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The expression of nuclear and membrane estrogen receptors in the European eel throughout spermatogenesis

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ABSTRACT

Estradiol (E_2) can bind to nuclear estrogen receptors (ESR) or membrane estrogen receptors (GPER). While mammals possess two nuclear ESRs and one membrane GPER, the European eel, like most other teleosts, has three nuclear ESRs and two membrane GPERs, as the result of a teleost specific genome duplication. In the current study, the expression of the three nuclear ESRs (ESR1, ESR2a and ESR2b) and the two membrane GPERs (GPERa and GPERb) in the brain-pituitary-gonad (BPG) axis of the European eel was measured, throughout spermatogenesis.

The eels were first transferred from freshwater (FW) to seawater (SW), inducing parallel increases in E_2 plasma levels and the expression of ESRs. This indicates that salinity has a stimulatory effect on the E_2 signalling pathway along the BPG axis.

Stimulation of sexual maturation by weekly injections of human chorionic gonadotropin (hCG) induced a progressive decrease in E_2 plasma levels, and different patterns of expression of ESRs and GPERs in the BPG axis. The expression of nuclear ESRs increased in some parts of the brain, suggesting a possible upregulation due to a local production of E_2. In the testis, the highest expression levels of the nuclear ESRs were observed at the beginning of spermatogenesis, possibly mediating the role of E_2 as spermatogonia renewal factor, followed by a sharply decrease in the expression of ESRs. Conversely, there was a marked increase observed in the expression of both membrane GPERs throughout spermatogenesis, suggesting they play a major role in the final stages of spermatogenesis.

1. Introduction

In male vertebrates, sex steroids, androgens, estrogens, and progestins, play significant roles in the control of spermatogenesis (Schulz and Miura, 2002), process in which diploid spermatogonia differentiate to mature haploid spermatozoa. Estrogens have been shown to be indispensable for the early spermatogenic cycle, controlling the spermatogonial stem cell renewal through its receptor (Miura et al., 1999; Miura and Miura, 2011). Estradiol (E_2), as all sex steroids is a small lipophilic hormone, which can diffuse through the cell membrane (Oren et al., 2004). E_2 can bind to intracellular nuclear estrogen receptors (ESRs) and modulates gene transcription (Mangelsdorf et al., 1995), which corresponds to the classic genomic mechanism of steroid action. Two nuclear ESRs, ESR1 and ESR2 (also named ERα or NR3A1, and ERβ or NR3A2, respectively), are present in mammals. They belong to the nuclear steroid receptor superfamily, as well as androgen, progestin, gluco- and mineralocorticoid receptors (Carson-Jurica et al., 1990; Laudet et al., 1992). Teleost species have at least three distinct ESR subtypes, including ESR1, ESR2a and ESR2b (Hawkins et al., 2000; Ma et al., 2000; Menuet et al., 2002), with ESR2a (also named ERβ2) and ESR2b (also named ERβ1) resulting from the third whole genome duplication (3R) event that occurred in teleost lineage (Hawkins et al., 2000; Lafont et al., 2015).

In addition to the classic genomic functions, E_2 can bind itself to membrane receptors, which activates intracellular signalling pathways through a fast, non-genomic action (for review see: Thomas, 2012, or Nelson and Habibi, 2013). In mammals, the former orphan receptor GPR30 was characterized as an E_2 membrane receptor, and is also called G-protein coupled estrogen receptor GPER (Filardo and Thomas, 2005; Filardo et al., 2007; for review see Prossnitz and Maggiolini, 2009). Two membrane GPERs have recently been observed in most teleosts including the eel, likely resulting from teleost 3R (Lafont et al., 2015).

The European eel (*Anguilla anguilla*) has a complex catadromous life cycle which includes a 5000–6000 km oceanic reproductive migration to reach its spawning site in an unknown area of the Sargasso sea. Eels

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are euryhaline fish which are subjected to high variations in salinity during their life cycle (Daverat et al., 2006). After their juvenile growth period in continental waters, eels change from yellow eels to prepubertal silver eels, future genitors that will undergo the transoceanic reproductive migration. In captivity, the reproductive cycle is still not closed, and long-term hormonal treatments (fish pituitary extracts for females, and human chorionic gonadotropin, hCG, for males) are required to induce sexual maturation in silver eels (Boëtius and Boëtius, 1967; Pérez et al., 2000; Asturiano et al., 2006; Gallego et al., 2012). This, together with the dramatic reduction in the wild European eel population (ICES, 2012) has increased the interest in deciphering the basic mechanisms controlling the reproduction of this species. Furthermore, the phylogenetical position of the European eel, branching at the base of teleosts, may provide insights into ancestral regulatory functions in teleosts, the largest group of vertebrates (Henkel et al., 2012a,b). As far as we know, this is the first study on male teleosts to look at the expression of the three nuclear (ESR1, ESR2a and ESR2b) and two membrane (GPERa, GPERb) estrogen receptors in the BPG axis throughout the spermatogenetic process.

2. Material and methods

2.1. Fish maintenance, hormonal treatments and sampling

Eighty male European eels (mean body weight 100 ± 6 g) were purchased from the fish farm Valenciana de Acuicultura, S.A. (Puzol, Valencia, Spain) and transferred to the Aquaculture Laboratory in the Polytechnic University of Valencia. The 80 males were randomly distributed and kept at 20 °C in two freshwater 200-L aquaria equipped with separated recirculation systems, thermostats/coolers, and covered to maintain constant darkness.

One group of 8 eels was anaesthetized with benzocaine (60 ppm) and sacrificed by decapitation in freshwater (FW). The rest of the fish were gradually acclimatized over the course of one week to seawater (37 ± 0.3‰ of salinity). Groups of 8 eels were anaesthetized and sacrificed by decapitation in seawater conditions (SW). Once a week for 8 weeks the rest of the fish were anaesthetized, weighed and injected with hCG (1.5 IU g^{-1} fish; Profasi, Serono, Italy), to induce the spermatogenesis as previously described by Pérez et al. (2000). Groups of 8 eels were anaesthetized and sacrificed by decapitation each week (W1–8) through the hormonal treatment. For the analysis of ESR expression through the spermatogenesis, the 8 latter groups have been redistributed to 4 groups based on their spermatogenic stage.

Total body weight and testis weight were recorded to calculate the gonadosomatic index [GSI = (gonad weight/total body weight)*100]. Blood samples were collected, centrifuged and stored at −20 °C until E_2 plasma level analysis. Testicular tissue samples were fixed in 10% formalin buffered at pH 7.4 for histological analysis.

Samples of anterior brain (dissected into three parts: olfactory bulbs, telencephalon, mes-/di-encephalon), pituitary and testis were stored in 0.5 ml of RNAlater (Ambion Inc., Huntingdon, UK) at −20 °C until extraction of total RNA.

Because eels stop feeding at the silver stage and throughout sexual maturation thee fish were not fed throughout the experiment. They were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC).

2.2. Gonadal histology

The formalin-fixed mid-part testis samples were dehydrated in ethanol, embedded in paraffin, sectioned to 5–10 μm thickness with a Shandom Hypercut manual microtome (Shandon, Southern Products Ltd., England), and stained using the haematoxylin and eosin method of National Diagnostic (www.nationaldiagnostics.com/histology/article/staining-procedures). Five slides per fish were observed with a Nikon Eclipse E-400 microscope, and pictures were taken with a Nikon DS-5M camera attached to the microscope (Nikon, Tokyo, Japan). The stages of spermatogenesis were determined according to the germ cell types present in the testis (Miura and Miura, 2011; Leal et al., 2009) their relative abundance, the degree of development of the seminal tubules and the sperm production of the male at the time of sacrifice (Morini et al., submitted for publication). The stages considered were: Stage SPGA: dominance of A spermatogonia, B spermatogonia present in low numbers; Stage SPGB/SPC: dominance of B spermatogonia and spermatocytes, in some cases low numbers of spermatids; Stage SD: dominance of spermatids, in some cases a small number of spermatozoa; Stage SZ: dominance of spermatozoa (Fig. 1).

