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 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 either sex. *In situ* hybridization of *esr*s along with germ cell (*vasa*) and gonadal somatic cell markers (*gsdf*, *3β-hsd* and *amh* for testicular, or *gsdf* for ovarian somatic cells) showed that in testis all three *esr*s were preferentially localized within interstitial fibroblasts composed of immature and mature Leydig cells, whereas in ovary they were differentially expressed in both follicular cells and oocytes. Quantitative real-time PCR analysis revealed a sexually dimorphic expression pattern of the three *esr* paralogues in testis and ovary. A significant increase in *esr2a* expression was identified in testis and *esr2b* in ovary, whereas *esr1* transcripts were elevated in both testis and ovary at February and March prior to the spawning period. The localization and sexually dimorphic expression of *esr* genes in gonads suggest 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 estradiol-17β (E_2), act directly to promote sex differentiation and gonadal maturation (Korach 1994). Estrogen action has been classically known 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 number of organisms from human (Keaveney *et al.* 1991) to nematode (Mimoto *et al.* 2007). The role of ERs in gonadal development has been elucidated from the phenotypic alterations of knock-out (KO) mice. The KO mice lacking ERα showed complete infertility in both male and female (Lubahn *et al.* 1993), whereas both male and female ERβ-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, suggesting the possible direct action by estrogen in gonadal cells in vertebrates. It has been known that the concentration of estrogen in testis and rete testis fluid far exceed 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 overview the localization of ER subtypes within gonads, O'Donnell *et al.* (2001) surmised the likely localization of ERs in adult rodent testis from numerous conflicting data: Leydig cells express both ERα and ERβ, whereas Sertoli cells and testicular germ cells (e.g., spermatogonia, pachytene spermatocytes and round spermatid) predominately expressed ERβ. The direct effects of estrogen on gonad development have been observed by 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 administrated with estrogen displayed increased numbers of undifferentiated and differentiating type-A spermatogonia (Kula 1988). Within rodent ovary, 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, ArKO mice that lack functional aromatase (Britt *et al.* 2001) - an alternative model of ER depletion - exhibit abnormal folliculogenesis as seminiferous tube-like structures due to apoptotic granulosa cells (Drummond *et al.* 2002, Drummond & Findlay 1999). Moreover, an *in vitro* culture study of mice ovarian follicle reported that ERβ, but not ERα, plays a direct role in folliculogenesis to facilitate follicle maturation including E_2 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 in gonadal development and the role of ERs are poorly understood (Shi *et al.* 2011). ER expression is first detected in teleosts at the embryonic stage, indicating an essential role of estrogen signaling for 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: ERα in the interstitial fibroblasts (the Leydig cell precursors) in testis of rainbow trout *Onchorhynchus mykiss* (Bouma & Nagler 2001), ERα in thecal, granulosa and interstitial cells in ovary and in Sertoli cells in testis of eelpout *Zoarces viviparus* (Andreassen *et al.* 2003), and ERα in granulosa cells in ovary and ERβ2 in cells in undifferentiated gonad in medaka *Oryzias latipes* (Chakraborty *et al.* 2011b). Therefore, information about localization patterns and expression profiles for ER subtypes in teleost gonadal cells is required 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 at two years old in aquaculture conditions. This is an important bottleneck for the cod farming industry, since it is associated with a significant reduction in somatic growth (Karlsen *et al.* 2006). Vitellogenesis generally starts in the fall 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 in several days intervals (Kjesbu 1994). The timing of sexual maturation and spawning period are strongly associated with a peak of the plasma E_2 levels at two- and three-year-old Atlantic cod under aquaculture conditions (Norberg *et al.* 2004). However, molecular mechanisms underlying the direct effect of E_2 signaling in 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 two-year-old Atlantic cod in land-based tanks and examined their annual reproductive cycle throughout one year. In this report, we focused on the estrogen receptors in gonads: ERα (*esr1*) and two paralogues of ERβ (*esr2a* and *esr2b*). In addition, we also analyzed the relative mRNA levels of androgen receptor (*ar*) which mainly acts in spermatogenesis (Shi *et al.* 2012), and cytochrome P450 aromatase (*cyp19a1a*) catalyzing the biosynthesis of estrogens (Johnsen *et al.* 2013) to determine their association with *esr* expression in gonads.

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 two-year old adults (initial weight of 1.1 ± 0.2 kg, mean ± standard deviation [SD], n = 18), they were transferred to three land-based flow-through tanks (40 m^3) at a density of approximately 100 individuals per tank from August 2009 until May 2010. Sea water (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 each tank (n = 24), euthanized by exposure to 0.5 $g \cdot L^{-1}$ tricaine methanesulfonate (Sigma-Aldrich, Oslo, Norway), measured total length (TL), total body weight (TW), gonad weight (GW) and calculated the gonadosomatic index (GSI, 100 × GW / TW (%)) at each sampling time point (August, November in 2009, February, March and May in 2010; Table 1). Differences in all numerical data each sampling point within the same sexes 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 prior sampling other tissues. Excised tissues were then rinsed three times with phosphate buffered saline (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 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 by hematoxylin-eosin. All procedures used in this study were in accordance with the guidelines set by the National Animal Research Authority (Forsøksdyrutvalget, 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, Nydalen, Sweden) as detailed elsewhere (Campos *et al.* 2010) and quantified by spectrophotometry with a NanoDrop ND-1000 (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 Quantitect reverse transcriptase kit (Qiagen, Nydalen, Sweden) as previously reported (Campos *et al.* 2010).

cDNA cloning of estrogen receptor (*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) (Flicek *et al.* 2013). The following protein sequences were used as query: i) gilthead seabream ERα (AAD31032.2), ERβ1 (AAD31033.1) and ERβ2 (CAE30470.1), and ii) rainbow trout ERα1 (NP_001117821.1), ERα2 (NP_001118030.1), ERβ1 (NP_001118225.1) and ERβ2 (NP_001118042.1). After extracting the corresponding genomic sequences (*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). PCR reactions

were performed as described (Nagasawa *et al.* 2012). 5' rapid amplification of cDNA ends (RACE) of *esr2a* used the gene-specific primers listed in Table 2 and the GeneRacer™ Kit with SuperScript™ III RT (Invitrogen, Carlsbad, CA, USA), 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 TBLASTN searches against the nr 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 sequences were aligned with corresponding orthologues in various species (Table S1) using MUSCLE (www.drive5.com). Poorly aligned or divergent regions were trimmed by Gblocks 0.91b (molevol.cmima.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 with 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%, 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 by a final elongation step of 72°C for 3 min. 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 (Oslo, Norway). Expression patterns were confirmed using two biological replicates of each sex.

In situ hybridization (ISH)

Spatial expression patterns of *esr* paralogues were determined by ISH, along with those of known germ cell and gonadal somatic cell markers reported in other teleosts. *Vasa* was used for detection of spermatogonia/spermatocytes in testis or oogonia/oocytes in ovary because of its well-conserved spatio-temporal expression pattern amongst teleosts, including Atlantic cod (Presslauer *et al*. 2012). The gonadal soma-derived factor (*gsdf*) was used to identify Sertoli cells in testis or follicular cells in ovary, since 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 (*3β-hsd*) as a marker, as reported in rainbow trout (Kobayashi *et al*. 1998), swamp eel (Lo Nostro *et al*. 2004) and Nile tilapia (Ruksana *et al*. 2010). The *gsdf* and *3β-hsd* genes were cloned from Atlantic cod in this study. Digoxigenin-labeled sense and anti-sense RNA probes of all genes examined (*esr1*, *esr2a*, *esr2b*, *vasa*, *gsdf*, *amh* and *3β-hsd*) were individually *in vitro* transcribed by T3 or T7 RNA polymerase (Roche) from corresponding DNA fragments amplified using the primers shown in Table 2. Tissue samples were obtained from two-year-old Atlantic cod reared at Mørkvedbukta Research Station. Testis (GSI = 0.4%) and ovary (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 testis and ovary 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 of all genes (*esr1*, *esr2a*, *esr2b*, *vasa*, *gsdf*, *amh* and *3β-hsd*) were individually hybridized at 70°C for 24 h

and then washed out with RNase A treatment (Sigma-Aldrich) as previously reported (Nagasawa *et al.* 2009). Sections were mounted with gelatin embedding medium (Sigma-Aldrich) and observed under a BX-51 microscope (Olympus, Oslo, Norway).

