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TITLE

Seeking the True Time: Exploring Otolith Chemistry as an Age-Determination Tool

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ABSTRACT

Fish otoliths’ chronometric properties make them useful for age and growth rate estimation in fisheries management. For the Eastern Baltic Sea cod stock (*Gadus morhua*), unclear seasonal growth zones in otoliths have resulted in unreliable age and growth information. Here, a new age estimation method based on seasonal patterns in trace elemental otolith

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incorporation was tested for the first time and compared with the traditional method of visually counting growth zones, using otoliths from the Baltic and North seas. Various trace elemental ratios, linked to fish metabolic activity (higher in summer) or external environment (migration to colder, deeper habitats with higher salinity in winter), were tested for age estimation based on assessing their seasonal variations in concentration. Mg:Ca and P:Ca, both proxies for growth and metabolic activity, showed greatest seasonality and therefore have the best potential to be used as chemical clocks. Otolith image readability was significantly lower in the Baltic than in the North Sea. The chemical (novel) method had an overall greater precision and percent agreement among readers (11.2%, 74.0%) than the visual (traditional) method (23.1%, 51.0%). Visual readers generally selected more highly contrasting zones as annuli whereas the chemical readers identified brighter regions within the first two annuli, and darker zones thereafter. Visual estimates produced significantly higher, more variable ages than did the chemical ones. Based on the analyses in our study, we suggest that otolith micro-chemistry is a promising alternative ageing method for fish populations difficult to age, such as the Eastern Baltic cod.

KEYWORDS

age estimation, Baltic Sea, *Gadus morhua*, LA-ICP-MS, otolith chemistry, seasonal patterns

1. INTRODUCTION

Accurate knowledge of the age composition of a fish population is essential for estimating stock parameters such as growth rate, age at maturity, recruitment, mortality, and population biomass-at-age. Therefore, “losing track of time,” meaning inaccurate age estimation, is problematic in the management of fish stocks (Andrews *et al.*, 2009; Beamish and McFarlane, 1983; 1995; Campana, 2001). This is the current situation for the Atlantic cod *Gadus morhua* L. 1758 in the eastern Baltic Sea (referred to hereafter as the Eastern Baltic cod stock (EBC)), for which increasingly uncertain ageing has led to failed analytical stock assessment with substantial consequences for management between 2014 and 2019 (ICES, 2014; 2017; 2019).

Fish age is often determined by examining otoliths, the calcium carbonate structures composing parts of the balance and the hearing system in the inner ear (Popper *et al.*, 2005). Otoliths grow through continuous accretion of material throughout the fish’s life, deposited in alternating opaque and translucent zones, which in temperate and boreal fishes generally correspond to fast growth during summer and reduced growth in winter (Pannella, 1971).

Atlantic cod in most of its range has clear, optically contrasting seasonal growth zones in its otoliths. In the case of the EBC, however, detecting seasonal zonation has become increasingly more complicated over time, such that traditional age estimation is no longer feasible (Hüssy *et al.*, 2016). The EBC otoliths can display a variety of visual patterns, from multiple, highly contrasting translucent rings to total absence of growth zonation (Figure 1), making it difficult for age readers to distinguish between false or true annuli or even their

presence. Potential causes have been summarized by Hüsey *et al.* (2016) and include prolonged spawning, complex hydrography and migrations across widely varying temperatures and salinities. A modelling comparison of otolith biomineralisation in Barents Sea cod versus North Sea cod showed that the formation of the translucent zones in cod otoliths can be attributed to low food intake concurrent with increased water temperature, and that small variations in feeding and temperature corresponded to low contrast growth zones (Fablet *et al.*, 2011). In the Baltic Sea, environmental and biological drivers that further exacerbate unclear seasonal zonation in cod otoliths appear to be factors that affect fish condition and metabolic activity, such as environmental stress caused by hypoxia (Casini *et al.*, 2016; Chabot and Claireaux, 2008), low prey availability (Casini *et al.*, 2016), parasite infestation (Eero *et al.*, 2015; Mehrdana *et al.*, 2014) and thiamine deficiency (Engelhardt *et al.*, 2020).

EBC expert groups of age readers within the International Council for the Exploration of the Sea (ICES) have attempted to standardise EBC age estimation and reduce uncertainty through international age calibrations (Hüsey *et al.*, 2016). Nevertheless, agreement and precision have not improved in over four decades, and without known-age reference samples it has not been possible to validate the accuracy of age estimations or develop alternative methods of age estimations based on fish length, otolith weight or otolith morphology (Hüsey *et al.*, 2009; Hüsey *et al.*, 2016; ICES, 2006). Therefore, alternative methods to age EBC are needed.

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As an otolith grows, trace elements are incorporated into its structure, reflecting the chemistry of the ambient water and/or physiological processes (Campana, 1999; Payan *et al.*, 2004; Thomas *et al.*, 2017). The chemical composition of the otolith thus may reveal information about the life history of the fish. Examples of systematic, sinusoidal temporal patterns, with peaks correlating with translucent zones for some trace elements and the reverse for others have been discovered in chemical profiles of some otoliths (Hughes *et al.*, 2016).

Attempts to use otolith chemistry as an aid to ageing cod are in their infancy. Western Baltic Sea cod otoliths, with clearly contrasting growth zones and high readability, were analysed to investigate whether temporal variations in the chemical profiles followed the visual pattern of opaque and translucent zones in a synchronized manner (Hüssy *et al.*, 2015). This concept was further tested on North Sea and EBC otoliths with sufficient readability and finally on unreadable EBC otoliths with no recognizable growth structure. Comparisons of opacity and chemistry profiles showed that chemical signals in EBC (i.e., seasonal variations in Zn, Cu, and Mg) were stronger than visual growth zones in EBC otoliths with low visual contrast (Hüssy *et al.*, 2015). These trace elements are all controlled by physiological processes such as growth, metabolism, reproduction, and phylogeny (Limburg and Elfman,

2010; Limburg *et al.*, 2018; Sturrock *et al.*, 2015; Thomas and Swearer, 2019; Watanabe *et al.*, 1997).

