Aquaculture 464 (2016) 323–330

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Enhancement of oogenesis/spermatogenesis in meagre *Argyrosomus regius* using a combination of temperature control and GnRHa treatments

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ARTICLE INFO

Article history:
Received 30 March 2016
Received in revised form 24 June 2016
Accepted 5 July 2016
Available online 05 July 2016

Keywords:
Argyrosomus regius
Meagre
Reproduction
Induced spawning
GnRHa
Egg quality

ABSTRACT

A multiple GnRHa injection method for inducing spawning of high fecundity and quality in meagre was optimized. Specifically, the study (a) examined how long females would continue spawning in response to consecutive, weekly GnRHa injections, if maintained at the natural spawning temperature, and (b) evaluated whether males can produce adequate amounts of good quality sperm for the same period, with the assistance of a GnRHa therapy. Combined with stable temperatures (19–20 °C) that are prevalent during the early spawning season (April–May) in the Mediterranean Sea, each weekly GnRHa injection induced ~2 consecutive spawns per week for a period of 17 weeks. Fish spawned consistently 2 days after treatment and produced high fecundity and egg quality (*i.e.* fertilization, hatching and early larval development). Maximum mean total fecundity (±S.D.) obtained was 1,415,000 ± 149,000 eggs kg^{-1} in 32 spawns, being the highest total season fecundity reported for meagre, and significantly higher than the estimated maximum potential fecundity for the species. At the same time, sperm of consistently high quality (*i.e.* initial motility, duration of motility and storage survival) was produced in response to treatment with multiple GnRHa implants, though towards the end of the experiment a significant reduction (ANOVA, $P \leq 0.05$) in spermatozoa density was observed. This protocol is expected to enhance greatly commercial production of meagre.

Statement of relevance: The present study reports on an optimized protocol for the induction of consistent and repeated spawning in meagre, based on the use of weekly GnRHa injections combined with stable temperatures that are prevalent at the early spawning season in the Mediterranean Sea. This protocol produced eggs of high fecundity and quality (*i.e.* fertilization, hatching and early larval development) for a period of >4 months, and it is expected to enhance greatly commercial production of meagre.

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1. Introduction

The aquaculture production of meagre *Argyrosomus regius* (Sciaenidae) has increased rapidly in the last decade, in part due to the development of effective spawning induction methods (Duncan et al., 2012; Duncan et al., 2013; Fernández-Palacios et al., 2014; Mylonas et al., 2015), since meagre rarely undergo spontaneous oocyte maturation, ovulation and spawning in captivity (Duncan et al., 2013; Gil et al., 2013; Mylonas et al., 2013b; Soares et al., 2015). Meagre exhibit an asynchronous or group-synchronous oocyte development pattern (Duncan et al., 2012; Gil et al., 2013; Schiavone et al., 2006) with a spawning season in the wild extending from late spring to early fall, depending on geographic location (Abou Shabana et al., 2012; González-Quirós et al., 2011). In aquaculture, experiments with GnRHa treatments were effective in inducing maturation of both wild-caught (Duncan et al., 2012) and hatchery-produced broodstocks (Fernández-Palacios et al., 2014; Mylonas et al., 2013a; Mylonas et al., 2015).

Both liquid injections and controlled-release delivery systems that release GnRHa for a prolonged period of time (Mylonas and Zohar, 2001) have been shown to be effective in inducing maturation and multiple spawns in females, and GnRHa injections are especially useful in species such as meagre, which rarely undergo spontaneous final maturation in captivity (Duncan et al., 2012; Duncan et al., 2013;

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http://dx.doi.org/10.1016/j.aquaculture.2016.07.006
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Fernández-Palacios et al., 2014; Mylonas et al., 2013a; Mylonas et al., 2015). In a recent study (Mylonas et al., 2015), the overall egg production and egg/larval quality did not differ significantly between GnRHa implants and multiple GnRHa injections (up to 7 weekly injections). However, the resulting differences in spawning kinetics and production characteristics showed that multiple GnRHa injections resulted in more consistent spawning results and better control of egg production, and this method may offer significant advantages to commercial aquaculture production (Mylonas et al., 2015). Similarly, advantages in the use of repeated injections have been noted in European sea bass *Dicentrarchus labrax*, which is also a species with group-synchronous ovarian development (Asturiano et al., 2000). In this species, batches of oocytes undergo maturation with a long interval between them (*e.g.*, 3–10 days) and it appeared that repeated GnRHa injections could be timed to induce the ovulation of distinct batches, resulting in more consistent spawns of higher fecundity and egg quality (Forniés et al., 2001; Mylonas et al., 2003).

Meagre in the Mediterranean spawn between April and July (Abou Shabana et al., 2012; Gil et al., 2013), and in captivity they have been reported to spawn spontaneously in water temperatures between 19 and 23 °C (Mylonas et al., 2013b; Soares et al., 2015). Gametogenesis was reported to stop in response to the onset of high temperatures by mid July and ovaries contained large numbers of apoptotic oocytes (Mylonas et al., 2013b; Soares et al., 2015). Successful spawning induction methods using hormonal therapies have been implemented at water temperatures between 18 and 23 °C (Duncan et al., 2013; Mylonas et al., 2013a; Mylonas et al., 2015). Recent studies have also employed constant "early spawning season" temperatures (19–21 °C) during the period of hormonal treatment, and demonstrated that the fish responded very well to this thermal regime (Mylonas et al., 2013a; Mylonas et al., 2015), pointing to the potential of constant temperatures to be used as a way to extent the reproductive season in this species.

