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Acknowledgments

This report is the result of a Workshop on Aging and Sexing American Eel held on November 30 and December 1, 2000, in Laurel, Maryland. The workshop was convened and organized by a Workshop Steering Committee composed of: Julie Weeder (Maryland Department of Natural Resources), Victor Vecchio (New York Department of Environmental Conservation), Gail Wippelhauser (Maine Department of Marine Resources), and Heather Stirratt (Atlantic States Marine Fisheries Commission).

Special thanks are extended to the session moderators, speakers, and participants whose commitment of valuable time and effort helped make the workshop a success. In particular, the Steering Committee would like to acknowledge Julie Weeder, Wendy Morrison, Dr. Keneth Olivera, and David Secor for moderating the sexing and aging breakout sessions. In addition, the Steering Committee would like to thank Commission staff Jeffrey Brust, Carrie Selberg, and Heather Stirratt for facilitating the two-day workshop.

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Overview

The purpose of the American Eel Sexing and Aging Workshop was to present current knowledge on sexing and aging techniques to the ASMFC's American Eel Technical Committee for application along the Atlantic coast. Technical Committee determination of which technique is most appropriate for employment in a coastwide stock assessment for American eel will follow a subsequent privately sponsored workshop to be for the purposes of technique validation.

This report summarizes the sexing and aging techniques presented during the workshop and seeks to document ASMFC American Eel Technical Committee discussions regarding these techniques.

Sexing Techniques

Gonad Squash Technique[1]

Sex can be identified in American eel using a sequence of criteria that were designed to distinguish the sex of both yellow and silver phase eel while minimizing the number of eel sacrificed. Male eel (from the Northeastern US) rarely exceed 400 mm in total length while female eel rarely undergo the silver metamorphosis before reaching 400 mm. This size cut-off is >99% effective in distinguishing the sexes of silver and yellow eel >400 mm. Yellow eel smaller than 400 mm require macroscopic examination of the gonads. Gonads are dissected from the eel and observed on a microscope slide using either a dissecting microscope or a compound scope at low 4X-10X magnification. Gonads are classified according to the descriptions of Beullens et al, 1997.

In the case of the smallest eel (usually <20 mm) a rudimentary histological examination (gonad squash method) can aid in distinguishing gonadal tissue types. In the squash method small sections of hanadal tissue are placed on a glass slide with a drop of aceto-carmine stain for 30 seconds and then pressed ("squashed") with a glass cover slip or a second glass slide. This process distinguishes lipid from the gonad and may show early goycyte stages.

Additional Literature Available for Technique Review:

Olivera, K. and J. D. McCleave. 2000. Variation in population and life history traits of the American eel, *Anguilla rostrata* in four rivers in Maine. U.S.A. Environmental Biology of Fishes 59:141-151.

Krueger, W.H., and K. Olivera. 1999. Evidence for environmental sex determination in the American eel, *Anguilla rostrata*. Environmental Biology of Fishes 55: 381-389.

Olivera, K. 1999. Life history characteristic and strategies of the American eel, *Anguilla rostrata*. Canadian Journal of Fisheries and Aquatic Sciences 56: 795-802.

Krueger, W.H., and K. Olivera. 1997. Sex, size and gonad morphology of silver American eel*, Anguilla rostrata*. Copeia 2: 415-420.

Olivera, K. 1997. Movements and growth rates of yellow phase American eels, *Anguilla rostrata*, in the Annaquatucket River, Rhode Island. Transactions of the American Fisheries Society, 126 (2): 638-646.

Beullens, K., E.H. Eding, P. Gilson, F. Ollevier, J. Komen & C.J.J. Richter. 1997. Gonadal differentiation, intersexuality and sex ratios of European eel (*Anguilla anguilla L.*)maintained in captivity. Aquaculture 153:1-2.

[1] Workshop participants discussed the fact that the squash method is most appropriate for sexing silver eels, whereas the histological technique is more definitive when sexing yellow eels.

