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Research paper

Endocrine regulation of long-term enhancement of spermiation in meagre (*Argyrosomus regius*) with GnRHa controlled-delivery systems

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ABSTRACT

Meagre (*Argyrosomus regius*) undergo spermatogenesis and spermiation when reared in captivity, but often produce low milt volumes, sometimes with reduced quality and for a limited time period. In the present study we a) compared the efficiency of gonadotropin releasing hormone agonist (GnRHa) implants versus injections on testicular stimulation and spermiation enhancement, b) investigated the effect of GnRHa on the endocrine spermiation regulation (sex steroid hormones), and c) evaluated a commercial induced spawning simulation scenario. Firstly, males (n = 5) were injected with 15.0 ± 0.2 μg GnRHa kg^{-1} (Injections) or implanted with 51.0 ± 5.1 μg GnRHa kg^{-1} (Implant) and compared their sperm production response. Secondly, the best hormonal treatment (Implant) was tested treating males (n = 8) with 57.5 ± 7.5 μg GnRHa kg^{-1} every 3 weeks for a period of 70 days. Milt production was improved by the GnRHa implants with only minor sperm quality alterations (improved sperm motility percentage). Elevated plasma testosterone (T) and 11-ketotestosterone (11-KT) levels were recorded in response to GnRHa implants, while no significant difference for 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) was observed. In the commercial induced spawning simulation, it was shown that meagre females are capable of on-demand induction of spawning at random intervals (5–21 days) using GnRHa injections, over a period of at least 2.5 months. During this period, spermiation enhancement was achieved with GnRHa implants every 3 weeks, producing sperm with stable, in general, quality and motility parameters. Percentage of motile cells, motility duration and density fluctuated significantly, but remained within levels that are considered appropriate for high fertilization success in this species.

1. Introduction

The availability of high-quality eggs and sperm is a prerequisite for the sustainable expansion of commercial aquaculture. In many commercially produced species, however, important reproductive dysfunctions hinder the efficient and reliable production of fertilized eggs (Mylonas et al., 2010). Although reproductive dysfunctions are most often seen in females (*e.g.* failure of oocyte maturation, ovulation and/or spawning) and cultured males usually complete spermatogenesis and spermiation, in some species the amount of good quality sperm produced proves to be limiting, both for spontaneous spawning and for artificial fertilization purposes (Mylonas et al., 2017). A variety of hormonal manipulations have been used in order to address these problems exhibited by male breeders.

The gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH) regulate testicular function through their action on the production of sex steroid hormones (Schulz et al., 2010). Synthesis and release of FSH and LH is controlled by hypothalamic hormones, mainly gonadotropin-releasing hormone (GnRH). In turn, the two gonadotropins regulate testicular function by stimulating the production of sex steroids (androgens, estrogens and progestins) and other growth factors (Miura and Miura, 2001; Schulz et al., 2010). Plasma levels of testosterone (T) and, mainly, 11-ketotestosterone (11-KT) increase gradually as spermatogenesis proceeds, reaching a maximum during spermiogenesis or just before the onset of spermiation, respectively (Vizziano et al., 2008). Then immature spermatozoa become mature under the influence of 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) or in some cases of 17,20β,21-trihydroxy-4-pregnen-3-one

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(17,20β,21-P or 20β-S) (Miura and Miura, 2003; Schulz et al., 2010).

Meagre (*Argyrosomus regius*) is one of the emerging species of the European aquaculture industry (Duncan et al., 2013) due to its excellent taste, low fat content and firm texture (Grigorakis et al., 2011; Monfort, 2010; Poli et al., 2003). It is a gonochoristic species with asynchronous or group – synchronous ovarian development (Abou Shabana et al., 2012; Duncan et al., 2012; Gil et al., 2013; Mylonas et al., 2013b; Schiavone et al., 2012) and in most cases females fail to undergo oocyte maturation at the completion of vitellogenesis, necessitating the use of exogenous hormones to induce maturation, ovulation and spawning (Duncan et al., 2012; Duncan et al., 2018; Fernández-Palacios et al., 2014; Mylonas et al., 2015; Mylonas et al., 2013a; Mylonas et al., 2016). The most current protocols for inducing spawning in meagre involve the use of weekly injections of GnRH agonists (GnRHa) in the females, which usually induce two spawns after 2 and 3 days (Duncan et al., 2018; Mylonas et al., 2015; Mylonas et al., 2016). This protocol when coupled with temperature control can be applied effectively for a period of at least 17 weeks (Mylonas et al., 2016).

Males on the other hand do undergo spermatogenesis and spermiation successfully in captivity, but often produce low volumes of milt (Mylonas et al., 2016; Ramos-Júdez et al., 2019; Santos et al., 2018) for a limited time period (Mylonas et al., 2016) and sometimes with reduced (Duncan et al., 2012) or variable quality (Mylonas et al., 2013a), and fall in the category of species with "poorly spermiating" males (Mylonas et al., 2017). To ensure adequate milt production, GnRHa has been administered either in controlled-release implants (Langer, 2019; Mylonas et al., 2013a; Mylonas et al., 2016) or in the form of repeated injections (Duncan et al., 2018; Soares et al., 2015) in all experiments inducing spawning, but a comparative study of these methods of spermiation enhancement has not been carried out so far and neither has a systematic study on the GnRHa enhancement effect of these treatments on the endocrine system and sperm quality parameters, in order to establish an optimized protocol that will result in high volumes of good quality sperm.

The aim of the present study was: a) to compare the efficiency of GnRHa implants versus multiple injections on testicular stimulation and spermiation enhancement in meagre in terms of sperm quantity and quality, b) to investigate the effect of GnRHa on the endocrine spermiation regulation, in terms of plasma sex steroid hormone production and c) to apply and evaluate the best method in a commercial scenario of on-demand induction of multiple spawnings with varying intervals between them, satisfying the potential needs of an industrial meagre hatchery.

2. Materials and methods

2.1. Broodstock maintenance

In the first experiment, a stock of hatchery-produced 4-year-old meagre was maintained in the Aqualabs facilities of the Institute of Marine Biology, Biotechnology and Aquaculture (IMBBC) at the Hellenic Centre for Marine Research (HCMR), Heraklion, Crete, Greece. The fish were maintained in a rectangular tank of 10-m^3 volume during the year, supplied with aerated well seawater and exposed to simulated natural thermal (16.2–21.4 °C) and photoperiodic conditions. The fish were individually tagged with passive integrated transponder (PIT) tags (AVID, UK). Feed (Vitalis Repro and Cal, Skretting S.A.) was given 5 days a week to apparent satiation. Monitoring of pH, dissolved oxygen (% saturation), NH_3-N and NO_2-N was conducted once a week. The second experiment was carried out 2 years later with a stock of hatchery-produced 6 and 8-year-old meagre maintained in the Aqualabs. The fish were maintained under the same conditions as above.

