Otolith growth and zone formation during first maturity and spawning of Atlantic cod (Gadus morhua)

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Otolith growth and zone formation during first maturity and spawning of

Atlantic cod (*Gadus morhua*)

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Abstract

Specific impacts of somatic growth, sexual maturation and spawning events on otolith zone formation in Atlantic cod (*Gadus morhua*) were assessed in a 33-mo tank experiment, using Barents Sea cod and Norwegian coastal cod. High and low feeding ration combinations were used to mimic environmental stressors in the field. For both stocks apparent macrostructural “spawning zones” in otoliths are registered in statutory stock monitoring programs to estimate age at maturity, thus adding key information to stock biomass assessments. We found that substantial energy investments in reproduction caused reductions in otolith growth and altered proportional width between translucent and opaque zones. These effects, however, were only significant among individuals with high reproductive investments, while otoliths from individuals with low investments did not differ from the ones for immatures. Reproduction may thus not necessarily induce spawning zones, and alternatively, “spawning zones” may not necessarily reflect reproduction. Altogether, this suggests that the individual energy level, as a premise for metabolic activity,
plays a key role in the formation of such zones, and thus is related to environmental conditions.

KEY WORDS: Otoliths · spawning zone · sexual maturation · Atlantic cod

Introduction

Biomineralization of fish otoliths involves rhythmic variations in the crystallization of calcium carbonate (CaCO$_3$) and the incorporation of organic matrix fibres (mainly proteins) from ionic and organic components in the endolymph (Allemand et al. 2007, Morales-Nin 2000, Wright et al. 2002). Physiological and environmental factors influence this process, both in respect to the incorporation of protein fibres causing the otolith to become opaque, and the overall accretion rate (growth rate). As a result, otoliths in many fish species have annual alternations between opaque and translucent zones (referred to as annuli), which are often used for age determination (Campana 2001, Fablet et al. 2011). Life history events may, however, induce irregular checks (i.e. a discontinuity in an otolith zone) or shifts in the periodicity of the annuli (Geffen et al. 2002).

Rollefsen (1933) was the first to associate onset of sexual maturation with transitions in the seasonal zone structure of otoliths in Atlantic cod (Gadus morhua). The outer zones differed from the inner ones (created prior to sexual maturation) by being comparably narrower, in which were referred to as “spawning zones”. The outer annuli had also a more distinct and often proportionally thicker translucent zone than the following opaque zone, compared to the annuli before sexual maturation where the opaque otolith zones dominated. This distinct change in otolith structure was seen in both Barents Sea (BS) cod (often referred to as Northeast Artic cod) and Norwegian coastal (NC) cod, while distinct stock differences in the innermost zone structure were also detected (Rollefsen 1933).
Rollefsen (1933) argued that only a major change in the life history, represented by sexual maturation and spawning, could cause such a noticeable transition in the otolith pattern. Otolith spawning zones became central in the further investigations on stock structure of the BS and NC cod stocks (Jørgensen 1990, Rollefsen 1934, 1935, 1953), and have been used to determine maturity ogives as essential information for stock assessments and management (ICES 2001, Yaragina et al. 2009, Zuykova et al. 2009).

Transitions in otolith macrostructure have been reported to correspond to first maturity also in other teleost species (Francis and Horn 1997, Pulliainen and Korhonen 1994, Reglero and Mosegaard 2006, Rijnsdorp and Storbeck 1995). However, there is a clear lack in knowledge about the precise relationship between otolith formation and reproduction, and as such highlighting the need for dedicated validation experiments. Reproduction has been hypothesized to affect the otolith mineralization through both endogenous physiological processes and changes in the physical environment, either directly, or indirectly due to behavioural actions (e.g. spawning migrations and reduced feeding). Prior to sexual maturation, energy is allocated to body compartments (muscle, liver and/or viscera) as storage sites or to growth in body size, while at sexual maturation a significant part of this energy needs to be allocated to gonad development and other reproductive costs (Dabrowski 1985, Jobling 1982, Kjesbu et al. 1991). These changed routes of energy flow are likely to affect otolith formation, as the metabolic rate is closely linked to otolith accretion rate and optical density (Høie et al. 2008, Mosegaard et al. 1988).

A more direct linkage between reproduction and otolith mineralization is indicated from studies on the relationships between gonad development (including spawning), composition of blood plasma and endolymph, and otolith chemical composition and morphometric structure (Kalish 1989, 1991, Woodhead 1968). Woodhead (1968) showed...
that seasonal changes in the concentration of plasma total calcium corresponded to the maturation cycle in Atlantic cod. This was further hypothesized to affect the calcification rate and thus the annual increments in bones and otoliths. Later studies on bearded rock cod (*Pseudophycis barbatus*), indicated that levels of calcium in the endolymph markedly declined with increasing gonadosomatic index (gonad over body weight; GSI), which again was linked to the higher production of calcium-binding proteins, in particular “yolk” (vitellogenin) (Kalish 1991). Although this may suggest a “hypomineralization” of otoliths during maturation of female fish, the comparable narrow translucent spawning zones found in both male and female Atlantic cod (e.g. Woodhead 1968) indicate that the formation of spawning zones were not influenced by gender. Additionally, as reproductive investment is well known to vary with environmental conditions, the role of external modulators (stressor) such as prey abundance and encountered temperatures, may also be important to evaluate.