Histological Technique

Eels longer than 40 cm (from tidal Maryland) are 100% female. Therefore, only eels shorter than 40 cm are sexed. Only fresh gonadal tissue is used. When gonads are obvious upon dissection of specimens, a small amount of gonadal tissue is removed. When gonads are not readily discernible due to the eel's small size, a cross section of the body 3-5 mm wide is taken. The first cut is made 10 mm anterior of the anal opening and the second cut 5 mm anterior of the first cut. Unneeded muscle tissue is often trimmed from the sample before preservation. Tissue is placed in a histological cassette and immediately preserved in 10% buffered formalin for a minimum of 72 hours. Cassettes are transported in formalin to the histology facility, where the tissue is dehydrated, decalcified, and embedded in paraffin. Also at the facility, thin tissue sections (3-4 Fm) are placed on microscope slides and stained with hematoxylin, then counterstained with eosin. A cover slide is then affixed to each slide. Gonadal tissue is later distinguished from other organs and the sex is determined.

Literature for Interpretation of Gonadal Tissue:

Beullens, K., Eding, E. H., Gilson, P., Ollevier, F., Komen, J. and C. J. J. Richter. 1997. Gonadal differentiation, intersexuality and sex ratios of European eel (Anguilla anguilla L.) maintained in captivity. Aquaculture 153: 135-150.

Todd, P. R. 1981. Morphometric changes, *gonad histology, and fecundity estimates in migrating New Zealand freshwater eels (*Anguilla spp.) *New Zealand Journal of Marine and Freshwater Research* 15: 155-170.

Aging Techniques

Embedding and Sectioning Technique (with Etching and Staining)[2]

The determination of age and growth of American eel requires the use of otolith microstructure analysis. The initial steps in preparing otoliths are dissection from the eel, cleaning (70% ethanol), and then dry storage. These otoliths are then embedded in epoxy and a transverse section (0.2 mm thick) through the nucleus that is perpendicular to the sulcus is made using a double bladed slow speed saw. The section is mounted on a glass slide and polished with a series of fine grade lapping films. Sections are then etched in 5% EDTA for 2-5 minutes and stained with 0.1% toluidine blue for 2-5 minutes to enhance annuli visibility by adding relief and color. Otoliths are examined wet and report staining is often necessary. This process results in otolith sections that can be used both counting and increment analysis.

Additional Literature Available for Technique Review:

Olivera, K. and J.D. McCleave. (2000) Variation in population and life history traits of the American eel, *Anguilla rostrata*, in four rivers in Maine, U.S.A. Environmental Biology of Fishes 59: 141-151.

Olivera, K. (1999) Life history characteristic and strategies of the American eel, *Anguilla rostrata*. Canadian Journal of Fisheries and Aquatic Sciences. 56: 795-802.

Olivera, K. (1996) Field validation of annular ring formation fo the American eel, *Anguilla rostrata*, using tetracycline treated otoliths. Fishery Bulletin (1): 186-189.

Grinding and Polishing Technique

Both sagittal otoliths are removed from each specimen, cleaned for five minutes in 10% bleach, and stored dry. One otolith is lightly bonded to a microscope slide with CrystalBond, a thermoplastic adhesive (aremco@aremco.com; www.aremco.com). The slide is heated on a hot plate and the adhesive is then heated to a liquid state on the slide. The otolith is placed in a convex position on the slide. The bottom and sides of the otolith are affixed to the slide, and the adhesive is then lightly drawn over the dorsal surface of the otolith, which fills in tiny crevices on the surface of the structure and provides great clarity. The otolith is examined under a dissecting microscope using both reflected and transmitted light and an external fiberoptic light source. It is sometimes possible to discern opaque zones on the otolith at this time. Viewing the underside of the otolith by turning the slide over is sometimes helpful. If annuli are not sufficiently visualized, the dorsal surface of the otolith is lightly polished on moistened 600 grit silicon carbide wet-dry sandpaper (available at hardware stores). Frequent examination of the otolith is necessary to ensure that sanding does not penetrate the primordium

[2] Workshop participants discussed the fact that the embedding and sectioning method is most appropriate for sexing older eels, whereas the grinding and polishing technique is more definitive when aging younger eels.

(core). Immersion oil is sometimes applied sparingly to the sanded surface to fill in newly exposed crevices. If further visualization is necessary, the slide is placed on the hot plate to liquefy the adhesive and the otolith is turned over. The entire process is repeated on the opposite surface.

Additional Literature Available for Technique Review:

Sinha, V. R. P. and J. W. Jones. 1967. On the age and growth of the freshwater eel (*Anguilla anguilla*). *J. Zool., Lond*. 153: 99-117.

