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Abstract: The flatfish Senegalese sole (Solea senegalensis) exhibits severe reproductive dysfunctions in captivity, which lead to diminished gamete quality and release in F1 generation cultured breeders. The aim of this study was to investigate the existence of a dopamine (DA) inhibitory tone influencing the reproductive process in this species. Four groups of mature Senegalese sole breeders were treated with 1) saline (controls, CNT), 2) the DA antagonist pimozide (PIM, 5 mg kg-1), 3) gonadotropin-releasing hormone agonist (GnRHa, 40 μg kg-1) and 4) a combination of PIM + GnRHa (COMB). Effects were evaluated on pituitary levels of GnRHs (ELISA), pituitary transcript levels of gonadotropin subunits (qPCR), plasma levels of sex steroids and vitellogenin (ELISA), gonad development (histology), spermiation and spawning performance. As expected, the GnRHa treatment induced spawning in females and stimulated testis maturation in males. In males, PIM did not affect pituitary GnRH content, but enhanced GnRHa-induced pituitary GPα transcripts and modified plasma androgen levels; moreover, PIM stimulated spermatogenesis and milt production and enhanced GnRHa-induced effects. In females, PIM did not affect pituitary and plasma endocrine parameters, and did not influence spawning performance of the broodstock, either alone or in the combination treatment. In conclusion, these data indicate the existence of a DA inhibitory tone in mature males, which would be absent or weakly expressed in females; the spawning induction efficacy of the GnRHa was not improved by co-treatment with PIM.

Dear editor,

Please find enclosed our article “Effects of in vivo treatment with the dopamine antagonist pimozide and gonadotropin-releasing hormone agonist (GnRHa) on the reproductive axis of Senegalese sole (Solea senegalensis)” by Guzmán et al., to be considered for publication in the journal GENERAL AND COMPARATIVE ENDOCRINOLOGY.

Please, do not hesitate to contact me for any further information. Sincerely,

Evaristo Mañanós Sanchez
Researcher

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

4

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24

25 **Abstract**

26 The flatfish Senegalese sole (*Solea senegalensis*) exhibits severe reproductive
27 dysfunctions in captivity, which lead to diminished gamete quality and release in F1
28 generation cultured breeders. The aim of this study was to investigate the existence of a
29 dopamine (DA) inhibitory tone influencing the reproductive process in this species.
30 Four groups of mature Senegalese sole breeders were treated with 1) saline (controls,
31 CNT), 2) the DA antagonist pimozide (PIM, 5 mg kg^{-1}), 3) gonadotropin-releasing
32 hormone agonist (GnRHa, 40 µg kg^{-1}) and 4) a combination of PIM + GnRHa (COMB).
33 Effects were evaluated on pituitary levels of GnRHs (ELISA), pituitary transcript levels
34 of gonadotropin subunits (qPCR), plasma levels of sex steroids and vitellogenin
35 (ELISA), gonad development (histology), spermiation and spawning performance. As
36 expected, the GnRHa treatment induced spawning in females and stimulated testis
37 maturation in males. In males, PIM did not affect pituitary GnRH content, but enhanced
38 GnRHa-induced pituitary GPα transcripts and modified plasma androgen levels;
39 moreover, PIM stimulated spermatogenesis and milt production and enhanced GnRHa-
40 induced effects. In females, PIM did not affect pituitary and plasma endocrine
41 parameters, and did not influence spawning performance of the broodstock, either alone
42 or in the combination treatment. In conclusion, these data indicate the existence of a
43 DA inhibitory tone in mature males, which would be absent or weakly expressed in
44 females; the spawning induction efficacy of the GnRHa was not improved by co-
45 treatment with PIM.

46

47 ***Key words***: FSH, LH, gonadotropin, GnRH, pimozide, dopamine, spawning,
48 spermiation, flatfish, Senegalese sole, *Solea senegalensis.*

49

50 **1. Introduction**

51 The brain-pituitary-gonad (BPG) axis regulates reproduction in fish, as in all
52 vertebrates. External and internal signals are integrated in the brain by hypothalamic
53 neurons producing the gonadotropin-releasing hormones (GnRHs) GnRH1, GnRH2 and
54 GnRH3 (Zohar et al., 2009). The GnRHs stimulate the pituitary synthesis and release of
55 the gonadotropins (GTHs) follicle stimulating hormone (FSH) and luteinizing hormone
56 (LH), which are critical modulators of gametogenesis and gonadal maturation, through
57 their actions on gonadal steroids and growth factors (Levavi-Sivan et al., 2009; Schulz
58 et al., 2009; Lubzens et al., 2009). In fish, in addition to the primary GnRH stimulatory
59 system, neurons secreting dopamine (DA) have been identified as an inhibitory system
60 over the reproductive axis. Dopamine exerts inhibitory actions on both the brain and
61 pituitary through reduction of GnRH synthesis and release, down-regulation of GnRH
62 receptors and interference with the GnRH-signal transduction pathways, thus affecting
63 GTH secretion from the pituitary (Peter and Yu, 1997; Popesku et al., 2008). Inhibitory
64 effects of DA have been clearly demonstrated in freshwater fishes, including cyprinids
65 (Yaron et al., 1995), silurids (De Leeuw et al., 1986; Silverstein et al., 1999) and
66 salmonids (Saligaut et al., 1999), as well as in tilapia (*Oreochromis spp.*) (Melamed et
67 al., 1998), European eel (*Anguilla anguilla*) (Vidal et al., 2004) and grey mullet (*Mugil*
68 *cephalus*) (Aizen et al., 2005), but have not been demonstrated in marine species
69 (Dufour et al., 2005).

70 When reared in captivity, most fishes exhibit reproductive dysfunctions, which is
71 thought to be caused by stress associated with intensive culture conditions (Zohar and
72 Mylonas, 2001). In many cases, the application of exogenous hormonal treatments is an
73 effective therapy to stimulate reproduction and induce spawning in captive fish

74 (Mylonas et al., 2009). One of the most common strategies is the administration of
75 GnRH agonists (GnRHa), via saline-diluted injections or slow-delivery implants. The
76 GnRHa treatment induces the synthesis and release of endogenous GTHs and stimulates
77 oocyte maturation (OM), ovulation and spermiation in fish (Mylonas and Zohar, 2001),
78 and has been used effectively in various flatfishes (Larsson et al., 1997; Clearwater and
79 Crim, 1998; Vermeissen et al., 1998, 2000; Mugnier et al., 2000; Guzmán et al., 2009a).
80 However, species expressing a strong DA inhibition require the co-administration of DA
81 antagonists with the GnRHa treatment, in order to remove the DA inhibitory tone and
82 permit the stimulatory action of GnRHa on the reproductive axis (Peter and Yu, 1997;
83 Mylonas et al., 2009). Combined treatment of GnRHa with DA antagonists, such as
84 pimozide (PIM) or domperidone, has been used successfully to stimulate reproduction in
85 cyprinids (Yaron et al., 1995; Mikolajczyk et al., 2004), catfishes (Silverstein et al.,
86 1999; Wen and Lin, 2004) and mullets (Arabaci and Sari, 2004; Aizen et al., 2005).
87 However, only a few studies have investigated the effects of DA on the reproductive
88 axis and the efficiency of combined DA antagonist-GnRHa treatments in marine fish.
89 Studies performed on gilthead seabream (*Sparus aurata*), Atlantic croaker
90 (*Micropogonias undulatus*) and red seabream (*Pagrus major*) showed that treatment
91 with PIM did not enhance the stimulatory effects of GnRHa on pituitary LH release
92 (Copeland and Thomas, 1989; Zohar et al., 1995; Kumakura et al., 2003). In European
93 sea bass (*Dicentrarchus labrax*), co-treatment of PIM with GnRHa did not improve
94 GnRHa-induced OM or spawning (Prat et al., 2001). Based on these studies, it has been
95 proposed that a DA inhibition is lacking in marine fish, but recent studies (Vidal et al.,
96 2004; Dufour et al., 2005) indicate that more work is needed to investigate the potential
97 existence of a DA inhibitory tone in the reproduction of marine fish species.

