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3 **Effects of in vivo treatment with the dopamine antagonist pimozide and**
4 **gonadotropin-releasing hormone agonist (GnRHa) on the reproductive axis**
5 **of Senegalese sole (*Solea senegalensis*)**

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24

25**Abstract**

26 The Senegalese sole (*Solea senegalensis*) is a flatfish that exhibits severe reproductive
27 dysfunctions in captivity. This study aimed at investigating the existence of a dopamine (DA)
28 inhibitory tone on the reproductive axes of this species. Four groups of Senegalese sole
29 breeders were treated with, saline (controls, CNT), the DA antagonist pimozide (PIM, 5 mg
30 kg⁻¹), gonadotropin-releasing hormone agonist (GnRHa, 40 µg kg⁻¹) or a combination of PIM
31 + GnRHa (COMB). Effects were evaluated on pituitary GnRH levels (ELISA), pituitary
32 gonadotropin subunit transcript levels (qPCR), plasma levels of sex steroids and vitellogenin
33 (ELISA), gonad development (histology), spermiation and egg production. The GnRHa
34 treatment induced egg release in females and stimulated testis maturation in males. In males,
35 PIM did not affect pituitary GnRH content, but enhanced GnRHa-induced pituitary GPα
36 transcripts and modified plasma androgen levels; moreover, PIM stimulated spermatogenesis
37 and milt production, both alone and combined with GnRHa. In females, PIM did not affect
38 pituitary and plasma endocrine parameters and did not affect egg production and fertilization
39 success of the broodstock, either alone or in the combined treatment. In conclusion, data
40 indicated the existence of a DA inhibition in mature males, which would be absent or weakly
41 expressed in females.

42

43 **Key words:** dopamine, FSH, GnRH, LH, pimozide, Senegalese sole, spawning, spermiation.

44

45 1. Introduction

46 The brain-pituitary-gonad (BPG) axis regulates reproduction in fish, as in all vertebrates.
47 External and internal signals are integrated in the brain by hypothalamic neurons producing
48 the gonadotropin-releasing hormones (GnRHs), GnRH1, GnRH2 and GnRH3 (Zohar et al.,
49 2009), that stimulates in the pituitary the synthesis and release of gonadotropins (GTHs),
50 follicle stimulating hormone (FSH) and luteinizing hormone (LH). The GTHs are the critical

51modulators of gametogenesis and gonadal maturation, through their actions on gonadal
52steroids and growth factors (Levavi-Sivan et al., 2009; Schulz et al., 2009; Lubzens et al.,
532009). In fish, in addition to the primary GnRH stimulatory system, neurons secreting
54dopamine (DA) have been identified as an inhibitory system over the reproductive axis.
55Dopamine exerts inhibitory actions on both the brain and pituitary through reduction of GnRH
56synthesis and release, down-regulation of GnRH receptors and interference with the GnRH-
57signal transduction pathways, thus affecting GTH secretion from the pituitary (Peter and Yu,
581997; Popesku et al., 2008). Inhibitory effects of DA have been clearly demonstrated in
59freshwater fishes, including cyprinids (Yaron, 1995), silurids (De Leeuw et al., 1986;
60Silverstein et al., 1999) and salmonids (Saligaut et al., 1999), as well as in tilapia
61(*Oreochromis spp.*) (Melamed et al., 1998), European eel (*Anguilla anguilla*) (Vidal et al.,
622004) and grey mullet (*Mugil cephalus*) (Aizen et al., 2005), but have not been demonstrated
63in marine fishes (Dufour et al., 2005).

64 When reared in captivity, most fish species exhibit reproductive dysfunctions, which are
65thought to be caused by the stress associated with intensive culture conditions and the absence
66of adequate environmental cues (Zohar and Mylonas, 2001). In many cases, the application of
67exogenous hormonal treatments is an effective therapy to stimulate reproduction and induce
68spawning in captive fish (Mylonas et al., 2009). One of the most common strategies is the
69administration of GnRH agonists (GnRHa), via saline-diluted injections or slow-delivery
70implants. The GnRHa treatment induces the synthesis and release of endogenous GTHs and
71stimulates oocyte maturation (OM), ovulation and spermiation in fish (Mylonas and Zohar,
722001), and has been used effectively in various flatfishes (Larsson et al., 1997; Clearwater and
73Crim, 1998; Vermeissen et al., 1998, 2000; Mugnier et al., 2000; Guzmán et al., 2009a).
74However, GnRHa treatments have been shown to be ineffective in some species and this was
75associated to the action of an endogenous DA inhibitory tone. In these species it is required

76the co-administration of GnRHa with DA antagonists, in order to remove the endogenous DA
77inhibition and permit the stimulatory action of GnRHa on the reproductive axis (Peter and Yu,
781997; Mylonas et al., 2009). Combined treatment of GnRHa with DA antagonists, such as
79pimozide (PIM) or domperidone, has been used successfully to stimulate reproduction in
80cyprinids (Yaron, 1995; Mikolajczyk et al., 2004), catfishes (Silverstein et al., 1999; Wen and
81Lin, 2004) and mullets (Arabaci and Sari, 2004; Aizen et al., 2005). But, only few studies
82have investigated the DA system in marine fishes, with no conclusive results. Studies
83performed on gilthead seabream (*Sparus aurata*), Atlantic croaker (*Micropogonias undulatus*)
84and red seabream (*Pagrus major*) showed no effect of PIM to enhance the stimulatory effects
85of GnRHa on pituitary LH release (Copeland and Thomas, 1989; Zohar et al., 1995;
86Kumakura et al., 2003). Similarly, In European sea bass (*Dicentrarchus labrax*), co-treatment
87of PIM with GnRHa did not improve GnRHa-induced OM or spawning (Prat et al., 2001).
88Based on these studies, it has been proposed that a DA inhibition would be lacking in marine
89fishes, although it has been suggested that more work is needed to investigate the potential
90existence of a DA inhibitory tone influencing reproduction in marine fish species (Vidal et al.,
912004; Dufour et al., 2005).

