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Reproductive development, GnRHa-induced spawning and egg quality of wild meagre (*Argyrosomus regius*) acclimatised to captivity

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Abstract The objective of the study was to acclimatise wild-caught meagre (*Argyrosomus regius*) to captivity to produce viable eggs for aquaculture production. Twelve meagre (3 males and 9 females, mean weight = 20 ± 7 kg) were caught and transported to a land-based facility on 26 October 2006. During, March to June 2007, all three males were spermiating and five of the nine females were in vitellogenesis with mean maximum oocyte diameter ≥550 μm. No spontaneous spawning was observed. Two hormone treatments, either a single injection of gonadotropin-releasing hormone agonist (GnRHa, 20 μg kg^{-1} for females and 10 μg kg^{-1} for males) or a slow-release implant loaded with the same GnRHa (50 μg kg^{-1} for females and 25 μg kg^{-1} for males), were used to induce spawning on three different dates on 26 March 2007, 4 May 2007 and 18 April 2008. From each spawning event, the following parameters were determined: fecundity, number of floating eggs, egg size, fertilisation and hatching success, unfed larval survival, and proximal composition and fatty acid profile of the eggs. In 2007, two females that were injected on 26 March and 4 May spawned a total of 5 times producing 9,019,300 floating eggs and a relative fecundity of 198,200 eggs kg^{-1} and two different females that were implanted on the same dates spawned 14 times producing 12,430,000 floating eggs and a relative fecundity of 276,200 eggs kg^{-1}. In 2008, a pair that was implanted spawned five times producing a total of 10,211,900 floating eggs and a relative fecundity of 527,380 eggs kg^{-1}. The latency period was 48–72 h. Parameters were compared between hormone treatments, date of hormone induction and parents determined by microsatellites. Percentage hatch and egg size were 70 ± 0.3% and 0.99 ± 0.02 mm, respectively, for GnRHa-implanted fish and were significantly higher ($P < 0.05$) compared to 30 ± 0.3% and 0.95 ± 0.03 mm, respectively, for injected fish. Few differences were observed in proximal composition and fatty acid profile and for all spawns mean (% dry weight) lipid content was 17.3 ± 3.0%, carbohydrate was 4.4 ± 1.9% and protein was 31.5 ± 6.4% and the essential fatty acids: Arachidonic acid (ARA, 20:4n-6) ranged between 0.9 and 1% (of total fatty acids), eicosapentaenoic acid (EPA 20:5n-3) 7.7–10.4% and docosahexaenoic acid (DHA 22:6n-3), 28.6–35.4%. All good quality spawns

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were obtained in the second and/or third spawn after GnRHa treatment, whereas all bad quality spawns were obtained either on the first spawn or after the fifth spawn. Both spawning protocols gave commercially viable (1,000,000+) numbers of good quality eggs that could form the basis of a hatchery production.

Keywords Meagre · *Argyrosomus regius* · Reproduction · Induced spawning · Egg quality

Introduction

A number of sciaenid species are established species for the aquaculture industry (FAO 2010), including the red drum (*Sciaenops ocellatus*) in the Americas (Gardes et al. 2000), the yellow croaker (*Larimichthys crocea*) in Asia (Chen et al. 2003). In 2009, production of red drum was 51,476 tons and yellow croaker was 66,021 tons (FAO 2011). Characteristics of red drum that have made the species successful in aquaculture include controlled spawning of good quality eggs in captivity, larvae that can be reared on an industrial scale using live feeds and formulated diets, and juveniles that mature reproductively after reaching harvest size and that exhibit good growth and feed conversion efficiency during ongrowing (Davis 1990a, b; FAO 2005–2010a; Mañanós et al. 2008). The meagre (*Argyrosomus regius*) has similar biological characteristics (Mateos-Velasco 2007; FAO 2005–2010b) and is a species with good aquaculture potential for the Mediterranean region (Quéméner et al. 2002), though production is still limited to a total of 4,112 tons in 2009 that was produced in Egypt, Spain, France, Italy and Portugal (FAO 2011).

Despite of this species aquaculture potential and rearing efforts by many commercial operations, little peer-reviewed information has been published on the cultured methods being used for meagre, and this has resulted in the dependence of the industry to just a few producers (FAO 2005–2010b), and at times a restricted supply of juveniles. The few peer-reviewed articles available and the many short communications presented in conferences have described general aspects of the aquaculture potential of meagre (Mateos-Velasco 2007), larval rearing (Cruz et al. 2007; Estévez et al. 2007; Fernández-Palacios et al. 2007; Hernández-Cruz et al. 2007; Rodríguez-Rúa et al. 2007; Roo et al. 2007), ongrowing and nutrition (Ortega and de la Gándara 2007; Vargas-Chacoff et al. 2007; Estévez et al. 2010) product quality (Garrido et al. 2007; Hernández et al. 2007), and some effort has also been given to the study of reproduction (Duncan et al. 2007; Grau et al. 2007; Schuchardt et al. 2007; Duncan et al. 2008; Estevez et al. 2009; Fernández-Palacios et al. 2009a, b, 2011a, b, Mylonas et al. 2011a). To date, there are no reports of spontaneous maturation and spawning in captivity. Commercial production relies on treating females in advanced stages of vitellogenesis with gonadotropin-releasing hormone agonists (GnRHa), either in the form of injections (Duncan et al. 2007; Grau et al. 2007; Duncan et al. 2008; Fernández-Palacios et al. 2009a, b, 2011a, b) or slow-release implants (Duncan et al. 2007; Grau et al. 2007; Duncan et al. 2008; Mylonas et al. 2011a).

In an effort to establish effective hormonal induction protocols for this species of great interest to the Mediterranean aquaculture industry, the present study describes the reproductive development, GnRHa-induced spawning and egg quality parameters of wild-caught meagre acclimatised to captivity.

