Quality Assessment of Past Spawning Mark Estimations from a Long-Term Survey in the Connecticut River Watershed

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Abstract

The calcified structures of fishes provide insight into their periodic growth rates and can be combined with other biological variables to identify metrics such as size or age at maturity and mortality rates. Collecting this information on growth and life history can help evaluate the success of conservation efforts and inform future management decisions for a species in need. However, before these life history data can be applied to larger stock assessments that direct management decisions, confidence in the validity of the data needs to be reported through metrics of accuracy and precision. For this report, we assessed the bias and precision of paired reader estimations of spawning marks on scales of Blueback herring (*Alosa aestivalis*) collected in the U.S. Fish & Wildlife (USFWS) Annual Adult River Herring Stock Assessment for the lower Connecticut River basin. The paired reads on scales from a total of 8,698 fish over the ten years of the long term monitoring program were evaluated for the annual presence of systematic bias and precision using a combination of qualitative (i.e., frequency tables) and quantitative (i.e., Evan's and Hoenig's Test of Symmetry, and Coefficient of Variation (CV) calculations) analyses. While seven out of the ten survey years had systematic bias detected by the tests of symmetry, only three years (2013, 2016, 2018) had imprecision values >10% CV threshold. Data were further categorized into specific age classes within survey years to increase our resolution on where bias and imprecision was most prevalent. While the ability for accurate bias detection was limited by sufficient sample sizes (>25 fish), average imprecision values increased with age, and median age classes (4 through 6) commonly had bias detected. However, the removal of insufficient age classes prior to calculating average annual CV did not significantly change the initial average. Lastly, 2023, which was the first year to implement a standardized training procedure prior to production estimating, had the highest precision for both the annual average and specific age classes compared to all prior survey years. This standardized training procedure will continue to be used by USFWS for the lower Connecticut River tributaries, and can be modified for other river systems. Overall, this report's results highlight the importance of assessing precision and encourage the standardization of spawning mark identification quality control and assurance for future studies. With more quality assessments and baseline information on precision and bias, there can be more beneficial discussion on defining thresholds and how to implement spawning history variability into catch curve analyses.

Introduction

The scales of anadromous and iteroparous clupeids provide insight into how old a fish is and how many times it has returned to spawn in freshwater throughout its lifespan (Cating 1953). When combined with other information such as species, sex, and size, important aspects of a population's reproductive potential can be inferred (Casselman 1990, Tomkiewicz et al. 2003). Life history data within and across cohorts can then be analyzed for both temporal and spatial demographic trends in the returning river-specific populations. Both age and spawning mark estimations are commonly used in stock assessments to determine metrics such as repeat spawner frequency and to analyze spawning stock biomass per recruit and total instantaneous mortality estimates (Z) (ASMFC 2012, 2017, 2024).

To apply estimated data within stock assessments, it is valuable to first evaluate the accuracy of the data (ASMFC 2012). Accuracy is the proximity of an estimate to the true known value (Campana et al. 1995). Using inaccurate estimations can introduce bias, potentially impacting conclusions and associated management decisions (Beamish and McFarlane 1983, 1987). For example, incorrect population estimates could lead to overexploitation, erroneous growth rates, and underestimations that can skew mortality estimates (Campana 2005). However, the accuracy of an estimation can only be determined if the estimation method has been validated for all life stages (Beamish and McFarlane 1983). This would require verifying each variation of spawning marks at different ages against actual observed spawning events, a challenging process due to the difficulty of confirming true spawning events. Since accuracy has not been determined, precision— the reproducibility of a given value—is a viable alternative for data validation (Campana et al. 1995). Assessing precision can improve the overall understanding of relative ease of estimating a value with a specific structure. It also allows for comparisons between estimators and grouping factors such as survey years or laboratory facilities (Campana et al. 1995).

There are two main sources of error in estimating spawning marks from scales: 1) process error, and 2) interpretation error (Campana 2005). Process error is based on the physical structures being examined. For example, removing scales from different areas on the fish can increase the risk of collecting damaged or regenerated scales that do not show the complete growth sequence (Abdu-Nabi 1983; Devries and Frie 1996). Not having the fish's whole life history can lead to underestimation. Method validation studies and uniform scale collection protocols (Elzey et al. 2015) are often implemented to reduce process errors. Alternatively, interpretation error is based on a reader's or laboratory's preparation and overall understanding of what they are estimating. Like process error, interpretation error can be systematically biased, but it can also be random (Campana 2005). In addition to uniform collection protocols and standardized training, frequent testing with reference collections made up of accurate or precise values has been recommended to avoid biased estimations (Buckmeier 2002). Conducting this testing before, during, and after production estimating ensures the reader's interpretation does not change over time (Campana 2005). Even with proper measures taken to reduce both process and interpretation error, reporting bias detected alongside precision levels is a best practice for quality assessment of estimation data, as both random and nonrandom bias between estimators will artificially decrease precision levels (Campana 1995).

Along the Atlantic coastline of the United States, the use of scales to determine both age and spawning history estimations of anadromous fishes, such as river herring (alewife *Alosa pseudoharengus* and blueback herring *A. aestivalis*), has continued with a lack of standardized quality reporting. The most transparent reporting has resulted from biannual scale reading workshops organized by the

Atlantic States Marine Fisheries Commission (ASMFC) from 2014–2018. These biannual workshops identified systematic bias and imprecision greater than Campana (2005)'s recommended coefficient of variation (CV) < 5% between participating laboratories' estimating age and spawning marks (ASMFC 2014, 2016, 2018). While recommendations have been proposed to minimize bias, the last workshop concluded that it was up to the individual programs to create training sets based on system-specific samples due to biogeographic variation impacting scale formation growth patterns (ASMFC 2018). In addition to individual training sets, it was also recommended that organizations maintain their own in-house quality assurance and control measures (ASMFC 2018). While many organizations continuing to collect river herring scales for age and spawning mark estimations have adopted or modified the Massachusetts Department of Marine Fisheries Age and Growth Laboratory's scale protocol for proper collection and interpretation (Elzey et al. 2015), the current quality assessments of scales primarily focuses on age estimations and lacks established thresholds for precision or bias detection for spawning mark estimations on a regional level.

The importance of quality assessment for estimated data and expanding long-term data series (ASMFC 2012) had been notably magnified for the Connecticut River as studies began suggesting a loss of repeat spawners and truncation in size and age classes compared to initial demographic work completed in the 1960s (Loesch 1969, Marcy 1969, Davis and Schultz 2009, Davis et al. 2016). As a result, the U.S. Fish & Wildlife Service (USFWS) began conducting the Annual Adult River Herring Stock Assessment, a boat electrofishing survey at five specific sites along the mainstem of the lower Connecticut River, in 2013 (Sprankle and Desmarais 2018). Since establishment, the USFWS survey has collected a suite of biological information including species, sex, body measurements, age, and spawning mark estimations on an annual subsample of returning river herring (Sprankle and Desmarais 2018). Like many long-term monitoring programs, it has relied on an annual rotation of seasonal employees to assist in data collection and processing. Two technicians are hired each spring and as of 2023, there have been 19 different technicians. Their primary duties on the survey involve the field collection and laboratory processing of river herring, as well as preparing and estimating spawning marks on scales. Before technicians began estimating spawning marks, they completed one-day informal training sessions on scale reading with the Connecticut Department of Energy and Environmental Protection (CT DEEP) diadromous biologist. There had been no scale reference collections for technicians to review or quality assessments completed within or throughout survey years to ensure proper scale interpretation until recent implementation in 2023. While the dataset has recently met the minimal time series requirement for analysis, the quality assessment is still needed before the spawning mark estimation data can be included in the coast-wide river herring stock assessments (ASMFC 2012, 2017).

This report analyzed paired reader data on spawning marks for the USFWS Annual Adult River Herring Assessment from 2013 to 2023 to guide the use of spawning mark data in stock assessments. Specifically, the objectives were to 1) visualize and quantify systematic bias between readers, 2) assess precision between readers based on identified bias, and 3) develop a science based best practices manual for scale preparation and estimations. Each metric was assessed across survey years and within age classes to determine what subsets of the data may be viable for use in stock assessments. The paired reader data from the 2023 survey was also assessed against other years as it was the first year the new training protocol and reference collection was implemented. A subsampling approach for reassessing the precision of past scale reads that had reader consensus is proposed at the end of this report, along with a description of the scale protocol and test set that was created in 2023.

Methods

Study Area

The Connecticut River flows 653 km from the Canadian border to the Long Island Sound in Old Lyme, Connecticut with a tidal boundary occurring 90 km from the mouth of the river (CRC 2020). In the early 2000s, it became a prime illustration of the historical river herring declines in New England as the Holyoke Dam, which is the first mainstem barrier that once passed 630,000 blueback herring in 1985, had passed an annual average of 867 ± 1178 blueback herring since 2002 (Sprankle 2023). This stark decrease in fish passage prompted Connecticut to implement a state moratorium in 2002, which is still in place today. Additionally, CT DEEP further reinforced adult monitoring and restoration programs based on video and electronic counters, juvenile index surveys, and translocation efforts to re-establish runs at historical spawning habitats.

For the USFWS Annual River Herring Stock Assessment survey, the study area includes five sites adjacent to the lower Connecticut River basin below the first mainstem dam in Holyoke, Massachusetts. This lower basin was determined as the cut off due to only one of the two target species (blueback herring) being known to pass above the dam at the Holyoke Fish Lift. Three sites were in Connecticut (Mattabesset River, Wethersfield Cove, Farmington River) and two were in Massachusetts (Westfield River and Chicopee River) (Sprankle and Desmarais 2018; Figure 1).

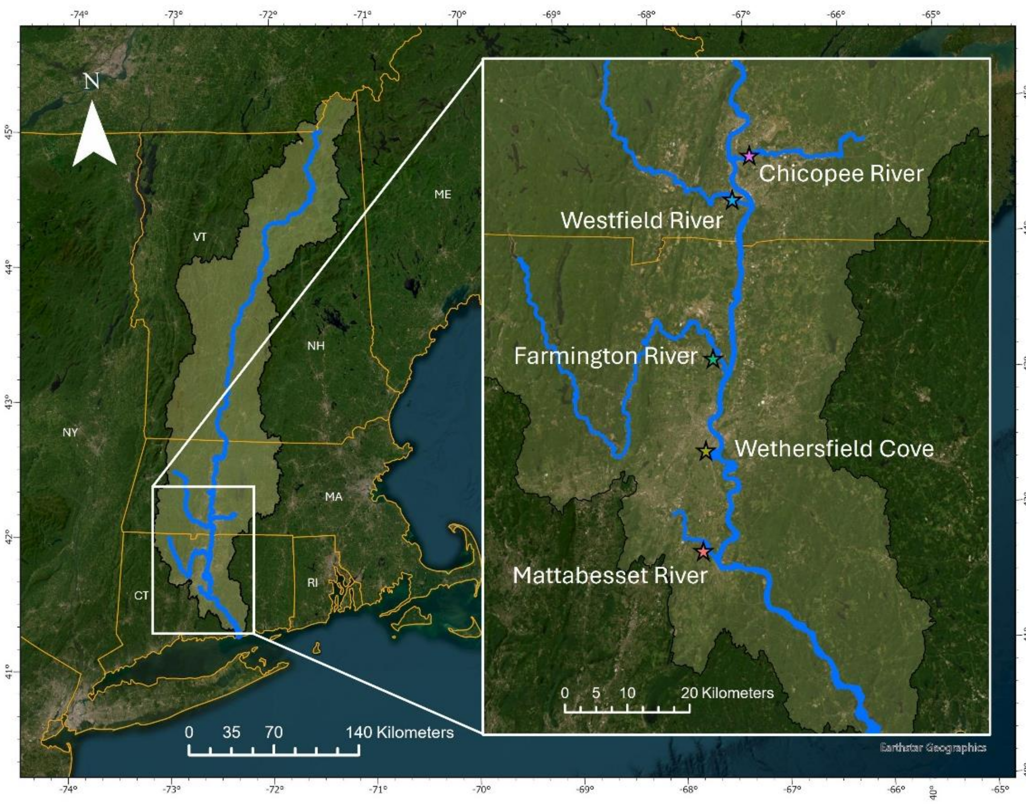

Figure 1. Map of the four tributaries and one cove along the mainstem Connecticut River in Massachusetts (MA) and Connecticut (CT) that are surveyed as part of the U.S. Fish & Wildlife Service Annual Adult River Herring Stock Assessment survey (Sprankle and Desmarais 2018). The stars indicate the approximate location where sampling occurred at each site. Surrounding states are listed as abbreviations: New York (NY), Rhode Island (RI), Vermont (VT), New Hampshire (NH), and Maine (ME).

Fish Collection

River herring data collected by USFWS from 2013 to 2023 (excluding 2020 due to limited resources during the global pandemic) were used. Annual sampling typically began at the end of March when the first fish arrived in the river and continued until the middle of June when fish had left all systems. Initial sampling dates occurred at the two most southern sites (Mattabesset River and Wethersfield Cove, CT) to capture the earlier running alewives which were typically present in the first weeks of April. Once blueback herring were observed at those southern sites around mid-April, the Farmington River site was added, followed by the remaining two northern sites to track the migration upriver (Figure 1, Sprankle and Desmarais 2018).

Fish were sampled once per week at each site with an electrofishing boat (Smith-Root®, Vancouver, Washington, USA). Each site visit consisted of five to seven "runs" or passes of the boat downstream in a serpentine direction, applying a pulsed direct current ("on" for five seconds, "off" for five seconds) to the water that temporarily stunned the fish for a total of 500 shock seconds. As fish rose to the water surface, they were netted with dip nets and transferred to live wells on the boat until the run was complete. Shock seconds were used to calculate catch per unit effort (CPUE in fish min^{-1}; Sprankle and Desmarais 2018). From all completed runs at a site, a cumulative target of 80 fish for further laboratory processing were identified to species (based on visual eye diameter), measured for total and fork length, weighed, sexed, evaluated for overall spawning condition (gravid, ripe/running, partially spent, and spent) based on expression of gametes, euthanized, and brought back to the laboratory on ice (IACUC #4348; Sprankle and Desmarais 2018). Fish above the target subsample were counted and sex, species, and body measurements (e.g., total length, fork length and weight) were recorded before they were released.

Laboratory Processing

Laboratory processing was conducted on fresh samples the day after field collection. Fishes were removed from ice and dissected for verification of species (alewife or blueback herring), based on peritoneum pigmentation (Berlinsky et al. 2015), and sex. Both sagittal otoliths were extracted for determining the age of the fish by an experienced biologist as part of the survey protocol. The estimated ages were used rather than scale-based ages for the remainder of this report due to higher accuracy at all ages (Elzey et al. 2015). Scales were removed by scraping a knife in the opposite direction of the scales, below the dorsal fin and above the lateral line on the left side (Figure 2). This region on the fish that displays complete growth patterns is commonly less damaged than other regions of the fish (Penttila and Dery 1988); this scale removal location is consistent with other protocols for river herring and American shad (*Alosa sapidissima*)(Elzey et al. 2015; GSMFC and ASMFC 2020). Scales were dried overnight, cleaned of mucus and other foreign debris with a warm water bath and scrubbing brushes, air dried, and then a set of eight scales were mounted between glass slides. In 2023, immersion in warm water was replaced with a ten minute bath in a 5% pancreatin solution. Pancreatin is a digestive enzyme aiding in protein breakdown that was first tested for scale cleaning by Whaley (1991) and has since been recommended as a preferred cleaning technique (Elzey et al. 2015; GSMFC and ASMFC 2020).