The experimental protocol was approved by the National Veterinary Service (PN 255,356 - ΑΔΑ: 6ΛΙ17ΛΚ-ΠΛΩ). All procedures were conducted in accordance to the "Guidelines for the treatment of animals in behavioral research and teaching" (Anonymous, 1998), the Ethical justification for the use and treatment of fishes in research: an update (Metcalfe and Craig, 2011) and the "Directive 2010/63/EU of the European parliament and the council of 22 September 2010 on the protection of animals used for scientific purposes" (EU, 2010).

2.2. Evaluation of reproductive stage and hormonal treatments

Evaluation of reproductive stage and selection of breeders was done in late April - early May after a 2-day starvation period. Fish were initially tranquilized in their tank with the use of clove oil (0.01 ml l^{-1}). Then, the fish were transferred to a separate tank for complete sedation with a higher concentration of clove oil (0.03 ml l^{-1}) (Mylonas et al., 2005). The fish were selected according to their spermiation condition –which is a measure of the available milt in the testes- after gentle abdominal pressure was applied, determined by a subjective scale from 0 to 3, as follows: Spermiation index S0 = no milt released, S1 = only a drop of milt released after multiple stripping attempts, S2 = milt was released easily after the first stripping attempt and S3 = copious amounts of milt released with very little pressure (Mylonas et al., 2016). Milt obtained from males at a spermiation condition of ≤S1 could not be used for sperm quality evaluation (too small a volume and often contaminated with urine or feces). To obtain milt for evaluation (50–100 μl), the genital pore was rinsed with freshwater and blot dried and care was taken after stripping to avoid contamination of samples with feces or urine. Milt was collected using a positive displacement pipette. The collected milt sample was stored in a 500-μl centrifuge tube, placed on ice and then transferred to 4 °C refrigerator until evaluation, immediately after the end of the sampling. Only males with a spermiation index of ≥S1 were considered eligible for hormonal treatment and were used in the experiment. Blood (2.5 ml) was collected from the fish at each sampling in order to estimate the concentration of sex steroids in the plasma. The same procedure (evaluation of spermiation condition, sperm quality, blood collection) was done at each sampling.

2.2.1. GnRHa implant versus injections

In the experiment comparing a GnRHa implant versus two injections spaced 12 days apart, male fish (n = 15; mean body weight (BW) ± SD, 3.76 ± 0.97 kg) were divided equally in three groups. Control fish were punched with an empty needle. Another group of males was injected with desGly^{10}, DAla^6, Pro^9-GnRH-NEthylamide (GnRHa, H-4070, Bachem, Switzerland) with an effective dose of 15.0 ± 0.2 μg GnRHa kg^{-1} BW (Injections) (Duncan et al., 2018; Fernández-Palacios et al., 2014; Ramos-Júdez et al., 2019). The injection was given in the sinus created by the epaxial musculature and the spinal cord, at the level of the posterior base of the dorsal fin. A third group of males was given a GnRHa implant (effective dose of 51.0 ± 5.1 μg GnRHa kg^{-1} BW) constructed with [Ethyl-Vinyl Acetate]-copolymer (Implant) (Mylonas et al., 2015; Mylonas et al., 2013a; Mylonas et al., 2016). After treatment, fish were placed in three separate 5-m^3 rectangular tanks supplied with aerated well seawater at 20.7 ± 0.1 °C and were exposed to simulated natural photoperiodic conditions. Fish were sampled on Days 0, 2, 5, 12 and 19 after the treatment. A 2nd GnRHa injection was administered on Day 12 to the Injection group only. At the final sampling, one representative fish (in regards to spermiation index) from each treatment group was sacrificed and gonadosomatic index (GSI) was calculated. Also, portions of the testes were fixed in a solution of 4% formaldehyde-1% glutaraldehyde for further histological processing.

2.2.2. Commercial scenario

In the next experiment we evaluated the efficacy of the best spermiation enhancement treatment from above in a commercial scenario of on-demand induction of multiple spawnings. The ovulation induction

protocol used a modification of an earlier study that showed that female meagre were capable of spawning reliably for a period of at least 17 weeks, in response to weekly injections of 15 μg GnRHa kg^{-1} body weight (Mylonas et al., 2016). In the present study we wanted to examine if meagre would spawn equally well if the GnRHa treatment was not done in regular (*i.e.* weekly) intervals. As in previous studies, females were selected for the experiment after examining a wet mount of an ovarian biopsy under a compound microscope (40 and 100x), and evaluating the stage of oogenesis (full vitellogenesis) and measuring the mean diameter of the largest, most advanced batch of vitellogenic oocytes (n = 10). Females were considered eligible for GnRHa treatment, if they had vitellogenic oocytes of > 550 μm in diameter and very little atresia present (Mylonas et al., 2015; Mylonas et al., 2013a; Mylonas et al., 2016) and were placed in each tank after a GnRHa injection of 15 μg GnRHa kg^{-1} BW. Male fish (n = 8, 6.2 ± 1.0 kg BW) were treated with GnRHa implants for an effective dose of 57.5 ± 7.5 μg GnRHa kg^{-1} BW and were divided equally in four 5-m^3 rectangular tanks (2 fish per tank) containing a single GnRHa-injected female (15 μg GnRHa kg^{-1}), supplied with aerated well seawater of 19.5 ± 0.1 °C and exposed to simulated natural photoperiodic conditions. For the GnRHa implant treatments of the males, dose variations were caused by the fact that implants are loaded with fixed amounts of GnRHa. Even though implants loaded with different amounts of GnRHa were used, it was still not possible to adjust the dose exactly to the different body weights of the fish. Fish (males and females) were sampled again on Days 7, 23, 28, 42, 63 and 70 after the first treatment. Females were injected with the same dose of a GnRHa injection at each sampling. Males were treated with the same GnRHa implants of the same dose approximately every 3 weeks (Days 23, 42 and 63). Spawning was monitored daily, and egg collection was done in a 250-L passive egg collectors supplied with water from the surface outflow of each tanks.

