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Spawning Induction of First-Generation (F1) Greater Amberjack *Seriola dumerili* in the Canary Islands, Spain Using GnRHa Delivery Systems

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Abstract: The development of a sustainable aquaculture industry requires the closing of the life-cycle of a potential species in captivity, and the establishment of breeding selection programs using hatchery-produced breeding stocks. The greater amberjack *Seriola dumerili* is a cosmopolitan pelagic species that has been considered as a good candidate for the species diversification of aquaculture production in the Mediterranean region. However, commercial production is still very limited due to bottlenecks in reproduction, larval rearing and management control during grow out. The aim of the present study was to examine the reproductive development of hatchery-produced greater amberjack and to develop a spawning induction protocol based on the use of gonadotropin releasing hormone agonist (GnRHa) in a controlled-release delivery system. The results showed that hatchery-produced greater amberjack undergo normal gametogenesis and can be induced to undergo maturation, ovulation and spawning after multiple administrations of GnRHa implants, over an extended spawning period lasting from May to September in the Canary Islands, Spain. The use of GnRHa-delivery systems resulted in multiple spawns of fertilized and viable eggs. Egg production was high and egg quality adequate for the implementation of larval rearing for commercial purposes. The handling required to administer the GnRHa implants during the prolonged spawning season did not result in any negative effect on the welfare and reproductive performance of the fish based on evaluation of several biochemical parameters. The developed reproduction control method shows great potential to advance the commercial production of greater amberjack, by enabling the use of hatchery-produced broodstocks for further breeding selection.

Keywords: *Seriola dumerili*; greater amberjack; induced spawning; GnRHa implants; broodstock; F1 fish; hatchery reared

1. Introduction

The greater amberjack *Seriola dumerili* is a cosmopolitan pelagic species [1] with high growth rate and excellent flesh quality [2–4]. It has been considered a good candidate for the species diversification of aquaculture production in the Mediterranean region [5]. However, commercial production is still very limited [2], due to bottlenecks in reproduction, larval rearing and health control during grow out. Recent research has evaluated the potential of wild-caught broodstock to mature and be induced to

spawn in captivity [6–8]. However, the development of a sustainable aquaculture industry requires the closing of the life-cycle of a potential species in captivity, and the establishment of breeding selection programs using hatchery-produced breeding stocks [9,10].

Reproductive dysfunctions in captive broodstock occur in many cultured species [11–13], including the greater amberjack [14–17], preventing commercial development of seed production [3,4]. In some species, reproductive dysfunctions are more severe in hatchery-produced broodstocks. This has been demonstrated in the Senegalese sole *Solea senegalensis* [18–23], the greenback flounder *Rhombosolea tapirina* [24] and the sharpsnout seabream *Diplodus puntazzo* [25]. In Senegalese sole, F1 females undergo maturation, ovulation and spawning, but the obtained eggs are not fertilized, which has been related to a critical reproductive dysfunction of the F1 males [26]. On the other hand, in the greenback flounder a delayed release of eggs by the females after ovulation has been blamed for the failure to produce fertilized eggs [24]. 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 dysfunction in the females or the lack of breeding behavior of the males [25]. The existence of a significantly lower reproductive success of captive-produced compared to wild-caught breeding animals has been reported in a great number of animal taxa with relevance not only in aquaculture production, but also in conservation biology and laboratory experimentation [27].

The most commonly observed reproductive dysfunctions in fish maintained in aquaculture facilities are the failure to undergo oocyte maturation (OM) after completion of vitellogenesis in females and the low production of sperm in males [26,28–30]. 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 [15,31–34]. In addition, spontaneous spawning has also been achieved in some individuals without any exogenous hormonal therapies [35]. However, production of fertilized eggs from hatchery-produced broodstock has not been successful so far in Europe, although vitellogenesis and OM has been achieved [36]. This is contrary to what has been reported for the greater amberjack in Japan [37] and the congener yellowtail kingfish *Seriola lalandi*, in which spontaneous spawning with fertilized eggs have been obtained successfully from F1 broodstock [38].

The objectives of the present study were to examine the reproductive development of hatchery-produced F1 generation greater amberjack, 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. The method described in this study shows great potential for the development of the aquaculture industry for greater amberjack, by enabling the use of hatchery-produced broodstocks for further breeding selection.