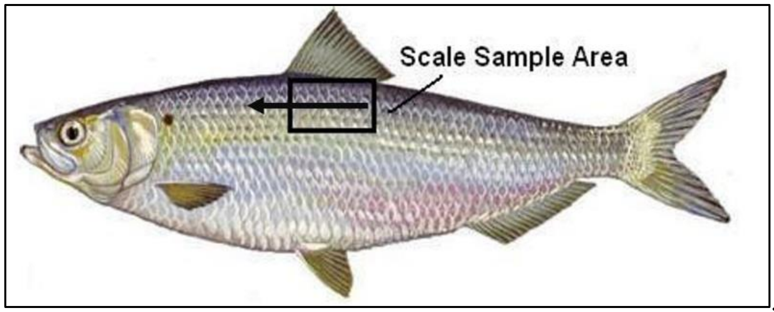

Figure 2. Diagram of a blueback herring *Alosa aestivalis* that displays the correct scale sampling area (black box) below the dorsal fin and above the lateral line. The black arrow pointed at the anterior end of the fish indicates the direction the knife is scraped to remove scales

Once mounted, scale slides from all fish were independently examined using a microfiche by the two seasonal technicians (hereafter, readers) for the presence of spawning marks. Spawning marks appear as fuzzy or jagged lines extending around the anterior portion of the scale where an annulus, or annual growth ring, would typically be observed (Moss 1946; Cating 1953; Judy 1961). These scar-like marks result from a fish reabsorbing calcium and other ions from their scales to supplement nutrients during a period of physiological stress where the fish most likely is not feeding ("Crichton effect", Simkiss 1974; Penttila & Dery 1988; GSMFC and ASMFC 2020). The amount of scale absorption and resulting appearance of a spawning mark is dependent on the age of a fish, migration distance, and temporal duration in freshwater systems (Cating 1953).

For this ten year survey, annual readers estimated the exact number of spawning marks seen and their confidence level in that estimation ranging from one (most confident) to five (least confident). The two readers then compared their original independent estimations and established a consensus spawning estimation for scales where original estimations differed.

Data Analysis

Only the spawning mark estimations from blueback herring scales were assessed due to the larger sample sizes in the survey years compared to alewives. Before bias analysis using the tests of symmetry, all random estimating error was assumed to be constant over the years. This assumption was due to no expectations for readers to have prior scale reading experience, and all readers following the same one day training procedure prior to 2023. This error assumption meant that all bias detected both visually with frequency tables and statistically with tests of symmetry would be nonrandom interpretation bias between the two annual readers.

Bias Detection

Frequency tables were created first to visually explore agreement on spawning mark estimations between the paired reads (Campana et al. 1995; McBride 2015). Two sets of tables were created: one set based on each survey year (2013–2019, 2021–2023, ten tables total, see Appendix A) and another set based on each age class by survey year (73 tables total, see Appendix B). Age class was used in addition to survey year as it has been hypothesized that older fish would disproportionately affect precision and detection of bias between readers (i.e., increasing amounts of theoretical spawning marks leading to increasing interpretation difficulties and error, Marriott et al. 2010). Frequency tables allowed a visual gauge of the presence of bias based on asymmetry on either side of the diagonal (exact agreement) compared to the other (i.e., one reader over- or under-estimating compared to the other).

To quantitatively evaluate reader disagreement for evidence of systematic bias, the diagonally pooled Evans & Hoenig's (1998) test of symmetry was applied to the paired reader data on spawning marks. The null hypothesis of the test is that specific observations that do not share agreement are randomly distributed among the readers and specific classifications (i.e., spawning mark estimations) (Evans and Hoenig 1998). A criterion of $\alpha < 0.05$ was used to support the rejection of the null hypothesis, indicating that there was an asymmetric, or nonrandom, distribution among cells off the central diagonal of the spawning mark frequency tables (i.e., exact agreement between readers). All bias calculations were conducted using R Statistical Software (v. 4.1.2; R Core Team 2023) with the ageBias() function in R package FSA (v0.9.5; Ogle et al. 2023).

For interpretation of the test of symmetry results, the Nesslage et al. 2022 simulation study was referenced as it expanded on the work of McBride (2015) to include the influence of sample sizes, associated trends, and number of age classes. Specifically, focus was placed on the simulation scenario of short-lived fish with a maximum age of five and decreasing trend in sample sizes because it reflected the five potential spawning mark categorizations (i.e., zero to four marks) present throughout the survey years and the decreasing number of fish with longer spawning histories. Together, the simulation studies displayed how random error (imprecision) and sample size would both need to be high for the Evans & Hoenig's test to incorrectly identify random aging error (imprecision) as bias (i.e., type one error). When bias (overestimating by one unit) was added to the simulation, the Evans & Hoenig's test was able to detect it 100% of the time at all sample sizes (minimum being 25 fish), even at the lowest level of random aging error (coefficient of variance of 5). Given this, the results of age classes with more than 25 fish were considered for further comparison with imprecision values.

Precision Assessment

Precision, or repeatability, of paired reader data was measured using Chang's (1982) Coefficient of Variation (CV). A CV of < 5 to 10% is often used as a rough indicator of ageing error for fish with moderate reading complexity for age (Campana 2005; McBride 2015). CV results were assessed with three thresholds: < 5%, < 7% (equivalent to 5% average percent error (APE); Beamish and Fournier 1981), and < 10%. Lower CV values indicated higher precision or agreement between readers (Campana 2005). CVs were calculated for overall survey years and for each age class within survey years. Additionally, annual average CVs were re-calculated with all age classes below the 25 fish minimum removed to assess the effect on annual imprecision. All precision calculations were conducted using R Statistical Software (v. 4.1.2; R Core Team 2023) with the agePrecision() function in R package FSA (v0.9.5; Ogle et al.. 2023).

Results

From all ten survey years, there were 8,698 blueback herring that had both otolith-derived age information and their scales independently examined by two readers. Annual sample sizes varied with an average of 870 fish (standard deviation (SD) 330), ranging from 362 (2013) to 1,461 fish (2019) (Table 1). Each annual survey had a set of two readers; however, a single reader from 2022 returned to the survey in 2023. This resulted in 19 different readers over the 10 years. Altogether, exact agreement, or consensus, on spawning mark estimations was ~83% out of the 8,698 fish and 10 sets of readers.

The sample sizes of age classes also varied both within and across years due to observations of strong and weak cohorts (Table 1). For example, the 2010 and 2011 cohorts mainly supported the returning populations from survey years 2013 to 2016, followed by 2014 and 2015 cohorts appearing in survey years 2017 to around 2021. The last notable cohorts were 2018 and 2019 which dominated as four and three year olds in 2022, then as four and five year olds in 2023 (Table 1). Fish from age classes two, eight, nine, and ten were not present in all years; however, when present all sample sizes were less than 25 fish (except for eight year olds in 2023, n = 40, Table 2).

Bias

There were differences in bias detection among survey years. From the frequency tables, an uneven distribution of disagreement between paired readers was visually observed for survey years 2013, 2015, 2016, 2017, and 2018 (Appendix A). Survey years 2014, 2019, and 2021 appeared to show less obvious or severe bias than the prior listed years, while survey years 2022 and 2023 appeared to show little to no evidence of bias present (i.e., even distribution of disagreement between readers) (Appendix A). The tests of symmetry also revealed that seven out of the ten years had systematic bias between readers ($p < 0.05$, Table 1). Bias was not detected in 2014, 2022, or 2023 ($p = 0.614, 0.616, 0.169$; Table 1).

Within survey years, differences in bias detection among age classes were also observed. Out of the 73 unique frequency tables between age classes and survey years (Appendix B), 28 tables had sample sizes below the 25 fish minimum and were disregarded from further bias analysis (Table 2). This exclusion comprised all survey years for age classes two, eight (except for 2023), nine, and ten (n = 23), as well as four different survey years of age class seven. Age class three and six also both had one survey year each that was below 25 fish. The remaining 45 tables with sufficient sample sizes ranged from 33 (age class seven in 2016) to 824 fish (age class four in 2019), with an average of 189 (SD 154) per age class and year (Table 2). Of these, 18 (~40%) had systematic bias detected by the tests of symmetry (Table 2). Overall, age class four and five, which were present in all ten survey years, had the highest bias detection rates (40% and 60%). Age class six followed with a bias detection rate of 44%, but only nine out of the 10 survey years had sufficient sample sizes for detection. Within survey years, the number of age classes with sufficient sample sizes varied, with a minimum of three and maximum of six different age classes present. Overall, the survey year 2018 had the highest detection rate for bias for age classes (n = 4, 100%) by year, while 2022 had no bias detected for any of the five age classes present (i.e., 3 through 7 all $n > 25$).

Table 1. Quality assessment based on bias and precision values for ten survey years. Bias detection was completed through calculating a chi-squared value (χ^2) and p-value (p) for the test of symmetry (Evans and Hoenig 1998); bolded p < 0.05 values indicate the detection of bias. Precision was measured with the coefficient of variation (CV). Total annual sample sizes (N) are listed. The numbers for each age class indicate the proportion (%) of that specific age class within a survey year. Colors indicate the hatch year of six different cohorts (yellow - 2010, blue - 2011, orange - 2014, green - 2015, purple - 2018, red - 2019). A row of dashed cells for survey year 2020 was included to reduce confusion on cohort tracking. The bottom row displays the annual average and standard deviation values for precision (coefficient of variation, CV), sample size (N), and the proportion of age classes present (zeros indicate absence of an age class and were included in the averaging).

Year	Test of Symmetry		Coefficient of Variation (CV)	Sample Size (N)	Age Class								
	χ^2	p			2	3	4	5	6	7	8	9	10
2013	27.4	**< 0.001**	10.99	362	0	32.6	30.11	22.93	13.81	0.28	0.28	0	0
2014	1.8	0.614	9.66	628	0	17.68	62.74	12.74	5.57	1.27	0	0	0
2015	8.6	**0.014**	8.87	496	0	4.44	33.47	47.38	9.68	3.83	1.21	0	0
2016	53.5	**< 0.001**	11.11	709	1.27	17.49	6.77	33.57	34.41	4.65	1.27	0.56	0
2017	44.5	**< 0.001**	6.91	1167	1.89	55.36	15.51	4.80	13.28	8.23	0.60	0.34	0
2018	56.6	**< 0.001**	11.38	988	0.20	44.94	36.64	8.91	1.42	5.77	2.02	0.10	0
2019	11.6	**0.003**	6.99	1461	0.21	12.59	56.4	23.07	4.93	1.44	1.03	0.21	0.14
2020	-	-	-	-	-	-	-	-	-	-	-	-	-
2021	11.2	**0.011**	6.16	920	0	30.33	7.93	13.7	41.96	5.54	0.33	0.11	0.11
2022	2.0	0.579	4.04	885	0.23	24.07	41.24	7.46	10.73	15.03	1.24	0	0
2023	3.5	0.170	3.67	1082	0.09	15.90	42.33	30.13	4.07	3.60	3.70	0.18	0
		Average	7.98	870	0.39	25.54	33.31	20.47	13.99	4.96	1.17	0.15	0.03
		Standard Deviation	2.86	330	0.65	15.54	18.95	13.56	13.49	4.27	1.07	0.18	0.05

Precision

Precision varied among survey years. The annual coefficient of variation (CV) values ranged from 3.67 (2023) to 11.38 (2018) across the ten years with an average of 7.98 (SD 2.86) (Table 1). In total, two years had a CV value of <5% (2022 and 2023), a total of five years had a CV value of <7% (prior listed, 2021, 2017, and 2019), and a total of seven years had a CV value of <10% (prior listed, 2015, and 2014). The remaining three survey years (2013, 2016, and 2018) had CV values >10% (Table 1).

Within survey years, age classes differed in terms of precision. Examining only the 45 unique age class and survey year categorizations that had sufficient sample sizes for bias detection (i.e., > 25 fish), the average CV value was 8.86 (SD 5.35) with a minimum of 0.27 (age three 2023, n = 172) and maximum of 25.1 (age six 2014, n = 35) (Table 2). Using the three thresholds, about 29% (n = 13) had <5% CV, 38% (n = 17 total) had <7% CV, and 60% (n = 27 total) had <10% CV. Lower CV values were commonly observed for younger age classes, larger sample sizes, and when bias was not detected (Table 2, Figure 2). Notably, 2022 and 2023 were the only survey years for all age classes (n >25 fish) to have CV values <10%, with the lowest CV value for an age class commonly from one of these two survey years (Table 2).

Table 2. Quality assessment of bias and precision values for ten survey years based on age classes present. The sample sizes of each annual age class are listed in parentheses (N), and the value above the parentheses is the coefficient of variation (CV) values. Dashes indicate that there were no fish present in the age class. All bolded values indicate CV >10% threshold and an asterisk (*) indicates p<0.05 for the test of symmetry (Evans and Hoenig 1998). Shaded boxes indicate age class sample sizes that were below 25 fish. The column Fish Removed displays the sum of fish removed from all age classes below 25 fish and imprecision values were re-calculated (Reduced N). For precision (CV), Total (CV value for all fish) , Reduced N (recalculated CV with samples < 25 fish removed), and △CV (difference between total and reduced CVs) are listed. Positive values in the △CV indicate a decrease in precision when smaller sample sizes were removed, while negative values indicate an increase in precision.

Year (N)	Age Class									Fish Removed	Precision (CV)		
	2	3	4	5	6	7	8	9	10		Total	Reduced N	△CV
2013 (362)	-	8.59 (118)	**10.16*** **(109)**	**12.02*** **(83)**	**17.21** **(50)**	0 (1)	0 (1)	-	-	2	10.99*	11.05*	0.06
2014 (628)	-	7.01 (111)	9.25 (394)	7.93* (80)	**25.08** **(35)**	**16.84** **(8)**	-	-	-	8	9.66	9.57	-0.09
2015 (496)	-	7.50 (22)	9.06* (166)	8.39 (235)	**10.33*** **(48)**	**11.34** **(19)**	8.08 (6)	-	-	47	8.87*	8.85*	-0.02
2016 (709)	0 (9)	4.71 (124)	8.45 (48)	**12.87*** **(238)**	**12.99*** **(244)**	**14.37** **(33)**	**16.91** **(9)**	7.07 (4)	-	22	11.11*	11.2*	0.09
2017 (1167)	2.14 (22)	2.37* (646)	**10.83*** **(181)**	**10.02** **(56)**	**16.75*** **(155)**	**12.52** **(96)**	6.73 (7)	**28.28** **(4)**	-	33	6.91*	6.92*	0.01
2018 (988)	0 (2)	7.76* (444)	**12.51*** **(362)**	**18.48*** **(88)**	**18.95*** **(14)**	**15.16*** **(57)**	**25.53*** **(20)**	0 (1)	-	37	11.38*	11.01*	-0.37
2019 (1461)	0 (3)	3.07* (184)	4.76 (824)	**11.11*** **(337)**	**18.24** **(72)**	**18.31** **(21)**	**14.73*** **(15)**	9.43 (3)	**14.14** **(2)**	44	6.99*	6.74*	-0.25
2021 (920)	-	1.35 (279)	3.87 (73)	5.09 (126)	9.57* (386)	**10.10** **(51)**	**28.28** **(3)**	0 (1)	**70.7** **(1)**	5	6.16*	6.02*	-0.14
2022 (885)	0 (2)	0.44 (213)	3.16 (365)	6.43 (66)	5.04 (95)	9.97 (133)	8.69 (11)	-	-	13	4.04	3.99	-0.05
2023 (1082)	0 (1)	0.27 (172)	1.90 (458)	6.22* (326)	9.81 (44)	7.53 (39)	6.87 (40)	**14.14** **(2)**	-	3	3.66	3.66	0

After removing age classes with limited samples sizes (i.e., < 25 fish), annual average CV values decreased, but did not result in any year meeting a lower precision threshold. The largest absolute change in CV was seen for 2018, in which removing 37 fish decreased the original CV from 11.38 to 11.01 (Table 2). Altogether, six years displayed a decrease in CV values (-0.02 to -0.37, removing 5 to 47 fish), one year (2023) saw no change in CV (only removed 3 fish total), and three years saw an increase in CV values (2017: CV +0.01 removed 33 fish; 2013: CV +0.06 removed 2 fish, 2016 CV +0.09 removed 22 fish) (Table 2).