Chisnall, B. L. and J. M. Kalish. 1993. Age validation and movement of freshwater eels (*Anguilla diffenbachii* and *A. australis*) in a New Zealand pastoral stream. *New Zealand Journal of Marine and Freshwater Research* 27: 333-338.

Issues of Bias, Precision, and Accuracy

ASMFC American Eel Aging Workshop
Secor and Morrison (CBL) Precision Exercise

Calibration

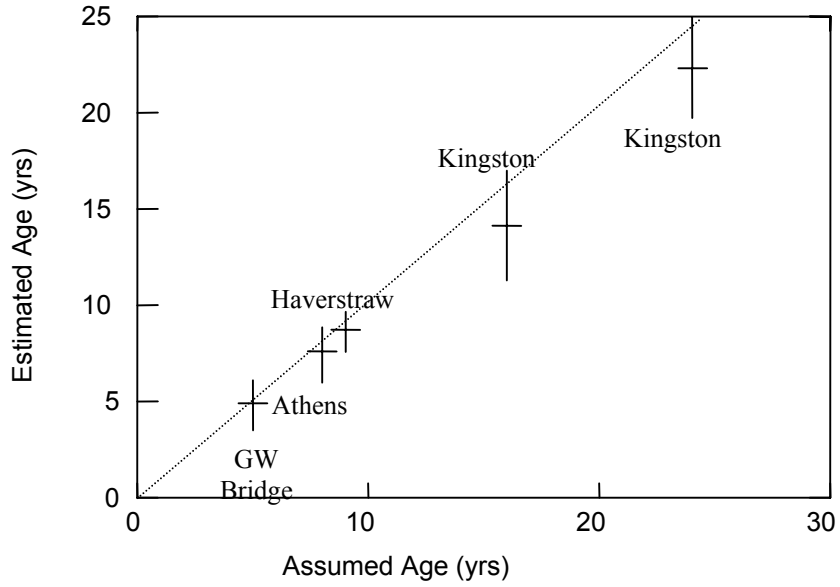
Thirteen ASMFC workshop participants independently interpreted 5 American eel otolith sections that were stained and etched. Means and s.e.s of their 13 counts are plotted against the assumed age (interpreted from single experienced CBL investigator). Identity line is shown. Precision among readers slightly decreased with assumed age. Yellow phase American eels were collected from the Hudson River; sites of collection are shown.

Precision

Comparison of 65 paired counts made by 13 ASMFC workshop participants of 5 otolith sections that were stained and etched by 13 readers:

Absolute Precision = 0.6±0.7years; range 0,3

Paired t-test for no difference in paired counts: P=0.90; no tendency to increase or decrease second count over first one.



Group Discussions

Discussion Questions

Each technique for assessing sex and age were discussed in terms of the following three primary questions:

1. What are the time limitations associated with this technique? How will time requirements affect state personnel's ability to conduct this technique?
2. What additional laboratory/field equipment is required to conduct this technique?
3. What are the funding requirements to perform this technique? Are monetary resources available at the state in question to support this technique?
4. What additional personnel requirement(s) (i.e. employee labor) is(are) associated with this technique?

Conclusions

Reproductive Samples

The squash method and the histology method were compared relative to the time, personnel, and funding required to implement each method. Samples for both methods can be collected cheaply and quickly in the field. Processing samples using the squash method is much quicker and much less costly than the histology method, but requires greater expertise to read the samples. Processing histological samples requires time and funding ($3-5.00/slide) to produce slides, but the samples are easier to interpret than samples from the squash method. Cost may be reduced ($1-2.00/slide) if samples are processed on site. Neither method requires substantial personnel. It was noted that all sampling costs and parameters are dependent on sample size, which will require extensive discussion.

Participants were not too optimistic on being able to collect and process reproductive samples of eels at this time. Eel samples are not a funding priority in most states, and there are little available resources to sample them voluntarily. Sampling now is voluntary, but may become a requirement in the future to support a stock assessment. The more data that is collected now, the less the requirements later. Participants were concerned with collecting data that may not be useful in the stock assessment, depending on the type of assessment chosen. Participants noted that above a certain size, there may not be a need to sample all females.

Age Samples

Similar questions were asked relative to collecting and processing American eel age samples. All methods are comparable in terms of overall cost and personnel needs, but differ where the labor and cost are concerned. Aging methods are comparable in results to approximately age 6. The choice of method used may be decided based on the survey objectives and the population being sampled.