2.3. Extraction and reverse-transcription

Total RNA of the testis, anterior brain parts and pituitary were isolated using a Trizol reagent (Life Technologies, Inc., Carlsbad, CA) as described by Peñaranda et al. (2013). RNA concentration was evaluated using a NanoDrop 2000C Spectrophotometer (Fisher Scientific SL, Spain). The testis RNA was treated using a DNase I of NucleoSpin RNA XS kit (Macherey-Nagel, Düren, Germany). Twenty μl cDNA were synthesized from 500 ng of testis total RNA, using a qScript cDNA Synthesis Kit (Quanta Bioscience, MD, USA). The brain parts and pituitary RNAs were treated using a DNase (gDNA Wipeout Buffer, Qiagen, Hilden, Germany). Using a Quantiscript Reverse Transcriptase (Qiagen, Hilden, Germany), 20 μl cDNA was synthesized from 500 ng of total RNA in the case of the olfactory bulb and pituitary, and from 1 μg in the case of the telencephalon and the mes-/diencephalon.

2.4. Gene expression analyses by quantitative real-time PCR

The quantitative real-time Polymerase Chain Reactions (qPCR) were carried out using specific qPCR primers for each European eel estrogen nuclear and membrane receptor (Lafont et al., 2015) and the Acidic ribosomal phosphoprotein P0 (ARP) (Weltzien et al., 2005) was used as the reference gene (Table 1).

2.4.1. Reference gene

The stability of the reference gene was determined using the BestKeeper program (Pfaffl et al., 2004), reporting a standard deviation (SD[±Cq]) lower than 1. The BestKeeper calculated that variations in the reference gene are based on the arithmetic mean of the Cq values. Genes with a SD value higher than 1 are defined as unstable. In the testis: SD = 0.83; $p < 0.05$ with a Cq geometric mean of 24.21 ± 1.77; in the brain and pituitary, olfactory bulb: SD = 0.81; telencephalon: SD = 0.48; mes-/diencephalon: SD = 0.58, pituitary: SD = 0.63; $p < 0.05$ and the Cq geometric mean of the olfactory bulb: 23.39 ± 1.76; telencephalon: 21.76 ± 1.40; mes-/diencephalon: 21.89 ± 1.49; pituitary: 22.34 ± 1.55.

2.4.2. SYBR Green assay

To determine the expression of each ESR and GPER gene, qPCR assays were performed using a model 7500 unit (Applied Biosystems; Foster City, CA, USA) with Maxima SYBR Green/ROX qPCR Master Mix (Fermentas Corp. Glen Burnie, MD, USA). The qPCR program used for all was an initial step of 50 °C for 2 min, followed by 95 °C for 10 min, and 40 cycles of 95 °C for 1 s and 60 °C for 10 s and 72 °C for 7 s. To evaluate assay specificity, the machine performed a melting curve analysis directly after PCR by slowly (0.1 °C/s) increasing the temperature from 68 to 95 °C, with a continuous registration of any changes in fluorescent emission intensity.

The total volume for each qPCR reaction was 20 μl, with 5 μl of diluted cDNA template, forward and reverse primers (250 nM each), and SYBR Green/ROX Master Mix (12 μl). The transcript levels were determined by the efficiency-adjusted relative quantification method described by Weltzien et al. (2005). Serial dilutions of the cDNA pool of the gonad tissues were run in duplicate and used for the standard

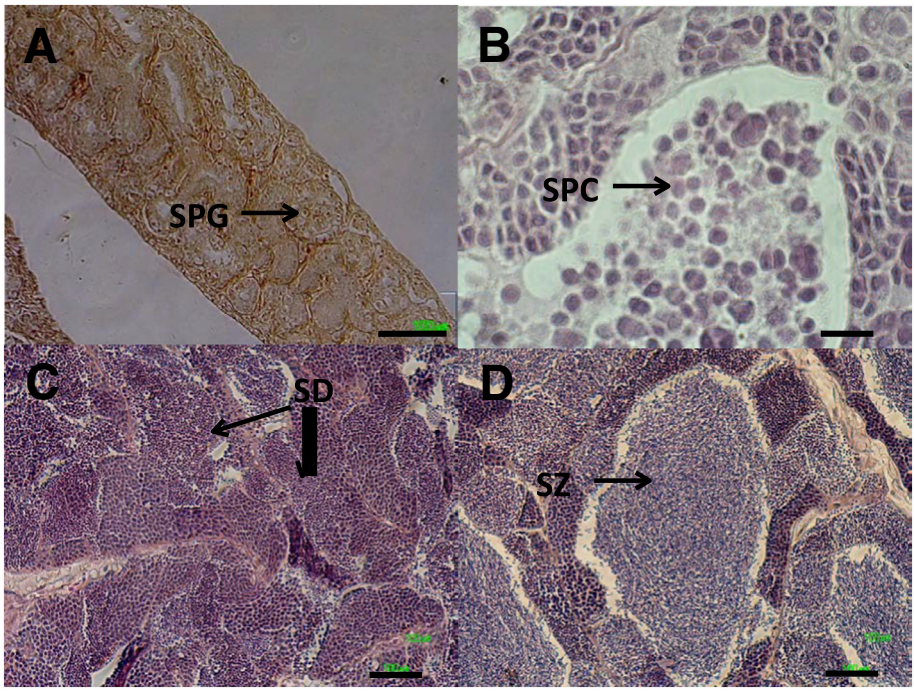


Fig. 1. Histological sections of European eel testis at different developmental stages during human chorionic gonadotropin (hCG) hormonal treatment. A: SPGA (spermatogonia A); B: SPGB/SPC (spermatogonia B/spermatocyte); C: SD (spermatid), D: SZ (spermiation). Scale bar: A = 100 μm; B = 10 μm, C, D = 50 μm; Cell types: SPG = spermatogonia; SPC: spermatocytes; SD: spermatids; SZ: spermatozoa.

curve to measure all ESRs and GPERs in the testis. Serial dilutions of the cDNA pool of the brain and pituitary tissues were used for the standard curve from which to measure all the ESRs and GPERs in the different parts of the brain and pituitary. A1/32 dilution of the standard curve was included in each run of the corresponding gene as a calibrator. Target and reference genes in unknown samples were run in duplicate PCR reactions. A non-template control (cDNA replaced by water) for each primer pair was run in duplicate on all plates. All ESR and GPER data were normalized to eel reference gene ARP. qPCR calculations were performed according to the Roche Applied Science protocol, Technical Note No. LC 13/2001, part 4 "Calibrator normalized relative quantification".

2.5. Immunoassays for E_2

Plasma concentrations of E_2 were measured by means of radioimmunoassay (RIA), according to the method described by Schulz (1985). Free (i.e. not conjugated) steroids were extracted from 200 μl plasma, with 4 ml diethyl ether after vigorously shaking for 4 min. The aqueous phase was frozen in liquid nitrogen, whereas the organic phase was transferred to a glass tube, evaporated in a water bath at 45 °C and then reconstituted by addition of 600 μl assay buffer, and then assayed for E_2. The assay characteristics and cross-reactivities of the E_2 antiserum were previously examined by Frantzen et al. (2004) and further validated for eel plasma by Mazzeo et al. (2014). The limit for the assay was 0.2 ng/ml. The inter- and intra-assay coefficients of variation (CV) for the E_2 assay were 9.40% (n = 4) and 8.39% (n = 11), respectively. The standard curve was made up of nine different concentrations of non-radioactive steroid, and ranged from 0.15 to 40 ng/ml. It was prepared by eight (1:1) serial dilutions in RIA buffer, starting at 40 ng/ml.

2.6. Statistics

Statistical analyses were performed to study the expression of ESRs and GPERs in the different tissues, as well as the E_2 plasma levels in FW, SW and throughout spermatogenesis (SPGA, SPGB/SPC, SD or SZ).

Two non-parametric tests were performed. Means between FW and SW conditions were compared by Mann-Whitney *U* test. Means between the stage of spermatogenesis were compared by Kruskal-Wallis ANOVA. Differences were considered significant when $p < 0.05$.

All statistical procedures were performed using Statgraphics Plus 5.1 (Statistical Graphics Corp., Rockville, MO, USA). Results are presented as mean ± standard error (SEM).

Table 1
Quantitative PCR primer sequences for nuclear estrogen receptors (*ESR1*, *ESR2a*, *ESR2b*) and membrane progestin receptors (*GPERa* and *GPERb*).