2. Results

2.1. Fish Condition

The initial weight of the males and females was significantly different, with the females weighing approximately 55% more than the males in May (Table 1). Fish from both sexes lost weight between May and July, with the loss being higher on average in males (Table 1). However, from July to September, three females weighed 8% less and three others increased their weight by approximately 6%. The weight of one male decreased by 16%, while the weight of the remaining males increased by 8%.

This weight loss coincided with a decrease of feed intake by the broodstock and an increase in water temperature (Figure 1). During the spawning period the seawater temperature increased from $20.4 \pm 0.3\,^{\circ}\text{C}$ (May) to $24.5 \pm 0.2\,^{\circ}\text{C}$ (September), and monthly feed intake (food per biomass) decreased from $3.4 \pm 0.3\%$ after the first sampling (May) to $2.1 \pm 1.1\%$ between the second and third sampling (June and July), and remained stable until September ($1.9 \pm 0.3\%$).

Table 1. Mean (±standard deviation (SD)) weight of F1 greater amberjack broodstock during the monitored reproductive period and mean (±SD) ratio of weight loss (%) at each sampling time versus the previous sampling.

| Month	Sex	*n*	Weight (kg) (Mean ± SD)	Weight Change (%) (Mean ± SD)
May	Females	7	23.3 ± 10.8	
	Males	7	14.9 ± 5.0	
July	Females	7	21.6 ± 10.2	−7.7 ± 5.0
	Males	7	13.3 ± 4.5	−10.5 ± 4.1
September	Females	6	23.9 ± 10.0	−1.4 ± 9.3
	Males	5	15.3 ± 4.7	1.2 ± 10.6
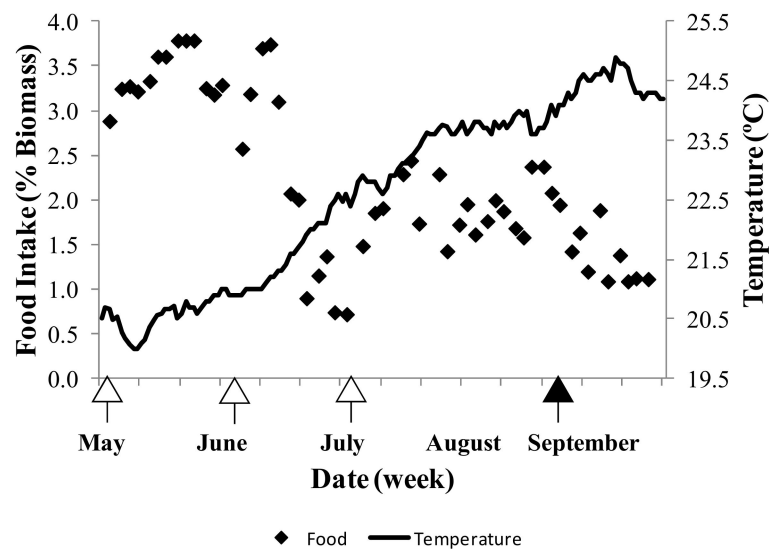
Figure 1. Temperature (solid line) and food intake (black rhombus) during the experimental period. White arrowheads in the *X*-axis indicate the GnRHa treatment and sampling times, and the black arrowhead indicates the final sampling time.

