Application and validation of otolith microstructure as a stock identification method in mixed Atlantic herring (*Clupea harengus*) stocks in the North Sea and western Baltic

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Clausen, L. A. W., Bekkevold, D., Hatifield, E. M. C., and Mosegard, H. 2007. Application and validation of otolith microstructure as a stock identification method in mixed Atlantic herring (*Clupea harengus*) stocks in the North Sea and western Baltic. – ICES Journal of Marine Science, 64: 377 – 385.

Herring (*Clupea harengus*) populations with different spawning times mix in ICES Division IIIa. For stock assessment, otolith microstructure analysis is used to determine the hatching season of individuals, classifying them into hatch type spring, autumn, or winter. The currently applied method uses visual inspection of season-specific daily increment pattern for the larval period. With this method, variability in individual microstructure and a lack of correspondence between hatch and spawning time may lead to classification error. We validate the visual inspection procedure in relation to these potential sources of error. Otoliths from spawning herring were first classified blindly and the results compared with spawning season. In all, 91% of classifications corresponded, and errors represented misclassifications mainly between autumn and winter spawners. However, the estimates may be biased if hatch and spawning times differ, and an objective method of hatch-time estimation based on linear modelling was employed, enumerating unbroken series of daily increments in 0-group herring hatched in different seasons. Visual inspection and objective estimation agreed in 89% of cases, and most of the errors were explained by overlapping hatch periods. Results show that herring older than the 0-group can be classified using multiple linear regression of hatch time on median increment width.

Keywords: fisheries management, herring assessment, otolith microstructure, stock identification, strayers.

Received 18 January 2006; accepted 27 November 2006; advance access publication 8 January 2007.

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Introduction

Atlantic herring (*Clupea harengus*) population dynamics are complex, and different stocks often display variation in life history and spawning season (Jennings and Beverton, 1991; McQuinn, 1997a) as well as genetic structuring (Bekkevold *et al.*, 2005; Mariani *et al.*, 2005). Herring perform extensive seasonal migrations between spawning, feeding, and wintering areas (Slotte, 1998), and different stock components often mix on feeding and wintering grounds (Rosenberg and Palmén, 1981; Wheeler and Winters, 1984; Husebø *et al.*, 2005; Ruzzante *et al.*, 2006). Estimation of individual population contributions to these mixed stocks has attracted considerable interest for management purposes (ICES, 2005), because the preservation of complex stock structures necessitates knowledge of how migratory components of various stocks overlap spatially and seasonally.

In the North Sea, management currently recognizes two main stocks: North Sea autumn spawners (NSAS) and winter-spawning Downs herring (ICES, 2004). These populations mix on nursery and feeding grounds in the North Sea as well as in ICES Division IIIa (Cushing, 1967; Rosenberg and Palmén, 1981; Hulme, 1995; Ruzzante *et al.*, 2006). Although meristic characters such as vertebral counts and otolith microstructure to some extent differ between the two groups (Cushing and Bridger, 1966; Hulme, 1995; Mosegaard and Madsen, 1996), little genetic differentiation has been identified between stock components (Mariani *et al.*, 2005). In contrast to the North Sea stock, western Baltic spring spawners (WBSS) comprise several genetically distinct populations spawning in Divisions IIIa, IIIb, and IIIc (Bekkevold *et al.*, 2005), of which the Rügen spawning component is assumed to be the largest and other components of relatively lesser significance. Although there may be population differences (Ruzzante *et al.*, 2006), these three stocks are collectively highly migratory, and NSAS and Downs juveniles as well as adults of WBSS origin migrate into Division IIIa, where they feed in mixed stocks. The large Norwegian spring-spawning (NSS) stock spawning along the west coast of Norway also migrates extensively (Slotte, 1998). However, an extensive literature search has not produced evidence for its migration into Division IIIa (Dragesund *et al.*, 1997; Slotte, 1998; Kvamme *et al.*, 2003; Husebø *et al.*, 2005).

Analysis of otolith microstructure is a powerful tool for determining life history trajectories, determination of hatch season and larval ambient environment being the key proxies for individual population affiliation. Differences in otolith growth trajectories between herring larvae experiencing different temperature and

feeding regimes have been identified in both field and laboratory studies (Moksness, 1992; Fossum and Moksness, 1993; Stenevik *et al.*, 1996; Folkvord *et al.*, 1997). Herring larvae hatched at different times of the year in the wild, experiencing different temperature and feeding regimes, display different patterns of primary increments in their otolith. Otolith microstructure has been used to identify larvae from NSAS and NSS stocks (Moksness and Fossum, 1991), and differences in the larval otolith microstructure have been identified too in adult herring (Zhang and Moksness, 1993), and used successfully to separate adult herring from NSAS, Downs, and WBSS spawning stocks at an individual level (Mosegaard and Madsen, 1996; ICES, 2004). For Division IIIa, the ICES Herring Assessment Working Group (HAWG) for the area south of 62°N has applied splitting keys to catches to separate NSAS and Downs herring from WBSS herring. Before 1996, the splitting key used by the HAWG was calculated from a sample-based mean vertebral count. In the period 1996–2001, splitting keys were constructed using information from a combination of vertebral count and otolith microstructure methods (ICES, 2001). From 2001 on, the splitting keys have been constructed solely using otolith microstructure methods (ICES, 2004).

