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Spawning performance and plasma levels of GnRHa and sex steroids in cultured female Senegalese sole (Solea senegalensis) treated with different GnRHa-delivery systems

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Abstract

The aquaculture of Senegalese sole (Solea senegalensis) is limited by the poor spawning performance of the F1 generation cultured broodstock. The present study compared the efficiency of two sustained-release delivery systems loaded with gonadotropin-releasing hormone agonist (GnRHa) and a single GnRHa injection on the stimulation of oocyte maturation (OM), sex steroid hormone secretion and spawning. Analysis with an ELISA showed that the administered GnRHa was no longer detectable in the plasma 3, 7 and 14 d after administration via a single injection, microspheres and an implant, respectively. All GnRHa treatments induced a transient elevation of testosterone (T) and 17β estradiol (E_2) plasma levels after 1-3 d. Only three spawns were obtained from controls on days 35, 41 and 42 of the study and ovarian biopsies showed abundant fully vitellogenic oocytes, but little OM. Fish given a GnRHa injection spawned sporadically and fist spawn was 20 days post treatment (dpt). In contrast, both GnRHa implants and microspheres stimulated OM and daily spawning from 4-5 dpt for a period of 28 d. Total fecundity in the broodstock treated with GnRHa-delivery systems was 15-22 times greater than controls and 3-5 times greater than GnRHa-injected fish. Although egg quality appeared to be high, no fertilization was obtained. The results suggest that GnRHa-delivery systems are highly efficient on stimulating OM and spawning in Senegalese sole, presumably through the long-term elevation of pituitary luteinizing hormone release, which in turn stimulated the appropriate changes in ovarian steroidogenesis. However, the effectiveness of the treatment was negated by possible dysfunctions of the male breeders or of the reproductive behavior of the broodstock.

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Key words: Solea senegalensis, flatfish, GnRHa delivery systems, spawning, oocyte maturation.

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1. Introduction

The Senegalese sole (Solea senegalensis) is a common flatfish in the Mediterranean Sea and Southern Atlantic Ocean (Imsland et al., 2003). In the last decade, there has been growing interest in the aquaculture of Senegalese sole, which has become a priority species for diversification in some European and Mediterranean countries (Imsland et al., 2003; Cañavate et al., 2005). A number of studies have reported on aspects of the reproduction in captivity of wild-caught Senegalese sole broodstock, including gonadal development, spawning performance and egg quality (Rodríguez, 1984; Dinis, 1986; Dinis et al., 1999; Mourente and Vázquez, 1996; Anguis and Cañavate, 2005). At present, advances in rearing techniques have allowed viable eggs to be obtained from natural spawning of wild-caught Senegalese sole (Dinis et al., 1999; Anguis and Cañavate, 2005). However, efforts to establish an expanding, sustainable and efficient aquaculture industry for Senegalese sole have been severely constrained by the failure of F1 cultured broodstock to reproduce (Howell et al., 2006). Spawning in cultured breeders is rare, and when it occurs fecundity is low and the eggs are unfertilized. This situation has prompted studies on the reproductive characteristics of cultured Senegalese sole that have provided information on gonad development, spawning and endocrine profiles (García-Lopez et al., 2006a, 2007; Guzmán et al., 2008). These studies demonstrated that cultured females undergo vitellogenesis normally, adequate levels of vitellogenin (VTG) and sex steroid hormones are present in the plasma and accumulation of yolk proteins into the growing oocytes takes place. Nevertheless, most females fail to undergo oocyte maturation (OM), ovulation and spawning, and most of the developing oocytes undergo apoptosis.

Failure to complete OM and ovulation is a common reproductive dysfunction in cultured fish, including flatfishes (Zohar, 1988; Zohar and Mylonas, 2001). In most

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cases, OM, ovulation and spawning can be stimulated through hormonal treatment with gonadotropins or gonadotropin-releasing hormone agonists (GnRHa), either in the form of liquid injections or sustained-release delivery systems (Mylonas and Zohar, 2001; Mañanos et al., 2008; Mylonas and Zohar, 2008). Treatment with GnRHa delivery systems has proven successful for the induction of multiple spawning in several flatfishes with asynchronous ovarian development and has both shown higher efficiency than a single GnRHa injection treatments and improved quality compared to naturally spawning females (Mañanos et al., 2008). For example, administration of GnRHa implants induced multiple ovulations in cultured southern flounder (Paralichthys lethostigma) (Berlinsky et al., 1996) and wild-caught summer flounder (Paralichthys dentatus) (Berlinsky et al., 1997). In the latter case, tank spawning occurred when fish were acclimatized in captivity for one year (Watanabe et al., 1998). In turbot (Scolphtalmus maximus), treatment with GnRHa implants induced multiple ovulations in 100% of the treated fish compared to 50% of controls, and reduced the inter-ovulation period (Mugnier et al., 2000). In yellowtail flounder (Pleuronectes ferrugineus), treatment with different GnRHa delivery systems increased the number of ovulations, fecundity and fertilization and hatching success (Larsson et al., 1997).

Finally, a recent attempt on hormonal induction of spawning in cultured Senegalese sole using a GnRHa implant or multiple GnRHa injections showed that both treatments induced repetitive spawning, but without any fertilization (Agulleiro et al., 2006).

Therefore, further study of the responses of cultured Senegalese sole to hormonal treatments is required for the development of an optimized spawning induction protocol.

The purpose of this study was to compare the efficacy of two sustained-release GnRHa delivery systems to a single injection treatment on the spawning performance of

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cultured Senegalese sole broodstock by evaluating (a) the resulting GnRHa plasma profile and the induced plasma 17β estradiol (E_2) and testosterone (T) levels, (b) their ovarian response, and (c) their spawning kinetics, fecundity and egg quality.

2. Materials and Methods

2.1. Broodstock management

Two-year old cultured Senegalese sole, obtained from natural spawns of wild Senegalese sole broodstock at the facilities of CIFPA "El Toruño" (Cádiz, Spain) in the spring of 2001, were transported to the Institute of Aquaculture of Torre la Sal (Castellon, Spain) in March 2003. Fish were tagged with passive integrated transponders (PIT tags, AVID, UK) and sexed by using a heterologous vitellogenin ELISA for European sea bass (Dicentrarchus labrax) (Mañanós et al., 1994). Sex was further confirmed during the next reproductive season by abdominal swelling in females and by feeling the shape of the testes in males.

Fish were reared in circular fibreglass tanks (3000 l, 1 m depth, 4.15 m^2) at a density of 3-4 kg m^2, without sand substrate, and exposed to the natural temperature and photoperiod regimes of the region. On 2004, water temperatures increased naturally from 16.4ºC to 22.4ºC (mean temperature of this period, 18.7±0.4ºC) from April 30th to June 21st, respectively, when the first and last spontaneous spawns were registered in the F1 broodstocks maintained at the Institute of Aquaculture of Torre la Sal. The experiment was initiated on April 19th and finished on May 17th, at water temperatures of 14.6ºC and 16.6ºC, respectively (mean temperature of this period, 16.1±0.2ºC). Tanks were covered with a thin shade mesh to reduce excessive light. Dissolved oxygen was checked regularly and was maintained at 6.5 ± 0.3 ppm. Tanks were supplied with flow-through seawater

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(salinity ~37 psu) at a flow rate of 400% day^{-1}, and were fitted with an overflow egg collector. Fish were fed ad libitum 5 days a week using dry pellets (ProAqua, Spain).

Handling of the fish for routine management and experimentation was always done according to the European Union Directive for the protection of animals used for experimental and other scientific purposes (EEC, 1986). All fish to be handled were anesthetised by immersion in 0.3 ml l^{-1} of 2-phenoxyethanol.

2.2. Experimental design and hormonal treatments

On January 2004, 84 2-year-old cultured females with a mean (± SD) body weight (BW) and total length (TL) of 898 ± 55 g and 37.8 ± 0.8 cm, respectively, and males of 839 ± 48 g BW and 36.9 ± 0.5 cm TL, respectively, were distributed in four experimental tanks, as described above (n=18-22 fish per tank), at a male:female sex ratio of 1:1 - 1:1.2 for acclimatization. To determine the best time for the initiation of the hormonal treatments, the external signs of ovarian maturation were examined carefully during spring, as described by Guzmán et al. (2008). Briefly, the ocular side of females was examined and classified as stage I, II or III, corresponding to a flat, swollen or highly swollen abdomen, respectively. In addition, examination of the blind side classified females as stage A or B, depending on the absence (A) or presence (B) of an apparent orange coloration along the ventral side. Thus, as the ovary developed the classification of the females maturational stage proceeded from I to III and from A to B.

