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3**Comparative effects of human chorionic gonadotropin (hCG) and**
4**gonadotropin-releasing hormone agonist (GnRHa) treatments on the**
5**stimulation of male Senegalese sole (*Solea senegalensis*) reproduction**

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25Abstract

26 The aquaculture of Senegalese sole (*Solea senegalensis*) is limited by the failure of
27cultured breeders (G1 generation) to produce fertilized spawning. Critical reproductive
28dysfunctions have been observed in both female and male Senegalese sole cultured
29breeders, including reduced fecundity and diminished sperm production. The present
30work aimed to study the effectiveness of different hormonal treatments on the
31stimulation of male reproduction. Male Senegalese sole cultured breeders were treated
32with 1) saline injections (controls), 2) gonadotropin-releasing hormone agonist
33(GnRHa) injections (25 $\mu\text{g kg}^{-1}$), 3) GnRHa slow release implants (40 $\mu\text{g kg}^{-1}$) or 4)
34human chorionic gonadotropin (hCG) injections (1000 IU kg^{-1}). Each group of males
35was placed in separated spawning tanks together with females treated with GnRHa
36implants.

37 All three hormonal treatments increased plasma testosterone (T) and 11-
38ketotestosterone (11-KT) levels and the gonadosomatic index (GSI), with highest
39effects exerted by the hCG treatment. Histological examination of the testes showed no
40effect of the GnRHa injection, but a clear stimulation of germ cell proliferation and
41testicular maturation by GnRHa implants and hCG injections. As expected, GnRHa
42implantation of females induced egg release in all experimental tanks and interestingly,
43female fecundity increased in tanks containing GnRHa- or hCG-treated males. A
44fertilized spawning was obtained only from the group containing hCG-treated males. In
45conclusion, hormonal treatments stimulated steroidogenesis and spermatogenesis in
46male Senegalese sole, with highest efficiency of the hCG multiple injection treatment.
47Female fecundity was affected by the hormonal treatment applied over the
48accompanying males, suggesting a pheromone communication between fish. However,

49none of the treatments seemed to be adequate in solving the problem of lack of fertilized
50spawning in cultured Senegalese sole broodstocks.

51

52**Keywords:** hCG, GnRHa, spermatogenesis, behaviour, flatfish, Senegalese sole.

53

541. Introduction

55 The Senegalese sole (*Solea senegalensis*) is a highly valuable flatfish that has become
56a priority species for the diversification of aquaculture in European and Mediterranean
57countries (Imslund et al., 2003). However, the establishment of an expanding,
58sustainable and efficient aquaculture industry is seriously constrained by the failure of
59cultured broodstocks (G1 generation; hatched and raised in captivity) to produce
60fertilized spawning (Howell et al., 2006, 2009). This has been correlated with
61reproductive dysfunctions detected in both male and female cultured breeders,
62evidenced by diminished sperm production in males and low fecundity in females
63(Mañanós et al., 2008). Cultured females complete vitellogenesis and show elevated
64plasma profiles of vitellogenin (VTG) and sex steroids during the reproductive season
65that correlated well with gonadal growth (Guzmán et al., 2008), but they often fail to
66complete oocyte maturation and ovulation, and most of postvitellogenic oocytes
67undergo atresia (García-López et al., 2007; Guzmán et al., 2009a). Cultured males
68complete spermatogenesis and show well correlated profiles of plasma androgen levels
69(García-López et al., 2006), but sperm production is reduced compared to wild-caught
70breeders, which is thought to limit significantly the fertilization success in cultured
71broodstocks (Cabrita et al., 2006).

