Artificial fertilization of Senegalese sole (*Solea senegalensis*): hormone therapy administration methods, timing of ovulation and viability of eggs retained in the ovarian cavity

I. Rasines^a, M. Gómez^a, I. Martín^a, C. Rodríguez^a, E. Mañanós^b, O. Chereguini^a

^a Spanish Oceanographic Institute, Santander Oceanographic Centre, Promontorio de San Martín, s / n. PO 240. 39080 Santander, Spain. ^b Research Council for Scientific Research (CSIC), Institute of Aquaculture of Torre la Sal (IATS-CSIC), 12595-Ribera de Cabanes, Castellón

Corresponding author: Inmaculada Rasines - Spanish Oceanographic Institute, Marine Aquaculture Plant "El Bocal" Barrio de Corbanera s / n, CP 39012 Santander, Spain. E-mail: inma.rasines@st.ieo.es Tel: +34942348397; fax: +34942323486

Abstract

One method to overcome the difficulties involved in reproducing first generation Senegalese sole that are born in captivity (F1) is to obtain gametes by stripping and then using them for artificial fertilization. The aim of this paper was to determine an appropriate protocol for hormonal induction of ovulation and artificial fertilization. To induce ovulation, two different GnRHa administration methods, weekly injections versus a single GnRHa implant, were compared. The number of spawns per female and the relative fecundity were significantly higher in females that received the hormonal therapy through repeated injection (6.3 ± 0.8 spawns per female and $574.9 \pm 67.2 \times 10^3$ eggs kg^{-1} BW) than those treated with a single implant (2.7 ± 0.6 spawns per female and $134.6 \pm 40.9 \times 10^3$ eggs kg^{-1} BW). The lapsed time to ovulation and the changes in fertilization and hatching rates of eggs that were retained in the ovarian cavity after ovulation were determined after GnRHa treatment. At 16°C, ovulation was detected at 41-44 h post-treatment in 70% of the females. The highest fertilization and hatching rates were obtained during the first 3 h after ovulation, and

they significantly decreased thereafter. The results suggested that the GnRHa injection was the most appropriate administration method for the induction of ovulation. Ovulated eggs inside the female were viable for a short time, and therefore it is important to determine the time of ovulation after hormonal treatment.

Keywords: *Solea senegalensis*, hormonal induction, artificial fertilization, egg quality

1. Introduction

Currently, marine fish aquaculture in Spain is dominated by three species: sea bass, *Dicentrarchus labrax*, gilthead seabream, *Sparus aurata*, and turbot, *Psetta maxima*. However, there is an increasing demand for new species to diversify the number of cultivated fish available on the market. The Senegalese sole, *Solea senegalensis*, is one of the leading candidates for the Mediterranean aquaculture industry due to the availability of appropriate zootechniques for all steps of the biological cycle (Imsland et al., 2003) and the high market price of this species; the Senegalese sole have the highest market price of all farmed fish sold in Spain (APROMAR, 2010).

One of the problems involved in the integral culture of Senegalese sole is the reproductive dysfunctions detected in animals born and reared in captivity (generation F1). Spontaneous spawns are scarce and primarily not fertilized; F2 larvae have only been obtained in a very few number of cases (Calvo et al., 2005). The reasons underlying the reproductive problems of F1 soles are still unknown. It has been suggested that these problems could be related to environmental factors and/or management in early larval stages, the lack of courtship of F1 broodstock and/or incorrect alimentation (Howell et al., 2009).

Captive breeding of many teleost species, especially flatfish, is based on the artificial fertilization of eggs that are obtained by stripping. Mañanós et al. (2009) reviewed several flatfish species, some consolidated in

aquaculture, for which this method is used (southern flounder *Paralichthys lethostigma*, summer flounder *Paralichthys dentatus*, Atlantic halibut *Hippoglossus hippoglossus*, starry flounder *Platichthys stellatus*, yellowtail flounder *Limanda ferruginea* and turbot *Psetta maxima*). The viability of gametes obtained from F1 Senegalese sole breeders has been previously demonstrated (Chereguini et al., 2007). Eggs obtained by stripping, after hormonal induction of ovulation, were successfully fertilized artificially with sperm from both wild (Chereguini et al., 2007) and F1 (author's observations) males. Therefore, a possible way of dealing with the lack of F2 Senegalese sole larvae for the industry could be to establish a suitable method for hormonal induction of ovulation and artificial fertilization for this species. At the fourth Working Group on sole culture held in Faro (Portugal), important advances in artificial fertilization techniques that have been made in China were presented. It was stressed that from a business perspective, it should be a priority to assess stripping techniques for obtaining eggs (Howell et al., 2009).

The success of hormonal therapies to induce ovulation depends highly on the type of hormone, the dose, the administration method and the maturational stage of the female. Hormonal therapies based on treatment with a synthetic agonist of gonadotropin-releasing hormone (GnRHa) are used to control the reproductive process of many species (Mañanós et al., 2009). The GnRHa can be administered by either injection or sustained-release delivery systems. An injection of saline-diluted GnRHa causes an acute release of GnRHa into the bloodstream and thus highly detectable GnRHa plasma levels for the first few hours after injection. In contrast, sustained-release delivery systems, such as implants, cause a slow release of GnRHa, and thus high plasma levels of GnRHa are maintained for a long period of time, i.e., several weeks, which may allow consecutive spawns to be obtained (Mylonas and Zohar, 2007). In Senegalese sole, GnRHa treatment has been shown to be effective for the induction of spawning, both when administered using implants (Agulleiro et al., 2006, Mañanós et al., 2007, Guzmán et al., 2009) or by repeated injections (Agulleiro et al., 2006).