Materials and methods

Broodstock management

Adult wild meagre were caught off the Algarve coast, Portugal, and 3 males and 9 females, with a mean weight 20 ± 7 kg, were transported to IRTA Sant Carles de la Ràpita on the 26 October 2006. The fish were acclimatised to captivity (Duncan et al. 2008) and held in the following conditions, six fish in each of two thermally isolated 18-m^3 rectangular covered tanks (3 × 6×0.9 m) in a recirculating system, a natural photoperiod with 50 lux of natural light and a controlled natural temperature cycle with a range between 14 and 25°C. Frozen sardines (Clupeidae, fish market, Sant Carles de la Ràpita, Tarragona, Spain) and squid *Loligo gahi* (from Falkland Islands, Congelados Marcos, Tarragona, Spain) were fed at 0.8–2.5% body weight per day (2:1 weight ratio of sardine to squid) 5 days a week. Beginning in February 2007, a commercially available broodstock diet (Vitalis Repro, Skretting, Burgos, Spain) was included at a 0.1% BW. During the period from February to October 2007, the number of fish decreased due to

Table 1 Wild meagre (*Argyrosomus regius*) broodstock data (weight, oocyte size or sperm motility: percentage and duration) at the time of GnRHa application in the 2007 trials and the dose applied as an implant (Imp) or injection (Inj)

Fish	Weight (kg)	Oocyte diameter (μm) or sperm motility (% and duration)		Hormone treatment (μg kg^{-1})	
		26/03/07	04/05/07	26/03/07	04/05/07
1♀	25–23	590 ± 60	1020 ± 60	54 (Imp)	48 (Imp)
2♀	21–21	630 ± 60	590 ± 60	52 (Imp)	48 (Imp)
3♀	19–18	410 ± 40	190 ± 10	None	None
4♀	27	270 ± 40	Genital closed	None	None
5♀	20–20	660 ± 70	610 ± 60	20 (Inj)	20 (Inj)
6♀	26–25	580 ± 40	580 ± 50	20 (Inj)	20 (Inj)
7♀	18–17.5	Genital closed	120 ± 30	None	None
8♀	30	Genital closed	Dead	None	
1♂	18–16.5	100% 5′25″	90% 5′28″	25 (Imp)	27 (Imp)
2♂	26	90% 5′00″	90% 4′30″	11 (Inj)	12 (Inj)
3♂	14	No sample	80% 5′09″	25 (Imp)	None

mortalities caused by injuries incurred by fish jumping inside the enclosed tanks and the over inflation of the swim bladder.

Spawnings

The maturity status of all fish present at the time of sampling (number of broodstock, n in brackets) was examined on the 26 March ($n = 3$ males, 8 females), 4 May ($n = 3$ males, 7 females) in 2007 and 15 June ($n = 3$ males, 6 females) in 2007, and on the 18 April ($n = 1$ male, 1 female) and 23 May ($n = 1$ male, 1 female) in 2008. On each date, all fish were anesthetised and sperm (from spermiating males) and ovarian samples were obtained. The maturity status of a female that died on the 26 February 2007 was also assessed and recorded. Sperm was collected in a 1-mL syringe while applying abdominal pressure and motility percentage, and duration was recorded for samples activated with sea water and observed at 100× magnification. Ovarian samples were obtained by inserting a plastic catheter (internal diameter of 1 mm) approximately 10–15 cm into the genital pore and applying a slight suction. Fresh ovarian samples were examined at 50× magnification, in clearing solution (6 mL:absolute ethanol, 3 mL:formalin, 2 mL:glycerol and 1 mL:acetic acid), and the diameter (accuracy of 20 μm) was recorded in two different analyses: (a) 100–150 randomly selected oocytes and (b) 30 of the largest vitellogenic oocytes. Females with oocytes of a diameter greater than 550 μm were treated with either a single GnRHa (des-Gly^{10}, [D-Ala^{6}]-gonadotropin-releasing hormone ethylamide) injection of approximately 20 μg kg^{-1} or were implanted with slow-release GnRHa implants of a dose of approximately 50 μg kg^{-1}, while mature males were treated with half these doses (Table 1). In 2007, two mature females were implanted on the 26 March and 4 May and were placed together in a tank with two males and two of the immature females. Two other mature females were injected with GnRHa on the same two dates and placed together in another tank with one spermiating male and the remaining immature females. In 2008, only a single pair (a male and a female) was available and the fish were given GnRHa implants (47.8 μg kg^{-1} for the female 7♀ and 16.1 μg kg^{-1} for the male 3♂) once on the 18 April.

After hormonal treatment, the external egg collectors were examined on a daily basis and spawned eggs were retrieved. Eggs were separated into floating (FE) and non-floating (NFE) in a measuring cylinder, and the volumes and number of eggs were recorded. Floating eggs were removed, washed thoroughly and subsequently kept in seawater that had been UV treated. The eggs were observed under a stereomicroscope to assess the morphology, fertilisation success and embryological stage and transferred to the laboratory where eggs were placed individually in a 96-well EIA plates (EIA plates, Nunc) (Shields et al. 1997; Giménez et al. 2006). One developing egg was

placed in each well using a Pasteur pipette and each well then filled with 200 µL autoclaved seawater, and the EIA plate was incubated in darkness at 17°C in a refrigerated incubator. Replicate samples of 20,000 eggs were also incubated in 35-L mesh baskets (150 µm nylon mesh) at 17 ± 1°C in order to compare hatching success to that obtained in the EIA plates. For the EIA plate samples, the eggs/larvae were inspected and counted on a daily basis, in order to calculate the hatching success, percentage survival after hatching, and mortality at 3 and 5 days post-hatching (dph), as well as time to 100% mortality. Considering that first feeding and the point of no-return in this species occur at 3 and 7 dph, respectively, high-quality batches (good) of eggs were considered those with a mortality of less than 25% at 5 dph and less than 10% from 3 to 5 dph, whereas low-quality batches (bad) of eggs were considered those with mortality of greater than 35% at 3dph (Giménez et al. 2006).

