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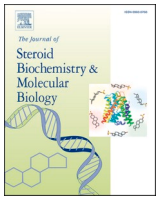

Evolutionary history and functional characterization of duplicated G protein-coupled estrogen receptors in European sea bass

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ABSTRACT

Across vertebrates, the numerous estrogenic functions are mainly mediated by nuclear and membrane receptors, including the G protein-coupled estrogen receptor (GPER) that has been mostly associated with rapid non-genomic responses. Although Gper-mediated signalling has been characterized in only few fish species, Gpers in fish appear to present more mechanistic functionalities as those of mammals due to additional gene duplicates. In this study, we ran a thorough investigation of the fish Gper evolutionary history in light of available genomes, we carried out the functional characterization of the two *gper* gene duplicates of European sea bass (*Dicentrarchus labrax*) using luciferase reporter gene transactivation assays, validated it with natural and synthetic estrogen agonists/antagonists and applied it to other chemicals of aquaculture and ecotoxicological interest. Phylogenetic and synteny analyses of fish *gper1 and gper1-like* genes suggest their duplication may have not resulted from the teleost-specific whole genome duplication. We confirmed that both sbsGper isoforms activate the cAMP signalling pathway and respond differentially to distinct estrogenic compounds. Therefore, as observed for nuclear estrogen receptors, both sbsGpers duplicates retain estrogenic activity although they differ in their specificity and potency (Gper1 being more potent and more specific than Gper1-like), suggesting a more conserved role for Gper1 than for Gper1-like. In addition, Gpers were able to respond to estrogenic environmental pollutants known to interfere with estrogen signalling, such as the phytoestrogen genistein and the anti-depressant fluoxetine, a point that can be taken into account in aquatic environment pollution screenings and chemical risk assessment, complementing previous assays for sea bass nuclear estrogen receptors.

1. Introduction

Estrogens are well known for their regulation of the reproductive system and the development of secondary sexual characteristics, but they also modulate other physiological processes, such as the cardiovascular, immune and nervous systems [1,2]. Across vertebrates, estrogenic actions are mainly mediated by binding to different types of estrogen receptors [3]. Specific transcription factors, the nuclear estrogen receptors (ESRs), have been mainly associated with the classical activation of gene expression by estrogens or estrogenic compounds. Contrariwise, membrane receptors such as the G protein-coupled estrogen receptors (GPERs) have been related to rapid, non-genomic estrogenic actions. Both types of receptors and their associated responses can act concomitantly, but the number of receptors differs among species as result of gene/genome duplication events reflecting a complex evolution within the vertebrate clade, and their activities and roles may differ between species [4].

Two rounds of whole genome duplication (WGD) events have been identified as major evolutionary drivers that shaped the genomes of vertebrates, including fish and tetrapods. In the case of nuclear estrogen receptors, two nuclear estrogen receptor subtypes, ESR1 and ESR2 have been identified and characterized in most tetrapod species [5]. Only one GPER subtype was identified in tetrapods, which was initially named as the orphan receptor GPR30 and subsequently demonstrated to bind and

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be activated by estrogens and, therefore, renamed as GPER1 [6].

The analysis of fish genomes revealed that teleosts experienced an additional WGD, designated as teleost-specific genome duplication (TSGD) or 3R-WGD [7]. The TSGD event was suggested to be at the origin of the additional duplication of nuclear estrogen receptors, although some paralogs may have been subsequently lost and only one of the duplicates was maintained, as it appears to be the case of Esr1 [8]. It was previously suggested that additional *gper* paralogs probably originated and were retained from the TSGD event, but phylogenetic trees were not informative enough to be fully conclusive and initial synteny analyses showed that this genomic region contains duplicated genes only in the case of the European eel [8–11]. Therefore, a more thorough investigation was needed including all currently available genomes.

Concerning the nuclear receptors, most teleosts present three Esr subtypes: one Esr1 (or Erα) and two Esr2 paralogs designated Esr2a (alternative names: ERβ, β1 or βa) and Esr2b (ERγ, β2 or βb), which are considered to have resulted from the TSGD [12–14]. In the European sea bass (*Dicentrarchus labrax*), complete cDNAs coding for Esr1, Esr2a and Esr2b were isolated and shown to have partially overlapping but distinct patterns of tissue distribution, regulation by estrogens and other hormones and transcriptional activity [14–16]. Two membrane estrogen receptor genes, designated *gpera* and *gperb* (or *gper1* and *gper1-like*) were recently identified in the sea bass: expression of *gpera* 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 [11]. Significant changes in *gpers* transcript expression were detected in the hypothalamus and pituitary of male and female sea bass throughout their annual reproductive season, especially for *gpera* [11]. In the skin scale barrier, *gperb* was the main form expressed and was up-regulated by estrogens, while in the liver both transcripts were downregulated [17]. Therefore, evidences from sea bass and other fish species have indicated that both Gpers differ in their patterns of tissue distribution, expression during ontogenesis and along the reproductive cycles as well as their regulation by estrogens [11, 17–20], but their functional characterization was not yet assessed.

The physiological roles of GPERs have been increasingly investigated for the last two decades. Results from *in vitro* and *in vivo* studies suggest that GPERs can be activated by 17β-estradiol (E2) to trigger various intracellular signalling pathways both in mammals and in fish [21–24], but information on the different functions of GPER paralogs in fish is scarce. It is known that they are expressed in diverse fish tissues, including reproductive tissues but also in the thymus, head kidney, gills, eye, heart and in the skin-scales barrier [8, 9, 11, 17, 19, 25]. This suggests that Gpers can contribute to the regulation of a wide variety of physiological responses. The expression of *gpers* in hypothalamus, pituitary and testis was shown to be regulated along the reproductive season in male and female European sea bass and European eel [11,25], which supports their role in the regulation of reproductive processes.

The complexity of the estrogenic regulatory system is also linked to the potential crosstalk between nuclear and membrane receptors, which to date has been mainly studied in mammals. This may include the regulation of expression of ESRs by GPERs or *vice-versa*, their physical interaction and their simultaneous signalling [22]. Several GPER autonomous actions have also been evidenced by functional assays using knock-out animals or GPER-specific agonists or antagonists such as G-1 and G-15, respectively [22]. In teleosts, Gpers are believed to influence nuclear Esr signalling depending on cell type or developmental stage, but the clear involvement of fish Esrs in Gper signalling has not yet been demonstrated. So far, functional studies carried out in a few teleost species (including the Atlantic croaker, Gilthead seabream, Japanese medaka and ricefield eel) showed Gper activation in response to E2 or to G1, namely through cAMP or protein-kinase activation [9,10,26,27]. Nevertheless, many aspects remain to be investigated to better understand the roles evolved by duplicated *gper* genes in teleost fish.

Hence, the aim of this study was to revise the evolutionary history of GPERs and to carry out the functional characterization of the two membrane estrogen receptors in the European sea bass. This is one of the most productive species in European aquaculture as well as a representative of the species-rich order of Perciformes and a widely used model in physiological studies, which has a sequenced and well annotated genome available and the advantage of featuring all five estrogen receptors. For this functional characterization, the sex steroid hormones 17β-estradiol (E2) and estriol (E3) were used as natural agonists, previously shown to activate sea bass Esrs [15,16]; G-1 and G-15 were used as synthetic agonist and antagonist, respectively [28,29]; and testosterone (T) as an androgenic hormone.

Furthermore, the synthetic estrogen ethinylestradiol (EE2), the phytoestrogen genistein (GEN) and the antidepressant fluoxetine (FLX) have been evaluated in this study as they have been previously demonstrated to activate or to interfere with estrogen signalling in fish [17,30–32]. EE2 has shown potent estrogenic and antiandrogenic activities in fish and activation of both nuclear and membrane estrogen receptors in Japanese medaka [10,30,33]. GEN is one of the most abundant phytoestrogens, shown to have either agonistic or antagonistic estrogenic effects in mammals or fish, *in vitro* and *in vivo* [17,34–37], and to differentially transactivate the three Esrs of the European sea bass [31]. Finally, FLX is one of the most prescribed antidepressants worldwide and a widespread aquatic micropollutant [38–40]. Although FLX is not a compound with an expected *a priori* estrogenic activity, it has been reported in mammals and fish to alter circulating levels of estrogen and to modify the signalling cascades and the expression of estrogen-responsive genes, such as different Esr forms [41–44]. In addition, FLX may act as an antagonist for sea bass nuclear Esr1 and Esr2b activation by E2 [45]. Including known endocrine disrupting chemicals (EDCs) into the functional characterization of the entire suite of sea bass estrogen receptors can give insights into their evolution and also contribute to improve the screening of potential estrogenic compounds, as emphasized within the Marine Strategy Framework Directive to achieve a good environmental status of the EU's marine waters.

