Nuclear and membrane progestin receptors in the European eel: characterization and expression in vivo through spermatogenesis

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Short title: Progestin receptors expression in the European eel.

Keywords: teleost, PGR, mPR, nPR

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Abstract

Characterization of all the progestin receptor genes (PRs) found in the European eel has been performed. There were five membrane PRs (mPRs): mPRα (alpha), mPRAL1 (alpha-like1), mPRAL2 (alpha-like2), mPRγ (gamma), mPRδ (delta) and two nuclear PRs (nPRs or PGRs): pgr1 and pgr2. In silico studies showed that the C and E(F) domains of Pgr are well conserved among vertebrates whereas the A/B domain is not. Phylogeny and synteny analyses suggest that eel duplicated pgr (pgr1 and pgr2) originated from the teleost-specific third whole genome duplication (3R). mPR phylogeny placed three eel mPRs together with the mPRα clade, being termed mPRα, mPRAL1 and mPRAL2, while the other two eel mPRs clustered with mPRγ and mPRδ clades, respectively.

The in vivo study showed differential expression patterns along the brain-pituitary-gonad axis. An increase in nPR transcripts was observed in brain (in pgr1) and pituitary (in pgr1 and pgr2) through the spermatogenesis, from the spermatogonia B/spermatocyte stage to the spermiation stage. In the testis, mPRγ, mPRδ and pgr2 transcripts showed the highest levels in testis with A spermatogonia as dominant germ cell, while the highest mPRα, mPRAL1 and mPRAL2 transcripts were observed in testis from spermiating males, where the dominant germ cell were spermatozoa. Further studies should elucidate the role of both nuclear and membrane progestin receptors on eel spermatogenesis.
1. Introduction

The European eel (*Anguilla anguilla*) have a complex catadromous life cycle. This includes a long reproductive migration across the Atlantic Ocean, for supposedly 6-7 month, to reach their spawning site in unknown areas of the Sargasso Sea. Before leaving the European coast, the silver eel reproductive development is blocked in a pre-pubertal stage until the 5000-6000 km oceanic reproductive migration can occur (Dufour et al., 1988). Because the pre-pubertal silver eels are the most advanced stage of the wild eels caught in river or coastal areas, it is difficult to simulate the variable environmental factors which would occur during the migration, and a long-term hormonal treatment (fish pituitary extracts for females, and human chorionic gonadotropin, hCG, for males) are currently necessary to mature eels in captivity (Asturiano et al., 2006; Gallego et al., 2012; Huang et al., 2009; Pérez et al., 2011). Besides its complex life cycle, the phylogenetical position of the European eel, branching at the basis of the teleosts, which are the largest group of vertebrates (Henkel et al., 2012a, 2012b), makes this species an excellent model to study the ancestral regulatory functions which are controlling reproduction.

In all vertebrates, progestins have a crucial function in gametogenesis. It is known that in male fish two progestins: 17α,20β-dihydroxy-4-pregnen-3-one (DHP) and/or 17α,20β,21-trihydroxy-4-pregnen-3-one (20βS) are the maturation-inducing steroids (MIS), mediating the process of sperm maturation and spermiation (see review Scott et al., 2010). However, it has also been demonstrated that progestins are involved in the early stages of the spermatogenesis in Atlantic salmon (*Salmo salar*), cod (*Gadus morhua*), and zebrafish (*Danio rerio*) (Chen et al., 2010, 2011, 2012, 2013). In Japanese eel, DHP has been demonstrated to be an essential factor for the meiosis initiation of the spermatogonia (Miura et al., 2006), but also to regulate final sperm maturation through
the increase of pH in the seminal plasma, which induces an increase in intracellular cAMP, making the sperm cells capable of motility and fertilization (Miura et al., 1995). Furthermore, it is known that progestins regulate spermiation in Salmonidae and Cyprinidae (Ueda et al., 1985), increase milt production in Moronidae (Asturiano et al., 2002) and Salmonidae (Baynes and Scott, 1985), and stimulate sperm motility in Anguillidae (Miura et al., 1995), Sciaenidae (Tubbs and Thomas, 2008) and Paralichthydae (Tan et al., 2014; Tubbs et al., 2011).

Progestins, as small lipophilic steroid hormones, can diffuse through the cell membranes (Oren et al., 2004) and bind to nuclear progestin receptors (nPRs or Pgrs) belonging to the nuclear steroid receptor family. Receptor activation leads to modulation of gene transcription and translation activity (Mangelsdorf et al., 1995), resulting in a relatively slow biological response. However, many progestin actions are non-genomic, and involve rapid activation of intracellular signal transduction pathways mediated by membrane progestin receptors (mPRs). The mPRs are 7-transmembrane receptors coupled to G-proteins, but they do not belong to the G protein coupled receptor (GPCR) superfamily. Instead, they are members of the progestin and adipocytokine receptor (PAQR) family (Tang et al., 2005; Thomas et al., 2007). Evidence has been obtained that steroid hormones, thyroid hormones, and vitamin D, similarly to water soluble signalling molecules, exert this rapid cell surface-initiated hormone action through binding to membrane receptors, which lead to the activation of intracellular second messenger pathways (Falkenstein et al., 2000; Norman et al., 2004; Revelli et al., 1998; Watson et al., 1999). The mPRs were first discovered and characterized in fish ovaries (Zhu et al., 2003a), and five isoforms (mPRα, mPRβ, mPRγ, mPRδ, mPRε) were subsequently identified in humans and other vertebrates (Peterson et al., 2013; Thomas and Pang, 2012; Zhu et al., 2003b). Both nPRs and mPRs are highly expressed
in testis (Hanna and Zhu, 2009; Ikeuchi et al., 2002) and brain (Thomas and Pang, 2012; Peterson et al., 2013), but the functions mediated by them are still unclear. In Japanese eel, progestin receptor 1 (pgr1) is expressed in testis germ cells, Sertoli cells, and testis interstitial cells, whereas progestin receptor 2 (pgr2) mRNA has been detected only in testis germ cells (Miura et al., 2006). According to Chen et al. (2012) the only nPR present in Atlantic cod testis is involved both in the beginning of spermatogenesis, mediating the mitotic proliferation of spermatogonia, and in the final spermatogenesis, in processes associated with the spermiation/spawning period. In Atlantic croaker (Micropogonias undulatus), the expression of the mPRα in all early to mid-spermatogenic cell types suggest its involvement in the regulation of early stages of spermatogenesis (Tubbs et al., 2010) but it has also been related with the induction of sperm hypermotility (Thomas et al., 2004).