Here, we develop and test a new approach to evaluate the utility of chemical profiles in otoliths for age validation by i) identifying useful elements for age estimation of cod in two study areas (Baltic Sea and North Sea) over time, ii) comparing the accuracy and precision of the visual vs. chemical age estimation method, iii) examining differences in how the visual and chemical age readers identify annuli, and iv) comparing the estimated annulus distance from otolith core between methods. The overall goal is to assess whether chemical ageing has the potential to improve age estimation of EBC.

2. MATERIALS AND METHODS

Fish otoliths were collected as part of annual research surveys as well as from commercial fisheries, all involving lethal sampling. No experiments were conducted, nor were surgical procedures performed. No procedures caused lasting harm to sentient fish, nor were sentient fish subjected to chemical agents that would induce neuromuscular blockade. The care and use of experimental animals complied with animal welfare laws and regulations set by the Swedish Government and the Swedish Board of Agriculture. The study followed guidelines and policies approved by the Swedish Board of Agriculture (permission to use animals for scientific purposes, licenses no. 31-1069/12 and 5.2.18-443/17) and permissions

from the Gothenburg Animal Ethic Committee for the specific surveys (licenses no. 255-2012 and 126-2017). Fishes sampled from commercial fisheries are not regarded as research animals according to Swedish legislation, as these animals primarily were caught and killed for other purposes. The samples used here were covered by permit from the Swedish Board of Agriculture (license no 6.7.18-13720/2019).

2.1. Data used and description of the study area

Cod otoliths from 1982 to 2018 were obtained from the archives of the Department of Aquatic Resources, Swedish University of Agricultural Sciences (SLU Aqua, Table 1). Samples (n=52) that were previously analyzed for another study (Limburg and Casini, 2018), were supplemented with otoliths (n=48) from the SLU Aqua archives. The material spanned the last four decades (1980s, 1990s, 2000s, 2010s), representing different levels in EBC biomass, body condition, hypoxia, salinity and events of major Baltic inflows (MBI) of oxygenated and saline water from the North Sea (Hansson *et al.*, 2017; Liblik *et al.*, 2018; Matthäus, 2006), (Table 1).

Two main study areas were selected for comparison (Figure 2). The eastern North Sea region, here specifically the Skagerrak (ICES Subdivision (SD) 20) and the Kattegat (SD 21), is a marine environment with a south-north salinity gradient from 17 to 35. The second region is the Baltic Sea (specifically the Baltic Proper, SDs 25-29), a brackish environment strongly

influenced by its large watershed area and limited water exchange with the North Sea, constrained by shallow and narrow straits, with salinity ranging from 7 in the north to 16 in the south (Furman *et al.*, 2014), (Figure 2). In the Baltic Sea, restricted water circulation and strong density stratification prevent oxygenated water from reaching the bottom layers (Diaz and Rosenberg, 2008), which in combination with eutrophication and global warming has created one of the largest anthropogenic “dead zones” in the world (Breitburg *et al.*, 2018).

2.2. Otolith chemical analyses

Trace elemental analyses were performed on transverse cross sections of cod otoliths with laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) at the Department of Geology at Lund University, Sweden and at the College of Environmental Science and Forestry at the State University of New York (SUNY-ESF) in Syracuse, New York USA. The otolith surface was pre-ablated to remove any contamination and subsequently laser ablated along a line transect running from edge to edge passing through the nucleus, following the longest axis. At the Lund facility, two-dimensional maps, colour-coded for each element or isotope representing concentration for single-matrix were created for three selected otoliths by using parallel adjoining line scans (Ulrich *et al.*, 2009). These two-dimensional maps verified that the seasonal patterns were real and provided a more complete understanding of trace elemental incorporation, since ring patterns as well as

inhomogeneities were readily seen (Figure S1). Details of configurations and settings for the LA-ICP-MS analyses are listed in Supporting Information Table 1.

A number of trace elements in otoliths are used as proxies for interpreting fish life history events. By combining these known proxies we have developed and tested additional unconventional proxies, based on fish ecology and physiology, to aid in age determination. Known proxies for the most commonly analysed trace elements in ratio to calcium (Ca) or other elements are listed in Table 2, and proposed proxies of less commonly analysed trace elements, unconventional ratios, and their interpretation are listed in Table 3.

2.3. Age estimation and readability ranking

Cod otoliths were aged with two different methods. Visual age determination was applied by six professional age readers from Denmark, Poland, Sweden and Russia, all of them experts in the traditional method of counting visible growth zones of the cross section broken at the nucleus. This method is based on the assumption of consistent formation of clearly contrasting opaque and translucent seasonal growth zones, corresponding to summer and winter, respectively (ICES, 2014). Chemical age determination is a novel method, based on the identification of seasonal maxima and minima in trace elemental uptake, and it was tested for the first time by three experts in both age reading and otolith microchemistry from Denmark, Sweden and the USA. The main differences between this study's approach

and (Hüssy et al., 2015) was that besides comparing increment opacity profiles with chemistry, we also compared the readers' subjective interpretations of visual growth zones versus seasonal variations in the chemical profiles.