Quantitative real-time PCR (qPCR)

Twenty-fold diluted cDNA samples synthesized as above, negative (minus reverse transcriptase) and no template (water) controls were used in duplicate. Gene-specific qPCR primers for the three *esr* paralogues were designed with 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 reaction 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 previously described (Campos *et al.* 2010). Specificity of the reactions was evaluated by melting curve analysis and further Sanger sequencing of amplicons. Amplification efficiencies were calculated by standard curves from a five-fold dilution series (1:1, 1:5, 1:25, 1:125 and 1:625) of pooled cDNA as detailed (Fernandes *et al.* 2008). Cycle threshold (C_T) values were determined with the LightCycler® 480 software using a level of fluorescence intensity arbitrarily set to one. Three endogenous reference genes, β-actin (*actb*), *arp* and eukaryotic elongation factor 1α (*eef1a*), were validated as reported (Fernandes *et al.* 2008, Nagasawa *et al.* 2012). Their geNorm stability values (*M*) were 0.812, 0.779 and 0.618 for *actb*, *arp* and *eef1a*, respectively. The most stable gene combination was found to be *arp* + *eef1a* with an average pairwise variation $V_{2/3}$ =0.258. Therefore, raw qPCR data of target genes was corrected by the geometric average of *arp* and *eef1a* quantities as normalization factors. Differences in relative expression level of three *esr*s, *ar* and *cyp19a1a* amongst different sampling time points or between sexes were

examined by Mann-Whitney tests on GraphPad Prism software, since 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 two-year-old Atlantic cod

Atlantic cod kept in land-based tanks throughout a year showed a clear reproductive cycle with significant increase of gonad weight (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). Spermiation was seen in some male individuals in February and March, whereas ovulation was seen in a few female individuals in May. The testis weight was 218-fold larger in February compared to August, whereas the ovary weight increased 25.5-fold from August to March. The GSIs for both sexes decreased significantly from March to May. Histological observations in gonads revealed 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 seen in February and March (Fig. 1C, D). After spermiation, 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 seen in August (Fig. 1F) and then oocytes with cortical alveoli were partially observed in November (Fig. 1G). Vitellogenic oocytes with yolk granules accumulating their cytoplasm were found mainly in February (Fig. 1H). Enlarged yolk granules and eccentric nucleus were seen in vitellogenic oocytes in March (Fig. 1I).

Post-ovulatory follicles and remained hydrated eggs in follicle sacs were found in May (Fig. 1J).

Three *esr* paralogues 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 amino acid (AA) sequences (479 AA) overlapped on 83% 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 in partial), C (84 AA), D (62 AA), E (240 AA) and F (64 AA). For ERβ1, the 532 AA partial sequence covered 94% region of 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), E (246 AA partial). In addition, the 61 AA ERβ2 partial sequence corresponded to 9% of full-length gilthead sea bream ERβ2 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 the all homologs of estrogen-related receptor alpha (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., *C. auratus*, *O. mykiss* and *S. denticulatus*) was observed. *G. morhua* ERα was grouped in the ERα clade. The two clades of teleost ERβ1 and ERβ2 displayed similar topologies. In the both 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*, *V. barbatulus*, *C. auratus* and *D. rerio*) showed an isolated subcluster from teleost ERβ1 and ERβ2.

Tissue distribution of *esr* transcripts

Esr1, *esr2a* and *esr2b* were differentially expressed across various tissues in both adult male and female Atlantic cod (Fig. 3). *Esr1* transcripts were mainly found in liver and gonads in both sexes, but prominent in testis and female liver. *Esr2a* transcripts were widely distributed in several tissues with varied expression levels. Nevertheless, *esr2a* transcripts exhibited a sex-dependent distribution in liver and gonads, similarly to *esr1*. Expression of *esr2a* was found in skin of male and blood of female, whereas *esr2a* transcripts were seen in the pronephros and excretory kidney in males but not much in females. *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 was more prominent in female than male liver, and all *esr* transcripts were consistently present in testis than in ovary, except for *esr2b.* In brain, low transcript levels of *esr1* in female and *esr2a* in male were observed but expression of *esr2b* was not detected.

Localization of *esr* transcripts in testis

The testis central region 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 visualized Sertoli cells surrounding germ cell cysts (Fig. 4E, F). A *3β-hsd* 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 *esr1* (Fig. 4H, I). These two *esr*s were preferentially observed in the testicular interstitial fibroblasts, whereas weak signals of *esr2b* were detected in some interstitial

fibroblasts (Fig. 4J). No hybridization signals were observed with sense probes (Supplementary figure 1).

Localization of *esr* transcripts in ovary

Sequential cross sections of ovary were prepared and used for histological analyses (Fig. 5A, 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 mid-vitellogenic oocytes (Fig. 5D), while a *gsdf probe* stained the follicular cell layer surrounding oocytes at all developmental stages (Fig. 5E). *Esr1* signals 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). *Esr2b* signals were detected in both follicular cells and the cytoplasm of all oocytes (Fig. 5H). No hybridization signals were observed in any of the cells with negative control probes (Supplementary figure 2).

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

Esr1 mRNA levels were up-regulated 12.6- and 7.0-fold from August to March in both testis and ovary, respectively (Fig. 6A). *Esr1* mRNA levels in testis in March were 5.0-fold higher than in ovary (Fig. 6A). A significant increase of *esr2a* mRNA level in testis was particularly evident in February and March ($P < 0.05$, Fig. 6B), but no significant alterations of *esr2a* in ovary were observed throughout the reproductive cycle. *Esr2a* mRNA level in testis was 13.3- and 12.0-fold higher than in ovary in February and March, respectively (Fig. 6B). *Esr2b* mRNA level in ovary was elevated up to 24.9-fold from August to March and then rapidly decreased by May, showing a similar temporal pattern to *esr1* in testis (Fig. 6C). In addition, *ar* mRNA levels in testis were elevated up to 6.5-fold from August to March and then

decreased in May, while *ar* expression in ovary had a modest peak in November (Fig. 6D). Remarkably, *cyp19a1a* transcript levels in ovary increased 37.5-fold from August to March, whereas *cyp19a1a* in testis was barely detected 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 the present study, we characterized three *esr* paralogues in Atlantic cod that were hitherto unknown in the teleost superorder Paracanthopterygii. The sexually dimorphic expression of *esr* transcripts in gonads with peaks prior to the spawning period throughout a reproductive cycle represent the first observation 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 *Carassius auratus* and phoenix barb *Spinibarbus 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. Their chromosomal localization *esr2a* and *esr2b* suggests that these paralogues arose from the 3R teleost-specific whole genome duplication event that occurred 350 million years ago (Meyer & Van de Peer 2005).

Teleost *esr*s exhibit a wide variety of tissue distribution patterns but with common E_2 target tissues associated with reproduction such as liver and gonads (Chen *et al.* 2011, Filby & Tyler 2005). In Atlantic cod, all three *esr*s transcripts were present in liver and gonad with

a sexual dimorphic expression pattern. Prominent expression of *esr*s in female liver compared to male's could be caused by higher endogenous E_2 level in female serum (Norberg *et al.* 2004). In gonads, *esr1* and *esr2a* were predominantly expressed in testis compared to ovary indicating an important role of both ERs on spermatogenesis, as suggested in other teleost species (Pinto *et al.* 2006, Zhu *et al.* 2008).

