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

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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 Paralichtydae (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 adipoQ 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

146 the Care and Use of Laboratory Animals of the Spanish Royal Decree 53/2013 on
147 protection of animals used for scientific purposes (BOE 2013). The protocol was
148 approved by the Committee on the Ethics of Animal Experiments of the Universitat
149 Politècnica de València (UPV) (Permit Number: 2014/VSC/PEA/00147). All efforts
150 were made to minimize animal suffering and stress.
151

152 **2.3. Gonadal histology**

153 To determine the maturational stage of the testis, formalin-fixed samples were
154 dehydrated in ethanol and embedded in paraffin. Sections of 5-10 μm thickness were
155 cut with a Shandom Hypercut manual microtome and stained with haematoxylin and
156 eosin. Slides were observed with a Nikon Eclipse E-400 microscope, and micrographs
157 were taken with a Nikon DS-5M camera attached to the microscope. Stages of
158 spermatogenesis were determined according to the germ cell types present in the testis
159 (Leal et al., 2009; Miura and Miura 2011) and their relative abundance, the degree of
160 development of the seminal tubules, the GSI and the sperm production by the male in
161 the week of the sacrifice. The stages considered were: Stage 1: A Spermatogonia
162 (SPGA): dominance of A spermatogonia, B spermatogonia present in low number,
163 presence/absence of lumen; mean GSI = 0.08 (0.0-0.36); Stage 2 Spermatogonia
164 B/Spermatocytes (SPGB/SPC): dominance of B spermatogonia, spermatocytes present,
165 in some cases low number of spermatids appeared, mean GSI = 0.80 (0.29-1.52); Stage
166 3 spermatids (SD): spermatids very abundant, some sperm cells could appear, mean GSI
167 = 4.02 (1.79-5.93); Stage 4 spermatozoa (SZ): spermatozoa was the dominant germ cell,
168 mean GSI =7.35 (3.41-12.8) (Fig. A).
169

170 **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 osteichthyans 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 adipoQ 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

196 adjusted. The JTT (Jones, Taylor and Thornton) protein substitution matrix of the
197 resulting alignment was determined using ProTest software (Abascal et al., 2005). Both
198 phylogenetic trees of nPRs and Progestin and adipoQ receptor were constructed based
199 on the sequence alignments, using the maximum likelihood method (PhyML software,
200 Stamatakis et al., 2008) with 1000 bootstrap replicates, and subsequently visualized
201 using treedyn (http://phylogeny.lirmm.fr/phylo_cgi/).
202
203 **2.4.3. Synteny analysis of nuclear progestin receptor genes**
204 Neighboring genomic regions of the duplicated eel nuclear progestin receptors, *pgr1*
205 and *pgr2*, were characterized manually on the European and Japanese eel genomic
206 databases, using CLC DNA Workbench 6 software and the GENSCAN Web Server
207 (http://genes.mit.edu/GENSCAN.html). BLAST analyses were performed in the
208 European and Japanese eel genomes to identify potential additional paralogs of the *pgr*
209 neighboring genes. Homologs of eel *pgr* neighboring genes were then identified, using
210 PhyloView of Genomicus v82.01, in other vertebrate genomes, *i.e.* human, zebra finch
211 (*Taeniopygia guttata*), spotted gar, zebrafish, stickleback (*Gasterosteus aculeatus*),
212 tilapia (*Oreochromis niloticus*) and fugu (*Takifugu rubripes*). BLAST analyses were
213 also performed to search potential *pgr* paralogs in the genomes of these species. For
214 each *pgr* neighboring gene family, when only one gene was annotated in all the above-
215 mentioned genomes, BLAST analyses were performed to search for potential additional
216 paralogs.
217
218 **2.5. Gene expression analyses by quantitative real-time PCR**
219 **2.5.1. Primers and reference gene**
220 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

246 conditions: 95 °C for 1 s, 60 °C for 30 s.

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

258

259 **2.5.3. Eel progestin receptors tissue distribution**

260 In order to investigate the tissue distribution of each PR mRNA expression in male and
261 female European eels; gonads (testes and ovaries) and somatic tissues (liver, heart, gill,
262 muscle, spleen, pectoral fin, posterior kidney, head kidney, brain, pituitary) were
263 collected from three immature male eels (mean body weight 118±14 g) from the fish
264 farm Valenciana de Acuicultura, S.A. (Puzol, Valencia; East coast of Spain) and three
265 wild female eels (mean body weight 608±35 g) captured by local fishermen in the
266 Albufera lagoon (Valencia, Spain). Total RNA was extracted following the method used
267 by Peñaranda et al. (2014), treated with DNase I (Turbo DNA-free; Ambion) at 37 °C
268 for 30 min, and reverse-transcripted using superscript III (Invitrogen) and random
269 hexamer primers on 1 µL total RNA, according to the manufacturer's protocol. All
270 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).

296 **3. Results**

297 **3.1. Characterization of progestin receptor genes**

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

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

314 Although both nPRs were composed of 8 exons, they shared only 25.38% sequence
315 identity at the amino acid level (Fig. B). The European and Japanese eel Pgr amino acid
316 sequence differed by 8 and 26 amino acids, for Pgr1 and Pgr2 respectively. The eel *pgr*
317 gene sequences could be subdivided into four domains (Fig. 1B) as described by Laudet
318 (1997). For Pgr1, the A/B domain was from residues 338 to 415 and the E(F) domain
319 was from residues 463 to 710 , and for Pgr2 the A/B domain was from residues 305 to
320 382, 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*

421 *latipes*), and stickleback (Hanna et al., 2010). Our BLAST analyses confirmed the
422 presence of a single *pgr* paralogon in these species, as well as in other teleosts such as
423 tilapia.
424 Phylogeny and synteny analyses allowed us to infer the origin of the duplicated eel *pgr*.
425 Phylogeny analysis clustered teleost PGR in two clades, one encompassing Japanese
426 and European eel PGR1, and the second one encompassing all other teleost PGR, with
427 Japanese and European eel PGR2 at the basis of this second clade.
428 Synteny analysis showed that the duplicated *pgr* genomic region in the teleosts
429 investigated is the result of the teleost specific 3R, suggesting that eel duplicated *pgr*
430 (*pgr1* and *pgr2*) originated from the 3R. The other teleosts investigated (zebrafish,
431 stickleback, tilapia and fugu) have conserved a single *pgr*, orthologous to eel *pgr2*. This
432 is in agreement with the phylogeny analysis clustering eel PGR2 with the other teleost
433 PGR. These results suggest that *pgr1* paralog would have been lost in the teleost
434 lineage, after the emergence of the basal teleost group of elopomorphs. Considering that
435 *pgr2* is located on the 3R-paralogon "b" in zebrafish, it could be named *pgrb,* according
436 to the official zebrafish nomenclature. Thus, eel *pgr1* and *pgr2* could be named *pgra*
437 and *pgrb*, respectively.
438 Other teleost species would have conserved *pgrb*, following Zebrafish Nomenclature.
439 Previous studies from our groups have shown that eels have conserved more 3R-
440 duplicated paralogs than other teleosts species. This is the case as well for *hox* genes
441 (Henkel et al., 2012a, 2012b), as for hormone and receptor genes such as leptin and its
442 receptors (Morini et al., 2015b) and estradiol receptors (Lafont et al., 2016).
443 Different from the origin of duplicated PGRs in the eel, PGRs duplication in other
444 species may be the result from tetraploidisation. Our phylogeny analysis indicated that
445 the two goldfish PGRs clustered together and inside the same PGR2 (PGRb) clade,

446 suggesting that they may result from the tetraploidisation. Thus, the goldfish *pgr*
447 paralogs could be named *pgrb1* and *pgrb2*, instead of *pgr1* and *pgr2*, respectively.
448 Similarly, the two PGR reported in *Xenopus laevis* (Liu et al., 2005), which clustered
449 together and inside the sarcopterygian PGR clade, may result from tetraploidisation of
450 this species.
451 The conservation of duplicated PGR may reflect evolutionary processes such as neo- or
452 sub-functionalisation. Regarding the PGR amino acid sequences, alignment clearly
453 showed that DBD and LBD domains were well conserved among vertebrates, whereas
454 A/B domain had lower identity. Almost all PGR residues critical for progestin binding
455 (Williams and Sigler, 1998) were conserved in the LBD domain of both Japanese eel
456 (Hanna et al., 2010; Ikeuchi, 2002; Todo et al., 2000) and European eel PGR1 and
457 PGR2.
458
459 **4.2. Multiple membrane progestin receptors in the eel**
460 We identified five membrane progestin receptors in the genomes of the European and
461 Japanese eels. The mPRs belong to the progestin and adipoQ receptor (PAQR) family.
462 This family includes five mPR subtypes: mPRα (PAQR7) first identified and
463 characterized in spotted seatrout (*Cynoscion nebulosus;* Zhu et al., 2003a), mPRβ
464 (PAQR8) and mPRγ (PAQR5) identified and characterized in humans and other
465 vertebrates (Zhu et al., 2003b), mPRδ (PAQR6) and mPRε (PAQR9), which respond to
466 progestins in yeast recombinant expression systems (Pang et al., 2013; Smith et al.,
467 2008; Thomas and Pang, 2012). This family also includes other proteins different from
468 membrane receptors: PAQR3, PAQR4, two adiponectin receptors (ADR1 and ADR2)
469 and two monocyte to macrophage differentiation proteins (MMD, MMD2) (Thomas et
470 al., 2007). In the present study, phylogenetic analyses included PAQR3 to PAQR9 in