In Atlantic cod, gonad growth typically starts in autumn (near autumnal equinox) and lasts for several months until spawning commences in spring (Kjesbu et al. 2010). This more or less coincides with the translucent zone formation in otoliths, although latitudinal and population specific differences in this timing has been documented (Høie et al. 2009). In addition to the energetic costs of gonad development, Rollefsen (1933) pointed out several other circumstantial factors associated with reproduction that could contribute to spawning zone formation, including spawning migration and occupancy in new habitats. Changes in the ambient temperature is another factor that may affect the otolith growth rate (Folkvord et al. 2004) and optical density (Neat et al. 2008). In the BS cod in particular, the long counternatant migration from the feeding grounds in the Barents Sea to the spawning grounds along the Norwegian coast not only exposes the fish to different environments, but
is also highly energetically costly (Jørgensen et al. 2008, Opdal et al. 2008). Moreover, their appetite is generally reduced prior to and during spawning events (Lambert and Dutil 2000, Skjæraasen et al. 2004), although sporadic feeding at the spawning grounds have been shown (Michalsen et al. 2008). Reduced food intake may consequently reduce the metabolic rate, which is linked to otolith growth (Mosegaard et al. 1988, Wright 1991), but could also reduce the protein incorporation in otoliths resulting in more translucent deposits (Hüssy and Mosegaard 2004). However, another potential effect on cod otolith opacity that would be more directly linked to reproduction, although not yet examined, is the major depletion of proteins (and other nutrients) from white muscle and liver which is required to support gamete maturation (Kjesbu et al. 1991).

In many iteroparous teleost fish, including Atlantic cod, skipped spawning may occur as a function of limited energy reserves (Folkvord et al. 2014, Rideout and Tomkiewicz 2011) and (or) due to an adaptive strategic investment in body growth to increase lifetime reproductive output, i.e. allowing for greater fecundities in subsequent years (Jørgensen et al. 2006). However, to our knowledge, “missing” spawning zones that could indicate years without reproduction (i.e. skipped spawning) have yet not been reported, despite the fact that this phenomenon is recognized as being highly prevalent in long-migratory gadoids such as BS cod and haddock (*Melanogrammus aeglefinus*) (Skjæraasen et al. 2015, Skjæraasen et al. 2012). Altogether, this indicates that circumstances other than physiological processes directly linked to reproductive efforts in certain cases may also be responsible for otolith macrostructural transitions and the production of narrow zones. Thus, at least in theory, unfavourable feeding conditions, lowered energy reserves and extended rest periods (which often are associated with skipping) could have the potential to impact otolith growth and opacity similarly as actual sexual maturation and spawning. This
Otolith zone formation during reproduction

highlights the necessity to examine and evaluate underlying mechanisms affecting otolith zone structure in a broader sense which includes effects of key life history events. This view is further emphasized since studies on advanced modelling of otolith growth has not yet addressed reproduction specifically (e.g. Fablet et al. 2011). Consequently, a more rigorous examination is required to document the validity of the earlier assumptions made by Rolffsen (1933) that sexual maturation and circumstances associated with spawning are causing the observed otolith features referred to as spawning zones.

The present study investigates experimentally how trade-offs between reproduction and body growth influence otolith zone formation patterns. Cod were kept under controlled environmental conditions at different feeding levels over two reproductive cycles (years with potential spawning) to manipulate the onset of sexual maturation and further reproductive investments. To verify potential reproduction induced macrostructural otolith patterns, the fish were regularly exposed to a fluorescent dye that induces age- and time specific marks in the otoliths. Otolith zone formation was then compared between individuals with different spawning history, considering also age and gender as explanatory factors. Finally, we tested for spawning effects on the otolith growth – body growth and otolith growth – body size relationships. To our knowledge, this is the first dedicated study to experimentally challenge this presumed close relationship between otolith zone formation and sexual maturation in a gadoid represented by Atlantic cod.

Material and methods

Pre-experimental rearing

Atlantic cod juveniles were obtained from Institute of Marine Research (IMR) Parisvatnet Field Station, Øygarden (60°38′N, 4°49′E), located northwest of Bergen,
Norway. Broodstock that produced these juveniles consisted of eight couples of wild caught BS cod and six couples of domesticated NC cod. After spawning and subsequent hatching in March 2011, the offspring were transferred to a 7 x 7 m wide and 3.5 m deep plastic bag held in an outdoor seawater mesocosm at the same site and reared under common garden conditions with natural light cycle and temperature fluctuations, initially 6°C in March to 14 °C in June. Live natural zooplankton (Blom et al. 1994) was freely offered followed by weaning on formulated feed pellets at about 30 dph (Gemma Micro 300, Skretting, Norway). In June, all juveniles were moved into onshore tanks. On 30 November 2011, 266 juveniles were injected with AEG® Passive Integrated Transponder (PIT) tags intramuscularly. Two weeks later the fish were transported to the IMR Matre Research Station (60°87°N, 5°58°E), northeast of Bergen, where the outlined experiment was carried out. Fin clips were taken from all fish and preserved in 96% ethanol for later genetic analysis to determine stock and “family” origin. Genotyping from DNA analyses of nine microsatellites and the Pan1 locus were performed at IMR Bergen according to methodology in Glover et al. (2010).

