Otolith microstructure analysis to resolve seasonal patterns of hatching and settlement in western Baltic cod

Sabine Rehberg-Haas[1,2], Cornelius Hammer[2], Nicola Hillgruber[3,4]*, Karin Hüssy[5], and Axel Temming[2,6]

[1]GMA – Society/Association for Marine Aquaculture Ltd, Hafentörn 3, D-25761 Büsum, Germany
[2]Thünen-Institute of Baltic Sea Fisheries, Johann Heinrich von Thünen-Institute, Alter Hafen Süd 2, D-18069 Rostock, Germany
[3]University of Alaska Fairbanks, School of Fisheries & Ocean Sciences, 17101 Point Lena Loop Road, Juneau, AK 99801, USA
[4]Thünen Institute of Fisheries Ecology, Wulfsdorfer Weg 204, D-22926 Ahrensburg, Germany
[5]National Institute of Aquatic Resources, Technical University of Denmark, Jaegersborg Allee 1, 2920 Charlottenlund, Denmark
[6]Institute for Hydrobiology and Fisheries Science, Olbersweg 24, D-22767 Hamburg, Germany

*Corresponding Author: tel: +49-4102-70860-16; fax: +49-4102-70860-10; e-mail: nicola.hillgruber@vti.bund.de

Previous studies showed that Baltic cod (*Gadus morhua*) settle to demersal life at a given size, while the annulus is formed seasonally, irrespective of size. The goal of this study was to examine the timing of check formation in juvenile Baltic cod otoliths to validate macrostructural ageing and to differentiate between true annuli and secondary structures such as settlement checks. Otoliths were collected from fish off Fehmarn Island in 2008 and 2009, and were examined for macrostructural and microstructural patterns using light and scanning electron microscopy. All fish examined were age-0. Back-calculation of hatch dates indicated hatching from April to June and from February to August in 2008 and 2009, respectively. Juveniles formed either one or two translucent rings. The first translucent ring started to form ∼3 months post-hatch and was interpreted as a settlement check, since it appeared to be a function of age and/or size and not season. Deposition of the second ring began in mid October to early November irrespective of fish size and/or age, thus indicating that this ring may represent the first annulus of Baltic cod. Both rings were clearly distinguishable in individuals hatched between February and May, but were merged in those fish where settlement coincided with the seasonally formed second ring.

Keywords: annulus, Baltic cod, daily increment analysis, *Gadus morhua*, settlement check.

Introduction

Baltic cod (*Gadus morhua*) is one of the top piscivorous fish species in the Baltic Sea, which together with Atlantic herring (*Clupea harengus*) and sprat (*Sprattus sprattus*) dominates the fish biomass of this ecosystem (Ojaveer *et al.*, 2010). In addition to its ecological importance, this species is also of great commercial relevance, with an annual harvest in 2009 of 15.3 kt in the western Baltic Sea (ICES Subdivisions 22–24) and 48.4 kt in the eastern Baltic Sea (ICES Subdivisions 25–32; ICES, 2010). For management, Baltic cod is divided into two separate stocks, the western and eastern stock (Bagge *et al.*, 1994). The reasons for this separation are, among others, differences in spawning time, as well as differing spawning and nursery areas. For the western stock, spawning continues from January until May, with peak spawning in March, while the eastern stock is characterized by a more extended spawning period from March until September (Bagge *et al.*, 1994), with peak spawning gradually changing from early May to mid June in the late 1970s and 1980s to the end of July in the 1990s (Wieland *et al.*, 2000). The main spawning areas of western Baltic cod are Fehmarn Belt, Kiel Bay, and, to a lesser degree, the Arkona Basin, while eastern Baltic cod spawn in the deep basins of the Baltic Sea, particularly the Bornholm Basin and, to a lesser degree, the Gdansk and the Gotland basins (Bagge *et al.*, 1994). The relationships between both stocks are still poorly understood, but different lines of evidence suggest a notable amount of mixing that might occur during the pelagic larval and juvenile stages (Hinrichsen *et al.*, 2001, 2009) and by adult migration (Otterlind, 1985).

Baltic cod, like many other marine fish species, undergo ontogenetic habitat changes from the larval and early juvenile pelagic stage to the juvenile demersal stage. Settling to the near-shore waters of the Baltic Sea could be viewed as a physiologically stressful experience that requires the fish to adjust to vastly differing hydrographic conditions and prey fields (Hüssy *et al.*, 1997, 2003). This transition to demersal, nearshore habitats also leads to a transition zone, i.e. a zone in which otolith structure notably changes (Kalish *et al.*, 1995). Specifically, movement from the pelagic to the demersal habitat in cod was observed as a zone of distinct changes in the otolith daily increment patterns from wide to narrow increments (Steffensen, 1980; Hüssy *et al.*, 2003), and this increment pattern change has been associated with settlement of pelagic Baltic cod juveniles to demersal habitats (Hüssy *et al.*, 2003). In light microscopy (LM), the closely spaced daily increments may be seen as a dark ring on the otoliths, and these settlement checks are not unique to Baltic cod, but have also been identified in other gadiform species such as white hake, *Urophycis tenuis* (Lang *et al.*, 1996), and other fish taxa such as tropical reef fish (Wilson and McCormick, 1997) or rockfish (Laidig, 2010). In combination with validated daily increments, these settlement checks can be used to estimate the duration of the pelagic larval and early juvenile phase and to determine the timing of settlement to demersal habitats.