72 Failure to undergo oocyte maturation and ovulation in females and reduced sperm
73production in males are common reproductive dysfunctions in fish maintained under
74captive conditions, either wild or cultured (Donaldson and Hunter, 1983; Zohar and
75Mylonas, 2001; Mañanós et al., 2008). In aquaculture, the most common strategy to
76stimulate gonad maturation, ovulation and spermiation in fish is the exogenous
77treatment with gonadotropin-releasing hormone agonists (GnRHa), either in the form of
78liquid injections or sustained-release delivery systems (Zohar and Mylonas, 2001;
79Mylonas and Zohar, 2001; Mylonas et al., 2009). The GnRHa based hormonal
80treatments have been successfully used in several flatfish species to induce spawning
81and spermiation, normally with highest efficiency exhibited by treatments using GnRHa
82slow-release delivery implants (Larsson et al., 1997; Mugnier et al., 2000; Vermeirssen
83et al., 2000; Moon et al., 2003). Although less widely used than GnRHa, another
84effective hormonal therapy is the treatment with human chorionic gonadotropin (hCG),
85usually administered through saline-diluted multiple injections. The hCG acts on the
86gonad and has been shown to stimulate spermatogenesis and sperm production in
87several fish species (Miura et al., 1991, 2002; Cacot et al., 2003; Schiavone et al., 2006).
88Interestingly, some studies have reported effects of hCG on breeding behaviour. In
89male mutton snapper (*Lutjanus analis*) a single hCG injection stimulated spermiation
90and induced high fertilization rates (Watanabe et al., 1998a). In Japanese eel (*Anguilla*
91*japonica*) weekly hCG injections stimulated active courtship behaviour before
92spermiation (Dou et al., 2007).

93 Several attempts have been undertaken to stimulate reproduction of cultured
94Senegalese sole broodstock through hormonal treatments, limited to the use of GnRHa-
95based therapies. In females, treatment with GnRHa through saline-diluted multiple

96injections (Agulleiro et al., 2006), slow-release microspheres (Guzmán et al., 2009a)
97and slow-release EVAc-implants (Agulleiro et al., 2006; Guzmán et al., 2009a) induced
98egg release, with highest efficiency of the slow-release delivery systems (Guzmán et al.,
992009a). Effects on males were less conclusive and showed that GnRHa injections and
100GnRHa implants, given alone or in combination with 11-ketoandrostenedione, slightly
101stimulated spermatogenesis and milt production (Agulleiro et al., 2006, 2007). In these
102studies all spawning obtained from both non-treated and GnRHa-treated fish were
103unfertilized, indicating that the tested hormonal protocols were not successful to induce
104fertilized tank spawning. It has been hypothesized that failure of cultured broodstock to
105produce fertilized spawning is highly related to male reproductive dysfunctions,
106inhibited breeding behaviour and failed synchronization of gamete release (Guzmán et
107al., 2009a; Howell et al., 2009).

108 The aim of this study was to investigate the comparative effectiveness of hCG and
109GnRHa based therapies on the stimulation of male reproduction in Senegalese sole.
110Treatment effects were analyzed on steroidogenesis (plasma androgen levels) and
111spermatogenesis (testis development) and correlated with spawning performance of the
112broodstock.

113

1142. Materials and Methods

1152.1. *Fish husbandry*

116 Cultured Senegalese sole, obtained from natural spawns of wild-caught broodstock at
117the facilities of CIFPA “El Toruño” (Cádiz, Spain) in spring 2001, were transported to the
118facilities of the Institute of Aquaculture of Torre la Sal (Castellón, Spain, 40° N 0° E) in
119March 2003. Fish were tagged with passive integrated transponder tags (PIT-tags, AVID).

Sex of the fish was determined by using a heterologous VTG ELISA (Mañanós et al., 1994) and was further confirmed during the reproductive period by abdominal swelling in females and by feeling the shape of the testis in males. Fish were housed in circular fibreglass tanks (3000 L, 1 m depth, 4 m²) without sand substrate, at a density of around 3 kg m⁻² and were exposed to the natural photoperiod and temperature regimes of the region. Tanks were covered with a thin shade mesh to reduce light incidence; maximum light intensity recorded on the water surface was 600 lux. Dissolved oxygen was checked regularly and ranged between 6.8 and 7.5 ppm.

The experiment was initiated on April 19th 2005 and terminated on May 31st 2005, under increasing water temperatures ranging from 15.3 °C to 21.0 °C (mean temperature of this period, 17.9 ± 0.3 °C). Tanks were fitted with overflow egg collectors and were supplied with flow-through seawater (salinity ~37‰) at a flow rate of 400% d⁻¹. Fish were fed to apparent satiation 5 days a week with both commercial pellets (Solea immunofeed, Proaqua s.a., Spain) and natural food, consisting of chopped fresh mussels and frozen squid.

