New species for EU aquaculture

Deliverable Report

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Lead Scientist preparing the Deliverable: Salvador Jerez (IEO)

Other Scientists participating: Virginia Martín (IEO), Constantinos C. Mylonas (HCMR), Yannis Fakriadis (HCMR), Maria Papadaki (HCMR)

Objective: The objective of the present deliverable was to develop a dose response of GnRHa implant therapy for the induction of spawning in F1 generation broodstock of greater amberjack in the eastern Atlantic. The deliverable is a report containing the results related to the use of the hormonal induction therapy with implants of different doses of GnRHa on reproductive performance, including egg release frequency, fecundity and egg quality parameters. In addition the deliverable includes data about the effect of the hormonal therapy on plasma levels of the sex steroid hormones and several hematological and biochemical parameters indicators of health and welfare such as plasma levels of triglycerides, cholesterol, protein, enzymes, cortisol, glucose, lactate and electrolytes.

Description: F1 Greater amberjack females were treated with different doses of GnRHa in consecutive years while the males were implanted with the same dose of GnRHa. Repetitive implants with Ethylene–Vinyl acetate (EVAc) GnRHa were applied during each spawning season, according to the planned dose. The effects of the spawning induction treatment were evaluated in terms of reproductive performance.

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Introduction

The greater amberjack *Seriola dumerili* is a cosmopolitan pelagic species (Paxton, et al., 1989), with high growth rate and flesh quality (FAO, 2016; Jover, et al., 1999; Mazzola, et al., 2000). It has been considered as a good candidate for the species diversification of aquaculture production in the Mediterranean region (Mylonas, et al., 2016), but commercial production of greater amberjack is still very limited (FAO, 2016) due to bottlenecks in terms of reproduction, larval rearing and fish health. Recent researches have evaluated the potential of wild caught breeders to mature and be induced to spawn in captivity (Mylonas & Zohar, 2009; Mylonas, et al., 2010; Mylonas, et al., 2017a) including the greater amberjack (Díaz, et al., 1997; Lazzari, et al., 2000; Micale, et al., 1999; Pastor, et al., 2000). These reproductive failures have prevented so far the commercial development of seed production of greater amberjack (Jover, et al., 1999; Mazzola, et al., 2000). In some species, reproductive failures are more severe in hatchery-produced broodstocks. This has been demonstrated in the Senegalese sole *Solea senegalensis* (Agulleiro, et al., 2007; Carazo, et al., 2013; Duncan, et al., 2013; Howell, et al., 2008; Morais, et al., 2016; Rasines, et al., 2012), the greenback flounder *Rhombosolea tapirina* (Pankhurst & Fitzgibbon, 2006) and the sharpsnout seabream *Diplodus puntazzo* (Papadaki, et al., 2018). In Senegalese sole, F1 females undergo maturation and spawn, but the obtained eggs are not fertilized, which has been related to a critical reproductive dysfunction of the F1 males (Mañanos, et al., 2009). On the other hand, in the greenback flounder a delayed release of eggs by the females after ovulation has been blamed for the failure of produce fertilized eggs (Pankhurst & Fitzgibbon, 2006). In sharpsnout seabream, females ovulate their eggs but they do not spawn them, though it is still not known if this is associated with a problem of the females or the lack of breeding behaviour of the males (Papadaki, et al., 2018). The existence of a significantly lower reproductive success of captive produced versus wild-caught breeding animals has been reported in a great number of animal taxa and it is of a great interest not only in aquaculture production but also in conservation biology and laboratory experimentation (Farquharson, et al., 2018).

The most commonly observed reproductive dysfunction in fish maintained in aquaculture facilities is the failure to undergo oocyte maturation (OM) after vitellogenesis is completed in females, and the production of lower quantity of sperm in males (Mañanos, et al., 2009; Mylonas & Zohar, 2001; 2007; Zohar & Mylonas, 2001a). Hormonal treatments using human chorionic gonadotropin (hCG) or gonadotropin-releasing hormone agonists (GnRHa) have been used successfully in several species to overcome the observed reproductive dysfunctions. In greater amberjack, wild-caught fish have been shown to complete gametogenesis in captivity, and spawning could be induced using different hormonal therapies (Fernández-Palacios, et al., 2015a; García, et al., 2001; Kozul, et al., 2001; Lazzari, et al., 2000; Mylonas, et al., 2004b). In addition, spontaneous spawning has also been achieved in some individuals without any exogenous hormonal therapies (Jerez, et al., 2006). However, fertilized eggs from hatchery produced, F1 generation breeders has not been succeeded so far, although vitellogenesis and OM has been achieved (Rodríguez-

Barreto, et al., 2014), contrary to what has been reported for yellowtail kingfish *Seriola lalandi*, a congener of the greater amberjack, in which spontaneous spawning with fertilized eggs have been obtained successfully from F1 broodstock (Setiawan, et al., 2016).

The objectives of the present deliverable were to examine the reproductive development of hatchery produced F1 generation greater amberjack, and to evaluate the potential of controlled-release GnRHa delivery systems (implants) to induce OM, spermiation and spawning of fertilized eggs, and to monitor spawning kinetics and gamete quality. To achieve these objectives, three different doses of GnRHa implants were proposed to treating the females in three successive years (50, 75 and 25 µg kg^{-1} in 2015, 2016 and 2017, respectively). The males would be implanted with the same GnRHa dose (30 µg kg^{-1}) each year.

Materials and Methods

Rearing was undertaken in the facilities of the Centro Oceanográfico de Canarias, Instituto Español de Oceanografía, Tenerife, Spain. At the beginning of the experiment, the broodstock consisted of 14 hatchery-produced fish, from eggs obtained from wild-caught breeders between 2005 and 2009. Fish were maintained during 2015 in two outdoor covered 50-m^3 tanks, supplied with well-water (10 renewals day^{-1}) at ambient water temperature and photoperiod until the beginning of the experiments on 13 May 2015 (Table 1). After the 1st GnRHa treatment, the selected fish were placed in a single outdoor covered raceway tank of 500 m^3 with continuous water supply (6 renewals day^{-1}) under natural photoperiod. Fish were fed three times per week to apparent satiation with raw fish. Measurements of temperature and water quality (Dissolved Oxygen, NH_3-N and NO_2-N) were conducted once per week throughout the year.

The fish were sampled monthly during the 2015 and 2016 spawning season (May, June, July and September). Fish were starved for two days prior to sampling and were tranquilized initially in their tank with the use of chlorobutanol (0.1 ml l^{-1}) and then transferred to an anesthetic bath for complete sedation with a higher concentration of chlorobutanol (0.3 ml l^{-1}). Fish were individually identified with PIT tags and biometric parameters of length and body weight were measured. Ovarian biopsies for the evaluation of oocyte development were obtained by inserting a plastic cannula (Pipelle de Cornier). A wet mount of the biopsy was examined first under a compound microscope (40 and 100x) to evaluate the stage of oogenesis and measure the mean diameter of the largest, most advanced vitellogenic oocytes (n = 10). A portion of the biopsy was also fixed in a solution of 4% formaldehyde-1% glutaraldehyde for further histological processing. Maturation of the males was examined by the release of sperm upon application of gentle abdominal pressure. If this was not possible, a sperm sample was obtained by inserting a plastic catheter into the genital pore. The collected sperm was stored on ice and then transferred to a 4°C refrigerator until evaluation. At each sampling, blood was collected from all fish from the caudal vessel using heparinized syringes, in order to measure sex steroid hormone concentrations and blood biochemical parameters. Blood was centrifuged at 1400 rpm for 20 min and plasma was collected, frozen in liquid nitrogen and stored at −80°C until hormonal and biochemical analysis.

Fish were treated with an Ethylene–Vinyl acetate (EVAc) GnRHa implant (Mylonas & Zohar, 2001) loaded with Des-Gly^{10}, D-Ala^6-Pro-$NEth^9$-mGnRHa (H-4070, Bachem, Switzerland) at the sampling times of May, June and July. 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. 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 intratesticular sperm was obtained from males.

Sperm quality parameters that were evaluated included (a) sperm density (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 density was estimated after a 2211-fold dilution with 0.9% saline using a Neubauer haemocytometer under a compound

light microscope at 400X magnification (in duplicate). Sperm motility and duration were evaluated on a microscope slide at 400X magnification after mixing 1 μl of sperm with a drop of seawater (~ 50 μl) in duplicate. Activated sperm samples were observed under a compound light microscope for the first time 10 sec 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.

