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Reproduction of hatchery-produced meagre *Argyrosomus regius* in captivity II. Hormonal induction of spawning and monitoring of spawning kinetics, egg production and egg quality

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ABSTRACT

The present study aimed at the development of an effective and efficient hormonal spawning induction method for meagre *Argyrosomus regius*, using controlled-release delivery systems loaded with gonadotropin-releasing hormone agonist (GnRHa). Three sets of spawning induction experiments were undertaken in the course of three years. In experiment 1, group spawnings were performed at different times during the reproductive period, in order to determine the most appropriate time for hormonal induction of spawning. Experiment 2 described in more detail the spawning kinetics of individual females induced to spawn at different times during the season, in order to obtain important information on spawning kinetics, batch fecundity and total fecundity in response to the hormonal treatment. In experiment 3, two similar broodstocks were used to obtain further production characteristics and monitor variations in egg/larval quality over the spawning period. The effective GnRHa doses used were 55–84 μg GnRHa kg^{-1} body weight in males and 46–92 μg GnRHa kg^{-1} in females. In general, successful spawning induction was achieved between early May and early June. Implantation with GnRHa induced spawning 2–3 d after treatment and produced an average (±SD) of 12 ± 5 spawns per broodstock or female over 3 weeks, most of them over consecutive days. Batch fecundity was extremely variable (mean of 6631 to 104,409 eggs kg^{-1}), with the largest batches occurring 3–4 d after treatment, and very low fecundity after 1 week from GnRHa treatment. As a result, the first 4 spawns of all spawning induction experiments constituted a mean of 75 ± 13% of the total relative fecundity. Overall mean total relative fecundity was variable at 380,780 ± 167,577 eggs kg^{-1}. Evaluation of the ovaries after the cessation of spawning, demonstrated that the females still contained large numbers of oocytes in full vitellogenesis, and it is hypothesized that these fish could spawn further in response to a second GnRHa implantation. Fertilization success was very high in all spawning induction experiments, with the overall mean being 89 ± 5%. Similarly, the produced eggs were of very high quality, based on the survival of the eggs during embryogenesis (>95%), hatching success (>99%) and larval survival 5 d after egg collection (>87%), resulting in an overall larva survival of 73% of the total number of eggs spawned. These studies demonstrate that the GnRHa implants are an effective and efficient method for the induction of spawning in meagre, leading to the production of eggs of high fecundity and quality.

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1. Introduction

The meagre *Argyrosomus regius* (Sciaenidae) is a species with great potential for the diversification of aquaculture production in the Mediterranean region (Duncan et al., 2013; Monfort, 2010). Its aquaculture production has increased rapidly in the last decade (Duncan et al., 2013), even though reproduction of meagre in captivity still remains a problem (Mylonas et al., 2013-this volume). With few exceptions, females do not mature in captivity (Duncan et al., 2013; Gil et al., 2013; Mylonas et al., 2013-this volume), and exogenous hormones need to be used to induce ovulation and spawning (Duncan et al., 2012).

Failure to undergo oocyte maturation is the most common reproductive dysfunction of cultured fishes (Mylonas et al., 2010). The failure is due to lack of luteinizing hormone (LH) release at the end of vitellogenesis (Mylonas et al., 1997, 1998). Therefore, agonists of gonadotropin-releasing hormone (GnRHa) have been used extensively in many fish species (Mañanos et al., 2009; Mylonas and Zohar, 2009; Mylonas et al., 2010). The exogenous GnRHa triggers the release of LH, which in turn stimulates the necessary changes in gonadal steroidogenesis that will ultimately result in oocyte maturation, ovulation and spawning (Lubzens et al., 2010). Both liquid injections and controlled-release delivery systems that release GnRHa for a prolonged period of

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time (Mylonas and Zohar, 2001) have been shown to be effective in inducing maturation in females, as well as enhancing sperm production in males (Mañanos et al., 2009; Mylonas and Zohar, 2009; Mylonas et al., 2010). Of particular advantage is the use of GnRHa delivery systems in fishes with a protracted spawning season and asynchronous or group synchronous ovarian development, which undergo many cycles of oocyte maturation in the course of many weeks (Guzmán et al., 2009; Kokokiris et al., 2005; Larsson et al., 1997; Zohar et al., 1995).

Meagre exhibit an asynchronous or group-synchronous oocyte development pattern (Duncan et al., 2012; Gil et al., 2013; Schiavone et al., 2012), a characteristic that is common with other sciaenids, (Grau et al., 2009; Mylonas et al., 2004a; Thomas et al., 1995). Initial experiments with GnRHa treatment were effective in inducing maturation of both wild-caught (Duncan et al., 2012) and hatchery-produced broodstocks (Fernández-Palacios et al., 2009b). While fertilization was very high (min of 80% with the great majority >90%), total relative fecundity in all spawning induction studies (Duncan et al., 2012, 2013; Fernández-Palacios et al., 2009a) has been significantly lower than the maximum potential reported for the species (Gil et al., 2013), especially when single GnRHa injections were used. In the latter case, the lower fecundity was due to the fact that a single GnRHa injection induced only 2–3 spawns (Duncan et al., 2012, 2013; Fernández-Palacios et al., 2009a), while the species has the capacity for multiple spawns (Gil et al., 2013; Mylonas et al., 2013-this volume). Other missing information for efficient management of meagre broodstocks includes the spawning kinetics of individual females, daily batch fecundity and total season fecundity. Such information is still missing because most spawning induction experiments were undertaken using group spawnings or single GnRHa injections.

The present study follows one on the reproduction of hatchery-produced meagre broodstocks (Mylonas et al., 2013-this volume) and describes a set of experiments aiming at the development of an effective and efficient hormonal spawning induction method using GnRHa delivery systems. The individual female egg production characteristics were described, and monitoring of egg and larval quality was undertaken over the course of the spawning induction period. The information obtained is expected to be useful for controlling reproduction and planning egg production of meagre in commercial aquaculture operations.

2. Materials and methods

2.1. Broodstock maintenance

Rearing was undertaken at the facilities of the Institute of Marine Biology, Biotechnology and Aquaculture (previously Institute of Aquaculture) of the Hellenic Centre for Marine Research (HCMR), Iraklion, Crete, Greece. Different broodstocks were used over three years (2010–2012) with fish coming from eggs produced in the hatchery in 2004, 2006 and 2007. During the year, fish were maintained in various tanks (9000–35,000 l in volume) and were exposed to controlled, simulated natural water temperature and photoperiod. Feeding was done 5 d per week to apparent satiation with industrial feed (Excel XL, Skretting S.A., Spain or Genesis, IRIDA, S.A., Greece) and 3 d per week with frozen squid during the expected reproductive season (February–July). For spawning induction, fish were maintained in 9000 l (group spawnings) or 5,000 l (individual female spawnings) Recirculation Aquaculture Systems (ACE, the Netherlands), under natural photoperiod and controlled temperature. Measurements of temperature and water quality (NH_3 and NO_2) were conducted once per week.

Three sets of spawning induction experiments were undertaken in the course of three years (Table 1). In experiment 1 (see Section 2.3 below), group spawnings were performed at different times during the reproductive period, in order to determine the most appropriate time for hormonal induction of spawning. Experiment 2 (Section 2.4) described in more detail the spawning kinetics of individual females induced to spawn at different times during the season, in order to obtain important information on spawning kinetics, batch fecundity and total fecundity in response to the hormonal treatment. In experiment 3 (Section 2.5), two similar broodstocks were used to obtain further production characteristics and monitor variations in egg/larval quality over the period fish were spawning in response to the GnRHa treatment.