The visual (traditional) and the chemical (novel) age estimation methods were compared in an age-reading exercise in SmartDots, a new internet-based platform for age calibration based on interpreting otolith images (ICES, 2018). SmartDots projects an otolith image, and tools are available to place marks on winter growth checks (annuli) along a pre-defined line from the core to the dorsal edge following the maximum growth axis. Participants were instructed to annotate annulus locations based on visual growth zones or chemistry, respectively. The opacity values for the otolith images, provided by SmartDots, ranged from 0 (black) = 0% to 255 (white) = 100%. We used the opacity values where readers marked the line transects to evaluate whether the methods differed in annotated annulus opacity. To normalize data, each otolith's opacity profile was scaled to the minimum and maximum opacity values as a percentage.

Ancillary information for each sample included date of catch, area (SD, Figure 2) and total length of the fish. Date of birth was set by convention to January 1st, hence if the fish was caught in the first quarter of the year, the edge of the otolith was counted as an annulus, irrespective of winter growth zone completion.

A ranking system for optical otolith image readability was developed to evaluate the certainty of visual age estimation (Table 4). Chemical age readers were asked to use the same criteria, and also to apply them to rank trace elemental ratios by their accuracy in identifying seasonal growth zones.

2.4. Otolith chemical analyses and ageing

Both the chemical profiles (transects) and the two dimensional maps (Figure S1), were assessed for seasonal patterns with the same approach, based on interpreting variations in trace elemental concentrations from the otolith core to the dorsal edge. Each ratio was ranked according to the readability scoring system (Table 4) to evaluate the reader's confidence in identifying annuli. The chemical profiles were overlaid on the otolith image in order for the reader to know where along the pre-drawn line in SmartDots to annotate the chemical annuli and to tease out where false (visual) annuli occurred and what they corresponded to chemically.

Figure 3 illustrates our chemical age estimation method. Here, the yellow vertical dashed lines correspond to chemical minima in Mg:Ca and Mn:Ca, interpreted as winter annuli associated with low metabolic activity, reduced growth and little to no hypoxia exposure, which occurs mainly in summer in the Baltic Sea (Limburg and Casini, 2018; Limburg et al., 2018). The seasonal patterns in these chemical profiles enabled an age estimation with high certainty,

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and thus were assigned a ranking score of 5. For P:Ca, a proxy for high metabolic activity and growth (Table 3), the seasonal pattern is less clear and the decrease in concentration between the first and second yellow dashed line, marked with a white arrow, could be interpreted as a potential annulus, which introduced an uncertainty of one year and hence the assigned ranking score of 4. The environmental proxy Sr:Ca increases with higher salinity and lower water temperature (Table 3); therefore the maxima in the chemical profiles might be interpreted as migration to colder offshore habitats with higher salinity during winter. The Sr:Ca profile in Figure 3 is unclear, displaying two peaks, which did not correspond to seasonal patterns of other ratios; readability is therefore ranked as moderate, and given a ranking score of 3. Our interpretation of the Sr:Ca profile in Figure 3 is that its variation correlates with migration patterns rather than with seasonality. The Ba:Ca ratio (Figure 3) displays a complex pattern. The elevated levels of Ba:Ca in early growth are interpreted as the fish residing in juvenile habitat close to coastal runoff sources in summer during the first two years, with chemical minima as annuli. The unclear pattern between the second and fifth yellow dashed lines (marked with white arrows) is difficult to interpret, as the Ba:Ca pattern at the fourth yellow line flips to the reversed pattern, now following the same pattern as Sr:Ca. This indicates a habitat switch and that the chemical maxima now instead should be interpreted as annuli, corresponding to winter residency in deeper water, where barium is upwelled (Table 2). Age estimation with Ba:Ca might vary by more than two years and hence is considered poor, and is assigned a score of 2. The Zn:Ca (Figure 3), concentration was elevated in the otolith core, but otherwise low with an irregular pattern, difficult to interpret and regarded as

unreadable with a ranking score of 1. The ratio Sr:Mg (Figure 3) enhances the signal of poor growth during winter by dividing the environmental proxy Sr (maxima during low temperature and high salinity) with the metabolic proxy Mg (minima during low growth and low metabolic activity). In this example, the complex pattern of Sr resulted in an uncertainty of one year for Sr:Mg and a ranking score of 4.

2.5. Data analysis

Chemical ratios useful for age estimations were identified using the ratio ranking score system (Table 4). ANOVA was conducted with R (R Development Core Team, 2019) to assess if the mean ranking scores for the ratios differed among readers, areas and over time (decades). The average ranking scores for the trace elemental ratios were plotted using R package “ggplot2” (Auguie, 2017; Wickham, 2016) and R package “gridExtra” (Auguie, 2017) for illustrating elemental ratios over time.

Out of the one hundred otoliths read by the age readers, four otoliths were judged unreadable by one of the visual readers and hence, estimates for these were not included in the statistical analyses. The statistical analyses of the age comparison followed the recommendations of the European Fish Ageing Network (EFAN), that account for the consistency among readers relative to the modal age (most common age of all age estimates per otolith sample) (Eltink, 2000). Due to the lower number of chemical readers (n = 3),

rounded mean age was used instead of modal age. We defined the degree of accuracy as the average percent of agreement (PA), of age readers' estimates agreeing with the modal age (or rounded mean age). PA was calculated as:

$$PA = 100 * \left(\frac{n \text{ samples agreed}}{n \text{ samples}} \right)$$

To account for the age range, precision errors were calculated to assess agreement among age readers within each sample and described by the average percent error (APE) and the average coefficient of variation (ACV). The difference between these measures is that ACV incorporates the standard deviation in the equation, whereas APE assumes proportional standard deviations of the readers' estimated age to the modal age (or rounded mean age) of the estimates (Beamish and Fournier, 1981; Chang, 1982; Eltink, 2000; Ogle, 2013). The APE and ACV were calculated as:

$$APE = \frac{\sum_{j=1}^n APE_j}{n} \text{ where } APE_j = 100 * \frac{\sum_{i=1}^R \frac{|x_{ij} - \bar{x}_j|}{\bar{x}_j}}{R}$$

$$ACV = \frac{\sum_{j=1}^n CV_j}{n} \text{ where } CV_j = 100 * \frac{\sqrt{\sum_{i=1}^R \left(\frac{(x_{ij} - \bar{x}_j)^2}{R - 1} \right)}}{\bar{x}_j}$$

where n is the number of otoliths and R is the number of age estimations per otolith. x_{ij} is the i^{th} age estimation for the j^{th} otolith, and \bar{x}_j is the modal age or rounded mean age for the j^{th} otolith. The APE was calculated using the R-package "fishR" (Ogle, 2013).