In rodents, several reports have shown that the spatial distribution of ER subtypes in testis varies significantly amongst 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 teleosts, there is very limited knowledge about the localization of ER mRNA or protein in adult gonads and their expression pattern is controversial (Andreassen *et al.* 2003, Bouma & Nagler 2001, Chakraborty *et al.* 2011a, Wu *et al.* 2001). The present study delivers significant insights on this matter by identifying the cells expressing *esr* paralogues in fish gonads for both sexes. In cod testis, all three *esr* transcripts were preferentially found in testicular interstitial fibroblasts, similarly 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, number of the *esr1*/*esr2a*-expressing interstitial fibroblasts was larger than that of *3β-hsd*-expressing interstitial fibroblasts (Fig. 3G-I), suggesting that *esr1*/*esr2a*-expressing cells in testicular interstitial fibroblasts could be both precursor and mature Leydig cells. Therefore, the main target cells of E_2 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 conflicting reports to our observation, which show predominant *esr* localization in fish mitotic or meiotic germ cells in

eelpout (Andreassen *et al.* 2003) and channel catfish (Wu *et al.* 2001), suggesting a diversity of E_2 signaling requirement on spermatogenic cells amongst teleosts. There are only two other reports describing the localization of *esr*s in teleost ovarian cells so far: *esr1* in eelpout (Andreassen *et al.* 2003) and *esr1*, *esr2a* and *esr2b* in medaka (Chakraborty *et al.* 2011a). In Atlantic cod, transcripts of the three *esr* genes were differentially localized in ovary 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 expressed in follicular cells of pre- to mid-vitellogenic oocytes with weak expression. In contrast, *esr2a* is predominantly expressed in cytoplasm of pre- and early-oocytes rather than their follicular cells. Furthermore, *esr2b* is mainly expressed in both the follicular layer and cytoplasm of oocytes. These observations in ovarian cells highlight the potential importance of all three *esr*s on follicular cell growth and differentiation during cod folliculogenesis, as suggested in rodents (Drummond *et al.* 1999, Drummond & Findlay 1999). Further studies are required to specify the ER function in fish oocytes.

It is noteworthy that Atlantic cod testis has a unique structure in each individual testicular lobe: undifferentiated spermatogonia and 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 by using ISH detection with a *gsdf* probe (upper right panel in Fig 3E). Similarly 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 cyst of advanced germ cells such as spermatocytes and spermatozoa. In contrast, *amh* transcripts were distributed in Sertoli cells with similar signal intensity regardless of germ cell developmental progression.

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

Gonadal *esr1* mRNA expression increased significantly in both testis and ovary as gonadal maturation progressed and decreased after the spawning period, resembling the results of *esr1* expression in orange-spotted grouper ovary at different developmental stages (Chen *et al.* 2011). A similar increase in mRNA level was also found for *esr2a* in testis and for *esr2b* in ovary. These sexually dimorphic expression patterns of *esr* paralogues along with the observed significant increase of their relative mRNA levels indicate that E_2 signaling via ERs may be essential for Leydig cells in testis and follicular cells in ovary during gonadal maturation period. Since *esr1/esr2a* are preferentially expressed in Leydig cells in cod testis and *esr1* is specifically expressed in the follicular layer in cod ovary, it is plausible that this increase in their mRNA levels be associated with Leydig cell proliferation prior to the spawning period and proliferation of follicular cells during oocyte growth and maturation, respectively. Recently, an *in vitro* study uncovered the novel pathway in direct function of estrogen in the marsupial mammal gonad: estrogen mediated with ER blocks the nuclear entry of SOX9 in gonadal somatic cells, preventing testicular gene activation pathway and permitting up-regulation of key female genes in ovarian development (Pask *et al.* 2010). Therefore, further studies may examine the suppression of *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 maintenance of spermatogenesis in testis (Ikeuchi *et al.* 2001). In cod, Almeida *et al.* (2009) reported that Atlantic cod plasma 11-ketotestosterone levels increased in February and March along with spermiogenesis and spermiation advancement, in agreement with GSI increase. Moreover, they have shown 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 towards the spawning season and significantly higher transcript levels in February and March along with the GSI increase in testis, suggesting 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 indicates the potential importance of androgen signaling in Sertoli cells to maintain spermatogenesis and spermiogenesis in cod. In contrast, we found only a modest peak of *ar* expression in ovary 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 ovary still remains to be investigated.

Aromatase is a key enzyme for converting androgens to estrogens in sexual development. It is encoded by a single gene in mammals, with exception of the domestic pig and other suiformes, which have multiple *cyp19* genes (Corbin *et al*. 2009). In contrast, teleosts have two *cyp19* genes: *cyp19a1a* is preferentially expressed in ovary, whereas *cyp19a1b* is found predominantly in brain (Tchoudakova & Callard 1998). Our current knowledge of seasonal changes of *cyp19a1a* expression in fish ovary throughout a reproductive cycle is still very limited (Rocha *et al.* 2009, Sampath Kumar *et al.* 2000, Johnsen *et al.* 2013). In Atlantic cod, *cyp19a1a* transcripts are constantly present in ovary at higher levels than in testis, with a significant expression increase in ovary but not in testis towards the spawning season. Importantly, these data suggest that *cyp19a1a* has potential as a

molecular indicator of ovarian maturation status in batch-spawning teleosts. It should be noted that the up-regulation pattern of mRNA levels for *cyp19a1a* and *esr1/esr2b* in ovary coincided with the increase of plasma E_2 levels in female cod reported by Norberg *et al.* (2004), suggesting that estrogen biosynthesis and its signaling via ERs are particularly important during the period prior to spawning. In conclusion, we identified three estrogen receptor subtypes in Atlantic cod, representing the superorder Paracanthopterygii, and found that *esr1*, *esr2a* and *esr2b* transcripts were detected mainly in liver and gonads of either sex. All three *esr* paralogues were predominantly localized within interstitial fibroblasts in testis, whereas they were differentially expressed in both follicular cells and oocytes in ovary. Throughout an annual reproductive cycle, the mRNA level of three *esr* genes, as well as *ar* and *cyp19a1a*, displayed sex-dependent up-regulation in gonads with a peak during the period prior to 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.

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 contributions

Conceived and designed the experiments: IB JMOF. Performed the experiments: KN CP LK. Analyzed the data: KN CP. Contributed reagents/materials/analysis tools: IB JMOF. Wrote the paper: KN CP LK IB JMOF.

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Figure legends

Fig. 1. Histology of representative testes and ovaries at different maturation status by hematoxylin-eosin staining in two-year-old Atlantic cod throughout annual reproductive cycle. A) Spermatogonia and cysts of primary spermatocytes. B) Cysts of spermatocytes. C, D) Cysts of spermatocytes and lobules containing spermatozoa. E) Gonadal myoid cells with remaining spermatozoa. F) Perinuclear stage oocytes with large circular nucleus and peripheral nucleoli. G) Oocyte with cortical alveoli, chorion and nucleus with detached nucleoli. H) Vitellogenic oocytes with yolk granules filling most of the cytoplasm and central large 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; yg, yolk granule. The scale bars represent 100 µm.

Fig. 2. Phylogenetic inference of ER subtypes found in vertebrates.

Arrowheads show positions of each ER subtype in Atlantic cod. Numbers at the nodes indicate posterior probability obtained from Bayesian analysis. GenBank accession numbers of all ERs can be found in Supplementary Table S1.

Fig. 3. Tissue distribution of three *esr* paralogues in two-year-old Atlantic cod adult tissues by semi-quantitative RT-PCR.

The three *esr* genes were differentially expressed amongst the tissues tested and showed a sexually dimorphic expression pattern (male GSI = 4.7%; female GSI = 1.5%). *Arp* was used as an endogenous reference gene, since it showed stable expression amongst all tissues in Atlantic cod.

Fig. 4. Localization of three *esr*s, *vasa*, *gsdf*, *3β-hsd* and *amh* transcripts in Atlantic cod testis.