Almost one third of the females were in early vitellogenic stage (eVg) in May (Figure 2a), but mostly the fish (*n* = 4) had fully vitellogenic (Vg) oocytes (Figure 2b), with a mean (±Standard Error of the Mean, SEM) diameter of the largest oocytes of 685 ± 21 µm (Figure 3). Some atresia (AT) was present, as well. Only females with oocytes >600 µm were considered eligible for spawning after GnRHa implantation. In June, four of the biopsied females again had mainly Vg oocytes, but also oocytes in maturation, with the largest oocytes having a mean diameter of 723 ± 47 µm (Figure 3), with also some AT. At the same sampling, one female was found with ovulated eggs. In July, three of the females had Vg oocytes (Figure 2c) in the ovary, with an increased percentage of AT. The mean diameter of the largest Vg oocytes was 697 ± 26 µm (Figure 3). The rest of the females had mainly ovulated eggs and primary or eVg oocytes at most. At the last sampling in September, most of the females (*n* = 4) were in Vg stage with a mean diameter of 650 ± 37 µm (Figure 3). Two of them had oocytes in OM (Figure 2d). No significant differences were found in the mean diameters of the largest Vg oocytes among the different samplings.
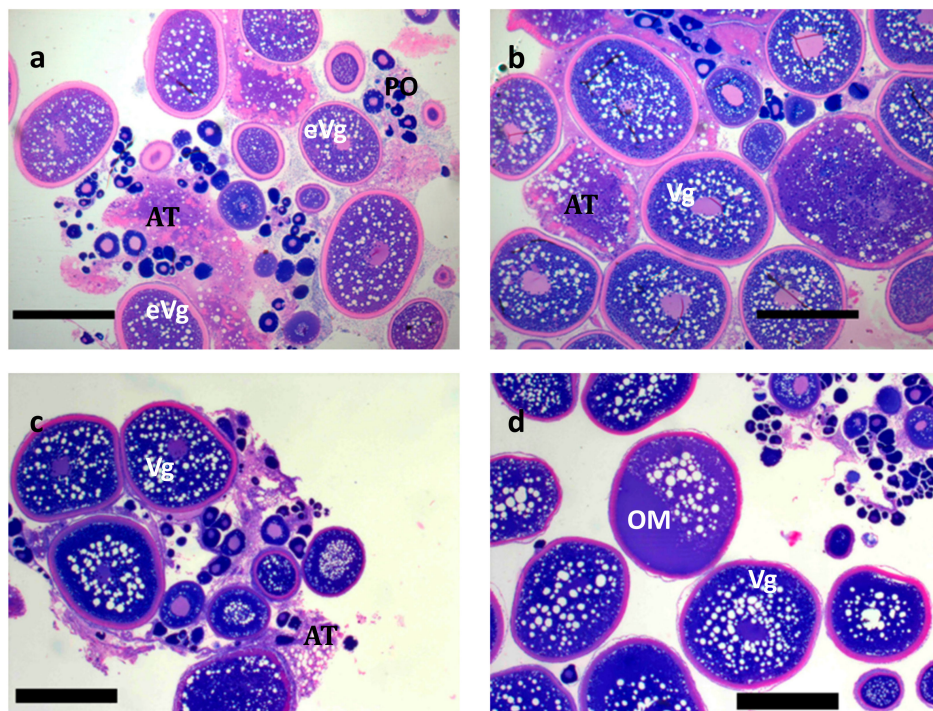
Figure 2. Ovarian biopsies from F1 greater amberjack maintained in land-based tanks in Tenerife, Canary Islands and processed for histology (**a**): Female in May in early Vitellogenesis (eVg) with a large number of primary oocytes (PO) as well as atresia (AT). (**b**): Female in May with oocytes in full Vg. (**c**): Female in mid-July, in full Vg. (**d**): Female in mid-September, having oocytes in oocyte maturation (OM) and Vg. Bar = 500 μm.
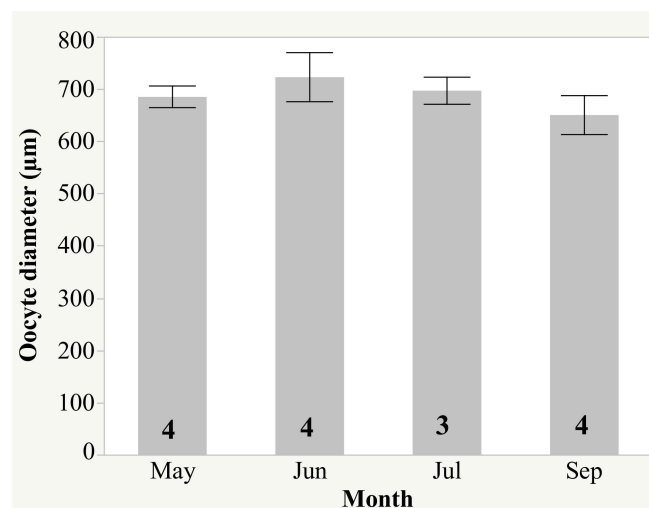
Figure 3. Mean (±Standard Error of the Mean, SEM) oocyte diameter of F1 greater amberjack (*n* inside the bars) that were expected to respond to given GnRHa implant, at the time of treatment (May–July) and at the conclusion of the experiment (September). No statistically significant differences were observed ($p < 0.05$).