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

2.2. *Experimental design and hormonal treatments*

On April 2nd 2005, when external signs of gonad maturation were clearly evident in the broodstock (Guzmán et al., 2008, 2009a), such as abdominal swelling in females and expressible milt in males, 56 fish were distributed homogeneously in four tanks (6 females

144and 8 males per tank), at a density of $3.6 \pm 0.1 \text{ kg m}^{-2}$. Fish were four years old, with a
145mean ($\pm$ SEM) body weight (BW) of females and males of $1,266.0 \pm 35.5 \text{ g}$ and $932.8 \pm$
146 33.7 g , respectively, and body length (BL) of $42.4 \pm 0.4 \text{ cm}$ and $39.3 \pm 0.4 \text{ cm}$,
147respectively.

148 On April 19th (day 0), four males were randomly selected (one male per tank),
149sacrificed and testis collected and processed for histological analysis. Thereafter, the
150following treatments were applied on males: (1) saline (0.9% NaCl) injections given on
151days 0 and 21 (group CNT, controls); (2) GnRHa injections ($25 \mu\text{g kg}^{-1}$) given on days 0
152and 21 (group GINJ); (3) GnRHa implants ($40 \mu\text{g kg}^{-1}$) given on days 0 and 21 (group
153GIMP), and (4) hCG injections (1000 IU kg^{-1}) given on days 0, 7, 14, 21, 28 and 35 (group
154hCG). To assure occurrence of egg release in the tanks, females from all experimental
155groups were treated with GnRHa-implants ($40 \mu\text{g kg}^{-1}$), given on days 0 and 21.

156 The drugs used were [D-Ala⁶, Pro⁹ Net]-LHRHa (Bachem, Switzerland) and hCG-
157Lepori 2500 (Farma-Lepori, Spain). For injections, both GnRHa and hCG were dissolved
158in saline. The GnRHa implants were manufactured from p[Ethylene-Vinyl acetate]
159copolymer (EVAc, Elvax; DuPont Chemical CO., DE) as 2-mm diameter x 3-mm
160cylinders (Zohar et al., 1990). Both injections and implants were applied in the dorsal
161musculature of the fish. The doses of GnRHa were based on previous studies in other
162fishes, including Senegalese sole (Mylonas and Zohar, 2001; Guzmán et al., 2009a). The
163dosage of hCG was based on previous hCG treatment protocols used in other fishes
164(Schiavone et al., 2006; Dou et al., 2007).

165 Females were only manipulated for GnRHa implant administration (days 0 and 21) and
166were not sampled throughout the experimental period. Males were sampled for blood at
167weekly intervals from day 0 (initiation of treatments). Blood (0.8 ml) was taken from the

caudal vasculature, using heparinised syringes and placed in ice-cold heparinised tubes. Plasma was obtained by centrifugation (3,000 g, 15 min, 4 °C) and stored at -20 °C until analysis for sex steroid hormones by ELISA. Males were not sampled for milt throughout the experiment, to avoid potential detrimental effects of milt collection on fertilization capacity (Suquet et al., 1992). After blood sampling on day 42, males were sacrificed by decapitation and testis removed and weighed for calculation of the gonadosomatic index (GSI = gonad weight x BW⁻¹ x 100). Transverse sections from the middle region of the dorsal and ventral lobes of the testis were dissected out and collected for histology.

2.3. Sex steroid ELISAs

For steroid analysis, plasma samples were first extracted by addition of ice cold methanol (methanol:plasma 6:1, v/v), shaken and centrifuged (3,000 g, 15 min, 4 °C). The pellet was re-extracted twice with 200 µl of methanol. Supernatants were pooled, dried and reconstituted in 0.1 M potassium buffer (pH 7.4). The levels of testosterone (T) and 11-ketotestosterone (11-KT) were quantified by ELISA using previously developed protocols (Rodríguez et al., 2000), further adapted and validated for analysis of plasma samples in Senegalese sole (Guzmán et al., 2008, 2009a,b). The sensitivities of the ELISAs, calculated as the lowest detection limit (Bo-2SD), were 8.8 and 0.4 pg ml⁻¹ for the T and 11-KT ELISA, respectively. The intra-(n=4) and inter-assay (n=8) coefficients of variation, at 50% of binding, were 6.1% and 11.3% for T, and 10.7% and 9.1% for the 11-KT ELISA.