Otolith-based assessment of stock affiliation (Mosegaard and Madsen, 1996) is based on the assumption that herring hatched in a specific season also spawn in that season (known both as the "pure stock concept" and "spawning time fidelity"). However, observations of autumn spawning on traditional spring-spawning sites and the sympatric existence of herring with different spawning times (Brophy and Danilowicz, 2002, 2003; Husebø *et al.*, 2005; Bekkevold *et al.*, in press) make a revisit of these assumptions appropriate now. The current study was initiated to analyse variability in the otolith microstructure pattern in post-larval 0-group herring, hatched during different seasons, to achieve a validation method independent of the assumptions behind the pure stock concept. Formation of the first annual translucent ring in herring otoliths coincides with winter stagnation of growth, and the cessation of daily increment formation (Arneri *et al.*, 1998). The 0-group herring, caught during their first growth period, were chosen according to the assumption that they would exhibit an assessable unbroken series of daily increments from the period after hatching until capture. We use information from larval increment patterns to develop an independent objective validation method that combines backtracking of the date of formation of the first primary increment with measurements of microstructure increment patterns and visual inspection of the larval otolith. Primary increments formed during the larval stage in herring are daily in Norwegian spring spawners (Moksness, 1992), so allowing back-calculation of hatch date by counting the daily increments in a fish from the edge to the centre and adding an estimated initial period with no daily increments (Moksness, 1992). However, it is unknown whether this procedure is valid across populations (Geffen, 1982; Folkvord *et al.*, 2000; Fox *et al.*, 2004).

Our study assesses the currently employed routine of identifying herring from different spawning stocks at an individual level by visually inspecting the larval otolith microstructure in both 0-group and adult spawning herring, using two approaches: (i) evaluating the accuracy of visual inspection, by assessing the extent to which hatch-type classification by visually inspecting otoliths from spawning herring collected in the North Sea (representing NSAS), English Channel (Downs), and the western Baltic (WBSS) corresponds with the respective spawning season of the individual fish, and (ii) evaluating the correspondence between visual hatch-type classification and backtracked hatch date in 0-group herring sampled from a mixed stock in Division IIIa, based on a linear modelling approach that uses objectively measured and enumerated larval otolith microstructure data.

We discuss the application of the results of visually inspecting otolith microstructure as a valid stock separation method in the light of the results from a quantitative objective validation method. We also infer the accuracy of the visual inspection and natural variability of the otolith microstructure methods in terms of the ability to indicate violated assumptions of, for instance, spawning time fidelity.

Material and methods

Validation by visual inspection of spawning herring assuming spawning time fidelity

Ripe-and-running (maturity stage 6) herring were sampled from collections from spawning sites in the North Sea, English Channel, and western Baltic (Table 1, Figure 1). We assumed there were no strays from extant populations with divergent hatching and spawning times in our sample. Otoliths were mounted with the sulcus side up in thermoplastic resin (Buehler Thermoplastic Cement no. 40–8100) at 150°C to facilitate grinding and polishing of both sides. The identity of each individual was coded in such a manner that readers were unable to detect from which population the fish originated. The order of the otoliths was set so that the three possible hatch types (spring, autumn, and winter) appeared randomly. The otoliths were polished using a series of grinding and polishing films with decreasing grain size from 30 to 0.3 μm, to optimize the visual resolution at a focal plane through the otolith's nucleus and a transect from this to the edge.

Table 1. Sampling of 0-group herring and spawning fish in the years 2001–2003.

State	Sampling year	Sampling month	Sampling area	Number of individuals
0-group	2001	August	IIIaN, IIIaS	12
		September	IIIaN	13
	2002	July	IIIaS	8
		September	IIIaS	22
	2003	July	IIIaN, IIIaS	5
		November	IIIaN	25
		December	IIIaN	23
Spawning	2001	November	English Channel	40
		December	English Channel	45
	2002	March	Sub.div.24	98
		April	IIIaN	1
		August	Sub.div. IVb	146
	2003	March	Sub.div. 24	192
		April	IIIaN	1
		August	Sub.div. IVb	91
		September	Sub.div. IVb	83

Sampling areas described in Figure 1.

