Morini, M.; Peñaranda, D.; Vilchez Olivencia, MC.; Tveiten, H.; Lafont, A.; Dufour, S.; Pérez Igualada, LM.... (2017). The expression of nuclear and membrane estrogen receptors in the European eel throughout spermatogenesis. Comparative Biochemistry and Physiology Part A Molecular & Integrative Physiology. 203:91-99. doi:10.1016/j.cbpa.2016.08.020

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

Marina Morini[1], David S. Peñaranda[1], M. Carmen Vílchez[1], Helge Tveiten[2], Anne-Gaëlle Lafont[3], Sylvie Dufour[3], Luz Pérez[1], Juan F. Asturiano[1,*]

[1]Grupo de Acuicultura y Biodiversidad. Instituto de Ciencia y Tecnología Animal (Edificio 7G). Universitat Politècnica de València, Camino de Vera s/n. 46022, Valencia, Spain.

[2]Norwegian Institute of Fisheries and Aquaculture, Muninbakken 9-13, Breivika, P.O. Box 6122 NO-9291 Tromsø, Norway.

[3]Muséum National d'Histoire Naturelle, Sorbonne Universités, Research Unit BOREA, Biology of Aquatic Organisms and Ecosystems, CNRS 7208, IRD 207, UPMC, UCN, UA, Paris, France.

*Corresponding author:
Dr. Juan F. Asturiano
Grupo de Acuicultura y Biodiversidad
Instituto de Ciencia y Tecnología Animal. Edificio 7G
Universitat Politècnica de València
Camino de Vera, s/n 46022 Valencia
Phone: +34 96 387 93 85
E-mail: jfastu@dca.upv.es

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.

Keywords: ESR, GPER, *Anguilla*, teleost, reproduction

1. Introduction

In male vertebrates, sex steroids, androgens, estrogens, and progestins, play significant roles in the control of spermatogenesis (Schultz 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., in press).

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 et al., 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., in press).

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 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

103 randomly distributed and kept at 20 °C in two freshwater 200-L aquaria equipped with
104 separated recirculation systems, thermostats/coolers, and covered to maintain constant
105 darkness.
106 One group of 8 eels was anaesthetized with benzocaine (60 ppm) and sacrificed by
107 decapitation in freshwater (FW). The rest of the fish were gradually acclimatized over
108 the course of one week to seawater (37±0.3‰ of salinity). Groups of 8 eels were
109 anaesthetized and sacrificed by decapitation in seawater conditions (SW). Once a week
110 for 8 weeks the rest of the fish were anesthetized, weighed and injected with hCG (1.5
111 IU g^{-1} fish; Profasi, Serono, Italy), to induce the spermatogenesis as previously
112 described by Pérez et al. (2000). Groups of 8 eels were anaesthetized and sacrificed by
113 decapitation each week (W1-8) through the hormonal treatment. For the analysis of
114 ESR expression through the spermatogenesis, the 8 latter groups have been redistributed
115 to 4 groups based on their spermatogenic stage.
116 Total body weight and testis weight were recorded to calculate the gonadosomatic index
117 [GSI = (gonad weight/total body weight)*100]. Blood samples were collected,
118 centrifuged and stored at -20 ºC until E_2 plasma level analysis. Testicular tissue samples
119 were fixed in 10% formalin buffered at pH 7.4 for histological analysis.
120 Samples of anterior brain (dissected into three parts: olfactory bulbs, telencephalon,
121 mes-/di-encephalon), pituitary and testis were stored in 0.5 ml of RNAlater (Ambion
122 Inc., Huntingdon, UK) at -20 ºC until extraction of total RNA.
123 Because eels stop feeding at the silver stage and throughout sexual maturation thee fish
124 were not fed throughout the experiment. They were handled in accordance with the
125 European Union regulations concerning the protection of experimental animals (Dir
126 86/609/EEC).
127

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, 2001; 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). 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

153 Wipeout Buffer, Qiagen, Hilden, Germany). Using a Quantiscript Reverse Transcriptase
154 (Qiagen, Hilden, Germany), 20 µl cDNA was synthesized from 500 ng of total RNA in
155 the case of the olfactory bulb and pituitary, and from 1 µg in the case of the
156 telencephalon and the mes-/diencephalon.
157
158 **2.4. Gene expression analyses by quantitative real-time PCR**
159 The quantitative real-time Polymerase Chain Reactions (qPCR) were carried out using
160 specific qPCR primers for each European eel estrogen nuclear and membrane receptor
161 (Lafont et al., in press) and the Acidic ribosomal phosphoprotein P0 (ARP) (Weltzien et
162 al., 2005) was used as the reference gene (Table I).
163
164 **2.4.1. Reference gene**
165 The stability of the reference gene was determined using the BestKeeper program
166 (Pfaffl et al., 2004), reporting a standard deviation (SD[±Cq]) lower than 1. The
167 BestKeeper calculated that variations in the reference gene are based on the arithmetic
168 mean of the Cq values. Genes with a SD value higher than 1 are defined as unstable. In
169 the testis: SD= 0.83; $p<0.05$ with a Cq geometric mean of 24.21±1.77; in the brain and
170 pituitary, olfactory bulb: SD= 0.81; telencephalon: SD= 0.48; mes-/diencephalon: SD=
171 0.58, pituitary: SD= 0.63; $p<0.05$ and the Cq geometric mean of the olfactory bulb:
172 23.39±1.76; telencephalon: 21.76±1.40; mes-/diencephalon: 21.89±1.49; pituitary:
173 22.34±1.55.
174
175 **2.4.2. SYBR Green assay**
176 To determine the expression of each ESR and GPER gene, qPCR assays were
177 performed using a model 7500 unit (Applied Biosystems; Foster City, CA, USA) with