Participants had several concerns regarding age sampling of eel. There was concern whether age samples from an estuarine population would be representative of a fresh water population, and if not, whether an age-length key would be relevant. There was also a concern whether biological sampling through ACCSP would be sufficient in terms of spatial coverage.

There was concern also about the cost and time required to process samples. Some of the equipment required to read samples would be cost preventive. It was suggested, however, that some of the equipment might be borrowed from other labs or agencies. There was some discussion on having a single or rotating "clearing house" for processing all samples. This way, not every agency would be required to purchase all necessary equipment and cost may be shared among the agencies.

It was noted that these issues are not specific to aging American eel. Many species require age samples to support stock assessments and fishery management plans. It was suggested that there should be an infrastructure to address age and other samples for multiple species. Participants proposed developing an ASMFC committee on age determination to address these issues. To support this initiative, the agencies involved with American eel will take an inventory of available aging equipment at their agency.

Agencies are currently capable of collecting and archiving American eel age samples until it becomes more feasible to process the samples, and most agencies agreed that they will do so. Participants are concerned that validation is still necessary. There is also a need for a discussion on sample size, which should be conducted by the stock assessment committee. Gerard Chauput (Canada) was suggested as a potential SAC member.

Appendices

Appendix 1: Workshop Agenda

ASMFC American Eel Sexing and Aging Workshop
November 30- December 1, 2000
Atlantic States Marine Fisheries Commission
Laurel, MD

November 30, 2000

1:00 – 1:30pm	Welcome/Opening Statements – Heather Stirratt (ASMFC)
1:30 – 2:30pm	Sexing Presentations (20 min. presentation; 10 min. question/answer period) 1. Ken Olivera (University of Massachusetts) ➢ Gonad Squash Technique 2. Julie Weeder (Maryland Department of Natural Resources) ➢ Histological Technique
2:30 – 2:45pm	Break
2:45 – 4:15pm	Hands On Break Out Sessions (Technical Committee members will be divided into two groups; each group will spend 45 minutes with a given technique/speaker and then move to the next technique station)
4:15 – 4:45pm	Group Discussion – Issues and Conclusions
4:45pm	Adjourn

December 1, 2000

10:00 – 10:30am	Opening Statements - Heather Stirratt (ASMFC)
10:30 – 12:00pm	Aging Presentations (20 min. presentation; 10 min. question/answer) 1. Ken Olivera (University of Massachusetts) ➢ Embedding and Sectioning Technique with Etching and Staining 2. David Secor / Wendy Morrison (Chesapeake Biological Laboratory) ➢ Transverse Sectioning Technique ➢ Micro-chemistry Analysis 3. Julie Weeder (Maryland Department of Natural Resources)

- Grinding and Polishing Technique

12:00 – 12:45pm	Lunch (Catered on site)
12:45 – 3:00pm	Hands On Break Out Sessions (Technical Committee members will be divided into three groups; each group will spend 45 minutes with a given technique/speaker and then move to the next technique station)
3:00 – 3:15pm	Issues of Bias, Precision, Accuracy (David Secor)
3:15 – 4:00pm	Group Discussion – Issues and Conclusions
4:00pm	Adjourn

Appendix 2: List of Workshop Participants

Participants	*Contact Information*
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John McClain	NJ FG&W Route 9 Mile Marker 51 PO Box 418 Port Republic, NJ 08241
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Jennifer Temple	RI DEM 1231 Succotash Rd. Wakefield, RI 02879
Heidi O'Riordan	NY DEC 205 Belle Mead Rd. East Setauket, NY 11733
Victor Vecchio	NY DEC 205 Belle Mead Rd. East Setauket, NY 11733

Julie Weeder	MD DNR 301 Marine Academy Dr. Stevensville, MD 21666
Gail Wippelhauser	ME DMR 21 State House Station Augusta, ME 04333
David Secor	University of Maryland PO Box 38 Solomons, MD 20688
Ken Olivera	University of Massachusetts 285 Old Westport Rd. North Dartmouth, MA 02747
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Jeffery Brust	ASMFC 1444 Eye St. NW Sixth Floor Washington, DC 2005
Carrie Selberg	
Heather Stirratt	

Appendix 3: Archiving and Storage Protocol (Biological Aging Samples)

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A special manuscript report of the Glenora Fisheries Station (GFS series)