At the expected onset of the spawning season, a passive egg collector was placed in the outflow of the spawning tank and checked daily, in order to collect the spawned eggs. Eggs were collected every morning 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 all the eggs in this sub- sample for the presence of a viable embryo using a stereoscope. The diameter of ten randomly collected eggs and their lipid droplet were measured using a binocular microscope equipped with a Nikon Digital Sight DS-Fi1 camera. Each spawning was incubated in a 90-l tank with gentle aeration and filtered water supply.

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. (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 they were 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 room and maintained for 5 days at 21 ± 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).

Embryo survival was calculated as the number of eggs having live embryos 1-d 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 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 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).

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) (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).

The enzyme-linked immunoassays (ELISA) used for the quantification of testosterone (T), 17β-estradiol (E_2), 11-keto testosterone (11-KT) and 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) in the plasma of fish were performed according to established methods (Bennett, et al., 1976; Rodríguez, et al., 2000; Nash, et al., 2000 and Cuisset, et al., 1994), with some modifications and using reagents from Cayman Chemical Company (USA). For the steroid extraction, 200 μl of plasma were extracted twice with 2 ml of diethyl ether. Extraction was done by vigorous vortexing (Vibramax 110, Heidolph, Germany) for 3 min. After vortexing, samples were frozen for 10 min at -80° C and the supernatant organic phase was collected in new tubes and was left to dry under a stream of nitrogen (Reacti-vap III, Pierce, Germany). Samples were reconstituted in 200 μl of Reaction Buffer.

Haematological parameters were estimated from fresh samples of blood. Total erythrocytes and leucocytes were determined by counting in 1/100 dilutions of blood in Natt and Herricks solution, using a Neubauer haemocytometer. Hematocrit count was carried out by capillary diffusion and centrifugation. Plasma levels of protein, triglycerides, cholesterol, glucose, lactate and enzymes (GPT, GOT, Alcaline phosphatase, Cholinesterase and amylase) were measured in duplicates by enzymatic colorimetric assays (Biosystems, Spain). Plasma concentrations of sodium (Mg-Uranylacetate Method), potassium (TPB-Na Method), and chloride (Thiocyanate-Hg. Colorimetric) were determined using standard spectrophotometric assays (Spinreact, Spain). Plasma cortisol level was analyzed by radioimmunoassay using ELISA kits (Arbor Assay, Michigan, USA).

Differences in spawning, egg and sperm quality and blood (biochemical and steroids) parameters were tested using one-way Analysis of Variance (ANOVA) followed by Tukey's post hoc test. Pearson's correlation coefficients were used to assess the relationships between some egg quality variables. Percentage data were Arcsine transformed prior to statistical analyses to normalize variances. Results are presented as mean ± standard deviation (SD), unless mentioned otherwise. In all statistical tests used, differences with a *P* value of less than 0.05 were considered statistically significant. Analyses were performed with the IBM SPSS statistics package (version 20.0 for Windows) and JMP 12.

Results from the 2015 reproductive season.

In 2015, the fish were sampled in May, June, July and September, and the selected fish, based on their reproductive status, were implanted with the required dose of GnRHa in May, June and July (**Table 1**).

Table 1. Number, mean weight (± SD; kg) and dose of GnRHa (µg kg^{-1} body weight) of implanted greater amberjack at each treatment time of 2015. All fish were treated with a GnRHa implant, and variations in the effective GnRHa dose were due to the fact that implants were loaded with fixed amounts of GnRHa.

Sex		Females			Males		
Sampling (Month)	Treatment	N		Dose (µg kg^{-1})	N		Dose (µg kg^{-1})
		Biopsied	Treated		Biopsied	Treated	
May	First	7	4 (29.1±10.2)	54 ± 11	7	7 (14.9±5.0)	68 ± 20
June	Second	7	4 (25.7±9.5)	54 ± 8	7	5 (17.0±4.3)	38 ± 4
July	Third	7	3 (29.7±9.5)	50 ± 5	7	6 (13.9±4.7)	40 ± 14
Septemb.		6			5		

The mean (± SEM) diameter of the largest vitellogenic oocytes of the females biopsied varied between 560 ± 80 and 760 ± 110 µm over the reproductive season, with higher mean values in June and July (**Fig.1**). The selected females (largest vitellogenic oocytes > 600 µm) were implanted with a dose of GnRHa about 50 µg GnRHa kg^{-1} body weight (in the form of EVAc implant) in three successive spawning induction trials (**Table 1**).

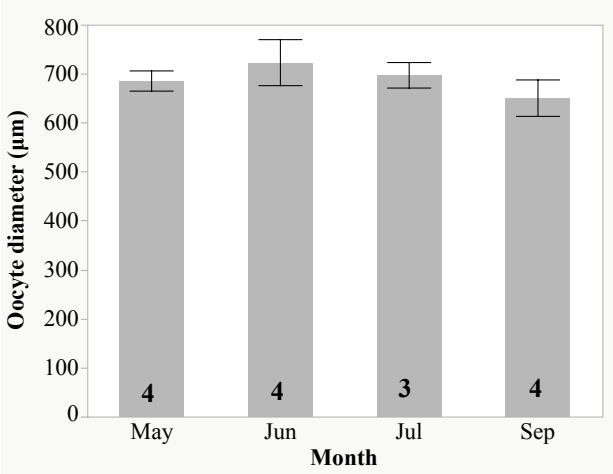

Figure 1. Mean (± SEM) oocyte diameter of the largest vitellogenic oocytes at each sampling in 2015. The number in the bars indicates the number of females that were selected for GnRHa treatment. No statistically significant differences were observed (P<0.05).

Mean sperm motility percentage was > 50% (**Fig. 2**) and remained unchanged throughout the monitored period, while the duration of sperm motility was significantly higher in May (4.35±1.12 min) than in June (2.44±0.24 min) (P<0.05). The mean sperm density was 30.8±6.8 x 10^9 spermatozoa ml^{-1} in May and 78.0±72.2 x 10^9 spermatozoa ml-^{1} in September, although with higher individual variability in September (**Fig. 2**).

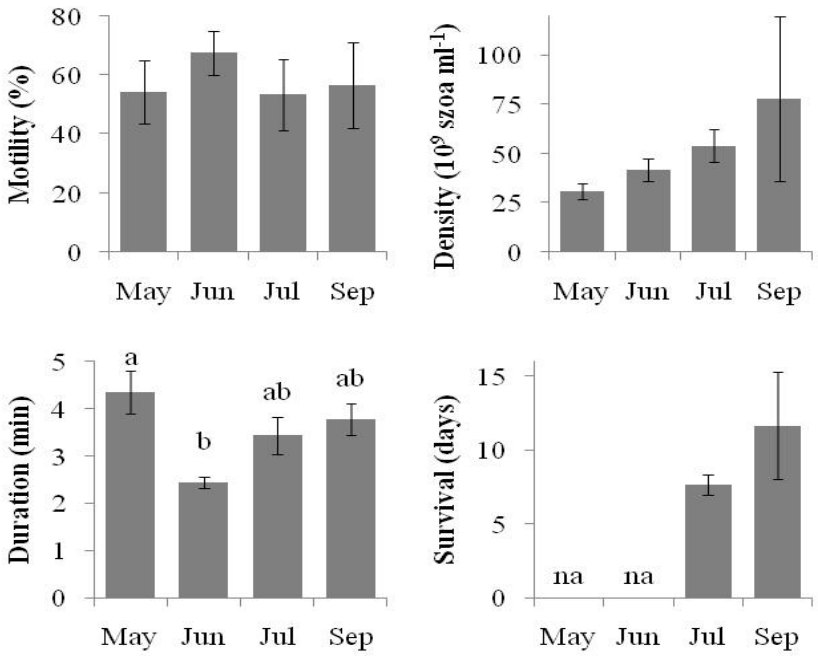

Figure 2. Mean (± SEM) sperm quality parameters of greater amberjack at different times during the reproductive season of 2015 (spermatozoa forward motility, density, duration of motility and maximum survival during storage at 4°C). Statistically significant differences among months are indicated by different lower-case letters ($P \leq 0.05$). na = not available.