2.2. Evaluation of reproductive stage and broodstock selection

To select the broodstocks for the spawning experiments, fish were tranquilized initially in their tank with the use of clove oil (0.01 ml l^{-1}) and then transferred to an anesthetic bath for complete sedation with a higher concentration of clove oil (0.03 ml l^{-1}) (Mylonas et al., 2005). Ovarian biopsies for the evaluation of oocyte development were obtained by inserting a glass cannula (Natelson tube) into the ovarian cavity, connected to a TygonR tubing and applying gentle aspiration. A wet mount of the biopsy was first examined under a compound microscope (40 and 100×) to evaluate the stage of oogenesis and measure the mean diameter of the largest, most advanced vitellogenic oocytes (n = 10). A portion of some biopsies was fixed in a solution of 4% formaldehyde-1% glutaraldehyde for further histological processing. Females were considered eligible for spawning induction if they contained oocytes in full vitellogenesis with a diameter of >550 μm (Duncan et al., 2012), with very little atresia present.

Male fish were considered eligible for spawning induction if they were in full spermiation, releasing substantial amounts of sperm upon application of gentle abdominal pressure (Mylonas et al., 2013-this volume). To obtain sperm for evaluation, the genital pore was carefully blot dried and gentle abdominal pressure was applied to force the sperm out of the testes, avoiding contamination of the samples with feces or urine. Sperm quality was evaluated for all males used in the spawning

Table 1
Biometric and treatment data of all meagre breeders used in the spawning induction studies, at the time of hormone administration. The mean oocyte diameter represents the largest vitellogenic oocytes at the time of treatment. All fish were treated with an EVAc GnRHa implant, and variations in the effective GnRHa dose were due to the fact that implants were loaded with fixed amounts of GnRHa.

		Females				Males		
Year—experiment	Date	No	Body weight (kg ± SD)	GnRHa dose (μg kg^{-1} ± SD)	Oocyte diameter (μm ± SD)	No	Body weight (kg ± SD)	GnRHa dose (μg kg^{-1} ± SD)
2010								
Group spawn 1	5 May 2010	3	9.2 ± 1.1	66 ± 27	630 ± 40	3	7.9 ± 0.7	52 ± 18
Group spawn 2	18 May 2010	4	8.1 ± 1.0	57 ± 5	590 ± 40	5	7.7 ± 0.9	53 ± 17
Group spawn 3	9 June 2010	4	5.0 ± 1.1	84 ± 4	620 ± 30	6	4.6 ± 0.7	67 ± 11
2011								
Individual spawn 1	4 May 2011	3	6.9 ± 1.4	74 ± 15	630 ± 30	6	6.7 ± 0.9	46 ± 6
Individual spawn 2	3 June 2011	3	11.8 ± 0.4	68 ± 2	650 ± 15	6	8.9 ± 0.9	64 ± 11
2012								
Broodstock 1	23 May 2012	4	6.1 ± 1.2	55 ± 10	660 ± 30	4	6.0 ± 0.9	85 ± 12
Broodstock 2	23 May 2012	4	5.6 ± 0.9	57 ± 8	590 ± 50	4	5.5 ± 0.7	92 ± 11

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experiments (except for group spawn 3 experiment in 2010), by looking at sperm density/concentration (number of spermatozoa ml^{-1} of milt), percentage of spermatozoa showing forward motility immediately after activation (initial sperm motility, %) and duration of forward sperm motility of at least 10% of the spermatozoa in the field of view (motility duration, min), using the methods described earlier (Mylonas et al., 2013-this volume). The gonad of one male each year was excised at the beginning of the spawning induction experiments, in order to evaluate histologically the status of spermatogenesis. A small fraction (1 cm^3) from the middle part of the testes was fixed in a solution of 4% formaldehyde-1% glutaraldehyde for further histological processing.

2.3. Group spawning experiments at different times of the reproductive season

Three group spawning induction experiments were undertaken in 2010 using different broodstocks (Table 1). Females were treated with an Ethylene–Vinyl acetate (EVAc) GnRHa implant (Mylonas and Zohar, 2001) loaded with Des-Gly^{10}, D-Ala^6-Pro-$NEth^9$-mGnRHa (H-4070, Bachem, Switzerland). There were variations in the effective GnRHa dose applied to each fish, due to the fact that implants are loaded with fixed amounts of GnRHa (Table 1). Even though combinations of two GnRHa implants loaded with different amounts of GnRHa were used when necessary, it was still not possible to adjust the dose exactly to the different body weights of the fish. At the time of GnRHa implantation, selected females were in advanced vitellogenesis and males were in full spermiation (Table 2). After treatment with GnRHa, fish were placed in 9000-l tanks and were allowed to spawn for 4 weeks. The temperature range during the experiments was 18.6–20.5 °C (group spawn 1), 19.2–20.5 °C (group spawn 2) and 19–20.2 °C (group spawn 3). One week after cessation of spawning, the fish were removed from the spawning tank, evaluated using ovarian biopsies and returned to a large communal tank (35 m^3) to recover.

2.4. Individualized female spawning experiments

Individualized female experiments were conducted at two different dates in 2011 (Table 1). On 4 May 2011, three females and six males were selected and treated with a GnRHa implant. Each female with two males were placed in separate 5000-l tanks immediately after treatment and were monitored for spawning for 4 weeks. The temperature range during the experiment was 19.3–19.4 °C. One week after cessation of spawning, the fish were removed from the spawning tanks, evaluated using ovarian biopsies and returned to a large communal tank (35 m^3) to recover. Another group of three females and six males were selected on 3 June 2011 and treated as described above (Table 1). The fish were placed in separate 5000-l tanks and were monitored for spawning for 4 weeks. The temperature range during the experiment was 19.4–20.4 °C. One week after cessation of spawning, the fish were removed from the spawning tanks, evaluated using ovarian biopsies and returned to a large communal tank (35 m^3) to recover.

2.5. Evaluation of production and egg/larval quality over the spawning season

Two similar stocks of meagre maintained under identical conditions (duplicates) were evaluated for their stage of reproductive development and were treated with a GnRHa implant on 23 May 2012 (Table 1). Each broodstock consisted of four females and four males maintained in 9000-l tanks. The temperature range during the spawning induction period was 18.2–18.7 °C. Three weeks later (14 June) all females were evaluated using ovarian biopsies, and were returned to the spawning tank and the occurrence of any further spawning was monitored for another 3 weeks. The temperature range during this period (14 June–8 July) was 18.4–18.8 °C. On 8 July (>6 weeks after GnRHa treatment), all fish were evaluated again using ovarian biopsies and were returned to a large communal tank (35 m^3) to recover.

2.6. Evaluation of egg/larval quality

A passive egg collector was placed in the outflow of each spawning tank, in order to collect the spawned eggs. Eggs were collected every morning (~12 h after spawning) into a 10-l bucket and their number (fecundity) was estimated by counting the total number of eggs in a sub-sample of 10 ml, after vigorous agitation. Fertilization success was evaluated at the same time by examining each of the eggs in this 10 ml sample for the presence of a viable embryo (usually at the blastula stage) using a stereoscope.

In order to monitor embryo and larval survival, eggs from each spawn were placed individually in 96-well microtiter plates (in duplicates) according to the procedure of Panini et al. (2001), with some modifications. Briefly, floating (~100% fertilized) eggs were taken in a 250-μm mesh filter and were rinsed with sterilized seawater and poured in a 2-l beaker. A Petri dish was used to scoop 100–200 eggs from the beaker. The Petri dish was then placed under a stereoscope and only fertilized eggs were taken one by one with a micropipette set to 200 μl and transferred to the wells of the microtiter plates (one egg per well). The microtiter plates were then covered with a plastic lid, placed in a controlled-temperature incubator and maintained for 5 d at 19 ± 0.5 °C. Using a stereoscope, embryonic and early larval development was evaluated once a day, recording the number of live embryos 24 h after egg collection (or ~36 h after spawning), hatched larvae (examined ~60 h after spawning) and viable larvae on day 5 after egg collection. At 18–20 °C, hatching of meagre eggs takes place in 44–56 h.

