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

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Abstract

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

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

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

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

1. Introduction

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

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

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

2. Materials and Methods

2.1. Fish husbandry

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

120Sex of the fish was determined by using a heterologous VTG ELISA (Mañanós et al.,
1211994) and was further confirmed during the reproductive period by abdominal swelling in
122females and by feeling the shape of the testis in males. Fish were housed in circular
123fibreglass tanks (3000 L, 1 m depth, 4 m^2) without sand substrate, at a density of around
1243 kg m^{-2} and were exposed to the natural photoperiod and temperature regimes of the
125region. Tanks were covered with a thin shade mesh to reduce light incidence;
126maximum light intensity recorded on the water surface was 600 lux. Dissolved oxygen
127was checked regularly and ranged between 6.8 and 7.5 ppm.

128 The experiment was initiated on April 19th 2005 and terminated on May 31st 2005,
129under increasing water temperatures ranging from 15.3 °C to 21.0 °C (mean temperature
130of this period, 17.9 ± 0.3 °C). Tanks were fitted with overflow egg collectors and were
131supplied with flow-through seawater (salinity ~37‰) at a flow rate of 400% d^{-1}. Fish
132were fed to apparent satiation 5 days a week with both commercial pellets (Solea
133immunofeed, Proaqua s.a., Spain) and natural food, consisting of chopped fresh mussels
134and frozen squid.

135 Handling of fish for routine management and experimentation was always done
136according to national and institutional regulations and the current European Union
137legislation on handling experimental animals (EEC 1986). All fish to be handled were
138anesthetised by immersion in 0.3 ml^{-1} of 2-phenoxyethanol.

139

140*2.2. Experimental design and hormonal treatments*

141 On April 2nd 2005, when external signs of gonad maturation were clearly evident in the
142broodstock (Guzmán et al., 2008, 2009a), such as abdominal swelling in females and
143expressible milt in males, 56 fish were distributed homogenously in four tanks (6 females

144and 8 males per tank), at a density of 3.6 ± 0.1 kg m^{-2}. Fish were four years old, with a 145mean (±SEM) body weight (BW) of females and males of 1,266.0 ± 35.5 g and 932.8 ± 14633.7 g, respectively, and body length (BL) of 42.4 ± 0.4 cm and 39.3 ± 0.4 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 μg kg^{-1}) given on days 0 152and 21 (group GINJ); (3) GnRHa implants (40 μg 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 μg 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 ºC) and stored at -20 ºC 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 ºC). 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)
192formalin for 48–96 h at room temperature. After rinsing in running tap water (16 h) and
193dehydration in ascending concentrations of ethanol, fragments were infiltrated and
194embedded in glycol methacrylate resin (Technovit 7100, Heraeus Kulzer, Germany).
195Sections were cut at 3 μm on a Supercut 2065 microtome (Reichert-Jung, Germany) and
196stained with methylene blue / azure II / basic fucsin (Bennett et al., 1976). Stained
197sections were examined and photographed on a Leitz Diaplan light microscope.
198 Photomicrographs were taken and analyzed from the two main regions of the
199Senegalese sole testes, cortex and medulla, and the stage of testicular development
200determined according to the germ cell types and their relative abundance (García-López et
201al. 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
205were collected for evaluation of fecundity and quality. Eggs were placed in graduated
206cylinders with 37 ‰ sea water and allowed to stand for a few minutes, to separate
207buoyant (viable) and sinking (dead) eggs. The volume of each fraction was recorded
208and fecundity further expressed as nº of eggs, considering that 1 ml of eggs corresponds
209approximately to 1,000 eggs (Guzmán et al., 2009a). Daily relative fecundity was
210calculated using the BW of the females at the previous sampling, and total relative
211fecundity was calculated at the end of the study. From each spawn, a sample of 50
212buoyant eggs was examined under a binocular microscope to determine egg morphology
213(size, shape, transparency and distribution of oil droplets) and fertilization success.