The objective of the present work was to optimize further the multiple GnRHa injection method for inducing spawning of high fecundity and quality in meagre, in combination with temperature manipulations, in order to extend the reproductive season. Specifically, the study (a) examined how long females would continue spawning in response to consecutive, weekly GnRHa injections, if maintained at the natural spawning temperatures, and (b) evaluated whether males can produce adequate amounts of good quality sperm for the same period, with the assistance of a hormonal therapy. We report here on an optimized protocol for the induction of spawning in meagre for a period of >4 months, based on the use of weekly GnRHa injections.

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. Fish came from eggs produced in the hatchery in 2004, 2006 and 2007. Feeding was done 5 days per week to apparent satiation with industrial feed (Skretting S.A., Spain and IRIDA, S.A., Greece). During the year and outside the period of the spawning induction experiments, fish were maintained in a large communal tank (10 m^3) exposed to a simulated natural photo-thermal regime (Fig. 1). Measurements of temperature and water quality (Dissolved Oxygen, NH_3-N and NO_2-N) were conducted once per week throughout the year. For spawning induction, single pairs of fish (one male and one female) were transferred to 5000-l Recirculation Aquaculture System (ACE, the Netherlands) supplied with seawater from a well, under simulated natural photoperiod, but controlled temperature ranging between 19 and 20 °C (Fig. 1). The maintenance of constant temperature was chosen based on previous experiments with meagre, showing that with the increase in summer temperature (>24 °C), the reproductive season was concluded (Mylonas et al., 2013b), while maintaining the temperature at spring levels (19–20 °C) resulted in fish sustaining vitellogenesis, and spermatogenesis and sperm production (Mylonas et al., 2015).

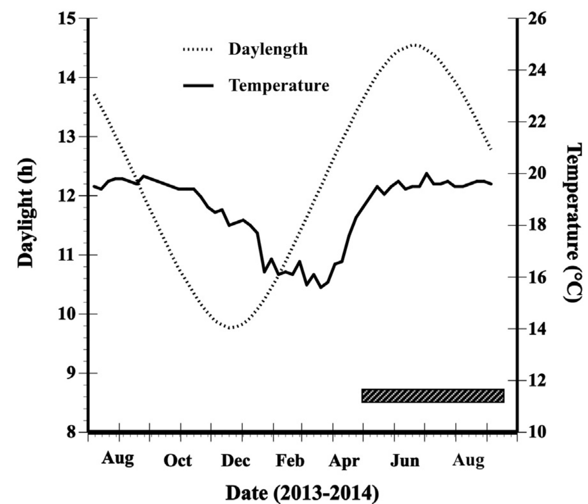

Fig. 1. Annual photoperiod and water temperature profile for the meagre broodstocks used in the spawning induction experiments (2013–2014). The horizontal bar at the bottom of the graph indicates the approximate duration of the spawning induction experiment.

2.2. Broodstock selection

To select the broodstocks for the spawning experiments, fish were starved 2 days prior to handling, 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 plastic cannula (Pipelle de Cornier, Laboratoire CCD, France) 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 batch of vitellogenic oocytes (n = 10). A portion of some biopsies was fixed in a solution of 4% formaldehyde-1% glutaraldehyde for histological processing. Females were considered eligible for spawning induction if they contained oocytes in full vitellogenesis with a diameter of >550 μm and very little atresia/apoptosis present (Mylonas et al., 2013a). Male fish were considered eligible for spawning induction, if they were in full spermiation (Spermiation Index ≥ 2, see later), releasing substantial amounts of sperm upon application of gentle abdominal pressure (Mylonas et al., 2013b).

2.3. Spawning induction experiments

Injections of GnRHa were administered once a week (every Monday) between 7 May and 28 August 2014 using four pairs of fish. Females (mean ± SD body weight 9.7 ± 1.0 kg) were treated with a GnRHa injection of 15 μg kg^{-1} (H-4070, Bachem, Switzerland). In order to enhance spermiation and ensure adequate amounts of sperm, males (7.9 ± 1.0 kg body weight) were treated at the start of the experiment with a 450 or 500 μg GnRHa implant (Mylonas et al., 2013a) for an effective dose of 40–60 μg kg^{-1} GnRHa. Implants of GnRHa were used instead of multiple injections, since previous work in European sea bass (*Dicentrarchus labrax*) demonstrated that GnRHa implants result in a more prolonged production of sperm (Rainis et al., 2003). If sperm production was considered low (Spermiation Index ≤ 1, see later) during any of the weekly injection times for the females, males were implanted again with GnRHa at the same doses. A single female with a single male were placed in separate 5000-l tanks connected to

overflow egg collectors and were allowed to spawn. If a female failed to spawn in response to 2 consecutive GnRHa injections, she was removed from the remaining experiment. The experiment was stopped when a cumulative total of two females (*i.e.*, 50%) failed to spawn in response to 2 consecutive injections. One week after the last GnRHa injection (122 days after the first GnRHa injection), all fish still in the experiment were removed from their spawning tanks, evaluated using ovarian biopsies and sperm collection, and were returned to a large communal tank (10 m^3) to recover. In addition, a male and a female were sacrificed to obtain the gonads for histological evaluation.