471 order to determine the relationship of eel mPRs characterized with other PAQR forms.
472 The resulting tree clustered the five eel mPRs in 3 groups: mPRα, mPRAL1 (alpha-
473 like1), mPRAL2 (alpha-like2) clustered with the mPRα/mPRβ clade, while eel mPRγ
474 and mPRδ clustered with mPRγ and mPRδ clade, respectively. The second part of the
475 phylogenetic tree showed that mPRε, PAQR3, PAQR4 evolutionally diversified from
476 other groups of mPRs, with PAQR3 clearly closer to PAQR4. It can be noticed that the
477 mPRα clade formed a paraphyletic group supported by low bootstrap values, which
478 included the mPRβ clade. According to our *in silico* and phylogenetic analyses, the
479 European and Japanese eel genomes seem devoid of mPRβ and mPRε. Both mPRAL1
480 and mPRAL2 could possibly be derived from eel mPRα as a result of a local eel specific
481 duplication; however, the low phylogenetic resolution does not allow to conclude on
482 this. Nevertheless, all eel mPRα or alpha-like are devoid of introns in their coding
483 region, similar to catfish mPRα and β, as described Kazeto et al. (2005). In our study, as
484 mPRδ and mPRγ lack any particular sequence signature, the nomenclature was only
485 based on phylogenetic analyses.
486 The eel is not the only teleost with mPR derived forms. In the pufferfish genome
487 database, Kazeto et al. (2005) found three uncharacterized forms (FmPRLP 1–3) with
488 FmPRLP 1 and 2 closely related to mPRα, whereas FmPRLP 3 shared high identity
489 with the β form. Thus, these derived mPR forms seem to be expressed only in teleost
490 species.
491 The presence of both nPRs and mPRs in actinopterygian and sarcopterygian members
492 suggests that they both arose around the same time, early in the vertebrate evolution,
493 coinciding with the appearance of critical steroidogenic enzymes (Thomas et al., 2007).
494 The emergence of both mPRs and nPRs in early vertebrates might suggest a
495 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, *IpmPRα* 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 *mPRα*, which is mainly expressed in the cerebellum of male eels and in the head kidney of female eels. In the seatrout, *mPRα* 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 *mPRα* tissues distribution among the different species investigated until now may indicate species-specific differences of the mPRα 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

521 female eel, while in males they were mainly expressed in the gonads and in other tissues
522 outside the BPG axis (*pgr1* in kidney and muscle, *pgr2* in the gill). The tissue
523 distribution of nPRs in the European eel is similar to what was found in the Japanese eel
524 (Ikeuchi et al., 2002). Nevertheless, the different nPR tissue distribution found among
525 teleosts may indicate species-specific differences of the nPR function.
526 Thus, eel nuclear and membrane PRs were expressed in the neuroendocrine and non-
527 reproductive tissues. Further analyses are required to determine the function of both
528 nuclear and membrane PRs and their potential interactions in some tissues from the
529 BPG axis were they were highly co expressed. However, our results suggest that these
530 receptors could be involved not only in reproduction, but also in other non-reproductive
531 functions.
532

533 **4.4. Expression of progestin receptors through spermatogenesis**

534 Neuroendocrine mechanisms regulated by progestins influence a wide variety of brain
535 functions. These mechanisms have been shown to be mediated by specific nPRs (Hanna
536 et al., 2010; Mani, 2008), or by mPRs (Sleiter et al., 2009).
537 Although nPRs and membrane mPRα and mPRβ are quite well studied in mammals,
538 information is lacking on the function of mPRγ, mPRδ and mPRε. The present study is
539 the first to report mRNA expression of five membrane and two nuclear PRs through
540 spermatogenesis in the fish brain-pituitary-gonad axis. In the anterior brain and
541 pituitary, mRNAs for all five mPR subtypes were constantly expressed. *mPRγ*, *mPRAL1*
542 and *mPRAL2* showed low expression in all the brain parts studied and the pituitary,
543 whereas *mPRα* was highly expressed; and *mPRδ* showed the greatest brain expression,
544 like in human brain (Pang et al., 2013; Thomas and Pang, 2012). In human, the mPRs
545 seem to be involved in the negative feedback of progesterone on the gonadotropin-

546 releasing hormone secretion (Sleiter et al., 2009; Thomas and Pang, 2012), while further
547 research is required to elucidate the specific signalling roles of mPRs in the eel brain
548 and pituitary.
549 Concerning the nPRs, our study showed very low expression of both *pgr* in different
550 brain parts, but high expression in the pituitary, which is similar to the nPR mRNA
551 pattern found by Pang et al. (2013) in humans. Furthermore, both nPRs in the pituitary
552 were up-regulated throughout the induced-hCG maturation, showing higher expression
553 from spermatogonia B /spermatocyte stage to spermatozoa stage, which correspond to
554 proliferating germ cell to full spermiation.
555 From both nPRs, only *pgr1* mRNA expression increased in all the brain parts through
556 spermatogenesis, corresponding with the plasma levels of DHP found in the European
557 eel, which significantly increased during the spermatogenesis (Fig. D, Peñaranda et al.,
558 2016). It should be noted that DHP is the main progestin in Japanese eel, showing
559 maximum affinity with PGR1 and PGR2 (Ikeuchi et al., 2002).
560 The observed profiles suggest that brain and pituitary nPRs could be involved in the
561 spermatogenesis process. PGR1 could be the main progestin receptor in the brain, while
562 both PGR1 and PGR2 could mediate DHP signaling in the pituitary through
563 spermatogenesis, as DHP plasma levels showed a similar profile to the expression of
564 both nuclear receptors in pituitary (Fig. D). This is supported by the fact that a
565 reproductive neuroendocrine role for DHP has been recently demonstrated in zebrafish,
566 where DHP exerted a Pgr-mediated direct stimulatory effect on *fshb* mRNA at pituitary
567 level (Wang et al., 2016).. However, in coho salmon (*Oncorhynchus kisutch*), no
568 evidence was found for DHP effects on gonadotropin gene expression in the pituitary
569 (Dickey et al., 1998). Nevertheless, further experimental studies will be necessary to

570 determine the cellular sites of expression of the progestin receptors in eel brain and
571 pituitary in order to infer their physiological role.
572 In cyprinids, DHP has a well-known role as pheromone released by females during final
573 oocyte maturation, which induces in males the courtship behavior, and increased LH,
574 steroid and milt production (see review of Scott et al., 2010). In the Chinese black
575 sleeper (*Bostrichthys sinensis*), Zhang et al. (2016) suggested that progestin receptors
576 may be involved in the detection of progestin in the olfactory rosette. In female eels, an
577 increase of DHP levels is observed before ovulation (Huertas et al., 2006), which can be
578 possibly released to the environment to act as a pheromone. In this sense, the increase of
579 *pgr1* in the olfactory bulb at spermiating stage suggests a pheromone action of
580 progestins which should be further investigated in this species.
581 Nuclear and membrane PRs are highly expressed in fish testis (Hanna and Zhu, 2009;
582 Ikeuchi et al., 2002). In Japanese eel, *PR I* (*pgr1*) was expressed in germ cells, Sertoli
583 cells and interstitial cells of testis, whereas *PR II* (*pgr2*) was detected only in germ cells
584 (Miura et al., 2006). The reproductive functions of the PRs have been well studied in
585 mammalian models, but less information is available in teleost fish. In Japanese eel,
586 progestins induce early spermatogonia to enter in the meiotic prophase (Miura et al.,
587 2006), further regulating sperm maturation (Miura et al., 1995; Schulz et al., 2010). In
588 other fish species, both Pgr and mPRα were suggested to play a role at the beginning of
589 the spermatogenesis and/or at the final sperm maturation (Chen et al., 2010, 2011, 2012;
590 Thomas et al., 2004; Tubbs and Thomas, 2008). Tubbs et al. (2010) showed that mPRα
591 was expressed in all testicular germ cell stages in Atlantic croaker, with an up-
592 regulation of mPRα in both ovaries and testes under gonadotropin control, most likely
593 mediated by increases in LH secretion at the end of the reproductive cycle. In European
594 eel testis, the three mPRalpha/alpha-like (*mPRα, 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

619 that PRs have differential functions during spermatogenesis depending on the teleost
620 species.
621 In conclusion, all the progestin receptor genes found in the European eel have been
622 described. Two nPR and five mPR genes were identified in the genome of the European
623 and the Japanese eel. The two nPRs showed the C and E(F) domains well conserved
624 among vertebrates, whereas the A/B domain showed lower degree of conservation.
625 Phylogenetic and syntenic analyses of nPR allow us to infer the origin of these
626 receptors, likely resulting of the teleost specific 3R. Phylogenetic analysis of mPRs
627 placed three eel mPRs together with the mPRα clade, called mPRα, mPRAL1 (alpha-
628 like1) and mPRAL2 (alpha-like2), while the other two eel mPRs clustered respectively
629 with mPRγ and mPRδ clades.
630 In the testis, two membrane (*mPRγ, mPRδ*) and one nuclear receptor (*pgr2*) transcripts
631 showed the highest levels in spermatogonia A stage, while the membrane receptors
632 alpha/alpha-like (*mPRα, mPRAL1* and *mPRAL2*) transcripts showed the highest levels at
633 spermatozoa stage. Further studies should elucidate the role of both nuclear and
634 membrane progestin receptors on eel spermatogenesis.
635

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645

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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), *mPRα* (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 ºC 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 *mPRα* (A), *mPRAL1* (B), *mPRAL2* (C), *mPRγ* (D), *mPRδ* (E) in different brain parts and pituitary of male eel kept at 20 ºC 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 *mPRα, 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.

Tables

Table 1. Quantitative PCR primer sequences for nuclear progestin receptors (*pgr1* and *pgr2*) and membrane progestin receptors (*mPRα, mPRγ, mPRδ, mPRAL1* and *mPRAL2*).

Name	Sequence (5'- 3')	Orientation	Length / Efficiency
pgr1	AGTTTGCCAATCTCCAGGTG	Forward	107bp Eff 2,04
	ATCAAACTGTGGCTGGCTCT	Reverse	
pgr2	GCCTCTGGATGTCACTACGG	Forward	95bp Eff 1,95
	CCGGCACAAAGGTAGTTCTG	Reverse	
mPRα	CTGTCGGAGACGGTGGACTT	Forward	151bp Eff 1,91
	CCAGGAAGAAGAAGGTGTAGTG	Reverse	
mPRγ	AAACAGCACCTTCCACCTGT	Forward	102bp Eff 2,02
	TGCAGAAACGGTAAGCCAAG	Reverse	
mPRδ	GCAGCTTCCAGATGACCAAT	Forward	147bp Eff 1,99
	GCAGCATGTAGACCAGCAGA	Reverse	
mPRAL1	CTGGCCTACATGAGCTTCAG	Forward	92bp Eff 2,01
	CCCACGTAGTCCAGGAAGAA	Reverse	
mPRAL2	CCTGGCGCTACTACTTCCTG	Forward	70bp Eff 2,07
	AGCAGGTGTGTCCAGACGTT	Reverse	

Table 2. Tissue distribution summary of progestin receptors in the European eel.