178 Maxima SYBR Green/ROX qPCR Master Mix (Fermentas Corp. Glen Burnie, MD,
179 USA). The qPCR program used for all was an initial step of 50 °C for 2 min, followed
180 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
181 s. To evaluate assay specificity, the machine performed a melting curve analysis
182 directly after PCR by slowly (0.1 ºC/s) increasing the temperature from 68 to 95 ºC,
183 with a continuous registration of any changes in fluorescent emission intensity.
184 The total volume for each qPCR reaction was 20 µl, with 5 µl of diluted cDNA
185 template, forward and reverse primers (250 nM each), and SYBR Green/ROX Master
186 Mix (12 µl). The transcript levels were determined by the efficiency-adjusted relative
187 quantification method described by Weltzien et al. (2005). Serial dilutions of the cDNA
188 pool of the gonad tissues were run in duplicate and used for the standard curve to
189 measure all ESRs and GPERs in the testis. Serial dilutions of the cDNA pool of the
190 brain and pituitary tissues were used for the standard curve from which to measure all
191 the ESRs and GPERs in the different parts of the brain and pituitary. A 1/32 dilution of
192 the standard curve was included in each run of the corresponding gene as a calibrator.
193 Target and reference genes in unknown samples were run in duplicate PCR reactions. A
194 non-template control (cDNA replaced by water) for each primer pair was run in
195 duplicate on all plates. All ESR and GPER data were normalised to eel reference gene
196 ARP. qPCR calculations were performed according to the Roche Applied Science
197 protocol, Technical Note No. LC 13/2001, part 4 “Calibrator normalized relative
198 quantification”.
199
200 **2.5. Immunoassays for E_2**
201 Plasma concentrations of E_2 were measured by means of radioimmunoassay (RIA),
202 according to the method described by Schulz (1984). 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).

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 ($p<0.05$),

253 with them being 12.5 and 4-fold higher respectively. GPERa and GPERb expression
254 levels then remained stable until the end of spermatogenesis.
255
256 **3.3. Pituitary estrogen receptor expressions**
257 In the pituitary, the expression of the three ESRs increased with the change from FW to
258 SW ($p<0.05$) (Fig. 5). The expression of ESR1, ESR2a and ESR2b was 7, 4 and 3-fold
259 higher respectively, in SW than in FW. During spermatogenesis, ESR1 and ESR2b
260 showed significant changes in the pituitary throughout testis development, while ESR2a
261 remained stable. ESR1 expression levels increased from stage SPGA to stage
262 SPGB/SPC ($p<0.05$), and then decreased from SPGB/SPC until the final maturation
263 stage SZ ($p<0.05$). ESR2b remained stable from stage SPGA to stage SPGB/SPC, and
264 then progressively decreased until the final maturation stage SZ.
265 Concerning the GPERs, the expression levels of both GPERa and GPERb increased
266 with the change from FW to SW ($p<0.05$), with them being 4 and 2-fold higher
267 respectively. GPERa and GPERb expression levels then remained stable until the end of
268 spermatogenesis.
269
270 **3.4. Testis estrogen receptor expressions**
271 In the testis, the expression levels of ESR1 and ESR2a increased with the change from
272 FW to SW ($p<0.05$), with them being 3 and 2-fold higher respectively (Fig. 6). In
273 contrast, the expression of ESR2b remained stable with the change of salinity. During
274 spermatogenesis, the expressions of the three ESRs decreased sharply from stage SPGA
275 to SPGB/SPC: the expressions of ESR1, ESR2a and ESR2b were 7, 9.5 and 9-fold
276 lower respectively, at stage SPGB/SPC compared to stage SPGA, then remained low
277 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.

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.

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 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 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

328 shown in males undergoing E_2 treatment. In the brain no significant change was
329 observed for any ESRs (Filby et al., 2006). In goldfish, the response of the ESRs to E_2
330 seems to depend on the stage of maturation (for review see Nelson and Habibi, 2013).
331 The increase in the E_2 receptor expression levels in SW observed in the male eels in this
332 experiment could also be the result of other hormones involved in SW tolerance. For
333 instance, the growth hormone (GH) is a pleiotropic hormone which regulates various
334 functions in teleosts, including SW acclimation (McCormick, 2001; Eckert et al., 2001).
335 In salmonids, growth hormone acts in synergy with cortisol to increase seawater
336 tolerance (McCormick, 2001). In the European eel, GH was shown to increase the
337 number of nuclear E_2 receptors, as seen in the liver (Messaouri et al., 1991; Peyon et al.,
338 1996). Future studies could investigate the potential effect of GH or other
339 osmoregulatory hormones on E_2 receptors in the BPG axis.
340
341 **4.2. Brain and pituitary estrogen receptor expression levels during induced**
342 **spermatogenesis**
343 Several studies have demonstrated that E_2 is an important regulatory factor in the brain,
344 due to the role it plays in the neuroendocrine system controlling reproductive functions
345 (for review see Beyer, 1999), but also because of its neurotrophic, neuroprotective and
346 organizational properties (Behl, 2002). In this study we observed a progressive decrease
347 in male European eel E_2 plasma levels during induced spermatogenesis, contrary to the
348 increase observed in experimentally matured female eels (Pérez et al., 2011).
349 Nevertheless, in both sexes, androgen (testosterone and 11-ketotestosterone) plasma
350 levels increase during induced maturation (Aroua et al., 2005; Peñaranda et al., 2010).
351 A local production of E_2 in the brain/pituitary could exert autocrine and/or paracrine
352 actions in these organs, throughout spermatogenesis. Although most teleosts possess

353 duplicated aromatase *cyp19a1* genes (enzyme responsible of the conversion of
354 androgens to estrogens), eels have a single *cyp19a1* expressed in the brain, pituitary and
355 gonads (Ijiri et al., 2003; Jeng et al., 2012a; Peñaranda et al., 2014). Jeng et al. (2012a),
356 showed that hCG in males strongly up-regulates aromatase immunoreactivity in the
357 brain and pituitary, leading to a local production of E_2. In rainbow trout (*Oncorhynchus*
358 *mykiss*), aromatase expression has been localized to the neuroendocrine regions which
359 also express ESRs, indicating that locally produced estrogens can affect neuroendocrine
360 functions through genomic effects in a paracrine way (Menuet et al., 2003; Diotel et al.,
361 2010). According to Pellegrini et al. (2005), in fish, E_2 may be also involved in the
362 regulation of neuro-glial communications in the hypothalamus and in the
363 neurohypophysis.
364 In this study, ESR2a expression levels in the pituitary remained stable throughout
365 spermatogenesis, whereas ESR1 and ESR2b expression levels decreased, until testis
366 development was complete. According to Lafont et al. (in press), ESR1 expression
367 levels in the pituitary of female European eels increased as the eels matured, showing a
368 difference in the expression of ESR1 between the sexes. ESR2a and ESR2b expression
369 levels remained unchanged in both sexes (Lafont et al., in press; this study).
370 In the brain, the expression levels of the three ESRs progressively increased in the mes-
371 /diencephalon until the spermatozoa stage. In the female European eel, only ESR1
372 levels increased in the forebrain with maturation, with ESR2a and ESR2b levels
373 remaining stable (Lafont et al., in press). These results again suggest a differential
374 regulation of the nuclear estrogen receptors during the maturation of male and female
375 eels. According to Jeng et al. (2012b), E_2 plasma levels may up-regulate the expression
376 of ESR1 in the brain of female Japanese eel. As E_2 plasma levels decreased throughout
377 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., in press). 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

