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Duplicated membrane estrogen receptors in the European sea bass (*Dicentrarchus labrax*): phylogeny, expression and regulation throughout the reproductive cycle

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Abstract

The numerous estrogen functions reported across vertebrates have been classically explained by their binding to specific transcription factors, the nuclear estrogen receptors (ERs). Rapid non-genomic estrogenic responses have also been recently identified in vertebrates including fish, which can be mediated by membrane receptors such as the G protein-coupled estrogen receptor (Gper). In this study, two genes for Gper, namely *gpera* and *gperb*, were identified in the genome of a teleost fish, the European sea bass. Phylogenetic analysis indicated they were most likely retained after the 3R teleost-specific whole genome duplication and raises questions about their function in male and female sea bass. *Gpera* expression was mainly restricted to brain and pituitary in both sexes while *gperb* had a widespread tissue distribution with higher expression levels in gill filaments, kidney and head kidney. Both receptors were detected in the hypothalamus and pituitary of both sexes and significant changes in *gpers* expression were observed throughout the annual reproductive season. In female pituitaries, *gpera* showed an overall increase in expression throughout the reproductive season while *gperb* levels remained constant. In the hypothalamus, *gpera* had a higher expression during vitellogenesis and decreased in fish entering the ovary maturation and ovulation stage, while *gperb* expression increased at the final atresia stage. In males, *gpers* expression was constant in the hypothalamus and pituitary throughout the reproductive cycle apart from the mid- to late testicular development stage when a significant up-regulation of *gpera* occurred in the pituitary. The differential sex, seasonal and subtype-specific expression patterns detected for the two novel *gper* genes in sea bass suggests they may have acquired different and/or complementary roles in mediating estrogens actions in fish, namely on the neuroendocrine control of reproduction.

Keywords: brain, evolution, G protein-coupled estrogen receptors, pituitary, reproduction, teleost

receptor; Fsh, follicle stimulating hormone; GPCRs, G-protein coupled receptors; Gper, G protein-coupled estrogen receptor; Gnrh, gonadotropin-releasing hormone; GSI, gonadosomatic index; HPG axis, Hypothalamus-Pituitary-Gonads axis; Lh, luteinizing hormone; qPCR, quantitative polymerase chain reaction. Protein and gene nomenclature followed that recommended by genenames.org and used for fish at http://zfin.org/; in this abbreviation list, for each case protein abbreviation is presented first followed by the corresponding gene abbreviation.

1. Introduction

Estrogens are a group of sex steroid hormones that are essential for reproductive functions in both female and male vertebrates. They are involved in the regulation of gonadotropin levels, spermatogenesis, oogenesis or vitellogenesis (in oviparous animals) and are in part responsible for the development of female secondary sex characteristics [1]. Estrogens are mainly produced in the ovaries of active females and may feedback on the hypothalamus and pituitary that contain a high density of nuclear estrogen receptors (Esrs), regulating the expression and release of gonatropin-releasing hormone (Gnrh) and gonadotropins Fsh (follicle-stimulating hormone) and luteinizing hormone (Lh) [1, 2]. Non-reproductive functions regulated by estrogens include cognitive and cardiovascular functions and skeletal homeostasis [3].

In the classical model of action, estrogens diffuse through the cell membrane and interact with specific intracellular nuclear receptors, two of which, Esr1 and Esr2, exist in terrestrial vertebrates while in fish three forms, one Esr1α and two Esr2s (expressed from duplicate genes) have been detected [4]. The resulting ligand-receptor complex binds to specific response elements in the promoter regions of target genes and regulates their transcription, a process that can take hours or days to be completed [5, 6]. Several alternative mechanisms of estrogen action have been described including indirect genomic effects through interaction with other transcription factors or rapid non-genomic effects initiated by binding to membrane receptors [5, 7] that can produce effects within minutes [8]. No consensus exists about the identity of the membrane receptors mediating non-genomic effects of estrogens and they have been attributed to membrane sub-populations of nuclear estrogen receptors or to novel membrane receptors such as the G protein-coupled estrogen receptor, Gper (formerly known as Gpr30). The multitude of signaling responses evoked by estrogens makes it likely that both nuclear and membrane bound receptor types contribute to the complexity of cellular mechanisms of estrogenic action [3, 7, 8]. The involvement of Gper in mediating estrogens actions was first proposed by Filardo et al. [9] when rapid estrogen activation of the second messenger cyclic AMP (cAMP) and of mitogen-activated

protein kinases (Erk1/2) was observed in a human breast cancer cell line that lacks Esrs. In 2005, Peter Thomas's and Prossnitz's groups independently demonstrated that human Gper displays high affinity estrogen binding and has the binding characteristics of a membrane estrogen receptor [10, 11]. Since then numerous studies have characterized Gper signaling, tissue expression and functions from fish to mammals [8, 12, 13], many by using a selective Gper agonist (G-1) that does not activate ERs or by using gene knockdown technologies.

In mammals, Gper is expressed in a variety of tissues and it appears to mediate estrogens protective effects on the nervous, immune and cardiovascular system and to be involved in the regulation of glucose, lipid and mineral metabolism [reviewed in 8, 13]. In the mammalian reproductive system, Gper has been associated with 17β-estradiol (E2) regulation of primary follicle formation and uterine proliferation and contraction in females and to proliferative or apoptotic pathways during spermatogenesis in males, and it is also implicated in endometrial, ovarian, breast, prostate and testicular cancers [8, 13].

In fish, Gper expression has been reported in brain, testis and in the ovary and a clear role in mediating E2 effects on meiotic arrest and inhibition of oocyte maturation has been established [14, 15]. We recently reported the existence of two Gper forms encoded by different genes in the European sea bass [16] and showed in liver and scales different patterns of tissue expression and regulation by E2 and the phytoestrogen genistein. The novel membrane receptor identified (designated *gperl*) was the preponderant estrogen receptor, together with the nuclear *esr2* form, expressed in sea bass and Mozambique tilapia scales [17]. This study suggested Gperl most likely mediates the rapid effects of E2 and estrogenic pollutants that were detected on scale enzyme activities related to mineral turnover. In the European eel, a basal teleost, two Gper forms have also been isolated that have a different tissue distribution and regulation in response to experimentally-induced maturation in both sexes [18, 19].

