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Three nuclear and two membrane estrogen receptors in basal teleosts, *Anguilla* sp.: Identification, evolutionary history and differential expression regulation

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ABSTRACT

Estrogens interact with classical intracellular nuclear receptors (ESR), and with G-coupled membrane receptors (GPER). In the eel, we identified three nuclear (ESR1, ESR2a, ESR2b) and two membrane (GPERa, GPERb) estrogen receptors. Duplicated ESR2 and GPER were also retrieved in most extant teleosts. Phylogeny and synteny analyses suggest that they result from teleost whole genome duplication (3R). In contrast to conserved 3R-duplicated ESR2 and GPER, one of 3R-duplicated ESR1 has been lost shortly after teleost emergence. Quantitative PCRs revealed that the five receptors are all widely expressed in the eel, but with differential patterns of tissue expression and regulation. ESR1 only is consistently up-regulated *in vivo* in female eel BPG-liver axis during induced sexual maturation, and also up-regulated *in vitro* by estradiol in eel hepatocyte primary cultures. This first comparative study of the five teleost estradiol receptors provides bases for future investigations on differential roles that may have contributed to the conservation of multiple estrogen receptors.

1. Introduction

Sex steroid hormones, such as estrogens, are essential for reproductive function, being involved, in females, in the regulation of oogenesis and vitellogenesis as well as the development of secondary sex characteristics, and, in males, in testicular development/spermatogenesis. They also feed back on brain and pituitary to regulate GnRH and gonadotropin expression and release (Campbell et al., 2004; Zohar et al., 2010). As a steroid, estrogen interacts with nuclear receptors, forming a hormone-receptor complex, translocated in the nucleus, where it activates promoter regions of target genes by binding to specific hormone response elements (ERE) (Mangelsdorf et al., 1995). These mechanisms, resulting in mRNA and protein synthesis, are described as classical genomic steroid actions, and considered as relatively slow (Thomas, 2012). The estrogen nuclear receptors ER belong to the nuclear receptor superfamily and are composed by six distinct regions corresponding to autonomous functional domains (for review: Aranda and Pascual, 2001; Nelson and Habibi, 2013). The N-terminal region (A/B), the most variable between species, contains a first activation function (AF1), which induces constitutive ligand-independent activation of the receptor. The C region, also known as the DNA-binding domain (DBD), has two zinc-finger structures essential for recognition and specific binding of the receptor to DNA. This region is the most highly conserved domain between ERs of different species. The D region, poorly conserved, is necessary for the maintenance of ER three-dimensional structure and also confers nuclear localization signals. The ligand-binding domain (LBD) is found in the E region and is responsible for transcriptional activation (AF-2) and dimerization. Finally, the F region denotes the end of the AF2 and continues to the C-terminus of the receptor.

The first ER to be characterized (ESR1, also named ERα and NR3A1) was isolated in human from breast cancer cell line MCF-7 in 1986 (Green et al., 1986). Ten years later, a second ER (ESR2, also named ERβ and NR3A2) was identified in mammals (rat: Kuiper et al., 1996; human: Mosselman et al., 1996). In teleosts, the first ER isolated (ESR1 or ERα) was reported in 1990 in rainbow trout (*Oncorhynchus mykiss*) (Pakdel et al., 1990), while the first ESR2 (ERβ) was identified in 1996 in Japanese eel (*Anguilla japonica*) (Todo et al., 1996). Subsequently, two ESR2 genes were reported in teleosts (goldfish, *Carassius auratus*: Ma et al., 2000; Tchoudakova et al., 1999; Atlantic croaker, *Micropogonias undulatus*: Hawkins et al., 2000; zebrafish, *Danio rerio:* Bardet et al., 2002; Menuet et al., 2002). These two ESR2 genes likely

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result from the third round of whole genome duplication (3R) that occurred in teleosts (Bardet et al., 2002; Hawkins et al., 2000).

It has been suggested that steroids could also interact with cell membrane receptors, inducing a fast non-genomic response via rapid activation of intracellular signal transduction pathways (Falkenstein et al., 2000; Norman et al., 2004; Revelli et al., 1998; Watson and Gametchu, 1999). Some rapid estrogen actions appear to be mediated by nuclear estrogen receptors expressed near the cell surface (Razandi et al., 1999; Watson et al., 1999). However, increasing evidence has demonstrated the involvement of specific membrane receptors in these rapid actions of estrogen (for review: Thomas, 2012). Evidence that the orphan G protein-coupled receptor, GPR30, cloned from human and rodent tissues (Carmeci et al., 1997; O'Dowd et al., 1998; Owman et al., 1996; Takada et al., 1997), functions as a membrane estrogen receptor date back to 2005 (Revankar et al., 2005; Thomas et al., 2005). This led to its renaming as G protein coupled estrogen receptor (GPER). Homologous GPER have since also been identified in teleosts (Atlantic croaker: Pang et al., 2008; zebrafish: Liu et al., 2009; Pang and Thomas, 2009; common carp, *Cyprinus carpio*: Majumder et al., 2015). While in genome databases two GPER genes have been recently annotated in some teleosts species, to our knowledge, this study in the European eel constitutes the first investigation of two GPER in teleosts, and in vertebrates.

Due to their phylogenetic position, as representative of a basal group of teleosts, Elopomorphs, *Anguilla* species may provide insights into ancestral regulatory functions in teleosts (Henkel et al., 2012a,b), the largest group of vertebrates. Furthermore, their striking biological cycle, with a blockade of sexual maturation as long as the reproductive oceanic migration is not performed, makes the eel a powerful model to investigate neuroendocrine mechanisms of puberty (Dufour et al., 2003; Rousseau et al., 2014). In addition to the complete cDNA sequence of *Anguilla japonica* ESR2a (ERβ2) (Todo et al., 1996), partial cDNA of two estrogen receptors, ESR1 and ESR2a, have already been isolated in European eel, *Anguilla anguilla* (Palstra and van den Thillart, 2010). In the present study, we report the occurrence of two ESR2 (ESR2a and ESR2b) in the European eel, as well as the complete sequence of ESR1. We also identified two GPERs (GPERa and GPERb). Phylogenetic and syntenic analyses allowed us to investigate the origin and evolutionary history of ER and GPER families in osteichthyans, with special focus on teleosts. In order to get insights into the potential process driving the conservation of multiple ER and GPER, we studied their tissue distribution and regulation during experimental maturation in the eel. We also investigated *in vitro* the regulation of their expression in the liver, after development of a simplified method for primary culture of eel hepatocytes.

2. Material and methods

2.1. Genome and database searches

2.1.1. Vertebrate ER and GPER

ER and GPER sequences from vertebrate species were retrieved from the Ensembl (http://www.ensembl.org/index.html) and NCBI (http://www.ncbi.nlm.nih.gov/) databases. The references are indicated in Supplementary Tables S1 and S2. Additional blasts were performed in the genomes of teleost species when only one copy of the target gene was annotated in the databases, in order to look for the potential presence of duplicated genes resulting from the teleost 3R.

2.1.2. Eel ER and GPER prediction

The TBLASTN algorithm of the CLC DNA Workbench software (CLC bio, Aarhus, Denmark) was used to predict the ER and GPER sequences in the European and Japanese eel draft genomes (Henkel et al., 2012a,b). Several actinopterygian sequences of ER and GPER were used as queries, including spotted gar, *Lepisosteus oculatus* (ESR1: XP_006625908.1, ESR2: XP_006632252.1, GPER: XP_006637125.1), zebrafish (ESR1: AAI62466.1, ESR2a: AAK16741.3, ESR2b: NP_851297.1, GPER: NP_001122195.1), medaka, *Oryzias latipes* (ESR1: ENSORLT00000018193, ESR2a: BAS02056.1, ESR2b: NP_001098172.1, GPERa: XP_004071380.1, GPERb: XP_011486455.1), Japanese eel (ESR2a: O13012.1) and European eel partial sequences (ESR1: ABU98636.1, ESR2a: ABF50552.1). The empirical nucleotide signature was used to predict the exons and splicing junctions from the genomic databases, *i.e.* introns begin with "GT" and end with "AG".

2.2. ER and GPER phylogenetic analyses

2.2.1. Sequence alignments

Multiple sequence alignments of the protein families were created using Clustal Omega (Sievers et al., 2011) included in SeaView version 4.4.2 (Gouy et al., 2010). The alignments were manually edited to adjust poorly aligned sequence stretches. Calculation of best amino acid substitution matrix was determined using the Protest software (Abascal et al., 2005). The JTT (Jones, Taylor and Thornton) protein substitution matrix was selected. Phylogenetic analyses of the resulting protein alignments were performed using the Maximum Likelihood method with 1000 bootstrap replicates (RaxML software (Stamatakis, 2014), www.phylo.org).

2.2.2. ER

Amino acid sequences of 66 osteichthyan ER were retrieved from NCBI and Ensembl databases (Table S1). The vertebrate species included sarcopterygians (tetrapods and a representative species of a basal group, a dipnoan, *Protopterus annedens*) and actinopterygians (teleosts and non-teleost actinopterygians, a chondrostean, Japanese sturgeon, *Acipenser schrenckii*, and two holosteans, spotted gar, and alligator gar, *Atractosteus tropicus*). ER-like sequences from protostomian species, the Pacific oyster, *Crassostrea gigas*, and the polychaete annelid ragworm, *Platynereis dumerilii*, were used as outgroup.

2.2.3. GPER

Amino acid sequences of 37 osteichthyan GPER were retrieved from NCBI and Ensembl databases (Table S2). The vertebrate species included sarcopterygians (tetrapods and a basal sarcopterygian, an actinistian, the coelacanth, *Latimeria chalumnae*), and actinopterygians (teleosts and a non-teleost actinopterygian, the spotted gar). The GPER sequence of a chondrichthyan (Elephant shark, *Callorhinchus milii*) was used as outgroup.