A key point for the success of artificial fertilization methods is the quality (viability) of the eggs that are obtained by stripping, which is highly dependent on the time elapsed between ovulation and egg collection. Collecting eggs (stripping) later than the appropriate time window after ovulation can lead to low fertility rates (Flajshans et al., 2007), high mortality rates of embryos and larvae and a high incidence of embryo deformities (Legendre et al., 2000, Aegerter and Jalabert, 2004) due to the loss of egg viability from overripening. It is therefore important to accurately predict the time of ovulation for each species after hormonal treatment (Mylonas et al., 2010) and to determine the time that the eggs can remain inside the female after ovulation with no significant reduction of egg quality (Bromage et al., 1994).

The aim of this study was to establish a protocol for the hormonal induction of ovulation and the artificial fertilization of Senegalese sole. We sought to address several questions: 1) what is the most appropriate administration method for hormonal induction of ovulation that provides the highest quality and quantity of eggs for artificial fertilization, 2) what is the amount time to ovulation after hormonal treatment, and 3) how long do the eggs that are retained in the ovarian cavity remain viable?

2. Material and methods

2.1. Experimental animals

The F1 broodstock of the Spanish Oceanographic Institute in Santander (northern Spain) was kept indoors in six tanks that were 4 to 14 m² and 1 m deep. The tanks had an open flow circuit (water renewal 33% tank hour⁻¹) and constant aeration. The density ranged between 2.6 and 5.8 Kg m⁻², and the sex ratio was 2-1♂:1♀. The photoperiod was artificial (16L: 8D) for the whole year. The light intensity was reduced by placing mesh shading over the tanks, which allowed a maximum light intensity of 50 lux to reach the water surface. The fish were fed 1.5% of the total biomass 6 days a week using commercially extruded feed (Vitalis Cal,

Skretting). The fish were tagged with passive integrated transponder tags (Pit-tags, AVID) on the dorsal musculature. Gonadal maturation of the broodstock was stimulated by manipulation of the thermoperiod. Fish were maintained under natural temperatures until the end of January, when the ambient temperature is approximately 13°C. At this time, the temperature was manipulated artificially to increase by 0.5°C every week. After approximately 6 weeks, when the temperature reached 16°C, the temperature was set-up to increase and decrease by 2°C every four and three days, respectively. This temperature fluctuation was selected to mimic the natural fluctuations recorded in the South Atlantic coast of Spain (IFAPA “el Toruño”, Cadiz, Spain), where these temperature fluctuations have been suggested as a major factor for the stimulation of natural spawning in wild Senegalese sole broodstocks (Anguis and Cañavate, 2005).

The fish were weighed and measured (total length) once a month, and the gonad stage was estimated by external examination of abdominal swelling according to the classification of Anguis and Cañavate (2005). From late March, females in stage III of ovarian development were selected for hormonal induction and artificial fertilization experiments.

The fish were always handled (routine management and experimentation) according to the European Union Directive (EEC, 1986) for the protection of Animals Used for Experimental and Other Scientific Purposes.

2.2. Hormone administration methods

Sixteen F1 females (1498 ± 131 g body weight, BW) were divided into three experimental groups that were placed in three 4 m² tanks with males (male:female ratio 1:1). The culture conditions (food, water, air, light and temperature fluctuations) were maintained as described above. The groups were: control (C, n = 4) consisting of the females that received an intramuscular injection of saline (0.15 ml kg⁻¹ BW); injection (IN, n = 6) consisting of females that were injected once a week with a dose of 25 µg kg⁻¹ BW of GnRHa ((des-

Gly¹⁰, D-Ala⁶)-LH-RH-ethyl-amide (Sigma)) dissolved in saline for four weeks; and implant (IM, n = 6) consisting of females that were treated with a single GnRHa implant at a dose of 50 µg kg⁻¹ BW. The GnRHa implants were 2-mm x 3-mm cylinders of a copolymer of ethylene-vinyl acetate (EVAc, Elvax, DuPont Chemical CO., USA) loaded with a GnRHa agonist (Mylonas and Zohar, 2001, Mylonas et al., 2007). The implants were inserted into the dorsal musculature of the fish using a pit tag syringe. For manipulation, the females were anesthetized with clove oil (40 ppm) for one minute.

Females were examined daily until the first ovulation was detected, and eggs were obtained by stripping. After that, the females continued to receive abdominal massages every two to three days depending on the degree of external abdominal swelling. The eggs were collected into a dry bowl, avoiding contamination by feces, urine and seawater. We recorded the number of spawns per female and the volume of eggs produced (ml). To determine the number of eggs ml⁻¹, 0.5 ml aliquots (triplicates) were taken from each batch of eggs using a 1 ml micropipette and were placed on a Boronov counting plate with sea water; the eggs were then counted under the binocular. Relative fecundity was calculated as the number of eggs kg⁻¹ BW.

For each spawn, the egg diameter of a sample of viable eggs (eggs perfectly spherical, translucent, without a perivitelline space and lipid droplets evenly spread throughout the egg) was determined. To measure egg diameter, a sample of 100 eggs from each spawn was placed on a watch glass with sea water and was photographed. Then, the diameter of 30 viable eggs in the image was measured using an image analyzer (Analysis ®. GmbH, 2001, Münster, Germany).