In the present study, we report the isolation of full-length cDNAs encoding the two sea bass Gpers, their phylogeny and their tissue distribution across adult male and female tissues, to give insight into

the evolution and function of two Gper forms in teleost fish. To test the hypothesis that they are involved in mediating the action of estrogens on reproduction via the hypothalamus - pituitary unit, we investigated the patterns of expression of the two *gpers* in male and female sea bass across their annual reproductive cycle.

2. Methods

2.1. Animals and sampling

The European sea bass used for cloning and evaluating the tissue distribution of *gpers* were obtained from local fish farms and maintained at Ramalhete Marine Station (Faro, Portugal) in 500-1000 L flow-through seawater tanks, under natural photoperiod (between 10:14 hours light:dark in winter, and 15:9 in summer) and natural temperatures (between 11ºC in winter and 25ºC in summer). Fish were fed once a day (1% wet fish weight) with commercial food pellets (Sparos, Portugal). Three-years-old sea bass (four adult females of 569-778 g and 36.5-42 cm total length and four adult males of 215-533 g and 28-38 cm) were collected in early October, at the beginning of gametogenesis. Fish were anesthetized with a lethal dose of tricaine methanesulfonate (MS 222, Sigma-Aldrich), washed with clean seawater and then measured and weighed. The following tissues were collected from sacrificed fish and immediately frozen in liquid nitrogen and stored at -80ºC until RNA extraction: eye, pituitary, brain, gonads (ovary and testis), liver, adipose tissue, head kidney, kidney, intestine (mid gut), gill filaments, scales and vertebra. Manipulation of animals was performed in compliance with international and national ethics guidelines for animal care and experimentation (Guidelines of the European Union Council, 86/609/EU). Animal maintenance and experimentation was carried out by certified investigators (DMP and PISP) and in certified experimental facilities, following national legislation of Portugal (DL 113/2013) under a 'group-1' license issued by the Veterinary General Directorate, Ministry of Agriculture, Rural Development and Fisheries of Portugal.

The expression of *gper* transcripts was characterized throughout the annual reproductive cycle in male and female sea bass pituitary and hypothalamus using RNA samples from the study of Alvarado et al., 2013 [20]. Briefly, two-years-old males and three-years-old females were sampled from August to late April, covering their first annual reproductive season from beginning of gametogenesis to rest. Anaesthetized fish were killed, pituitary and brain were collected and then the hypothalamus dissected out. Tissue samples were snap frozen and stored at -80 ºC until RNA extraction. The developmental stage of the gonads was determined by histology, following the classifications previously described for this species [21, 22]. This classification was used to divide collected pituitary and hypothalamus samples into the following developmental stages for male (Stage I, immature stage; Stage II, early recrudescence; Stage III, mid recrudescence; Stage IV, late recrudescence; Stage V, full spermiating testes and Stage VI, rest) and female (PV, previtellogenesis; V, vitellogenesis; M, maturation-ovulation and A, atresia), as described in Alvarado et al. 2013 [20].

2.2. RNA extraction and cDNA synthesis

Total RNA for tissue distribution was extracted from frozen tissues using a Maxwell 16 robot and Maxwell 16 SEV total RNA purification kit (Promega, Southampton, UK). Initially all tissues were subjected to mechanical disruption using an Ultra Turrax homogenizer (IKA, Germany) equipped with a dispersing element S25N–8-G or S25N-8G-ST (for scales). To facilitate RNA extraction vertebra were first pulverized with a hammer before mechanical disruption. Total RNAs from the pituitary and hypothalamus collected during the sea bass annual reproductive cycle were extracted using Maxwell 16 LEV kits, as described in Alvarado et al., 2013 [20]. RNA integrity and purity was analyzed by 1% agarose gel electrophoresis and RNA was quantified in a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA). For both sample panels (tissue distribution or annual cycle), total RNA (1-8 µg, depending on the tissue) was treated with DNase (DNA-free kit, Ambion, UK) and cDNA syntheses were carried out in 20 µl reactions containing 500 ng of DNase-treated RNA, 200 ng of

random hexamers (Jena Biosciences, Germany), 100 U of RevertAid reverse transcriptase and 8 U of Ribo-Lock RNase Inhibitor (Fermentas, Thermo Fisher). Reactions were incubated for 10 min, at 25 ºC, and then 60 min at 42 ºC, followed by enzyme inactivation for 10 min, at 70 ºC, and storage at -20 ºC until use.

2.3. Identification and cloning of sea bass Gpers

Teleost Gper amino acid sequences retrieved from Ensembl (http://www.ensembl.org/index.html) and NCBI (http://www.ncbi.nlm.nih.gov/) databases (Table S1) were used as queries to search the sea bass genome at http://seabass.mpipz.de/ [23], using BLAT [24]. Two *gper* genes were identified, initially designated as *gper* and *gperl* [16, 17] and now renamed *gpera* and *gperb*, based on the phylogenetic tree results (see below). Specific primers were designed to amplify and clone the complete coding regions of *gpera* and *gperb* (Table 1) after retrieving their predicted genomic sequences.

Table 1 - List of primers used in this study to clone the full-length coding region of each sea bass *Gper* and for gene expression analyses by quantitative RT-PCR.