The first spawn in 2015 occurred between 1 and 2 days after each GnRHa treatment (**Fig. 3**). A total of 52 spawns were obtained during a period of 72 days (**Table 2**). The number of spawns and fecundity obtained after successive GnRHa implantations decreased. Moreover, the spawning events were concentrated more around the application of each GnRHa treatment. After the 1st treatment, fish spawned 29 times. However, after the 2nd treatment, a total of 15 spawns were recorded during the first 16 days and no eggs were collected the following days. The eggs released after the 3rd GnRHa treatment were collected from 8 spawning events that were obtained during the following 9 days. The highest daily relative fecundity recorded was 5,539 eggs kg^{-1} fish after the 2nd GnRHa treatment, but the total egg production was higher after the 1st treatment, *i.e.* 60,540 eggs kg^{-1} fish compared to 40,180 eggs kg^{-1} fish after the 2nd treatment (**Table 2**). No significant differences were found for daily relative fecundity between the three treatment periods. Almost 15 million eggs were produced from the three successive GnRHa applications.

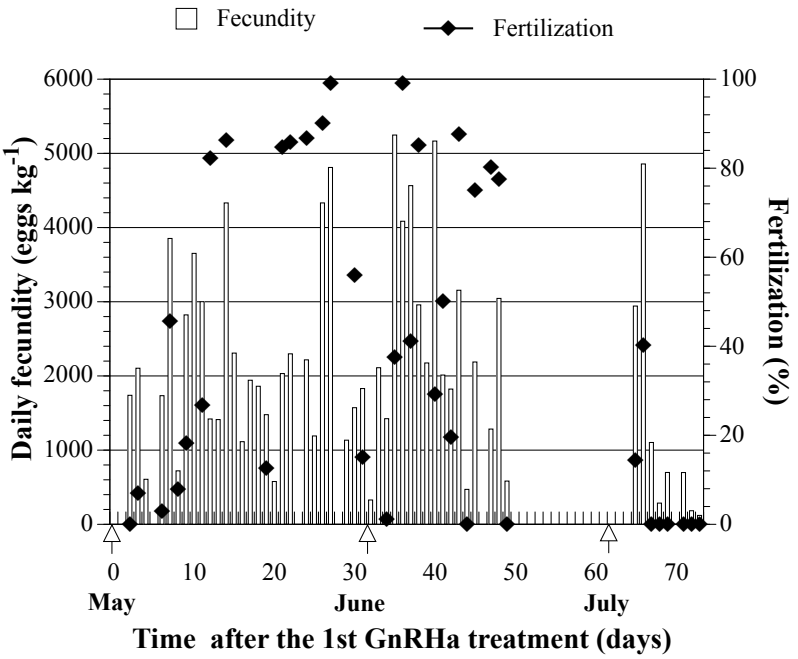

Figure 3. Daily fecundity (eggs kg^{-1}) and fertilization (%) of greater amberjack in response to three GnRHa implants in 2015 (white arrows on the X-axis).

Table 2. Egg production (mean ± SEM were appropriate) of greater amberjack induced to spawn using three GnRHa implant treatments at different times during the reproductive season. No statistically significant differences were observed (P<0.05) between the means obtained after different GnRHa treatments.

Treatment	Spawns (n)	Eggs $spawn^{-1}kg^{-1}$	Total eggs kg^{-1} (x1000 eggs)	Total eggs ($x10^6$ eggs)
1	29	2087±218	60.54	7.05
2	15	2828±420	42.42	6.55
3	8	1895±827	15.16	1.35

Mean fertilization changed through the spawning period after each treatment, with the highest values after the 1st and 2nd GnRHa treatment, and a significant decrease after the 3rd treatment (P<0.05) (**Fig. 4**). On the other hand, no significant differences were observed in hatching, 1-day embryo survival and 3-day larval survival after successive GnRHa treatment. Mean fertilization and hatching success exhibited similar trends during the three spawning periods, reaching their highest values after the 2nd GnRHa treatment and a significant decrease after the 3th treatment (**Fig. 4**).

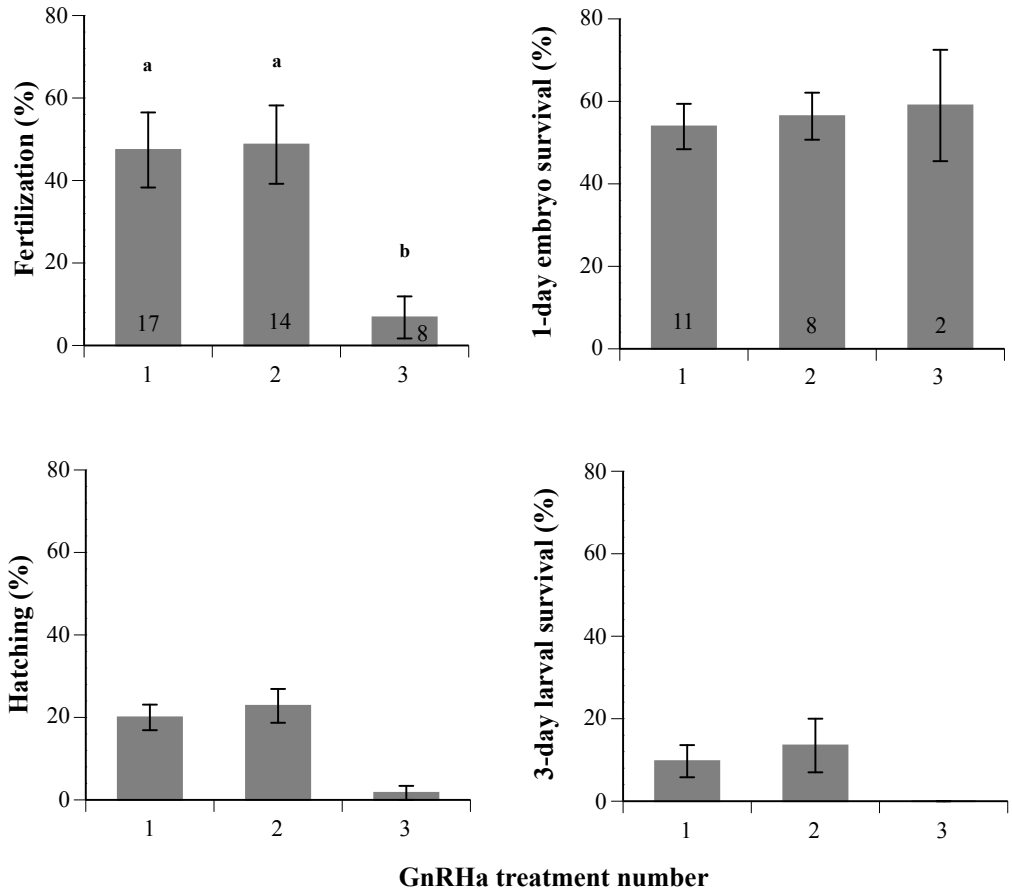

Figure 4. Mean (± SEM) egg quality parameters after the three GnRHa implantations. Numbers in bars indicate the number of samples that constitute each mean. The number of samples of each mean for hatching (%) and 3-day larval survival (%) are the same as for 1-day embryo survival. Statistically significant differences between GnRHa implantations are indicated by different lower-case letters (P = 0.05).

Female plasma E_2 levels were high at the beginning of the spawning period (May), although with high individual variability, as indicated by the high values of SEM (**Fig. 5**). Testosterone in the females was low along the spawning season and increased significantly (P<0.05) at the final sampling. Plasma 17,20β-P remained below 1 ng ml^{-1} during the spawning period. In males, both plasma T and 11-KT levels followed a decreasing trend from May to July, while they increased significantly in September (P<0.05). On the other hand, 17,20β-P increased gradually during the spawning season, with the highest levels in September.