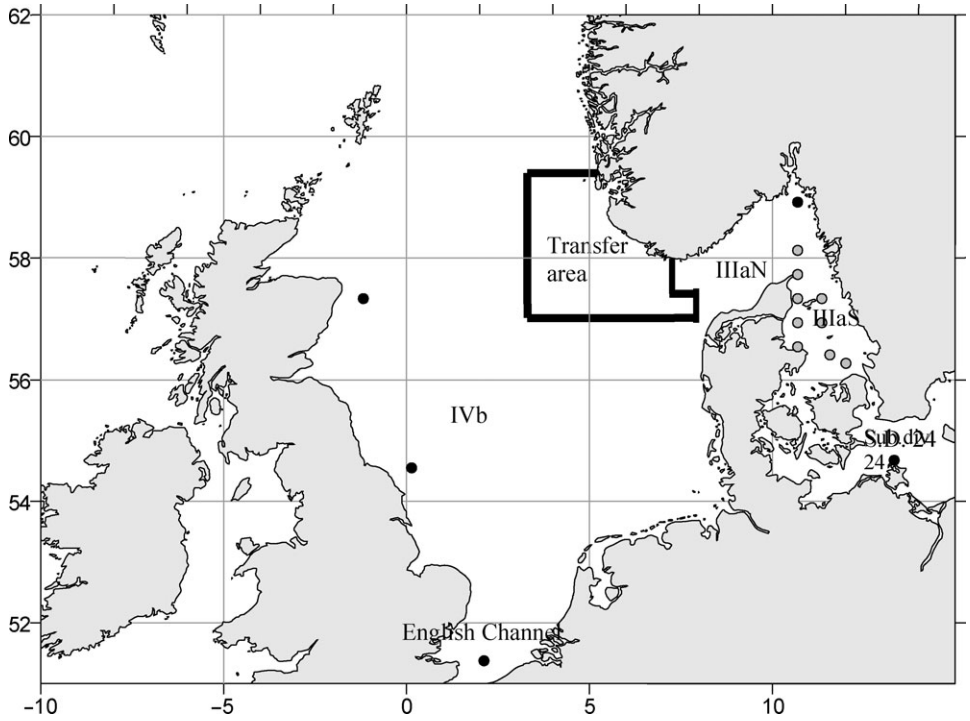

Figure 1. Sampling locations for spawning herring (black dots) and 0-group herring (grey dots). Sampling year and months are listed in Table 1.

Hatch type was estimated for all 697 herring examined. Visual inspection was performed by two experienced readers using a Leica™ DMLB compound light microscope with objective lenses of 20× and 40× magnification and a long distance between focus and lens to facilitate viewing of the otolith microstructure through a microscope slide 1.5 mm thick. The readers assigned a hatch type of either spring, autumn, or winter to all otoliths by visual inspection, following the normal laboratory guidelines presented in Table 2. Through experience, the readers have been able to calibrate their perception of distances in the view field of the microscope, such that they know (approximately) the scale of measurement at the two magnifications used. The zone where incremental widths increased from <2 to >2.5 μm was used as a marker for the onset of increased spring growth conditions (Mosegaard *et al.*, 2001). Otoliths considered as unreadable by one or both readers were disregarded for that comparison. The accuracy of the visual inspection of hatch type was calculated as the correspondence between the assigned hatch type and the season in which the fish spawned, assuming that all individuals exhibited spawning time fidelity.

Table 2. General guidelines followed when determining hatch type by visual inspection. All otolith types are variable, and the appearance depends on the stock and exact timing of hatch.

Hatch type	Criteria for visual inspection of the otolith microstructure
Spring	Wide increments, rapidly increasing in width very close to the centre of the otolith Highly variable Early-hatched fish exhibit increments rapidly increasing from a width of 2 to >4 μm Later-hatched fish have relatively wide increments of about 4 μm already 20–40 μm from the nucleus
Autumn	Otolith increments <2.5 μm wide are found >200 μm from the centre All increments appear to have close-to-constant widths
Winter	Otolith increments gradual increase from about 1 μm width about 10 μm from the centre to >3 μm wide at a distance of 150 μm from the centre The increase in increment widths accelerates at about 200 μm from the centre

Validation by image analysis of otolith microstructure pattern in 0-group herring

A search in the DIFRES database on 0-group herring (fish caught before the onset of the first annual otolith winter ring) from Sub-divisions IIIaN and IIIaS and the transfer area in the North Sea (Figure 1) between 2001 and 2003 was made, and 108 fish were selected from different locations within the area (Table 1, Figure 1).

The otoliths of these herring were retrieved from archives, remounted, and inspected visually following the same procedure as described earlier for otoliths from the spawning populations. After preparation, all otoliths could be classified as autumn, winter, or spring hatch types. Following visual inspection, images of 0-group herring sagittae ($n = 108$) were digitized; each otolith was analysed by taking several pictures following the longest axis along the postrostrum. Measurements of otolith microstructure were made with a Leica 350 F digital camera and ImagePro™ 5.0 image-analysis package for Windows™. Increment widths were measured automatically using the Caliper tool in ImagePro, along a profile of grey values and using a profile bandwidth of 10 μm.