Objective	Gene name abbreviation	Primer name[a]	Primer sequence (5´-3´)	Ta[b]	bp[c]
Cloning	*gpera*	GPERa_F1	ATGGAAGTGCAGACAACCTCTC	58	1065
		GPERa_R1	TCACACCTCTGACACTTCGCTC		
	gperb	GPERb_F1	ATGGAGAATCACTTGTCTGTTAAAC	57	1241
		GPERb_R3	GAAATGGTAGGCATGGATGG		
RT-PCR	*gpera*	GPERa_RT_F1	GCCACCCTTCTCCCTTTCACC	62	157
		GPERa_RT_R1	TTCGCCCAATCAGAGAGTAGCAT		
	gperb	GPERb_RT_F1	ACAGCAGCGTCTTCTTCTTAACC	60	122
		GPERb_RT_R1	AGATGAGGACACCCAGATAAGGCAG		
Reference genes	*ef1α*	Fw	GACACAGAGACTTCATCAAG	58	114
		Rv	GTCCGTTCTTAGAGATACCA		
	18s	Fw	TGACGGAAGGGCACCACCAG	60	158
		Rv	AATCGCTCCACCAACTAAGAACG		

[a] F denote "forward" primers and R denote "reverse" primers. [b] Optimized annealing temperature used for each pair of primers. [c] Amplicon size in base pairs (bp)

While the *gpera* gene (DLAgn_00191960) was predicted to be encoded by a single exon, the *gperb* (DLAgn_00100480) gene prediction indicated it contained an intron at the end of the coding region, and differed from the longer, single-exon transcript predicted in NCBI for this gene (Accession number

FQ310507.3; see Fig. S1). For this reason, only one pair of primers was designed to amplify the *gpera* coding region ("Gpera_F1" and "Gpera_R1") while several pairs of primers were designed to verify the gene structure of *gperb* and clone the full-length cDNA and possible alternative transcripts (Fig. S1). Samples used for gene cloning were tested using sea bass cDNAs from several tissues (testis, gill, ovary, brain, kidney and head kidney) and successful amplification of a full-length *gperb* transcript was achieved with the primers "Gperb_F1" and "Gperb_R3" in adult sea bass testis (Table 1, Fig. S1). To clone the full-length *gper* cDNAs, reverse transcription-polymerase chain reactions (RT-PCR) of 25 µL contained 1 µL of cDNA, 10 pmol of each primer, 50 µM dNTPs and 0.5 U DreamTaq DNA Polymerase (Fermentas) were performed. Cycling conditions were 5 min at 95 ºC followed by 35 cycles of denaturation for 20 s at 95 ºC, 20 s of annealing at the optimized temperature for each primer pair (Table 1) and 1 min of extension at 72 ºC, followed by a final extension of 5 min at 72 ºC. Amplified targets were gel-purified, inserted into pGEM-T Easy (Promega, UK) and their identity was confirmed by sequencing. Positive clones for the two *gpers* were re-sequenced to give at least 3-fold coverage. The final sea bass *gper* cDNA sequences were assembled using the CAP contig assembling program [25]. A proofreading DNA polymerase was used to confirm the full-length *gperb* cDNA gene structure and sequence, which was amplified by RT-PCR in a total volume of 20 µL containing 1 µL of testis cDNA, 10 pmol of each primer "Gperb_F1" and "Gperb_R3", 200 µM of each dNTP and 0.4 U Phusion® Hot Start DNA Polymerase (Finnzymes), in 5x Phusion® HF Buffer. Cycling conditions were 3 min at 98 ºC, 35 cycles of 10 s at 98 ºC, 30 s at 60 ºC and 50 s at 72 ºC, followed by 10 min at 72 ºC.

2.4. Sequence characterization and phylogenetic analysis

The genomic organization of sea bass *gpera* and *gperb* genes was characterized by aligning the cloned full-length cDNA with the genomic sequence using ClustalW v.2.0 [26]. Multiple sequence alignments of deduced amino acid (aa) sequences for sea bass Gper with those of human (*Homo sapiens*), spotted

gar (*Lepisosteus oculatus*), zebrafish (*Danio rerio*) and European eel (*Anguilla anguilla*) (accession numbers presented in the legend of Fig. 1) were carried out using ClustalW and edited using GeneDoc version 2.7.0 [27]. The deduced sea bass Gper protein sequences were analyzed using TMHMM v. 2.0 [28] to predict transmembrane regions.

The deduced aa sequences of the sea bass Gpers were used as queries to search for Gpers in vertebrates, using tBLASTN against publicly available DNA databases (Ensembl for genomes and Genbank for mRNAs and expressed sequence tags) or BLASTP against the protein database in Genbank [29].

The phylogenetic tree was built using the deduced aa sequences between the seven transmembrane domains (7TM) in Gpers, aligned in ClustalW 2.0, and PROTTEST version 2.4 [30] was used to select the model of protein evolution that best fitted the dataset. The final tree was constructed using the maximum likelihood (ML) method in PhyML 3.0 [31] with 100 bootstrap replicates using a JTT substitution model with a discrete gamma distribution of rates among sites with 4 categories. Additional Gper sequences used in phylogenetic analyses included sequences from sarcopterygians (tetrapods and a basal sarcopterygian, an actinistian, the coelacanth, *Latimeria chalumnae*) and actinopterygians (teleosts and a non-teleost actinopterygian, the spotted gar). The Gper sequence of a chondrichthyan (Elephant shark, *Callorhinchus milii*) was used as outgroup.

2.5. Analysis of gpers gene expression

Quantitative real time RT-PCR (qPCR) was carried out to measure transcript abundance of *gpers* in several tissues and in the hypothalamus and pituitary collected from several time points during the annual reproductive cycle. The relative standard curve method and EvaGreen chemistry were used, as previously described [32]. Reactions were performed in duplicate and contained 1x Sso Fast EvaGreen Supermix (Bio-Rad), 300 nM of each specific primer (Table 1) and 2 µL of each cDNA (diluted 1:5) in a final volume of 15 µL. Initially, to determine the relative abundance of *gpera* and *gperb* transcripts in each tissue, pools of cDNAs from several individual samples (n = 3 - 4 male or female per tissue)

were analyzed. Expression of *gpera* and *gperb* was then quantified by qPCR in individual cDNA samples of tissues that contained detectable levels of expression. For the reproductive cycle, 3-6 males and 4-8 females were used per sampling point.