Embryo survival was evaluated the day after egg collection (1 d), and was calculated as the number of eggs having live embryos / the

Table 2
Spermiation data (mean ± SD) from the male meagre used in the various spawning induction experiments using GnRHa implants, at the time of hormone administration (see Table 1). There were no statistically significant differences among means of any parameter within each year's experiments (one-way ANOVA), but there were significant differences among the three years (see Fig. 2).

Year—experiment	Date	Males (n)	Sperm density (szoa ml^{-1})	Sperm motility (initial %)	Motility duration (min)
2010					
Group spawn 1	5 May 2010	3	$14.5 \pm 8.3 \times 10^9$	70 ± 10	1.5 ± 0.2
Group spawn 2	18 May 2010	4	$10.7 \pm 7.9 \times 10^9$	80 ± 10	1.8 ± 0.1
Group spawn 3	9 June 2010	4	n/a	n/a	n/a
2011					
Individual spawn 1	4 May 2011	6	$19.0 \pm 5.4 \times 10^9$	50 ± 30	0.8 ± 0.3
Individual spawn 2	3 June 2011	6	$24.1 \pm 6.7 \times 10^9$	60 ± 20	1.1 ± 0.2
2012					
Broodstock 1	23 May 2012	4	$27.1 \pm 1.0 \times 10^9$	95 ± 5	3.3 ± 0.2
Broodstock 2	23 May 2012	4	$28.1 \pm 4.6 \times 10^9$	90 ± 10	3.3 ± 0.3
Overall mean (±SD)			$20.6 \pm 7.1 \times 10^9$	75 ± 20	2.0 ± 1.1

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number of fertilized eggs initially loaded in the microtiter plates. Hatching success was calculated as the number of hatched larvae / the number of live 1-d embryos, and 5-d larval survival was calculated as the number of live larvae 5 d after egg collection / the number of hatched larvae. Estimating percentage survival (%) by using in the denominator the number of individuals that survived to the previous developmental stage is considered as a more independent evaluation of survival within specific developmental stages, without the potential of a masking effect of the previous stage (Mylonas et al., 1992, 2004b). In order to develop egg and larval quality indicators that could be used in commercial hatcheries to predict the performance of a batch of meagre eggs obtained after hormonal spawning induction, the existence of correlations among fecundity, fertilization, embryo survival, hatching and larval survival were examined.

2.7. Histological analysis

Before embedding in methacrylate resin (Technovit 7100®, Heraeus Kulzer, Germany) ovarian biopsies were dehydrated in gradually increasing ethanol solutions (70–96%). Serial sections of 3 μm were obtained with a microtome (Reichert Jung, Biocut 2035, Germany). Sections were stained with Methylene Blue (Sigma, Germany)/Azure II (Sigma, Germany)/Basic Fuchsin (Polysciences, USA) according to Bennett et al. (1976). Sections were examined under a light compound microscope (Nikon, Eclipse 50i) and photographed with a digital camera (Jenoptik progress C12 plus).

2.8. Statistical analysis

Differences in mean initial sperm motility (%), motility duration (min) and spermatozoa concentration between males used in different spawning experiments or years were evaluated using one-way ANOVA, followed by Duncan's New Multiple Range (DNMR) test when appropriate, at a minimum of $P \leq 0.05$. Differences in mean daily relative fecundity and fertilization success among broodstocks induced to spawn at three different times (2010, Section 2.3) were examined using one-way ANOVA at a minimum of $P \leq 0.05$. Differences in mean daily relative fecundity, total relative fecundity and fertilization success among individual females induced to spawn at two different dates (2011, Section 2.4) were examined using two-way ANOVA with females and GnRHa treatment date as factors, at a minimum of $P \leq 0.05$. Differences in mean daily relative fecundity, fertilization and hatching success, and 5-day larval survival between two broodstocks induced to spawn at the same time and over the course of the study (2012, Section 2.5) were examined using two-way ANOVA with broodstock and week after GnRHa treatment as factors at a minimum of $P \leq 0.05$, followed by DNMR when appropriate. Correlation between egg quality parameters was examined using Simple Regression analysis. Data was examined for normality in the distribution of variances, in order to comply with the prerequisites of ANOVA. All analyses were performed with a linear statistics software (SuperAnova, Abacus Concepts, Berkeley, CA, USA). Results are presented as mean ± SEM, unless mentioned otherwise.

3. Results

Biopsies of selected females exhibited vitellogenic oocytes of different diameters, as well as primary oocytes and oocytes at the cortical alveoli stage (Fig. 1A). Oocytes had a spherical shape and a dark color, and upon histological examination the germinal vesicle (gv) was visible in a central location and the ooplasm was filled with lipid droplets and yolk globules, whereas a small number of cortical alveoli were localized at the periphery of the oocyte, adjacent to a thick zona radiata (Fig. 1B). The mean (±SD) diameter of the largest vitellogenic oocytes of the females selected for the experiments varied between 590 ± 40 and 660 ± 30 μm (Table 1). The effective dose of GnRHa given to the females in the various spawning induction experiments (in the form of an EVAc implant) varied between 55 ± 10 and 84 ± 4 μg GnRHa kg^{-1} body weight (Table 1).