2. Material and methods

2.1. Phylogenetic analysis

The amino acid sequences of GPERs from different vertebrate species used for alignments and phylogenetic analysis were extracted from the Ensembl Genome Browser (http://www.ensembl.org; 02/2022) or from NCBI (http://blast.ncbi.nlm.nih.gov; 02/2022) and the accession numbers can be found in Supplementary Table S1. The original designations used for each sequence were maintained in the table and phylogenetic tree, while the sea bass Gper forms were designated as Gper1 and Gper1-like according to their position in the phylogenetic tree (see results). Following established organism specific nomenclature rules for genes/proteins the notations *GPER*/GPER were used for tetrapods while fish forms were designated as *gper*/Gper. Complete amino acid sequences were aligned using MUSCLE (MUltiple Sequence Comparison by Log-Expectation; [46]), and phylogenetic analysis was performed using the Molecular Evolutionary Genetics Analysis Software (MEGA 11; [47]). The evolutionary history was inferred using the Maximum Likelihood method based on the Jones–Taylor–Thornton matrix-based model [48]. A discrete Gamma distribution was used to model evolutionary rate differences among sites [5 categories (+G, parameter = 0.7389)]. Gaps or missing data were deleted pairwise. One thousand bootstrapping replicates were used to assess the robustness of the inferred nodes of the tree.

2.2. Synteny analyses

Synteny analyses were carried out using information extracted from Ensembl genome assemblies (http://www.ensembl.org) and the Genomicus browser (https://www.genomicus.biologie.ens.fr/), of

human (*Homo sapiens*) GRCh37, chicken (*Gallus gallus*) WASHUC2, western clawed frog (*Xenopus tropicalis*) JGI_4.2, spotted gar (*Lepisosteus oculatus*) LepOcu1, Asian bonytongue (*Scleropages formosus*) fSclFor1.1 and European seabass (*Dicentrarchus labrax*) dlabrax2021. In the case of the European eel (*Anguilla anguilla*) the genome assembly from NCBI (fAngAng.pri; https://www.ncbi.nlm.nih.gov/home/genomes/) was used.

2.3. Expression plasmids

The coding sequences of sea bass Gpera (renamed as sbsGper1) and Gperb (renamed as sbsGper1-like) with accession numbers MF508726 and MF508727, respectively, were amplified by PCR with iProof High-Fidelity proofreading DNA Polymerase (Bio-Rad, Hercules, USA), using previously isolated full-length transcripts of the respective *gpers* as templates [11]. Specific primers were modified to include restriction enzymes compatible with the pcDNA3.1/V5-His TOPO expression vector (Invitrogen, Carlsbad, USA). To isolate the 1065-bp coding sequence of sbsGper1, the primers used were sbsGPERxpF1 (5′-GCGAAGCTTATTATGGAAGTGCAGAC-3′) and sbsGPERxpR1 (5′-CGCGCGGCCGCTTCACACCTCTGACAC-3′) including the recognition sites for *Hind*III and *Not*I, respectively. For 1215-bp coding-sequence of sbsGper1-like, the primers used were sbsGPERLxp F1 (5′-GCGAAGCTTATGGAGAATCACTTGTC-3′) and sbsGPERLxp R2 (5′-CGCGCGGCCGCTTAAATAGGTGAAGA-3′), also containing restriction sites for *Hind*III and *Not*I. The Gper1 and Gper1-like coding sequences were amplified in a total volume of 20 μL containing 1 μL of template plasmid, the original clones of *sbsgpers* [11] cloned in pGEMT-easy and diluted 1:500. The reaction mixture also contained 10 pmol of each primer, 200 μM of each dNTP and 0.4 U of iProof DNA Polymerase. Cycling conditions were 3 min at 98 °C, 35 cycles of 10 s at 98 °C, 30 s at 55 °C and 40 s at 72 °C, followed by a final extension of 5 min at 72°C. The purified PCR products were digested with *Hind*III and *Not*I and ligated into a *Hind*III/*Not*I digested pcDNA3.1/V5-His TOPO vector. Their identity, orientation and the lack of sequence errors was confirmed by sequencing each construct from both ends, with a 3-fold coverage, and each construct was used to transfect HEK 293 cells (ATCC reference CRL-1573) used in the transactivation assays.

2.4. Transactivation assays

HEK 293 cells, which do not contain endogenous GPER, were used to express sbsGper1 and sbsGper1-like. Cells were seeded in 6-well plates (at ~6.25×10^5 cells / well) and cultured in Dulbecco modified Eagle medium (DMEM) GlutaMAX (Thermo Fisher, Waltham, USA) supplemented with 10% v/v charcoal-stripped foetal bovine serum (FBS) and 100 U/ml of penicillin and streptomycin, at 37 °C in a 5% CO_2 incubator. After reaching 75–80% confluence, cells were co-transfected using Lipofectamine 3000 (Invitrogen) with the following constructs: the pCRE-Luc reporter plasmid (1.6 μg; BD Clontech, Palo Alto, CA) that contains the firefly luciferase gene under the control of a promoter with cAMP Response Element (CRE) binding sites, and 0.750 μg of the pcDNA3.1 expression constructs containing either sbsGper1 or sbsGper1-like coding regions. After 15 h, the medium containing plasmid/Lipofectamine 3000 was removed and cells were resuspended in phenol red-free DMEM/F12 supplemented with charcoal-treated FBS and reseeded in Corning™ 96-well flat clear bottom white plates. After 6 h, media containing different concentrations of 17β-estradiol (E2, 10^{-9} to 10^{-5} M), estriol (E3, 10^{-9} to 10^{-5} M), testosterone (T, 10^{-9} to 10^{-5} M), G-1 (10^{-8} and 10^{-7} M), G-15 (10^{-7} M), ethinylestradiol (EE2, 10^{-10} to 10^{-7} M), genistein (GEN, 10^{-9} to $1{,}25\times10^{-7}$ M), or fluoxetine (FLX, 8×10^{-11} to $1{,}6\times10^{-9}$ M) prepared in ethanol were added. The final concentration of the ethanol solvent was 0.1% in all preparations, including the controls. The pollutant concentrations (EE2, GEN, FLX) were chosen according to levels found in the environment or accumulated in fish tissues [49–51]. After incubation at 37 °C for 18–24 h, luciferase activities were determined directly on the plates using the Steady-Glo Luciferase Assay System (Promega, Madison, USA) following the manufacturer's instructions. The light emitted was measured in a Tecan Ultra Evolution microplate reader (Tecan Group Ltd., Männedorf, Switzerland) and expressed as relative light units (RLU), designated as Relative Luciferase Activity values in the figures. All transfections were analysed with triplicate measurements in at least two independent experiments.

2.5. Statistics

Data are presented as mean ± SEM of relative luciferase activity values, which were statistically analysed using one-way ANOVA followed by the Tukey pairwise multiple comparison method, in GraphPad Prism 8 (GraphPad software, Inc). When the test of equal variance failed (Bartlett's test), ANOVA on ranks (Kruskal-Wallis non-parametric test) was performed followed by a pair-wise multiple comparison procedure (*i.e.*, the Dunn's test). The significance level was set at a P value of ≤ 0.05.

3. Results

3.1. Phylogenetic and structural analysis

Based on the alignment of forty Gper amino-acid sequences from Actinopterygii and Sarcopterygii representative species (using zebrafish G Protein-Coupled Receptor 182, Gpr182, as the outgroup), a phylogenetic tree was generated using the Maximum Likelihood method (Fig. 1). Sea lamprey (*Petromyzon marinus*) *gper1*, as a representative species of an ancient vertebrate lineage, clustered before the divergence of Gnathostomes. The phylogenetic tree clearly separated gnathostome *gpers* in two major clades: *gper1* from Actinopterygii, Sarcopterygii and Chondrichthyes clustered together, and *gper1-like* from teleosts in another clade. In the phylogenetic tree build from *gper* gene sequences in the Ensembl genome browser (www.ensembl.org; [52]) two clades are also observed, one for all the *gper1* genes and another for the *gper1-like* of teleosts (Suppl. Fig. S1).

The predicted amino acid sequence of the European sea bass Gper1 gave the highest sequence identity (97%) to that of Seriola (*Seriola dumerilii*) and Gilthead seabream (*Sparus aurata*) Gper1, while Gper 1-like had the highest sequence identity (78%) with the seabream Gper-like form. Only one *gper* gene is found in the Sarcopterygian genomes, designated as *gper1*. The analysis of the phylogenetic tree Gper1 clade (Fig. 1) also revealed that mammals (represented by human and mouse), birds (represented by chicken), amphibia (represented by Xenopus), Chondrichthyes (represented by elephant shark and whale shark) and the non-teleost bony fish coelacanth were clearly separated from the other chordate Gpers, especially teleosts. Interestingly, the Gper1b of the eel, previously classified as Gper1-like, was found to be more similar to other Gper1 (being clearly placed in this clade) than to Gper1-like forms, which indicates that a second *gper1* gene exists in the European eel.

An alignment of the amino-acid sequences from sixteen teleost fish Gpers (Fig. 2) shows that the structure of Gper1 forms is much more conserved than that of Gper1-like. Notably, the cysteine residue in TM1 and the tyrosine residue from the DRY-triplet typically conserved in Gpers are not conserved in most of the teleost Gper1-like, with the exception of the first cysteine in seabream. In addition, all analysed Gper1-like are predicted to have a longer C-terminal tail, also including the forms not represented in the alignment, which is the most divergent region among species. Both eel Gpers are more similar to other Gper1 than to Gper1-like, in agreement with their position in the phylogenetic tree (Figs. 1 and 2).