2.3. Sperm quality evaluation

2.3.1. GnRHa implant versus injections

The sperm quality parameters that were evaluated during the first experiment included: (a) sperm density (number of spermatozoa ml^{-1} of milt), (b) survival of spermatozoa under cold storage at 4 °C (spermatozoa survival, days), (c) initial percentage of spermatozoa showing forward motility immediately after activation (spermatozoa motility, %) and (d) duration of forward spermatozoa motility of ≥5% of the spermatozoa in the field of view (motility duration, min). Spermatozoa density was estimated after a 2121-fold dilution with 0.9% saline using a Neubauer haemocytometer under 200X magnification (in duplicate) under a compound light microscope. In order to estimate spermatozoa survival time (days), milt was stored at 4 °C for the days after collection and was examined every other day for spermatozoa motility until forward motility was reduced to < 5%, as was explained for duration of forward motility earlier. Spermatozoa motility was evaluated on a microscope slide (400X magnification) after mixing 1 μl of milt with a drop of about 50 μl of saltwater (in duplicate). Activated sperm samples were observed under the compound light microscope (Nikon, Eclipse 50i) 10 sec after activation. Spermatozoa motility (%) was determined subjectively using increments of 10%.

2.3.2. Commercial scenario

In the commercial simulation experiment sperm quality parameters that were evaluated as above included: (a) sperm density, (b) survival of spermatozoa under cold storage at 4 °C, (c) duration of forward spermatozoa. Additionally, milt samples were assessed using computer-assisted sperm analysis (CASA, ISAS, Spain). Milt samples were activated in seawater (1:334) to obtain 200–300 cells in the field and spermatozoa movement was recorded on a disposable counting chamber with a fixed depth (Leja) using a digital camera (The Imaging Source DMK 22BUC03) with a resolution of 744x480 pixels at 30 fps attached to a light microscope (Zeiss Primo Star) under x200 magnification, using dark field microscopy, immediately after milt collection. The analyzed parameters were curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP) (μm s^{-1}), motile cells, progressive cells (> 80% straightness - STR), rapid cells and STR (%). The software settings were adjusted to: 1 to 90 μm for head area; VCL < 10 μm s^{-1} to classify a spermatozoon as immotile; and spermatozoa were considered rapid when VCL was higher than 100 μm s^{-1}.

2.4. Evaluation of egg quality

Egg collectors were checked daily. For each spawn, date, collection time and developmental stage were recorded in order to identify different spawns. Eggs were collected and transferred into a 10-l bucket. Their number (fecundity) was estimated by counting the total number of eggs in a sub-sample of 10 ml collected with a pipette after vigorous agitation. Fertilization percentage was evaluated at the same time by examining each egg in the subsample for the presence of viable embryos.

2.5. Plasma steroid hormone evaluation

Plasma steroid hormone concentrations were quantified with the use of enzyme-linked immunosorbent assays (ELISAs) (Cuisset et al., 1994; Nash et al., 2000; Rodríguez et al., 2000), validated for meagre, with the following sensitivity and accuracy characteristics: The minimum levels of detection for T, 11-KT and 17,20β-P, at 90% of binding, were 0.5, 0.5 and 1 pg of steroid concentration, whereas the intra- (n = 5) and inter-assay (n = 5) coefficients of variation, at 50% of binding, were 3.6% and 9.6% for T, 3.4% and 7.9% for 11-KT and 3.1% and 9.8% for 17,20β-P, respectively. Reagents used for the ELISAs were purchased from Cayman Chemical Company (Ann Arbor, Michigan, USA). For running in the ELISAs, plasma (200–300 μl) was extracted twice with diethyl ether (1:10 vol), followed by vigorous vortexing (Vibramax 110, Heidolph, Germany) for 3 min. After decanting of the organic phase, drying of the supernatant was done under a stream of nitrogen (Reacti-vap III, Pierce, USA). Samples were reconstituted in assay buffer before analysis.

2.6. Histological analysis

For histological processing, testes were dehydrated in increasing ethanol concentrations (70–96%) and embedded in methacrylate resin (Technovit 7100®, Heraeus Kulzer, Germany). Serial sections (4 μm) were obtained with a microtome (Leica RM 2245, Germany) and stained with Methylene Blue (Sigma, Germany)/Azure II (Sigma, Germany)/Basic Fuchsin (Polysciences, USA) according to Bennett et al. (Bennett et al., 1976). After drying and covering with a coverslip, section slides were examined under a light compound microscope (Nikon, Eclipse 50i) and photographed with a digital camera (Jenoptik progress C12 plus).

2.7. Statistical analysis

Mean differences in the measured parameters (sperm motility, duration, density, survival, percentage of motile, progressive and rapid cells, VCL, VSL, VAP, STR, T, 11-KT and 17,20β-P) were tested either using a two-way repeated measures analysis of variance (ANOVA) for the GnRHa implant versus injections experiment or a one-way repeated measures ANOVA for the commercial scenario trial, followed by Tukey's HSD post hoc test. Differences in spermiation index for both experiments were tested using the nonparametric Friedman's test followed by Tukey's post hoc test. Data were transformed accordingly (arcsine transformation for percentages and $\log_{10}$ transformation for the rest continuous parameters) to meet the ANOVA assumptions, if not normally distributed. No differences were observed among the three

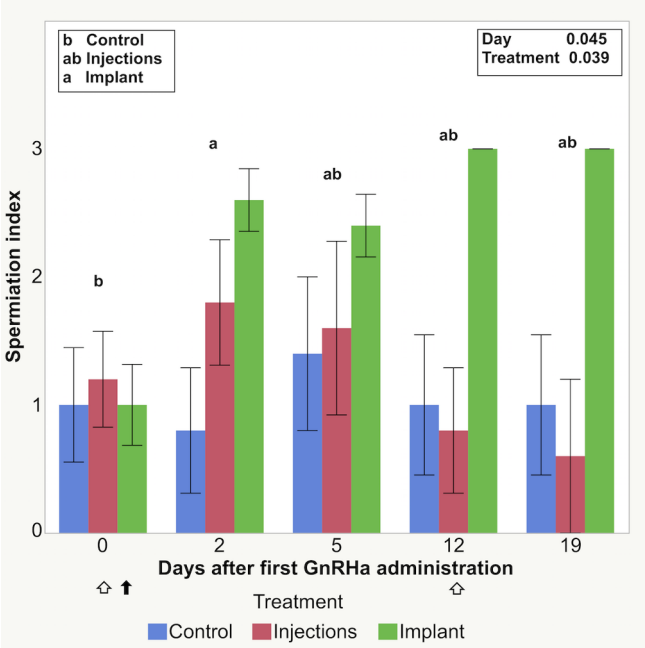

Fig. 1. Mean (± SEM) spermiation index of meagre (*Argyrosomus regius*) treated with GnRHa injections or implant (n = 5 individuals in each group). Lowercase letters in the legend or above the sample means indicate statistically significant differences among the different treatment groups or sampling times after the first GnRHa administration (Friedman's test, Tukey's HSD, $P \leq 0.05$). Arrows on the x-axis indicate the time of GnRHa administration (open thick – Injections, solid thin –Implant).

hormone treatment groups in Day 0 (one-way ANOVA). A level of $P \leq 0.05$ was set as minimum statistical significance for the post hoc test. Statistical analyses were performed with JMP 12 (SAS Institute Inc., Cary, NC, USA). Results are presented as mean ± standard error (SEM), unless mentioned otherwise.