A

Male	*mPRα*	*mPRAL1*	*mPRAL2*	*mPRγ*	*mPRδ*	*pgr1*	*pgr2*
liver	-	+	-	-	-	-	-
heart	-	+	+	-	-	-	-
gill	-	+	+	+	-	-	++
muscle	-	+	+	-	+++	+++	-
spleen	-	+	+	-	-	-	-
fins	-	+	+	+	+	-	+
post. kidney	-	+	+	+++	-	+++	-
head kidney	+	+	++	+	-	-	-
gonad	-	+	+	+	-	+++	+++
olfactory bulb	-	+	+	-	+	+	+
telencephalon	-	+	+	-	+	+	+
mes-/diencephalon	-	+	+	+	+	+	+
cerebellum	+++	+++	+++	-	++	+	-
medulla oblongata	-	+	+	-	+	+	+
pituitary	+	+	+	+	-	-	+

1012

B

Female	*mPRα*	*mPRAL1*	*mPRAL2*	*mPRγ*	*mPRδ*	*pgr1*	*pgr2*
liver	+	++	++	-	-	-	+
heart	+	++	++	-	+	-	+
gill	+	+	+	+	+	-	+
muscle	-	+	+	-	-	-	-
spleen	+	++	++	-	-	-	-
fins	+	++	++	+	+	-	+
post. kidney	+	+	+	+	-	-	+
head kidney	+++	+	+++	-	-	-	+
gonad	+	++	+	+++	-	-	+
olfactory bulb	+	++	+++	-	+	+	+
telencephalon	+	++	++	-	+	+	+++
mes-/diencephalon	+	++	++	-	++	+	+
cerebellum	+	+++	+++	-	+	+++	+
medulla oblongata	+	++	++	-	+++	+	++
pituitary	+	++	++	+	+	+	+++

1013

1014 Progestin receptor mRNA expression in immature male (A), and in immature
1015 female (B) (n = 3). Data are normalised to eel ARP. "+" or "-" symbols indicate relative
1016 differences between tissues of each receptors.

1017

1018

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

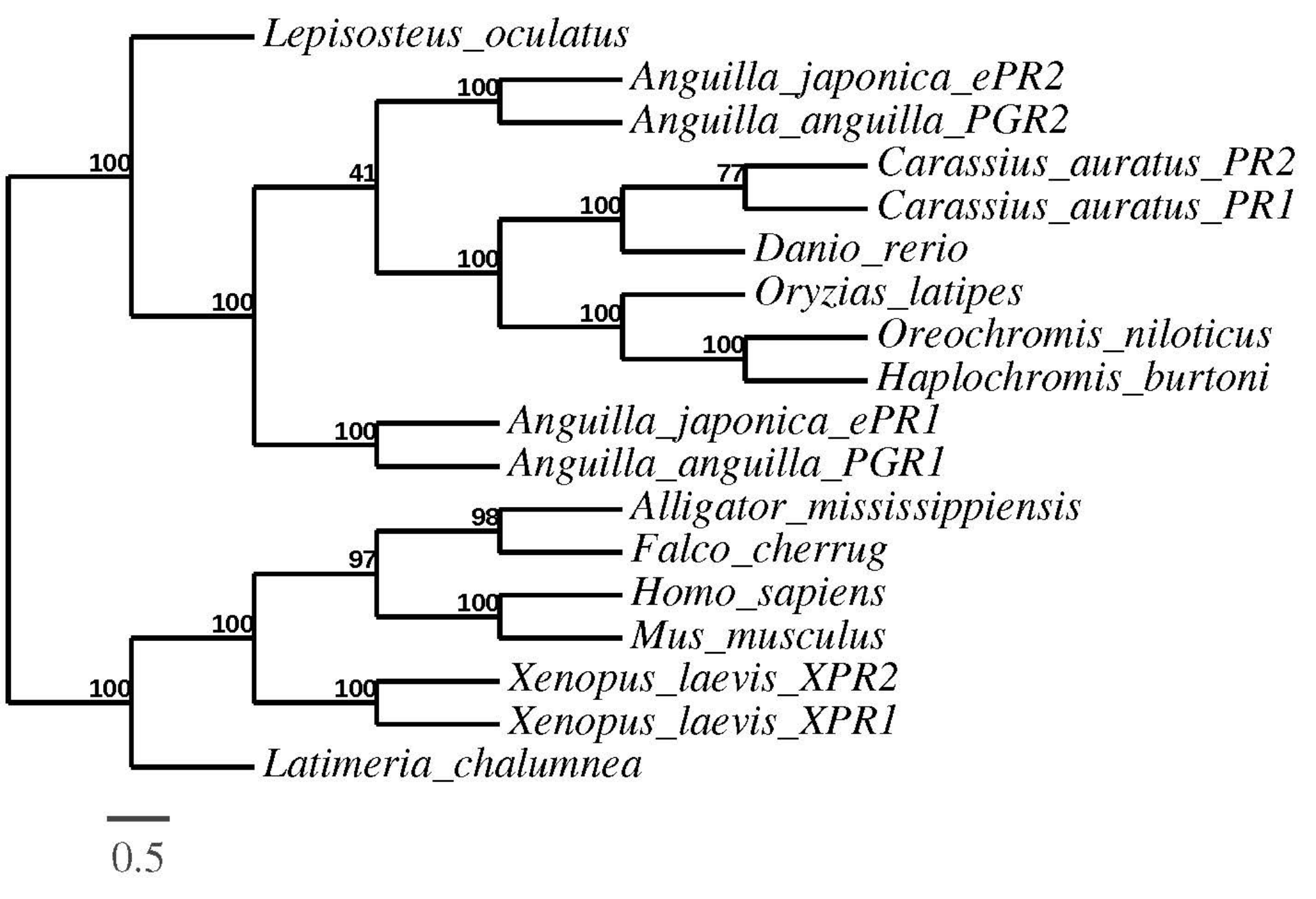
Fig B. Multiple sequence alignment of the European eel nPRs at amino acid level The functional domains A/B, C (DNA binding domain), D, E/F (Ligand binding domain) are indicated with dark arraw above the amino acid sequence alignment.

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)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Alligator_mississippiensis
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Homo_sapiens
Mus_musculus
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Xenopus_laevis_XPR1
Latimeria_chalumnea
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41
77
100
100
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98
97
100
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100
0.5