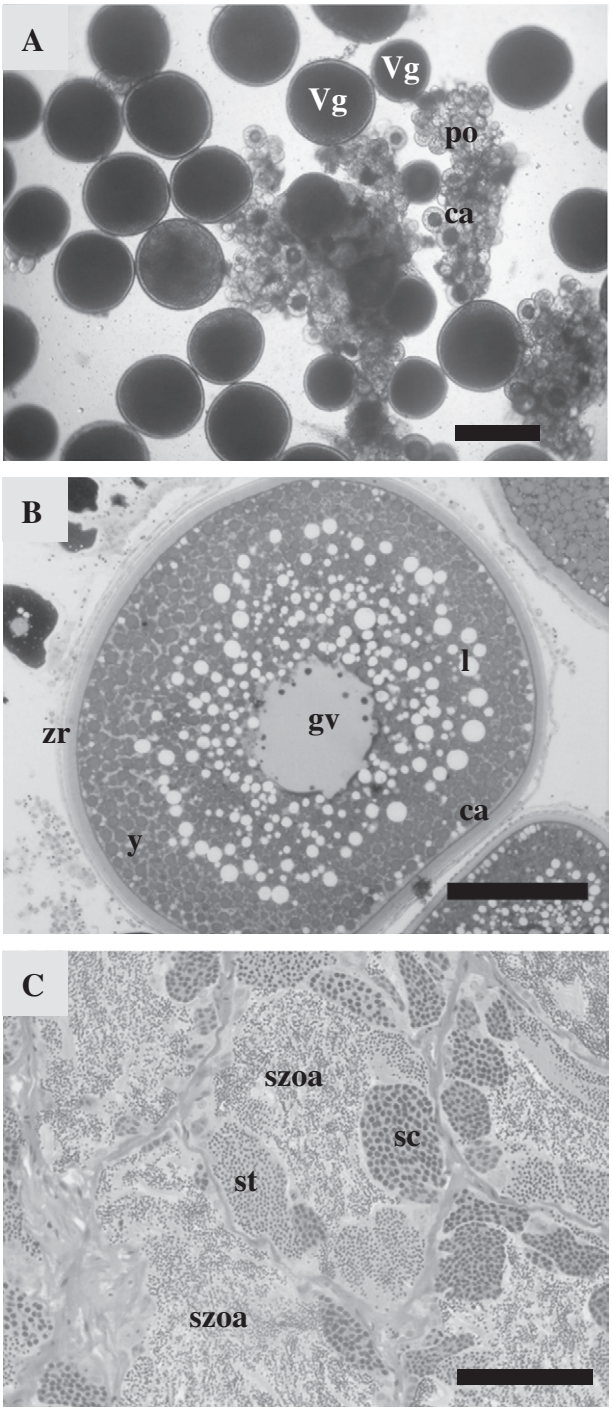

Fig. 1. Microphotographs of representative wet mounts (A) or histological sections of meagre oocytes from ovarian biopsies (B), or excised testes (C) obtained at the time of GnRHa treatment of the various experiments. In addition to vitellogenic oocytes at different sizes (Vg), the ovarian biopsies contained also primary oocytes (po) and oocytes at the cortical alveoli stage (ca). The fully vitellogenic oocytes had a thick zona radiata (zr), a centrally located nucleus (germinal vesicle, gv), a number of lipid droplets (l) and yolk globules (y) dispersed throughout the cytoplasm and a small number of cortical alveoli (ca) along the periphery. The males were in full spermiation and the testes contained large numbers of spermatozoa (szoa), but also spermatocysts with spermatids (st) and spermatocytes (sc) at different stages of development. Bar = 500 μm (A), 200 μm (B) or 100 μm (C).

Histological examination of spermiating males at the time of spawning induction indicated the presence of large numbers of spermatozoa

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in the testes, together with spermatocysts with germ cells are at different stages of development (Fig. 1C). The effective dose of GnRHa given to the males in the various experiments (in the form of an EVAc implant) varied between 46 ± 6 and 92 ± 11 μg GnRHa kg^{-1} (Table 1). Sperm quality at the time of induction was variable (Table 2). Within each year, there were no significant differences in the mean sperm quality parameters of males in the different spawning experiments (Table 2), but there were significant differences in all measured parameters among three years of the study (Fig. 2).

3.1. Group spawning experiments at different times of the reproductive season

The first spawning induction experiment with a group of fish (5 May 2010), resulted in multiple spawns beginning 3 d after GnRHa treatment and lasting for 24 d (Fig. 3). After a series of four consecutive spawns, 6 d passed before the next spawns were obtained. Maximum batch fecundity was observed in the second spawn with a relative batch fecundity of 88,278 eggs kg^{-1} and 87% fertilization success. At the second experiment (18 May 2010), spawning begun 2 d after GnRHa treatment (Fig. 3). Maximum relative batch fecundity was again on the second spawn with batch fecundity of 133,333 eggs kg^{-1} and 94% fertilization success. Again, after a series of 4 consecutive spawns, spawning ceased for a while, but this time lasted for only 2 d, and more spawning events were observed in this trial, with fewer days without spawning. At the third experiment (9 June 2010), spawning began again 2 d after GnRHa treatment, with maximum batch fecundity occurring at the third spawn (Fig. 3). After six consecutive spawns, spawning paused for 10 d and a single spawn was obtained again 18 d after treatment. In all three spawning induction experiments, relative batch fecundity exhibited a rapid decrease after reaching its maximum a few days after GnRHa treatment. As a result, batch fecundity exhibited very high variability (Table 3). Total relative fecundity for the three experiments varied between 266,860 and 479,636 eggs kg^{-1}. Fertilization success remained high throughout the spawning periods (Fig. 3) and mean values ranged between 83 ± 17 and 87 ± 6% for the three experiments (Table 3). No significant differences were observed in relative batch fecundity ($P = 0.19$) or in fertilization success among the three spawning induction experiments ($P = 0.75$).

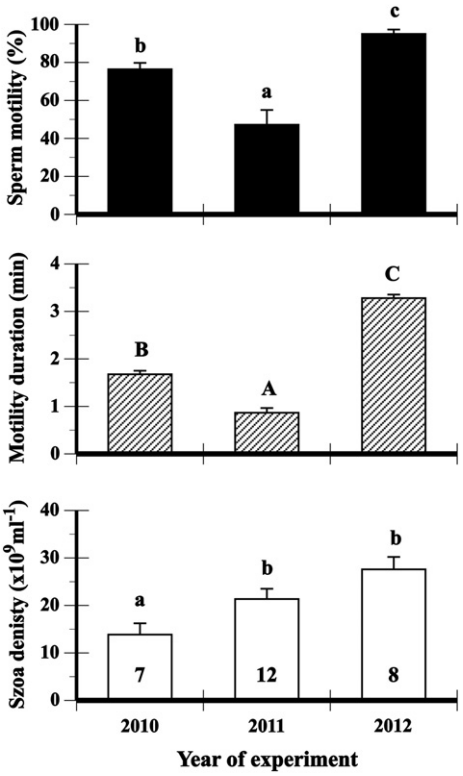

Fig. 2. Mean (±SEM) initial sperm motility (%), motility duration (min) and spermatozoa concentration/density ($\times 10^9$ szoa ml^{-1}) of meagre sperm used for the spawning induction experiments between 2010 and 2012. The sample number is shown inside the mean bars of the "Spermatozoa density" graph. Statistically significant differences among years are indicated by different lower case ($P \leq 0.05$) or capital ($P \leq 0.01$) letter superscripts (ANOVA, DNMR).

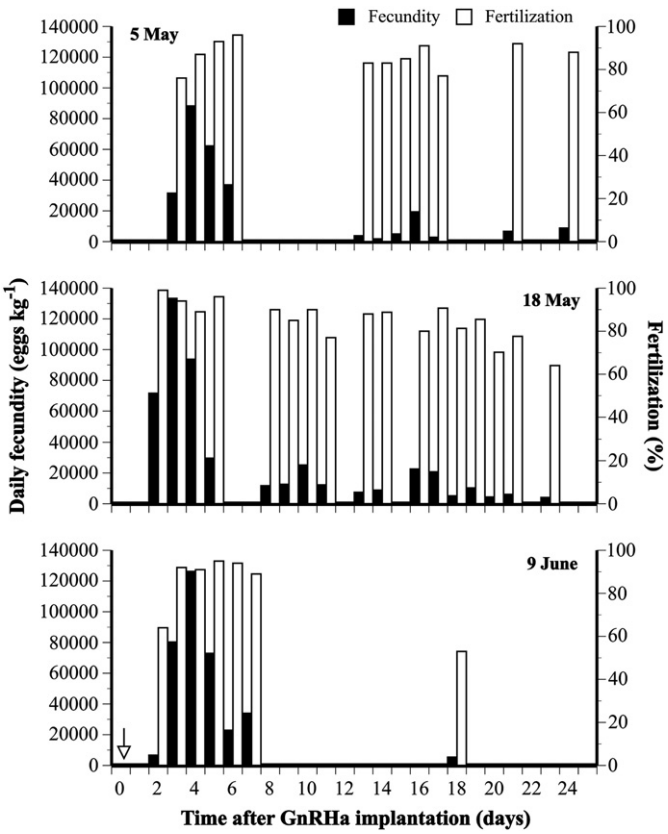

Fig. 3. Daily relative fecundity (eggs kg^{-1} female body weight) and fertilization success (%) of three meagre broodstocks induced to spawn at different times (5 May, 18 May, and 9 June 2010) during the reproductive season using a GnRHa implant (arrow).