2.4. Evaluation of sperm quality

To obtain sperm for evaluation, the genital pore was rinsed, 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. Small volumes of sperm (50–100 μl) were collected using a positive displacement pipette (Gilson, M100) in order to avoid influencing the quantity and/or quality of sperm during spawning. The collected sperm sample was stored in a 500 μl micro-centrifuge tube placed on ice and then transferred to a 4 °C refrigerator until evaluation.

Spermiation condition was evaluated based on the presence and ease of sperm release upon application of gentle abdominal pressure (Mylonas et al., 2013b). Spermiation condition (Spermiation Index) was reported on a subjective scale from 0 to 3, with S0 = no sperm released, S1 = only a drop of sperm released after multiple stripping attempts, S2 = sperm was released easily after the first stripping attempt and S3 = copious amounts of sperm release with very little pressure. Only ≥S2 males released enough sperm to be collected for evaluation. Sperm quality parameters that were evaluated included (a) sperm concentration (number of spermatozoa ml^{-1} of sperm), (b) initial percentage of spermatozoa showing forward motility immediately after activation (sperm motility, %), (c) duration of forward sperm motility of ≥5% of the spermatozoa in the field of view (motility duration, min) and (d) survival of sperm during storage at 4 °C (sperm survival, days). Sperm concentration was estimated after a 21 to 4221-fold dilution (depending on spermatozoa density) with 0.9% saline using a Neubauer haemocytometer under 200× magnification (in duplicate) under a compound light microscope (Nikon, Eclipse 50i). Sperm motility (% spermatozoa showing forward motility) and motility duration (min) were evaluated on a microscope slide (400× magnification) after mixing 1 μl of sperm with a drop of about 50 μl of saltwater (in duplicate). Activated sperm samples were observed under the compound light microscope for the first time 10 s after activation. Sperm motility was determined subjectively using increments of 10% and sperm was considered immotile when <5% of the spermatozoa were exhibiting forward motility. Sperm was stored at 4 °C for the following days, and was examined every other day for motility, until no forward motility was observed. The survival time (days) for each sample was considered as the day before the sample was found to have lost all its motility capacity.

2.5. 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 5 or 10 ml (depending on the total number of eggs), after vigorous agitation. Fertilization success was evaluated at the same time by examining each of the eggs in this 5 or 10 ml sample for the presence of a viable embryo (usually at the blastula stage) using a stereoscope.

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 (almost 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 days at 19 ± 0.5 °C. Using a stereoscope, embryonic and early larval development was evaluated once a day for 5 days. The number of (a) live embryos was recorded 1 day after egg collection (or ~36 h after spawning, day 1), (b) hatched larvae was recorded 2 and 3 days after egg collection (>60 h after spawning) and (c) viable larvae was recorded 4 and 5 days after egg collection (~yolk sack absorption). For reference, hatching of meagre eggs takes place in 44–56 h at 18–20 °C.

Embryo survival was calculated as the number of eggs having live embryos 1 days after egg collection / 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 days 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 was 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; Mylonas et al., 2004a).

2.6. 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 (Leica RM 2245, 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).

2.7. Statistical analysis

Differences in mean relative fecundity and egg performance parameters (fertilization success, embryonic development, hatching and 5-d larval survival) among GnRHa injections (different times) were examined using two-way ANOVA with GnRHa injection number (n = 17) and spawn number after injection (n = 3) as factors, at a minimum P ≤ 0.05, followed by Duncan's New Multiple Range (DNMR) test at P ≤ 0.05, when appropriate. Differences in mean oocyte diameter and sperm quality parameters (sperm density, sperm motility, motility duration and sperm survival) at the time the females were given the weekly GnRHa injections were examined using one-way ANOVA with time as a factor, at a minimum P ≤ 0.05, followed by Duncan's New Multiple Range (DNMR) test at P ≤ 0.05, when appropriate. 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

Mean (±SEM) oocyte diameters at the onset of the study were 590 ± 10 μm, and throughout the study ranged between 550 ± 9 μm and 620 ± 6 μm (Fig. 2), with some small, but statistically significant variations (ANOVA, P = 0.04). Large numbers of vitellogenic oocytes, with very little atresia/apoptosis, could be seen in the biopsies of all four females until week 5, of three females until week 16 and of two females until week 18 – one week after the last GnRHa injection (Fig. 3).

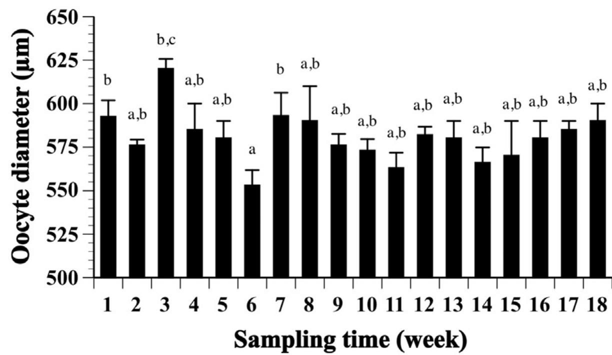

Fig. 2. Mean (+SEM) oocyte diameter of ovarian biopsies obtained from females (n = 1–4) treated with consecutive weekly GnRHa injections (n = 17) for a period of 125 days between May and August 2014. There were statistically significant variations during the experiment, and significantly different means are indicated by different letter superscripts (ANOVA, DNMR, P ≤ 0.05).