Name	Sequence (5′-3′)	Orientation	Tm	Reference	Accession number	Primer efficiency	Amplicon size (bp)
ARP	GTGCCAGCTCAGAACACTG	Forward	56.36	Weltzien et al. (2005)	AAV32820	2.14	107
	ACATCGCTCAAGACTTCAATGG	Reverse	60.09				
ESR1	GCCATCATACTGCTCAACTCC	Forward	58.20	Lafont et al. (2015)	CUH82767	2.04	75
	CCGTAAAGCTGTCGTTCAGG	Reverse	59.32				
ESR2a	TGTGTGCCTCAAAGCCATTA	Forward	58.71	Lafont et al. (2015)	CUH82768	2	68
	AGACTGCTGCTGAAAGGTCA	Reverse	57.16				
ESR2b	TGCTGGAATGCTGCTGGT	Forward	59.93	Lafont et al. (2015)	CUH82769	1.95	120
	CCACACAGTTGCCCTCATC	Reverse	58.44				
GPERa	CAACTTCAACCACCGGGAGA	Forward	61.81	Lafont et al. (2015)	CUH82770	1.99	165
	TGACCTGGAGGAAGAGGGACA	Reverse	62.86				
GPERb	CAACCTGAACCACACGGAAA	Forward	60.36	Lafont et al. (2015)	CUH82771	1.97	162
	TGACCTGGAAGAAGAGGGACA	Reverse	60.59				

3. Results

Male eel ESRs (ESR1, ESR2a and ESR2b) and GPERs (GPERa and GPERb) expressions were studied in the BPG axis after the transfer from FW to SW (before hCG treatment) and throughout hormonally induced spermatogenesis (SPGA, SPGB/SPC, SD and SZ stages). E_2 plasma levels were also measured.

3.1. Histological observation

The different spermatogenic stages were determined based on histological analyses of European eel testis during hCG hormonal treatment. Mean GSI were calculated for each spermatogenic stage: SPGA: GSI = 0.07 ± 0.02; SPGB/SPC: GSI = 0.74 ± 0.1; SD: GSI = 3.65 ± 0.4; SZ: GSI = 7.89 ± 0.4. Spermiating males were observed from the fifth week of hCG treatment (W5).

3.2. Brain estrogen receptor expressions

In all the brain parts, the expressions of the three ESRs (ESR1, ESR2a, ESR2b) increased from FW to SW (Figs. 2, 3 and 4) (9.75, 4.7 and 6.7-fold higher in SW in the olfactory bulb, respectively; 8, 5, and 3.7-fold higher in SW in the telencephalon, respectively; and 7.8, 3.8 and 3.5-fold higher in SW in the mes-/diencephalon, respectively) ($p < 0.05$).

During spermatogenesis, ESRs mRNA in the brain remained stable until the end of the experiment, except in the mes-/diencephalon, where the expression of the three ESRs increased ($p < 0.05$), with them being 1.9, 2 and 1.7-fold higher respectively at SZ than at SPGA.

Concerning the GPERs, the expression levels of GPERa in the telencephalon and GPERb in the mes-/diencephalon decreased with the change from FW to SW ($p < 0.05$), with them being 12.5 and 4-fold higher respectively. GPERa and GPERb expression levels then remained stable until the end of spermatogenesis.

3.3. Pituitary estrogen receptor expressions

In the pituitary, the expression of the three ESRs increased with the change from FW to SW ($p < 0.05$) (Fig. 5). The expression of ESR1, ESR2a and ESR2b was 7, 4 and 3-fold higher respectively, in SW than in FW. During spermatogenesis, ESR1 and ESR2b showed significant changes in the pituitary throughout testis development, while ESR2a remained stable. ESR1 expression levels increased from stage SPGA to stage SPGB/SPC ($p < 0.05$), and then decreased from SPGB/SPC until the final maturation stage SZ ($p < 0.05$). ESR2b remained stable from stage SPGA to stage SPGB/SPC, and then progressively decreased until the final maturation stage SZ.

Concerning the GPERs, the expression levels of both GPERa and GPERb increased with the change from FW to SW ($p < 0.05$), with them being 4 and 2-fold higher respectively. GPERa and GPERb expression levels then remained stable until the end of spermatogenesis.

3.4. Testis estrogen receptor expressions

In the testis, the expression levels of ESR1 and ESR2a increased with the change from FW to SW ($p < 0.05$), with them being 3 and 2-fold higher respectively (Fig. 6). In contrast, the expression of ESR2b remained stable with the change of salinity. During spermatogenesis, the expressions of the three ESRs decreased sharply from stage SPGA to SPGB/SPC: the expressions of ESR1, ESR2a and ESR2b were 7, 9.5 and 9-fold lower respectively, at stage SPGB/SPC compared to stage SPGA, then remained low until stage SZ ($p < 0.05$).

Neither GPERa nor GPERb expression levels altered significantly between FW and SW, but they did increase progressively and markedly throughout spermatogenesis ($p < 0.05$), with levels being 14.2 and 15.2-fold higher at stage SZ than at stage SPGA.

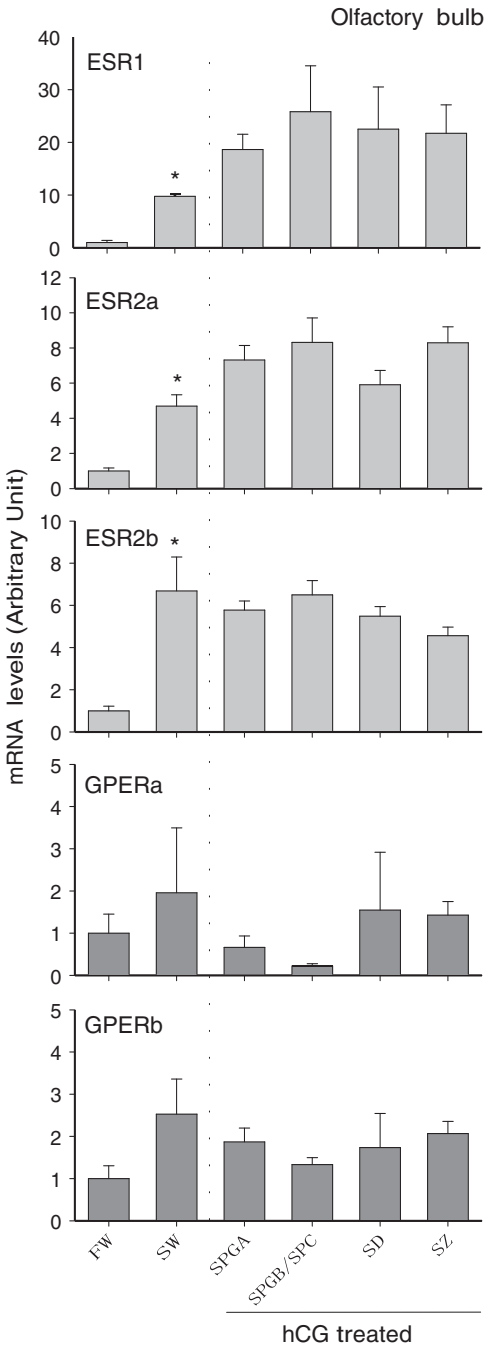
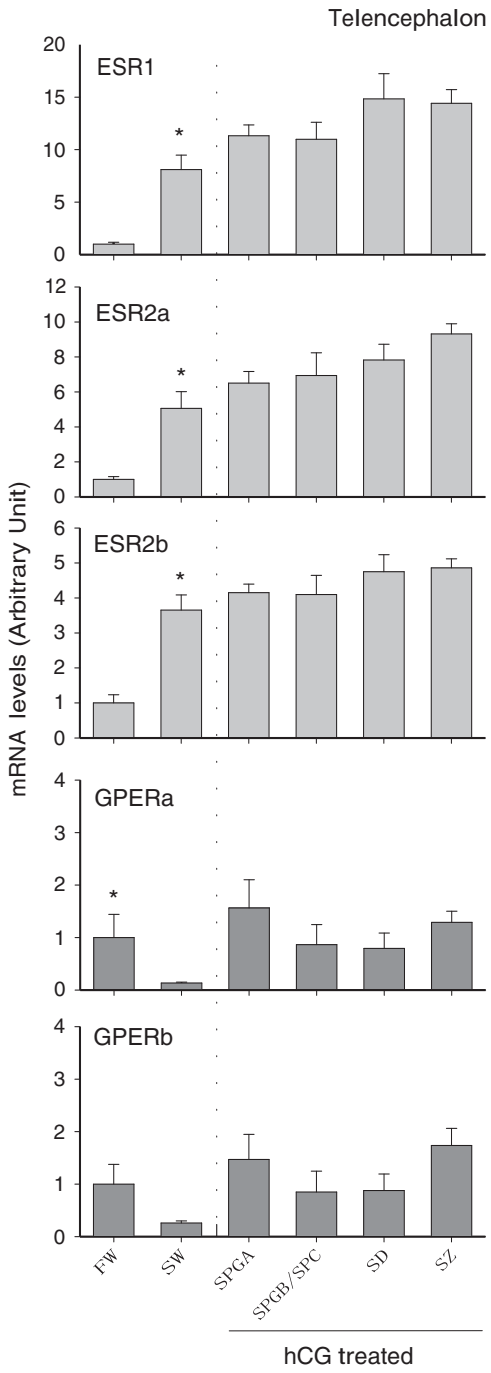