**Experimental conditions**

The experimental design aimed at producing cod (with chemically marked otoliths) showing different feeding, growth and spawning histories during a 33-mo trial that included two reproductive cycles (with spawning seasons in spring 2013 and 2014). On 20 January 2012, at the age of 9 months, 240 juveniles in the size range 18-31 cm and 55-379 g (on average 24 cm and 157 g) were immersed in 100 mg/L Alizarin Red S (AZ) (VWR International) seawater solution for 24 hours, with aeration and oxygen supply, leaving a fluorescent mark in the otoliths reflecting experimental start (Li et al. 2008). The fish were
subsequently split into two groups of “high” and “low” feeding rate, each in 1000 L tanks. The tanks were kept indoor under artificial light with simulated natural photoperiod. Water was pumped from a depth of 90 m in the local fjord (Matrefjorden), which held a seasonally variable temperature with a mean of 8.8 ± 0.5°C (±SD) and a salinity of approximately 34 psu throughout the whole experiment. The flowrate was adjusted to approximately 30 L/min per tank. To maintain approximately the same fish densities (<30 kg/m²), each feeding group was split into two, and later three replicated tanks. However, 11 months into the trial period, this criterion required that they were all transferred and reunited in large indoor tanks (16 000 L, one tank per feeding regime), where water supply was set to 400-500 L/min per tank, but otherwise unchanged environmental conditions.

The high ration group was fed *ad libitum* four days per week, while the low feeding group was given restricted rations corresponding to 50 % of the feed consumed per biomass of the former group. Consumption in the high group was calculated by subtracting non-eaten pellets (collected in spill water) from given rations. To ensure that less competitive individuals also got their chance to eat but sticking to the overall feeding regime, the amount of food provided each time to the low feeding group was increased by limiting the number of feeding days per week to only two. The following two equations for *ad libitum* specific growth rate (SGR) were consulted to adjust feeding ratios; i) $SGR = (0.5735T^{0.5} - 0.1934 - 0.02001T)$ by Björnsson and Steinarsson (2002) and ii) $SGR = 0.216 + 0.297T - 0.000538T^3$ by Jobling (1988), where W is whole body weight and T experimental temperature (see below). The best fitted equation in a given situation was used to predict body growth in the next two months period. By including also observed food conversion ratio (FCR) in the preceding two months, feed ratios were firstly estimated for the high feeding group and then adjusted for the low feeding group. The fish were initially fed with 5...
mm formulated pellets (Amber Neptun, Skretting, Norway), and subsequently 7- and 9-mm pellets as body size got bigger.

The same staining procedure used at the start of the experiment was repeated every six months throughout the experiment. In total, six AZ marks (AZ1 – AZ6) were induced, in mid-January and mid-July from 2012 to 2014, associated with regular fish size measurements. Two 12-month periods of special interest, hereby referred to, for the sake of simplicity, as potential reproductive cycles (RC), were defined from July 2012 to June 2013 (RC1), and July 2013 to June 2014 (RC2) (Table 1). Each RC was further split into two 6-month periods: July-Dec and Jan-June, all periods in correspondence to the otolith AZ mark intervals. To aim at increasing the variability in reproductive outputs, half of all individuals from each feeding regime were transferred to the other feeding regime in July 2013, i.e. at the interphase between RC1 and RC2. This design generated four feeding histories; low-low, low-high, high-low, and high-high (Table 1). To reduce biases in otolith interpretations resulting from edge effects, sufficient otolith growth after RC2 was ensured by keeping the fish under unchanged feeding regimes for additionally three months, terminating the experiment on 17 October 2014. The experiment was approved by the local ethics committee at the IMR and was carried out in accordance with national and international guidelines for animal welfare (e.g. www.norecopa.no) at the IMR Matre Research Station. The station is authorized for animal experimentation by the Norwegian Food Safety Authority (facility 110). Atlantic cod is not considered to be an endangered species in Norway (national IUNC redlist: www.biodiversity.no).

Fish measurements and gonad sampling
Fish were measured for weight ($W$, ± 1.0 g) and total length ($TL$, ± 0.5 cm) at the start and every second month throughout the experiment (in total 18 sampling times), including at the day of termination. At each sampling point, fish were anesthetized in batches by immersing 3-5 fish at the time in seawater containing 100 mg/L MS-222 (Finquel™), with subsequent recovery in well-aerated water. Ovarian catherization (“biopsy”) was performed in January, March and May in both RCs (at age 2 and 3 years) for automatic (Thorsen and Kjesbu 2001) and microscopic (Kjesbu 1991) stage determination, while male fish were stripped to indicate maturity and time of milt release. Prior to each spawning season, ultrasonography was also carried out to determine sex and stage of gonad maturity in accordance to methodology of Karlsen and Holm (1994), using a Kretz Combison 301 apparatus. SGR were calculated for each sampling interval by the equation:

$$SGR = \left( \frac{\ln W_2 - \ln W_1}{t_2 - t_1} \right) \times 100$$

where $W_1$ is body weight at time $t_1$, and $W_2$ at time $t_2$, and $t_2 - t_1$ is number of days between each sampling point.

**Otolith preparation**

In total, 95 sagittal otoliths were extracted from individuals that experienced both reproductive cycles, while otoliths from fish that were sacrificed earlier in the experiment for various experimental reasons ($n = 52$), health issues ($n = 70$) (cf. strict welfare instructions), or cannibalism ($n = 23$) were not included in any further analyses. The otoliths were rinsed in distilled water, photographed whole and weighed (on Sartorius ME 235S Genius). The left otoliths (right otolith in a limited number of cases where the left was deformed by vaterite crystals) were embedded in epoxy resin and hardener (Struers A/S,
Ballerup, Denmark) and cut through the core in 400-500 µm thick transverse sections (using an Isomet 1000 low-speed saw; Buehler Inc., Lake Bluff, IL), which were mounted on glass slides and ground with a series of abrasive papers to a thickness of approximately 300 µm before being polished (McCurdy et al. 2002). Multiple overlapping digital images (TIFF, 2560 × 1920 pixels) were captured of each otolith section to cover its whole plane, using a Nikon® Digital Sight DS-Fi2 camera mounted on a Zeiss Axioskop 2 with a 0.63 × adapter and 4 × magnification, DS-U3 controller, and NIS-Elements F (v4.00.06) software. At each frame position, two images were captured, one under reflected white light and the other with fluorescent epi-illumination. Both image sets of the whole otolith were merged using Adobe® Photoshop CS5 software, and manually checked for being accurately positioned.