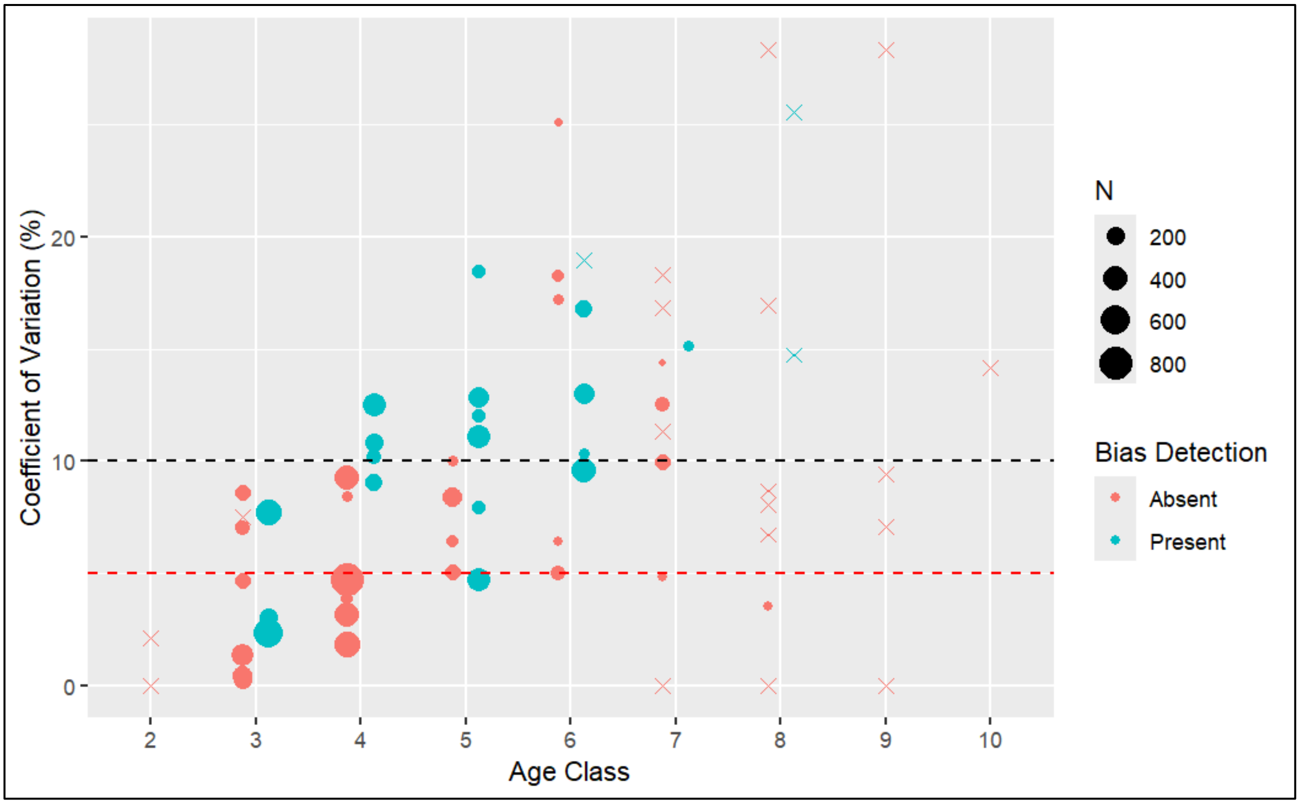

Figure 3. Precision values (Coefficient of Variation, CV) across age classes including sample size (N) (Xs represent sample sizes < 25 fish) and bias detection through color.

Discussion

Past and Future Implications for Spawning Estimations

To date, there has been no validation study for annulus or spawning mark formation on the scales of river herring. In the past, Cating's (1953) ageing method for American shad scales has often been cited for river herring. However, multiple studies (Marcy 1969; McBride et al. 2005; Duffy et al. 2011) have provided evidence against its universal use for all species in the genus *Alosa* due to varying life history strategies across their biogeographic ranges. The lack of scale validation at all sexually mature ages precludes the ability to assess accuracy of spawning mark estimations, yet precision can be evaluated to assess the quality of estimates within a survey. This report is the first qualitative and quantitative assessment of the bias and precision of paired spawning mark estimations collected annually by the USFWS Annual Adult River Herring Assessment.

Systematic, or nonrandom, bias can be introduced by overestimating by one spawning mark or underestimation by 10%, and all types of bias can artificially reduce precision levels between readers (Campana 2005). The tests of symmetry used in this report resulted in seven out of ten survey years detecting systematic bias between readers. While the tests did not identify the type of bias detected, all survey years had above the minimum sample size (n = 250 fish) for a population of five age classes necessary to sufficiently detect more elusive bias such as underestimations by 10% (Nesslage et al. 2022). Regardless of the type, frequent detection of bias over the years suggested a potential insufficiency of the scale interpretation training for annual technicians and further highlighted the need for robust inhouse training recommended by past aging workshops (ASMFC 2014, 2016, 2018).

Concerns about the potential lack of training consistency were expressed after the 2022 field season and before completion of this report. Due to these concerns, a new scale training protocol (Appendix C) was developed prior to the 2023 field season, which consisted of detailed examples of different spawning marks for reference, and a test set based on scale samples from past years. The technicians completed the new training manual prior to production estimating, and 2023 appeared in this report as having the highest precision and lowest bias of any year. The higher precision in 2023 supports the use of this new scale manual and reference collection implemented for standardized training and sufficient interpretation. However, one of the technicians in 2023 had returned from 2022. This second year in the position violates the assumption of readers having no prior scale reading experience. While the experience reading scales most likely had a positive impact on the 2023 results, the use of reference collections and uniform training allowed the inexperienced second reader to achieve comparable results.

In conjunction with the bias detection, it was assumed that the same years would display decreased levels of precision. On the contrary, CV calculations revealed that most years (70%) had precision < 10% CV threshold which has been categorized as acceptable for longer lived species (Nesslage et al. 2022). One main observation from these annual calculations was that higher precision (CV < 7%) was observed in the latter half of the survey years apart from 2018. Furthermore, the two most recent years of the survey, 2022 and 2023, even met the strictest threshold of CVs < 5%. This increase in precision over time is difficult to interpret as there was no documented change in scale processing or training procedures from 2013 to 2022. While it is assumed workflow and resources may have improved since the survey was established in 2013, the annual change in technicians does not support a systematic or directional change in precision. The most convincing interpretation of this increase in precision over time is related to the average annual sample size between the first and second five-year spans (i.e., 2013 to 2017 was 672.4 fish, while 2018 to 2023 was 1,067.2 fish). More fish present represent more opportunities for readers to gain interpretation experience and agree on spawning estimates that would increase overall precision.

Incorporating age class into the annual survey year analysis revealed a secondary effect on precision and bias in scale reading, with older age classes having higher imprecision and bias. This was not a surprising observation as it is common for older fish to be the primary sources of reader disagreement (Marriott et al. 2010). This disagreement can be primarily linked to the decreasing growth rates at older ages that result in less scale material between years (Beamish and McFarlane 1987; Casselman 1990; Maceina and Sammons 2006). With less scale laid down between years, spawning marks have the potential to erode back on previous marks and be unidentifiable as multiple, different spawning events (McBride et al. 2005).

Imprecision at older age classes may also be explained by the higher potential number of spawning events at older ages. For example, there are only two estimation potentials for a three year old fish: zero or one spawning mark. However, a six year old fish could theoretically be returning for the first time or up to its fifth time to spawn depending on when it reached sexual maturity (i.e., age 2 males were observed in six out of the ten survey years presented). While the potential of skipped spawning events has not been confirmed for river herring, these events have been observed with their marine cousins, Atlantic herring (*Clupea harengus*) (Engelhard and Heino 2005, 2006; Kennedy et al. 2010). If skipped spawning events occur within anadromous river herring populations, it could continue to cause issues for readers and support higher variability for ages over four years old.

Acknowledging the decrease in precision with increasing age, an alternative to exact spawning mark estimations is to simplify agreement into a binary variable of presence or absence of spawning marks (i.e., both readers see no marks signifying a virgin fish vs both readers see at least one mark signifying a repeat spawner). This simplification of spawning categories is a common practice when assessing repeat spawner frequencies of a population. The difficulties for readers to agree exactly on spawning marks would be alleviated and theoretically increase precision values; however, it decreases the overall specificity and value of the data. Without an exact number of spawning marks estimated, information about age-at-maturity (if there is associated age information) and number of spawning events is lost, which has been proven to do well at accounting for variation in life history (Billard 2020).

Similar to the interpretation of annual survey years, the sample size of age classes was reviewed as a potential explanation for perceived trends in precision and bias. Generally, sample sizes followed the normal curved age structure, with many fish at median ages (three through six years old) and few on the tailed ends (seven years old and above). However, the explanation based on age class sample sizes did not hold as removing age classes with less than 25 fish did not change the original precision values or bias detection for a survey year. While the largest cumulative removal was 44 fish (41 being age seven and above), the largest change in CV was by 0.37 which did not result in a new precision threshold being met. Altogether, this examination of age classes within annual survey years highlighted that there would not be an advantage to removing older fish at low sample sizes if the CV did not originally meet a defined precision threshold.

Lastly, the impact of the presence and strength of cohorts over time on precision and bias between readers was considered. The 2011 cohort appeared through seven survey years and there were five years in which the associated age class had bias detected and or a CV > 10%. Recent multi-lake studies within New England have concluded that adult run densities influence juvenile densities (Devine et al. 2021), and juvenile growth is both density- and resource- dependent (Devine et al. 2024). This potential variability in early growth rate of the offspring from a strong cohort year could continue to influence growth rates later in life and impact the age that a cohort reaches sexual maturity. Expanding on the cohort strength pattern, it was observed that 2018 contained age classes consisting of four strong cohorts (i.e., 2011, 2012, 2014, and 2015). Examining the age classes within this year revealed that out of the five age classes present with more than 25 fish (i.e., three to seven), all had bias detected and only age three fish fell with the maximum CV < 10% threshold. While this is one observation from the data, it may suggest that the presence of strong cohorts or a common environmental effect that follows cohorts could negatively impact precision over multiple years.

Using a survey-specific subsample approach

Although it is acknowledged that accuracy of spawning estimations could not be assessed in this report, re-evaluating the reproducibility of the 7,311 blueback herring that technicians independently agreed on for spawning estimations through a third reader review can function as a proxy for accuracy. Such a re-evaluation can quantify a level of confidence in the estimations and further justify the inclusion or exclusion of data in future stock assessment models. Instead of re-reading all 7,311 fish that had consensus on spawning estimations, a third reader can review a subset of the fish (Table 3).

The number of fish that need to be reviewed can be calculated based on Cochran's (1977) calculation for simple random sample size of given populations.

$$n_0 = \frac{Z^2 * p * (1 - p)}{E^2}$$

The formula considers three main variables. A specified alpha or confidence level (i.e., 1-∝, if ∝ = 0.05 then 95%) that is used to calculate the critical score Z (∝ = 0.05 results in Z = 1.96). The variable (p) is the expected proportion of a given population, and (E) is the margin of error or maximum allowable error or risk in the estimate (Cochran 1977). While a 5% margin of error is often considered acceptable for categorical data, the selected value can be tailored with prior knowledge and goals of the study, (i.e., decreasing the value when a higher degree of precision is necessary) (Bartlett et al. 2001). A similar decision process can be completed for selecting confidence level values (i.e., choosing 0.75 compared to 0.95).

Table 3. Overall total sample sizes (N) of all specific age classes and consensus spawning estimations from 2013 to 2023. A dash in a cell indicates that no fish were present for that classification. Using the subsampling calculation with confidence level set to 80%, a total of 1654 scale slides (n) need to be re-read out of the original 7,311.

		Female		Male	
Age	Spawning Marks	Total (N)	Subsample (n)	Total (N)	Subsample (n)
2	0	-	-	38	0
3	0	551	10	1534	18
	1	8	5	43	13
4	0	657	92	1279	119
	1	162	64	466	101
	2	4	3	17	5
5	0	229	96	421	119
	1	155	77	338	107
	2	56	32	113	45
	3	1	1	7	4
6	0	106	63	183	83
	1	115	67	212	91
	2	59	39	105	53
	3	11	8	21	12
7	0	32	26	47	34
	1	49	38	105	65
	2	19	16	47	34
	3	14	12	12	10
	4	-	-	1	1
8	0	8	8	10	10
	1	14	13	19	18
	2	11	11	13	12
	3	3	3	2	2
	4	2	2	-	-
9	0	-	-	1	1
	1	2	2	4	4
	2	-	-	1	1
	3	2	2	1	1
10	1	-	-	1	1
	Total	2270	690	5041	964

For finite populations such as a sample of fish all categorized as a specific spawning estimation, the calculated sample size $n0$ cannot exceed <5% of the population size N (Bartlett et al. 2001). If the sample size $n0$ exceeds this threshold, a finite population correction factor (FPC) is applied to adjust the sample size (Cochran 1977).

$$n = \frac{n_0}{1 + \frac{n_0 - 1}{N}}$$

A single subsample will be defined for each specific spawning estimation category (ranging from zero to four) with an age class and sex (i.e., for an example calculation see Figure 3). While it was not assessed in this report, separating data by sex when possible is common for stock assessments (ASMFC 2012, 2017, 2024) due to the importance of female fecundity, specifically for larger and older individuals (Hixon et al. 2014, Marjadi et al. 2019), and possible variation in average age at maturity between sexes. For the USFWS survey specifically, the annual average proportion of females was 31% (SD 6) over all years (Sprankle and Desmarais 2023).

Of all age 4 female fish collected from the USFWS survey of Connecticut River over 10 years, a total of 823 fish had two readers agree on spawning estimations (i.e. 0, 1, 2).

To calculate the subsamples of each consensus estimation for female age 4 fish, we defined the (E) as 0.05, and set the alpha level (∝) to 0.20, which is used to calculate the Z score.

$$Z = \Phi^{-1}\left(\frac{1-(1-0.20)}{2}\right)$$

$$Z \approx 1.28$$

For the virgin fish (Consensus = 0) subsample, the (p) would be 79.8% (Table A).

$$n_0 = \frac{(1.28)^2 * (0.79) * (1-0.79)}{(0.05)^2}$$

$$n_0 = 105.89$$

Because the subsample ($n_0 =$ 105.89) exceeds the more than 5% of N (657 * 0.05 = 32.85), the finite population correction factor (FPC) was used to correctly adjust the sample size.

$$n = \frac{n0}{1+\frac{n0-1}{N}}$$

$$n = \frac{105.898}{1+\frac{105.89-1}{657}}$$

$$n = 91.32$$

Following these steps for each Consensus group, a third reader would have a subsample of 92 scales from first time spawners, 64 scales from second time spawners, and 3 scales to re-read for the subsample of age 4 female fish (Table A).

Table A. The number and proportion of fish that make up each spawning estimation consensus read for age 4 female fish from the USFWS survey data 2013 to 2023.

Consensus	0	1	2
Number of Fish	657	162	4
p (%)	79.8	19.7	0.50

Figure 4. Example of using Cochran's (1977) sample size calculation and finite population correction factor (FPC) to identify the number of samples to reassess for spawn history estimations.

The results of this report also highlight how the subsample calculations of specific categories (i.e., age classes and survey years) can be modified based on past precision and bias detection. For example, the confidence level for three and four year olds could be set to a lower value (i.e. CI 0.75 vs 0.95) as those age classes displayed the highest precision. Depending on the importance of older individuals within a study, confidence level could be increased to ensure all older fish are reviewed due to their overall infrequency in populations and low precision levels.

Training manual and reference collection

As mentioned previously, establishing a scale reading manual with a reference collection for future uniform training at the USFWS Connecticut River Fish and Wildlife Conservation Office (Appendix C) was one of the main objectives of this project. The guide begins with an in-depth protocol on scale collection and the updated cleaning process. The protocol is then followed by descriptions and photo examples of basic scale structures (i.e., baseline, annulus, stress marks, transverse grooves) and explanations on how to identify and differentiate between these structures on the scales. Within the photo examples, there are references to scales that display zero, one, two, and three spawning marks. Three main characteristics (i.e., depth, length, and frequency/consistency of marks) were also identified to help distinguish genuine spawning marks.

The guide also includes a training and test set of scales. The training set is made up of eight scale slides that initially exposes readers to examples of both "good" and "bad" quality scales they may come across while reading. Each slide in the training set has an associated explanation for what past readers and experts have concluded it to be. The test set consists of 44 scale slides that readers individually read and provide spawning estimations on. The reader's estimations are then assessed for accuracy and precision through the calculation of percent agreement (PA) and coefficient of variation (CV). The target for the test set is a score of >80% percent agreement and <10% for CV. Any incorrect scales are discussed with the reader prior to reading scales from the current field season. This test set is completed by the readers at both the beginning and end of the season for confirmation of consistent spawning mark interpretation.