A) Excised testis from two-year-old Atlantic cod (GSI = 0.4%). Dashed line indicates the region sampled for ISH analysis. a; anterior, p; posterior. B) Schematic representations of a testicular lobe connected to efferent duct (left panel in B) and its cross section image (right panel in B). Dashed line indicates the section location. The area enclosed by the black box in B indicates the position of low magnification fields (insets in C-J). C-J) Hematoxylin staining (C) and ISH staining with sense or anti-sense probes of *vasa* (D), *gsdf* (E), *amh* (F), *3β-hsd* (G), *esr1* (H), *esr2a* (I) and *esr2b* (J). C-J are high magnification fields taken from the adjacent area of insets. White arrowheads indicate spermatogonia. SC; spermatocytes. Arrows indicate the position of signals that were weakly detected in interstitial fibroblasts. The bars represent 5 cm (A), 100 μm (C-J) and 200 μm (insets in C-J).

Fig. 5. Localization of three *esr*s, *vasa* and *gsdf* transcripts in Atlantic cod ovary.

A) Excised ovary from two-year-old Atlantic cod (GSI = 1.3%). The region sampled for ISH analysis is indicated by the dashed line. a; anterior, p; posterior. B) Schematic representation of cross section of ovary. The area enclosed by the black box in B indicates the position of low magnification fields (insets in C-H).C-H) Hematoxylin staining (C) and ISH staining with anti-sense probes of *vasa* (D), *gsdf* (E), *esr1* (F), *esr2a* (G) and *esr2b* (H). C-H are high magnification fields taken from the adjacent area of insets.The bars represent 5 cm (A), 100 μm (C-H) and 200 μm (insets in C-H).

Fig. 6. Quantification of three *esr*s, *ar* and *cyp19a1a* in gonads of Atlantic cod throughout the reproductive cycle.

Black bars and white bars show the relative mRNA expression in testis and ovary, respectively. Transcript levels were quantified by real-time PCR. Error bars show standard error of mean on each sampling time point. Different superscript letters indicate significant

differences within the same sex throughout the reproductive cycle (capital letters: testis; lower-case letters: ovary; $n = 25$). Asterisks (*) indicates significant differences between testis and ovary at a particular sampling point at $P < 0.05$ ($n = 5$).

Supplementary Figure 1. Hematoxylin and ISH staining with sense or anti-sense probes of *vasa*, *gsdf*, *amh*, *3β-hsd*, *esr1*, *esr2a* and *esr2b* in testis. The bars represent 200 µm.

Supplementary Figure 2. Hematoxylin and ISH staining with sense or anti-sense probes of *vasa*, *gsdf*, *esr1*, *esr2a* and *esr2b* in ovary. S1; pre-vitellogenic oocyte, S2; early-vitellogenic oocyte and S3; mid-vitellogenic oocyte. The bars represent 50 µm (high magnification views of hematoxylin staining, right panel) and 200 µm (other panels).

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

Sex	Sampling point	TL (cm)	TW (g)	GW (g)	GSI (%)	*n*
Male	August 2009	44.3 ± 0.7 [a]	1050.0 ± 75.8 [a]	1.0 ± 0.5 [a]	0.1 ± 0.0 [a]	9
	November 2009	50.7 ± 1.0 [b]	1566.7 ± 92.5 [ab]	37.9 ± 6.9 [a]	2.5 ± 0.4 [a]	9
	February 2010	50.9 ± 1.4 [b]	1746.2 ± 158.0 [b]	218.1 ± 27.9 [b]	12.4 ± 1.0 [b]	8
	March 2010	53.6 ± 1.7 [b]	2346.0 ± 211.6 [c]	198.0 ± 25.9 [b]	11.2 ± 1.2 [b]	9
	May 2010	51.4 ± 1.3 [b]	1452.3 ± 121.2 [b]	12.1 ± 1.4 [a]	0.9 ± 0.1 [a]	7
Female	August 2009	45.2 ± 1.0 [a]	1143.3 ± 65.7 [a]	15.9 ± 1.8 [a]	1.4 ± 0.1 [a]	9
	November 2009	51.5 ± 0.9 [b]	1563.6 ± 72.5 [a]	38.9 ± 3.2 [a]	2.5 ± 0.2 [a]	14
	February 2010	52.6 ± 1.0 [b]	2098.2 ± 134.1 [b]	231.4 ± 21.1 [b]	11.2 ± 1.0 [b]	14
	March 2010	53.8 ± 1.0 [b]	1855.0 ± 119.9 [ab]	405.1 ± 51.7 [c]	18.3 ± 1.7 [c]	16
	May 2010	53.6 ± 0.9 [b]	1726.6 ± 83.1 [ab]	234.5 ± 58.6 [b]	12.9 ± 2.8 [b]	14

Values with the different lowercase letters indicate significant differences within the same sex ($P < 0.05$).

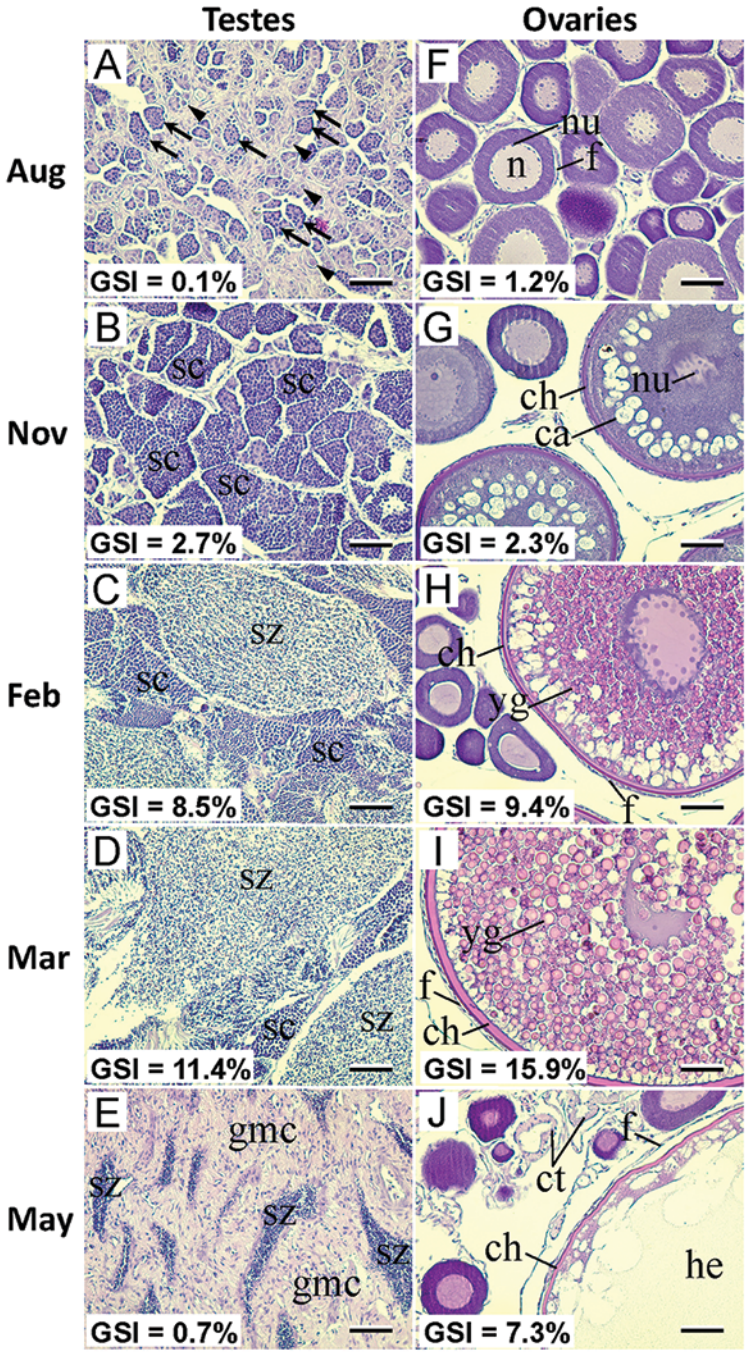
Table 2. Primers used for cDNA cloning, semi-quantitative RT-PCR, quantitative real-time PCR (qPCR) and *in situ* hybridization. Gene names, GenBank accession numbers, PCR efficiencies (E, %), coefficient of determination values (R^2) and amplicon sizes (bp) are indicated.

