Sexually dimorphic transcription of estrogen receptors in cod gonads throughout a reproductive cycle

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Abstract
The role of sex steroid regulation in gonadal maturation is a very complex process that is far from being fully understood. Hence, we have investigated seasonal changes in gonadal expression of estrogen receptors (ERs) in Atlantic cod (Gadus morhua L.), a batch spawner, throughout the annual reproductive cycle. Three nuclear ER partial cDNA sequences (esr1, esr2a, and esr2b) were cloned and all esr transcripts were detected mainly in liver and gonads of fish of both sexes. In situ hybridization of esrs along with germ cell (vasa) and gonadal somatic cell markers (gonadal soma-derived factor (gsdf), 3β-hydroxysteroid dehydrogenase (3βhsd), and anti-Müllerian hormone (amh) for testicular, or gsdf for ovarian somatic cells) showed that all three esrs were preferentially localized within interstitial fibroblasts composed of immature and mature Leydig cells in testis, whereas they were differentially expressed in both follicular cells and oocytes in ovary. Quantitative real-time PCR analysis revealed a sexually dimorphic expression pattern of the three esr paralogs in testis and ovary. A significant increase in esr2a expression was identified in testis and of esr2b in ovary, whereas esr1 transcripts were elevated in both testis and ovary in February and March before the spawning period. The localization and sexually dimorphic expression of esr genes in gonads indicate a direct function of estrogen via ERs in gonadal somatic cell growth and differentiation for Leydig cell in testis and follicular cells in ovary throughout the annual reproductive cycle in Atlantic cod.

Introduction
Estrogens, mainly 17β-estradiol (E₂), act directly to promote sex differentiation and gonadal maturation (Korach 1994). Estrogen action has been classically shown to act in liver via specific nuclear estrogen receptors (ERs), which act as ligand-dependent DNA-binding transcription factors (Dahlman-Wright et al. 2006) in order to stimulate vitellogenin and choriogenin production during oocyte growth in vitellogenic stages for all vertebrates studied (Kazeto et al. 2011). Two distinct subtypes of nuclear ERs (ERα and ERβ) have been cloned in a number of organisms from humans (Keaveney et al. 1991) to nematodes (Mimoto et al. 2007). The role of ERs in gonadal
Sex-biased expression of cod esr genes

K Nagasawa and others

Expression of esr is first detected in teleosts at the embryonic stage, indicating an essential role of estrogen signaling in larval development (Lassiter et al. 2002). Despite the paramount importance of estrogen signaling mediated by ERs in fish gonadal development, information about the precise localization of ERs is still limited to a few fish species: ERs in the interstitial fibroblasts (the Leydig cell precursors) in testis of rainbow trout *Oncorhynchus mykiss* (Bouma & Nagler 2001), ERz in thecal, granulosa, and interstitial cells in ovary and in Sertoli cells in testis of eelpout *Zoarces viviparus* (Andreassen et al. 2003), and ERs in granulosa cells in ovary and ERβ2 in cells in undifferentiated gonad in medaka *Oryzias latipes* (Chakraborty et al. 2011a). Therefore, information about localization patterns and expression profiles for ER subtypes in teleost gonadal cells is required in order to understand their potential role in regulating gene transcription and gonadal development process in teleosts.

Atlantic cod (*Gadus morhua*) is a cold-water marine fish species that has an annual reproductive cycle (Kjesbu 1989). Importantly, precocious sexual maturation (also known as early puberty) often occurs in both sexes in Atlantic cod when they are 2 years old under in aquaculture conditions. This is an important bottleneck for the cod farming industry, as it is associated with a significant reduction in somatic growth (Karlsen et al. 2006). Vitellogenesis generally starts in the autumn and subsequent reproduction occurs from February to April along the Norwegian coastline. During the 50–60-day spawning period, females can release up to 17–19 batches of eggs at intervals of several days (Kjesbu 1994). The timing of sexual maturation and the spawning period are strongly associated with a peak of the plasma E2 levels in 2- and 3-year-old Atlantic cod under aquaculture conditions (Norberg et al. 2004). However, molecular mechanisms underlying the direct effects of E2 signaling on gonadal maturation and batch spawning have been poorly investigated, even if the final maturation process by maturation-inducing steroids has been studied ex vivo (Tveiten et al. 2010). To deepen our understanding of the role played by estrogen regulation in gonadal maturation, we kept 2-year-old Atlantic cod under aquaculture conditions. This is an important bottleneck in Atlantic cod when they are 2 years old under in aquaculture conditions. This is an important bottleneck for the cod farming industry, as it is associated with a significant reduction in somatic growth (Karlsen et al. 2006).

In fish, the major target organs of estrogens are gonads, liver, pituitary, and brain (Filby & Tyler 2005). However, the direct effect of estrogen on gonadal development and the role of ERs are poorly understood (Shi et al. 2011).

Development has been elucidated from the phenotypic alterations of knockout (KO) mice. KO mice lacking *Esr1* showed complete infertility in both males and females (Lubahn et al. 1993), whereas both male and female *Esr2-KO* animals developed normally, albeit with reduced ovulation efficiency (Krege et al. 1998).