Reactions were run for 40 cycles using the cycling conditions recommended by the supplier and the optimized annealing temperatures for *gpera/gperb* primers (Table 1). All qPCR reactions had a single product melt curve and primer specificity was confirmed by sequencing the amplicons. No amplification products were obtained when reverse transcriptase was omitted from the cDNA synthesis reactions (negative RT control), confirming the absence of genomic DNA contamination. Standard curves prepared from serial dilutions of quantified amplicons for each gene were included in all qPCR plates to permit product quantification and for determination of the reaction efficiency, which ranged between 97% and 103% with $R^2 > 0.99$.

The stability of two reference genes (18S ribosomal RNA sub-unit, *18s*, and elongation factor 1a, *ef1α*) was evaluated for both tissue panels, by analyzing the Cts (threshold cycles) using RefFinder (available at 150.216.56.64/referencegene.php) that integrates the geNorm, Normfinder, BestKeeper and Comparative Ct methods [33-36]. Since both reference genes were stable, all data was normalized by the geometric mean of these two genes. Copy number of target or reference genes were calculated using the following equation: number of copies $= (X/NA)/(Y \times 1 \times 10^9 \times 650)$, where X is the initial template amount (ng of the amplicon fragment), NA is Avogadro's number, Y is the template length (bp of each amplicon), and 650 (Da) is the average weight of a base pair [37, 38]. *Gper* normalized expression was calculated by dividing the obtained gene copy number for the samples of the tissue distribution and the annual cycle panels by the geometric mean of the two reference genes.

2.6. Statistical Analysis

Results are expressed as the mean ±standard error of the mean (SEM). Statistical differences between groups at each sampling point of the annual reproductive cycle were analyzed by one-way analysis of

variance (one-way ANOVA, SigmaStat v.3.50, Systat Software, USA) followed by a post-hoc Tukey test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Identification of two estrogen membrane receptors in sea bass

Two *gper* genes were identified in the sea bass genome: DLAgn_00191960 gene (*gpera*; formerly designated as *gper*) in position LG8:11487608-11488672 and DLAgn_00100480 gene (*gperb*; formerly designated as *gperl*) in position LG1B:5202057-5203200, and both were annotated as "g-protein coupled estrogen receptor 1-like".

The predicted coding sequence (CDS) of European sea bass *gpera* was 1065 bp in length, it consisted of a single exon coding for a 354 aa protein and was isolated from adult sea bass testis cDNA. This form shared the highest sequence similarity with previously reported *gpers* in other teleost species [19, 39, 40] and has been deposited in Genebank (accession number MF508726). For sea bass *gperb*, two gene structure predictions were available from public databases (Fig. S1). In the sea bass genome database, the DLAgn_00100480 coding region was 1026 bp and was interrupted by a predicted intron of 118 bp followed by the last 5 nucleotides containing the termination codon "TGA", encoding a predicted protein of 341 aa. The gene prediction in the NCBI database (positions 3849949-3851163 of the chromosomal sequence with Accession FQ310507) showed a coding sequence on a single exon of 1215bp, ending with a termination codon "TAA" and encoding a predicted protein of 404 aa. A survey of different sea bass tissues using different primer combinations for possible *gperb* transcripts (Fig. S1) resulted in the isolation of a full-length transcript of 1241 bp. This transcript was isolated from adult sea bass testis and contained the single exon coding sequence of 1215 bb, confirming the *gperb* structure (deposited in Genebank with Accession No. MF508727).

Analysis of the deduced aa sequences of Gpera and Gperb revealed that both contained seven highly conserved transmembrane domains, which is the signature of GPCRs (G-protein coupled receptors)

(Fig. 1). The eight cysteines typical of vertebrate Gpers were also conserved in sea bass Gpers (Fig. 1). The exception to this was the substitution of the first cysteine by serine in sea bass Gperb (in the first transmembrane domain; Fig.1). Substitutions were also found in the same position of Gperb in several other teleost species (substitution to serine in *O. niloticus*, *M. zebra, O. latipes* and *G. aculeatus* and to glycine in *X. maculatus* and in *P. reticulata*; data not shown, observed in the multisequence alignment carried out for the phylogenetic analysis). The European eel was the only teleost analyzed that has conserved this cysteine residue (Fig.1).
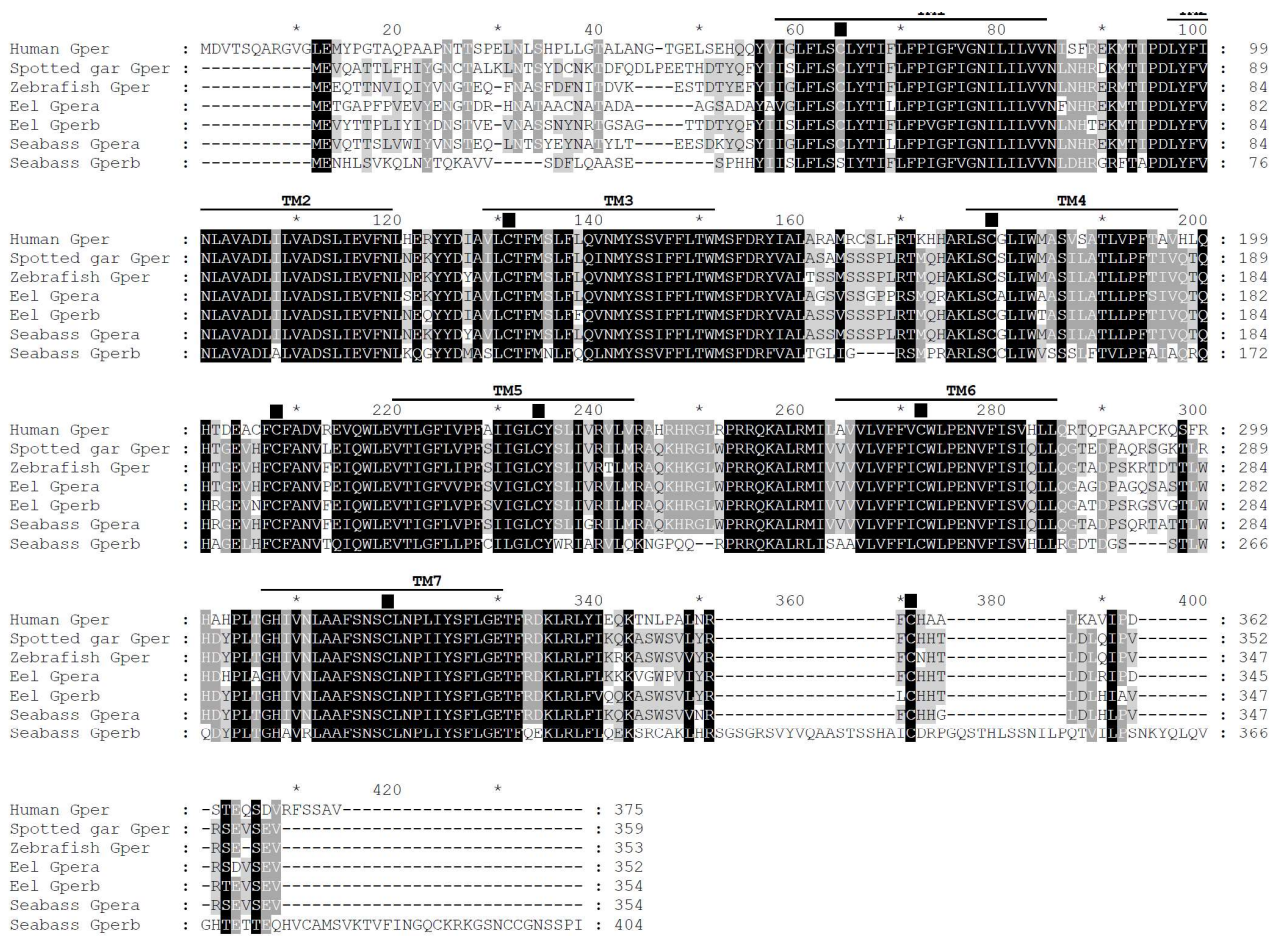
Fig. 1 – The deduced amino acid sequence of Gpera and Gperb contain conserved features typical of GPCRs. The figure shows the multiple sequence alignment (MSA) of the sea bass deduced Gpera and Gperb proteins with human Gper (NP_001496.1), spotted gar Gper (ENSLOCP00000022201), zebrafish Gper (NP_001122195.1) and the European eel Gpera (CUH82770.1) and Gperb (CUH82771.1) proteins. Amino acid conservation between the proteins in different species is shaded and the transmembrane domains (TM1-7) predicted by comparison to mammalian and fish Gpers are indicated with solid lines above the MSA. The cysteine residues typically conserved in Gpers are indicated by a black square above the MSA (the first cysteine in sea bass Gperb is replaced by serine).