Biometry and biochemical analysis

Samples of floating eggs (in the blastula/gastrula stage of development) and newly hatched larvae were taken for biometry and individual dry weight determination. Egg diameter and larval standard length were measured under a stereomicroscope using an image analyser (AnalySIS, SIS Gmbh, Germany). Around 50 eggs and 20 larvae were sampled, placed in beakers and euthanised using a lethal concentration of 3-amino benzoate methane sulphonate (MS 222, 1,000 mg l^{-1}). Dry weight (DW) determination was carried out by rinsing the eggs and larvae with distilled water to remove salt and then oven-drying at 60°C for 24 h.

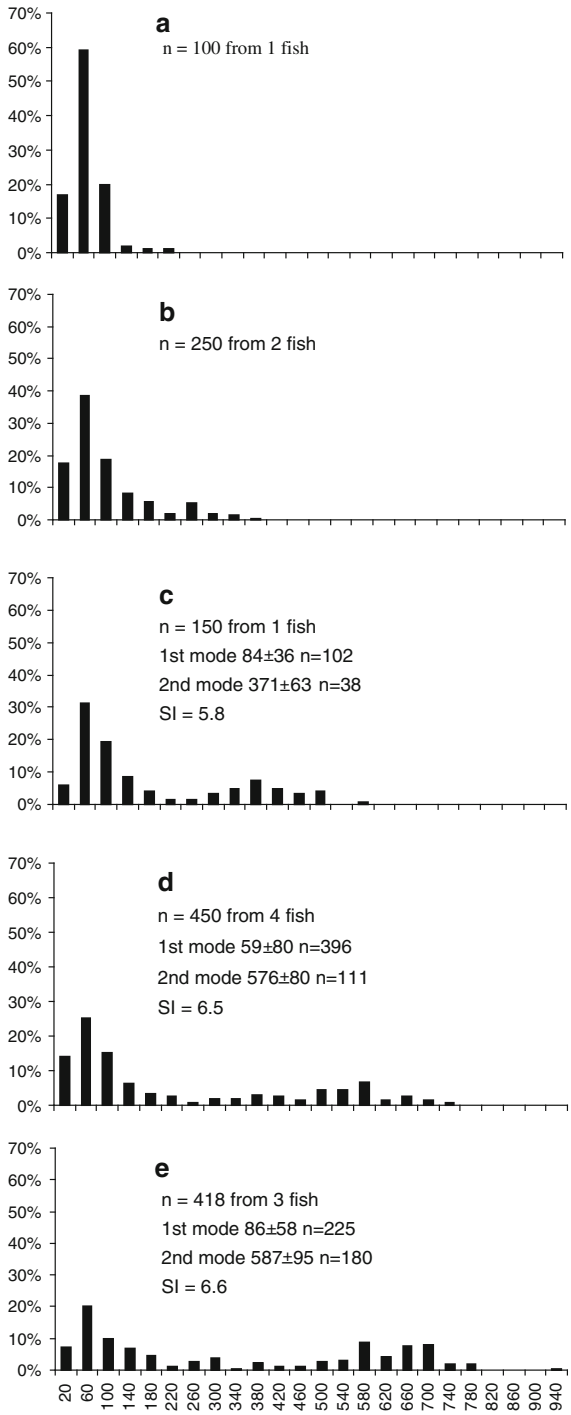
For biochemical analysis samples of about 1 g of eggs and newly hatched larvae were taken in cryovials and frozen immediately at −80°C until analysis. Protein and carbohydrates were analysed in triplicates after 5 min disruption with an Ultra turrax T-25 (Ika, Germany) and 1 min sonication (Vibracell, Sonics, USA) by means of Lowry et al. (1951) and Dubois et al. (1956) methods. Total lipids were extracted from samples by homogenisation in chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene (BHT) (Folch et al. 1957). Fatty acid methyl esters (FAME) were prepared from total lipid by acid-catalysed transesterification using 2 mL of 1% H_2SO_4 in methanol plus 1 mL toluene (Christie 1982), and FAME were then extracted and purified (Tocher and Harvie 1988). Fatty acid methyl esters were separated and quantified by gas–liquid chromatography (Thermo Trace GC, Thermo Finningan, Milan, Italy) using a 30 m × 0.25 mm ID capillary column (BPX 70, SGE Europe Ltd., UK) utilising on-column injection at 50°C and flame ionisation detection at 250°C. Helium was used as carrier gas (1.2 mL min^{-1} constant flow rate) and temperature programming was from 50 to 180°C at 40°C min^{-1} and then to 225°C at 2°C min^{-1}. Individual methyl esters were identified by comparison with known standards (Supelco Inc., Madrid, Spain) and a well-characterised fish oil and quantified by means of the response factor to the internal standard, 21:0 fatty acid, added prior to transmethylation, using a Chrompack program (Thermo Finnigan, UK).

Parentage analysis with microsatellites

Extraction of DNA was made from fin and blood samples from the broodstock and from pools of 1-day-old larvae using "High Pure Genomic DNA Isolation kit" (Aquasolutions Biotech). Pools of larvae were analysed from the following spawns: 29, 30 March; 1, 2, 4, 9, 10, 11, 12, 17 April; and 7, 9 May 2007 from the implanted fish, and 31 March and 2 April 2007 from injected fish. The samples were genotyped using 10 microsatellite loci isolated from the species (Andree et al. 2010) in a single multiple PCR. The PCR products were analysed with fluorescence in a genetic sequencer ABI3130 (Applied Biosystems), and allele length was determined with the software GeneMapper V. 4.0. (Applied Biosystems). Parentage was determined by visually comparing allele exclusion between potential parents and off-spring pools.