Abstract. Removal, cleaning, drying, and archival storage of fish otoliths are detailed. The advantages and disadvantages of various methods are reviewed, with discussion of cause and effect. The pros and cons of air-dried versus aqueous storage methods are considered. Currently it appears to be best simply to air-dry otoliths and store them in polypropylene microcentrifuge vials. Both otoliths should be removed, but only one should be prepared and interpreted, using present procedures, and only those procedures that provide detailed, high-quality, high-resolution preparations and images should be considered. The second otolith should be archived so that it will be available for future analyses, and if chemical analysis is envisaged, then otoliths could be specially wrapped (e.g., in Parafilm) to prevent contamination during storage.

Fish otoliths, or sagittae, are calcareous accretions that are routinely used to study growth, age, and chemical environmental history of fish. Otoliths must be properly cleaned and stored if the best-quality samples and images are to be available for subsequent analyses. Over the years, problems have arisen concerning the collection, storage, and preparation of these structures. Various procedures have been used and studied, specific advantages and disadvantages have been detected, and procedures have been refined and improved. This is a review of procedures and associated problems, with recommendations acquired through considerable practical experience.

I will not provide specific details concerning the location of otoliths or their removal, although several methods are routinely practiced, depending upon species, and specific benchmarks on the cranium and head area have been developed for extracting otoliths easily, quickly, and consistently. Some prefer to use a method that involves a vertical cut with a sharp knife from the tip of the snout through to the back of the cranium, exposing the otoliths on either side of the incision. This works well in species that have lightly ossified heads, including American eels. Others prefer to make a cross-sectional incision through the top of the cranium. In small fish, this is done with sharp, pointed, surgical scissors. In large fish, this cross-sectional cut can be made with a sharp or serrated knife, sawing through the top of the cranium, no deeper than to the top of the

brain. In both cases, the cranium is split open by applying firm pressure with one hand on the anterior of the head while the other hand grasps the nape and the trunk just behind the head, breaking open the cranium. This method works well for most species, and in medium to large eels, the benchmark for this transverse cut is from 5 to 8 mm ahead of the back of the cranium. A similar method that works well in some species involves making a cross-sectional cut by etching the bottom of the cranium with a sharp or serrated knife after the gills have been removed. This method minimizes the apparent external mutilation of the carcass.

Regardless of the method of removal, it is important that both otoliths be extracted and that they be cleaned while they are still moist. In species that have relatively small otoliths, such as American eels, the otoliths are removed with fine forceps. They can be placed on soft, absorbent tissue or, as we routinely do, on the top of the extractor's other hand. The otoliths are then teased away from the soft tissue and out of the otolitic sac, if present, and away from any mucoid material. The otolith is then rolled under the tip of the finger to rub away any remaining mucus and moisture. Otoliths can then be rinsed in distilled water, although this is not routinely done for general growth and age interpretation studies.

Otoliths are stored in labelled vials. Microcentrifuge tubes are inexpensive and practical storage vials. For most species, we use 2-mL ClickSeal Microcentrifuge tubes (National Scientific Supply Company Inc., 250 York Park, Claremont, CA 91711-4883; phone 415-459-6070). These polypropylene tubes come in various sizes from several sources. Those described above are chemically inert and do not contaminate the otoliths for most current chemical analytical techniques. A wide range of microcentrifuge vials is available. The vials we use have a pebbled writing surface that can be easily labeled with a fine, permanent, waterproof marker. We leave the vials open for 24 hours so that the otoliths dry completely before the tops are closed. This is critically important because the vials are airtight, and if they are sealed while the otoliths are still moist, bacterial action and decomposition will start. This is accelerated if the otoliths are dirty but will still occur if the otoliths are clean, because they contain a protein matrix. This bacterial action and deposition produces carbon dioxide, which produces a moist, acidic condition that causes decalcification of the edge of the otolith. This damages the otolith and should be avoided.

If otoliths are especially small or if there is concern over contamination, we wrap them in Parafilm M laboratory film (American National Can, Greenwich, CT 06836). The wrapped otolith is then placed in the vial, making it easy to locate and remove from the film. The organic wrap protects larger otoliths from contamination, which may be important if they are to be used for chemical analyses. Currently we archive all otoliths in an air-dried state.

The vials can be stored in specially purchased trays or boxes with multiple compartments holding 100, 200, or up to 500 vials. Once the vials are closed, they can be placed in envelopes, although this becomes rather bulky.