When the first external signs of female maturation were observed (stages II-B and III-B), females (n=6) and males (n=6) were randomly selected for a more detailed determination of the stage of gonadal development. At this time, ovarian biopsies examined using a binocular microscope demonstrated the presence of a significant batch of post-vitellogenic oocytes with an oocyte diameter of ~500 μm, together with less

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developed oocytes at earlier stages of vitellogenesis. All males were spermiating, releasing sperm by abdominal pressure, and the sperm was considered to be of good quality, having >80% motile spermatozoa upon activation with seawater. On the next day (April 19th 2004, day 0), all fish were anesthetised, weighted and sampled for blood. Blood was taken from the caudal vasculature using heparinized syringes and placed in ice-cold heparinized tubes. Plasma was obtained by centrifugation (3000 g, 15 min., 4 ºC) and stored at -20 ºC for hormone analysis. Thereafter, fish were treated as follows: (a) In tank 1, Control fish (CNT) were injected with a 0.9% NaCl saline solution (9 females and 10 males); (b) in tank 2 a single GnRHa injection at a dose of 5 µg kg^{-1} BW (INJ-5, 6 females and 5 males) or a single GnRHa injection at a dose of 25 µg kg^{-1} BW (INJ-25, 6 females and 5 males); (c) in tank 3 a GnRHa-loaded implant at a dose of 10 µg kg^{-1} BW (IMP-10, 6 females and 5 males) or 50 µg kg^{-1} BW (IMP-50, 6 females and 5 males); in tank 4 GnRHa-loaded microspheres at a dose of 20 µg kg^{-1} BW (MIC-20, 6 females and 5 males) or 100 µg kg^{-1} BW (MIC-100, 6 females and 5 males).

For all hormonal treatments, the agonist [D-Ala^6, Pro^9 Net]-LHRH (Bachem, Switzerland) was used. For injection, GnRHa was dissolved in 0.9% NaCl saline. Implants were manufactured from Ethylene-Vinyl acetate copolymer (EVAc, Elvax; DuPont Chemical CO., USA) as 2-mm diameter x 3-mm cylinders (Zohar et al., 1990). Microspheres were prepared from biodegradable poly-[fatty acid dimmer-sebasic dimmer] (Mylonas et al., 1995). All three GnRHa treatments were applied in the dorsal musculature. The doses of GnRHa injection, implant and microsphere treatments were based on previous studies with other fishes, including flatfishes (Zohar and Mylonas, 2001).

To determine the effect of GnRHa treatment on spawning performance, the occurrence of spawning was checked daily, and the morphology and fertilization of the

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produced eggs was examined using a binocular microscope (see further subsection). The effect of the treatments on oocyte diameter frequency and plasma levels of GnRHa, E_2 and T was determined for each fish, thus obtaining data on the effect of the low and high doses of the particular treatment. At 1, 3, 7, 14, 21 and 28 days post-treatment (dpt), females were anesthetised, and the following procedures completed: BW and TL were recorded; stage of maturation classified according to external signs and a blood sample of 0.8 ml was collected. At 7, 14, 21 and 28 dpt ovarian biopsies were also obtained.

2.3. Ovarian biopsies

The ovarian biopsy obtained from each individual female was split into two sub-samples; one used fresh for immediate observation under the binocular microscope to determine oocyte diameter and morphology, and the other processed for histological analysis using light microscopy. The fresh biopsy sample was placed in a Petri dish and the diameter of a sample of 50 oocytes determined under the binocular microscope, using an ocular micrometer. Then, the sample was dispersed with a clearing solution of ethanol:formalin:acetic acid (6:3:1) and the oocytes classified morphologically in five stages (Table 1, Fig. 1): (i) primary oocytes were small (50 - 150 µm) translucent oocytes, (ii) cortical alveoli oocytes contained vesicles forming distinct granular rings, (iii) vitellogenic oocytes had an opaque and uniform cytoplasm with no indication of yolk clarification, (iv) post-vitellogenic oocytes exhibited germinal vesicle migration and a granular ooplasm indicating yolk clarification, and (v) hydrated oocytes exhibited a total clarification and coalescence of oil drops (Table 1).

For histological analysis, the other ovarian subsample was placed in fixative (4% formaldehyde, 1% gluteraldehyde), embedded in glycol methacrylate resin (Technovit

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7100, Heraeus Kulzer, Germany) and cut into 3-µm sections, before staining with methylene blue/ azure II/ basic fuchsin (Bennet et al., 1976).

2.4. Spawning performance

Once eggs were collected, the fecundity of the fish was determined volumetrically and then converted to number of eggs, considering that 1 ml contains approximately 1,000 eggs. The relation between spawning volume and number of eggs was determined by counting 0.5 ml egg samples (n=29) from eight spawns obtained throughout the spawning period (April 30th to June 14th), given a value of 1.033±69 eggs ml^{-1}. Spawning quality was first evaluated by separating buoyant eggs from non-buoyant eggs, in an adapted volumetric cylinder filled with seawater at 37 psu and the volume of each recorded. Buoyant eggs were then incubated for 48 h to determine embryo development and hatching success. From each spawn, a sample of 50 buoyant eggs was examined using a binocular microscope to determine egg diameter, morphology and fertilization success. Daily relative fecundity was calculated using the weight of the females at the previous sampling, and total relative fecundity was calculated at the end of the spawning period.

2.5. Hormonal analyses

For steroid analysis, plasma samples were first extracted with alcohol. Ice-cold methanol was added to the plasma (6:1, v/v), shaken and centrifuged (3,000 g, 15 min, 4ºC). 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 concentrations of E_2 and T were quantified by ELISA, using a protocol previously validated for Senegalese sole plasma samples (Guzmán et al., 2008). The sensitivities of the ELISAs,

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calculated as Bo-2 SD (maximum binding minus twice the standard deviation), were 5.2 pg ml^{-1} and 8.8 pg ml^{-1}, for the E_2 and T ELISA, respectively. The intra- (n=4) and inter-assay (n=8) coefficients of variation, at 50% of binding, were 5.8% and 6.3% for the E_2, and 6.1% and 11.3% for the T ELISA.

A specific ELISA developed recently for the GnRHa loaded in the delivery systems (Mylonas et al., 2007) was validated for the quantification of GnRHa in plasma samples of Senegalese sole. The sensitivity of the assay, determined as the lowest detection limit (Bo - 2SD), was 51.3 pg ml^{-1}, whereas the amount of GnRHa that caused 50% of binding (ED50) was 340 pg ml^{-1}. Intra-assay variability (IA), tested by measuring replicates of the same sample in a single ELISA plate was 3.4% (n=4). The inter-assay variability (IE), tested by analyzing the same sample in different ELISAs, analyzed by different operators and at different days, was 5.8% (n=10). Prior to subjecting them to the ELISA, plasma samples were extracted according to a protocol for extraction of small peptides (King et al., 1995). Briefly, acetone:acetic acid 2N (1:1, v/v) was added to the plasma (5:1, v/v), vortexed, let stand for 10 min on ice and centrifuged (16,000 g, 30 min. 4ºC). The pellet was re-extracted twice with acetone:acetic acid 2N. The supernatants were pooled, vacuum dried and reconstituted with distilled water. The extract was lyophilized and resuspended in 0.01 M sodium phosphate buffer (PBST, pH 7.4, containing 0.9% NaCl and 0.05% Tween-20), centrifuged (3,000 g, 15 min. 4º C) and the supernatant stored at -20ºC until analysis by ELISA.

2.6. Statistical analyses

The expression of ELISA results was performed after linearization of the sigmoid standard curve using the logit transformation (logit (Bi/ Bo) = ln(Bi - NSB/Bo -NSB)), where Bi represents the binding of each point, Bo the maximum binding and NSB the

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non-specific binding. The parallelism between regression curves was tested by a Student's t test. Dependence and association between variables was analyzed by simple linear regression and correlation index, respectively. The Pearson product-moment was used to examine the significance of regressions.

Differences in relative daily fecundity, egg buoyancy and egg size were examined using One-way ANOVA followed by the Student-Newman-Keuls (SNK) multiple comparison procedure, with significance levels of $P<0.05$. Differences in plasma levels of E_2, T and GnRHa were examined using Two-way ANOVA followed by the HolmSidak test, with significance levels of $P<0.05$. Normality and homogeneity of variances were tested by the Kolmogorov-Smirnov and Bartlett methods, respectively. Data are expressed as mean ± standard error of the mean (S.E.M.).

3. Results

3.1. In vivo release kinetics of the GnRHa delivery systems.

An ELISA method was developed and validated for analysis of GnRHa plasma levels in Senegalese sole, using the agonist [D-Ala^6, Pro^9-NEthylamide]-LHRH (GnRHa) for the standard curve and for production of the specific antibodies (AbGnRHa). The characteristics of sensitivity, precision, specificity and accuracy of the GnRHa ELISA were carefully checked. The precision of the GnRHa ELISA was tested by means of the intra- and inter-assay coefficient of variations. The specificity of the assay was tested by cross-reaction of the AbGnRHa with GnRHa-free plasma from Senegalese sole, which showed absence of immunoreaction at dilutions lower than 1/10 (Fig. 2). The accuracy of the assay was tested by spike-recovery tests, checking also the parallelism between the standard curve and GnRHa-free Senegalese sole plasma spiked with increasing doses of GnRHa. The calculated recovery rate was 78.0 ± 8.6 %.