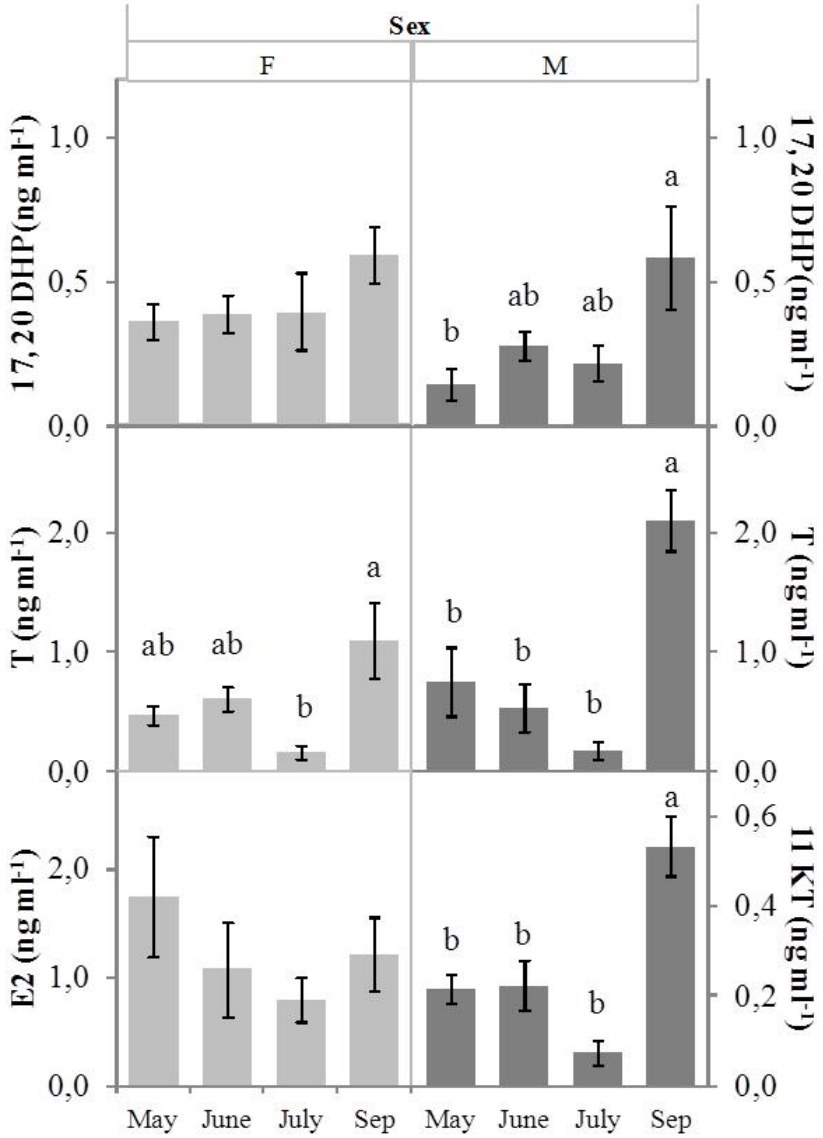

Figure 5. Plasma levels (means ± SD) of E2, T and 17, 20 DHP in female (F) and plasma levels of 11 KT, T and 17, 20 DHP in male (M) greater amberjack broodstock. Different letters indicate significant differences (P<0.05).

The studied blood parameters remained constant along the study and only the number of erythrocytes and plasma levels of protein, cholesterol, alkaline phosphatase and amylase changed during the experimental period (**Tables 3, 4)**. In both, females and males, a significant gradual decrease (*P*<0.05) in the number of erythrocytes was observed along the spawning season reaching the lowest level in September. On the contrary, alkaline phosphatase increased gradually till peaking in September**.** In females, plasma levels of protein and amylase showed slight changes during the experimental period being lower in September compared to the previous samplings (*P*<0.05) (**Table 3** and **Fig. 6)**. Male plasma levels of cholesterol were high in June and July decreasing significantly in September (*P*<0.05) (**Table 4)**. No significant differences (*P*<0.05) in cortisol levels were observed along the spawning season although a trend to diminish was observed at the end of the spawning season (September) in females and males. Regarding other secondary responses to stress, no differences were found in glucose and lactate; however sodium showed lower values at the end of the spawning season in males (*P*<0.05).

Table 3. Erythrocytes (10^4/mm^3), leucocytes (10^3/mm^3), hematocrit (%), triglycerides (mg/dl), cholesterol (mg/dl), protein (g/l), glucose (mg/dl), ALT/GPT (U/L), AST/GOT (U/L), alkaline phosphatase (U/L), cholinesterase (U/L), amylase (U/L), cortisol (ng/ml), lactate (mg/dl), sodium (mg/dl), potassium (mg/dl) in blood from female amberjack during experimental spawning period. Values are means ± SEM. Different letters indicate significant differences (ANOVA, P<0.05).

	May		June		July		September	
Erythrocytes	347.78 ± 118.50	a	275.62 ± 72.75	a	149.31 ± 79.41	b	128.07 ± 52.50	b
Leucocytes	866.54 ± 475.47		653.21 ± 341.54		573.00 ± 260.08		694.54 ± 247.66	
Hematocrit	45 ± 10		52 ± 13		35 ± 15		37 ± 11	
Triglycerides	226.18 ± 58.52		172.14 ± 129.94		206.25 ± 104.28		221.44 ± 147.89	
Cholesterol	226.18 ± 58.52	ab	336.33 ± 170.63	a	275.65 ± 93.44	ab	177.86 ± 90.10	b
Protein	39.85 ± 10.41	ab	44.20 ± 12.60	a	49.93 ± 14.27	a	28.51 ± 8.68	b
Glucose	94.57 ± 26.27		74.94 ± 31.33		100.39 ± 34.85		107.84 ± 53.51	
ALT/GPT	12.92 ± 3.08		14.72 ± 7.55		13.53 ± 6.35		21.60 ± 11.25	
AST/GOT	23.96 ± 16.48		32.71 ± 27.36		31.03 ± 24.76		14.11 ± 4.33	
Alcaline phosphatase	63.11 ± 12.60	c	89.74 ± 20.90	bc	105.22 ± 19.49	b	142.50 ± 31.62	a
Cholinesterase	288.81 ± 235.19		186.44 ± 42.00		235.80 ± 125.87		243.72 ± 40.21	
Amylase	10.97 ± 2.69		15.09 ± 4.24		13.26 ± 17.99		1.98 ± 1.44	
Cortisol	10.82 ± 2.66		11.89 ± 4.30		32.79 ± 8.76		7.69 ± 3.37	
Lactate	39.20 ± 9.71		38.82 ± 7.34		40.09 ± 13.15		37.37 ± 11.84	
Sodium	435.57 ± 18.12	a	415.45 ± 11.67	a	516.06 ± 123.03	a	381.40 ± 10.11	b
Potassium	22.92 ± 6.61		15.98 ± 1.81		20.53 ± 7.39		14.41 ± 2.68	

Table 4. Erythrocytes (10^4/mm^3), leucocytes (10^3/mm^3), hematocrit (%), triglycerides (mg/dl), cholesterol (mg/dl), protein (g/l), glucose (mg/dl), ALT/GPT (U/L), AST/GOT (U/L), alkaline phosphatase (U/L), cholinesterase (U/L), amylase (U/L), cortisol (ng/ml), lactate (mg/dl), sodium (mg/dl), potassium (mg/dl) in blood from male amberjack during experimental spawning period. Values are means ± SEM. Different letters indicate significant differences (ANOVA, P<0.05).

Males	May		June		July		September	
Erythrocytes	426.25 ± 15.93	a	256.25 ± 19.44	b	197.75 ± 36.79	bc	140.50 ± 17.45	c
Leucocytes	92.79 ± 16.40		71.33 ± 13.90		50.63 ± 10.89		58.25 ± 9.35	
Hematocrit	43.00 ± 4.00		56.00 ± 5.00		42.00 ± 9.04		21.00 ± 11.00	
Triglycerides	98.01 ± 5.27		129.04 ± 46.27		236.38 ± 49.35		169.38 ± 40.80	
Cholesterol	209.20 ± 15.76	ab	285.51 ± 17.84	a	265.11 ± 35.76	a	151.47 ± 17.45	b
Protein	35.98 ± 3.56		40.75 ± 2.87		49.08 ± 6.06		30.80 ± 5.54	
Glucose	91.32 ± 8.94		55.53 ± 16.27		99.10 ± 14.65		101.85 ± 21.66	
ALT/GPT	12.78 ± 2.15		11.67 ± 1.32		14.44 ± 3.31		19.63 ± 0.19	
AST/GOT	12.92 ± 3.79		15.00 ± 3.82		40.08 ± 11.32		13.33 ±	
Alcaline phosphatase	65.18 ± 5.25	b	105.95 ± 7.83	ab	103.06 ± 9.45	ab	147.62 ± 24.26	a
Cholinesterase	197.41 ± 10.97		219.35 ± 0.00		179.86 ± 10.75		257.73 ± 25.91	
Amylase	9.88 ± 0.78	b	15.36 ± 4.39	a	7.90 ± 0.41	b	1.98 ± 0.41	c
Cortisol	6.61 ± 0.69		17.86 ± 8.44		35.28 ± 15.39		4.90 ± 1.92	
Lactate	42.79 ± 5.21		45.41 ± 2.94		40.95 ± 1.70		35.97 ± 10.08	
Sodium	441.71 ± 9.17	a	414.56 ± 14.23	ab	531.77 ± 46.09	ab	382.95 ± 1.73	b
Potassium	24.62 ± 3.56		15.69 ± 1.39		18.69 ± 3.05		14.99 ± 2.03	