Image analysis

Due to otoliths with missing AZ marks, only 73 of the 95 photographed otoliths could be used in the further analyses. Image annotations of AZ marks and the transitions between opaque and translucent zones were placed along the dorsal axis of the transversal section, using ImageJ software v.1.50i (Rasband 2016) (Fig. 1). Zone widths and the proportion of each zones type between successive AZ marks were then reported. The dorsal axis was expected to reflect growth in fish length, in addition to being an important area of the otolith section with regards to standard age estimation methods (Li et al. 2008). The associated macrostructure was also found in pilot tests to be clearer and show fewer examples of merged/overlapping increments (i.e. due to irregular lobe formations) than in the distal and the ventral axes.

Statistics
GLM Repeated Measures ANOVA (RM-ANOVA) were used to test for differences in W and SGR (recorded in two-month intervals). This statistical technique was also used on otolith growth, proportion opaque otolith, and TL increase per six-month interval, corresponding to the periodicity of AZ marking in RC1 and RC2. The data were split into two overall data categories (Table 1): 1) “repeat” (spawned in RC1 and RC2), “late” (immature in RC1 and first-time spawning in RC2), “skipped” (spawned in RC1 but not in RC2); and 2) within each RC, individuals with “zero” (< 0 %], “minor” (0-11 %] and “major” (> 11 %) weight loss during spawning. However, the skipped group could not be reliably tested against the repeat and the late groups due to low N (Table 1). The weight loss during spawning was based on accumulated weight loss registrations during the spawning season between the sampling points in mid-January and mid-May relative to the January body weight. The median weight loss (11 %) was used as separation criterion between members of the minor and major groups. In all RM-ANOVA analyses, potential effects from gender and population were tested by including the variables as interacting factors before excluded if found not significant. The relationships between otolith growth and body growth, or alternatively, otolith growth and body size (mean TL) per six-month AZ interval were tested cross-sectionally (between spawning groups per RC) and longitudinally (within each spawning group over both RCs) with GLM ANCOVA. Mean TL was here successively based on the geometric mean of measurements at the second and fourth month within each half-year period. Differences in mean otolith growth was also tested between spawning groups with GLM ANOVA. A significance level of \( p < 0.05 \) was used for all tests. All statistical analyses were carried out in Statistica® 13 (Dell Inc. 2015).

**Results**
Food-induced variability in body growth and reproductive histories

In the high feeding group 54 of 60 individuals sexually matured in RC1, while at that time only 14 of 39 of those in the low feeding group, i.e. receiving 50% less food. Growth trajectories of early mature (ending up as repeat spawners) and late mature fish (ending up as first-time spawners) were typically parallel throughout the experiment but with higher body weight (W) for the former category ($F_{17, 1088} = 1.66$, $p < 0.05$, RM-ANOVA, Fig. 2a, b).

This difference in W started appearing already during age 1 year, where repeat spawners showed a higher SGR than late mature individuals ($F_{4, 368} = 3.49$, $p < 0.01$, RM-ANOVA) (Fig. 2c, d). The corresponding information for “skippers” was indicative only due to few individuals (Table 1). From March to April in RC1, the estimated SGR of repeat spawners dropped markedly compared to late spawners, a feature being more noticeable in females than males. A boost in SGR was apparent in late mature individuals (i.e. late group, n = 30) early in RC2 (Fig. 2c, d). This jump was mostly attributed to the transfer of fish from the low- to high-feeding tank, whereas members of the late group (immatures in RC1) was overrepresented with 14 of 20 transferred individuals (Table 1). On the other hand, the relocation of 29 fish from high to low feeding, dominated by 25 sexually mature individuals that repeated spawning in RC2, resulted in a relatively low SGR of the repeat group (n = 66) through the summer at age 2 years. These feeding related effects in SGR were, however, normalized soon after all remaining individuals became sexually mature, as seen around October in RC2 (when vitellogenesis typically occurs in cod, cf. Introduction), and SGR of repeat and late spawners became similar ($F_{5, 325} = 1.15$, $p = 0.33$, RM-ANOVA). The switch from high to low feeding resulted in only two fish skipping spawning in RC2, while all others spawned. Despite the group of fish held under restricted feeding in both RC1 and RC2, no one was sexually immature when the experiment ended. Although female spawning
resulted in larger weight loss than for males, and thus different SGR trajectories ($F_{16,1024} = 5.25, p < 0.001$, RM-ANOVA), the relative difference in body growth patterns between the spawning groups were regarded to be similar for both genders.