The objective of this study was to characterize all the progestin receptor genes (from nuclear and membrane receptors) in the European eel, as well as to study their gene expression profiles during the spermatogenesis process in the BPG axis, in order to have a first approach to understand the role of the progestin signaling on European eel spermatogenesis.

2. Material and methods

2.1. Fish maintenance, hormonal treatments and sampling

One hundred European eel males (mean body weight 100±6 g) from the fish farm Valenciana de Acuicultura, S.A. (Puzol, Valencia; East coast of Spain) were transferred to the Aquaculture Laboratory in the Polytechnic University of Valencia. They were randomly distributed and kept in freshwater, in two 200-L aquaria (approximately 50 males per aquarium), equipped with separated recirculation systems,
thermostats/coolers, and covered to maintain constant shadow.

The fish were gradually acclimatized for one week to sea water (37±0.3‰ of salinity) and kept at 20 °C during the whole experimental period. Then, to induce the sex maturation, the eels were treated with weekly intraperitoneal injections of human chorionic gonadotropin (hCG, Profasi, Serono, Italy; 1.5 IU g⁻¹ fish) during 8 weeks, as previously described by Gallego et al. (2012).

Groups of 5-8 eels were anaesthetized with benzocaine (60 ppm) and sacrificed by decapitation before the start of the hormonal treatment in freshwater conditions (after arrival to the laboratory), and in sea water conditions (one week after sea water acclimation), and later each week of the hormonal treatment. Morphometric parameters such as total body weight and testis weight were recorded to calculate individual gonadosomatic indices [GSI = (gonad weight/total body weight)*100] (Pankhurst, 1982). For histological analysis, testicular tissue samples were fixed in 10% formalin buffered to pH 7.4 with phosphate buffer.

All samples of brain, pituitary, testis, liver, gill, muscle, spleen, pectoral fin, heart, posterior kidney and head kidney were stored in 0.5 ml of RNAlater (Ambion Inc., Huntingdon, UK) at -20 ºC until extraction of total RNA (Peñaranda et al., 2010). The brain was dissected into five parts: olfactory bulbs, telencephalon, mes-/diencephalon, cerebellum, and medulla oblongata.

2.2. Ethics amendment

As the eels stop feeding at the silver stage and throughout sexual maturation, they were not fed during the experiment and were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC). This study was carried out in strict accordance with the recommendations in the Guide for
the Care and Use of Laboratory Animals of the Spanish Royal Decree 53/2013 on protection of animals used for scientific purposes (BOE 2013). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Universitat Politècnica de València (UPV) ( Permit Number: 2014/VSC/PEA/00147). All efforts were made to minimize animal suffering and stress.

2.3. Gonadal histology

To determine the maturational stage of the testis, formalin-fixed samples were dehydrated in ethanol and embedded in paraffin. Sections of 5-10 µm thickness were cut with a Shandom Hypercut manual microtome and stained with haematoxylin and eosin. Slides were observed with a Nikon Eclipse E-400 microscope, and micrographs were taken with a Nikon DS-5M camera attached to the microscope. Stages of spermatogenesis were determined according to the germ cell types present in the testis (Leal et al., 2009; Miura and Miura, 2011) and their relative abundance, the degree of development of the seminal tubules, the GSI and the sperm production by the male in the week of the sacrifice. The stages considered were: Stage 1: A Spermatogonia (SPGA): dominance of A spermatogonia, B spermatogonia present in low number, presence/absence of lumen; mean GSI = 0.08 (0.0-0.36); Stage 2 Spermatogonia B/Spermatocytes (SPGB/SPC): dominance of B spermatogonia, spermatocytes present, in some cases low number of spermatids appeared, mean GSI = 0.80 (0.29-1.52); Stage 3 spermatids (SD): spermatids very abundant, some sperm cells could appear, mean GSI = 4.02 (1.79-5.93); Stage 4 spermatozoa (SZ): spermatozoa was the dominant germ cell, mean GSI = 7.35 (3.41-12.8) (Fig. A).

2.4. Identification of progestin receptor sequences
2.4.1. European and Japanese eel genome database analyses

All the genomic sequences of nPRs and mPRs were retrieved from European and Japanese eel genomes by performing TBLASTN algorithm of the CLC DNA Workbench software (CLC bio, Aarhus, Denmark) (Henkel et al., 2012a, 2012b). The exons and splicing junctions were predicted using the empirical nucleotidic splicing signatures, i.e.: intron begins with ‘‘GT’’ and ends with ‘‘AG’’. The following peptide sequences were used as queries: *Danio rerio* mPRα (acc. number AY149121.1), *Carassius auratus* mPRα (AB122987.1), *C. auratus* mPRγ (AB284132.1), *C. auratus* mPRδ (AB284133.1), *Oreochromis niloticus* mPRγ (XM_003456742), *Anguilla japonica* ePR1 (AB032075.1), *A. japonica* ePR2 (AB028024.1). The percentage of European eel PR identity was calculated with Sequences Identities And Similarities (SIAS) server (imed.med.ucm.es/Tools/sias.html).

2.4.2. Phylogenetic analysis of nuclear and membrane progestin receptors

With the aim to obtain a better understanding of nPR and mPR family evolution, phylogenetic analyses were performed on osteichthyanas of key-phylogenetical positions: mammalians; sauropsids (birds and reptiles); a representative of an early sarcopterygian, the coelacanth (*Latimeria chalumnae*); the non-teleost actinopterygian spotted gar (*Lepisosteus oculatus*); the European and Japanese eels, as members of an early group of teleosts (elopomorphs), and other teleosts.

Two unrooted phylogenetic trees were constructed with amino acid sequences of known or predicted sequences of nPRs and progestin and adipocytokine receptor (PAQR) family (For accession/ID number, see Supplemental Table A). The sequences were retrieved from NCBI or ENSEMBL, first aligned using Clustal Omega (Sievers et al., 2011) with seaview 4.5.4 software (http://doua.prabi.fr/software/seaview), and later manually...
adjusted. The JTT (Jones, Taylor and Thornton) protein substitution matrix of the resulting alignment was determined using ProTest software (Abascal et al., 2005). Both phylogenetic trees of nPRs and Progestin and adipoQ receptor were constructed based on the sequence alignments, using the maximum likelihood method (PhyML software, Stamatakis et al., 2008) with 1000 bootstrap replicates, and subsequently visualized using treedyn (http://phylogeny.lirmm.fr/phylo cgi/).