Systematic under- or overestimation of ages compared to the modal age (or rounded mean age) was expressed as the relative bias:

$$Relative\ bias = 100 * \frac{x_j - \bar{x}_j}{\bar{x}_j}$$

Differences between methods were tested with the R package “lme4” (Bates *et al.*, 2015) and “nlme” (Pinheiro J, 2019) using a nested linear mixed-effects model (LME). Age was the response group, method the fixed effect, and sample nested within method and reader within sample the random effects, allowing for variances to differ and to estimate the variances within the random effects. To quantify the proportion of variance explained by the total model as well as for the fixed and random effects within the model, an R^2 value was estimated with the R package “MuMin” (Barton, 2019) where higher values corresponded to better fit (Nakagawa and Schielzeth, 2013).

Differences in mean otolith image readability ranking score for methods, areas (Baltic vs. North Sea) and decades were analysed with LME. The ranking scores were the response variables, with method, area or decade as the fixed effect for each respective model, and sample was treated as a random effect nested within the fixed effects.

We used the R package “psycho” (Makowski, 2018) to perform a repeated measures ANOVA to predict the opacity per method for each annulus (1-7) separately, with opacity as the response group, method as the fixed effect and sample number and method as the random effects. By adding method (*i.e.*, visual or chemical) as both fixed and random effects, we grouped the repeated measures by specifying method as the average regression coefficients, allowing both random slopes and intercepts for each sample with individual deviations from the mean. For annuli > 8, the sample sizes were too small for reliable estimates.

To predict differences in annotated annulus distance from the otolith core per method and area, we extracted the distances from SmartDots and used the R package “psycho” (Makowski, 2018) to perform a repeated measures ANOVA. We compared each annulus separately, with distance (log transformed) as the response group, method as the fixed effect and sample number and age reader as the random effects. Mean distances from the otolith core were estimated by method, and subtracted differences were plotted. The significance level, $P < 0.05$ was used in all statistical analyses to reject the null hypothesis of no difference between means.

3. RESULTS

3.1. Chemical ranking

All three chemical readers assigned the highest mean score value for Mg:Ca and the second highest rank for P:Ca, consistently for both study areas. Overall, the ranked mean scores of proxies for metabolic activity and physiology, i.e., Mg:Ca (4.3) and P:Ca (3.8), were higher than the environmental proxies Sr:Ca (2.6) and Ba:Ca (2.7). Due to the influences of both environment (hypoxia) and metabolism (growth), Mn:Ca (3.3) scored in between the two previous groups (Figure 4). Notably, Mn:Ca in the more intensely hypoxic Baltic Sea scored higher (3.46) than in the North Sea (3.18). No significant differences were detected among readers (ANOVA, $F_{2,39} = 1.173$, $R^2 = 0.057$, $P > 0.05$), areas (ANOVA, $F_{1,110} = 0.261$, $R^2 = 0.002$, $P > 0.05$) or decades (ANOVA, $F_{3,108} = 0.748$, $R^2 = 0.020$, $P > 0.05$).

Ranking scores over time in the Baltic and North seas (Figure 5) show that the metabolic proxies (Mg:Ca and P:Ca) remained high over the last four decades, whereas the environmental proxies (Sr:Ca and Ba:Ca) have been consistently lower throughout the time series. Mn:Ca, the proxy for both environment (hypoxia exposure) and metabolic activity (somatic growth) falls in between. The spatial and temporal consistency confirms that the ratios Mg:Ca and P:Ca are consistently considered as easier to interpret. (Figure 5). All ratios ranking scores over time are listed in the Supporting Information Table 2.

3.2. Age comparison within and between methods

The percent agreement (PA) for the visual method was lower at 50.2% (Baltic Sea) and 54.2% (North Sea) compared to the chemical method with 74.2% and 73.3% respectively (Table 5). Precision errors (APE and ACV) for the visual method were more than double those of the chemical method and the average relative bias indicated an overall threefold systematically higher under- or overestimation for the visual method than for the chemical method. The visual method's age estimations ranged from one to twelve years and the chemical method from one to nine years (Table 5).

Visual modal age estimates were plotted against the chemical method's rounded mean age estimates (Figure 6), showing that visually determined age estimates were generally higher than corresponding age estimates by the chemical method.

Nested linear mixed effects models of age (years) as a function of method (fixed effect) and sample within method, and reader within sample (random effects), showed that 3.79% of variance was explained by the fixed effect and the remainder by the random effects (full model $R^2 = 0.95$). The model estimated approximately three times greater variance and significantly higher average age estimates for the visual method (mean = 5.06, standard deviation (SD) = 1.10, confidence interval (C.I.) = 4.39, 5.73), than for the chemical method (mean = 4.43, SD = 0.35, C.I. = 4.16, 4.71), (LME, $F_{1,894}$, $R^2 = 0.95$, $P < 0.001$).