403 expression pattern throughout spermatogenesis. The highest expression levels were
404 measured at stage SPGA. All three ESR expression levels then sharply decreased at
405 stage SPGB/SPC, and remained low until the end of spermatogenesis.
406 When studying Japanese eel, Miura et al. (1999) discovered that E_2 plays an important
407 role in spermatogonial renewal. They demonstrated that low concentrations of E_2 act in
408 the primary stages of spermatogonia through receptors present in Sertoli cells,
409 stimulating and maintaining spermatogonia proliferation prior to the progression of
410 further stages of spermatogenesis. The high expression levels of all ESRs at stage
411 SPGA that we observed corroborates the proposed role of estrogens as a spermatogonial
412 renewal factor. In the European eel, the parallel regulation of the three ESRs suggests
413 that the role of E_2 as a spermatogonial renewal factor is mediated by ESR1, ESR2a and
414 ESR2b.
415 In the testis, the expression pattern of GPERs during spermatogenesis was notably
416 different from that of ESRs. The expression levels of both GPERs were low at the
417 beginning of the spermatogenesis (SPGA stage), and increased sharply until the end of
418 spermatogenesis. These results suggest that both GPERa and GPERb may play a role in
419 the final sperm maturation process. The high expression levels of both GPERs at the
420 spermatozoa stage suggests that these receptors may are mainly localized in the germ
421 cells in the eel. Nevertheless, as GPER was shown to be localized in the Leydig and
422 Sertoli cells, spermatogonia, spermatocytes and spermatozoa of humans, in the
423 spermatogonia cell line of mice (*Mus musculus*), and in the Sertoli cells, pachytene
424 spermatocytes and round spermatids of rats (*Rattus norvegicus*) (Luconi et al., 2004;
425 reviewed by Correia et al., 2015), it cannot be excluded that this may be due to Sertoli
426 cell proliferation in the final sperm maturation process.
427 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). 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

453 teleost. The presence of all these estrogen receptors in theBPG axis suggests an
454 implication in the control of male eel reproduction. Our results support the evidence that
455 the regulation of eel ESRs and GPERs expressions are tissue and stage-specific, as
456 shown by the different expression profiles found in the different BPG tissues throughout
457 spermatogenesis. It appears likely that testes ESRs play a role in spermatogonia
458 renewal, while testes GPERs are mainly involved in the end of spermatogenesis. The
459 three ESRs and both GPERs in the brain and pituitary may control neuroendocrine
460 functions, mediating the autocrine or paracrine actions of locally-produced estrogen,
461 during the entire process of eel spermatogenesis. Finally, the expression of both ESRs
462 and GPERs in the BPG axis indicates a possible cooperation between genomic and non-
463 genomic estrogen actions in the control of reproduction.
464

465 **Acknowledgements**

466 Funded by the Spanish Ministry of Science and Innovation (REPRO-TEMP project;
467 AGL2013-41646-R) and IMPRESS (Marie Sklodowska-Curie Actions; Grant
468 agreement nº: 642893). M.C. Vílchez has a predoctoral grant from UPV PAID
469 Programme (2011-S2-02-6521), M. Morini has a predoctoral grant from Generalitat
470 Valenciana (Programa Grisolía). D.S. Peñaranda was supported by MICINN and UPV
471 (PTA2011-4948-I)..
472

473 **References**

474 Asturiano, J.F., Marco-Jiménez, F., Pérez, L., Balasch, S., Garzón, D.L., Peñaranda,
475 D.S., Vicente, J.S., Viudes-de-Castro, M.P., Jover, M., 2006. Effects of hCG as
476 spermiation inducer on European eel semen quality. Theriogenology 66, 1012–1020.
477 Aroua, S., Schmitz, M., Baloche, S., Vidal, B., Rousseau, K., Dufour, S., 2005.
478 Endocrine evidence that silvering, a secondary metamorphosis in the eel, is a
479 pubertal rather than a metamorphic event. Neuroendocrinology 82, 221–232.

480 Behl, C., 2002. Oestrogen as a neuroprotective hormone. Nat. Rev. Neurosci. 3, 433–
481 442.
482 Beyer, C., 1999. Estrogen and the developing mammalian brain. Anat Embryol 199,
483 379–390.
484 Boëtius, I., Boëtius, J., 1967. Studies in the European eel, *Anguilla anguilla* (L.).
485 Experimental induction of the male sexual cycle, its relation to temperature and other
486 factors. Meddelser fra Danmarks Fiskeri- og Havunderogelser. 4, 339–405.
487 Carreau, S., Wolczynski, S., Galeraud-Denis, I., 2010. Aromatase, oestrogens and
488 human male reproduction. Phil. Trans. R. Soc. B. 365, 1571–1579.
489 Carson-Jurica, M.A., Schrader, W.T., O'malley. B.W., 1990. Steroid Receptor Family:
490 Structure and Functions. Endocr. Soc. 11, 201–220.
491 Correia, S., Cardoso, H.J., Cavaco J.E., Socorro, S., 2015. Oestrogens as apoptosis
492 regulators in mammalian testis: angels or devils? Expert Rev. Mol Med. 17: e2.
493 Daverat, F., Limburg, K.E., Thibault, I., Shiao, J.C., Dodson, J.J., Caron, F., Tzeng,
494 W.N., Iizuka, Y., Wickström, H., 2006. Phenotypic plasticity of habitat use by three
495 temperate eel species, *Anguilla anguilla, A. japonica* and *A. rostrata*. Mar. Ecol.
496 Prog. Ser. 308, 231–241.
497 Diotel, N., Page, Y.L., Mouriec, K., Tong, S.K., Pellegrini, E., Vaillant, C., Anglade, I.,
498 Brion, F., Pakdel, F., Chung, B.C., Kah, O., 2010. Aromatase in the brain of teleost
499 fish: expression, regulation and putative functions. Front Neuroendocrinol 31, 172–
500 192.
501 Eckert, S.M., Yada, T., Shepherd, B.S., Stetson, M.H., Hirano, T., Grau, E.G., 2001.
502 Hormonal Control of Osmoregulation in the Channel Catfish *Ictalurus punctatus*.
503 Gen. Comp. Endocrinol. 122, 270–286.
504 Filardo, E., Thomas, P., 2005. GPR30: a seven-transmembrane-spanning estrogen
505 receptor that triggers EGF release. Trends Endocrinol. Metab. 16, 362–367.
506 Filardo, E., Quinn, J., Pang, Y., Graeber, C., Shaw, S., Dong, J., Thomas, P., 2007.
507 Activation of the Novel Estrogen Receptor G Protein-Coupled Receptor 30 (GPR30)
508 at the Plasma Membrane. Endocrinology 148, 3236–3245.
509 Filby, A.L., Thorpe, K.L., Tyler, C.R., 2006. Multiple molecular effect pathways of an
510 environmental oestrogen in fish. J. Mol. Endocrinol. 37, 121–134.
511 Frantzen, M., Arnesen, A.M., Damsgård, B., Tveiten, H., Johnsen, H.K., 2004. Effects
512 of photoperiod on sex steroids and gonad maturation in Artic charr. Aquaculture 240,
513 561–576.