2.4.3. Synteny analysis of nuclear progestin receptor genes

Neighboring genomic regions of the duplicated eel nuclear progestin receptors, pgr1 and pgr2, 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). BLAST analyses were performed in the European and Japanese eel genomes to identify potential additional paralogs of the pgr neighboring genes. Homologs of eel pgr neighboring genes were then identified, using PhyloView of Genomicus v82.01, in other vertebrate genomes, i.e. human, zebra finch (Taeniopygia guttata), spotted gar, zebrafish, stickleback (Gasterosteus aculeatus), tilapia (Oreochromis niloticus) and fugu (Takifugu rubripes). BLAST analyses were also performed to search potential pgr paralogs in the genomes of these species. For each pgr 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.

2.5. Gene expression analyses by quantitative real-time PCR

2.5.1. Primers and reference gene

The quantitative real-time Polymerase Chain Reactions (qPCR) were carried out using
the Acidic ribosomal phosphoprotein P0 (ARP): ARPfw: GTG CCA GCT CAG AAC
ACT G; ARPrv: ACA TCG CTC AAG ACT TCA ATG G (Morini et al., 2015a) as
reference gene because its mRNA expression has been shown to be stable during
experimental maturation (Weltzien et al., 2005). The qPCR expression stability of the
reference gene was determined using the BestKeeper program [(Pfaffl et al., 2004),
reporting a standard deviation (SD[±Cq]) lower than 1. The BestKeeper calculated
variations in the reference gene are based on the arithmetic mean of the Cq values.
Genes with a SD value higher than 1 are defined as unstable. In the testis: SD= 0.82;
p<0.05 with a Cq geometric mean of 24.14±1.76; in the brain and pituitary, olfactory
bulb: SD= 0.81; telencephalon: SD= 0.35; mes-/diencephalon: SD= 0.46, pituitary: SD=
0.62; p<0.05 and a Cq geometric mean of olfactory bulb: 23.51±1.76; telencephalon:
21.95±1.28; mes-/diencephalon: 22.02±1.37; pituitary: 22.39±1.54.

European eel progestin receptor specific qPCR primers (Table 1) were designed based
on in situ full-length European eel coding sequences. All the primers were designed on
two different exons, in order to avoid amplification of potential genomic contamination,
and all the primers were tested on genomic DNA and RNA to confirm that potentially
contaminant was not amplified. All primers were designed using Primer3 Software
(Whitehead Institute/Massachusetts Institute of Technology, Boston, MA, USA) and
were purchased from Integrate DNA Technology, Inc. (IDT, Coralville, IA).

2.5.2. SYBR Green assay
To determine the expression of each progestin receptor gene, qPCR assays were
conducted as previously described by Peñaranda et al. (2013) using a model 7500 unit
(Applied Biosystems; Foster City, CA, USA). After an initial activation of Taq
polymerase at 95 °C for 10 min, 40 PCR cycles were performed at the following cycling
conditions: 95 °C for 1 s, 60 °C for 30 s.

The total volume for PCR reaction was 20 µl, performed from 5 µl of diluted (1:20 for the nPRs; 1:40 for the mPRs) DNA template, forward and reverse primers (250 nM each), and SYBR Green/ROX Master Mix (12 µl). Transcript levels were determined using an efficiency-adjusted relative quantification method as described by Peñaranda et al. (2014). Serial dilutions of cDNA pool of gonad tissues were run in duplicate and used as standard curve for both nPRs and for three mPRs (mPRγ, mPRAL1 and mPRAL2). Serial dilutions of cDNA pool of brain tissues were used as standard curve for mPRα and mPRδ. One of these dilutions was included in each run of the corresponding gene as a calibrator. Target and reference genes in unknown samples were run in duplicate PCR reactions. Non-template control (cDNA replaced by water) for each primer pair was run in duplicate on all plates.

2.5.3. Eel progestin receptors tissue distribution

In order to investigate the tissue distribution of each PR mRNA expression in male and female European eels; gonads (testes and ovaries) and somatic tissues (liver, heart, gill, muscle, spleen, pectoral fin, posterior kidney, head kidney, brain, pituitary) were collected from three immature male eels (mean body weight 118±14 g) from the fish farm Valenciana de Acuicultura, S.A. (Puzol, Valencia; East coast of Spain) and three wild female eels (mean body weight 608±35 g) captured by local fishermen in the Albufera lagoon (Valencia, Spain). Total RNA was extracted following the method used by Peñaranda et al. (2014), treated with DNase I (Turbo DNA-free; Ambion) at 37 °C for 30 min, and reverse-transcripted using superscript III (Invitrogen) and random hexamer primers on 1 µL total RNA, according to the manufacturer’s protocol. All tissues were analyzed by qPCR.
2.5.4. Progestin receptor gene expression profiles during artificial maturation

To study the regulation of the nPRs and mPRs during European eel artificial maturation, total RNA of testis, olfactory bulbs, telencephalon, mes-/diencephalon and pituitary from hCG treated male silver eels was isolated using Trizol reagent (Life Technologies) as described by Peñaranda et al. (2013). Testis RNA was treated and purified with DNase I of NucleoSpin RNA XS kit (Macherey-Nagel, Düren, Germany). Twenty µl of cDNA were synthesized from 500 ng of testis total RNA, using qScript cDNA Synthesis Kit (Quanta Bioscience, MD, USA).

Olfactory bulbs, telencephalon, mes-/diencephalon and pituitary RNA were treated with deoxyribonuclease (gDNA Wipeout Buffer, Qiagen), from 500 ng of total RNA for the olfactory bulb and pituitary, or from 1 µg for the telencephalon and the mes-/diencephalon. First-strand cDNA was synthesized in 20 µl reactions using Quantiscript Reverse Transcriptase (Qiagen). RNA concentration was evaluated by using a NanoDrop 2000C Spectrophotometer (Fisher Scientific SL, Spain).