Fig. 2. Expression in the olfactory bulb of the male European eel ERs (*ESR1*, *ESR2a* and *ESRb2*) and GPERs (*GPERa* and *GPERb*) in freshwater (FW, n = 6) and sea water conditions (SW, n = 5), and through the stages of testis development. Means are given ± SEM. Differences were considered significant when $p < 0.05$. Asterisks indicate significant differences between FW and SW condition. SPGA = Spermatogonia A stage (n = 8), SPG/SPC = Spermatogonia B/Spermatocyte stage (n = 11), SD = Spermatid stage (n = 9), SZ = Spermatozoa stage (n = 18). See main text for description of development stages.

3.5. Estradiol plasma levels

E_2 plasma levels increased significantly with the change from FW to SW (Fig. 7), with them being 4-fold higher in SW than in FW ($p < 0.05$). In hCG treated males, E_2 plasma level decreased progressively until stage SZ ($p < 0.05$), and were 2-fold lower at stage SZ than at stage SPGA. By the end of spermatogenesis, E_2 plasma thus returned to very similar levels to those recorded in the initial FW stage.



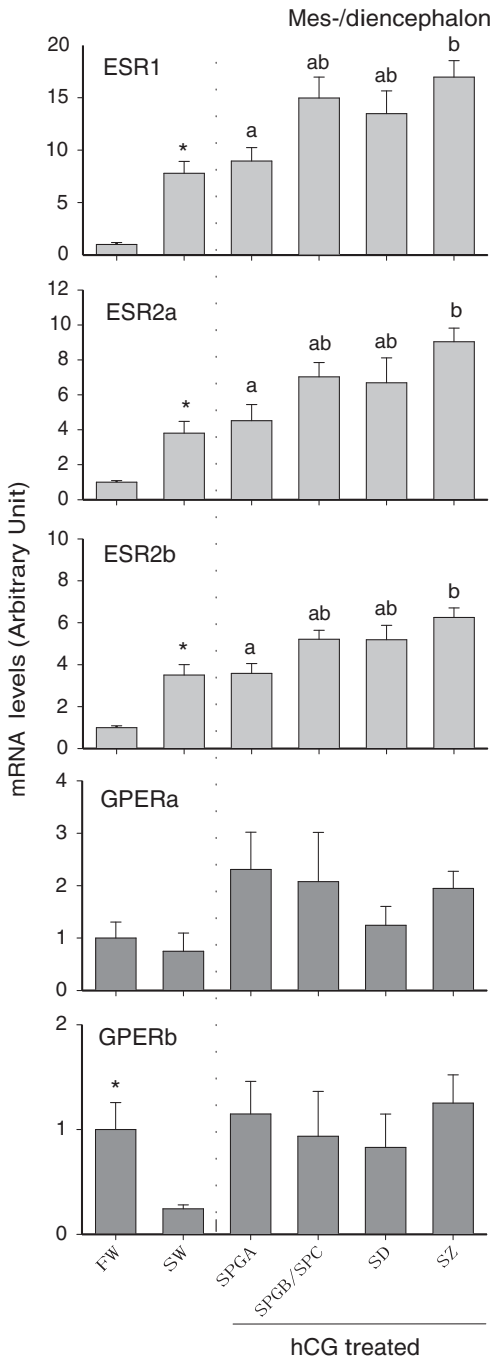


Fig. 3. Expression in the telencephalon of the male European eel ERs (*ESR1*, *ESR2a* and *ESRb2*) and GPERs (*GPERa* and *GPERb*) in freshwater (FW, n = 7) and sea water conditions (SW, n = 6), and through the stages of testis development. Means are given ± SEM. Differences were considered significant when $p < 0.05$. Asterisks indicate significant differences between FW and SW condition. SPGA = Spermatogonia A stage (n = 9), SPGB/SPC = Spermatogonia B/Spermatocyte stage (n = 11), SD = Spermatid stage (n = 10), SZ = Spermatozoa stage (n = 21). See main text for description of development stages.

Fig. 4. Expression in the mes/-diencephalon of the male European eel ERs (*ESR1*, *ESR2a* and *ESRb2*) and GPERs (*GPERa* and *GPERb*) in freshwater (FW, n = 7) and sea water conditions (SW, n = 6), through the stages of testis development. Means are given ± SEM. Differences were considered significant when $p < 0.05$. Asterisks indicate significant differences between FW and SW condition; small letters indicate significant differences though the stages of development (hCG treated). SPGA = Spermatogonia A stage (n = 9), SPGB/SPC = Spermatogonia B/Spermatocyte stage (n = 12), SD = Spermatid stage (n = 10), SZ = Spermatozoa stage (n = 23). See main text for description of development stages.

4. Discussion

4.1. Effect of salinity on estradiol and its receptors

Before any hormonal treatment, the E_2 plasma levels of immature male European eels increase sharply with the change from FW to SW (37 g/l), suggesting that salinity plays a role in sex steroidogenesis. These results match those from previous studies, where the increase in salinity to SW conditions augmented E_2 plasma levels in both male (Peñaranda et al., 2016) and female eels (Quérat et al., 1987). According to Quérat et al. (1987), the E_2 plasma level was higher in SW than in FW, in both hypophysectomized as well as in intact female silver European eels, which suggests that an extra-pituitary mechanism is at work modulating levels of E_2 with the transfer to SW. The increase of E_2 in SW may be related to the osmoregulation, as relation between osmoregulation and reproduction has been demonstrated. For instance in

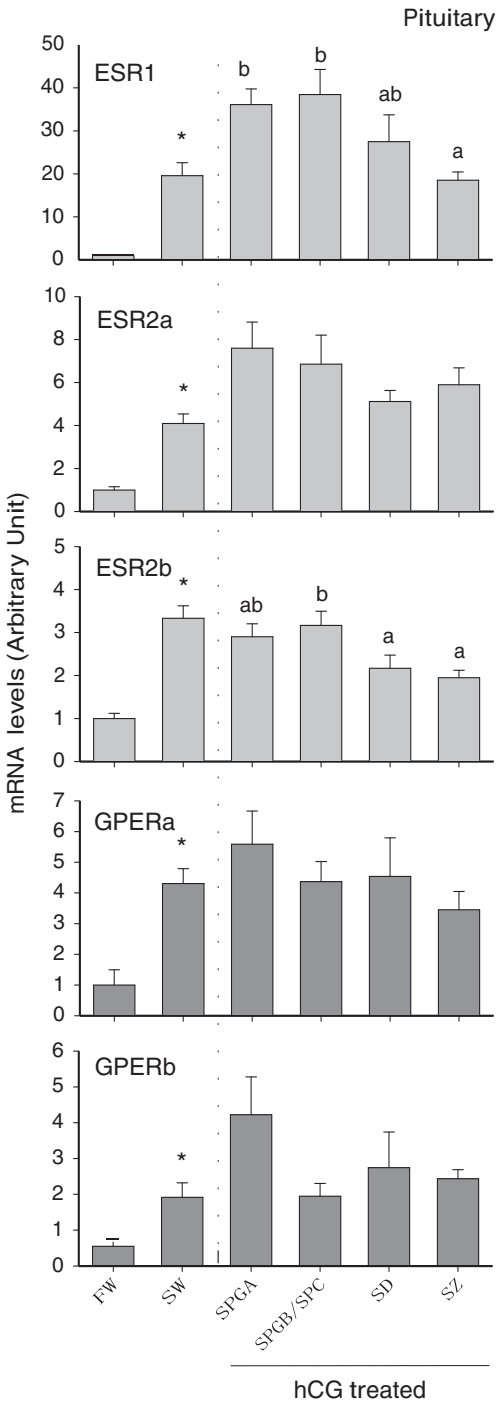


Fig. 5. Expression in the pituitary of the male European eel ERs (*ESR1*, *ESR2a* and *ESRb2*) and GPERs (*GPERa* and *GPERb*) in freshwater (FW, n = 5) and sea water conditions (SW, n = 6), and through the stages of testis development. Means are given ± SEM. Differences were considered significant when $p < 0.05$. Asterisks indicate significant differences between FW and SW condition; small letters indicate significant differences though the stages of development (hCG treated). SPGA = Spermatogonia A stage (n = 7), SPGB/SPC = Spermatogonia B/Spermatocyte stage (n = 12), SD = Spermatid stage (n = 8), SZ = Spermatozoa stage (n = 19). See main text for description of development stages.