For the estimation of the annual age of fish it is necessary to evaluate the accuracy of ageing. Irrespective of the method, prior to using either macrostructural analysis to determine annual age or microstructural analysis to reveal daily age, a proper validation of the periodicity of the growth zone has to be conducted. Different methods are available for this validation, and some of them have been applied successfully for Atlantic cod. For example, daily increment deposition has been validated for Atlantic cod larvae, with daily increment formation starting on the day of hatch and rings being deposited daily thereafter (Clemmesen and Doan, 1996). For juveniles, daily increment deposition was validated in laboratory-reared juvenile Atlantic cod by assessing the number of daily increments deposited within a known time span (Hüssy *et al.*, 2003). Since microstructural increments are deposited with a daily frequency in this species, determining the exact age in days can therefore be used to validate macrostructural age estimates (Waldron and Kerstan, 2001). In addition, determining the exact daily age of individuals may also allow discerning between possible 'false' and 'true' annuli (Waldron and Kerstan, 2001).

Accurate and precise age determination is of critical importance for proper management of fish stocks, because age forms the basis for many population demographics, such as growth rates, mortality rates, and year-class strength (Campana, 2001; Reeves, 2003). The recognition that fish age can be estimated through counting the annuli, i.e. the periodic deposition of one opaque and one translucent zone (Kalish *et al.*, 1995), on the otoliths has resulted in a worldwide increasing use of ageing routines (Campana and Thorrold, 2001); the opaque zone is generally associated with a period of high growth rate, while the translucent zone is viewed as the 'winter' ring, i.e. a zone of reduced growth rate. The formation of these zones is still not clearly understood and has been attributed to different external factors, such as water temperature (Pannella, 1980; Campana and Hurley, 1989; Otterlei *et al.*, 2002) and food ration (Campana and Hurley, 1989), as well as the developmental

state of the fish (Otterlei *et al.*, 2002) and endogenous rhythms (IBACS, 2006). With the increasing use of otoliths for ageing purposes, it also became apparent that in many fish species, ageing precision (i.e. repeatability) and/or accuracy (i.e. closeness between measurement and true value) may be limited, leading to large inconsistencies in age determination (e.g. Waldron and Kerstan, 2001; de Pontual *et al.*, 2006; La Mesa, 2007; Hüssy, 2010). For Baltic cod, this problem is particularly pronounced due to the appearance of apparently 'irregular annuli' at young ages and has led to the establishment of the Study Group for Aging of Baltic Cod (SGABC) by the ICES in 1996 (DECODE, 2006) to define and ultimately resolve the age reading problem. To date, it is apparent that problems with precision and accuracy still exist and that the definition, location, and quantity of ring structures may vary not only between different ageing institutes, but also between single age readers (ICES, 2006). One reason for these inconsistencies appears to be the identification of the first annulus in Baltic cod, which is often hampered by the occurrence of additional translucent zones on the otolith that are erroneously identified as annuli. These false annuli may arise from the physiological strain experienced by juvenile Baltic cod during settlement (Steffensen, 1980; DECODE, 2006). Consequently, any attempts to resolve ageing problems in Baltic cod must also include a better understanding of size and age of these pelagic 0-group juvenile Baltic cod at the onset of the formation of the first translucent zone during the time of settlement from a pelagic habitat to a benthic and nearshore habitat utilization (Hüssy *et al.*, 2003).

In this study, microstructural scanning electron microscopy (SEM) observations of daily increments were used to differentiate between true annuli and secondary translucent zones (e.g. settlement checks) detected with LM. In contrast to LM, SEM can provide a higher accuracy of daily increment counts because it achieves a higher resolution, thus revealing even narrow daily increments that might be undetectable with LM alone (Jones and Brothers, 1987). The goal of this study was to examine the timing of the formation of otolith marks in western Baltic cod to validate macrostructural ageing and thus reliably identify the first annulus. Specifically, the tasks for this study were to (i) determine the number of translucent zones displayed on juvenile Baltic cod otoliths using LM; (ii) determine the number of daily increments and estimate juvenile age using SEM; (iii) back-calculate the dates of onset of hatching and translucent ring formation; and (iv) back-calculate the range of length-at-age of juvenile Baltic cod at the onset of the formation of translucent zones.

