Differential involvement of the three nuclear estrogen receptors during oogenesis in European sea bass (*Dicentrarchus labrax*)[1]

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Running title: Estrogen receptors in female European sea bass

Summary sentence: The three sea bass Esr subtypes have different ligand affinities and tissue expression patterns. In liver, only sbEsr1 and sbEsr2b play a role in estradiol-17β induced vitellogenesis. In ovary, sbEsr2b appears to be involved in vitellogenesis while sbEsr1 and sbEsr2a seem to be implicated in maturation. All are localized in somatic and germ cells. Follicle-stimulating hormone regulates *sbesr2b* but not *sbesr1* and *sbesr2a* transcription.

Keywords: estrogen/estrogen receptor; female reproduction; ovary; vitellogenesis; teleost; immunolocalization; gonadotropins; transcriptional activation

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Abstract

Estrogens are involved in a wide range of processes in vertebrate reproduction through ligand-activation of their specific cognate receptors. In most teleosts, three nuclear estrogen receptor subtypes have been identified (Esr1, Esr2a and Esr2b). Differences in ligand binding affinity and seasonal expression patterns in reproductive tissues among these Esr subtypes suggest distinct roles during oogenesis, vitellogenesis and spermatogenesis. This study focuses on the role of the Esr subtypes in European sea bass (*Dicentrarchus labrax*) oogenesis and their endocrine regulation. The coding genes of the three Esr subtypes are highly expressed in reproduction-related tissues such as pituitary, gonad and liver. Quantification of *esr1*, *esr2a* and *esr2b* expression in the ovary and liver during a whole reproductive cycle showed different patterns depending on stage and subtype, suggesting differential roles of the three receptors in the regulation of oogenesis and vitellogenesis. Esr2a and Esr2b also showed differences in transcriptional activity and ligand affinity when functionally characterized in HEK293 cells. Finally, for the first time in teleosts, the localization of the three Esr subtypes in ovarian follicles and their regulation by gonadotropins is described. Immunodetection of the receptors revealed different distribution patterns in follicular cells and various subcellular locations of the oocyte. Gonadotropin stimulation of ovarian follicles in different stages of vitellogenesis showed a consistent induction of *esrb2b* expression by Fsh. All together these data reinforce the hypothesis that each estrogen receptor plays a specific role in oogenesis.

1. Introduction

Estrogens are involved in the regulation of a wide range of processes in vertebrates, including reproduction and multiple non-reproductive functions in the neuroendocrine, skeletal and immune systems [1]. Estrogens are also implicated in multiple disorders and diseases including obesity, metabolic disorder, cancer, osteoporosis, endometriosis and the development of fibroids [2, 3]. In teleost fishes, estrogens are involved in the regulation of ovarian differentiation, vitellogenesis and testicular development through autocrine, paracrine and endocrine actions [4-6]. Gonadotropins regulate estrogen synthesis in the ovarian follicle, and in turn, estrogens regulate GnRH and gonadotropin expression and release in the brain and pituitary [7].

Estrogens exert their functions through ligand-activation of their specific cognate receptors. Two main classes of estrogen receptors have been reported in vertebrates: membrane-associated estrogen receptors (GPER), which are G-protein coupled receptors which mediate rapid non-genomic actions through intracellular signaling pathways [8], and nuclear estrogen receptors (ESR), transcription factors that bind to estrogen response elements (EREs) on gene promoters to regulate their expression [9].

Nuclear estrogen receptor proteins are formed by six functionally independent domains, termed A to F from the amino to the carboxyl terminus [10]. The N-terminal region (A/B domain) contains the first cell-type and promoter specific activation function (AF-1) and is one of the most variable regions among species [11]. Domain C or DNA Binding Domain (DBD) and domain E or Ligand Binding Domain (LBD) are the most conserved domains among the ESRs of different species. The DBD contains two zinc fingers involved in receptor binding to specific palindromic sequences of DNA, known as EREs, and the LBD contains the second promoter activation function (AF-2) and specific sequences for binding ligands and co-regulators. The intermediary D domain, located between the DBD and the LBD, is less

conserved but important for the maintenance of the ESR structure. Finally, the F domain is located in the C-terminal region and, although its function is not fully defined, it has a role in transcription modulation and interactions with agonists/antagonists [12].

Two nuclear ESR subtypes, ESR1 and ESR2 (also named ERα or NR3A1, and ERβ or NR3A2, respectively) have been identified and characterized in most tetrapod species [13]. These two ESR subtypes have different tissue expression patterns and bind estrogens with different affinities [14] resulting in different physiological roles. Studies in the female reproductive system, using specific agonists [15] or knockout mice for each receptor [16-19], show that ESR1 has an important role in ovarian function and uterine growth, while ESR2 is more involved in follicular growth and has an inhibitory action on the ESR1-mediated response in the uterus.

Three Esr subtypes have been identified in most teleost species, Esr1, Esr2a (also named Esrβ1 or Esrβa) and Esr2b (also named Esrβ2 or Esrβb), of which Esr2a and Esr2b coding genes arose from the whole genome duplication event specific of the teleost lineage [20-24]. Moreover, a second form of Esr1, Esr1b, was identified in rainbow trout (*Oncorhynchus mykiss*) and other tetraploid species [25, 26].

In fish different roles for the Esr subtypes during oogenesis, vitellogenesis and testicular development have been suggested based on their differences in binding affinity [24, 27-32], in gene expression between male and female tissues [20, 23, 31, 33-36], and in seasonal expression patterns in reproductive tissues such as gonad, liver and brain [23, 36-39]. Indeed, knockdown studies in goldfish hepatocytes, demonstrated that Esr1 is the main subtype involved in vitellogenesis induction whereas Esr2 subtypes regulate *esr1* expression in the liver [40]. In addition, loss of Esr2a and Esr2b in female zebrafish knockouts led to an arrest of folliculogenesis at the previtellogenic stage followed by female to male sex reversal [41].

The different spatiotemporal expression patterns exhibited by the *esr* subtypes indicate a tight regulation of their expression during the reproductive cycle. Diverse studies have demonstrated that estradiol-17β (E_2) exerts autoregulation on nuclear estrogen receptor expression [23, 32, 38, 42, 43]. However, there is still a lack of information on gene expression regulation in gonads in different reproductive stages and on the effect of endocrine mediators such as gonadotropins. Only recently, it has been observed that treatment of zebrafish follicular cells with human chorionic gonadotropin, at different doses and times, decrease the expression of all *esr* subtypes [38].

In a previous study, a complete Esr1 cDNA (sbERα) and partial cDNAs for Esr2a (sbERβ1) and Esr2b (sbERβ2) were isolated in European sea bass (*Dicentrarchus labrax*) [20], an aquacultured marine teleost used for years as model in reproductive physiology. Functional characterization of sbEsr1 showed that E_2 is the most potent inducer of its transcriptional activity. The expression of sea bass *esr*-subtypes localized in *lhβ*- and *fshβ*-expressing cells in the pituitary, providing anatomical evidence for direct estrogenic feedbacks on gonadotropic cells [30, 44]. However, the information on sbEsr2a and sbEsr2b is scarce and little is known about the significance of the presence of all Esr subtypes in reproductive tissues.

This study aims at investigating the differential roles of the Esr subtypes in European sea bass oogenesis and their endocrine regulation. The complete cDNAs coding for sea bass Esr2a and Esr2b were isolated and the transcriptional activity of both receptors was functionally characterized. In addition, we have studied the seasonal expression patterns of all Esr-subtype genes and the specific localization of the receptors in the ovary of a teleost fish, for the first time. Finally, the endocrine regulation of *esr* expression by gonadotropins was investigated using *in vitro* ovarian cultures.

2. Material and methods

100 *2.1. Animal and tissue samplings*

Adult female European sea bass (*Dicentrarchus labrax*) were obtained from stock raised at the Instituto de Acuicultura de Torre la Sal (IATS) facility. Fish were anesthetized with an overdose of ethyl 3-aminobenzoate methanesulfonate (MS-222; 300-400 mg/l; Sigma-Aldrich) and euthanized by decapitation in accordance with Spanish Royal Decree (53/2013) 105 and European legislation (2010/63/EU) for the protection of animals used for experimentation. The protocol used to euthanize the animals was approved by the IATS Ethics Committee (Register Number 09-0201), under the supervision of the Secretary of State for Research, Development and Innovation of the Spanish Government. Ovaries were collected for *in vitro* tissue culture. For each animal, the stages of ovarian development were determined by 110 histological analysis following previously established criteria [45]. Ovarian and liver tissue from adult females was sampled monthly (n=5 fish /month) during a complete reproductive cycle. The tissues from adult males and females (n= 2 pooled animals/sex/tissue), as well as the follicular cells used for gene expression analysis were from previous studies where the full description of total RNA extraction and cDNA synthesis can be found [46, 47].

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2.2. Cloning of sea bass nuclear estrogens receptors

Full-length cDNAs for European sea bass nuclear estrogen receptors sbEsr2a and sbEsr2b were isolated by reverse transcription (RT) followed by PCR. Ovaries from two females were pooled prior to extraction and 50 to 100 mg of tissue were homogenized in a FastPrep™ 120 device (Qbiogene, Inc., Irvine, CA). Total RNA was extracted using Maxwell™ 16 LEV simplyRNA Tissue Kit (Promega Corp.) on a Maxwell™ 16 Instrument (Promega Corp.). For cDNA synthesis, 2 µg of total RNA were reverse-transcribed using Superscript III (Invitrogen Corp., Carlsbad, CA) and random hexamers as primers, following the manufacturer's instructions. Full length *sbesr2a* and *sbesr2b* were amplified with specific forward (*sbesr2a* =

5'- GCAGATATCGAGAGCGTCTGTATTTGCAATG-3' and *sbesr2b* = 5'-GCAGATATCCAGTAGATTCTCCCTGACATG-3') and reverse (*sbesr2a* = 5'-ACGTCTAGACTGCAAGTTTGGTCTGTCTGCAG-3' and *sbesr2b* = 5'-ACGTCTAGACTCTTCTTGATCTTTGGGG-3') primers using Easy-A Hi-Fi Cloning Enzyme (Agilent technologies, Inc.). These primer sequences were obtained from searches against the sea bass genome database (http://seabass.mpipz.mpg.de/index.html) using available partial sequences of sea bass *esrs* as queries [20].

All PCR products were cloned into pGEM-T Easy vector (Promega Corp., Madison, WI) and sequenced on an automatic ABI 3100 Genetic Analyser (Applied Biosystems, Foster City, CA), using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). After sequence confirmation, *sbesr2a* and *sbesr2b* were cloned into the *Eco*RV/*Xba*I sites of the expression vector pcDNA3 (Life Technologies, Inc.) to perform transactivation assays.