Importantly, these training and test sets are not comprised of scales from river herring with known spawning histories because no such collections currently exist and methods of estimating spawning history on scales have not been validated for either species. While Judy (1961) used physical markers (i.e. pelvic fin removals of juveniles) to track American shad and validate Cating's (1953) method of estimating age and spawning marks on scales of age four through six year olds, issues with its lack of protocol relating to blind estimating have been raised and recent studies discourage its universal use for all clupeids (McBride et al. 2005; Duffy et al. 2011). As scale validation methods continue to be listed as a priority research need (ASMFC 2012, 2018), future validation efforts for river herring could draw insight from the study design and methods used for other anadromous species. For instance, previous studies with Steelhead *Oncorhynchus mykiss* involved trapping pre-spawn individuals at passage structures, physically marking or tagging them, and later recapturing post-spawners (Hernandez et al. 2014; Copeland et al. 2018). This approach could be relevant for known river herring spawning sites equipped with similar passage and trapping structures for collection pre- and post- spawning.

To work around the absence in known demographic data, multiple experts with experience reading spawning marks read through and verified estimations for both the training and test sets. With the expert verification, scales were included, and it is assumed that the identified spawning estimations in the test set will assess both precision and bias of readers (Campana 2005). In the future, the digitization of both sets to ensure long-term availability may be considered to facilitate exchanges with other laboratories to increase standardization and unification. Overall, this training manual and reference collection will function as quality control and assurance for the future survey years of the USFWS Annual River Herring Assessment.

Conclusions

Overall, this report provides an assessment of nonrandom bias and precision of past spawning mark estimations, and its publication aims to encourage and support the quality assessment efforts of other organizations. While past ASMFC scale reading workshops (2014, 2016, 2018) have listed defining thresholds of precision as a priority, there was no clear answer for what threshold would be most informative for decisions about removing samples. As suggested by McBride et al. (2015) and Nesslage et al. (2022), decisions on imprecision thresholds are primarily study- and modeling-specific. Based on the report's results, a CV value of < 10 % seemed appropriate for many of the survey years and age classes within years. It would be helpful for future assessments to report information on age classes and sample sizes alongside precision and bias values to give a more holistic view of reader disagreement. Lastly, this report highlights shortcomings in the validation and interpretation of spawning mark formation on scales, and encourages the implementation of uniform training within organizations collecting river herring spawning mark estimations.

Additional Resources

Supplemental material: McBride, R. S. 2015. Diagnosis of paired age agreement: a simulation of accuracy and precision effects. ICES Journal of Marine Science 72: 2149–2167. https://doi.org/10.1093/icesjms/fsv047. to review of methods for precision and bias

Ogle, D.H., Doll, J.C., Wheeler, A.P., & Dinno, A. 2023. FSA: Simple Fisheries Stock Assessment Methods. R package version 0.9.5. https://CRAN.R-project.org/package=FSA. for data analysis with R and R Studio

Sutherland, S. (2022, May 6). Precision Templates for Ageing. NOAA Fisheries. https://www.fisheries.noaa.gov/resource/tool-app/precision-templates-ageing for data analysis with Microsoft Excel

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Appendix A: Frequency Tables by Survey Year

Table A.1: Frequency table of the paired spawning mark estimations of 362 blueback herring *Alosa aestivalis* from 2013.

	Reader 2				
Reader 1	0	1	2	3	4
0	165	13	2	-	-
1	39	75	4	-	-
2	7	17	30	1	-
3	2	1	3	3	-
4	-	-	-	-	-

Table A.2: Frequency table of the paired spawning mark estimations of 628 blueback herring *Alosa aestivalis* from 2014.

	Reader 2				
Reader 1	0	1	2	3	4
0	247	49	6	-	-
1	43	205	6	-	-
2	4	18	31	2	-
3	-	2	5	8	-
4	1	-	-	1	-

Table A.3: Frequency table of the paired spawning mark estimations of 496 blueback herring *Alosa aestivalis* from 2015.

	Reader 2				
Reader 1	0	1	2	3	4
0	122	40	6	-	-
1	12	170	14	2	-
2	1	23	79	5	1
3	-	-	8	11	-
4	-	-	-	1	1

Table A.4: Frequency table of the paired spawning mark estimations of 709 blueback herring *Alosa aestivalis* from 2016.

	Reader 2				
Reader 1	0	1	2	3	4
0	229	62	23	2	-
1	11	163	45	1	-
2	6	14	110	8	-
3	1	-	8	21	-
4	-	1	-	2	2

Table A.5: Frequency table of the paired spawning mark estimations of 1167 blueback herring *Alosa aestivalis* from 2017.

	Reader 2				
Reader 1	0	1	2	3	4
0	742	96	11	3	-
1	24	185	21	2	-
2	-	20	49	-	-
3	-	-	5	8	1
4	-	-	-	-	-

Table A.6: Frequency table of the paired spawning mark estimations of 988 blueback herring *Alosa aestivalis* from 2018.

	Reader 2				
Reader 1	0	1	2	3	4
0	566	121	19	1	-
1	49	145	25	1	-
2	6	9	25	11	1
3	-	-	-	7	1
4	1	-	-	-	-

Table A.7: Frequency table of the paired spawning mark estimations of 1461 blueback herring *Alosa aestivalis* from 2019.

	Reader 2				
Reader 1	0	1	2	3	4
0	1004	104	10	-	-
1	63	194	16	2	-
2	5	20	31	7	-
3	-	-	1	4	-
4	-	-	-	-	-

Table A.8: Frequency table of the paired spawning mark estimations of 920 blueback herring *Alosa aestivalis* from 2021.

	Reader 2				
Reader 1	0	1	2	3	4
0	591	55	4	1	-
1	41	181	16	2	-
2	1	4	20	2	-
3	-	-	-	2	-
4	-	-	-	-	-

Table A.9: Frequency table of the paired spawning mark estimations of 885 blueback herring *Alosa aestivalis* from 2022.

	Reader 2				
Reader 1	0	1	2	3	4
0	556	27	1	1	-
1	33	204	6	-	-
2	2	7	38	2	-
3	-	-	2	6	-
4	-	-	-	-	-

Table A.10: Frequency table of the paired spawning mark estimations of 1082 blueback herring *Alosa aestivalis* from 2023.

	Reader 2				
Reader 1	0	1	2	3	4
0	828	27	5	-	-
1	30	136	20	-	-
2	3	2	23	3	-
3	-	-	2	3	-
4	-	-	-	-	-

Appendix B: Frequency Tables by Survey Years and Age Classes

Table B.1: Frequency table of the paired spawning mark estimations of 9 blueback herring *Alosa aestivalis* that are age 2 from 2016.

	Reader 2				
Reader 1	0	1	2	3	4
0	9	-	-	-	-
1	-	-	-	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.2: Frequency table of the paired spawning mark estimations of 22 blueback herring *Alosa aestivalis* that were age 2 from 2017.

	Reader 2				
Reader 1	0	1	2	3	4
0	21	1	-	-	-
1	-	-	-	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.3: Frequency table of the paired spawning mark estimations of 2 blueback herring *Alosa aestivalis* that were age 2 from 2018.

	Reader 2				
Reader 1	0	1	2	3	4
0	2	-	-	-	-
1	-	-	-	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.4: Frequency table of the paired spawning mark estimations of 3 blueback herring *Alosa aestivalis* that were age 2 from 2019.

	Reader 2				
Reader 1	0	1	2	3	4
0	3	-	-	-	-
1	-	-	-	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.5: Frequency table of the paired spawning mark estimations of 2 blueback herring *Alosa aestivalis* that were age 2 from 2022.

	Reader 2				
Reader 1	0	1	2	3	4
0	2	-	-	-	-
1	-	-	-	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.6: Frequency table of the paired spawning mark estimation of 1 blueback herring *Alosa aestivalis* that were age 2 from 2023.

	Reader 2				
Reader 1	0	1	2	3	4
0	1	-	-	-	-
1	-	-	-	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.7: Frequency table of the paired spawning mark estimations of 118 blueback herring *Alosa aestivalis* that were age 3 from 2013.

	Reader 2				
Reader 1	0	1	2	3	4
0	86	6	-	-	-
1	14	11	-	-	-
2	1	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.8: Frequency table of the paired spawning mark estimations of 111 blueback herring *Alosa aestivalis* that were age 3 from 2014.

	Reader 2				
Reader 1	0	1	2	3	4
0	90	11	-	-	-
1	4	5	-	-	-
2	1	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.9: Frequency table of the paired spawning mark estimations of 22 blueback herring *Alosa aestivalis* that were age 3 from 2015.

	Reader 2				
Reader 1	0	1	2	3	4
0	16	2	1	-	-
1	-	3	-	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.10: Frequency table of the paired spawning mark estimations of 124 blueback herring *Alosa aestivalis* that were age 3 from 2016.

	Reader 2				
Reader 1	0	1	2	3	4
0	110	7	2	1	-
1	-	3	-	-	-
2	-	1	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.11: Frequency table of the paired spawning mark estimations of 646 blueback herring *Alosa aestivalis* that were age 3 from 2017.

	Reader 2				
Reader 1	0	1	2	3	4
0	608	25	1	-	-
1	6	6	-	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.12: Frequency table of the paired spawning mark estimations of 444 blueback herring *Alosa aestivalis* that were age 3 from 2018.

	Reader 2				
Reader 1	0	1	2	3	4
0	359	43	1	-	-
1	22	14	-	-	-
2	4	1	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.13: Frequency table of the paired spawning mark estimations of 184 blueback herring *Alosa aestivalis* that were age 3 from 2019.

	Reader 2				
Reader 1	0	1	2	3	4
0	170	10	-	-	-
1	2	2	-	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.14: Frequency table of the paired spawning mark estimations of 279 blueback herring *Alosa aestivalis* that were age 3 from 2021.

	Reader 2				
Reader 1	0	1	2	3	4
0	270	3	-	-	-
1	5	1	-	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.15: Frequency table of the paired spawning mark estimations of 213 blueback herring *Alosa aestivalis* that were age 3 from 2022.

	Reader 2				
Reader 1	0	1	2	3	4
0	208	1	-	-	-
1	1	3	-	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.16: Frequency table of the paired spawning mark estimations of 172 blueback herring *Alosa aestivalis* that were age 3 from 2023.

	Reader 2				
Reader 1	0	1	2	3	4
0	168	1	-	-	-
1	0	3	-	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.17: Frequency table of the paired spawning mark estimations of 109 blueback herring *Alosa aestivalis* that were age 4 from 2013.

	Reader 2				
Reader 1	0	1	2	3	4
0	58	3	1	-	-
1	13	26	-	-	-
2	2	5	1	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.18: Frequency table of the paired spawning mark estimations of 394 blueback herring *Alosa aestivalis* that were age 4 from 2014.

	Reader 2				
Reader 1	0	1	2	3	4
0	138	32	4	-	-
1	30	165	4	-	-
2	1	9	11	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.19: Frequency table of the paired spawning mark estimations of 166 blueback herring *Alosa aestivalis* that were age 4 from 2015.

	Reader 2				
Reader 1	0	1	2	3	4
0	73	23	1	-	-
1	5	57	2	-	-
2	-	2	3	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.20: Frequency table of the paired spawning mark estimations of 48 blueback herring *Alosa aestivalis* that were age 4 from 2016.

	Reader 2				
Reader 1	0	1	2	3	4
0	29	7	-	-	-
1	1	10	-	-	-
2	-	1	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.21: Frequency table of the paired spawning mark estimations of 181 blueback herring *Alosa aestivalis* that were age 4 from 2017.

	Reader 2				
Reader 1	0	1	2	3	4
0	88	32	-	1	-
1	6	51	3	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.22: Frequency table of the paired spawning mark estimations of 362 blueback herring *Alosa aestivalis* that were age 4 from 2018.

	Reader 2				
Reader 1	0	1	2	3	4
0	182	58	4	-	-
1	25	82	-	-	-
2	1	6	3	-	-
3	-	-	-	-	-
4	1	-	-	-	-

Table B.23: Frequency table of the paired spawning mark estimations of 824 blueback herring *Alosa aestivalis* that were age 4 from 2019.

	Reader 2				
Reader 1	0	1	2	3	4
0	653	38	2	-	-
1	38	89	-	-	-
2	2	2	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.24: Frequency table of the paired spawning mark estimations of 73 blueback herring *Alosa aestivalis* that were age 4 from 2021.

	Reader 2				
Reader 1	0	1	2	3	4
0	53	2	-	-	-
1	4	14	-	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.25: Frequency table of the paired spawning mark estimations of 365 blueback herring *Alosa aestivalis* that were age 4 from 2022.

	Reader 2				
Reader 1	0	1	2	3	4
0	257	10	1	-	-
1	13	82	-	-	-
2	-	-	2	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.26: Frequency table of the paired spawning mark estimations of 458 blueback herring *Alosa aestivalis* that were age 4 from 2023.

	Reader 2				
Reader 1	0	1	2	3	4
0	395	4	-	-	-
1	13	44	-	-	-
2	1	-	2	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.27: Frequency table of the paired spawning mark estimations of 83 blueback herring *Alosa aestivalis* that were age 5 from 2013.

	Reader 2				
Reader 1	0	1	2	3	4
0	12	1	-	-	-
1	10	25	2	-	-
2	3	6	20	-	-
3	-	-	2	2	-
4	-	-	-	-	-

Table B.28: Frequency table of the paired spawning mark estimations of 80 blueback herring *Alosa aestivalis* that were age 5 from 2014.

	Reader 2				
Reader 1	0	1	2	3	4
0	17	2	-	-	-
1	7	33	-	-	-
2	-	6	13	-	-
3	-	-	2	-	-
4	-	-	-	-	-

Table B.29: Frequency table of the paired spawning mark estimations of 235 blueback herring *Alosa aestivalis* that were age 5 from 2015.

	Reader 2				
Reader 1	0	1	2	3	4
0	29	12	2	-	-
1	7	93	11	-	-
2	1	16	56	-	-
3	-	-	5	3	-
4	-	-	-	-	-

Table B.30: Frequency table of the paired spawning mark estimations of 238 blueback herring *Alosa aestivalis* that were age 5 from 2016.

	Reader 2				
Reader 1	0	1	2	3	4
0	60	35	7	-	-
1	9	81	13	-	-
2	1	2	27	-	-
3	-	-	-	3	-
4	-	-	-	-	-

Table B.31: Frequency table of the paired spawning mark estimations of 56 blueback herring *Alosa aestivalis* that were age 5 from 2017.

	Reader 2				
Reader 1	0	1	2	3	4
0	11	7	1	-	-
1	1	28	2	-	-
2	-	2	4	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.32: Frequency table of the paired spawning mark estimations of 88 blueback herring *Alosa aestivalis* that were age 5 from 2018.

	Reader 2				
Reader 1	0	1	2	3	4
0	18	16	7	-	-
1	2	28	9	-	-
2	-	1	7	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.33: Frequency table of the paired spawning mark estimations of 337 blueback herring *Alosa aestivalis* that were age 5 from 2019.

	Reader 2				
Reader 1	0	1	2	3	4
0	164	42	5	-	-
1	16	76	7	-	-
2	3	8	15	1	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.34: Frequency table of the paired spawning mark estimations of 126 blueback herring *Alosa aestivalis* that were age 5 from 2021.

	Reader 2				
Reader 1	0	1	2	3	4
0	62	8	-	-	-
1	5	41	1	-	-
2	-	-	9	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.35: Frequency table of the paired spawning mark estimations of 66 blueback herring *Alosa aestivalis* that were age 5 from 2022.

	Reader 2				
Reader 1	0	1	2	3	4
0	29	4	-	-	-
1	5	26	-	-	-
2	-	-	2	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.36: Frequency table of the paired spawning mark estimations of 326 blueback herring *Alosa aestivalis* that were age 5 from 2023.

	Reader 2				
Reader 1	0	1	2	3	4
0	220	16	5	-	-
1	11	48	10	-	-
2	1	1	13	1	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.37: Frequency table of the paired spawning mark estimations of 50 blueback herring *Alosa aestivalis* that were age 6 from 2013.