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

Egg incubation and larval rearing was performed in a closed re-circulation sea water system (salinity ~37‰), filtered through 10 and 20 µm cartridge filters (Cuno Incorporated, Meriden, CT) and UV sterilized. Tanks were exposed to the natural photoperiod and temperature regimes of the region. Air diffusers were installed in the centre and perimeter of the tanks to ensure gentle and continuous aeration. Dissolved oxygen (6.7 ± 0.2 ppm), pH (7.5 – 8.2), and nitrites and ammonium water levels (< 0.5 ppm) were checked regularly. Cleaning of the tanks was carefully performed by siphoning the bottom of the tank every other day. From 2 to 7 days post hatching (dph), larvae were fed on rotifers (*Brachionus plicatilis*) (5 individuals ml^{-1} d^{-1}); during this period microalgae (*Isochrysis galbana*) ($2.5x10^5$ cells ml^{-1} d^{-1}) were added to the larval rearing tanks. From 5 to 40 dph, specimens were fed with an increasing ratio of newly hatched *Artemia salina* nauplii (1-7 individuals ml^{-1} d^{-1}), decreasing thereafter up to 70 dph. From 10 to 70 dph, specimens were co-fed with an increasing ratio of commercial dried pellets (0.5–30 g m^{-2} d^{-1}, Solea immunofeed, Proaqua, Spain). From 70 dph onward, juveniles were exclusively fed with commercial dried pellets; ratio was adjusted daily depending on remaining food observed in the bottom of the tank.

2.6. Statistics

Data are expressed as mean ± standard error of the mean (SEM). Statistical differences in plasma levels of T and 11-KT were examined using two-way Analysis of Variance (ANOVA, treatment *versus* sampling point) followed by the Holm-Sidak test, 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.

3. Results

3.1. Plasma levels of sex steroids

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

3.2. Testicular development

The testes of males at the day of initiation of the treatments were at the functional maturation stage, as expected at this time of the year (spermiation period). At the end of the experimental period (day 42), the testes from control males showed a similar maturation stage, characterized by a low number of early developing germ cells (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

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

Despite the similar treatment applied to females, total fecundity was higher in those tanks containing hormone-treated males (Fig. 5). Total fecundity increased 1.2-, 2.0- and 1.9-fold in groups GINJ, GIMP and hCG, respectively, with respect to controls (group CNT).

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

4. Discussion

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

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

The hCG treatment showed higher potency than GnRHa-based protocols on the stimulation of androgen release, as showed by highest plasma levels of 11-KT and T throughout the experimental period. The higher efficiency of the hCG treatment on androgen release might be related to the different organs targeted by hCG and GnRHa. The GnRHa acts on the pituitary of the treated fish to stimulate the secretion of the fish's own gonadotropins, which in turn induce steroidogenesis in the gonad, while the hCG acts directly on the gonad to stimulate steroidogenesis and thus, its effects are not dependent on the activation of the pituitary and/or the amount of pituitary gonadotropin stores (Miura et al., 1991; Zohar and Mylonas 2001). In the flatfish greenback flounder (*Rhombosolea tapirina*), a single hCG injection (1,000 IU kg-1) was more effective than a GnRHa injection (100 μg kg^{-1}) in increasing 11-KT and T plasma levels at 2 d post-treatment (Pankhurst and Poortenaar, 2000). On the other hand, a double hCG injection procedure (62.5 + 187.5 IU kg^{-1}) was ineffective at stimulating androgen release in male dab (*Limanda limanda*, Pleuronectiforme), but this was likely associated to the low hCG dose used (Canario and Scott 1991). A multiple hCG injection protocol has not been assayed in flatfishes, but weekly injections of 1,500 UI kg^{-1} were highly effective in stimulating 11-KT secretion in the European eel (*Anguilla anguilla*) (Peñaranda et al., 2009). Stimulation of androgen release by GnRHa treatment has been previously reported in several flatfishes, such as the winter flounder (*Pleuronectes americanus*) (Harmin et al., 1995), plaice (*Pleuronectes platessa*) (Vermeirssen et al., 1998), starry flounder (*Platichthys stellatus*) (Moon et al., 2003) and the Senegalese sole (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 leaded 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

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

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

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

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

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

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

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598

Table 1 Spawning characteristics of the four experimental groups of cultured Senegalese sole breeders. Females from all groups were treated with GnRHa implants; the accompanying males were treated with saline (controls, CNT), GnRHa injections (GINJ), GnRHa implants (GIMP) or hCG injections (hCG). Data are expressed as mean ± 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 ± 1,420	8,740 ± 229	9,390 ± 1,660	10,750 ± 2,170
Egg buoyancy (%)	23.8 ± 1.4	27.9 ± 6.5	17.6 ± 3.6	32.1 ± 4.8
Hatching success (%)	0	0	0	4.75 ± 4.75

599

Figure legends

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

Figure 2. Photomicrographs of cross-sections of Senegalese sole testes from males treated with saline, CNT (A,B), GnRHa injections, GINJ (C,D), GnRHa implants, GIMP (E,F) and hCG injections, hCG (G,H). Photomicrographs on the left (A,C,E,G) are from the cortex and on the right (B,D,F,H) from the medulla region. Abbreviations: spg, spermatogonia; spc, spermatocyte; spd, spermatid; spz, spermatozoon. Scale bars: 100 μm.