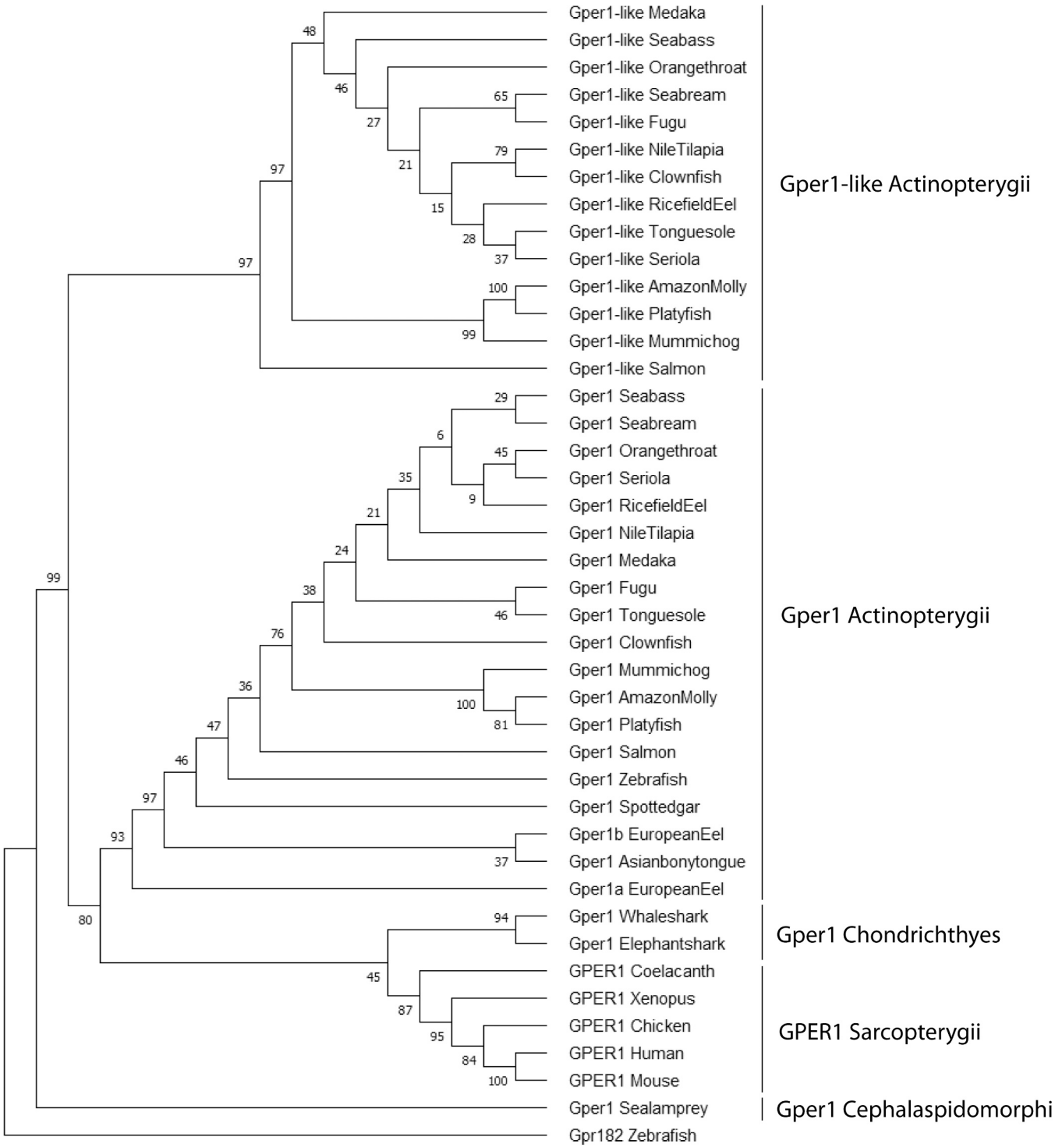

Fig. 1. Rooted phylogenetic tree of 41 vertebrate GPER sequences constructed using the Maximum Likelihood method with *gpr182* from zebrafish used as outgroup. Bootstrap support values for each node are shown as a percentage. See Supplementary Table S1 for Accession numbers of the sequences from Ensembl and NCBI databases.

3.2. Gene environment analysis: synteny

To understand the evolutionary relationship between *gper1* and *gper1-like* genes we analysed the conservation of synteny (*i.e.*, the gene environment) around both genes in different species including tetrapods, non-teleost fish and teleosts (Fig. 3). The Asian bonytongue (*Scleropages formosus,* "Sfo" in Fig. 3) was chosen as the reference species for the *gper* genomic region in this syntenic analysis because i) it represents an ancient lineage of teleosts that emerged soon after the TSGD [53], and ii) it has no *gper-like* gene, excluding the possibility that this gene appeared in the TSGD event. The European seabass ("Dla" in Fig. 3) was used as teleost reference genome since all the teleost genomes we analysed gave similar results in the synteny. The syntenic analyses revealed that there are conserved blocks of genes (represented in different colours in Fig. 3B) around *gper1* in all the studied species. In Xenopus ("Xtr"), chicken ("Gga") and the non-teleost fish spotted gar

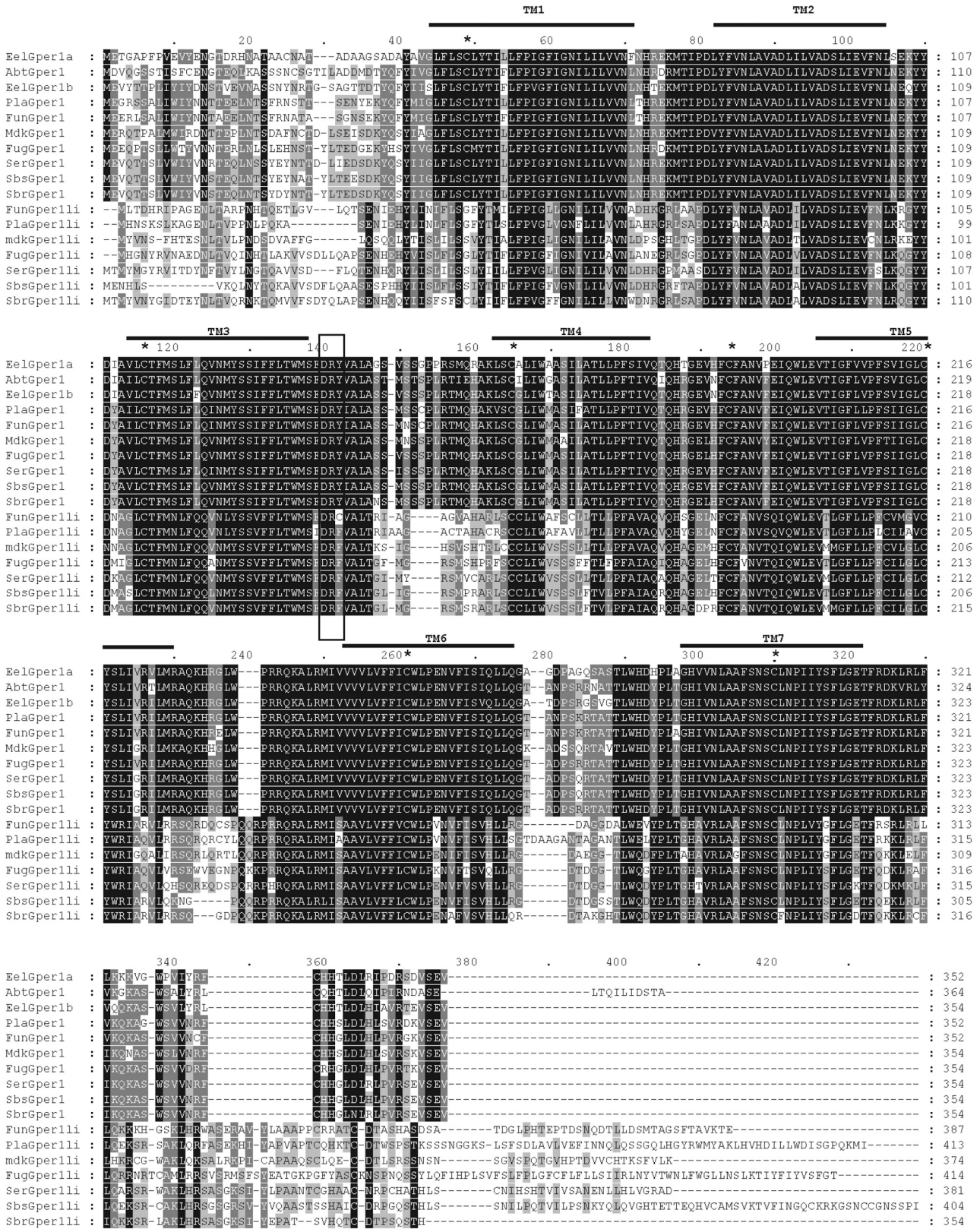

Fig. 2. Multiple sequence alignment (MSA) of the seabass Gper with that of other representative teleosts. See Supplementary Table S1 for all accession numbers and scientific names. The alignment was edited in Genedoc with shading in group configuration mode and shows fully conserved residues boxed in black, residues conserved in some of the species in grey and non-conserved residues in white. Gaps introduced to optimize the alignment are shown with dashes. 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 an asterisk above the alignment. The characteristic DRY residues are enclosed by a rectangle. Species abbreviations: Aan, *A. Anguilla*; Sfo, *S. formosus*; Xma, *X. maculatus*; Fhe, *F. heteroclitus*; Ola, *O. latipes*; Tru, *T. rubripes*; Sdu, *S. dumerilii*; Dla, *D. labrax and* Sau, *S. aurata*.