3.3 Otolith image readability ranking

No significant difference in otolith image readability ranking was found between methods (LME, $F_{1,898}$, $R^2=0.47$, $P > 0.05$). Comparing areas indicated significantly higher mean ranking scores for the North Sea (mean = 3.94, SD = 0.86, C.I. = 3.53, 4.36)), vs. the Baltic Sea (mean = 3.15, SD = 0.84, C.I. = 3.03, 3.28)), indicating that otoliths from EBC are more difficult to read (LME, $F_{1,898}$, $R^2 = 0.33$, $P < 0.001$). The fixed effect of area accounted for approximately 9.48% of the total variation in ranking scores, whereas the random effect of group (sample nested within area) accounted for approximately 24.23% and the whole model of 33.37%. There were no significant differences among decades for the Baltic Sea indicating that otolith readability has been consistently low over time, corresponding to uncertainty in age estimation which might vary by two or more years (LME, $F_{3,716}$, $R^2=0.20$, $P > 0.05$). The low sample size ($n = 5$ per decade) in the North Sea made it difficult to make any reliable estimates (full model $R^2 = 0.52$).

3.4 Annulus opacity

In general, readers selected annuli with declining opacity values with age (annulus), along the line transects regardless of method. The decline was steeper for the chemical method and the discrepancy between methods generally increased with age. The highest mean opacity values were estimated for annulus 1 at 81% for the chemical method and 73% for the visual method and the lowest for annulus 8 with 16% for the

chemical and 41% for the visual method. The chemical method estimated significantly higher average opacity values for annuli 1 and 2 than the visual method ($P < 0.05$; Figure 7). In contrast, for annuli 4, 6, 7 and 8, the average opacity values for the chemical method were significantly lower than for the visual method ($P < 0.05$). There was no significant difference in opacity between methods for annulus 3 and 5. The effect of method was small for annuli 1 to 6, but moderate for annulus 7 and strong for annulus 8. Hence, the random effect of sample grouped by method accounted for most of the variance of opacity in the model. The chemical annuli generally occurred within opaque zones for annuli 1 to 3, to thereafter switch to correspond to thin dark translucent zones for annuli 4 to 8. The visual readers generally selected more pronounced, but not necessarily darker, translucent zones, which chemically corresponded to hypoxia exposure, leading to suppressed growth during summer.

3.5 Annulus distance from otolith core

Repeated measures ANOVA showed that visual core-to-annulus distances were shorter ($P < 0.05$) for annuli 3 - 8 in Baltic samples compared to chemically determined ones, but not for annuli 1 and 2 ($P > 0.05$). In the North Sea, there were no significant differences between methods for annuli 1 - 6 ($P > 0.05$). Visual readers thus interpret more translucent zones as annuli compared to chemical readers in the Baltic Sea, starting at annulus 3 in the Baltic Sea (Figure 8).

4. DISCUSSION

The inability to standardize traditional age readings, in this case for the Eastern Baltic cod (EBC), (Hüssy *et al.*, 2015; 2016), called for the development of alternative ageing methods, such as the chemical method presented here. This study may be the first of its kind aimed at developing ageing criteria using otolith microchemistry; namely, conducting a formal age reading comparison with traditional methods based on visually identified annuli. We have taken a novel approach of using knowledge about ecology and environment (external influences on otolith chemistry) on the one hand, and physiology (internal influences) on the other. We have then systematically tested both single and combinations of elemental ratios to determine which ones were most suitable for age determination. Chemically-based age estimates resulted in greater agreement and less variation than age estimates by experienced visual readers, all of whom are experts in this skill.

Increasingly, otolith chemistry is being examined as a means to validate difficult-to-age fish. Early work explored the use of radionuclides such as $^{210}\text{Pb}/^{226}\text{Ra}$ to age long lived, deep-sea fishes (Campana *et al.*, 1990; Fenton *et al.*, 1991) This was followed by radiocarbon dating, which relies on the strong temporal changes in ^{14}C worldwide after nuclear bomb testing in the 1950's and 1960's (Campana, 1997; Campana, 1999; Campana, 1998; Kalish *et al.*, 1996; Kalish *et al.*, 1997). However, cod is not a long lived fish, hence radiocarbon dating is

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inappropriate as an ageing method. Moreover, otolith oxygen and carbon isotopic ratios relate to the isotopic equilibria in the water (Campana, 1999; Degens *et al.*, 1969). At constant salinity, the fractionation of stable oxygen isotopes is assumed to be temperature dependent: $\delta^{18}\text{O}$ under those circumstances enables reconstruction of seasonal changes in water temperature and has been applied as a method for age validation of different species with calcified chronological structures (Høie and Folkvord, 2006; Judd *et al.*, 2018; Upton *et al.*, 2012). For the EBC, however, complex migration patterns, water vertical stratification and strong salinity gradients from south to north, combined with hydrographic variations over time, complicate the interpretation of water temperature as a proxy for season and thus age from metrics such as $\delta^{18}\text{O}$. Nevertheless, some combinations of trace elements and isotopes (e.g., $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$) may serve to amplify seasonal signals in otoliths. For example, Wurster and Patterson (2003) found seasonal variation in $\delta^{13}\text{C}$ in Holocene *Aplodinotus grunniens* otoliths that suggested it was a metabolic proxy, subsequently verified experimentally by (Chung *et al.*, 2019).

The highest ranked trace elemental ratios, and hence most useful for chemical age estimation, were Mg:Ca and P:Ca. These ratios are strongly affected by fish metabolic activity (Avigliano *et al.*, 2015; Izzo *et al.*, 2018; Limburg *et al.*, 2011; Limburg *et al.*, 2018; Martin and Thorrold, 2005; Serre, 2018; Sturrock *et al.*, 2015; Thomas *et al.*, 2017). They reflect seasonal variations in otolith trace elemental uptake more consistently than the

environmental proxy ratios of Sr:Ca and Ba:Ca, which seem to be linked to migration behaviour and habitat shifts.