If the otoliths were not clean when they were removed, this material oxidizes as it dries, forming a brown soft-tissue residue on the surface of the otolith. It is very difficult to remove the dried soft tissue, but cleaning can be attempted by soaking the otoliths for several hours in tepid water with a small amount of detergent. The otoliths can be handled with fine forceps and brushed with a soft toothbrush. If the groove, or sulcus,

remains dirty, it may be necessary to rub a needle along the sulcus, followed by additional brushing. If soft tissue has dried on the otoliths, the bond of embedding and mounting media is affected. Most importantly, the precise interpretation of the growth on the edge is affected, making it difficult to assign the growth on the edge to the current or previous year, particularly in samples caught near the beginning of the growing season.

Otoliths are composed of pure calcium carbonate in the form of aragonite. They are a calcareous accretion, neither bone nor tissue. Otoliths have been variously stored, but they should not be placed directly in scale envelopes because they are brittle and easily cracked, and this makes them useless for shape studies and for certain preparations for growth and age interpretation procedures. In addition, many kraft papers contain elements related to the pulping and bleaching process that could chemically contaminate the otolith.

Some have recommended that fish should be frozen with the otoliths left intact. Short-term freezing is probably not a problem. I have no evidence that freezing otoliths either in the fish or after extraction causes any detrimental effect for routine growth and age studies. However, freezing and thawing could affect the moisture in the otolith. The major concern is in thawing and whether the fish are properly frozen and is related to soft-tissue decomposition, which will produce carbon dioxide and acidic conditions, resulting in decalcification. It is important that there be minimal soft-tissue decomposition before the otoliths are removed. This is especially critical when working with very small larval fish. If larval fish are left unrefrigerated at ambient summer temperatures for only a few hours, decomposition can occur and otoliths can completely dissolve and disappear, leaving only the otolitic sac, devoid of its calcareous accretion. This emphasizes the importance of keeping the fish chilled or frozen at all times when the otolith is intact in the cranium and emphasizes that the otolith should be removed promptly if the carcass is to be left at ambient summer or room temperatures.

Some mount otoliths directly on glass slides, using thermal resin or epoxy. This assists in handling small otoliths but, in the case of permanent mounting with epoxy, limits future grinding and sectioning procedures. If thermal resin is used, otoliths can be removed, although this requires heat and alcohol washes, which, if used to excess, can dehydrate the otoliths.

Although many store otoliths in aqueous solutions, we prefer that they be air-dried under ambient conditions. Initially, when I was still trying to develop methods for otolith preparation procedures, I stored one otolith in liquid. I felt that one otolith should be held in as natural an aqueous condition as possible. This was an attempt to retain optical density in the otolith that was typical of removal and the intact condition. We developed and tested many solutions and came to rely upon 60% to 65% glycerol, which produced an isotonic aqueous solution where moisture was neither drawn from nor added to the otolith. Otolitic fluid would be ideal; however, it would need to be preserved to prevent bacterial action. Therefore, glycerol was chosen. More aqueous solutions of glycerol appear to clear the otolith somewhat, whereas higher concentrations of glycerol appear to dehydrate the otolith, making it slightly more opaque.

Over the years, we have had some problems with archiving otoliths in an isotonic solution of glycerol. Initially we made up the glycerol with distilled water. Over time, archived otoliths developed a brown sheen, suggesting that they had been dirty when stored. However, we knew that they had been clean and white when they were removed

and stored. Electron microprobe analysis confirmed that the chemistry of the edge of the otolith had changed and that the natural organic protein matrix had been exposed, producing the brown color. The edge of the otolith had decalcified. We realized that the calcium carbonate in the matrix had dissolved and that the organic matrix, which was impervious to acidic conditions that had developed in the isotonic solution, had been exposed. The isotonic solution had become somewhat acidic.

Over the years, we have had a number of inquiries concerning this occurrence in aqueous solutions. In this case, it occurred because the 60% glycerol had been made with distilled water, which was naturally acidic and demineralized the edge of the otolith. Subsequently we have added a drop of calcium carbonate or some marble chips to buffer the solution. This solved the problem but produced a more chemically artificial solution than we desired.

If aqueous solutions are used, they should not be acidic; depending upon the type and amount of water used, many become so naturally. They will dissolve the otolith until enough calcium has been removed to buffer the solution. The extent of the decalcification will be a function of either the acidity or the volume of the solution.