**Life history impacts on otolith zonation patterns**

The onset of translucent otolith zone formation (annuli) occurred shortly after January in both years. This applied both to sexually immature and mature fish. The only skipper surviving to the end of the experiment showed a notable delay in the timing of the third translucent zone, i.e. until July in the year of skipping in RC2 (near the AZ6 mark) (Fig. 3). No consistent differences were found in proportion opaque otolith deposits nor in absolute otolith growth between groups of repeat spawners and late matures in RC1 or RC2 ($F_{3,177} = 0.052, p = 0.98$, and $F_{3,177} = 1.61, p = 0.19$, RM-ANOVA, respectively) (Fig. 4a, c), but their associated growth in TL differed ($F_{3,174} = 6.14, p < 0.001$, RM-ANOVA) (Fig. 4e) (see also tests above for W). However, repeat spawners had a faster otolith growth ($p < 0.05$, GLM) in the autumn of RC1, i.e. at the time they entered sexual maturation, although the proportion of opaque otolith deposits was similar to the late mature group ($p = 0.31$, GLM). A clear seasonality in the proportion of opaque otolith deposits (versus translucent otolith deposits) was evident in both spawning groups: opaque deposits generally dominated during autumn growth, while opaque and translucent deposits were equally represented in spring (Fig. 4a).

Otolith growth, however, was less impacted by seasonal fluctuations, but exhibited an overall decrease with time ($F_{3,177} = 60.91, p < 0.001$, RM-ANOVA) (Fig. 4c).

**Spawning weight loss and otolith zonation patterns**
Individuals with a relatively major loss in spawning weight in spring (indicating high investment in gamete production) showed a larger decline in proportion opaque otolith (opacity), otolith growth and body growth, during the period from autumn (July-Dec) to spring (Jan-Jun) in both RCs (Fig. 4b, d, f). The interaction between this degree of loss in W (zero, minor or major) was significant for all of these three test parameters in RC1 (proportion opaque otolith: $F_{2, 78} = 4.71, p < 0.05$; otolith growth: $F_{2, 78} = 12.15, p < 0.001$, and body growth: $F_{2, 77} = 20.94, p < 0.001$, RM-ANOVAs), but less obvious in RC2 (proportion opaque otolith: $F_{2, 59} = 2.87, p = 0.06$; otolith growth: $F(2, 59) = 2.06, p = 0.014$; and body growth: $F_{2, 59} = 2.63, p = 0.08$, RM-ANOVAs). However, on an annual scale no such effects of spawning weight loss were detected on the expression of these three considered variables (RC1: $0.30 < p < 0.85$, RC2: $0.26 < p < 0.61$, RM-ANOVAs). Nevertheless, the combined interpretation of the otolith growth with the proportion of opaque otolith (vs. translucent otolith) showed that the major spawning weight loss resulted in wider opaque zone in autumn, and narrower translucent zones in the spring than those with minor and zero weight loss.

Otolith growth – body growth relationship

The body growth (TL) – otolith growth relationship differed significantly between immature and first-time spawners in autumn ($p < 0.05$, GLM ANCOVA) (Fig. 5a), but not in spring ($p = 0.052$, GLM ANCOVA) (Fig. 5c). Mean otolith growth did however not differ between these two categories in either half of the year ($p = 0.11$ and $0.22$, respectively, GLM ANOVA). Moreover, the growth relationship between body and otolith in the autumn seemed affected by age. Comparing first-time spawners in RC1 against those that first spawned in RC2 (being one year older) showed that otolith growth was less affected by
elevated body growth in RC2 than in RC1 (p < 0.05, GLM ANCOVA). In spring, however, first-time spawners of the two cycles were not significantly different in terms of body growth dependency (p = 0.15, GLM ANCOVA) or otolith growth (p = 0.49, GLM test). Potential effects of spawning history did not seem to affect otolith growth of first-time vs. repeat spawners in RC2, either in terms of the body growth–otolith growth relationship (autumn: \( p = 0.91 \), spring: \( p = 0.21 \), GLM ANCOVAs), or the otolith growth (autumn: \( p = 0.18 \); spring: \( p = 0.66 \), GLM tests). Body size (TL) per se did not significantly influence otolith growth (autumn: \( p = 0.38 \), spring: \( p = 0.35 \), GLM ANCOVAs) (Fig. 5b, d).

Gender and stock effects

No gender-related differences could be detected in either the proportion of opaque otolith deposits, otolith growth, or body growth (all \( p > 0.05 \)). Although females were found to have higher spawning weight loss (relative to body weight) than males (\( F_{1,139} = 62.13, p < 0.001 \), GLM), gender did not significantly interact with the relationships between either of the above-mentioned variables and groups tested (repeat spawners and late matures: \( p > 0.17 \), RM-ANOVAs; zero, minor and major spawning weight loss: \( p > 0.13 \), RM-ANOVAs). BS cod did not differ from NC cod in either proportion opaque otolith deposits, otolith growth or body growth (\( p > 0.11 \), RM-ANOVAs). Likewise, stock characterization did not play any significant role when testing for interactions between the repeat vs. the late group (all, \( p > 0.22 \), RM-ANOVAs).

Discussion

The assumptions that the observed transition in seasonal zone pattern in otoliths of adult BS and NC cod is linked to the onset of sexual maturation, and that the subsequent
annuli termed “spawning zones” represents actual spawning events (Rollefsen 1933), has not been rigorously examined previously. Rollefsen (1933) rightfully pointed out that changes in the ambient environment due to lengthy spawning migrations and different spawning habitats may have contributed to the formation of spawning zones. These changes were not mimicked in the current experiment since all spawners and non-spawners were kept under the same environmental conditions, in line with natural fluctuations in a Norwegian fjord at 60°N. Therefore, potential effects on otolith zone formation that were linked to sexual maturation, spawning events and associated change in body metrics could be directly examined in this extensive study that lasted nearly three years and included two reproductive cycles. To emphasize variability in body growth, we introduced varying feed levels as additional important stressors, which proved to be successful in creating different responses in the fish groups established.