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Females treated with a GnRHa injection had detectable GnRHa plasma levels at 1 dpt, and at 1 and 3 dpt for the low and high dose, respectively (Fig. 3). Females treated with a GnRHa implant maintained detectable and sustained plasma GnRHa levels from 1 to 14 dpt, without any significant differences between the low and high dose. Females treated with GnRHa microspheres exhibited an initial burst of GnRHa at 1 pdt, with the levels declining progressively thereafter, but remaining detectable 7 dpt.

3.2. Plasma steroid levels

Control females had similar plasma profiles of E_2 and T during the 4-week experimental period, with initially low levels that increased to peak concentrations at 14 d after the initiation of the experiment, and decreased thereafter (Fig. 4). All GnRHa treatments induced significant increases of E_2 and T at 1 dpt relative to Controls ($P<0.05$). This was maintained until 3 dpt for E_2. After the initial rise, the plasma E_2 profile in GnRHa-injected females resembled that of Controls, whereas both GnRHa-implant and GnRHa-microsphere treated females showed a gradual decrease. Also, after 7 dpt, plasma T levels in all GnRHa-treated groups resembled that of Controls.

3.3. Induction of oocyte maturation and spawning

Control females did not show clear variations in oocyte size distribution during the 4 week experimental period, with most of the population below 650 μm in diameter, consisting of oocytes at various stages of vitellogenesis and maturation (Fig. 5). Hydrated oocytes were only found in one female at 14 dpt and two females at 21 and 28 dpt., but always at low incidence (< 5% of total). In general, females treated with GnRHa by injection showed a similar oocyte size distribution to Controls. However, 4 females of the

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low dose GnRHa injection group possessed hydrated oocytes at weeks 2 and 3 pt, coincident with the recorded spawning events.

In contrast, treatment with both GnRHa implants and microspheres (high and low doses) caused a clear differentiation of the oocyte size distribution relative to Controls (Fig. 5). Beginning one week after treatment and throughout the experiment, post-vitellogenic and hydrated oocytes (diameter >650 µm) were present. All females from these groups appeared to have spawned at least once, as evidenced by the presence of ovulated eggs in their biopsies and a high volume of ovarian fluid. Daily fecundities decreased at 28 dpt in GnRHa-implanted females and 21 dpt in females treated with GnRHa-microspheres. At this time, there were still significant numbers of hydrated oocytes in the biopsies, together with apoptotic oocytes that were very abundant at 28 dpt in the fish treated with GnRHa microspheres. Fish from the Control tank showed sporadic spawning of low fecundity in May and June (Fig. 6). The GnRHa injection treatment caused no apparent effect on spawning, with some late spawning detected in May-June. In contrast, both sustained-release GnRHa delivery systems induced spawning from 4 and 6 dpt, respectively. In both cases, a clear rhythm of daily spawning was established until day 26-28 pt, then continuing with sporadic spawning during the next month presumably not under the direct influence of the GnRHa treatments.

All three GnRHa treatments improved spawning performance with respect to Controls (Table 2). All treatments increased the number of spawnings, the spawning period and daily fecundity, with respect to Controls. Total fecundity was increased 4-fold by the GnRHa injection and 22- and 15-fold by the GnRHa implant and microsphere treatments, respectively, with respect to Controls. Egg buoyancy was similar in GnRHa treated fish, but all eggs were unfertilized, based on the absence of embryos 24 h after spawning. No differences in mean egg diameter were found among treatments (Table 2). However, a

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significant decrease in egg size was observed in the GnRHa implanted fish along the spawning period ($P < 0.01$, Fig. 7). This correlation between decreasing egg size and the date of spawning became highly significant when considering the first 4 weeks after treatment, the period of induced spawning under direct GnRHa influence, were the fish treated with GnRHa microspheres also exhibited a significant reduction of egg size with time. There were no significant changes in egg diameter during the experiment in spawns from the Control or GnRHa-injected tanks.

4. Discussion

Currently, the emerging aquaculture industry for the Senegalese sole is entirely dependent on the spawning of wild-caught individuals (Dinis et al., 1999; Imsland et al., 2003; Howell et al., 2006). The expansion and optimization of this industry requires the development of protocols for the control of reproduction of cultured broodstock (F1 generation). The present work studied the efficacy of three GnRHa-based spawning induction therapies on cultured Senegalese sole, by comparing injection with two sustained-release delivery systems (Mylonas and Zohar, 2001). This is the first report on in vivo release kinetics of GnRHa treatments in a warm-water flatfish and the results may be applicable to related species. The ELISA developed for this purpose showed optimal characteristics of precision, accuracy and specificity, and its sensitivity was comparable to previously developed GnRHa radioimmunoassays, which were 62 pg ml^{-1} (Sherwood et al., 1988) and 50 pg ml^{-1} (Mylonas et al., 1997a). The exogenously administered GnRHa in Senegalese sole was cleared from the circulation at different rates, depending on the administration method, and was 3 d for injection, 14 d for microspheres and 21 d for implants. This pharmacokinetic information is of interest in determining the duration of the GnRHa in vivo action and the adequate timing for

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potential repetitive treatments (Mylonas et al., 2004). However, the clearance of exogenous GnRHa from the blood may vary due to administration method and dose, but also due to water temperature and the metabolism of the species (Zohar and Mylonas, 2001; King and Pankhurst, 2004). For example, in winter flounder (Pleuronectes americanus) GnRHa given as a single injection was cleared from the circulation in 3 d (Harmin and Crim, 1993), whereas in common dentex (Dentex dentex) in only 24 h (Greenwood et al., 2001). When administered via an implant, plasma GnRHa levels in Atlantic halibut (Hippoglossus hippoglossus) peaked at 6 dpt and then declined slowly for 1 month (Vermeirssen et al., 2000), while in common dentex peaked at 10 dpt and remained detectable for 24 d (Greenwood et al., 2001). Administration of GnRHa via microspheres resulted in peak GnRHa plasma levels at 5 dpt in the North Sea plaice (Pleuronectes platessa) and remained detectable for 43 d (Vermeirssen et al., 1998), whereas in gilthead seabream (Sparus aurata) peaked immediately after implantation and became undetectable after 7 d (Mylonas and Zohar, 2001). A higher dose for the microspheres was used compared to the implants, since microspheres were expected to result in a longer release duration compared to the implants (8 vs 4 weeks) (Mylonas et al., 1995). Therefore, in order to achieve similar plasma GnRHa levels for at least the first 4 weeks of the study, a twice as high dose was used. However, the opposite was observed, with microspheres releasing for a much less period of time.

Plasma levels of both E2 and T increased after all three GnRHa treatments, indicating stimulation of gonadotropin-mediated sex steroid secretion by the gonad. Previous studies with other fishes reported a similar sex steroid increase soon after GnRHa treatment. For example, female yellowtail flounder treated with GnRHa implants or microspheres showed increased plasma E2 levels 4 dpt (Larsson et al., 1997). In stripped trumpeter (Latris lineata), GnRHa implantation increased T and E2

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levels at days 1 and 2, respectively (Morehead et al., 1998), whereas in captive white bass (Morone chrysops) both E2 and T plasma levels peaked 18 h after GnRHa treatment (Mylonas et al., 1997). After the initial GnRHa induced rise of sex steroid secretion, the plasma levels of E2 decreased steadily in Senegalese sole treated with both GnRHa implants and microspheres, but remained elevated in controls and GnRHa injected fish. This could be related to the observed differences in spawning between groups. A study performed in the North Sea plaice (Pleuronectes platessa), showed that treatment with a GnRHa implant caused an initial rise of plasma T and E2 levels 4 dpt and a sharp decrease thereafter, which was correlated with a concomitant increase of plasma progestogens and the stimulation of OM (Scott et al., 1999). In the present study, the decreased plasma levels of E2 in Senegalese sole treated with implants and microspheres could be correlated with the steroidogenic shift to the production of progestogens (Senthilkumaran et al., 2004), related to the observed stimulation of OM and spawning in these group of fish, as compared to controls and GnRHa injected Senegalese sole.

Saline-treated Senegalese sole in the present study produced some spawns of poor quality in May and early June. This was expected, since spawning of cultured breeders at our facilities coincides with the main natural spawning period of wild-caught Senegalese sole broodstock (Guzmán et al., 2008). Egg production by cultured broodstock is always less frequent and of lower fecundity than that of wild breeders. In the present study, the fecundity of the Controls was 150-fold lower than reported for wild-caught broodstocks (Anguis and Cañavate, 2005), reflecting the fact that only one third of females ovulated, producing small numbers of hydrated oocytes (<5% of the total oocyte population).