2.6. Statistics

Normality of each variable was first checked. Variables that did not have a normal distribution were log-transformed and their normality was checked again. Then, data were analyzed by analysis of variance (one-way ANOVA), using the Student–Newman–Keuls test to compare means. Variance homogeneity was checked with the Bartlett test. Differences were considered significant when p<0.01. All statistical procedures were performed using Statgraphics Plus 5.1 (Statistical Graphics Corp., Rockville, MO, USA). Results are presented as mean ± standard error (SEM).
3. Results

3.1. Characterization of progestin receptor genes

The complete Coding Domain Sequence (CDS) of two nPRs were retrieved from the European eel genome. Furthermore, the complete CDS of four mPR genes (mPrα, mPRAL2, mPRγ, mPRδ) were retrieved from both European and Japanese eel genomes, and the complete mPRAL1 CDS was retrieved from the Japanese eel genome while the partial corresponding sequence was retrieved from the European eel genome. To characterize the nPRs and mPRs of European and Japanese eels in the eel genomes, two phylogenetic trees were constructed, one for the nPRs family and another with part of the PAQR family (PAQR3-9). Concerning nPRs phylogenetic analyses (Fig. 1A), the early sarcopterygian coelacanth clustered at the basis of the monophyletic sarcopterygians nPR group, and eel Pgr1 and Pgr2 branched with the spotted gar at the basis of the monophyletic actinopterygian nPR group, constituting an actinopterygian nPR clade as sister clade of the sarcopterygian nPR.

The complete European eel pgr1 CDS was a 2133bp sequence, the resulting predicted amino acid sequence consisted of 711 aa (GenBank accession number AFV13730.1), and complete European eel pgr2 CDS was a 2028bp sequence, the resulting predicted amino acid sequence consisted of 676 aa (GenBank accession number AFV13731.1).

Although both nPRs were composed of 8 exons, they shared only 25.38% sequence identity at the amino acid level (Fig. B). The European and Japanese eel Pgr amino acid sequence differed by 8 and 26 amino acids, for Pgr1 and Pgr2 respectively. The eel pgr gene sequences could be subdivided into four domains (Fig. 1B) as described by Laudet (1997). For Pgr1, the A/B domain was from residues 338 to 415 and the E(F) domain was from residues 463 to 710, and for Pgr2 the A/B domain was from residues 305 to 382, and the E(F) domain was from residues 428 to 675.
The PAQR phylogeny is composed of vertebrate PAQR3 to PAQR9 protein sequences (Fig. 2), and was divided into two major monophyletic groups: the first comprising mPRα (PAQR7), mPRβ (PAQR8), mPRγ (PAQR5) and mPRδ (PAQR6) from the mPR subfamily, and the second clustering three other members of the PAQR family (PAQR9, PAQR3, PAQR4). Three eel mPRs were placed together within the mPRα clade, and were called mPRα, mPRAL1 (alpha-like1), mPRAL2 (alpha-like2). The two other eel mPRs (mPRγ, mPRδ) clustered together with their respective mPR types amongst vertebrate representatives.

The complete CDS of the mPRs (mPRα, mPRAL1, mPRAL2, mPRγ and mPRδ,) were 1059, 1077, 1055, 1071 and 1005 bp, respectively, giving open reading frames (ORF) of respectively 353, 359, 351, 357 and 335 aa sequences. The mPRα, mPRAL1 and mPRAL2 forms were devoid of introns, while the mPRγ and mPRδ forms comprised 7 and 5 introns, respectively.

The predicted European eel mPRs showed a similar structure with the same exon number as the corresponding predicted Japanese eel amino acid sequences. European eel mPRα, mPRγ, mPRδ and mPRAL2 only differed from 2, 6, 2 and 17 aa respectively, when compared with the corresponding Japanese eel complete sequences; and mPRAL1 differed from 5 aa when compared with the corresponding Japanese eel partial sequence (Fig. C). However, eel mPRs showed very low sequence identity. Higher percentages of identity were found between the mPRα, mPRAL1 and mPRAL2, with 55 to 62% percentage of identity. Both mPRγ and mPRδ showed very low sequence identity (about 30%) with other mPRs. A seven transmembrane structure was predicted for eel mPRα, mPRγ, mPRAL1 and mPRAL2 subtypes with TMpred (http://www.ch.embnet.org/software/TMPRED_form.html). Although the mPRδ protein was predicted as possessing five transmembrane domains with this software,
INTERPROSCAN 5 (www.ebi.ac.uk/Tools/pfa/iprscan/) predicted seven transmembrane domains (Fig. 3).

3.2. Syntenic analyses of nuclear progestin receptor genes

To better understand the evolutionary history of the PGRs, we compared the genomic regions that encompass eel pgr1 and pgr2 with homologous regions in other vertebrate genomes such as sarcopterygians (human, zebra finch), teleosts (zebrafish, stickleback, tilapia and fugu) and a non-teleost actinopterygian (spotted gar) (Fig. 4). Comparative analyses of the small scaffolds of European and Japanese eel genomes allowed us to retrieve five pgr neighboring gene families: yap1, cep126, angptl5, trpc6 and arhgap42. As for pgr genes, these neighboring genes are duplicated in the European and Japanese eels with the exception of angptl5. The single paralog of angptl5 is located on the genomic region of eel pgr1. The other angptl5 paralog has been lost on the genomic region of eel pgr2. The eel pgr neighboring genes are also located in the pgr genomic regions of all vertebrate species investigated in this study, which supports the orthology of the vertebrate pgr genes. The synteny analysis shows that the pgr genomic region has been duplicated in teleosts, likely as a result of the teleost-specific third whole genome duplication (3R). As eels, the other teleosts have conserved duplicated yap1, trpc6 and arhgap42 genes, and only a single angptl5 gene. In contrast to the eels, the other teleosts investigated have lost the pgr1 gene, located in the eels on the same paralogon as the single angptl5 gene and also lost one cep126 gene on this paralogon. Zebrafish has further lost one yap1 gene on this paralogon. The single pgr gene conserved by zebrafish, stickleback, tilapia and fugu, is orthologous to eel pgr2. In zebrafish, this pgr2 gene is located on the 3R-paralogon “b” according to the Official Zebrafish Nomenclature Guidelines (http://zfin.org).
3.3. Tissue distribution of progestin receptors mRNA in the European eel

Tissue distribution of all nPR and mPR transcripts revealed a differential expression in male and female European eel (Table 2, Fig. 5).