3.2. Phylogenetic analysis of Gpera and Gperb

A phylogenetic tree was constructed using the maximum likelihood method (Fig. 2), based on the alignment of 29 Gper sequences from fish to tetrapods. The analysis clustered the Gper sequences into two main branches, the Sarcopterygian Gper branch and a branch containing Actinopterygian Gpers. This Actinopterygian branch was organized into two branches that corresponded respectively to Gpera and Gperb and revealed that in a number of teleost fish species the *gper* gene duplicated and persisted. The spotted gar (*Lepisosteus oculatus*) Gper branched in a basal position in relation to the teleost sequences and the coelacanth (*Latimeria chalumnae*) Gper branched at the base of Sarcopterygian, as expected. Based on these results, the nomenclature of the two previously reported partial sea bass Gpers transcripts (*gper* and *gperl*) was modified to *gpera* and *gperb*, respectively.
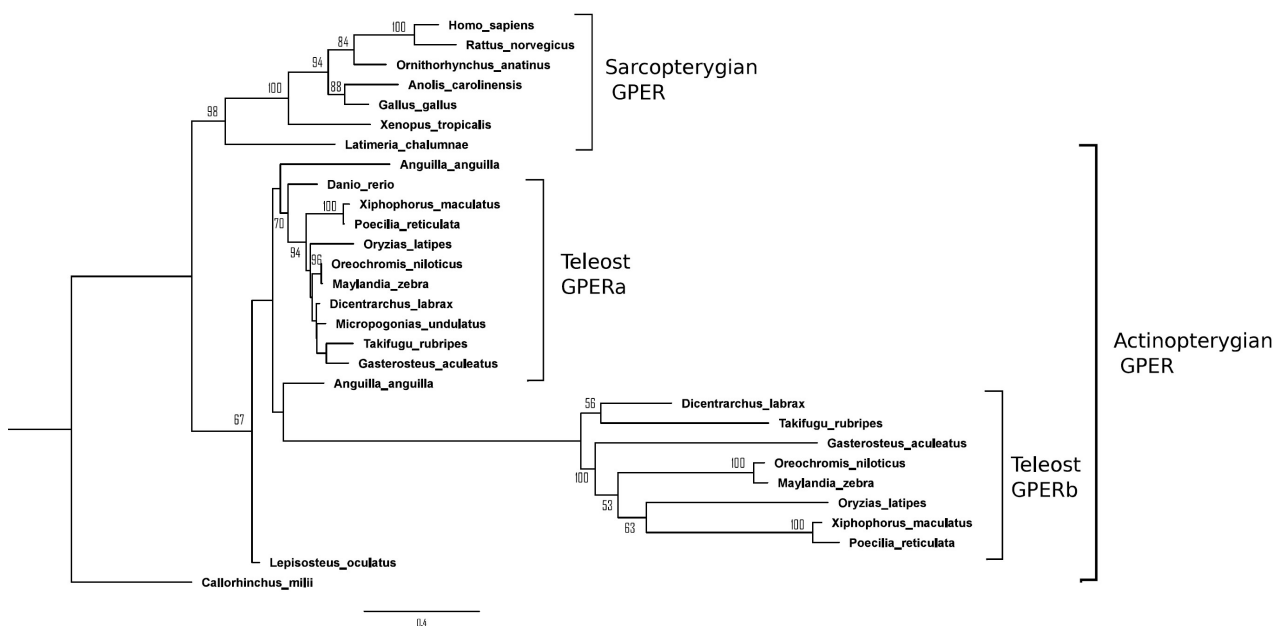

Fig. 2 – Phylogenetic analysis suggests that a duplication of Gper occurred in teleosts. The figure shows the phylogenetic tree of 29 Gper amino acid sequences from fish and tetrapods, performed using the Maximum likelihood method with 100 bootstrap replicates (for the references of sequences see table S1). The *Callorhinchus milii* (elephant shark) Gper was used to root the tree. The bootstrap support for each fork are indicated.