A/B
C
D
E(F)
European eel PGR1
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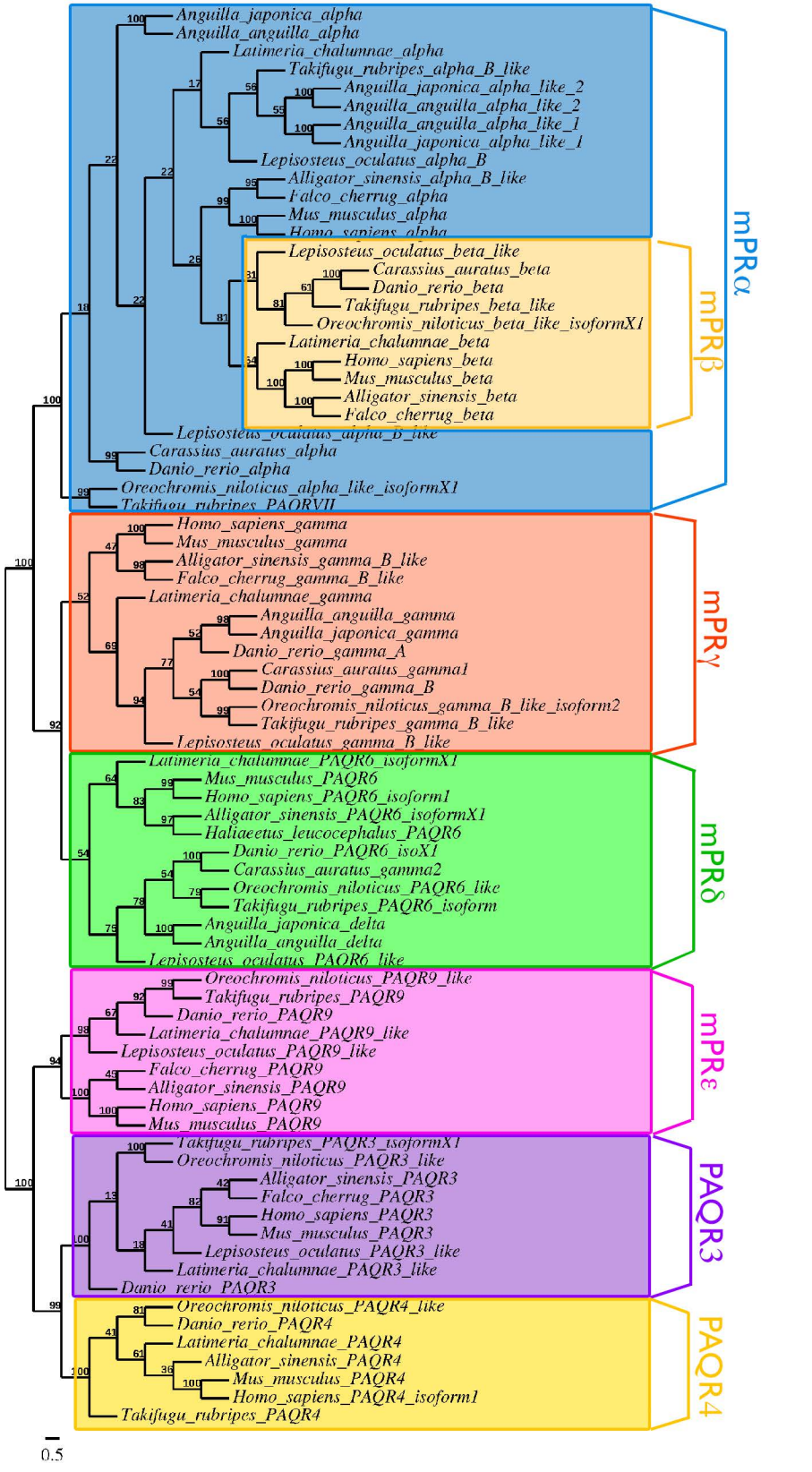
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Danio_rerio_beta
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Takifugu_rubripes_PAQR9
Danio_rerio_PAQR9
Latimeria_chalumnae_PAQR9_like