2.4. Testicular histology

191 Fragments of testicular tissue were fixed in 4% phosphate-buffered (0.1 M, pH 7.2)
192 formalin for 48–96 h at room temperature. After rinsing in running tap water (16 h) and
193 dehydration in ascending concentrations of ethanol, fragments were infiltrated and
194 embedded in glycol methacrylate resin (Technovit 7100, Heraeus Kulzer, Germany).
195 Sections were cut at 3 μ m on a Supercut 2065 microtome (Reichert-Jung, Germany) and
196 stained with methylene blue / azure II / basic fuchsin (Bennett et al., 1976). Stained
197 sections were examined and photographed on a Leitz Diaplan light microscope.

198 Photomicrographs were taken and analyzed from the two main regions of the
199 Senegalese sole testes, cortex and medulla, and the stage of testicular development
200 determined according to the germ cell types and their relative abundance (García-López et
201 al. 2005, 2006).

202

203 2.5. *Egg collection, incubation and larval rearing conditions*

204 Egg collectors were checked daily (09:00 h) to detect the presence of eggs; eggs
205 were collected for evaluation of fecundity and quality. Eggs were placed in graduated
206 cylinders with 37 ‰ sea water and allowed to stand for a few minutes, to separate
207 buoyant (viable) and sinking (dead) eggs. The volume of each fraction was recorded
208 and fecundity further expressed as n° of eggs, considering that 1 ml of eggs corresponds
209 approximately to 1,000 eggs (Guzmán et al., 2009a). Daily relative fecundity was
210 calculated using the BW of the females at the previous sampling, and total relative
211 fecundity was calculated at the end of the study. From each spawn, a sample of 50
212 buoyant eggs was examined under a binocular microscope to determine egg morphology
213 (size, shape, transparency and distribution of oil droplets) and fertilization success.

214 Buoyant eggs were incubated for 48 h to determine hatching rates. Hatched larvae were
215 transferred to 150 L flat-bottom circular tanks.

216 Egg incubation and larval rearing was performed in a closed re-circulation sea water
217 system (salinity ~37‰), filtered through 10 and 20 µm cartridge filters (Cuno
218 Incorporated, Meriden, CT) and UV sterilized. Tanks were exposed to the natural
219 photoperiod and temperature regimes of the region. Air diffusers were installed in the
220 centre and perimeter of the tanks to ensure gentle and continuous aeration. Dissolved
221 oxygen (6.7 ± 0.2 ppm), pH (7.5 – 8.2), and nitrites and ammonium water levels (< 0.5
222 ppm) were checked regularly. Cleaning of the tanks was carefully performed by
223 siphoning the bottom of the tank every other day. From 2 to 7 days post hatching (dph),
224 larvae were fed on rotifers (*Brachionus plicatilis*) (5 individuals ml⁻¹ d⁻¹); during this
225 period microalgae (*Isochrysis galbana*) (2.5×10^5 cells ml⁻¹ d⁻¹) were added to the larval
226 rearing tanks. From 5 to 40 dph, specimens were fed with an increasing ratio of newly
227 hatched *Artemia salina* nauplii (1-7 individuals ml⁻¹ d⁻¹), decreasing thereafter up to 70
228 dph. From 10 to 70 dph, specimens were co-fed with an increasing ratio of commercial
229 dried pellets (0.5–30 g m⁻² d⁻¹, Solea immunofeed, Proaqua, Spain). From 70 dph
230 onward, juveniles were exclusively fed with commercial dried pellets; ratio was
231 adjusted daily depending on remaining food observed in the bottom of the tank.

232

233 2.6. Statistics

234 Data are expressed as mean $\pm$ standard error of the mean (SEM). Statistical
235 differences in plasma levels of T and 11-KT were examined using two-way Analysis of
236 Variance (ANOVA, treatment *versus* sampling point) followed by the Holm-Sidak test,
237 with a significance level of $p < 0.05$. Statistical differences on GSI, daily relative

fecundity and egg buoyancy were examined using a one-way ANOVA, followed by the Holm-Sidak test (significance level of $p < 0.05$). Normality and homogeneity of the variance were tested by the Kolmogorov-Smirnov and Bartlett methods, respectively.