One female failed to spawn in response to GnRHa injections on weeks 6 and 7, and was thus removed from the study. Another female failed to spawn in response to GnRHa injections on weeks 16 and 17, at which time the experiment was concluded, since 50% of the females failed to spawn for two consecutive weeks.

The GnRHa injected females spawned for up to 17 consecutive weeks (2 out of 4 females), most of the times spawning both on the 2nd and 3rd day after each weekly injection (Fig. 4). The first spawns obtained on the 2nd day after each injection had significantly higher fecundity compared to the second spawns obtained on the 3rd day after each injection (ANOVA, P < 0.001). Overall, there was no significant effect of injection number on mean fecundity (ANOVA, P = 0.83), but there was a slight negative linear correlation (n = 32, R^2 = 0.38, P < 0.01, not shown) between the 3rd day fecundity values and GnRHa injection number, suggesting a drop in fecundity of the second spawn obtained after the GnRHa injections, as time progressed. Fertilization success was high during the experiment (Fig. 4), without any significant effect of either GnRHa injection number/week (ANOVA, P = 0.16) or spawn number after each injection (ANOVA, P = 0.21), and with the exception of the 2nd spawn of the last GnRHa injection (54%) it was always >80% and most of the times >90%.

Embryonic development was very high overall and did not differ significantly between eggs obtained in the first or second spawn after the GnRHa injections (data not shown), in terms of 24-h embryo survival (ANOVA, P = 0.54), hatching (ANOVA, P = 0.50) or 5-d larval survival (ANOVA, P = 0.80). Similarly, there were no significant differences over the course of the study in response to the consecutive GnRHa injections (data not shown), in terms of 24-h embryo survival (ANOVA, P = 0.99), hatching (ANOVA, P = 0.88) or 5-d larval survival (ANOVA, P = 0.33), even after 17 weekly injections of GnRHa. Mean 24-h embryo survival for the 17 weekly spawns ranged between 60 and 97%, hatching between 88 and 99%, or 5-d larval survival between 89 and 99%.

Sperm production appeared to decrease after week 3, based on the reduction in Spermiation Index from S2 to S1 in two males (data not shown). Therefore, all males were implanted again with a GnRHa implant. Spermiation Index varied from S1 to S3 in subsequent samplings, and between weeks 4 and 15 at least one male was given another GnRHa implant at each sampling. Spermatozoa density varied significantly during the study, though without a consistent trend (Fig. 5). During the last few weeks, spermatozoa density was reduced significantly compared to the seasonal maximum levels, and on the sampling for week 15 it averaged only 0.05 × 10^9 spermatozoa ml^{-1}, a value that was 400× lower than the mean at the beginning of the study. A GnRHa implantation at this time increased the Spermiation Index (data not shown) and the spermatozoa density of sperm obtained from all males the following two weeks. The other sperm quality parameters (spermatozoa motility, motility duration and sperm survival during storage) were variable during the 17 weeks of the study, but without any statistically significant differences or a consistent trend (Fig. 5). As long as sperm could be collected (Spermiation Index ≥ 2), it appeared that spermatozoa motility, duration of motility and sperm survival during storage remained statistically unchanged during the study. The exception may be on the sampling of week 15, when the very low spermatozoa density could be related to much lower values for all other sperm quality parameters, though this could not be confirmed statistically. Although the GSI of the sacrificed male at the end of the study (week 18) was only 0.8%, the testes contained not only