3. Results

3.1. GnRHa implant versus injections

Mean spermiation index was very low (S1) at the start of the GnRHa Implant vs Injections experiment, and most fish did not produce adequate milt for sperm quality evaluations (Fig. 1). Treatment with GnRHa implant increased significantly milt production compared to the Control but not to the Injections group (Friedman's test, $P = 0.039$, Tukey's HSD, $P \leq 0.05$). A significant increase of milt production was observed on Day 2 after GnRHa treatment (Friedman's test, $P = 0.045$, Tukey's HSD, $P \leq 0.05$). The fish from the Implant group showed increased spermiation index throughout the study, while the injected males exhibited a gradual reduction in spermiation towards the final sampling, even after a 2nd GnRHa injection administered on Day 12.

Plasma T was higher in the GnRHa Implant group compared to the Control (two-way ANOVA, $P < 0.001$, Tukey's HSD, $P \leq 0.05$), while on Day 2 we observed the highest T level of the experiment (Fig. 2**A**). Plasma 11-KT was significantly higher in the Implant group compared both to the Control and the Injections group (two-way ANOVA, $P < 0.001$, Tukey's HSD, $P \leq 0.05$) (Fig. 2**B**). Plasma 17,20β-P was not found to be different among the three treatment groups (two-way ANOVA, $P = 0.586$) (Fig. 2**C**).

Regarding sperm quality parameters, sperm motility was only marginally different among the treatment groups (two-way ANOVA, $P = 0.046$), but not enough to differentiate from each other (Tukey's HSD, $P \leq 0.05$), and was lower on Day 5 compared to Day 2 (two-way ANOVA, $P = 0.003$, Tukey's HSD, $P \leq 0.05$) (Fig. 3**A**). Sperm motility duration, density and survival under cold storage were not different among the three experimental groups (Fig. 3**B-3D**).

The GSI of the sacrificed males from the Control, Injections and Implant treatment groups was 0.33%, 0.59% and 1.56%, respectively, having a spermiation index of S0, S0 and S3, respectively (data not shown). The histology sections of the testes were compatible with the overall milt production, with the control and injected fish showing increased proportion of somatic tissue and spermatogonia compared to the Implant treated fish which contained lobules full with spermatozoa, but also a number of spermatocysts in active spermatogenesis (Fig. 4).

3.2. Commercial scenario

In the commercial simulation experiment, females spawned 1–3 times after each GnRHa injection and produced 50,565 ± 5,818 eggs kg^{-1} with 77 ± 6% fertilization (Fig. 5**A**). Treatment of males with the GnRHa implants increased significantly spermiation index 7 days after the first GnRHa treatment and maintained milt production to these levels up to the end of the experiment after the repeated administrations (Friedman's test, $P = 0.011$, Tukey's HSD, $P \leq 0.05$) (Fig. 5**B**). A trend towards increased milt production was observed 7 days after the 2nd treatment as well, but not after the 4th treatment.

Plasma T (one-way ANOVA, $P = 0.002$, Tukey's HSD, $P \leq 0.05$) (Fig. 6**A**) and 11-KT (one-way ANOVA, $P = 0.015$, Tukey's HSD, $P \leq 0.05$) (Fig. 6**B**) increased significantly 7 days after the first GnRHa treatment and 11-KT remained at the same levels for the whole experiment, while T decreased on Day 42 and remained unchanged thereafter. Treatment with GnRHa implants did not seem to affect the plasma levels of 17,20β-P since in after both the 1st and 2nd administrations no change was found, however, a difference between day 42 and 70 was observed (one-way ANOVA, $P = 0.033$, Tukey's HSD, $P \leq 0.05$) (Fig. 6**C**).

The percentage of motile cells remained unchanged almost throughout the second experiment, however, a difference between day 42 and 70 was observed (one-way ANOVA, $P = 0.020$, Tukey's HSD, $P \leq 0.05$) (Fig. 7**A**). The percentage of progressive cells (data not shown) was significantly different among the different samplings after the first GnRHa administration (one-way ANOVA, $P = 0.040$), but not enough to differentiate from each other (Tukey's HSD, $P \leq 0.05$), while no differences were observed in the percentage of rapid. Duration of sperm motility was different among the samplings (one-way ANOVA, $P < 0.001$, Tukey's HSD, $P \leq 0.05$) and an increasing trend 7 days after each sampling was observed (Fig. 7**B**), a pattern that was also observed in sperm density (one-way ANOVA, $P = 0.005$, Tukey's HSD, $P \leq 0.05$) (Fig. 7**C**). No changes were observed in sperm survival at cold storage (one-way ANOVA, $P = 0.153$) (Fig. 7**D**). The VCL, VSL, VAP and STR (data not shown) remained unchanged (one-way ANOVA, $P > 0.05$) throughout the second experiment and their values were 91 ± 5 μm sec^{-1}, 59 ± 3 μm s^{-1}, 79 ± 4 μm s^{-1} and 77 ± 2% , respectively.

4. Discussion

As it is generally the case for fish reproduction in captivity, meagre females have received much more research attention than males, due to the more apparent reproductive dysfunctions they display, including lack of oocyte maturation, ovulation and spawning. Males have received less attention, since non-spermiating males are rarely found during the spawning period. However, reproductive dysfunctions such as decreased milt quantity, deteriorated sperm quality and lack of spawning behavior can severely affect fertilized egg production and quality, and should not be overlooked (Duncan et al., 2012; Mylonas et al., 2013b). In the context of expanding the spawning period of meagre, as one of the emerging species of the European aquaculture, and after the establishment of a reliable weekly spawning induction protocol for females (Duncan et al., 2018; Mylonas et al., 2015), the