3.2. Individualized female spawning experiments

In both spawning induction experiments with individualized females, the first spawns occurred 2 d after GnRHa treatment (Figs. 4 and 5). Maximum batch fecundity was again in the early spawns (1–3), ranging between 56,204 and 189,014 eggs kg^{-1} (Table 3). As before, fertilization success remained high throughout the spawning periods and mean values among females ranged between 85 ± 3 and 95 ± 9% (Table 3). In the first experiment, females spawned daily for 5 d and then either stopped completely or resumed spawning after a pause of 2–3 d (Fig. 4). On the contrary, in the second experiment females spawned in consecutive days for long periods, ranging between 7 and 15 d (Fig. 5). As in the group spawning experiments above, batch fecundity exhibited a rapid decrease after reaching its maximum a few days after GnRHa treatment. As a result, batch fecundity exhibited very high variability among individual females, ranging between 189,014 and 4286 eggs kg^{-1} (Table 3). Total relative fecundity also varied among females (168,127–699,394 eggs kg^{-1}), with the females of the second experiment that spawned more times having significantly higher total egg production ($P = 0.012$). No statistically significant

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Table 3
Egg production data from all spawning induction experiments with meagre induced to spawn with a GnRHa implant (see Table 1). Statistically significant differences among means of a parameter within each year's experiments are indicated by different letter superscripts (one or two-way ANOVA, $P < 0.05$), while the superscript "ns" indicates that no significant differences were observed.

Year—experiment	Date	Females (n)	Spawns (n)	Relative batch fecundity (egg kg^{-1})			Total fecundity (egg kg^{-1})	Fertilization (mean ± SD%)
				Min	Max	Mean ± SD		
2010								
Group spawn 1	5 May 2010	3	11	1737	88,278	24,260 ± 28,439^{ns}	266,860	87 ± 6^{ns}
Group spawn 2	18 May 2010	4	17	4074	133,333	28,214 ± 36,565	479,636	85 ± 9
Group spawn 3	9 June 2010	4	7	5404	126,263	49,784 ± 44,943	348,485	83 ± 17
2011								
Individual spawn 1a	4 May 2011	1	5	12,895	56,204	33,625 ± 18,726^{ns}	168,127	89 ± 7^{ns}
Individual spawn 1b	4 May 2011	1	10	8759	85,036	28,540 ± 26,786	285,401	85 ± 13
Individual spawn 1c	4 May 2011	1	6	4286	104,714	59,381 ± 39,296	356,286	87 ± 7
		Individual spawn 1 means				38,562 ± 31,070^{ns}	269,938^{a}	86 ± 10^{a}
Individual spawn 2a	3 June 2011	1	14	9775	189,014	49,957 ± 53,521^{ns}	699,394	93 ± 5^{ns}
Individual spawn 2b	3 June 2011	1	10	18,926	120,248	52,207 ± 39,279	522,066	95 ± 6
Individual spawn 2c	3 June 2011	1	19	6585	103,252	30,415 ± 24,465	577,886	95 ± 6
		Individual spawn 2 means				41,845 ± 39,753^{ns}	599,782^{b}	94 ± 6^{b}
2012								
Broodstock 1	23 May 2012	4	16	369	66,598	12,567 ± 20,016^{ns}	201,066	82 ± 26^{a}
Broodstock 2	23 May 2012	4	17	133	75,556	16,669 ± 23,077	283,378	96 ± 3^{b}
Overall production data								
	mean ± SD		12 ± 5	6631 ± 5689	104,409 ± 37,344	35,056 ± 15,485	380,780 ± 167,577	89 ± 5

difference was found either among females within experiments or between experiments in relative batch fecundity ($P = 0.92$ and $P = 0.72$, respectively). Similarly, no significant difference was found among females within experiments in fertilization success ($P = 0.89$), but in the second experiment mean (±SD) fertilization success was significantly higher ($P < 0.01$) than in the first (Table 3).

3.3. Evaluation of egg production and egg/larval quality over the spawning season

In both broodstocks induced to spawn at the same date (23 May 2012), the first spawns occurred 2 d after GnRHa treatment and

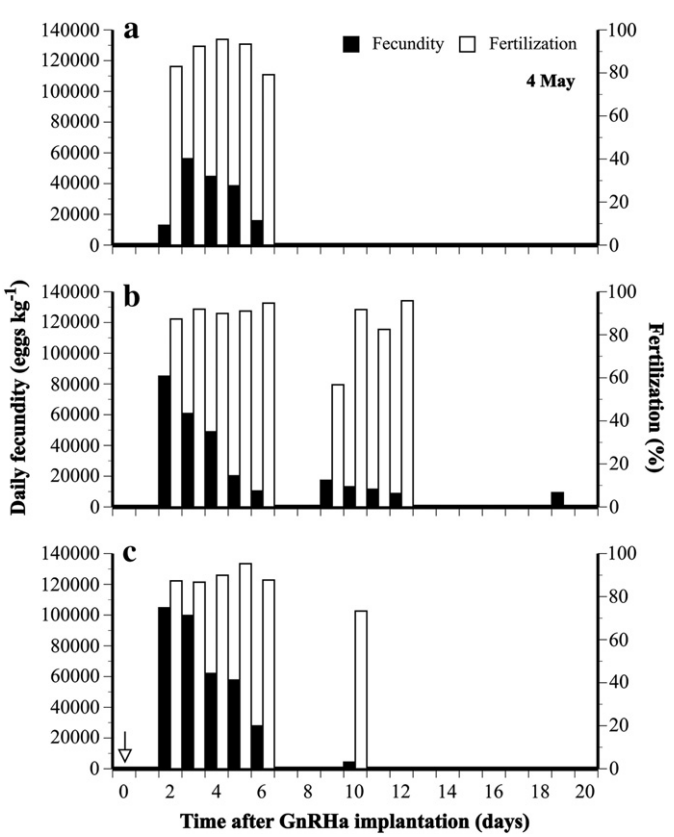

Fig. 4. Daily relative fecundity (eggs kg^{-1} female body weight) and fertilization success (%) of three individual meagre females (1a–c) induced to spawn at 4 May 2011, using a GnRHa implant (arrow). Each female was maintained separately together with 2 spermiating and GnRHa-implanted males.

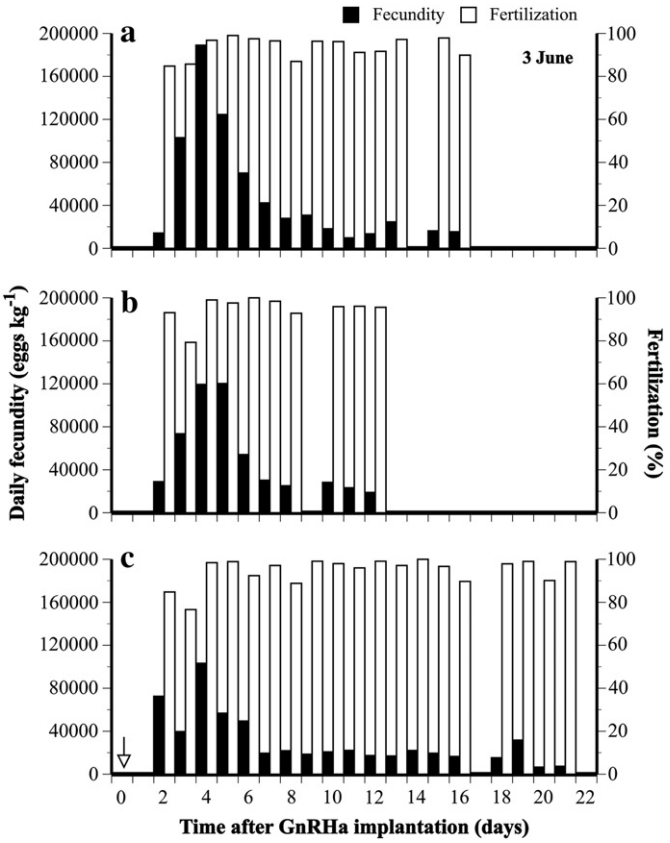

Fig. 5. Daily relative fecundity (eggs kg^{-1} female body weight) and fertilization success (%) of three individual meagre females (2a–c) induced to spawn at 3 June 2011, using a GnRHa implant (arrow). Each female was maintained separately together with 2 spermiating and GnRHa-implanted males.