The Caliper tool was set to identify the onset of an increment as the point at which the grey values changed towards lower values at the fastest rate. The process was monitored by an expert reader, and if the program produced obviously erroneous increments (e.g. because of cracks in the otolith), these were altered manually to fit the real increments. In cases where the increments were not sufficiently clear to be identified by the Caliper tool or by eye, the distance from the last visible increment to the next visible increment was measured. A minimum acceptable increment width was set at 0.5 μm, to filter out the segments where false or no daily rings were visible along the measurement axis. All measurements were transferred to an MS Excel™ spreadsheet. Areas with no detectable ring structures were occasionally found in the trajectory from the otolith centre to the edge. As these areas would appear as abnormally broad increments but only represent 1 d, a running median value $m_i = \mathrm{MED}(w_{i-2}, \ldots, w_{i+2})$ was applied as a smoother to yield a robust estimate of increment width at distance from centre. This median was then used to estimate duration in days (d_i) between observed increments w_i, as $d_i = w_i/m_i$, independent of whether these were true daily increments or just zones with several unreadable daily increments. A median over five successive increments was enough to screen out all unreadable areas. This was indicated by the fact that no median increment exceeded 7 μm in the first 200 μm from the centre, and no median at all was more than 14 μm wide. Further, only five successive pairs of medians out of 28 300 had more than a 50% change in width between them.

Initial otolith increment position after hatch in herring larvae subjected to suboptimal growth conditions (as in autumn and winter) is sometimes not discernible. Therefore, measurements closer than 10 μm from the centre were disregarded following the findings in Folkvord *et al.* (2004). In 94% of otoliths, the first measurable increment was formed at a radius (R_1) less than 25 μm from the centre. To estimate the number of increments formed in the zone between 10 μm from the centre and R_1, otolith initial growth rate was analysed as a function of day of formation. As a measure of early otolith growth rate, the median increment width of the first six measured increments (m) was regressed vs. Julian day number minus 200 at 25 μm from the centre (J) in a quadratic relationship: $m = 1.67 + 0.0081J + 0.000037J^2$ ($r^2 = 0.75$, $p < 0.0001$, $n = 108$). The value of −200 is applied to achieve the most monotonic quadratic function and gives the best display of the different seasons' growth patterns. Assuming that increments are daily, this relationship was then used to extrapolate the number of days in the unreadable zone from 10 μm to the first measurable increment [number of days $= (R_1 - 10)/m$]. The total estimated age at catch was then subtracted from the Julian day of the catch to obtain the Julian day of first possible ring formation (10 μm from the centre), which was then used as a proxy for hatch date, neglecting the possible initial period of very slow otolith growth after hatch (Folkvord *et al.*, 2004). This estimated hatch date, based on counts plus additional zones with an estimated number of increments, is hereafter referred to as the back-calculated hatch date h.

As this method is only applicable to fish with a potentially unbroken series of daily increments, e.g. 0-group herring, we analysed how shorter series (segments) of measured increment widths would estimate h. Owing to variable resolution in the segment 0–15 μm from the centre, this area was neglected in the analysis.

Starting from a distance of 15 μm from the centre, the otolith trajectory was divided into k segments of 30 μm width, and each k segment's median increment width (m_z) was used as independent variable in a multiple regression analysis ($z = 1$ for 15–45 μm; $z = 2$ for 45–75 μm, etc.). Both original and natural log-transformed values [$\ln(m_z)$] were explored. Stepwise regression analysis was performed to obtain a selection of significant coefficients from the total array of coefficients corresponding to all k segments (a, b_1, b_2, ..., b_k). The estimation of hatch date from the multiple regression may be expressed as $h = a + \sum(b_j \times f(m_j))$, where f is the untransformed or ln-transformed median increment width and j is an index of the subset of measured segments giving a significant linear combination for the estimation of hatch date h.

Summer, when very few herring have hatched, constitutes a natural separation between fish hatched in spring and fish hatched in autumn. A good separation was found by letting summer start at Julian date 189 − 365 = −176. The distribution of h was then analysed using the cumulative frequency distribution from $h = -176$ to 189. A plot of the data for the 108 herring (Figure 2) suggested the existence of three major aggregations in time (from approximately −150 to −70; −25 to 70; and 90 to 150). Assuming normal distributions of the three clusters, the number of individuals (N_k), the mean (μ_k) hatch date, and its standard deviation (σk) for each cluster (k) were estimated by the minimum sum of squares (SSQ) method:

$$\mathrm{Min(SSQ)} = \mathrm{Min}\left[\sum_i^{108}\left[\frac{\mathrm{Rank}(h_i)}{108} - \frac{\Phi(h_i,\mu_1,\sigma_1)\times N_1 + \Phi(h_i,\mu_2,\sigma_2)\times N_2 + \Phi(h_i,\mu_3,\sigma_3)\times N_3}{108}\right]^2\right]$$

Here, Φ is the cumulative normal distribution with estimated mean μ_k and standard deviation σ_k, and N_k is the estimated number of individual fish in the kth of the three hatch groups (autumn, winter, or spring).