When interpreting the seasonal variations in concentration of the metabolic proxies Mg:Ca and P:Ca for age estimation, it is imperative to evaluate whether the fish's metabolic activity could have been affected by hypoxia or other unknown outside stressors, such as starvation, disease, etc. In the case of intense hypoxia exposure, which results in high concentrations of Mn:Ca (Figure S2), we have observed that the Mg:Ca and P:Ca levels appeared to attenuate simultaneously (Limburg and Casini, 2019). By inspecting the Sr:Ca and Ba:Ca chemical profiles we evaluated whether it was possible to determine if the fish during the hypoxic exposure was located in cold, deep offshore habitats with high salinity (winter) or in warmer coastal habitats with lower salinity (summer). Thus, by combining the different ratios and comparing the periodic patterns of both metabolic and environmental proxies we could increase the certainty of our age estimations. Hence we recommend to include the environmental proxies Sr:Ca and Ba:Ca in the chemical analyses as an important aid in age estimation when the metabolic proxies do not display readable seasonal signals.

For Mn:Ca, which is a proxy for both hypoxia and growth (Limburg *et al.*, 2015), the interpretation is more complex. Seasonal maxima may reflect intense summer hypoxia, but also in some cases Mn:Ca appears to have been incorporated while fish occupied deep

hypoxic habitats during winter displaying the reversed pattern compared to Mg:Ca. In the event of major Baltic water inflows such as in the early 1990s, 2003, and 2014, the Mn:Ca signal drops flat as the water becomes oxygenated (Figure S3). This is a disadvantage regarding the possibility of counting maxima and minima in the chemical profile for age estimation, but is also an advantage as these can be used as temporal reference points for age validation, similar to dendrochronology (Cook and Kairiukstis, 2013). The synchrony in chemical profiles' seasonal signals can be combined to achieve greater certainty of the chemical age estimation, and will be the subject of future investigations.

The visual age estimates were made by highly experienced age readers, who have up to several decades of experience and cross-training, while the chemical agers never had a single calibration exercise, nor any documented manual. In age reading exercises such as this, the acceptance criteria for the percent of agreement (PA) vary depending on species, age and area. For most species the criterion for acceptance of age agreement is set to 80% (Eltink, 2000; Vitale, 2019), but for species difficult to age, such as the European eel *Anguilla Anguilla* L. 1758, acceptable agreement is 70% (SLU-Aqua, 2012). Our study suggests that chemical ageing (PA = 74%) reached the required level of agreement (PA > 70%) for difficult to age species, serving as a more accurate age estimation method than the visual method (PA = 51%) for both the EBC and North Sea cod. The average coefficient of variation (ACV) for the visual method (24.2%) was more than double that of the chemical method (11.2%), although both methods exceeded the generally accepted reference point of 5% (Campana,

2001). In routine age estimation, there are seldom otolith samples with known age available, therefore precision and agreement do not necessarily identify the true age. Validation with daily increments or otolith marked tag/recapture samples is an important part to obtain a correct age determination, and will be forthcoming for EBC.

One factor to consider is that the visual readers employed in the study are experts in ageing actual otoliths under a microscope, and not from photographic images. When working with the actual samples, the reflected light can be adjusted to optimize the contrast between growth rings, which is not possible for images. The resolution and quality of the images therefore varied which could have had an effect on the analyses. However, trials that use actual otoliths and circulate samples between age readers also encounter variation with different microscopes and light sources, and find that these differences affect the visual interpretation. Reading from images meant that all visual readers age estimated under the identical optical circumstances, which was also true for all nine readers when ranking the otolith image readability.

Otolith image readability scored significantly lower in the Baltic Sea than in the North Sea, with no significant difference over time. This came as no surprise due to the historical difficulties of age estimation of the EBC (Hüssy *et al.*, 2016).

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Chemical age readers annotated annuli at significantly brighter (more opaque) zones than visual readers for the first two annuli, perhaps due to lower contrasting differences within the central part of the otolith compared to the outer regions. There was generally a high agreement among the chemical age readers in these annuli, because of very clear seasonal “minima” in Mg:Ca and Mn:Ca. Often (although not always) the first chemical annulus coincided with a visible ring, but surprisingly (and counterintuitively for experienced age readers), the second and third chemical annuli occurred in the middle of large opaque zones. For annuli 4, 6, 7, and 8 the opposite pattern occurred where chemical readers noted a coincidence of chemical annuli with thinner, significantly darker (more translucent) bands in older individuals, suggesting a more consistent growth cycle, possibly affected by sexual maturation and spawning migration at age > 3, linked to ontogenetic habitat occupation. Young cod are distributed in shallower water than older individuals (Cohen, 1990; Uzars and Plikshs, 2000), and may therefore be exposed to warmer temperatures in summer, which leaves a pronounced translucent zone (Fablet *et al.*, 2011; Høie *et al.*, 2009) interpreted as an annulus by the visual readers. In occasions of intense summer hypoxia with high Mn:Ca concentrations, false translucent thin rings or thicker zones were often visible within the corresponding part of the chemical profile, especially between annuli 1 to 3. These have been observed in other species (e.g., alewife *Alosa pseudoharengus*, (Limburg *et al.*, 2015; Monteiro Pierce *et al.*, 2019)). This would explain the visual method’s lower opacity for the 1- and 2-year-old age classes compared to the chemical method. Once the fish mature and

migrate to deeper water to spawn in summer (Cohen, 1990; Uzars and Plikshs, 2000), the pattern is reversed.