Gene	GenBank	5′ → 3′ forward primer	5′ → 3′ reverse primer	Purpose
esr1	JX178935	TGCAGTCCCTGGGCAGTGGGTCCACCA	TGACCTCGGTGTACGGCCGGTTCATCT	cloning/ISH
—	—	AGGCAGCTGGAGAACAGGACGTCGCC	TGGGCGTCCAGCATCTCCAGCAGCAGGT	cloning/ISH
—		AAAGGAGGTATGCGCAAGG	TGACCTCGGTGTACGGCCGGTTCATCT	RT-PCR/qPCR
esr2a	JX178936	GGACACTGACATGGACTGAAGGAGTA	TGCAGCTGGAGAACTGGTTGCTCCCGCT	cloning (5'RACE)
—	—	CCTTACACCGACCTGGGCCATGACT	GCATGATGTGGGCGTCCAGCATCTC	cloning/ISH
—	—	TTGGTATGGCAACCTCCTCCCCAA	GGCCGTAGAAGGGCACGGCGCCAA	cloning
—	—	CCTTACACCGACCTGGGCCATGACT	ACGACACCACACTCTCCTCGCTGTCCT	RT-PCR/qPCR
esr2b	JK993476	CAGATCGCTGCTGTCGTCCAATAAGA	CCGTAGTGGTACCCGGACGCGTAGTCCT	cloning/RT-PCR/I
ar	FJ268742	CGATATGTTCCCAGGAATGAG	GGTGGTTCTGTTTACCTGCTG	qPCR
cyp19a1	DQ402370	ACAACAACAAGTACGGCAGCAT	GTAGAGGAGCTGCTGAGGATGAG	qPCR
actb	EX739174	TGACCCTGAAGTACCCCATC	TCTTCTCCCTGTTGGCTTTG	qPCR
arp	EX741373	TGATCCTCCACGACGATGAG	CAGGGCCTTGGCGAAGA	RT-PCR/qPCR
eef1a	EX721840	CACTGAGGTGAAGTCCGTTG	GGGGTCGTTCTTGCTGTCT	qPCR
vasa	HM451456	CTGCGTGCGTCCAGTGGTGTTGTA	TCTTTCTGGAGTCCGTGGAGGCAA	ISH
gsdf	KC204828	ATGACAGTCCTGCTGGGCTCTTCCAT	ACAGCCACACTGGCGGGTCAGCACCG	ISH
3β-hsd	KC204829	ATGTCTCTGAGTGGGGACGTGTGTCT	TGCCTTCTGGTAGCCGAAGCTGAACG	ISH
amh	JN802292	TCCTACACCATCCCGGTGTCTTCAGG	TCAGCGGCAGCCACATTCTTTGGCA	ISH

Table S1. Genbank accession numbers of *esr* orthologues and *esr*-related genes.

Gene		*esr1a*	*esr1b*	*esr2a*	*esr2b*	*esrra*
Species/ common name						
Acanthopagrus schlegelii	black porgy	AAL82743.1	—	AAL82742.1	ABY60988.1	—
Amphiprion melanopus	fire clownfish	ADJ96332.1	—	ADJ96333.1	ADJ96331.1	—
Carassius auratus	goldfish	AAL12298.1	AAR17610.1	AAD26921.1	AAF35170.1	—
Danio rerio	zebrafish	NP_694491.1	—	NP_777287.2	NP_851297.1	NM_212955.1
Dicentrarchus labrax	European seabass	CAD43599.1	—	CAD33851.1	CAD33852.1	—
Epinephelus coioides	orange-spotted grouper	ADK90033.1	—	ADK90034.1	ADK90035.1	—
Fundulus heteroclitus	mummichog	AAT72914.1	—	AAU44352.1	AAU44353.1	—
Gadus morhua	Atlantic cod	JX178935	—	JX178936	—	—
Gasterosteus aculeatus	stickleback	BAF96738.1	—	ENSGACP00000009971*	ENSGACP00000000274*	—
Haplochromis burtoni	African cichlid	AAR82891.1	—	ABI18966.1	ABI18967.1	—
Homo sapiens	human	NP_000116.2	—	NP_001428.1	—	NP_004442.3
Micropogonias undulatus	Atlantic croaker	AAG16713.1	—	AAG16711.1	AAG16712.1	—
Micropterus salmoides	largemouth bass	AAG44622.2	—	AAO39210.1	AAO39211.1	—
Mus musculus	mouse	NP_031982.1	—	NP_997590.1	—	NP_031979.2
Oncorhynchus mykiss	rainbow trout	NP_001117821.1	NP_001118030.1	NP_001118225.1	NP_001118042.1	—
Oreochromis niloticus	Mozambique tilapia	CAK95869.1	—	CAK95870.1	ABE73151.1	—
Oryzias latipes	medaka	BAA25900.1	—	BAB79705.1	NP_001121984.1	—
Sebastes schlegelii	Korean rockfish	ACN39246.2	—	ACN38898.3	ADR73047.1	—
Sparus auratus	gilthead seabream	AAD31032.2	—	AAD31033.1	CAE30470.1	—
Spinibarbus denticulatus	phoenix barb	ABF56051.1	ABU41681.1	ABF56052.1	ABU41682.1	—
Varicorhinus barbatulus	Taiwan shoveljaw carp	CAD67996.3	—	CAC85366.1	CAD67997.1	—