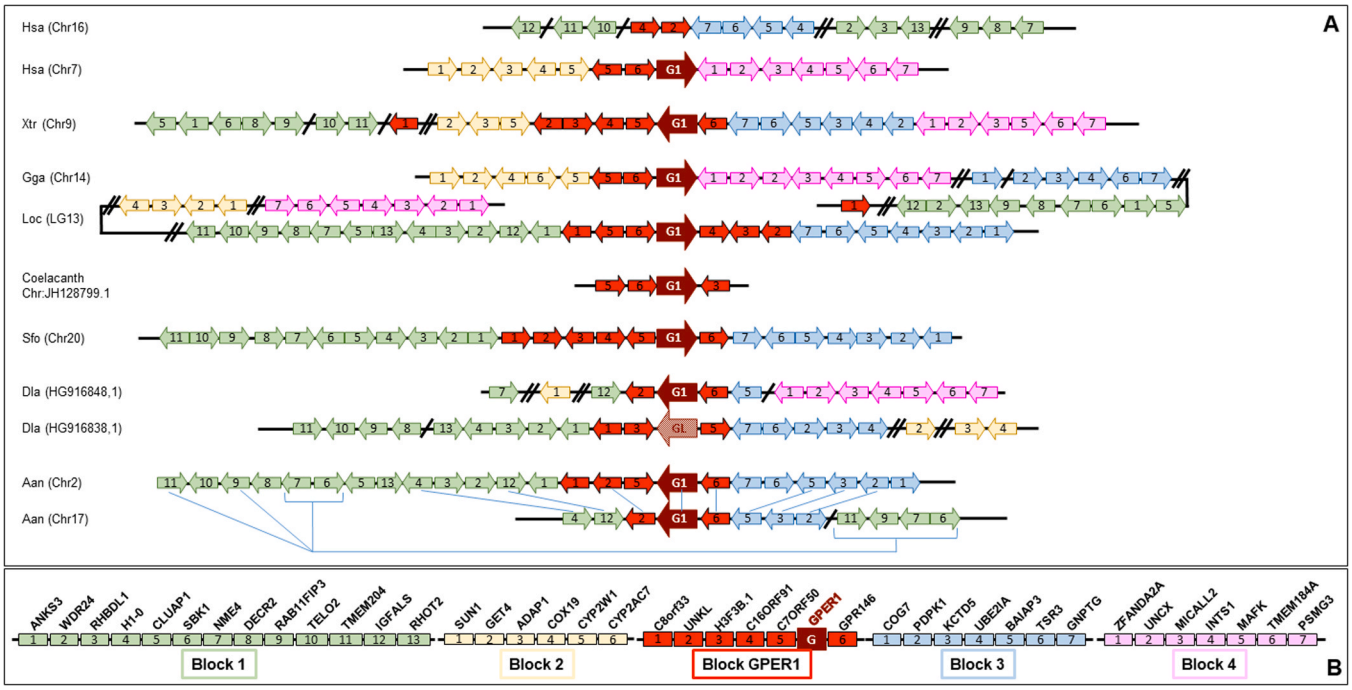

Fig. 3. Synteny of *gper1* containing regions in representative vertebrate genomes. A) Synteny of *gper1* genes neighbour regions in representative vertebrate genomes. Each gene is represented by a coloured arrow that indicates orientation of transcription (with the *gper1* genes represented at a central position by "G1" and teleost *gper1-like* gene labelled as "GL" inside the correspondent arrow). Numbers in brackets represent the chromosomes (Chr), linkage groups (LG) or scaffolds. A vertical bar between two genes indicates that they are not contiguous and a double bar indicates a larger chromosome region among them. The name of the genes in this synteny can be seen in Fig. 3B. Species abbreviations: Aan, *A. Anguilla*; Dla, *D. labrax*; Gga, *G. gallus*; Hsa, *H. sapiens*; Loc, *L. oculatus*; Sfo, *S. formosus* and Xtr, *X. tropicalis*. B) Different genes in the chromosomic region of *gper1* (indicated by a red arrow in the center) from different species. The genes are numbered and grouped by blocks and organized as groups of genes that are segregated together. Each block is identified by a different colour, including the block of genes that are close to *gper1* in all the species (in red), with the name of each gene shown above the arrows and the numbers represented in the scheme for each gene indicated inside the respective arrow. Duplicated blocks of genes in the two chromosomes of *Anguilla anguilla* are connected by blue lines.

("Loc") all these blocks are located in the same chromosome, but with different proximity to the GPER1 block. In human and in more advanced teleosts, these blocks of genes are split into two different chromosomes, but with different distributions (Fig. 3A). In the European sea bass, as representative of teleosts, block 1 and block 3 are accompanying *gper1-like* (orange arrow), while block 4 is near *gper1*. In addition, genes from block 2 and block GPER1 are distributed between both chromosomes. In the human genome, block 1 and 3 are in one chromosome and blocks 2 and 4 in another chromosome, and in both cases are neighbour genes of the GPER1 block, which is distributed in 2 chromosomes as in the sea bass genome, although in this case only one GPER1 gene exists. A different scenario among the teleost genomes analysed is the European eel ("Aan" in the lower panel), since unlike all the other species this one contains two *gper1* genes. Furthermore, some of the conserved neighbouring genes were also duplicated as would correspond with duplications of the whole genome (Fig. 3A).

3.3. Functional characterization of sea bass membrane estrogen receptors

3.3.1. Activation of sea bass membrane receptors by natural steroids

The functionality of sbsGper1 and sbsGper1-like was tested in a mammalian cell line by expressing both receptors together with a cAMP-responsive luciferase construct as a reporter. Both receptors were significantly activated by different concentrations of the natural ligands E2 and E3 and also by T (Fig. 4), although their patterns of response differed for each receptor. E2 was the ligand that induced the highest cAMP-driven luciferase activity, both through Gper1 and Gper1-like, which was obtained for a concentration of 10^{-8} M of E2. Without expression of either sbsGper1 or sbsGper1-like, the cells did not respond to E2 treatment (see in Suppl. Fig. S2 the luciferase levels for cells transfected with the empty vector, that did not significantly differ from those of control, non-exposed cells).

Both receptors exhibited a non-monotonic concentration-response relationship with a bell-shaped curve in response to E2-binding, characterized by significant stimulations at lower concentrations (starting at 10^{-9} M of E2) followed by loss of significant stimulation at higher concentrations, including and above 10^{-6} M for Gper1 and 10^{-7} M for Gper1-like (Fig. 4). The natural estrogen E3 significantly activated both Gpers at almost all concentrations from 10^{-9} to 10^{-5} M, without any evidence for a concentration-dependent response. T was the steroid with less efficiency in inducing cAMP-driven transcriptional activity of the luciferase reporter plasmid. Nevertheless, T effectively activated Gper1 between 10^{-9} M and 10^{-6} M, and less potently Gper1-like for which only the 10^{-8} M dose was able to produce a significant activation of pCRE-Luc (Fig. 4).