Lepisosteus_oculatus_PAQR9_like
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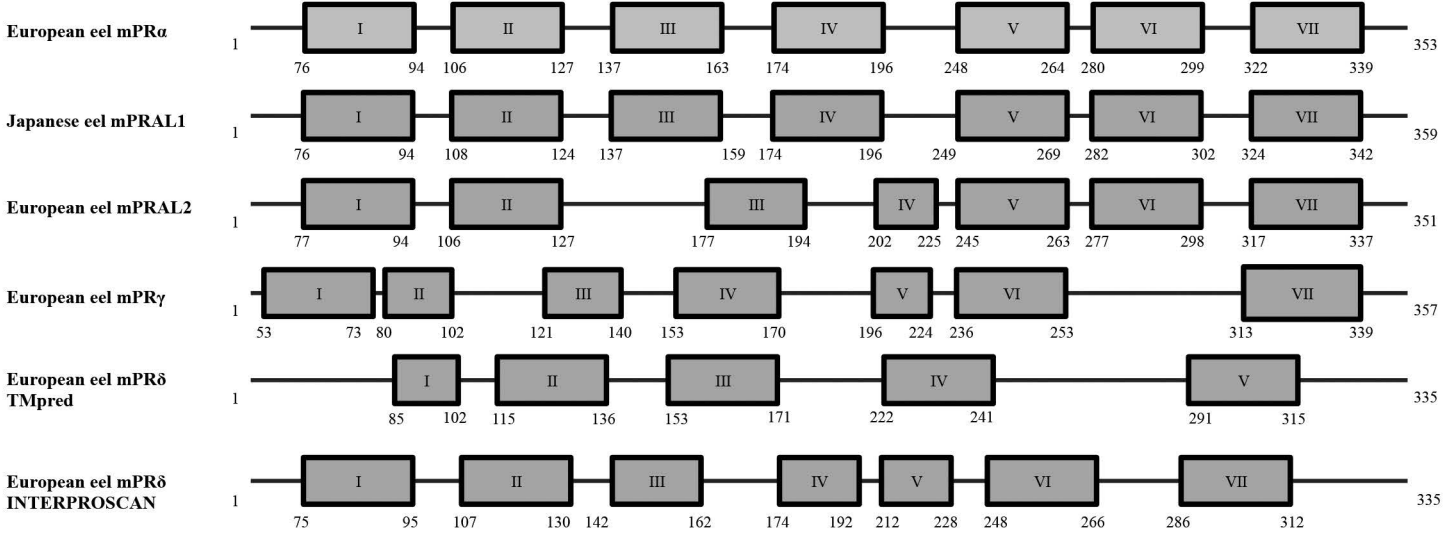
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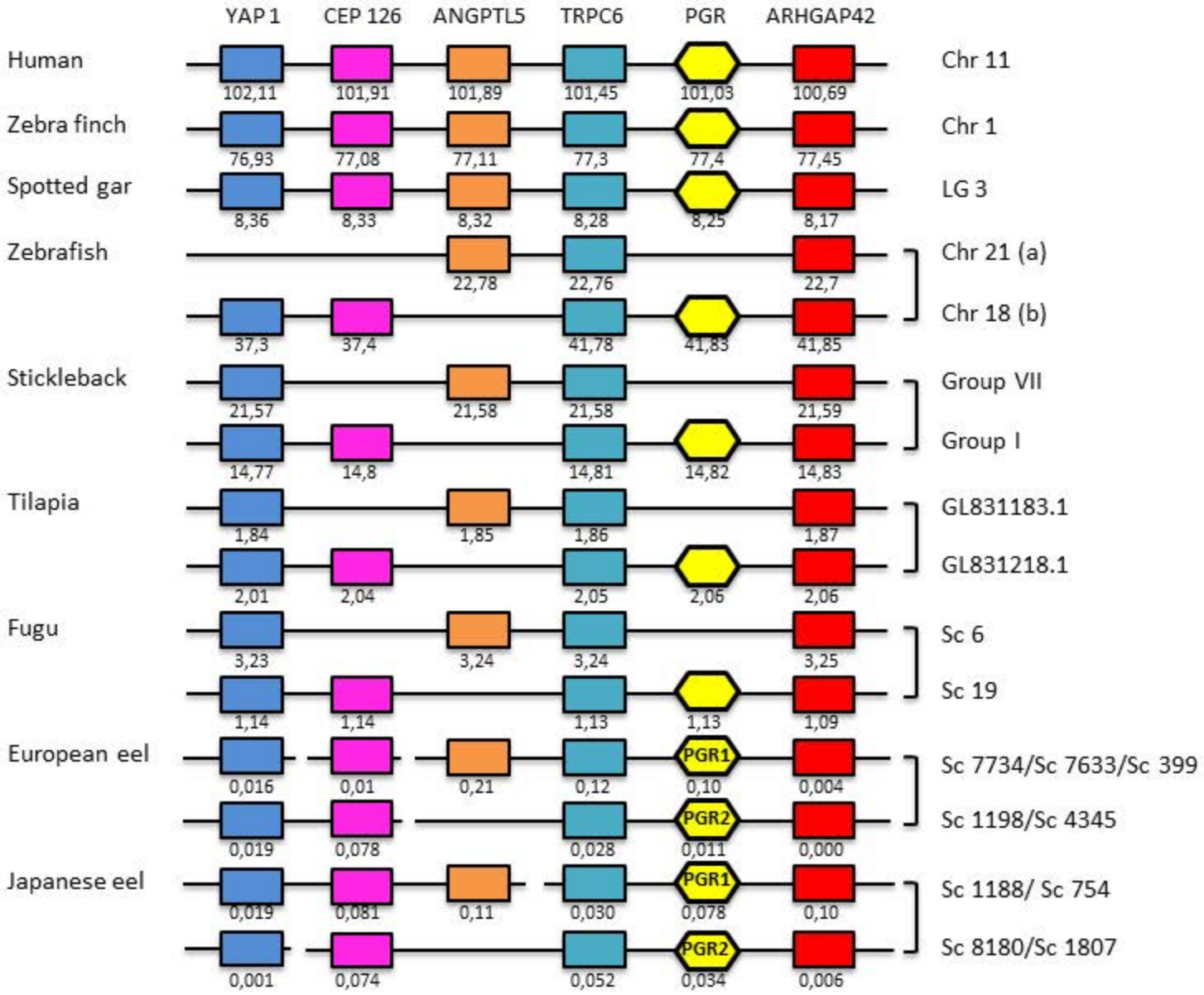
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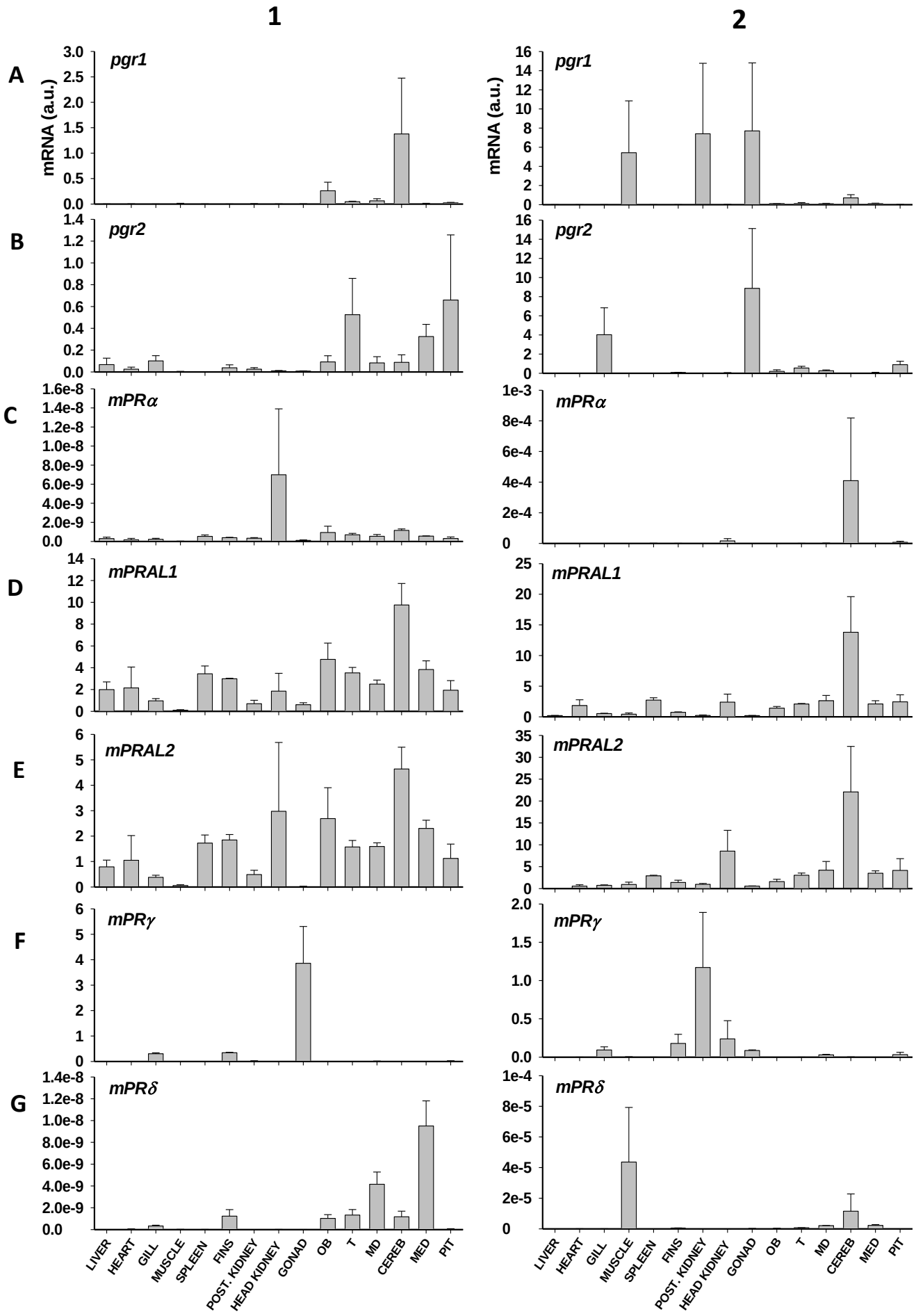