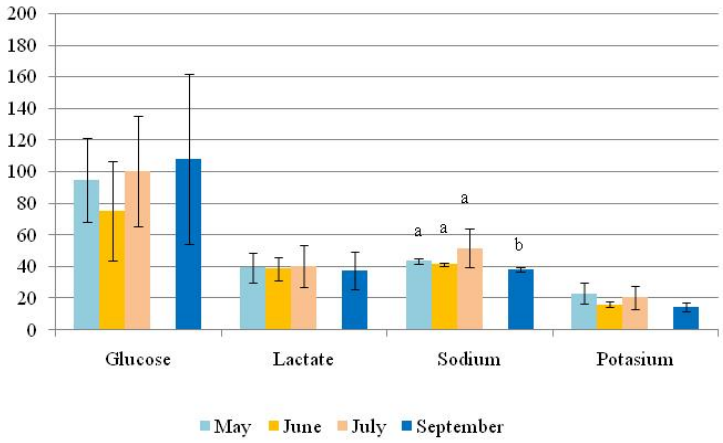

Figure 6. Mean (± SEM) plasma glucose (mg dl^{-1}), lactate (mg dl^{-1}), sodium (mg dl^{-1}) and potassium (mg dl^{-1}) in female broodstock of greater amberjack during 2015 spawning period (ANOVA, $P<0.05$).

Results from the 2016 spawning period

In 2016, the number of available broodstock was reduced to eight (3 males and 5 females) (**Table 5**) after some mortality in the previous year. The fish were sampled in June, July, August, September, and October, and the selected fish were again implanted with the required GnRHa dose at three different times from June to September (**Table 5**). The selected females were administered an effective dose of ~75 µg GnRHa kg^{-1} body weight. The GnRHa dose for the males was the same as in 2015**.**

Table 5. Number and mean weight (± SD) of implanted greater amberjack at each treatment time of 2016.

Sex		Females			Males		
Sampling (Month)	Treatment	N		Dose (µg kg^{-1})	N		Dose (µg kg^{-1})
		Biopsied	Treated		Biopsied	Treated	
June	First	5	5 (20.1±6.8)	91.3 ± 5.7	3	3 (15.3±4.8)	59.9 ± 4.3
July	Second	5	5 (18.5±6.2)	99.4 ± 12.4	3	3 (14.4±4.2)	63.4 ± 1.4
August	Third	5	5 (18.8±6.0)	97.3 ± 11.0	3	3 (15.4±4.9)	59.6 ± 1.7
Septemb.	Fourth	5	2 (14.4±5.0)	92.4 ± 7.6	3	3 (15.2±4.6	60.5 ± 2.7
October		5			3		

The mean oocyte diameter of the largest vitellogenic oocytes during the three samplings ranged between 571 µm in July to 776 µm in October, but no significant differences were observed (**Fig. 7**). Sperm motility was 40-80% (**Fig. 7**) and remained unchanged throughout the monitored period, while the duration of sperm motility was significantly higher in September (3.9±0.2 min) than in June (1.8±0.1 min) and August (1.8±0.2 min) ($P<0.05$). The sperm density ranged from 0.86 ±1.28 x 10^{11} spermatozoa ml^{-1} in July and 2.24±1.82 x 10^{11} spermatozoa ml^{1} in October, but no significant differences were observed during the monitored period. The number of spawns was 20 after the 1st treatment, 23 after the 2nd treatment, 17 after the 3rd treatment and 1 after the final treatment (**Fig. 8**).

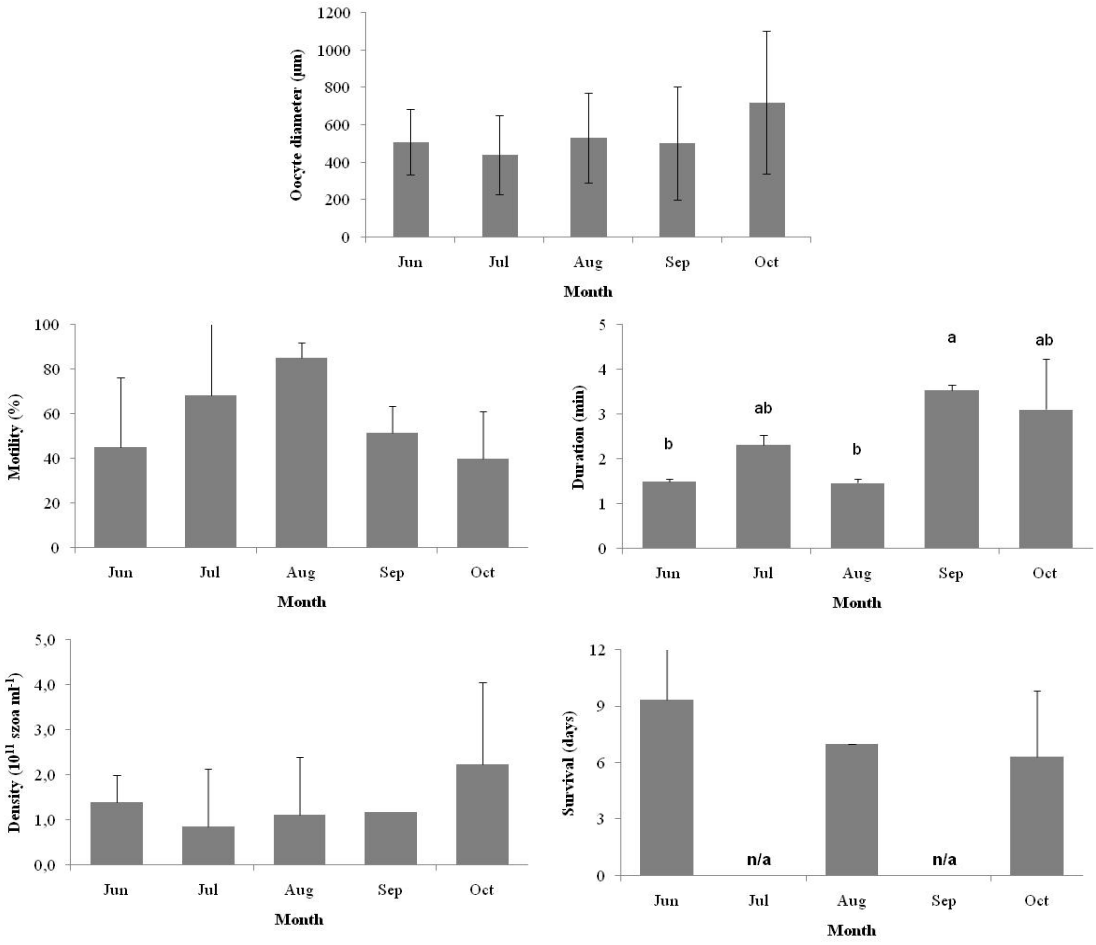

Figure 7. Mean (± SEM) oocyte diameter of the largest vitellogenic oocytes (upper graph) and sperm quality parameters of greater amberjack at different times during the 2016 spawning period (spermatozoa forward motility, density, duration of motility and maximum survival during storage at 4°C). Statistically significant differences among months are indicated by different lower-case letters ($P \leq 0.05$). na = not available

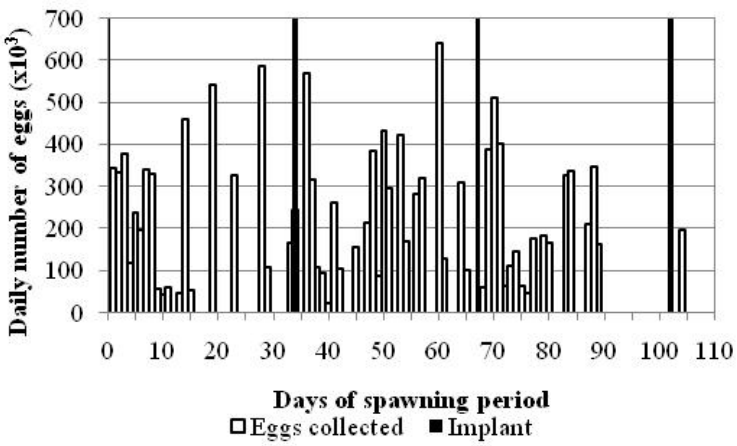

Figure 8. Daily fecundity (x10^3 eggs) during 2016. The black bars indicate different GnRHa treatments.