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In contrast, GnRHa treatments induced significantly more spawning events. Although a single GnRHa injection was relatively ineffective, both implant and microspheres induced daily spawning for a period of around 3 weeks. Injected fish showed a slightly higher fecundity than Controls, although this might not have been caused directly by the hormonal treatment, because the first spawning was observed at 21 dpt, which was 17 d after exogenously administered GnRHa had disappeared from the bloodstream in these fish. Also, the observation of the ovarian biopsies in the injected females did not reveal significant signs of induced maturation in the first two weeks following treatment, and total fecundity and number of spawns per female, although lower than the Control fish of this study, were similar to those obtained in previous years in cultured Senegalese sole broodstock at the same facilities (Guzmán et al., 2008). Other studies in Senegalese sole using a single GnRHa injection have also reported absence of induced spawning or the occurrence of a few spawns of non-viable eggs (Dinis et al., 1999; Agulleiro et al., 2006). Similarly, in the common sole (Solea solea) a single GnRHa injection accelerated ovulation and induced spawning, but did not increase fecundity or egg quality compared to Controls (Ramos, 1986). Although the repetitive GnRHa injection method has been used effectively in other species, the frequent handling causes significant stress that can have detrimental effects on the timing of ovulation, egg quality (Campbell et al., 1992) or broodstock health (Harmin and Crim, 1992; Zohar and Mylonas, 2001).

Treatment with GnRHa implants or microspheres induced daily spawning and increased total fecundity by 22- and 15-fold, respectively. These sustained release GnRHa administration methods have been used successfully for the induction of spawning in other flatfishes. For example, treatment with GnRHa implants in Atlantic halibut induced ovulation and spawning, and increased relative fecundity slightly

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compared to untreated fish (Mazorra de Quero et al., 2000). Similarly, GnRHa implants were very effective in stimulating ovulation in turbot (Scophtalmus maximus) (Mugnier et al., 2000). The only study testing the effect of GnRHa microspheres in a flatfish (i.e., the yellowtail flounder) showed similar stimulatory effects to the GnRHa implants, inducing multiple ovulations and increasing by ~2-fold egg production compared to untreated females (Larsson et al., 1997). Recently, GnRHa implants have been tried in Senegalese sole and showed induction of spawning and a 3-fold increase in relative fecundity with a high dose of 50 μg kg^{-1}, but not with a low dose of 5 - 25 μg kg^{-1} (Agulleiro et al., 2006). In the present study, unfortunately, it was not possible to ascertain if females implanted with low or a high GnRHa dose contributed equally to the total fecundity of the tank. However, the analysis of individual ovarian biopsies taken from GnRHa implanted females revealed that both high-dose and low-dose implants caused a similar stimulation of OM, increasing the proportion of post-vitellogenic, hydrated and ovulated oocytes, suggesting induction of spawning with both doses of implants. The different results obtained with the GnRHa implants in Senegalese sole in our study compared to that of Agulleiro et al. (2006) could be due to a different stage of maturity of the females at the moment of treatment, since those authors reported absence of post-vitellogenic oocytes at the time of implantation. These data could suggest that the optimal stage for treatment Senegalese sole with GnRHa would be when females contain oocytes with diameters of around 550 μm. This optimum oocyte size for initiation of treatment is consistent with previous studies with southern flounder and summer flounder (Berlinsky et al., 1996, 1997).

The ovarian biopsies taken before the hormonal treatments showed oocytes of all diameters and stages of development. This confirmed that ovarian development in Senegalese sole is asynchronous (Tyler and Sumpter, 1996), as previously determined

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in naturally spawning wild Senegalese sole (Rodríguez, 1984) and wild-caught Senegalese sole maintained in captivity (García-López et al., 2006b). Treatment with both sustained release GnRHa delivery systems induced oocyte growth in all females, stimulating maturation of the largest oocyte batches (diameter increase from 550 µm to 950 µm) and growth of other smaller oocyte batches. Biopsies taken during the 4-week sampling period when spawning occurred almost daily, showed a multimodal oocyte diameter frequency distribution, which indicates that Senegalese sole may continuously recruit vitellogenic oocytes to maturation, similar to other asynchronous flatfishes such as the summer flounder (Watanabe et al., 1998) and greenback flounder (Sun and Pankhurst, 2004). This observation is also consistent with the observation of high vitellogenin plasma levels in Senegalese sole females throughout the spawning period (Guzmán et al., 2008).

After 3-4 weeks from treatment with GnRHa implants or microspheres, when circulating GnRHa levels were undetectable and spawning was reduced to sporadic events of low fecundity, ovarian biopsies revealed accumulation of hydrated and post-ovulated oocytes, together with the first signs of oocyte apoptosis. Considering the dates of the experiment, this moment still corresponded to the reported mid-spawning period of Senegalese sole and may point to an inhibition of spawning in the absence of exogenous GnRHa. It has been recently observed that the administration of a second GnRHa implant after 3 weeks restored daily spawning in 48 h (Guzmán, Mylonas and Mañanós, unpublished results).

In spite of the success in inducing OM, ovulation and spawning by sustained release GnRHa treatments, there was a complete failure of fertilization, as observed earlier in both GnRHa-induced and spontaneous spawning of cultured Senegalese sole (Agulleiro et al., 2006; Guzmán et al., 2008). One possible explanation for this result would be the

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absence or alteration of courtship and spawning behaviour of the cultured breeders, a critical process for successful reproduction. In greenback flounder and southern flounder, spawning occurs when a male swims close to the female, with genital pores together, and lifts the female toward the water surface, at which time eggs and sperm are released simultaneously (Smith et al., 1999; Pankhurst and Fitzgibbon, 2006). In common sole, a similar behaviour has been described, in which pairs formed on the tank floor swam together towards the water surface where the gamete release took place simultaneously (Baynes et al., 1994). Recent video-recording studies in cultured Senegalese sole have shown that in tanks containing GnRHa implanted fish, egg release might take place in the absence of any courtship or spawning behaviour (N. Duncan, IRTA, Spain, personal communication). In other flatfishes, such as the flounder (Paralichthys orbignyanus) (Bambill et al., 2006) or summer flounder (Watanabe et al., 1998; Watanabe and Carroll, 2001), the high variability in egg fertilization success has been attributed to a low participation of males in spawning events. Another factor influencing fertilization success could be the inhibition or delayed spawning after ovulation, leading to overripening of the eggs (Berlinsky et al., 1996), a process that can be especially rapid in warm-water species (Bromage et al., 1994; Zohar and Mylonas, 2001).

There was a reduction of the size of the eggs spawned after GnRHa implant or microsphere treatment as spawning progressed. Variations in egg diameter during the natural spawning season have been previously described in wild Senegalese sole (Dinis et al., 1999), in its congener common sole (Baynes et al., 1993) and in other multiple-spawning fishes (Hinckey, 1990; McEvoy and McEvoy, 1991; Kjesbu et al., 1996). In both GnRHa-treated and untreated turbot, egg size decreased concomitantly with egg batch number, but without any differences in egg quality (Mugnier et al., 2000). Also,

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in GnRHa-treated European sea bass a reduction in egg size was observed in consecutive spawns, but without any variations in egg quality (Forniés et al., 2001). The underlying reason for decreased egg size along the spawning season in many fishes is unclear.

In conclusion, the present results indicated that cultured Senegalese sole female breeders may be induced to undergo OM and spawn spontaneously in a daily rhythm after treatment with sustained administration of GnRHa, through implants or microspheres. However, such hormonal treatment is not sufficient to solve the problem of complete lack of egg fertilization observed in cultured (F1 generation) broodstock, which needs to be investigated further, focusing on breeding behaviour and/or male fish function.

Acknowledgments

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ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

References

Agulleiro, M.J., Anguis, V., Cañavate, J.P., Martínez-Rodríguez, G., Mylonas, C.C., Cerdá, J., 2006. Induction of spawning of captive reared Senegalese sole (Solea Senegalensis) using different delivery systems 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.

Bambill, G.A., Masakazu, O., Radonic, M., López, A.V., Müller, M.I., Boccanfuso, J.J., Bianca, F.A., 2006. Broodstock management and induced spawning of flounder (Paralichthys orbignyanus) (Valenciennes, 1893) under a closed recirculated system. Revista de Biología Marina y Oceanográfica 41, 45-55.

Baynes, S.M., Howell, B.R., Beard, T.W., 1993. A review of egg production in captive sole, Solea solea (L). Aq. Fish. Mang. 24, 171-180.

Baynes, S.M., Howell, B.R., Beard, T.W., Hallam, J.D., 1994. A description of spawning behaviour of captive Dover sole Solea solea (L). Neth. J. Sea Res. 32, 271-275.

Bennett, H.S., Wyrick, A.D., Lee, S.W., McNeil, J.H., 1976. Science and art in preparing tissues embedded in plastic for light microscopy, with special reference to glycol methacrylate, glass knives, and simple stains. Stain Technol. 51, 71- 94.