2.3. Phylogenetic analysis

The amino acid sequences of different vertebrate species used for alignments and phylogenetic analysis were extracted from Ensembl (Ensembl Genome Browser, http://www.ensembl.org) or NCBI (http://blast.ncbi.nlm.nih.gov) (Supplemental Table S1). Complete amino acid sequences were aligned using MUSCLE (MUltiple Sequence Comparison by Log-Expectation) [48], and phylogenetic analysis was performed using the Molecular Evolutionary Genetics Analysis Software (MEGA 5) [49]. The evolutionary history was inferred by using the Maximum Likelihood method based on the Jones–Taylor–Thornton matrix-based model [50]. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.7389)). Gaps or missing data were pairwise deleted. One thousand bootstrapping replicates were used to assess the robustness of the inferred nodes of the tree.

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2.4. Transactivation assays for estrogen receptors

Human embryonic kidney (HEK) 293 cells were used to express sea bass *esr2a* and *esr2b*. Cells were seeded in 6-well plates (~6.25 x 10^5 cells / well) and cultured in Dulbecco modified Eagle medium (DMEM) GlutaMAX (Life Technologies, Inc.) supplemented with
155 10% v/v charcoal-stripped foetal bovine serum (FBS), and 100 U/ml of penicillin and streptomycin, at 37 ºC in a 5% CO_2 incubator. After reaching 75-80% confluence, cells were co-transfected using Lipofectamine 2000 (Invitrogen, Inc.) with the following constructs: pERE-TK-Luc (1600 ng), which contains the firefly luciferase (Luc) gene under the control of ERE binding sites; pcDNA3 expression constructs containing *sbesr2a* or *sbesr2b* (750 ng);
160 and the pRL-TK plasmid (Promega) (100 ng), which constitutively expresses the *Renilla reniformis* luciferase gene, to normalize transfection. After 15 h, the medium containing plasmid/Lipofectamine 2000 was removed, and cells were resuspended in phenol red-free DMEM/F12 supplemented with charcoal FBS, and reseeded in 96 well plates (Corning). After 6 h, media containing E_2, 16α,17β-estriol (E_3) or testosterone (T) prepared in ethanol at
165 concentrations ranging between 10^{-5} M and 10^{-10} M were added. The final concentration of the ethanol vehicle was 0.1%. After incubation at 37 ºC for 24-36 h, luciferase activities were determined directly on the plates using the Dual-Glo Luciferase Assay System (Promega) following the manufacturer's instructions. The light emitted was measured in a Tecan Ultra Evolution microplate reader (Tecan Group, Ltd.) and expressed as relative light units (RLU).
170 Luciferase activity was calculated as the quotient between RLU of firefly luciferase and RLU of *Renilla reniformis* luciferase. The EC50 value (concentration causing a half-maximal response) and Emax value (maximal response obtained) of each agonist were calculated from the concentration-response curve by fitting a logistic equation to the data by nonlinear

regression analysis. The Emax for each steroid is expressed relative to that of the E_2, which was considered as endogenous full agonist and whose Emax was set as 100.

2.5. Steroid immunoassay

Ovarian estradiol was measured by a conventional enzyme immunoassay (EIA), validated for sea bass in our laboratory [51]. The assay uses specific rabbit antiserum against E_2, whose specificity was described in a previous work, [52], and E_2 acetylcholinesterase conjugate (E-AChE, Cayman Chemical, MI, USA) as tracer. The EIA protocol was similar to that previously developed for testosterone determination [53]. Tissue samples were homogenized in 2% NaCl (20 µl / mg of tissue) using the FastPrep instrument and 120 µl of the homogenate was extracted with methanol (Panreac Quimia S.A., Spain). The organic solvent was evaporated and the dry extract was reconstituted in assay buffer (EIA buffer, Cayman Chemical MI, USA). The assay was performed in a final volume of 150 µl in 96-well microtiter plates coated with mouse anti- monoclonal rabbit IgG as secondary antibody (Clone RG-16, Sigma-Aldrich, Inc). Each component, E-AChE tracer (0.083 UE/ml), anti-E_2 rabbit antiserum (diluted to 1:2,500,000) and E_2 standards (ranging from 80 ng/ml to 0.039 ng/ml) or samples were added in a final volume of 50 µl. Plates were incubated overnight at 37 ºC. After incubation, plates were rinsed and color development was performed by the addition of 200 µl of Ellmans reagent in each well and incubation under constant gentle stirring for 2 h at 20 ºC in the dark. Optical density was read at 405 nm using a microplate reader (Bio-Rad microplate reader model 3550). The sensitivity of the assay was around 0.156 ng/ml (Bi/B0 = 90%) and half-displacement (Bi/B0 = 50%) occurred around 1.90 ng/ml.

2.6. Tissue culture

For *in vitro* tissue cultures, early- and late-vitellogenic ovaries of adult sea bass sacrificed in November or December, respectively, were rapidly immersed in ice-cold Sea Bass Ringer (SBR; according to [54] containing 0.5% bovine serum albumin (BSA, fraction V, Sigma-Aldrich, Inc.), 100 U/ml penicillin/streptomycin and 250 ng/ml Amphotericin B (Life Technologies, Inc.). Ovaries were maintained on ice and dissected into small fragments using a razor blade. Ovarian fragments were transferred to 96- or 48-well plates at about 15 mg (early-vitellogenic) or 20 mg (late-vitellogenic) per well containing 0.1 ml or 0.2 ml of SBR and pre-incubated for 1 h at 21 ºC while shaking (100 rpm). After pre-incubation, the medium was replaced with 0.1 ml or 0.2 ml of fresh SBR containing 300 ng/ml of recombinant single-chain sea bass Fsh (rFsh), single-chain sea bass Lh (rLh) (350 ng/mL) [51] or DMEM control medium. Incubations with the hormones were carried out in triplicate for 24 h (early-vitellogenic) or 6, 24 and 48 h (late-vitellogenic). After incubation, the medium was collected and the gonadal fragments were deep-frozen in liquid nitrogen and stored at −80 °C until RNA extraction. Three independent experiments were carried out on three different females.

2.7. Quantitative real-time PCR

The gonad expression pattern of *sbesr1, sbesr2a* and *sbesr2b* genes during oogenesis, as well as in the fragments from the ovarian cultures, was determined by quantitative real-time PCR (qPCR). Ovarian mRNA extraction and cDNA synthesis of the seasonal samples were as previously described [55]. Total RNA from ovarian culture fragments was isolated from ≈ 15 mg of frozen tissue using the FastPrep Instrument (Qbiogene Inc., Irvine, CA) for homogenization, and Maxwell™ 16 LEV simplyRNA Tissue Kit (Promega Corp.) on a Maxwell™ 16 Instrument (Promega Corp.) for RNA extraction. Liver total RNA extraction for the seasonal expression study was extracted from ≈ 30 mg of frozen tissue as described above. The purity and concentration of the RNA was verified by NanoDrop UV-Vis

Spectrophotometer (Thermo Fisher Scientific, Inc.). All qPCR assays were run in duplicate for each sample on 96-well plates using the CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Inc) with default settings. Data were captured and analyzed with CFX Manager Software (version 3.1). Optimized amounts of primers for the target genes [56] were mixed with 1 µl of non-diluted cDNA and 1x PyroTaq EvaGreen qPCR Master Mix (Cultek) for a final reaction volume of 20 µl. Ten-fold serial dilutions of pooled cDNA samples were included as standard curves. The average value of the correlation coefficients (R^2) of the standard curves for all gene assays was 0.99. qPCR efficiencies ranged from 0.95 to 1.00. The melting curves generated at the end of each reaction produced a single peak for each gene, confirming primer specificity. For each experimental sample, the relative transcript level of target and endogenous reference genes was determined from the appropriate standard curve, whose initial number of copies were arbitrarily assigned, and data were normalized against appropriate reference genes whose expression values remained stable among experimental samples. These reference genes were elongation factor 1 alpha (*ef1-alpha*) for ovarian and liver seasonal samples, and *18s* ribosomal RNA for ovarian culture fragments, whose qPCR assays were optimized previously [55].

2.8. Immunohistochemistry

For immunohistochemistry, ovarian samples were fixed with 4% paraformaldehyde overnight at 4 ºC and subsequently dehydrated and embedded in paraffin. Sections of approximately 5 µm in thickness were deparaffinised in xylene, hydrated in decreasing concentrations of ethanol and washed twice with double distilled water. Antigen retrieval treatment was carried out with Tris-EDTA Buffer (10 mM Tris Base, 1 mM EDTA Solution, 0.05% Tween 20, pH 9.0) at 95 ºC for 15 min. After antigen treatment, slides were allowed to cool at room temperature, washed twice with Tris-buffered saline - 0.05% Triton X-100 (TBST) and

blocked with 3% normal goat serum, 1% BSA in TBST for 2 h. After blocking, slides were incubated overnight at 4 ºC with Esr subtype specific rabbit polyclonal antibodies developed based on the sea bream (*Sparus aurata*) amino acid sequences [57, 58] diluted in 3% normal goat serum, 1% BSA in TBST. Sections were then washed twice in TBST and immersed in 0.5% hydrogen peroxide for 15 min to quench endogenous peroxidase activity. Then, the slides were incubated for 1 h at room temperature with secondary goat anti-rabbit IgG-horseradish peroxidase conjugated (GAR-HRP, Sigma-Aldrich) diluted 1/250 in 3% normal goat serum, 1% BSA in TBST. Detection of immunostaining was performed using 3,3'-diaminobenzidine (DAB, ACROS Organics) for 1-5 minutes. Nuclei were counterstained with 25% hematoxylin (Sigma-Aldrich) for 10 seconds. For immunofluorescence, goat-Alexa-594-coupled anti-rabbit IgG secondary antibody (1:300; Life Technologies Corp) was incubated for 1 h at room temperature in 3% normal goat serum / 1% BSA in TBST. Slides incubated without primary antibody served as negative controls. Slides were mounted using Prolong Gold antifade reagent (Life Technologies, Eugene OR, USA). Sections were examined and photographed with a Nikon Eclipse E600 imager microscope (Nikon Instruments Europe) or under an Olympus FluoView FV1000 confocal laser-scanning microscope, and images were processed with the FV10-ASW FluoView software (Olympus Corporation, Tokyo, Japan). Images from control sections were taken with the same fluorescence intensity and exposure as those used for the test sections.