2.3. Synteny analysis of actinopterygian GPER genomic regions

Neighboring genomic regions of the duplicated eel GPER were characterized manually on the European and Japanese eel genomic databases, using CLC DNA Workbench 6 software and the GENSCAN Web Server (http://genes.mit.edu/GENSCAN.html). The genes located in the same scaffolds as GPER were identified. Potential paralogs of these genes were searched in the European and Japanese eel genomes. Their homologs were then identified in the other actinopterygian genomes, using Genomicus PhyloView of Genomicus v78.01. GPER genomic regions were compared between a non-teleost actinopterygian, the spotted gar, and teleosts (zebrafish, medaka, stickleback, *Gasterosteus aculeatus*, fugu, *Takifugu rubripes*, tilapia, *Oreochromis niloticus*, platyfish *Xiphophorus maculatus*, and amazon molly, *Poecilia formosa*). For each GPER neighboring gene family, when only one gene was annotated in all

the above-mentioned genomes, BLAST analyses were performed to search for potential additional paralogs.

2.4. In vivo experiments

2.4.1. Ethics statement

Experimental maturations were conducted on farmed female European eels, at a DTU Aqua research facility at Lyksvad Fishfarm, Vamdrup, Denmark. All fish were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC). Eel experimental protocol was approved by the Animal Experiments Inspectorate (AEI), Danish Ministry of Food, Agriculture and Fisheries (permit number: 2010/561-1783).

Female European eels used in this study were at the pre-pubertal stage prior to experiments. As eels undergo a natural fasting period from the pre-pubertal silver stage to the end of the sexual maturation, they were not fed during treatment. All eels were anesthetized using ethyl p-aminobenzoate (benzocaine; Sigma–Aldrich, Germany), before tagging, handling and sacrifice. All efforts were made to minimize animal handling and stress.

2.4.2. Experimental maturation

Farmed female eels, raised from glass eels to large size ~65–85 cm at a commercial eel farm, using freshwater aquaculture recirculation systems (RAS) at ~25 °C, were selected and transferred to the research facility. Selection criteria included size and weight comparable to natural silvering females. At the facility, eels were acclimatized to saltwater (36‰) and temperature (20 °C) in 300 L tanks in RAS systems. The experimental protocol was established according to Butts et al. (2014). Thus, female eels received weekly injections of salmon pituitary extract (SPE Argent Chemical Laboratories, Washington, USA) at 18.75 mg/kg body weight for four months to induce vitellogenesis, followed by one dihydroxyprogesterone (17α,20ß-dihydroxy-4-pregnen-3-one; Sigma–Aldrich Denmark A/S) injection at 2 mg per/kg body weight to induce final oocyte maturation and ovulation. 10 control eels were sacrificed at the beginning of the experiment and 10 matured eels at the end of the experiment, after ovulation. Anterior brain (including olfactory bulbs, telencephalon, and di-/mesencephalon), pituitary, liver and ovary were sampled in RNAlater (Ambion) and stored at −20 °C until RNA extraction.

2.4.3. Tissue distribution

ER and GPER tissue distributions were analyzed on eight farmed female silver eels (mean weight 352 ± 38 g) acquired from Gebr. Dil import–export B.V. (Akersloot, The Netherlands). The following tissues were sampled in RNAlater (Ambion Inc., Austin, TX, USA) and stored at −20 °C until RNA extraction: olfactory bulbs, telencephalon, di-/mes-encephalon (including the hypothalamus), *cerebellum* and *medulla oblongata*, pituitary, eye, gills, liver, heart, spleen, peri-visceral adipose tissue, intestine, muscle and ovary.

2.4.4. RNA extraction

Extraction of total RNA was performed using mechanical homogenization (TissueLyser II, Qiagen, Hilden, Germany). Brain parts, pituitary, gills, heart, spleen and intestine were extracted using the Qiagen RNeasy Mini Kit (Qiagen). A deoxyribonuclease I treatment, using the Qiagen RNase-free DNase Set, was applied during the procedure, according to the manufacturer instructions. Eye, liver, adipose tissue, muscle and ovary were extracted using Trizol reagent (Invitrogen SARL, Cergy Pontoise, France) according to the manufacturer's instructions. After extraction, a final deoxyribonuclease I (Roche Ltd., Basel, Switzerland) treatment was applied to each sample. RNA quantifications have been performed in duplicates using a nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, USA).

2.5. European eel ER and GPER cDNA cloning

Pituitary, ovary and liver total RNA, from control and matured European eels, were used for cDNA cloning of European eel ERs and GPERs. One microgram of RNA was reverse transcribed using SuperScript III First Strand cDNA Synthesis Kit (Invitrogen) for PCR, or using SMART RACE cDNA amplification kit (Clontech Laboratories Inc., Palo Alto, CA., USA) for 3′ and 5′ RACE PCR.

Primers were designed using the Primer3 Software (Whitehead Institute/Massachusetts Institute of Technology, Boston, MA), and purchased from Eurofins (Elsersberg, Germany) (Table S3). PCR primer sequences were designed from the partial sequences predicted from the European and Japanese draft genomes.

PCRs were performed as follows: an initial step of polymerase activation for 3 min at 94 °C; then 2 cycles with 30 s at 94 °C, 30 s at 66 °C, 2 min at 72 °C; 2 cycles with 30 s at 94 °C, 30 s at 64 °C, 2 min at 72 °C; 2 cycles with 30 s at 94 °C, 30 s at 62 °C, 2 min at 72 °C; 2 cycles with 30 s at 94 °C, 30 s at 60 °C, 2 min at 72 °C; 3 cycles with 30 s at 94 °C, 30 s at 58 °C, 2 min at 72 °C; 3 cycles with 30 s at 94 °C, 30 s at 57 °C, 2 min at 72 °C; 2 cycles with 30 s at 94 °C, 30 s at 56 °C, 2 min at 72 °C; 3 cycles with 30 s at 94 °C, 30 s at 55 °C, 2 min at 72 °C; 20 cycles with 30 s at 94 °C, 30 s at 54 °C, 2 min at 72 °C, and a single final extension step of 5 min at 72 °C.

The RACE PCR with 5′cDNA or 3′cDNA as templates were performed as follows: an initial step of polymerase activation for 3 min at 94 °C; then 10 cycles with 30 s at 94 °C, 30 s at 70 °C, 3 min at 72 °C; then 25 cycles with 30 s at 94 °C, 30 s at 68 °C, 3 min at 72 °C; and a single final extension step of 5 min at 72 °C.

PCR products of appropriate estimated size were isolated with the QIAquick gel extraction Kit (Qiagen, Hilden, Germany), directly sequenced at Cochin Sequencing Platform (Paris, France), or cloned using the TOPO® TA Cloning® Kit (Invitrogen) before sequencing.

2.6. Eel hepatocyte primary culture

2.6.1. Dispersion and culture

The protocol for eel hepatocyte primary cell culture, developed in the present study, was adapted from Peyon et al. (1993), and simplified. In the previous protocol, collagenase was applied *in vivo*, using liver perfusion. In the present protocol, the collagenase treatment was applied *in vitro* directly on eel liver slices. This *in vitro* enzymatic treatment was adapted from the trypsin treatment, we had previously developed for eel pituitary primary cell cultures (Montero et al., 1996).

Livers from female farmed silver eels were dissected out, washed in calcium-free and magnesium-free Dulbecco's phosphate-buffered saline (DPBS) (Gibco, Invitrogen SARL, Cergy Pontoise, France) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 250 ng/mL fungizone (Gibco) (DPBS-sup), and placed into a petri dish with DPBS-sup. Livers were cut into small pieces with a sterile razor blade. The small pieces were further cut with a Mcilwain Tissue Chopper (Stoelting Co., Wood Dale, IL) into 1 mm^3 slices, and washed several time in DPBS-sup, in order to eliminate the maximum of red blood cells. Cell dispersion was performed using enzymatic and mechanical procedures. Liver slices were incubated at 25 °C in a solution of collagenase (Sigma) at 0.5 mg/mL DPBS-sup for 30 min with slow shaking. Enzyme digestion was stopped by removing the collagenase solution and gently washing the liver slices several times in DPBS-sup. The liver slices were then mechanically dispersed in DPBS-sup by repeated passages through a plastic transfer pipette (Falcon, Duscher, Brumath, France). Cell suspensions were filtered

through 70 μM nylon mesh filter, and harvested by centrifugation at 200*g* for 10 min. The pellet was re-suspended in DPBS-sup, and the number of cells was counted with a Malassez cytometer in the presence of trypan blue (Sigma) to assess cell viability. Cells were then harvested by centrifugation at 200*g* for 10 min and re-suspended in serum-free culture medium (CM: Medium 199 with Earle's salt, sodium bicarbonate, 100 U/mL penicillin, 100 μg/mL streptomycin, 250 ng/mL fungizone (Gibco)). Cells were plated on 96-well plates (60,000 cells/well) pre-coated with poly-L-lysine (Sigma), and incubated at 18 °C under 3% CO_2 and saturated humidity.

2.6.2. In vitro estradiol treatments

Treatments started on Day 0, 24 h after hepatocyte plating. Estradiol (E2: Sigma) stock solution (10^{-2} M) was prepared in ethanol and stored at −20 °C. E2 stock solution was further diluted in culture medium on each day of treatment, just before addition to the culture wells. Solutions of E2 at 10^{-5}, 10^{-6}, 10^{-8}, and 10^{-10} M were prepared and added to the cells on days 0, 3, 6, and 10. Ethanol final concentration in the culture wells never exceeded 0.001%. Control wells were treated with the similar final concentration of ethanol. Cultures were stopped on Days 3, 7, 10, and 12. The effects of treatments were tested in three independent experiments performed on different cell preparations from different batches of fish. Figures display the results of a representative experiment.