98 The Senegalese sole (*Solea senegalensis*, Pleuronectiforme, Soleidae) is a highly
99 valuable marine flatfish that has become a priority species for European and Mediterranean
100 aquaculture (Imsland et al., 2003). However, the establishment of an efficient
101 aquaculture industry is constrained by the failure of cultured broodstock (F1 generation)
102 to reproduce (Howell et al., 2006, 2009). Spawning of cultured breeders is rare, and
103 when it occurs fecundity is low and eggs are unfertilized. Recent studies have shown
104 that cultured females complete vitellogenesis and show adequate profiles of plasma
105 vitellogenin (VTG) and sex steroid levels, but as the reproductive cycle proceeds most
106 females fail to undergo OM, ovulation and spawning, and most of post-vitellogenic
107 oocytes undergo apoptosis (García-López et al., 2007; Guzmán et al., 2008, 2009a). On
108 the other hand, cultured males complete spermatogenesis and sperm maturation and
109 show normal androgen plasma profiles, but sperm production is low compared to wild-
110 caught breeders, affecting the fertilization success (García-López et al., 2006; Cabrita et
111 al., 2006). These reproductive dysfunctions have been observed in most captive-reared
112 flatfish species (Larsson et al., 1997; Mazorra de Quero et al., 2000ab; Mañanós et al.,
113 2008). A few studies have tested the use of hormonal therapies to stimulate
114 reproduction in Senegalese sole, and with limited success. For example, treatment with
115 GnRHa injections or GnRHa slow-delivery systems stimulated OM in females and
116 induced spawning, but the eggs were not fertilized (Agulleiro et al., 2006, Guzmán et
117 al., 2009a); and results from GnRHa stimulation of male sperm production were
118 inconclusive (Agulleiro et al., 2006, 2007). To date, no attempts have been made to
119 investigate the existence of a DA inhibition on the reproductive axis of Senegalese sole
120 or other flatfish species, and determine the potential efficiency of treatments with DA
121 antagonists. Nevertheless, anatomical studies on Senegalese sole suggested the

122 existence of a DA inhibitory system in this species. Immunoreactivity to tyrosine
123 hydroxylase (TH), a rate-limiting enzyme in DA synthesis, has been detected in the
124 preoptic area of the brain and TH-immunoreactive fibres extend into the proximal *pars*
125 *distalis* of the pituitary, where gonadotrophs are located (Rendón et al., 1997;
126 Rodríguez-Gómez et al., 2000), suggesting DA activity in the brain and pituitary in this
127 species.

128 The present study investigated for the first time in a flatfish species the influence of
129 DA on the reproductive axis and the potential of combined treatment with GnRHa and
130 DA antagonist on the stimulation of reproduction in Senegalese sole. The effects of in
131 vivo treatments with GnRHa and PIM (a D2-receptor antagonist), both alone and in
132 combination, were evaluated in mature males and females by analyzing pituitary content
133 of GnRHs (GnRH1, GnRH2 and GnRH3), pituitary transcript levels of gonadotropin
134 subunits (FSHβ, LHβ and the common glycoprotein α, GPα) and plasma levels of sex
135 steroids (estradiol, testosterone and 11-ketotestosterone) and VTG, and were correlated
136 with gonadal maturation, spermiation and spawning performance.
137

138 **2. Materials and Methods**

139 *2.1. Fish husbandry*

140 Senegalese sole juveniles (F1 generation), obtained from natural spawns of wild-
141 caught broodstock (spring 2003) at "Stolt Sea Farm s.a." (La Coruña, Spain), were
142 transported to the facilities of the Spanish Institute of Oceanography (IEO, Vigo, Spain) in
143 March 2004. Fish were tagged with passive integrated transponder tags (PIT tags,
144 AVID). Sex of the fish was further determined at puberty by abdominal swelling in
145 females and by feeling the shape of the testis in males. Fish were reared in rectangular

146 fibreglass tanks (4000 l, 1 m depth, 4 m^2) without sand substrate, at a density of around
147 3 kg m^{-2} and were exposed to the natural temperature and photoperiod regime of the
148 region.
149 The experiment was initiated on April 24th 2006 and terminated on May 19th 2006,
150 under increasing water temperatures ranging from 16.5 °C to 18.1 °C (mean temperature
151 of this period, 17.2 ± 0.2 °C). Dissolved oxygen was checked regularly and ranged
152 between 6.8 and 7.5 ppm. Tanks were fitted with overflow egg collectors and were
153 supplied with flow-through seawater (salinity 36‰) at a flow rate of 400% d^{-1}. Fish
154 were fed to apparent satiation 3 d a week using commercial dry pellets (Trout) and semi-
155 dry pellets made at the facility with raw fish, squid and mussel meat.
156 Handling of the fish for routine management and experimentation was always done
157 according to national and institutional regulations and the current European Union
158 legislation on handling experimental animals (EEC, 1986). All fish to be handled were
159 anesthetised by immersion in 0.3 ml l^{-1} of 2-phenoxyethanol.
160
161 *2.2. Experimental design and hormonal treatments*
162 On April 11th 2006, when external signs of gonad maturation were clearly evident
163 (Guzmán et al., 2008, 2009a), such as abdominal swelling in females and expressible milt
164 in males, 96 fish were distributed homogenously in eight 4.000 l tanks, at a sex ratio of 1:1
165 and a density of 3.2 ± 0.1 kg m^{-2}. The experiment was run on duplicates, with two tanks
166 per treatment group (4 groups, n = 12 fish per tank). Fish were three years old, with a
167 mean (± S.E.M.) body weight (BW) of females and males at 1216.0 ± 38.5 g and 981.3 ±
168 30.2 g, respectively, and body length (BL) of 40.4 ± 0.5 cm and 38.8 ± 0.5 cm,
169 respectively.

170 On April 24th 2006 (day 0), 8 females and 8 males were randomly selected (2 females
171 and 2 males per tank), anesthetised and sampled for blood and milt. Thereafter, the
172 following treatments were applied: (1) control treatment groups (CNT) were injected with
173 saline (0.9% NaCl) on days 0 and 24; (2) treatment with PIM (PIM) was given as 3
174 injections (5 mg kg^{-1}) on days 0, 10 and 24; (3) treatment with GnRHa (GnRHa) was given
175 as a single implant (40 μg kg^{-1}) on day 0 and injection (25 μg kg^{-1}) on day 24, and (4) the
176 combined PIM + GnRHa treatment (COMB) was applied as for the GnRHa and PIM
177 groups above on days 0, 10 and 24.
178 The drugs used were the long-acting DA D2-receptor antagonist PIM (Sigma-Aldrich)
179 and the [D-Ala^6, Pro^9 Net]-LHRHa (Bachem, Switzerland). For injections, both PIM and
180 GnRHa were dissolved in saline. The GnRHa implants were manufactured from
181 p[Ethylene-Vinyl acetate] copolymer (EVAc, Elvax; DuPont Chemical CO., DE) as 2-mm
182 diameter x 3-mm cylinders (Zohar et al., 1990). Both injections and implants were applied
183 in the dorsal musculature of the fish. The doses of PIM and GnRHa were based on
184 previous studies in other fishes, including Senegalese sole (Zohar and Mylonas, 2001;
185 Guzmán et al., 2009a).
186 From the two groups of replicated experimental tanks, only one was sampled for blood
187 (days 0, 10 and 25), sperm (days 0 and 25) and tissues (sacrifice, day 25), whereas the
188 other duplicate of each treatment group were left “undisturbed” (only manipulated for
189 hormone administration) throughout the experimental period. The fecundity and quality of
190 the eggs from each spawn was checked daily in all tanks. As described later, the
191 spawning data obtained from both duplicates (“sampled” and “undisturbed”) of each
192 experimental group were very similar, indicating that blood and sperm sampling did not
193 influence the spawning performance of the broodstock.