3.3.2. Agonists and antagonists of sea bass membrane estrogen receptors

In this study, we have tested the membrane receptors with two synthetic molecules, G-1 and G-15, reported to be agonist and antagonist, respectively, of the human GPER (Fig. 5). G-1 transactivated both Gper1 and Gper1-like although with different potency: it significantly activated Gper1 at 10^{-7} M, whereas it was able to activate Gper1-like at a lower concentration (10^{-8} M). Therefore, G-1 behaved as a weak agonist with Gper1, as compared to E2, *i.e.*, requiring higher concentrations for a significant cAMP-mediated transactivation (10^{-7} M) and reaching significantly lower luciferase levels compared to E2. However, G-1 behaved as an agonist as potent as E2 (with the minimum effective concentration also at 10^{-8} M) with Gper1-like resulting in similar luciferase transactivation levels. The reported human GPER1 antagonist G-15 also acted as an antagonist for both sea bass receptors, Gper1 and Gper1-like. G-15 was able to reduce receptor activation in E2-stimulated cells transfected with either Gper1 or Gper1-like, lowering their

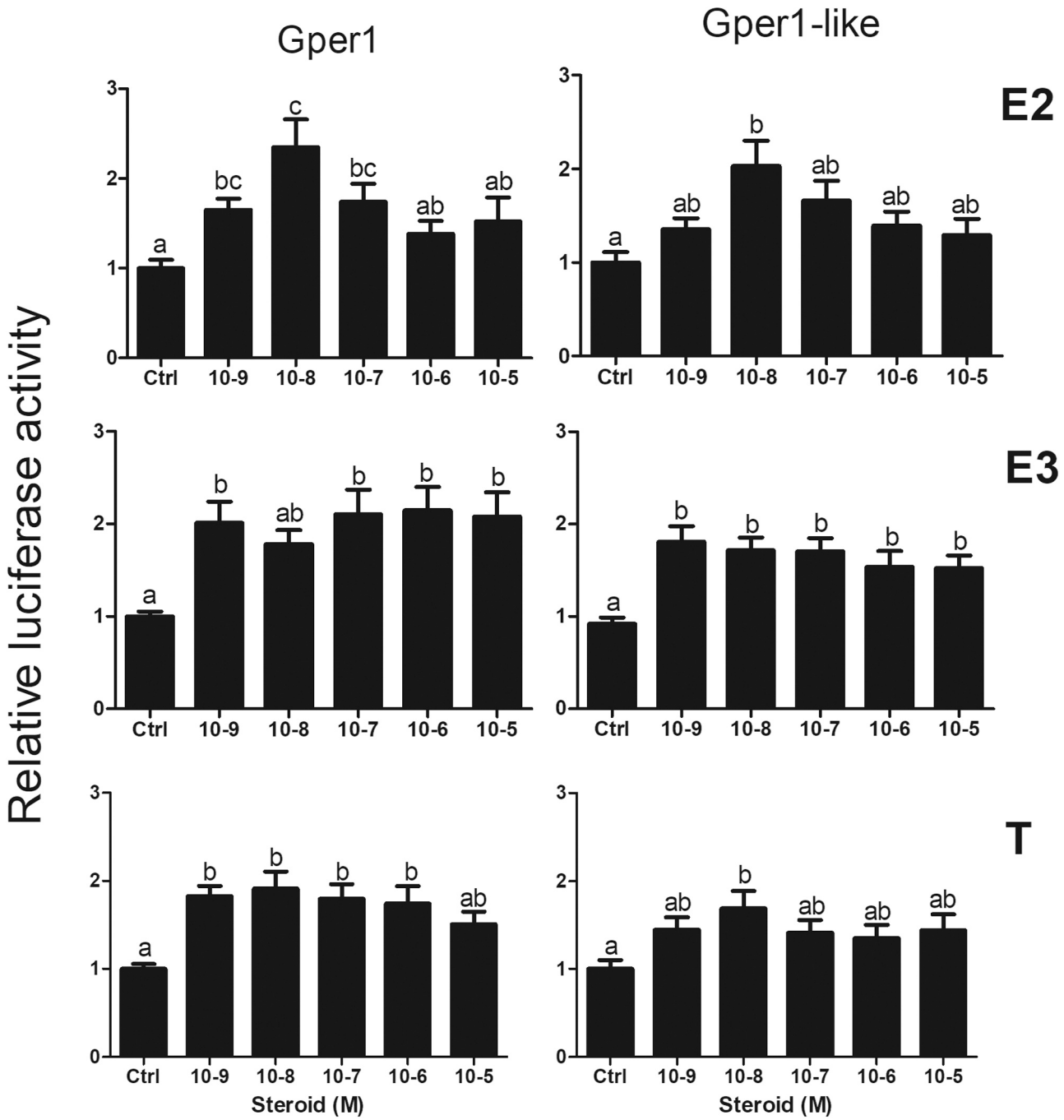

Fig. 4. Luciferase Activity of HEK293 cells co-transfected with either of the membrane estrogen receptor of seabass (Gper1 or Gper1-like) and the pCRE-Luc reporter plasmid following exposure to E2, E3 and T. Induction is expressed in relative light units (RLUs), relative to the control cells (not exposed to the compounds) that were set as 1. Bars present the mean ± SEM of at least two independent experiments performed with triplicate measurements. Different superscript letters represent significantly different values (ANOVA, Tukey post-hoc or Kruskal-Wallis, $p \leq 0.05$).

luciferase activity to the same levels as the non-treated control (Fig. 5).

3.3.3. Activation of Gpers by synthetic steroids

Both seabass Gper1 and Gper1-like were stimulated by 10^{-8} M E2 used as positive control, but only Gper1-like was significantly activated by the synthetic estrogen EE2 (Fig. 6), at concentrations ranging from 10^{-9} to 10^{-7} M EE2. On the other hand, the sbsGper1 was not significantly activated by this anthropogenic steroid, although there was a slight activation at the 10^{-8} M dose (reaching values non-significantly different from those obtained in the induction of the receptor by E2).

3.3.4. Activation of Gpers by fluoxetine

The antidepressant FLX activated both receptors with Gper1-like showing a higher sensitivity, as it was activated by all tested concentrations of FLX (8×10^{-11} M to 8×10^{-9} M). Gper1 was only significantly activated at the lowest dose of 8×10^{-11} M (Fig. 7). When activating Gper1-like, FLX elicited a luciferase activity comparable to the one achieved by the positive control (E2 at 10^{-8} M), while the activation of sea bass Gper1 by FLX was significantly lower compared to E2.

The mixture of FLX at 8×10^{-11} M with both tested concentrations of E2 (10^{-8} and 10^{-9} M) was able to activate both Gper1 and Gper1-like at comparable levels of those achieved by E2 alone, although the

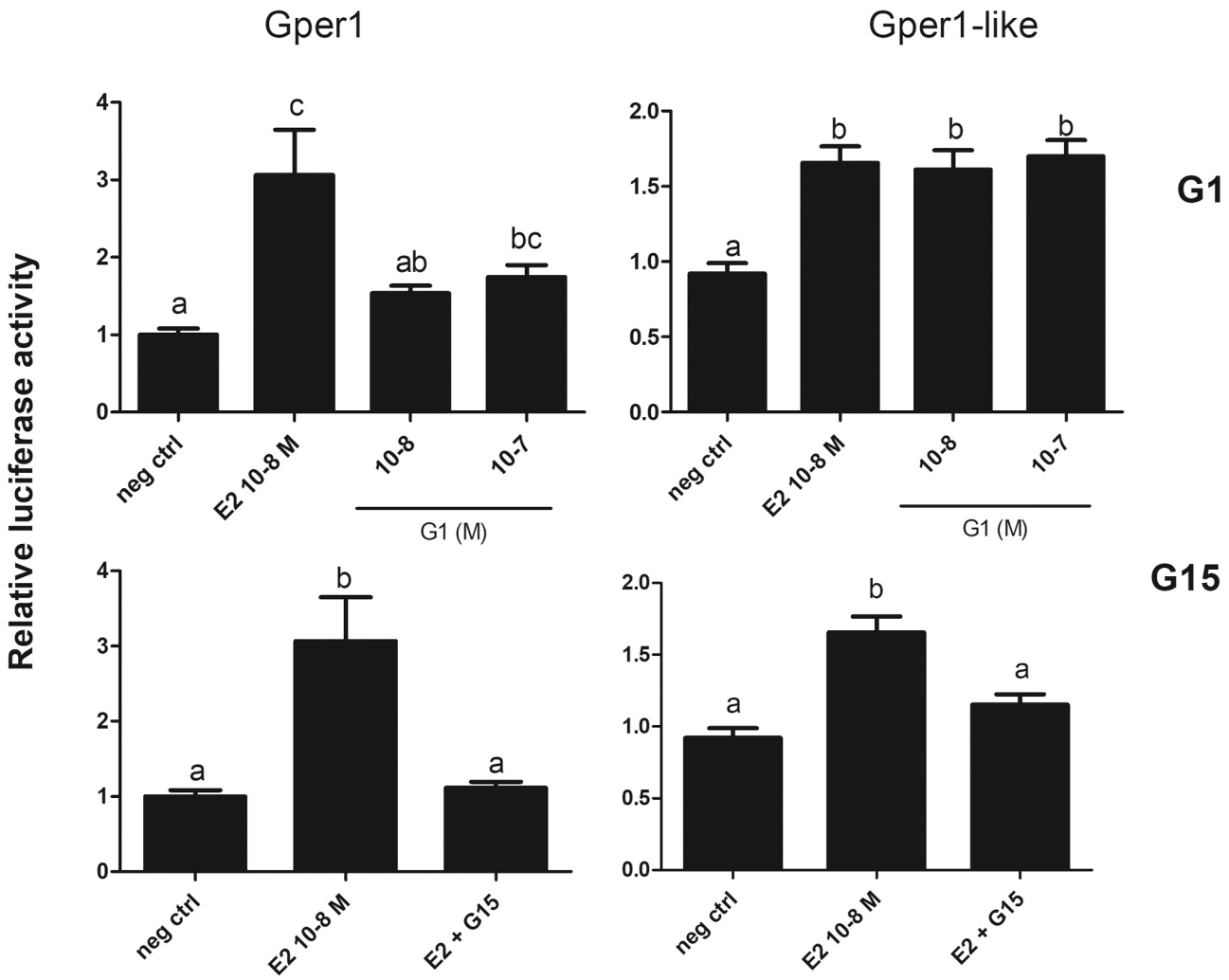

Fig. 5. Luciferase Activity of HEK293 cells co-transfected with each membrane estrogen receptor of seabass (Gper1 and Gper1-like) and the pCRE-Luc reporter plasmid, in response to the exposure to G-1 or with a mixture of G-15 (10^{-7} M) and E2. E2 at 10^{-8} M was used as positive control. Induction is expressed relative to the control cells (not exposed to the compounds) that were set as 1, in relative light units (RLUs). Bars present the mean ± SEM of at least two independent experiments performed with triplicate measurements. Different superscript letters represent significantly different values (ANOVA, Tukey post-hoc $p \leq 0.05$).

combination did not show any synergetic or antagonistic effect of FLX on the E2 transactivation in the cAMP-reporter assay (Fig. 7).