514 Gallego, V., Mazzeo, I., Vílchez, M.C., Peñaranda, D.S., Carneiro, P.C.F., Pérez, L.,
515 Asturiano, J.F., 2012. Study of the effects of thermal regime and alternative
516 hormonal treatments on the reproductive performance of European eel males
517 (*Anguilla anguilla*) during induced sexual maturation. Aquaculture 354–355, 7–16.
518 Hawkins, M.B., Thornton, J.W., Crews, D., Skipper, J.K., Dotte, A., Thomas, P., 2000
519 Identification of a third distinct estrogen receptor and reclassification of estrogen
520 receptors in teleosts. PNAS 97, 10751–10756.
521 Henkel, C.V., Burgerhout, E., de Wijze, D.L., Dirks, R.P., Minegishi, Y., Jansen, H.J.,
522 Spaink, H.P., Dufour, S., Weltzien, F.A., Tsukamoto, K., van den Thillart, G.E.,
523 2012a. Primitive duplicate Hox clusters in the European eel's genome. PLoS One
524 7:e32231.
525 Henkel, C.V., Dirks, R.P., de Wijze, D.L., Minegishi, Y., Aoyama, J., Jansen, H.J.,
526 Turner, B., Knudsen, B., Bundgaard, M., Hvam, K.L., Boetzer, M., Pirovano, W.,
527 Weltzien, F.A., Dufour, S., Tsukamoto, K., Spaink, H.P., van den Thillart, G.E.,
528 2012b. First draft genome sequence of the Japanese eel, *Anguilla japonica*. Gene
529 511, 195–201.
530 ICES, International Council for the Exploration, report of the 2012 session of the Joint
531 EIFAC/ICES Working Group on Eels (WGEEL).
532 Ijiri, S., Kazeto, Y., Lokman, P.M., Adachi, S., Yamauchi, K., 2003. Characterization of
533 a cDNA encoding P-450 aromatase (CYP19) from Japanese eel ovary and its
534 expression in ovarian follicles during induced ovarian development. Gen. Comp.
535 Endocrinol. 130, 193–203.
536 Jeng, S.R., Yueh, W.S., Pen, Y.T., Gueguen, M.M., Pasquier, J., Dufour, S., Chang,
537 C.F., Kah, O., 2012. Expression of Aromatase in Radial Glial Cells in the Brain of
538 the Japanese Eel Provides Insight into the Evolution of the cyp191a Gene in
539 Actinopterygians. PLoS One 7, e44750.
540 Jeng, S.R., Pasquier, J., Yueh, W.S., Chen, G.R., Lee, Y.H., Dufour, S., Chang, C.F.,
541 2012b. Differential regulation of the expression of cytochrome P450 aromatase,
542 estrogen and androgen receptor subtypes in the brain-pituitary-ovarian axis of the
543 Japanes eel (*Anguilla japonica*) reveals steroid dependent and independent
544 mechanisms. Gen. Comp. Endocrinol. 175, 163–172.
545 Kazeto, Y., Ijiri, S., Matsubara, H., Adachi, S., Yamauchi, K., 2000. Cloning of 17b-
546 Hydroxysteroid Dehydrogenase-I cDNAs from Japanese Eel Ovary. Biochem.
547 Biophys. Res. Commun. 279, 451–456.

548 Lafont, A.G., Rousseau, K., Tomkiewicz, J., Dufour, S., in press. Three nuclear and two
549 membrane estrogen receptors in basal teleosts, *Anguilla* sp.: identification,
550 evolutionary history and differential expression regulation. Gen. Comp. Endocrinol.
551 Doi: 10.1016/j.ygcen.2015.11.021.
552 Laudet, V., Hänni, C., Coll, J., Catzeflis, C., Stéhelin, D., 1992. Evolution of the nuclear
553 receptor gene family. EMBO J. 11, 1003–1013.
554 Leal, M.C., Cardoso, E.R., Nóbrega, R.H., Batlouni, S.R., Bogerd, J., França, L.R.,
555 Schulz, R.W. Histological and stereological evaluation of zebrafish (*Danio rerio*)
556 spermatogenesis with an emphasis on spermatogonial Generations. Biol Reprod.
557 2009; 81: 177–187.
558 Liu, X., Zhu, P., Sham, K.W.Y., Yuen, J.M.L., Xie, C., Zhang, Y., Liu, Y., Li, S.,
559 Huang, X., Cheng, C.H.K., Lin, H., 2009. Identification of a Membrane Estrogen
560 Receptor in Zebrafish with Homology to Mammalian GPER and Its High Expression
561 in Early Germ Cells of the Testis. Biol. Reprod. 80, 1253–1261.
562 Luconi, M., Francavilla, F., Porazzi, I., Macerola, B., Forti, G., Baldi, E., 2004. Human
563 spermatozoa as a model for studying membrane receptors mediating rapid
564 nongenomic effects of progesterone and estrogens. Steroids 69, 553–559
565 Lundqvist, H., Borg, B., Berglund, I., 1989. Androgens impair seawater adaptability in
566 smolting Baltic salmon (*Salmo salar*). Can. J. Zool. 67, 1733–1736.
567 Ma, C.H., Dong, K.W., Yu, K.L., 2000. cDNA cloning and expression of a novel
568 estrogen receptor beta-subtype in goldfish (*Carassius auratus*). Biochim. Biophys.
569 Acta 1490, 145–152.
570 Madsen SS, Mathiesen AB, Korsgaard B. 1997. Effects of 17β-estradiol and 4-
571 nonylphenol on smoltification and vitellogenesis in Atlantic salmon (*Salmo salar*).
572 Fish Physiol. Biochem. 17:303–312.
573 Mangelsdorf, D.F., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K.,
574 Blumberg, B., Kastner, P., Mark, M., Chambon, P, Evans, R.M., 1995. The nuclear
575 receptor superfamily: the second decade. Cell 83, 835–839.
576 Mazzeo, I., Peñaranda, D.S., Gallego, V., Baloche, S., Nourizadeh-Lillabadi, R.,
577 Tveiten, H., Dufour, S., Asturiano, J.F., Weltzien, F.A., Pérez, L., 2014. Temperature
578 modulates the progression of vitellogenesis in European eel. Aquaculture 434, 38–
579 47.
580 McCormick, S.D., Naiman, R.J., 1985. Hypoosmoregulation in an anadromous teleost:
581 influence of sex and maturation. J. Exp. Zool. 234, 193–198.