Males and females in May, June and July were implanted with a GnRHa implant at a dose of ~50 μg GnRHa kg^{-1} body weight in the successive spawning induction treatments (Table 2). In June and July, more females were given a GnRHa implant than were eligible for spawning based on their maximum Vg oocyte diameters (Figure 3), because the measurements of the oocytes were done later in the laboratory once the fish sampling was completed.

Table 2. Number and mean (±SD) oocyte diameter (μm) of biopsied greater amberjack (N), and number, mean weight (±SD) and dose of GnRHa (μg kg^{-1} body weight) of implanted greater amberjack at each treatment/sampling time. All treated fish (N) were given a GnRHa implant, and slight 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 ± 5.1)	53.9 ± 10.9	7	7 (14.9 ± 1.9)	67.9 ± 20.3
June	Second *	7	7 (23.3 ± 4.1)	54.4 ± 8.5	7	5 (16.9 ± 1.9)	38.5 ± 4.1
July	Third	7	6 (23.9 ± 3.6)	52.7 ± 4.6	7	6 (13.9 ± 1.9)	39.9 ± 14.5
September		6			5		

* Dose estimated using the individual weights in May.

Mean sperm motility was >50% (Figure 4) 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 × 10^9 spermatozoa mL^{-1} in May and 78.0 ± 72.2 × 10^9 spermatozoa mL^{-1} in September, although with elevated individual variability in September (Figure 4).
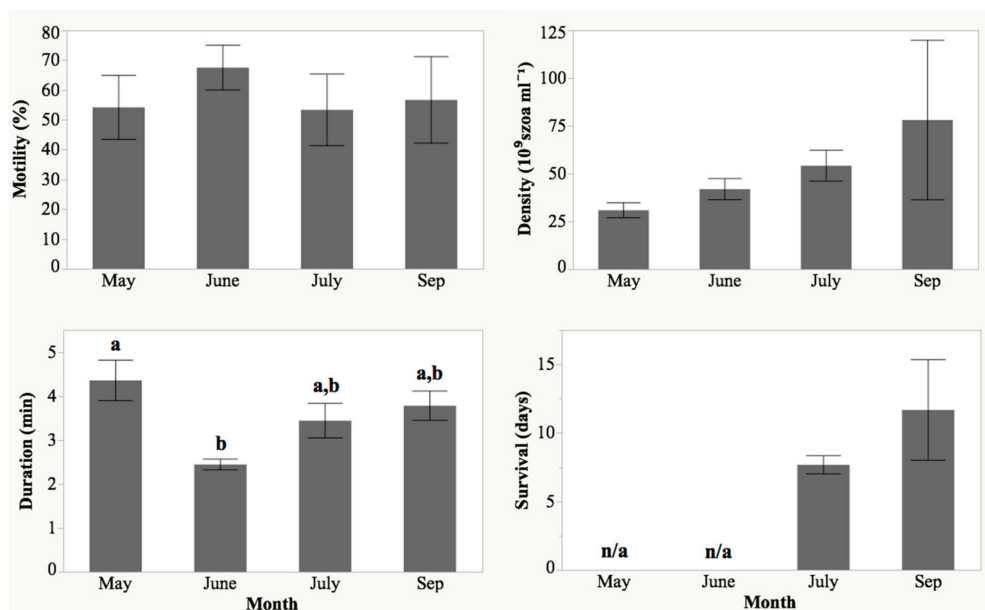

Figure 4. Mean (±SEM) sperm quality parameters of F1 greater amberjack at different times during the reproductive season (spermatozoa (szoa) forward motility, density, duration of motility and maximum survival during storage at 4 °C). Statistically significant differences among sampling times are indicated by different lower-case letters (a, b) ($p \leq 0.05$). n/a = not available.