Statistics

The variance of the data was given as standard deviation (SD) of the mean. Biochemical data were from three replicates per sample. One-way ANOVA or a *t* test was used to compare the dependant variables (number of eggs, percentage fertilisation, percentage hatch, egg size, larval survival and biochemical data) for the four independent variables—hormone treatment, parents, spawning induction date and egg quality that is, for hormone treatment implant-induced spawns were compared with injection-induced



◀ **Fig. 1** Frequency distribution of oocyte diameters in ovarian biopsies taken from meagre (*Argyrosomus regius*) broodfish having different maximum oocyte diameters. **a** Taken on the 04/05/07 from 7♀ maximum diameter of 220 μm, **b** taken on the 26/03/07 from 4♀ and on the 04/05/07 from 3♀, maximum diameter of 340–400 μm, **c** taken on the 26/03/07 from 3♀, maximum diameter of 580 μm, **d** taken on the 26/03/07 from 6♀ and on the 04/05/07 from 2♀, 5♀, 6♀ maximum diameter of 680–760 μm, **e** taken on the 26/03/07 from 1♀, 2♀, 5♀ maximum diameter of 800–940 μm. SI = separation index

spawns, for parents spawns from different parents were compared, for spawning induction dates spawns were compared from different induction dates and for egg quality spawns grouped as "good" and "bad" quality were compared. Some data sets from total number of eggs, percentage fertilisation, percentage larval survival and biochemical data that failed normality or the Levene Median equal variance test were compared with the Kruskal–Wallis or Mann–Whitney test. Where differences were observed a pair-wise comparison of means was applied using Tukey's test. Sigma Stat 3.1 package (Systat Software Inc., Germany) was used for all statistical analyses, and a value of $P \leq 0.05$ was considered to indicate statistical significance. Bimodality of distributions of oocyte diameter was described (mean, SD, population size and separation Index between modes) with the Bhattachara's method using FISAT II (FAO-ICLARM Fish Stock Assessment Tools, version 1.2.2 2000–2005, FAO, Rome, Italy). A separation index greater than 2 was considered to indicate the modes were separate (Gayanilo et al. 2005).

Results

Maturation

Spermiating males and females in vitellogenesis were observed from March through to June. On the 26 February 2007, a female that died had an average oocyte diameter of vitellogenic oocytes of 570 ± 90 μm. On both the 26 March and 4 May 2007, four females had a mean diameter of vitellogenic oocytes greater than 580 ± 40 μm and the other females had mean oocyte diameters less than 410 ± 40 μm or the genital pore was closed and an ovarian biopsy could not be taken (Table 1). The distributions of the oocyte diameter from fish with maximum oocyte diameters below 400 μm were skewed to the right (Fig. 1a, b), and the distributions from fish with maximum diameters above 580 μm were bimodal with a separation index of over 5 (Fig. 1c–e). The fish that had an upper mode with a mean of over 570 μm (Fig. 1d, e) were induced to

spawn. One female (2♀) examined on the 4 May 2007 had ova that had not been spawned and were being broken down and reabsorbed (distribution not included in Fig. 1). On the 26 March 2007, two of the three males had flowing sperm, and on the 4 May 2007, all three males had flowing sperm (Table 1). Sperm had motility above 80% and motility duration ranged between 4′30″ and 5′28″ mins. On the 15 June, all females were immature or exhibited gonadal atresia (regression), and only two males were spermiating, and the sperm was of reduced quality, 50 and 40% motility and duration of 4′29″ and 1′32″ mins. In 2008, on 18 April, the remaining males (3♂) and females (7♀) were in advances stages of maturity and the male had sperm of 90% motility and motility duration of 5′59″ mins and the female had vitellogenic oocytes with a mean diameter of 550 ± 50 μm. On the 23 May, the male had sperm with 10% motility and motility lasted only 35″ sec and the ovarian sample from the female contained all stages of oocyte development from primary oocytes to ovulated ova being broken down, and 5% of the oocytes were vitellogenic with a mean diameter of 810 ± 110 μm. During the period close to when the fish were reproductively mature, February–July, a total of eight fish died and a further two individuals died in October. All mortalities were caused by injuries due to the broodstock jumping from the tank during husbandry operations ($n = 3$) or over inflation of the swim bladder ($n = 6$). During autopsies of the dead fish no parasitic or bacterial infections were observed in association with or that explained the over inflation of the swim bladder. Dates of mortality (26 February; 28 April; 12, 15, 19 and 24 June; 9 July; 15 and 30 October) were not associated with any particular husbandry task such as revision of maturity status and hormone application, however, during June temperatures increased from 16 to 25°C.

Spawning

After GnRHa treatment on the 26 March 2007, the two injected females (5♀ and 6♀) spawned 3 times in a period of 4 days and produced 7,221,000 floating eggs, whereas the two implanted females (1♀ and 2♀) spawned 10 times over a period of 20 days and produce 9,515,000 floating eggs (Fig. 2). Spawning was less frequent and with lower fecundity after the second hormone application on the 4 May in 2007, and the two injected females spawned twice to produce 1,789,300 floating eggs compared to the two implanted females that spawned four times to produce 2,915,000 floating eggs (Fig. 2). The total number of floating eggs spawned from injected fish was 9,019,300, resulting in a relative fecundity of 198,200 eggs kg^{-1} of female body weight and from the implanted fish was 12,430,000, resulting a relative fecundity of 276,200 eggs kg^{-1}. In 2008, five spawns were collected after GnRHa implantation to give a total fecundity of 10,211,900 floating eggs and a relative fecundity of 527,380 eggs kg^{-1}. The first spawn from the two treatments induced on the 26 March 2007 and which were held at temperatures that oscillated between 11.9 and 14.9°C was collected in the afternoon, 72 h after treatment. The first spawns for the fish treated on the 4 May 2007 (temperature of 15–16°C) and on the 18 April 2008 (temperature of 17.2–18.1°C) were collected in the morning, less than 48 h after treatment. Generally, both hormone treatments produced a series of 2–4 spawns in excess of 1,000,000 eggs $spawn^{-1}$ during the period of 2–5 days after treatment. Thereafter, injected fish did not spawn again, while implanted fish continued to spawn a number of spawns that were generally <1,000,000 eggs $spawn^{-1}$. The reduction in the number of eggs spawned over time from hormone implantation was described by a significant exponential decay regression analysis with $R^2 = 0.72$ (data not shown).