The specificity and lack of crossreactivity of the anti-Esr antibodies was analyzed by immunocytochemistry in COS-7 cells expressing each of the three sea bass Esrs (Supplemental Material and Methods; Supplemental Fig. S1, S2 and S3)

2.9. Statistical analyses

Data are presented as the mean ± SEM and statistical differences were evaluated using one-way or two-way ANOVA followed by the Holm-Sidak pair-wise multiple comparison method using SigmaStat 3.5 (SYSTAT Software, Inc.). Data were log-transformed in order to comply with normality. When the test of equal variance failed, ANOVA on ranks (Kruskal-Wallis non parametric test) was performed followed by a pair-wise multiple comparison procedure (the Dunn method). Criteria for significance level were a p-value of <0.05.

3. Results

3.1. Cloning of esr2a and esr2b and phylogenetic analysis

The complete cDNAs encoding sbEsr2a and sbEsr2b have now been isolated from ovary. The Esr2a and Esr2b coding regions consists of 2,066 bp (*esr2a*) and 2,088 bp (*esr2b*), which encode proteins of 561 and 662 amino acids with predicted molecular masses of 62.7 and 73.3 kDa, respectively. These sequences were deposited in GenBank with the following accession numbers KY968340 (*esr2a*) and KY968341 (*esr2b*).

Multiple sequence alignments of tetrapod Esr2 sequences showed that both sea bass sequences contain the domains that are characteristic of nuclear steroid receptors, namely A/B, C (DBD), D (hinge region), E (LBD) and F (C-terminus) (Supplemental Fig. S4). The DBD of both sbEsr2a and sbEsr2b contains conserved characteristics of this region as the eight cysteines of the zinc finger motifs, P-box and D-box. Both LBDs also contain the characteristic AF-2 motif (important for transactivation function) and residues important for ligand and co-factor biding, with the exception of two residues involved in ligand binding that differ in sbEsr2b and in most of the available fish Esr2b. These residues correspond to Val398, which changes to isoleucine in fish, and Glu402, a negatively charged amino acid, which changes to a lysine, a positively charged amino acid. Less conserved regions correspond to the hinge region, which is involved in DBD rotation, and the C-terminus involved in

transcription modulation and binding to agonists/antagonists [12] (Supplemental Fig. S4).The global percentage of identity between sbEsr2a and sbEsr2b sequences was 59%. The least conserved regions, which are longer in Esr2b than in Esr2a, share an identity of 41.7% for the hinge region and 27.6% for the C-terminus, while the two most conserved regions DBD and LBD are 90.9% and 75.5% identical, respectively (Supplemental Fig. S5). Comparison between the sea bass receptors and other teleost Esr2 subtypes and tetrapod ESR2 showed for Esr2a, percent identities in the DBD that ranged from 88.3 to 94.9% and in the LBD from 68.1 to 95.4% (Supplemental Fig. S5A). In the case of Esr2b, percent identities ranged between 94.8 and 100% in the DBD and 67.1 and 95.8% in the LBD (Supplemental Fig. S5B). Maximum likelihood phylogenetic analysis of estrogen receptor polypeptide sequences supported the existence of two distinct subclusters for sbEsr1 and sbEsr2 with robust separation (99% probability) between Actinopterygian and Sarcopterygian sequences. Two subclades corresponding to Esr2a and Esr2b exist in teleosts but not in basal Actinopterygii such as the spotted gar (*Lepisosteus oculatus*) and amur sturgeon (*Ascipenser schrenckii*), indicating an origin that can be placed during the teleost specific whole genome duplication period. Sea bass estrogen receptors clustered together with Esr2a and Esr2b from other teleost species (Fig. 1).

3.2. Functional characterization of Esr2a and Esr2b

The transcriptional activity of sbEsr2a and sbEsr2b was tested in HEK293 cells by transiently expressing the receptor constructs together with a plasmid containing the luciferase gene driven by an estrogen responsive element (ERE) (Fig. 2). Cells were stimulated with 10^{-8} M E_2 and luciferase activity was measured 24 h after stimulation. The results show that E_2 induced the transcriptional activity of sbEsr2a and, more strongly, of sbEsr2b. In the absence of E_2 no increase in relative luciferase activity was observed for any of the receptors, which had the same luciferase activity as the empty expression vector (Fig. 2A). To investigate

ligand specificity, transfected cells were stimulated with increasing concentrations of various steroid hormones for each receptor. A clear dose-response activation of the ERE promoter was observed when sbEsr2a was stimulated with E_2 or E_3, while no activation was observed with T. In contrast, in the case of sbEsr2b a dose-response activation of the ERE promoter was observed with all the steroids tested including T (Fig. 2B). The patterns of response to the tested estrogens differed for each receptor (Table 1). As a dose-response curve for both receptors exhibited a different maximal agonist effect for each steroid, Emax values were used to measure the relative efficacy of partial agonists (Strange, 2008). E_2 was the most efficient inducer of activity and considered as full agonist for both receptors. However, when EC_{50} values were compared, E_2 was more potent inducing sbEsr2b than sbEsr2a. As a partial agonist, E_3 was less effective at activating any of the receptors, although the relative efficacy was lower for sbEsr2a (Emax = 30%) than for sbEsr2b (Emax = 60%). T had no effect on sbEsr2a but, in contrast, was also a partial agonist for sbEsr2b (Emax = 43%).

3.3. Tissue distribution of sea bass esr1, esr2a and esr2b

The distribution of *sbesr1*, *sbesr2a* and *sbesr2b* transcripts in different tissues and organs of adult male and female sea bass was determined by RT-qPCR (Fig. 3). *Sbesr1* had similar expression patterns for males and females and was mainly expressed in pituitary. However, some expression was also observed in brain, gonad and head kidney (Fig. 3A). Transcript levels of *sbesr2a* were higher in males than in females and were mainly detected in pituitary, gonad and kidney in both sexes (Fig. 3B). In both sexes *sbesr2b* was expressed in pituitary, gonad, liver, intestine and kidney; however, while expression levels in kidney were higher in males, gonad levels were higher in females (Fig. 3C). Levels of *sbesr1* mRNA in isolated ovarian follicular cells were similar to that observed in female gonad; while *sbesr2a* and

sbesr2b expression levels were much lower in follicular cells than in the gonad as a whole (Fig. 3).

3.4. Expression pattern of sea bass esr1, esr2a and esr2b during ovarian development

Changes of *sbesr1, sbesr2a* and *sbesr2b* expression were determined by RT-qPCR in ovaries of adult female sea bass at different stages of maturation during a whole reproductive cycle (Fig. 4). Since European sea bass is a species that displays group-synchronous ovarian development, ovarian stages were defined according to the predominant and most advanced follicles present in the ovary [45]. Both, *sbesr1* (Fig. 4A) and *sbesr2a* (Fig. 4B) ovarian transcript levels were low during previtellogenesis, and increased during vitellogenesis, with a more pronounced accumulation of mRNAs during the maturation stage, and then decreased significantly during atresia. In contrast, *sbesr2b* mRNA levels in the ovary showed a bimodal expression pattern. They increased abruptly at the beginning of vitellogenesis, reaching its highest expression at this stage, and then decreased significantly in advanced vitellogenesis and post-vitellogenesis. A second peak in expression respect to the initial previtellogenic stage occurred during oocyte final maturation and finally reached the lowest levels during atresia (Fig. 4C).

3.5. Seasonal expression patterns of sea bass esr1, esr2a and esr2b in liver

Changes in the expression of the three estrogen receptors in liver were determined during a whole reproductive cycle, and were related with the stages of ovarian development in the same animals (Fig. 5). Levels of *sbesr1* mRNA were low during previtellogenesis and significantly increased in early vitellogenesis, continuing to rise during advanced- and post-vitellogenesis, peaked in the maturation-ovulation stage before decreasing abruptly during atresia (Fig. 5A). Transcript levels of *sbesr2a* in the liver were constant throughout the annual

cycle with no significant changes observed (Fig. 5B). Basal expression levels of *sbesr2b* mRNA were found to be higher than those of *sbesr1* and *sbesr2a*, indeed the expression of *sbesr2b* was stable at all the stages, and a statistically significant increase in expression was only observed during early vitellogenesis (Fig. 5C).

3.6. Ovarian E_2 levels during the reproductive cycle of sea bass

Previous studies described the circulating levels of E_2 in female sea bass plasma during an annual cycle [55], but the E_2 levels in ovarian tissue were not investigated. Now, the analysis of E_2 accumulation in female ovaries throughout the reproductive cycle shows a gradual increase of E_2 during vitellogenesis that peaked during post-vitellogenesis. They decreased sharply during maturation/ovulation and reached their lowest levels during atresia (Fig. 6).

3.7. Immunolocalization of sea bass Esr1, Esr2a and Esr2b in the ovary

Immunolocalization was carried out to investigate the localization of Esr subtypes in sea bass ovarian samples (Fig. 7-8). The three Esrs were detected both in the follicular cells and in the oocyte (see Supplemental Fig. S6 for representative stained sections). More specifically, Esr1 (Fig. 7A, B) and Esr2a (Fig. 7C, D) were detected mainly in post-vitellogenic and mature follicles. They localized in the continuous cell layer surrounding the oocyte, which corresponds to granulosa cells, in the oocyte plasma membrane and in the ooplasma surrounding subcellular structures. Esr2b was strongly detected in discontinuous cells of the outer follicle layer, which corresponds to theca cells, in all follicle types (Fig. 8) but only slightly in granulosa cells of mature follicles (Fig. 8B). Inside the oocyte, Esr2b gave a very strong signal in small vesicles of early- and vitellogenic oocytes (Fig. 8A, C, D), whereas in mature oocytes the signal was more widespread in the ooplasma (Fig. 8B).

3.8. Gonadotropin regulation of sbesr1, sbesr2a and sbesr2b expression

The specific seasonal expression patterns of *sbesr1*, *sbesr2a* and *sbesr2b* observed in ovary suggest that they are highly regulated at transcriptional level. In a first experiment, the effects of Fsh on early-vitellogenic ovarian explants were studied as Fsh is the main endocrine regulator during this developemntal stage [55, 59]. After *in vitro* incubations of these explants with 300 ng/ml of rFsh for 24 h, the transcripts levels of *sbesr2b* significantly increased, whereas no effect was observed on *sbesr1* and *sbesr2a* (Fig. 9A).