Concerning nPRs (Fig.5.1A-B and Fig.5.2A-B), pgr2 was highly expressed in the pituitary and both pgr1 and pgr2 were highly expressed in the brain of female eel, while in male eel they were highly expressed in the testis and other tissues outside the brain-pituitary-gonad (BPG) axis (pgr1 in kidney and muscle, pgr2 in the gill).

Concerning mPRs (Fig.5.1C-G and Fig.5.2C-G), both mPRAL1 and mPRAL2 were detected in all tissues studied, and the highest expression was in the cerebellum, both in male and female eels. For instance, in male eel, cerebellum mPRAL2 was 5-fold more expressed than in the pituitary, and 8,000-fold more expressed than male mPRγ. In contrast, the receptors mPRα and mPRδ were lowly expressed in the tissues of male and female eel. mPRα was mainly expressed in the cerebellum of male eel and in the head kidney of females. mPRδ expression was detected in the muscle and in different brain parts of male eels, while in females it was detected in the gill, fins, and highly in the brain parts. Finally, the receptor mPRγ was mostly expressed in the gonads and in peripheral tissues (gill, fins, posterior-/head kidney) both in male and female eels.

3.4. Brain and pituitary progestin receptors mRNA expression during spermatogenesis

Significant variations were found in nPR gene expression levels through the BPG axis during the course of spermatogenesis (Fig. 6). The pgr1 mRNA transcripts increased in the olfactory bulbs (OB) and telencephalon (T) (Fig. 6) through the spermatogenesis (p<0.01), and in mes-diencephalon (MD) and pituitary, pgr1 mRNA showed lower
levels at SPGA stage than in further spermatogenic stages. *pgr2* pituitary gene expression showed the same profile as *pgr1*, but in the forebrain it did not show variations during the spermatogenesis (Fig. 6).

Regarding to mPRs, none of them showed significant differences in brain and pituitary throughout the spermatogenesis (Fig. 7).

3.5. **Testis progestin receptor mRNA expression during spermatogenesis**

In the testis (Fig. 8), different PR expression profiles were observed during the course of spermatogenesis. The highest variations were observed in the *mPRα*, being 50-fold higher at SZ stage than at SPGA stage, followed by *mPRAL1* and *mPRAL2*, being both 12-fold higher at SZ stage than at SPGA. The other two mPRs (*mPRγ* and *mPRδ*) showed lower variations, in an opposite profile, decreasing from the SPGA to SPGB/SPC and later stages (p<0.01).

Regarding the nPRs (Fig. 8A-B), *pgr1* mRNA expression was stable during testis development (Fig. 8A), while *pgr2* mRNA expression decreased from SPGA to SPGB/SPC stage, and then kept stable to SZ stages (Fig. 8B). *pgr2* mRNA levels thus showed an opposite profile compared to the pituitary.

4. **Discussion**

4.1. **Duplicated nuclear progestin receptors in the eel**

In this study, we identified duplicated nuclear progestin receptor genes (*pgr1* and *pgr2*) in the genomes of the European and Japanese eels, in agreement with the previous cloning of two progestin receptors from Japanese eel testis (ePR1 and ePR2 (Ikeuchi et al., 2002; Todo et al., 2000)). In contrast, a single PGR has been reported in pufferfish (*Takifugu rubripes*), tetraodon (*Tetraodon nigroviridis*), zebrafish, medaka (*Oryzias*
latipes), and stickleback (Hanna et al., 2010). Our BLAST analyses confirmed the
presence of a single pgr paralogon in these species, as well as in other teleosts such as
tilapia.
Phylogeny and synteny analyses allowed us to infer the origin of the duplicated eel pgr.
Phylogeny analysis clustered teleost PGR in two clades, one encompassing Japanese
and European eel PGR1, and the second one encompassing all other teleost PGR, with
Japanese and European eel PGR2 at the basis of this second clade.
Synteny analysis showed that the duplicated pgr genomic region in the teleosts
investigated is the result of the teleost specific 3R, suggesting that eel duplicated pgr
(pgr1 and pgr2) originated from the 3R. The other teleosts investigated (zebrafish,
stickleback, tilapia and fugu) have conserved a single pgr, orthologous to eel pgr2. This
is in agreement with the phylogeny analysis clustering eel PGR2 with the other teleost
PGR. These results suggest that pgr1 paralog would have been lost in the teleost
lineage, after the emergence of the basal teleost group of elopomorphs. Considering that
pgr2 is located on the 3R-paralogon “b” in zebrafish, it could be named pgrb, according
to the official zebrafish nomenclature. Thus, eel pgr1 and pgr2 could be named pgra
and pgrb, respectively.
Other teleost species would have conserved pgrb, following Zebrafish Nomenclature.
Previous studies from our groups have shown that eels have conserved more 3R-
duplicated paralogs than other teleosts species. This is the case as well for hox genes
(Henkel et al., 2012a, 2012b), as for hormone and receptor genes such as leptin and its
receptors (Morini et al., 2015b) and estradiol receptors (Lafont et al., 2016).
Different from the origin of duplicated PGRs in the eel, PGRs duplication in other
species may be the result from tetraploidisation. Our phylogeny analysis indicated that
the two goldfish PGRs clustered together and inside the same PGR2 (PGRb) clade,
suggesting that they may result from the tetraploidisation. Thus, the goldfish pgr paralogs could be named pgrb1 and pgrb2, instead of pgr1 and pgr2, respectively. Similarly, the two PGR reported in *Xenopus laevis* (Liu et al., 2005), which clustered together and inside the sarcopterigian PGR clade, may result from tetraploidisation of this species. The conservation of duplicated PGR may reflect evolutionary processes such as neo- or sub-functionalisation. Regarding the PGR amino acid sequences, alignment clearly showed that DBD and LBD domains were well conserved among vertebrates, whereas A/B domain had lower identity. Almost all PGR residues critical for progestin binding (Williams and Sigler, 1998) were conserved in the LBD domain of both Japanese eel (Hanna et al., 2010; Ikeuchi, 2002; Todo et al., 2000) and European eel PGR1 and PGR2.