Interestingly, there have been a considerable number of reports that show predominant ER transcripts in gonads rather than other organs, indicating the possible direct action of estrogen in gonadal cells in vertebrates. It is known that the concentration of estrogen in testis and rete testis fluid far exceeds that in male serum in various species (Hess 2000), indicating a central role for estrogen may function in testicular and epididymal cells (O’Donnell et al. 2001). To obtain an overview of the localization of ER subtypes within gonads, O’Donnell et al. (2001) surmised the likely localization of ERs in adult rodent testis from numerous items of conflicting data: Leydig cells express both ERα and ERβ, whereas Sertoli cells and testicular germ cells (e.g. spermatogonia, pachytene spermatocytes, and round spermatids) predominately expressed ERβ. The direct effects of estrogen on gonad development have been observed following administration of diethylstilbestrol (DES) to rats. DES exposure revealed that estrogen plays an inhibitory role in Leydig cell proliferation (Abney & Myers 1991) and Sertoli cell maturation (Sharpe et al. 1998). In addition, there are several lines of evidence showing that estrogen has a stimulatory effect on germ cells. In particular, a report in which neonatal rats were treated with estrogen displayed increased numbers of undifferentiated and differentiating type A spermatogonia (Kula 1988). Within rodent ovaries, both ERα and ERβ are expressed in granulosa cells in follicles (Drummond et al. 1999). Administration of estrogen to hypophysectomized rats resulted in a stimulatory effect on granulosa cell growth and differentiation (Drummond & Findlay 1999). In addition, androgen receptor (Ar) KO mice that lack functional aromatase (Britt et al. 2001) – an alternative model of ER depletion – exhibit abnormal folliculogenesis with seminiferous tube-like structures due to apoptotic granulosa cells (Drummond & Findlay 1999, Drummond et al. 2002). Moreover, an in vitro culture study of mouse ovarian follicles reported that ERβ, but not ERα, plays a direct role in folliculogenesis to facilitate follicle maturation including E2 production and ovulation (Emmen et al. 2005).

In fish, the major target organs of estrogens are gonads, liver, pituitary, and brain (Filby & Tyler 2005). However, the direct effect of estrogen on gonadal development and the role of ERs are poorly understood (Shi et al. 2011).
Materials and methods

Fish husbandry and sample collection

Atlantic cod were hatched and reared at Mørkvedbukta Research Station (University of Nordland, Norway). In 2009, when the fish were 2-year-old adults (initial weight of 1.1 ± 0.2 kg, mean ± s.d., n = 18), they were transferred to three land-based flow-through tanks (40 m³) at a density of ~100 individuals/tank from August 2009 to May 2010. Seawater (7.4 ± 0.4 °C) was pumped from 200 m depth. A commercial diet (Amber Neptun, Skretting AS, Stavanger, Norway) was provided daily at 5% (w/w) body weight of the fish by automatic belt feeders. Eight fish were taken from Norway) was provided daily at 5% (w/w) body weight of the fish by automatic belt feeders. Eight fish were taken from each tank (n = 24), killed by exposure to 0.5 g/l tricaine methanesulfonate (Sigma–Aldrich), total length, total body weight (TW), and gonad weight (GW) were measured, and the gonadosomatic index (GSI, 100 × GW/TW (%)) was calculated at each sampling time point (August, November in 2009, and February, March, and May in 2010; Table 1). Differences in all numerical data for each sampling point within fish of the same sex were determined using a Tukey’s multiple comparison test using the GraphPad Prism Software, when the one-way ANOVA was significant. Normality and equal variance conditions were met, and the significance level was set at P < 0.05. In order to minimize blood contamination, the fish were exsanguinated after terminal anesthesia by cutting their gill arches before sampling of other tissues. Excised tissues were then rinsed three times with PBS. For semi-quantitative RT-PCR, brain, gill, heart, head kidney, kidney, liver, spleen, stomach, midgut, testis (GSI = 4.7%), ovary (GSI = 1.5%), skeletal muscle, skin, and blood were collected, snap-frozen in liquid nitrogen, and stored at −80 °C for subsequent RNA extraction. For histological observations and quantitative real-time PCR (qPCR) analysis, testes (n = 5) and ovaries (n = 5) were sampled from individual fish at each sampling time point, fixed with 4% paraformaldehyde (PFA)/PBS at 4 °C for 24 h, or snap-frozen and stored at −80 °C respectively. PFA-fixed gonads were then washed, embedded in paraffin, sectioned at 4.5-μm thickness, and stained with hematoxylin–eosin. All procedures used in this study were in accordance with the guidelines set by the National Animal Research Authority (Forskningsdyrvalget, Norway) and approved by the Faculty of Biosciences and Aquaculture (University of Nordland, Norway) Ethics Committee.

RNA extraction and cDNA synthesis

Total RNA was extracted from various tissues using QIAzol lysis reagent (Qiagen) as detailed elsewhere (Campos et al. 2010) and quantified by spectrophotometry with a NanoDrop ND-1000 instrument (Thermo Scientific, Saven & Werner AS, Kristiansand, Norway). RNA integrity was assessed by electrophoresis on a 1% (w/v) agarose gel. Total RNA (1 μg) was transcribed to cDNA using a Quantitect reverse transcriptase kit (Qiagen) as reported previously (Campos et al. 2010).

cDNA cloning of ER (esr) genes in Atlantic cod

In order to identify genomic sequences that contain putative coding regions for esr genes in Atlantic cod, TBLASTN searches were performed in Ensembl (http://www.ensembl.org/) against the cod genome assembly (release 69, October 2012; Flieck et al. 2013). The following protein sequences were used as queries: i) gilt-head sea bream ERα (AAD31032.2), ERβ1 (AAD31033.1), and ERβ2 (CAE30470.1) and ii) rainbow trout ERα1 (NP_001117821.1), ERα2 (NP_00118030.1), ERβ1 (NP_00118225.1), and ERβ2 (NP_00118042.1). After extracting the corresponding genomic sequences