194 Blood (0.8 ml) was taken from the caudal vasculature using heparinised syringes and
195 placed in ice-cold heparinised tubes containing aprotinin (0.15 IU ml^{-1}, Sigma). Plasma
196 was obtained by centrifugation (3000 *g*, 15 min, 4ºC) and stored at -20ºC for until analysis
197 for sex steroid hormones and VTG. For milt collection, the urogenital pore was first wiped
198 off with a piece of paper, and milt was collected within a syringe by gentle abdominal
199 pressure on the area of the testis. Syringes containing the milt samples were immediately
200 placed on ice and maintained refrigerated until determination of sperm parameters (milt
201 volume, sperm concentration and sperm motility). After blood and sperm sampling on day
202 25, fish were sacrificed by decapitation for dissection of tissues. Pituitary glands were
203 collected, frozen immediately in liquid nitrogen and stored at -80ºC until analysis of GnRH
204 content and GTH subunit transcript levels. In males, testes were removed and weighed for
205 calculation of the gonadosomatic index (GSI = gonad weight x BW^{-1} x 100). Transverse
206 sections from the middle region of the dorsal and ventral lobes of the testes were dissected
207 out and collected for histology.
208
209 *2.3. Gonadotropin subunit real-time quantitative PCRs (qPCRs)*
210 Transcript levels of Senegalese sole FSHβ, LHβ and GPα subunits were quantified
211 by specific qPCRs (Guzmán et al., 2009b). Briefly, plasmids containing the specific
212 subunit insert were linearized and used as templates for gene-specific RNA standard
213 synthesis. Total RNA isolated from a pool of Senegalese sole pituitaries, using Tri-
214 Reagent (Sigma) and treated with DNase (RQ1 RNAse-free DNase, Promega), served as
215 standard for 18s RNA. The amount of each RNA standard was determined using
216 RiboGreen RNA quantification kit (Molecular Probes, Eugene, OR).

217 For sample analysis, total RNA was isolated from each sample using Tri-Reagent,
218 according to the manufacturer's instructions, treated with DNase and quantified using a
219 Nano Drop ND-1000 Spectophotometer. The RNA standards (target and reference) and
220 RNA from each sample were simultaneously reverse-transcribed into cDNA using
221 random hexamers and MMLV reverse-transcriptase (Promega) and diluted 5-fold in
222 sterile water. Real-time analysis was carried out on an ABI Prism 7700 Sequence
223 Detection System. Reactions were performed in a 20 µl volume containing cDNA
224 generated from 10 ng of original RNA template, 200 nM gene-specifics primers (Table
225 1), and 10 µl of SYBR Green PCR core reagent (Applied Biosystem). Amplification
226 reactions were carried out at 50 ºC for 2 min, 95 ºC for 10 min, and 40 cycles of 95 ºC
227 for 15 sec and 60 ºC for 60 sec. After the qPCR reaction, copy number for unknown
228 samples was determined by comparing C_T (threshold cycles) values to the specific
229 standard (run in every plate) and normalized to the amount of 18s RNA in each sample.
230 Amplification efficiencies (%) for the qPCRs, obtained from validation assays using
231 serially diluted reverse-transcribed RNA, were 102.3 ± 2.1, 102.5 ± 2.0 and 96.8 ± 3.5
232 for FSHβ, LHβ and GPα, respectively. The lowest standard point was 100
233 copies/reaction for all three transcripts (FSHβ, LHβ and GPα).
234
235 *2.4. GnRH ELISAs*
236 Peptide levels of GnRH1, GnRH2 and GnRH3 were measured in the same
237 pituitaries used for GTH mRNA analysis, from the protein fraction obtained after RNA
238 extraction. Proteins were precipitated with iso-propanol (1:2, v/v) and pellets dried and
239 reconstituted in 200 µl PBST buffer (10 mM phosphate buffer, 0.9% NaCl, 0.05%
240 Tween-20, pH 7.2). Acetic acid 4N was added to each sample (1:1, v/v), incubated for

241 10 min at 80 ºC and centrifuged (13,000 g, 30 min, 4ºC). Pellets were re-extracted twice
242 with acetic acid 4N. Supernatants were pooled, vacuum dried and reconstituted with 0.1
243 M potassium phosphate buffer, pH 7.4.

244 Levels of GnRH1, GnRH2 and GnRH3 were measured in each reconstituted sample
245 using specific ELISAs for each GnRH form. The ELISA protocol was based on that
246 developed for gilthead seabream (Holland et al., 1998) and used further in other species
247 (Rodríguez et al., 2000; Andersson et al., 2001; Holland et al., 2001; Steven et al.,
248 2003), including the Senegalese sole (Guzmán et al., 2009b). The sensitivities of the
249 ELISAs, determined as the GnRH dose giving 80% of binding, were 6 pg well^{-1} for
250 GnRH1, 7 pg well^{-1} for GnRH2 and 2 pg well^{-1} for GnRH3. Cross-reactivities, calculated
251 at 50% of binding, were below 0.6% except for the GnRH3 assay, which displayed 3.7%
252 cross-reactivity with GnRH2.

253

254 *2.5. Vitellogenin and sex steroid ELISAs*

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

261 For steroid analysis, plasma samples were first extracted by addition of ice cold
262 methanol (methanol:plasma 6:1, v/v), shaken and centrifuged (3,000 g, 15 min, 4ºC).
263 The pellet was re-extracted twice with 200 µl of methanol. Supernatants were pooled,
264 dried and reconstituted in 0.1 potassium buffer (pH 7.4). The levels of estradiol (E2),

265 testosterone (T) and 11-ketotestosterone (11-KT) were quantified by ELISAs using a
266 protocol validated previously for Senegalese sole plasma (Guzmán et al., 2008, 2009a).
267 The sensitivities of the ELISAs, calculated as the lowest detection limit (Bo-2SD), were
268 5.2 pg ml^{-1}, 8.8 pg ml^{-1}, and 0.4 pg ml^{-1} for the E2, T and 11-KT ELISA, respectively.
269 The intra-(n=4) and inter-assay (n=8) coefficients of variation, at 50% of binding, were
270 5.8% and 6.3% for E2, 6.1% and 11.3% for T, and 10.7% and 9.1% for the 11-KT
271 ELISA.
272

273 *2.6. Testicular histology*

274 Fragments of testicular tissue were fixed in 4% phosphate-buffered (0.1M, pH 7.2)
275 formalin for 48–96 h at room temperature. After rinsing in running tap water (16 h) and
276 dehydration in ascending concentrations of ethanol, fragments were infiltrated and
277 embedded in Leica Historesin (2-hydroxi-ethylmethacrylate; Reichert-Jung, Germany).
278 Sections were cut at 3 µm on a Supercut 2065 microtome (Reichert-Jung, Germany) and
279 stained with Harris' Haematoxylin-Eosin. Stained sections were examined and
280 photographed on a Leitz Diaplan light microscope.
281 Photomicrographs were taken and analyzed from the two main regions of the Senegalese
282 sole testis, cortex and medulla (García-López et al., 2005). The stage of testicular
283 development was determined according to the germ cell types and their relative
284 abundance (García-López et al., 2005, 2006).
285

286 *2.7. Sperm parameters*

287 The volume of milt expressed by each male was measured using a micropipette and
288 standardized according to BW. Sperm concentration was measured using a Neubauer

289 haemocytometer after 1:400 dilution in 1% formalin. Total sperm production was
290 calculated as milt volume (µl kg^{-1} BW) x sperm concentration (spermatozoa ml^{-1}). For
291 determination of sperm motility, milt samples (1 µl) were first diluted 1:5 in 200 mOsm
292 kg^{-1} Ringer's solution (Chereguini et al., 1997) and then activated by addition of 36‰
293 sea water (19 µl). Motility was examined under light contrast microscopy
294 (magnification 400 x) and scored by five different operators from 0 to 5, according to
295 the percentage of moving cells (score 0: no movement, 1: 0-20%, 2: 20-40%, 3:40-60%,
296 4: 60-80% and 5: 80-100%); the motility of each sample was the mean value of the five
297 scores (Chereguini et al., 1997).
298
299 *2.8. Spawning performance*
300 The occurrence of spawning was checked twice daily (09:00 and 17:00 h) and eggs
301 were collected for evaluation of fecundity and quality. Eggs were transferred to
302 calibrated cylinders filled with 36‰ sea water and were allowed to stand in order to
303 separate buoyant (viable) and sinking (dead) eggs, determining the volume fraction of
304 each type. Total fecundity (buoyant and sinking eggs) was further expressed as nº of
305 eggs, considering that a volume of 1 ml of eggs corresponds to 1,000 eggs (Guzmán et
306 al., 2009a). Daily relative fecundity was calculated using the BW of the females at the
307 previous sampling, and total relative fecundity was calculated at the end of the study.
308 From each spawn, a sample of 50 buoyant eggs was examined under a binocular
309 microscope to determine morphology and fertilization success. Buoyant eggs were
310 incubated for 48 h to determine embryo development and hatching success.
311
312 *2.9. Statistics*

Data are expressed as mean ± standard error of the mean (S.E.M.). Statistical differences among the four experimental groups per sampling time 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 evolution of steroid and VTG plasma levels over time. Differences in daily fecundity and egg buoyancy data between duplicate tanks of each group were analyzed by a t-test with a significance level of $p<0.05$. Normality and homogeneity of 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 (logit (Bi/ Bo) = ln(Bi - NSB/Bo -NSB)), where Bi represents the binding of each point, Bo the maximum binding and NSB the non-specific binding.