The microsatellite-based paternity analysis indicated that two of the spawns (30 March and 1 April 2007) in the tank where the fish were injected involved both of the females (5♀ and 6♀) and the single male (2♂). The analysis of the eggs obtained from implanted fish indicated that the two females spawned on different dates with the same male. One female and the male (1♀ and 1♂) were assigned to eight spawns (29, 30 March and 1, 2, 4, 9, 10, 17 April) that produced 8,715,000 floating eggs, which accounted for the majority of the eggs collected after the induction on the 26 March. The second female and the same male (2♀ and 1♂) were assigned to four spawns (11, 12 April and 7, 9 May) that produced 4,764,500 floating eggs, which accounted for the majority of the eggs collected after the induction on the 4 May. There were no significant differences in the mean number of eggs spawned between type of hormone applications (implant and injection), amongst date of induction (26 March 2007, 4 May

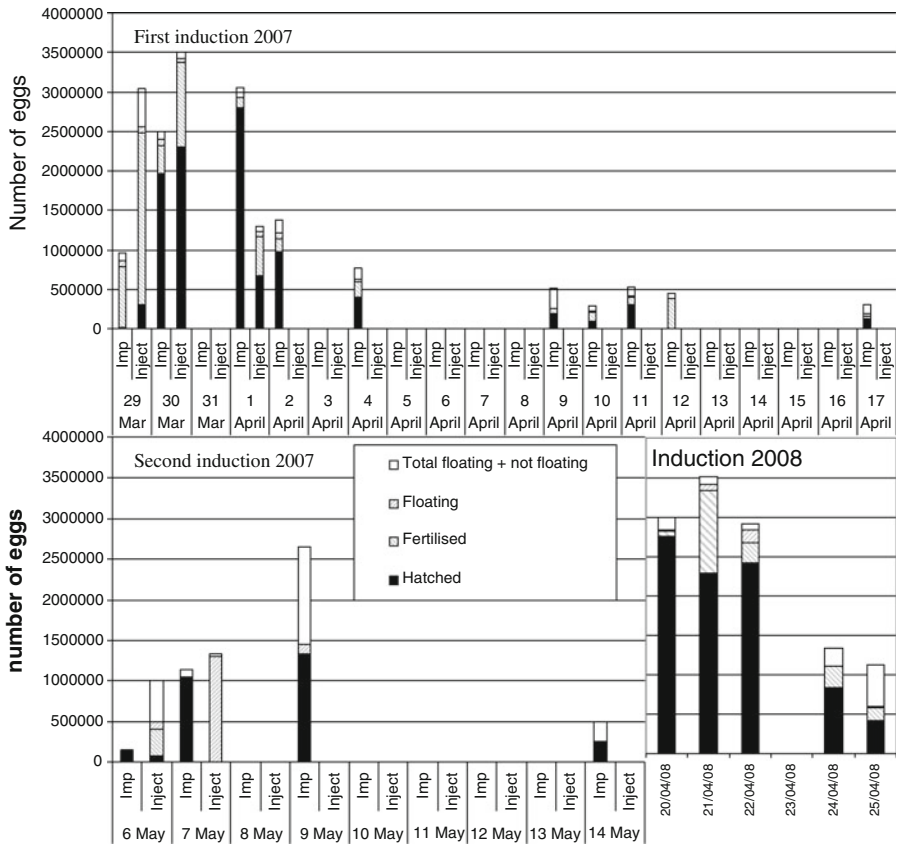


Fig. 2 Daily egg production of meagre (*Argyrosomus regius*) broodstock induced to spawn with a GnRHa injection (Inj) or implant (Imp). *Black bars* indicate number of eggs spawned that hatched, *grey bars* with left to right slope indicate number of fertilised eggs, *grey bars* with slope right to left indicate number of floating eggs and *open bars* indicate total number of eggs spawned

2007 or 18 April 2008) or amongst parents (four parental combinations: 1♀ + 1♂; 2♀ + 1♂; 5♀ + 6♀ + 2♂; and 7♀ + 3♂). The mean total number of eggs per spawn was 1,552,500 ± 982,000 eggs, and mean number of floating eggs was 1,344,000 ± 943,500.

Egg quality

Percentage hatch (Table 2) and egg diameter (data not shown) was significantly higher ($P < 0.05$) from fish that were implanted compared to fish that were injected with GnRHa. Percentage hatch and egg diameter were 70 ± 0.3% and 0.99 ± 0.02 mm, respectively, for implanted fish compared to 30 ± 0.3% and 0.95 ± 0.03 mm for injected fish. There were no significant differences amongst treatments for percentage fertilisation (mean of all spawns was 91 ± 9%) or amongst parents and date of spawning for percentage hatch and egg diameter. Although there was considerable variation between spawns, there were no significant differences for lipid, carbohydrate or protein content of spawned eggs amongst hormone treatment, date of spawning and parents and the respective mean lipid, carbohydrate and protein content of all spawns expressed in percentage dry weight were 17.3 ± 3.0, 4.4 ± 1.9 and 31.5 ± 6.4. There was also considerable variation between fatty acid (FA) content of different spawns. Except for a significant difference in total lipids, no differences were observed between the eggs from implanted fish treated on the 26 March 2007 and 18 April 2008, but differences were observed between eggs from fish that were implanted on the 26 March 2007 and 4 May 2007 for the following parameters: 16:0, total saturated FA, total monosaturated FA, 18:2n-6, total n-6 FA, 18:3n-3, total n-3 FA and total

Table 2 Fertilisation and hatching success (%) of wild meagre (*Argyrosomus regius*) eggs obtained after hormonal treatment with GnRHa (implant or injection) and larval survival (%) after 3 and 5 days of starvation in 96-well EIA plates