A knife-edge separation of individual hatch season ($S_{aut} = 9$, $S_{win} = 12$, $S_{spr} = 16$) was calculated by the highest probability of belonging to a specific period. The ability of $f(m_z)$, from the different measured segments, to estimate hatch season S_k was explored using stepwise linear regression, with $\alpha = 0.05$ for parameters staying in the model. Classification success was compared between analyses of segments in two different otolith areas, dependent on experience in routine preparation of the larval otolith centre in adult herring: (i) when the centre remains intact with visible segments from 15 to 225 μm ($z = 1, 2, \ldots, 7$) and

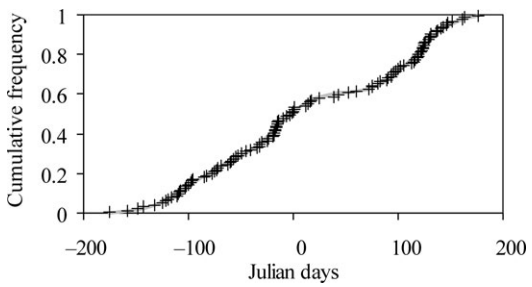

Figure 2. Cumulative distribution of back-calculated hatch dates in 0-group herring from counts of daily increments along a transect from the otolith centre to the edge (plus signs, raw data; line, cumulative sum of three estimated normal distributions).

Table 3. Accuracy of visual inspection of hatch type in spawning herring assumption spawning time fidelity.

Visual inspection	Sampling season		
	Spring (%)	Autumn (%)	Winter (%)
Spring	97	1	0
Autumn	2	92	32
Winter	1	7	68

(ii) in cases of overgrinding, where usually only increments >300 μm from the centre are visible.

Results

Validation by visual inspection of spawning herring assuming spawning time fidelity

The accuracy of visual inspection of hatch type is presented in Table 3. Assuming that herring exhibited spawning time fidelity to hatch date, the accuracy of visual inspection was high with an overall correct classification of 91%. Herring collected ripe-and-running in winter (November–December) were most difficult to classify, with a misclassification rate of 32%, whereas individuals collected in spring (March–June) were of a hatch type determined with the lowest misclassification rate of 3%. The most apparent pattern in the misclassification was that spawning herring from autumn and winter were most frequently confused with each other, whereas spring spawners were assigned equally often to winter or autumn hatch type when misclassified (Table 3).

Validation by image analysis of otolith microstructure pattern in 0-group herring

Most otoliths had whole unbroken transects of daily increments from the start of measurement (at an average 20 μm from the centre) to the edge of the otolith (95% of the otoliths had 92% of the transect complete without interruption). The distribution of back-calculated hatch dates is shown in Figure 3, in which the smooth curves are the fitted normal distributions of hatch dates based on the backtracked number of days from catch. The back-calculated hatch dates fell within three well separated groups, winter, spring, and autumn. However, some overlap between groups was evident, especially between the autumn and winter

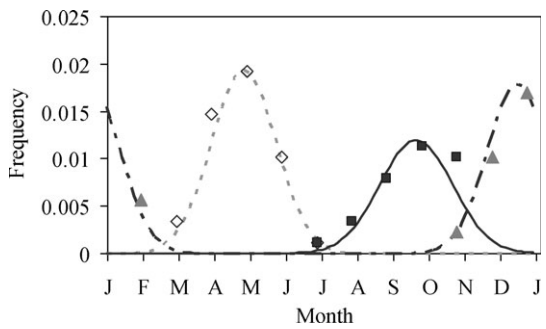

Figure 3. Back-calculated hatch date distributions of 0-group herring analysed by counts of daily increments along a transect from the otolith centre to the edge. Observed data are grouped by 30 d intervals (symbols), and the corresponding normal density distributions (lines) are estimated by minimum sum of squares based on cumulative raw data (see Figure 2) (squares, autumn; triangles, winter; diamonds, spring).

Table 4. Accuracy of visual inspection of hatch type in 0-group herring when compared with the back-calculated hatch season based on micro-increment enumeration.

Visual assigned hatch type	Back-tracked hatch season		
	Spring (%)	Autumn (%)	Winter (%)
Spring	95	0	3
Autumn	0	90	17
Winter	5	10	80

hatch date groups. The periods for the hatching seasons were defined by normal distribution, spring being from 18 February to 9 July, autumn from 9 July to 5 November, and winter from 5 November to 18 February.