3. Results

3.1. Pituitary content of GnRHs

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

3.2. Pituitary transcript levels of gonadotropin subunits

The transcript levels of each GTH subunit (FSHβ, LHβ and GPα) in the pituitary were analyzed on day 25 from the initiation of treatments, 24 h after the last hormone

337 administration (Fig. 2). In males, GnRHa increased pituitary GPα transcript levels, but had
338 no effect on levels of FSHβ and LHβ transcripts. Treatment with PIM alone did not affect
339 GTH transcript levels, with respect to controls, but enhanced GnRHa-induced GPα
340 transcript levels, as showed by statistically significant differences between GnRHa and
341 COMB treatment groups. In females, GnRHa treatment induced an elevation of LHβ and
342 GPα transcript levels, but not those of FSHβ, with respect to controls. Treatment with PIM
343 alone did not affect GTH transcripts, nor did it modify GnRHa-induced levels in the
344 combined treatment.

345

346 *3.3. Plasma levels of sex steroids and vitellogenin*

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

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

358

359 *3.4. Testicular development*

360 The males of the control group showed testes at the stage of functional maturation
361 (stage IV), as expected by the time of the year (spermiation period), characterized by
362 low numbers of early developing germ cells (spermatogonia (spg) and spermatocytes
363 (spc)), very high abundance of spermatids (spd) in the cortex and low to intermediate
364 number of spermatozoa (spz) within the medullar efferent duct system (Fig. 4A,B). The
365 cortical seminiferous lobules were fully filled by spd, with some sporadic spz (Fig. 4A).
366 The medullar efferent ducts, surrounded by interstitial tissue, were of small diameter
367 and were filled by spd and spz; the spz being clearly distinguishable by a strongly
368 basophilic small head and a large flagellum (Fig. 4B).

369 In general, all three hormonal treatments (PIM, GnRHa and COMB) stimulated
370 spermatogenesis and spermiogenesis; the testes of males from the three hormone-treated
371 groups were at the stage of advanced functional maturation. The GnRHa treatment
372 increased the presence of cysts containing early developing germ cells (spg and spc) at
373 the distal part of the cortical seminiferous lobules and reduced the number of spd in the
374 lumen of the lobules (Fig. 4E). Such reduction was accompanied by a relative increase
375 in the abundance of spz accumulated in the medullar efferent ducts (Fig. 4F). The PIM
376 treatment induced similar germ cell dynamics than the GnRHa treatment, although in
377 the testis of PIM-treated males the presence of spg and spc in the cortical seminiferous
378 lobules (Fig. 4C) and ripe spz in the medullar efferent ducts (Fig. 4D) was higher. The
379 testes of males that received the combined treatment (COMB) showed the highest
380 abundance of cysts containing spg and spc in the cortex and lowest abundance of spd
381 (Fig. 4G); a high occurrence of empty seminiferous lobules was observed in the cortex,
382 as a consequence of transformation of batches of spd into spz. The efferent duct system

383 in the medulla was highly developed and showed the highest abundance of spz (Fig.
384 4H). No effect of the treatments was observed on the GSI (data not shown).
385

386 *3.5. Sperm parameters*

387 Milt volume and sperm density was determined in males on days 0 and 25 from
388 initiation of treatments (Fig. 5). In controls, milt volume, sperm density and consequently,
389 total sperm production, did not change from 0 to 25 days. The GnRHa treatment induced a
390 slight 1.9-fold increase in milt volume (no statistical difference from controls) but
391 significantly reduced sperm density ($p<0.05$). Total sperm production in GnRHa-treated
392 males was similar to controls. Treatment with PIM increased milt volume 3.5-fold
393 compared to controls ($p<0.05$) and was shown to enhance the GnRHa-induced increase in
394 milt volume 2.7-fold, when COMB and GnRHa groups were compared ($p<0.05$). As
395 observed with GnRHa, the increase in milt volume in both PIM and COMB groups
396 compared to controls was accompanied by diminished sperm density, although these
397 differences were only significant ($p<0.05$) for the COMB treatment. Total sperm
398 production in both PIM and COMB groups was slightly increased 2.5-fold compared to
399 controls, although these differences were not statistically significant. The PIM treatment
400 was shown to increase GnRHa effects on total sperm production 3.3-fold, when COMB
401 and GnRHa groups were compared ($p<0.05$). Sperm motility ranged from 40 to 80%
402 among individual males and was similar among all control and hormone-treated fish (data
403 not shown).
404

405 *3.6. Spawning performance*

406 No differences in daily mean fecundity, egg buoyancy or egg morphology were
407 observed between duplicate tanks of each group, indicating a similar spawning behaviour
408 among the "sampled" and "undisturbed" populations (data not shown). No spawning was
409 detected in any of the tanks before the initiation of treatments on day 0 or the end of
410 treatments on day 24 (Fig. 6). The control fish did not spawn during the experimental
411 period, while PIM-treated fish showed some sporadic spawning. The GnRHa treatment
412 induced daily spawning from day 3 until day 15; this spawning kinetics was not modified
413 by co-treatment with PIM (COMB). The GnRHa and COMB treatment showed similar
414 number of spawns, daily and total fecundity, and egg buoyancy (Table 2). Eggs examined
415 under the binocular showed a similar morphology in all groups and the absence of egg
416 fertilization.
417
418 **4. Discussion**
419 This study investigated, for the first time in a marine flatfish, the potential
420 inhibitory effects of dopamine (DA) on the endocrine regulation of the reproductive axis
421 of the Senegalese sole. The results did not show clear effects of the DA D2-receptor
422 antagonist pimozide (PIM) treatment on pituitary GnRH content, or pituitary FSHβ and
423 LHβ transcript levels. However, in males, PIM alone stimulated androgen plasma
424 levels, testicular maturation and sperm production, and enhanced GnRHa-induced
425 effects on these parameters, suggesting that in fact a DA inhibitory tone may be active in
426 the regulation of the reproductive axis in this fish.
427 The lack of effect of PIM on the pituitary content of GnRHs indicates that DA is not
428 affecting GnRH release from nerve terminals and GnRH accumulation in the pituitary.
429 This is in contrast with a previous study performed in the goldfish (*Carassius auratus*),

430 which reported an increase in immunoreactive GnRH in discrete parts of the brain and
431 pituitary 24 h after in vivo treatment with a PIM injection (Yu and Peter, 1990). These
432 authors suggested a DA action on both brain GnRH neurons and pituitary gonadotrophs
433 influencing GTH secretion, supporting further studies that demonstrated a strong DA
434 inhibition on the reproductive axis of cyprinids (Yaron, 1995; Peter and Yu, 1997). The
435 present study indicates that a DA inhibition on pituitary GnRH secretion through DA D2-
436 receptors is not active in mature Senegalese sole.