Table 1  Total length (TL), total body weight (TW), gonad weight (GW), and gonadosomatic index (GSI, 100 × GW/TW) of Atlantic cod sampled throughout a sexual maturation cycle

<table>
<thead>
<tr>
<th>Sex</th>
<th>Sampling point</th>
<th>TL (cm)</th>
<th>TW (g)</th>
<th>GW (g)</th>
<th>GSI (%)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>August 2009</td>
<td>44.3 ± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1050.0 ± 75.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.0 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.1 ± 0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>November 2009</td>
<td>50.7 ± 1.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1566.7 ± 92.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>37.9 ± 6.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.5 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>February 2010</td>
<td>50.9 ± 1.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1746.2 ± 158.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>218.1 ± 27.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.4 ± 1.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>March 2010</td>
<td>53.6 ± 1.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2346.0 ± 211.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>198.0 ± 25.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.2 ± 1.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9</td>
</tr>
<tr>
<td>Female</td>
<td>May 2010</td>
<td>51.4 ± 1.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1452.3 ± 121.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.1 ± 1.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.9 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>August 2009</td>
<td>45.2 ± 1.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1143.3 ± 65.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.9 ± 1.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.4 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>November 2009</td>
<td>51.5 ± 0.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1563.6 ± 72.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.9 ± 3.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.5 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14</td>
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<tr>
<td></td>
<td>February 2010</td>
<td>52.6 ± 1.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2098.2 ± 134.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>231.4 ± 21.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.2 ± 1.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14</td>
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<td></td>
<td>March 2010</td>
<td>53.8 ± 1.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1855.0 ± 119.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>405.1 ± 51.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.3 ± 1.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14</td>
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<tr>
<td></td>
<td>May 2010</td>
<td>53.6 ± 0.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1726.6 ± 83.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>234.5 ± 58.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.9 ± 2.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14</td>
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</tbody>
</table>

Values with different lower-case letters display significant differences within the same sex (P < 0.05).
(esr1, GeneScaffold_4271; esr2a, GeneScaffold_1546; and esr2b, GeneScaffold_26), putative cDNA sequences were obtained with the Augustus gene prediction software (http://augustus.gobics.de/) and used to design specific primer sets (Table 2). PCRs were performed as described by Nagasawa et al. (2012). For 5’ RACE of esr2a, the gene-specific primers listed in Table 2 and the GeneRacer Kit with SuperScript III RT (Invitrogen) were used according to the manufacturer’s instructions. Specific amplicons were separated by electrophoresis, purified, cloned, and sequenced as detailed elsewhere (Nagasawa et al. 2012).

Bioinformatic analysis

Identity of cod esr cDNA sequences was confirmed by TBLASTN searches against the non-redundant database at NCBI BLAST (www.ncbi.nlm.nih.gov). For phylogenetic analysis, Atlantic cod cDNA sequences were extended by in silico cloning using the Ensembl genome browser and their deduced amino acid (AA) sequences were aligned with corresponding orthologs in various species (Supplementary Table 1, see section on supplementary data given at the end of this article) using MUSCLE (www.drive5.com). Poorly aligned or divergent regions were trimmed using Gblocks 0.91b (molevol.cmi.ma.csic.es). The resulting multiple sequence alignment was used for Bayesian (MrBayes v3.1.2, mrbayes.csit.fsu.edu) phylogenetic reconstruction as detailed elsewhere (Fernandes et al. 2010). A graphical representation of the phylogenetic tree was obtained using the PhyloWidget (www.phylowidget.org).

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was performed with cDNAs from the various tissues mentioned above (male, GSI = 4.7% and female, GSI = 1.5%) using gene-specific primer sets (Table 2). Thermocycling parameters were 94 °C for 3 min, followed by 35 cycles (25 cycles for acidic ribosomal protein (arp)) of 30 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C, with a final elongation step of 72 °C for 3 min.