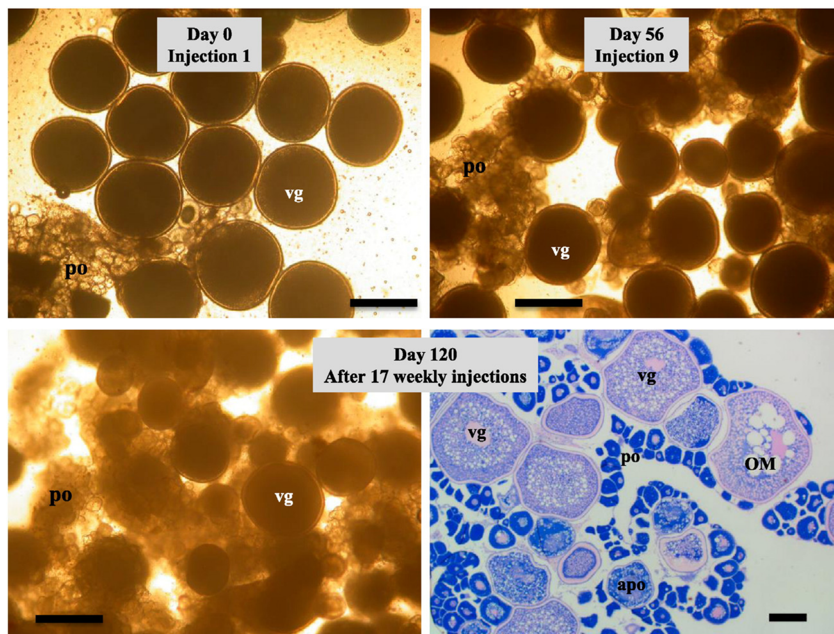

Fig. 3. Wet mount and histological evaluations of representative ovarian biopsies obtained from meagre injected weekly with GnRHa, prior to injection 1 and 9, and a week after injection 17. apo = apoptotic, Vg = vitellogenic, OM = oocyte maturation, po = primary oocyte. The black bar in the microphotographs of the wet mounts and histological section indicate 500 and 200 µm, respectively.

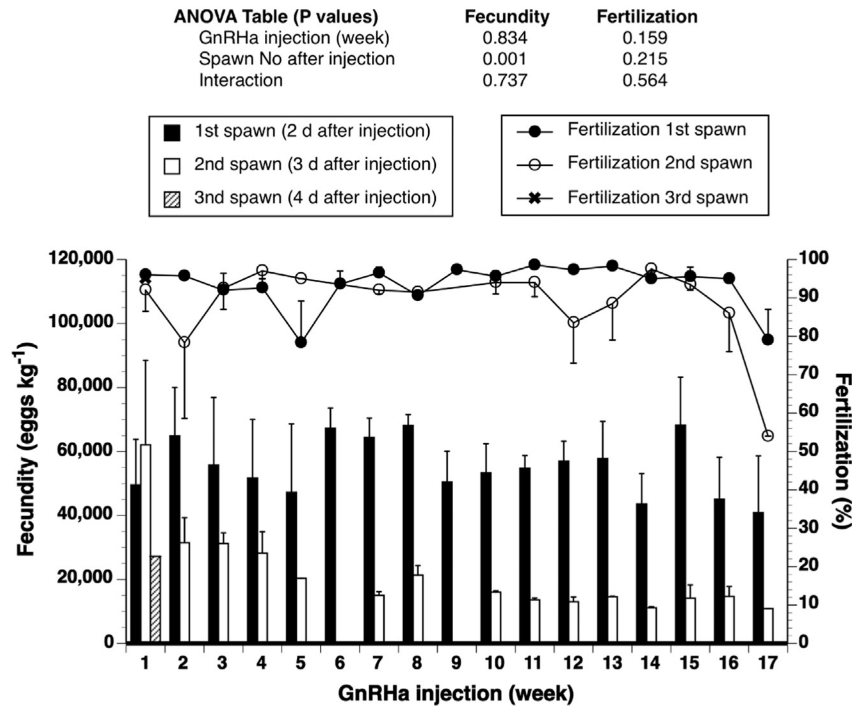

Fig. 4. Mean (±SEM) daily batch relative fecundity and fertilization success of individual meagre females (n = 1–4) induced to spawn with multiple GnRHa injections (n = 17, once every week) during 2014. The first GnRHa treatment was administered on 8 May 2014. The two-way ANOVA (GnRHa injection number *vs* Spawn number after each injection) indicated the existence of a significant effect of Spawn number after injection (P = 0.001) on fecundity, while there were no other significant effects (either on fecundity or fertilization). Linear regression analysis indicated the existence of a significant negative relation between GnRHa injection number and fecundity for the 2nd spawn data (n = 32, R^2 = 0.37, P = 0.001, data not shown).

spermatozoa, but also spermatocysts with germ cells at various stages of development (Fig. 6).

4. Discussion

Corroborating recent results (Fernández-Palacios et al., 2014; Mylonas et al., 2015), multiple weekly GnRHa injections in the present study have been very effective in inducing consistent and repeated spawning in meagre, producing high fecundity and egg quality (*i.e.* fertilization, hatching and early larval development). Combined with stable temperatures (19–20 °C) that are prevalent during the early spawning season (April–May) in the Mediterranean Sea, each weekly GnRHa injection induced ~2 spawns per week for a period of 17 weeks. Although the mean diameter of the largest vitellogenic oocytes prior to each GnRHa injection varied significantly during the experiment, there was no consistent trend and at all times females contained oocytes >550 μm in diameter. At this size, meagre oocytes are considered to be post-vitellogenic and capable of undergoing maturation and ovulation (Duncan et al., 2013). The present results suggest that after each GnRHa-induced spawning, the ovaries continued vitellogenesis and produced another batch of post-vitellogenic oocytes ready for maturation, within a period of 4–5 days after spawning. This process