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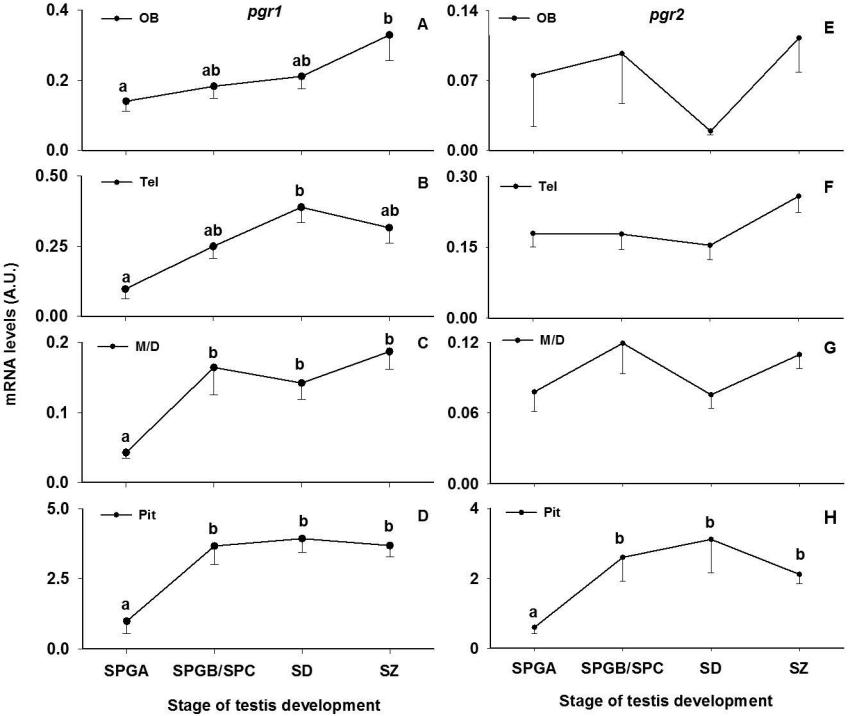
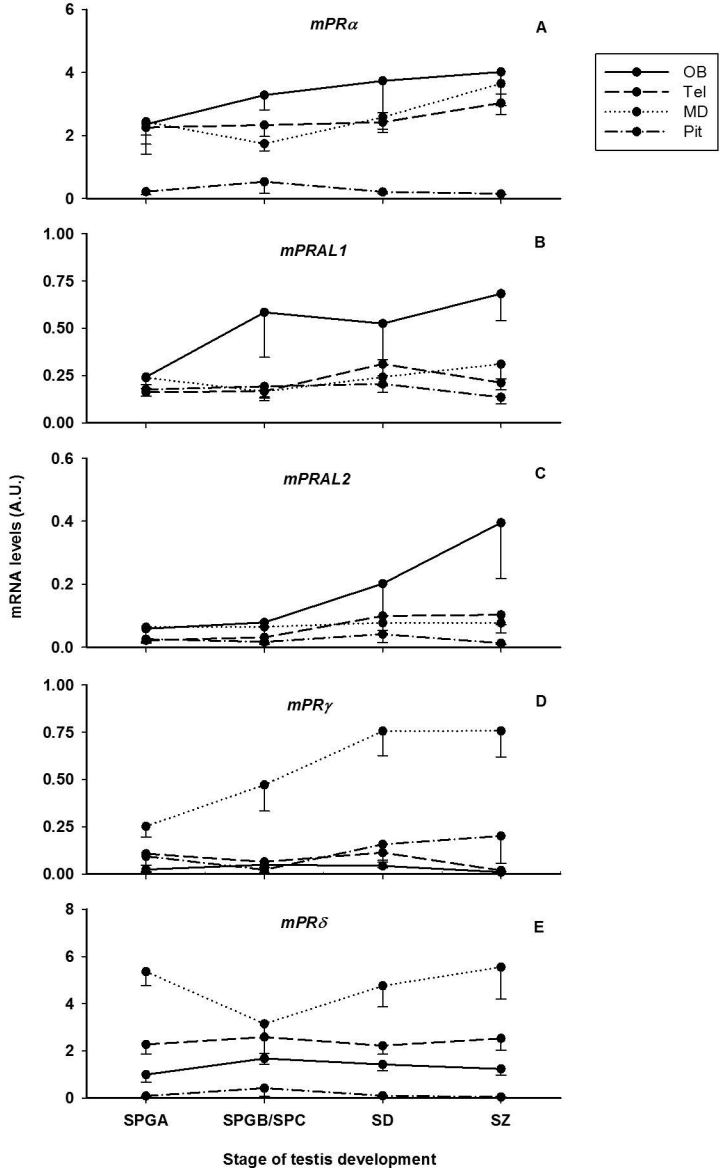
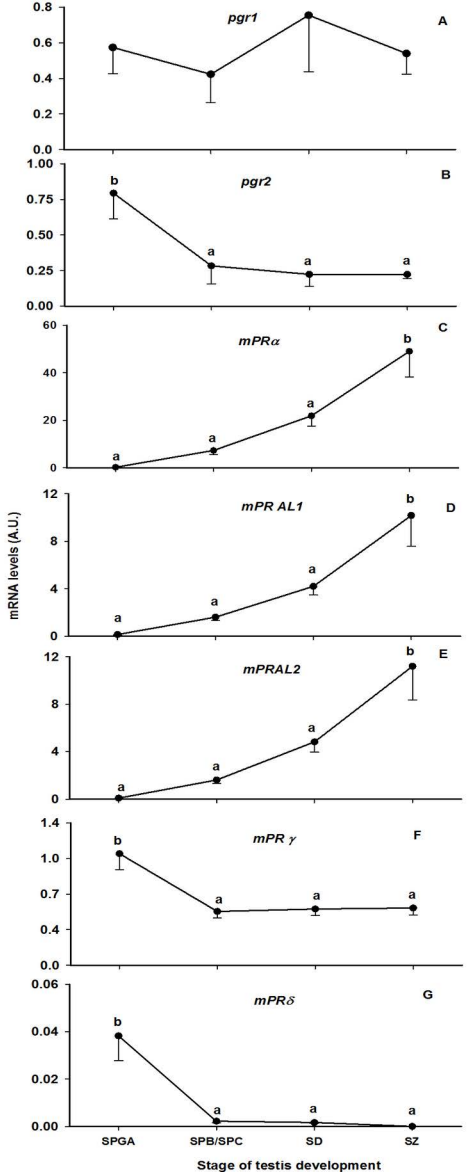
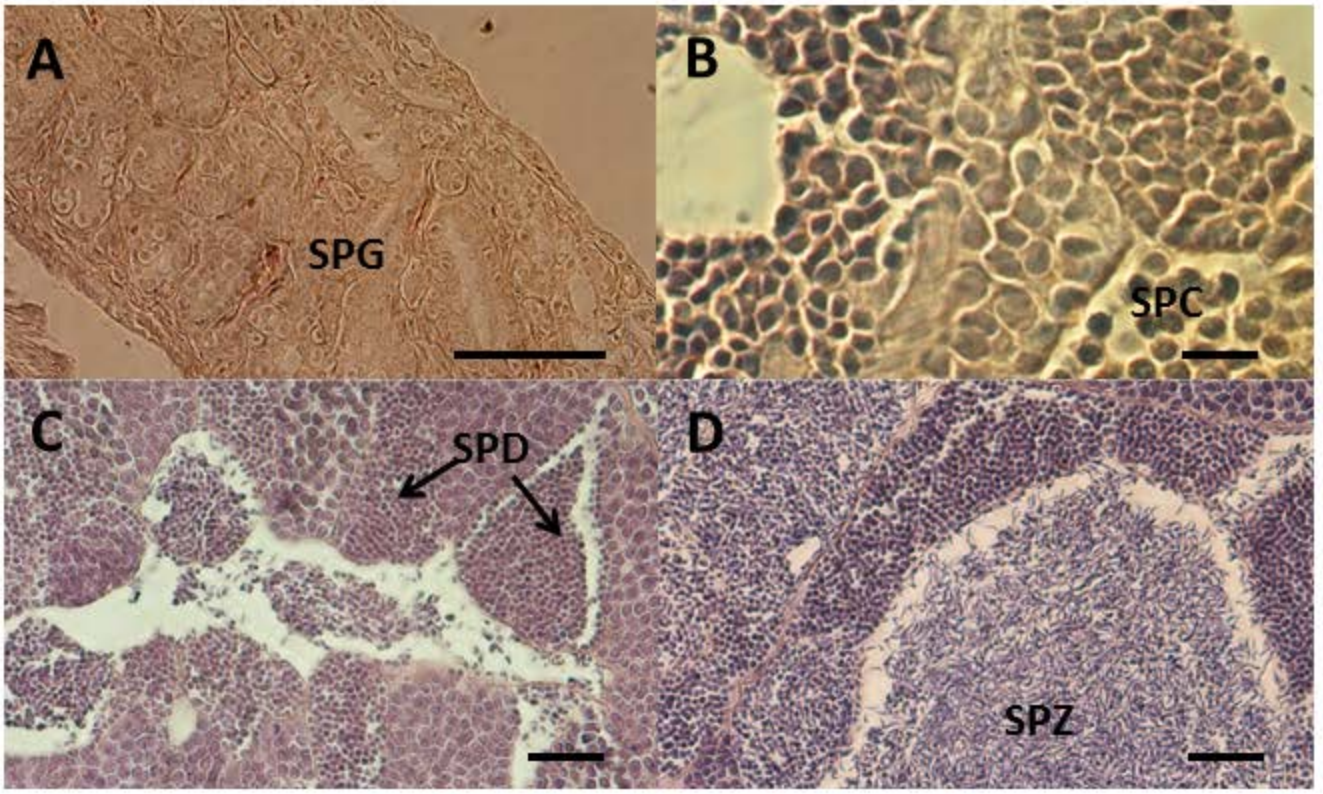
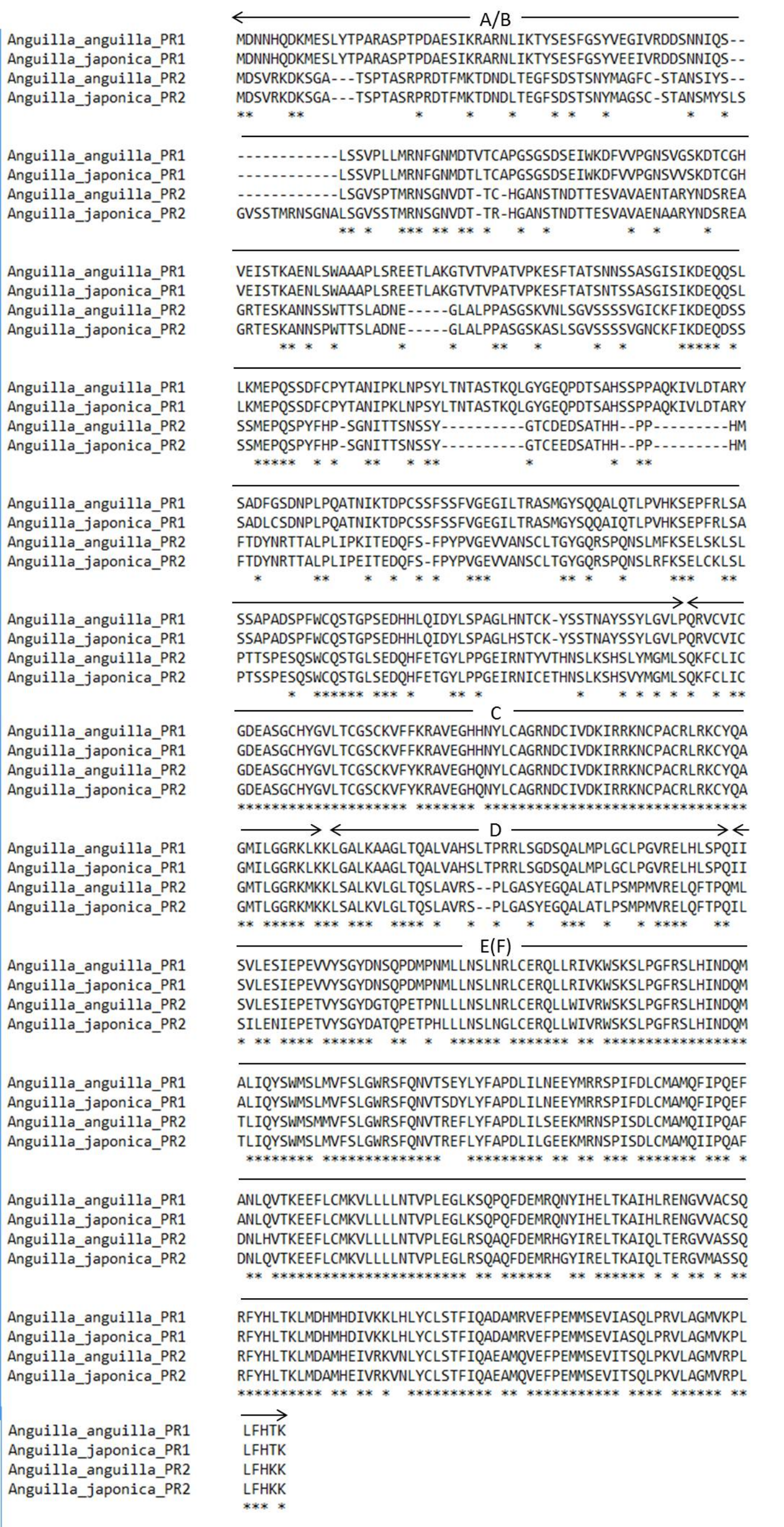
HEAD KIDNEY
GONAD
OB
T
MD
CEREB
MED
PIT