92 The Senegalese sole (*Solea senegalensis*, Pleuronectiforme, Soleidae) is a highly valuable
93marine flatfish that has become a priority species for European and Mediterranean aquaculture
94(Imsland et al., 2003). However, the establishment of an efficient aquaculture industry is
95constrained by the failure of cultured broodstock (F1 generation) to reproduce (Howell et al.,
962006, 2009). Spawning of cultured breeders is rare, and when it occurs fecundity is low and
97eggs are unfertilized. Recent studies have shown that cultured females complete
98vitellogenesis and show adequate profiles of plasma vitellogenin (VTG) and sex steroid levels
99but, as the reproductive cycle proceeds, most females fail to undergo OM, ovulation and
100spawning, and most of post-vitellogenic oocytes undergo apoptosis (García-López et al.,

1012007; Guzmán et al., 2008, 2009a). On the other hand, cultured males complete
102spermatogenesis and sperm maturation and show normal androgen plasma profiles, but sperm
103production is low compared to wild-caught breeders, which is thought to affect fertilization
104success (García-López et al., 2006; Cabrita et al., 2006). These reproductive dysfunctions
105have been observed in most captive-reared flatfish species (Larsson et al., 1997; Mazorra de
106Quero et al., 2000ab; Mañanós et al., 2008). A few studies have tested the use of hormonal
107therapies to stimulate reproduction in Senegalese sole but, for the moment, with limited
108success. Treatment of Senegalese sole breeders with GnRHa, given as injection or via slow-
109delivery systems, were effective in stimulating sex steroid release, OM and egg release in
110females (Agulleiro et al., 2006, Guzmán et al., 2009a) and slightly, steroidogenesis and sperm
111production in males (Agulleiro et al., 2006, 2007) but in all cases, spawned eggs were
112unfertilized, indicating a failure of the GnRHa treatment to induce fertilized tank spawning in
113the broodstock. Preliminary experiments with human-chorionic gonadotropin (hCG) have
114shown a higher efficiency of hCG than GnRHa treatments on the stimulation of
115steroidogenesis, spermatogenesis, testes maturation and sperm production in male Senegalese
116sole breeders and production of few fertilized spawning in broodstock containing hCG-treated
117males (Guzmán et al., unpublished results).

118 To date, no attempts have been made to investigate the influence of a DA inhibition on
119the reproductive axis of Senegalese sole or in any other flatfish species, and determine the
120potential efficiency of DA antagonist treatments on the stimulation of reproduction and
121spawning in flatfishes. There are anatomical evidences suggesting the existence of a DA
122inhibitory system in Senegalese sole. Immunoreactivity to tyrosine hydroxylase (TH), a rate-
123limiting enzyme in DA synthesis, has been detected in the preoptic area of the brain and TH-
124immunoreactive fibres were shown to extend into the proximal *pars distalis* of the pituitary,

125where gonadotrophs are located (Rendón et al., 1997; Rodríguez-Gómez et al., 2000),
126suggesting DA activity in the brain and pituitary of Senegalese sole.

127 The present study aimed at investigating, for the first time in a flatfish species, the
128existence of an endogenous DA inhibition on the endocrine reproductive axis in Senegalese
129sole and its influence on the efficiency of GnRHa-based spawning induction hormonal
130therapies. The effects of in vivo treatments with GnRHa and PIM (a D2-receptor antagonist),
131both alone and in combination, were evaluated in mature male and female Senegalese sole
132breeders, by analyzing pituitary content of GnRHs (GnRH1, GnRH2 and GnRH3), pituitary
133transcript levels of gonadotropin subunits (FSH β , LH β and the common glycoprotein α , GP α)
134and plasma levels of sex steroids (estradiol, testosterone and 11-ketotestosterone) and VTG,
135and were correlated with testicular maturation, spermiation, egg production and fertilization
136success.

137

1382. Materials and Methods

1392.1. *Fish husbandry*

140 Senegalese sole juveniles (F1 generation), obtained from natural spawns of wild-caught
141broodstock (spring 2003) at “Stolt Sea Farm s.a.” (La Coruña, Spain), were transported to the
142facilities of the Spanish Institute of Oceanography (IEO, Vigo, Spain) in March 2004. Fish were
143tagged with passive integrated transponder tags (PIT tags, AVID). Sex of the fish was
144determined at puberty by abdominal swelling in females and by feeling the shape of the testis
145in males. Fish were reared in rectangular fibreglass tanks (4,000 l, 1 m depth, 4 m²) without
146sand substrate, at a density of around 3 kg m⁻² and were exposed to the natural temperature
147and photoperiod regime of the region.

148 The experiment was initiated on April 24th 2006 and terminated on May 19th 2006, under
149increasing water temperatures ranging from 16.5 °C to 18.1 °C (mean temperature of this

150period, 17.2 ± 0.2 °C). Dissolved oxygen was checked regularly and ranged between 6.8 and
1517.5 ppm. Tanks were fitted with overflow egg collectors and were supplied with flow-through
152seawater (salinity 36‰) at a flow rate of 400% d⁻¹. Fish were fed to apparent satiation 3 d a
153week using commercial dry pellets (LE-5 Elite from Skretting Co.) and semi-dry pellets made
154at the facility with raw fish, squid and mussel meat.

155 Handling of the fish for routine management and experimentation was always done
156according to national and institutional regulations and the current European Union legislation
157on handling experimental animals (EEC, 1986). All fish to be handled were anaesthetised by
158immersion in 0.3 ml l⁻¹ of 2-phenoxyethanol.

159

1602.2. *Experimental design and hormonal treatments*

161 On April 11th 2006, when external signs of gonad maturation were clearly evident (Guzmán
162et al., 2008, 2009a), such as abdominal swelling in females and expressible milt in males, 96 fish
163were distributed homogeneously in eight 4,000 l tanks, at a sex ratio of 1:1 and a density of $3.2 \pm$
1640.1 kg m⁻². The experiment was run on duplicates, with two tanks per treatment group (4 groups,
165n = 12 fish per tank). Fish were three years old, with a mean ($\pm$ S.E.M.) body weight (BW) of
1661,216.0 $\pm$ 38.5 g and 981.3 $\pm$ 30.2 g for females and males, respectively, and body length (BL)
167of 40.4 $\pm$ 0.5 cm and 38.8 $\pm$ 0.5 cm for females and males, respectively.