### Table 2

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank</th>
<th>Forward primer (5’ → 3’)</th>
<th>Reverse primer (5’ → 3’)</th>
<th>Purpose</th>
<th>E</th>
<th>R²</th>
<th>Size (bp)</th>
</tr>
</thead>
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<td>esr1</td>
<td>JX178935</td>
<td>TGCAGTCCCTGGG-CAGTGGGTCACCA</td>
<td>TGAACCTCGGTG-TACGGCGGTCTCATCT</td>
<td>Cloning/ISH</td>
<td>–</td>
<td>–</td>
<td>822</td>
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<tr>
<td></td>
<td>–</td>
<td>AGGCCAGCTGGAGAACAG-GACGTCGCA</td>
<td>TGGGGTCCAGACTCTCCAG-CAGCAAGT</td>
<td>Cloning/ISH</td>
<td>–</td>
<td>–</td>
<td>643</td>
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<tr>
<td></td>
<td>–</td>
<td>AAAGGAGTATTGCGCAAGG</td>
<td>TGAACCTCGGTG-TACGGCGGTCTCATCT</td>
<td>RT-PCR/qPCR</td>
<td>83.0</td>
<td>0.999</td>
<td>307</td>
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<tr>
<td>esr2a</td>
<td>JX178936</td>
<td>GGACACTGACATGGACT-GAGGAGTA</td>
<td>TGGCACTGGAAA-GAACTGGTTGCTCCCGCT</td>
<td>Cloning (5’ RACE)/ISH</td>
<td>–</td>
<td>–</td>
<td>655</td>
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<tr>
<td></td>
<td>–</td>
<td>CTTACACCGCGCTGGGCCCAT-GACT</td>
<td>GCAGTAATGGAGGG-CACGGGCCCAA</td>
<td>Cloning</td>
<td>–</td>
<td>–</td>
<td>1438</td>
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<tr>
<td></td>
<td>–</td>
<td>TTGATAGTGGACACTTCTCCCTCC-CAA</td>
<td>GCCGGTTAGAAAGGG-CACGGGCCCAA</td>
<td>Cloning</td>
<td>–</td>
<td>–</td>
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<td>esr2b</td>
<td>JK993476</td>
<td>CAGATCGCTGCTGCTCCCAA-TAGA</td>
<td>CAGATCGCTGCTGCTCCCAA-TAGA</td>
<td>Cloning/RT-PCR/ISH/qPCR</td>
<td>85.4</td>
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<td>304</td>
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<td>ar</td>
<td>FJ268742</td>
<td>CGTATGTCGTTCTGCAGTTAAGAGA</td>
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<td>QPCR</td>
<td>92.3</td>
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<td>ACAAAACAAATGACGAG-CATCAT</td>
<td>QPCR</td>
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<td>152</td>
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<td>actb</td>
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<td>TGCACCCTGAGTACCCAC-CTGCT</td>
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<td>QPCR</td>
<td>86.0</td>
<td>0.991</td>
<td>76</td>
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<tr>
<td>arp</td>
<td>EX741373</td>
<td>GACCGCGTTGGGACGCG-AGATG</td>
<td>GACCGCGTTGGGACGCG-AGATG</td>
<td>QPCR</td>
<td>82.0</td>
<td>0.996</td>
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<tr>
<td>eef1a</td>
<td>EX721840</td>
<td>CTGGGATGCTGCGCTGGAGTCTG</td>
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<td>QPCR</td>
<td>91.6</td>
<td>0.994</td>
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<td>vasa</td>
<td>HM451456</td>
<td>CAGATGGGCGGATGAGAG-CATG</td>
<td>CAGATGGGCGGATGAGAG-CATG</td>
<td>QPCR</td>
<td>88.9</td>
<td>0.995</td>
<td>142</td>
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<td>gsd</td>
<td>KC204828</td>
<td>ATGAGTCTGTCGTAACCCGTCTTCC-CT</td>
<td>ATGAGTCTGTCGTAACCCGTCTTCC-CT</td>
<td>ISH</td>
<td>–</td>
<td>–</td>
<td>600</td>
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<tr>
<td>amh</td>
<td>JN802292</td>
<td>TCTTATCCAACCTCACCTCCCCCTGTGCTT-CAGG</td>
<td>TCTTATCCAACCTCACCTCCCCCTGTGCTT-CAGG</td>
<td>ISH</td>
<td>–</td>
<td>–</td>
<td>786</td>
</tr>
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</table>
PCR products were separated by electrophoresis on a 1% (w/v) agarose gel and visualized with the Kodak gel documentation system v.4.0.5. Expression patterns were confirmed using two biological replicates of each sex.

In situ hybridization
Spatial expression patterns of esr paralogs were determined by in situ hybridization (ISH), along with those of known germ cell and gonadal somatic cell markers reported in other teleosts. The gene vasa was used for detection of spermatagonia/spermatocytes in testes or oogonia/oocytes in ovaries because of its well-conserved spatio-temporal expression pattern among teleosts, including Atlantic cod (Presslauer et al. 2012). The gonadal somatic-derived factor (gsdf) was used to identify Sertoli cells in testes or follicular cells in ovaries, as it is a proven marker of these cells in rainbow trout (Sawatari et al. 2007), medaka (Shibata et al. 2010), and zebrafish (Gautier et al. 2011). In addition to gsdf, anti-Müllerian hormone (amh) has been used as a Sertoli cell marker in zebrafish (Rodriguez-Mari et al. 2005). Leydig cells were detected using 3β-hydroxysteroid dehydrogenase (3βhdsd) as a marker, as reported for rainbow trout (Kobayashi et al. 1998), swamp eel (Lo Nostro et al. 2004), and Nile tilapia (Rukasna et al. 2010). The gsdf and 3βhdsd genes were cloned from Atlantic cod in this study. Digoxigenin-labeled sense and anti-sense RNA probes for all genes examined (esr1, esr2a, esr2b, vasa, gsdf, amh, and 3βhdsd) were individually in vitro transcribed using T3 or T7 RNA polymerase (Roche) from corresponding DNA fragments amplified using the primers listed in Table 2. Tissue samples were obtained from 2-year-old Atlantic cod reared at Mørkvedbukta Research Station. Testes (GSI=1.3%) and ovaries (GSI=1.3%) were fixed with Bouin’s solution (Sigma–Aldrich) and 4% PFA/PBS respectively at 4°C for 12–24 h, washed out with nuclease-free water, dehydrated with an ascending ethanol series, and then embedded in paraffin. Cross sections of testes and ovaries samples were prepared at 4-μm thickness, attached onto polylysine-coated glass slides (Polysine, VWR international, Leuven, Belgium), and used for ISH analysis as reported by Fernandes et al. (2006) with slight modifications. Sense and anti-sense probes for all genes (esr1, esr2a, esr2b, vasa, gsdf, amh, and 3βhdsd) were individually hybridized at 70°C for 24 h and then washed out with RNase A treatment (Sigma–Aldrich) as reported previously (Nagasawa et al. 2009). Sections were mounted on gelatin embedding medium (Sigma–Aldrich) and observed under a BX-51 microscope (Olympus).