4.2. Multiple membrane progestin receptors in the eel

We identified five membrane progestin receptors in the genomes of the European and Japanese eels. The mPRs belong to the progestin and adipoQ receptor (PAQR) family. This family includes five mPR subtypes: mPRα (PAQR7) first identified and characterized in spotted seatrout (*Cynoscion nebulosus*; Zhu et al., 2003a), mPRβ (PAQR8) and mPRγ (PAQR5) identified and characterized in humans and other vertebrates (Zhu et al., 2003b), mPRδ (PAQR6) and mPRε (PAQR9), which respond to progestins in yeast recombinant expression systems (Pang et al., 2013; Smith et al., 2008; Thomas and Pang, 2012). This family also includes other proteins different from membrane receptors: PAQR3, PAQR4, two adiponectin receptors (ADR1 and ADR2) and two monocyte to macrophage differentiation proteins (MMD, MMD2) (Thomas et al., 2007). In the present study, phylogenetic analyses included PAQR3 to PAQR9 in
order to determine the relationship of eel mPRs characterized with other PAQR forms. The resulting tree clustered the five eel mPRs in 3 groups: mPRα, mPRAL1 (alpha-like1), mPRAL2 (alpha-like2) clustered with the mPRα/mPRβ clade, while eel mPRγ and mPRδ clustered with mPRγ and mPRδ clade, respectively. The second part of the phylogenetic tree showed that mPRε, PAQR3, PAQR4 evolutionally diversified from other groups of mPRs, with PAQR3 clearly closer to PAQR4. It can be noticed that the mPRα clade formed a paraphyletic group supported by low bootstrap values, which included the mPRβ clade. According to our in silico and phylogenetic analyses, the European and Japanese eel genomes seem devoid of mPRβ and mPRε. Both mPRAL1 and mPRAL2 could possibly be derived from eel mPRα as a result of a local eel specific duplication; however, the low phylogenetic resolution does not allow to conclude on this. Nevertheless, all eel mPRα or alpha-like are devoid of introns in their coding region, similar to catfish mPRα and β, as described Kazeto et al. (2005). In our study, as mPRδ and mPRγ lack any particular sequence signature, the nomenclature was only based on phylogenetic analyses.

The eel is not the only teleost with mPR derived forms. In the pufferfish genome database, Kazeto et al. (2005) found three uncharacterized forms (FmPRLP 1–3) with FmPRLP 1 and 2 closely related to mPRα, whereas FmPRLP 3 shared high identity with the β form. Thus, these derived mPR forms seem to be expressed only in teleost species.

The presence of both nPRs and mPRs in actinopterygian and sarcopterygian members suggests that they both arose around the same time, early in the vertebrate evolution, coinciding with the appearance of critical steroidogenic enzymes (Thomas et al., 2007). The emergence of both mPRs and nPRs in early vertebrates might suggest a complimentary relationship between the two receptor systems, leading to a wide range
of progestin mechanism of action and multiple possible responses of progestin target cells.

4.3. Differential tissue distribution of progestin receptors

In the present study, different expression patterns were found for all the receptors in male and female European eel. This results could have been affected by the different physiological/maturational stage of males and females, as male eels were probably more immature than females, being in farmed/yellow stage, while female eels were in silver stage, considered as the onset of puberty in this species (Aroua et al. 2005). Male and female eel progestin receptors showed differential expression patterns when compared with the corresponding genes in other species. In female catfish, IpmpRa transcript is expressed in all the tissues (Kazeto et al., 2005) which is similar to the eel tissue distribution of mPRAL1 or mPRAL2, but is different to the eel mPRa, which is mainly expressed in the cerebellum of male eels and in the head kidney of female eels. In the seatrout, mPRa gene is expressed in the brain, pituitary and gonads (Zhu et al., 2003a), in the zebrafish in testis, ovary and head kidney (Kazeto et al., 2005), and in humans in gonads and kidney (Zhu et al., 2003b). Thus, the different mPRa tissues distribution among the different species investigated until now may indicate species-specific differences of the mPRa function.

The eel mPR subtypes showed a wide distribution, and even mRNA co-expression of some subtypes was observed in a few tissues, such as kidney, similar to what is found in human (Zhu et al., 2003b). Finally, we found that tissue distributions of both mPRALs were ubiquitous in male and female eels.

Concerning the nPRs, both pgr1 and pgr2 subtypes showed tissue-specific and sex-related expression. Both were mainly expressed in the pituitary and in the brain in
female eel, while in males they were mainly expressed in the gonads and in other tissues outside the BPG axis (*pgr1* in kidney and muscle, *pgr2* in the gill). The tissue distribution of nPRs in the European eel is similar to what was found in the Japanese eel (Ikeuchi et al., 2002). Nevertheless, the different nPR tissue distribution found among teleosts may indicate species-specific differences of the nPR function. Thus, eel nuclear and membrane PRs were expressed in the neuroendocrine and non-reproductive tissues. Further analyses are required to determine the function of both nuclear and membrane PRs and their potential interactions in some tissues from the BPG axis were they were highly co expressed. However, our results suggest that these receptors could be involved not only in reproduction, but also in other non-reproductive functions.

4.4. Expression of progestin receptors through spermatogenesis

Neuroendocrine mechanisms regulated by progestins influence a wide variety of brain functions. These mechanisms have been shown to be mediated by specific nPRs (Hanna et al., 2010; Mani, 2008), or by mPRs (Sleiter et al., 2009).

Although nPRs and membrane mPRα and mPRβ are quite well studied in mammals, information is lacking on the function of mPRγ, mPRδ and mPRε. The present study is the first to report mRNA expression of five membrane and two nuclear PRs through spermatogenesis in the fish brain-pituitary-gonad axis. In the anterior brain and pituitary, mRNAs for all five mPR subtypes were constantly expressed. *mPRγ, mPRAL1* and *mPRAL2* showed low expression in all the brain parts studied and the pituitary, whereas *mPRα* was highly expressed; and *mPRδ* showed the greatest brain expression, like in human brain (Pang et al., 2013; Thomas and Pang, 2012). In human, the mPRs seem to be involved in the negative feedback of progesterone on the gonadotropin-
releasing hormone secretion (Sleiter et al., 2009; Thomas and Pang, 2012), while further research is required to elucidate the specific signalling roles of mPRs in the eel brain and pituitary.

Concerning the nPRs, our study showed very low expression of both pgr in different brain parts, but high expression in the pituitary, which is similar to the nPR mRNA pattern found by Pang et al. (2013) in humans. Furthermore, both nPRs in the pituitary were up-regulated throughout the induced-hCG maturation, showing higher expression from spermatogonia B /spermatocyte stage to spermatozoa stage, which correspond to proliferating germ cell to full spermiation.