168 On April 24th 2006 (day 0), 8 females and 8 males were randomly selected (2 females and 2
169males per tank), anaesthetised and sampled for blood and milt. Thereafter, the following
170treatments were applied: (1) control treatment groups (CNT) were injected with saline (0.9%
171NaCl) on days 0 and 24; (2) treatment with pimozide (PIM) was given as 3 injections (5 mg kg⁻¹)
172on days 0, 10 and 24; (3) treatment with GnRH α (GnRH α) was given as an implant (40 μ g kg⁻¹)
173on day 0 and injection (25 μ g kg⁻¹) on day 24, and (4) the combined PIM + GnRH α treatment
174(COMB) was applied as for the GnRH α and PIM groups above on days 0, 10 and 24.

175 The drugs used were the long-acting DA D2-receptor antagonist PIM (Sigma-Aldrich) and
176the [D-Ala⁶, Pro⁹ Net]-LHRHa (Bachem, Switzerland). For injections, both PIM and GnRHa
177were dissolved in saline. The GnRHa implants were manufactured from p[Ethylene-Vinyl
178acetate] copolymer (EVAc, Elvax; DuPont Chemical CO., DE) as 2-mm diameter x 3-mm
179cylinders (Zohar et al., 1990). Both injections and implants were applied in the dorsal
180musculature of the fish. The doses of PIM and GnRHa were based on previous studies in other
181fishes, including Senegalese sole (Zohar and Mylonas, 2001; Guzmán et al., 2009a).

182 From the two groups of replicated experimental tanks, only one was sampled for blood (days
1830, 10 and 25), milt (days 0 and 25) and tissues (sacrifice, day 25), whereas the other duplicate of
184each treatment group was left “undisturbed” (only manipulated for hormone administration)
185throughout the experimental period. The fecundity and quality of the eggs from each spawn was
186checked daily in all tanks. As described later, egg production data obtained from both
187duplicates (“sampled” and “undisturbed”) of each experimental group were very similar,
188indicating that blood and milt sampling did not influence the spawning performance of the
189broodstock.

190 Blood (0.8 ml) was taken from the caudal vasculature using heparinised syringes and placed
191in ice-cold heparinised tubes containing aprotinin (0.15 IU ml⁻¹, Sigma). Plasma was obtained by
192centrifugation (3,000 g, 15 min, 4°C) and stored at -20°C until analysis for sex steroid hormones
193and VTG (ELISA). For milt collection, the urogenital pore was first wiped off with a piece of
194paper, and milt was collected within a syringe by gentle abdominal pressure on the area of the
195testis. Syringes containing milt samples were immediately placed on ice and maintained
196refrigerated until determination of sperm parameters (milt volume, sperm concentration and
197sperm motility). After blood and milt sampling on day 25, fish were sacrificed by decapitation
198for dissection of tissues. Pituitary glands were collected, frozen immediately in liquid nitrogen
199and stored at -80°C until analysis for GnRH content (ELISA) and GTH subunit transcript levels

200(qPCR). In males, testes were removed and weighed for calculation of the gonadosomatic index
201($GSI = \text{gonad weight} \times BW^{-1} \times 100$). Transverse sections from the central region of the dorsal
202and ventral lobes of the testes were dissected out and collected for histology.

203

2042.3. *Gonadotropin subunit real-time quantitative PCRs (qPCRs)*

205 Transcript levels of Senegalese sole FSH β , LH β and GP α subunits were quantified by
206specific qPCRs (Guzmán et al., 2009b). Briefly, plasmids containing the specific subunit
207insert were linearized and used as templates for gene-specific RNA standard synthesis. Total
208RNA isolated from a pool of Senegalese sole pituitaries, using Tri-Reagent (Sigma) and
209treated with DNase (RQ1 RNase-free DNase, Promega), served as standard for 18s RNA.
210The amount of each RNA standard was determined using RiboGreen RNA quantification kit
211(Molecular Probes, Eugene, OR).

212 For sample analysis, total RNA was isolated from each pituitary sample using Tri-
213Reagent, according to the manufacturer's instructions, treated with DNase and quantified
214using a Nano Drop ND-1000 Spectrophotometer. The RNA standards (target and reference)
215and RNA from each sample were simultaneously reverse-transcribed into cDNA using
216random hexamers and MMLV reverse-transcriptase (Promega) and diluted 5-fold in sterile
217water. Real-time analysis was carried out on an ABI Prism 7700 Sequence Detection System.
218Reactions were performed in a 20 μ l volume containing cDNA generated from 10 ng of
219original RNA template, 200 nM gene-specific primers (Table 1), and 10 μ l of SYBR Green
220PCR core reagent (Applied Biosystem). Amplification reactions were carried out at 50 °C for
2212 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 sec and 60 °C for 60 sec. After the
222qPCR reaction, copy number for unknown samples was determined by comparing C_T
223(threshold cycles) values to the specific standard (run in every plate) and normalized to the
224amount of 18s RNA in each sample.

225 Amplification efficiencies (%) for the qPCRs, obtained from validation assays using
226 serially diluted reverse-transcribed RNA, were 102.3 ± 2.1 , 102.5 ± 2.0 and 96.8 ± 3.5 for
227 FSH β , LH β and GP α , respectively. The lowest standard point was 100 copies/reaction for all
228 three transcripts (FSH β , LH β and GP α). The intra- (n=4) and inter-assay (n=6) coefficients of
229 variation, at 10^6 copies/reaction, were 0.4% and 7.6% for FSH β , 0.2% and 8.2% for LH β , and
230 0.1% and 5.5% for GP α .

231

232 2.4. GnRH ELISAs

233 Peptide levels of GnRH1, GnRH2 and GnRH3 were measured over the same pituitary
234 samples used for GTH mRNA analysis. The protein fraction obtained after RNA extraction
235 was precipitated with iso-propanol (1:2, v/v) and pellets dried and reconstituted in 200 μ l
236 PBST buffer (10 mM phosphate buffer, 0.9% NaCl, 0.05% Tween-20, pH 7.2). Acetic acid
237 4N was added to each sample (1:1, v/v), incubated for 10 min at 80 °C and centrifuged
238 (13,000 g, 30 min, 4°C). Pellets were re-extracted twice with acetic acid 4N. Supernatants
239 were pooled, vacuum-dried and reconstituted in 0.1 M potassium phosphate buffer, pH 7.4,
240 for analysis.