	Implant				Injection			
Spawning Date	Fertilization rate (%)	Hatching rate (%)	Larval survival (%) 3 dph	5 dph	Fertilization rate (%)	Hatching rate (%)	Larval survival (%) 3 dph	5 dph
29/3/07	90	2±2.1	17±28.9	0.0	97	12±10.5	54±17.4	39±10.7
30/3/07	97	82±2.8	91±1.0	86±4.6	98	67±5.0	84±4.5	75±1.8
1/4/07	100	9±2.2	95±1.5	88.±5.8	95	55±0.2	92±0.3	79±7.4
2/4/07	93	80±1.9	82±3.6	55±4.1				
4/4/07	96	64±9.2	61±2.2	41±22.1				
9/4/07	99	74±6.5	94±2.8	84±5.1				
10/4/07	96	41±3.1	86±2.9	59±11.4				
11/4/07	96	72±7.4	87±5.9	76±0.8				
12/4/07	100	70±16.3	ND	ND				
17/4/07	82	61	84	77				
6/5/07	95	89	ND	ND	82	15	88	65
7/5/07	100	100	1	46	0	0	0	0
9/5/07	92	92	100	95				
14/5/07	93	93	89	18				
20/4/08	99	97±8.8	79±1.6	68±2.4				
21/4/08	98	67±6.3	71±3.6	63±6.4				
22/4/08	94	85±23.7	86±9.8	85±9.8				
24/4/08	100	75±1.8	83±3.2	82±3.2				
25/4/08	96	69±6.4	43±3.7	41±4.6				

Shaded in grey are the spawns considered as "bad", and in unshaded boxes are the spawns considered as "good". Unmarked spawns were of intermediate quality. In 2007, the fish were treated with GnRHa twice (on 26 March and 4 May), whereas in 2008 only once (18 April). The dashed lines separate spawns from different GnRHa application. dph = days post hatch, ND = no data

PUFA (Table 3). Differences were also observed for 16:0, 16:1 n-7 and 22:5 n-3 between eggs from fish implanted and injected on the 26 March 2007 and for total lipids between eggs from fish implanted and injected on the 4 May 2007 (Table 3). Five spawns were classified as "good" egg quality (mortality rate less than 25% at 5 dph and less than 10% mortality from 3 to 5 dph), and five spawns were classified as "bad" egg quality (mortality rates greater than 35% at 3 dph), but no significant differences were observed between these groups in nutritional parameters, proximal analysis and lipid classes. All other spawns were of intermediate quality between the good and bad spawns. All good spawns were the second and/or third spawn after GnRHa treatment and all bad quality spawns were either the first spawn or the fifth + spawn

Table 3 Average total lipid content (mg g^{-1} DW), total fatty acid content (mg g^{-1} lipids) and fatty acid composition (percentage total fatty acids, %TFA) of eggs spawned from wild meagre (*Argyrosomus regius*) that were induced to spawn with GnRHa implant or injection on the 26 March and 4 May in 2007 and 18 April in 2008

Fatty acids (% TFA)	Implant		Injection		
	26 March 07 ($N = 10$)	4 May 07 ($N = 3$)	18 April 08 ($N = 5$)	26 March 07 ($N = 3$)	4 May 07 ($N = 2$)
16:0	$21.4 \pm 2.9^{a,A}$	16.6 ± 2.2^{B}	21.6 ± 2.9^{A}	16.2 ± 2.8^{b}	15.7 ± 2.0
18:0	3.6 ± 0.6	4.4 ± 0.9	3.0 ± 0.5	3.7 ± 0.4	4.0 ± 0.8
Total saturated[1]	27.5 ± 3.4^{B}	23.2 ± 2.2^{A}	28.6 ± 3.1^{B}	22.3 ± 5.1	20.7 ± 2.2
16:1n-7	10.5 ± 0.8^{a}	6.9 ± 2.8	8.7 ± 1.2	6.6 ± 1.7^{b}	4.8 ± 0.6
18:1n-9	13.6 ± 2.0	14.0 ± 2.4	13.6 ± 2.7	13.9 ± 1.6	13.8 ± 2.9
Total monounsaturated[2]	28.9 ± 2.3^{B}	25.0 ± 1.2^{A}	26.5 ± 1.5^{AB}	26.5 ± 4.0	23.1 ± 4.9
18:2n-6	2.6 ± 0.6^{A}	3.7 ± 1.0^{B}	1.8 ± 0.1^{A}	3.4 ± 0.9	4.0 ± 1.3
20:4n-6 (ARA)	0.9 ± 0.2	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.4	1.0 ± 0.1
Total n-6[3]	3.9 ± 0.8^{A}	5.1 ± 0.6^{B}	3.6 ± 0.3^{A}	4.3 ± 0.7	5.2 ± 1.7
18:3n-3	0.8 ± 0.1^{A}	1.4 ± 0.2^{B}	0.7 ± 0.1^{A}	1.2 ± 0.6	1.1 ± 0.5
20:5n-3 (EPA)	7.7 ± 1.9	9.8 ± 1.2	7.7 ± 0.8	10.4 ± 1.9	9.2 ± 2.1
22:5n-3	2.1 ± 0.4^{a}	2.1 ± 0.3	1.6 ± 0.4	2.8 ± 0.5^{b}	2.3 ± 0.3
22:6n-3 (DHA)	28.6 ± 3.8	31.9 ± 0.6	29.7 ± 3.4	31.4 ± 5.8	35.4 ± 2.1
Total n-3[4]	39.8 ± 5.7^{A}	46.7 ± 1.3^{B}	41.3 ± 4.2^{A}	46.9 ± 8.3	51.1 ± 5.4
Total PUFA	43.6 ± 5.6^{A}	51.7 ± 1.2^{B}	44.9 ± 4.4^{A}	51.2 ± 9.0	56.3 ± 7.0
Total lipids/DW (mg g^{-1})	192.7 ± 27.3^{A}	$161.1 \pm 64.2^{B,a}$	140.9 ± 33.6^{B}	161.1 ± 11.4	187.5 ± 12.2^{b}
FAMES/DW (mg g^{-1})	700.8 ± 120.9	560.0 ± 113.9	684.7 ± 176.8	659.9 ± 122.6	576.5 ± 18.2

Different superscript letters indicate significant differences ($P < 0.05$) between the type of hormonal induction (lower case letters) and between months using the same type of hormonal induction (capital letters)

[1] Includes 14:0 and 22:0

[2] Includes 18:1n-7, 20:1n-9, 22:1n-11

[3] Includes 22:5n-6

[4] Includes 18:4n-3

(Table 2). In 2007, eggs were used for experimental rearing of meagre larvae and juveniles (Estévez et al. 2007; Fernández-Palacios et al. 2007; Hernández-Cruz et al. 2007; Roo et al. 2007), and in 2008, eggs were also used by a commercial marine fish hatchery to produce 380,000 meagre juveniles.