Trade-offs between body growth and reproduction and influences on age at first maturity are well documented in Atlantic cod (Folkvord et al. 2014, Lambert and Dutil 2000). As for many other temperate spring spawning species, cod typically build up energy reserves during summer and autumn before speeding up investment in gonad development at winter time, i.e. during January – March before spawning (Jobling 1982). In agreement with this, the present results showed reductions in both SGR and body length growth among spawning fish in each reproductive cycle (RC) (i.e. from autumn to spring), while body growth increased throughout RC1 in immatures. Due to a general correspondence between body growth and otolith size (Campana and Neilson 1985, Secor and Dean 1989), the costs of reproduction at the expense of body growth could potentially also reduce otolith growth. Even so, the otolith growth rates declined among both late mature and repeat spawners throughout the experiment and did not differ significantly in either RC1 or RC2.
Furthermore, the proportion of opaque deposits, or “opacity”, did not differ between the two groups, which both displayed a strong and almost identical seasonal alternation. The overall similarity in otolith formation between repeat spawners and late mature fish points in the direction that reproduction per se does not markedly affect otolith formation. The unrestricted feeding of most repeat spawners causing high somatic growth rates prior to, and during, the first maturation did not, however, increase otolith growth and the proportion of opaque deposits. This could therefore suggest reproduction-related uncoupling of the somatic growth – otolith growth relationship, at least in one of the groups.

Despite limited differences in otolith growth and opacity between spawners (i.e. repeat spawners) and immatures (i.e. late matures) in RC1, the degree of spawning weight loss (or “spawning effort”) as a proxy for energy loss due to reproduction influenced otolith formation. A major spawning effort led to stronger seasonal alternations of otolith growth and opacity compared to a minor or zero effort. Although this effect was most obvious in RC1, in which both sexually immature and mature fish were present, similar trends were also seen among the first-time and repeat spawners in RC2. The clear reductions in otolith growth and opacity from autumn to spring in fish exhibiting major spawning weight loss correspond well with the fact that loss of somatic weight and energy during reproduction, in relative terms to body size, negatively correlate with postspawning condition in cod (Lambert and Dutil 2000). The trade-offs between reproduction and body growth clearly suggest that higher investments in gonadal development and spawning, in relative terms, deplete energy reserves at the expense of somatic growth. The present data therefore suggests that reduced body growth during reproduction as a consequence of high loss of somatic mass size relative to body size, along with the corresponding energy depletion,
Otolith zone formation during reproduction affects otolith growth and opacity. To evaluate the effects of spawning on the overall otolith zone structure, both otolith growth and the proportion of each zone type (opaque vs. translucent), should be interpreted in concert. In the autumn, high spawning effort resulted in high otolith growth with predominantly opaque deposits, which could contribute to a wider and opaquer zone, while in the spring low otolith growth with predominantly translucent deposits could lead to a narrower and more translucent zone. This was not in accordance with the reported characters of a spawning zone, where the opaque zone becomes significantly narrower and the translucent zones more clear and sometimes wider than the opaque zone (Rollefsen 1933). The otolith growth patterns in fish with low spawning effort (or gonad investment) did not distinguish as much from the sexually immature fish as in fish with high investments. Consequently, first-time spawning cod, which exhibit lower fecundity and produce smaller eggs, and thus experience less somatic costs (Trippel 1998, Trippel et al. 2014), may not impact the otolith structure sufficiently to induce recognizable spawning zones. Altogether, these results indicate that high reproductive investments exert stronger impacts on the otolith growth structure by changing the relative widths of opaque vs. translucent zone, and possibly also the timing of the zone deposits compared to cod with lower investments or no reproduction.

The lack of differences in otolith growth between repeat spawners and fish maturing late (being immature in RC1) despite significant higher somatic growth in the repeat group could indicate an uncoupling between fish growth and otolith growth. Other studies have shown changes in the otolith size – fish size relationship during ontogenetic changes in early life history of bluefish (Pomatomus saltatrix) (Hare and Cowen 1995) and at onset of sexual maturity in North Sea plaice (Plaunonectes platessa) (Rijnsdorp and Storbeck 1995). Also, sexual maturation has previously been suggested to rapidly reduce annulus width in orange
Otolith zone formation during reproduction

...roughy (*Hoplostethus atlanticus*), although somatic growth rates could not be individually recorded in the field (Francis and Horn 1997). Environmental factors may also induce changes to the otolith growth – fish growth relationship. Both water temperature above the optimum for somatic growth (Mosegaard et al. 1988) and starvation as such (Tzeng and Yu 1992) induce uncoupling in the growth relationship between fish length and otolith. In cases of insufficient feeding, uncoupling takes place first after the metabolic rate has declined to a resting level and the somatic growth is arrested (Wright et al. 2001). Common for all cases is that otolith growth generally continues at some level, even when somatic growth declines or fully ceases for shorter periods (e.g. Wright et al. 1990). Presently, neither temperature nor feeding levels suggested that an uncoupling of the otolith growth – body growth relationship should occur: throughout the experiment temperature was identical in all tanks and remained below the level necessary for maximum somatic growth rate in both juveniles and adults (Björnsson et al. 2001), and furthermore, all fish were offered sufficient feeding to avoid starvation. On the other hand, signs of uncoupling between somatic and otolith were found in the transition from immature (in RC1) to mature (in RC2) suggesting that onset of sexual maturation may influence the otolith growth rate independently of somatic growth.