	Reader 2				
Reader 1	0	1	2	3	4
0	8	3	1	-	-
1	2	13	2	-	-
2	1	6	8	1	-
3	2	1	1	1	-
4	-	-	-	-	-

Table B.38: Frequency table of the paired spawning mark estimations of 35 blueback herring *Alosa aestivalis* that were age 6 from 2014.

	Reader 2				
Reader 1	0	1	2	3	4
0	2	3	2	-	-
1	2	2	2	-	-
2	2	3	5	1	-
3	-	1	2	6	-
4	1	-	-	1	-

Table B.39: Frequency table of the paired spawning mark estimations of 48 blueback herring *Alosa aestivalis* that were age 6 from 2015.

	Reader 2				
Reader 1	0	1	2	3	4
0	3	3	2	-	-
1	-	11	1	1	-
2	-	2	16	4	-
3	-	-	-	5	-
4	-	-	-	-	-

Table B.40: Frequency table of the paired spawning mark estimations of 244 blueback herring *Alosa aestivalis* that were age 6 from 2016.

	Reader 2				
Reader 1	0	1	2	3	4
0	19	12	11	-	-
1	1	59	28	1	-
2	5	8	70	6	-
3	-	-	8	14	-
4	-	1	-	1	-

Table B.41: Frequency table of the paired spawning mark estimations of 155 blueback herring *Alosa aestivalis* that were age 6 from 2017.

	Reader 2				
Reader 1	0	1	2	3	4
0	10	23	8	-	-
1	7	60	10	1	-
2	-	8	23	-	-
3	-	-	3	2	-
4	-	-	-	-	-

Table B.42: Frequency table of the paired spawning mark estimations of 14 blueback herring *Alosa aestivalis* that were age 6 from 2018.

	Reader 2				
Reader 1	0	1	2	3	4
0	1	3	-	-	-
1	-	4	2	1	-
2	-	-	2	1	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.43: Frequency table of the paired spawning mark estimations of 72 blueback herring *Alosa aestivalis* that were age 6 from 2019.

	Reader 2				
Reader 1	0	1	2	3	4
0	13	11	2	-	-
1	6	20	4	1	-
2	-	6	6	1	-
3	-	-	1	1	-
4	-	-	-	-	-

Table B.44: Frequency table of the paired spawning mark estimations of 386 blueback herring *Alosa aestivalis* that were age 6 from 2021.

	Reader 2				
Reader 1	0	1	2	3	4
0	190	38	3	-	-
1	23	103	15	1	-
2	-	4	7	1	-
3	-	-	-	1	-
4	-	-	-	-	-

Table B.45: Frequency table of the paired spawning mark estimations of 95 blueback herring *Alosa aestivalis* that were age 6 from 2022.

	Reader 2				
Reader 1	0	1	2	3	4
0	21	2	-	-	-
1	4	38	-	-	-
2	1	3	23	2	-
3	-	-	-	1	-
4	-	-	-	-	-

Table B.46: Frequency table of the paired spawning mark estimations of 44 blueback herring *Alosa aestivalis* that were age 6 from 2023.

	Reader 2				
Reader 1	0	1	2	3	4
0	19	3	-	-	-
1	2	11	3	-	-
2	1	-	2	1	-
3	-	-	1	1	-
4	-	-	-	-	-

Table B.47: Frequency table of the paired spawning mark estimation of 1 blueback herring *Alosa aestivalis* that was age 7 from 2013.

	Reader 2				
Reader 1	0	1	2	3	4
0	1	-	-	-	-
1	-	-	-	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.48: Frequency table of the paired spawning mark estimations of 8 blueback herring *Alosa aestivalis* that were age 7 from 2014.

	Reader 2				
Reader 1	0	1	2	3	4
0	-	1	-	-	-
1	-	-	-	-	-
2	-	-	2	1	-
3	-	1	1	2	-
4	-	-	-	-	-

Table B.49: Frequency table of the paired spawning mark estimations of 19 blueback herring *Alosa aestivalis* that were age 7 from 2015.

	Reader 2				
Reader 1	0	1	2	3	4
0	1	-	-	-	-
1	-	4	-	1	-
2	-	2	3	1	1
3	-	-	2	3	-
4	-	-	-	1	-

Table B.50: Frequency table of the paired spawning mark estimations of 33 blueback herring *Alosa aestivalis* that were age 7 from 2016.

	Reader 2				
Reader 1	0	1	2	3	4
0	2	-	3	1	-
1	-	7	3	-	-
2	-	2	11	1	-
3	-	-	-	1	-
4	-	-	-	1	1

Table B.51: Frequency table of the paired spawning mark estimations of 96 blueback herring *Alosa aestivalis* that were age 7 from 2017.

	Reader 2				
Reader 1	0	1	2	3	4
0	4	7	1	1	-
1	4	36	6	1	-
2	-	9	19	-	-
3	-	-	2	5	1
4	-	-	-	-	-

Table B.52: Frequency table of the paired spawning mark estimations of 57 blueback herring *Alosa aestivalis* that were age 7 from 2018.

	Reader 2				
Reader 1	0	1	2	3	4
0	3	1	4	-	-
1	-	14	8	-	-
2	1	-	9	10	1
3	-	-	-	6	-
4	-	-	-	-	-

Table B.53: Frequency table of the paired spawning mark estimations of 21 blueback herring *Alosa aestivalis* that were age 7 from 2019.

	Reader 2				
Reader 1	0	1	2	3	4
0	1	3	1	-	-
1	1	4	-	-	-
2	-	3	5	2	-
3	-	-	-	1	-
4	-	-	-	-	-

Table B.54: Frequency table of the paired spawning mark estimations of 51 blueback herring *Alosa aestivalis* that were age 7 from 2021.

	Reader 2				
Reader 1	0	1	2	3	4
0	16	4	1	-	-
1	4	19	-	1	-
2	-	-	4	1	-
3	-	-	-	1	-
4	-	-	-	-	-

Table B.55: Frequency table of the paired spawning mark estimations of 133 blueback herring *Alosa aestivalis* that were age 7 from 2022.

	Reader 2				
Reader 1	0	1	2	3	4
0	37	10	-	1	-
1	9	50	6	-	-
2	1	3	10	-	-
3	-	-	1	5	-
4	-	-	-	-	-

Table B.56: Frequency table of the paired spawning mark estimations of 39 blueback herring *Alosa aestivalis* that were age 7 from 2023.

	Reader 2				
Reader 1	0	1	2	3	4
0	10	2	-	-	-
1	2	18	3	-	-
2	-	-	2	-	-
3	-	-	1	1	-
4	-	-	-	-	-

Table B.57: Frequency table of the paired spawning mark estimation of 1 blueback herring *Alosa aestivalis* that was age 8 from 2013.

	Reader 2				
Reader 1	0	1	2	3	4
0	-	-	-	-	-
1	-	-	-	-	-
2	-	-	1	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.58: Frequency table of the paired spawning mark estimations of 6 blueback herring *Alosa aestivalis* that were age 8 from 2015.

	Reader 2				
Reader 1	0	1	2	3	4
0	-	-	-	-	-
1	-	2	-	-	-
2	-	1	1	-	-
3	-	-	1	-	-
4	-	-	-	-	1

Table B.59: Frequency table of the paired spawning mark estimations of 9 blueback herring *Alosa aestivalis* that were age 8 from 2016.

	Reader 2				
Reader 1	0	1	2	3	4
0	-	1	-	-	-
1	-	-	-	-	-
2	-	-	2	1	-
3	1	-	-	3	-
4	-	-	-	-	1

Table B.60: Frequency table of the paired spawning mark estimations of 7 blueback herring *Alosa aestivalis* that were age 8 from 2017.

	Reader 2				
Reader 1	0	1	2	3	4
0	-	1	-	-	-
1	-	3	-	-	-
2	-	-	3	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.61: Frequency table of the paired spawning mark estimations of 20 blueback herring *Alosa aestivalis* that were age 8 from 2018.

	Reader 2				
Reader 1	0	1	2	3	4
0	1	-	3	1	-
1	-	3	6	-	-
2	-	1	4	-	-
3	-	-	-	-	1
4	-	-	-	-	-

Table B.62: Frequency table of the paired spawning mark estimations of 15 blueback herring *Alosa aestivalis* that were age 8 from 2019.

	Reader 2				
Reader 1	0	1	2	3	4
0	-	-	-	-	-
1	-	2	4	1	-
2	-	-	4	3	-
3	-	-	-	1	-
4	-	-	-	-	-

Table B.63: Frequency table of the paired spawning mark estimations of 3 blueback herring *Alosa aestivalis* that were age 8 from 2021.

	Reader 2				
Reader 1	0	1	2	3	4
0	-	-	-	1	-
1	-	2	-	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.64: Frequency table of the paired spawning mark estimations of 11 blueback herring *Alosa aestivalis* that were age 8 from 2022.

	Reader 2				
Reader 1	0	1	2	3	4
0	2	-	-	-	-
1	1	5	-	-	-
2	-	1	1	-	-
3	-	-	1	-	-
4	-	-	-	-	-

Table B.65: Frequency table of the paired spawning mark estimations of 40 blueback herring *Alosa aestivalis* that were age 8 from 2023.

	Reader 2				
Reader 1	0	1	2	3	4
0	14	1	-	-	-
1	2	12	3	-	-
2	-	1	5	1	-
3	-	-	-	1	-
4	-	-	-	-	-

Table B.66: Frequency table of the paired spawning mark estimations of 4 blueback herring *Alosa aestivalis* that were age 9 from 2016.

	Reader 2				
Reader 1	0	1	2	3	4
0	-	-	-	-	-
1	-	3	1	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.67: Frequency table of the paired spawning mark estimations of 4 blueback herring *Alosa aestivalis* that were age 9 from 2017.

	Reader 2				
Reader 1	0	1	2	3	4
0	-	-	-	1	-
1	-	1	-	-	-
2	-	1	-	-	-
3	-	-	-	1	-
4	-	-	-	-	-

Table B.68: Frequency table of the paired spawning mark estimation of 1 blueback herring *Alosa aestivalis* that was age 9 from 2018.

	Reader 2				
Reader 1	0	1	2	3	4
0	-	-	-	-	-
1	-	-	-	-	-
2	-	-	-	-	-
3	-	-	-	1	-
4	-	-	-	-	-

Table B.69: Frequency table of the paired spawning mark estimations of 3 blueback herring *Alosa aestivalis* that were age 9 from 2019.

	Reader 2				
Reader 1	0	1	2	3	4
0	-	-	-	-	-
1	-	-	1	-	-
2	-	-	1	-	-
3	-	-	-	1	-
4	-	-	-	-	-

Table B.70: Frequency table of the paired spawning mark estimation of 1 blueback herring *Alosa aestivalis* that was age 9 from 2021.

	Reader 2				
Reader 1	0	1	2	3	4
0	-	-	-	-	-
1	-	1	-	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.71: Frequency table of the paired spawning mark estimations of 2 blueback herring *Alosa aestivalis* that were age 9 from 2023.

	Reader 2				
Reader 1	0	1	2	3	4
0	1	-	-	-	-
1	-	-	1	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-

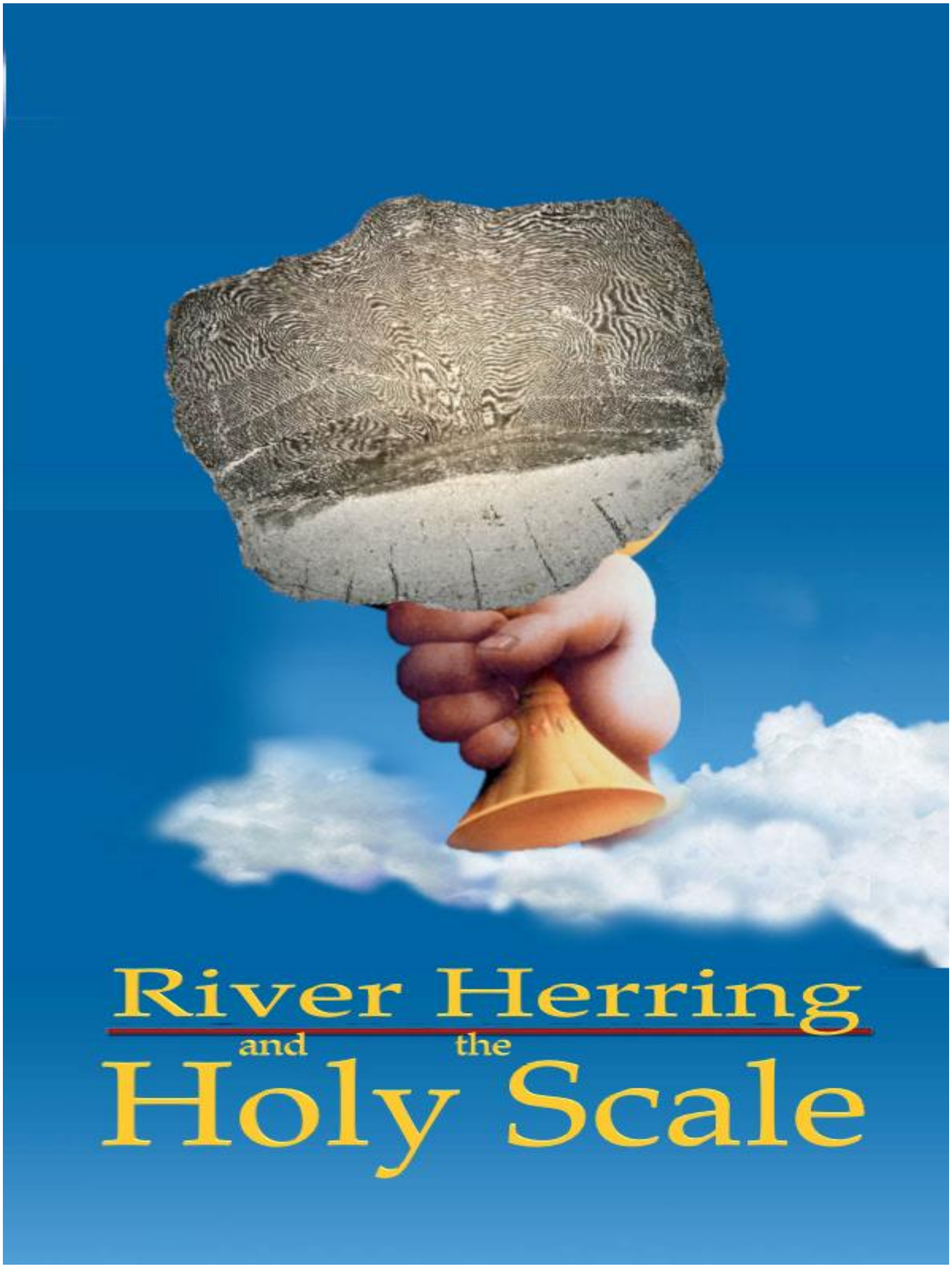
Table B.72: Frequency table of the paired spawning mark estimations of 2 blueback herring *Alosa aestivalis* that were age 10 from 2019.

	Reader 2				
Reader 1	0	1	2	3	4
0	-	-	-	-	-
1	-	1	-	-	-
2	-	1	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Table B.73: Frequency table of the paired spawning mark estimation of 1 blueback herring *Alosa aestivalis* that was age 10 from 2021.

	Reader 2				
Reader 1	0	1	2	3	4
0	-	-	-	-	-
1	-	-	-	-	-
2	1	-	-	-	-
3	-	-	-	-	-
4	-	-	-	-	-

Appendix C: River Herring and the Holy Scale



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Scale Reading Protocol established in the Spring of 2023

Jacqueline Stephens, U.S. Fish and Wildlife Service Pathways Intern

Introduction

The purpose of this protocol is to introduce and uniformly teach scale interpretation for the life history of anadromous river herring. As you read through this protocol, you will begin to understand the difficulties associated with scale reading and why a standardized form of teaching and reading is necessary to confidently apply spawning mark data in statistical analyses for population assessments of returning river herring. The term "river herring" encompasses two alosine species, Blueback herring *Alosa aestivalis* and Alewife *Alosa pseudoharengus*. While their scales may have slight differences in appearance, this protocol can be referenced for both species' scales.