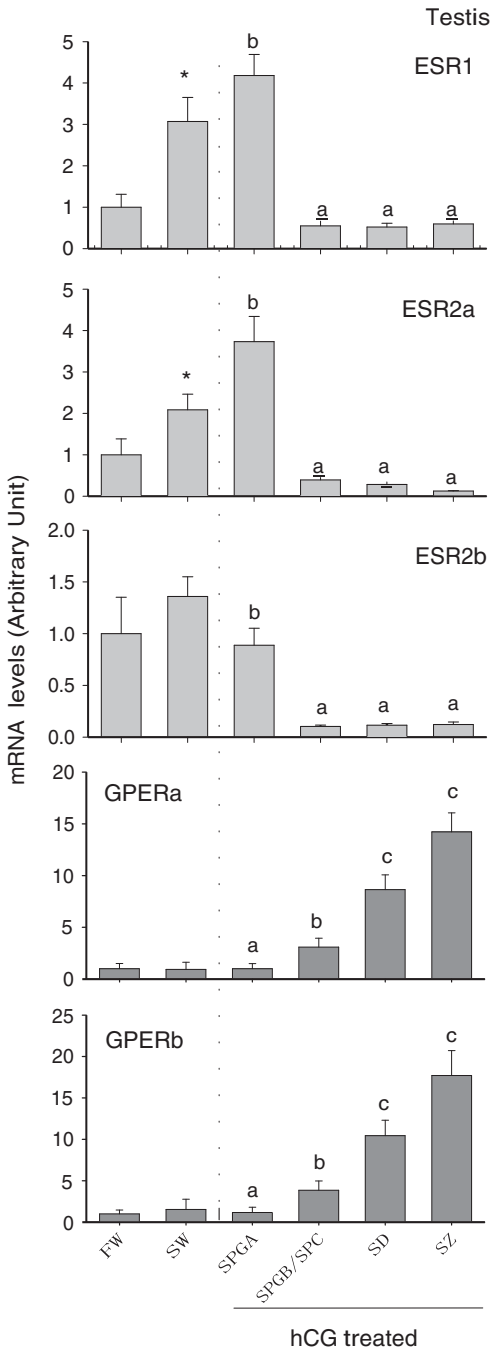


Fig. 6. Expression in the testis of the male European eel ERs (*ESR1*, *ESR2a* and *ESRb2*) and GPERs (*GPERa* and *GPERb*) in freshwater (FW, 5) and sea water conditions (SW, 7), and through the stages of testis development. Means are given ± SEM. Differences were considered significant when $p < 0.05$. Asterisks indicate significant differences between FW and SW condition; small letters indicate significant differences though the stages of development (hCG treated). SPGA = Spermatogonia A stage (n = 11), SPGB/SPC = Spermatogonia B/Spermatocyte stage (n = 14), SD = Spermatid stage (n = 10), SZ = Spermatozoa stage (n = 24). See main text for description of development stages.

salmon, an anadromous species, there is evidence of a negative relationship between sexual maturation and SW adaptability (for review, see McCormick and Naiman, 1985; Lundqvist et al., 1989; Staurnes et al., 1994; Madsen et al., 1997). E_2 is shown to be inversely correlated with pretransfer gill Na+/K+-ATPase activity: E_2 may have an important role during the development, as elevated plasma E_2 levels has a deleterious effect on hypo-osmoregulatory physiology (Madsen et al., 1997). In contrary, the catadromous European eel may respond in the opposite way to its oceanic salinity changes, attending to its reproductive migration in SW.

The change from FW to SW induced variation of E_2 receptor expression through the BPG axis. ESR1, ESR2a and ESR2b expression levels increased in the anterior brain and in the pituitary, as well as GPERa and GPERb in the pituitary, and ESR1 and ESR2a in the testis. In contrary, GPERa increased in the telencephalon and GPERb increased in the

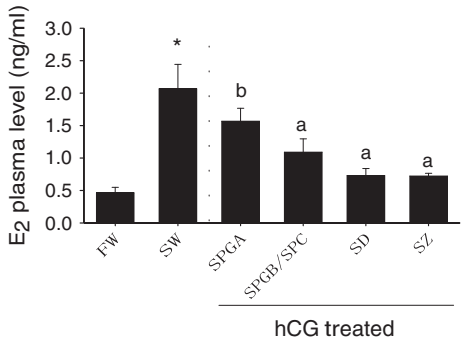


Fig. 7. Estradiol (E_2) plasma level of male European eel in freshwater (FW, n = 6) and sea water conditions (SW, n = 5), and through testis of development stage. Means are given ± SEM. Differences were considered significant when $p < 0.05$. Asterisks indicate significant differences between FW and SW condition; small letters indicate significant differences though the stages of development (hCG treated). SPGA = Spermatogonia A stage (n = 10), SPGB/SPC = Spermatogonia B/Spermatocyte stage (n = 15), SD = Spermatid stage (n = 10), SZ = Spermatozoa stage (n = 25). See main text for description of development stages.

mes-/diencephalon. These results suggest that the increase in salinity, in the absence of any hormonal treatment, affects the expression of reproductive genes along the gonadotropic axis of the male European eel.

The parallel increases in E_2 plasma levels and E_2 receptors could reflect a positive autoregulation by E_2 of the expression of its receptors. In teleosts, the ESRs appeared to be differentially regulated by E_2, according to tissue, stage of maturation, gender and species. In the Japanese female eel, E_2 treatment induces an up-regulation of ESR1 but not of ESR2 (Jeng et al., 2012b). In the European eel, while ESR and E_2 showed the same expression pattern with the change of salinity, ESR expression levels in brain and pituitary remained high during the spermatogenesis whereas E_2 plasma levels sharply decreased through the spermatogenesis. In the fathead minnow (*Pimephales promelas*), E_2 treatment induces an up-regulation of ESR1 in the testis and a down-regulation in the ovaries, a down-regulation of ESR2b in both male and female gonads, while ESR2a does not appear to be affected. In the pituitary, the three ESRs are up-regulated in females but no significant differences (ESR1, ESR2b) or decreases (ESR2a) have been shown in males undergoing E_2 treatment. In the brain no significant change was observed for any ESRs (Filby et al., 2006). In goldfish, the response of the ESRs to E_2 seems to depend on the stage of maturation (for review see Nelson and Habibi, 2013).

The increase in the E_2 receptor expression levels in SW observed in the male eels in this experiment could also be the result of other hormones involved in SW tolerance. For instance, the growth hormone (GH) is a pleiotropic hormone which regulates various functions in teleosts, including SW acclimation (McCormick, 2001; Eckert et al., 2001). In salmonids, growth hormone acts in synergy with cortisol to increase seawater tolerance (McCormick, 2001). In the European eel, GH was shown to increase the number of nuclear E_2 receptors, as seen in the liver (Messaouri et al., 1991; Peyon et al., 1996). Future studies could investigate the potential effect of GH or other osmoregulatory hormones on E_2 receptors in the BPG axis.

4.2. Brain and pituitary estrogen receptor expression levels during induced spermatogenesis

Several studies have demonstrated that E_2 is an important regulatory factor in the brain, due to the role it plays in the neuroendocrine system controlling reproductive functions (for review see Beyer, 1999), but also because of its neurotrophic, neuroprotective and organizational properties (Behl, 2002). In this study we observed a progressive decrease in male European eel E_2 plasma levels during induced spermatogenesis, contrary to the increase observed in experimentally matured female eels (Pérez et al., 2011). Nevertheless, in both sexes, androgen (testosterone and 11-ketotestosterone) plasma levels increase during induced maturation (Aroua et al., 2005; Peñaranda et al., 2010).