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maximum fecundity was observed in the second spawn, 3 d after GnRHa treatment (data not shown). A total of 16–17 spawns were obtained from the two broodstocks, with decreasing fecundity over time and some variation in fertilization success (Table 3, Fig. 6). Relative batch fecundity decreased significantly ($P < 0.001$) after the first week after GnRHa treatment (Fig. 6). Among the two broodstocks, total relative fecundity as well as relative batch fecundity was similar ($P = 0.52$). Fertilization success exhibited significant differences between the two broodstocks over time ($P < 0.01$), caused mainly from significantly different values between the two broodstocks in the spawns during week 3 after GnRHa treatment (Fig. 6). No significant differences in hatching success were observed between broodstocks ($P = 0.07$) or over time ($P = 0.19$) after GnRHa treatment (Fig. 6). Larval survival 5-d after egg collection did not differ in the two broodstocks ($P = 0.13$), but was significantly lower ($P < 0.001$) for the spawns obtained during week 3 after treatment (Fig. 6).

There was a slight ($P = 0.08$) correlation between fertilization success and relative batch fecundity (data not shown), which was due to the existence of lower fertilization success in spawns of extremely low fecundity values (*i.e.*, <5000 eggs kg^{-1}), which were obtained in the latter spawns in the experiment. On the other hand, there were significant correlations ($P < 0.01$) between 1-d embryo survival and fertilization success, and between hatching success and 1-d embryo survival (data not shown). However, in the case of both of these correlations, the statistical significance was due exclusively to the existence of two spawns with extremely low fertilization success (<50%). Therefore, for egg batches with fertilization success >50% and 1-d embryo survival >90%, which were the vast majority of the spawns, there were no significant correlations among these parameters. Finally, larval survival 5 d after spawning was not correlated to hatching success ($P < 0.3$, data not shown).

Examination of ovarian biopsies 3 weeks after GnRHa treatment of the two meagre broodstocks–while females were still spawning–indicated that the ovaries contained large numbers of vitellogenic oocytes with little atresia (Fig. 7). The mean (±SD) diameter of the largest vitellogenic oocytes (650 ± 40 μm) in females of both broodstocks was similar to the initial diameter prior to treatment (Table 1). Further histological evaluation of these biopsies demonstrated that the oocytes were indeed viable, with no indication of oocyte atresia. Examination of ovarian biopsies and histological sections from females after a further 3 week period–but this time without any spawning during this period–again showed that the ovaries contained large numbers of vitellogenic oocytes with little atresia and of similar mean oocyte diameter (620 ± 50 μm) to the oocytes of females when they were induced to spawn >6 weeks before (Fig. 7).

4. Discussion

Administration of GnRHa implants was demonstrated to be an effective and efficient method for inducing oocyte maturation, ovulation and spawning in hatchery-produced meagre broodstocks, catering specifically to the asynchronous nature of ovarian function in this species. In response to this treatment, fish produced up to 19 daily spawns with very high fertilization success. The number of spawns varied within

ANOVA Table (P values)	Factor		
	Week	Broodstock	Week x Broodstock
Daily fecundity	0.001	ns	ns
Fertilization	0.01	0.01	0.01
Hatching	ns	ns	ns
5-day larval survival	0.001	ns	ns

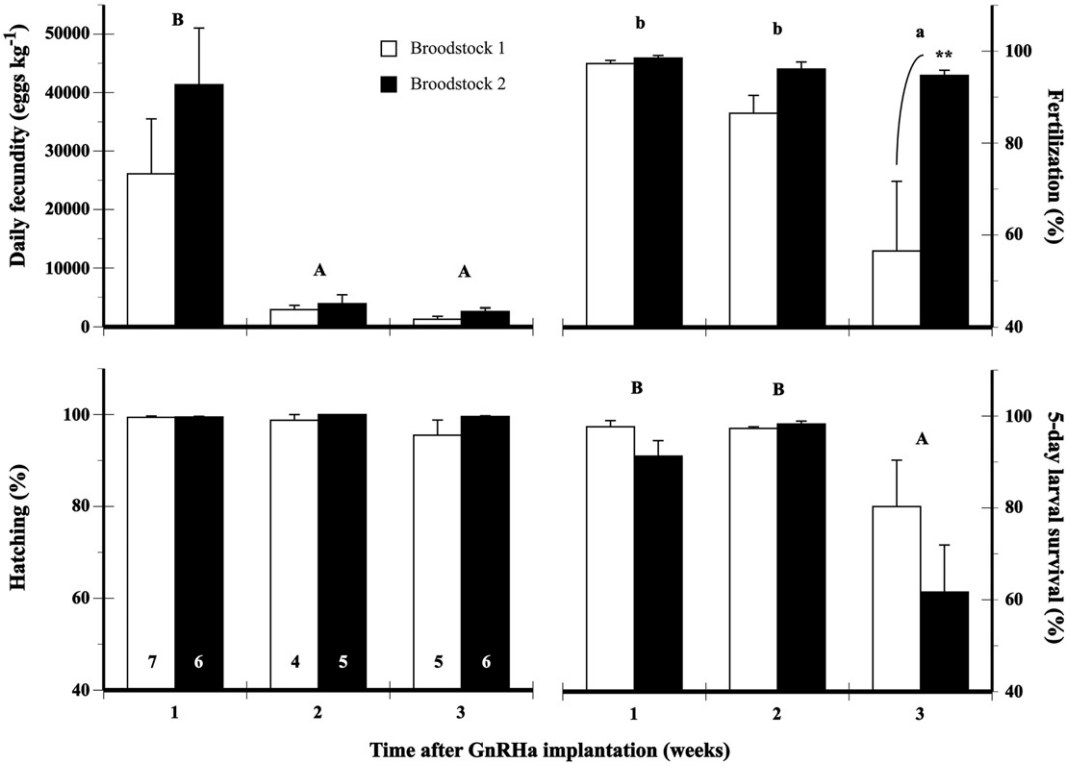

Fig. 6. Mean (±SEM) daily egg production (eggs kg^{-1} female body weight) and survival of the eggs/larvae obtained from two meagre broodstocks (4 females, 4 males per broodstock) according to week after induction of spawning with a GnRHa implant (23 May 2012). The number of spawns for each mean is indicated inside the bars in the "Hatching (%)" graph. The results of the statistical analysis (2-way ANOVA) are indicated on the table above. Significant differences between weeks (DNMR) regardless of broodstock are indicated by small ($P < 0.05$) or capital letter superscripts ($P < 0.01$). Double asterisks (**) indicate significant differences ($P < 0.01$, mean comparisons) between broodstocks within week.