The overall variation in annulus width depended primarily on differences in individual growth rate, but when assessing variations within individual samples, it became apparent that for the visual readers, a major source of variation was disagreement on which translucent zones that should be interpreted as true annuli. On average, from annulus 3 onward, the visual readers interpreted a higher number of annuli than the chemical readers. The chemical method provided on average lower age estimations than the visual method, but the variation was large and visual ages were both older and younger than those assigned chemically. It was not possible to draw any conclusions on differences in absolute growth rate estimates between the methods as the samples were not collected to representatively estimate growth rates, which are moreover expected to vary between areas and over the four analysed decades. For the chemical method, the variation depended on which elemental ratios were selected for annotating the annulus and also the temporal lags in ratios' maxima and minima relative to each other. These maxima and minima, although displaying similar patterns, were not exactly synchronized in distance from the otolith core (Figure 3), therefore reflecting different mechanisms of temporal trace elemental uptake in the otolith biomineralisation process. This was especially true for the ratios Mg:Ca, Mn:Ca, and P:Ca. Mn:Ca would often decline prior to Mg:Ca or P:Ca, perhaps due to cessation of hypoxia exposure (Figure 3). Mg:Ca was generally higher in the early years of the fish's life

to decline, displaying an attenuated seasonal pattern with age (Limburg *et al.*, 2018), while the level of P:Ca remained high or even increased with age in some cases (Figure 3).

Although Mg:Ca received a higher average ranking score than P:Ca, solving the question of which ratios correspond to the “true time” demands further validation studies based on *e.g.*, daily increments and samples from tag/recapture experiments.

5. CONCLUSIONS

The results of the analyses in this study lead us to conclude that the chemical method provides more accurate age estimates than the traditional ageing method for the Eastern Baltic cod. Techniques and instrumentation used for otolith chemical analyses have developed rapidly in recent decades. Although still fairly expensive and time-consuming, advances in preparation technique, instrumentation and data reduction software have already sped up the process. In the case of Eastern Baltic cod and other species difficult to age, it should be feasible to use otolith chemical ageing for at least a subset of otoliths as part of the routine age determination to achieve time series of age composition and growth rates, in order to provide higher certainty of the data provided for analytical assessment. Additionally, otolith chemical age determination on a subset of samples may also enhance the ability of visual readers to interpret optical structures in the future. More work is needed in this direction and for validating this technique, but otolith chemical ageing shows great promise as a means to obtain truer ages for this stressed stock of cod, as well as other

difficult-to-age species and populations. We suggest that other researchers consider adopting similar lines of reasoning (ecological and physiological factors) for use of otolith chemistry as an aid to age determination.

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CONTRIBUTIONS

The work is original and all authors have approved the submitted manuscript. YH, KL and KH conceived the study design; YH, KL, KH, RS, A-M P, SL, AK, KR and JÖ collected the data; YH, KL, KH and MC analysed the data and YH, KL, KH and MC wrote the manuscript. All authors have critically reviewed the manuscript and given comments.

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TABLE 1 Description of the *Gadus morhua* otolith sample collection. MBI = Major Baltic

Inflow

Time period	North Sea (n)	Baltic Sea (n)	Length range (mm)	Characteristics of the Baltic Sea during the last four decades
1980s	5	20	245 – 789	High cod biomass, salinity and body condition. Moderate MBIs in November 1982 and January 1983.
1990s	5	20	490 - 973	Low cod biomass, salinity and hypoxia. Very strong MBI in January 1993 and moderate in September 1997.
2000s	5	20	170 - 820	Low cod biomass, increasing hypoxia and a strong MBI in January 2003.
2010s	5	20	274 - 600	Low cod biomass, low body condition and increasing hypoxia. Very strong MBI in December 2014 and moderate MBIs during winter time in 2015 to 2016 and 2016 to 2017.
Total	20	80	170 - 973	

TABLE 2 Known proxies of trace elemental isotopes and ratios analysed in the *Gadus morhua* otoliths, with literature references

Chemical analyte	Known proxies	References
^{88}Sr	Salinity, temperature and migration Higher Sr concentration is an indicator of colder offshore habitat with higher salinity.	(Chowdhury and Blust, 2001; Elsdon and Gillanders, 2002; Kraus and Secor, 2004; Limburg, 2004; Martin and Thorrold, 2005; Nelson <i>et al.</i> , 2017; Stanley <i>et al.</i> , 2015; Townsend <i>et al.</i> , 1995)
^{138}Ba	Habitat and migration. High concentrations close to coast due to riverine input of Ba rich particles that dissolve during estuarine mixing.	(Limburg <i>et al.</i> , 2011; Secor <i>et al.</i> , 2001; Thorrold <i>et al.</i> , 2001; Walther and Limburg, 2012)
^{24}Mg , ^{25}Mg , ^{26}Mg	Metabolic activity and somatic growth rate High concentration is an indicator of high metabolic activity and somatic and otolith growth.	(Avigliano <i>et al.</i> , 2015; Limburg <i>et al.</i> , 2011; Limburg <i>et al.</i> , 2018; Martin and Thorrold, 2005; Sturrock <i>et al.</i> , 2015)

⁵⁵ Mn	Hypoxic exposure and somatic growth	(Limburg <i>et al.</i> , 2011; Mohan and Walther, 2016; Mohan <i>et al.</i> , 2015)
⁶⁴ Zn	Habitat indicator, physiologically controlled, growth, seasonality, spawning indicator for females. Generally elevated in the core of the EBC otolith	(Halden and Friedrich, 2018; Limburg and Elfman, 2010; Sturrock <i>et al.</i> , 2015) (Limburg and Casini, 2018)
⁶³ Cu	Condition and temperature, Generally elevated in the core of the EBC otolith	(Sturrock <i>et al.</i> , 2015) (Limburg and Casini, 2018)
³¹ P	Metabolic activity, physiologically regulated, seasonality, otolith growth	(Hüssy, 2018; Thomas <i>et al.</i> , 2017)
Mn:Mg	Indicator of exposure to hypoxia (Mn) with correction of growth effects (Mg)	(Limburg and Casini, 2018)