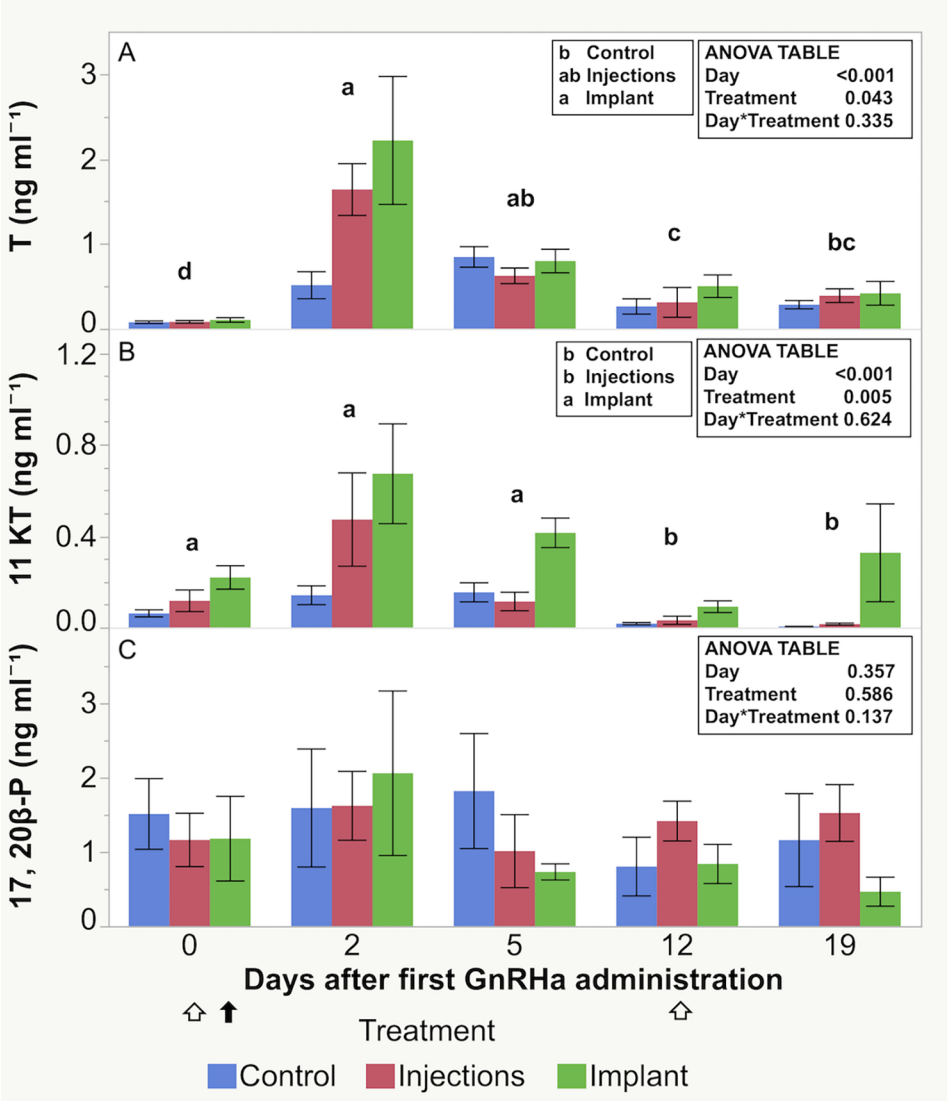

Fig. 2. Mean (± SEM) plasma A. testosterone (T), B. 11-ketotestosterone (11-KT) and C. 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) in male meagre (*Argyrosomus regius*) treated with GnRHa injections or implant (n = 5 individuals in each group). Lowercase letters in the legends or above the sample means indicate statistically significant differences among the different treatment groups or sampling times after the first GnRHa administration (two-way ANOVA, Tukey's HSD, $P \leq 0.05$). Arrows on the x-axes indicate the time of GnRHa administration (open thick – Injections, solid thin – Implant).

efforts of the present study were focused on stimulating long term spermiation in meagre males, in order to prolong the spawning season and obtain good quality sperm.

The GnRHa implant administration enhanced spermiation in meagre and increased milt production compared to non-treated Controls. GnRHa implanted fish maintained the highest spermiation index for almost 3 weeks, in contrast to the injected fish, which increased their milt production for only a week, while a second injection did not improve sperm production. Milt production was also improved by GnRHa implantation for > 2 months, after a GnRHa administration of about every 3 weeks. In a similar study, milt production was maintained for 17 weeks in meagre after repeated GnRHa implant administration, but in that study each male was treated with a different frequency, based on spermiation index (Mylonas et al., 2016). In a previous experiment in aquaculture conditions, meagre maintained the highest spermiation index for only a month at the peak of the breeding season without any hormonal stimulation (Mylonas et al., 2013b), showing that milt production could be a limiting factor for the expansion of the spawning season of this species, if no hormonal therapies are used. On a comparative spermiation enhancement study in European seabass (*Dicentrarchus labrax*) GnRHa implants of 20 μg kg^{-1} significantly increased the expressible milt production compared to control and GnRHa injected fish (Rainis et al., 2003), showing a similar response pattern to the hormonal treatments with the current study. Implants loaded with GnRHa were also used successfully to increase milt volume in the starry flounder (*Platichthys stellatus*), a species with a usually viscous milt production (Moon et al., 2003), yellowtail flounder (*Pleuronectes ferrugineus*) (Clearwater and Crim, 1998) and in the Atlantic cod (*Gadus morhua*) (Garber et al., 2009).

Regarding sex steroid levels, GnRHa implants significantly increased both T and 11-KT, while GnRHa injections affected only plasma T levels. Plasma T and 11-KT are known to increase gradually as spermatogenesis proceeds, and then decrease at spermiation (Schulz et al., 2010). In meagre males, elevated 11-KT was observed before the onset of full spermiation period while two peaks of T were recorded, one at the middle of the spawning season and a second one at the end of the spawning season (Mylonas et al., 2013b). Higher 11-KT level in the Implant group was observed for 19 days of the comparative experiment. Testicular 11-KT is considered the main reproductive steroid in males regulating spermatogenesis (Schulz et al., 2010) and was possibly related both with the increased milt production of the Implant treated males and the active spermatogenesis that was observed in the