582 McCormick, 2001. Endocrine Control of Osmoregulation in Teleost Fish. Amer. Zool.
583 41,781–794.
584 Menuet, A., Pellegrini, E., Anglade, I., Blaise, O., Laudet, V., Kah, O., Pakdel, F., 2002.
585 Molecular characterization of three estrogen receptor forms in zebrafish: binding
586 characteristics, transactivation properties, and tissue distributions. Biol. Reprod. 66,
587 1881–1892.
588 Menuet, A., Anglade, I., le Guevel, R., Pellegrini, E., Pakdel, F., Kah, O., 2003.
589 Distribution of Aromatase mRNA and protein in the Brain and Pituitary of female
590 Rainbow Trout: Comparison with Estrogen Receptor α. J. Comp. Neurol. 462, 180–
591 193.
592 Messaouri, H., Baloche, S., Hardy, A., Leloup-Hatey, J., and Burzawa-Gerard, E., 1991.
593 Hepatic 17β-estradiol receptors and vitellogenesis in *Anguilla anguilla* L. after
594 treatment by 17β-estradiol alone or associated with bovine growth hormone. Gen.
595 Comp. Endocrinol. 82, 238.
596 Miura, T., Miura, C., Ohta, T., Nader, M.R., Todo, T., Yamauchi, K., 1999. Estradiol-
597 17β stimulates the renewal of spermatogonial stem cells in males. Biochem. Biophys.
598 Res. Com. 264, 230–234.
599 Miura C, Miura T. 2011. Analysis of spermatogenesis using an eel model. Aqua-BioSci.
600 Monogr. 4, 105–129.
601 Morini, M., Peñaranda, D.S., Vílchez, M.C., Gallego, V., Nourizadeh-Lillabadi, R.,
602 Asturiano, J.F., Weltzien, F.A., Pérez, L., 2015. Transcript levels of the soluble
603 sperm factor protein phospholipase C zeta 1 (PLCζ1) increase through induced
604 spermatogenesis in European eel. Comp. Biochem. Physiol. A 187: 168–176.
605 Morini, M., Peñaranda, D.S., Vílchez, M.C., Gallego, V., Nourizadeh-Lillabadi, Lafont,
606 A.G., Dufour, S., Asturiano, J.F., Weltzien, F.A., Pérez, L. Nuclear and membrane
607 progestin receptors in the European eel: characterization and expression *in vivo*
608 through spermatogenesis. PLoS One, submitted.
609 Nelson, E.R., Habibi, H.R., 2013. Estrogen receptor function and regulation in fish and
610 other vertebrates. Gen. Comp. Endocrinol. 192, 15–24.
611 Olde, B., Leeb-Lundberg, L.M.F., 2009. GPR30/GPER1: searching for a role in
612 estrogen physiology. Trends Endocrinol. Metab. 20, 409–416.
613 Oren, I., Fleishma, S.J., Kessel, A., Ben-Tai, N., 2004. Free diffusion of steroid
614 hormones across biomembranes: a simplex search with implicit solvent calculations,
615 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., 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.

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.

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., 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. Part 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.

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. Miura, T., 2002. Spermatogenesis and its endocrine regulation. Fish Physiol. Biochem. 26, 43–56.

Simpson, E.R., 2004. Aromatase: biologic relevance of tissue-specific expression. Semin. Reprod. Med. 22, 11–23.

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.

Table legend

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

Figure legends

Figure 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

Figure 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.

Figure 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.

Figure 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.

Figure 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.

Figure 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.

Figure 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

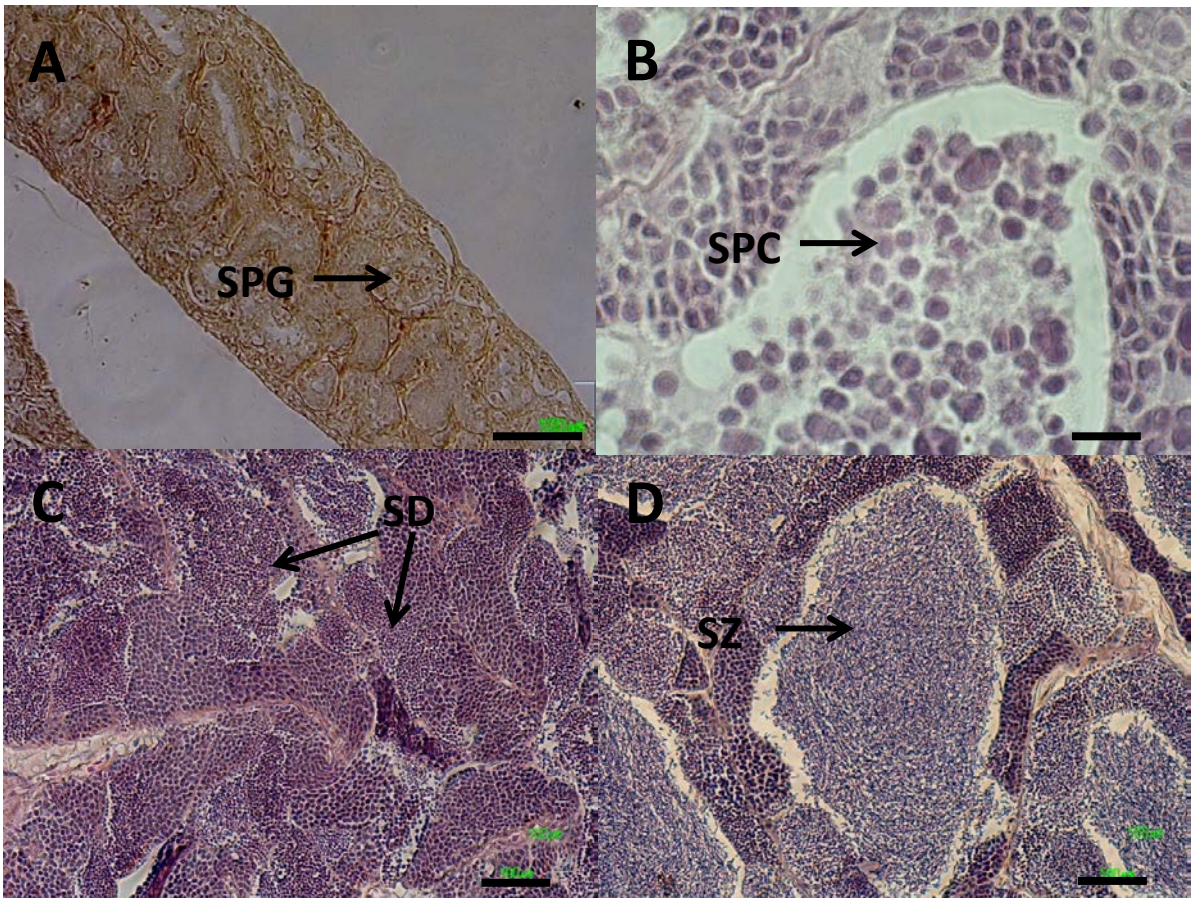
752 (n=15), SD= Spermatid stage (n=10), SZ= Spermatozoa stage (n=25). See main text for
753 description of development stages.
754