In a second experiment post-vitellogenic ovarian explants were incubated with 300 ng/ml of rFsh or rLh, since Lh is also playing a role at this stage [55]. No differences in *sbesr1* or *sbesr2a* expression were found in the gonadotropin treated samples after 24 h or 48 h compared with controls, whereas only rFsh, but not rLh, stimulated *sbesr2b* expression. However, when observing the response to gonadotropin treatments over shorter time periods (6 h), a pronounced increase in the expression levels of all *esr* subtypes was detected with both rFsh and rLh treatments (Fig. 9B).

4. Discussion

In the present study, starting from the previous partial cloning of type 2 *esrs* [20], we have isolated the complete coding cDNAs of European sea bass *esr2a* and *esr2b* and performed a molecular and functional characterization of sea bass Esr subtypes, with special emphasis on their role in the ovary. Moreover, for the first time in teleosts, the localization of the different estrogen receptor subtypes in ovarian follicles and their regulation by gonadotropins are described. The results support their different roles in reproductive functions.

In agreement with a previous study [20], sbEsr2a and sbEsr2b have the typical domain structure of nuclear steroid receptors (A to F domains), with highly conserved DNA and ligand binding domains [60, 61], and the least conserved A/B, D and F domains, which are

involved in ligand-independent transcriptional activity, nuclear receptor co-factor binding and transcription modulation and interactions with agonists/antagonists, respectively [12, 62-65]. These least conserved regions are longer in sbEsr2b than in sbEsr2a, which is more similar to tetrapod Esr2. Indeed, only sbEsr2b showed amino acid differences with the tetrapod LBD domain. Sequence identities between the sea bass Esr2 subtypes were lower than sequence identities between sea bass Esr2a or Esr2b and their corresponding counterparts from other species. Maximum likelihood phylogenetic analysis showed four major clades containing tetrapod and fish *esr1* and *esr2*, with two subclades for *esr2* in the teleost lineage where *sbesr2a* and *sbesr2b* were grouped, confirming previous findings using the Neighbor-Joining method [20]. In addition, the separation of ancient fish (spotted gar and amur sturgeon) *esr* genes from the teleost lineage supports the hypothesis that *esr2a* and *esr2b* arose from the teleost-specific whole genome duplication event [29]. Genome duplication results in the emergence of duplicated paralogous genes that, when both copies remain functional, can divide the functions of the original copy (subfunctionalization) or acquire new roles (neofunctionalization). The pattern of amino acid divergence between sbEsr2b and the other two Esr subtypes results in charge differences in the LBD that may imply differences in ligand and co-factor affinities, as observed in other teleost species [66]. In addition, the higher divergence of Esr2b than Esr2a from their tetrapod ortholog Esr2, suggests that, in this case, Esr2b may have acquired new functions.

The transcriptional activity of sbEsr1 analyzed in a previous work showed that it is activated by all doses of estradiol and estriol, and high doses of estrone and testosterone, when it is expressed in HEK293 cells [30]. By contrast, in other teleost species, testosterone had no effect on Esr1 [24, 31, 66, 67]. When assaying the sbEsr2 subtypes in this work, the same cell system (HEK293) was used as for sbEsr1 to facilitate a comparison of the results and to avoid differences in transcriptional activities that depend on cell type [68]. Both sbEsr2 subtypes

were transcriptionally active in an estrogen dependent manner, but sbEsr2b had higher activity than sbEsr2a, unlike what has been observed in other teleost species, in which they had similar levels [27, 32, 66] or Esr2a was more active than Esr2b [24, 28]. Ligand specificity also differed between Esr2 subtypes; sbEsr2a and sbEsr2b were both efficiently activated by E_2, but E_3 was more effective at activating sbEsr2b (60% of Emax) than sbEsr2a (30% of Emax). In the same vein, T induced transcriptional activity in sbEsr2b, as also observed for sbEsr1 [30], but not for sbEsr2a. In other teleost species, T has no effect on Esr transcriptional activity and E_2 was the most potent inducer of all Esr subtypes [24, 28, 29, 31, 67]. These differences in transcriptional activity and ligand affinity between Esr2 subtypes in teleost species could result from differences in the LBDs sequences, as discussed above.

In teleosts, as in mammals, *esr1, esr2a* and *esr2b* have different tissue expression patterns [14, 23, 31, 69, 70]. In sea bass, the three subtypes are highly expressed in reproduction-related tissues such as pituitary or gonads, confirming the results of a previous work using semi-quantitative RT-PCR [20]; some expression was also observed in other tissues, such as liver or kidney, in line with the different known functions that estrogens play in vertebrates [1]. As shown by our own and others observations, in teleosts the expression of *esr1* and *esr2b* is higher in female than in male liver [20, 35, 36, 69, 71, 72]. In addition, we observed that the expression of these two receptors in female sea bass liver increased during vitellogenesis, and the expression of *esr1* further increases in maturation, resembling the profile levels of vitellogenin and plasma E_2 in this same species [55, 73, 74]. These results suggest that, in sea bass, E_2-induced vitellogenesis involves both Esr subtypes, although the constant high expression levels detected for *sbesr2b* throughout the reproductive cycle may indicate the existence of post-transcriptional regulation events for this receptor, such as the already described mRNA stabilization [75, 76].. In addition, screening assays for estrogenic effects in medaka demonstrate that estrogens increase *esr1* expression in the liver [77]. It is

plausible that sbEsr2b modulates the expression of *sbesr1* and its action on vitellogenesis, as observed with knockdown experiments in goldfish, which showed that Esr2 subtypes induce the expression of *esr1* [40]. In some teleost species, including sea bass, the expression of *esr2a* in liver is almost undetectable, suggesting that Esr2a is not involved in vitellogenesis regulation [33, 35, 78].

Comparison of the tissue expression pattern of teleost *esr2a* and *esr2b* shows that *esr2a* is highly expressed in pituitary and weakly expressed in liver, whereas *esr2b* has the opposite profile [23, 72, 79], this work). Considering that tetrapod *esr2* is expressed in both tissues [80-83], teleost-specific duplication probably resulted in sub-functionalization of the two subtypes.

The three *sbesr* subtypes show substantial expression in gonads. Although there are differences according to sex and subtype, the consistent expression of the *esrs* in testis suggests an important role for these receptors in spermatogenesis, as observed in other species [36, 84, 85]. The sea bass *esrs* have a specific seasonal expression pattern in ovary, where the expression levels of *sbesr1* and *sbesr2a* strongly increased at the end of vitellogenesis and peaked during maturation, differing from *sbesr2b*, whose expression increased with the onset of vitellogenesis, similar to that observed in Korean rockfish and European eel [23, 71, 72]. In contrast, in other teleosts, levels of *esr1* were more similar to those of *esr2b*, increasing during vitellogenesis, whereas the levels of *esr2a* do not change at any time during the reproductive cycle [33, 36]. In addition, high levels of E_2 were found in vitellogenic ovaries of sea bass, reaching maximum production in post-vitellogenesis and decreasing during maturation to reach the lowest levels in atresia. Plasmatic E_2 in these same animals shows a different profile - the highest levels are reached during post-vitellogenesis but are maintained during maturation and atresia [55]. These profiles suggest a paracrine/autocrine role for E_2 in the ovary during vitellogenesis, and an endocrine role involving negative feedback in other

organs during later stages. Taken together, these results point to the involvement of E_2 and estrogen receptors in the regulation of ovarian development in teleosts, as described in knockout studies in zebrafish [41], with a differential function of each receptor depending on the species. In mammals, estrogen receptor subtypes have different roles in ovarian growth as observed by its specific localization in different female reproductive tissues [86] and confirmed in knockout experiments in rodents, where ERα (ESR1) knockout produces infertility and inhibits ovarian follicle maturation whereas ERβ (ESR2) knockout produce subfertile animals with follicles at all stages of development [16-18].

In teleosts, the expression sites of the estrogen receptor genes in the ovary have been described in some species [34-36, 87]. However, localization of the Esr protein is described only for Esr1 in Ricefield eel [88]. In our study, we found that all receptor subtypes are present in vitellogenic ovaries, although with different localization sites in the oocyte and the follicular cells. In Ricefield eel, positive immunoreactivity for Esr1 was found in the membranes of oocytes in different stages [88]. In yellow croaker *esr1* mRNA was detected in pre- and early-vitellogenic oocytes [35], while in other fish species *esr1* expression was only detected in follicular cells [34, 36, 38, 87]. In sea bass, Esr1 localized to both oocyte plasma membrane and the cytoplasm of granulosa cells in advanced- and post-vitellogenic follicles. This location is similar to that found for sbEsr2a, but this receptor was also strongly detected surrounding subcellular structures of the oocyte. By contrast, strong sbEsr2b signals were found in theca cells of early vitellogenic follicles and in small vesicles of the oocyte, which might correspond to vitellogenic globules. In advanced vitellogenesis, Esr2b was also found in granulosa cells. Altogether, these localizations are consistent with the expression sites of *esr2a* and *esr2b* in the oocyte and the follicular layer described for other fish species, [35, 36]. The extranuclear localization found in the sea bass Esrs suggests that estrogen receptors can exert functions other than transcriptional regulation, which implies rapid activation of

intracellular pathways or interaction with the G-protein coupled estrogen receptors Gper [89], recently described in sea bass [90]. In mammals, there is evidence that nuclear steroid receptors exist in multiple subcellular locations, including plasma membrane, mitochondria, endoplasmic reticulum and other cellular organelles, (see review: [91]; and in fish, cytoplasmic and/or perinuclear location of Esrs was found in tilapia and sea bream scales [57]. The differences in localization among receptor subtypes, together with their expression pattern in the ovary, suggest different roles for each receptor, with Esr1 and Esr2a being more relevant during follicular maturation and Esr2b both during follicle growth and maturation. However, further studies are needed to define the specific function of each receptor in ovarian development.