Mean fertilization and hatching exhibited similar trends during the three spawning periods, reaching their highest values in the second period (July) (**Fig. 9**). However, no significant differences were observed between the three spawning periods after successive GnRHa treatment.

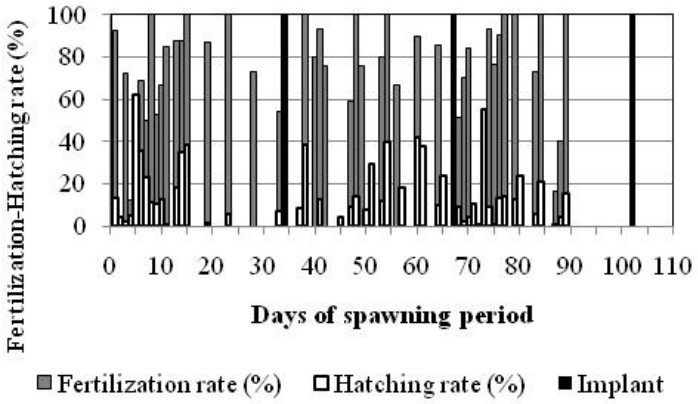

Figure 9. Daily fertilization and hatching success (%) during 2016. The black bars indicate different treatments.

Results from the 2017 spawning period

In 2017, only 3 males and 1 female (mean weight ± SD, 17.3 ± 6.9 kg) were available, and were maintained in an outdoor covered circular tank of 50 m^3 with continuous water supply under natural photoperiod. At the expected onset of the spawning season (May 2017), a passive egg collector was placed in the outflow of the spawning tank and checked daily, in order to monitor the occurrence of any spontaneous spawning. According to the plan, the fish was supposed to be treated in the middle of June with the lowest dose of GnRHa proposed in the DOW (~25 µg GnRHa kg^{-1} fish). However, spontaneous spawns were obtained before the GnRHa treatment and it was decided not to handle the fish, in order to obtain valuable data on the kinetics, fecundity and egg quality of spontaneous spawns from captive F1 broodstock. In a period of 125 days (more than 4 months), 21 spawns were collected with 4-10 days interval among spawns (**Fig. 10**). The larger intervals among the spawns were observed from mid July to mid August (**Fig. 11**).

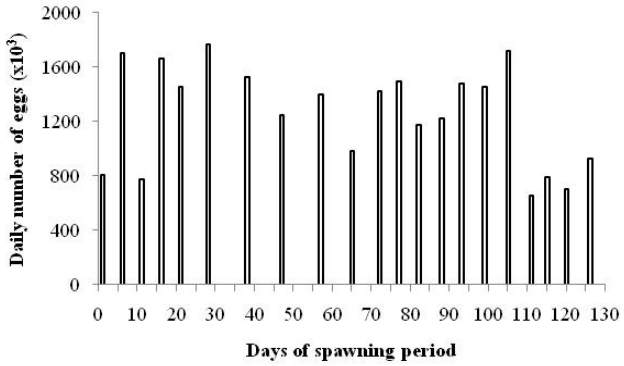

Figure 10. Daily fecundity (x10^3 eggs) during the 2017 spawning period.

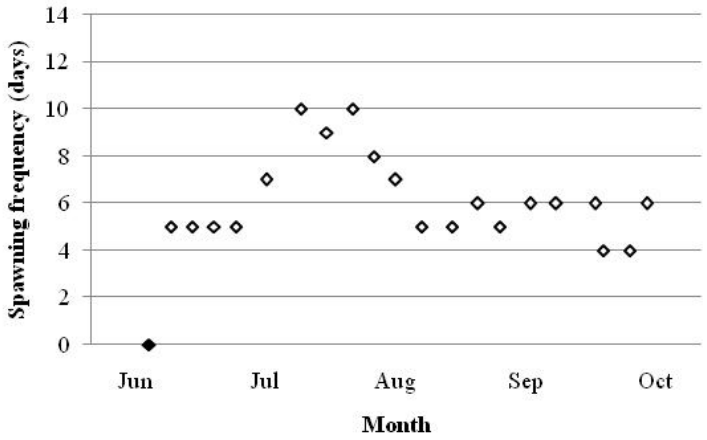

Figure 11. Spawning interval between spawnings during 2017. The solid diamond indicates the first spawn.

Fertilization was high throughout the reproductive season, but hatching success was quite variable (**Fig. 12**).

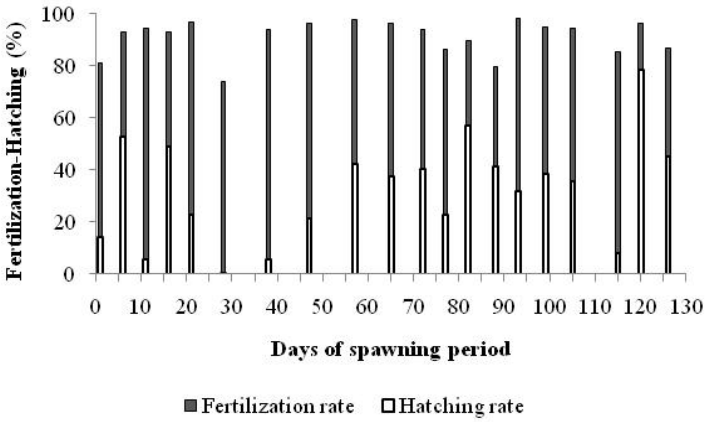

Figure 12. Daily fertilization and hatching success (%) during 2017 spawning period.

Discussion

In the Mediterranean region, the ovaries of wild-caught greater amberjack have batches of oocytes at distinct stages of development with diameters of 120-400 µm in early vitellogenesis (Marino, et al., 1995), mid-fully viotellogenic oocytes of 400-600 µm at the beginning of the spawning season in May (Lazzari, et al., 2000) and the largest diameter of vitellogenic oocytes of 650-750 µm in June (Kozul, et al., 2001; Mylonas, et al., 2004b). However, even after completing vitellogenesis and reaching the appropriate oocyte sizes, a considerable number of females fail to mature and ovulate, and hormonal treatments are necessary to induce spawning in captivity. The efficiency of the hormonal treatments depends on the stage of ovarian development at the time of treatment, the hormone type, the dose and the method of application. Captive-reared wild greater amberjack have spawned successfully after been induced with human Chorionic Gonadotropin (hCG) injections when females had vitellogenic oocytes of 550-600 µm in diameter (Díaz, et

al., 1997; Kozul, et al., 2001), and after GnRHa injection (Fernández-Palacios, et al., 2015a) or implantation (Mylonas, et al., 2004b) when oocytes were at 500 and 650 µm in diameter, respectively. In the present study, some of the F1 females underwent full vitellogenesis and had larger oocytes than reported before for wild-caught individuals, and were appropriate to be induced for spawning during all sampling times during the 2015 and 2016 reproductive periods. This demonstrates that under these rearing conditions and hormonal treatments, F1 greater amberjack undergo normal oogenesis and maintained their vitellogenic production for an extended period of time, as it is customary for this species in the subtropical area of the Canary Islands (Fernández-Palacios, et al., 2015a; Jerez, et al., 2006). This is the first study demonstrating that F1 generation greater amberjack do undergo normal gametogenesis in captivity and are capable of producing fertilized eggs, after exogenous hormonal therapy for the induction of oocyte maturation, ovulation and spawning. In addition, in 2017 the only surviving female spawned spontaneously for an extended period of time, even without any hormonal treatment.