Visual inspection of 0-group otoliths gave an overall correct classification of 89% when the classification by visual inspection was compared with the back-calculated hatch season of individual fish (Table 4). The misclassification pattern repeated the pattern seen in the pure stock samples, the most frequently confused hatch types being those of autumn and winter. Autumn-hatched herring were classified as winter-hatched in 10% of the fish analysed, and winter-hatched herring were classified as autumn-hatched by visual inspection in 17% of those analysed.

The large overlap between autumn and winter hatch seasons and the poor fit of the later autumn-hatched herring (Figure 3), together with the pattern of greater misclassification by visual inspection between autumn- and winter-hatched fish than between either of these two and spring-hatched fish (Table 4) led to further examination of the division between autumn and winter hatching seasons. The seasons were subjectively forced to fixed periods so that the classical start of winter was applied, categorizing winter hatch as from 1 December to 18 February, spring from 18 February to 9 July, autumn from 9 July and 5 November, and late autumn from 5 November and 1 December. Using these four categories, the visual inspection results were re-analysed, revealing that 6% of the misclassified winter hatch types fell within the period late autumn (Table 5). The subjective categorization of hatch season did not affect classification of spring hatch types, whereas classification success of winter hatch types increased.

Although three well separated hatch date groups were found, there was a significant within-group difference in mean hatch date for both autumn ($p = 0.04$) and winter ($p = 0.0018$) groups among the three sampling years (2001–2003). The development of increment width with distance from the otolith centre is shown for the four hatch types (as determined by increment counts to winter, spring, autumn, and late autumn) in Figure 4. The spring-hatched herring clearly separated from the remaining hatch types

Table 5. Accuracy of visual inspection of hatch type in 0-group herring when compared with the back-calculated hatch season with defined periods for spring, autumn, late autumn, and winter.

Visual assigned hatch type	Back-tracked hatch season			
	Spring (%)	Autumn (%)	Late autumn (%)	Winter (%)
Spring	96	0	0	4
Autumn	0	86	4	10
Winter	5	4	6	85

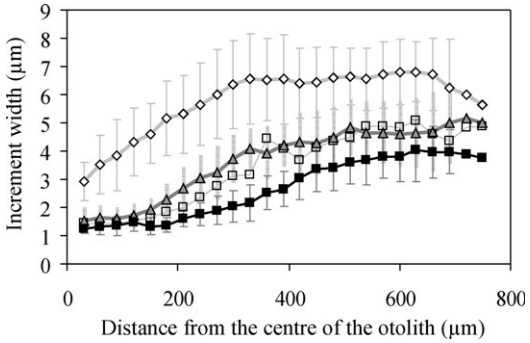

Figure 4. Development of micro-increment width in the first 750 μm from the centre of the otolith for spring-, autumn-, late-autumn-, and winter-hatched 0-group herring (bars indicating ± 1 s.d. are not given for late autumn, to enhance readability). (diamonds, spring; closed squares, autumn; open squares, late autumn; triangles, winter).

by exhibiting increments wider than 2 μm from the beginning of measurements, and the increments continued to increase in width over the whole measurement transect, levelling out at approximately 6 μm at a distance 400 μm from the centre. The increment width development in autumn-, late-autumn-, and winter-hatched herring overlapped, especially in the first part of the measurement transect. At a distance 150 μm from the centre, the three types can be separated, the late-autumn hatch type being on the line of gradual change from autumn to winter type.

To aid hatch type determination by visual inspection in these hatch types that are more difficult to separate, a series of segments along a transect from the otolith centre towards the edge was selected, and the separation ability of the increment widths in these segments were tested using a multiple regression analysis.

When median increment widths, m_z, from segments 1–7 of the otolith (i.e. the area from 15 to 225 μm from the centre) were analysed, the linear combination $S_k = 7.8 + 1.3 \times \ln(m_1) + 1.6 \times \ln(m_5) + 4.8 \times \ln(m_6) - 0.74 \times m_6$ ($r^2 = 0.88$) exhibited the best fit (Figure 5). However, a large number of other segment combinations also gave a good prediction of hatch season, with segment 5 (135–165 μm from the centre) often showing up as the major influence. When segments with $k > 10$ were analysed, the best combination was $S_k = 1.6 + 2.6 \times \ln(m_{11}) + 3.0 \times \ln(m_{14}) + 0.45 \times m_{20}$ ($r^2 = 0.76$), with segment 11 (315–345 μm from the centre) generally having the highest influence in different combinations.

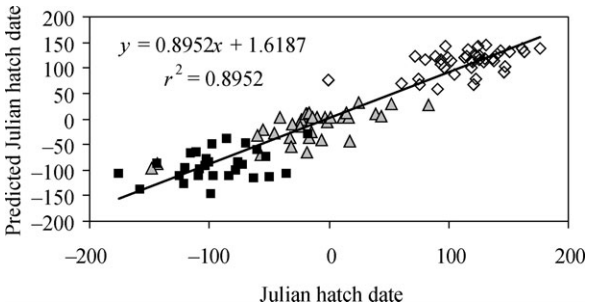

Figure 5. Relationship between back-calculated hatch date and predicted hatch date based on multiple regression analysis of otolith micro-increment measurements (m_z) from segments 1–7 of the otolith, i.e. the area from 15 to 225 μm from the centre. Fish were further assigned hatch season by visual inspection (squares, autumn; triangles, winter; diamonds, spring).