The abrupt increase in the ovarian expression of the *esr* genes during the vitellogenesis and maturation stages suggests strict regulation of these genes at the transcriptional level. In teleosts, several studies have shown that *esrs* are closely regulated by E_2 [23, 32, 38, 42, 43, 92]. This effect of E_2 varies with the target tissue and receptor subtype, but in general, E_2 exposure increases the expression of *esr1* while it has no effect on *esr2* subtypes. The exception being zebrafish where *esr2a* and *esr2b* expression is suppressed in follicular cells and hepatocytes [24, 38, 75]. As E_2 production is under endocrine control by gonadotropins, it can be deduced that these hormones may also participate in the transcriptional regulation of the *esrs*. In fact, in mammals, expression of nuclear steroid receptors is subjected to regulation by gonadotropins [93-95]. In female sea bass, comparison of the seasonal profiles of gonadotropin plasma levels with those of *esr* expression suggests that these hormones are among the factors controlling *esr* expression during ovarian development [55, 59]. Here, we show that rFsh stimulation of early-vitellogenic ovarian explants results in a significant increase of *sbesr2b* expression. rFsh also induces the production of E_2 in vitellogenic ovarian explants [96] and, in turn, E_2 increases plasma vitellogenin levels [97]. These results, together

with the specific localization of sbEsr2b in small granules in the cytoplasm of vitellogenic oocytes, supports the hypothesis that sbEsr2b is probably involved in oocyte growth. In post-vitellogenic/mature ovarian explants, there is a rapid increase of expression of the three *esr*-subtypes 6 h after stimulation with rFsh, and of *esr1* and *esr2a* after rLh treatment. Plasma levels of Lh start to increase during post-vitellogenesis and peak during maturation, in parallel with Lhr expression in the ovary [55]. These results are in agreement with the ovarian expression peak observed in the maturation stage for the three *esr* subtypes. However, this rapid expression increase disappears at 24 h treatment for *sbesr1* and *sbesr2a*, probably by the action of E_2 that tends to decrease Esr expression in ovarian explants (data not shown). Nevertheless, rFsh-induced expression of *sbesr2b* is maintained over time, which may indicate a role of this receptor in advanced stages of ovarian development and a lack of repression by E_2. There are few reports investigating the effect of gonadotropins on nuclear steroid receptor expression in the teleost ovary [38, 98], which does not allow for comparisons, and none of them analyzes the effect of homologous gonadotropins on *esr* expression in intact ovarian follicles.

In conclusion, in this first complete molecular and functional characterization of sbEsr2a and sbEsr2b we provide the first data in teleosts about the localization of all estrogen receptor subtypes in ovary and their regulation by gonadotropins. The tissue distribution of *sbesr1*, *sbesr2a* and *sbesr2b* confirms their roles in vitellogenesis and ovarian development. Specific expression profiles in the liver for each subtype strongly suggest that sbEsr1 plays a direct role in inducing vitellogenesis, while sbEsr2b regulates its action. In the ovary, the expression of the different *sbesr* subtypes is differentially regulated both *in vivo* during development and *in vitro* by gonadotropins. Finally, the localization of the estrogen receptor in the ovarian follicles differs depending on the developmental stage and receptor subtype. Although further

studies are needed to define the specific role for each receptor, the present findings suggest a different and novel function in ovarian development for each paralog.

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Figure legends

Fig. 1. Phylogenetic tree of aligned amino acid sequences of vertebrate estrogen receptor (ESR) subtypes (see Supplemental Table S1 for Ensembl and GenBank accession numbers). The evolutionary history was inferred using the Maximum Likelihood method based on the JTT matrix-based model [50]. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.7497)). The percentage of times each branching was obtained, out of 1000 bootstrap iterations, is indicated at each node. Branches with less than 50% support have been collapsed. The grouping of clades and genes is indicated.

Fig. 2. Functional characterization of sbEsr2a and sbEsr2b using luciferase reporter assays in HEK293 cells. (A) Fold induction in luciferase activity of sbEsr2a and sbEsr2b transfected cells exposed to 10nM E_2. Data are expressed relative to the empty vector that was set as 1.

Bars with asterisks are significantly different ($P < 0.001$). (B) Ligand-induced transactivation of sbEsr2a and sbEsr2b after incubation of cells with increasing doses of various steroid hormones. Percentage of response is given relative to the maximal dose of E_2 set as 100% and the minimal dose of T set as 0%. Luciferase activity in each case was calculated as relative light units (RLU) of induced firefly LUC/ RLU of constitutive *Renilla* LUC. Data represents the mean ± SEM of three independent experiments with triplicates for each condition.

Fig. 3. Tissue distribution of sea bass *esr1* (A), *esr2a* (B) and *esr2b* (C) expression detected by RT-qPCR. White bars represent female sea bass tissues, black bars male sea bass tissues. Tissues: Cer, cerebellum; Tel, telencephalon; O te, optic tectum; Htha, hypotalamus; Pit, pituitary; Gon, gonad; Liv, liver; Int, intestine; Spl, spleen; Kid, kidney; HKid, head kidney; Fat; Hea, heart; Mus, muscle; Skin; Gill; Eye; Fol cel, follicular cells.

Fig. 4. Expression pattern of *sbesr1* (A), *sbesr2a* (B) and *sbesr2b* (C) during annual ovarian development, determined by RT-qPCR. Data represent relative mean expression (± SEM), normalized to *ef1a* RNA expression, in ovaries at different developmental stages: pre-vitellogenesis (prevtg) (n = 15), early vitellogenesis (evtg) (n = 10), late post-vitellogenesis (late-postvtg) (n = 7), maturation-ovulation (mat-ovul) (n = 14) and atresia (atre) (n = 15). Statistical differences ($p < 0.001$) are indicated with different letters above the bars.

Fig. 5. Seasonal expression pattern of *sbesr1* (A), *sbesr2a* (B) and *sbesr2b* (C) in liver determined by RT-qPCR. Data represent relative mean expression (± SEM) normalized to *ef1a* expression in liver at different developmental stages: pre-vitellogenesis (prevtg) (n = 15), early vitellogenesis (evtg) (n =13), late post-vitellogenesis (late-postvtg) (n = 9), maturation-

ovulation (mat-ovul) (n = 9) and atresia (atre) (n = 7). Statistical differences ($p < 0.001$) are indicated with different letters above the bars.

Fig. 6. Levels of E2 in ovary of sea bass during their first sexual maturation season showing different stages of ovarian development (see Fig.6). Data are means ± SEM. Statistical differences ($p < 0.001$) are indicated with different letters above the bars.

Fig. 7. Immunolocalization of sbEsr1 and sbEsr2a in post-vitellogenic ovaries. Photomicrographs of (A) late-vitellogenic and (B) post-vitellogenic follicles where sbEsr1 is localized in granulosa cells, the plasma membrane and the cytoplasm. Photomicrographs of (C (detail), E) late-vitellogenic and (D) post-vitellogenic follicles where sbEsr2a is localized in granulosa cells, the plasma membrane and the cytoplasm. Control sections of post-vitellogenic ovaries without primary antibody, (F) secondary antibody goat-Alexa-594-coupled anti-rabbit IgG, (G) secondary antibody goat anti-rabbit IgG- HRP conjugated and developed with DAB.

Fig.8. Immunolocalization of sbEsr2b in sea bass ovaries at different developmental stages. (A) Fluorescent photomicrograph of early-vitellogenic ovaries, where sbEsr2b is localized in theca cells (white arrows) and inside small vesicles in the cytoplasm. (B) Photomicrograph of post-vitellogenic ovaries, where sbEsr2b is strongly localized in theca (white arrows) and weakly in granulosa cells. (C, D) Photomicrographs of two early-vitellogenic follicles showing Esr2b localization in small vesicles in the cytoplasm (black asterisks) and in theca cells (black arrows).

Fig. 9. *In vitro* effect of sea bass rFsh or rLh on *sbesr1*, *sbesr2a* and *sbesr2b* transcript levels in early-vitellogenic ovarian explants after 24 h of treatment (A) and post-vitellogenic ovarian explants 6, 24 h and 48h after gonadotropin treatment (B). Data (means ± SEM: n=3 separate experiments with 4 different replicates) were determined by RT-qPCR and normalized to *18s* ribosomal RNA expression. Bars with asterisk are significantly different (* $P < 0.05$; ** $P < 0.01$ *** $P < 0.001$) and *ns* indicates no significant differences.

Supplemental Data

Supplemental Materials and Methods

Immunocytochemistry of transfected COS-7 cells

African green monkey kidney fibroblast-like (COS-7) were cultured in DMEM with GlutaMAX (Life Technologies, Inc.) supplemented with 10% FBS and 100 U/ml penicillin and streptomycin, at 37 ºC in a 5% CO_2 incubator. Cells were transiently transfected with 2.2 µg of either pcDNA3-*sbesr1* [30], pcDNA3-*sbesr2a* or pcDNA3-*sbesr2b* and 0.2 µg of pEGFP-N3 (Clontech) using Lipofectamine 2000 (Invitrogen, Inc.). Fifteen hours after transfection, cells were reseeded on Poly-L-Lysine treated sterile cover slips. The next day, cells were washed with PBS and fixed in 4% paraformaldehyde in PBS pH 7.4 for 10 min. Permeabilization was done in PBS containing 0.25% Triton X-100 for 10 minutes. Blocking was done for 1 h with 5% FBS in PBS. Then, cells were incubated with 1:600 anti-Esr1, anti-Esr2a or anti-Esr2b [57, 58] in blocking solution overnight at 4ºC. Antibody solution was washed in PBS and incubated for 1 h with the secondary antibody Alexa Fluor 594 goat anti-rabbit IgG (Invitrogen) diluted 1:2000 in blocking buffer. After washing with PBS, nuclei were stained with Hoescht 33342 (10 µg/mL in PBS) and cover slips were mounted on slides using Prolong Gold antifade reagent (Life Technologies, Eugene OR, USA). Slides were

photographed with a Nikon Eclipse E600 imager microscope (Nikon Instruments Europe) using the ProgRes CapturePro software (Jenoptik AG, Jena Germany). Observation of green fluorescence protein (GFP) was used to identify transfected cells.

Legends to supplemental figures

Supplemental Fig. S1. Immunocytochemistry of anti-Esr1 (red) in transfected COS-7 cells producing sea bass Esr1, Esr2a or Esr2b. GFP presence (green) identifies transfected cells. Nuclei were counterstained with DAPI (blue).

Supplemental Fig. S2. Immunocytochemistry of anti-Esr2a (red) in transfected COS-7 cells producing sea bass Esr1, Esr2a or Esr2b. GFP presence (green) identifies transfected cells. Nuclei were counterstained with DAPI (blue).

Supplemental Fig. S3. Immunocytochemistry of anti-Esr2b (red) in transfected COS-7 cells producing sea bass Esr1, Esr2a or Esr2b. GFP presence (green) identifies transfected cells. Nuclei were counterstained with DAPI (blue).