Material and methods
Field sampling

Samples for this study were collected near the main spawning area of the western Baltic cod stock at different stations in the nearshore waters of Fehmarn Island in the western Baltic Sea, Subdivision 22 (Figure 1). All juvenile Baltic cod were sampled by commercial fishers using traditional coastal eel traps (poundnets) at a maximum depth of 3–4 m. In 2008, a total of 121 juvenile Baltic cod were collected between October 11 and December 11, and in 2009 a total of 136 fish were collected between November 19 and December 8. In 2008, fish were frozen immediately after capture for future analysis, while in 2009 some fish were kept in tanks for a maximum of 19 days at 10°C under a restricted
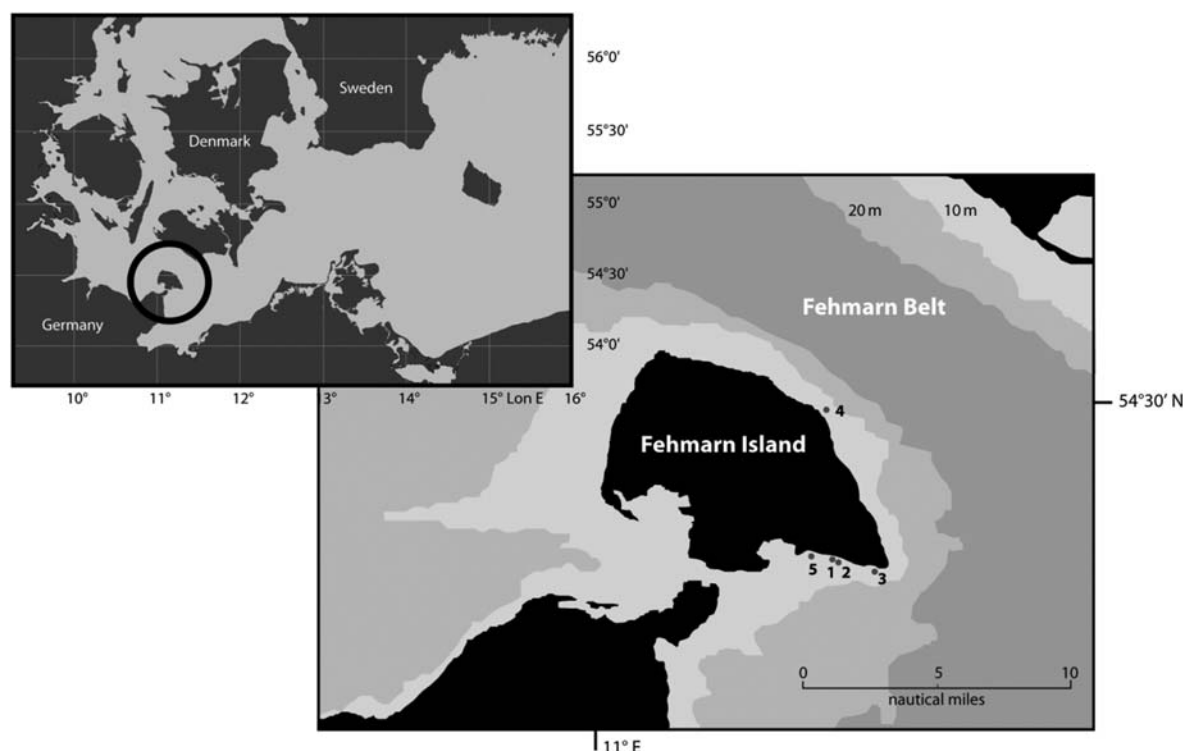
Figure 1. Map of Fehmarn Island, western Baltic Sea, indicating poundnet sampling stations (1–5) for juvenile Baltic cod in 2008 and 2009. Grey shading indicates the 20 m and 10 m bathymetry isoline.

feeding regime in a 12 h light/12 h dark cycle before being frozen for subsequent otolith analysis.

Otolith preparation

In the laboratory, each fish was thawed, the total length was measured to the nearest 0.1 cm, the total wet weight was measured to the nearest 0.1 g, and both sagittal otoliths (hereafter otoliths) were excised. For this study, both otoliths were used.

For LM, otoliths were embedded in polyester-resin (GTS® polyester-resin + MEKP-hardener) with the sulcus facing down and were cut under water with two parallel sawing blades (ATM Brilliant® 250) into 0.5 mm sections encompassing the otolith core. Under a binocular stereomicroscope, an image was taken of each otolith section with a high-definition camera (Leica® DC300, 3132 × 2328 pixels). Otolith sections were removed from the resin and fixed on a microscope slide with thermoplastic glue. Both sides were ground with abrasive paper with decreasing grain size using a lapping wheel (Presi Mecapol® P260) and polished with 3 μm paper (266× Imperial PSA, 3M®). Analysis of sections was conducted with ImagePro (Version 6.0) at magnifications of ×40 and ×400. ImagePro was used to combine single views of different focal planes into one picture, which was later used for comparison with SEM images.

For SEM, the middle section of the otolith was cut out and mounted with thermoplastic glue on an SEM stub with the transverse plane facing up. Each otolith was ground with abrasive paper of decreasing grain size and polished with 0.3 aluminium oxide polishing powder to reveal the internal structure. All otoliths were rinsed in water and dried in air before etching. Otoliths were etched for 6–7 min using EDTA (ethylenediaminetetraacetic acid; Karakiri, 1990); a subsample of 28 otoliths from 2009 was instead etched with 5% acetic acid solution for 2–3 min (Karakiri, 1990). Before examination with an LEO 1515® scanning electron microscope, all samples were gold-sputter coated under vacuum. For each otolith, one overview and several detailed images were taken and analysed with ImagePro (Version 6.0). For each overview image, two axes were drawn and measured to the nearest 0.01 μm, from the nucleus to the dorsal edge and from the nucleus to the ventral edge of the otolith.

Age and length estimates

Annual ages of Baltic cod were estimated from translucent annuli counts with LM. For both years of the study, a comparative age reading exercise was conducted on the same set of otolith images by expert age readers of the Johann Heinrich von Thünen-Institute at the Institute of Baltic Sea Fisheries in Rostock and at the Institute of Sea Fisheries in Hamburg.

Daily age was estimated by counting the number of increments for each 100 μm section of the SEM images. In addition, daily increment counts were used to estimate hatch date and age, and date at the onset and end of unstructured zone formation. In this study, unstructured zones were defined as those regions on the otoliths that contained no discernible daily increments as seen by SEM. Correspondence between translucent LM zones and the occurrence of unstructured SEM zones was examined. A close spatial match between these sections demonstrated that they were indeed the same structures and thus for the remainder of this study were treated as translucent zones. To validate annual age of Baltic cod, daily increment estimates were compared with the number of annuli. If increments in a given 100 μm section were

unreadable, the number of increments for that section was extrapolated by using the mean number of daily increments from the two neighbouring sections (Waldron and Kerstan, 2001). Daily age was estimated by summing up all counted and extrapolated increments of one axis and calculating the mean of both axes. If >33% of the sections of one axis were unreadable or damaged, daily age estimates were based on increment counts of the remaining axis only. Daily increment counts from the nucleus to the start of an unstructured (i.e. translucent) zone were recorded, the distance was measured to the nearest 0.1 μm, daily increment counts from the nucleus to the end of the unstructured zone were extrapolated as described above, and the width of the unstructured zones was measured.