241

2423. Results

2433.1. Plasma levels of sex steroids

244 Males treated with GnRHa injections (group GINJ) showed significantly ($p < 0.05$)
245 higher levels than controls at weeks 1 and 2 for both androgens (Fig. 1). Treatment with
246 GnRHa implants increased significantly ($p < 0.05$) plasma levels of 11-KT and T at week 1
247 and levels remained elevated compared to controls for 5 and 6 weeks, respectively
248 ($p < 0.05$); levels of 11-KT in GnRHa implanted males were significantly higher than those
249 in GnRHa injected males at weeks 4 and 5, whereas those of T were higher from 3 weeks
250 onwards. The hCG treatment caused the highest increase in plasma androgens levels; both
251 T and 11-KT in hCG treated males were significantly higher ($p < 0.05$) than controls at all
252 sampling points (except day 0). Levels of 11-KT in hCG treated males were significantly
253 ($p < 0.05$) higher than all other groups at weeks 3, 5 and 6, whereas those of T were higher
254 at weeks 5 and 6.

255

2563.2. Testicular development

257 The testes of males at the day of initiation of the treatments were at the functional
258 maturation stage, as expected at this time of the year (spermiation period). At the end of
259 the experimental period (day 42), the testes from control males showed a similar
260 maturation stage, characterized by a low number of early developing germ cells
261 (spermatogonia, spg) and spermatocytes (spc), a high abundance of spermatids (spd) in the

cortex and low to intermediate number of spermatozoa (spz) within the medullar efferent duct system (Fig. 2A,B). At this time, testes from GnRHa injected males showed similar morphological features than controls, with few spermatocysts with spc and abundant spd in the cortical seminiferous lobules (Fig. 2C) and ripe spz in the medullar efferent duct system (Fig. 2D). The testes from GnRHa implanted and hCG treated males showed morphological differences to those from CNT and GnRHa injected males, with clear signs of stimulated spermatogenesis, mostly on hCG treated males. Both treatments stimulated the proliferation of germinal cysts containing spc all along the cortical region, while clutches of spd transforming into spz became larger and more abundant (Fig. 2E,G). In the medulla, both treatments caused an increment in the lumen size of the efferent ducts, highly evident on hCG treated males, together with an increased amount of spz within the medullar duct system (Fig. 2F,H).

All three hormonal treatments increased the GSI with respect to controls (Fig. 3), although differences were only significant ($p<0.05$) in GnRHa implanted and hCG treated males.

3.3. *Spawning and F2 larvae production*

As expected, GnRHa implants induced daily egg production in females from all experimental tanks, with two clear peaks associated with the moments of implant administration (Fig. 4). The response on daily fecundity was higher for the first GnRHa implant (day 0) than for the second implant given 3 weeks later (day 21). Also, the latency period was longer after the first GnRHa implant compared to the second one; spawning started 3-5 days after the first implantation in the different tanks, compared to only 2 days after the second implantation. No differences in daily fecundity or egg buoyancy were

286observed among groups (Table 1). Egg morphology, in terms of egg shape and size,
287transparency and distribution of oil droplets, was similar in all groups.

288 Despite the similar treatment applied to females, total fecundity was higher in those
289tanks containing hormone-treated males (Fig. 5). Total fecundity increased 1.2-, 2.0- and
2901.9-fold in groups GINJ, GIMP and hCG, respectively, with respect to controls (group
291CNT).

292 Egg fertilization did not occur except for the hCG group that produced one fertilized
293spawn on day 33. This spawn consisted on a total of 24,000 eggs, from which 4,000 eggs
294were buoyant (16.7% buoyancy) and showed 100% of fertilization. Fertilized eggs were
295incubated for 48 h and showed a hatching success of 95%; larval survival after the
296weaning period was 80%. This F2 generation Senegalese sole was further reared and
297showed normal growth rates, with BW and BL of 71.9 ± 2.4 g and 17.0 ± 0.2 cm,
298respectively, at one year of age.