Discussion

To ensure conservation of herring population diversity in the North Atlantic, all stock components and their natural migration patterns should be considered in compiling scientific advice on the fishery (Stephenson, 2001). Therefore, obtaining high levels of precision in the input data to the assessment of mixed stocks is warranted. The good agreement between the hatch type assigned by visual inspection and the sampling season of spawning herring observed in this study confirms visual inspection of larval otolith microstructure in spawning herring as a valid method of discriminating between hatch types. However, despite the high level of correspondence between assigned hatch type and spawning season, some variation was observed among the readers' classification results. A lack of correspondence between estimated hatch and spawning season was mainly discriminating between autumn- and winter-spawned herring. An explanation for the misclassifications may be found in a possible straying of fish not exhibiting spawning time fidelity (McQuinn, 1997b; Slotte, 1998, 2001). For studies of population structuring, the identification of fish straying among populations with different spawning seasons is of focal interest, but their potential existence also presents a problem concerning validation of the otolith microstructure method. The otolith microstructure of straying fish may not be detected because the apparent variability of the otolith microstructure may be too high to allow detection of the phenomenon. If, on the other hand, a specific hatch period gives rise to highly variable otolith microstructure, some fish may be falsely identified as originating from populations exhibiting different spawning seasons. The misclassification rates observed in this work were higher when gonad stage was used as an indicator of spawning time, than when using back-tracked hatch dates (Tables 3 and 4). This could suggest some spawning season straying. However, it could also be related to a natural variability in the larval otolith microstructure formed after hatch, as well as potentially overlapping spawning seasons.

The variation between reader-assigned hatch type and gonad stage indicated that spawning time may be affected by both within- and among-reader variation, influenced perhaps by insufficient training and/or lack of quality control during the reading process. This issue was not investigated in our study. However, both readers in this study have been tested already for reader consistency and exhibit good correspondence between hatch type determination (ICES, 2005).

The quantitative objective classification method of hatch types in 0-group herring developed here provides an opportunity to calibrate the visual inspection of hatch types in all herring. Inclusion of possible variation in environmental influence on otolith microstructure allows for variability in the pattern within each hatch type. The underlying assumption for this approach is that primary increments are sufficiently close to being daily for back-calculation of hatch season in 0-group herring to be possible. A further development of the method assumes that increment measurements along radii at specific distances from the otolith core reflect season- and area-specific environmental conditions during the larval growth phase, so permitting the use of otolith microstructure patterns to identify offspring from different spawning populations.

Although it was not intended to estimate the absolute hatch date, but rather to estimate the hatch season with reasonable

accuracy, the validation technique presented here has two important prerequisites: knowledge of the timing of formation of the first daily increment and of the successive daily deposition of micro-increments in the larval otolith. The formation of the first discernible daily increment in herring larvae coincides with the onset of first feeding at the start of post-yolk-sac growth (Moksness, 1992; Høie *et al*., 1999). This takes place in herring around 10–19 d from hatching, depending on the population in question (Fox *et al*., 2004). However, growth rate and temperature strongly influence the formation of the first discernible increment (Høie *et al*., 1997; Folkvord *et al*., 2000; Pavlov *et al*., 2000; Fox *et al*., 2004). Folkvord *et al*. (2004) found no increase in size of sagittae from herring larvae reared at 4°C up to 30 d, whereas herring reared at 12°C showed sagittal growth after 9 d. We calculated initial undetectable increment widths by a general curvilinear relationship from the fish with clearest otolith patterns. However, otolith no-growth under abnormal environmental conditions could not be detected using our methods. Adding a variable number of days to the counts of daily increments, depending on some uncertain environmental forcing, would make calculation of absolute age more uncertain than necessary for our purpose. In this study, it is likely that the ages of the winter hatch type herring were underestimated, because these fish would have experienced the lowest post-hatch temperatures of the three hatch types. However, estimates for fish hatched during autumn could also have been lower than the actual ages, depending on specific hatch time and annual variation in temperature.