To assess egg quality, fertilization and hatching rates were calculated after artificial fertilization (AF) with cryopreserved sperm. The collection, evaluation and cryopreservation of the sperm and the AF were performed with slight modifications, as described for turbot by Chereguini et al. (2003). The sperm was obtained by gentle abdominal pressure of the testis. Prior to obtaining the sperm, the area around the genital

papilla was washed with distilled water and dried with absorbent paper. The sperm of each male was collected in a 1 ml syringe and kept on ice, at approximately 0°C, until use. To determine sperm motility, the sperm sample was diluted 1:5 in 200 mOsm kg⁻¹ immobilizing Ringer solution and then activated by the addition of sea water to a final dilution of 1:20 (Chereguini et al. 2003). For AF experiments, cryopreserved sperm was used because of its higher availability and constant quality. Taking into account that the volume of milt obtained from Senegalese sole males is very limited (Cabrita et al., 2006, Chereguini et al., 2006), for the AF experiments, pooled sperm from 3-5 males with good motility (classified as 3-5 according to the scale of Sánchez-Rodríguez 1975, cited in Chereguini et al., 2003) was used. Straws of 0.5 ml (IMV, L'Aigle, France) were filled with 50 µl of semen and 100 µl of Mounib solution modified according to Legendre & Billard (cited in Chereguini et al. 2003), which contained DMSO and BSA as cryoprotectants. The sperm mix was frozen on liquid nitrogen vapor by placing the straws over a 5 cm height catamaran-shaped polystyrene device that was floating in liquid nitrogen. Straws were placed horizontally over the floater for 7 min and then plunged directly into the liquid nitrogen. Finally, the straws were stored in a liquid nitrogen container (Air Liquide GT 21) until use for AF. The straws for each pool were identified by color bar codes. The sperm were thawed by immersing the straws in a 40°C water bath for 7 sec. For AF, triplicates of 1 ml of eggs from each spawn were placed in a 30 ml plastic beaker, and 30 µl of cryopreserved sperm and 1 ml of seawater (16°C and 35 ppt salinity) were added successively for sperm activation and fertilization. After 3 min, 25 ml of seawater was added to the beaker. One straw was used to fertilize the triplicates of each spawn. After 105 min of incubation, the eggs were gently mixed, and the samples were examined under a binocular to determine fertilization rates (number of eggs with blastomeres/total number of eggs). The eggs were then placed in 1 L polyester incubators (conical bottom) in a filtered (1 µm Cuno filters) open flow-through sea water system. After two days of incubation at 19°C, hatching rates were determined in each incubator as the number of larvae / total number of eggs.

2.3. Timing of ovulation and viability of eggs retained in the ovarian cavity

Fifteen F1 females (1278 ± 120 g BW) were injected with $25 \mu\text{g kg}^{-1}$ BW of GnRHa at 12 a.m. The water temperature of the tank was kept at 16°C from the day before hormonal treatment until the end of the experiment. The fish were checked for ovulation every 3 h, beginning 32 h after injection, by applying soft abdominal pressure. When ovulation was detected (time 0 h post-ovulation, hpo), a small portion of the ovulated eggs was gently stripped from the ovarian cavity. Abdominal pressure was then applied at 3, 6, 9 and 12 hpo. For each female, each batch of eggs was immediately fertilized using the same pool of cryopreserved sperm, to avoid sperm variability. Fertilization and hatching rates were determined for each AF trial.

2.4. Protocol of hormonal induction of ovulation and stripping for artificial fertilization

The results of the experiments described in sections 2.2 and 2.3 allowed for the selection of optimized conditions for the hormonal induction of ovulation and for the timing of egg collection (stripping) after ovulation. The optimized protocol was applied to F1 females in stage III at 16°C . Females were successfully induced to ovulate, and the buoyancy of the eggs was assessed. Egg buoyancy ((ml of floating eggs/total ml of eggs) $\times 100$) was determined by placing 20 ml of eggs in a 100 ml graduated cylinder filled with seawater (35 ppt salinity and 16°C temperature) and measuring the volume of floating and sinking eggs. AF was then performed, and the fertilization and hatching rates were determined.

2.5. Statistical analysis

All values are expressed as the mean $\pm$ standard error of the mean (S.E.M.). Data normality and homogeneity of variance were analyzed using the Kolmogorov-Smirnov and Levene methods, respectively. Differences were examined using ANOVA or the equivalent nonparametric Kruskal-Wallis and Mann-Whitney tests with

significance levels of $p < 0.05$. The possible relationships between the parameters measured were analyzed using a Pearson correlation or the Spearman test.

3. Results

3.1 Hormone administration method

The number of spawns per female and the relative fecundity was significantly higher in the females that were treated with repeated injection compared to the implanted or control females (Table 1). In the experiment, a total of 5.1 million eggs were obtained from the females of the IN group, 1.3 million eggs from the IM group and 0.3 million eggs from the C group. Only one female died during the experiment 13 days after receiving the implant. In all cases, the response occurred two days after the hormonal treatment (Figures 1 and 3).

In the IN group, eggs were obtained after the first two injections from all females, after the third injection from five females, and after the fourth injection only from females 3 and 5 (Figure 1). During the experimental period, an average of 6.3 spawns per female were obtained (Table 1). The average relative fecundity decreased significantly after the first three injections (Figure 2).

For the implanted females, the largest amount of eggs was obtained after two days of treatment, and only females 10, 11 and 12 were able to produce small quantities of eggs over the 30 day experimental period (Figure 3). During the whole period, an average of 2.7 spawns per female was obtained (Table 1). There was no difference in relative fecundity between the IN and IMP groups (223.0 ± 57.8 and 137.6 ± 40.9 eggs kg^{-1} , respectively) when the first two week period was considered, which corresponded to administration of the two first injections (IN group) or the implant (IMP group).

A female in the control group produced a spawn of 132000 eggs kg^{-1} and two days later another small amount of overripe eggs. Another control female also produced 11400 eggs kg^{-1} .