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

Figure 4. Daily fecundity (x1,000 eggs kg^{-1} female biomass) in the four experimental tanks of cultured Senegalese sole broodstock. All females were treated with GnRHa implants, administered on day 0 and 21 (indicated by white arrowheads); the accompanying males were treated with saline (controls, CNT), GnRHa injections (GINJ), GnRHa implants (GIMP) or hCG injections (hCG), given the days indicated by black arrowheads. The quantity of buoyant (black bars) and sinking (white bars) eggs was determined for each spawn. No spawning was detected in any group after day 37 from the initiation of treatments.

Figure 5. Total fecundity (x1,000 eggs kg^{-1} female biomass) in the four experimental tanks of cultured Senegalese sole broodstock. All females were treated with GnRHa implants; the accompanying males were treated with saline (controls, CNT), GnRHa injections (GINJ), GnRHa implants (GIMP) or hCG injections (hCG).

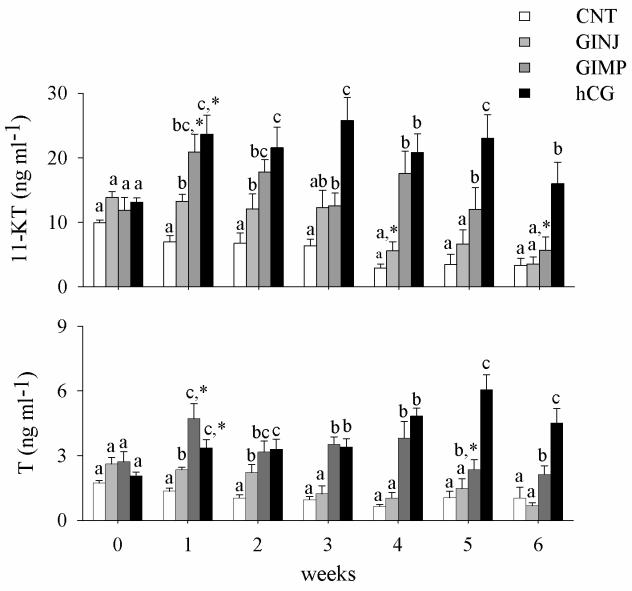

Figure 1

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Top

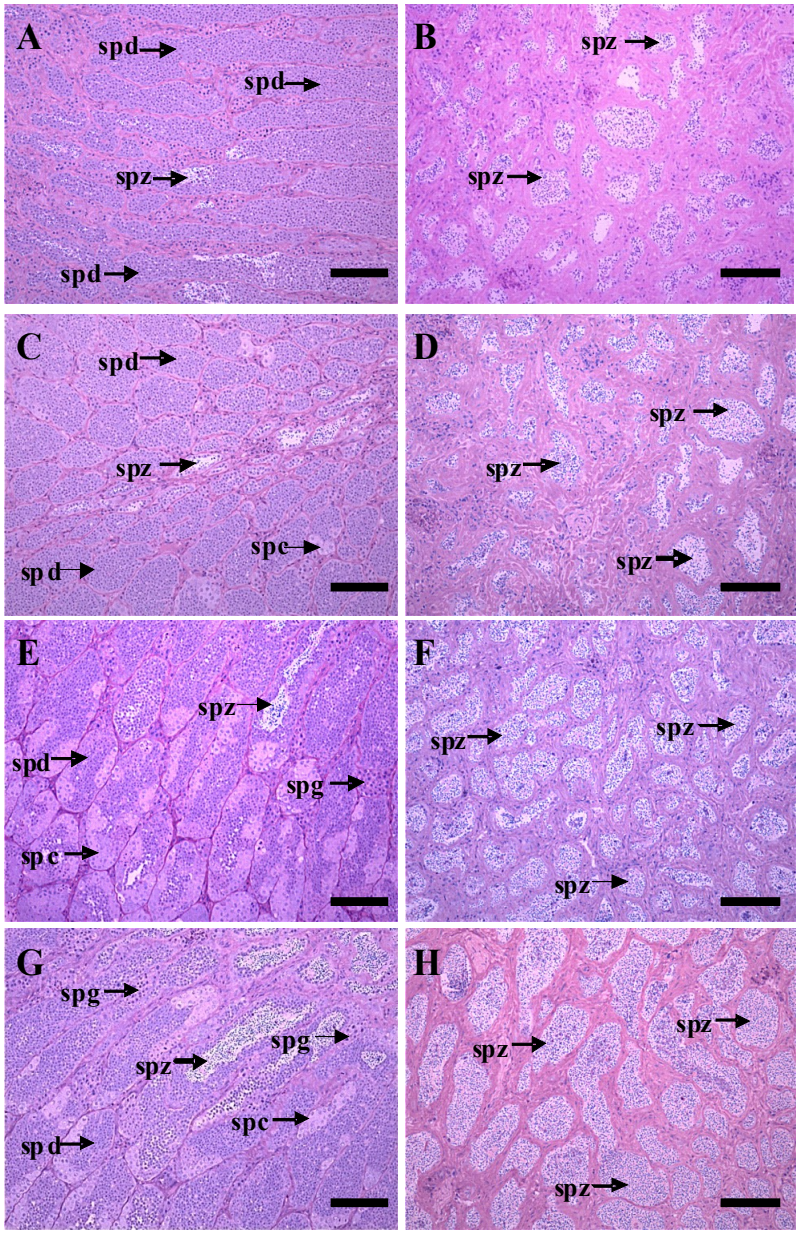

Figure 2

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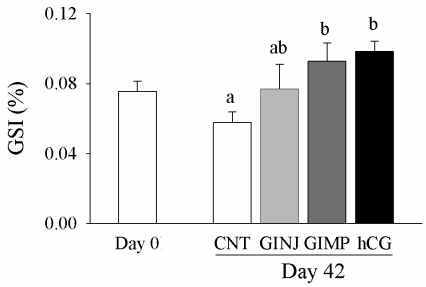

Figure 3

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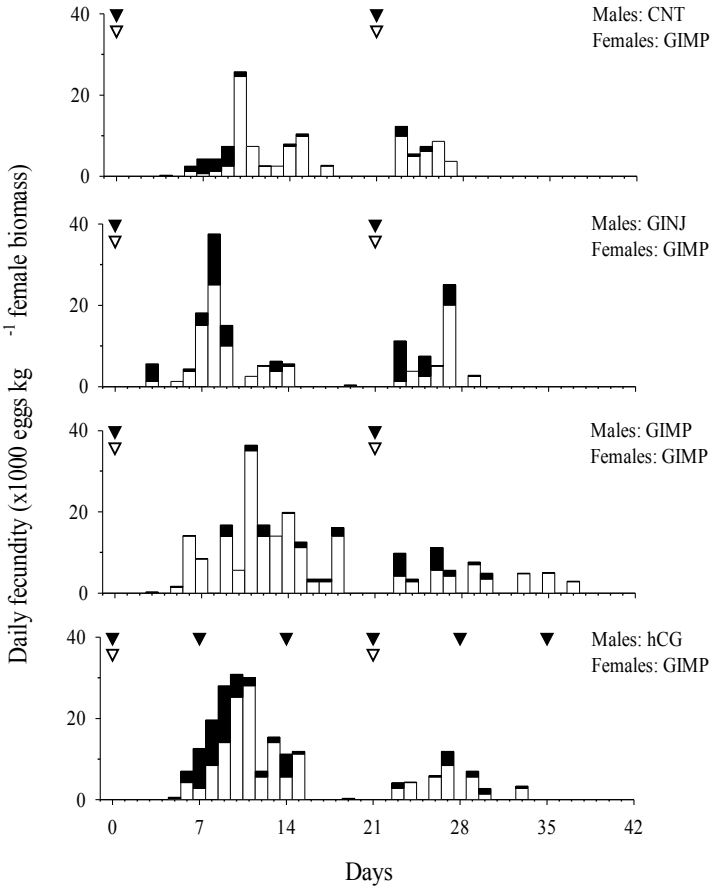
Males: CNT
Females: GIMP
Males: GINJ
Females: GIMP
Males: GIMP
Females: GIMP
Males: hCG
Females: GIMP
Daily fecundity (x1000 eggs kg -1 female biomass)
Days
0
7
14
21
28
35
42
20
40

Figure 4

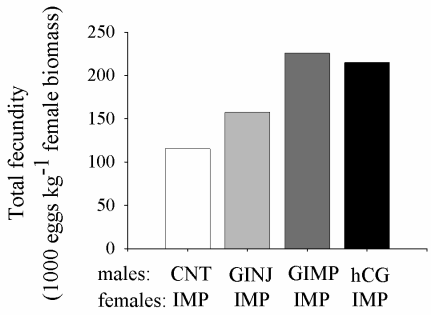
Total fecundity
(1000 eggs kg-1 female biomass)
250
200
150
100
50
0
males: CNT GINJ GIMP hCG
females: IMP IMP IMP IMP

Figure 5

644