Sperm could not be collected by abdominal pressure at any sampling time, even though the fish were in spermiating condition and sperm samples were taken using a catheter introduced into the genital pore. The same situation was observed in captive male greater amberjack broodstocks that were held either in tanks or in sea cages in Greece (Fakriadis, et al., 2017; Mylonas, et al., 2017b; Zupa, et al., 2017a; Zupa, et al., 2017c). Similar to other fast swimming pelagic fishes, such as the Atlantic bluefin tuna (*Thunnus thynnus*), the abdominal wall of greater amberjack is thick and very muscular, and this probably makes it very difficult to apply adequate pressure to the internal organs and the testes (Mylonas, et al., 2007; Zupa, et al., 2013), in order to release sperm by stripping. In addition, in captive-reared fish is very common that males produce lower amounts of sperm, often of high sperm density (Mylonas & Zohar, 2001; Zohar & Mylonas, 2001b), making it difficult to obtain sperm with abdominal pressure. In general, treatments with GnRHa increase the milt volume by stimulating seminal plasma production, but often with a proportional decrease in sperm density (Mylonas, et al., 1998; Vermeirssen, et al., 1998). Sustained-release GnRHa-delivery systems have been used successfully to induce an overall increase both to the expressible sperm and spermatozoa production in various cultured fishes (reviewed by Mylonas and Zohar, 2001), enhance the quality and the quantity of sperm produced (Clearwater & Crim, 1998; Mylonas, et al., 1997; Rainis, et al., 2003; Sorbera, et al., 1996; Vermeirssen, et al., 1998), including wild-caught greater amberjack (Mylonas, et al., 2004b). In the present study, we did not observe a significant increase in sperm production in response to the GnRHa treatment over the course of the monitoring period that would translate into easier collection of sperm using abdominal pressure.

Similarly, there were in general no differences in the sperm quality parameters of the males during the present study, showing similar values to wild-caught GnRHa-treated greater amberjack reported earlier (Mylonas, et al., 2004b). A gradual reduction in the sperm motility duration during the reproductive season was observed in another study with wild-caught breeders in the eastern Mediterranean Sea (Zupa, et al., 2017a). However, as water temperatures do not rise to the same levels in the summer in the Canary Islands, apparently greater amberjack remain in spermiating condition for a much longer period of time, reported from May to October (Fernández-Palacios, et al., 2015a; Jerez, et al., 2006). In the present study, contradictory results were observed in sperm motility duration since a decreasing trend was observed in 2015 and an increasing trend in 2016, respectively. The sperm density in GnRHa-treated F1 males showed an increasing trend over the course of the 2015 and 2016 spawning periods. Different effects of GnRHa-delivery systems have been obtained in other species. For example, in GnRHa-treated Atlantic salmon *Salmo salar*, the total expressible milt increased and sperm density or motility did not change (Goren, et al., 1995), while in Atlantic halibut *Hippoglossus hippoglossus* (Mazorra de Quero, et al., 2000; Vermeirssen, et al., 2000) and common dentex *Dentex dentex* (Greenwood, et al., 2001), the sperm motility was enhanced slightly and the sperm density decreased. On the contrary, GnRHa implants were ineffective in enhancing sperm production in both flatfish *Paralichthys lethostigma* and *Paralichthys dentatus* (Berlinsky, et al., 1997; Berlinsky, et al., 1996). Therefore, the application of GnRHa delivery systems for inducing and enhancing spermiation in greater amberjack requires more research in order to determine if a different mode, time of application or dose are required for a successful enhancement of milt production, as has been demonstrated in many other fishes (Mylonas, et al., 2017a).

Spontaneous and induced spawning of wild-caught greater amberjack has been obtained from May to October in the Canary Islands (Fernández-Palacios, et al., 2015a; Jerez, et al., 2006), but only from May to July in the Mediterranean Sea, where summer temperatures are much higher (Grau, et al., 1992; Lazzari, 1991). In the present study, a total of 52 spawns were obtained from GnRHa-treated F1 greater amberjack in 2015 and 61 in 2016, with the number obtained after each consecutive treatment decreasing gradually. The use of GnRHa for inducing and increasing spawning frequency is well-known (Mylonas, et al., 2010; Zohar & Mylonas, 2001a) and it has been reported also in wild-caught greater amberjack (Fernández-Palacios, et al., 2015b; Mylonas, et al., 2004b), longfin yellowtail *Seriola rivoliana* (Roo, et al., 2014), and F1 yellowtail kingfish (Setiawan, et al., 2016). In this study, the gradual decrease in the number of spawns obtained after each implantation could be related to the vitellogenic capacity of the ovary, and to potentially a determinant type of fecundity characteristics of this species. In terms of a comparison between GnRHa injections and implants, recent studies in wild-caught greater amberjack have shown that a larger number of spawns can be expected in response to successive GnRHa implants compared to injections (Fakriadis, et al., 2017; Fernández-Palacios, et al., 2015b; Mylonas, et al., 2004b), due to lower stress inflicted on the breeders and a longer period of hormonal stimulation (Carolsfeld, et al., 1988). In the Canary Islands, 38 spawns during 156 days without any hormonal treatment have been obtained from wild-caught broodstock, with an estimated spawning frequency of 6-7 days^{-1} (Jerez *et al*., 2006), and 22 spawns during 123 days from wild-caught broodstock injected with GnRHa every 10 days (Fernandez-Palacios *et al*., 2015). In the present study, a total of 21 spontaneous spawns of untreated F1 greater amberjack broodstock were collected during 125 days in 2017 and the spawning frequency varied between 4 and 10 days.

The total number of eggs obtained from GnRHa-implanted F1 females in 2015 (14.95 x 10^6 eggs) were similar to that obtained from natural spawning of wild-caught greater amberjack reported previously (14.31 x 10^6 eggs)(Jerez, et al., 2006), and three times higher than that (4.96 x 10^6 eggs) obtained from wild-caught females treated with multiple GnRHa injections in the Canary Islands (Fernández-Palacios, et al., 2015b). In 2016, the egg production was even higher (22.60 x 10^6 eggs). Further to this, the total number of eggs per kg of female obtained in this study using successive GnRHa implants was two times higher than the one obtained from wild-caught females in the Canary Islands treated with successive injections of GnRHa (Fernández-Palacios, et al., 2015b) and six times higher than the eggs obtained spontaneously from wild-caught females of greater amberjack not treated with any hormones (Jerez, et al., 2006). Comparable results have been recorded in the Mediterranean Sea where the spawning season is shorter, in wild-caught greater amberjack treated with hCG or GnRHa, in the form of injections or implants, (García, et al., 2001; García-Gómez & De la Gándara, 2003; Mylonas, et al., 2004b). In the present study, in 2017 the only female produced 25.00 x 10^6 eggs during the spawning period with no treatment. In terms of relative fecundity, the fish produced 95.32 x 10^3 eggs kg^{-1} fish in 2015, 234.59 x 10^3 eggs kg^{-1} fish in 2016 and 939.00 x 10^3 eggs kg^{-1} fish in 2017. In the Mediterranean, broodstock treated with GnRHa implants produced 102.40 x 10^3 eggs kg^{-1} fish and GnRHa injections 26.52 x 10^3 eggs kg^{-1} fish, respectively (Fakriadis, et al., 2017).

In some species, spawning induction using GnRHa has been reported to have a negative effect on egg quality (Bobe & Labbé, 2010). For example, GnRHa implantation has been associated with decreased egg buoyancy, fertilisation and number of viable eggs, as well as smaller oil globule diameters (Agulleiro, et al., 2006; Bonnet, et al., 2007; Forniés, et al., 2000; Garber, et al., 2009; Mugnier, et al., 2000), a situation that was observed also using F1 yellowtail kingfish (Setiawan, et al., 2016). The fertilization success obtained from hormonally treated wild-caught greater amberjack has been highly variable, ranging between 16-50% (Kozul, et al., 2001) and 58-99% (Hamasaki, et al., 2009) for hCG injected fish, and 22% to 96.01 ± 6.50 % for GnRHa implanted (Mylonas, et al., 2004b) and injected (Fernández-Palacios, et al., 2015a) fish, respectively. In the latter study the sex ratio used was higher (2♂:1♀) and it might have improved the fertilization success (Tachihara, et al., 1993). In the present study, the GnRHa implanted F1 greater amberjack produced eggs with fertilization success of 41.9 ± 6.2 % and 75.1 ± 26.4 %, in 2015 and 2016, respectively, values similar to those obtained from spontaneous spawning of wild-caught greater amberjack maintained in our large communal tanks (61.75 ± 3.60 %) at similar sex ratio (Jerez *et al*., 2006). In 2017, the higher fertilization rate (> 85%) obtained could be related to a higher sex ratio (3♂:1♀) as well as a lower tank volume (50 m^3). The hatching success in 2015 (18.6 ± 12.4 %) and 2016 (15.9 ± 14.5 %) were similar to those obtained in natural spawning (16.49 ± 3.03 %, Jerez *et al*., 2006) and slightly lower than the

one obtained in 2017 (32.6 ± 20.0 %), but always were lower than those obtained in wild-caught greater amberjack injected with hCG (52.3-97.2 %) (Hamasaki *et al*., 2009) and GnRHa in the Mediterranean (69.7 ± 4.3%) (Fakriadis, et al., 2017) and Canary islands (92.58 %) (Fernandez-Palacios *et al*., 2015). Thus, the successive GnRHa implants used in this study to induce maturation and spawning of F1 greater amberjack were an adequate treatment to produce fertilized eggs. With further optimization, this method may be a reliable and highly efficient way to obtain eggs from hatchery-produced broodstocks maintained in aquaculture facilities, enabling the development of a sustainable greater amberjack industry.