Quantitative real-time PCR
Twenty-fold diluted samples of cDNA synthesized as described above were used in duplicate, as were negative (minus reverse transcriptase) and no template (water) controls. Gene-specific qPCR primers for the three esr paralogs were designed using the GenScript Real-time PCR Software (www.genscript.com) to span at least one intron/exon border whenever possible (Fernandes et al. 2008). The qPCR was run at 95°C for 15 min, followed by 45 cycles of 15 s at 94°C, 20 s at 60°C, and 20 s at 72°C using LightCycler 480 SYBR Green I Master chemistry (Roche) on a LightCycler 480 (Roche), as described previously (Campos et al. 2010). Specificity of the reactions was evaluated by melting curve analysis and further by Sanger sequencing of amplicons. Amplification efficiencies were calculated by standard curves from a fivefold dilution series (1:1, 1:5, 1:25, 1:125, and 1:625) of pooled cDNA as detailed by Fernandes et al. (2008). Cycle threshold (Ct) values were determined using the LightCycler 480 Software with a level of fluorescence intensity arbitrarily set at 1. Three endogenous reference genes, β-actin (actb), arp, and eukaryotic elongation factor 1α (eef1αa), were validated as reported by Fernandes et al. (2008) and Nagasawa et al. (2012). Their geNorm stability values (M) were 0.812, 0.779, and 0.618 for actb, arp, and eef1αa respectively. The most stable gene combination was found to be arp+eef1αa with an average pairwise variation V2/3 = 0.258. Therefore, raw qPCR data of target genes were corrected using the geometric average of arp and eef1αa quantities as normalization factors. Differences in relative expression levels of three genes esr, ar, and cyp19a1a among different sampling time points or between sexes were examined by Mann–Whitney U tests using the GraphPad Prism Software (San Diego, CA, USA), as the data were not normally distributed. The significance level was set at P<0.05.

Results
Gonadal maturation status throughout an annual reproductive cycle in 2-year-old Atlantic cod
Atlantic cod kept in land-based tanks throughout a year showed a clear reproductive cycle with a significant increase in GW (Table 1) and differentiation of germ cells in both testes and ovaries (Fig. 1). Throughout a reproductive cycle, testis and ovary weights reached a peak in February (GSI = 12.4 ± 1.0%, n = 8) and March (GSI = 18.3 ± 1.7%, n = 16) respectively (Table 1). Spermatiation was seen in some male individuals in February and March,
Figure 1
Histology of representative testes and ovaries at different maturation status using hematoxylin–eosin staining in 2-year-old Atlantic cod throughout an annual reproductive cycle. (A) Spermatogonia and cysts of primary spermatocytes. (B) Cysts of spermatocytes. (C and D) Cysts of spermatocytes and lobules containing spermatozoa. (E) Gonadal myoid cells with remaining spermatozoa. (F) Perinuclear stage oocytes with a large circular nucleus and peripheral nucleoli. (G) Oocyte with cortical alveoli, chorion, and nucleus with detached nucleoli. (H) Vitellogenic oocyte with yolk granules filling most of the cytoplasm and a large central nucleus. (I) Vitellogenic oocyte with enlarged yolk granules, eccentric nucleus, and chorion. (J) Hydrated egg in follicle sac with post-ovulatory follicle. Arrowheads, spermatogonia; arrows, primary spermatocytes; ca, cortical alveoli; ch, chorion; ct, connective tissue; f, follicle layer; gmc, gonadal myoid cells; he, hydrated egg; n, nucleus; nu, nucleolus; SC, spermatocytes; SZ, spermatozoa; and yg, yolk granule. The scale bars represent 100 μm. A full colour version of this figure is available at http://dx.doi.org/10.1530/JME-13-0187.
whereas ovulation was seen in a few female individuals in May. The testis weight was 218-fold larger in February compared with August, whereas the ovary weight increased by 25.5-fold from August to March. The GSIs for both sexes decreased significantly from March to May. Histological observations of gonads revealed the maturation and differentiation status of germ cells: in testes, spermatogonia and several cysts of primary spermatocytes were observed in immature testis in August (Fig. 1A). In November, testes were mainly filled with the cysts of spermatocytes (Fig. 1B), and lobules containing spermatozoa were subsequently observed in February and March (Fig. 1C and D). After spermatiation, a number of gonadal myoid cells were preferentially observed in testis with remaining spermatozoa in lobules (Fig. 1E). In ovaries, perinuclear stage oocytes were mainly observed in August (Fig. 1F) and then some oocytes with cortical alveoli were observed in November (Fig. 1G). Vitellogenic oocytes with yolk granules accumulating in their cytoplasm were found mainly in February (Fig. 1H). Enlarged yolk granules and eccentric nuclei were observed in vitellogenic oocytes in March (Fig. 1I). Post-ovulatory follicles and remaining hydrated eggs in follicle sacs were found in May (Fig. 1J).