2.6.3. RNA extraction

Total RNA was directly extracted in wells using the Cell-to-cDNA II kit (Ambion Inc.) according to the manufacturer's instructions. Cells were washed with sterile DPBS (Gibco) and lysed with Cell Lysis II Buffer (80 μL/well). The RNA solutions were treated with RNase-free DNase I (Roche), and stored at −80 °C until Reverse Transcription.

2.7. Quantitative PCR (qPCR)

2.7.1. Design of qPCR primers

Specific qPCR primers for the three European eel estrogen nuclear receptors (ESR1, ESR2a, ESR2b) and the two European eel estrogen membrane receptors (GPERa, GPERb) were designed using the Primer3 Software. For ER, the forward and reverse primers were designed on two different exons in order to avoid a potential genomic DNA contamination of the tissue samples. For GPER, as the coding sequence of the genes are composed by a single exon, both primers were designed on the same exon. In order to confirm that there was no genomic contamination, a negative control RNA template was used in GPER qPCR, to ensure that no amplification was obtained. Primers for vitellogenin (Vtg) and beta-actin have been previously designed (Aroua et al., 2007; Pierron et al., 2009). All primers were purchased from Eurofins. The sequences are indicated in Supplementary Table S3.

2.7.2. qPCR protocol

For qPCR analyses, 500 ng of tissue total RNA, or 5 μL of cell culture RNA solution, were reverse transcribed using SuperScriptIII First Strand cDNA Synthesis Kit (Invitrogen). The qPCR analyses were performed with a Lightcycler (Roche, Ltd. Basel, Switzerland), using SYBR Green I sequence-unspecific detection. Each reaction contained 4 μL of diluted cDNA template (1/6 dilution), 2 μL of SYBR Green master mix, and 1 μL of each forward and reverse specific primers (0.5 pmol each at final concentration). The following program was applied for each gene: a polymerase activation step of 10 min at 95 °C, followed by 41 to 51 cycles of 10 s of denaturizing at 95 °C, 5 s of annealing at 60 °C, 6 s of elongation at 72 °C. The program ended with a melting curve analysis by slowly increasing the temperature (0.1 °C/s) from 68 °C to 95 °C, with a continuous registration of changes in fluorescent emission intensity. This step aimed at ensuring the presence of only a single specific amplified product.

The specificity of the primers was verified by sequencing the single amplification product obtained by qPCR, for each of the specific primer couples (GATC Biotech Ltd.). To further assess the specificity of the qPCR primers, each couple was tested for its inability to amplify the transcripts of the other receptor genes. PCR were conducted to obtain amplicons of ESR1, ESR2a, ESR2b, GPERa, and GPERb. Each amplicon overlapped the regions containing the qPCR primers of the other paralogs. Each qPCR primer couple, specific to a receptor, was not able to amplify the amplicons of the other receptors.

All tissue and cell culture samples were analyzed in duplicate. Each qPCR run contained a non-template control for each primer couple, by substituting the cDNA template with water. Serial dilutions of each specific amplicon were used as a standard curve. One dilution was included in each run as a calibrator. Normalization of the qPCR data was performed using total RNA content for tissue distributions, and eel beta-actin as a reference gene for comparison of experimental groups (experimental maturation and cell cultures).

2.8. Statistical analysis

Results are given as mean ± SEM. Means were compared by Student's *t*-test using Instat (GraphPad Software Inc., San Diego, Calif., USA).

3. Results

3.1. Identification of five estrogen receptors in the eel

BLAST analyses were conducted in order to retrieve sequences from the European and Japanese eel draft genomes. Several actinopterygian sequences, including spotted gar, zebrafish, and medaka, were used as queries (Tables S1 and S2). Three partial estrogen nuclear receptors, named ESR1, ESR2a, ESR2b, and two partial estrogen membrane receptors, named GPERa, GPERb, were predicted in each of the two eel draft genomes. The ESR2a sequence predicted in the Japanese eel genome corresponds to the sequence ESR2 (O13012.1) already published (Todo et al., 1996). Specific primers were designed from the five partial sequences predicted in the European eel genome. PCR and RACE PCR analyses were conducted on European eel tissue samples in order to obtain the complete coding DNA sequences (CDS).

3.1.1. Estrogen nuclear receptors

The CDS of European eel ESR1 (ERα) was a 1758 bp sequence composed by at least 8 exons (LN879034). In their current version, the European and Japanese eel draft genomes do not contain the complete 5′ end of the ESR1 sequence. This part of European eel ESR1 sequence was obtained from RACE PCR. When compared with other actinopterygian (spotted gar; zebrafish; tetraodon, *Tetraodon nigroviridis*; medaka; tilapia) and sarcopterygian (human, *Homo sapiens*; opossum, *Monodelphis domestiqua*; chicken, *Gallus gallus*; frog, *Xenopus tropicalis*) ESR1 sequences, we suggested that the 491 first nucleotides of the sequence that we could amplify corresponded to the first exon of ESR1 sequence, and therefore that the complete CDS is made of 8 exons. The resulting amino acid sequence consisted of 586 aa, and shared 51.2 percent identity with human ESR1, 63 percent with spotted gar ESR1, and 57 percent with zebrafish ESR1 (Table 1).

Table 1
Comparison of nuclear estrogen receptor amino acid sequences. Percentages of identity were calculated using LALIGN software (http://www.ch.embnet.org/software/LALIGN_form.html).

	hESR1	sgESR1	zESR1	eeESR1	hESR2	sgESR2	zESR2a	zESR2b	eeESR2a	eeESR2b
hESR1	–									
sgESR1	57.1	–								
zESR1	48.5	54.0	–							
eeESR1	51.2	63.0	57.0	–						
hESR2	46.3	47.5	46.5	46.2	–					
sgESR2	45.0	46.9	46.3	45.9	55.9	–				
zESR2a	44.5	42.0	43.4	42.6	48.5	54.9	–			
zESR2b	45.4	44.4	43.4	44.9	52.4	61.1	53.7	–		
eeESR2a	46.4	45.0	44.3	45.7	55.1	66.0	59.7	62.2	–	
eeESR2b	42.8	40.9	40.2	40.7	47.0	53.7	50.8	54.3	62.9	–

The CDS of European eel ESR2a (ERβ2) was a 1716 bp sequence composed by 8 exons (LN879035). The resulting amino acid sequence consisted of 572 aa, and shared 55.1 percent of identity with human ESR2, 66.0 percent with spotted gar ESR2, 59.7 percent with zebrafish ESR2a, and 62.2 percent with zebrafish ESR2b (Table 1). The CDS of European eel ESR2b (ERβ1) was a 1860 bp sequence composed by 9 exons (LN879036). The resulting amino acid sequence consisted of 620 aa, and shared 47.0 percent identity with human ESR2, 53.7 percent with spotted gar ESR2, 54.3 percent with zebrafish ESR2b, and 50.8 percent with zebrafish ESR2a (Table 1). European eel ESR1 sequence presented 45.7 and 40.7 percent of sequence identity with European eel ESR2a and ESR2b, respectively. The two European eel ESR2 shared 62.9 percent sequence identity (Table 1).

The three nuclear receptors of the European eel contained the classical domains of the nuclear receptor superfamily (Fig. S1). The most conserved domain was the C domain (DBD) with 86.7 and 84.0 percent of identity between European eel ESR1 and ESR2a, and between European eel ESR1 and ESR2b, respectively, and with 93.3 percent of identity between European eel ESR2a and ESR2b (Table 2). The three nuclear receptors contained eight conserved cysteines, localized in the DBD, and conserved in vertebrates. The ligand binding domain (E domain) was also highly conserved with 62.4 percent of identity between European eel ESR1 and each of the two ESR2 subtypes, and 77.8 percent between ESR2a and ESR2b (Table 2). The least conserved domain was the F domain with 26.7 percent identity between European eel ESR1 and ESR2a, 16.7 percent between European eel ESR1 and ESR2b, and 34.9 percent between the two European eel ESR2 subtypes (Table 2).

Table 2
Amino acid comparisons of the different domains of the nuclear estrogen receptors. Percentages of identity were calculated using LALIGN software (http://www.ch.embnet.org/software/LALIGN_form.html).

	ESR1	ESR2a	ESR2b
A/B			
ESR1	–		
ESR2a	23.7	–	
ESR2b	20.8	43.3	–
C (DBD)			
ESR1	–		
ESR2a	86.7	–	
ESR2b	84.0	93.3	–
D			
ESR1	–		
ESR2a	31.9	–	
ESR2b	29.2	56.0	–
E (LBD)			
ESR1	–		
ESR2a	62.4	–	
ESR2b	62.4	77.8	–
F			
ESR1	–		
ESR2a	26.7	–	
ESR2b	16.7	34.9	–

3.1.2. Estrogen membrane receptors

The CDS of European eel estrogen membrane receptor "a" (GPERa), orthologous to the one previously reported in some teleosts (Pang et al., 2008; Pang and Thomas, 2009) (see discussion), was a 1056 bp sequence composed by a single exon (LN879037). The resulting amino acid sequence consisted of 352 aa, and shared 63.3 percent of identity with human GPER, 78.8 percent with spotted gar GPER, and 79.7 percent with zebrafish GPER (Table 3). The CDS of European eel estrogen membrane receptor "b" (GPERb), was a 1062 bp sequence composed, as for GPERa, by a single exon (LN879038). The predicted amino acid sequence consisted of 354 aa, and shared 63.2 percent identity with human GPER, 84.7 percent with spotted gar GPER, and 84.5 percent with zebrafish GPER (Table 3). Both sequences contained seven highly conserved transmembrane domains classically described for GPCR (G protein-coupled receptor), and eight cysteines conserved among vertebrate GPER (Fig. S2).