3.3. Tissue distribution of sea bass gpera and gperb

The tissue distribution of both *gpera* and *gperb* transcripts was investigated and their expression was observed in a wide range of adult sea bass tissues from both sexes (Fig. 3). *Gpera* mRNA levels were

higher in pituitary and brain from both sexes while *gperb* had a more widespread distribution. In the

gonads, dimorphic expression was suggested with higher expression in testis compared to ovary for

both *gpers*. The highest expression levels for *gperb* were found in gill filaments followed by head

kidney and kidney in both sexes and in male intestine.
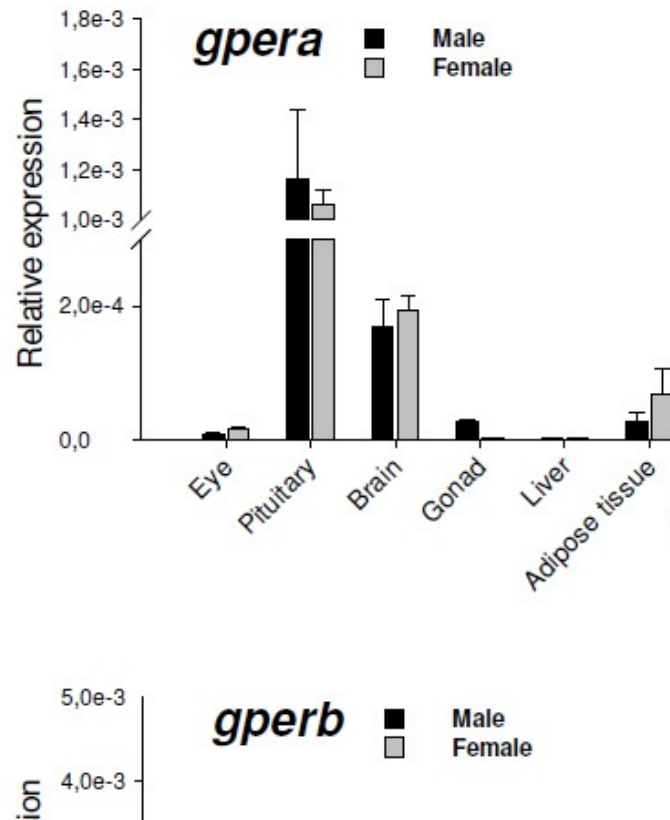
Fig. 3 – *Gpera* is more abundant in pituitary and brain from both sexes while *gperb* has a more widespread tissue distribution. Sea bass *gpera* (A) and *gperb* (B) transcript expression were measured in a panel of tissues from both sexes using qPCR and normalized by dividing calculated gene copy number by the geometric mean of the two reference genes *18s* and *ef1α*. Data are presented as mean ± SEM (n = 3-4).
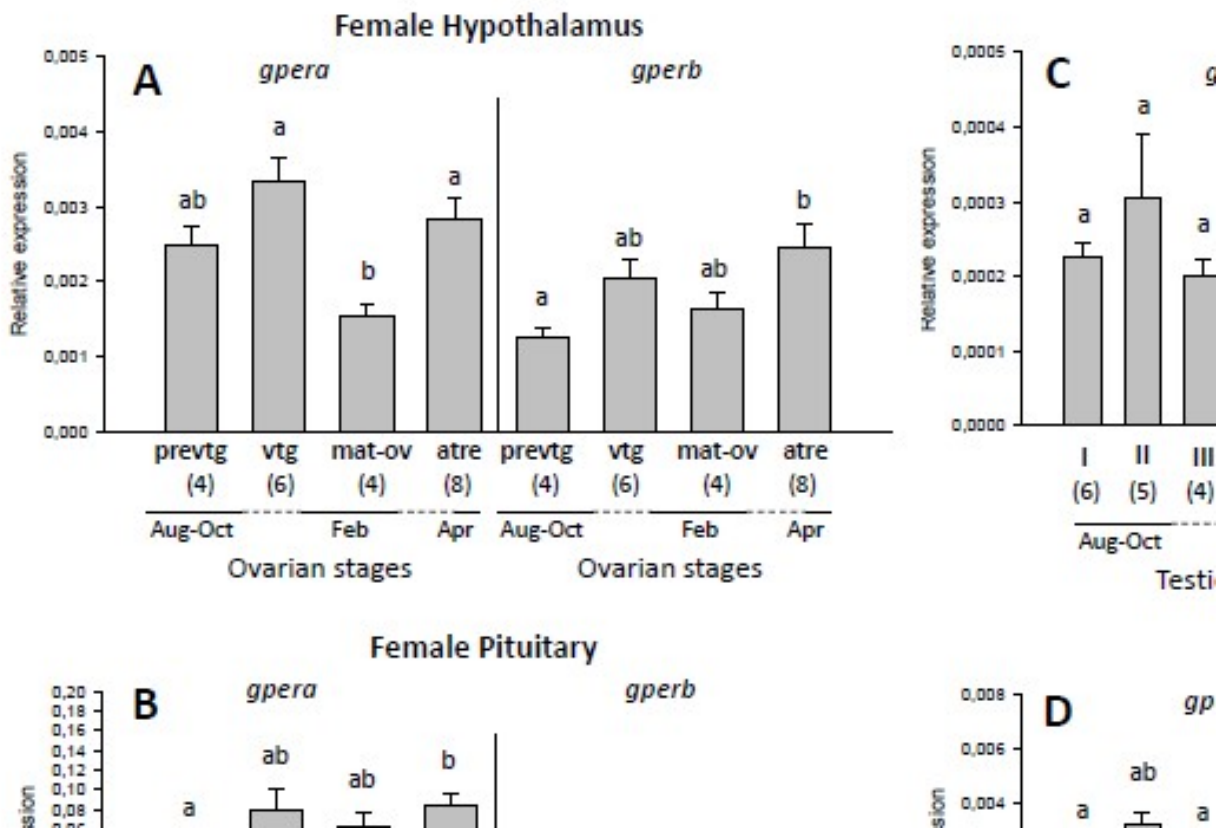
3.4. Gpera *and* gperb *transcript expression throughout the reproductive cycle*