The relatively higher growth rate of individuals that matured already in RC1 (i.e. repeat group) compared to the immatures (i.e. late group) were mainly caused by the two different feeding regimes. In general, fish with high feeding histories and high body growth, sexually matured a year before those undergoing restricted feeding causing poor growth. Food consumption was also generally higher after onset of sexual maturation of the repeat group, although appetite has been found to be reduced in the weeks before and during spawning (Fordham and Trippel 1999). However, this first scenario contrasts to the natural...
conditions of stocks where long-range spawning migrations may cause sexually mature individuals to spend less time on active feeding, or have fewer chances of encountering suitable prey, (i.e. BS cod Bergstad et al. 1987). Although stomach analyses on mature BS cod have indicated sporadic feeding at the spawning grounds in Lofoten, the frequency of empty stomachs was clearly higher compared to non-spawning cod (Michalsen et al. 2008). Meanwhile, non-reproductive cod typically spend the winter period feeding in the Barents Sea (Johannesen et al. 2015) prioritizing growth to maximize fitness through future reproductive success (Folkvord et al. 2014, Heino and Kaitala 1999, Kjesbu et al. 1998).

Considering the positive relationships between feeding level and metabolism (Jobling 1981), and between metabolism and otolith growth (Mosegaard et al. 1988, Wright 1991), it is likely that otolith growth will lower in periods with reduced feeding levels during reproduction. Restricted feeding has also been shown to reduce the opacity in cod otoliths, due to declined incorporation of organic compounds (i.e. protein fibres) in the otoliths (Dannevig 1956, Hüsey and Mosegaard 2004, Hüsey et al. 2004, Høie et al. 2008).

Consequences of an overall reduction in feeding in years with reproduction may therefore lead to both narrower otolith annuli and a shorter period with opaque otolith deposits, in correspondence with the characteristics of spawning zones. Bearing in mind that spawners are likely to feed less than immatures under natural feeding conditions, the present results where otoliths did not differ between spawners and immatures although food consumption where higher in the maturing individuals, suggests that reproduction in natural conditions could still lead to narrower and more translucent otolith zones. However, feeding effects on otoliths should not serve as a proxy for reproduction. Some mature individuals may also continue feeding during spawning (Michalsen et al. 2008) and may not necessarily form spawning zones. Moreover, low feeding and depletion of energy reserves is not necessarily
associated with reproduction. Natural temporal or spatial variations in prey availability 
(Gjøsæter et al. 2009), or other environmental factors that could lead to nutritional fatigue 
and reduced metabolism (e.g. disease or parasitism), could also potentially influence otolith 
growth and opacity in immature fish similarly as in spawning fish.

Skipped spawning frequently occurs in many iteroparous fish species (Rideout and 
Tomkiewicz 2011), and low food availability and insufficient energy reserves has been 
suggested to be an important factor for why skipping happens (Rideout et al. 2000, 
Skjæraasen et al. 2009). As mentioned above, these factors may influence the otolith zone 
structure in a similar manner as reproduction. In fact, there have been relatively few 
observations of “missing” spawning zones in BS and NC cod otoliths (J. Godiksen, personal 
communication), although skipped spawning is reported to frequently occur in BS cod 
(Skjæraasen et al. 2012). In keeping with the overall results, this indicates that spawning 
zones are not a diagnostic character for actual reproduction. The typical narrow and 
translucent annuli in sexually mature cod is therefore likely to rather reflect the metabolic 
rate which is coupled to the available energy storage and food availability.

Higher reproductive investments in females than in males is a common 
phenomenon, as production of eggs is more costly (Jobling 1982, Tocher 2003). Gender was 
however generally not found to significantly impact otolith zone formation in the present 
results, although spawning weight reductions indicated that females invested more energy 
in reproduction. In accordance with the present results, previous experiments on cod have 
demonstrated higher reductions in female body and liver weight, as proxies for energy 
invested in reproduction (Karlsen et al. 1995, Karlsen et al. 2006). Considering that higher 
spawning weight loss is presently found progressively to affect otolith formation, the 
differences in gonad investments between gender suggests that the clarity (or expression)
of a spawning zone could depend on gender, implying that female otoliths should feature
more distinct spawning zones.

The lack of difference in otolith zone formation between BS and NC cod in the
experiment could indicate that genetic effects alone do not play a major role in otolith
growth. However, otoliths collected from these two stocks north of 62°N diverged
considerably in terms of relative zone width (Rollefson 1933, Stransky et al. 2008).

Latitudinal differences in the periodicity of otolith zone formation has been demonstrated
between southern and northern cod stocks (Høie et al. 2009), where differences in
temperatures and feeding conditions could play a role. In addition, varying proximity to
spawning areas may also be important. While NC cod spawn more locally in fjords and along
the coast (Godø 1995), BS cod have a particularly long and energetically demanding
migration (Jørgensen et al. 2008) from the feeding areas in the Barents Sea to the spawning
areas in Lofoten (Bergstad et al. 1987), or even further south (Godø 1983), which ultimately
may impact the otoliths differently due to environmental differences. The comparison of
stock responses in otolith growth dynamics must be made with caution, however, as the
experimental setup could not mimic the typical environmental conditions of both stocks at
the same time, and for practical reasons involved higher average growth and temperature
than under natural conditions. This in consideration, the present findings suggest that
reproduction alone does not impact otoliths differently between individuals of the two
populations.

