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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

José M. Guzmán [a], Jesús Ramos [a], Constantinos C. Mylonas [b], Evaristo L. Mañanós [a,*]

[a] Institute of Aquaculture of Torre la Sal, Spanish Council for Scientific Research (CSIC), 12595-Ribera de Cabanes, Castellón, Spain
[b] Institute of Aquaculture, Hellenic Center for Marine Research, P.O. Box 2214, Iraklion 71003, Crete, Greece

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ABSTRACT

The aquaculture of Senegalese sole (*Solea senegalensis*) is limited by the failure of cultured breeders (F1 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.

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 (F1 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).

Failure to undergo oocyte maturation and ovulation in females and reduced sperm production in males are common reproductive dysfunctions in fish maintained under captive conditions, either wild or cultured (Donaldson and Hunter, 1983; Mañanós et al., 2008; Zohar and Mylonas, 2001). In aquaculture, the most common strategy to stimulate gonad maturation, ovulation and spermiation in fish is the exogenous treatment with gonadotropin-releasing hormone agonists (GnRHa), either in the form of liquid injections or sustained-release delivery systems (Mylonas and Zohar, 2001; Mylonas et al., 2009; Zohar and Mylonas, 2001). The GnRHa based hormonal

* Corresponding author. Tel.: +34 964 319500; fax: +34 964 319509.
E-mail address: evaristo@iats.csic.es (E.L. Mañanós).

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treatments have been successfully used in several flatfish species to induce spawning and spermiation, normally with highest efficiency exhibited by treatments using GnRHa slow-release delivery implants (Larsson et al., 1997; Moon et al., 2003; Mugnier et al., 2000; Vermeirssen et al., 2000). Although less widely used than GnRHa, another effective hormonal therapy is the treatment with human chorionic gonadotropin (hCG), usually administered through saline-diluted multiple injections. The hCG acts on the gonad and has been shown to stimulate spermatogenesis and sperm production in several fish species (Cacot et al., 2003; Miura et al., 1991, 2002; Schiavone et al., 2006). Interestingly, some studies have reported effects of hCG on breeding behavior. In male mutton snapper (*Lutjanus analis*) a single hCG injection stimulated spermiation and induced high fertilization rates (Watanabe et al., 1998a). In Japanese eel (*Anguilla japonica*) weekly hCG injections stimulated active courtship behavior before spermiation (Dou et al., 2007).

Several attempts have been undertaken to stimulate reproduction of cultured Senegalese sole broodstock through hormonal treatments, limited to the use of GnRHa-based 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 behavior 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). 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 fiberglass tanks (3000 L, 1 m depth, 4 m^2) 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 lx. 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 anesthetised 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 homogenously in four tanks (6 females and 8 males per tank), at a density of 3.6 ± 0.1 kg m^{-2}. Fish were four years old, with a mean (±SEM) body weight (BW) of females and males of 1266.0 ± 35.5 g and 932.8 ± 33.7 g, respectively, and body length (BL) of 42.4 ± 0.4 cm and 39.3 ± 0.4 cm, respectively.

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

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

Females were only manipulated for GnRHa implant administration (days 0 and 21) and were not sampled throughout the experimental period. Males were sampled for blood at weekly 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 (3000 *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 × BW^{-1} × 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 (3000 *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 (Rodriguez 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