Length-at-age was estimated to determine the length distribution of Baltic cod at the start of translucent zone formation using the following linear regression model, with y representing length-at-age (cm) and x representing age expressed as the number of daily rings, NDR (Fey and Linkowski, 2006):

$$y = 0.734 \times NDR - 23.732; \; r^2 = 0.908$$

Data analysis

Statistical analyses were carried out with the statistical package R (R Development Core Team, 2009). Differences in the annual age estimates between readers from different institutes were tested using a Wilcoxon signed-rank test, a non-parametric test based on paired observations. A comparison of the estimated annual age distribution obtained by the independent readers was tested using a one-sided Wilcoxon signed-ranks test. For all statistical tests, a p-value <0.05 was considered statistically significant.

Results

General catch information

In 2008, 121 juvenile Baltic cod were caught and processed for macrostructural age determination. In addition, a subsample of ten fish was used for microstructural analysis in a first exploratory comparative study. Captured and retained fish ranged in size from 12.5 to 32.5 mm total length ($TL_{mean} = 18.4$ mm) and from 17.7 to 294.5 g total wet weight ($TW_{mean} = 64.5$). In 2009, a full-scaled comparative analysis of otolith macro- and microstructure was conducted with a total of 136 juvenile Baltic cod ranging in size from 14.1 to 27.1 mm total length ($TL_{mean} = 18.6$ mm) and from 25.9 to 186.4 g total weight ($TW_{mean} = 60.4$ g). It should be noted that samples for this study were not taken randomly; instead, care was taken to select only juvenile Baltic cod of an approximate size <30 cm total length to include all potential 0-group fish.

Macrostructural ageing

Annual ages assigned to juvenile Baltic cod by expert age readers ranged from 0 to 2 years for 2008 and from 0 to 1 year for 2009. Age estimates for the 2008 samples differed significantly between both readers (two-sided Wilcoxon signed-rank test, $p < 0.05$); specifically, reader 1 assigned age-0 to 8.3%, age-1 to 90.9%, and age-2 to 0.8% of the 2008 samples, while reader 2 identified 8.3% of the sample as age-0 and 90.9% as age-1. Thus, age estimates by reader 1 were significantly higher than those by reader 2 (one-sided Wilcoxon signed-rank test, $p < 0.05$). In contrast,

for 2009 samples, both readers identified 74.3% of the otoliths as age-0 and the remaining 25.7% as age-1.
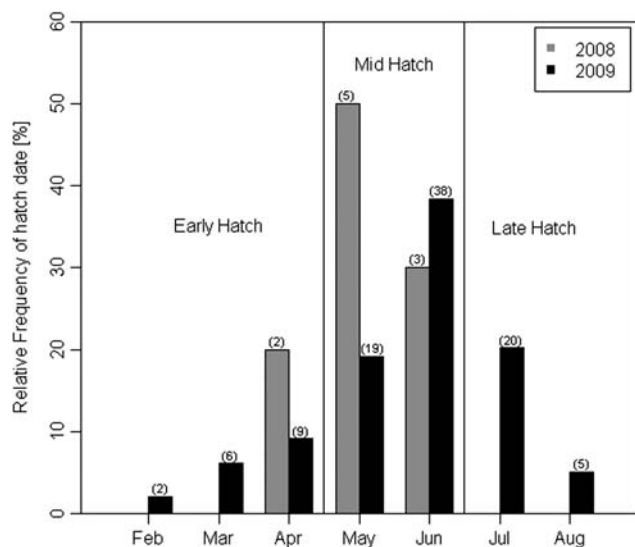
Microstructural ageing

SEM analysis of daily increments was conducted for a subsample of otoliths in 2008 ($n = 10$) and for all readable otoliths in 2009 ($n = 99$); some otoliths had to be excluded from this analysis because of an unreadable crystalline appearance or damage sustained during storage and processing. Sampled juvenile Baltic cod otoliths had a mean number of daily increments of 191 ($\pm$ 22) and 168 ($\pm$ 40) in 2008 and 2009, respectively (Table 1). Thus, daily age estimates showed all individuals examined in this study to be age-0.

Back-calculation

Hatch dates, reported as day of the year (DOY), for juvenile Baltic cod were calculated using the number of daily increments and the date of capture (Figure 2). Hatching ranged from April until June with a mean hatch date of 13 May (DOY = 132 $\pm$ 22) in 2008 and from February until August with a mean hatch date of 7 June (DOY = 158 $\pm$ 37) in 2009. For further analyses, otolith samples were grouped into three hatch groups based on back-calculated hatch dates, namely early hatch from February to April, mid hatch from May to June, and late hatch from July to August

Table 1. Summary of microstructural otolith analysis of juvenile Baltic cod from 2008 and 2009, with n indicating the sample size and NDR the mean ($\pm$ s.d.), minimum (min), and maximum (max) number of daily rings.

Year	n	NDR_{mean} (s.d.)	NDR_{min}	NDR_{max}
2008	10	191 (22)	167	225
2009	99	168 (40)	94	310

Figure 2. Monthly relative frequency distribution of hatch dates of juvenile Baltic cod in 2008 and 2009 back-calculated from the date of capture using microstructural otolith analysis. Numbers in parentheses indicate the sample distribution per month with an overall sample size of $n = 10$ and $n = 99$ in 2008 and 2009, respectively.

(Figure 2). For 2008, the hatch distribution was 20% for early hatch, 80.0% ($n = 8$) for mid hatch, and 0% for late hatch. In contrast, in 2009, 17% ($n = 17$) of the analysed cod otoliths belonged to the early hatch group, 58% ($n = 57$) to the mid hatch group, and the remaining 25% ($n = 25$) to the late hatch group, respectively.