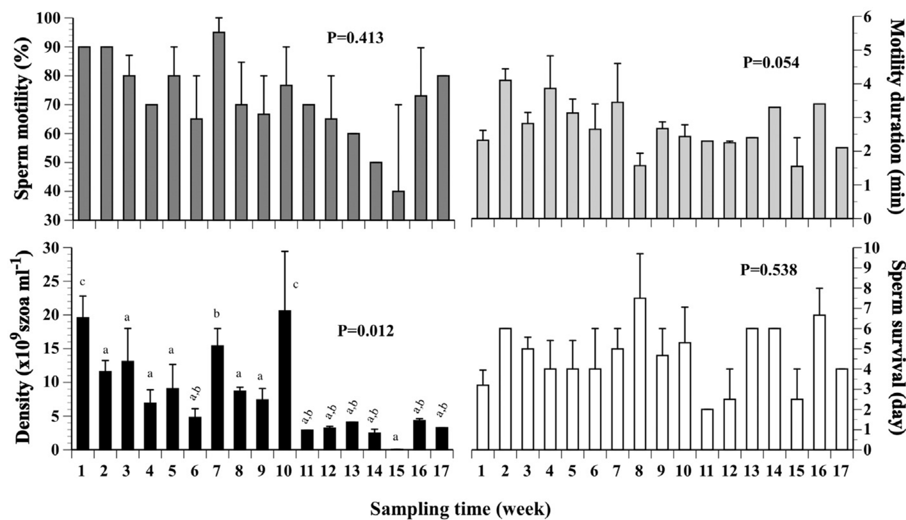

Fig. 5. Mean (+SEM) sperm quality of meagre (n = 1–4) at the times females were injected with GnRHa (once a week). All males were given an initial GnRHa implant at the beginning of the experiment, and then as needed when sperm production was considered inadequate. Data were analyzed using one-way ANOVA and the resulting P values are indicated on the graphs. Statistically different means are indicated by different letter superscripts (ANOVA, DNMR, P ≤ 0.05).

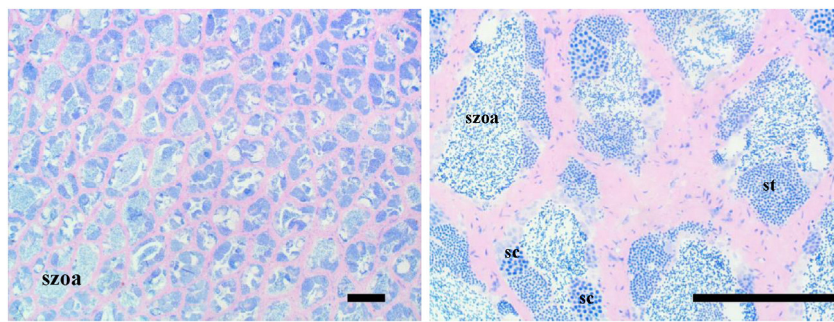

Fig. 6. Histological sections of a testis from meagre at the end of the spawning experiment, after 17 weeks of spawning one to three times per week. Fish were given GnRHa implants at different times during the experiment, as needed. szoa = spermatozoa, sc = spermatocytes, st = spermatids. The black bar in the microphotographs indicates 200 μm.

continued for 17 weeks. In fact, the histological evaluation of the ovary of the two fish that spawned after the last GnRHa injection, showed that more vitellogenic oocytes and very little atresia were present at this time. So, meagre were capable of undergoing vitellogenesis for a very long period from May to August, with repeated cycles of oocyte maturation in response to weekly GnRHa injections. In the males, the use of GnRHa implantations maintained adequate sperm volume for at least 17 weeks, as demonstrated by the high fertilization success.

In nature, meagre in the Mediterranean region spawn between April and July (Abou Shabana et al., 2012; Gil et al., 2013), while in the Atlantic Ocean (Gulf of Cádiz, Spain) they spawn between March and September (González-Quirós et al., 2011; Prista et al., 2014). Although the ambient water temperature has not been reported in all studies, optimal temperatures for growth and development of meagre are considered to be between 17 and 21 °C (Cárdenas, 2010). These temperatures are in agreement with the prevailing water temperatures in the Mediterranean region at the onset and peak of the meagre spawning period in April–May, and in some years up to the middle of June. Also in captivity, meagre have been reported to spawn spontaneously in water temperatures between 19 and 23 °C (Mylonas et al., 2013b; Soares et al., 2015), while successful spawning inductions using hormonal therapies have been reported at water temperatures between 18 and 23 °C (Duncan et al., 2013; Mylonas et al., 2013a; Mylonas et al., 2015). So, it seems that reproductive development (gametogenesis, maturation and spawning) stops in response to the onset of high water temperatures in the summer, which in the Mediterranean Sea reach 26–28 °C by mid July. In the present study, the exposure of fish to constant "early spawning" temperatures was conducive to gametogenesis and allowed the GnRHa to induce the recruitment of multiple batches of oocytes into vitellogenesis, oocyte maturation and ovulation. Maintaining water temperatures constant during the spawning season has been shown in other fish species to extend the period during which the fish spawn naturally or are capable to respond to hormonal treatment and undergo oocyte maturation, ovulation and spawning. For example, the natural spawning season of gilthead seabream (*Sparus aurata*) under natural Mediterranean Sea conditions extends for 100–150 days between December and April (Mylonas et al., 2011; Zohar et al., 1995). However, maintaining fish in a simulated natural photoperiod and a constant 19 °C (early spring temperature) extended the spawning period to 160–210 days from January to June (unpublished data). In striped bass (*Morone saxatilis*) males, maintaining broodstock at constant spawning temperatures (18 °C) extended the spermiation period into late July (as opposed to May), though the same effect was not observed in the females (Clark et al., 2005). Also, greater amberjack (*Seriola dumerili*) in the Canary Islands (Spain) where the water temperature during the summer spawning season does not increase >2–3 °C, have a prolonged spawning season from June to October (Fernández-Palacios et al., 2015). On the contrary, in the Mediterranean Sea where summer peak temperatures reach 28 °C, the same species spawns only between late May to early July (Mylonas et al., 2004b). Hence, it seems that dissociating the thermal and photoperiodic regime of broodstock, by preventing the natural rise in water temperature during late Spring and Summer, may allow for a prolongation of the reproductive season.