*Sequences were obtained from Ensembl genome browser.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Ovaries
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Nov
Feb
Mar
May
A
F
B
G
C
H
D
I
E
J
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GSI = 1.2%
GSI = 2.7%
GSI = 2.3%
GSI = 8.5%
GSI = 9.4%
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sc
sz
gmc
nu
n
f
ch
ca
yg
ct
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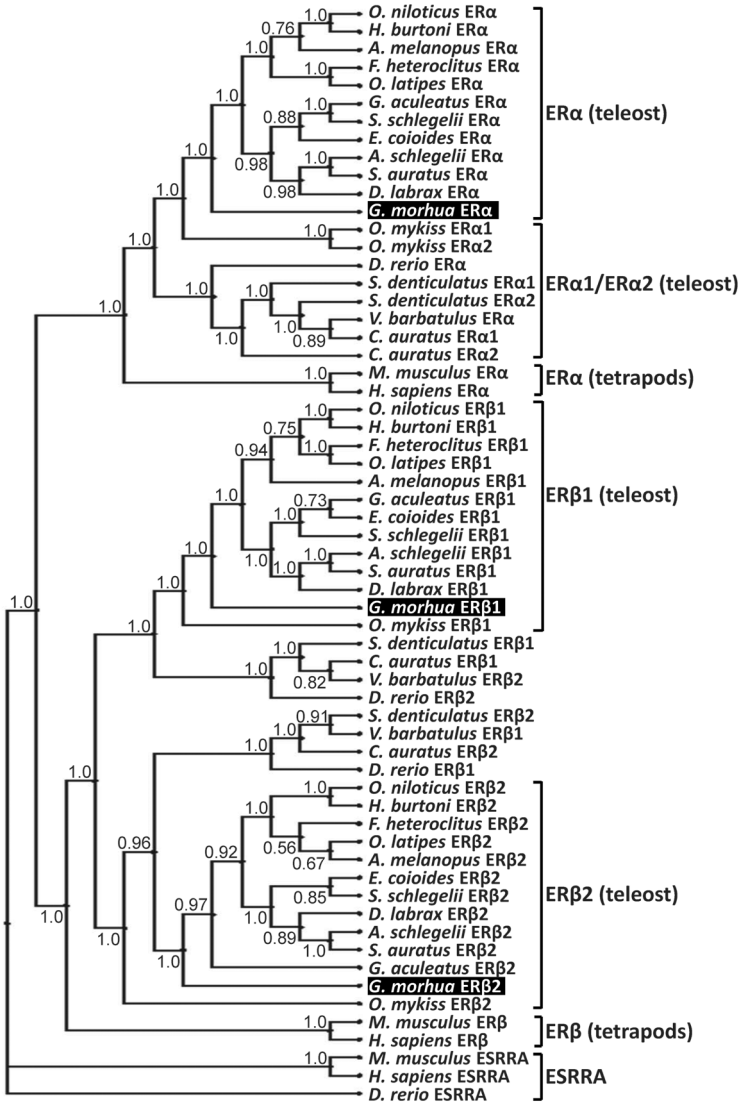
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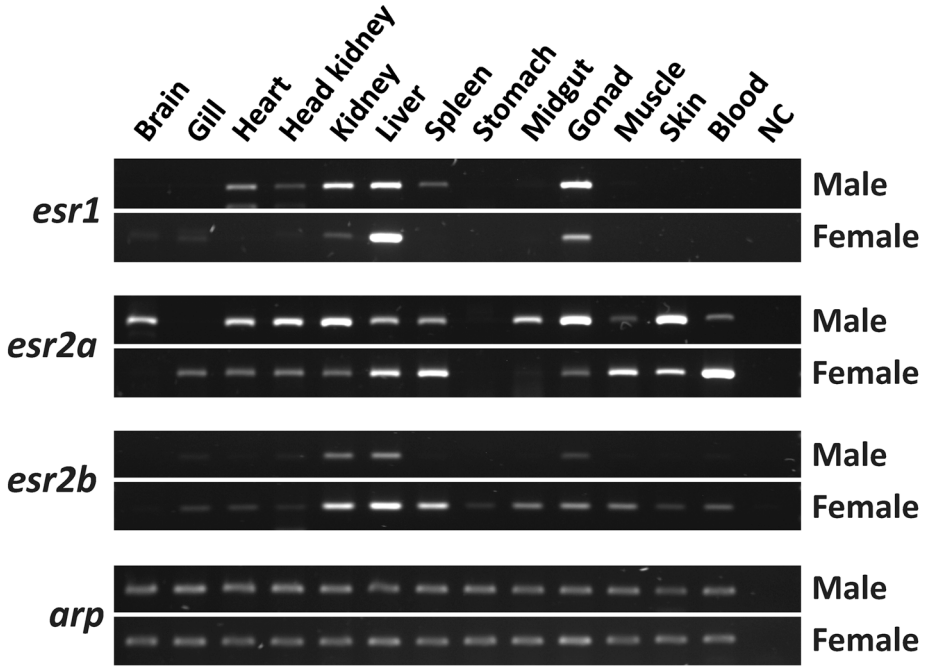
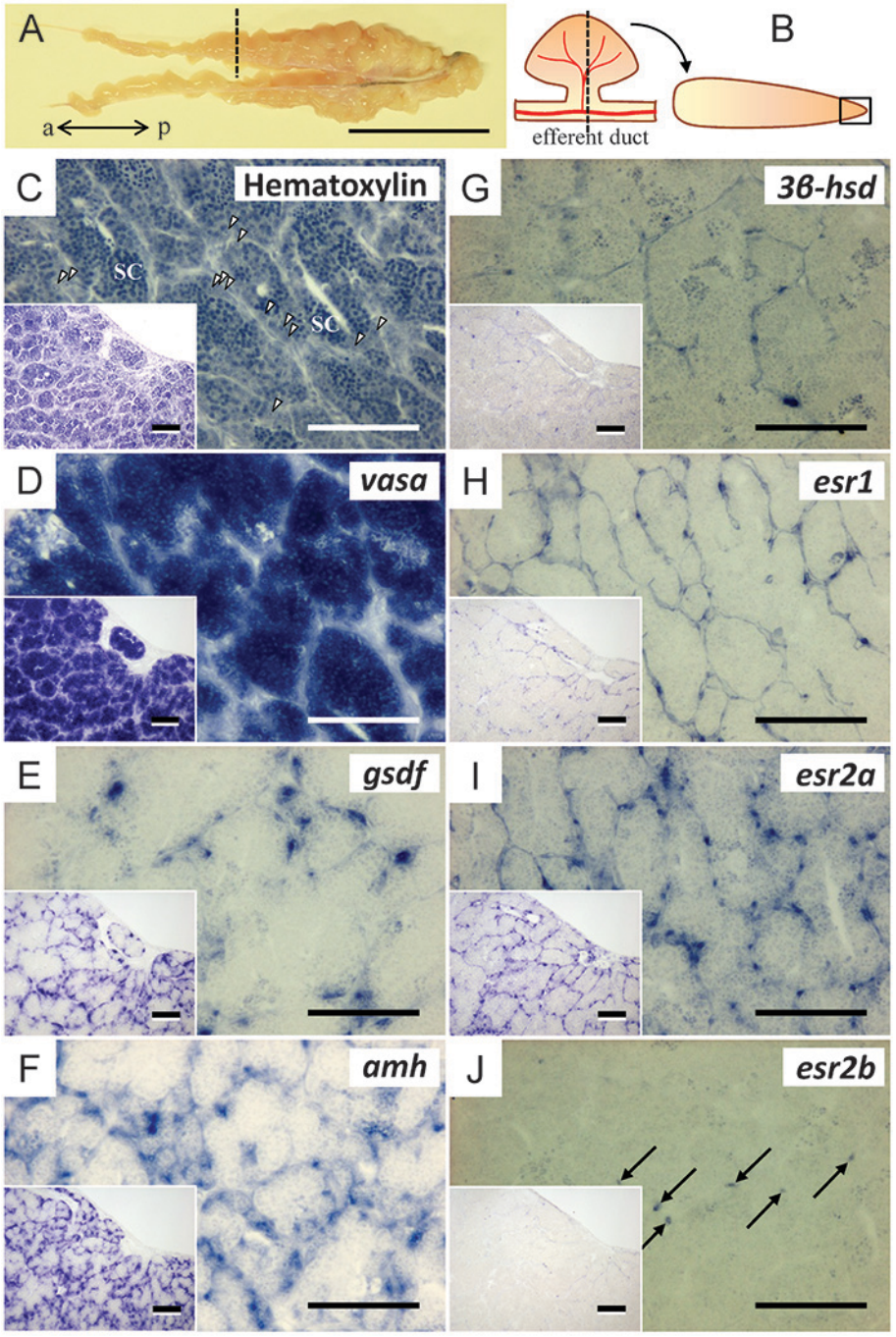
H. burtoni ERα
A. melanopus ERα
F. heteroclitus ERα
O. latipes ERα
G. aculeatus ERα
S. schlegelii ERα
E. coioides ERα
A. schlegelii ERα
S. auratus ERα
D. labrax ERα
G. morhua ERα
ERα (teleost)
O. mykiss ERα1
O. mykiss ERα2
D. rerio ERα
S. denticulatus ERα1
S. denticulatus ERα2
V. barbatulus ERα
C. auratus ERα1
C. auratus ERα2
ERα1/ERα2 (teleost)
M. musculus ERα
H. sapiens ERα
ERα (tetrapods)
O. niloticus ERβ1
H. burtoni ERβ1
F. heteroclitus ERβ1
O. latipes ERβ1
A. melanopus ERβ1
G. aculeatus ERβ1
E. coioides ERβ1
S. schlegelii ERβ1
A. schlegelii ERβ1
S. auratus ERβ1
D. labrax ERβ1
G. morhua ERβ1
O. mykiss ERβ1
ERβ1 (teleost)
S. denticulatus ERβ1
C. auratus ERβ1
V. barbatulus ERβ2
D. rerio ERβ2
S. denticulatus ERβ2
V. barbatulus ERβ1
C. auratus ERβ2
D. rerio ERβ1
O. niloticus ERβ2
H. burtoni ERβ2
F. heteroclitus ERβ2
O. latipes ERβ2
A. melanopus ERβ2
E. coioides ERβ2
S. schlegelii ERβ2
D. labrax ERβ2
A. schlegelii ERβ2
S. auratus ERβ2
G. aculeatus ERβ2
G. morhua ERβ2
O. mykiss ERβ2
ERβ2 (teleost)
M. musculus ERβ
H. sapiens ERβ
ERβ (tetrapods)
M. musculus ESRRA
H. sapiens ESRRA
D. rerio ESRRA
ESRRA