A comparison of SEM and LM otolith images demonstrated that the location of unstructured SEM zones coincided with that of translucent LM rings (Figure 3). Consequently, for subsequent analyses, unstructured zones of SEM images were regarded as translucent rings and the dates of their formation were back-calculated (Figure 4). Juvenile Baltic cod otoliths displayed one or two translucent zones. Stratification into hatch groups indicates that the number of translucent zones may be a function of hatch date. In 2008, the subsample of ten analysed cod otoliths were either early hatch or mid hatch, and displayed only one translucent zone (Figure 4). In 2009, the majority of the late hatch group (92%; $n = 23$) and of the mid hatch group (91.2%; $n = 57$) also exhibited only one translucent zone. Among the early hatch fish, however, the majority (76.5%; $n = 13$) showed two translucent zones.

The mean duration of the first translucent ring was 42 days in 2008 and 36 days in 2009, while the mean deposition period of the second translucent ring was 32 days in 2009. The time of the formation of the first translucent ring in 2008 ranged from 6 September to 10 October and from 2 September to 4 October for cod from the early hatch and mid hatch groups, respectively. In 2009, deposition of the first translucent ring occurred from 16 July to 12 August, from 11 September to 6 October, and

Figure 3. Microphotographs of the left and right otolith of the same Baltic cod individual as viewed with SEM (a) and LM (b). The white bar indicates the location of the corresponding translucent zone on both images.

from 28 September to 21 October for the early, mid, and late hatch groups, respectively. The deposition period of the second translucent zone was from 12 October to 8 November for early hatch, from 21 October to 5 November for mid hatch, and from 17 October to 3 November for late hatch, respectively.

The total length of juvenile Baltic cod at the start and end of the translucent ring formation was estimated using a linear regression of length as a function of daily age (Fey and Linkowski 2006). Considering both years of this study, minimum estimated length of juvenile Baltic cod at the start of formation of the first ring was 5.0 cm total length, and first ring formation was completed at 8.8 cm total length (Table 2). Formation of the second translucent ring of the late hatch group occurred at a total length of 5.4–6.7 cm and therefore overlaps with the size ranges of the formation of the first ring. In contrast, total lengths at formation of the second translucent ring of juvenile Baltic cod are considerably greater, with a range of 12.5–14.7 cm and 9.2–10.2 cm for the early and mid hatch group, respectively (Table 2). Both age and total length at ring formation declined with hatch date.

## Discussion
Comparative ageing
Comparative age reading revealed large inconsistencies in the annual ages assigned to juvenile Baltic cod by two independent age readers. The main problem associated with the annual ageing, however, was not the lack of visibility of translucent rings, but rather the interpretation of these otolith structures as true or false rings and therefore the correct identification of the first annulus. For the 2008 samples, significant differences in ageing were primarily caused by one reader counting every translucent ring as an annulus, while the other reader chose to ignore some of the translucent ring structures, based on his/her conviction of the existence of false rings. For the same reasons, inconsistent age readings were previously observed by the SGABC, which identified two different ageing protocols, the western and eastern age reading school (ICES, 2004). For the 2009 samples, differences in ageing results between readers were less pronounced, primarily because both expert age readers followed the same approach to ageing. However, this comparative ageing exercise clearly demonstrated the lack of precision and the high degree of subjectivity currently associated with the ageing of Baltic cod, differing between countries, which so far have not been resolved, and emphasized the need for age validation in Baltic cod and research into the nature of checks on the otoliths and the sequence of their formation.