From both nPRs, only pgr1 mRNA expression increased in all the brain parts through spermatogenesis, corresponding with the plasma levels of DHP found in the European eel, which significantly increased during the spermatogenesis (Fig. D, Peñaranda et al., 2016). It should be noted that DHP is the main progestin in Japanese eel, showing maximum affinity with PGR1 and PGR2 (Ikeuchi et al., 2002).

The observed profiles suggest that brain and pituitary nPRs could be involved in the spermatogenesis process. PGR1 could be the main progestin receptor in the brain, while both PGR1 and PGR2 could mediate DHP signaling in the pituitary through spermatogenesis, as DHP plasma levels showed a similar profile to the expression of both nuclear receptors in pituitary (Fig. D). This is supported by the fact that a reproductive neuroendocrine role for DHP has been recently demonstrated in zebrafish, where DHP exerted a Pgr-mediated direct stimulatory effect on fshb mRNA at pituitary level (Wang et al., 2016). However, in coho salmon (Oncorhynchus kisutch), no evidence was found for DHP effects on gonadotropin gene expression in the pituitary (Dickey et al., 1998). Nevertheless, further experimental studies will be necessary to
determine the cellular sites of expression of the progestin receptors in eel brain and pituitary in order to infer their physiological role.

In cyprinids, DHP has a well-known role as pheromone released by females during final oocyte maturation, which induces in males the courtship behavior, and increased LH, steroid and milt production (see review of Scott et al., 2010). In the Chinese black sleeper (*Bostrichthys sinensis*), Zhang et al. (2016) suggested that progestin receptors may be involved in the detection of progestin in the olfactory rosette. In female eels, an increase of DHP levels is observed before ovulation (Huertas et al., 2006), which can be possibly released to the environment to act as a pheromone. In this sense, the increase of *pgrl* in the olfactory bulb at spermiating stage suggests a pheromone action of progestins which should be further investigated in this species.

Nuclear and membrane PRs are highly expressed in fish testis (Hanna and Zhu, 2009; Ikeuchi et al., 2002). In Japanese eel, *PR I (pgrl)* was expressed in germ cells, Sertoli cells and interstitial cells of testis, whereas *PR II (pgr2)* was detected only in germ cells (Miura et al., 2006). The reproductive functions of the PRs have been well studied in mammalian models, but less information is available in teleost fish. In Japanese eel, progestins induce early spermatogonia to enter in the meiotic prophase (Miura et al., 2006), further regulating sperm maturation (Miura et al., 1995; Schulz et al., 2010). In other fish species, both Pgr and mPRα were suggested to play a role at the beginning of the spermatogenesis and/or at the final sperm maturation (Chen et al., 2010, 2011, 2012; Thomas et al., 2004; Tubbs and Thomas, 2008). Tubbs et al. (2010) showed that mPRα was expressed in all testicular germ cell stages in Atlantic croaker, with an up-regulation of mPRα in both ovaries and testes under gonadotropin control, most likely mediated by increases in LH secretion at the end of the reproductive cycle. In European eel testis, the three mPRalpha/alpha-like (*mPRa, mPRAL1* and *mPRAL2*) showed the
same expression pattern as in the Atlantic croaker, increasing progressively during spermatogenesis, and being maximum at spermatozoa stage. These results suggest an implication of these receptors on the regulation of the spermiogenesis in the eel testis, according to what it was demonstrated in the Atlantic croaker, seatrout and southern flounder (Thomas et al., 2009; Tubbs, 2007; Tubbs and Thomas, 2008, 2009). This suggestion is also supported by the fact that in Japanese eel it was demonstrated an effect of DHP on sperm motility and pH of the sperm duct, related with the final sperm maturation (Miura et al., 1991).

In contrast with the three mPRalpha/alpha-like, mPRγ, mPRδ and pgr2 showed an opposite profile, with high expression in the testis during the spermatogonia A stage, showing a fast decrease onwards, until spermatozoa stage. It is known that the ratio germ cells/somatic cells increase during the spermatogenesis. Thus, if there is a constant expression of one gene in somatic cells, but the gene is not expressed in the germ cells, a decrease in the expression of that gene through spermatogenesis would be observed. However, the pgr2 only expressed in the germ cells of the Japanese eel testis (Miura et al., 2006). Thus, the decrease in pgr2 expression transcript levels observed in the present work was probably due to a real decrease in pgr2 expression in the germ cells, from SPGB stage onwards. The possible role of pgr2 (and mPRγ, mPRδ) on eel spermatogonial function should be further studied.

The zebrafish showed the same Pgr expression profile as eel mPRγ, mPRδ and pgr2, with strong expression observed in spermatogonia and early spermatocyte stages (Hanna et al., 2010). Nevertheless, in cod, the expression of the pgr mRNA varies in an opposite way, reaching peak levels in spawning testes (Chen et al., 2012). This suggests
that PRs have differential functions during spermatogenesis depending on the teleost species.

In conclusion, all the progestin receptor genes found in the European eel have been described. Two nPR and five mPR genes were identified in the genome of the European and the Japanese eel. The two nPRs showed the C and E(F) domains well conserved among vertebrates, whereas the A/B domain showed lower degree of conservation. Phylogenetic and syntenic analyses of nPR allow us to infer the origin of these receptors, likely resulting of the teleost specific 3R. Phylogenetic analysis of mPRs placed three eel mPRs together with the mPRα clade, called mPRα, mPRAL1 (alpha-like1) and mPRAL2 (alpha-like2), while the other two eel mPRs clustered respectively with mPRγ and mPRδ clades.

In the testis, two membrane (mPRγ, mPRδ) and one nuclear receptor (pgr2) transcripts showed the highest levels in spermatogonia A stage, while the membrane receptors alpha/alpha-like (mPRα, mPRAL1 and mPRAL2) transcripts showed the highest levels at spermatozoa stage. Further studies should elucidate the role of both nuclear and membrane progestin receptors on eel spermatogenesis.