Acknowledgements

This protocol would not have been possible without the guidance from the experts listed below and the review of their associated state protocols:

Ken Sprankle – U.S. Fish & Wildlife Service Connecticut River Fish & Wildlife Conservation Office

Scott Elzey – Massachusetts Division of Marine Fisheries

Wes Eakin – New York Department of Environmental Conservation

Kevin Job – Connecticut Department of Energy and Environmental Protection

Rebecca Colby – Massachusetts Division of Wildlife

Kyle Hubbard and Rogue Brock – American Conservation Experience (ACE) Technicians 2023

Initial Scale Processing

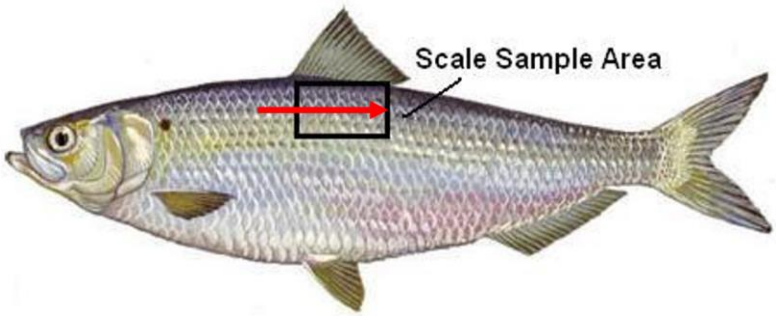
Scale Collection

Each fish will have a unique sample ID associated with it:

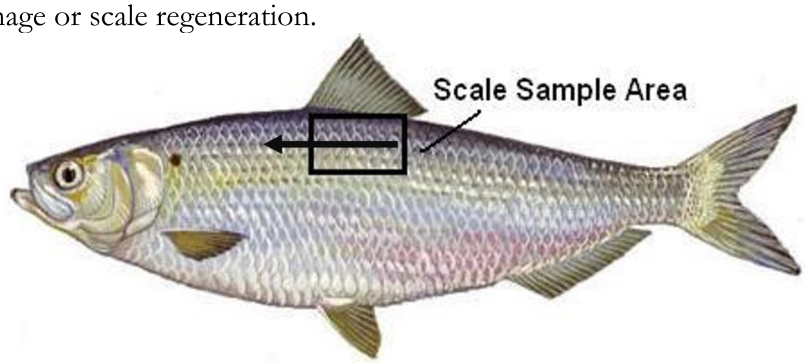
Sampling date (MMDDYY), Site code (MTB, WFC, FRM, WFD, or CCP) and fish number (i.e., 001 – 080)

Example. 052622WFC045 indicates that the fish was collected on May 26th, 2022, at Wethersfield Cove and it was the 45 fish of the total subsample.

1. Check the knife to make sure no scales are present on it to avoid cross-contamination from past sample processing.
2. Prior to scale removal, use knife to scrape away (**with the direction of scales**) any dirt or mucus coating the scales.



3. Wipe off mucus from the knife.
4. Now in the opposite direction of the scales (**against them**), scrape the knife across the area of the fish just ventral of the dorsal fin and above the lateral line. Avoid areas of obvious damage or scale regeneration.



5. Take approximately 20 scales and place into a scale envelope with the corresponding sample ID written on it.
6. Clean the knife by dipping knife in water and wiping off ***all*** scales to avoid cross contamination, and proceed with processing.

Scale Preparation

1. Make a Pancreatin solution.
    a. In a beaker, combine 500mL of water and 3.5 grams of Pancreatin powder.
    b. Place beaker on stir plate and add metal pill.
    c. Turn stir plate on and let solution mix for approximately 10 minutes or until solution looks uniform and powder is fully dissolved.
2. Place approximately 10 scales into an empty petri dish. Try to avoid picking regenerated scales (see page 7).
3. Fill the petri dish with 25mL of Pancreatin solution.
4. Soak scales for 5 minutes, occasionally swishing the solution around in the dish.
5. Remove all scales from the dish using forceps and place in a secondary petri dish about halfway full of water to rinse solution off scales.
6. Once rinsed, dry scales by placing them on a paper towel and flipping repeatedly or dabbing with more paper towel until dry.
7. Arrange dried scales on a glass slide (2 rows of 4 scales all facing the same direction/orientation), top with second glass slide (so that scales are sandwiched between slides), and tape around the ends so slides are firmly pressed together.
8. Label the slide with the corresponding sample ID (do not cover the scales).
9. After each sample, it is IMPERATIVE that no scales remain in either dish.
    a) Visually inspect the pancreatin solution dish for missed scales.
    b) Empty, rinse and refill the water petri dish before moving on to the next sampling to avoid cross- contamination of scales.

NOTE: the 25 mL of Pancreatin can be used 3 times before the digestive properties begin to diminish; after three sets of scales have been cleaned, dump it out and add another 25mL from the beaker.

Scale Reading Equipment

While the goal of this protocol is to promote uniformity, there is no right answer to the question of what equipment is the best to use for scale reading. The microfiche, a machine that gained popularity in the 1960s for its storage capacity of documents and other media, has commonly been used for scale reading. However, advancements in its design and mechanics have been made over the years. Electric versions of the microfiche have become popular with some state agencies. There are positive and negative arguments that can be debated for which is better: the classic microfiche vs the electronic, including the ease in information sharing, difference in perspectives, and physical storage of scale readings. In the end, the machine you use may ultimately come down to prior resources and availability.

The scale photos seen throughout this protocol are of a microfiche screen, taken on an iPhone SE.

Scale Reading with a Microfiche: Background

Scale Structures

Fish scales are derived from the dermis and grow concentrically around their focus. The anterior portion of the scale (darker seen below) is embedded within dermis, while the posterior is exposed to the environment.

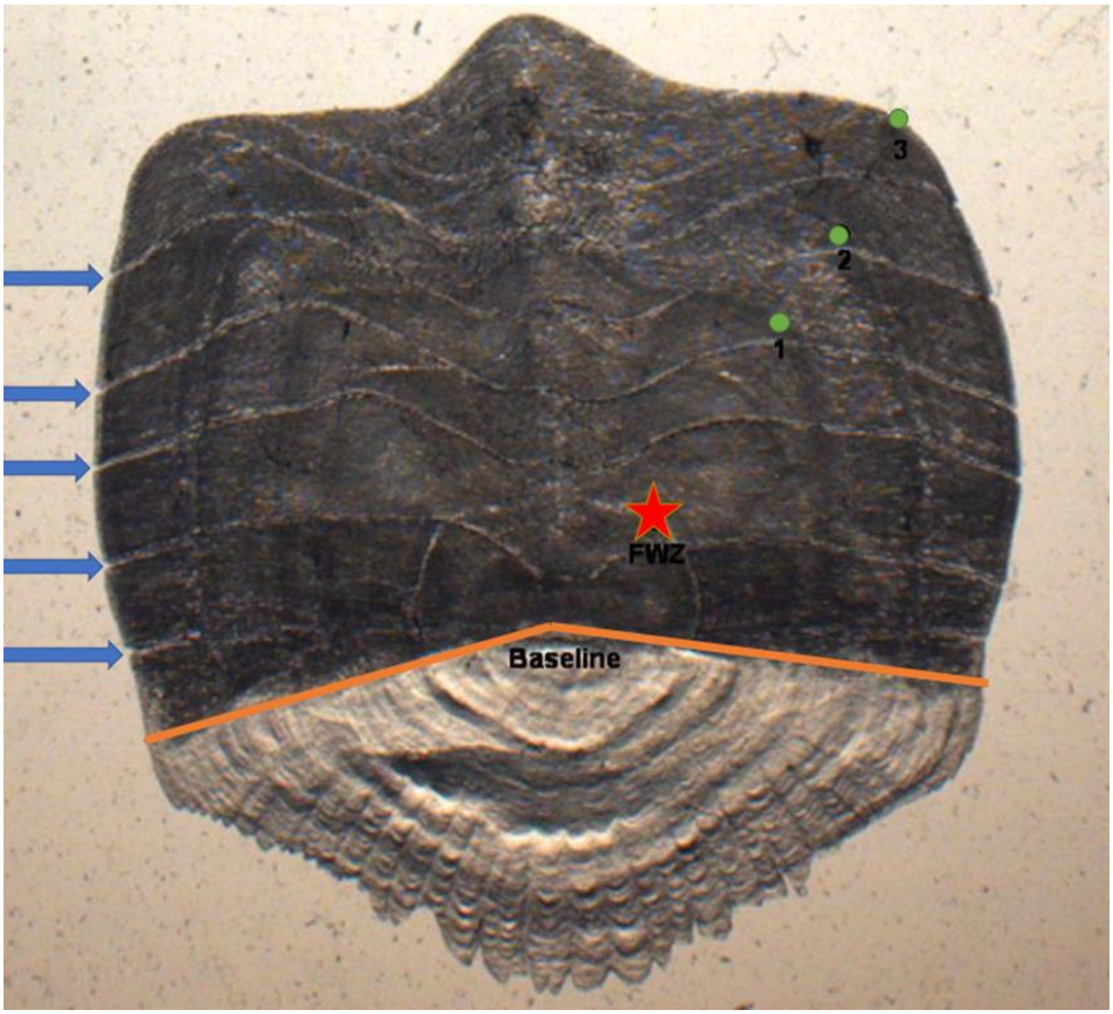


Figure 1: Blueback herring (*Alosa aestivalis*) scale with structures highlighted for identification. The photograph is originally Figure 8 from the Massachusetts Division of Marine Fisheries Age and Growth Laboratory: Fish Aging Protocols (Elzey et al. 2015).

Baseline (orange line): First transverse groove that separates the anterior and posterior sides, the baseline is straight across for alewife scales and angled (see above) for blueback herring scales.

Transverse grooves (blue arrows): Distinct horizontal lines across scale that allow for flexibility

Freshwater Zone (FWZ, red star): First dark band commonly mistaken for the first annulus; they are more difficult to distinguish on blueback herring scales than alewife. They are not present in all individuals (small proportion for alewives).

Annulus (green circles): Continuous, concentric smooth band or line that travels around the scale and must pass through the baseline (i.e., present on both the anterior and posterior sides of the scale)

Fish growth slows during cold periods (winter months) and causes breakages in the circuli which can be interpreted as an annulus. Multiple annuli can become crowded together at the edge of the scale or re-absorb over each other but will separate back out beneath the baseline.

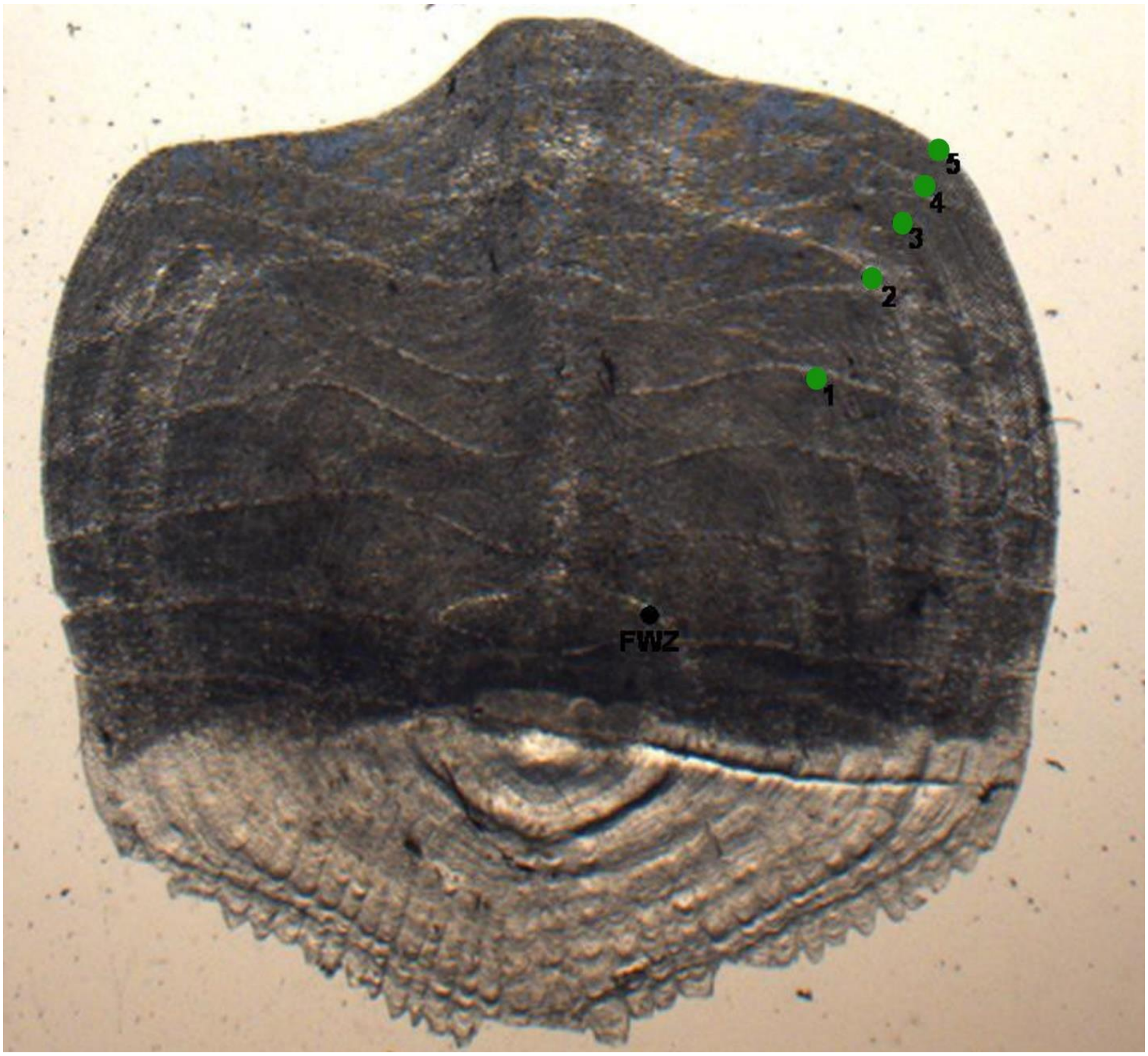


Figure 2: Blueback herring (*Alosa aestivalis*) scale with freshwater zone (FWZ) and annuli marked (green circles), specifically displaying how the second annulus typically appears the "strongest" in blueback herring scales. The photograph is Figure 11 from the Massachusetts Division of Marine Fisheries Age and Growth Laboratory: Fish Aging Protocols (Elzey et al. 2015).

What are Spawning Marks?

Each spawning mark appears as a degraded annulus (i.e., a jagged line where the smooth line of an annulus is expected). The jagged appearance is usually seen ½ - ¾ way up the annulus from the anterior (above the baseline) portion of the scale (see Figures 5–11), although in some cases the degradation can affect the entire annulus.

Spawning marks on river herring scales occur due to the physiological stress involved in migrating upriver to their natal freshwater environments from the Atlantic ocean. Freshwater is ion deficient compared to seawater (i.e., calcium), so fish are believed to supplement ion intake through the re-absorption of their scales. Because this re-absorption is an internal process, spawning marks do not cross over the baseline and therefore do not appear on the part of the external scale as annuli do.

The terms 'erosion' and 'reabsorption' may be used interchangeably when discussing spawning marks and the associated scale degradation.

Distinguishing between a Stress Mark and a Spawning Mark

It is important to understand how to differentiate between a stress mark and a spawning mark as the migration into freshwater to spawn is not the only time adult river herring experience physiological stress. Use these three classifications to help differentiate between the two types of marks:

1. Depth and Strength of the Mark –

A spawning mark will usually be "deeper" and "stronger" because the migration to freshwater can be extremely strenuous and extend over a few weeks depending on the fish. Some questions to ask:

Is it clearly jagged even when taking a few steps away from the microfiche?

Can you still see the mark as you increase and or decrease the focus of the microfiche?

2. Length of the Mark –

As mentioned in the prior section, the jagged appearance of a spawning marks will be seen ½ - ¾ way up the annulus from the anterior (above the baseline) portion of the scale. The "longer" the mark extends, the more confident you can be that it is a spawning mark. The simple question to ask:

Does the mark extend more than the ½ way up from the baseline?