Age validation
Age validation, i.e. the confirmation of the accuracy of an ageing method, is a necessary requirement to determine the absolute age of fish. Among other methods, the deposition of daily increments is used to confirm the formation of the first annulus and thus validate ageing (Quiñonez-Velázquez, 1999; Waldron and Kerstan, 2001; La Mesa, 2007). For Atlantic cod larvae and juveniles, daily increment deposition had previously been validated (Clemmesen and Doan, 1996; Hüssy *et al.*, 2003), thus allowing the use of daily increment counts as a means of annual age validation for this species. Consequently, daily increment counts were used in this study to validate annual ages assigned to juvenile Baltic cod. In contrast to the annual ageing results, all juvenile Baltic cod examined as part of this study were age-0 fish. In
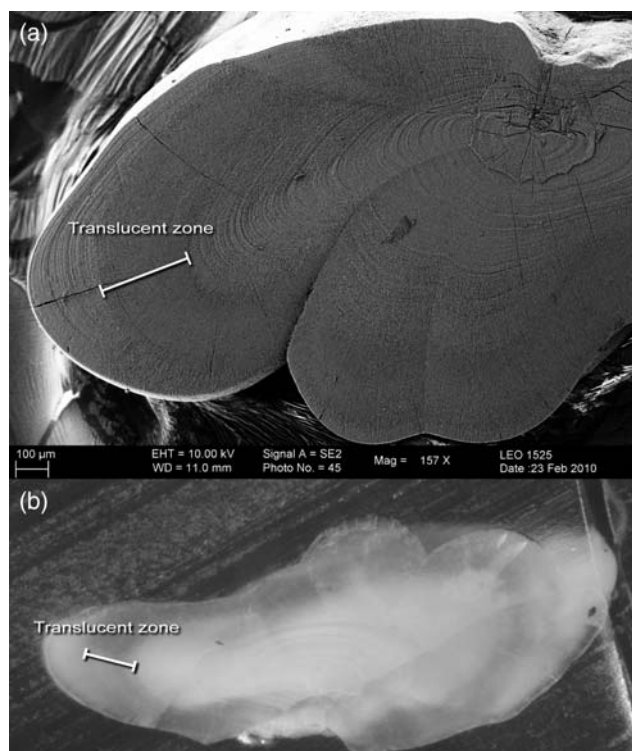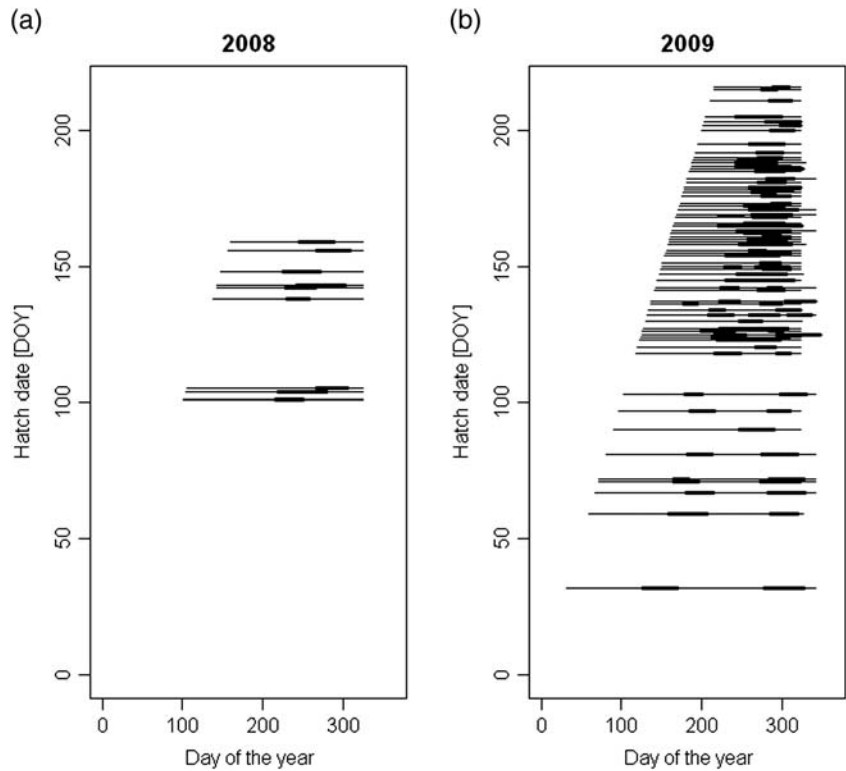
Figure 4. Summary of the timing of life history events for all juvenile Baltic cod samples from 2008 (a) and 2009 (b) examined for daily increments and zone formation. Each horizontal line represents the life line of a single Baltic cod juvenile, starting with the back-calculated hatch date and ending with the catch date. Bold lines mark the period of translucent ring formation.

Table 2. Age, presented in number of daily rings (NDR), and total length at formation of translucent rings for juvenile Baltic cod from two study years (2008, 2009) and three hatch groups (early, mid, and late).

Year	Hatch group	n	Age (NDR) at formation of first ring; [start–end (s.d.$_{start}$–s.d.$_{end}$)]	Total length range (cm) at formation of first ring (start–end)	Age (NDR) at formation of second ring [start–end (s.d.$_{start}$–s.d.$_{end}$)]	Total length range (cm) at formation of second ring (start–end)
2008	All	10	104–146 (24.4–28.1)	5.9–8.8	–	–
	Early	2	146–180 (46.3–47.4)	8.8–11.2	–	–
	Mid	8	97–129 (10.7–15.6)	5.4–7.6	–	–
2009	All	99	91–127 (21.5–24.7)	5.0–7.5	168–202 (40.1–47.7)	10.4–12.8
	Early	17	107–134 (24.1–27.8)	6.1–8.0	198–225 (24.4–29.4)	12.5–14.7
	Mid	57	98–123 (19.6–21.1)	5.5–7.2	151–166 (22.9–24.6)	9.2–10.2
	Late	25	73–96 (15.2–16.7)	3.7 – 5.3	97–115 (25.5–16.3)	5.4–6.7

Total lengths at the start and end of ring formation were calculated from linear regression models (Fey and Linkowski, 2006). n = sample size; s.d. = standard deviation.

contrast, results from the comparative macrostructural ageing were very inconsistent and led to overestimation of age. While underestimating the age of fish is known, particularly from those species that attain very high ages (e.g. Campana *et al.*, 1990), over-estimating ages is often the result of difficulties in distinguishing between true and false annuli in juvenile fish (de Pontual *et al.*, 2006). Overestimating the age of fish can have far-reaching consequences for management, because age is routinely used for age–length keys (ALKs) that are constructed annually for the assessment of fish stocks. Incorrect ALKs will subsequently intro-duce errors into estimates of other input data, such as catch-at-age, maturity-at-age, and any age-structured catch per unit of effort indices (Reeves, 2003). For example, in European hake (*Merluccius merluccius*) from the Bay of Biscay, an overestimate of age and subsequent underestimate of growth rates was identified

through a tagging study and otolith microstructure analysis (de Pontual *et al.*, 2006). A subsequently conducted simulation study demonstrated that faster growth rates in hake may result in substantially lower stock biomass and higher fishing mortality (Bertignac and de Pontual, 2007). This clearly demonstrates the need for age validation to ensure accurate age data for the assess-ment of managed fish stocks.

At the date of capture (late October–mid December), age-0 ju-venile cod exhibited a mean total size of ∼18.5 cm. With back-calculated hatch dates ranging from March to August, captured juveniles had a maximum growth period of 7 months. In a study on Atlantic cod, Bolz and Lough (1988) concluded that an average Atlantic cod hatched on 15 March could be expected to reach a length of 26.1 cm by 1 January, and in a follow-up study Atlantic cod <32 cm were considered 0-group fish (Lough *et al.*,

2006). Considering that growth tends to be reduced in winter, a mean length of 26.1 cm is well above mean lengths observed for fish in this study, thus supporting the concluded age-0 for fish examined here. Similarly, juvenile Baltic cod reaching a maximum length of 30 cm were classified as age-0 fish (Bingel, 1972; Berner and Borrmann, 1985).