pgr1
pgr2
OB
Tel
M/D
Pit
A
B
C
D
E
F
G
H
a
ab
b
0.4
0.2
0.0
0.14
0.07
0.50
0.25
0.30
0.15
0.12
0.06
5.0
2.5
4
2
SPGA
SPGB/SPC
SD
SZ
mRNA levels (A.U.)
Stage of testis development


mPRα
A
mPRAL1
B
mPRAL2
C
mPRγ
D
mPRδ
E
OB
Tel
MD
Pit
mRNA levels (A.U.)
SPGA
SPGB/SPC
SD
SZ
Stage of testis development


pgr1
A
0.8
0.6
0.4
0.2
0.0
pgr2
B
1.00
0.75
0.50
0.25
0.00
b
a
a
a
mPRα
C
60
40
20
0
b
a
a
a
mPR AL1
D
12
8
4
0
b
a
a
a
mPRAL2
E
12
8
4
0
b
a
a
a
mPR γ
F
1.4
1.0
0.7
0.4
0.0
b
a
a
a
mPRδ
G
0.06
0.04
0.02
0.00
b
a
a
a
mRNA levels (A.U.)
SPGA
SPB/SPC
SD
SZ
Stage of testis development


A
SPG
B
SPC
C
SPD
D
SPZ


A/B
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Anguilla_japonica_PR1 MDNNHQDKMESLYTPARASPTPDAESIKRARNLIKTYSESFGSYVEEIVRDDSNNIQS--
Anguilla_anguilla_PR2 MDSVRKDKSGA---TSPTASRPRDTFMKTDNDLTEGFSDSTSNYMAGFC-STANSIYS--
Anguilla_japonica_PR2 MDSVRKDKSGA---TSPTASRPRDTFMKTDNDLTEGFSDSTSNYMAGSC-STANSMYSLS
Anguilla_anguilla_PR1 ----------LSSVPLLMRNFGNMDTVTCAPGSGSDSEIWKDFVVPGNSVGSKDTCGH
Anguilla_japonica_PR1 ----------LSSVPLLMRNFGNMDTLTCAPGSGSDSEIWKDFVVPGNSVVSKDTCGH
Anguilla_anguilla_PR2 ----------LSGVSPTMRNSGNVDT-TC-HGANSTNDTTESVAVAENTARYNDSREA
Anguilla_japonica_PR2 GVSSTMRNSGNALSGVSSTMRNSGNVDT-TR-HGANSTNDTTESVAVAENAARYNDSREA
Anguilla_anguilla_PR1 VEISTKAENLSWAAAPLSREETLAKGTVTVPATVPKESFTATSNNSSASGISIKDEQQSL
Anguilla_japonica_PR1 VEISTKAENLSWAAAPLSREETLAKGTVTVPATVPKESFTATSNTSSASGISIKDEQQSL
Anguilla_anguilla_PR2 GRTESKANNSSWTTSLADNE-----GLALPPASGSKVNLSGVSSSSVGICKFIKDEQDSS
Anguilla_japonica_PR2 GRTESKANNSPWTTSLADNE-----GLALPPASGSKASLSGVSSSSVGNCKFIKDEQDSS
Anguilla_anguilla_PR1 LKMEPQSSDFCPYTANIPKLNPSYLTNTASTKQLGYGEQPDTSAHSSPPAQKIVLDTARY
Anguilla_japonica_PR1 LKMEPQSSDFCPYTANIPKLNPSYLTNTASTKQLGYGEQPDTSAHSSPPAQKIVLDTARY
Anguilla_anguilla_PR2 SSMEPQSPYFHP-SGNITTSNSSY----------GTCDEDSATHH--PP---------HM
Anguilla_japonica_PR2 SSMEPQSPYFHP-SGNITTSNSSY----------GTCEEDSATHH--PP---------HM
Anguilla_anguilla_PR1 SADFGSDNPLPQATNIKTDPCSSFSSFVGEGILTRASMGYSQQALQTLPVHKSEPFRLSA
Anguilla_japonica_PR1 SADLCSDNPLPQATNIKTDPCSSFSSFVGEGILTRASMGYSQQAIQTLPVHKSEPFRLSA
Anguilla_anguilla_PR2 FTDYNRTTALPLIPKITEDQFS-FPYPVGEVVANSCLTGYGQRSPQNSLMFKSELSKLSL
Anguilla_japonica_PR2 FTDYNRTTALPLIPEITEDQFS-FPYPVGEVVANSCLTGYGQRSPQNSLRFKSELCKLSL
Anguilla_anguilla_PR1 SSAPADSPFWCQSTGPSEDHHLQIDYLSPAGLHNTCK-YSSTNAYSSYLGVLPQRVCVIC
Anguilla_japonica_PR1 SSAPADSPFWCQSTGPSEDHHLQIDYLSPAGLHSTCK-YSSTNAYSSYLGVLPQRVCVIC
Anguilla_anguilla_PR2 PTTSPESQSWCQSTGLSEDQHFETGYLPPGEIRNTYVTHNSLKSHSLYMGMLSQKFCLIC
Anguilla_japonica_PR2 PTSSPESQSWCQSTGLSEDQHFETGYLPPGEIRNICETHNSLKSHSVYMGMLSQKFCLIC
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Anguilla_anguilla_PR1 GDEASGCHYGVLTCGSCKVFFKRAVEGHHNYLCAGRNDCIVDKIRRKNCPACRLRKCYQA
Anguilla_japonica_PR1 GDEASGCHYGVLTCGSCKVFFKRAVEGHHNYLCAGRNDCIVDKIRRKNCPACRLRKCYQA
Anguilla_anguilla_PR2 GDEASGCHYGVLTCGSCKVFYKRAVEGHQNYLCAGRNDCIVDKIRRKNCPACRLRKCYQA
Anguilla_japonica_PR2 GDEASGCHYGVLTCGSCKVFYKRAVEGHQNYLCAGRNDCIVDKIRRKNCPACRLRKCYQA
D
Anguilla_anguilla_PR1 GMILGGRKLKKLGALKAAGLTQALVAHSLTPRRLSGDSQALMPLGCLPGVRELHLSPQII
Anguilla_japonica_PR1 GMILGGRKLKKLGALKAAGLTQALVAHSLTPRRLSGDSQALMPLGCLPGVRELHLSPQII
Anguilla_anguilla_PR2 GMTLGGRKMKKLSALKVLGLTQSLAVRS--PLGASYEGQALATLPSMPMVRELQFTPQML
Anguilla_japonica_PR2 GMTLGGRKMKKLSALKVLGLTQSLAVRS--PLGASYEGQALATLPSMPMVRELQFTPQIL
E(F)
Anguilla_anguilla_PR1 SVLESIEPEVVYSGYDNSQPDMPNMLLNSLNRLCERQLLRIVKWSKSLPGFRSLHINDQM
Anguilla_japonica_PR1 SVLESIEPEVVYSGYDNSQPDMPNMLLNSLNRLCERQLLRIVKWSKSLPGFRSLHINDQM
Anguilla_anguilla_PR2 SVLESIEPETVYSGYDGTQPETPNLLLNSLNRLCERQLLWIVRWSKSLPGFRSLHINDQM
Anguilla_japonica_PR2 SILENIEPETVYSGYDATQPETPHLLLNSLNGLCERQLLWIVRWSKSLPGFRSLHINDQM
Anguilla_anguilla_PR1 ALIQYSWMSLMVFSLGWRSFQNVTSEYLYFAPDLILNEEYMRRSPIFDLCMAMQFIPQEF
Anguilla_japonica_PR1 ALIQYSWMSLMVFSLGWRSFQNVTSDYLYFAPDLILNEEYMRRSPIFDLCMAMQFIPQEF
Anguilla_anguilla_PR2 TLIQYSWMSMMVFSLGWRSFQNVTREFLYFAPDLILSEEKMRNSPISDLCMAMQIIPQAF
Anguilla_japonica_PR2 TLIQYSWMSLMVFSLGWRSFQNVTREFLYFAPDLILGEEKMRNSPISDLCMAMQIIPQAF
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Anguilla_japonica_PR1 ANLQVTKEEFLCMKVLLLLNTVPLEGLKSQPQFDEMRQNYIHELTKAIHLRENGVVACSQ
Anguilla_anguilla_PR2 DNLHVTKEEFLCMKVLLLLNTVPLEGLRSQAQFDEMRHGYIRELTKAIQLTERGVVASSQ
Anguilla_japonica_PR2 DNLQVTKEEFLCMKVLLLLNTVPLEGLRSQAQFDEMRHGYIRELTKAIQLTERGVMASSQ
Anguilla_anguilla_PR1 RFYHLTKLMDHMHDIVKKLHLYCLSTFIQADAMRVEFPEMMSEVIASQLPRVLAGMVKPL
Anguilla_japonica_PR1 RFYHLTKLMDHMHDIVKKLHLYCLSTFIQADAMRVEFPEMMSEVIASQLPRVLAGMVKPL
Anguilla_anguilla_PR2 RFYHLTKLMDAMHEIVRKVNLYCLSTFIQAEAMQVEFPEMMSEVITSQLPKVLAGMVRPL
Anguilla_japonica_PR2 RFYHLTKLMDAMHEIVRKVNLYCLSTFIQAEAMQVEFPEMMSEVITSQLPKVLAGMVRPL
Anguilla_anguilla_PR1 LFHTK
Anguilla_japonica_PR1 LFHTK
Anguilla_anguilla_PR2 LFHKK
Anguilla_japonica_PR2 LFHKK

```
                                    1
Anguilla_anguilla_mPRalpha          MATVVMEQIG RLFINVQQLR QIPRLLETAF PTLPCTVKAA DVPWVFREPH
Anguilla_japonica_mPRalpha          MATVVMEQIG RLFINVQQLR QIPRLLETAF PTLPCTVKAA DVPWVFREPH
Anguilla_japonica_mPRalpha_like_1   MATIVMERIG RLFISLQQVR QVPRMLTEAA PSAPGTLRDS EVPRFFREHH
Anguilla_anguilla_mPRalpha_like_1   ---------- ---------- ---------- ---------- ----------
Anguilla_anguilla_mPRalpha_like_2   MATIVMERLG HLFINLQQVR QVAQVLREAV PSIPGTLRAS EVPAFFREPY
Anguilla_japonica_mPRalpha_like_2   MATIVMERLG HLFINLQEVR QVAQVLREAV PSIPGTLRAS EVPVFFREPY
Anguilla_anguilla_mPRgamma          ---------- ---------- ------MLSL IKLPRVFTIN QVPKVFHEDG
Anguilla_japonica_mPRgamma          ---------- ---------- ------MLSL IKLPRVFTIN QVPKVFHEDG
Anguilla_anguilla_mPRdelta          ---------- ---------- ---------- ------MPCY SVLKVFQEDG
Anguilla_anguilla_mPRdelta          ---------- ---------- ---------- ------MPCY SVLKVFQEDG

                                   51
Anguilla_anguilla_mPRalpha          ILAGYRPPDQ SWRYYGLTLF QRHNEAVNVW THLLAALVIL VKFRQLSETV
Anguilla_japonica_mPRalpha          ILAGYRPPDQ SWRYYGLTLF QRHNEAVNVW THLLAALVIL VKFRQLSETV
Anguilla_japonica_mPRalpha_like_1   IHGGYRPLGR PWRYYFLSLF RRHNETVNVW THLLGALLVL LRAGRLAETV
Anguilla_anguilla_mPRalpha_like_1   -------LGR PWRYYVLSLF RRHNETVNVW THLLGALLVL QRAGRLAETV
Anguilla_anguilla_mPRalpha_like_2   IHSGYRAPDL AWRYYFLSLF QRHNETVNVW THLLGALLIL ATSLRLAETV