241 Levels of GnRH1, GnRH2 and GnRH3 were measured simultaneously in each
242 reconstituted sample using specific ELISAs for each GnRH form. The ELISA protocol was
243 based on that developed for European sea bass and gilthead seabream GnRHs (Kah et al.,
244 1994; Holland et al., 1998) and further used in these and other species (Rodríguez et al., 2000;
245 Andersson et al., 2001; Holland et al., 2001; Steven et al., 2003), including the Senegalese
246 sole (Guzmán et al., 2009b). The sensitivities of the ELISAs, determined as the GnRH dose
247 giving 80% of binding, were 6 pg well⁻¹ for GnRH1, 7 pg well⁻¹ for GnRH2 and 2 pg well⁻¹ for
248 GnRH3. Cross-reactivities, calculated at 50% of binding, were below 0.6% except for the
249 GnRH3 assay, which displayed 3.7% cross-reactivity with GnRH2. The intra- (n=5) and inter-

250 assay (n=4) coefficients of variation, at 30-50% of binding depending on the assay, were 6% and
251 7% for GnRH1, 8% and 11% for GnRH2, and 4% and 8% for GnRH3 (Holland et al., 1998).

252

253 2.5. *Vitellogenin and sex steroid ELISAs*

254 Plasma levels of VTG were measured by homologous ELISA, using purified Senegalese
255 sole VTG as standard and specific antibodies against Senegalese sole VTG (Guzmán et al.,
256 2008). The sensitivity, checked by means of the lowest detection limit (Bo-2SD, maximum
257 binding minus twice the standard deviation) was 3.6 ng ml⁻¹. Intra- and inter-assay
258 coefficients of variation, checked at 50% of binding, were 6.7% (n=12) and 9.8% (n=29),
259 respectively.

260 For steroid analysis, plasma samples were first extracted by addition of ice cold methanol
261 (methanol:plasma 6:1, v/v), shaken and centrifuged (3,000 g, 15 min, 4°C). The pellet was re-
262 extracted twice with 200 µl of methanol. Supernatants were pooled, air-dried and
263 reconstituted in 0.1 potassium buffer (pH 7.4). The levels of estradiol (E2), testosterone (T)
264 and 11-ketotestosterone (11-KT) were quantified by ELISA, using a protocol previously
265 validated for Senegalese sole plasma (Guzmán et al., 2008, 2009a). The sensitivities of the
266 ELISAs, calculated as the lowest detection limit (Bo-2SD), were 5.2 pg ml⁻¹, 8.8 pg ml⁻¹, and
267 0.4 pg ml⁻¹ for the E2, T and 11-KT ELISA, respectively. The intra-(n=4) and inter-assay
268 (n=8) coefficients of variation, at 50% of binding, were 5.8% and 6.3% for E2, 6.1% and
269 11.3% for T, and 10.7% and 9.1% for the 11-KT ELISA.

270

271 2.6. *Testicular histology*

272 Fragments of testicular tissue were fixed in 4% phosphate-buffered (0.1M, pH 7.2)
273 formalin for 48–96 h at room temperature. After rinsing in running tap water (16 h) and
274 dehydration in ascending concentrations of ethanol, fragments were infiltrated and embedded

275in Leica Historesin (2-hydroxi-ethylmethacrylate; Reichert-Jung, Germany). Sections of 3 μ m
276were cut on a Supercut 2065 microtome (Reichert-Jung, Germany) and stained with Harris'
277Haematoxylin-Eosin. Stained sections were examined and photographed on a Leitz Diaplan
278light microscope.

279 The stage of testicular development was determined according to the methodology
280previously described for the study of the morphology and developmental stages of the
281Senegalese sole testes (García-López et al., 2005, 2006). This is based on a qualitative
282analysis of the presence of the different germ cell types and their relative abundance and was
283considered adequate for the particular type of testicular development exhibited by this species.
284Briefly, the Senegalese sole exhibits a semi-cystic type of spermatogenesis in which
285spermatids accumulate in the lumen of the cortical seminiferous lobules and transform into
286spermatozoa in a group-synchronous fashion (García-López et al., 2005); successive
287spermatozoa batches are produced and accumulated in the medullar efferent ducts and
288eventually released during each spawning event (García-López et al., 2006). This type of
289spermatogenic development implies a high heterogeneity in the histological observations of
290nearly located seminiferous lobules, with some lobules filled with only spermatids, some other
291with both spermatids and spermatozoa at different percentages and other adjacent lobules
292appearing empty or containing few residual spermatids and spermatozoa. Similar
293heterogeneity is observed when analyzing the presence of cysts containing type B
294spermatogonia and primary spermatocytes among different areas of a histological section.
295Owing to this, histological analysis of testicular sections is best performed through a
296qualitative than a quantitative approach that would require counting of cell types in selected
297seminiferous lobules, leading to biased results due to sample heterogeneity (García-López et
298al., 2006).

299 For histological analysis of the testes collected from males of the four experimental
300 groups, six sections from each individual were selected, three from each testicular lobule,
301 separated 100 μm between them. From each section, photomicrographs were taken and
302 analyzed from the two main regions of the testes, the cortex, where germ cells proliferate and
303 differentiate and the medulla, the efferent duct system where spermatogenic production is collected
304 and stored. Photomicrographs were analyzed and the relative abundance of each germ cell
305 type scored as, 0) absent or very low, 1) low, 2) intermediate, 3) high, and 4) very high, and
306 used to estimate the degree of maturation of the sample (García-López et al., 2006).

307

308 2.7. *Sperm parameters*

309 The volume of milt expressed by each male was measured using a micropipette and
310 standardized according to BW. Sperm concentration was measured using a Neubauer
311 haemocytometer after 1:400 dilution in 1% formalin. Total sperm production was calculated
312 as milt volume ($\mu\text{l kg}^{-1}\text{ BW}$) x sperm concentration (spermatozoa ml^{-1}). For determination of
313 sperm motility, milt samples (1 μl) were first diluted 1:5 in 200 mOsm kg^{-1} Ringer's solution
314 (Chereguini et al., 1997) and then activated by addition of 36‰ sea water (19 μl). Motility
315 was examined under light contrast microscopy (magnification 400 x) and scored by three
316 different operators from 0 to 5, according to the percentage of moving cells (score 0: no
317 movement, 1: 0-20%, 2: 20-40%, 3: 40-60%, 4: 60-80% and 5: 80-100%); motility for each
318 sample was considered the mean value of the three scores (Chereguini et al., 1997).