AF was carried out for 30 spawns: 24 from the injected females, 5 from the implanted females and 1 from a control female. The overall fertilization and hatching rates were $16.6 \pm 2.5\%$ and $3.9 \pm 1.6\%$, respectively.

Because the number of spawns from the two groups subjected to the different GnRH α administration methods was different, fertilization and hatching rates were only compared from those spawns that were obtained two days after the first treatment (implantation and the first injection). The fertilization and hatching rates were low in both cases ($13.6 \pm 2.8\%$ and $0.09 \pm 0.0\%$ for the IM group, $15.2 \pm 2.9\%$ and $5.5 \pm 2.0\%$ for the IN group), and there were no statistically significant differences between them.

The number of eggs ml^{-1} and the diameter of eggs that were obtained by hormonal induction were 1493 ± 26 and 1056.4 ± 38.7 mm, respectively. No significant differences were found for these parameters between the IN and IMP groups. However, the weight of the female was negatively correlated with the number of eggs ml^{-1} (Spearman Rho = -0.449, $p = 0.000$) and was positively correlated with egg diameter (Spearman Rho = 0.513, $p = 0.000$).

3.2. Timing of ovulation and viability of eggs retained in the ovarian cavity.

The GnRH α injection induced ovulation in ten females, which was detected at 41-44 h after treatment in 70% of the females, with a total range of 38 to 50 h depending on the female. Fertilization rates were high at both the time of ovulation (0 hpo) and at 3 hpo ($32.0 \pm 4.1\%$). It then decreased significantly from 6 hpo onwards (Figure 4). In most of the AF trials, irregular and/or asynchronous egg divisions were observed after 6 hpo.

The hatching rates were similar at 0 and 3 hpo (13.1 ± 2.7 and $19.9 \pm 4.1\%$, respectively) and significantly higher than those obtained at other times post-ovulation (Figure 4).

3.3. Protocol of hormonal induction to ovulation and stripping for artificial fertilization

The optimized protocol was established as: hormonal induction of females at maturation stage III by injection of GnRHa at a dose of $25 \mu\text{g kg}^{-1}$ BW at 12 a. m., with a water temperature of 16°C and egg collection carried out by stripping 44 h after treatment. Using this protocol, 19 spawns were obtained. Fertilization and hatching rates were 36.7 ± 4.5 and 18.5 ± 3.6 , respectively. It is worth noting that AF trials performed with eggs that showed 0% floatability produced fertilization and hatching rates ranging from 6.1 to 44.4% and 0 to 31.6%, respectively.

4. Discussion

This study showed that a repeated GnRHa injection protocol was more effective than the single GnRHa implant method for stimulating egg production by stripping without compromising egg quality. The use of hormonal treatments to induce ovulation and to allow egg collection by stripping is a common practice in the artificial fertilization protocols that are used for several fish species (Mañanós et al., 2009). Hormonal therapies based on the administration of GnRHa through sustained release implants or injections are highly effective for inducing ovulation and spermiation and, thus, helpful for solving reproductive dysfunctions that have been described for most cultured fishes. Regarding Senegalese sole, both GnRHa implants and repeated injections have been shown to induce tank spawning in F1 broodstocks (Agulleiro et al., 2006, Mañanós et al., 2007, Guzmán et al., 2009). Additionally, GnRHa injections have been shown to induce egg production in F1 females after stripping (Chereguini et al., 2007). Weekly GnRHa injections allowed egg collection by stripping in response to at least three consecutive injections; the number of stripped eggs decreased greatly

after the fourth injection, which is in accordance with previous results of Senegalese sole (Agulleiro et al., 2006) and European sea bass *Dicentrarchus labrax* (Mylonas et al., 2003). In the present work, treatment with GnRHa implants induced ovulation in all females but only three females (7, 10 and 12) produced large spawns. In black sea bass, the relative fecundity that was obtained by stripping was similar in females that received a single treatment of GnRHa implant or a single injection (Berlinsky et al., 2005). Similarly, in European sea bass, the relative fecundity that was obtained by a single injection or implant was similar (Forniés et al., 2001), whereas multiple injections produced the highest relative fecundities (Mylonas et al., 2003). In turbot and yellowtail flounder, GnRHa implantation and subsequent stripping was shown to induce multiple spawns and, thus, it was considered an effective method for concentrating and synchronizing spawns and reducing the interval between spawns (Larsson et al., 1997, Mugnier et al., 2000). Similarly, consecutive spawning was obtained for grouper and striped trumpeter after GnRHa implantations (Morehead et al., 1998, Marino et al., 2003). When the same total dose of hormone was provided (two injections or one implant), the relative fecundity was similar. Therefore, a second implant or an implant of higher dose hypothetically would improve relative fecundity; however, then the advantages of using implants could be lost (implants reduce handling, avoid adverse effects on the fish's health and require less work (Mugnier et al., 2000, Marino et al., 2003, Mylonas and Zohar, 2007)) or a poor egg quality could be obtained. This aspect remains to be investigated.

The relative fecundity that was obtained in the present study was different than that obtained by Agulleiro et al. (2006) and Guzmán et al. (2009). The differences could be due to the technique that was used for obtaining the eggs (tank spawning versus stripping), different doses and/or stages of maturation (Agulleiro et al. 2006) or different female sizes (Guzmán et al. 2009). The kinetics of egg production were also different; spawns were obtained at 3-4 days after treatment in Agulleiro et al. (2006) and at 4-5 days in Guzmán et al. (2009), whereas here, the eggs were obtained by stripping after 2 days of hormone treatment. This longer time to

respond to the hormone treatment may be because F1 sole females delay the spontaneously release of ovulated eggs.