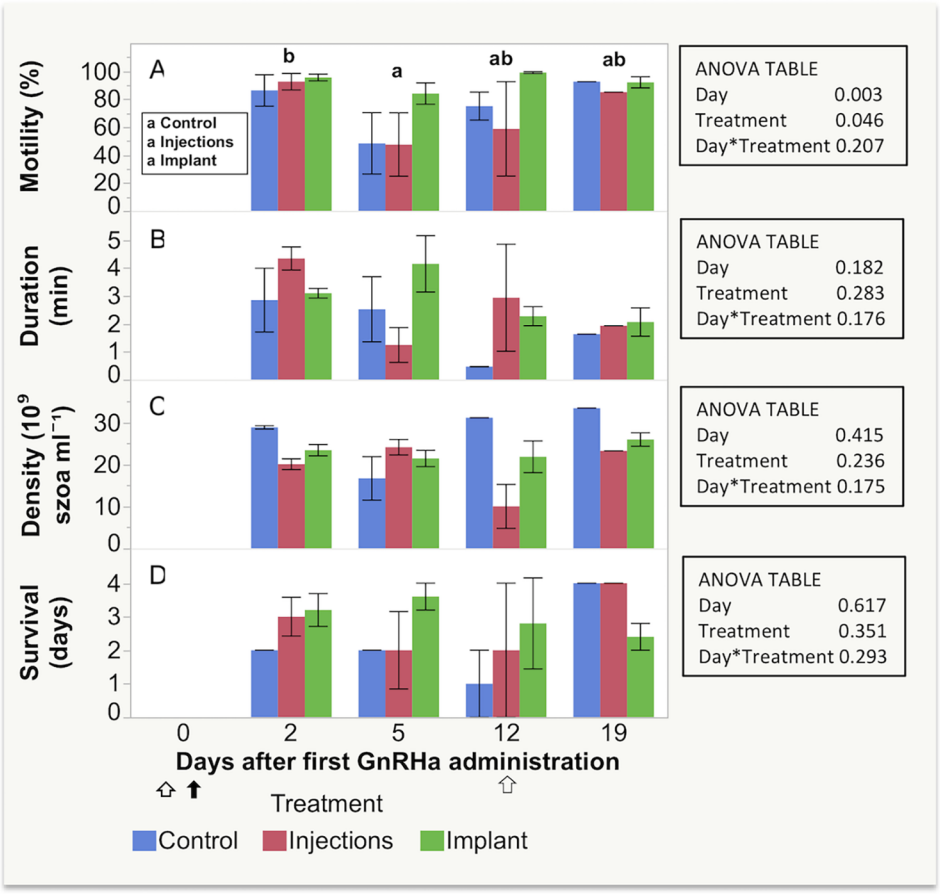

Fig. 3. Mean (± SEM) A. spermatozoa motility (%), B. motility duration (min), C. density (x10^9 spermatozoa ml^{-1}) and D. survival under cold storage at 4 °C (days) of sperm collected from male meagre (*Argyrosomus regius*) treated with GnRHa injections or implant (n = 5 individuals in each group). Lowercase letters in the legends or above the sample means indicate statistically significant differences among the different treatment groups or sampling times after the first GnRHa administration (two-way ANOVA, Tukey's HSD, $P \leq 0.05$). Arrows on the x-axes indicate the time of GnRHa administration (open thick – Injections, solid thin – Implant).

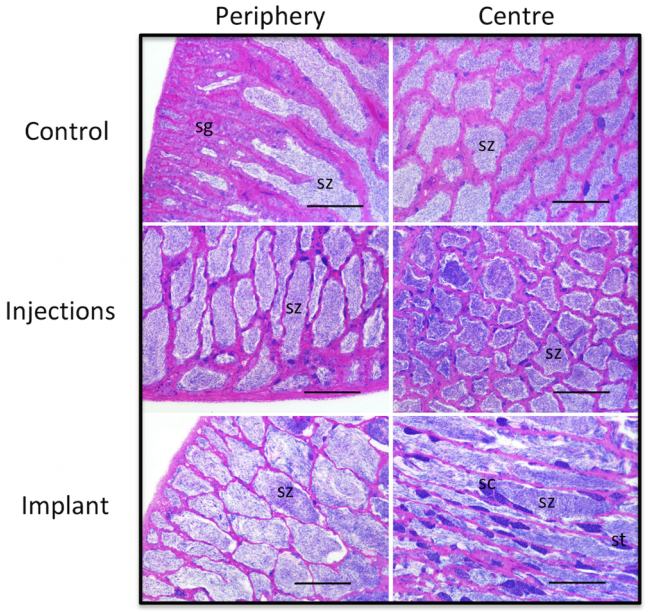

Fig. 4. Microphotographs of testes histological sections of meagre (*Argyrosomus regius*) treated with GnRHa injections or implant, at the final day (Day 19) of the experiment. sg: spermatogonia, sc: spermatocyte, st: spermatid, sz: spermatozoa. Bars = 200 μm.

histological sections. High levels of 11-KT were observed after a GnRHa treatment with controlled-release delivery systems in striped bass (*Morone saxatilis*) (Mylonas et al., 1997b), but not in white bass (*Morone chrysops*) (Mylonas et al., 1997a). Also elevated plasma levels of both T and 11-KT after treatment with GnRHa implants were also observed in slightly spermiating greenback flounder (*Rhombosolea tapirina*), together with increased milt production, as in the current study (Lim et al., 2004). However, an elevation of these plasma androgens was not associated with the significant increase in milt volume obtained after treatment with GnRHa controlled-release systems in some other teleosts (Clearwater and Crim, 1998; Mañanos et al., 2002; Mylonas et al., 1997a). Instantaneously measured plasma androgen levels may not correlate well with the significant GnRHa induced increases in milt volume, which is a parameter reflecting the cumulative production of sperm by the testes.

Contrary to plasma androgens in the present study, no significant difference in 17,20β-P was observed among the different treatment groups, although this steroid has been shown to be well-related with milt production in many fish species (Mylonas et al., 1997a; Scott et al., 2010), but not in others (Clearwater and Crim, 1998; Mañanos et al., 2002). On the other hand, in another species of the family Sciaenidae, the spotted seatrout (*Cynoscion nebulosus*), it has been shown that 20β-S is more potent in inducing oocyte maturation than 17,20β-P (Thomas et al., 1997). In males of the same species, a 20β-S receptor was found in testicular and sperm plasma membranes and its binding on these membranes was increased after gonadotropin stimulation *in vitro*, leading to the assumption that 20β-S, and not 17,20β-P, is the maturation inducing steroid, at least for this species (Thomas et al., 1997), and possibly for other Sciaenidae species -such as meagre as well.