Berlinsky, D.L., King, W.V., Smith, T.I.J., Hamilton II, R.D., Holloway Jr., J., Sullivan, C.V., 1996. Induced ovulation of Southern flounder Paralichthys lethostigma using gonadotropin releasing hormone analogue implants. J. World Aquacult. Soc. 27, 143-152.
ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Berlinsky, D.L., William, K., Hodson, R.G., Sullivan, C.V., 1997. Hormone induced spawning of summer flounder Paralichthys dentatus. J. World Aquacult. Soc. 28, 79-86.

Bromage, N.R., Bruce, M., Basavaraja, N., Rawa, K., Shield, 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.

Campbell, P. M., Pottinger, T. G., Sumpter, J. P. 1992. Stress reduces the quality of gametes produced by rainbow trout. Biol. Reprod. 47, 1140-1150.

Cañavate, J.P., 2005. Opciones del lenguado senegalés (Solea senegalensis) para diversificar la acuicultura marina. Actas X Congreso Nacional de Acuicultura, Gandía, Spain, October 2005. Universidad Politécnica de Valencia, pp. 340-347.

Dinis, M.T., 1986. Quatre Soleidae de l'Estuarie du tage. Reproduction et Croissance. Essai d'Elevage de Solea senegalensis Kaup 1858. Thèse d'État èn-Sciences Naturelles, Université de Bretagne Occidentale, France.

Dinis, M.T., Ribeiro, L., Soares, F., Sarasquete, C., 1999. A review on the cultivation potential of Solea senegalensis in Spain and in Portugal. Aquaculture 176, 27-38. EEC,

1986. Council Directive 86/609 EEC for the protection of animals used for experimental and other scientific purposes. Official J. L358 (18/12/1986), 1-28.

Forniés, M. A., Mañanós, E. L., Carrillo, M., Rocha, A., Laureau, S., Mylonas, C.C., Zohar, Y., Zanuy, S., 2001. Spawning induction of individual sea bass females (Dicentrarchus labrax) using different GnRHa delivery systems. Aquaculture 202, 221-234.

García-López, A., Fernández-Pasquier, V., Couto, E., Canario, A.V.M., Sarasquete, C., Martínez-Rodríguez, G., 2006a. Testicular development and plasma sex steroid

ACCEPTED MANUSCRIPT

levels in cultured male Senegalese sole Solea senegalensis Kaup. Gen. Comp. Endocrinol. 147, 343-351.

García-López, A., Anguis, V., Couto, E., Canario, A.V.M., Cañavate, J.P., Sarasquete, C., Martínez-Rodríguez, G., 2006b. Non-invasive assessment of reproductive status and cycle of sex steroid levels in a captive wild broodstock of Senegalese sole Solea senegalensis (Kaup). Aquaculture 254, 583-593.

García-López, A., Couto, E., Canario, A.V.M., Sarasquete, C, Martínez-Rodríguez, G., 2007. Ovarian development and plasma sex steroid levels in cultured female Senegalese sole Solea senegalensis. Comp. Biochem. Physiol. 146A, 342-354.

Greenwood, L.M., Scott, A.P., Vermeirssen, E., Le Gac, F., Mylonas, C.C. and Pavlides, M., 2001. Plasma steroids in mature common dentex (Dentex dentex) stimulated with a gonadotropin releasing hormone agonist. Gen. Comp. Endocrinol. 123, 1-12.

Guzmán, J.M., Norberg, B., Ramos, J., Mylonas, C.C., Mañanós, E.L., 2008. Vitellogenin, plasma sex steroids and spawning performance of cultured female Senegalese sole (Solea senegalensis). Gen. Comp. Endocrinol. 156, 285-297.

Harmin, S.A., Crim, L.W., 1992. Gonadotropin releasing-hormone analog (GnRH-A) induced ovulation and spawning in female winter flounder, Pseudopleuronectes americanus (Walbaum). Aquaculture 104, 375-390.

Harmin, S.A., Crim, L.W., 1993. Influence of gonadotropin hormone-releasing hormone analog (GnRH-A) on plasma sex steroid profiles and milt production in male winter flounder, Pseudopleuronectes americanus (Walbaum). Fish Physiol. Biochem. 10, 399-407.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Hinckley, S. 1990. Variation of egg size of walleye pollock Theragra chalcogramma with a preliminary examination of the effect of egg size on larval size. Fish. Bull. 88, 471-483.

Howell, B.R., Cañavate, J.P., Prickett, R., Conceicão, L.E.C., 2006. The Cultivation of Soles, Report of the 3rd Workshop held at CIFPA El Toruño, Cadiz, Spain. CICEM El Toruño, Cadiz, Spain, pp. 35.

Imsland, A.K., Foss, A., Conceicão, 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.

King, H.R., Pankhurst, N.W., 2004. Effect of maintenance at elevated temperatures on ovulation and luteinizing hormone releasing hormone analogue responsiveness of female Atlantic salmon (Salmo salar) in Tasmania. Aquaculture 223, 583-597.

King, J.A., Millar, R.P., Vallarino, M., Pierantoni R. 1995. Localization and characteriztion of gonadotropin-releasing hormones in the brain, gonads and plasma of a dipnoi (lungfish, Protopterus annectens). Regulatory Peptides 57, 163-174.

Kjesbu, O.S., Solemdal, P, Bratland, P., M. Fonn, M. 1996. Variation in annual egg production in individual captive Atlantic cod (Gadus morhua). Can. J. Fish. Aquat. Sci. 53, 610-620.

Larsson, D.G.J., Mylonas, C.C., Zohar, Y., Crim, L.W., 1997. Gonadotropin releasing hormone-analogue (GnRH-A) advances ovulation and improves the reproductive performance of a cold-water batch-spawning teleost, the yellowtail flounder (Pleuronectes ferrugineus). Can. J. Aquat. Fish. Sci. 54, 1957-1964.

Mañanós, E., Nuñez, J., Zanuy, S., Carillo, M., Menn, F.L., 1994. Sea bass (Dicentrarchus labrax L.) vitellogenin. II: Validation of an enzyme-linked immunosorbent assay (ELISA). Comp. Biochem. Physiol. 107B, 217-23.

ACCEPTED MANUSCRIPT

Mañanos, E., Duncan, N., Mylonas, C.C., 2008. Reproduction and control of ovulation, spermiation and spawning in cultured fish. In: Cabrita, E., Robles, V., Herráez, M.P. (Eds.), Methods in Reproductive Aquaculture: Marine and Freshwater Species, CRC Press, Taylor and Francis Group, Boca Raton, pp. 3-80.

Mazorra de Quero, C., Shields, R.J., Scott, A.P., Mylonas, C.C., Zohar, Y. and Bromage, N., 2000. Effects of GnRHa implants on female Atlantic halibut Hippoglossus hippoglossus spawning performance. Proceedings Aqua 2000, Nice, France, May 2000. European Aquaculture Society, p. 454.

McEvoy, L.-A., and McEvoy, J. 1991. Size fluctuations in the eggs and newly hatched larvae of captive turbot (Scophthalmus maximus). J. Mar. Biol. Assoc. U.K. 71, 679-690.

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.

Mourente, G., Vázquez, R., 1996. Changes in the content of total lipid, lipid classes and their fatty acids of developing eggs and unfed larvae of the Senegal sole, Solea senegalensis Kaup. Fish Physiol. Biochem. 15, 221-235.

Mugnier, C., Gaignon, J.L., Lebegue, E., Fostier, A. and Breton, B. (2000) Induction and synchronization of spawning in cultivated turbot (Scopthalmus maximus L.) broodstock by implantation of sustained-release GnRH-a pellet. Aquaculture 181, 241-255.

Mylonas, C.C., Tabata, Y., Langer, R., Zohar, Y., 1995. Preparation and evaluation of polyanhydride microspheres containing gonadotropin-releasing hormone (GnRH), for inducing ovulation and spermiation in fish. J. Controlled Release 35, 23-34.

ACCEPTED MANUSCRIPT

Mylonas, C.C., Scott, A.P., Vermeirssen, E.L.M., Zohar, Y., 1997a. Changes in plasma gonadotropin II and sex-steroid hormones, and sperm production of striped bass after treatment with controlled-release gonadotropin-releasing hormone agonist-delivery systems. Biol. Reprod. 57, 669-675.

Mylonas, C.C., Magnus, Y., Klebanov, Gissi, A., Zohar, Y., 1997b. Reproductive biology and endocrine regulation of final oocyte maturation of captive white bass. J. Fish Biol. 51, 234-250.

Mylonas, C.C., Zohar, Y., 2001. Use of GnRHa-delivery systems for the control of reproduction in fish. Rev. Fish Biol. Fish. 10, 463-491.

Mylonas, C.C., Papandroulakis, N., Smboukis, A., Papadaki, M., Divanach, P., 2004. Induction of spawning of cultured greater amberjack (Seriola dumerili) using GnRHa implants. Aquaculture 237, 141-154.

Mylonas, C.C., Bridges, C.R., Gordin, H., Belmonte Ríos, 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 (GnRHa) implants for the artificial control of reproductive maturation in captive-reared Atlantic bluefin tuna (Thunnus thynnus thynnus). Rev. Fisheries Sci. 15, 183-210.