Supplemental Fig. S4. Multiple amino acid alignment of European sea bass Esr2a and Esr2b with other known vertebrate counterparts using ClustalW (see Supplemental Table S1 for Ensembl and GenBank accession numbers). Conserved amino acids are highlighted in black (identical) or grey (similar). Conserved DNA- and ligand-binding domains are underlined. Asterisks indicate the 8 cysteine residues of the zinc-fingers motif of the DBD. The P- and D-boxes from the DBD, hinge region, and the AF-2 motif from the LBD are indicated with lines above the alignment. Aminoacids involved in estradiol binding and co-activator interaction are marked with hash or plus symbols, respectively. Hsa: *Homo sapiens*, Mmu: *Mus*

musculus, Xtr: *Xenopus tropicalis*, Loc: *Lepisosteus oculatus*, Dre: *Danio rerio*, Dla: *Dicentrarchus labrax*, Sau: *Sparus aurata.*

Supplemental Fig. S5. Percent identities in the DNA- and ligand-binding domains (DBD and LBD) of the sea bass Esr2a (A) or Esr2b (B) with other sequences from the ESR2 subfamily.

Supplemental Fig. S6. Representative stained sections of sea bass ovaries at (A) early vitellogenesis and (B) advanced vitellogenesis. The oocyte (o) shows the zona radiata (zr) in the periphery and it is surrounded by the follicular cells, namely a continuous inner layer of granulosa cells (gc) and an outer thecal layer (tc).

Table 1.

Transcriptional activities of sea bass nuclear estrogen receptors

Emax(%)	Esr2a	Esr2b
E2	100	100
E3	30	60
T	23	43

E2: 17β-Estradiol, E3: Estriol, T: testosterone

	E2 EC_{50} (M)	95%CI (M)
Esr2a	2,556e-007	1,298e-007 to 5,032e-007
Esr2b	1,725e-008	6,252e-009 to 4,759e-008

95%CI : 95% Confidence Interval of EC_{50}.