Sex steroid hormone levels have been shown to be quite low in meagre during the natural spawning season in captivity, both in females and in males, but these relatively low levels do not seem to prevent the process of vitellogenesis or spermatogenesis (Mylonas et al., 2013b). Mean cortisol levels have been also shown to be low in meagre, compared to other fish species, and these low levels were attributed to the specific metabolic needs and the habitat of each species (Fanouraki

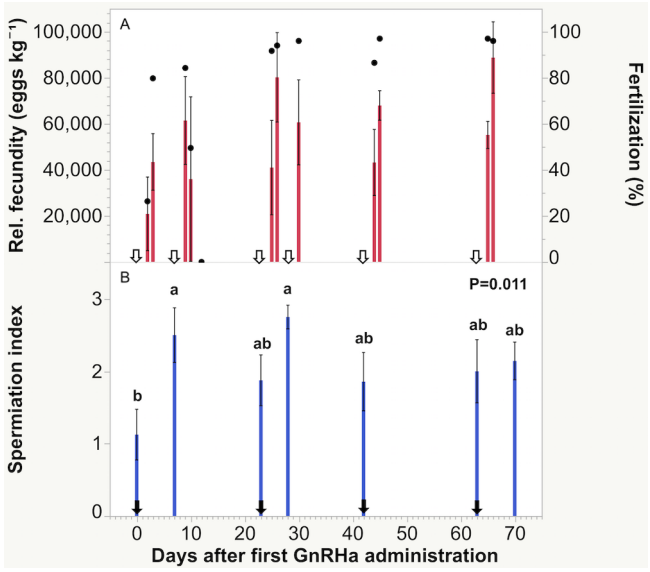

Fig. 5. A. Mean (± SEM) daily relative fecundity (bars, eggs kg^{-1} female) and fertilization success (circles, %) of meagre (n = 4) (*Argyrosomus regius*) females after repeated GnRHa injections at various intervals (open arrows). B. Mean spermiation index (± SEM) of male meagre (n = 8) at different days after repeated GnRHa implant administration (solid arrows). Lowercase letters above the spermiation index means indicate statistically significant differences among sampling times after the first GnRHa administration (Friedman's test, Tukey's HSD, P ≤ 0.05).

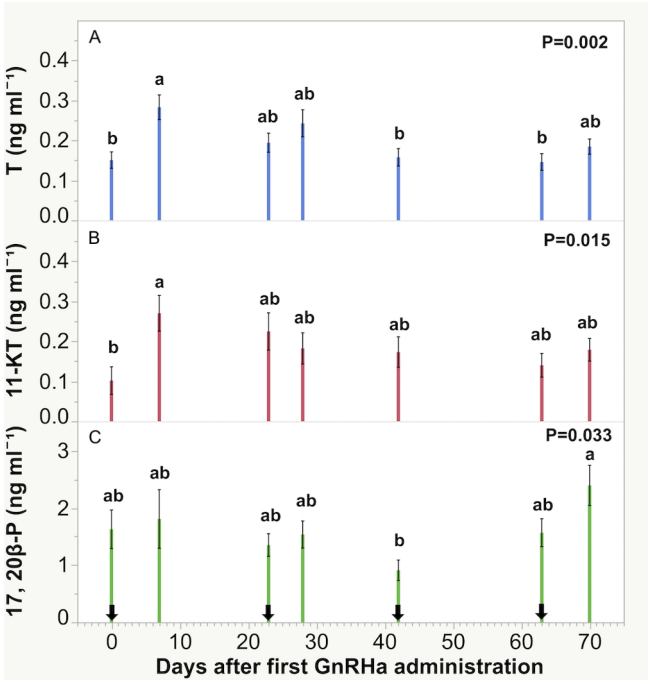

Fig. 6. Mean (± SEM) plasma A. testosterone (T), B. 11-ketotestosterone (11-KT) and C. 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) concentrations of male meagre (*Argyrosomus regius*) (n = 8) at different days after consecutive GnRHa implant administration (solid arrows). Lowercase letters above the plasma hormone means indicate statistically significant differences among sampling times after the first GnRHa administration (one-way ANOVA, Tukey's HSD, P ≤ 0.05).

et al., 2011). Nevertheless, the increase in both androgens after GnRHa treatment, coupled with the increase in milt production and the fact that the testes of the implanted fish continued to exhibit spermatogenic activity at the end of the experiment, suggest that low sex steroid concentrations do have an effect on spermiation and their increase after GnRHa treatment is related to the achievement of a long term full spermiation period in meagre.

Regarding sperm quality parameters in the GnRHa implant versus injections comparative experiment, no differences between the treatment groups were observed, showing that GnRHa treatments did not have an influence on sperm quality parameters. This is a typical phenomenon reported in many fishes, demonstrating that enhancement of spermiation with exogenous hormones is not usually followed by either improvements or deteriorations in sperm quality parameters (Mylonas et al., 2017). Compared to values reported during monitoring the reproductive season of meagre in captivity (Mylonas et al., 2013b), both sperm motility and motility duration in the present study were higher, while sperm density was at the same levels with previous studies (Mylonas et al., 2013b; Ramos-Júdez et al., 2019).

The testes of the Control and Injections group exhibited thickened connective tissue concomitantly with only a few scattered spermatocysts with spermatocytes and spermatids, typical of regressing testes in this species (Prista et al., 2014; Zupa et al., 2020), as well as other teleosts (Rainis et al., 2003). Although large numbers of spermatozoa were visible in the histological sections, meagre exhibiting this stage of reproductive development may or may not express milt upon application of abdominal pressure (Prista et al., 2014). On the contrary, the histological sections of the testes of the implanted fish showed active spermatogenesis with a higher number of spermatocysts containing spermatocytes, spermatids, as well as free spermatozoa; also this group's GSI was similar to values reported for "spawning-capable" fish (Prista et al., 2014). Therefore, as it was shown in an earlier study, treatment with GnRHa implants resulted in the maintenance of spermatogenesis, with the production of more spermatozoa over a prolonged period of time in meagre (Mylonas et al., 2016).

In the commercial simulation experiment, females spawned repeatedly and consistently after each GnRHa injection, as in the established induction protocol for oocyte maturation, ovulation and spawning using weekly injections (Duncan et al., 2018; Mylonas et al., 2015; Mylonas et al., 2016). To simulate a typical situation in a commercial hatchery, in the current study random intervals (5 to 21 days) between the GnRHa injections were used and the relative fecundity was similar (Mylonas et al., 2016) or lower (Duncan et al., 2018; Mylonas et al., 2015) to what was reported previously, while fertilization success was at the same levels (Mylonas et al., 2015) or lower (Duncan et al., 2012; Duncan et al., 2018; Mylonas et al., 2016). This suggests that allowing a period longer than 1 week to pass between consecutive GnRHa injections may at times result in a reduction in vitellogenesis and the recruitment of oocytes, resulting in lower fecundity when fish are induced to mature. Still, the results demonstrate that meagre are capable of on-demand induction of spawning over a period of > 2 months using injections of GnRHa, with the production of high quality eggs. We believe this protocol offers significant benefits to commercial operations.