Mylonas, C.C., Zohar, Y., 2008. Controlling reproduction in aquaculture. In: Burnell, G., Allan, G. (Eds.), New Technologies in Aquaculture: Improving Production Efficiency, Quality and Environmental Management, Woodhead Publishing Ltd, Cambridge, UK, pp. 001-038.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Pankhurst, N.W., Fitzgibbon, Q.P., 2006. Characteristics of spawning behaviour in cultured greenback flounder Rhombosolea tapirina. Aquaculture 253, 279-289.

Poortenaar, C.W. and Pankhurst, N.W., 2000. Effect of luteinizing hormone-releasing hormone analogue and human chorionic gonadotropin on ovulation, plasma and ovarian levels of gonadal steroids in greenback flounder Rhombosolea tapirina. J. World Aqua. Soc. 31, 175-185.

Ramos, J., 1986. Luteinizing hormone-releasing hormone analogue (LH-RHa) induces precocious ovulation in common sole (Solea solea L.). Aquaculture 54, 185-190.

Rodríguez, R.B., 1984. Biología y cultivo de Solea senegalensis Kaup 1858 en el Golfo de Cádiz. PhD Thesis, Universidad de Sevilla, Spain.

Scott, A.P., Witthames, P.R., Vermeirssen, E.L.M., Carolsfeld, J., 1999. Prolonged-release gonadotrophin-releasing hormone analogue implants enhance oocyte final maturation and ovulation, and increase plasma concentrations of sulfated C21 steroids in North Sea plaice. J. Fish Biol. 55, 316-328.

Senthilkumaran, B., Yoshikuni, M., Nagahama, Y., 2004. A shift in steroidogenesis occurring in ovarian follicles prior to oocyte maturation. Mol. Cell. Endocrinol. 215, 11-18.

Sherwood, N.M., Crim, L.W., Carolsfeld, J., Walters, S.M., 1988. Sustained hormone release. I. Characteristics of in vitro release of gonadotropin-releasing hormone analogue (GnRH-A) from pellets. Aquaculture 74, 75-86.

Smith, T.I.J., McVey, D.C., Jenkins,W.E., Denson, M.R., Heyward, L.D., Sullivan, C.V., Berlinsky, D,L., 1999. Broodstock management and spawning of southern flounder, Paralichthys lethostigma. Aquaculture 176, 87-99.

Sun, B., Pankhurst, N.W., 2004. Patterns of oocyte growth, vitellogenin and gonadal steroids concentrations in greenback flounder. J. Fish Biol. 64, 1399-1412.

ACCEPTED MANUSCRIPT

Tyler, J.R. and Sumpter, J.P., 1996. Oocyte growth and development in teleosts. Rev. Fish Biol. Fish. 6, 287-318.

Vermeirssen, E.L.M., Scott, A.P., Mylonas, C.C., Zohar, Y., 1998. Gonadotropin-releasing hormone agonist stimulates milt fluidity and plasma concentrations of 17,20β-dihydroxylated and 5β-reduced, 3α-hydroxylated C21 steroids in male plaice (Pleuronectes platessa). Gen. Comp. Endocrinol. 112, 163-177.

Vermeirssen, R.L.M., Shileds, R.J., Mazorra de Quero, C., Scott, A.P., 2000. Gonadotropin-releasing hormone agonist raises plasma concentrations of progestogens and enhances milt fluidity in male Atlantic halibut (Hippoglossus hippoglossus). Fish Phisiol. Biochem. 22, 77-87.

Watanabe, W.O., Ellis, E.P., Ellis, S.C. and Feeley, M.W., 1998. Progress in controlled maturation and spawning of summer flounder (Paralichthys dentatus) broodstock. J. World Aqua. Soc. 29, 393-404.

Watanabe, W.O., Carroll, P.M., 2001. Progress in controlled breeding of summer flounder, Paralichthys dentatus, and southern flounder, P. lethostigma. J. App. Aquaculture 11, 89-111.

Zohar, Y., 1988. Gonadotropin releasing hormone in spawning induction in teleosts: Basic and applied considerations. In: Zohar, Y. and Breton, B. (Eds.), Reproduction in Fish: Basic and Applied Aspects in Endocrinology and Genetics. INRA Press, Paris, pp. 47-62.

Zohar, Y., Pagelson, G., Gothilf, Y., Dickhoff, W. W., Swanson, P., Duguay, S., Gombotz, W., Kost, J., and Langer, R., 1990. Controlled release of gonadotropin releasing hormones for the manipulation of spawning in farmed fish. Proc. Int. Congr. Controlled Release Bioact. Mater. 17, 51-52.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Zohar, Y., Mylonas, C.C., 2001. Endocrine manipulations of spawning in cultured fish: from hormones to genes. Aquaculture 197, 99-136.

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Table 1. Classification of Senegalese sole oocyte development based on diameter and morphology. See text for further description.

Oocyte Stage	Range (µm)	Mean diameter (µm)
Primary	< 150	110 ± 10
Cortical alveoli	150 - 250	170 ± 10
Vitellogenic	250 - 500	440 ± 30
Post-vitellogenic	500 - 650	590 ± 10
Hydrated	>650	880 ± 90

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Table 2. Spawning characteristics of cultured Senegalese sole treated with, saline (Controls, CNT), GnRHa injection (INJ), GnRHa implant (IMP) or GnRHa microspheres (MIC).
Spawning was recorded daily from the day of treatment (April 19th) until the end of the natural spawning season (June 21st). Data are expressed as mean ± SEM.

	CNT	INJ	IMP	MIC
Nº of spawns	5	13	26	26
Spawning period[1] (days)	9	32	39	37
Total relative fecundity[2] (eggs Kg^{-1})	9,720	41,960	208,800	141,880
Daily[2] relative fecundity (eggs Kg^{-1})	1,940 ± 930	3,230 ± 870	8,030 ± 1,760	5,720 ± 1,310
Egg buoyancy (%)	58 ± 10	34 ± 8	40 ± 5	27 ± 5
Egg size[3] (µm)	979 ± 3	971 ± 6	979 ± 1	978 ± 1
Hatching success (%)	0	0	0	0

[1] Spawning period was the period between the first and last spawn.
[2] Total and daily relative fecundity was calculated based on female biomass.
[3] Egg size is the mean egg diameter of the floating eggs (n=50 eggs per spawn).

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Figure Legends

Figure 1. Histological sections of Senegalese sole oocytes obtained from ovarian biopsies. (A) Primary oocytes (PO), oocytes at the cortical alveoli stage (CA) and vitellogenic oocytes (V). (B) Vitellogenic oocyte with the germinal vesicle (GV) in a central position, some lipid droplets (L), yolk globules (Y), a clearly defined zona radiata (Z) and the follicular layer (F). (C) Post-vitellogenic oocyte with the GV migrated to the periphery, and the lipid droplets coalescing into a larger mass. The yolk globules are still in a dispersed form. (D) Apoptotic oocyte. Bars represent 100 μm.

Figure 2. Validation of the GnRHa ELISA for Senegalese sole plasma samples. The GnRHa standard curve (black circles) shows parallelism with GnRHa spiked Senegalese sole plasma (white circles) and serially diluted plasma from a GnRHa-treated Senegalese sole (black triangles). Plasma from untreated (GnRHa free) Senegalese sole (black squares) did not exhibit cross-reaction.

Figure 3. Mean (± SEM) plasma GnRHa levels in female Senegalese sole (n=6) after a single treatment with different GnRHa administration methods: injection (INJ), implant (IMP) or microspheres (MIC). Each administration method was tested at high (white circles) and low (black circles) GnRHa doses. Sampling points exhibiting non-detectable GnRHa plasma levels are indicated with ND. Control fish were treated with saline and had undetectable GnRHa plasma levels throughout the experiment (not shown). Letters indicate significant differences (ANOVA, $P<0.05$) between sampling days within treatment group, indicated in

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capitals for the high dose and in small letters for the low dose. The asterisk "*" indicates significant differences between doses, within treatment group ($P < 0.05$).

Figure 4. Evolution of mean (±SEM) plasma levels of 17ß-estradiol (E2) and testosterone (T) in female Senegalese sole (n=6), treated with saline (Control, CNT), GnRHa injection (INJ), GnRHa implant (IMP) or GnRHa microspheres (MIC). The GnRHa treatments were applied at high (white circles) and low (black circles) doses. In each graph, different letters indicate significant differences (ANOVA, $P<0.05$) between sampling days within treatment group, indicated in capitals for the high dose and in small letters for the low dose. The asterisk "*" indicates significant differences ($P<0.05$) with respect to the control group for each sampling point. The "#" indicates significant differences ($P<0.05$) between doses, within treatment group.