No differences were found between the implanted and injected females in terms of egg diameter, fertilization and hatching rates. These results are consistent with those obtained for the European sea bass and black sea bass (Forniés et al., 2001, Berlinsky et al., 2005).

The time between ovulation and stripping is very important for successful artificial fertilization because the eggs retained inside the female deteriorate and become overripe. Thus, the possibility of obtaining viable embryos after artificial fertilization is highly reduced. Therefore, for each species, an accurate prediction of the ovulation after hormonal treatment is necessary so that stripping is performed when the eggs are still viable (Mylonas et al., 2010). The time between hormonal induction and ovulation depends on the species, the maturational stage of the treated female (Takushima et al., 2003), the type of hormone (Drori et al., 1994), the dose (Sahoo et al., 2008), the temperature (Fortuny et al., 1988, Drori et al., 1994, Arabaci et al., 2001, Phelps et al., 2007) and time of the day when treatment is administered (Ohta et al., 1996). As shown in the present study, female Senegalese sole at stage III of maturation that were treated with a single GnRHa injection of 25 $\mu\text{g kg}^{-1}$ BW at 16°C were induced to ovulate in 41-44 h, ranging between 38 and 50 h. The variability in the ovulation time could be caused by slight differences in the stage of gonadal maturation among the females. The stage III of maturation was defined according to the scale proposed by Anguis and Cañavate (2005). This method for determining the state of maturity is based on visual examination of the external appearance of the females. This is a quick and easy method to perform in fish facilities and useful for purposes of management to follow the evolution of gonadal maturation of the broodstock and to predict the spawning period. However, this method may be not accurate enough for the precise selection of F1 females to be used for hormonal induction and artificial fertilization trials. This variability in the ovulation time may also be intrinsic to the

species. Phelps et al. (2007) have cited a number of species for which the time between hormonal induction and ovulation is predictable, whereas for other species it is not.

The period of time that the eggs remain viable inside the female after ovulation varies highly between species, for example, 4-6 days in *Salmo gairdneri* (Springate et al., 1984), 2 h in channel catfish, *Ictalurus punctatus* (Phelps et al., 2007) and 1 h in the hybrid sunshine bass (Mylonas et al., 1996). This "overripening" of eggs may be due to hypoxia (Stevens, 1966 quoted in Springate et al., 1984), changes in membrane permeability (Hirose et al., 1977) or a decrease in ATP stores that affects embryonic development (Boulckbache et al., 1989, cited in Legendre and Otémé, 1995). In the present study, at 16 °C, the viability of the eggs was maintained for at least 3 h after ovulation, with a clear reduction in fertilization and hatching rates 6 h after ovulation. This relatively short period of time has also been found for species such as channel catfish and sunshine bass, as mentioned above, and other species like the African catfish *Heterobranchus longifilis* (Legendre and Otémé, 1995), sábalo *Prochilodus platensis* (Fortuny et al., 1988) and the Asian catfish *Pangasius hypophthalmus* (Legendre et al., 2000). The temperature at which females are kept during the post-ovulatory period is an important factor that determines the duration of egg viability (Van der Kraak and Pankhurst, 1996, Phelps et al., 2007). The duration of egg viability generally increases at lower temperatures (Woynarovich and Horvath, 1980 quoted in Phelps et al., 2007). The present study was performed at 16°C, which is the reference temperature for the thermal fluctuations that are used to stimulate gonad maturation and spawning in Senegalese sole broodstock (Anguis and Cañavate, 2005). However, spontaneous spawning of Senegalese sole broodstock has been detected at water temperatures of 13°C (Anguis and Cañavate, 2005). It would be interesting to investigate the effect of hormonal induction at lower temperatures (<16°C) for Senegalese sole because this could increase the period of egg viability inside the female and consequently could increase the time window in which stripping can be performed for the purpose of artificial fertilizations. Hormonal induction of ovulation at low temperatures may also extend the lag time between hormonal injection and ovulation. Six hours after ovulation, the hatching rate declined more dramatically than the

fertilization rate, although irregular and/or asynchronous divisions were observed in many of the fertilized eggs. It has also been observed that in *Inimicus japonicus*, *Anguilla japonica* and *Clarias batrachus*, hatchability was affected more than the fertilization (Ohta et al., 1996, Takushima et al., 2003, Sahoo et al., 2008). Other studies on eggs from marine species have shown that a good fertilization rate lasts longer than the normal developmental capacity of the embryos, and thus the fertilization rate only is not sufficient to show the embryo development success (Kjørsvik et al., 1990).

Previous studies of Senegalese sole have shown that spawned eggs from F1 breeders are in most cases not fertilized. Mañanós et al. (2007) suggested that this problem was primarily related to a critical reproductive dysfunction of the F1 males. This suggestion was based on results that were obtained from studies over mixed sole broodstock, which showed that viable fertilized spawning could be obtained from populations that consisted of F1 females and wild males but not from those populations with the opposite composition (F1 males with wild females). However, it can also be considered that the lack of fertilized spawning from F1 breeders is caused by a delayed release of eggs after ovulation. The present study showed that the overripening period of Senegalese sole is short (3-6 h), and thus it would support the idea that an egg fertilization problem in F1 sole broodstock tanks could be associated with the release of overripe eggs from the F1 females due to a delayed egg release after ovulation. This possibility has already been suggested for Senegalese sole (Guzmán et al., 2008), as it has been for another flatfish, the greenback flounder *Rhombosolea tapirina* (Pankhurst and Fitzgibbon, 2006).