319

320 2.8. *Egg production*

321 The presence of eggs released from the broodstock tanks was checked twice daily (09:00
322 and 17:00 h) and were collected for evaluation of fecundity and quality. Eggs were
323 transferred to calibrated cylinders filled with 36‰ sea water and were allowed to stand for a

few minutes to separate buoyant (viable) and sinking (dead) eggs and the volume of each fraction determined. Total fecundity (buoyant and sinking eggs) was further expressed as n° of eggs, considering that in Senegalese sole a volume of 1 ml of eggs corresponds to approximately 1,000 eggs (Guzmán et al., 2009a). Daily relative fecundity was calculated using the BW of the females at the previous sampling, and total relative fecundity was calculated at the end of the study. From each spawn, a sample of 50 buoyant eggs was examined under a binocular microscope to evaluate morphological characteristics, such as, egg size and shape, transparency, distribution of oil globules and fertilization rate. Buoyant eggs were incubated for 48 h to determine embryo development and hatching success.

2.9. Statistics

Data are expressed as mean $\pm$ standard error of the mean (S.E.M.). Statistical differences among experimental groups per sampling point were examined using a one-way ANOVA, followed by Student-Newman-Keuls (SNK) multiple comparison procedure, with a significance level of $p < 0.05$. Statistical differences among different sampling times within the control group were also examined using a one-way ANOVA, to determine the temporal evolution of sex steroid and VTG plasma levels. Differences in daily fecundity and egg buoyancy data between duplicated tanks of each group were analyzed by a t-test with a significance level of $p < 0.05$. Normality and homogeneity of the variance were tested by the Kolmogorov–Smirnov and Bartlett methods, respectively. The expression of ELISA results was performed after linearization of the sigmoid standard curve using the logit transformation ($\text{logit} (B_i / B_o) = \ln(B_i - \text{NSB}) / (B_o - \text{NSB})$), where B_i represents the binding of each point, B_o the maximum binding and NSB the non-specific binding.

3. Results

3493.1. Pituitary content of GnRHs

350 The pituitary peptide levels of GnRH1, GnRH2 and GnRH3 were analyzed on day 25
351 from the initiation of treatments, 24 h after the last hormone administration (Fig. 1). Levels of
352 GnRH3 in all pituitary samples were below the detection limit of the assay (<21 pg/pituitary)
353 and thus, were considered undetectable (data not shown). No effect ($p < 0.05$) of the treatments
354 was observed on any GnRH form, in either males or females.

355

3563.2. Pituitary transcript levels of gonadotropin subunits

357 The transcript levels of each GTH subunit (FSH β , LH β and GP α) in the pituitary were
358 analyzed on day 25 from the initiation of treatments, 24 h after the last hormone administration
359 (Fig. 2). In males, GnRHa increased pituitary GP α transcript levels, but had no effect on FSH β
360 and LH β transcript levels. Treatment with PIM alone did not affect GTH transcript levels, with
361 respect to controls, but enhanced GnRHa-induced GP α transcript levels, as showed by
362 statistically significant differences between GnRHa and COMB treatment groups. In females,
363 GnRHa treatment induced an elevation of LH β and GP α transcript levels, but not those of FSH β ,
364 with respect to controls. Treatment with PIM alone did not affect GTH transcripts, nor did it
365 modify GnRHa-induced levels in the combined treatment.

366

3673.3. Plasma levels of sex steroids and vitellogenin

368 Levels of sex steroids and VTG were analyzed on 0, 10 and 25 days after initiation of
369 treatments (Fig. 3). In controls, the evolution of plasma steroid levels over time showed that in
370 males both 11-KT and T decreased from 0 to 25 days, whereas in females both E2 and T were
371 maintained high from 0 to 10 days but decreased on day 25; plasma VTG levels in females did
372 not change over time.

373 In males, GnRHa increased both 11-KT and T plasma levels on day 25, with respect to
374controls. The PIM treatment increased T plasma levels and reduced GnRHa-induced 11-KT and
375T plasma levels on day 25. In females, no effect of the treatments was detected on E2 and T
376plasma levels, but plasma VTG was significantly ($p<0.05$) increased on day 10 in GnRHa-treated
377animals, both in the GnRHa and COMB groups. The PIM treatment did not affect basal or
378GnRHa-induced VTG plasma levels.

379

3803.4. Testicular development

381 The males of the control group showed testes at the stage of functional maturation, as
382expected by the time of the year (spermiation period). This is characterized by low numbers
383of early developing germ cells (spermatogonia (spg) and spermatocytes (spc)), very high
384abundance of spermatids (spd) in the cortex and low to intermediate number of spermatozoa
385(spz) within the medullar efferent duct system (Fig. 4A,B). The cortical seminiferous lobules
386were fully filled by spd, with some sporadic spz (Fig. 4A). The medullar efferent ducts,
387surrounded by interstitial tissue, were of small diameter and were filled by spd and spz; the
388spz being clearly distinguishable by a strongly basophilic small head and a large flagellum
389(Fig. 4B).

390 The testes of males from the three hormone-treated groups (PIM, GnRHa and COMB)
391were at the stage of advanced functional maturation and showed clear signs of stimulation of
392spermatogenesis compared to control males. The PIM treatment increased the presence of
393cysts containing early developing germ cells (spg and spc) at the distal part of the cortical
394seminiferous lobules and reduced the number of spd in the lumen of the lobules (Fig. 4C).
395Such reduction was accompanied by a relative increase in the abundance of spz accumulated
396in the medullar efferent ducts (Fig. 4D). The GnRHa treatment induced similar germ cell
397dynamics than the PIM treatment, although in the testis of GnRHa-treated males the presence

398of spg and spc in the cortical seminiferous lobules (Fig. 4E) and ripe spz in the medullar
399efferent ducts (Fig. 4F) was lower. The testes of males that received the combined treatment
400(COMB) showed the highest abundance of cysts containing spg and spc in the cortex and
401lowest abundance of spd (Fig. 4G); a high occurrence of empty seminiferous lobules was
402observed in the cortex, as a consequence of transformation of batches of spd into spz. The
403efferent duct system in the medulla was highly developed and showed the highest abundance
404of spz (Fig. 4H).