Brain
Gill
Heart
Head kidney
Kidney
Liver
Spleen
Stomach
Midgut
Gonad
Muscle
Skin
Blood
NC
esr1
Male
Female
esr2a
Male
Female
esr2b
Male
Female
arp
Male
Female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B
efferent duct
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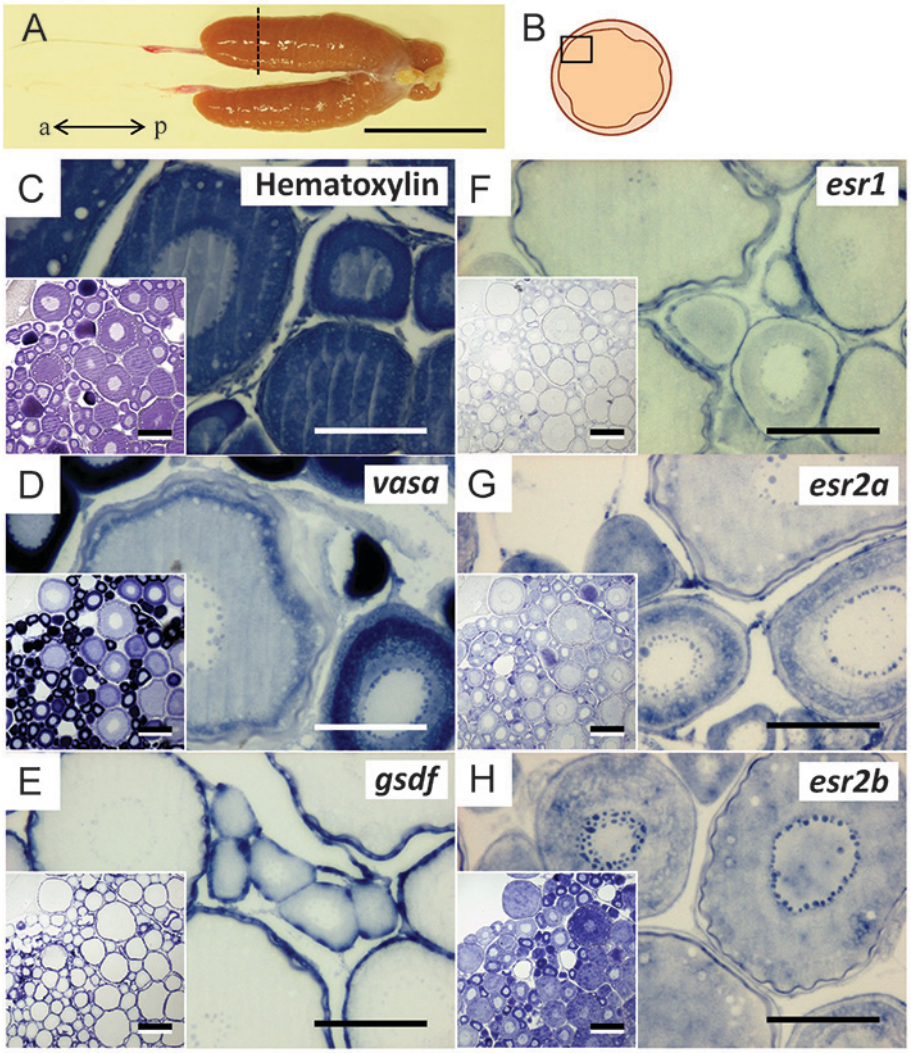
esr2a
F
amh
J
esr2b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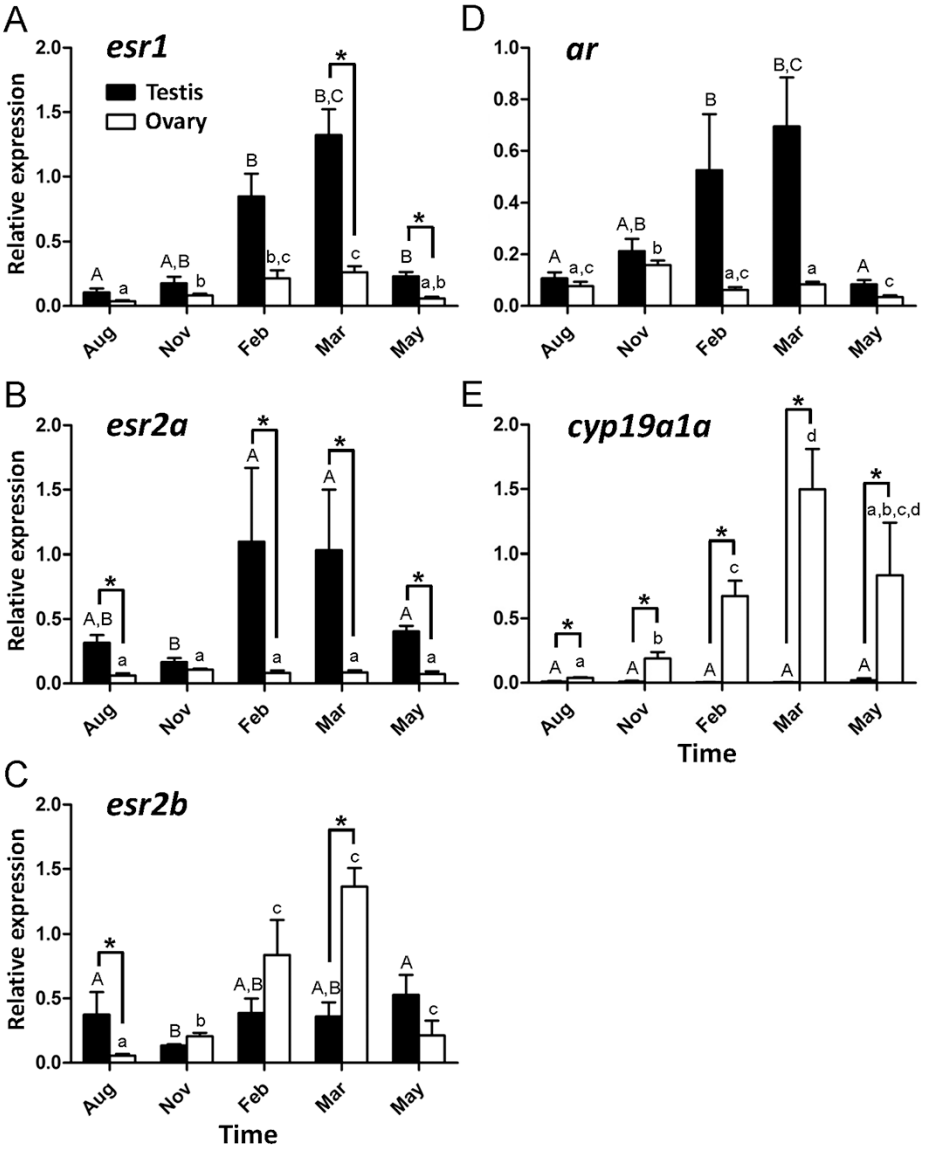
A
esr1
Testis
Ovary
Relative expression
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0.5
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A,B
b
B
b,c
B,C
c
B
a,b
Aug
Nov
Feb
Mar
May
D
ar
1.0
0.8
0.6
0.4
0.2
0.0
A
a,c
A,B
b
B
a,c
B,C
a
A
c
B
esr2a
A,B
a
B
a
A
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A
a
A
b
A
c
A
d
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c
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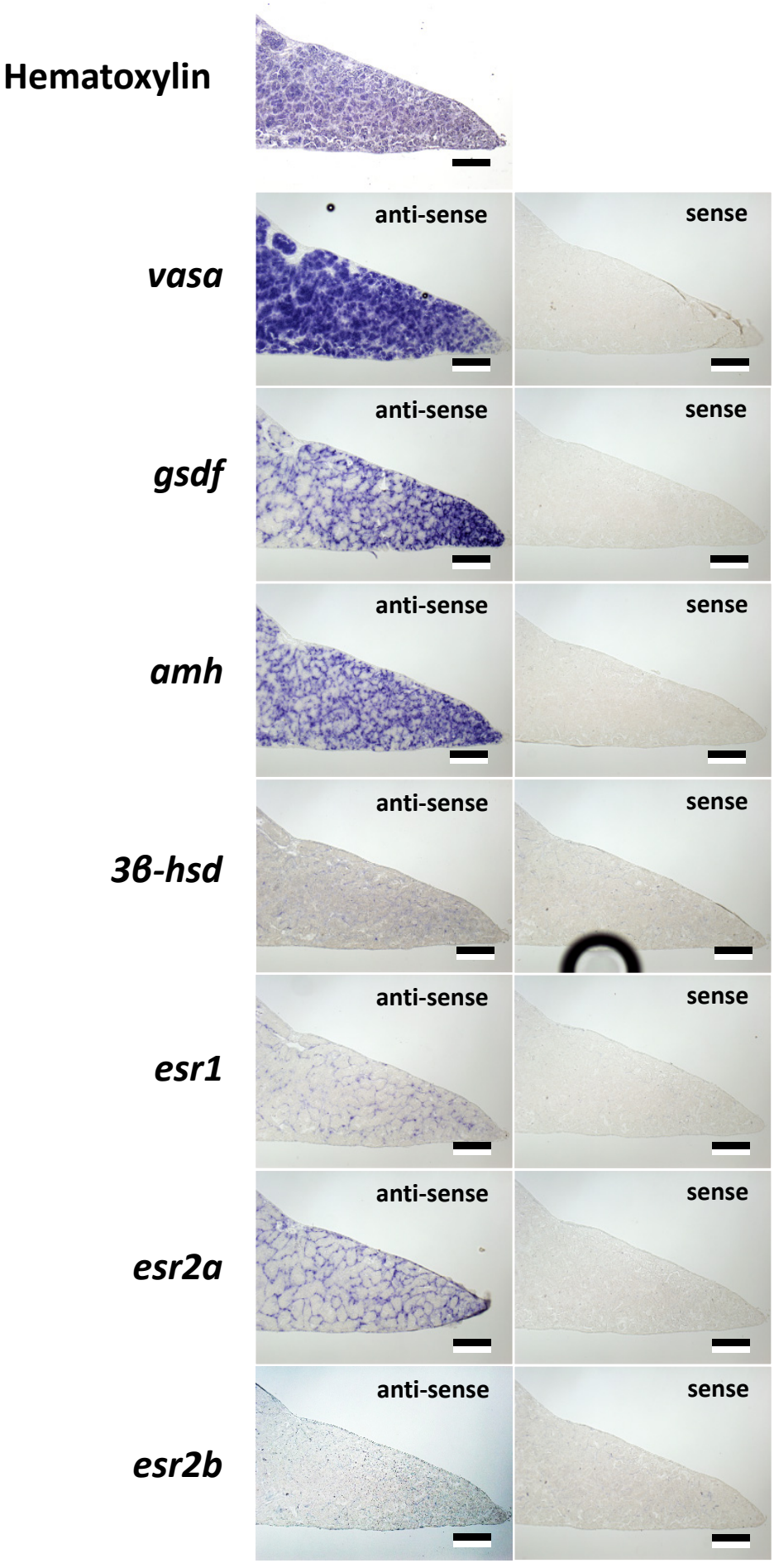
c
A
c
Time