437 Evaluation of the hormone treatment effects on the transcription of pituitary GTH
438 subunits showed that in females, the GnRHa treatment increased transcript levels of
439 LHβ (and also GPα), but not of FSHβ. This result indicates that transcription of LHβ
440 and FSHβ genes in Senegalese sole are differentially regulated by GnRHa. Temporal
441 differences in the transcription of GTH β-subunits have been described previously in
442 other fishes (Yaron et al., 2003; Zohar et al., 2009). For example, pituitary samples
443 collected from mature European sea bass 18 h after a single GnRHa injection showed
444 increased transcript levels of LHβ, but not FSHβ (Mateos et al., 2002). Similarly, red
445 seabream implanted with a GnRHa-pellet had increased pituitary transcript levels of
446 LHβ, but not FSHβ, at 10 and 20 days after treatment (Kumakura et al., 2003). In
447 striped bass (*Morone saxatilis*), GnRHa injection induced a rapid increase of LHβ at 6 h
448 post-treatment, while FSHβ transcript levels were only increased after 24 h (Hassin et
449 al., 1998). These results indicate temporal differences in GnRHa-induced stimulation of
450 FSHβ and LHβ transcription, which might vary depending on the species. In the present
451 study, pituitaries were collected at a single sampling point 24 h after the last hormone
452 treatment, and so, it is possible that an increment change in FSHβ gene expression at
453 other times post-treatment might occur. Similarly, this study cannot rule out an effect of

454 GnRHa on FSHβ and LHβ gene expression in males, which might be evidenced at other
455 sampling points.

456 The PIM treatment alone had no effect on basal FSHβ or LHβ transcript levels and
457 the co-treatment did not modify further the GnRHa effects, neither in female nor in male
458 Senegalese sole breeders. The absence of a stimulatory effect of PIM on GTH β-
459 subunits indicates that a DA inhibitory tone is not acting over pituitary GTH
460 transcription and is not interfering with the GnRHa-induced stimulation. Previous
461 studies on other fish species have also failed to demonstrate a DA inhibition on pituitary
462 GTH transcriptional rates. In cultured tilapia pituitary cells, in vitro treatment with DA
463 had no effect on steady state mRNA levels of LHβ throughout 36 h exposure period,
464 suggesting no influence of DA on LHβ gene transcription (Melamed et al., 1996). In
465 female red seabream, in vivo treatment with the DA D2-receptor antagonist
466 domperidone did not enhance the stimulatory action of GnRHa on LHβ or FSHβ gene
467 expression (Kumakura et al., 2003). In mature male striped bass, PIM had not effect on
468 pituitary transcript levels of FSHβ and LHβ in fish pre-treated with GnRHa and T
469 (Hassin et al., 2000). Further research will be necessary to demonstrate an inhibitory
470 action of DA on gene transcription of GTHs in Senegalese sole and other marine fishes.

471 Despite the absence of an effect of PIM on GTH β-subunit transcription, a
472 dopaminergic action on pituitary GTH synthesis in Senegalese sole cannot be ruled out,
473 as treatment of males with PIM increased slightly GPα transcript levels and enhanced
474 clearly the GnRHa-induced stimulation of GPα transcripts. Studies in rainbow trout
475 (*Oncorhynchus mykiss*) have suggested that production of GPα subunit acts as a rate-
476 limiting factor in the synthesis of FSH (Naito et al., 1991, 1997). In the absence of
477 immunoassays for FSH and LH in Senegalese sole, it was not possible to analyze

478 pituitary or plasma levels of FSH and LH in this study and thus, the elevation of GPα
479 transcript levels could not be correlated with concomitant increases in FSH and/or LH
480 protein levels. It has to be considered that the GPα subunit is common to both GTHs
481 (FSH and LH) and the thyroid stimulating hormone (TSH) and thus, the observed
482 stimulation of GPα transcription by PIM could be related to, 1) an increase in the
483 synthesis of GTHs (FSH and/or LH) and/or, 2) the synthesis of TSH. To date, there are
484 no studies on DA effects on TSH gene expression or TSH protein synthesis and thus,
485 this hypothesis remains to be investigated (McKenzie et al., 2009). On the other hand,
486 some studies might support a DA action on GTH synthesis, either directly or by
487 enhancing gonadotrophic cell responsiveness to the GnRH stimulus. In cultured tilapia
488 pituitary cells, treatment with the DA D2-receptor agonist quinpirole suppressed LH
489 release and GnRH receptor mRNA levels, indicating an inhibitory effect on GnRH
490 receptor synthesis (Levavi-Sivan et al., 2004). Similar evidence was obtained in African
491 catfish (*Clarias gariepinus*) (De Leeuw et al., 1988) and goldfish (De Leeuw et al.,
492 1989), where exposure of pituitary fragments to the DA agonist apomorphine was
493 followed by a decrease in GnRH binding capacity. In European eel, TH-
494 immunoreactive fibers have been found to innervate pituitary LH-secreting cells, which
495 provided an anatomical support for a role of DA on the inhibition of LH synthesis in this
496 species (Vidal et al., 2004). Likewise, TH-immunoreactive fibers have been found in
497 the *pars distalis* of the pituitary of Senegalese sole, where both FSH and LH-producing
498 cells are located (Rendón et al., 1997; Rodríguez-Gómez et al., 2000; Guzmán et al.,
499 2009b). This anatomical data, showing a potential dopaminergic innervation of
500 gonadotrophic cells, would support a direct effect of DA on GTH synthesis in this
501 species. In addition, as mentioned previously for the effects of GnRHa treatment,

502 effects of PIM on FSHβ or LHβ transcription may be evidenced at sampling times not
503 included in this study.
504 Plasma levels of sexual steroids were not affected by any of the treatments in
505 females. In males, however, PIM alone stimulated T levels (day 25), probably through
506 unblocking of endogenous DA inhibition of GTH action on testicular steroidogenesis
507 (Schulz et al., 2009). Unexpectedly, PIM decreased the GnRHa-induced elevation of
508 plasma T and 11-KT levels in the co-treatment. The explanation for this is unclear and
509 remains to be elucidated. It could be hypothesized that the low plasma levels of 11-KT
510 and T in males of the combined treatment are a reflection of a more advanced stage of
511 testicular maturation in these fish, compared to the other groups. This advanced
512 maturational stage would be associated with a shift in the steroidogenic pathway,
513 resulting in decreased release of androgens and a corresponding increase in production
514 of progestogens (Nagahama, 1994; Vermeissen et al., 2000). This is supported by the
515 histological analyses, which showed the most advanced maturation of the testes in the
516 COMB-treated males.
517 Histological analysis of the testes and quantification of sperm parameters revealed
518 that treatment with PIM greatly stimulated testicular maturation and sperm production
519 in male Senegalese sole breeders and further enhanced GnRHa-induced effects in the
520 combined treatment. These data are the first clear evidence of the existence of a DA-
521 mediated inhibition of endogenous and GnRHa-stimulated gonadal maturation in a
522 marine flatfish. The dopaminergic action over gonadal development is probably a
523 GTH-mediated sex steroid mechanism; such a mechanism has been demonstrated in
524 freshwater fish species, but not in marine fishes (Dufour et al., 2005). Previous studies
525 have demonstrated a DA inhibition of GTH release, in the absence of a concomitant

526 effect on pituitary GTH synthesis. For example, the D2-receptor agonist bromocryptine
527 inhibited plasma FSH and LH levels in mature trout, but did not affect pituitary FSH
528 and LH cell content (Vacher et al., 2000). Similarly, in cultured tilapia pituitary cells
529 DA had no effect on steady state levels of the LHβ mRNA, but significantly reduced
530 LH release (Melamed et al., 1996). Based on this, catecholaminergic drugs in
531 combination with GnRHa have been used to increase plasma levels of LH and milt
532 production in goldfish (Sokolowska et al., 1988; Roelants et al., 2000) and swamp eel
533 (*Synbranchus marmoratus*) (Ravaglia et al., 1997). The detected effect of DA on
534 spermatogenesis and sperm parameters in Senegalese sole is likely exerted through an
535 inhibitory action on pituitary FSH and LH synthesis and release, but this should be
536 confirmed in future studies. On the other hand, the possibility of a direct action of PIM
537 on the testes should not be discarded. Transcripts of DA D2-receptors have been found
538 in the ovary of tilapia (Levavi-Sivan et al., 2005) and grey mullet (Nocillado et al.,
539 2006), suggesting a direct DA action on the fish gonad. Although DA D2 receptors
540 have not been demonstrated in the fish testes, studies in mammals have shown that DA
541 may participate in the proliferation and/or differentiation of male germ cells by acting
542 through testicular DA D2-receptors (Otth et al., 2007).