TABLE 3 Proposed additional proxies, assessed with the chemical age estimation method for the Eastern Baltic *Gadus morhua*

Chemical	Proposed proxies
analyte	Unpublished hypotheses by Yvette Heimbrand and Karin Limburg
^{43}Ca	Positively correlated with water temperature
^{138}Ba	Winter habitats for adults, influenced by Ba that re-dissolves in deep anoxic environments.
Sr:Ba	The relationship between either offshore/onshore during the juvenile state or offshore and deep hypoxic adult habitats.
^{55}Mn	Seasonal patterns reflecting elevated hypoxia, mostly in summer but occasionally in winter.
Sr:Mg	Indicator of winter; Sr increases when the EBC migrate offshore to deeper, colder habitats with higher salinity. Mg decreases with decreased metabolic activity and somatic growth during winter.
Sr:Mn	Sr increases when the EBC migrate offshore to colder habitats with higher salinity. Seasonal patterns of Mn reflects elevated hypoxia, mostly in summer but occasionally in winter.
Mg:Mn	Indicator of growth and metabolic activity (Mg) with respect to exposure to hypoxia (Mn).

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TABLE 4 Ranking system for clarity of annual patterns in otolith images of *Gadus morhua*, chemical variables and reader estimate of readability

Ranking score	Readability	Description of annual growth zones and the certainty of the age estimation
5	VERY GOOD	Very clearly marked and contrasting annual growth zones and seasonal patterns. Age estimation with high certainty.
4	GOOD	Distinct contrasting annual growth zones and seasonal patterns. Age estimation quite certain but might vary with one year
3	MODERATE	Partly clear annual growth zones and seasonal patterns. Age estimation uncertain and might vary with two year.
2	POOR	Indistinct annual growth zones and seasonal patterns. Age estimation poor and might vary with more than two year.
1	UNREADABLE	No recognizable structures of annual growth zones or seasonal patterns. Age estimation is not feasible.

TABLE 5 Statistical evaluation of the method comparison of ageing *Gadus morhua*.

Areas	Both Areas (n=100)		Baltic Sea (n=80)		North Sea (n=20)	
	Chemical	Visual	Chemical	Visual	Chemical	Visual
Age estimation method						
Average percent agreement (%)	74.0	51.0	74.2	50.2	73.3	54.2
Average percent agreement SE (%)	14.0	9.5	11.5	10.7	15.4	15.2
Average percent error (%)	8.4	17.0	7.7	16.3	11.1	20.1
Average coefficient of variation (%)	11.2	23.1	10.3	21.7	14.5	28.6
Average relative bias (%)	3.0	9.4	2.1	4.6	6.7	28.0
Average relative bias SE (%)	21.6	34.4	19.4	32.8	23.4	48.6
Mean age (years)	4.4	5.1	4.6	5.3	3.7	4.2
Age range (years)	1-9	1-12	1-9	1-12	1-7	1-8

LIST OF FIGURE CAPTIONS

FIGURE 1 Photo montage to illustrate the differences in otolith growth zones for *Gadus morhua* from different regions. Left: a North Sea *Gadus morhua* otolith with strong contrasting growth zones; middle: an Eastern Baltic *Gadus morhua* otolith displaying multiple false translucent annuli; right: a mostly opaque Eastern Baltic *Gadus morhua* otolith lacking clear zonation (Photo montage: Y. Heimbrand)

FIGURE 2 Sampling areas: the eastern North Sea (subdivision 20 and 21) and the Baltic Sea (subdivision 25-29), showing the salinity gradient. Samples for the ageing comparison of *Gadus morhua* were collected within the ovals

FIGURE 3 Photo montage illustrating the laser transect from the *Gadus morhua* otolith core to the dorsal edge and the chemical profiles of different elemental ratios combined and compared for chemical maxima and minima for age estimation. The arrows indicate uncertainties for the age estimation and yellow vertical dashed lines correspond to chemical readers' consensus. Hence, this otolith was chemically aged as five years old. The visual readers' age estimations varied between four and seven years. Otolith image readability by all readers varied between two and five

FIGURE 4 Mean ranking scores, based on data from both study areas for trace elemental ratio's usefulness as age estimation tools of *Gadus morhua*

FIGURE 5 Average ranking scores over time (1980s – 2010s) for *Gadus morhua* in the Baltic and North Seas for the metabolic proxies (Mg:Ca and P:Ca), the environmental proxies (Sr:Ca and Ba:Ca) and Mn:Ca, proxy for both environment (hypoxic exposure) and metabolic activity (somatic growth)

FIGURE 6 Age bias plot illustrating the differences in age estimation of *Gadus morhua* between methods. The visual method's modal age estimates (y-axis) are plotted against the chemical method's rounded mean age estimates (x-axis)

FIGURE 7 Boxplot of image opacity in percent per annotated annulus for the *Gadus morhua* otoliths for the visual and chemical methods. To normalize data, each otolith's opacity profile is scaled to the minimum and maximum opacity values along the pre-defined line where readers' annuli were annotated. Box = 25th and 75th percentiles; horizontal line = median value; whiskers = 1.5 times interquartile range above 75th percentile or below 25th percentile, points = outliers > 1.5 times and < 3 times the interquartile range outside the end of each box. Pairwise comparisons marked with a star indicate significant differences between methods ($p < 0.05$) and non-significant differences with a cross

FIGURE 8 Differences in mean annulus distance from the *Gadus morhua* otolith core on the dorsal side of the otolith \pm SE, compared between methods in the Baltic Sea and North Sea, subtracting the chemical method from the visual method estimates