Altogether, the study could not fully support earlier statements in the literature that
actual reproduction affects growth and opacity of cod otoliths to such an extent that the
annual zone formation would differ between reproductive and non-reproductive
individuals. However, seasonal differences in the otolith growth and opacity increased with
degree of loss of energy due to the act of spawning. This indicate that high relative fecundity
could result in changes to the relative zone width within an otolith annulus, while fish with
low relative fecundity (e.g. first-time spawners) may not induce features that are
distinguishable from otoliths of immature fish. In addition, otolith zone structure of non-
reproductive individuals (i.e. immature fish and skippers) that were generally subjected to
low feeding levels did not differ from otoliths of spawning fish with high feeding levels.
Moreover, the observed relationship between otolith and fish growth rates suggests that
better feeding conditions of immature fish and reduced feeding in maturing fish, could
amplify differences in the otolith zone structure such that a spawning zone would
distinguish more (i.e. appear narrower and more translucent). This would depend on food
intake, somatic growth and body energy reserves, however, and seems not to relate directly
to actual reproduction. We may therefore conclude that otolith zone structures in BS and
NC cod do not unambiguously indicate reproduction, and that the term spawning zone
could be inaccurate.

Although the otolith zone formation in experimentally reared fish is not directly
comparable to that of wild cod, the findings in this study suggest that a relatively low
spawning effort, such as in many first-time spawners, may result in less distinct “spawning
zones” than in more experienced spawners. A potential consequence of this could be an
overestimation of age-at-maturity, and thus, an underestimation of spawning stock biomass
due to undetected spawning zones when otoliths are interpreted. Moreover, as skipped
spawning is likely to induce similar otolith zonation as spawners, we therefore recommend
that information on frequency of skipped spawning (e.g. Skjæraasen et al. 2012) are
considered if spawning zones are used for example in estimations of spawning stock
biomass. We also recommend further field investigations on the relationships between
otolith zone formation and past and present reproduction, in individually marked and tagged wild BS cod, which upon recapture could allow for a direct validation of the so-called spawning zones, and particularly provide information on the readability of spawning zones in first-time spawners. Until further knowledge is gained on this topic, we recommend that the apparent regular and narrow increments in otoliths of cod, and possibly other marine fish, are interpreted as a minimum estimate of the years since onset of sexual maturity, but not necessarily the number of years with reproduction.

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Figure and table captions

Fig. 1. Dorsal growth axis of a transversally sectioned Atlantic cod otolith, captured under reflected light and UV-light, visualized as overlaid images (using Adobe Photoshop CS5 software). Alizarin (AZ) stains indicate six-month otolith growth during the experiment, from staining in January and July. The dorsal edge (DE) corresponds to experimental end. The line indicates annotated annuli; opaque zones (white) and translucent zones (TZ, black). Blue arrows exemplify “break points” of the annotation axis; one at the onset of first annulus and the other one at the AZ1 mark. Scale bar = 1 mm.

Fig. 2. Means (± 95% CI) of body weights (a, b) and specific growth rate (SGR) (c, d) of Atlantic cod females (a, c) and males (b, d) in the three spawning groups: repeat (spawned in RC1 and RC2, circle and solid line); late immature in RC1 and spawned RC2, square and long-dashed line); and skip (spawned in RC1 and skipped spawning in RC2, triangle and short-dashed line).

Fig. 3. Examples of proportional dorsal otolith growth patterns (opaque = white, translucent = grey) from three Atlantic cod individuals with different reproductive histories: repeat spawners; skip spawners; and late matures. Six alizarin (AZ) stains (thin (red) bands, in sequence from AZ1 to AZ6) with six-month otolith growth between January and July from age 1 to age 3.5 years. The two reproductive cycles (RC) during the trial are indicated for individuals that spawned (blue) or did not spawn (hatched red, either immature or skipped spawning). The top panel is the real otolith image of the corresponding otolith annotations beneath from a repeat spawner (female NC cod, TL = 58 cm).
Fig. 4. Means (± CI) of proportion opaque otolith (a, b), (dorsal) otolith growth (c, d), and body length growth (e, f) in 6-month intervals (autumn and spring) in experimental reproductive cycle 1 (RC1) and 2 (RC2). Individuals are categorized by their spawning history (a, c, e): repeat (circle and solid line); late (square and dashed line), and, alternatively, by year-specific spawning weight loss (%) (b, e, f): zero (≤ 0 %) (triangle and dashed line), minor (0-11 % weight loss) (square and long-dashed line) and major (>11 % weight loss) (circle and solid line).

Fig. 5. Otolith growth rate corresponding to body growth rate (a, c) and geometric mean body length (b, d) from July to January (autumn) (a, b) and from January to July (spring) (c, d) in reproductive cycle 1 (RC1): immature (open triangles and long-dashed line) and first-time spawning (open circle and long-dashed line), and in RC2: first-time spawning (filled circle and solid line), repeat spawning (filled square and short-dashed line) and skipping (filled triangle, no line). Linear trend lines indicate the relationship in growth rate between otolith and body while 95% confidence ellipses indicate distribution of otolith growth rate – body length.

Table 1. The experimental design of feeding regimes and the groups of different spawning histories during two reproductive cycles (RCs) of Atlantic cod. Five six-month intervals were generated in correspondence to six alizarin (AZ) stain marks in the otoliths, and the outcome of N otoliths used in the further analyses.
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<table>
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<th>AZ marks Months Age</th>
<th>RC1 1-2 Jan-June 1</th>
<th>2-3 July-Dec 1</th>
<th>3-4 Jan-June 2</th>
<th>4-5 July-Dec 2</th>
<th>5-6 Jan-June 3</th>
<th>Group category</th>
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<td>Low</td>
<td>H-H</td>
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<tr>
<td></td>
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<td>Low</td>
<td>Low</td>
<td>High</td>
<td>High</td>
<td>H-L</td>
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<td>First-time spawning</td>
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<td>Repeat spawning</td>
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<td>Repeat spawning</td>
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