A local production of E_2 in the brain/pituitary could exert autocrine and/or paracrine actions in these organs, throughout spermatogenesis. Although most teleosts possess duplicated aromatase *cyp19a1* genes (enzyme responsible of the conversion of androgens to estrogens), eels have a single *cyp19a1* expressed in the brain, pituitary and gonads (Ijiri et al., 2003; Jeng et al., 2012a; Peñaranda et al., 2014). Jeng et al. (2012a), showed that hCG in males strongly up-regulates aromatase immunoreactivity in the brain and pituitary, leading to a local production of E_2. In rainbow trout (*Oncorhynchus mykiss*), aromatase expression has been localized to the neuroendocrine regions which also express ESRs, indicating that locally produced estrogens can affect neuroendocrine functions through genomic effects in a paracrine way (Menuet et al., 2003; Diotel et al., 2010). According to Pellegrini et al. (2005), in fish, E_2 may be also involved in the regulation of neuro-glial communications in the hypothalamus and in the neurohypophysis.

In this study, ESR2a expression levels in the pituitary remained stable throughout spermatogenesis, whereas ESR1 and ESR2b expression levels decreased, until testis development was complete. According to Lafont et al. (2015), ESR1 expression levels in the pituitary of female European eels increased as the eels matured, showing a difference in the expression of ESR1 between the sexes. ESR2a and ESR2b expression levels remained unchanged in both sexes (Lafont et al., 2015; this study).

In the brain, the expression levels of the three ESRs progressively increased in the mes-/diencephalon until the spermatozoa stage. In the female European eel, only ESR1 levels increased in the forebrain with maturation, with ESR2a and ESR2b levels remaining stable (Lafont et al., 2015). These results again suggest a differential regulation of the nuclear estrogen receptors during the maturation of male and female eels. According to Jeng et al. (2012b), E_2 plasma levels may up-regulate the expression of ESR1 in the brain of female Japanese eel. As E_2 plasma levels decreased throughout spermatogenesis in male eels, the expression of ESRs does not seem to respond to a peripheral E_2 production but rather to a local production in the brain. Nevertheless, other factors may be involved in the increase in the expression of ESR1, ESR2a and ESR2b in the brain. An interaction between androgen and estrogen in endocrine tissues has already been demonstrated (Panet-Raymond et al., 2000), and a cross talk between androgens and estrogens and their receptors has been highlighted in mice bone (Kousteni et al., 2001). Finally, according to Larsson et al. (2002), both androgens and estrogens are involved in the physiological regulation of brain androgen receptors in the Atlantic croaker (*Micropogonias undulatus*), another teleost species, during the reproductive cycle. In the male European eel, we cannot rule out an interaction between androgens and the regulation ESR1, ESR2a and ESR2b expressions in the brain.

Concerning GPERs, they are both expressed in the brain and in the pituitary of male European eel, just as previously seen in the female (Lafont et al., 2015). These results suggest GPERs in the eel brain are likely to play a role in the reproductive system of both sexes. These two studies on eel are so far the only ones to look at duplicated GPERs. Previous studies on zebrafish and humans have discovered GPER expressed in different brain regions, including regions that control reproduction and sex behavior (Liu et al., 2009; reviewed by Olde and Leeb-Lundberg, 2009). We did not observe any major change in the expression of GPERs in the brain and pituitary during spermatogenesis.

ESRs and GPERs are co-expressed in all the brain parts and in the pituitary of male as well as female European eels. Further analyses are required but these results may suggest potential interactions between nuclear and membrane E_2 receptors.

4.3. Testis estrogen receptor expression during spermatogenesis

The three ESRs were shown to be expressed in the eel testis, and with the same expression pattern throughout spermatogenesis. The

highest expression levels were measured at stage SPGA. All three ESR expression levels then sharply decreased at stage SPGB/SPC, and remained low until the end of spermatogenesis.

When studying Japanese eel, Miura et al. (1999) discovered that E_2 plays an important role in spermatogonial renewal. They demonstrated that low concentrations of E_2 act in the primary stages of spermatogonia through receptors present in Sertoli cells, stimulating and maintaining spermatogonia proliferation prior to the progression of further stages of spermatogenesis. The high expression levels of all ESRs at stage SPGA that we observed corroborates the proposed role of estrogens as a spermatogonial renewal factor. In the European eel, the parallel regulation of the three ESRs suggests that the role of E_2 as a spermatogonial renewal factor is mediated by ESR1, ESR2a and ESR2b.

In the testis, the expression pattern of GPERs during spermatogenesis was notably different from that of ESRs. The expression levels of both GPERs were low at the beginning of the spermatogenesis (SPGA stage), and increased sharply until the end of spermatogenesis. These results suggest that both GPERa and GPERb may play a role in the final sperm maturation process. The high expression levels of both GPERs at the spermatozoa stage suggests that these receptors may are mainly localized in the germ cells in the eel. Nevertheless, as GPER was shown to be localized in the Leydig and Sertoli cells, spermatogonia, spermatocytes and spermatozoa of humans, in the spermatogonia cell line of mice (*Mus musculus*), and in the Sertoli cells, pachytene spermatocytes and round spermatids of rats (*Rattus norvegicus*) (Luconi et al., 2004; reviewed by Correia et al., 2015), it cannot be excluded that this may be due to Sertoli cell proliferation in the final sperm maturation process.

It is well known that in female fish GPER mediates the E_2-induced meiotic arrest of oocytes (Pang et al., 2008; Pang and Thomas, 2009). Nevertheless, its role in male fish has still yet to be explored. In adult male zebrafish (*Danio rerio*), a RT-PCR analysis of gene expression in the isolated purified early and late germ cells revealed that GPER is mainly expressed in early germ cells of the testis, including the spermatogonia and spermatocytes, suggesting GPER may play a part in mediating estrogen action early on in spermatogenesis (Liu et al., 2009). In contrast, according to their expression profile during spermatogenesis, European eel GPERs (GPERa and GPERb) may have a major role in the final sperm maturation process. This suggests that GPER have differential functions during spermatogenesis depending on the teleost species. In humans, although the role of estrogens in spermatogenesis is still unclear, decreased spermatozoa numbers and motility in men who were genetically deficient in aromatase were observed, suggesting aromatase/estrogens play a role not only during the development and maintenance of spermatogenesis, but also in the final maturation of spermatozoa (Carreau et al., 2010; reviewed by Correia et al., 2015). Accordingly, studies have demonstrated the presence of sex steroid membrane receptors in human spermatozoa and have shown that the effects of estrogens and progesterone on human ejaculated spermatozoa may be mediated by GPERs and membrane progestin receptors (mPRs) respectively (Revelli et al., 1998; Luconi et al., 2004; Carreau et al., 2010). Similarly, in the European eel, sex steroid membrane receptors may be involved in final sperm maturation. We recently found high expression levels of mPRs in the final stage of spermatogenesis in male eel testis (Morini et al., submitted for publication). This study suggests that, both in the eel as well as in humans, progestin and estrogen membrane receptors have are implicated in the completion of spermatogenesis.

In conclusion, this is the first study to describe the expression levels of five estrogen receptors (three nuclear ESRs and two membrane GPERs) along the BPG axis of a male teleost. The presence of all these estrogen receptors in theBPG axis suggests an implication in the control of male eel reproduction. Our results support the evidence that the regulation of eel ESRs and GPERs expressions are tissue and stage-specific, as shown by the different expression profiles found in the different BPG tissues throughout spermatogenesis. It appears likely that testes ESRs play a role in spermatogonia renewal, while testes GPERs are mainly involved in the end of spermatogenesis. The three ESRs and both GPERs in the brain and pituitary may control neuroendocrine functions, mediating the autocrine or paracrine actions of locally-produced estrogen, during the entire process of eel spermatogenesis. Finally, the expression of both ESRs and GPERs in the BPG axis indicates a possible cooperation between genomic and non-genomic estrogen actions in the control of reproduction.

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References

Aroua, S., Schmitz, M., Baloche, S., Vidal, B., Rousseau, K., Dufour, S., 2005. Endocrine evidence that silvering, a secondary metamorphosis in the eel, is a pubertal rather than a metamorphic event. Neuroendocrinology 82, 221–232.

Asturiano, J.F., Marco-Jiménez, F., Pérez, L., Balasch, S., Garzón, D.L., Peñaranda, D.S., Vicente, J.S., Viudes-de-Castro, M.P., Jover, M., 2006. Effects of hCG as spermiation inducer on European eel semen quality. Theriogenology 66, 1012–1020.