A key issue regarding artificial fertilization is the availability of a method to determine the quality of the stripped eggs, which should be quick and easy to perform. The manipulator should be able to predict if the eggs can be fertilized and develop normal embryos. This is a critical point for Senegalese sole, considering the limited amount of sperm that can be obtained by abdominal massage and the importance of using this valuable sperm only for high quality egg batches. Various parameters, such as buoyancy, size, transparency, the

distribution of lipid droplets, and the pH of the ovarian fluid, have been used to estimate egg quality before fertilization (Lahnsteiner et al., 2009). In the present study, egg buoyancy was not a good indicator of egg quality, as the parameters were not correlated. The parameter buoyancy is often used in marine fish hatcheries to determine the quality of spawning because good quality eggs of pelagic species float better than poor quality eggs. However, there are a number of species, such as halibut, for which egg buoyancy has not been demonstrated to be related to egg quality (Bromage et al., 1994). Similarly, it was observed in the present study that stripped eggs exhibiting 0% floatability produced high fertilization and hatching rates after artificial fertilization, which suggests that egg buoyancy may not be a good indicator of egg quality in Senegalese sole.

Artificial fertilization carried out with eggs that were obtained according to the established protocol (induced by injection of GnRHa at 12 a.m. at doses of 25 mg kg⁻¹ BW and a temperature of 16°C; stripping was performed at 44 hours after induction) resulted in average fertilization and hatching rates of 36.7 and 18.5, respectively, which are higher than those obtained in the experiment of the hormone therapy administration method. In addition, the fertilization and hatching rates that were achieved through artificial fertilization were similar to those obtained for wild IEO broodstock if we consider the total volume of eggs produced (33% and 25%, respectively, for the period 2007-2011; unpublished data).

In conclusion, the artificial fertilization of eggs that are obtained by stripping after the hormonal induction of ovulation may be an appropriate method for obtaining second-generation larvae of farmed Senegalese sole. More research is needed to optimize the induction and artificial insemination protocols, especially the sperm:egg ratio, considering that this species is oligospermic.

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Bibliografía

- Aegerter, S., Jalabert, B., 2004. Effects of post-ovulatory oocyte ageing and temperature on egg quality and on the occurrence of triploid fry in rainbow trout, *Oncorhynchus mykiss*. *Aquaculture* 231, 59-71.
- Agulleiro, M. J., Anguis, V., Cañavate, J.P., Martínez-Rodríguez, G., Mylonas, C.C., Cerdà, J., 2006. Induction of spawning of captive-reared Senegal sole (*Solea senegalensis*) using different administration methods for gonadotropin-releasing hormone agonist. *Aquaculture* 257, 511-524.
- Anguis, V., Cañavate, J.P., 2005. Spawning of captive Senegal sole (*Solea senegalensis*) under a naturally fluctuating temperature regime. *Aquaculture* 243, 133-145.
- APROMAR, 2010. La acuicultura marina de peces en España 2009. Asociación Empresarial de Productores de Cultivos Marinos, Chiclana (Cádiz), Spain.
- Arabaci, M., Cagiran, H., Sari, M., 2001. Induction of spawning in common carp (*Cyprinus carpio*) using LHRHa ([D-Ser (tBu)₆, Pro⁹-Net]-LHRH) combined with haloperidol: effects of different treatment time and determination of latency period dependence on temperature. *Turkish J. Fish. Aquat. Sci.* 1, 1-5.
- Berlinsky, D.L., King, V. W., Smith, T.I.J., 2005. The use of luteinizing hormone releasing hormone analogue for ovulation induction in black sea bass (*Centropristis striata*). *Aquaculture* 250, 813-822.
- Bromage, N., Bruce, M., Basavaraja, N., Rana, K., Shields, R., Young, C., Dye, J., Smith, P., Gillespie, M., Gamble, J., 1994. Egg quality determinants in finfish: the role of overripening with special reference to the timing of stripping in the Atlantic halibut *Hippoglossus hippoglossus*. *J. World Aquacult. Soc.* 25, 13-21.
- Cabrita, E., Soares, F., Dinis, M.T., 2006. Characterization of Senegalese sole, *Solea senegalensis*, male broodstock in terms of sperm production and quality. *Aquaculture* 261, 967-975.

- Calvo, A., Gil, J., Moure, M., Álvarez, C.M., Cañavate, J.P., Anguís, V., 2005. Primeros resultados de puestas naturales obtenidas en reproductores de primera generación (F1) de lenguado senegalés (*Solea senegalensis*). Proceedings of X Congreso Nacional de Acuicultura. Universidad Politécnica de Valencia, Gandía, Spain, pp. 506-507.
- Chereguini, O., Anguis, V., Peleteiro, J. B., Guzmán, J.M., 2006. Sperm quality in Senegal sole *Solea senegalensis* (Kaup 1958): motility, density and response to hormonal treatments. Proceedings Aqua 2006, Florence, Italy, May 2006. World Aquaculture Society and European Aquaculture Society, p. 164.
- Chereguini, O., García de la Banda, I., Herrera, M., Martínez, C., De la Hera, M., 2003. Cryopreservation of turbot *Scophthalmus maximus* (L.) sperm: fertilization and hatching rates. Aquacult. Res. 34, 739–747.
- Chereguini, O., Rasines, I., Anguis, V., Cal, R., Martín, I., Rodríguez, C., Guzman, J.M., Mylonas, C.C., Mañanós, E., 2007. Primeras fecundaciones artificiales en lenguado senegalés cultivado (Generación F1). Proceedings of XI Congreso Nacional de Acuicultura. Xunta de Galicia, Vigo, Spain, vol. II, pp. 1435-1438.
- Drori, S., Ofir, M., Levavi-Sivan, B., Yaron, Z., 1994. Spawning induction in common carp (*Cyprinus carpio*) using pituitary extract or GnRH superactive analogue combined with metoclopramide: analysis of hormone profile, progress of oocyte maturation and dependence on temperature. Aquaculture 119, 393-407.
- Flajshans, M., Kohlmann, K., Ráb, P., 2007. Autotriploid tench *Tinca tinca* (L.) larvae obtained by fertilization of eggs previously subjected to postovulatory ageing *in vitro* and *in vivo*. J. Fish Biol. 71, 868-876.
- Forniés, M. A., Mañanós, E., Carrillo, M., Rocha, A., Laureau, S., Mylonas, C.C., Zohar, Y., Zanuy, S., 2001. Spawning induction of individual European sea bass females (*Dicentrarchus labrax*) using different GnRHa-delivery systems. Aquaculture 202, 221-234.
- Fortuny, A., Espinach Ros, A., Amutio, V.G., 1988. Hormonal induction of final maturation and ovulation in the sábalo, *Prochilodus platensis* Holmberg: Treatments, latency and incubation times and viability of ovules retained in the ovary after ovulation. Aquaculture 73, 373-381.
- Guzmán, J. M., Norberg, B., Ramos, J., Mylonas, C.C., Mañanós, E.L., 2008. Vitellogenin, steroid plasma levels and spawning performance of cultured female Senegalese sole (*Solea senegalensis*). Gen. Comp. Endocrinol. 156, 285-297.