405No effect of the treatments was observed on the GSI. The GSI values were 0.075 ± 0.01 in
406control males and 0.088 ± 0.015 , 0.094 ± 0.008 and 0.093 ± 0.009 in males from the GnRHa,
407PIM and COMB groups, respectively.

408

4093.5. *Sperm parameters*

410 Milt volume and sperm concentration was determined on days 0 and 25 from initiation of
411treatments (Fig. 5). In controls, milt volume, sperm concentration and consequently, total sperm
412production, did not change from 0 to 25 days. The GnRHa treatment induced a slight 1.9-fold
413increase in milt volume (no statistical differences from controls) and significantly reduced sperm
414concentration ($p < 0.05$). Total sperm production in GnRHa-treated males was similar to controls.
415Treatment with PIM increased milt volume compared to controls ($p < 0.05$), both alone (3.5-fold)
416and in the combined treatment (5.2-fold); the COMB treatment caused a significant ($p < 0.05$) 2.7-
417fold increase in milt volume compared to the GnRHa group. As observed with GnRHa, the
418increase in milt volume in both PIM and COMB groups was accompanied by diminished sperm
419concentration, although these differences were only significant ($p < 0.05$) for the COMB
420treatment. Total sperm production in both PIM and COMB groups was slightly (not statistically
421different) increased 2.5-fold compared to controls. Sperm motility ranged from 40 to 80%
422among individual males and was similar among all control and hormone-treated fish.

423

4243.6. *Egg production*

425 No differences in daily mean fecundity, egg buoyancy or egg morphology were observed
426between duplicate tanks of each experimental group. No spawning was detected in any of the
427tanks before the initiation of treatments on day 0 or the end of treatments on day 24 (Fig. 6). The
428control group did not spawn during the experimental period, while some sporadic batches of eggs
429of low fecundity were collected from the PIM group. The GnRHa treatment induced daily egg
430release from day 3 until day 15; this kinetic was not modified by co-treatment with PIM
431(COMB). The GnRHa and COMB treatment showed similar number of spawns, daily and total
432fecundity, and egg buoyancy (Table 2). Eggs examined under the binocular showed a similar
433morphology in all groups and the absence of fertilization.

434

4354. **Discussion**

436 This study investigated, for the first time in a marine flatfish species, the potential
437inhibitory effects of dopamine (DA) on the endocrine regulation of the reproductive axis in
438Senegalese sole. The results showed no effects of the DA D2-receptor antagonist pimozide
439(PIM) on pituitary GnRH content, or pituitary FSH β and LH β transcript levels but in males,
440PIM stimulated plasma androgen levels, testicular maturation and sperm production,
441suggesting that in fact a DA inhibitory tone may be active in the regulation of the reproductive
442axis in this fish species.

443 The lack of effect of PIM on the pituitary content of GnRHs would indicate that DA is not
444affecting GnRH release from nerve terminals and thus, GnRH accumulation in the pituitary. A
445DA inhibition on GnRH secretion through pituitary DA D2-receptors would not be active in
446mature Senegalese sole. This is in contrast with a previous study performed in the goldfish
447(*Carassius auratus*), which reported an increase in immunoreactive GnRH in discrete parts of the

448 brain and pituitary 24 h after in vivo treatment with a PIM injection (Yu and Peter, 1990). These
449 authors suggested a DA action on both brain GnRH neurons and pituitary gonadotrophs
450 influencing GTH secretion, supporting further studies that demonstrated a strong DA inhibition
451 on the reproductive axis of cyprinids (Yaron, 1995; Peter and Yu, 1997).

452 Evaluation of the hormone treatment effects on the transcription of pituitary GTH
453 subunits showed that in females, the GnRHa treatment increased transcript levels of LH β (and
454 also GP α), but not of FSH β . This result indicates that transcription of LH β and FSH β genes in
455 Senegalese sole are differentially regulated by GnRHa. Temporal differences in the
456 transcription of GTH β -subunits have been described previously in other fishes (Yaron et al.,
457 2003; Zohar et al., 2009). For example, pituitary samples collected from mature European sea
458 bass 18 h after a single GnRHa injection showed increased transcript levels of LH β , but not
459 FSH β (Mateos et al., 2002). Similarly, red seabream implanted with a GnRHa-pellet had
460 increased pituitary transcript levels of LH β , but not FSH β , at 10 and 20 days after treatment
461 (Kumakura et al., 2003). In striped bass (*Morone saxatilis*), GnRHa injection induced a rapid
462 increase of LH β at 6 h post-treatment, while FSH β transcript levels were only increased after
463 24 h (Hassin et al., 1998). These results indicate temporal differences in the GnRHa-induced
464 stimulation of FSH β and LH β transcription, which might vary depending on the species. In
465 the present study, pituitaries were collected at a single sampling point 24 h after the last
466 hormone treatment, and so, it is possible that an increment change in FSH β gene expression at
467 other times post-treatment might occur. Similarly, this study cannot rule out an effect of
468 GnRHa on FSH β and LH β gene expression in males, which might be evidenced at other
469 sampling points.

470 The PIM treatment alone had no effect on basal FSH β or LH β transcript levels and the co-
471 treatment did not modify further the GnRHa effects, neither in female nor in male Senegalese
472 sole breeders. Similar results have been previously found in other fish species using both in

473 vitro and in vivo treatments with DA antagonists (tilapia: Melamed et al., 1996; striped bass:
474 Hassin et al., 2000; red seabream: Kumakura et al., 2003), suggesting the absence of a DA
475 inhibitory tone on pituitary GTH transcription. Nevertheless, a dopaminergic action on
476 pituitary GTH synthesis in Senegalese sole cannot be ruled out because, interestingly,
477 treatment of males with PIM slightly increased GP α transcript levels and clearly enhanced
478 GnRHa-induced stimulation of GP α transcripts. Studies in rainbow trout (*Oncorhynchus*
479 *mykiss*) have suggested that production of GP α subunit acts as a rate-limiting factor in the
480 synthesis of FSH (Naito et al., 1991, 1997). In the absence of immunoassays for FSH and LH
481 in Senegalese sole, it was not possible to determine pituitary or plasma FSH and LH levels
482 and thus, the elevation of GP α transcript levels could not be correlated with concomitant
483 increases in FSH and/or LH protein levels. It should be considered that the GP α subunit is
484 common to both GTHs (FSH and LH) and the thyroid stimulating hormone (TSH) and thus,
485 the observed stimulation of GP α transcription by PIM could be related to an increase in the
486 synthesis of GTHs (FSH and/or LH) and/or to the synthesis of TSH. To date, there are no
487 studies on DA effects on TSH gene expression or TSH protein synthesis and thus, this
488 hypothesis remains to be investigated (McKenzie et al., 2009). In contrast, some studies
489 might support a DA action on GTH synthesis, either directly or by enhancing gonadotrophic
490 cell responsiveness to the GnRH stimulus. In European eel, TH-immunoreactive fibers have
491 been found to innervate pituitary LH-secreting cells, providing an anatomical support for a
492 role of DA on the inhibition of LH synthesis in this species (Vidal et al., 2004). Likewise,
493 TH-immunoreactive fibers have been found in the *pars distalis* of the pituitary of Senegalese
494 sole, where both FSH and LH-producing cells are located (Rendón et al., 1997; Rodríguez-
495 Gómez et al., 2000; Guzmán et al., 2009b). These anatomical data, showing a potential
496 dopaminergic innervation of gonadotrophic cells, would support a direct effect of DA on
497 pituitary GTH synthesis in these species. In addition, as mentioned previously for the GnRHa