543 Spawning in females was induced by the GnRHa treatment, as previously observed
544 in hormone induced Senegalese sole broodstock (Agulleiro et al., 2006; Guzmán et al.,
545 2009a). The GnRHa treatment also increased VTG plasma levels, probably through a
546 GnRHa-induced stimulation of E2 release, although in this study no effects of GnRHa
547 were observed on plasma E2 and T levels analyzed at 10 and 25 days. This was
548 probably due to the short-lived nature of the rise in GnRHa-induced plasma E2 levels,
549 which was previously shown to last for only 3 days after treatment (Guzmán et al.,

550 2009a). Spawning performance and plasma levels of steroids and VTG, clearly showed
551 a lack of a PIM effect on females, suggesting again the absence of an inhibitory
552 dopaminergic action on the reproductive axis of female Senegalese sole breeders.
553 Previous studies on other marine fishes have also failed to detect an effect of
554 dopaminergic drug treatments on spawning. For example, treatment of European sea
555 bass with the DA antagonist domperidone, either alone or combined with GnRHa, had
556 no effect on spontaneous or GnRHa-induced OM or spawning (Prat et al., 2001). In
557 contrast, in species expressing a strong DA inhibitory tone, DA antagonists stimulate
558 OM and spawning when given as single treatments, and enhance GnRHa-induced
559 effects in combined treatments (Yaron et al., 1995; Wen and Lin, 2004; Aizen et al.,
560 2005). The results from the present study indicate that a DA inhibition does not explain
561 the absence of spontaneous tank spawning in cultured Senegalese breeders.

562 Despite the observed stimulation of sperm production by all three hormonal
563 treatments and egg release by the GnRHa and COMB treatments, there was a complete
564 lack of egg fertilization in the spawnings obtained from all broodstock tanks. This is
565 consistent with what has been repeatedly observed in Senegalese sole cultured (F1)
566 broodstock, both for spontaneous and GnRHa-induced spawning (Agulleiro et al., 2006;
567 Guzmán et al., 2008, 2009a). The present study showed that the lack of egg fertilization
568 persist in broodstock treated with PIM, and suggests no action of DA on the
569 mechanisms leading to the fertilization of eggs. Previous studies in Senegalese sole
570 have suggested that lack of egg fertilization in tank spawning of cultured broodstock
571 might be due to an inhibition of courtship and sexual behaviour (Guzmán et al., 2008,
572 2009b; Howell et al., 2009). Recent video-recording studies in cultured Senegalese sole
573 have shown that GnRHa-induced egg release in females is produced in a total absence of

574 courtship between males and females, suggesting no participation of males and, thus, no
575 fertilization of the released eggs (N. Duncan, IRTA, Spain, personal communication).
576 As in other fishes, the mechanisms of sexual behaviour are critical in flatfish species for
577 synchronization of gamete release and successful egg fertilization. In flounder
578 (*Paralichthys orbignyanus*) and summer flounder (*Paralichthys dentatus*), the high
579 variability in egg fertilization success has been clearly associated with a low
580 participation of males in spawning events (Watanabe et al., 1998; Watanabe and Carroll,
581 2001; Bambill et al., 2006).

582 In conclusion, blockage of the endogenous DA action by treatment with PIM was
583 shown to stimulate spermatogenesis, testicular maturation and sperm release in mature
584 male Senegalese sole. In addition, some stimulatory effects of PIM on the endocrine
585 reproductive axis were demonstrated, suggesting the existence of a DA inhibitory tone
586 regulating male reproduction in this species. On the other hand, no effects of PIM were
587 seen in females, indicating the absence or weak expression of a DA inhibition in
588 females. Despite the stimulatory effects of PIM on males and GnRHa on males and
589 females, the lack of egg fertilization in all experimental tanks suggests that a DA
590 inhibition would not be the main reason underlying the failure of cultured Senegalese
591 sole broodstock to produce fertilized spawning.

592

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600

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859

860

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

Transcript	Primer	Nucleotide sequence	Amplicon size (bp)
FSHβ	ssFSHf_q	5’ GGACCCAAACTACATCCATGAAC 3’	60
	ssFSHr_q	5’ CAGTCCCCGTTACAGATCACCTGTCT 3’	
LHβ	ssLHf_q	5’ CGGTGGAGACGACCATCTG 3’	61
	ssLHr_q	5’ GGTATCTTGATGACGGGATCCTT 3’	
GPα	ssGPf_q	5’ ACGGGCTGTGAGAAATGCA 3’	56
	ssGPr_q	5’ GGATGCTCCCTGGAGAACAA 3’	
18s[1]	18Sf_q	5’ GGTACTTTCTGTGCCTACCATGGT 3’	61
	18Sr_q	5’ CCGGAATCGAACCCTGATT 3’	

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

861

862

863

Table 2. Spawning characteristics 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 ± SEM.

	CNT	**PIM**	**GnRHa**	**COMB**
Nº 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 ± 0.6 ^{a}	20.0 ± 3.5 ^{b}	14.6 ± 3.0 ^{b}
Egg buoyancy (%)	0	19.4 ± 8.0	12.9 ± 1.8	18.8 ± 5.0
Fertilization success (%)	0	0	0	0

864

865

866 **Figure legends**

867

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

874

875 **Figure 2.** Pituitary transcript levels of gonadotropin subunits (FSHβ, LHβ and
876 GPα) in male and female Senegalese sole breeders treated with saline (CNT), pimozide
877 (PIM), GnRHa (GnRHa) and the combined PIM + GnRHa treatment (COMB). Levels
878 were normalized to Senegalese sole 18s. Pituitaries were collected 24 h after the last
879 treatment. Different letters indicate significant differences ($p<0.05$) among treatments.
880 Data are expressed as mean ± SEM (n= 6).

881

882 **Figure 3.** Plasma levels of 11-ketotestosterone (11-KT) and testosterone (T) in
883 male and estradiol (E2), T and vitellogenin (VTG) in female Senegalese sole breeders
884 treated with saline (CNT), pimozide (PIM), GnRHa (GnRHa) and the combined PIM +
885 GnRHa treatment (COMB). Plasma samples were collected on days 0, 10 and 25 from
886 the initiation of treatments. Different capital letters indicate significant differences
887 ($p<0.05$) among sampling times within control fish. Different small letters indicate
888 significant differences ($p<0.05$) among treatments within sampling points. Data are
889 expressed as mean ± SEM (n=6).

890

891 **Figure 4.** Photomicrographs of cross-section of Senegalese sole testis from males

892 of the four experimental groups: controls (A,B), PIM (C,D), GnRHa (E,F) and COMB

893 (G,H). Photomicrographs on the left (A,C,E,G) are from cortex and on the right

894 (B,D,F,H) from medulla. Abbreviations: spg, spermatogonia; spc, spermatocyte; spd,

895 spermatid; spz, spermatozoon. Scale bars: 40 µm.

896

897 **Figure 5.** Milt volume, sperm density and total sperm production in male

898 Senegalese sole breeders treated with saline (CNT), pimozide (PIM), GnRHa (GnRHa)

899 and the combined PIM + GnRHa treatment (COMB). Milt samples were collected on

900 days 0 and 25 from the initiation of treatments. Different letters indicate differences

901 ($p<0.05$) among treatments within sampling points. Data are expressed as mean ± SEM

902 (n=6).

903

904 **Figure 6.** Daily spawning of cultured Senegalese sole broodstock (shown for one

905 of the duplicate tanks) treated with saline (CNT), pimozide (PIM), GnRHa (GnRHa)

906 and the combined PIM + GnRHa treatment (COMB). Treatment of PIM was

907 administered as 3 injections (days 0, 10 and 24) and GnRHa as a single implant (day 0)

908 and injection (day 24). The quantity of buoyant (black bars) and sinking (white bars)

909 eggs was determined for each spawn. No spawnings were detected in any group after

910 day 16 from the initiation of treatments.