Behl, C., 2002. Oestrogen as a neuroprotective hormone. Nat. Rev. Neurosci. 3, 433–442.

Beyer, C., 1999. Estrogen and the developing mammalian brain. Anat. Embryol. 199, 379–390.

Boëtius, I., Boëtius, J., 1967. Studies in the European eel, *Anguilla anguilla* (L.). Experimental induction of the male sexual cycle, its relation to temperature and other factors. Meddelser fra Danmarks Fiskeri- og Havunderogelser 4, pp. 339–405.

Carreau, S., Wolczynski, S., Galeraud-Denis, I., 2010. Aromatase, oestrogens and human male reproduction. Philos. Trans. R. Soc. B 365, 1571–1579.

Carson-Jurica, M.A., Schrader, W.T., O'malley, B.W., 1990. Steroid receptor family: structure and functions. Endocr. Rev. 11, 201–220.

Correia, S., Cardoso, H.J., Cavaco, J.E., Socorro, S., 2015. Oestrogens as apoptosis regulators in mammalian testis: angels or devils? Expert Rev. Mol. Med. 17, e2.

Daverat, F., Limburg, K.E., Thibault, I., Shiao, J.C., Dodson, J.J., Caron, F., Tzeng, W.N., Iizuka, Y., Wickström, H., 2006. Phenotypic plasticity of habitat use by three temperate eel species, *Anguilla anguilla*, *A. japonica* and *A. rostrata*. Mar. Ecol. Prog. Ser. 308, 231–241.

Diotel, N., Page, Y.L., Mouriec, K., Tong, S.K., Pellegrini, E., Vaillant, C., Anglade, I., Brion, F., Pakdel, F., Chung, B.C., Kah, O., 2010. Aromatase in the brain of teleost fish: expression, regulation and putative functions. Front. Neuroendocrinol. 31, 172–192.

Eckert, S.M., Yada, T., Shepherd, B.S., Stetson, M.H., Hirano, T., Grau, E.G., 2001. Hormonal control of osmoregulation in the channel catfish *Ictalurus punctatus*. Gen. Comp. Endocrinol. 122, 270–286.

Filardo, E., Thomas, P., 2005. GPR30: a seven-transmembrane-spanning estrogen receptor that triggers EGF release. Trends Endocrinol. Metab. 16, 362–367.

Filardo, E., Quinn, J., Pang, Y., Graeber, C., Shaw, S., Dong, J., Thomas, P., 2007. Activation of the novel estrogen receptor G Protein-coupled Receptor 30 (GPR30) at the plasma membrane. Endocrinology 148, 3236–3245.

Filby, A.L., Thorpe, K.L., Tyler, C.R., 2006. Multiple molecular effect pathways of an environmental oestrogen in fish. J. Mol. Endocrinol. 37, 121–134.

Frantzen, M., Arnesen, A.M., Damsgård, B., Tveiten, H., Johnsen, H.K., 2004. Effects of photoperiod on sex steroids and gonad maturation in Arctic charr. Aquaculture 240, 561–576.

Gallego, V., Mazzeo, I., Vílchez, M.C., Peñaranda, D.S., Carneiro, P.C.F., Pérez, L., Asturiano, J.F., 2012. Study of the effects of thermal regime and alternative hormonal treatments on the reproductive performance of European eel males (*Anguilla anguilla*) during induced sexual maturation. Aquaculture 354–355, 7–16.

Hawkins, M.B., Thornton, J.W., Crews, D., Skipper, J.K., Dotte, A., Thomas, P., 2000. Identification of a third distinct estrogen receptor and reclassification of estrogen receptors in teleosts. PNAS 97, 10751–10756.

Henkel, C.V., Burgerhout, E., de Wijze, D.L., Dirks, R.P., Minegishi, Y., Jansen, H.J., Spaink, H.P., Dufour, S., Weltzien, F.A., Tsukamoto, K., van den Thillart, G.E., 2012a. Primitive duplicate Hox clusters in the European eel's genome. PLoS One 7, e32231.

Henkel, C.V., Dirks, R.P., de Wijze, D.L., Minegishi, Y., Aoyama, J., Jansen, H.J., Turner, B., Knudsen, B., Bundgaard, M., Hvam, K.L., Boetzer, M., Pirovano, W., Weltzien, F.A., Dufour, S., Tsukamoto, K., Spaink, H.P., van den Thillart, G.E., 2012b. First draft genome sequence of the Japanese eel, *Anguilla japonica*. Gene 511, 195–201.

ICES, 2012. International Council for the Exploration, report of the 2012 session of the Joint EIFAC/ICES Working Group on Eels (WGEEL).

Ijiri, S., Kazeto, Y., Lokman, P.M., Adachi, S., Yamauchi, K., 2003. Characterization of a cDNA encoding P-450 aromatase (CYP19) from Japanese eel ovary and its expression in ovarian follicles during induced ovarian development. Gen. Comp. Endocrinol. 130, 193–203.

Jeng, S.R., Yueh, W.S., Pen, Y.T., Gueguen, M.M., Pasquier, J., Dufour, S., Chang, C.F., Kah, O., 2012a. Expression of aromatase in radial glial cells in the brain of the Japanese eel