299

3004. Discussion

301 Results showed that androgen release and spermatogenesis in cultured male
302Senegalese sole breeders were stimulated by GnRH α and hCG treatments, with highest
303potency shown by the hCG treatment. Interestingly, treatment of males with GnRH α
304implants and hCG injections enhanced the fecundity of the accompanying females,
305suggesting a pheromone communication between the two sexes. A single batch of
306fertilized eggs was obtained from the tank of hCG treated males, giving rise to the first
307reported F2 generation larvae from cultured Senegalese sole broodstock. Although this
308result is encouraging, the outcome of this study demonstrates the necessity for further

309research to understand and hopefully, solve the problem of failed fertilization in G1
310Senegalese sole broodstock.

311 The hCG treatment showed higher potency than GnRHa-based protocols on the
312stimulation of androgen release, as showed by highest plasma levels of 11-KT and T
313throughout the experimental period. The higher efficiency of the hCG treatment on
314androgen release might be related to the different organs targeted by hCG and GnRHa.
315The GnRHa acts on the pituitary of the treated fish to stimulate the secretion of the
316fish's own gonadotropins, which in turn induce steroidogenesis in the gonad, while the
317hCG acts directly on the gonad to stimulate steroidogenesis and thus, its effects are not
318dependent on the activation of the pituitary and/or the amount of pituitary gonadotropin
319stores (Miura et al., 1991; Zohar and Mylonas 2001). In the flatfish greenback flounder
320(*Rhombosolea tapirina*), a single hCG injection (1,000 IU kg⁻¹) was more effective
321than a GnRHa injection (100 µg kg⁻¹) in increasing 11-KT and T plasma levels at 2 d
322post-treatment (Pankhurst and Poortenaar, 2000). On the other hand, a double hCG
323injection procedure (62.5 + 187.5 IU kg⁻¹) was ineffective at stimulating androgen
324release in male dab (*Limanda limanda*, Pleuronectiforme), but this was likely associated
325to the low hCG dose used (Canario and Scott 1991). A multiple hCG injection protocol
326has not been assayed in flatfishes, but weekly injections of 1,500 UI kg⁻¹ were highly
327effective in stimulating 11-KT secretion in the European eel (*Anguilla anguilla*)
328(Peñaranda et al., 2009). Stimulation of androgen release by GnRHa treatment has been
329previously reported in several flatfishes, such as the winter flounder (*Pleuronectes*
330*americanus*) (Harmin et al., 1995), plaice (*Pleuronectes platessa*) (Vermeirssen et al.,
3311998), starry flounder (*Platichthys stellatus*) (Moon et al., 2003) and the Senegalese sole
332(Agulleiro et al., 2006, 2007). Taken together, these and the present study confirm the

stimulatory effect of GnRHa on 11-KT and T release, likely through the action of GnRHa-induced pituitary secretion of endogenous gonadotropins (Yaron et al., 2003).

Testicular development and maturation was highly stimulated by the hCG treatment and to a lesser extent, by GnRHa implants. The hCG treatment induced a marked proliferation of spermatocytes on the cortical seminiferous lobules and an increase in the lumen size of the medullar efferent ducts, which were filled with an increased amount of spermatozoa. These data are the first evidence of a stimulatory action of hCG treatments on testicular development in a flatfish species. The observed proliferation of germinal cysts containing spermatocytes in the cortical seminiferous lobules from testes of hCG-treated male Senegalese sole would indicate an active meiotic process which fuelled spermatid differentiation and led to a higher spermatozoa production. These features are similar to those previously described in fully mature cultured male Senegalese sole during mid and late spermatogenesis (García-López et al., 2006). In the present study, the hCG-induced stimulation of spermatogenesis was consistent with the long lasting stimulation of 11-KT plasma levels in hCG-treated males. This androgen is the major sex steroid hormone regulating testicular development in fish, promoting both germ cell proliferation and spermiogenesis (Amer et al., 2001; Schulz et al., 2009). An stimulatory effect of hCG treatments on germ cell proliferation and spermatozoid differentiation have been previously reported in European sea bass (*Dicentrarchus labrax*) (Schiavone et al. 2006), European eel (Peñaranda et al., 2009) and Japanese eel (Miura et al., 1991; Miura et al., 2002). Although displaying a lower potency than the hCG treatment, GnRHa implants also stimulated spermatogenesis in male Senegalese sole concomitantly with increased 11-KT plasma levels. This is in agreement with a

356previous study in male Senegalese sole which showed GnRHa-induced increase in the
357percentage of developing germ cells and ripe spermatozoa (Agulleiro et al., 2007).