Anguilla_japonica_mPRalpha_like_2   IHNGYRAPDL AWRYYFLSLF QRHNETVNVW THLLGALFIL ATSLRLAETV
Anguilla_anguilla_mPRgamma          IISGYRHPCS SATDCVLSLF QLTNETLNIW THFLPTWYFL YKLLTVVLMQ
Anguilla_japonica_mPRgamma          IISGYRHPCS SATDCVLSLF QLTNETLNIW THFLPTWYFL YKLLTVVLMQ
Anguilla_anguilla_mPRdelta          IISGYRHPHS SALDCILSSF QMTNETVNIW THFLPTWYFL WRFSVLCSSL
Anguilla_japonica_mPRdelta          IISGYRHPHS SALDCILSSF QMTNETVNIW THFLPTWYFL WRFSVLCSSL

                                  101
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Anguilla_japonica_mPRalpha          DFLRDAHALP LFIVLLSAFT YLSCSAAAHL LSAKSELSHY TFFFLDYVGV
Anguilla_japonica_mPRalpha_like_1   DFGGDPHAWP LLVLLLSSLA YMSFSAVAHL LAARSEFCHF AFFFLDYVGV
Anguilla_anguilla_mPRalpha_like_1   DFAGDPHAWP LLVLLLSSLA YMSFSAVAHL LAAKSEFCHF AFFFLDYVGV
Anguilla_anguilla_mPRalpha_like_2   DFAGDAHAWP LLLLLGSGLT YMLFSVAAHL LSARSPLHHH ALYFLDYTGV
Anguilla_japonica_mPRalpha_like_2   DFAGDAHAWP LLLLLGSALT YMLFSVAAHL LSARSPLHHH AFYFLDYSGV
Anguilla_anguilla_mPRgamma          DAWRDVFTWP LLVFLVSACM YPLASSCAHT FSTMSARARH VCFFFDYGAL
Anguilla_japonica_mPRgamma          DAWRDVFTWP LLVFLVSACM YPLASSCAHT FSTMSARARH VCFFFDYGAL
Anguilla_anguilla_mPRdelta          DFLSESYTWP LLVYMLLICL YPFTSSCAHI FSTMSAESRH ICYFFDYGAL
Anguilla_anguilla_mPRdelta          DFLSESYTWP LLVYMLLICL YPFTSSCAHI FSTMSAESRH ICYFFDYGAL

                                  151
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Anguilla_japonica_mPRalpha          AVYQYGSALA HYYYAIEEDW HARVRGCFLP AAAFLAWLSC A-GCCYGKLE
Anguilla_japonica_mPRalpha_like_1   AQYQYGSAVA HFYYAAEEGW HRRVRGVFMP AAALLCCLSC L-GCCYGKYR
Anguilla_anguilla_mPRalpha_like_1   AQYQYGSAVA HFYYAAEEGW HRRVRGVFMP AAALLCCLSC L-GCCYGKYR
Anguilla_anguilla_mPRalpha_like_2   ALYQYASAAV HFHYAAEPGW RGGAQGASLA LAALLSLVFC L-GGCCTKLG
Anguilla_japonica_mPRalpha_like_2   ALYQYASAAV HFYYAAEPGW RGGAQGASLV LAALLSLVFC L-GSCCTKLG
Anguilla_anguilla_mPRgamma          SFYSLGSAIT YSAYVFPDKW VNSTFHLYYI PIAVFNTIIC TALACYSRLG
Anguilla_japonica_mPRgamma          SFYSLGSAIT YSAYVIPDKW VNSTFHLYYI PIAVLNTIIC TALACYSRLG
Anguilla_anguilla_mPRdelta          SLYSLGCAIS YGSYVIPDCW VNTWLHRNFV VIAISNTLFC TSLSCYSRF-
Anguilla_anguilla_mPRdelta          SLYSLGCAIS YGSYVIPDCW VNTWLHRNFV VIAISNTLFC TSLSCYSRF-

                                  201
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Anguilla_japonica_mPRalpha          SRRL------ --------PK AAHKLFQVVP SGLAYCLDIS PVLHRIHGCS
Anguilla_japonica_mPRalpha_like_1   NHSL------ --------PP WVRKVGQVAP SSLAYAWDTS PVFHRVLSRG
Anguilla_anguilla_mPRalpha_like_1   NHSL------ --------PP WVRKVGQVAP SSLAYAWDTS PVFHRVLSRG
Anguilla_anguilla_mPRalpha_like_2   ---G------ --------PL WARGVWQLLP CALAYAWDSA PIFHRLSTCL
Anguilla_japonica_mPRalpha_like_2   ---G------ --------PL WVRGVWQLLP CALAYAWDSA PIFHRLSTCL
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Anguilla_japonica_mPRgamma          LPFLQYNHDT IKRFPECQTP KYGRSLRVLA FAYPYLFDNI PVFYRIFVCA
Anguilla_anguilla_mPRdelta          ---------- ----LELQFP CKSKVLRTAA FVYPFIFDNI PLFHRLLLCC
Anguilla_anguilla_mPRdelta          ---------- ----LELQFP CKSKVLRTAA FVYPFIFDNI PLFHRLLLCC

                                  251
Anguilla_anguilla_mPRalpha          L---RGCADP AVDYHRCQVL FFLVSAYFFA FPHPERWFPG RCDFIGQGHQ
Anguilla_japonica_mPRalpha          L---RGCADP AVDYHRCQVL FFLVSAYFFA FPHPERWFPG RCDFIGQGHQ
Anguilla_japonica_mPRalpha_like_1   LGPGGGGGDP ALPFHCGQVA FFLSSALFFT QPVPERWLPG RCDFLGQGHQ
Anguilla_anguilla_mPRalpha_like_1   LGLGA-GGDP ALPFHCGQVA FFLSSALFFT QPVPERWLPG RCDFLGQGHQ
Anguilla_anguilla_mPRalpha_like_2   P---TCADDE AGRYHGAQVA LFLLSAVFFT WPVPERWFPG RCDLLPQGHQ
Anguilla_japonica_mPRalpha_like_2   P---TCADDE AGRYHGAQVV FFLLSAVFFT WPMPERWFPG RCDLLPQGHQ
Anguilla_anguilla_mPRgamma          G--EGCTDNG TNTLHYWHTS LAFLTGFLFA THLPERLAPG CFDYFGHSHQ
Anguilla_japonica_mPRgamma          G--EGCTDNG TNTLHYWHTS LAFLTGFLFA THLPERLAPG CFDYFGHSHQ
Anguilla_anguilla_mPRdelta          G--GSCSHNE ALPSYHYHLM FAFLTCFLYT SHLPERLAPG RFDYIGHSHQ
Anguilla_anguilla_mPRdelta          G--GSCSHNE ALPSYHYHLM FAFLTCFLYT SHLPERLAPG RFDYIGHSHQ

                                  301
Anguilla_anguilla_mPRalpha          VFHVFLVLCT LVQIEAVRLD YGTRRALYQR LHGDLAHDS- VALFVFTA-C
Anguilla_japonica_mPRalpha          VFHVFLVLCT LVQIEAVRLD YGTRRALYQR LHGDLAHDS- VALFVFTA-C
Anguilla_japonica_mPRalpha_like_1   LFHVLLVLCT LCQIHASHLD YLGRRPLYSR LHGEGDARFF LALFAATGLA
Anguilla_anguilla_mPRalpha_like_1   LFHALLVLCT LCQIHASHLD YLGRRPLYSR LHGEGDARFF LALFAATGLA
Anguilla_anguilla_mPRalpha_like_2   VFHVLVVLCT FSQIRASHLD YLQRRALYAP A-GPQAPRLL LGLFAALALS
Anguilla_japonica_mPRalpha_like_2   VFHVLVVLCT FCQIRASHLD YLQRRALYAP A-GPQAPRLL IGLFAALALS
Anguilla_anguilla_mPRgamma          LFHVCGIIGT LFQMQAIEMD MTLRRQWLLV HAP---PITF ANTIGAGLLC
Anguilla_japonica_mPRgamma          LFHVCGIIGT LFQMQAIEMD MTLRRQWLLV HAP---PITF ANTIGAGLLC
Anguilla_anguilla_mPRdelta          LFHICAVVGT HFQMEALLAD MTSRRGWMMT HSA---IPSF LGSVGALALG
Anguilla_anguilla_mPRdelta          LFHICAVVGT HFQMEALLAD MTSRRGWMMT HSA---IPSF LGSVGALALG

                                  351
Anguilla_anguilla_mPRalpha          CSA------- -LTAFYMRQR VRALLRDKEE ----------
Anguilla_japonica_mPRalpha          CSA------- -LTAFYMRQR VRALLRDKEE ----------
Anguilla_japonica_mPRalpha_like_1   CAA------- -I-AAFMAGK VRRLLDRRDR SK--------
Anguilla_anguilla_mPRalpha_like_1   CAA------- -I-AAFMAGK VRRLLDRRDR SK--------
Anguilla_anguilla_mPRalpha_like_2   CAI------- -T-TILMTRR AQHLIGQKHK ----------
Anguilla_japonica_mPRalpha_like_2   CAI------- -T-AILMTRR AQHLIGQKHK RS--------
Anguilla_anguilla_mPRgamma          VLFSLCVIYT FSLPLYYALA KRGKRNRR-- PKTPARASN-
Anguilla_japonica_mPRgamma          VLFSLCVIYT FSLPLYCAPA KRGKRNRR-- HKTPARASN-
Anguilla_anguilla_mPRdelta          LLLNMGLIGF FSATLLWAPQ HTALQHSTNN QQCPIDCKDK
Anguilla_japonica_mPRdelta          LLLNMGLIGF FSATLLWAPQ HTALQHSTNN QQCPIDCKDK
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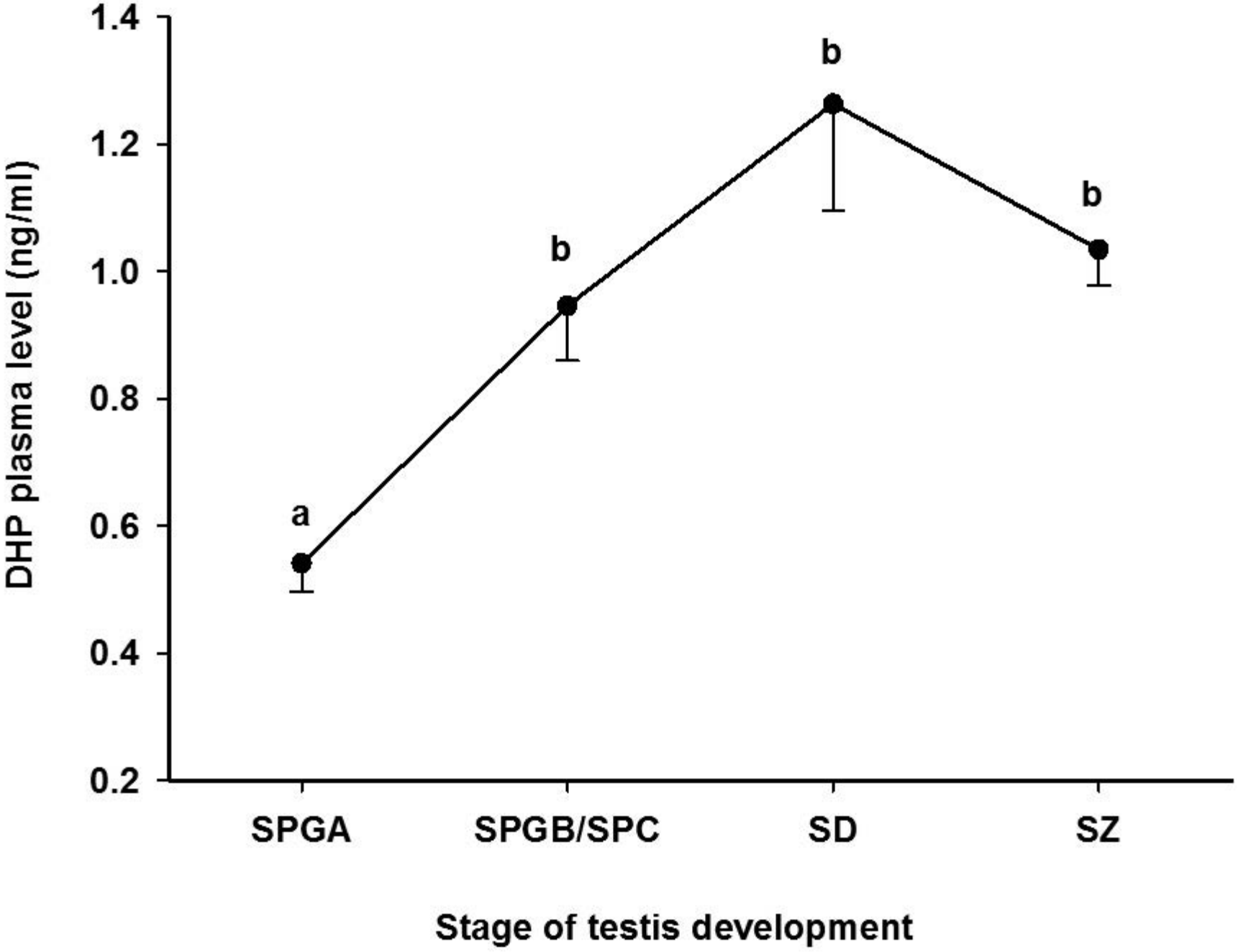
1.4
1.2
1.0
0.8
0.6
0.4
0.2
DHP plasma level (ng/ml)
a
b
b
b
SPGA
SPGB/SPC
SD
SZ
Stage of testis development