Since expression of both *gpers* was detected in the brain and pituitary of both male and female adult

sea bass, their expression was evaluated in pituitary and hypothalamus across the annual reproductive

cycles in order to investigate their possible involvement in the central control of reproduction in sea

bass. In female sea bass both *gper* forms were expressed in the hypothalamus (Fig. 4A) and pituitary

(Fig. 4B) throughout the entire reproductive cycle. In the hypothalamus, *gpera* decreased significantly

in fish in the ovary maturation-ovulation stage relative to fish in vitellogenesis, restoring its levels at the final atresia stage, while *gperb* tended to increase along the reproductive cycle and was most abundant in females with eggs in atresia (Fig. 4A).

In female pituitaries (Fig. 4B), a similar pattern was observed for *gpera* which also had a higher expression in the atresia stage, while abundance of *gperb* did not vary significantly along the reproductive season. Both *gper* forms were also expressed in male hypothalamus (Fig. 4C) and pituitary (Fig. 4D) throughout the entire reproductive cycle, although significant changes in expression were only detected for *gpera* in the pituitary and it increased when fish progressed from mid to late testicular development (stage III to IV; Fig. 4D).

Fig. 4 – *Gpera* and *gperb* are regulated in the hypothalamus and pituitary of both sexes during the reproductive cycle. Transcript expression of sea bass *gpera* and *gperb* was analyzed by qPCR in female (A) and male (C) hypothalamus and in female (B) and male (D) pituitary during the reproductive cycle. For details about the ovarian and testicular development stages see section 2.1. Transcript levels were normalized by dividing calculated gene copy number by the geometric mean of the two reference genes *18s* and *ef1α*. Data are presented as mean ± SEM. The numbers in parentheses represent the number of individuals analyzed at each sampling point. Different letters above the bars indicate significant differences between gonadal stages.

4.   Discussion

In the present study we isolated the full-length cDNAs for two *gper* genes (*gpera* and *gperb*) previously detected in European sea bass [16]. Phylogenetic analysis showed that the sea bass *gper* genes have been originated from the teleost specific whole genome duplication (3R), which occurred approximately 400 million years ago. These findings are in agreement with those observations in the European eel [19, 41]. Although duplicate *gper* genes have been identified in other teleost species, they are not present in the spotted gar, a representative of the ray-finned fish that preceded the teleost radiation, or the coelacanth, a basal lobe-finned fish which did not undergo tetraploidization. Only a single *gper* gene has been identified in zebrafish and in Atlantic croaker [15, 39, 40], indicating likely loss of the second copy occurred in these species [19]. The conservation of the 7TM domain structure and cysteines typical of Gpers [15, 19] in teleosts with duplicate *gper* genes suggests the duplicate *gpers* are likely to be functional. Interestingly, the cysteine in the first TM domain of teleost Gperb was substituted by other small non-polar amino acids (serine or glycine), although it was conserved in the basal teleost, European eel. It would be interesting to investigate if these changes affect receptor structure and consequently teleost Gperb function.

The tissue distribution analysis show that *gpera* is highly expressed in pituitary and brain, as reported in the European eel *gpera* [19]. The brain was also one of the main sites of expression of the single *gper* gene in the Atlantic croaker and zebrafish [15, 39]. In sea bass, the preponderance of *gpera* relative to *gperb* in the pituitary and brain of both males and females suggested a role in mediating rapid estrogen actions in these tissues. In this context, we decided to further investigate their expression patterns in these tissues across the male and female annual reproductive cycles (see below).

Gpera was also expressed at lower levels in other tissues including the testis, where rapid estrogen effects on androgen production have previously been reported associated with estrogen-binding properties of testicular membrane preparations in Atlantic croaker, although the receptor(s) involved

were not identified [42]. The ovary is a tissue where a clear role for *gpers* has been established in the E2-inhibition of meiotic arrest in the Atlantic croaker and zebrafish [14], at the oocyte maturation stage. In the present study, low expression levels were detected in sea bass ovary of the analyzed females, which were sampled at the beginning of the reproductive cycle. To complement these results, it would be of interest to investigate the expression of *gpera* receptor in sea bass ovaries, sampled at other stages of the female reproductive cycle.

Sea bass *gperb* had a more widespread distribution in the analyzed tissues, with highest expression in tissues related to osmoregulation such as the gill filaments, kidney and intestine. This suggests that *gperb* could be one of the mediators of observed E2 effects on calcium uptake in gill filaments and intestine, contributing to the described increase in calcium plasma levels in response to both physiological increases in circulating E2 during the female reproductive season and to exogenous estrogen treatments [7, 43]. Possible effects in the regulation of other aspects of mineral homeostasis and possible interaction of Gpers with mineralocorticoid receptors (MR), as described in human breast cancer cells [44], remain to be investigated. The high expression of sea bass *gperb* in both male and female head kidney suggests *gperb* could be the membrane receptor mediating effects of estrogen action. In fact estrogen effects on the head kidney adaptive immune function at different life stages in fish have been previously suggested based on Gper expression analysis and the effects of the Gper selective agonist G-1 [45-49].