3.2. Phylogeny analyses

3.2.1. ER phylogeny analysis

Based on a 66 osteichthyan ER amino-acid sequence alignment, and assuming the Pacific oyster, *C. gigas*, and ragworm, *P. dumerilii*, ER-like sequences as outgroup, a phylogenetic tree was generated using the Maximum Likelihood method (Fig. 1). This analysis clustered the osteichthyan ER sequences into two well-supported clades, the ESR1 and ESR2 clades. Each one of these two clades was composed of a sarcopterygian and an actinoptergian group. The ER sequences of non-teleost actinopterygians, the chondrostean Japanese sturgeon, *A. schrenckii*, and the two holostean spotted gar, *L. oculatus*, and alligator gar, *A. tropicus*, branched basis to their respective actinopterygian ESR1 and ESR2 clades, in accordance with the phylogenetical positions of these actinopterygian species. Among teleosts, the eel ESR1 sequence, *A. anguilla* ESR1, branched at the base of a single group encompassing all teleost

Table 3
Comparison of membrane estrogen receptors amino acid sequences. Percentages of identity were calculated using LALIGN software (http://www.ch.embnet.org/software/LALIGN_form.html).

	hGPER	sgGPER	zGPER	eeGPERa	eeGPERb
hGPER	–				
sgGPER	66	–			
zGPER	63.7	86.6	–		
eeGPERa	63.3	78.8	79.7	–	
eeGPERb	63.2	84.7	84.5	79.2	–

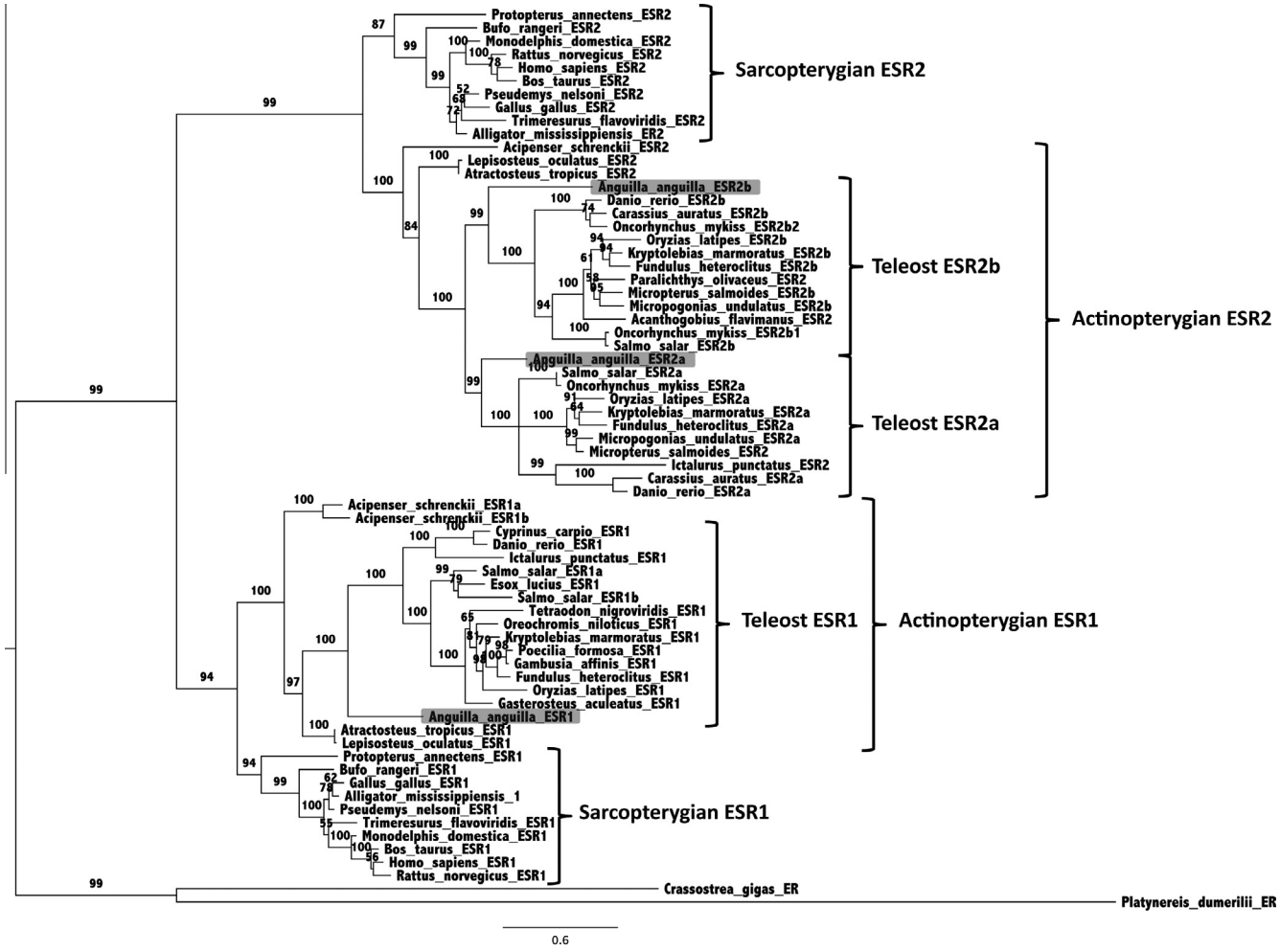

Fig. 1. Consensus phylogenetic tree of osteichthyan nuclear estrogen receptors. Phylogenetic analysis of 66 osteichthyan ESR amino acid sequences was performed using the Maximum Likelihood method, with 1000 bootstrap replicates (for the references of sequences, see Table S1). The number shown at each branch node indicates in percentage the bootstrap value. Only values above 50% are indicated. The tree was rooted using ER-like sequences from protostomian species, the Pacific oyster, *Crassostrea gigas*, and the polychaete ragworm, *Platynereis dumerilii*, as outgroup. The sarcopterygian and teleost clades are indicated on the figure.

ESR1 sequences. In contrast, teleost ESR2 sequences were separated in two well-supported clades, the ESR2a and ESR2b clades, with each of the two eel ESR2 sequences, *A. anguilla* ESR2a and *A. anguilla* ESR2b, branching at the base of the ESR2a and ESR2b clades, respectively. The two ESR2 clades in teleosts, including the eel, likely resulted from 3R, as previously proposed by other authors (Hawkins et al., 2000; Bardet et al., 2002). The names ESR2a/ESR2b for teleost duplicated ESR2 are given in accordance to the official nomenclature already established in zebrafish (Official Zebrafish Nomenclature Guidelines; http://zfin.org). ESR2a corresponds to ERβ2, ERβ-II, or ERβb, and ESR2b to ERβ1, ERβ-I, ERβa or ERγ, in some previous studies in teleosts. From the phylogenetic analysis, we could classify our two eel ESR2 sequences and name them eel ESR2a and eel ESR2b, based on their orthology to the other teleost ESR2a and ESR2b, respectively.

3.2.2. GPER phylogeny analyses

Based on a 37 osteichthyan GPER amino-acid sequence alignment, and assuming elephant shark sequence, *C. milii* GPER, as outgroup, a phylogenetic tree was generated using the Maximum Likelihood method (Fig. 2). This analysis clustered the osteichthyan GPER sequences in two clades, an actinopterygian and a sarcopterygian. The GPER sequence of an actinistian, the coelacanth, *L. chalumnae*, branched at the basis of the sarcopteryian clade, in agreement with the phylogeny. Similarly, the single GPER sequence of the non-teleost actinopterygian spotted gar, *L. oculatus,* branched basal to all teleost sequences. Two GPER sequences are present in various teleost species, and clustered in two clades, GPERa and GPERb. The teleost GPERb clade displayed longer branch lengths as compared to the shorter branch lengths of all the other osteichthyan GPER sequences. The two eel GPER sequences branched basal and independently from the two GPER clades of the other teleost sequences. From this phylogenetic analysis, we could suggest that the two GPER were issued from 3R, but we could not classify our two eel GPER sequences as GPERa and GPERb sequences.

3.3. GPER synteny analyses

To further resolve the origin and the nomenclature of the two eel GPER, we performed a synteny analysis on the GPER genomic region of several actinopterygians: spotted gar, medaka, zebrafish, stickleback, fugu, tilapia, platyfish, Amazon molly, Japanese eel and European eel. From the Japanese and European eel draft genomes, we could reconstruct the genomic environment of the two eel GPER. The spotted gar was chosen as the reference species for

the GPER neighboring genes in this synteny analysis. The GPER genomic region contains the following neighboring genes: PDPK1, KCTD5, UNKL, C16ORF91, ANKS3, WDR24, WDR90, NLRC3, XPO6, NME4, DECR2, RAB11FIP3, TLEO2, TMEM204, IFT140, CCDC78, and FAM173A (Fig. 3). Synteny analysis suggests that this genomic region had been duplicated in teleosts as a result of 3R. The GPER genes were duplicated in all the teleost species investigated, except for the zebrafish, while only a single GPER was present in the non-teleost actinopterygian spotted gar.

Concerning GPER neighboring genes, a single copy of all these genes was present in the genome of the non-teleost actinopterygian, spotted gar. For PDPK1 and KCTD5, duplicated genes were present in the European and Japanese eels and in the zebrafish, while only a single copy had been conserved in the other teleost genomes. In the zebrafish, the single conserved copy of 3R-duplicated GPER is located on the same genomic region as PDPK1a, and KCTD5a (Fig. 3). The zebrafish single GPER is thus located on 3R-paralogon "a". In accordance with the official zebrafish nomenclature for 3R-duplicated genes (http://zfin.org), we therefore named "GPERa" all the teleost GPER orthologous to zebrafish GPER. The second teleost GPER was named GPERb. Several of the GPER neighboring genes were duplicated only in the eel, such as UNKL, XPO6, NME4, RAB11FIP3, and TMEM204. Concerning C16ORF91, ANKS3, WDR24, WDR90, NLRC3, DECR2, TLEO2, IFT140, CCDC78, and FAM173A, a single copy of these genes was present in all the teleost genomes investigated, including both eel species. This suggests that one 3R-duplicated copy of all these genes has been lost in extant teleosts. In the eel, the genomic region of the GPER gene located on the scaffold 92.1 of the Japanese eel, and 173.1 of the European eel, contains the single conserved copy of C16ORF91, ANKS3, WDR24, WDR90, NLRC3, DECR2, TLEO2, IFT140, and CCDC78, as the genomic region of GPERb of the other teleosts investigated. This suggests that this eel GPER gene is orthologous to teleost GPERb. In the same way, in the eel, the genomic region of the GPER gene located on the scaffold 818.1 of the Japanese eel, and on the contig 11650.1 of the European eel contains the single conserved copy of FAM173A, as the genomic region of GPERa of the other teleosts, suggesting that this eel GPER is orthologous to teleost GPERa. According to their orthology to the other duplicated teleost GPER, we named eel GPER, GPERa and GPERb.