provides insight into the evolution of the cyp191a gene in Actinopterygians. PLoS One 7, e44750.
Jeng, S.R., Pasquier, J., Yueh, W.S., Chen, G.R., Lee, Y.H., Dufour, S., Chang, C.F., 2012b. Differential regulation of the expression of cytochrome P450 aromatase, estrogen and androgen receptor subtypes in the brain-pituitary-ovarian axis of the Japanes eel (*Anguilla japonica*) reveals steroid dependent and independent mechanisms. Gen. Comp. Endocrinol. 175, 163–172.
Kousteni, S., Bellido, T., Plotkin, L.I., O'Brien, G.A., Bodenner, D.L., Han, L., Han, K., DiGregorio, G.B., Katzenellenbogen, J.A., Katzenellenbogen, B.S., Roberson, P.K., Weinstein, R.S., Jilka, R.L., Manolagas, S.C., 2001. Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. Cell 104, 719–730.
Lafont, A.G., Rousseau, K., Tomkiewicz, J., Dufour, S., 2015. Three nuclear and two membrane estrogen receptors in basal teleosts, *Anguilla* sp.: identification, evolutionary history and differential expression regulation. Gen. Comp. Endocrinol. 235, 177–191.
Larsson, D.G.J., Sperry, T.S., Thomas, P., 2002. Regulation of androgen receptors in Atlantic croaker brains by testosterone and estradiol. Gen. Comp. Endocrinol. 128, 224–230.
Laudet, V., Hänni, C., Coll, J., Catzeflis, C., Stéhelin, D., 1992. Evolution of the nuclear receptor gene family. EMBO J. 11, 1003–1013.
Leal, M.C., Cardoso, E.R., Nóbrega, R.H., Batlouni, S.R., Bogerd, J., França, L.R., Schulz, R.W., 2009. Histological and stereological evaluation of zebrafish (*Danio rerio*) spermatogenesis with an emphasis on spermatogonial generations. Biol. Reprod. 81, 177–187.
Liu, X., Zhu, P., Sham, K.W.Y., Yuen, J.M.L., Xie, C., Zhang, Y., Liu, Y., Li, S., Huang, X., Cheng, C.H.K., Lin, H., 2009. Identification of a membrane estrogen receptor in zebrafish with homology to mammalian GPER and its high expression in early germ cells of the testis. Biol. Reprod. 80, 1253–1261.
Luconi, M., Francavilla, F., Porazzi, I., Macerola, B., Forti, G., Baldi, E., 2004. Human spermatozoa as a model for studying membrane receptors mediating rapid nongenomic effects of progesterone and estrogens. Steroids 69, 553–559.
Lundqvist, H., Borg, B., Berglund, I., 1989. Androgens impair seawater adaptability in smolting Baltic salmon (*Salmo salar*). Can. J. Zool. 67, 1733–1736.
Ma, C.H., Dong, K.W., Yu, K.L., 2000. cDNA cloning and expression of a novel estrogen receptor beta-subtype in goldfish (*Carassius auratus*). Biochim. Biophys. Acta 1490, 145–152.
Madsen, S.S., Mathiesen, A.B., Korsgaard, B., 1997. Effects of 17β-estradiol and 4-nonylphenol on smoltification and vitellogenesis in Atlantic salmon (*Salmo salar*). Fish Physiol. Biochem. 17, 303–312.
Mangelsdorf, D.F., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., Evans, R.M., 1995. The nuclear receptor superfamily: the second decade. Cell 83, 835–839.
Mazzeo, I., Peñaranda, D.S., Gallego, V., Baloche, S., Nourizadeh-Lillabadi, R., Tveiten, H., Dufour, S., Asturiano, J.F., Weltzien, F.A., Pérez, L., 2014. Temperature modulates the progression of vitellogenesis in European eel. Aquaculture 434, 38–47.
McCormick (Ed.), 2001. Endocrine control of osmoregulation in teleost fishAm. Zool. 41, 781–794.
McCormick, S.D., Naiman, R.J., 1985. Hypoosmoregulation in an anadromous teleost: influence of sex and maturation. J. Exp. Zool. 234, 193–198.
Menuet, A., Pellegrini, E., Anglade, I., Blaise, O., Laudet, V., Kah, O., Pakdel, F., 2002. Molecular characterization of three estrogen receptor forms in zebrafish: binding characteristics, transactivation properties, and tissue distributions. Biol. Reprod. 66, 1881–1892.
Menuet, A., Anglade, I., le Guevel, R., Pellegrini, E., Pakdel, F., Kah, O., 2003. Distribution of aromatase mRNA and protein in the brain and pituitary of female rainbow trout: comparison with estrogen receptor α. J. Comp. Neurol. 462, 180–193.
Messaouri, H., Baloche, S., Hardy, A., Leloup-Hatey, J., Burzawa-Gerard, E., 1991. Hepatic 17β-estradiol receptors and vitellogenesis in *Anguilla anguilla* L. after treatment by 17β-estradiol alone or associated with bovine growth hormone. Gen. Comp. Endocrinol. 82, 238.
Miura, C., Miura, T., 2011. Analysis of spermatogenesis using an eel model. Aqua BioSci. Monogr. 4, 105–129.
Miura, T., Miura, C., Ohta, T., Nader, M.R., Todo, T., Yamauchi, K., 1999. Estradiol-17β stimulates the renewal of spermatogonial stem cells in males. Biochem. Biophys. Res. Commun. 264, 230–234.
Morini, M., Peñaranda, D.S., Vílchez, M.C., Gallego, V., Nourizadeh-Lillabadi, Lafont, A.G., Dufour, S., Asturiano, J.F., Weltzien, F.A., Pérez, L., 2016. Nuclear and membrane progestin receptors in the European eel: characterization and expression *in vivo* through spermatogenesis. PLoS One (submitted for publication).
Nelson, E.R., Habibi, H.R., 2013. Estrogen receptor function and regulation in fish and other vertebrates. Gen. Comp. Endocrinol. 192, 15–24.
Olde, B., Leeb-Lundberg, L.M.F., 2009. GPR30/GPER1: searching for a role in estrogen physiology. Trends Endocrinol. Metab. 20, 409–416.
Oren, I., Fleishma, S.J., Kessel, A., Ben-Tai, N., 2004. Free diffusion of steroid hormones across biomembranes: a simplex search with implicit solvent calculations. Biophys. J. 87, 768–779.
Panet-Raymond, V., Gottlieb, B., Beitel, L.K., Pinsky, L., Trifiroa, M.A., 2000. Interactions between androgen and estrogen receptors and the effects on their transactivational properties. Mol. Cell. Endocrinol. 167, 139–150.
Pang, Y., Thomas, P., 2009. Involvement of estradiol-17β and its membrane receptor, G protein coupled receptor 30 (GPR30) in regulation of oocyte maturation in zebrafish, *Danio rerio*. Gen. Comp. Endocrinol. 161, 58–61.
Pang, Y., Dong, J., Thomas, P., 2008. Estrogen signaling characteristics of Atlantic croaker G protein-coupled receptor 30 (GPR30) and evidence it is involved in maintenance of oocyte meiotic arrest. Endocrinology 149, 3410–3426.
Pellegrini, E., Menuet, A., Lethimonier, C., Adrio, F., Gueguen, M.M., Tascon, C., Anglade, I., Pakdel, F., Kah, O., 2005. Relationships between aromatase and estrogen receptors in the brain of teleost fish. Gen. Comp. Endocrinol. 142, 60–66.
Peñaranda, D.S., Pérez, L., Gallego, V., Jover, M., Tveiten, H., Baloche, S., Dufour, S., Asturiano, J.F., 2010. Molecular and physiological study of the artificial maturation process in European eel males: from brain to testis. Gen. Comp. Endocrinol. 166, 160–171.
Peñaranda, D.S., Mazzeo, I., Hildahl, J., Gallego, V., Nourizadeh-Lillabadi, R., Pérez, L., Asturiano, J.F., Weltzien, F.A., 2013. Molecular characterization of three GnRH receptors in the European eel, *Anguilla anguilla*: tissue-distribution and changes in transcript abundance during artificially induced sexual development. Mol. Cell. Endocrinol. 369, 1–14.
Peñaranda, D.S., Mazzeo, I., Gallego, V., Hildahl, J., Nourizadeh-Lillabadi, R., Pérez, L., Weltzien, F.A., Asturiano, J.F., 2014. The regulation of aromatase and androgen receptor expression during gonad development in male and female European eel. Reprod. Domest. Anim. 49, 512–521.
Peñaranda, D.S., Morini, M., Tveiten, H., Vílchez, M.C., Gallego, V., Dirks, R.P., van den Thillart, G.E.E.J.M., Pérez, L., Asturiano, J.F., 2016. Temperature modulates the testis steroidogenesis in European eel. Comp. Biochem. Physiol. A 197, 58–67.
Pérez, L., Asturiano, J.F., Tomás, A., Zegrari, S., Barrera, R., Espinós, J.F., Navarro, J.C., Jover, M., 2000. Induction of maturation and spermation in the male European eel: assessment of sperm quality throughout treatment. J. Fish Biol. 57, 1488–1504.
Pérez, L., Peñaranda, D.S., Dufour, S., Baloche, S., Palstra, A.P., van den Thillart, G.E.E.J.M., Asturiano, J.F., 2011. Influence of temperature regime on endocrine parameters and vitellogenesis during experimental maturation of European eel (*Anguilla anguilla*) females. Gen. Comp. Endocrinol. 174, 51–59.
Peyon, P., Baloche, S., Burzawa-Gérard, E., 1996. Synthesis induced by 17b-Estradiol in primary culture of female silver eel (Anguilla anguilla L.) hepatocytes. Potentiating Effect of Growth Hormone on Vitellogenin. Gen. Comp. Endocrinol. 102, 263–273.
Pfaffl, M.W., Tichopad, A., Prgomet, C., Neuvians, T.P., 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper-Excel-based tool using pair-wise correlations. Biotechnol. Lett. 26, 509–515.
Prossnitz, E.R., Maggiolini, M., 2009. Mechanisms of estrogen signaling and gene expression via GPR30. Mol. Cell. Endocrinol. 308, 32–38.
Quérat, B., Nahoul, K., Hardy, A., Fontaine, Y.A., Leloup-Hâtey, J., 1987. Plasma concentrations of ovarian steroids in the freshwater European silver eel (*Anguilla anguilla*): effects of hypophysectomy and transfer to sea water. J. Endocrinol. 114, 289–294.
Revelli, A., Massobrio, M., Tesarik, J., 1998. Nongenomic actions of steroid hormones in reproductive tissues. Endocr. Rev. 19, 3–17.
Schulz, R.W., 1985. Measurement of five androgens in the blood of immature and maturing male rainbow trout, Salmo gairdneri (Richardson). Steroids 46, 717–726.
Schulz, R.W., Miura, T., 2002. Spermatogenesis and its endocrine regulation. Fish Physiol. Biochem. 26, 43–56.
Staurnes, M., Sigholt, T., Gulseth, O.A., Elliassen, R., 1994. Effects of maturation on seawater tolerance of anadromous Arctic char. Trans. Am. Fish. Soc. 123, 402–407.
Thomas, P., 2012. Rapid steroid hormone actions initiated at the cell surface and the receptors that mediate them with an emphasis on recent progress in fish models. Gen. Comp. Endocrinol. 175, 367–383.
Weltzien, F.A., Pasqualini, C., Vernier, P., Dufour, S., 2005. A quantitative real-time RT-PCR assay for European eel tyrosine hydroxylase. Gen. Comp. Endocrinol. 142, 134–142.