- Guzmán, J. M., Ramos, J., Mylonas, C.C., Mañanós, E. L., 2009. Spawning performance and plasma levels of GnRHa and sex steroids in cultured female Senegalese sole (*Solea senegalensis*) treated with different GnRHa-delivery systems. *Aquaculture* 291, 200-209.
- Hirose, K., Ishida, R., Sakai, K., 1977. Induced ovulation of ayu using human chorionic gonadotropin (HCG), with special reference to changes in several characteristics of eggs retained in the body cavity after ovulation. *Bull. Japan. Soc. Sci. Fish.* 43, 409-416.
- Howell, B., Conceição, L., Prickett, R., Cañavate, P., Mañanos, E., 2009. Sole farming: nearly there but not quite ? A report of 4th Workshop on the Cultivation of Soles. *Aquaculture Europe* 34, 24-27.
- Imsland, A. K., Foss, A., Conceição, L.E.C., Dinis, M.T., Delbare, D., Schram, E., Kamstra, A., Rema, P., White, P., 2003. A review of the culture potential of *Solea solea* and *S. senegalensis*. *Rev. Fish Biol. Fish.* 13, 379-407.
- Kjørsvik, E., Mangor-Jensen, A., Holmefjord, I., 1990. Egg quality in fishes. *Adv. Mar. Biol.* 26, 71-113.
- Lahnsteiner, F., Soares, F., Ribeiro, L., Dinis, M.T., 2009. Egg quality determination in teleost fish, in: Cabrita, E., Robles, V., Herraez, M.P., (Eds.), *Methods in reproductive aquaculture. Marine and Freshwater Species*. CRC Press, Boca Raton, pp. 149-180.
- Larsson, D.G.J., Mylonas, C.C., Zohar, Y., Crim, L.W., 1997. Gonadotropin-releasing hormone analogue (GnRH-a) induces multiple ovulations of high-quality eggs in a cold-water, batch-spawning teleost, the yellowtail flounder (*Pleuronectes ferrugineus*). *Can. J. Fish. Aquat. Sci.* 54, 1957-1964.
- Legendre, M., Otémé, Z., 1995. Effect of varying latency period on the quantity and quality of ova after hCG-induced ovulation in the African catfish, *Heterobranchius longifilis* (Teleostei, Clariidae). *Aquat. Living Resour.* 8, 309-316.
- Legendre, M., Slembrouck, J., Subagja, J., Kristano, A.H., 2000. Ovulation rate, latency period and ova viability after GnRH- or hCG-induced breeding in the Asian catfish *Pangasius hypophthalmus* (Siluriformes, Pangasiidae). *Aquat. Living Resour.* 13, 145-151.
- Mañanós, E., Duncan, N., Mylonas, C., 2009. Reproduction and control of ovulation, spermiation and spawning in cultured fish, in: Cabrita, E., Robles, V., Herraez, M.P., (Eds.), *Methods in reproductive aquaculture. Marine and Freshwater Species*. CRC Press, Boca Raton, pp. 3-80.