Non-reproductive effects have also been reported and *gperb*, together with the nuclear estrogen receptor *esr2a*, appear to be the main estrogen receptors in sea bass and Mozambique tilapia scales [16, 17]. In agreement with previous reports, in this study *gperb* and not *gpera* was detected in sea bass scales. In sea bass scales, both *gperb* and *esr2a* were shown to be up-regulated by E2 and the phytoestrogen genistein, and so *gperb* is a good candidate for mediating their effects on mineral homeostasis, as some of these effects are rapid and consistent with mediation through membrane receptors [7, 17, 50]. Both *gpera* and *gperb* were also expressed at low levels in sea bass vertebra of

both sexes. In mammals, Gper is expressed in osteocytes, osteoclasts, osteoblasts and chondrocytes and Gper-deficiency models and the use of Gper agonists demonstrate its involvement in estrogen-induced bone growth and development [13]. It will be interesting to investigate in the future the expression of Gper duplicates in fish bone and their possible role in this tissues homeostasis.

Considering that both *gpera* and *gperb* are expressed in sea bass pituitary and brain and that the brain-pituitary-gonad axis (HPG) is where the main neuropeptides related to reproduction are localized [51, 52], we analyzed the receptors expression in pituitary and hypothalamus throughout the annual reproductive season of this species. In males, both *gpers* were expressed at constant levels in the hypothalamus. Similarly, gonadotropin-releasing hormone 1 (*gnrh-1*) [20] also did not change in the same animals at this brain level and it has been proposed to play a continuous role in maintaining spermatogenesis throughout the reproductive season [53]. However, a significant increase was detected for *gpera* expression in the pituitary, and its expression increased from mid- to late recrudescence and remained constant through advanced spermatogenesis (spermiation and rest stages). Although circulating E2 levels remain relatively low and constant in male sea bass at these stages [54], the shift in *gpera* expression coincides with reported increases in pituitary expression and circulating levels of gonadotropin (Fsh and Lh), important regulators of spermatogenesis and gametogenesis [20, 54-56]. The pituitary expression of *gpers* supports the idea that they may mediate estrogens effects in this tissue along the gonadal cycle. Whether the increase in *gpera* at the last stages of testicular maturation is related to possible functions regulating gonadotropin synthesis or release remains to be investigated.

In female hypothalamus a slight increase in *gpera* expression was observed in the vitellogenesis stage followed by a significant decrease when fish entered the maturation-ovulation stage while *gperb* expression increased throughout the cycle. In the pituitary, *gpera* expression increased throughout the cycle reaching significantly higher levels at the atresia stage while *gperb* did not vary significantly. These differential patterns of expression in both tissues suggest that *gpera* and *gperb* may have

acquired different functions in the central neuroendocrine regulation of the female sea bass reproductive cycle.

The higher expression levels of hypothalamic *gpera* in the vitellogenesis stage coincides with the highest levels of Fsh in plasma, which acts on the ovaries to stimulate E_2 synthesis and gametogenesis [56-58]. It also coincides with the highest E2 plasma levels that stimulate hepatic synthesis of vitellogenin that accumulates in the yolk of growing oocytes [22, 55, 57, 59]. The highest levels of *gperb* were found in female sea bass hypothalamus at advanced stages of ovarian development when high hypothalamic expression of *gnrh-1*, the main hypophysiotropic form in sea bass [60] occurred in the same animals [20]. This coincided with high circulating levels of Lh, the major regulator of late gametogenesis, and of maturation inducing hormones [55, 61, 62]. Taken together these results suggest *gpera* mediates estrogens effects in the hypothalamus at the beginning of the female sea bass reproductive cycle, while *gperb* mediates estrogens effects mainly at later stages of oogenesis. In experimentally induced maturation of female European eels the duplicate *gpers* also had a differential response with *gpera* being up-regulated in the anterior brain and both forms down-regulated in the pituitary [19]. In summary, the results from both eel and sea bass suggest the duplicate *gpers* have acquired different functions in reproduction.

Both negative and positive feedback effects of sex steroids have been reported on regulating gonadotropin levels in the fish HPG axis although the sense of this regulation appears to depend on the species or the timing [2]. In sea bass, both testosterone and estradiol were shown to significantly decrease pituitary Fsh (gene expression and protein release) *in vivo* and *in vitro* [63-65], suggesting a negative feedback regulatory loop exists for gonadal sex steroids on sea bass pituitary Fsh. Nuclear estrogen receptors were shown to be expressed in Fsh and Lh producing cells in sea bass pituitary and the *esr2b* form was down-regulated by E2 treatment in castrated animals, thus suggesting a direct participation of nuclear Esrs on the control of gonadotropin hormone synthesis [66, 67]. Future localization and functional studies should establish if the two sea bass *gpers* can also mediate E2 effects

on pituitary gonadotropin production and other neuroendocrine functions and if rapid non-genomic mechanisms are involved. Future studies will also evaluate if and how hypothalamic/pituitary *gper* expression is regulated by E2. We have already reported the down regulation of both *gpera* and *gperb* in immature sea bass liver and the up regulation of *gperb* in the scales, where *gpera* was not expressed [16]. Moreover, it is also possible that *gpers* mediate estrogenic effects not related to reproduction in the brain of both males and females. These may include mediation of rapid E2 effects as observed on zebrafish radial glial cells, that are also rich in expression of nuclear estrogen receptors and aromatase [68], or actions associated with neuroprotection and behavior as reported in both sexes in mammals [69-71].

In summary, we report the identification of two estrogen membrane receptor genes *gpera* and *gperb* in sea bass and in other teleost fish, which probably arose during the teleost specific whole genome duplication (3R). The two receptors had a differential tissue distribution with *gpera* strongly expressed in brain and pituitary while *gperb* had a more widespread distribution with prevalence in gills and other osmoregulatory tissues. This study also revealed that both transcripts were expressed in pituitary and hypothalamus during the entire reproductive cycle in both male and female sea bass. In both male and female sea bass, significant differences in transcript abundance were found for *gpera* and *gperb* that may be related to their involvement in the estrogenic control of particular reproductive phases during the annual reproductive cycle, namely the pituitary secretion of gonadotropins or other hormones. The tissue distribution and patterns of regulation of the two duplicate *gpers* supports their role in mediating non-classical actions of estrogen in responsive tissues, with a more widespread target range for *gperb*, and possible functional specialization of the two forms between tissues and stages of the reproductive cycle.

Acknowledgments

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