The spermiation index increased significantly after the first GnRHa implantation in the commercial simulation experiment, when most fish were barely expressing enough milt to be identified as males. Thereafter, in response to GnRHa implantation every 3 weeks, spermiation index –which is a subjective measure of the available milt in the testes- was maintained unchanged for a period of > 2 months. The percentage of motile spermatozoa showed an increasing trend on Day 42 and significantly decreased on Day 70, not showing a logical pattern of change. This change coincided with an exactly opposite pattern of 17,20β-P increase. To our knowledge, sperm motility characteristics are connected with seminal plasma pH, which in turn can be affected by the 17,20β-P (Clearwater and Crim, 1998; Moon et al., 2003). Unfortunately, seminal plasma changes were not recorded during this experiment.

Motility duration increased significantly one week after the second

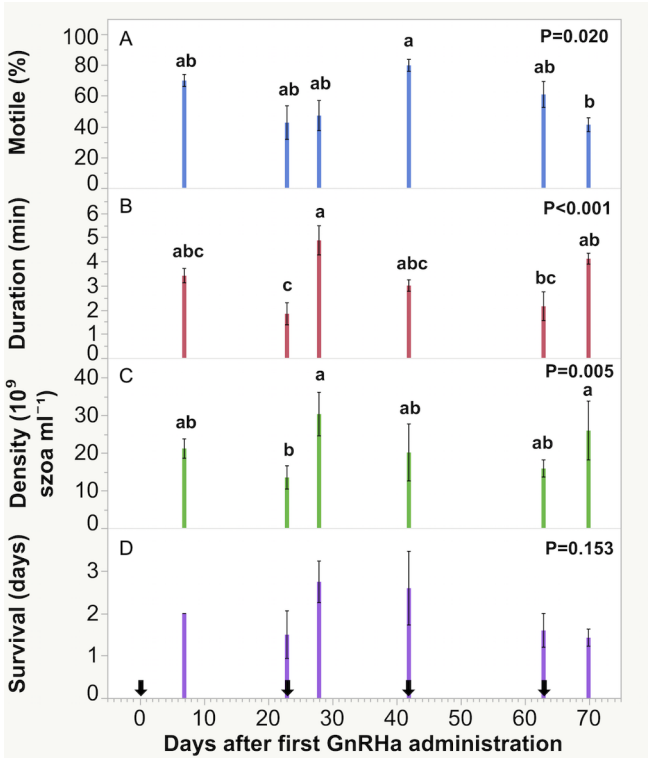

Fig. 7. Mean (± SEM) A. motile cells (%), B. motility duration (min), C. density (x10^9 szoa ml^{-1}) and D. survival under cold storage at 4 °C (days) of sperm collected from meagre (*Argyrosomus regius*) at different days after consecutive GnRHa implant administration (solid arrows). Lowercase letters above the sperm quality parameters means indicate statistically significant differences among sampling times after the first GnRHa administration (one-way ANOVA, Tukey's HSD, $P \leq 0.05$).

GnRHa implantation compared to the day of administration, when a sampling took place. An increase in sperm motility duration after GnRHa treatment delivered with cholesterol pellets or microspheres was recorded in the yellowtail flounder (*Pleuronectes ferrugineus*) (Clearwater and Crim, 1998), however usually motility duration is not affected by exogenous hormonal treatments (Mylonas et al., 2017). On the other hand, it is common for hormonal treatments to result in a reduction of sperm density (Mylonas et al., 2017), which is caused by a hydration of the testes due to increased seminal fluid production (Scott et al., 2010). However, in the current study sperm density remained at almost the same levels during a period of 2.5 months, with a temporary increase one week after the 2nd and a lesser one after the 4th GnRHa administration. This finding matches the histological sections of the Implant group testes of the first study, where active spermatogenesis was observed compared to the rest of the groups. On the contrary, after repeated GnRHa implantation in meagre over a period of 17 weeks, sperm density decreased 10 weeks after the first treatment (Mylonas et al., 2016), showing that spermatogenesis, spermiogenesis and spermiation can be "boosted" for only a limited period of time, and not indefinitely.

Computer assisted sperm analysis was used in the present study to monitor a number of sperm motion parameters using an objective, sensitive and accurate technique (Mylonas et al., 2017). The VAP from the present study was similar to the one recorded in an *in vitro* fertilization experiment in meagre, but the percentage of motile cells was higher, especially on Days 7 and 42 (Ramos-Júdez et al., 2019), and was also higher compared to the recorded values from a study dealing with the first maturation of meagre males (Schiavone et al., 2012). Interestingly, the GnRHa injection that was used to enhance spermiation in the first study, was shown to extend the period of time that spermatozoa maintained high levels of motility and velocity for 24-h after GnRHa administration, before a significant decrease occurred (Ramos-Júdez et al., 2019), and these enhanced values were considered appropriate for *in vitro* fertilization trials (Ramos-Júdez et al., 2019; Santos et al., 2018). Spermatozoa swimming speed is correlated with fertilization success (Gallego and Asturiano Juan, 2019; Kowalski and Cejko, 2019) as was reported for the Atlantic salmon (*Salmo salar*) (Gage et al., 2004), Atlantic cod (Rudolfsen et al., 2008) and gilthead seabream (*Sparus aurata*) (Beirão et al., 2011). In the latter study, apart from high swimming speed (VCL ~ 100 μm sec^{-1}), high linearity (LIN ~ 70%) was also considered as a significant factor of the final hatching success, perhaps through increased fertilization success, showing that speed is not the only prerequisite for fertilization (Beirão et al., 2011). Here, VCL was most of the time at the same levels with the latter study, while STR (STR = VSL/VAP while LIN = VSL/VCL) was even higher.

In conclusion, the present study demonstrated that spermiation enhancement in meagre can be achieved with the use of GnRHa implants given every 3 weeks. Consecutive implantations stimulated sperm production for a period of up to 2.5 months, while sperm quality parameters were not affected, indicating that milt of enhanced quantity and standard quality can be produced for a long spermiation period. This may effectively prolong, even slightly, the meagre spawning season for aquaculture purposes. The endocrine profile of GnRHa treated fish demonstrated that both androgen levels rise after GnRHa administration and can remain elevated with the use of repeated treatments, although their specific role on the process of spermiation regulation in meagre is still unclear. Conducting an experiment in the future to compare different doses of GnRHa implants, as well as the time of administration, will improve our knowledge and allow us to deliver an even more comprehensive protocol for the management of male fish in aquaculture conditions.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2020.113549.

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