Acknowledgements

This work was supported by the Spanish Ministry of Science and Innovation (SPERMOT project; AGL2010-16009; REPRO-TEMP project, AGL2013-41646-R), and IMPRESS (Marie Sklodowska Curie Actions – Innovative Training Network; Grant agreement nº: 642893). M.C. Vilchez has a predoctoral grant from UPV PAID Programme (2011-S2-02-6521), M. Morini has a predoctoral grant from Generalitat Valenciana (Programa Grisolía), D.S. Peñaranda was supported by MICINN and UPV
Grants to attend meetings were funded by COST Office (COST Action FA1205: AQUAGAMETE).
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Figure legends

Fig. 1. PGR consensus phylogenetic tree (A) and comparison of PGR structure (B). The phylogenetic tree was constructed based on the amino acid sequences of nuclear progestin receptor (for the references of each sequence see Table A) using the Maximum Likelihood method with 1000 bootstrap replicates. The number shown at each branch node indicates the bootstrap value (%). The functional domains A/B, C (DNA binding domain), D, E/F (Ligand binding domain) are schematically represented, with the numbers of amino acid residues indicated below.

Fig. 2. Consensus phylogenetic tree of vertebrate PAQR family. The phylogenetic tree was constructed based on the amino acid sequences of members of PAQR family, including PAQR3 to PAQR9 (for the references of each sequence see Table A) using the Maximum Likelihood method with 1000 bootstrap replicates. The number shown at each branch node indicates the bootstrap value (%).

Fig. 3. Comparison of membrane European and Japanese eel PRs. The 7 transmembrane domains are schematically represented for each eel mPR; with the two different eel mPRδ representation possible according to the predict program used.

Fig. 4. Conserved genomic synteny of vertebrate pgr. Genomic synteny maps comparing pgr and neighboring genes from human, non-teleost actinopterygian (spotted gar), and teleost species including the two eel pgr (pgr1 and pgr2) genomic regions, are represented. The pgr genomic region has been duplicated in teleost species, likely as a result of the teleost specific third round of genome duplication. The duplicated pgr1 paralog has been conserved in the eels but lost in the other teleosts studied. Genes are named after their human orthologs according to the Human Genome Naming Consortium (HGNC). 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 human, and their positions are indicated in 10^6 base pairs. The detailed genomic locations of the genes are given in Table B.
Fig. 5. **Tissue distribution of progestin receptors in the European eel.** Progestin receptor mRNA expression in immature female (1), and in immature male (2) of pgr1 (A), pgr2 (B), mPRa (C), mPRγ (D), mPRδ (E), mPRAL1 (F), mPRAL2 (G) mRNA expression. Data are normalised to eel ARP. Values are presented as means ± SEM (n = 3). OB: olfactory bulb, T: Telencephalon, M/D: Mes-/Diencephalon, CEREB: cerebellum, MED: medulla oblongata, PIT, pituitary.

Fig. 6. **Expression of European eel nuclear progestin receptors.** mRNA expression of pgr1 (A-D) and pgr2 (E-H) in different brain parts and pituitary of male eel kept at 20 °C during experimental maturation. Data are normalised to eel ARP. Means are given ± SEM. Significant differences (p<0.01, n=6-12) between tissues. SPGA= Spermatogonia A stage, SPGB/SPC= Spermatogonia B/Spermatocytes stage, SD= Spermatid stage, SZ= Spermatozoa stage. See main text for definition of gonad developmental stages. OB: olfactory bulb, T: Telencephalon, M/D: Mes-/Diencephalon, PIT: pituitary.

Fig. 7. **Expression of the eel membrane progestin receptors.** mRNA expression of mPRa (A), mPRAL1 (B), mPRAL2 (C), mPRγ (D), mPRδ (E) in different brain parts and pituitary of male eel kept at 20 °C during experimental maturation. Data are normalised to eel ARP. Means are given ± SEM (n=6-17). SPGA= Spermatogonia A stage, SPGB/SPC= Spermatogonia B/Spermatocytes stage, SD= Spermatid stage, SZ= Spermatozoa stage. See main text for definition of gonad developmental stages.

Fig. 8. **Expression of the European eel nuclear and membrane progestin receptors.** mRNA expression of pgr1 and pgr2 (A-B) and mPRa, mPRAL1, mPRAL2, mPRγ, mPRδ (C-G) during experimental maturation in fish testis kept at 20 degrees. Data are normalised to eel ARP. Means are given ± SEM. Significant differences (p<0.01; n=6-17). SPGA= Spermatogonia A stage, SPGB/SPC= Spermatogonia B/Spermatocytes stage, SD= Spermatid stage, SZ= Spermatozoa stage. See main text for definition of gonad developmental stages.
## Table 1. Quantitative PCR primer sequences for nuclear progestin receptors (*pgr1* and *pgr2*) and membrane progestin receptors (*mPRα*, *mPRγ*, *mPRδ*, *mPRAL1* and *mPRAL2*).

<table>
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<tr>
<th>Name</th>
<th>Sequence (5’- 3’)</th>
<th>Orientation</th>
<th>Length / Efficiency</th>
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## Table 2. Tissue distribution summary of progestin receptors in the European eel.

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</tbody>
</table>
Progestin receptor mRNA expression in immature male (A), and in immature female (B) ($n = 3$). Data are normalised to eel ARP. “+” or “−” symbols indicate relative differences between tissues of each receptors.
Appendices

Fig A. Histological sections of eel testis at different developmental stages during human chorionic gonadotropin (hCG) hormonal treatment  
A: SPGA (spermatogonia A); B: SPGB/SPC (spermatogonia B/spermatocyte); C: SPD (spermatid), D: SPZ (spermiation)  
Scale bar: A=100 µm; B= 10 µm, C, D= 25 µm;  
Cell types: SPG= spermatogonia; SPC: spermatocytes; SPD: spermatids; SPZ: spermatozoa  
See main text for definition of gonad developmental stages

Fig B. Multiple sequence alignment of the European eel nPRs at amino acid level  
The functional domains A/B, C (DNA binding domain), D, E/F (Ligand binding domain) are indicated with dark arrow above the amino acid sequence alignment.

Fig C. Multiple sequence alignment of the European eel mPRs at amino acid level  
Exons are indicated in dark grey or light grey

Fig D. 17α,20β-dihydroxy-4-pregnen-3-one (DHP) plasma level through spermatogenesis

Table A. Accession number of sequences used for phylogenetic analyses

Table B. Names, references and locations of the genes used in the PGR synteny analysis (Fig 4)