3.4. Tissue distribution of the five estrogen receptors in female European eels

In order to investigate and compare the expression of the five estrogen receptor transcripts in the eel, we developed specific qPCR assays for European eel ESR1, ESR2a, ESR2b, GPERa and GPERb. We assessed that there was no cross reaction in the qPCR assays between the three nuclear receptors, nor between the two membrane receptors.

The nuclear receptor ESR1 was widely expressed in female European eel tissues, including the five different parts of the brain, the pituitary and the various peripheral organs investigated, such as eyes, gills, heart, intestine, adipose tissue, liver muscle, spleen and ovary (Fig. 4A). The two nuclear receptors ESR2 were detectable in all the tissues with a differential expression. ESR2a was highly expressed only in the pituitary, while ESR2b was highly expressed in pituitary, adipose tissue and ovary (Fig. 4B). The two membrane receptors were also detectable in all the tissues and presented a differential expression. The highest expression of GPERa was found in the pituitary and ovary, while the highest expression of GPERb was found in pituitary and gills (Fig. 4C). For each of the five estrogen receptors, the highest expression was found in the pituitary (Fig. 4A–C).

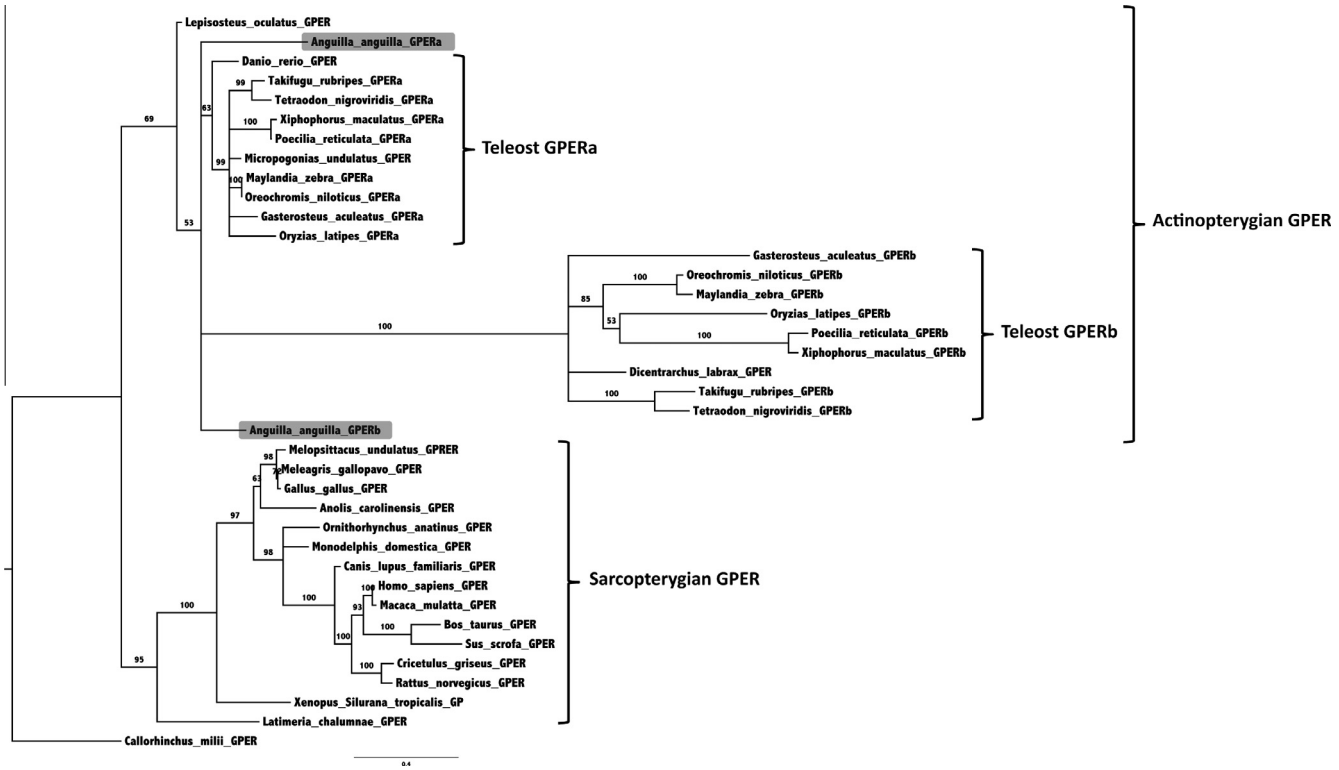

Fig. 2. Consensus phylogenetic tree of osteichthyan membrane estrogen receptors. Phylogenetic analysis of 37 osteichthyan GPER amino acid sequences was performed using the Maximum Likelihood method, with 1000 bootstrap replicates (for the references of sequences, see Table S2). The number shown at each branch node indicates in percentage the bootstrap value. Only values above 50% are indicated. The tree was rooted using GPER sequence from the chondrichthyan elephant shark, *Callorhinchus milii*, as outgroup. The sarcopterygian and teleost clades are indicated on the figure.

Fig. 3. Conserved genomic synteny of actinopterygian membrane estrogen receptor (GPER). Genomic synteny map comparing GPER and their neighboring genes from a non-teleost actinopterygian (spotted gar), and teleost species including the two eel GPER (GPERa and GPERb) genomic regions, are represented. The GPER genomic region has been duplicated in teleost species, likely as a result of the teleost specific third round of genome duplication. Orthologs of each gene are represented in the same color and displayed in the same column. The genes reproduced in this figure are not necessarily presented in the same order as they appear on the chromosomes and scaffolds, except for spotted gar, and their positions are indicated in 10^6 base pairs. A broken line symbolizes the location of a gene on different scaffolds or contigs. Concerning European eel GPERa neighboring genes, as they are located on multiple small scaffolds and contigs in the current draft genome, their positions were not indicated on the figure. The detailed genomic locations of all the genes are given in Supplementary Table S4.

3.5. In vivo regulation of the five estrogen receptors during experimental maturation in female European eels

The five estrogen receptor transcripts were all expressed in the organs involved in the reproductive function, such as in the BPG-Liver axis. To investigate their potential regulation in relation to reproduction, we analyzed their expression during experimentally induced reproduction in female European eels (Fig. 5). In the anterior brain, nuclear receptor ESR1 (2.8-fold, *i.e.* 2.8×; $n = 10$/group; $P < 0.0001$) and membrane receptor GPERa (1.7×; $n = 10$/group; $P < 0.0001$) transcripts were up-regulated, while no significant changes were observed for ESR2a, ESR2b, nor GPERb (Fig. 5A). In the pituitary, the nuclear receptor ESR1 expression was up-regulated (4.8×; $n = 9$/group; $P = 0.0006$), while the expression of the nuclear receptor ESR2b (0.7×; $n = 9$/group; $P = 0.0015$) and of the two duplicated membrane receptors, GPERa (0.6×; $n = 9$/-group; $P = 0.0041$) and GPERb (0.5×; $n = 9$/group; $P = 0.0008$), were down-regulated (Fig. 5B). In the ovary, the expression of nuclear receptor ESR1 was up-regulated (5.6×; $n = 10$/group; $P = 0.0068$), with no regulation of any of the other estrogen receptors (Fig. 5C). Finally, in the liver, the expression of nuclear receptor ESR1 was up-regulated (2.6×; $n = 10$/group; $P = 0.0005$), while the expression of nuclear receptors ESR2a (0.1×; $n = 10$/group; $P < 0.0001$), ESR2b (0.2×; $n = 10$/group; $P < 0.0001$), and membrane receptor GPERb (0.1×; $n = 10$/group; $P = 0.0013$) were down-regulated (Fig. 5D). These results indicate a differential regulation in the expression of the five estrogen receptors during experimentally induced reproduction in females, with an up-regulation of nuclear receptor ESR1 in all the tissues of the BGP-Liver axis.