Figure 1

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Fig. 1

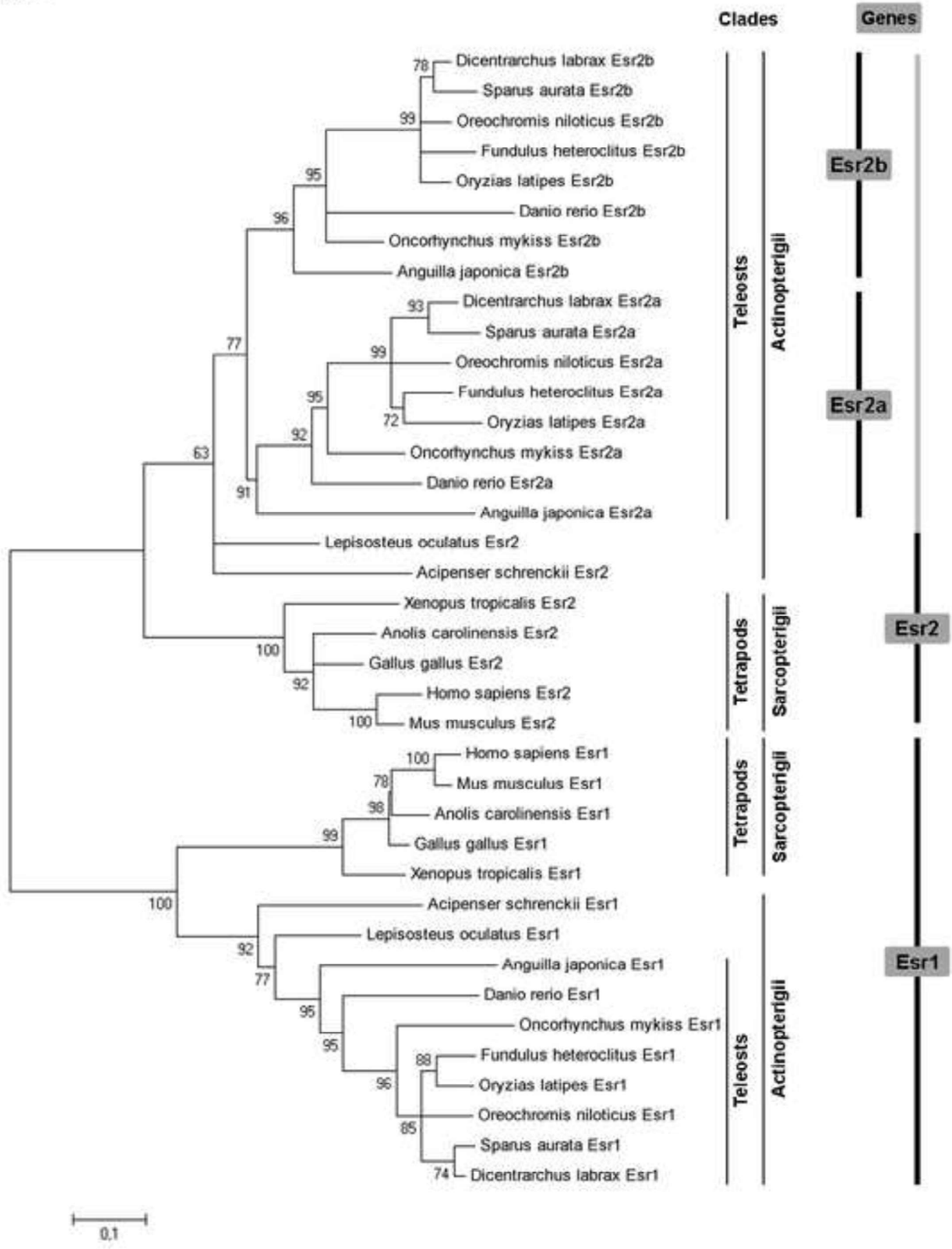

Fig. 2

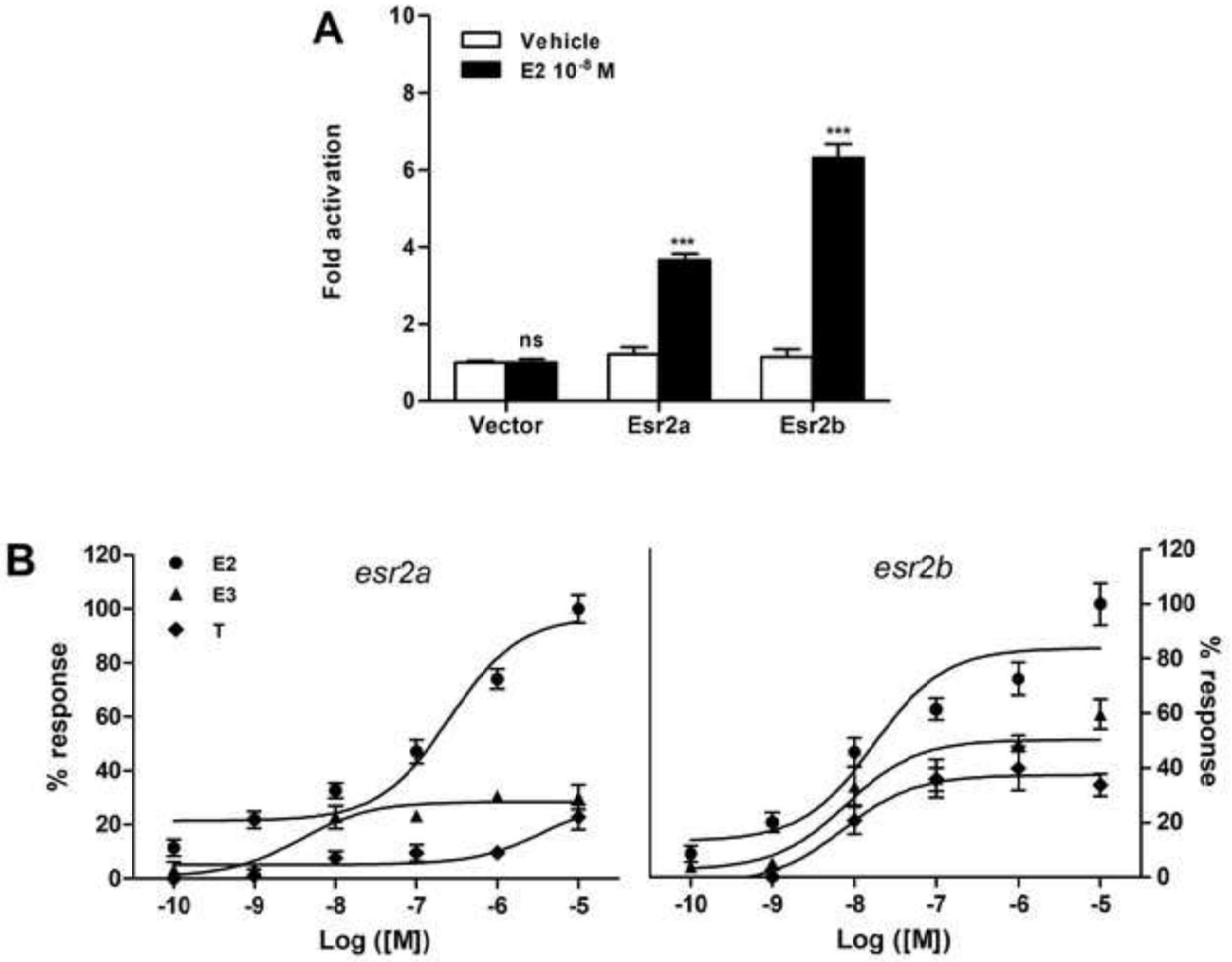

Figure 3

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Fig. 3

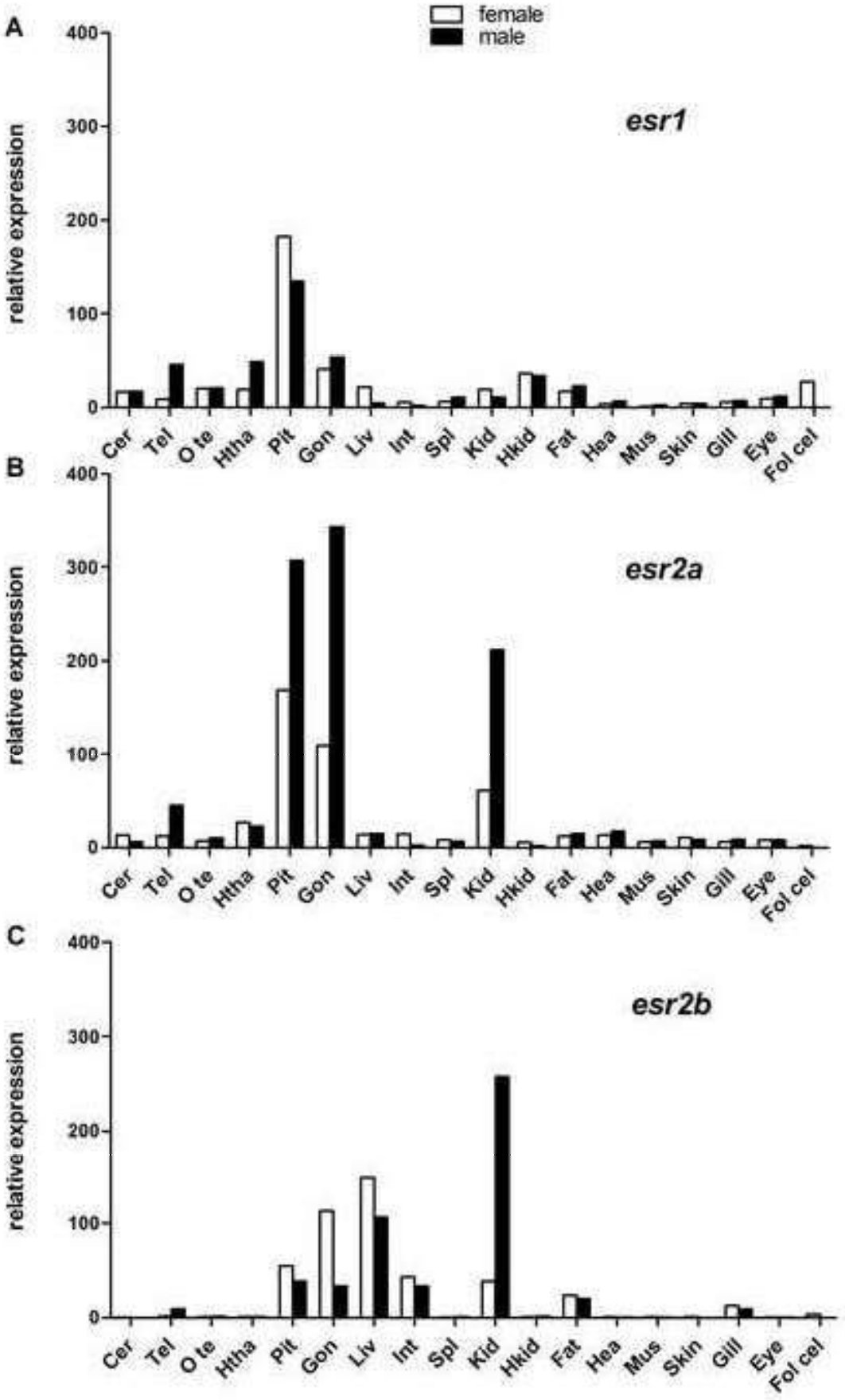

Figure 4

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Fig. 4

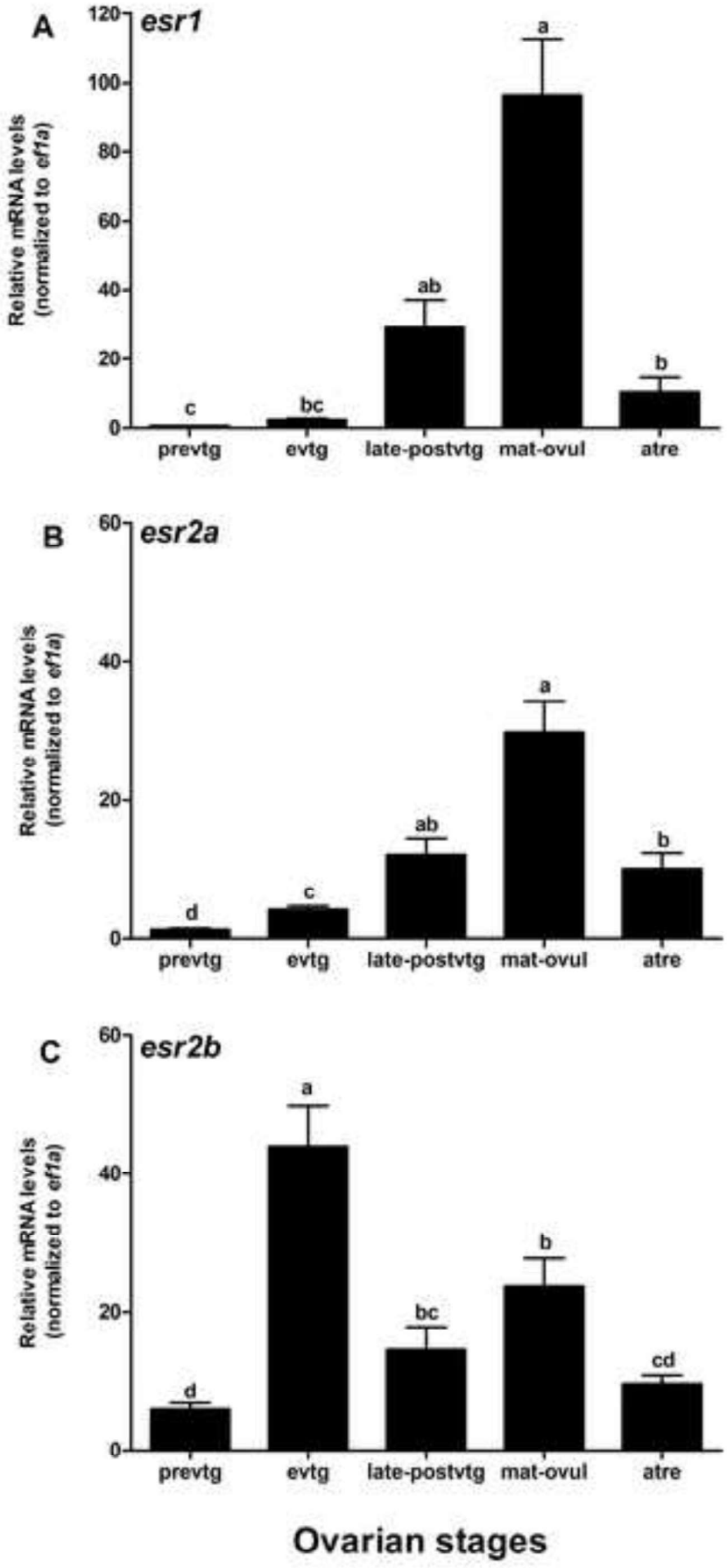