### Three esr paralogs in Atlantic cod

Partial cDNA sequences of three esr genes in Atlantic cod were obtained using a combination of *in silico* and experimental cloning: esr1 (JX178935), esr2a (JX178936), and esr2b (JK993476). Partial ERα AA sequences (479 AA) overlapped on 83% of the region of full-length orange-spotted grouper ERα with 81% similarity and 75% identity, and five conserved essential domains of ERs were identified: A/B (98 AA (partial)), C (84 AA), D (62 AA), E (240 AA), and F (64 AA). For ERβ1, the 532 AA partial sequence covered 94% of the region of the full-length Japanese flounder ERβ with 83% similarity and 74% identity and contained the essential domains A/B (157 AA), C (82 AA), D (47 AA), and E (246 AA (partial)). In addition, the 61 AA ERβ2 partial sequence corresponded to 9% of full-length gilt-head bream ERβ and comprised A/B (24 AA in (partial)) and C (37 AA in (partial)) domains. Bayesian analysis using *in silico*-extended cod sequences containing the E-domain produced a consensus phylogenetic tree that displayed a clear separation of ERα, ERβ1, and ERβ2 subtypes into three main clades, whereas all the homologs of estrogen-related receptor α (ESRRA) were grouped outside of all ER clades (Fig. 2). In the teleost ERα clade, an isolated subcluster containing ERα1 and ERα2 from a few teleost species (e.g. Carassius auratus, O. mykiss, and Spinibarbus denticulatus) was observed. G. morhua ERα was grouped in the ERα clade. The two clades of teleost ERβ1 and ERβ2 displayed similar topologies. In both the teleost ERβ1 and ERβ2 clades, G. morhua ERβ1 and ERβ2 were most closely related to O. mykiss ERβ1 and ERβ2 respectively. Unexpectedly, some Cyprinidae species (e.g. S. denticulatus, Varicorhinus barbatulus, C. auratus, and Danio rerio) showed an isolated subcluster distinct from teleost ERβ1 and ERβ2.

#### Tissue distribution of esr transcripts

The genes esr1, esr2a, and esr2b were differentially expressed across various tissues in both adult male and female Atlantic cod (Fig. 3). The esr1 transcripts were mainly found in liver and gonads in both sexes, but prominent in testes and in livers of females. The esr2a
transcripts were widely distributed in several tissues with varied expression levels. Nevertheless, the esr2a transcripts exhibited a sex-dependent distribution in liver and gonads, similar to esr1. Expression of esr2a was found in skin of males and blood of females, whereas the esr2a transcripts were seen in the pronephros and excretory kidney in males but not much in females. The esr2b transcripts had a broad distribution in female tissues, while their expression was found in fewer male tissues, including testis and liver. Notably, expression of esr genes in liver was more prominent in females than in males and all esr transcripts were consistently present at higher levels in testis than in ovary, except for esr2b. In brain, low transcript levels of esr1 in females and esr2a in males were observed but expression of esr2b was not detected.

Localization of esr transcripts in testis

The central region of the testes was sampled for histological analyses (Fig. 4A) and sequential cross sections of the testicular lobe were prepared (Fig. 4B). Hematoxylin staining showed the presence of spermatogonia and spermatocytes (Fig. 4C). ISH with a vasa probe specifically stained germ cells (Fig. 4D), while staining with gsdf or amh probes affected Sertoli cells surrounding germ cell cysts (Fig. 4E and F). A 39hsd probe detected Leydig cells in the interstitial tissue between lobule segments (Fig. 4G). There was no visible difference in localization between esr1 and esr2a in testis, even though the staining intensity of esr2a was higher than that of esr1 (Fig. 4H and I). These two esrs were preferentially observed in the testicular interstitial fibroblasts, whereas weak signals for esr2b were detected in some interstitial fibroblasts (Fig. 4J). No hybridization signals were observed with sense probes (Supplementary Figure 1, see section on supplementary data given at the end of this article).

Localization of esr transcripts in ovary

Sequential cross sections of ovary were prepared and used for histological analyses (Fig. 5A and B). Several developmental stages of oocytes could be visualized by hematoxylin staining (Fig. 5C). ISH with a vasa probe strongly stained pre- and early-vitellogenic oocytes rather than midvitellogenic oocytes (Fig. 5D), while a gsdf probe stained the follicular cell layer surrounding oocytes at all developmental stages (Fig. 5E). Signals for esr1 were predominantly observed in follicular cells, (Fig. 5F), whereas, esr2a signals were mainly detected in the follicular cell layer and the cytoplasm of pre- and early-vitellogenic oocytes (Fig. 5G). In both follicular cells and the cytoplasm of all oocytes, esr2b signals were detected (Fig. 5H). No hybridization signals were observed in any of the cells with negative control probes (Supplementary Figure 2, see section on supplementary data given at the end of this article).

Sexually dimorphic expression of ar, cyp19a1a, and esr genes during a reproductive cycle

The levels of esr1 mRNA were upregulated by 12.6- and 7.0-fold from August to March in both testes and ovaries respectively (Fig. 6A). In March, esr1 mRNA levels in testes were 5.0-fold higher than those in ovaries (Fig. 6A). A significant increase in esr2a mRNA level in testes was particularly evident in February and March (P<0.05; Fig. 6B), but no significant alterations in esr2a in ovaries were observed throughout the reproductive cycle. The level of esr2a mRNA in testes was 13.3- and 12.0-fold higher than that in ovaries in February and March respectively (Fig. 6B). The level of esr2b mRNA in ovaries was elevated up to 24.9-fold from August to March and then rapidly decreased by May, showing a temporal pattern similar to esr1 in testis (Fig. 6C). In addition, ar mRNA levels in testes were elevated up to 6.5-fold from August to March and then decreased in May, while ar expression in ovaries had a modest peak in November (Fig. 6D). Remarkably, cyp19a1a transcript levels in ovaries increased by 37.5-fold from August to March, whereas cyp19a1a was barely detected in testes throughout the reproductive cycle (Fig. 6E).
Discussion