3.6. In vitro regulation of estrogen receptors in eel hepatocyte primary cultures

To further investigate the differential regulation of estrogen receptors in the eel, we developed *in vitro* hepatocyte culture. The cells were treated with estradiol and the expression of Vtg,

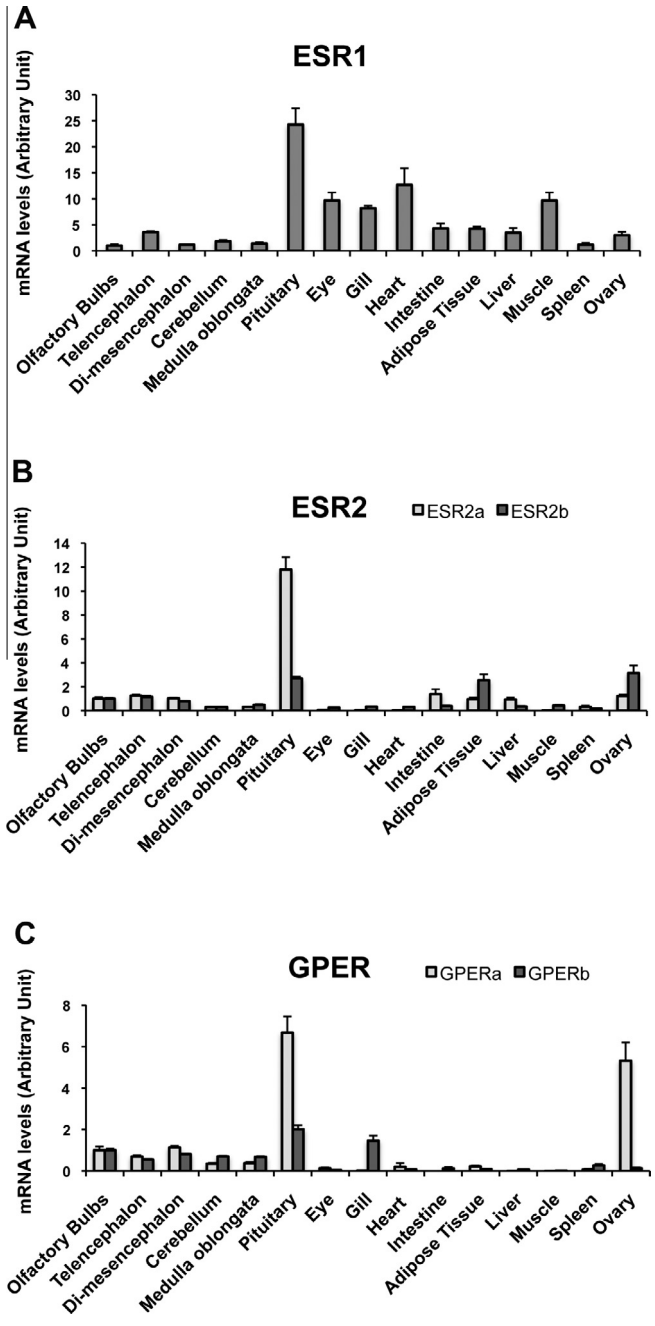

Fig. 4. Tissue distributions of the expression of the three nuclear estrogen receptors (ESR1, ESR2a, ESR2b) and the two membrane estrogen receptors (GPERa, GPERb) in the European eel. Messenger RNA levels for eel ESR1 (Fig. 4A), eel ESR2a and ESR2b (Fig. 4B), and eel GPERa and GPERb (Fig. 4C) were measured by qPCR and normalized to the amount of total RNA. Each bar represents the mean ± SEM from 8 female eels.

ESR1, ESR2a, ESR2b, GPERa, GPERb were analyzed by qPCR (Fig. 6). GPERa expression was under the level of detection and could not be measured in cultured hepatocytes. Different concentrations of estradiol (10^{-5}, 10^{-6}, 10^{-8}, 10^{-10} M) were tested over 3, 7, 10 and 12 days of culture (data not shown), and the concentration of 10^{-6} M was selected as the most efficient concentration. Vtg expression level was significantly ($P < 0.0001$) and time dependently increased after 3 days (820×), 7 days (1080×), 10 days (23,490×), and 12 days (63,400×) of estradiol treatment (Fig. 6A). The expression level of estrogen nuclear receptor ESR1 was also significantly up-regulated after 3 days (1.51×; $P = 0.0060$), 7 days (2.32×; $P < 0.0001$), 10 days (2.68×; $P < 0.0001$), and 12 days

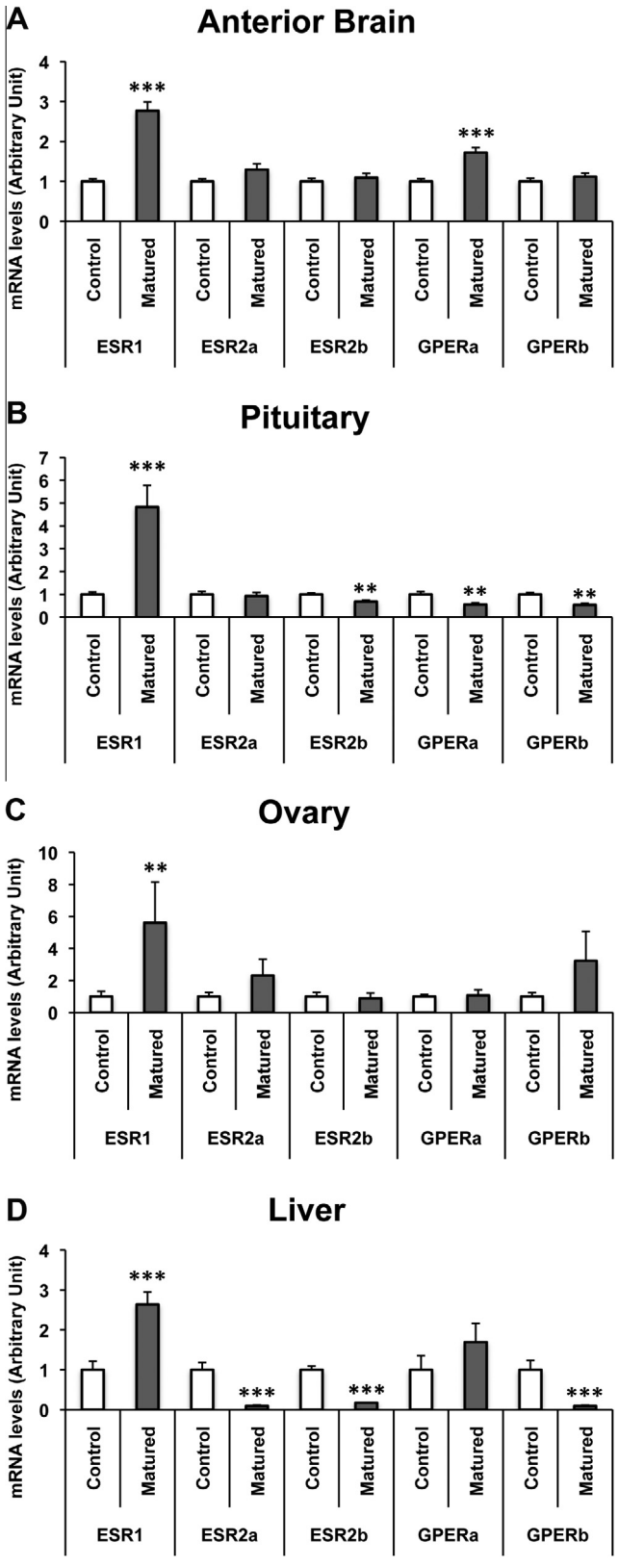

Fig. 5. Regulation of the expression of the three nuclear and two membrane estrogen receptors in female eels in the BPG-liver axis during experimental maturation. The mRNA levels of eel ESR1, ESR2a, ESR2b, GPERa, and GPERb were measured in the anterior brain (Fig. 5A), the pituitary (Fig. 5B), the ovary (Fig. 5C) and the liver (5D), by qPCR and normalized to beta-actin mRNA. Each bar represents the mean ± SEM from 10 control and 10 matured female eels. Significant differences between the two eel groups were analyzed by Student's *t*-test; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

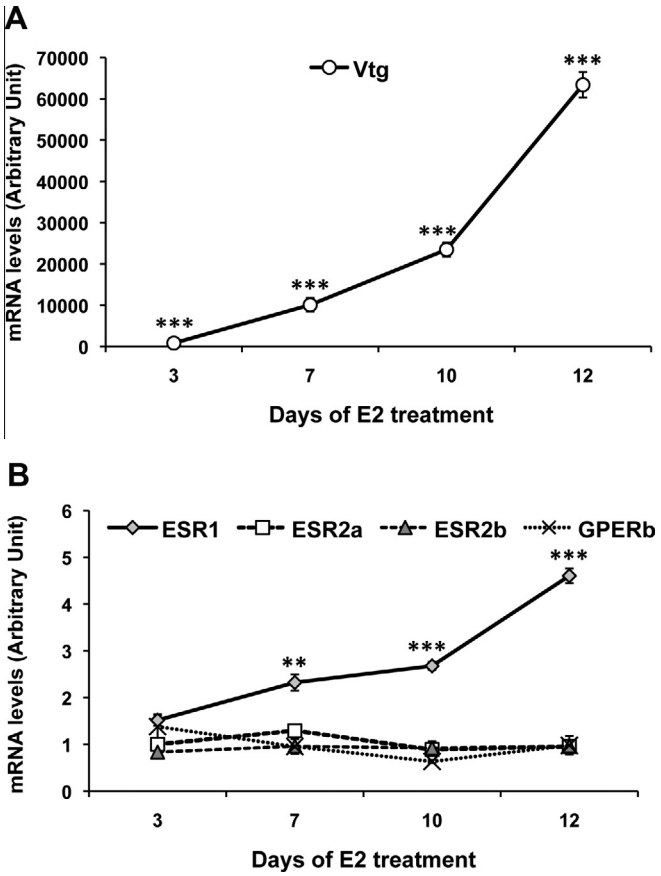

Fig. 6. *In vitro* regulation of the expression of Vtg and of the three nuclear and two membrane estrogen receptors in E2-treated eel hepatocyte primary culture. Hepatocytes were incubated with E_2 at 10^{-6} M. E2 treatments were added to the cells on days 0, 3, 6 and 10. Hepatocyte incubation was stopped after 3, 7, 10, and 12 days of treatment, respectively. The mRNA levels of eel Vtg (Fig. 6A), ESR1, ESR2a, ESR2b, and GPERb (Fig. 6B) were measured by qPCR and normalized to beta-actin mRNA. GPERa mRNA levels were not detectable throughout the experiment. Each bar represents the mean ± SEM from 5 well replicates. For each gene, the expression in control wells was normalized to an arbitrary value of 1. Significant differences between E_2-treated and control cells were analyzed by Student's *t*-test; $^{**}P < 0.01$, $^{***}P < 0.001$.

(4.60×; $P < 0.0001$) of treatment (Fig. 6B). In contrast, no significant regulation was observed for the other estrogen nuclear (ESR2a, ESR2b) and membrane (GPERb) receptors, throughout the experiment. GPERa expression remained undetectable after estradiol treatments.