Figures

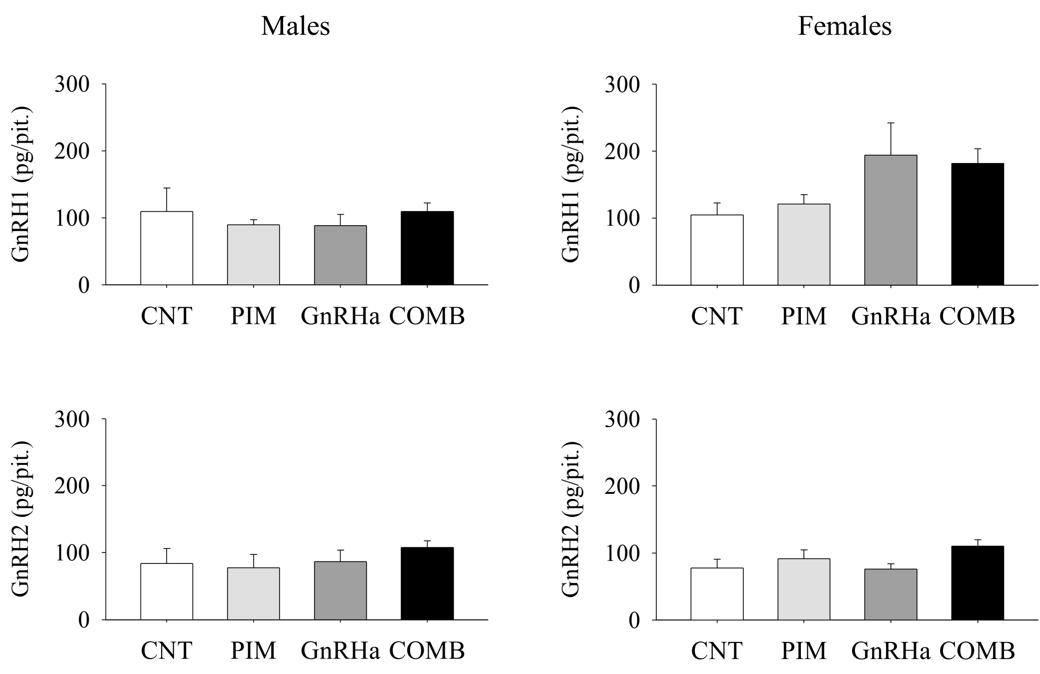

Figure 1

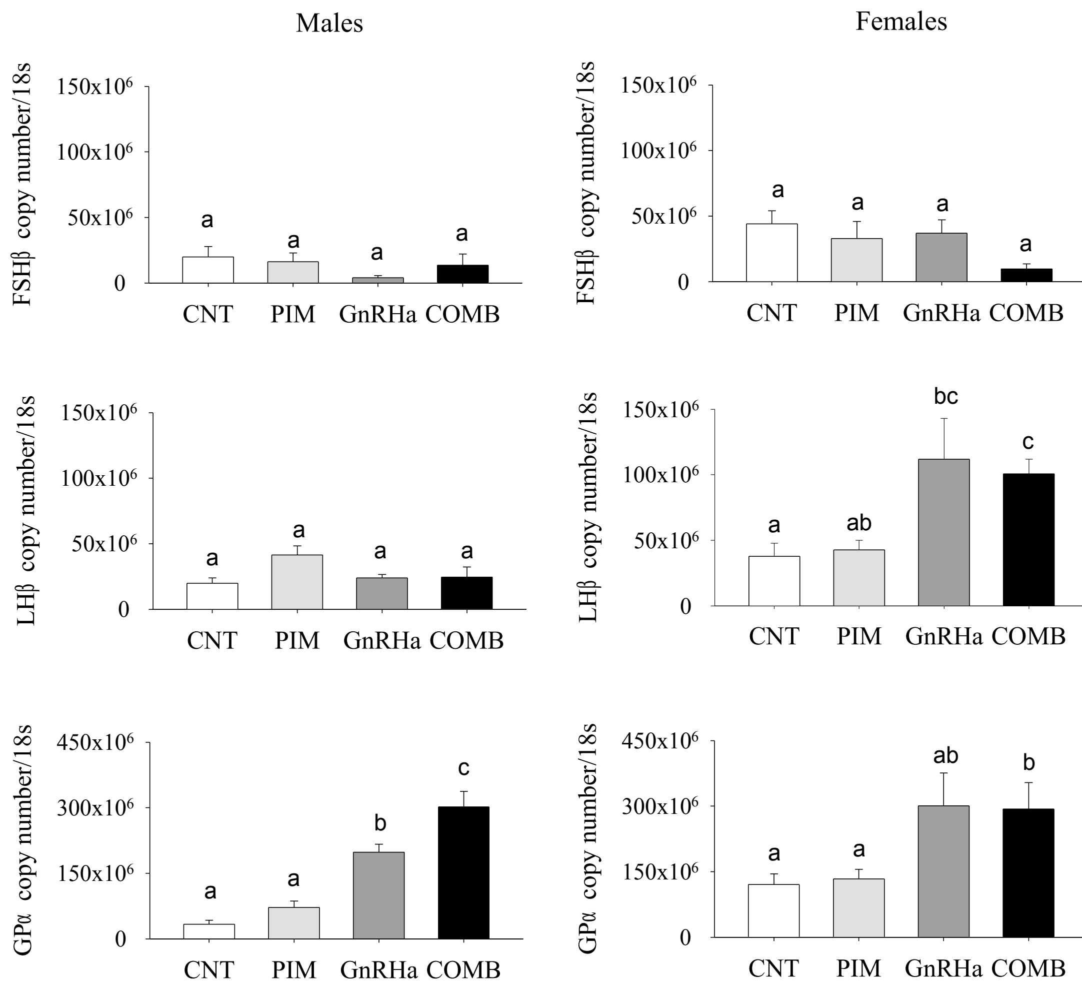

Figure 2

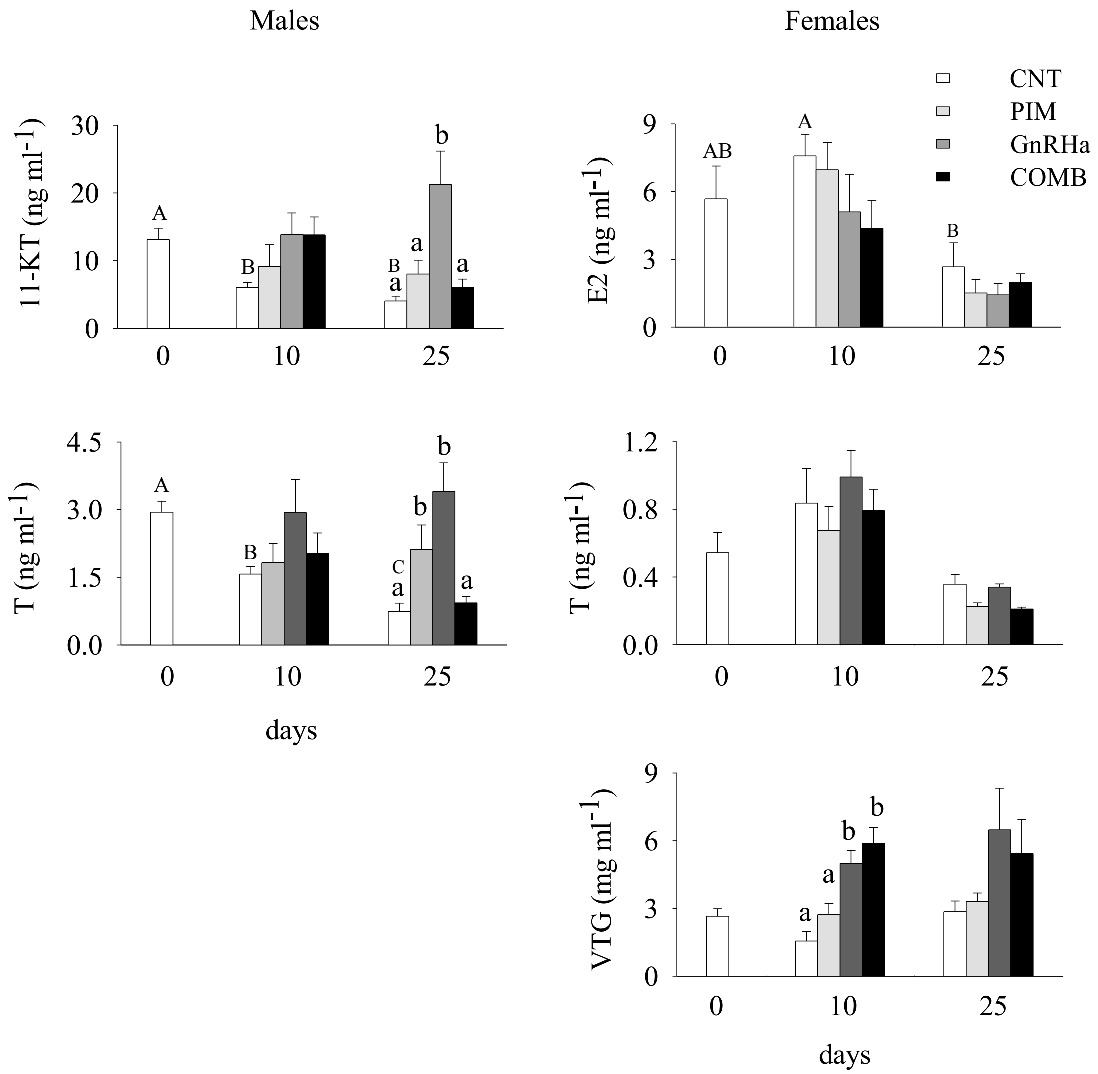

Figure 3