Knowledge about the direct functions of estrogen in gonadal cells, and particularly the role of ER subtypes, is still limited to a few mammal species. In this study, we characterized three esr paralogs in Atlantic cod that were hitherto unknown in the teleost superorder Paracanthopterygii. The sexually dimorphic expression of esr transcripts in gonads with peaks before the spawning period throughout a reproductive cycle represents the first observation, to our knowledge, in a batch spawner.
In teleost fish, two subtypes of ERβ (ERβ1 and ERβ2 - also named ERγ) have been identified in distant taxa. In contrast, most fish species have only ERα, but two ERα isoforms (ERα1 and ERα2) have recently been reported in salmonids (rainbow trout; Nagler et al. 2007) and cyprinids (goldfish C. auratus and phoenix barb S. denticulatus; Zhu et al. 2008). Atlantic cod has a single ERα subtype, supporting the hypothesis that the two ERα subtypes (ERα1 and ERα2) reported in goldfish, rainbow trout, and phoenix barb are derived from the recent tetraploidization event that is thought to have occurred in the salmonid and cyprinid lineages. Within the ERβ subtype, esr2a and esr2b were in silico mapped on different scaffolds in the Atlantic cod genome. The chromosomal localization of esr2a and esr2b indicates that these paralogs arose from the 3R teleost-specific whole-genome duplication event that occurred 350 million years ago (Meyer & Van de Peer 2005).

Teleost esrs exhibit a wide variety of tissue distribution patterns but with common E2 target tissues associated with reproduction such as liver and gonads (Filby & Tyler 2005, Chen et al. 2011). In Atlantic cod, all three esr transcripts were present in liver and gonads with a sexual dimorphic expression pattern. Prominent expression of esrs in livers of females compared with livers of males could be caused by the higher endogenous E2 level in female serum (Norberg et al. 2004). In gonads, esr1 and esr2a were predominantly expressed in testes compared with ovaries indicating an...
important role of both ERs in spermatogenesis, as proposed for other teleost species (Pinto et al. 2006, Zhu et al. 2008).

In rodents, several studies have demonstrated that the spatial distribution of ER subtypes in testis varies significantly among species, as reviewed by O’Donnell et al. (2001), but there is a consensus that both ERα and ERβ in rodent ovary are expressed in granulosa cells of follicles (Drummond & Findlay 1999). In testis, there is very limited knowledge about the localization of ER mRNA or protein in adult gonads and their expression pattern is controversial (Bouma & Nagler 2001, Wu et al. 2001, Andreassen et al. 2003, Chakraborty et al. 2011b). This study delivers significant insights into this matter by identifying the cells expressing esr paralogs in fish gonads for both sexes. In cod testis, all three esr transcripts were preferentially found in testicular interstitial fibroblasts, similar to the localization of rainbow trout ERα protein in testis (Bouma & Nagler 2001) but not in the Sertoli cells surrounding spermatogonia. It has been reported that rainbow trout ERα protein is expressed in interstitial fibroblasts containing Leydig cell precursors and is involved in differentiation of precursors into mature Leydig cells (Bouma & Nagler 2001). In Atlantic cod testis, the number of the esr1/esr2a-expressing interstitial fibroblasts was larger than that of the 3βhsd-expressing interstitial fibroblasts (Fig. 3G, H and I), indicating that esr1/esr2a-expressing cells in testicular interstitial fibroblasts could be both precursor and mature Leydig cells. Therefore, the main target cells of E2 via ERs in Atlantic cod testicular cells could be Leydig cells, and ERs (particularly in esr1 and esr2a) may be involved in growth and maturation of Leydig cells to maintain their steroidogenesis. Nevertheless, there are reports of results conflicting with our observations, which show predominant localization of esr in fish mitotic or meiotic germ cells in eelpout (Andreassen et al. 2003) and channel catfish (Wu et al. 2001), indicating a diversity of E2 signaling requirements of spermatogenic cells among teleosts. There are only two other reports describing the localization of esrs in teleost ovarian cells so far: esr1 in eelpout (Andreassen et al. 2003) and esr1, esr2a, and esr2b in medaka (Chakraborty et al. 2011b). In Atlantic cod, transcripts of the three esr genes were differentially localized in ovaries even though they were all observed in follicular cells surrounding mid-vitellogenic oocytes. Some degree of conservation in esr expression patterns may be noted between cod and the above two species. First, esr1 is preferentially weakly expressed in follicular cells of pre- to mid-vitellogenic oocytes. In contrast, esr2a is predominantly expressed in the cytoplasm of pre- and early-vitellogenic oocytes rather than their follicular cells. Furthermore, esr2b is mainly expressed in both the follicular layer and cytoplasm of oocytes. These observations of ovarian cells highlight the potential importance of all three esrs in follicular cell growth and differentiation during cod folliculogenesis, as suggested for rodents (Drummond & Findlay 1999, Drummond et al. 1999). Further studies are required to specify the function of ER in fish oocytes.