498treatment, effects of PIM on FSH β or LH β transcription may be evidenced at sampling times
499not included in this study.

500 Plasma levels of sex steroids were not affected by any of the treatments in females but in
501males, PIM increased plasma T levels (day 25), probably through unblocking endogenous DA
502inhibition of GTH action on testicular steroidogenesis (Schulz et al., 2009). Unexpectedly,
503PIM decreased the GnRHa-induced elevation of plasma T and 11-KT levels in the co-
504treatment. The explanation for this is unclear but it could be hypothesized that the low plasma
505levels of T and 11-KT in males of the combined treatment were in fact a reflection of a more
506advanced stage of testicular maturation in these fish, compared to the other groups. This
507advanced maturational stage would be associated with a shift in the steroidogenic pathway,
508resulting in decreased release of androgens and a corresponding increase in production of
509progestogens (Nagahama, 1994; Vermeissen et al., 2000). This is supported by the
510histological analyses, which showed the most advanced maturation of the testes in the COMB-
511treated males.

512 Histological analysis of the testes and quantification of sperm parameters revealed that
513PIM stimulated testicular maturation and sperm production in male Senegalese sole breeders.
514These data are the first evidence on the existence of an endogenous DA system inhibiting
515gonad maturation in a marine flatfish species. The dopaminergic action on gonadal
516development is probably a GTH-mediated sex steroid mechanism; as it was previously
517demonstrated in freshwater fish species (Dufour et al., 2005). Previous studies have shown an
518inhibitory effect of DA on GTH release from pituitary cells, with no concomitant effects on
519GTH synthesis (tilapia: Melamed et al., 1996; rainbow trout: Vacher et al., 2000), which
520would be in accordance with our present results on the absence of an effect of PIM on FSH β
521and LH β transcript levels. On another hand, direct effects of PIM on the testes should not be
522discarded. Transcripts of DA D2-receptors have been found in the ovary of tilapia (Levavi-

523Sivan et al., 2005) and grey mullet (Nocillado et al., 2006), suggesting a direct DA action on
524the fish gonad. Although DA D2 receptors have not been demonstrated in the fish testes,
525studies in mammals have shown that DA may participate in the proliferation and/or
526differentiation of male germ cells by acting through testicular DA D2-receptors (Otth et al.,
5272007).

528 As expected, treatment of female Senegalese sole breeders with GnRHa implants induced
529daily egg release for two weeks, similarly as observed in previous studies on hormonal
530induction of spawning in cultured Senegalese sole (Agulleiro et al., 2006; Guzmán et al.,
5312009a). The GnRHa treatment also increased VTG plasma levels, probably through a
532GnRHa-induced stimulation of E2 release; although in this study no effects of GnRHa were
533observed on plasma E2 and T levels analyzed at 10 and 25 days. This was probably due to the
534short-lived nature of the rise in GnRHa-induced plasma E2 levels, which was previously
535shown to last for only 3 days after treatment (Guzmán et al., 2009a). Results from both egg
536production and steroid and VTG plasma levels clearly showed a lack of effect of PIM on
537females, suggesting again the absence of an inhibitory dopaminergic action on the
538reproductive axis of female Senegalese sole breeders. Previous studies on other marine fishes
539have also failed to detect an effect of dopaminergic drug treatments on spawning. For
540example, treatment of European sea bass with the DA antagonist domperidone, either alone or
541combined with GnRHa, had no effect on spontaneous or GnRHa-induced OM or spawning
542(Prat et al., 2001). In contrast, in species expressing a strong DA inhibitory tone, DA
543antagonists stimulated OM and spawning when given as single treatments, and enhanced
544GnRHa-induced effects in combined treatments (Yaron, 1995; Wen and Lin, 2004; Aizen et
545al., 2005). The results from the present study indicate that an endogenous DA inhibition
546would not be the cause for the absence of spontaneous egg release in cultured Senegalese
547breeders.

548 Despite the observed stimulation of sperm production by all three hormonal treatments
549 and induced egg release by the GnRHa and COMB treatments, there was a complete lack of
550 egg fertilization in all broodstock tanks. This has been repeatedly observed in Senegalese sole
551 cultured (F1) broodstocks, both from spontaneous and GnRHa-induced spawning (Agulleiro
552 et al., 2006; Guzmán et al., 2008, 2009a). The present study showed that the lack of egg
553 fertilization persists in broodstock treated with PIM, suggesting no action of DA on the
554 mechanisms leading to the fertilization of eggs. Previous studies in Senegalese sole have
555 suggested that lack of egg fertilization in tank spawning of cultured broodstock might be due
556 to an inhibition of courtship and sexual behaviour (Guzmán et al., 2008, 2009b; Howell et al.,
557 2009). Recent video-recording studies in cultured Senegalese sole have shown that GnRHa-
558 induced egg release in females is produced in a total absence of courtship between males and
559 females, suggesting no participation of males and, thus, no fertilization of the released eggs
560 (Dr. N. Duncan, personal communication). As in other fishes, the mechanisms of sexual
561 behaviour are critical in flatfish species for synchronization of gamete release and successful
562 egg fertilization. In flounder (*Paralichthys orbignyanus*) and summer flounder (*Paralichthys*
563 *dentatus*), the high variability in egg fertilization success has been clearly associated with a
564 low participation of males in spawning events (Watanabe et al., 1998; Watanabe and Carroll,
565 2001; Bambill et al., 2006).