755 **Table I**

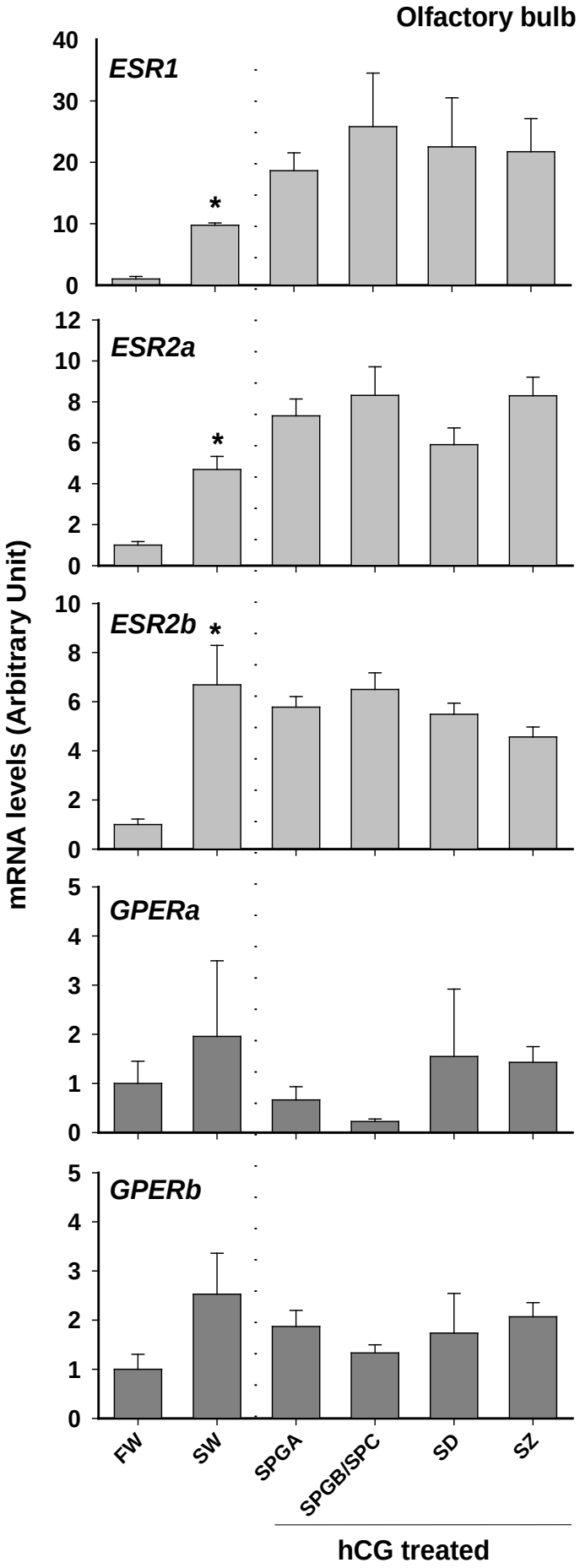
Name	Sequence (5'- 3')	Orientation	Tm	Reference	Accession number	Primer efficicency	Amplicon size (bp)
ARP	GTGCCAGCTCAGAACACTG	Forward	56.36	Weltzien et al., 2005	AAV32820	2.14	107
	ACATCGCTCAAGACTTCAATGG	Reverse	60.09				
ESR1	GCCATCATACTGCTCAACTCC	Forward	58.20	Lafont et al., in press	CUH82767	2.04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