It is noteworthy that Atlantic cod testes have a unique structure in each individual testicular lobe: undifferentiated spermatogonia and the most advanced germ cells are distributed in the periphery of the lobe and in the vicinity of the collecting duct respectively (Almeida et al. 2008). Interestingly, this gradient distribution of different germ cell types was also visualized in our study using the ISH detection system with a gsdf probe (upper right panel in Fig. 4E). Similar to rainbow trout (Sawatari et al. 2007), Atlantic cod gsdf was highly expressed in the Sertoli cells surrounding undifferentiated and/or early spermatogonia rather than the Sertoli cells surrounding cysts of advanced germ cells such as spermatocytes and spermatzoa. In contrast, amh transcripts...
were distributed in Sertoli cells with similar signal intensities regardless of germ cell developmental progression.

To the best of our knowledge, this is the first paper examining seasonal changes in all three esr transcripts in both testes and ovaries of a batch spawner throughout an annual reproductive cycle. At several time points, the three esr paralogs were differentially expressed in male vs female gonads. The results of GSI changes and histological observations of gonads enabled us to assume that their main spawning period may be April and revealed a clear annual reproductive cycle in 2-year-old Atlantic cod under the experimental conditions used, with a similar sexual maturation process to that reported by Karlsen et al. (2006).

Gonadal esr1 mRNA expression increased significantly in both testes and ovaries as gonadal maturation progressed and decreased after the spawning period, resembling the results of esr1 expression in orange-spotted grouper ovaries at different developmental stages (Chen et al. 2011). A similar increase in mRNA level was also found for esr2a in testes and for esr2b in ovaries. These sexually dimorphic expression patterns of esr paralogs along with the observed significant increase in their relative mRNA levels indicate that E2 signaling via ERs may be essential for Leydig cells in testes and follicular cells in ovaries during gonadal maturation period. As esr1/esr2a genes are preferentially expressed in Leydig cells in cod testes and esr1 is specifically expressed in the follicular layer in cod ovaries, it is plausible that this increase in their mRNA levels may be associated with Leydig cell proliferation before the spawning period and proliferation of follicular cells during oocyte growth and maturation respectively. Recently, an in vitro study uncovered the novel pathway of the direct function of estrogen in the marsupial mammal gonad: the function of estrogen mediated by ER blocks the nuclear entry of SOX9 into gonadal somatic cells, preventing the testicular gene activation pathway and allowing upregulation of key female genes in ovarian development (Pask et al. 2010). Therefore, further studies may examine the suppression of the sox9 transcription pathway by activated ER in gonadal somatic cells in fish.

AR plays a key role in androgen action and it is essential for the maintenance of spermatogenesis in testis (Ikeuchi et al. 2001). Almeida et al. (2009) reported that Atlantic cod plasma 11-ketotestosterone levels increased in February and March along with the advancement of spermiogenesis and spermiogenesis, in agreement with an increase in GSI. Moreover, they showed that ar mRNA was prominently localized in Sertoli cells regardless of growing spermatogonial clones. In our study, we observed a gradual increase in ar expression in testis toward the spawning season and significantly higher transcript levels in February and March along with the increase in GSI in testis, indicating that the number of Sertoli cells is increasing at this point. Taken together with the concomitant increase in plasma androgen levels (Almeida et al. 2009), our data indicate the potential importance of androgen signaling in Sertoli cells for maintaining spermatogenesis and spermiogenesis in cod. In contrast, we found only a modest peak of ar expression in ovaries in November, implying proliferation of the follicle cells and the epithelial cells of the ovigerous lamellae, as reported in ovary of Japanese eel Anguilla japonica (Tosaka et al. 2010). It should be noted that the molecular function of AR in teleost ovaries still remains to be investigated.

Aromatase is a key enzyme for converting androgens to estrogens during sexual development. It is encoded by a single gene in mammals, with exception of the domestic pig and other suiforms, which have multiple cyp19 genes (Corbin et al. 2009). In contrast, teleosts have two cyp19 genes: cyp19a1a is preferentially expressed in ovaries, whereas cyp19a1b is found predominantly in brain (Tchoudakova & Callard 1998). Our current knowledge of seasonal changes of cyp19a1a expression in fish ovaries throughout a reproductive cycle is still very limited (Sampath Kumar et al. 2000, Rocha et al. 2009, Johnsen et al. 2013). In Atlantic cod, cyp19a1a transcripts are constantly present at higher levels in ovaries than in testes, with a significant increase in expression in ovaries but not in testes towards the spawning season. Importantly, these data indicate that cyp19a1a has potential as a molecular indicator of ovarian maturation status in batch-spawning teleosts. It should be noted that the upregulation pattern of mRNA levels for cyp19a1a and esr1/esr2b in ovaries coincided with the increase in plasma E2 levels in female cod reported by Norberg et al. (2004), indicating that estrogen biosynthesis and its signaling via ERs are particularly important during the period before spawning. In conclusion, we identified three ER subtypes in Atlantic cod, representing the superorder Paracanthopterygii, and found that esr1, esr2a, and esr2b transcripts were detected mainly in liver and gonads of fish of both sexes. All three esr paralogs were predominantly localized within interstitial fibroblasts in testes, whereas they were differentially expressed in both follicular cells and oocytes in ovaries. Throughout an annual reproductive cycle, the mRNA level of three esr genes, as well as ar and cyp19a1a, displayed sex-dependent upregulation in gonads with a peak during the period before spawning, indicating their importance in
preparation for this event, perhaps through activation of ER-responsive genes and proliferation of Leydig/Sertoli and follicular cells. These findings provide new insights into the role of ERs in gonadal development during the reproductive cycle of batch-spawning teleosts.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JME-13-0187.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
I B and J M O F conceived and designed the experiments. K N and C P analyzed the data. I B and J M O F wrote the paper.

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