In some studies, lower embryo and larval survival were recorded at the first spawns after hormonal treatment (Fernández-Palacios, et al., 2015a; Kozul, et al., 2001). On the contrary, in the present study the lowest fecundity and quality of eggs were obtained after the third GnRHa treatment at the latter part of the reproductive season. Additionally, at the same treatment period the 3-day larval survival was lower than the one recorded using wild-caught greater amberjack injected with GnRHa (Fernández-Palacios, et al., 2015a) or hCG (Hamasaki, et al., 2009). A reduction in embryo and larval survival may occur towards the end of the spawning period in fishes with asynchronous ovarian development (Mihelakakis, et al., 1995; Mihelakakis, et al., 2001). The consecutive treatments of F1 greater amberjack with GnRHa implants in both 2015 and 2016 progressively stimulated the release of eggs and the fertilization success was correlated with spawn number and daily fecundity, reaching the highest values at the peak of the spawning period. The high fertilization success could indicate a progressive synchronization between sexes during the spawning season. The eggs and oil droplet diameters obtained were similar to those obtained from natural spawning of wild-caught greater amberjack in our facilities (Jerez, et al., 2006). Interestingly, the lower egg diameters were recorded when higher numbers of eggs were released in the present study. It has been suggested in F1 yellowtail kingfish (Setiawan, et al., 2016) that the GnRHa treatment could result in the production of smaller eggs due to the premature ovulation of oocytes before completion of vitellogenesis.

The GnRHa delivery systems have been tested in a wide variety of wild and hatchery-produced fish to induce the natural progression of plasma steroid increases associated with OM and spermiation, through increases in circulating luteinizing hormone (LH) levels (Mylonas & Zohar, 2001). Plasma sex steroid levels provide indicative information on reproductive performance of fish and they have been studied in wild greater amberjack (Mandich, et al., 2004; Zupa, et al., 2017c) and yellowtail kingfish (Poortenaar, et al., 2001). In wild greater amberjack females, levels of T and E_2 showed a similar profile and were positively correlated during the spawning season, and were maximum at the start of the spawning season (Mandich, et al., 2004). Yellowtail kingfish females showed similar kinetics in plasma sex steroids, with T as precursor for the synthesis of E_2, and reaching the highest levels during vitellogenesis, while 17,20β-P peaked in females with ovaries undergoing OM (Poortenaar, et al., 2001). In the present study, however, plasma sex steroids in F1 greater amberjack females were considerably lower than that of wild females (Mandich, et al., 2004; Zupa, et al., 2017c), but closer to the captive females (Zupa, et al., 2017c), and no significant changes after the successive GnRHa implants were observed. In fact, E_2 and 17,20β-P levels were similar between May and September, and only T increased significantly in September. There was a positive relation between individual levels of T and E_2, and between T and 17,20β-P. The unchanged levels of E_2 and 17,20β-P in this study could reflect the appropriate size and stage of oocyte development at the time of implantation with GnRHa, while the higher level of T in September could be related to the end of E_2 synthesis, as vitellogenesis was coming to an end. The E_2 and T levels covariate during the spawning season and T levels in plasma increase when is not used to obtain E_2 by aromatization (Kagawa, et al., 1982; Rinchard, et al., 1993).

In wild greater amberjack males (Mandich, et al., 2004), the changes in T and 11 KT plasma levels were related to testis development, reaching the highest levels during spermatogenesis and in males with milt, respectively. In yellowtail kingfish males, the plasma concentrations of T and 11KT were significantly elevated in spermiating males, but not during spermatogenesis, while plasma concentrations of 17,20β-P did not change with different testis maturation stage (Poortenaar, et al., 2001). In the present study, sex steroid levels in F1 greater amberjack males did not change significantly after successive GnRHa implants, but showed the highest levels in September, two months after the last treatment. Individual levels of T were positively correlated with 11-KT and 17,20β-P. However, there were no significant relations between sperm

parameters and the sex steroids analysed. The absence of significant changes in sex steroids with the successive implants between May and July are in agreement with the absence of significant differences in sperm quality parameters in the experiment. In contrast, the sexual hormone levels started to increase in September, reaching the highest levels, possibly in preparation for the next spawning. This fact would be according to the large spawning season of greater amberjack in the Canary Islands. Moreover, the oocyte size and sperm quality observed in May are consistent with previously cited values for mature greater amberjack. Specifically, there is evidence that 17,20β-P is involved in spermiation, but also in the initiation of meiosis, and two peaks can be recorded in some species (Scott, et al., 2010).

Analysis of haematological and biochemical parameters in blood is a valuable tool that can be used as an effective index to monitor fish health and pathological changes. Both haematological and biochemical parameters obtained in the present experiment were within the normal range for greater amberjack, compared to previous studies (Uyan et al., 2009; Dawood et al., 2015; Hossain et al., 2017). Most blood parameters studied remained constant along the study and only erythrocytes, protein, cholesterol and amylase were lower at the end of the spawning season.

During chronic stress in fish in culture, there are often characteristically high circulating levels of cortisol. In the present study, no significant differences in cortisol levels were observed along the spawning season, although a decreasing trend was observed at the end of the spawning season (September) in males and females. The primary stress response in fish is known to further trigger and lead to sequential secondary responses (*e.g.* increases in glucose, lactate, decreases in plasma sodium and potassium). In this study, no differences were found in glucose and lactate; however sodium showed lower values at the end of the spawning season in males. The absence of significant changes in haematological and biochemical parameters suggests that the physiological condition of F1 greater amberjack breeders seems largely unaffected by the repeated treatment with implants of GnRHa.

In summary, the present study showed that hatchery-produced F1 greater amberjack undergo normal gametogenesis and can be induced to undergo maturation, ovulation and spawning using GnRHa delivery systems of 50 and 75 μg kg^{-1}. Egg production is high and egg quality adequate for the implementation of larval rearing for commercial purposes. The use of consecutive GnRHa-delivery systems over a long reproductive period resulted in multiple spawns of fertilized and viable eggs. In addition to inducing OM after vitellogenesis is completed in females, the positive results obtained could be due to successful synchronization of gamete release between males and females, but also to the stimulation of egg release by the females at the appropriate time after ovulation (Mylonas, et al., 2004b). Despite that repetitive handling required to administer the implants of GnRHa during the prolonged spawning season of F1 greater amberjack in the Canary Islands, the present study demonstrated that there was no negative effect on the welfare and reproductive performance of the fish, and seems to be an appropriate method for cultured fish in terms of welfare status. Additionally, spontaneous spawning of F1 greater amberjack in tanks in the Canary Islands is possible, as it was shown in the 2017 spawning season. The successful reproduction of F1 greater amberjack broodstock, is a step towards the industrial aquaculture production of this valuable species, demonstrating that hatchery produced fish may be selected according to desired traits and then allowed to undergo full gametogenesis in captivity and then induced to spawn using the tested hormonal therapies. Implementing breeding selection is expected to further enhance the production parameters and profitability of the greater amberjack aquaculture industry.

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Deviations

The lowest dose (25 µg kg^{-1}) that was scheduled to be used in the 2017 spawning period was not applied at the end, since (a) only a single female was available at that time due to mortalities over the previous years and (b) the female started to produce eggs spontaneously with no hormonal treatment. This was a great chance to obtain spawning and egg quality data of spontaneous spawns in captivity, which are not available so far in F1 broodstocks.

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