Figure 5

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Fig. 5

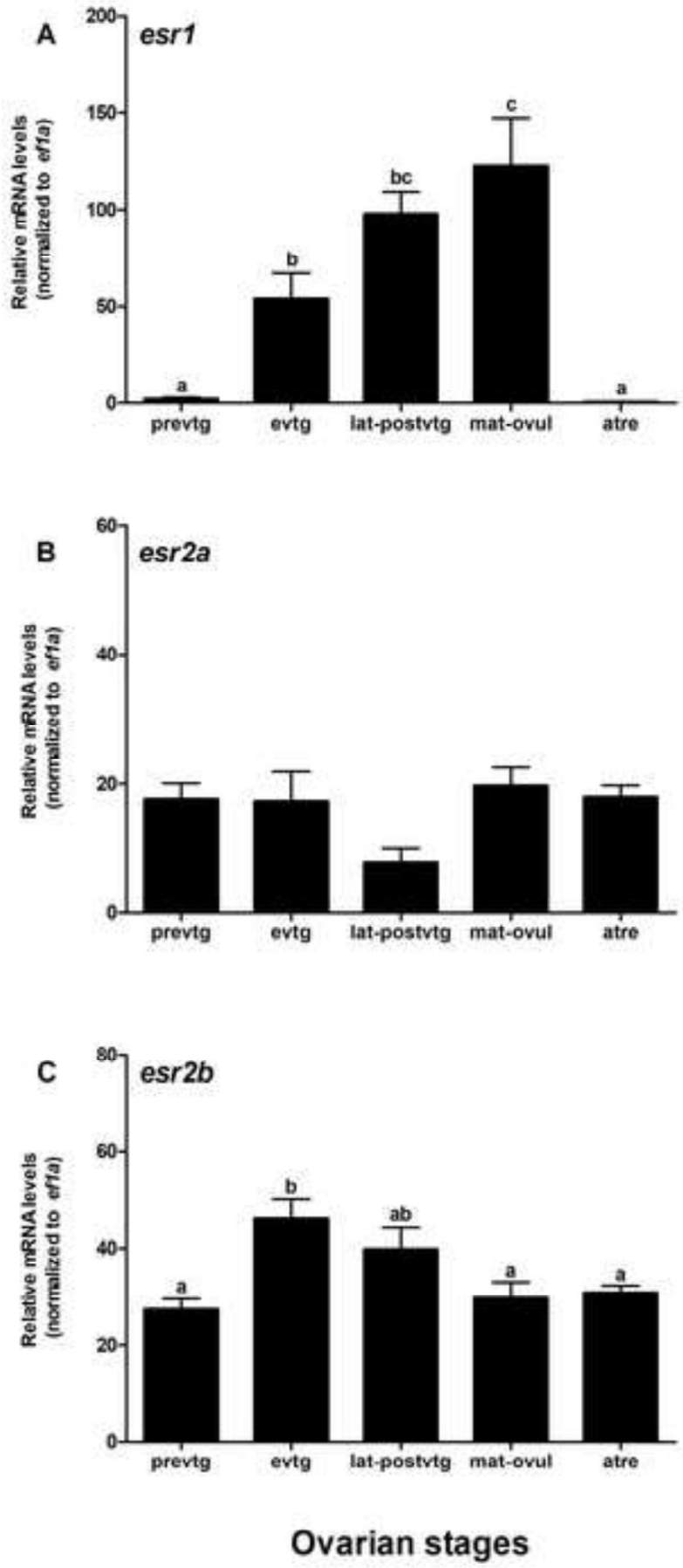

Fig. 6

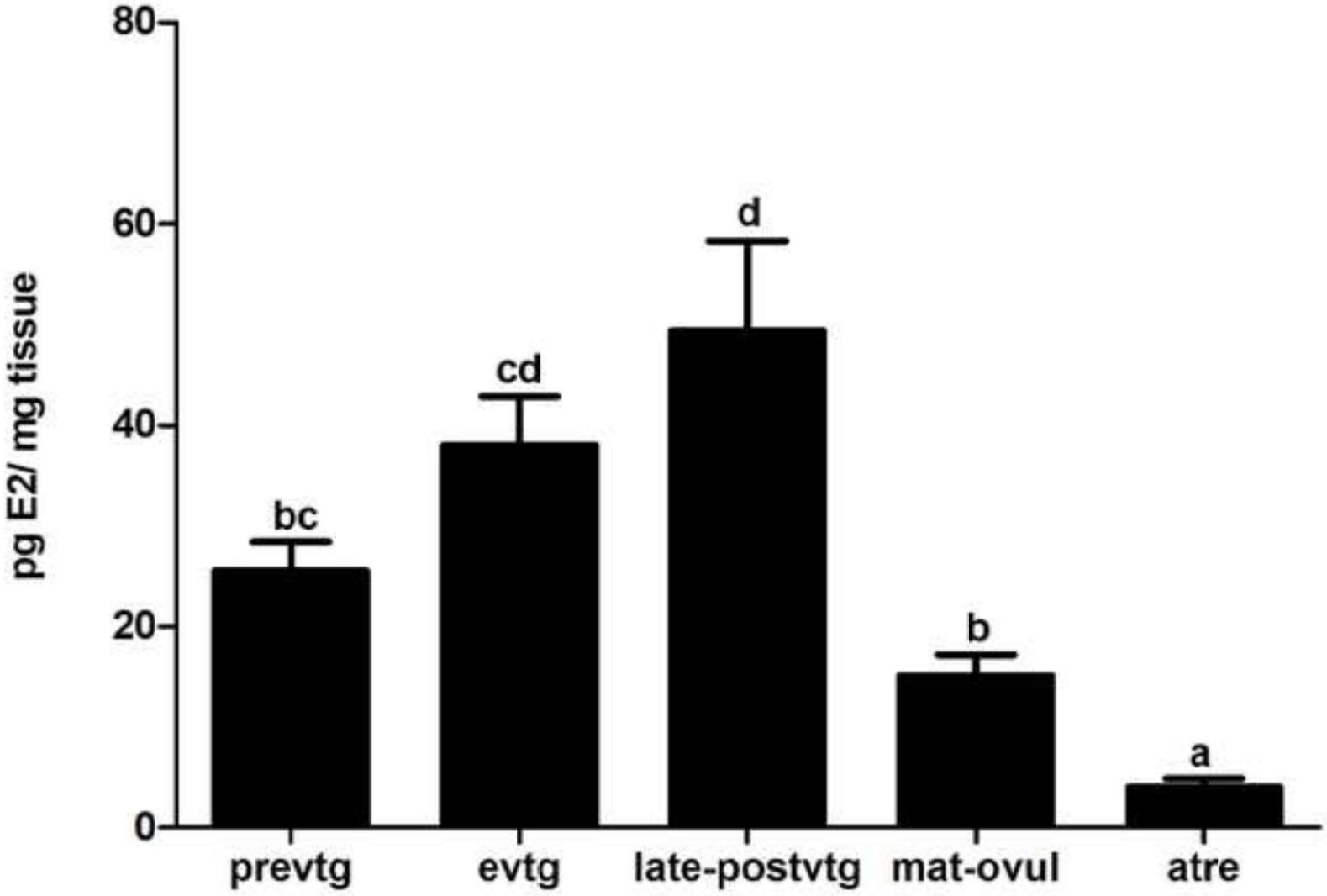

Figure 7

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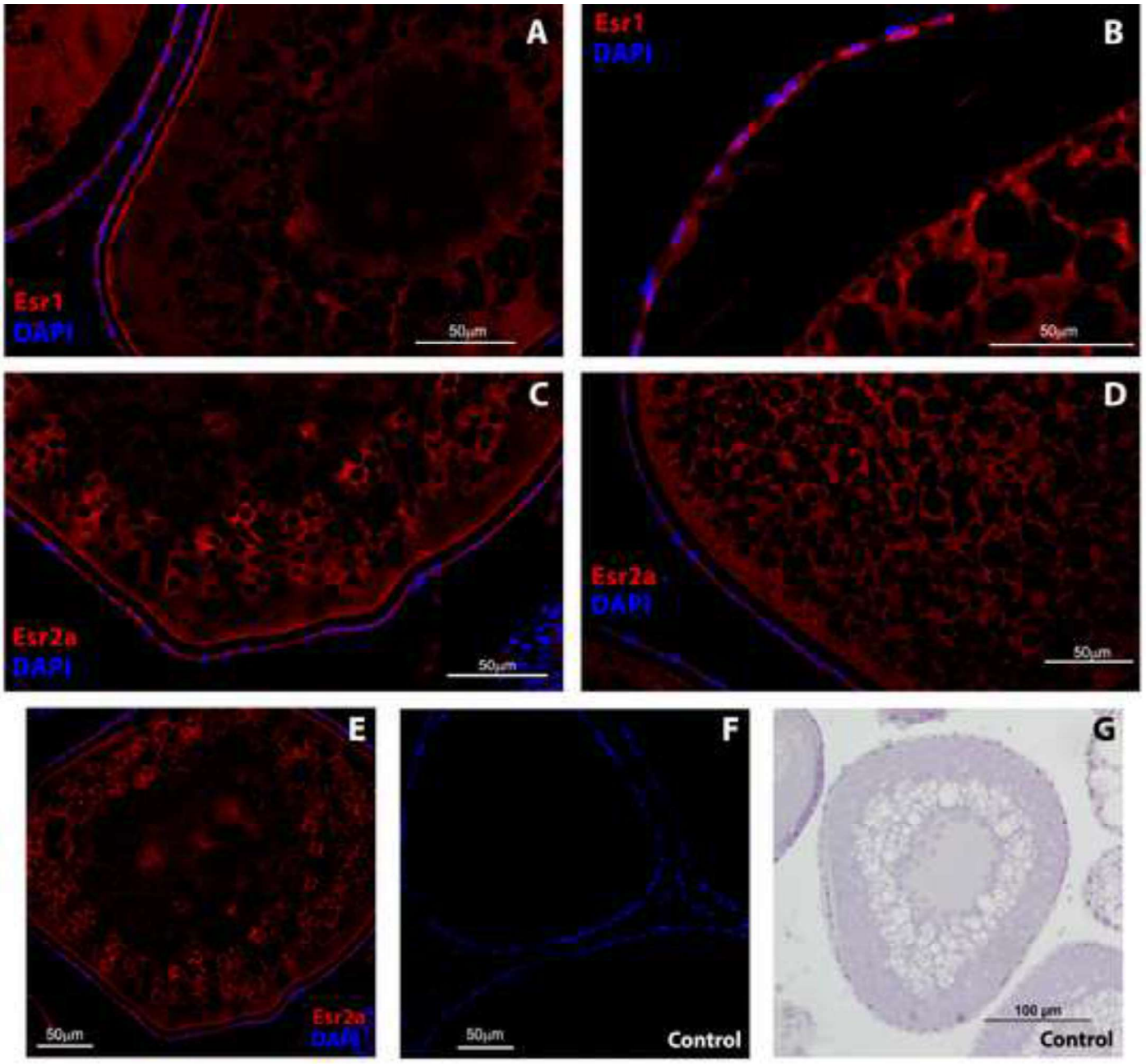

Fig. 7

Figure 8

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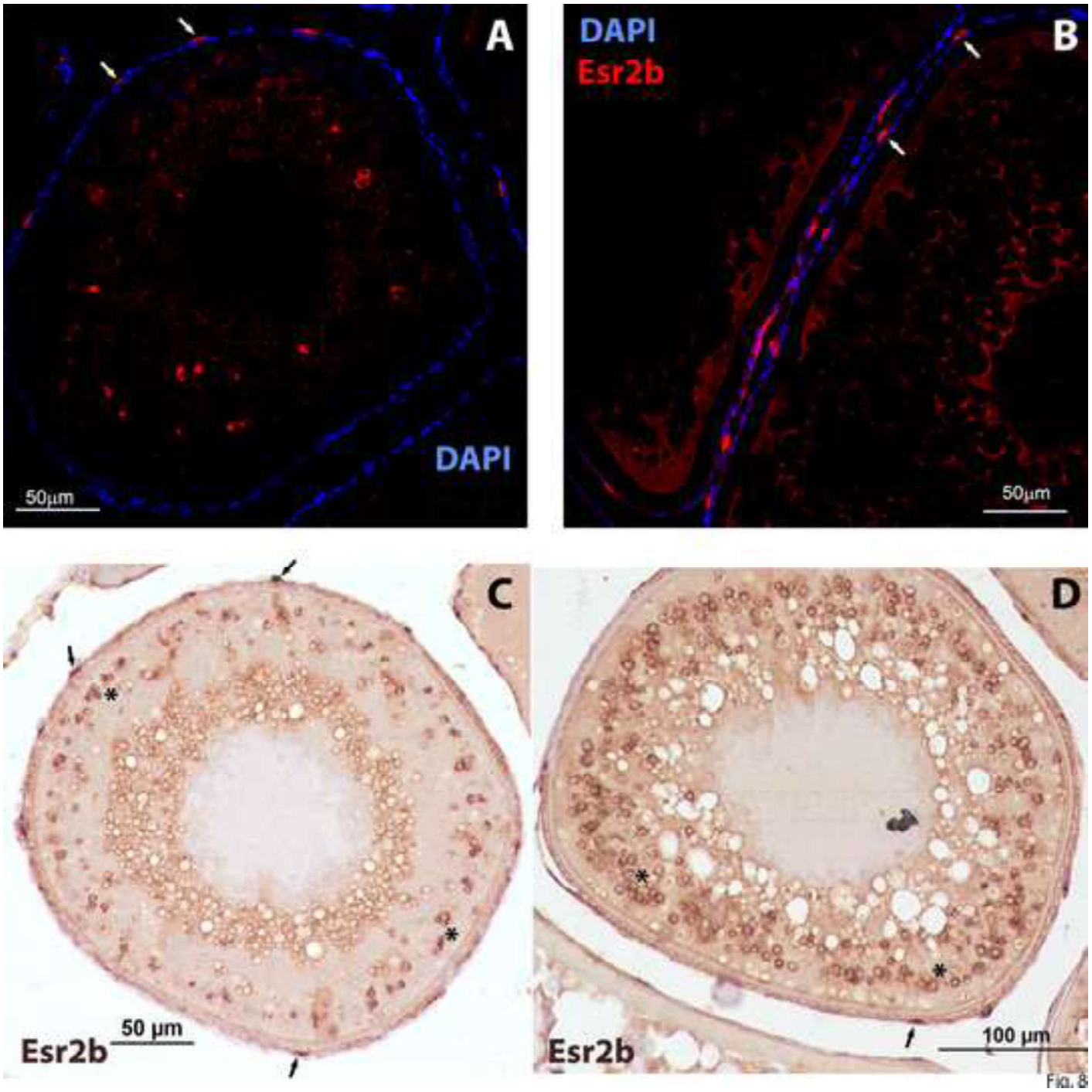

Fig. 8

Fig. 9