3. Frequency/ Consistency on all Scales –

Spawning marks should be seen in the same spot on multiple scales. The rule of thumb is to have the spawning mark present on at least ½ of all readable scales present. Ask:

How many scales is the mark present on? Is it in all the same spots on the all the scales?

Scale Reading with a Microfiche: Example Scales

A. Regenerated Scale

Regenerated scales rapidly grow in place of original scales that are lost or damaged. Due to this rapid regeneration to restore the protective function, regenerated scales do not display the entire life history of the fish and should be disregarded when estimating age or spawning marks. Notice the unorganized circuli in the central focus of the regenerated scale (Figure 3), as if an eraser was used on it.

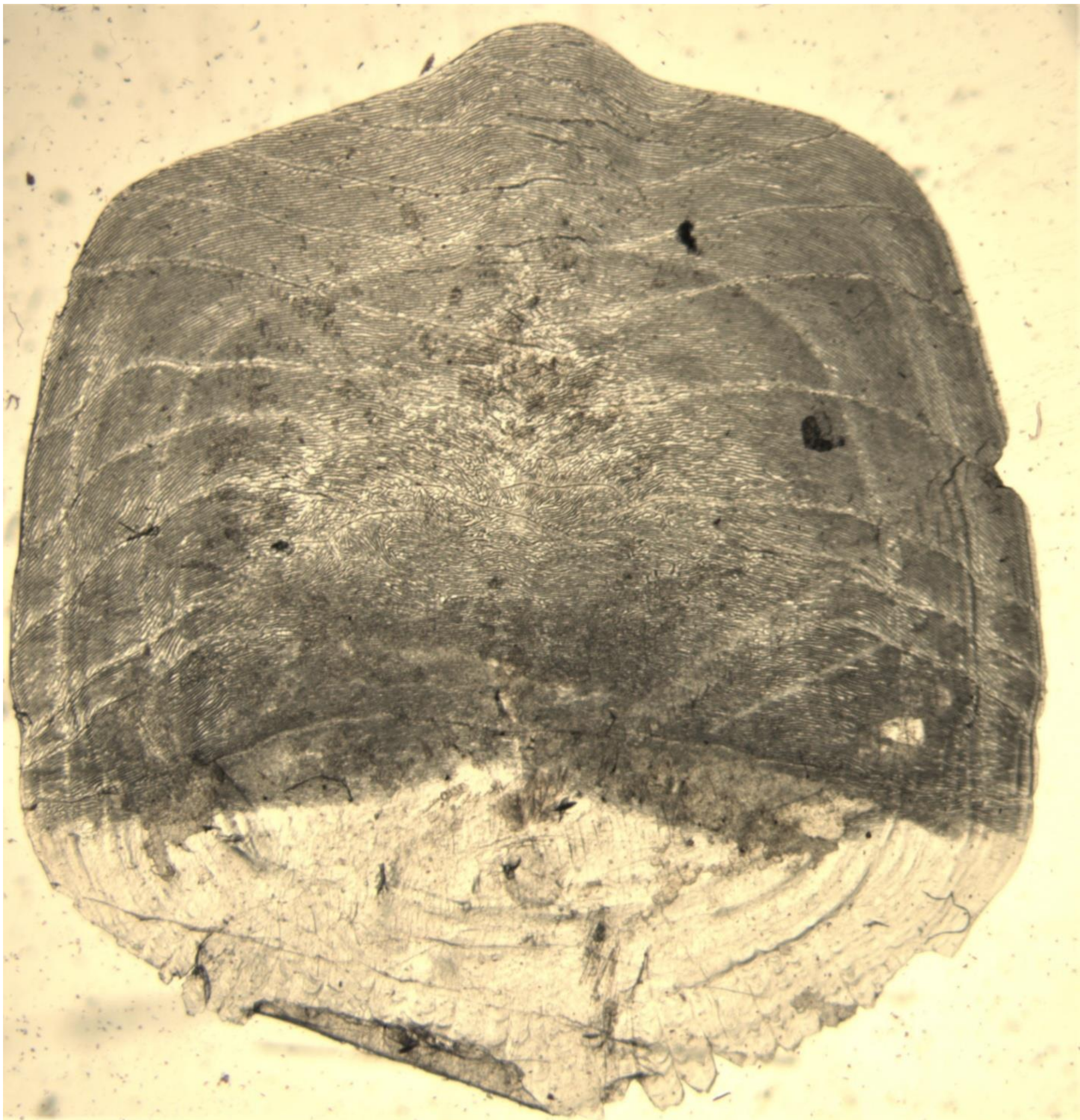


Figure 3: Regenerated alewife (*Alosa pseudoharengus*) scale provided by Rebecca Colby.

B. Virgin Spawner Scale – 0 Spawning Marks

The scale below displays clean lines of annuli but no jagged lines of spawning marks (Figure 4). The lack of spawning marks indicates that this fish was returning to spawn for the first time in freshwater when caught.

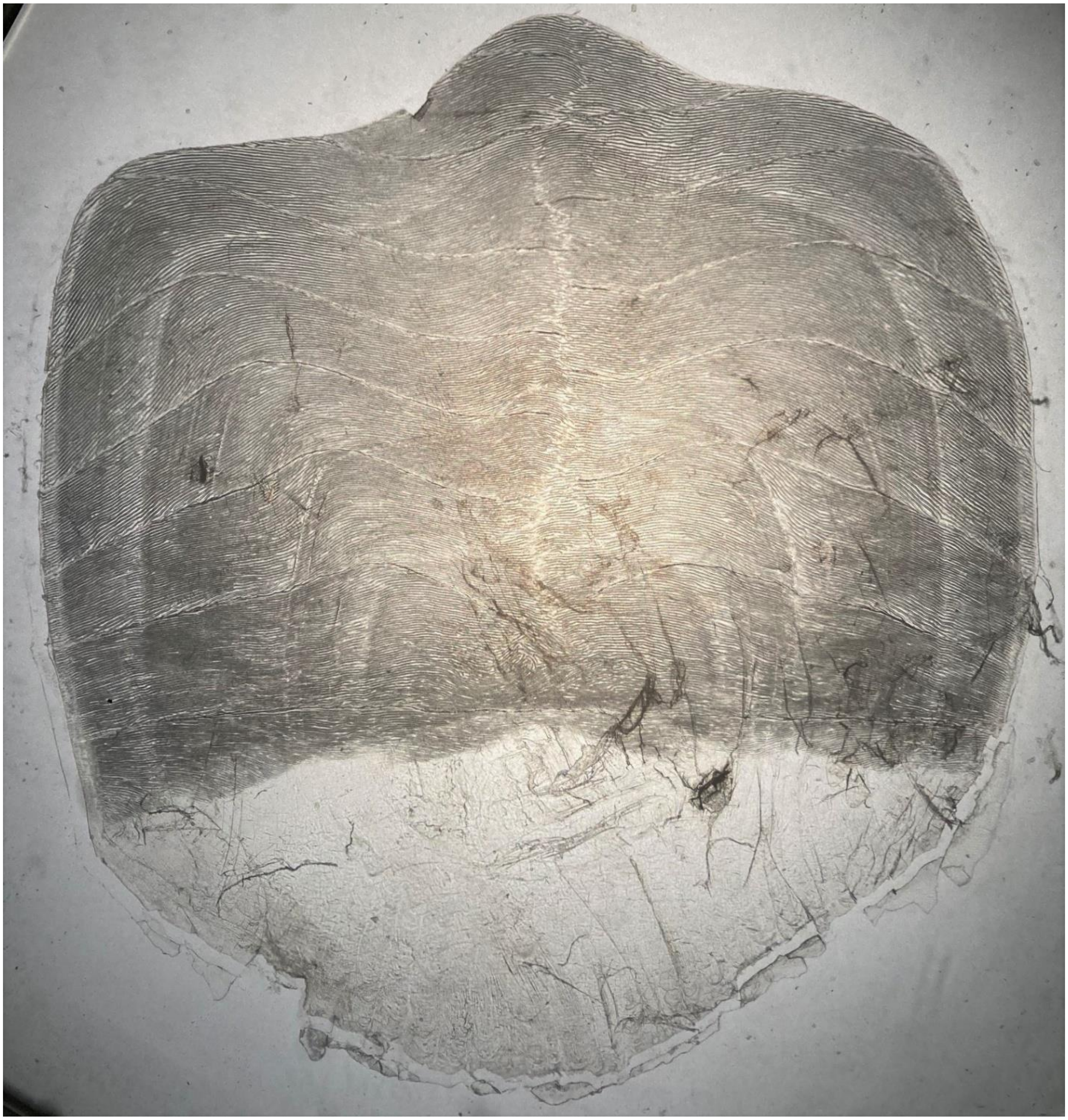


Figure 4: Blueback herring (*Alosa aestivalis*) scale from a virgin spawner collected in the USFWS Annual Adult River Herring Stock Assessment survey (Sprankle and Desmarais 2018).

C. Repeat Spawner Scale – 1 Spawning Mark

The blue arrows on the scale below (Figure 5) display a spawning mark (i.e., jagged line) that concentrically travels around the scale. Based on the sampling date and capture location, it can be assumed that this fish was returning for the second time to spawn.

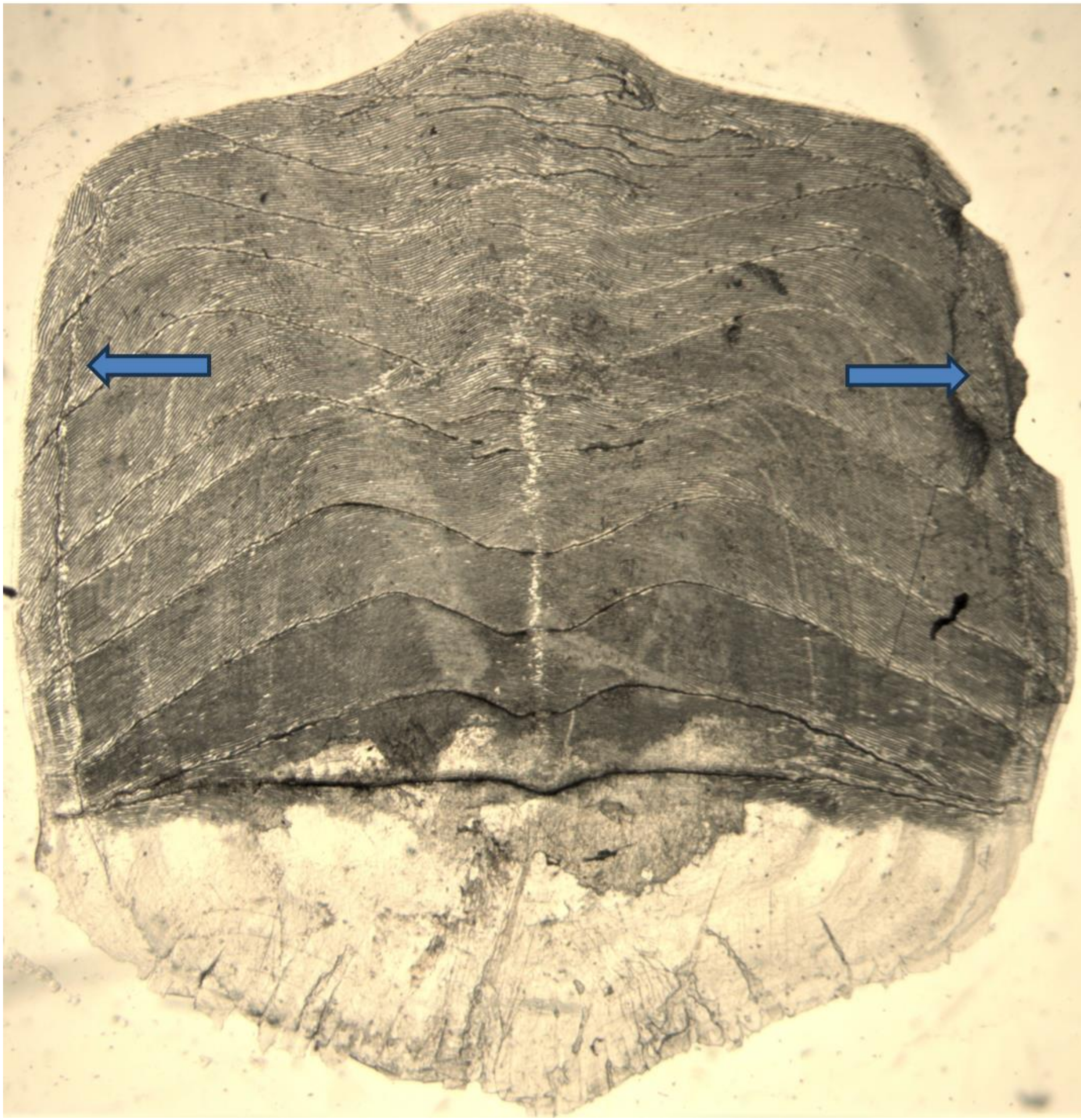


Figure 5: Alewife (*Alosa pseudoharengus*) scale displaying one spawning mark (blue arrows) from a repeat spawner provided by Rebecca Colby.

D. Repeat Spawner Scale – 2 Spawning Mark

The scale below displays two distinct spawning marks (i.e., jagged lines) that concentrically travels around the scale. Based on the sampling date and capture location, it can be assumed that this fish was returning for the third time to spawn.

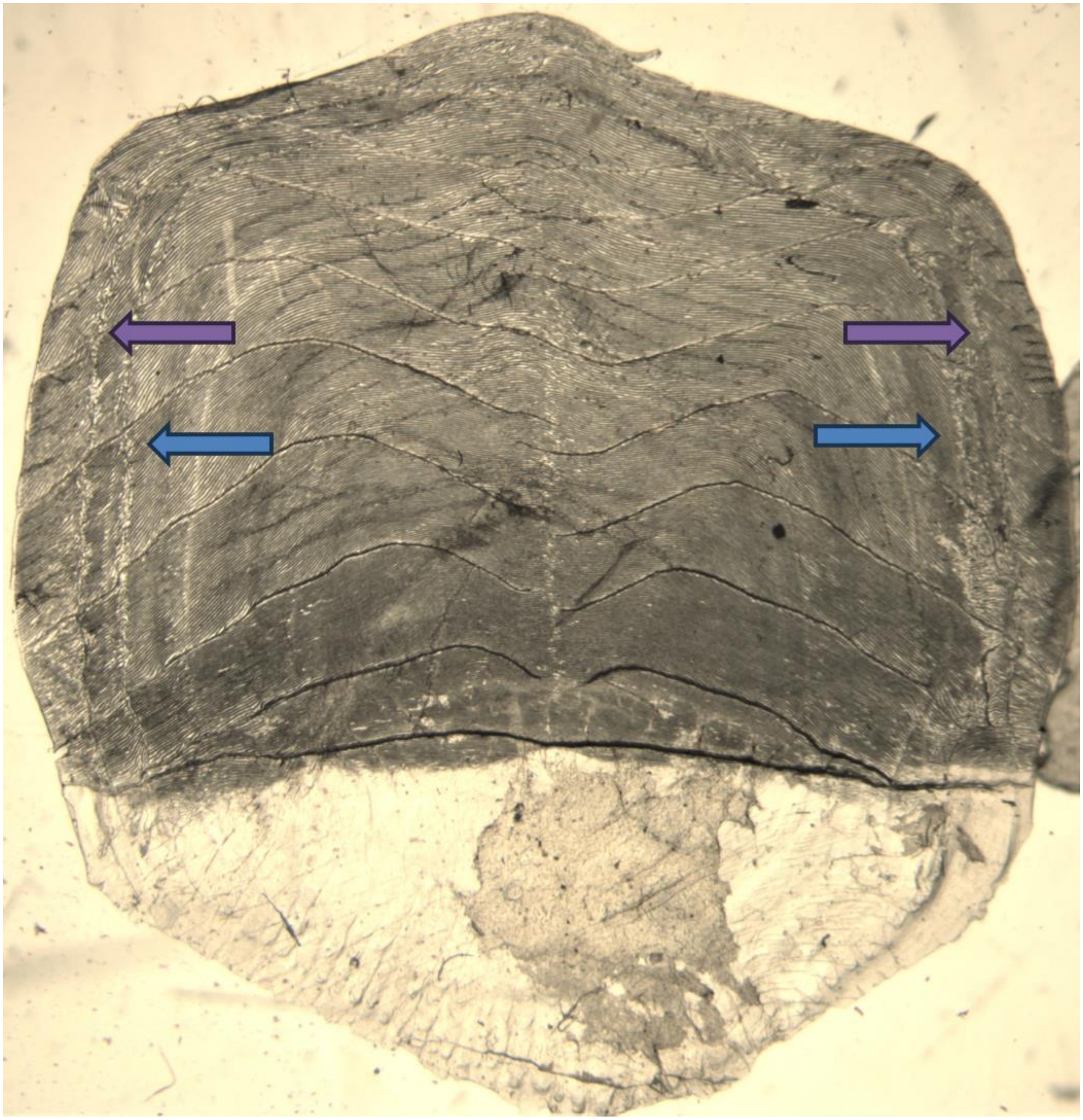


Figure 6: Alewife (*Alosa pseudoharengus*) scale displaying two spawning mark (blue arrows point to the first mark, and purple arrows to the second mark) from a repeat spawner provided by Rebecca Colby.