358 A fertilized spawning was obtained from the broodstock tank containing females
359treated with a double GnRHa implant and males treated for long-term with hCG injections
360(day 33 after initiation of treatments), giving rise to the first reported F2 generation of
361Senegalese sole. The hatching rate, survival and growth characteristics of this F2
362generation fish were similar to those previously described for G1 Senegalese soles
363(Dinis et al., 1999), indicating that egg quality from G1 breeders may be as good as that
364of wild-caught breeders. The obtaining of a single batch of fertilized eggs in the present
365study points out to the persistent problem of cultured Senegalese breeders to produce
366fertilized eggs. The absence of fertilization in this study was somehow unexpected,
367considering that both GnRHa and hCG treatments were highly successful to induce
368maturation and egg release in females and spermiogenesis in males. Previous studies in
369cultured Senegalese sole have described a total absence of fertilization in both
370spontaneous and GnRHa-induced spawning (Agulleiro et al., 2006; García-López et al.,
3712007; Guzmán et al., 2008, 2009a). It is unlikely that failed fertilization be related to
372bad quality of gametes, because recent studies have demonstrated that gametes obtained
373from cultured Senegalese sole breeders by stripping are able to produce egg fertilization
374in vitro, with fertilization and hatching rates similar to those obtained with gametes
375from wild breeders (Chereguini et al., 2007; author's unpublished results). Also, it has
376been repeatedly determined that egg and sperm samples obtained from cultured breeders
377showed similar quality parameters (binocular examination and CASA analysis) than
378those from wild breeders (Cabrita et al., 2006). A more likely possibility, as it has been
379previously suggested (Guzmán et al., 2009a; Howell et al., 2009), is that absence of

380fertilized tank spawning in cultured broodstock would be related to an inhibition of
381breeding behaviour or courtship and thus failure of synchronized gamete release.
382Recent video-recording studies have shown that treatment of cultured Senegalese sole
383broodstock with GnRHa implants induce egg release in females in the absence of any
384evident breeding behaviour (N. Duncan, IRTA, Spain, personal communication). A
385similar situation has been described in other flatfish species. The low or absent
386fertilization success observed in the summer flounder (*Paralichthys dentatus*) and
387flounder (*Paralichthys orbignyanus*) have been associated to a low participation of
388males during courtship (Watanabe et al., 1998b; Watanabe and Carrol 2001; Bambill et
389al., 2006).

390 Interestingly, female fecundity was enhanced by the hormonal treatment applied to
391the accompanying males. Females accompanied by GnRHa-implanted or hCG-injected
392males exhibited higher fecundities (aprox. 2-fold) than those females accompanied by
393control (saline-injected) males. This is in agreement with previous studies performed in
394cultured Senegalese sole broodstock, where a consistent increase of fecundity was
395observed in females sharing tanks with hormone-stimulated males, as compared to other
396non-treated broodstock tanks (E. Mañanós, unpublished results). These results suggest
397a communication between fish of the population and that males may exert a stimulatory
398action on females, stimulating oocyte maturation, ovulation and egg release. It is
399known that male and female fish can release a wide range of sex steroids into the water,
400which exert effects on the reproductive behavior and physiology in their conspecifics
401(Munakata and Kobayashi, 2009). In fact, male pheromones have been used to induce
402ovulation and spawning in zebrafish (*Brachydanio rerio*) (Lambert et al., 1986), Pacific
403herring (*Clupea harengus pallasii*) (Carosfeld et al., 1997) and goldfish (*Carassius*

404*auratus*) (Sorensen et al., 2005). It has also been described that hormonal treatments
405increase the releasing rate of pheromones into the water, as demonstrated in male tench
406(*Tinca tinca*) treated with a combined GnRHa and dopamine antagonist treatment
407(Pinillos et al., 2002) and goldfish treated with hCG injections (Sorensen et al., 2005). It
408could be hypothesized that the observed enhancement of female fecundity in the present
409study was caused by exposure to enhanced pheromone secretion from the
410accompanying hormone-treated males.