566 In conclusion, blockage of an endogenous DA inhibitory system by treatment with PIM
567 was shown to stimulate spermatogenesis, testicular maturation and sperm production in
568 mature male Senegalese sole. In addition, some stimulatory effects of PIM on the endocrine
569 reproductive axis were demonstrated, suggesting the existence of a DA inhibitory tone
570 regulating male reproduction in this species. On another hand, no effects of PIM were seen in
571 females, indicating the absence or weak expression of a DA inhibition in females. Despite the
572 stimulatory effects of PIM on males and GnRHa on males and females, the lack of egg

573fertilization in all experimental tanks suggests that a DA inhibition would not be the main
574reason underlying the failure of cultured Senegalese sole broodstock to produce fertilized
575spawning.

576

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Table 1. Gene-specific primers and amplicon size (bp) for each transcript in the qPCR assays.

Transcript	Primer	Nucleotide sequence	Amplicon size (bp)
FSH β	ssFSHf_q	5' GGACCCAAACTACATCCATGAAC 3'	60
	ssFSHr_q	5' CAGTCCCCGTTACAGATCACCTGTCT 3'	
LH β	ssLHf_q	5' CGGTGGAGACGACCATCTG 3'	61
	ssLHr_q	5' GGTATCTTGATGACGGGATCCTT 3'	
GP α	ssGPf_q	5' ACGGGCTGTGAGAAATGCA 3'	56
	ssGPr_q	5' GGATGCTCCCTGGAGAACAA 3'	
18s ¹	18Sf_q	5' GGTACTTTCTGTGCCTACCATGGT 3'	60
	18Sr_q	5' CCGGAATCGAACCCTGATT 3'	

¹Gene-specific primer designed from the Senegalese sole 18s complete gene sequence, available at Gene Bank (**EF126042.1**).

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Table 2. Egg production of cultured Senegalese sole broodstock treated with, saline (controls, CNT), pimozide (PIM), GnRHa (GnRHa) and the combined PIM + GnRHa treatment (COMB). Different letters indicate significant differences ($p < 0.05$) among treatments. Daily fecundity and egg buoyancy data are expressed as mean $\pm$ SEM.

	CNT	PIM	GnRHa	COMB
N° of spawns	0	6	13	13
Total fecundity (eggs kg ⁻¹)x10 ³	0	12.4	260.7	189.8
Daily fecundity (eggs kg ⁻¹)x10 ³	0	2.1 $\pm$ 0.6 ^a	20.0 $\pm$ 3.5 ^b	14.6 $\pm$ 3.0 ^b
Egg buoyancy (%)	0	19.4 $\pm$ 8.0	12.9 $\pm$ 1.8	18.8 $\pm$ 5.0
Hatching success (%)	0	0	0	0

812

813Figure legends

814

815 **Figure 1.** Pituitary content of GnRHs in male and female Senegalese sole breeders
816treated with saline (CNT), pimozone (PIM), GnRHa (GnRHa) and the combined PIM +
817GnRHa treatment (COMB). Levels of GnRH3 were undetectable in all samples (not shown).
818Pituitaries were collected 24 h after the last treatment (25 days from initiation of treatments).
819No significant differences ($p<0.05$) were found among treatments for any GnRH form. Data
820are expressed as mean $\pm$ SEM ($n=6$).

821

822 **Figure 2.** Pituitary transcript levels of gonadotropin subunits (FSH β , LH β and GP α) in
823male and female Senegalese sole breeders treated with saline (CNT), pimozone (PIM),
824GnRHa (GnRHa) and the combined PIM + GnRHa treatment (COMB). Levels were
825normalized to Senegalese sole 18s. Pituitaries were collected 24 h after the last treatment.
826Different letters indicate significant differences ($p<0.05$) among treatments. Data are
827expressed as mean $\pm$ SEM ($n= 6$).

828

829 **Figure 3.** Plasma levels of 11-ketotestosterone (11-KT) and testosterone (T) in male and
830estradiol (E2), T and vitellogenin (VTG) in female Senegalese sole breeders treated with
831saline (CNT), pimozone (PIM), GnRHa (GnRHa) and the combined PIM + GnRHa treatment
832(COMB). Plasma samples were collected on days 0, 10 and 25 from the initiation of
833treatments. Different capital letters indicate significant differences ($p<0.05$) among sampling
834times within control fish. Different small letters indicate significant differences ($p<0.05$)
835among treatments within sampling points. Data are expressed as mean $\pm$ SEM ($n=6$).

836

837 **Figure 4.** Photomicrographs of cross-section of Senegalese sole testis from males of the
838 four experimental groups: controls (A,B), PIM (C,D), GnRHa (E,F) and COMB (G,H).
839 Photomicrographs on the left (A,C,E,G) are from the cortex and on the right (B,D,F,H) from
840 the medulla region. Abbreviations: spg, spermatogonia; spc, spermatocyte; spd, spermatid;
841 spz, spermatozoon. Scale bars: 40 μ m.

842

843 **Figure 5.** Milt volume, sperm concentration and total sperm production in male
844 Senegalese sole breeders treated with saline (CNT), pimozone (PIM), GnRHa (GnRHa) and
845 the combined PIM + GnRHa treatment (COMB). Milt samples were collected on days 0 and
846 25 from the initiation of treatments. Different letters indicate differences ($p < 0.05$) among
847 treatments within sampling points. Data are expressed as mean $\pm$ SEM (n=6).

848

849 **Figure 6.** Daily fecundity of cultured Senegalese sole broodstock (shown for one of the
850 duplicate tanks) treated with saline (CNT), pimozone (PIM), GnRHa (GnRHa) and the
851 combined PIM + GnRHa treatment (COMB). Treatment of PIM was administered as 3
852 injections (days 0, 10 and 24) and GnRHa as a single implant (day 0) and injection (day 24).
853 The quantity of buoyant (black bars) and sinking (white bars) eggs was determined for each
854 spawn. No egg release was detected in any group after day 16 from the initiation of
855 treatments.

Figure 1

Figure 2

Figure 3

Top

Figure 4

Figure 5

Figure 6