Figure 5. Evolution of oocyte diameter frequency distribution in female Senegalese sole treated with saline (Control, CNT), GnRHa injection at 5 or 25 µg kg^{-1} (INJ 5, INJ 25), GnRHa implant at 10 or 50 µg kg^{-1} (IMP 10, IMP 50) and GnRHa microspheres at 20 or 100 µg kg^{-1} (MIC 2, MIC 10). Each histogram corresponds to the average oocyte frequency distribution from the females of the corresponding group. The number of biopsied females at each sampling point is indicated in brackets. The diameter ranges (dotted lines) correspond to different oocyte development stages (Table 2, Fig. 1), categorized as primary oocytes (P), cortical alveoli (C), vitellogenic (V), post-vitellogenic (M) or hydrated (H).

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Figure 6. Daily relative fecundity (egg kg^{-1} female biomass) of cultured Senegalese sole after a single treatment (day 0) with saline (Control, CNT) or GnRHa administrated as an injection (INJ), implant (IMP) or microspheres (MIC). The quantity of buoyant (black bars) and sinking (white bars) eggs was determined for each spawning.

Figure 7. Correlation of the mean diameter of buoyant eggs (n=50 spawns) with day of spawning of Senegalese sole treated with saline (Control, CNT), GnRHa injection (INJ), GnRHa implant (IMP) or GnRHa microspheres (MIC). Regression lines were fitted for the entire spawning season of 60 days (dotted line). For the groups IMP and MIC a second regression line (solid line) was fitted on the 0 to 28 day period, considered to be the period under direct GnRHa influence. Correlation coefficients (r) and significance of the regressions (P) are indicated in each graph (in bold for the second regression in groups MIC and IMP).

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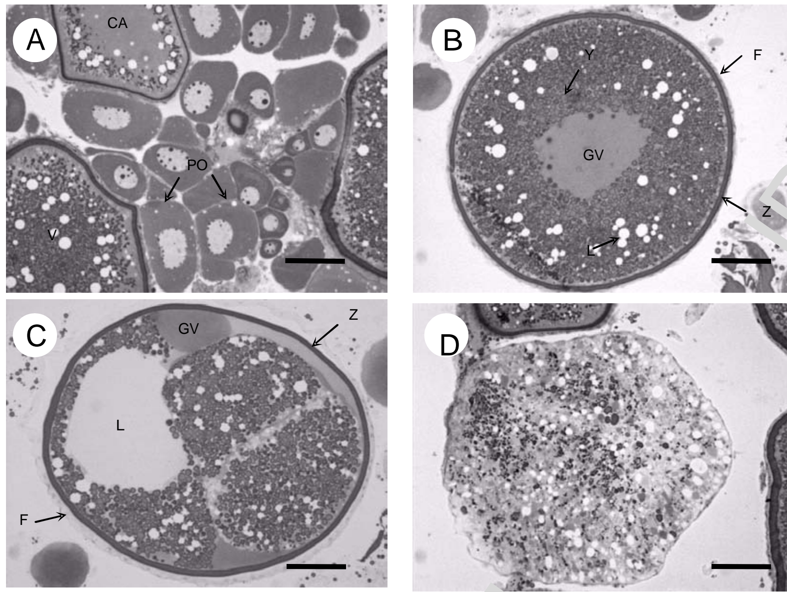

Fig. 1

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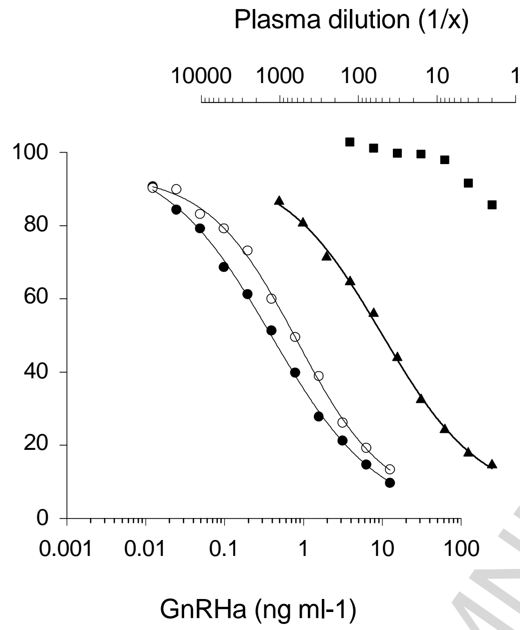

Fig. 2

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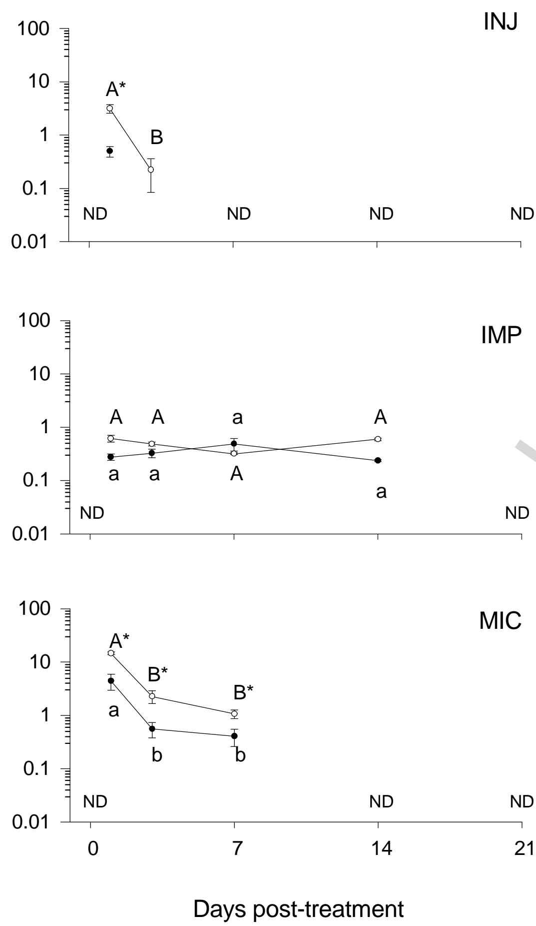

Fig. 3

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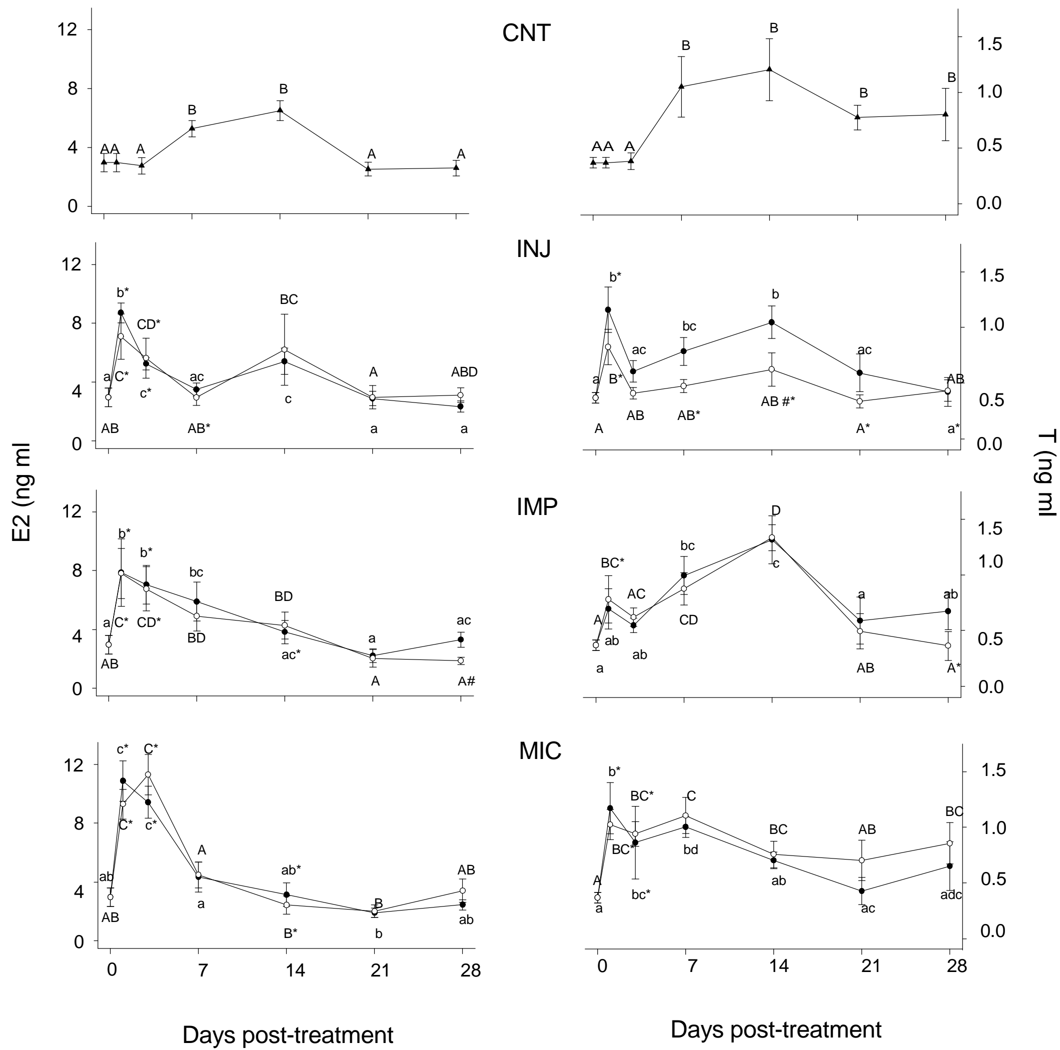