411 In conclusion, treatment of cultured male Senegalese sole with GnRHa implants or
412multiple hCG injections was effective in stimulating androgen release and testicular
413development, with higher potency exhibited by the hCG treatment. Both GnRHa and
414hCG treatments over males increased the fecundity of the accompanying GnRHa-
415implanted females, suggesting an effective pheromonal communication between sexes.
416However, despite the hormone-induced stimulation of spermiation and enhancement of
417fecundity, only a single batch of fertilized eggs was obtained from the hCG-treated
418group. The results of the present study indicate that hormonal therapies for Senegalese
419sole need to be further optimized and consider the role and stimulatory effects of
420hormonal treatments on sexual behaviour.

421

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Table 1 Spawning characteristics of the four experimental groups of cultured Senegalese sole breeders. Females from all groups were treated with GnRH α implants; the accompanying males were treated with saline (controls, CNT), GnRH α injections (GINJ), GnRH α implants (GIMP) or hCG injections (hCG). Data are expressed as mean $\pm$ SEM.

Male treatment	CNT	GINJ	GIMP	hCG
Female treatment	GIMP	GIMP	GIMP	GIMP
No of spawns	17	18	24	20
Daily fecundity (eggs kg ⁻¹)	6,790 $\pm$ 1,420	8,740 $\pm$ 229	9,390 $\pm$ 1,660	10,750 $\pm$ 2,170
Egg buoyancy (%)	23.8 $\pm$ 1.4	27.9 $\pm$ 6.5	17.6 $\pm$ 3.6	32.1 $\pm$ 4.8
Hatching success (%)	0	0	0	4.75 $\pm$ 4.75

Figure legends

601

602**Figure 1.** Plasma levels of 11-ketotestosterone (11-KT) and testosterone (T) in male
603Senegalese sole treated with saline (controls, CNT), GnRHa injections (GINJ), GnRHa
604implants (GIMP) or hCG injections (hCG). Plasma samples were collected weekly from
605the initiation of treatments (day 0). Different letters indicate significant differences
606(ANOVA, H-S, $p < 0.05$) among treatments within sampling points. Asterisks “*”
607indicate significant differences (ANOVA, H-S, $p < 0.05$) to the previous sampling point
608within a treatment. Data are expressed as mean $\pm$ SEM (n=8).

609

610**Figure 2.** Photomicrographs of cross-sections of Senegalese sole testes from males
611treated with saline, CNT (A,B), GnRHa injections, GINJ (C,D), GnRHa implants,
612GIMP (E,F) and hCG injections, hCG (G,H). Photomicrographs on the left (A,C,E,G)
613are from the cortex and on the right (B,D,F,H) from the medulla region. Abbreviations:
614spg, spermatogonia; spc, spermatocyte; spd, spermatid; spz, spermatozoon. Scale bars:
615100 μ m.

616

617**Figure 3.** Gonadosomatic index (GSI) in male Senegalese sole treated with saline
618(controls, CNT), GnRHa injections (GINJ), GnRHa implants (GIMP) or hCG injections
619(hCG). Testes were collected on day 0 (n = 4) and 42 (n = 8) from the initiation of
620treatments. Different letters indicate statistical differences (ANOVA, H-S, $p < 0.05$)
621among treatments within the sampling point on day 42. Data are expressed as mean $\pm$
622SEM (n=8).

623

624**Figure 4.** Daily fecundity ($\times 1,000$ eggs kg^{-1} female biomass) in the four experimental
625tanks of cultured Senegalese sole broodstock. All females were treated with GnRHa
626implants, administered on day 0 and 21 (indicated by white arrowheads); the
627accompanying males were treated with saline (controls, CNT), GnRHa injections
628(GINJ), GnRHa implants (GIMP) or hCG injections (hCG), given the days indicated by
629black arrowheads. The quantity of buoyant (black bars) and sinking (white bars) eggs
630was determined for each spawn. No spawning was detected in any group after day 37
631from the initiation of treatments.

632

633**Figure 5.** Total fecundity ($\times 1,000$ eggs kg^{-1} female biomass) in the four experimental
634tanks of cultured Senegalese sole broodstock. All females were treated with GnRHa
635implants; the accompanying males were treated with saline (controls, CNT), GnRHa
636injections (GINJ), GnRHa implants (GIMP) or hCG injections (hCG).

637

Figure 1

Top

Figure 2

640

Figure 3

641

642

Figure 4

Figure 5