The formation of daily increments in embryonic stages of herring has not been confirmed (McGurk, 1984; Moksness *et al*., 1987). However, for stages following the absorption of the yolk sac, otolith micro-increments are formed on a daily basis (McGurk, 1987; Moksness and Wespestad, 1989; Moksness and Fossum, 1991; Moksness, 1992). Growth rate, however, seems to influence the deposition of daily increments. Several studies have demonstrated non-daily increment deposition in herring larvae with a growth rate <0.4 mm d^{-1} (Geffen, 1982; McGurk, 1984; Folkvord *et al*., 2000; Pavlov *et al*., 2000; Fox *et al*., 2004). Autumn-spawned herring larvae may exhibit growth rates below this value (Munk and Christensen, 1990; Johannesen *et al*., 2000), and it is therefore likely that non-daily rates of micro-increment formation in these fish could lead to underestimating the absolute age, as seen in Feet *et al*. (2002) and Fox *et al*. (2004). Notwithstanding these uncertainties, the prerequisites of the validation approach in this study are fulfilled to the extent necessary for hatch type estimation, because the intention was to place the herring within a spawning season, not to estimate their precise hatch date. Despite the possibility of underestimation of absolute age in the 0-group herring in this study, the back-calculated hatch date distributions confirm the trimodal distribution of the peak periods of spawning in winter, spring, and autumn (Figure 3).

The overlapping seasons of the autumn- and winter-spawning herring (Zijlstra, 1969; Burd and Howlett, 1974) and the gradual change in otolith microstructure from autumn hatch type through late-autumn hatch type to winter hatch type identified here appeared to result in classification of late-hatched autumn spawners as winter hatch types by visual inspection, following the guidelines currently applied. Comparing the visual inspection results in Tables 4 and 5, it is clear that most autumn-hatched herring misclassified using the classic division of hatch seasons (Table 4) were represented by fish hatched late in autumn. Therefore, visual inspection of hatch types may fail to classify individuals hatched in the periods of overlapping spawning seasons. This has potential consequences for historically splitting catches between winter and autumn hatch types for herring caught in the Skagerrak and Kattegat. However, recent efforts to separate the winter-spawning Downs component from autumn-spawning components in the North Sea (ICES, 2005) may not be affected by this problem. Downs herring otolith microstructure has been reported to be >2 μm wide at a distance of 100 μm from the nucleus (Mosegaard and Madsen, 1996), more than observed for winter-hatched herring in the present study. Consequently, it is possible that winter-hatched herring analysed in this study (i.e. the archived 0-group herring from Sub-area IIIa) did not originate from the Downs population, but from a different population of winter spawners, e.g. from the western Baltic (Bekkevold *et al*.in press).

NSS herring daily increments start at a mean increment width of 1.5 μm at the centre and gradually increase to 2.5 μm around 100 μm from the centre (Figure 2 in Husebø *et al*., 2005). This pattern is different from that observed for spring spawners in the present study, for which the mean increment width was 3 μm at 25 μm from the centre, increasing to >4 μm at 100 μm from the centre (Figure 4). The autumn spawners analysed by Husebø *et al*. (2005) also showed an increment development pattern different from that in this study.

The appearance of the otolith microstructure is much influenced by the environmental conditions, such as temperature (Folkvord *et al*., 2004) and food availability (Johannessen *et al*., 2000), experienced in the first larval phase, so caution is necessary if environmental regimes in the spawning areas change over time. The observed separation of hatch type in the present study was performed on 0-group herring sampled from year classes 2001, 2002, and 2003, and because the sampling year does have an effect on the pattern of otolith microstructure within each hatch type, a more comprehensive analysis is needed before extrapolation to other year classes can be made. This suggests the need for an annual analysis of 0-group herring otolith microstructure to update the calibration criteria for separation of hatch type by visual inspection of otolith microstructure.

In addition to the otolith microstructure patterns formed during the larval period, otolith increments formed during the juvenile growth phase may also be used to identify offspring from different spawning seasons. When the calibration sample is sufficiently large, it is possible to select a subset of segments and apply those to classify hatch types, applying a multiple linear regression model as demonstrated in the present study (Figure 5). Measurements of such defined segments provide quality checks during routine visual inspection and can aid as an additional tool to visual inspection when overgrinding of an otolith precludes application of the routine method.

The objective separation method based on median increment width of segments of otolith microstructure in the juvenile growth phase validates the use of visual inspection for hatch type separation of both juvenile and adult herring. However, it is an improvement on the visual inspection method in two ways. First, the objectivity increases the reliability of hatch type estimation of readers regardless of experience and precision level. Second, the dampening down of the inherent natural variability in otolith microstructure patterns within each hatch type by using median measurements in segments reduces misclassification errors.

As the quality and precision of hatch type estimates determined by visual inspection has depended on individual skills and

experience, the need for standardization, objective control, and statistical evaluation is obvious in improving the reliability of the output. The method developed here facilitates an objective determination of hatch type, which makes standardization and quality assurance and quality control less complicated.

Acknowledgements

We thank Meinhard Poulsen, Mikael van Deurs, Agnete Hedegaard, and Stina Bjørk Stenersen Hansen for their help with the laboratory work. The project was partly funded by the EU project HERGEN (QLRT–2000–01370).

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doi:10.1093/icesjms/fsl036