Hematoxylin
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sense
gsdf
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amh
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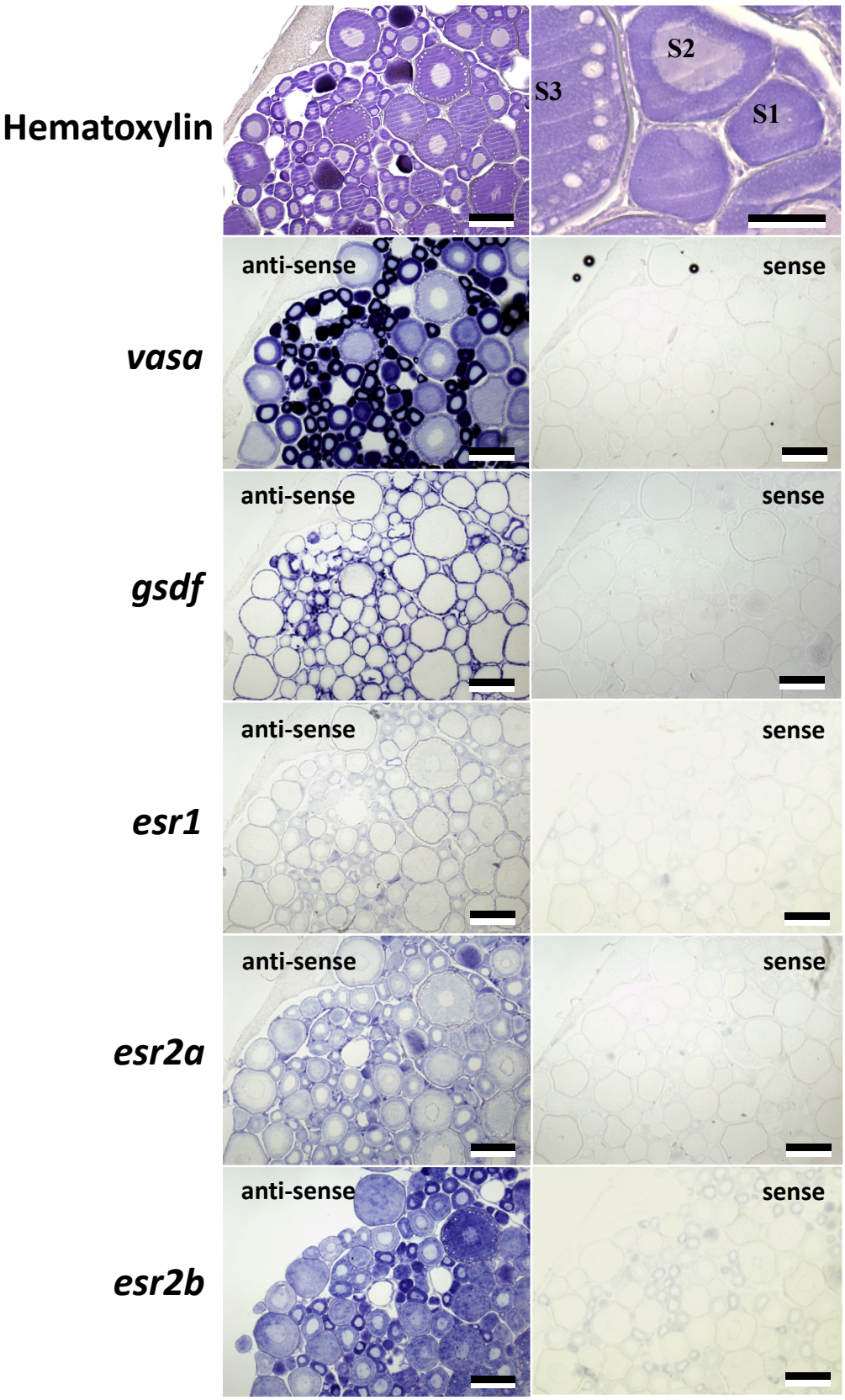
Hematoxylin
S2
S3
S1
vasa
anti-sense
sense
gsdf
anti-sense
sense
esr1
anti-sense
sense
esr2a
anti-sense
sense
esr2b
anti-sense
sense