E. Repeat Spawner Scale – 3 Spawning Marks

The scale below displays three distinct spawning marks (i.e., jagged lines) that concentrically travels around the scale. The presence of three spawning marks indicates that this fish was returning to spawn for the fourth time in freshwater when caught.

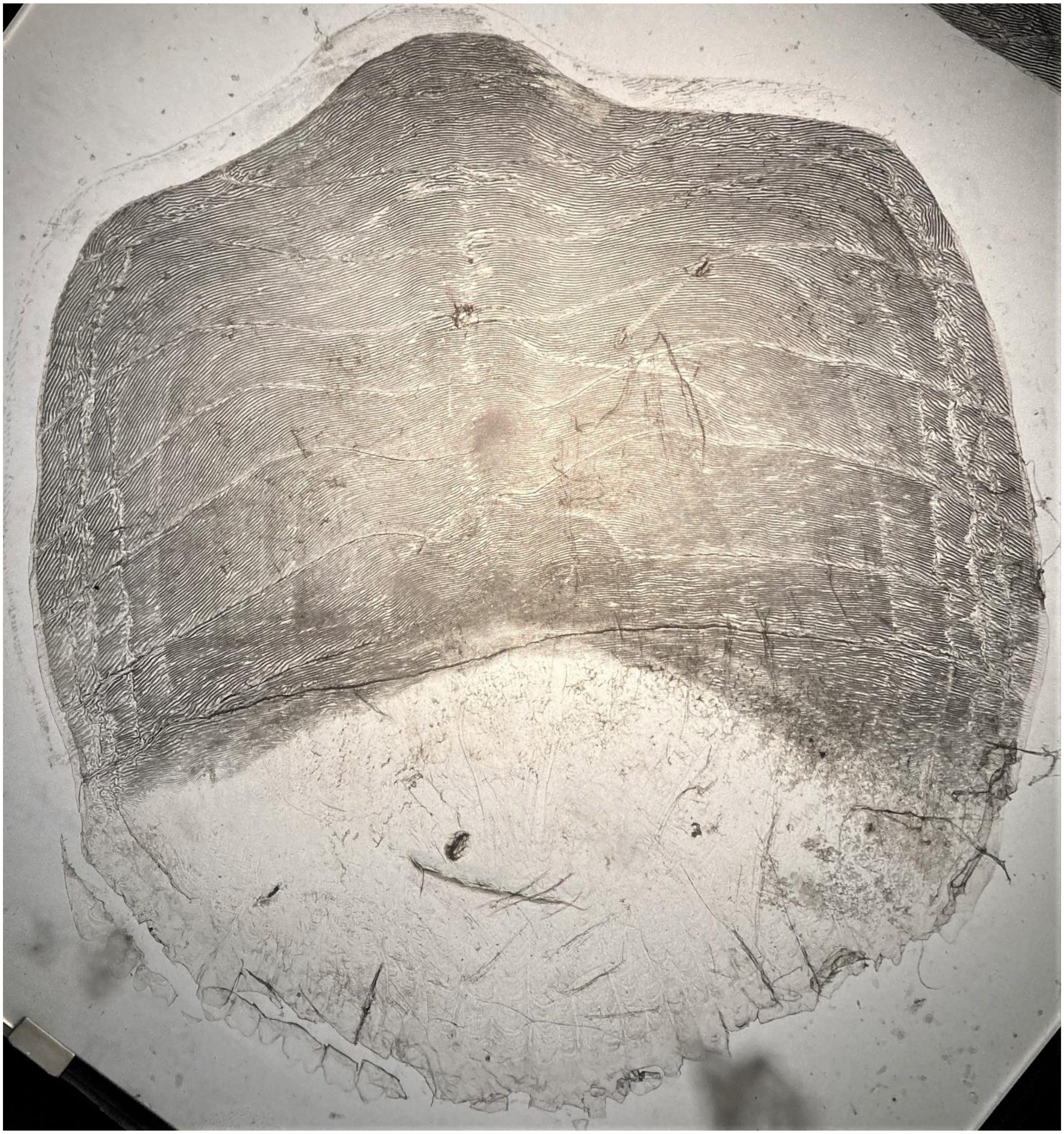


Figure 7: Blueback herring (*Alosa aestivalis*) scale from a repeat spawner collected in the USFWS Annual Adult River Herring Stock Assessment survey (Sprankle and Desmarais 2018).

Figure 8: The left (A.) and right (B.) side of the scale in Figure 7 display three spawning marks.

F. Examples of Bell Shaped Scales

The scales below displays how scale reabsorption from the formation of spawning marks can create a bell shape (think of a belt being cinched across the baseline, Figure 9 and 10).

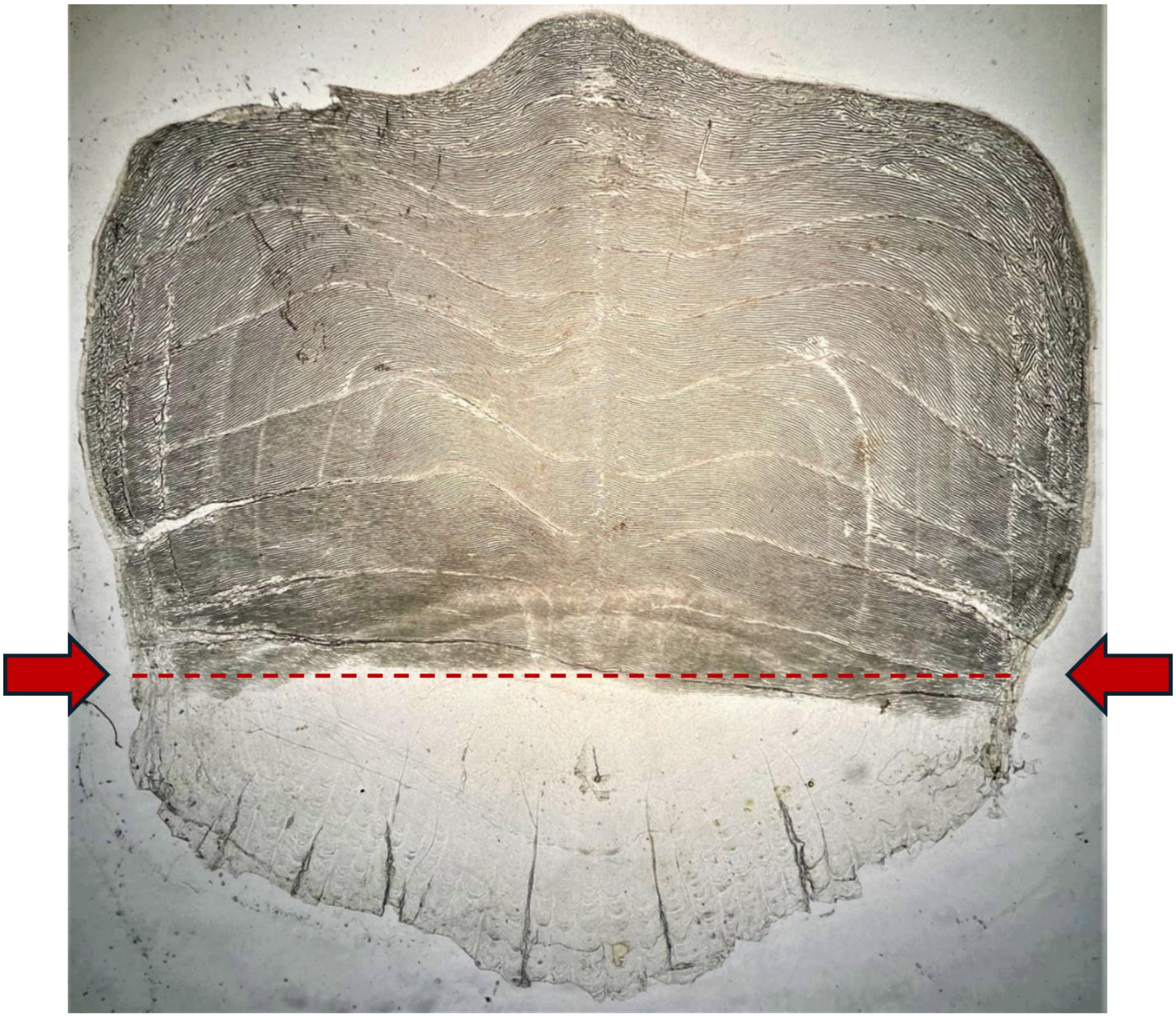


Figure 9: Alewife (*Alosa pseudoharengus*) scale from a repeat spawner collected in the USFWS Annual Adult River Herring Stock Assessment survey (Sprankle and Desmarais 2018). The red arrows point out where the bell shape caused by spawning mark formation is seen and the red dashed line indicates the baseline.

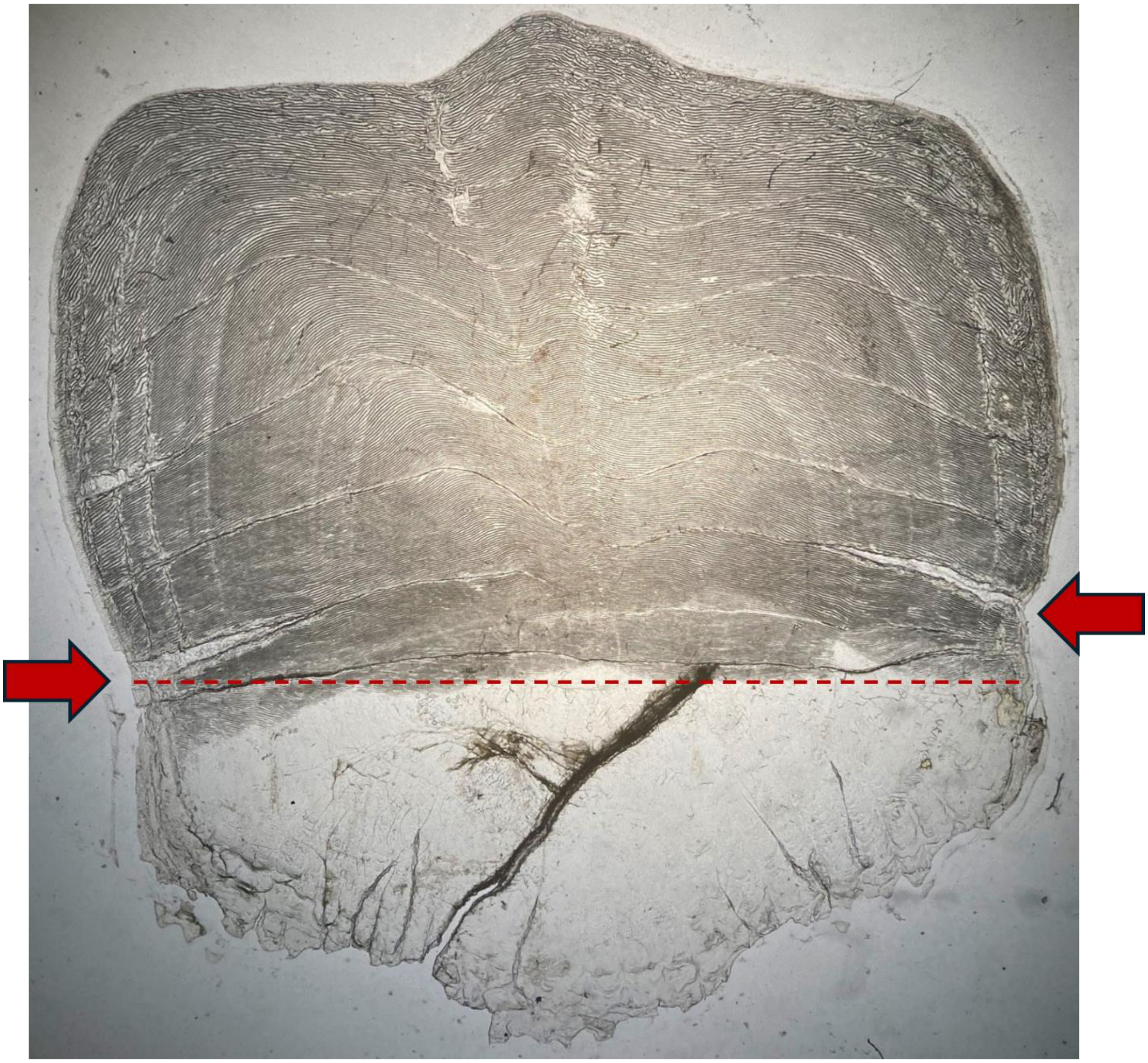


Figure 10: Alewife scale (*Alosa pseudoharengus*) from a repeat spawner collected in the USFWS Annual Adult River Herring Stock Assessment survey (Sprankle and Desmarais 2018). The red arrows point out where the bell shape caused by spawning mark formation is seen and the red dashed line indicates the baseline.

G. Example of Spawning Marks > ½ - ¾ way up Anterior Scale:

Figure 11: Scale from a repeat spawner collected in the USFWS Annual Adult River Herring Stock Assessment survey (Sprankle and Desmarais 2018) showing the three spawning marks (blue arrow) more than half up the anterior portion of the scale starting at the baseline.

Scale Reading with a Microfiche: Filling out the Datasheet

Depending on the datasheet you are using to record your reading, different information may be listed. However, listed below are the types of information usually present and should be recorded either electronically or on paper:

Fish ID number

Reader Initials

Date of Reading

Spawning Mark Read (0,1,2,3,4)

Confidence in Read (No confidence: 5 4 3 2 1 : Highest Confidence)

Notes (anything you find important to say, ex. "number of regenerated scales", "different sized scales")

Because of the struggle between confidence in estimation and scale readability (not being confident in a score just because the scale is hard to read, i.e., no reader would be confident in their estimations if the scale is just poor), some state protocols for scale reading have also adopted a readability code seen below.

Readability Code	Description and analysis consequence
A- Unreadable	Omit sample from analysis.
B- Very difficult to read	Age estimate differences between readers are expected to be >2 year for young, and >4 yrs for old fish (>10 yrs). Agreement on age may be difficult to reach, in which case sample should be classified as A and omitted from the analysis.
C- Fair readability	Age estimates between readers should be within 2 years in young, and within 4 years in old fish (>10 yrs). Agreement after second reading is expected after some discussion.
D- Good readability	Age estimates between readers should be within 1 year for young, to 2 years in old fish (>10 years). Agreement after second reading is expected without much discussion.
E- Excellent readability	Age estimates between readers should be the same.

Scale Reading Review

Spawning marks appear as jagged lines where straight, clean lines of an annulus would typically be.

Past Spawning Hints: look for bell shaped scales and broken transverse grooves (i.e., meets with one annulus but does not appear on the other side of the annulus)

Use the three classifications for identifying spawning marks:

1. Depth and Strength of the Mark
2. Length of the Mark - seen at least ½ - ¾ way up the anterior (above the baseline) portion of scale
3. Frequency/ Consistency - presence on > 1/2 of the readable scales, and on both sides of scales

Always IGNORE dirty, regenerated, or different sized scales!

- Scales on a fish can be knocked out of place and cause the core to continue to grow off center = IGNORE THESE SCALES
- If the scales are different shapes or sizes, they were probably removed from different area on the fish = IGNORE THESE SCALES

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