4. Discussion

In this study, we identified five estrogen receptors in the eel, three nuclear (ESR1, ESR2a, ESR2b) and two membrane (GPERa, GPERb). Comparison with other teleost species suggests that this would be a common feature to most extant teleosts. As compared to other vertebrates, teleosts thus possess a higher number of estrogen receptors. Phylogeny and synteny analyses show that this larger number would result from teleost whole genome duplication (3R). To our knowledge, this study represents the first comparative investigation of tissue expression and regulation of these five nuclear and membrane estrogen receptors in a teleost species.

4.1. Evolutionary history of nuclear estrogen receptors in osteichthyans

The presence of two types of nuclear receptors, ESR1 and ESR2, is a common feature among osteichthyans (for review: Eick and Thornton, 2011). These two types are present in sarcopterygians, including representative species of a basal group, dipnosteans, and in actinopterygians, including representatives of basal groups, chondrosteans and holosteans. Our phylogeny analysis positions each of the three eel nuclear estrogen receptor sequences at the basis of the three teleost clades ESR1, ESR2a, ESR2b, respectively (Fig. 1). This is consistent with the basal position of elopomorphs in the phylogeny of teleosts, and has also been observed in our previous phylogeny analyses of various gene families (Morini et al., 2015; Pasquier et al., 2012, 2011). The presence of two ESR2 in the eel, as in most teleosts, while a single ESR2 is present in non-teleost actinopterygians, the chondrostean Japanese sturgeon, and the holosteans spotted gar and alligator gar, as well as in sarcopterygian species, strengthens the current hypothesis that ESR2a and ESR2b sub-types likely result from the teleost 3R (Bardet et al., 2002; Hawkins et al., 2000). The duplicated copies have been conserved in most extant teleosts, such as in cypriniformes, goldfish (Ma et al., 2000; Tchoudakova et al., 1999), zebrafish (Menuet et al., 2002), fathead minnow (Filby and Tyler, 2005), Taiwan shoveljaw carp, *Varicorhinus barbatulus* (Fu et al., 2008), common carp (Katsu et al., 2013), in salmoniformes, rainbow trout (Nagler et al., 2007), in perciformes, European sea bass, *Dicentrarchus labrax* (Halm et al., 2004), Atlantic croaker (Hawkins et al., 2005; Hawkins and Thomas, 2004), largemouth bass, *Micropterus salmoides* (Sabo-Attwood et al., 2004), orange-spotted grouper, *Epinephelus coioides* (Chen et al., 2011), yellow croaker, *Larimichthys crocea* (Chen et al., 2015), in beloniformes, medaka (Chakraborty et al., 2011), or in scorpaeniformes, Korean rockfish, *Sebastes schlegeli* (Mu et al., 2013).

In contrast to the presence of two distinct teleost ESR2 clades, teleost ESR1 sequences cluster in a single clade in our phylogenetic analysis. This suggests the absence of a 3R-issued second ESR1 in extant teleosts. One of the 3R-duplicated ESR1 would have been lost shortly after teleost emergence, before the emergence of elopomorphs (Fig. 7A). A second ESR1 receptor has been identified in some salmonids (Nagler et al., 2007), and cyprinids (Zheng et al., 2013; Zhu et al., 2008). These duplicated ESR1 receptors likely result from additional genome duplication events (4R) that occurred specifically in these groups (Glasauer and Neuhauss, 2014).

4.2. Evolutionary history of membrane estrogen receptors in osteichthyans

As for ESR2, two genes coding the membrane receptor GPER are present in the eel, as in most teleost species, while only a single gene is present in the non-teleost actinopterygian spotted gar and in sarcopterygian species. These two copies likely result from 3R. The two eel GPER possess the classical seven transmembrane domains, highly conserved among vertebrates (Fig. S2). The two eel GPER sequences branch basal to the whole GPER teleost clade, but independently from the GPERa and GPERb sub-clades, which include the other teleost sequences (Fig. 2). The GPER phylogenetic tree displays longer branch lengths for the teleost GPERb as compared to all the other GPER, including the two eel sequences. This suggests that teleost GPERb sequences may have diverged more rapidly than the other GPER sequences, shortly after elopomorph emergence.

Contrary to the situation for ESR, the GPER phylogenetic analyses that we conducted in this study were not informative enough to allow us to classify the two eel GPER into the teleost subclades GPERa and GPERb. Towards this aim, we performed synteny analysis of GPER genomic regions in representative species of actinopterygians. The synteny analysis suggests that the whole region containing GPER would have been duplicated in teleosts, probably as a result from 3R. As we already reported (Henkel

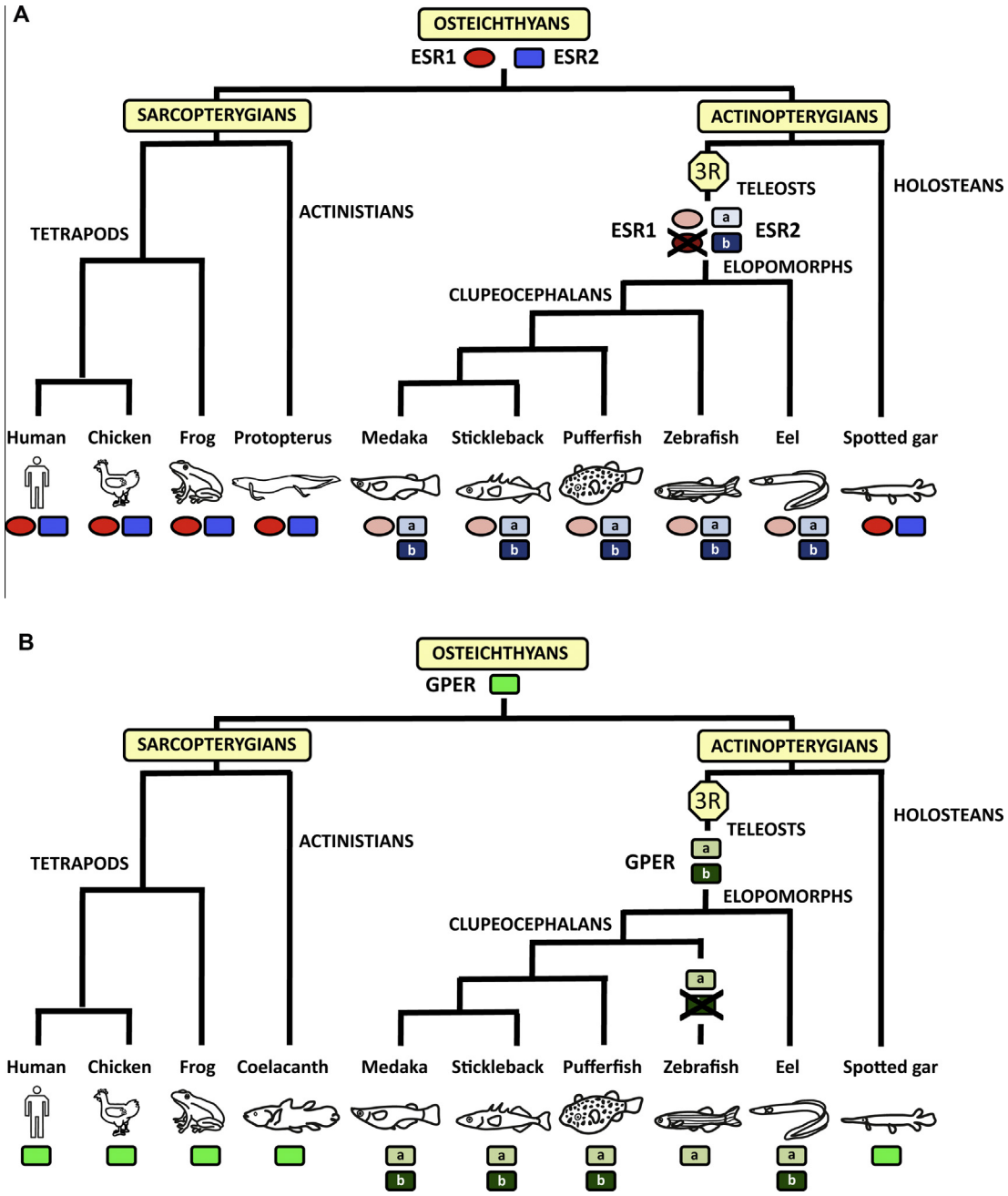

Fig. 7. Proposed evolutionary scenario of ESR and GPER genes in osteichthyans. Fig. 7A: Osteichthyan ancestor possessed two ESR genes, ESR1 and ESR2. These genes were duplicated in teleosts, as a result of the teleost-specific third whole genome duplication event (3R), specific to this group. ESR1b would have been lost during teleost radiation, shortly after 3R duplication and before elopomorph emergence. ESR1 genes are symbolized by a red oval. Duplicated ESR1 genes, resulting from teleost 3R, are symbolized by light and dark red ovals, respectively. ESR2 genes are symbolized by a blue rectangle. Duplicated ESR2 genes, resulting from 3R, are symbolized by light and dark blue rectangles, respectively. Gene losses are indicated by a black cross. Fig. 7B: Osteichthyan ancestor possessed one GPER gene. These genes were duplicated in teleosts, as a result of the teleost-specific third whole genome duplication event (3R), specific to this group. GPERb would have been lost in some specific group such as in zebrafish. GPER genes are symbolized by a green rectangle. Duplicated GPER genes, resulting from teleost 3R, are symbolized by light and dark green rectangles, respectively. Gene losses are indicated by a black cross.

et al., 2012a; Morini et al., 2015; Pasquier et al., 2012), eels seem to have retained more duplicated copies of genes than other teleost species. This may be related to the phylogenetic position of elopomorph, basal among teleosts (Chen et al., 2014). For instance, we could characterize, in GPER genomic regions of the European and Japanese eels, two genes for IFT140, TLEO2, NME4, XPO6, and UNKL, while a single copy is present in all the other teleosts investigated, as in non-teleost actinopterygians and sarcopterygians. This suggests that 3R-duplicated copies of these genes would have been lost in teleosts after the elopomorph emergence. For some genes, such as KCTD5, two copies remain in the European and Japanese eels, as well as in the zebrafish, suggesting that the duplicated copies of these genes may have been lost after the clupeocephalan emergence. Concerning GPER, our synteny analysis helped us to classify the two eel genes. We considered the eel GPER located in the genomic synteny of C16ORF91, ANKS3, WDR24, WDR90, NLRC3, DECR2, TLEO2, IFT140, and CCDC78 as orthologous to teleost GPERb, and the eel GPER located in the genomic synteny of FAM173A as orthologous to telost GPERa. The two eel GPER were therefore named accordingly, eel GPERb and eel GPERa. Our synteny analysis strengthens the hypothesis of a 3R duplication leading to the presence of two GPER genes in the eel as in most teleosts.