Discussion

Under the holding conditions described in the present study, wild meagre were observed to reach advanced stages of maturity, late vitellogenesis and spermiation. A number of individuals were induced to spawn successfully with either GnRHa implants or injections, producing eggs of sufficient quantity and quality for commercial scale production of juveniles. The spawning of eggs of sufficient quality and quantity in captivity is a prerequisite for the development of the sustainable aquaculture of a species.

For fish, such as meagre, that apparently exhibits a reproductive dysfunction during vitellogenesis and do not mature, ovulate and spawn spontaneously in captivity, hormone therapies such as the application of GnRHa have been used successfully in the past decades (Zohar and Mylonas 2001; Mañanós et al. 2008; Mylonas et al. 2011b). It has been identified that two of the most important aspects of a successful hormone therapy are dose and oocyte size, that is, stage of ovarian development (Ibarra-Castro and Duncan 2007; Mañanós et al. 2008). In the present study, doses were selected based on doses that were

successfully used in other species (Mañanós et al. 2008), particularly sciaenides (Thomas et al. 1995) and the experience of fish biologists (the authors and Dr. A. Grau, La Palma, Mallorca, Spain). The oocyte diameter selected (530 μm) was based on the observed oocyte morphology, for example, round and uniform shape, thick and multilayered zona radiata and dark cytoplasm indicating the presence of yolk and lipid inclusions, and the prediction given by the multi-species regression equation of minimum oocyte size for successful hormone-induced spawning against egg size (Mañanós et al. 2008). In other species, when the oocyte size was adequate, lower than optimal GnRHa doses gave a low spawning frequency and higher than optimal doses gave reduced egg quality (Ibarra-Castro and Duncan 2007). The high egg quantity and quality in the present study indicated that the doses (in the case of both GnRHa implants and injection) and oocyte size were close to optimal.

However, the quantity and quality of eggs from injected fish was lower than the values obtained from implanted fish. Eggs from injected fish had significantly lower percentage hatch (29.6 ± 0.3% 70.4 ± 0.3%) and egg diameter (0.95 ± 0.03 mm compared to 0.99 ± 0.02 mm) and relative fecundity was lower, but not significantly different (198,200 eggs kg^{-1} compared to 276,200 eggs kg^{-1}). The differences may indicate that the concentration of GnRHa in the blood circulation achieved by the implant was closer to optimal compared to the higher dose achieved by the single injection, even if the absolute amount of GnRHa contained in the implant was higher than in the injection. The release of GnRHa from the implant lasts for many weeks, but the actual amount that is released at a given time is certainly less (maximum 15% on day 0, declining gradually thereafter) than the bolus amount of 20 μg received by the injected fish (Mylonas and Zohar 2001; Guzmán et al. 2009). Therefore, the concentration of GnRHa in circulation on the very first 1–2 days after treatment is significantly higher in injected fish compared to the implanted fish, but these lower levels of GnRHa are maintained for many days in the implanted fish, where in the injected ones GnRHa is cleared from the circulation within the first week after treatment (Mañanos et al. 2002; Guzmán et al. 2009). Other studies that also achieved successful spawning used 50 μg kg^{-1} GnRHa implants (Mylonas et al. 2011a), a single injection of 0.15 μg kg^{-1} of GnRHa or 0.5 mL kg^{-1} of ovaprim® (Grau et al. 2007). Fernández-Palacios et al. (2009a, b, 2011a, b), tested doses from 1 to 50 μg kg^{-1} of GnRHa and found that the optimal single injection dose for smaller (5–7 kg) captivity-reared meagre was 15 μg kg^{-1}. These studies support both suggestions that (1) the present study used a close to optimal dose for the injection and (2) that the injection dose may have been higher than optimal. However, it should not be discounted that the observed differences in egg quality could have been due to variation between individuals. The number of females used was low and due to the limited number of males, the group that was treated with the injections contained only one male and poor spawns may be related to poor performance of the male, especially considering that the spawn on the 7 May 2007 was not fertilised. However, there were no significant differences in egg quality parameters assigned to different parents, and this observation indicates that the difference in quality was not the result of differences between individuals.

Generally, the percentage of the different lipid classes in the eggs of meagre were similar to the levels observed in other marine perciformes such as sciaenid, mulloway (*Argyrosomus japonicus*), (personal communication Cross, E., Cowden, K., National Marine Science Centre, Coffs Harbour, Australia); Moronidae, European sea bass (*Dicentrarchus labrax*) (Navas et al. 1997); Sparidae, common dentex (*Dentex dentex*) (Giménez et al. 2006); Latridae, striped trumpeter (*Latris lineate*) (Bransden et al. 2007) and marine species from other orders; Gadiformes, Gadidae, cod (*Gadus morhua*) (Salze et al. 2005) and Pleuronectiformes, Scophthalmidae, turbot (*Psetta maxima*) (Silversand et al. 1996). Studies on essential fatty acids and egg and larval quality have been focused on arachidonic acid (ARA, 20:4n-6), eicosapentaenoic acid (EPA 20:5n-3) and docosahexaenoic acid (DHA 22:6n-3) (Sargent 1995; Tocher 2010). The levels of ARA in meagre eggs (0.9–1%) were at the lower end of the range of ARA values observed in the selected species (above) that ranged from 1.1% in sea bass (Navas et al. 1997) to 3% in cod (Salze et al. 2005) and turbot (Silversand et al. 1996). The levels of EPA in meagre eggs (7.7–10.4%) were intermediate compared to the range for the selected species that ranged from 4.4% in common dentex (Giménez et al. 2006) to 15.5% in cod (Salze et al. 2005). The levels of DHA in meagre eggs (28.6–35.4%) were at the higher

end of the range for the selected species, which ranged from 20.3% in sea bass (Navas et al. 1997) to 27.6% in cod (Salze et al. 2005). Some differences were observed in lipid classes between meagre implanted to spawn on the 26 March 2007 and 4 May 2007. The parentage analysis showed that these eggs were predominantly from two different females (1♀ spawning after the 26 March 2007 and 2♀ spawning after the 4 May 2007) indicating that this may represent a difference between two individuals. The spawning in 2008 was from a third female, and apart from the total lipids, there were no differences when compared to eggs spawned after the hormonal induction on the 26 March 2007, which indicated that the nutritional status of eggs spawned from a female after 6 months in captivity were similar to those from a female held in captivity for 1.5 years with a diet of sardines, squid and 0.1% BW supplement of Vitalis Repro (Skretting).