Fig. 4

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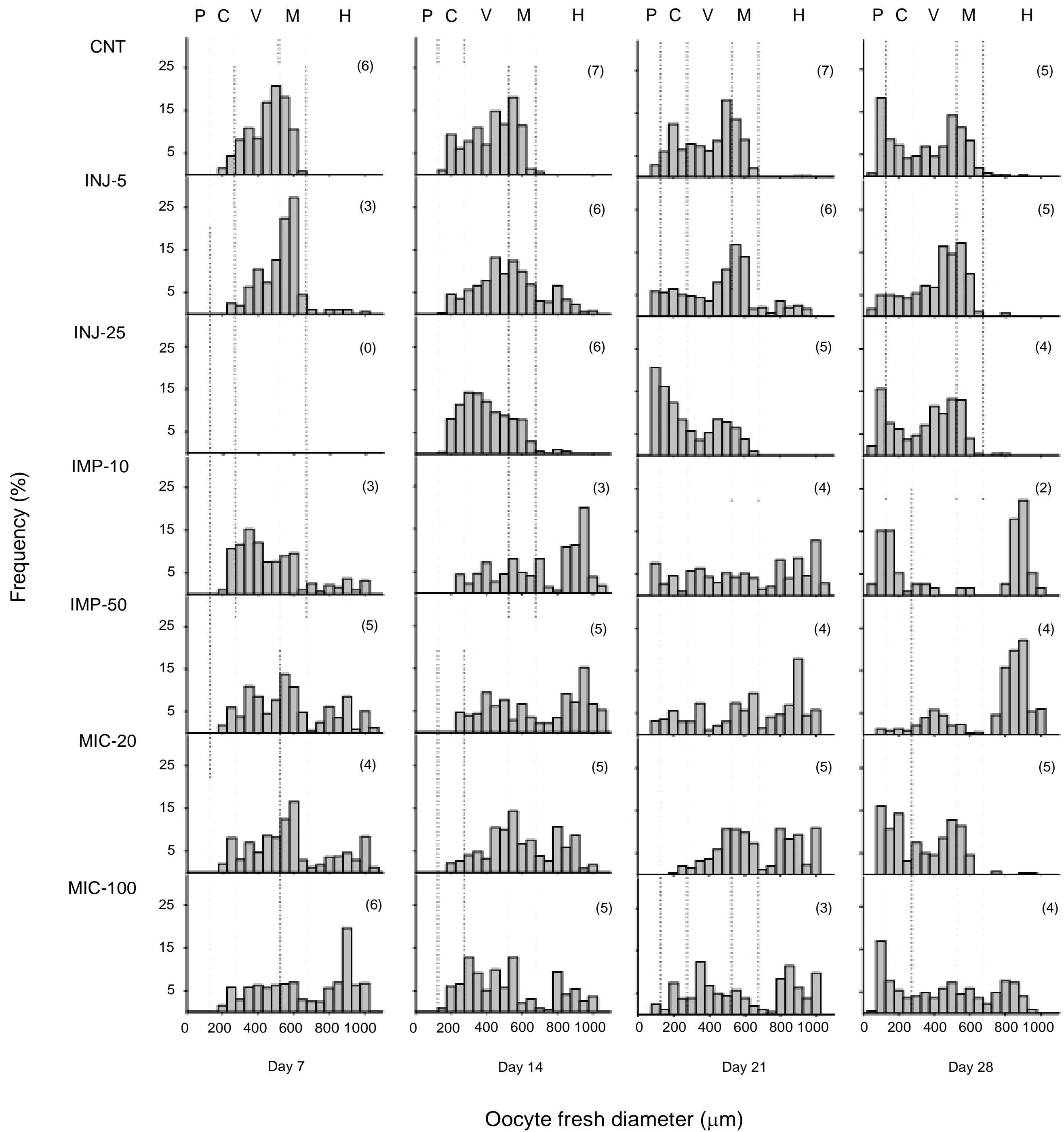

Fig. 5

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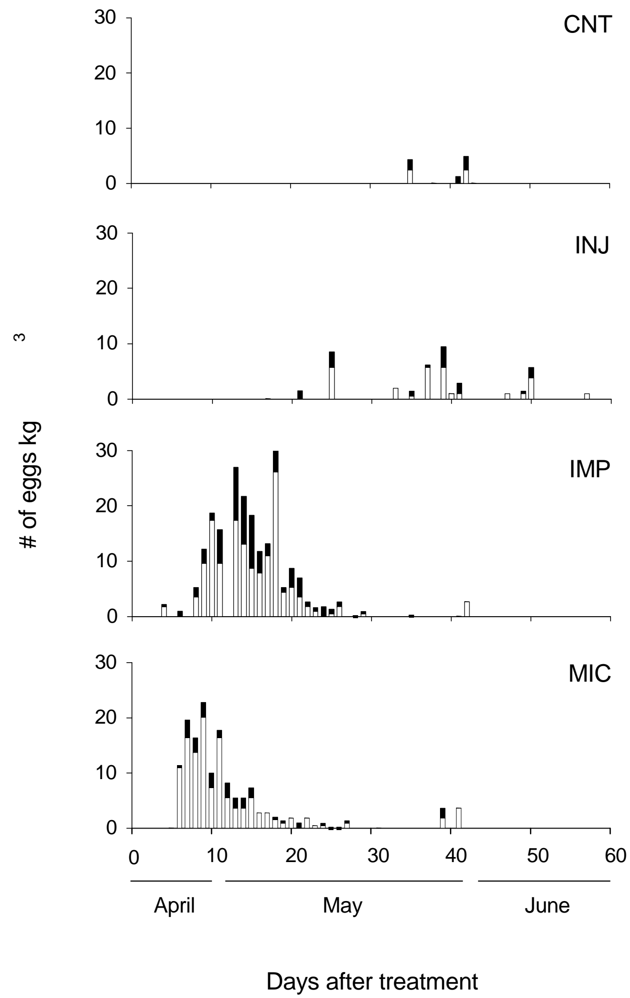

Fig. 6

ACCEPTED MANUSCRIPT

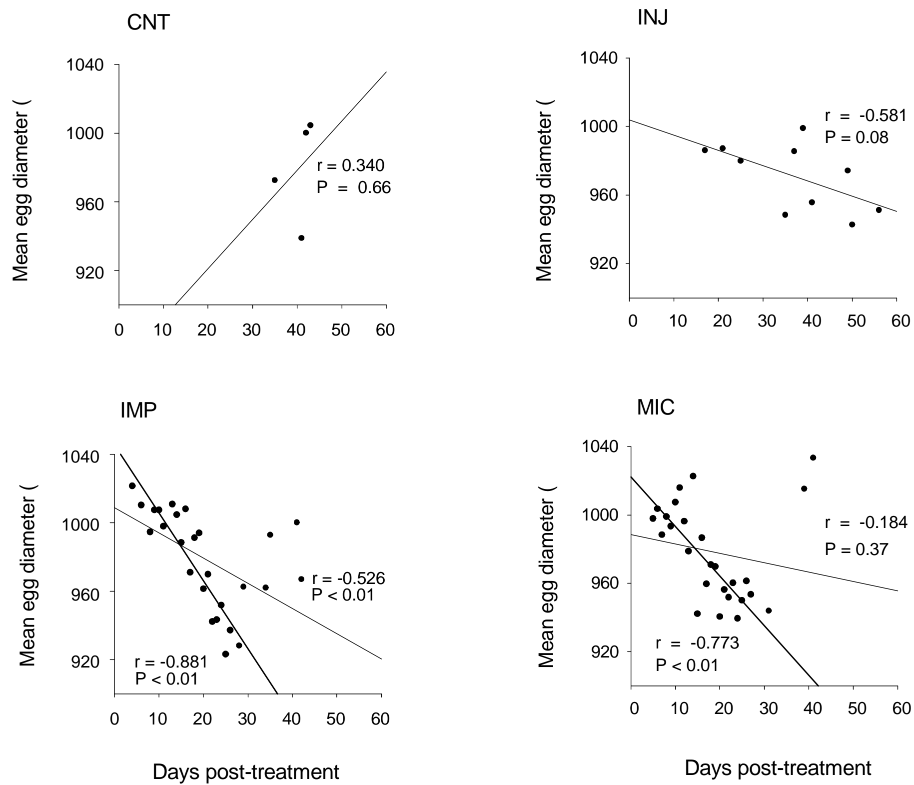

Fig. 7

EPTE