In all the teleost genomes investigated so far, two GPER sequences have been identified, except for the zebrafish that possess only a single GPER. The loss of the second copy of GPER in zebrafish likely represents a lineage specific event (Fig. 7B).

4.3. Differential expression and regulation of the five estrogen receptors in the BPG axis of female European eels

The five estrogen receptors transcripts are widely expressed in the European eel, with high expression levels in the organs of the brain-pituitary-gonad (BPG) axis.

4.3.1. Pituitary differential expression and regulation of the five estrogen receptors

The highest tissue expression for each of the five eel estrogen receptors was observed in the pituitary, making this organ a major target for estrogens. Previous studies from our group, using primary cultures of eel pituitary cells, demonstrated direct effects of estradiol on the pituitary expression of gonadotropins, LH and FSH (Aroua et al., 2007). The present study suggests that the five nuclear and membrane estrogen receptors may be involved in estrogen regulatory actions on pituitary cells.

During eel sexual maturation, we observed an up-regulation of the expression of ESR1 in the pituitary, while ESR2b, GPERa and GPERb expression levels were decreased. The up-regulation of ESR1 is in accordance with the results obtained in the Japanese eel (Jeng et al., 2012), suggesting an increasing role played by ESR1 in E2 feedback during sexual maturation. In mammals, estradiol feedbacks at the pituitary level also appear to be primarily mediated by ESR1 (Couse et al., 2003; Wintermantel et al., 2006).

In the European eel, membrane receptors could also be involved in E2-feedback on the pituitary. To our knowledge, pituitary expression of GPER has not yet been reported or investigated in teleosts. However, in mammals, GPER is well expressed in pituitary cells and may be involved in rapid non-genomic actions of estrogens (for review: Chimento et al., 2014). It is suggested that, in addition to nuclear receptor ESR1, GPER may also participate in mediating the feedback action of estrogens in pituitary during reproduction (for review: Chimento et al., 2014). In bovine, GPER is expressed in the pituitary gonadotrope cells and involved in the rapid estradiol feedback leading to the suppression of GnRH-stimulated LH release (Rudolf and Kadokawa, 2013). Future studies should aim at characterizing the pituitary cell types expressing the five estrogen receptors in the eel.

4.3.2. Brain differential expression and regulation of the five estrogen receptors

The present study shows that the five estrogen receptors are all expressed in the brain of female European eels, and may thus mediate classical genomic as well as fast non-genomic actions of estradiol. During sexual maturation, the expression of the nuclear receptor ESR1 and the membrane receptor GPERa were up-regulated in the anterior brain, suggesting increasing roles for these two receptors. In teleosts, as in other vertebrates, the brain is a major target organ for sex steroids involved in the neuroendocrine control of reproduction (Zohar et al., 2010). Estradiol reaches the brain after peripheral secretion by the gonads, but it may also be locally produced via brain steroidogenesis (for review: Coumailleau et al., 2015). In teleosts, besides exerting feedback regulatory effects on the activity of the brain-pituitary gonadotrope axis, one major role of estradiol is the regulation of neurogenesis by glial cells (Lin et al., 2015; Nagarajan et al., 2013; Pellegrini et al., 2015; Xing et al., 2014). Previous data in eels indicated that estradiol exerts a positive feedback on mGnRH (mammalian GnRH) expression by anterior brain neurons and also on brain aromatase expression by glial cells (Jeng et al., 2012; Montero et al., 1995). This suggests important roles of brain estradiol receptors in both neuroendocrine and neurogenesis functions in the eel. In the zebrafish, nuclear and membrane estrogen receptors have been shown to be involved in neurogenesis (Diotel et al., 2013; Froehlicher et al., 2009; Griffin et al., 2013; Shi et al., 2013). Identification of brain cell types expressing the estrogen receptors in the eel will further clarify this issue.

4.3.3. Ovarian differential expression and regulation of the five eel estrogen receptors

The five receptors are also all expressed in the ovary. In teleosts as in other vertebrates, the ovary constitutes both the main site of estradiol production and a major target of estradiol. In the ovary, only ESR1 transcript was significantly up-regulated during eel experimental maturation, suggesting an increased role of this nuclear receptor during eel gonadal development. In various teleost, such as goldfish, orange-spotted grouper, and rainbow trout, ESR1 expression is also strongly increased during ovarian maturation (Chen et al., 2011; Choi and Habibi, 2003; Nagler et al., 2000). ESR1 has thus been described as the predominant form of ESR in the regulation of ovarian development (Chen et al., 2011; Filby and Tyler, 2005; Lynn et al., 2008; Nagler et al., 2000). Several studies in teleosts have evidenced an up-regulation of ESR1 during sexual maturation, or induced by estradiol, while the regulation of the other receptor types varies depending on the species, suggesting differential roles during ovarian maturation (for review: Nelson and Habibi, 2013). In tilapia, European sea bass, largemouth bass, and orange-spotted grouper, ESR2a and ESR2b have been suggested to be involved in the early stages of ovarian development (Blázquez et al., 2008; Chen et al., 2011; Sabo-Attwood et al., 2004; Tsai et al., 2003). In mammals, ESR are differentially expressed in ovarian cell types and differentially regulated (for review: Britt and Findlay, 2002; Drummond and Fuller, 2010). For instance, in hamster, ESR2 is predominantly expressed in granulosa cells, while ESR1 is mainly expressed in interstitial and thecal cells (Yang et al., 2002).

Both GPERa and GPERb were found to be expressed in the eel ovary, but we observed no significant change in their expression during experimental maturation. In teleosts, a major role of GPER has been suggested in the inhibition of female oocyte final maturation (for review: Thomas, 2012). In zebrafish and Atlantic croaker, estrogens were shown to exert a non-classical action, via cell surface receptors, to inhibit oocyte final maturation (Pang et al., 2008; Pang and Thomas, 2009; Peyton and Thomas, 2011). Furthermore, GPER morpholino injection into zebrafish oocytes blocked the inhibitory effect of estrogen on oocyte maturation (Pang et al., 2008), while ESR morpholino injection had no effect (Pang and Thomas, 2010). These data strongly support that the non-genomic role of estrogens on the inhibition of oocyte maturation is mediated by GPER in teleosts. In zebrafish and Atlantic croaker, only a single GPER gene has been evidenced (Pang et al., 2008; Pang and Thomas, 2009). In both species, this GPER is orthologous to teleost GPERa (Fig. 2). Future studies should aim at investigating the potential differential roles of the two teleost GPER in oocyte final maturation.

4.4. Differential expression and regulation of the five eel estrogen receptors in the liver of female European eels

In teleosts, as in other oviparous vertebrates, the liver is the central organ of vitellogenin synthesis in females (Wahli, 1988). This phospho-lipo-glyco-protein, produced under the control of estradiol, is released into the circulation and incorporated into the oocytes to form the yolk, a crucial source of nutriment for the embryonic development and larval survival (Wahli, 1988; Wang and Lou, 2006). The five estrogen receptors are expressed

in the liver of female European eels, but GPERa and GPERb levels are very low. During sexual maturation, we observed an up-regulation of only ESR1. The setup of a protocol for primary culture of eel hepatocytes allowed us to demonstrate concomitant up-regulation of Vtg and ESR1 expression under estradiol treatment, with no change in the expression of the other estrogen receptors. This estradiol-induced up-regulation of both Vtg and ESR1 has also been evidenced in several other vertebrate species, suggesting that ESR1 is the major mediator of liver vitellogenesis (goldfish: Nelson et al., 2007; Marlatt et al., 2008; rainbow trout: Pakdel et al., 1991; largemouth bass: Sabo-Attwood et al., 2004). However, in the goldfish, while only ESR1 is up-regulated in the liver by estradiol, the role of ESR2 remains primordial (Nelson and Habibi, 2010). Using RNAi methodology, these authors indeed demonstrated that ESR2 subtypes are required for basal and E2-induced ESR1 expression (Nelson and Habibi, 2010). Similarly, in the rainbow trout, the use of ESR1 and ESR2 agonists suggests that both ESR1 and ESR2 are involved in the E2-induction of Vtg (Leaños-Castañeda and Van Der Kraak, 2007). Further studies should also aim at investigating the potential roles of membrane estrogen receptors in these pathways.

In conclusion, in the European eel we identified three nuclear ESR1, ESR2a, ESR2b and two membrane GPERa, GPERb, estrogen receptors. Two ESR2 receptors and two GPER receptors are also present in most extant teleosts and likely result from teleost-specific whole genome duplication, 3R. The five estrogen receptors are all widely expressed in the eel, but show some differential tissue distribution and expression regulation. ESR1 is the only receptor to be constantly up-regulated in the BPG-liver axis during eel induced sexual maturation, as well as up-regulated *in vitro* by estradiol, as shown in eel hepatocyte primary cultures. This study opens new research avenues on the potential differential roles of duplicated ESR2 and GPER, which may represent driving forces that led to the conservation of multiple estrogen receptors in the European eel, as in most extant teleosts.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2015.11.021.

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