In the past, we archived one otolith in this aqueous isotonic solution of 60% glycerol and stored the other air-dried. However, we now recommend that both otoliths be stored dry: one loose in the microcentrifuge vial and the other, for future research purposes, wrapped in Parafilm. The latter is not necessary if only current growth and age preparation and procedures are anticipated. Currently we hold the wrapped otolith in reserve or use it for chemical analysis. We think it especially important that only one otolith be prepared using current procedures. We are convinced that much more powerful and sophisticated procedures will be devised and that the other otolith should be archived for the future. It should not be standard procedure to prepare the second otolith simply to obtain another interpretation if preparation or interpretation methods are imprecise and not well defined. Before otoliths are ground, sectioned, or altered, we routinely capture their planar shape with a camera digitizer and weigh them.

Any exposure to formaldehyde will cause the otolith to decalcify and become chalky, soft, and opaque, affecting optical density and zonation. This usually destroys the ability to decipher cyclic growth and age.

Some otoliths and fish are stored in alcohol. If storage is short-term, the alcohol can be removed from the otolith by rinsing in distilled water. This appears to have no detrimental effect for most routine preparation and interpretation procedures. However, when storage is long-term, the alcohol is difficult to remove and can affect certain embedding and preparation procedures and permanently affect optical density. If otoliths have been stored in isotonic glycerol, it can usually be removed by rinsing in either water or alcohol. If storage was long-term, the glycerol appears to penetrate the otolith more thoroughly and is more difficult to remove and may affect certain preparations and embedding and mounting procedures.

Because aqueous solutions create abnormal conditions, we now prefer to work with otoliths that have simply been air-dried; but prolonged air drying, especially in very low humidity and high ambient temperature, may result in the loss of bound water and cause some minor internal fracturing. If possible, otoliths should be stored in relatively low or constant humidity and temperature. Internal fracturing related to natural contraction and expansion of the otolith increases in extremely dry or fluctuating

humidity, which, over the long term, effects the loss of bound water, creating fractures and fissures. The otoliths of some species, especially eels, appear to be more prone to this than others. If strongly exothermic compounds are used as embedding or mounting media, they can cause substantial dehydration and cracking in the otolith. All this affects the quality of the preparation and the image that is produced.

It is especially important to know the difference between good and poor quality preparations and images. Only the best storage and preparation techniques should be used, because in this technology, results depend greatly upon the quality of the preparations and images produced.

This is not meant to be an exhaustive review of the subject but is simply provided to convey insights and experiences that we have acquired over the years, with a discussion of the methods and procedures that work well, using current interpretation procedures and technology.

Appendix 4: Archiving and Storage Protocol (Biological Sexing Samples)

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Storage of tissue that has not been histologically prepared and mounted on slides:

- Collect gonad or cross section of eel. Tissues should be no thicker than 5 millimeters.

- Place individual tissues in labeled cassettes (Fisher 15-182-500E) or loose in individually labeled screw-cap jars.

- Place tissues in 15-20 volumes of 10% neutral (pH 7) buffered formalin (NBF) for 7 days at room temperature. (Example: If a tissue specimen occupies ca.1.5ml of volume then it requires 22.5 - 30ml of fixative just for itself. Additional tissues placed in the same container require equivalent additional fixative volume to maintain a ratio of 15-20 times the total tissue volume.) Agitate jars containing loose tissue to ensure all surfaces are wetted with formalin.

- For short-term storage (<= 2 months), store in 10% neutral buffered formalin sufficient to submerge specimen(s).

- For long-term storage place in 95:5 70% ethanol : glycerin sufficient to submerge specimen(s).
  DO NOT USE ISOPROPANOL OR RUBBING ALCOHOL.
  Drugstore glycerin is acceptable.

- If ethanol is not available, place tissues in fresh 95:5 10% NBF : glycerin for long-term storage.

- Do not allow tissues to freeze.

Storage of tissue that has been histologically prepared by the laboratory and that which has been mounted on slides:

- Keep slides away from extremes of heat and cold. Avoid direct contact between cover slips. Samples will remain stable indefinitely.

- Investigate archiving of paraffin blocks containing unused tissue by the histology facility.

Appendix 5: Sexing and Aging Presentations (CD-Rom)