3.3.5. *Activation of Gpers by genistein*

GEN was able to significantly activate both Gpers at 10^{-9} M, but not at the highest concentrations tested up to 1.25×10^{-7} M (Fig. 8). In mixture with E2, similar results as found for FLX were obtained for Gper1 activation. However, in the case of Gper1 and Gper1-like stimulated by E2 combined with genistein, a slight antagonism is suggested since the combined treatments gave slightly lower (although not statistically different) luciferase levels compared to the compounds alone, with the combined treatment luciferase levels becoming not significantly higher than the control.

4. Discussion

In the present study, we have shown that the teleost Gper duplicates most likely did not originate from the TSGD, contrary to what has been suggested in previous analyses [8,9,11]. This conclusion was obtained by combining phylogenetic, synteny and multiple sequence alignments of forty-one *gper* genes of available genomes from Actinopterygii and Sarcopterygii. Neither can we conclude that the duplicates arose from an ancient WGD event, due to a complex evolutionary story, but we can affirm that the duplicated *gper1-like* shows more divergent sequences among species, suggesting it may have suffered lower evolutionary pressure after duplication, probably associated to neofunctionalization. Moreover, we could demonstrate that both European seabass Gper1 and Gper1-like are functional and can act as G-protein coupled receptors through the cAMP signalling pathway, in response to both natural and synthetic estrogens as well as other steroids. Nevertheless, the two duplicate forms exhibit differences in their transcriptional activity levels and ligand potency/efficacy, reinforcing the hypothesis of distinct physiological roles for Gper1 and Gper1-like in fish. Finally, we could show that both Gpers can also be activated by exogenous substances. Indeed, environmental pollutants known to interact with the nuclear Esrs also have the capacity to interfere with the two Gpers of fish. Their impacts on different pathways and physiological endpoints regulated by the five estrogen receptors present in fish should, therefore, be taken into account in the environmental risk assessment of EDCs.

4.1. *Teleost gper evolutionary history*

In recent years, the exponential increase of genomic data availability enabled ever deeper investigations into phylogenetic relations, such as the origin of hormone receptors. Although there have been some recent phylogenetic studies on *gper* genes [8,9,11], due to better fish genome

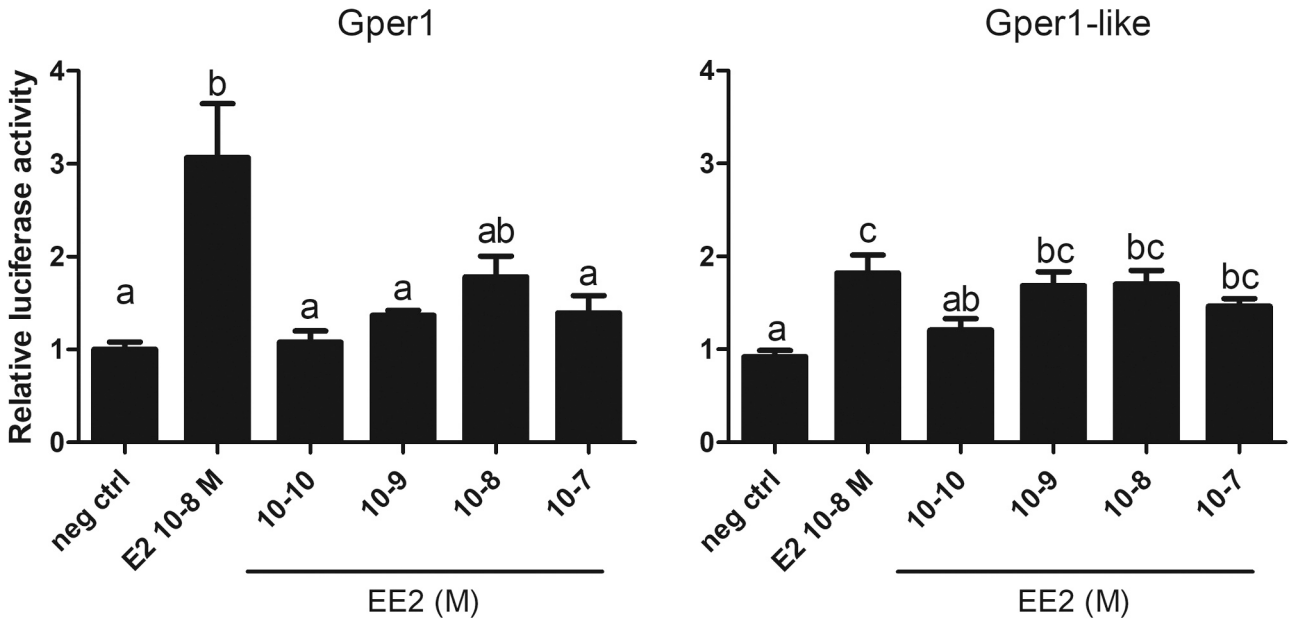

Fig. 6. Luciferase Activity of HEK293 cells co-transfected with each membrane estrogen receptors of seabass (Gper1 and Gper1-like) and the pCRE-Luc reporter plasmid, in response to the exposure to EE2. E2 at 10^{-8} M was used as positive control. Induction is expressed relative to the control cells (not exposed to the compounds) that were set as 1, in relative light units (RLUs). Bars present the mean ± SEM of at least two independent experiments performed with triplicate measurements. Different superscript letters represent significantly different values (ANOVA, Tukey post-hoc or Kruskal-Wallis, $p \leq 0.05$).

coverage we could include a higher number of sequences in our study, as well for key species at the basis of fish radiation. Contrary to what has been suggested in previous studies [8–11], our phylogenetic analysis, coinciding with the analysis available at Ensembl (constructed as consensus of four different methodologies and using all available genome sequences; Suppl. Fig. S1) and the synteny analysis (showing no conserved gene environment between these duplicates) do not support the hypothesis that the *gpers* originated in the TSGD event. Phylogenetic analysis would support that they originated from one of the two WGD-events that occurred in the course of chordate evolution, and then one of the duplicates could have been lost in tetrapods and, therefore, in the branch of Sarcopterygii only one of the *gper* genes exists. However, this hypothesis is not supported by our synteny analysis. In addition, the European eel *gper1a* and *gper1*b seem to have resulted from a later WGD in a common ancestor of freshwater Anguilliformes [54], as the two European eel *gpers* are included in the same clade as the *gper1* of other teleosts in the phylogenetic analysis and clearly separated from the *gper1-like*. It is worth mentioning the case of the ricefield eel where both Gpers cluster with the Gpers from other teleosts, differently from the European eel. This may be related to the fact that the ricefield eel is "eel-like" but does not belong to the Anguilliformes order as the European eel, but rather belongs to the Synbranchiformes order for which their Gpers appear to be more closely related to the other teleosts. In line with this result, in previous phylogenetic trees [11,25], the European eel *gper1b* was also not included in the same clades as the other teleost *gper1-like* genes. This finding is further corroborated by the current multiple sequence alignment, in which both European eel Gpers are more conserved in relation to teleost *gper1* than they are to *gper1-like*, reinforcing the results from our previous alignment [11] in which only two *gper1-like* annotated sequences (seabass and the European eel) had been included. Thus, the European eel duplicates appear to represent a different duplication compared to that originating the teleost *gper1* and *gper1-like* genes, which show clearly divergent sequences between them. These differences include the first cysteine residue and the tyrosine residue of the DRY-triplet sequence, known to be highly conserved among numerous GPCRs. This triplet is likely to be involved in signal transduction [55] and is conserved across all Gper1 sequences, except in the Gper1-like of teleosts. In addition, the highly divergent C-terminal tail previously reported for the sbsGper1-like and hypothesised to play a role in different intracellular signalling of this receptor [11], was confirmed for all other available teleost Gper1-like sequences. Taken together, these results indicate a divergence of the sequence between Gper1 and Gper1-like, possibly affecting protein function, which does not occur in the case of both European eel Gpers. In a previous syntenic analysis [8,9] several of the GPER neighbouring genes were duplicated only in the European eel and not in other species, which supports the hypothesis of *gper1b* from European eel being a specific duplication, more similar to *gper1* than to the *gper1-like* from teleost. This is confirmed by our synteny in which the European eel shows several duplications of the neighbouring genes, which may have originated from another WGD that occurred in a common ancestor of freshwater eels or a lineage specific rediploidization after 3R-WGD [54]. Our syntenic analyses suggest that *Gper1-like* did not originate in the 3R-WGD (TSGD), since the basal teleost Asian bonytongue that emerged after this duplication [53] has only one *gper1* sequence, in conserved synteny with *gper1* from tetrapods and non-teleost fish. The genomic region of *gper1* may have suffered a chromosomic fission with several recombinations in more advanced teleosts that do not include Osteoglossomorpha (Asian bonytongue) and Elopomorpha (eel), leading to the specific duplication of the *gper1* gene in teleosts. In fact, this region around *gper1* also underwent chromosomic fission in very distant species, as the *Homo sapiens*, although with no gene duplication in this case. Therefore, both teleost *gper* duplicates probably have not resulted from the teleost-specific whole genome duplication and they should be named as *gper1* and *gper1-like*, as it was decided to name for the European sea bass in light of these results.