Hatch date distribution

Back-calculation of hatch dates of juvenile Baltic cod indicated a hatching period ranging from April until June in 2008 and from February until August in 2009. This suggests that juvenile Baltic cod examined as part of this study may have originated from different spawning groups. Specifically, western Baltic cod are known as spring spawners with a spawning period from January to May, while eastern Baltic cod are viewed as summer spawners with a spawning period from May until August, with the highest egg abundances in July and August (Bagge *et al.*, 1994). Assuming an incubation duration of 12–23 days at temperatures ranging between 3.4 and 8.7°C (Thomson and Riley, 1981; Kühn, 1998), spawning events that produced juvenile Baltic cod examined in this study may have occurred between January and August. This may either be the result of mixing of eastern and western Baltic cod stocks or may indicate a more protracted spawning season for western Baltic cod than previously assumed. Mixing of eastern and western Baltic cod during the juvenile stage may be the result of wind-induced surface transport and thermohaline mixing that lead to dispersal of pelagic eggs and larvae (Hinrichsen *et al.*, 2001) and of directed juvenile migration towards favourable settling sites. However, while an expansion of western Baltic cod juveniles into eastern Baltic areas was demonstrated based on length–frequency distributions (Oeberst, 2000), a similar expansion of eastern Baltic cod juveniles, as suggested by hatch data from this study, has not been shown to date.

Otolith chemistry and its effects on etching

The chemical composition of fish otoliths and therefore the balance of organic and inorganic compounds varies throughout the otolith, with translucent zones consisting mainly of inorganic calcium carbonate, i.e. aragonite and calcite (Degens *et al.*, 1969), whereas opaque zones contain comparatively more organic protein, which is dominated by the collagen-like protein otolin-1 (Murayama *et al.*, 2002). Differences in transparency of otolith structures viewed by LM can therefore be explained by the enhanced optical density of organic components. Similarly, daily growth increments are also the result of two chemically differing zones (bipartite structure), with the broader incremental, translucent zone consisting particularly of aragonite (Mugiya *et al.*, 1981) and the narrower discontinuous, opaque zone containing more otolin (Degens *et al.*, 1969). Because of differences in the chemical composition, translucent and opaque zones have differential response timing to etching with weak acids, such as EDTA used here, which remove protein material at a higher rate than calcified material (Mugiya *et al.*, 1981). Since translucent zones, which consist mostly of inorganic material, are insufficiently affected by EDTA solution, daily increments may not be revealed. Translucent zones may thus appear 'unstructured' when viewed by SEM, supporting our observation that translucent LM zones were congruent with unstructured SEM zones.

Timing and size of translucent zone formation

In this study, dates of commencement of formation of the first translucent zone occurred progressively later with later hatch dates. Since the onset of the formation of the first translucent ring across all individuals ranged over a period of 4 months, formation of the first ring was independent of season, but instead appeared to be dependent on fish age and/or size. The narrow size and age range suggests that rather than representing an annulus, the first translucent zone might represent a settlement check and thus be the result of the physiologically stressful transition of pelagic juveniles to a demersal lifestyle. This assumption is supported by similar size and age ranges reported in other studies on cod. For example, Atlantic cod on Georges Bank begin a gradual settlement process of 1–1.5 months at an approximate age of 3 months and a total length of 4.0–6.0 cm (Bolz and Lough, 1988) and, similarly, in Nova Scotia, Atlantic cod settled at 3.5–6.0 cm (Tupper and Boutilier, 1995). In the Norwegian Skagerrak area, Atlantic cod also displayed a very narrow check prior to the annulus that was formed in fish of ~4.8 cm length and was interpreted as a settlement check (Gjøsæter and Danielssen, 2011). Similarly, Atlantic cod at Trinity Bay in Newfoundland also had a mostly pelagic diet up to a size of 6.0 cm, after which they rapidly switched to benthic prey, suggesting a settlement to a demersal lifestyle at this size (Lomond *et al.*, 1998). It was suggested that the rapid change in diet might be determined by fish settling out when they reach a sufficiently large gape size to ingest the relatively larger benthic prey species (Lomond *et al.*, 1998). In the Baltic Sea, a similar settlement size range of 4.0–6.0 cm total length (Hüssy *et al.*, 1997; Nielsen *et al.*, 1997; Oeberst and Böttcher, 1998) was observed for eastern Baltic cod, and this size range corresponded well to the size of increment pattern changes observed on the otoliths of demersal juvenile Baltic cod (Hüssy *et al.*, 2003) and in this study. Finally, the diet composition of Baltic cod from the Arkona and Bornholm basins revealed predominantly benthic prey at an age of 145 days (Oeberst and Böttcher, 1998); considering a gradual transition to a demersal life stage of ~1 month, an observation of mostly benthic prey thus indicates the end of this transition period and is therefore in good agreement with ages at onset of transition as determined here. Thus, the lack of a seasonal pattern combined with a narrow size and/or age at onset of translucent zone formation strongly suggest that this first translucent zone on the otoliths of juvenile Baltic cod represents a settlement check.