756
757

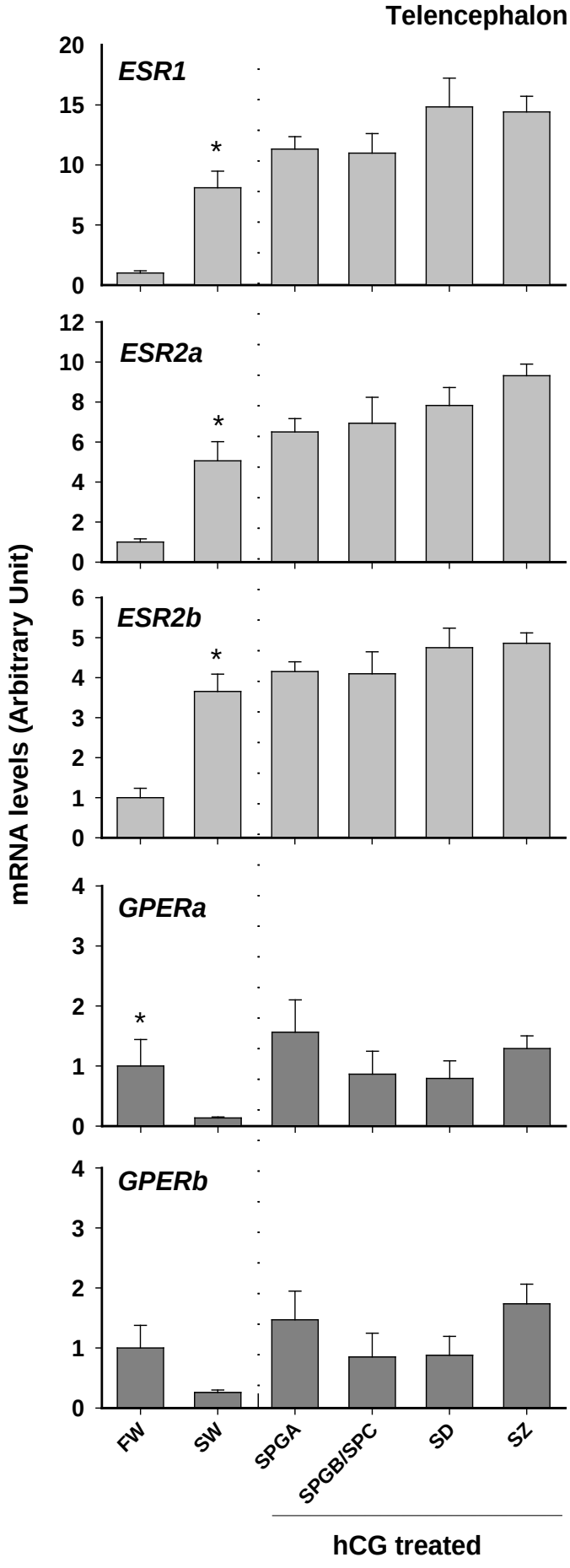
Figure 1



762
763 **Figure 2**
764

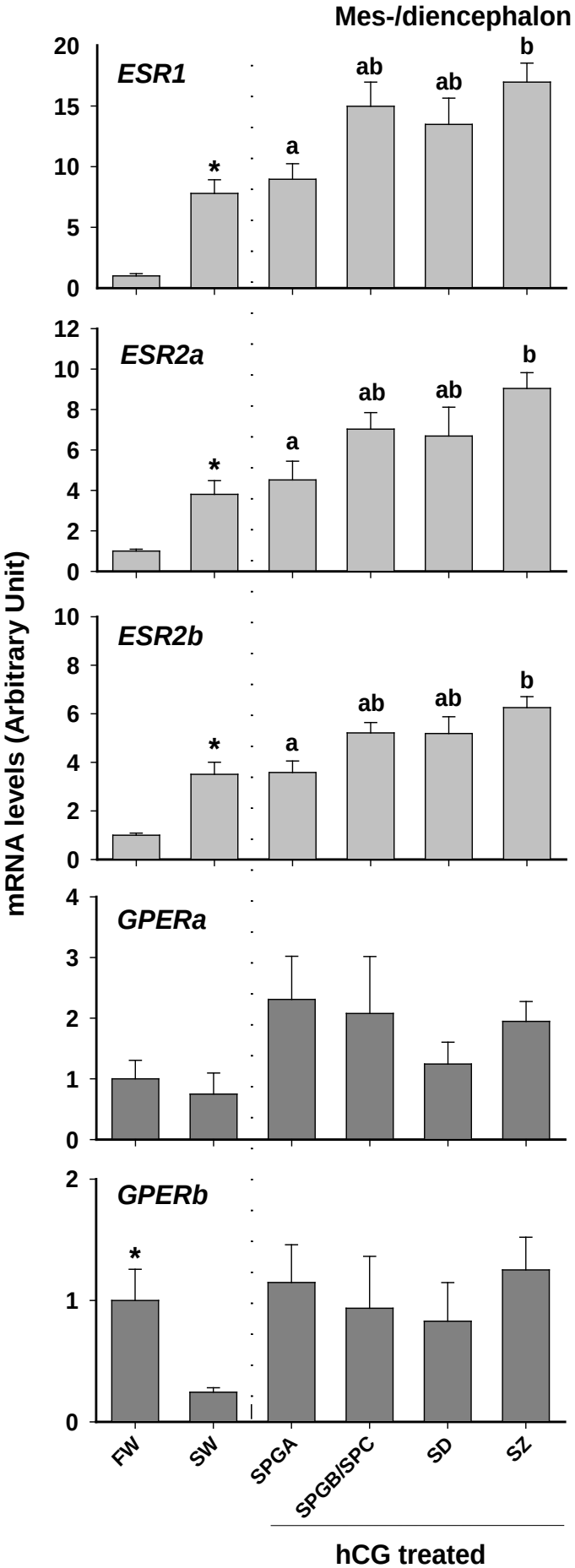


765
766 **Figure 3**

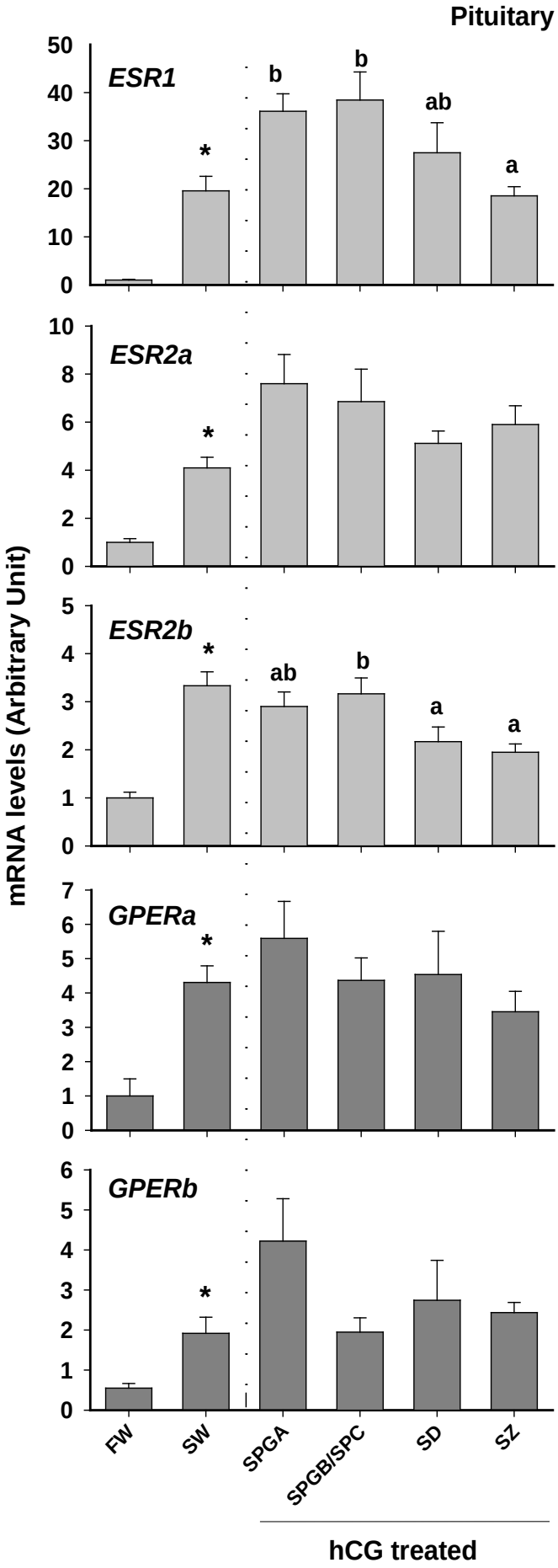


768 **Figure 4**

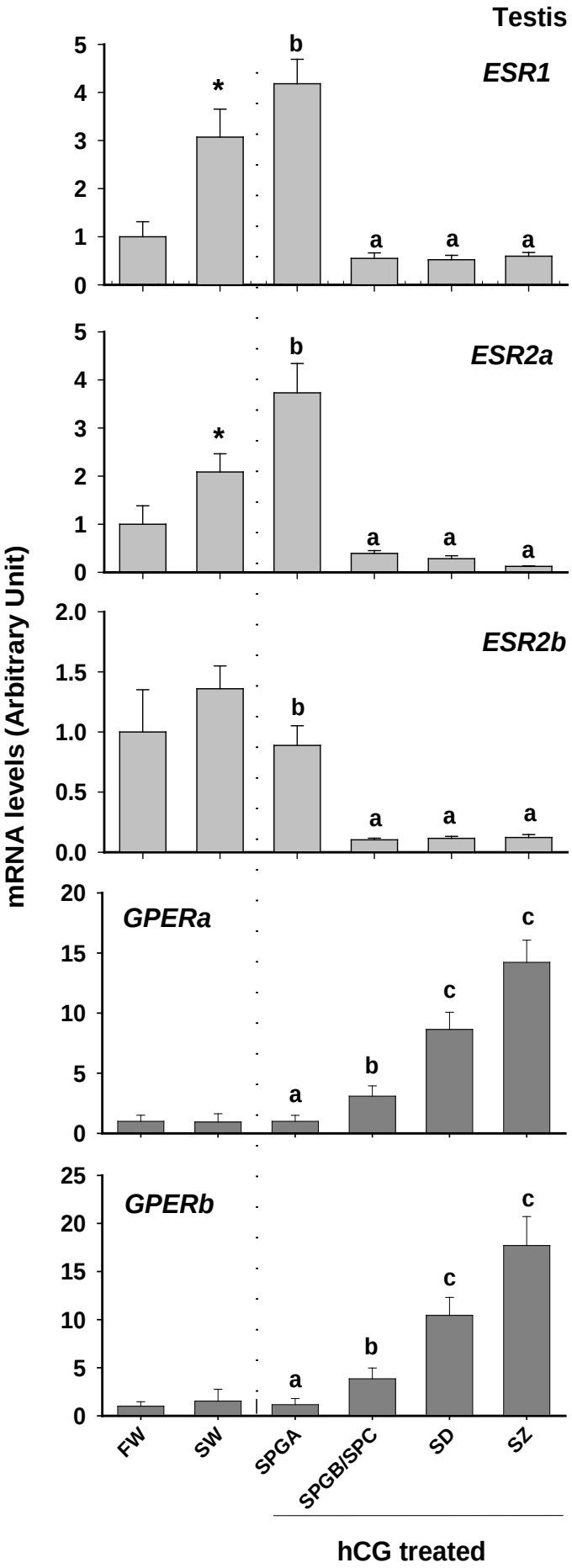
769



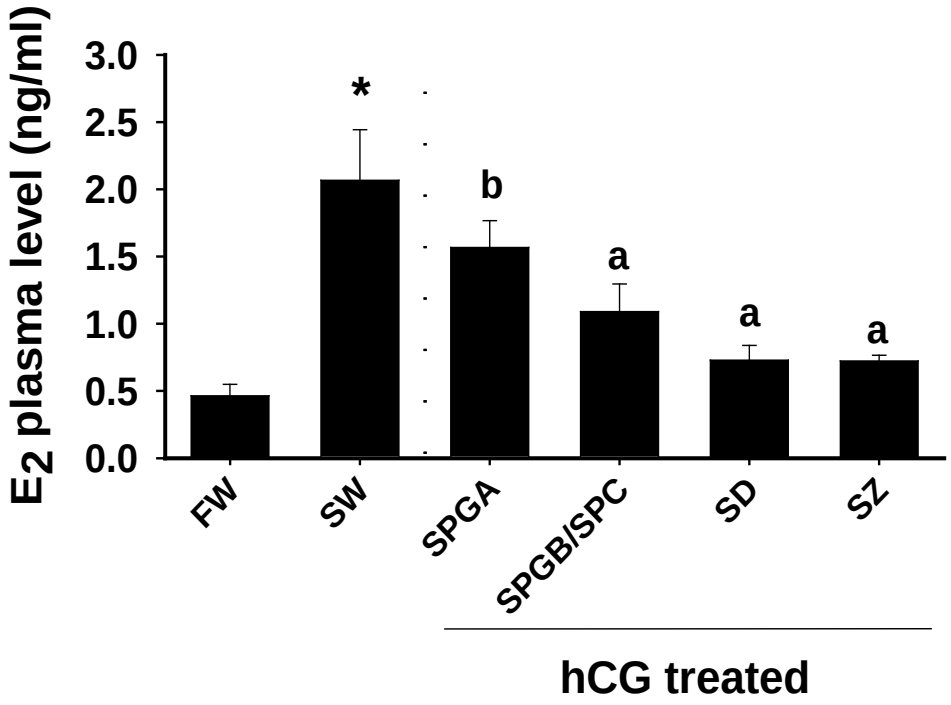
771 **Figure 5**


Pituitary
ESR1
ESR2a
ESR2b
GPERa
GPERb
mRNA levels (Arbitrary Unit)
FW
SW
SPGA
SPGB/SPC
SD
SZ
hCG treated

773 **Figure 6**


Testis
ESR1
ESR2a
ESR2b
GPERa
GPERb
mRNA levels (Arbitrary Unit)
FW
SW
SPGA
SPGB/SPC
SD
SZ
hCG treated

774 **Figure 7**



775