4.2. *European sbsGpers functional characterization*

In the present study we also demonstrated that European seabass Gper1 and Gper1-like are functional G-protein coupled receptors acting, at least in part, *via* the cAMP signalling pathway. Both Gpers could activate transcription directed by a cAMP-response element in an estrogen-dependent manner. Gper activation by E2 had previously been demonstrated in some teleost species using different assays [9,10,26,27,56], but the ricefield eel and the Japanese medaka were the only species

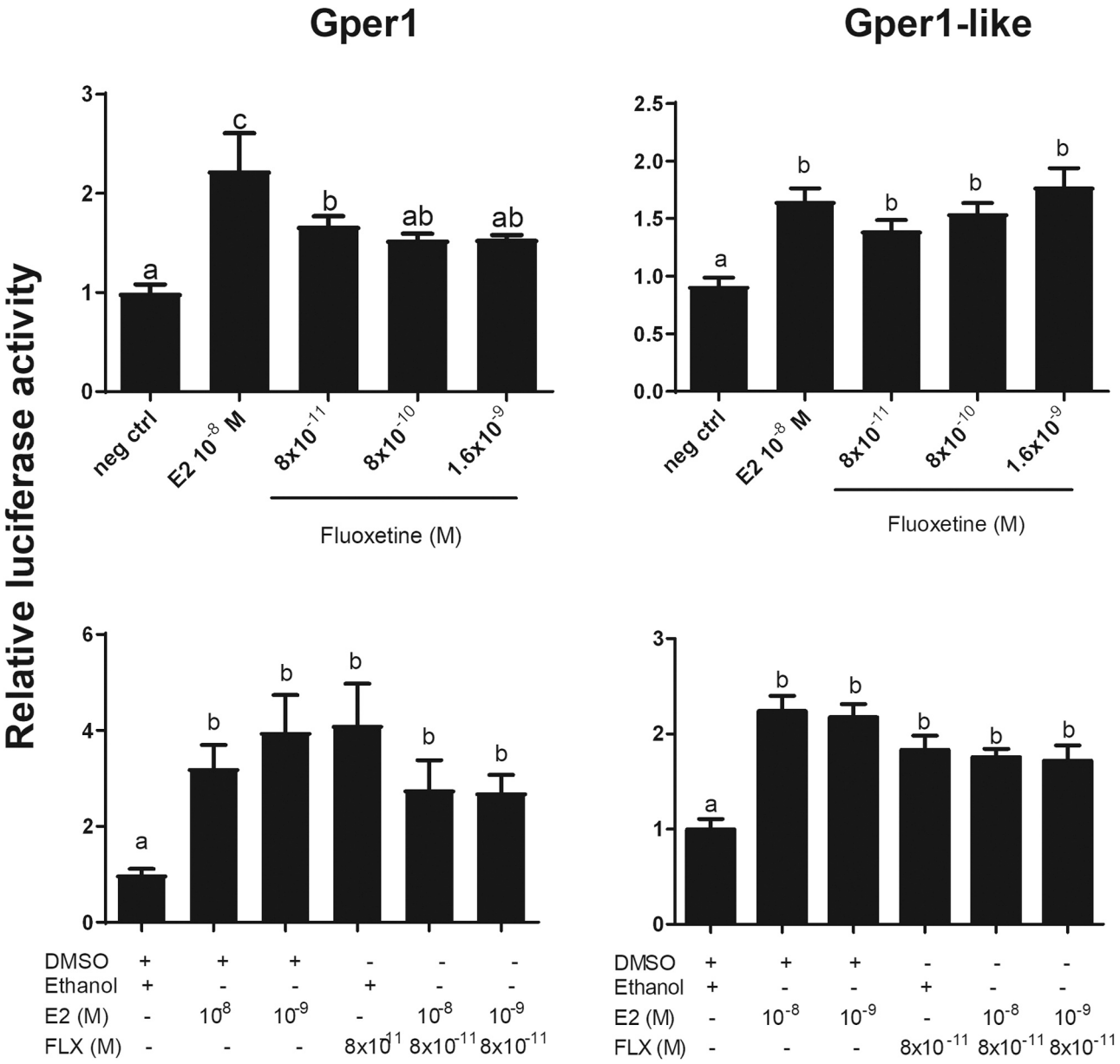

Fig. 7. Luciferase Activity of HEK293 cells co-transfected with each membrane estrogen receptors of seabass (Gper1 and Gper1-like) and the pCRE-Luc reporter plasmid, in response to the exposure to Fluoxetine (FLX) alone or in mixture with E2. E2 at 10^{-8} M or at 10^{-9} M were used as positive control. Induction is expressed relative to the control cells (not exposed to the compounds), which were set as 1, in relative light units (RLUs). Bars present the mean ± SEM of at least two independent experiments performed with triplicate measurements. + represents presence and - represents absence of each compound. Different superscript letters represent significantly different values (ANOVA, Tukey post-hoc, $p \leq 0.05$).

where the functional characteristics of the two duplicate Gpers were compared so far, measuring estrogens impacts on cAMP-driven transcription/cAMP production or in protein-kinase phosphorylation, respectively [9,10]. The effective doses for cAMP-driven reporter gene activation were within the same ranges (10 −100 nM) using sea bass or ricefield eel Gpers [9], but while in the ricefield eel the activation of both Gpers was more similar, sbsGper1 was slightly more potent than sbsGper1-like, also achieving significant activation by E2 at 1 nM. Interestingly, when using E2-BSA as ligand for the ricefield eel Gpers, similar activations compared to E2 alone were obtained, further supporting that the majority of the cAMP-driven transcriptional activation was induced at the membrane [9].

In this study, we also demonstrated cAMP-driven transactivation with both sbsGpers and E3, considered a weak natural estrogen in the activation of nuclear Esrs [*e.g.* 15, 16], which had an activity starting at 1 nM with both sbsGpers (similar range to E2, starting at 10 nM with sbsGper1-like and at 1 nM with sbsGper1). To date, only one study investigated the interaction of E3 with fish Gpers [26], demonstrating its binding to the Atlantic croaker Gper1 (in agreement with the results of the present study), although with weaker affinity than that of E2. However, E3 was not capable of significant binding to human GPER1 or increasing cAMP in its presence, contrary to E2 [6]. Interestingly, both seabass Gpers were also capable of cAMP-driven transcription activation by T, contrary to what was observed in humans and in Atlantic croaker, where no specific binding of T for Gper1 or for plasma membranes of Gper-transfected cells was detected [6,26]. Previous studies also demonstrated that the sea bass nuclear estrogen receptors can be activated by T [15,16], suggesting a greater versatility for European seabass receptors. Concerning the specific agonist and antagonist of human GPERs, G-1 [28] and G-15 [29], respectively, G-1 behaved as a weak