Spawns were classified into "good" and "bad" spawns based on larval survival (Giménez et al. 2006). There were no significant differences in nutritional parameters (proximal analysis and lipid classes) between the "good" and "bad" quality eggs, indicating that the bases for the poor egg quality was not nutritional. However, a pattern of "good" and "bad" egg quality was observed where good spawns were the second or/and third spawn after GnRHa treatment and bad spawns were either the first spawn or the fifth + spawn. This pattern may be related to an accelerated development of the oocytes in relation to the GnRHa treatment, resulting in an initially poor quality before quality improved. This effect has also been proposed in relation to advanced spawning of eggs with reduced quality due to higher than optimal hormone doses (Mylonas et al. 1992; Barbaro et al. 1997; Mugnier et al. 2000; Ibarra-Castro and Duncan 2007) and treatment of fish with smaller than optimal oocyte size (Crim and Glebe 1984; Duncan et al. 2003).

Two patterns of spawning were observed, where (1) large spawns (>million eggs) occurred over a period of less than 7 days after hormone application and (2) over an extended period of more than 7 days, initially large spawns (>million eggs) followed by smaller spawns (<million eggs). All hormone treatments induced the first spawn 48–72 h after the application of GnRHa, and during the first 7 days, three to five spawns were obtained. After this 7-day period, no further spawns were obtained, in the injected fish. On the contrary, in the two implant-induced trials in 2007, smaller spawns (<million eggs) occurred after this initial period after an interruption of 4 days. Mylonas et al. (2011a), working with smaller captivity-reared fish, also induced spawning with GnRHa implants of 50 μg kg^{-1} and obtained daily spawning for 5–19 days, but with a similar pattern in number of eggs spawned per day with 70% of the eggs being spawned during the first 4 spawns after which smaller spawns were obtained. The lower frequency of days when spawning occurred in the present study may indicate that the wild fish were less adapted to holding conditions compared to the cultured stock (Mylonas et al. 2011a). These spawning patterns from meagre can be compared with those obtained using similar implants for European seabass (Fornies et al. 2001) which has been classified as having group-synchronous batch oocyte development (Mayer et al. 1990) and gilthead sea bream (Barbaro et al. 1997) classified as having asynchronous oocyte development (Mylonas et al. 2011b). Both the present study and Mylonas et al. (2011a) exhibited a decline in the number of eggs spawned per day after the implant was administered, which was similar to that observed in implanted European seabass (Fornies et al. 2001). However, it should be noted that a decline in daily number of eggs spawned was also observed in implanted gilthead seabream, but bream spawned 40% of the eggs during the first 10 days (Barbaro et al. 1997). The frequency of days when spawning occurred in the present study was more similar to the frequency of spawning observed for European seabass (Fornies et al. 2001), while the daily spawning obtained for meagre by Mylonas et al. (2011a) was the same as that obtained for gilthead sea bream (Barbaro et al. 1997).

The frequency histograms of oocyte size suggest that the meagre has a group-synchronous batch development. In fish that exhibited a maximum oocyte diameter of <400 μm, the distribution of oocyte diameter was skewed, and in fish that exhibited an maximum oocyte diameter >400 μm, the distributions were bimodal. A bimodal frequency distribution of oocyte diameter is indicative of group-synchronous oocyte development (Wallace and Selman 1981; Tyler and Sumpter 1996) and, for example, European sea bass oocyte distributions were observed to be bi- and tri-modal as oocyte size increased during the spawning period (Mayer et al. 1990). It has been suggested that a prolonged constant release of GnRHa, such as with the implant used in the present study, may not be an

appropriate hormone induction treatment for a group-synchronous batch spawner such as the European sea bass (Fornies et al. 2001; Mylonas et al. 2003). The results from the present study, albeit on a few individuals ($n = 3$ implanted females and $n = 2$ injected females) suggested that an injection resulted in a few spawns of >1,000,000 eggs, compared to implants that gave both large spawns and also many smaller spawns. An acute injection that gave a few predictable spawns of >1,000,000 eggs in the week after hormone application would probably be favoured by a commercial hatchery, as it may allow for a better planning of the larval rearing activities.

The wild meagre broodstock appeared to acclimatise to the described captive conditions, both in terms of feeding response and maturation. After 21 days, the fish exhibited an active feeding response, eating readily 0.8–2.5% BW. All males matured with flowing sperm and five out of nine females matured to advanced stages of vitellogenesis. However, from February to July 2007, when the broodstock had advanced stages of maturity, six fish died from injuries sustained from jumping and from over-inflation of the swim bladder. Autopsies did not reveal any pathogen in association with the mortalities. Similar large wild meagre, treated in a similar way, but held in a 50 m^3 D-ended raceway did not exhibit any mortality due to swim-bladder inflation (personal observations). It appeared that the mature fish had a different behaviour, and this combined with the holding conditions particularly tank size and possibly the husbandry practices associated with evaluating maturity resulted in increased stress that was manifested by the fish jumping and over-inflating the swim bladder which caused the mortalities.

In conclusion, the study described two hormone induction protocols that resulted in the production of commercially viable quantities (i.e. +1,000,000 eggs per spawn) of good quality eggs (i.e. +75% survival of fertilised eggs and larvae to 5 dph). However, aspects such as the pattern of quality of spawns and differences in egg quality between independent variables indicated that hormonal induction protocols could be improved, particularly the GnRHa injection. Oocyte development was of the group-synchronous mode and the spawning results indicated that an acute injection may be favoured by commercial hatcheries, due to the larger amount of eggs produced within a shorter period of time, even if the total fecundity of the injected fish was less than that of the implanted fish.

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