Settlement of Baltic cod in the waters surrounding Fehmarn Island is a protracted process, ranging from June until October, thus forcing freshly settled juvenile cod to experience a vastly differing physical and biological rearing habitat. Also, since fish settled at approximately the same age and/or size, it has to be concluded that juveniles originated from different spawning populations. In Atlantic cod from Nova Scotia, freshly settled juveniles were shown to be site-attached and to defend their territories (Tupper and Boutilier, 1995). Those fish that settled earliest or at larger sizes held larger territories than juveniles arriving later in the season, and these larger fish maintained their size advantage throughout the study (Tupper and Boutilier, 1995). Similarly, early spawned haddock (*Melanogrammus aeglefinus*) had a higher probability of surviving to the autumn than later spawned individuals (Lapolla and Buckley, 2005). If these results are transferable to the Baltic Sea, they suggest that earlier

settlement of cod in the waters of Fehmarn Island might confer a competitive advantage to certain hatch groups, possibly even resulting in differential survival probabilities.

Commencement of the deposition of the second translucent ring occurred during a very narrow period of ~1 month, ranging from mid October to early November, suggesting a seasonally influenced deposition of the second translucent ring. This was supported by the fact that fish from all three hatch groups deposited this second ring at the same time of the year, but at different ages and sizes. Thus, the second translucent ring may represent the first annulus of juvenile Baltic cod. It should be noted, though, that our results indicate that the start of deposition of this first annulus occurred in early winter (October–November). While these results are not very different from those of some studies that also noted the formation of a translucent zone in late summer/autumn to early winter for Atlantic cod from the Skagerrak (Gjøsæter and Danielssen, 2011) and for North Sea cod (Pilling *et al.*, 2007), other studies predicted the formation to occur later in the season (i.e. January–March; CODYSSEY, 2007). However, previous studies indicated that the inception of the deposition of the first annulus in juvenile Baltic cod might occur earlier in the season than the deposition of subsequent annuli (Bingel, 1972). Also, the duration of the second translucent ring ranged between 12 and 51 days and was therefore much shorter than expected for a true annulus. On the one hand, the premature cessation of ring formation might be partially explained by the fact that of the 99 fish examined in 2009, a total of 21 fish were held and fed in captivity for 2–19 days before processing them for subsequent otolith analysis. On the other hand, it was suggested that the formation of a translucent zone might not only be the result of metabolic stress due to reduced temperature and food, but might also be due to the combined demands of reproduction, growth, and migration (Pilling *et al.*, 2007); consequently, 0-group Baltic cod, which are sexually immature and do not undergo spawning migrations, might display a comparatively reduced translucent zone formation.

Schematic model of translucent zone formation

Based on SEM analyses in connection with LM age reading, it was demonstrated that age-0 Baltic cod form either one or two translucent rings during the first year of their life. The main reason for variability in the number and timing of formation of otolith zones appears to be the extended spawning period of cod in the Baltic Sea (Bagge *et al.*, 1994). Specifically, it was shown that the first translucent ring deposited ~3 months post-hatch on the otoliths of age-0 Baltic cod was formed in response to the gradual settlement process of juveniles from a pelagic to a demersal life. A second translucent ring that started to form in mid October to early November may represent the true first annulus of Baltic cod as this ring was deposited independently of size or age of the fish. These two rings are clearly distinguishable in most individuals of the early and mid hatch groups. In later hatched fish, a clear distinction between these two otolith marks disappears due to overlapping timing of a non-periodic and a periodic structure (Figure 5).

Conclusions

In summary, the results of this study clearly indicate the deposition of two translucent zones on the otoliths of age-0 Baltic cod. The first ring is formed in response to the settlement
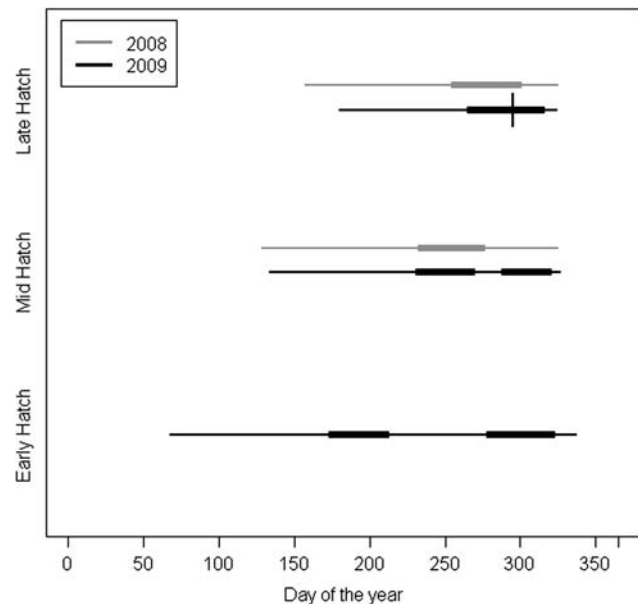
Figure 5. Schematic model of lifespan lines combined for three hatch groups in two years of the study, 2008 and 2009, and the timing of translucent ring formation. Bold lines mark the period of translucent ring formation.

process, while the second ring was a seasonally induced check, which may represent the first annulus.

Because of the protracted spawning season of Baltic cod, however, a distinction between the two otolith structures is no longer possible when settlement occurs at a time similar to the formation of the second ring, thus resulting in only one distinctly identifiable translucent zone. Consequently, it is this variability in ring deposition in Baltic cod that leads to the large degree of uncertainty in ageing these fish. Future research is clearly needed to examine further the sequence of ring formation on the otoliths of age-0 western Baltic cod to identify seasonal patterns of commencement and cessation of the formation of the first annulus. In addition, this study demonstrated that settlement of Baltic cod was independent of season but instead was a function of age and/or size of the fish, thus resulting in a protracted settling period in the waters of Fehmarn Island. This observation raises intriguing questions that warrant further investigation regarding the survival probability of individuals from different hatch groups and stocks of Baltic cod in the western Baltic Sea.

Acknowledgements

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