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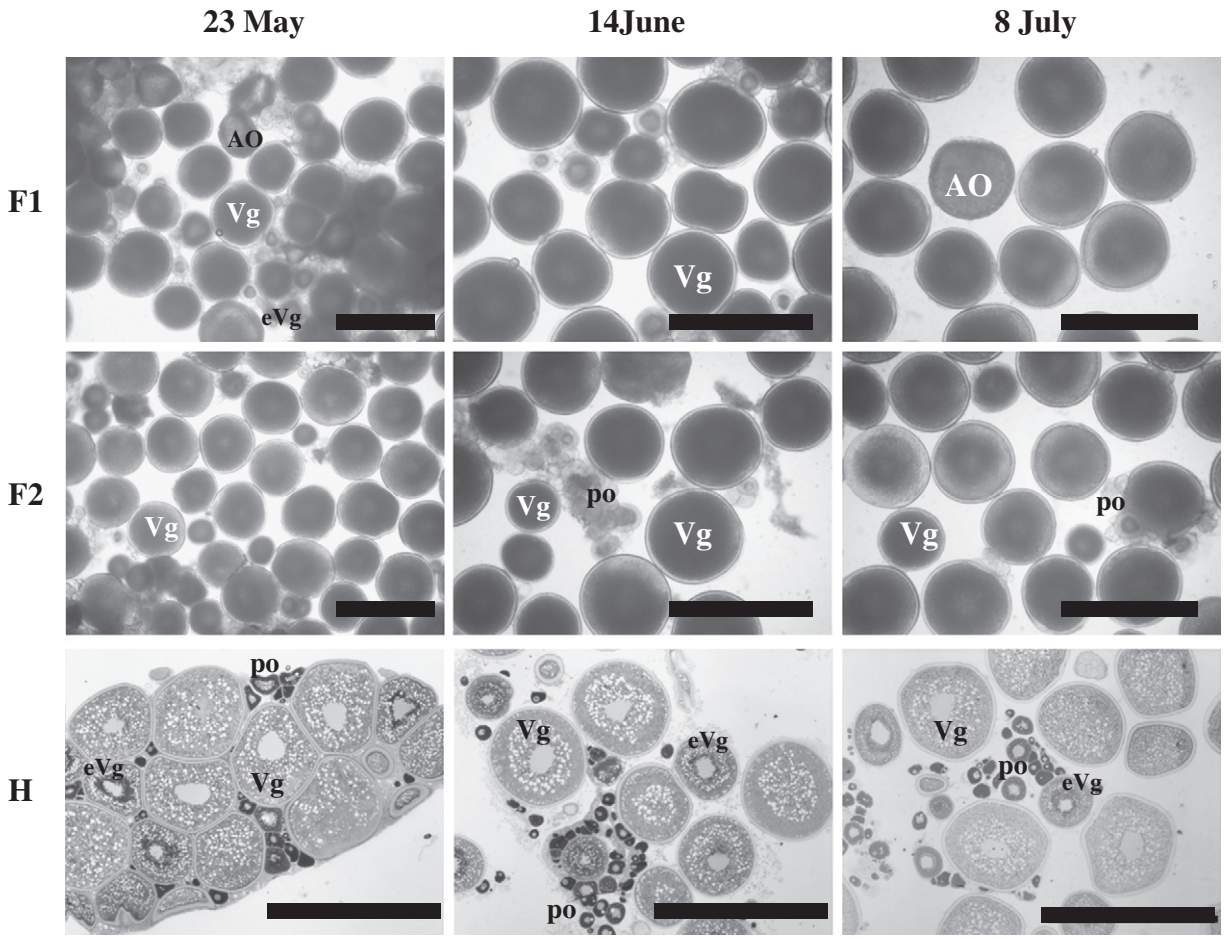

Fig. 7. Microphotographs of wet mounts from two females (F1 and F2) and histological sections (H) of representative meagre oocytes from ovarian biopsies obtained before and after spawning in response to treatment with a GnRHa implant (see Fig. 5) at 23 May 2012, prior to treatment with GnRHa; at 14 June 2012, 3 weeks after almost daily spawning; at 8 July, after >3 weeks without any further spawning. A large number of vitellogenic oocytes (Vg) of various diameters dominated the biopsies at all three sampling times. A small number of atretic oocytes (AO) was present also, along with primary oocytes (po) and oocytes at early vitellogenesis (eVg). Bar = 1000 μm.

year and spawning trial/broodstock, but without a statistically significant effect that could point to an effect of time of GnRHa administration or broodstock size on the number of spawns that can be produced by meagre. Furthermore, the fertilization success during the three years was very high, as compared with other hormonal spawning inductions with meagre (*i.e.*, >80%, Duncan et al., 2012, 2013), with the exception of the individual spawning experiment carried out in early May vs early June. Thus, successful spawning with high fertilization success can be induced in meagre using GnRHa implants throughout the natural spawning period (Mylonas et al., 2013-this volume). Nevertheless, based on the present studies, it appears that more spawns may be expected if meagre are treated with GnRHa later in the spawning season. In a previous study (Mylonas et al., 2013-this volume), vitellogenic oocytes of a size appropriate for hormonally induced spawning were observed already in April, but although the increases in oocyte diameter that occurred during May were not statistically significant, spontaneous spawning of a few females from the stock was obtained in June.

After GnRHa implantation, meagre began spawning 2–3 d later, as reported earlier for this species (Duncan et al., 2012) and its relative the shi drum *Umbrina cirrosa* (Mylonas et al., 2004a), and females produced consecutive daily spawns for 4–9 d when spawning in groups and for 5–15 d when spawning individually. In most cases, spawning resumed again after 1–2 d and consecutive daily spawns were obtained until complete cessation, while less frequently the fish spawned intermittently during the experiment. The total number of daily spawns obtained was by far the highest reported for meagre to date (Duncan et al., 2013). In a recent study with wild-caught captive meagre (Duncan et al., 2012), GnRHa implants induced spawning for only 2–3 consecutive daily spawns, and very limited spawning was observed thereafter — 7 spawns of extremely low fecundity. The differences with the present study could not be attributed to a higher GnRHa dose–since it was similar in both studies–but could be related to the fact that hatchery-produced broodstocks were better acclimatized to the captive environment and performed better in response to the hormonal treatment. The only other available study with hatchery-produced broodstocks used GnRHa injections and produced only 1–2 spawns, although further injections induced another set of 1–2 spawns each (Fernández-Palacios et al., 2009b). As meagre do not spawn readily in captivity, the natural spawning kinetics and rhythm of the females are currently unknown. In the only study reporting spontaneous spawning of meagre, two females in a stock of seven appeared to have participated in a total of 7 spawns during 15 d, but it could not be determined unequivocally if both or only one of the females participated in each spawn (Mylonas et al., 2013-this volume). Based on the results of the present study, it is obvious that meagre can produce a large number of consecutive spawns over many weeks.

Batch fecundity in response to GnRHa implantation was extremely variable within a broodstock or individual females with a maximum daily egg production of 104,409 eggs kg^{-1} and minimum of 6631 eggs kg^{-1}. Maximal fecundity was generally seen 3–4 d after treatment, and decreased steadily and significantly thereafter. The longer a female spawned–*i.e.*, the more spawns it produced–the smaller the batch fecundity became. When fish were induced to spawn using single GnRHa injections, the fecundity obtained in the 1–2 spawns

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that usually ensued was similar (Duncan et al., 2012; Fernández-Palacios et al., 2011), as it was in the present study for the 2nd–3rd egg batches. Very limited information exists on the daily fecundity of spontaneously spawning females, but the existing data pointed to a daily batch fecundity of 137,500–170,000 eggs kg^{-1} (Mylonas et al., 2013-this volume). However, as it relates to hormonally induced spawning, batch fecundity may depend on the hormonal treatment mode (GnRHa injection vs implant) and in the case of GnRHa implants on the time after implantation. It is also likely that the first surge of GnRHa from the implant would result in a rise of LH release from the pituitary stores; the increased LH level would, in turn, induce oocyte maturation and ovulation of post vitellogenic oocytes already present in the ovary. During the 2–3 d spawing pause, the LH stores in the pituitary were probably replenished, and new generations of oocytes entered vitellogenesis — a process that requires a time consuming depletion of energy stores from the entire body. The resumption of spawning with lower fecundity probably reflects the potential rate of the processes involved in producing post-vitellogenic oocytes ready to mature and ovulate. A gradual reduction in batch fecundity was also observed in the European sea bass *Dicentrarchus labrax*, which exhibits a group synchronous ovarian development. In this species, batch fecundity was maximal in the first spawn induced by a GnRHa injection (Mylonas et al., 2003). Subsequent injections produced further single spawns and each spawn had significantly lower fecundity, usually about half the fecundity of the previous spawn (Mylonas et al., 2003). However, the European sea bass produced only 3–4 spawns, while meagre have been shown to be able to produce up to 19 spawns after GnRHa implantation (present study) or 18 spawns after multiple GnRHa injections (Fernández-Palacios et al., 2009b).