- Mañanós, E., Ferreiro, I., Bolón, D., Guzmán, J.M., Mylonas, C.C., Riaz, A., 2007. Different responses of Senegalese sole *Solea senegalensis* broodstock to a hormonal spawning induction therapy, depending on their wild or captive-reared origin. Proceedings of Aquaculture Europe 07, Istanbul, Turkey, October 2007. European Aquaculture Society, pp. 330-331.
- Marino, G., Panini, E., Longobardi, A., Mandich, A., Finoia, M.G., Zohar, Y., Mylonas, C.C., 2003. Induction of ovulation in captive-reared dusky grouper, *Ephinephelus marginatus* (Lowe, 1834) with a sustained-release GnRH α implant. Aquaculture 219, 841-858.
- Morehead, D. T., Pankhurst, N.W., Ritar, A.J., 1998. Effect of treatment with LHRH analogue on oocyte maturation, plasma sex steroid levels and egg production in female striped trumpeter *Latris lineata* (Latrididae). Aquaculture 169, 315-331.
- Mugnier, C., Guennoc, M., Lebegue, E., Fostier, A., Breton, B., 2000. Induction and synchronisation of spawning in cultivated turbot (*Scophthalmus maximus* L.) broodstock by implantation of a sustained-released GnRH-a pellet. Aquaculture 181, 241-255.
- Mylonas, C.C., Bridges, C., Gordin, H., Belmonte, A., García, A., De la Gándara, F., Fauvel, C., Suquet, M., Medina, A., Papadaki, M., Heinisch, G., De Metrio, G., Corriero, A., Vassallo-Agius, R., Guzmán, J.M., Mañanos, E., Zohar, Y., 2007. Preparation and administration of Gonadotropin-releasing Hormone agonist (GnRH α) implants for the artificial control of reproductive maturation in captive-reared Atlantic Bluefin Tuna (*Thunnus thynnus thynnus*). Rev. Fish. Sci. 15, 183-210.
- Mylonas, C.C., Fostier, A., Zanuy, S., 2010. Broodstock management and hormonal manipulations of fish reproduction. Gen. Comp. Endocrinol. 165, 516-534.
- Mylonas, C.C., Magnus, Y., Gissis, A., Klebanov, Y., Zohar, Y., 1996. Application of controlled-released, GnRH α -delivery systems in commercial production of white bass x striped bass hybrids (sunshine bass), using captive broodstocks. Aquaculture 140, 265-280.
- Mylonas, C.C., Sigelaki, I., Divanach, P., Mañanós, E., Carrillo, M., Afonso-Polyviou, A., 2003. Multiple spawning and egg quality of individual European sea bass (*Dicentrarchus labrax*) females after repeated injections of GnRH α . Aquaculture 221, 605-620.
- Mylonas, C.C., Zohar, Y., 2001. Use of GnRH α -delivery systems for the control of reproduction in fish. Rev. Fish Biol. Fish. 10, 463-491.

- Mylonas, C.C., Zohar, Y., 2007. Promoting oocyte maturation, ovulation and spawning in farmed fish, in: Babin, P.J., Cerdá, J., Lubzens, E., (Eds.), *The Fish Oocyte: from basic studies to biotechnological applications*. Springer, Dordrecht, pp. 437-474.
- Ohta, H., Kagawa, H., Tanaka, H., Okuzawa, K., Hirose, K., 1996. Changes in fertilization and hatching rates with time after ovulation induced by 17, 20 β -dihydroxy-4-pregnen-3-one in the Japanese eel, *Anguilla japonica*. *Aquaculture* 139, 291-301.
- Pankhurst, N. W., Fitzgibbon, Q.P., 2006. Characteristics of spawning behaviour in cultured greenback flounder *Rhombosolea tapirina*. *Aquaculture* 253, 279-289.
- Phelps, R. P., Hastey, R., Pendetar, A., Linley, L., Papanikos, N., Dunham, R.A., 2007. Effects of temperature on the induced spawning of channel catfish and the production of channel x blue catfish hybrid fry. *Aquaculture* 273, 80-86.
- Sahoo, S. K., Giri, S.S., Chandra, S., 2008. Effects of latency periods and injection doses with carp pituitary extract on spawning performance and egg quality of Asian Catfish, *Clarias batrachus* (Linn.). *J. Appl. Aquacult.* 20, 295-303.
- Springate, J.R.C., Bromage, N.R., Elliott, J.A.K., Hudson, D.L., 1984. The timing of ovulation and stripping and their effects on the rates of fertilization and survival to eying, hatch and swim-up in the rainbow trout (*Salmo gairdneri* R.). *Aquaculture* 43, 313-322.
- Takushima, M., Nozaki, R., Kadamura, K., Yasumoto, S., Soyano, K., 2003. Induced ovulation using LHRHa and artificial fertilization in devil stinger, *Inimicus japonicus*. *Fish Physiol. Biochem.* 28, 521-522.
- Van der Kraak, G., Pankhurst, N.W., 1996. Temperature effects on the reproductive performance of fish, in: Wood, C.M., McDonald, D.G., (Eds.), *Global Warming: Implications for freshwater and Marine Fish*. Cambridge University Press, Cambridge, pp. 159-176.

Fig.1. Spawning Kinetics (10^3 Eggs kg^{-1}) of individual sole females after treatment with multiple injections of GnRH α . Arrow heads indicate the day of treatment.

Fig. 2. Relative fecundity (Mean $\pm$ S.E.M.) after each weekly injection of GnRH α . Bars with different letter superscripts were significantly different.

Fig.3. Spawning Kinetics (10³Eggs kg⁻¹) of individual sole females after treatment with GnRH α administered through an implant. Arrow heads indicate the day of treatment.

Fig. 4. Fertilization and hatching rates at different times after ovulation (hpo = hours post ovulation). Bars with different letter superscripts were significantly different.

Table 1. Weight of females, number of spawns per female and relative fecundity obtained in each treatment, expressed as mean $\pm$ standard error. The data in columns with different letter superscripts were significantly different. (IN= injection, IM= implant, C= control)

Treatment	Weight (g)	N° spawns/♀	Relative fecundity n° eggs/kg ♀ (x1000)
IN	1565 $\pm$ 276	6.3 $\pm$ 0.8 ^a	574.9 $\pm$ 67.2 ^c
IM	1450 $\pm$ 178	2.7 $\pm$ 0.6 ^b	134.6 $\pm$ 40.9 ^d
C	1470 $\pm$ 260	0.8 $\pm$ 0.5 ^b	39.5 $\pm$ 35.8 ^d

Highlights

Artificial fertilization of Senegalese sole eggs.

We have established a protocol for hormonal induction of ovulation and stripping.

Repeated injections were more effective than a single implant.

Ovulation was detected at 41-44h post hormonal treatment. Ovulated eggs inside the females were viable for 3 h.