The annual potential fecundity of meagre has been estimated histologically from ovaries of wild breeders from the fishery, as well as from hatchery-produced breeders (Gil et al., 2013). Hatchery-produced meagre had a potential annual fecundity $F_p = 0.0012\ W_s - 1.434$, where F_p is the fecundity (millions) and W_s is the body weight (g). According to this formula, reared meagre may produce ~1 million eggs kg^{-1} per reproductive season. The study also demonstrated that for a given body weight, potential annual fecundity was significantly higher in hatchery-produced breeders compared to breeders from the wild (Gil et al., 2013). Currently, it is not well known what is the "normal" annual fecundity of meagre in captivity, since meagre rarely spawn spontaneously in captivity (Mylonas et al., 2013b; Soares et al., 2015). For example, in the first reported spontaneous spawning of meagre (Mylonas et al., 2013b), only two of seven females in the broodstock appeared to participate in a total of six spawning events with fecundities ranging between 38,000 and 92,000 eggs kg^{-1} $spawning^{-1}$, and a mean total season (*i.e.* annual) fecundity of 376,000 eggs kg^{-1}. In a more recent study, a broodstock of 10 breeders (sex ratio not reported) of a mean weight of 2.2 kg was recorded to spawn 5 times, producing only ~80 g of eggs (we estimate ~100,000 eggs) (Soares et al., 2015), presumably the result of a single female spawning at each spawning event. This would mean that fecundity was a mere 45,000 eggs kg^{-1} $spawning^{-1}$. And if we assume that the same female spawned each time, then the total season fecundity would be 225,000 eggs kg^{-1}. Based on the estimations of potential annual fecundity in captive and wild meagre reported earlier (Gil et al., 2013), it becomes apparent that the amount of eggs produced in the rare occasions where meagre spawn spontaneously in captivity is only a very small fraction of the fish's potential.

More fecundity data have been reported from studies using either GnRHa injections or implants. For example, hatchery-produced meagre treated with a single GnRHa implant produced total fecundities ranging between 201,000 and 479,000 eggs kg^{-1} in a total of 7–17 daily spawning events (Mylonas et al., 2013a). In the same study, females spawning individually in separate tanks after a single GnRHa implant, produced a total of 168,000 to 699,000 eggs kg^{-1} in a total of 5–19 daily spawning events. Also, a single GnRHa implant in wild-caught breeders, resulted in 14 consecutive spawns with a total fecundity of 276,200 eggs kg^{-1}, while a single injection produced 198,200 eggs kg^{-1} in 5 consecutive spawns (Duncan et al., 2012). In all these mentioned studies it is sure that females did not spawn their maximum potential of eggs, since spawning lasted for only 3 weeks, and females still contained large numbers of viable vitellogenic oocytes at the end of spawning. Higher total fecundities ranging between 432,000 and 896,000 ± eggs kg^{-1} in a total of 12–26 daily spawning events have been reported from meagre given three consecutive GnRHa implants (Mylonas et al., 2015). In the same study, meagre treated with weekly injections of GnRHa produced total fecundities of 614,000 or

1,402,000 eggs kg^{-1} after 5 or 7 injections, respectively, in a total of 10–16 daily spawning events. Finally, in a hatchery-produced broodstock in the Canary Islands (Spain), females given 3 consecutive GnRHa injections produced a mean fecundity of 247,000 eggs kg^{-1} inj^{-1} (Fernández-Palacios et al., 2014), for an estimated total reproductive season fecundity of 741,000 eggs kg^{-1} in a total of 6 daily spawns. In comparison, in the present study fish produced a total fecundity of 1,415,000 eggs kg^{-1} in 32 spawns over 17 weeks and consecutive GnRHa injections, the highest total season fecundity reported to date for meagre, and significantly higher than the reported maximum potential fecundity for the species (Gil et al., 2013). All these studies demonstrated that meagre can respond to multiple hormonal treatments and produce eggs over a long period of time, which may depend more on the ambient water temperature and hormonal treatment, than on the determinate potential fecundity of the species (Gil et al., 2013). This may be a characteristic of Sciaenid species, since red drum (*Sciaenops ocellatus*) that were held under continuous environmental conditions to mimic the spawning period, spawned continuously for a total of seven years resulting in 360 spawns for a total of 250 million eggs from two females (Thomas et al., 1995).