The dramatic reduction in batch fecundity after the first week from GnRHa implantation–and the first 3–4 spawns–in meagre could result from the significant reduction in release of GnRHa from the implants after ~7 d (Mylonas et al., 2007). However, the implant can still release smaller amounts of GnRHa for at least 3 weeks, after which time they become completely depleted (Mylonas and Zohar, 2001). As a result of these GnRHa release kinetics, plasma LH was probably lower after a week from GnRHa implantation and resulted in the production of smaller batches of maturing eggs. Spawning stopped in all experiments 3 weeks after GnRHa implantation, when no more GnRHa was released by the implants. As plasma LH concentration was not measured in the present study, this hypothesis cannot be confirmed at this stage. Nevertheless, the results obtained here and from other published works on spawning induction of meagre with GnRHa implants or injections, point to our ability to manipulate the spawning kinetics and batch fecundity of meagre in aquaculture and adapt them to the needs of particular situations, by selecting GnRHa implants or multiple injections. For example, if an aquaculture operation has broodstock that are difficult to handle repetitively (*e.g.*, very large wild-caught fish, fish maintained in very large tanks or in sea cages, and inexperienced personnel) or if the hatchery requires a relatively steady supply of even small batches of eggs, then GnRHa implants would be appropriate for spawning induction. On the contrary, if handling of the fish repeatedly is not a problem (*e.g.*, smaller hatchery-produced fish, fish maintained in small tanks, personnel is very experienced in broodstock handling, *etc.*) and the hatchery requires large batches of eggs at specific times during the season, then use of GnRHa injections would be a more appropriate solution. The latter option has been investigated recently in order to establish the optimal interval for repeated GnRHa injections regarding batch fecundity and egg/larval survival, and it was shown that an interval of 7 d resulted in the highest fecundity and egg/larval survival (Duncan et al., 2013; Fernández-Palacios et al., 2011).

The overall mean (±SD) total relative fecundity obtained in the spawning induction experiments here was 380,780 ± 167,577 eggs kg^{-1} and, although it was variable among different years and experiments, it compares well with other studies with meagre. For example, use of GnRHa implants in large wild-caught broodstock resulted in the production of 276,000–527,000 eggs kg^{-1} in different years (Duncan et al., 2012). Using GnRHa injections resulted in the production of 198,200–359,000 eggs kg^{-1}, depending on the number of injections applied (Duncan et al., 2012, 2013). It is apparent from these studies that GnRHa implants produce larger numbers of eggs than GnRHa injections, through the induction of more spawns. It was also interesting to note that the highest total fecundity was obtained when females were allowed to spawn individually, as opposed to spawning in groups. It is not known at this stage whether this is a result of the natural breeding behavior of the species and its requirements (*e.g.*, pair vs group spawning). In terms of total reproductive season fecundity, a histological estimation in fish from the wild indicated a value of ~900,000 eggs kg^{-1} for a 20 kg female and for hatchery-produced fish fecundity was similarly estimated to 850,000 eggs kg^{-1} for a 5 kg female (Gil et al., 2013). It is clear from the above, that fish in the present study–as well as in other spawning induction studies–did not spawn their maximal egg potential. This conclusion was also reached based on the evaluation of the ovarian biopsies of the GnRHa implanted females after the cessation of spawning, which demonstrated that the females still contained large numbers of oocytes in mid and advanced vitellogenesis, with very little atresia. It is appropriate to speculate that further implantation with GnRHa could have induced further cycles of oocyte maturation, ovulation and spawning, and this is the focus of a current study (unpublished data).

Fertilization success was very high in all spawning induction experiments. Although some significant variations were observed between experiments in different years, mean fertilization was always >80% and in most cases >90%. Such very high fertilization percentages have been reported in all other spawning induction studies with meagre (Duncan et al., 2012; Fernández-Palacios et al., 2009a) and appear to be a characteristic of the species. Fertilization success in spawning induction studies in other fishes have been significantly less, and the mean (±SD) value in GnRHa-injected European sea bass was 71 ± 11% (Mylonas et al., 2003), <50% in GnRHa-implanted greater amberjack *Seriola dumerili* (Mylonas et al., 2004c), <40% in GnRHa-injected red drum *Sciaenops ocellatus* (Gardes et al., 2000), and 65% in GnRHa-implanted shi drum (Mylonas et al., 2004a). Although meagre hardly undergo maturation in captivity, they perform very well in response to hormonal treatment producing eggs with very high fertilization capacity.

Males too seem to perform well following GnRHa treatment fertilizing, fertilizing the vast majority of the spawned eggs. Although it was not the objective of the present study, important information has been gathered over three years on the spermiation characteristics of hatchery-produced males. Sperm production and quality characteristics of the males at the time of GnRHa treatment were similar among fish used in each year's spawning induction experiments, but significant differences were observed between years. Based on the overall high fertilization success obtained in all spawning induction experiments, it is safe to conclude that sperm characteristics of cultured meagre–after GnRHa implantation–were more than adequate for the production of fertilized eggs. Sperm density was similar to a recent study of the annual reproductive cycle in meagre (Mylonas et al., 2013-this volume) and was also similar to other Mediterranean marine fish (see review by Suquet et al., 1994), including the shi drum (13–26 × 10^9 spermatozoa ml^{-1}) (Mylonas et al., 2004a). The percentage of spermatozoa exhibiting forward motility was variable over the years, but values were similar to another published report of meagre (53–74%) (Schiavone et al., 2012), as well as the shi drum (70–80%) (Mylonas et al., 2004a). Regarding the duration of sperm motility, very large variations existed between the years, but most of the time values were similar to values reported for this species (0.57–1.33 min) (Mylonas et al., 2013-this volume; Schiavone et al., 2012).

Further to very high fertilization success, the present spawning induction experiments demonstrated that the produced eggs were in general of very high quality, based on the survival of the eggs during embryogenesis, hatching success and larval survival 5 d after egg collection. Some reduction in larval survival was documented in week 3 of

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spawning, but it cannot be explained based on the other data obtained, namely fertilization or hatching success. Fish were feeding well throughout the spawning experiments, so it is unlikely that the reduction in larval survival was caused by a nutrient deficiency of the eggs that completed vitellogenesis and were ovulated at the end of the experiment. A cumulative stress factor acting on the females and influencing indirectly the progeny through maternal transfer during early larval development may be a possibility (Bobe and Labbé, 2010; Schreck, 2010), but the nature of this factor is unknown at this stage. The finding of a reduction in larval survival in the last spawns in response to GnRHa implantation may support the multiple GnRHa injection method in meagre, and it needs further evaluation.

In conclusion, the present study demonstrated that meagre responds quite positively to hormonal induction of spawning with the use of GnRHa implants, producing a large number of daily spawns with high fecundity at the beginning and decreasing thereafter. The produced eggs were of high quality in terms of fertilization and hatching success, and early larval survival.

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