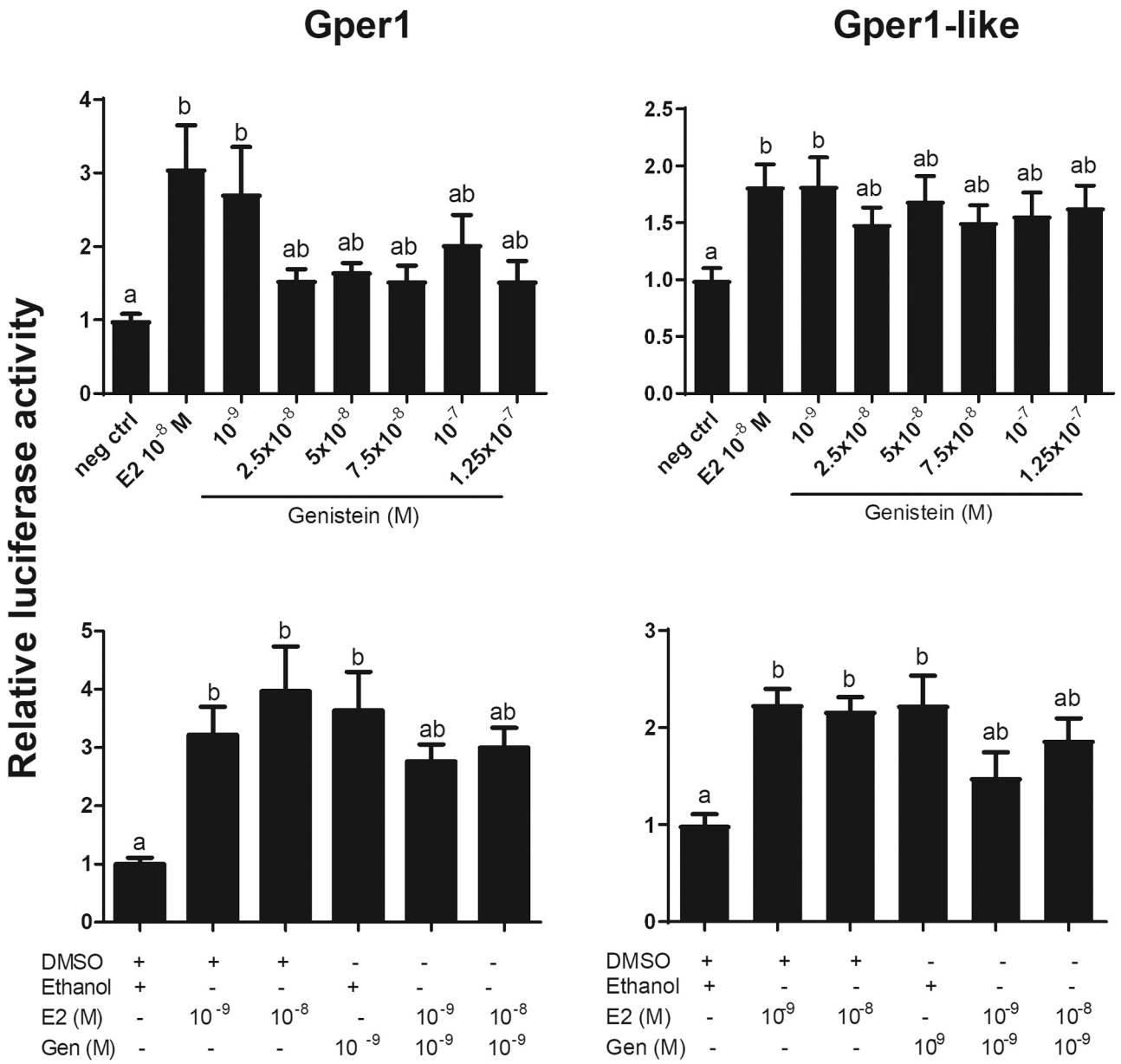

Fig. 8. Luciferase Activity of HEK293 cells co-transfected with each membrane estrogen receptor of seabass (Gper1 and Gper1-like) and the pCRE-Luc reporter plasmid, in response to the exposure to Genistein (GEN) alone and in mixture with E2. E2 at 10^{-8} or at 10^{-9} M were used as positive control. Induction is expressed relative to the control cells (not exposed to the compounds), which were set as 1, in relative light units (RLUs). Bars present the mean ± SEM of at least two independent experiments performed with triplicate measurements. + represents presence and - represents absence of each compound. Different superscript letters represent significantly different values (ANOVA, Tukey post-hoc, $p \leq 0.05$).

agonist with sbsGper1 when compared to E2 while with Gper1-like it was as strong as E2, and G-15 acted as an antagonist for both sbsGpers. This corroborates that ligand specificity and activity differ between both Gpers. Gper1 has approximately twice the activity of Gper1-like as observed in medaka [10], but not in ricefield eel [9]. Together with the differences in their amino acid sequence and the more divergent C-terminal domain for Gper1-like, this suggests differential physiological roles for both membrane receptors in teleosts, making it likely that Gper1-like have acquired neofunctionalization. The expression of both *gper1* and *gper1-like* mRNA were detected in reproductive and non-reproductive tissues with differential patterns in European eel [8,25], European sea bass [11,19], Grass carp [57] and ricefield eel [9]. In the European eel both *gper* receptors had a widespread tissue distribution and generally similar regulation of transcript expression in the hypothalamus-pituitary-gonads (HPG) axis along the maturation of female or male fish [8,25]. For the other teleost species, differential patterns of tissue distribution and differential regulation across different developmental or reproductive stages were detected between the two receptors [9–11,17–19,57], reinforcing the hypothesis that the two teleost *gper* duplicates have evolved different physiological functions.

In addition, it has been demonstrated in humans that GPER signalling can interact with that of nuclear estrogen receptors by direct protein interactions or by affecting *Esr* expression levels, although these complex interactions may vary according to cell type and developmental stage [22]. Following the demonstration that both sbsGpers are functional, it will be interesting in the future to further investigate their potential interactions, possible regulation of expression levels and relative roles in estrogen-mediated actions or disruption.

4.3. Evaluating chemicals estrogenicity using the sbsGper transactivation assays

Regarding potential estrogenic compounds, the synthetic estrogenic drug and widespread environmental pollutant EE2 [58] was able to activate Gper1-like to comparable levels as those induced by E2, but no significant activation of Gper1 was achieved using the cAMP-driven transcriptional activation as an endpoint. Both the phytoestrogen GEN and the antidepressant FLX were able to transactivate *via* the two sbsGpers, with Gper1-like appearing to be more activated by the tested pollutants than Gper1. In medaka, EE2 also activated both membrane estrogen receptors although it was more potent in activating Gper1-like than Gper1 [10], in line with our results in sea bass. Differential transactivation by nuclear estrogen receptor duplicates stimulated with EE2 has also been observed in zebrafish [30]. In addition, as in our study, in 48-hpf zebra fish embryos EE2 was less potent than E2 on GPER than on ESR2 [59]. GEN has been previously demonstrated to be a weak ligand that activates zebrafish nuclear estrogen receptors with no particular differences in sensitivity among the three receptors [60,61]. In sea bass, GEN has shown a higher potency activating both Esr2 forms, being slightly higher for Esr2a than for Esr2b [31]. Nevertheless, all sea bass receptors had lower transactivation activity when stimulated with genistein than with E2, as has previously been described for the zebrafish Esrs. In the present study, GEN activated both Gpers with similar cAMP-driven transactivation levels as the positive control E2. In mammals it was also able to bind and activate GPERs at similar levels than E2 [62,63], suggesting this phytoestrogen can strongly influence Gper-mediated estrogenic actions. This result is relevant in the context of pollution as well as animal and human nutrition, since this natural phytoestrogen can be found as a xenoestrogen in the aquatic environments; its levels are also increasing in aquaculture fish feeds (due to inclusion of increasing proportions of plant ingredients to improve production sustainability) [34,35], and in human nutrition (due to the popularity of plant-based diets and supplements) [64]. In addition, GEN is also receiving greater attention as a potential anti-inflammatory agent for autoimmune diseases, notably the multiple sclerosis [65].

Finally, we provide evidence that in sea bass the highly prescribed pharmaceutical antidepressant and environmental pollutant FLX [40,66] can also act *via* Gper-mediated pathways by activating both Gpers, especially Gper1-like. In contrast, FLX alone was not able to activate any of the sea bass nuclear receptors [45] but could interfere with their activation by E2. In the present study, GEN and FLX showed a trend for weak antagonism of both Gpers when combined with E2; the luciferase levels obtained in combined treatments were not statistically significant from those obtained for the stimulation with E2 alone but GEN rendered the E2 activation of Gper1/Gper1-like to be no longer statistically different from the control. Globally, sea bass Gpers are activated by xenoestrogens and, as observed with steroid hormones, have different activity and ligand affinity for each of the EDCs tested.

5. Conclusions

More comprehensive availability of sequenced genomes allowed us to perform a deep study on the origin of *gpers*. Contrary to what was previously suggested, we could neither find any evidence supporting the origin of teleost *gper1* and *gper1-like* as result of the TSGD, nor of their origin in more ancient genome duplications. Our data would rather support the origin of these duplicated genes as result of a translocation event between chromosomes happening in some teleost branches after TSGD. Notwithstanding, the evolutionary history of the *gpers* seems to be very complex, and further availability and advanced analyses of additional genomes may help to elucidate the exact origin of both duplicates in the future. The present study is one of the first studies to extensively characterize the functional characteristics of two fish Gpers and to test different estrogenic and steroid compounds and also different compounds with / or affecting estrogenic activity. We clearly demonstrated that Gpers act *via* the cAMP signalling pathway and respond differentially to the different estrogenic compounds, suggesting more conserved roles for Gper1 than for Gper1-like. The latter demonstrates that Gpers are able to respond to different endocrine-disrupting compounds, a point that should be taken into account in aquatic environment pollution screenings and chemical risk assessment, complementing previous assays for sea bass nuclear estrogen receptors [31,32].

Author statement

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript.

Declaration of Competing Interest

The authors have no competing interests to declare.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jsbmb.2023.106423.

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