From the studies on the use of GnRHa injections and implants carried so far in meagre (including the present one), it seems clear that multiple GnRHa injections result in a more prolonged and consistent spawning response, and the highest fecundity reported (Fernández-Palacios et al., 2014; Mylonas et al., 2015). From a physiological-endocrine point of view, the weekly GnRHa injections may be more appropriate and in line with the natural ovulation rhythm and spawning events of meagre. The GnRHa release from the GnRHa implants is maximal soon after administration to the fish, and the plasma levels decrease gradually over a period of 1–3 weeks (Mylonas and Zohar, 2001; Mylonas et al., 2007). In response to this sustained elevation in plasma GnRHa, the pituitary releases luteinizing hormone (LH) - the gonadotropin responsible for oocyte maturation and ovulation (Rosenfeld et al., 2007) - also in a sustained fashion (Mañanos et al., 2002; Mylonas and Zohar, 2001). In general, plasma LH remains elevated for as long as GnRHa remains in circulation (Mylonas et al., 1997a; Mylonas et al., 1998; Mylonas and Zohar, 2001; Mylonas et al., 2007). In females with synchronous ovarian development, such as the striped bass, these sustained elevations in plasma LH are necessary to support the process of oocyte maturation, which requires 5–10 days to be completed (Mylonas et al., 1998). Also in fish with asynchronous ovarian development and a daily spawning rhythm, such as the gilthead seabream, the sustained elevations in plasma LH induced by GnRHa implants (Mylonas and Zohar, 2001) induced daily spawning of high quality eggs for a prolonged period of time, (Zohar et al., 1995). Therefore, treatment with GnRHa implants may be an efficient method to support oocyte maturation and induce the expected (*i.e.* "natural") spawning kinetics in fishes with either synchronous or asynchronous ovarian development.

However, the species-specific natural profile of LH during oocyte maturation may be different (slightly or significantly) than what the GnRHa implants induce. In general, plasma LH is very low during vitellogenesis and it increases sharply during oocyte maturation (Gothilf et al., 1997; Mylonas et al., 1997b). This increase in plasma LH may last for many days in species with synchronous ovarian development, such as striped bass and common carp (*Cyprinus carpio*) (Yaron, 1995) and may last for a few weeks in cold-water species, such as salmon and trout (*Oncorhynchus* spp) (Prat et al., 1996; Zohar and Billard, 1984). On the contrary, in species with asynchronous ovarian development and daily spawning such as the gilthead seabream, LH rises and falls on a daily basis, thus inducing oocyte maturation of a batch of post-vitellogenic oocytes (Gothilf et al., 1997). Therefore, the prolonged release of LH induced by the implants appears to be relevant to the natural ovulation cycles of not only synchronous species, but also some asynchronous species, even if their natural LH profile is significantly different.

Based on very recent work (unpublished data) and the few published reports with spontaneous spawning of meagre in captivity (Mylonas et al., 2013b; Soares et al., 2015), the natural ovulatory cycle has a period of 2–10 days. So, the GnRHa injections given every 7 days are more successful in inducing oocyte maturation, perhaps because they mimic better the natural spikes in plasma LH that accompany the maturation-ovulation cycle of each successive batch of oocytes. Similarly, GnRHa injections spaced 7–14 days apart have been more appropriate for the induction of multiple spawns in the European seabass, than GnRHa implants (Forniés et al., 2001; Mylonas et al., 2003). As with meagre, the European seabass spawns multiple times in captivity with an ovulatory cycle of 1–2 weeks (Asturiano et al., 2000). So, in species with rather long ovulatory cycles (many days), the necessary spikes of LH may be induced better with appropriately timed GnRHa injections, thus inducing multiple spawns.

In regards to the males in the present study, the use of repeated GnRHa implantations in combination with stable "early spawning" water temperatures maintained adequate sperm production for at least 17 weeks, as demonstrated by the high fertilization success of the spawned eggs. Sperm density varied significantly during the study with a tendency to decline as the study proceeded, but still the amount of sperm produced was enough to give high fertilizations. As long as sperm could be collected, the quality of the sperm (motility parameters such as percentage forward motility, motility duration and motility survival during storage) was comparable to other studies (Mylonas et al., 2013b) and remained unchanged over the experimental period. Even at the last sampling (week 18) the sacrificed male had a GSI similar to what has been reported in the wild at the peak of the reproductive season (0.7%, Abou Shabana et al., 2012). The testes contained not only spermatozoa in the tubules, but also spermatocysts with germ cells at earlier stages of development, though the hypertrophied somatic tissue showed some signs of the end of the spawning season (Mylonas et al., 2016; Rainis et al., 2003). These results suggest that the fish were still undergoing spermatogenesis and had the potential for producing more spermatozoa.

In conclusion, the present study reports on an optimized protocol for the induction of consistent and repeated spawning in meagre, based on the use of weekly GnRHa injections combined with stable temperatures (19–20 °C) that are prevalent at the early spawning season (April–May) in the Mediterranean Sea. At the same time, sperm of high quality - though of diminishing spermatozoa density - was produced in response to treatment with multiple GnRHa implants when spermiation was diminished. This protocol produced eggs of high fecundity and quality (*i.e.* fertilization, hatching and early larval development) for a period of >4 months, and it is expected to enhance greatly commercial production of meagre.

Acknowledgements

This study has been supported in part under the framework of the European Union Seventh Framework Programme project DIVERSIFY (KBBE-2013-07 single stage, GA 603121) titled "Exploring the biological and socio-economic potential of new/emerging fish species for the expansion of the European aquaculture industry" awarded to CCM and ND.

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