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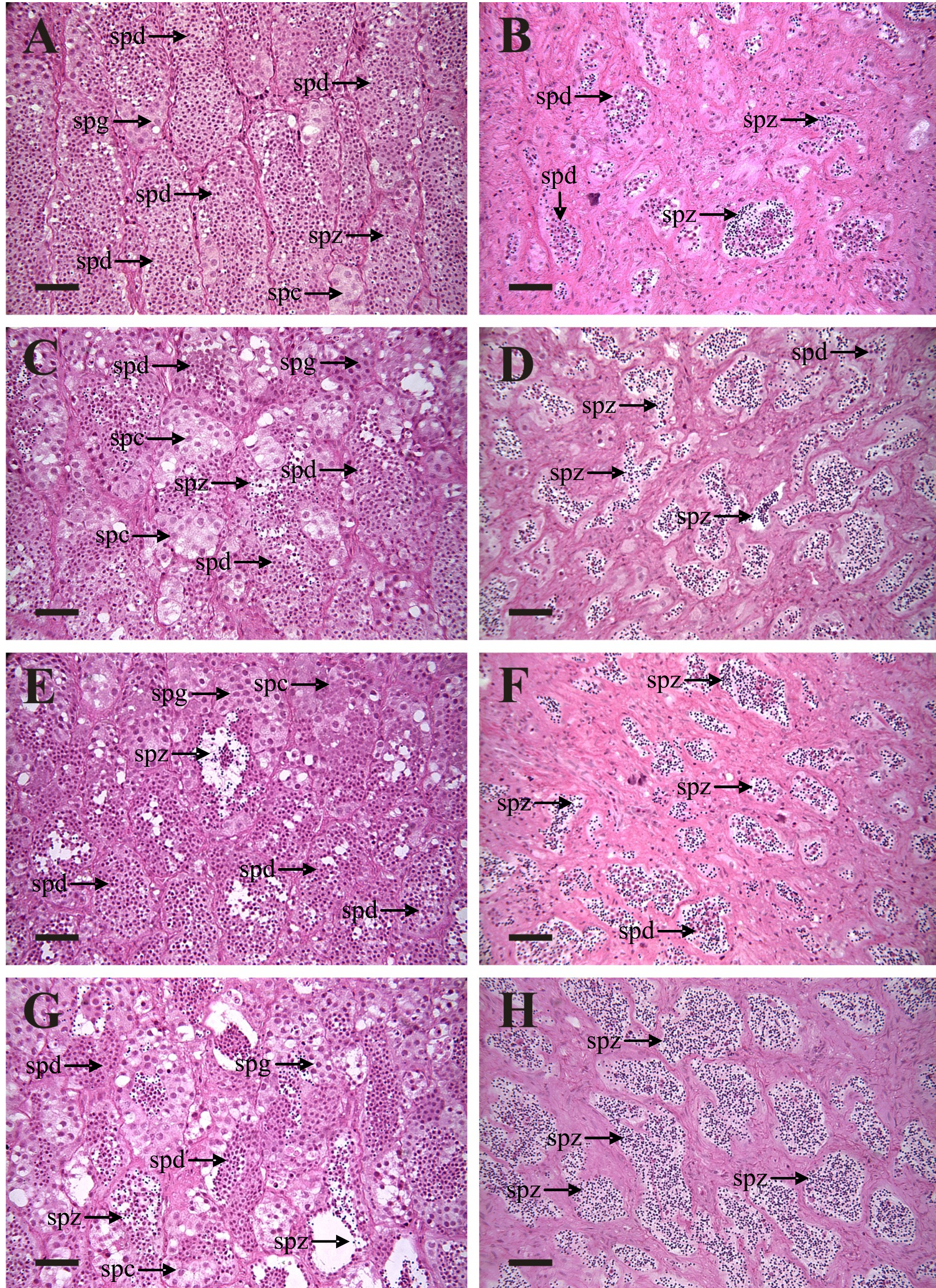

Figure 4

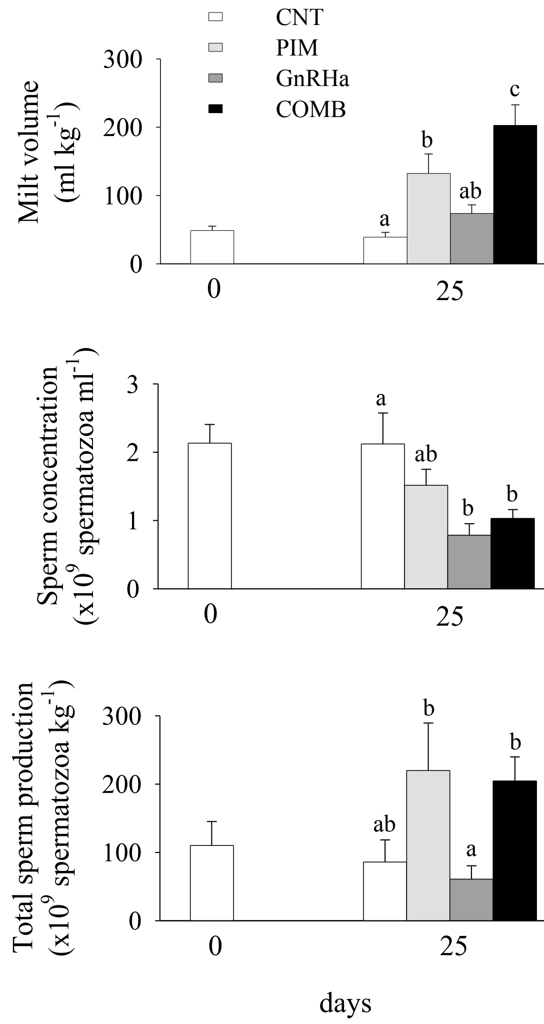

Figure 5

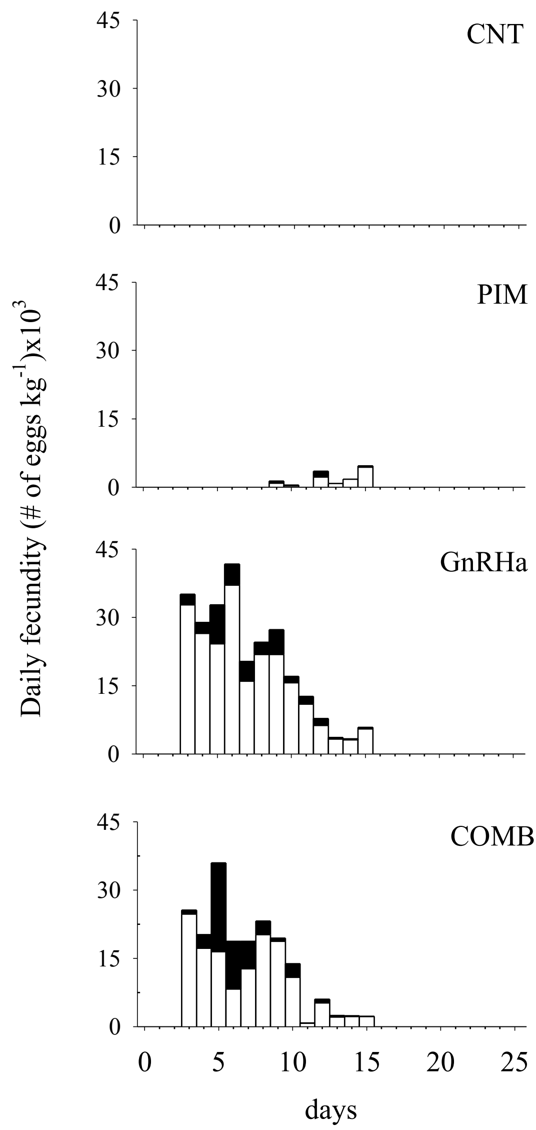

Figure 6