2.2. Spawning and Egg Quality

The first spawn occurred between one and two days after each GnRHa treatment (Figure 5). A total of 52 spawns were obtained during a period of 72 days (Table 3). The number of spawns and fecundity obtained after successive GnRHa implantations decreased over time and the spawning events were concentrated more and more around the application of each GnRHa treatment. In the period after the first treatment, the fish spawned 29 times. After the second treatment, a total of 15 spawns were recorded during the first 16 days, and no eggs were collected during the following

days. The eggs released after the third GnRHa treatment were collected from eight spawning events that were obtained during the first nine days.
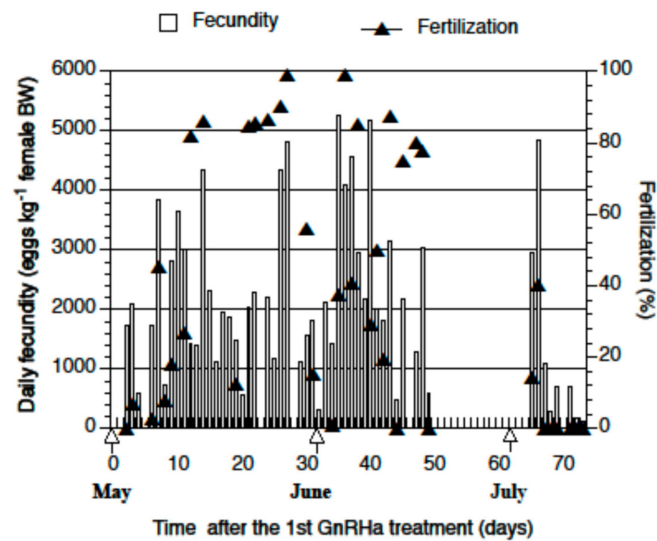

Figure 5. Daily fecundity (eggs kg^{-1} female body weight (BW)) and fertilization (%) of F1 greater amberjack in response to three treatments with GnRHa implants. The white arrows on the *X*-axis indicate the times of the GnRHa treatments.

Table 3. Egg production (mean ± SEM where appropriate) of F1 greater amberjack induced to spawn using a GnRHa implant treatment at different times during the reproductive season (May–July). No statistically significant differences were observed among the means of relative batch fecundity obtained after the three GnRHa treatments ($p < 0.05$).
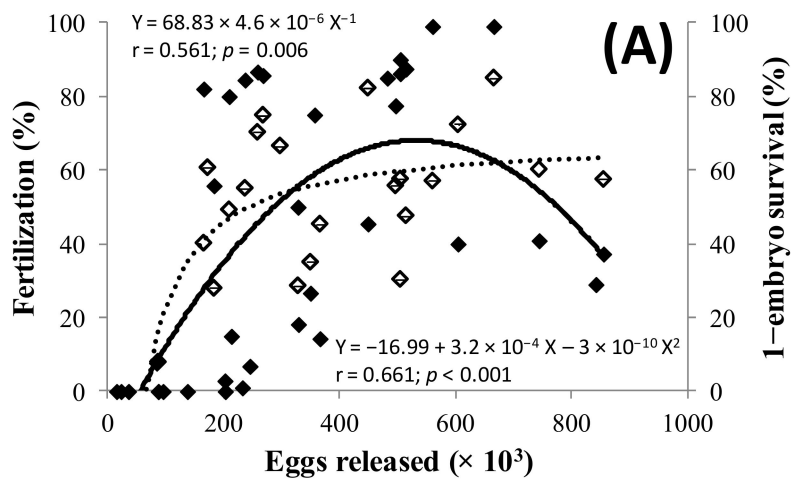
Treatment	Spawns (*n*)	Eggs Spawn^{-1} kg^{-1}	Total Eggs kg^{-1} (×1000 Eggs)	Total Eggs (×10^6 Eggs)
1	29	2087 ± 218	60.54	7.05
2	15	2828 ± 420	42.42	6.55
3	8	1895 ± 827	15.16	1.35

The highest daily relative fecundity that was recorded was 5539 eggs kg^{-1} fish after the second GnRHa treatment (Figure 5), but the total egg production was higher after the first treatment; that is, 60,540 eggs kg^{-1} fish compared to 40,180 eggs kg^{-1} fish after the second treatment (Table 3). No significant differences were found in daily relative fecundity among the three treatment periods. Almost 15 million eggs were produced from the three successive GnRHa applications.

Mean fertilization changed through the spawning period after each treatment, reaching its highest values after the first and the second GnRHa treatment, while a significant decrease was observed after the third treatment ($p < 0.05$) (Figure 6). On the other hand, no significant differences were observed in hatching, one-day embryo survival and three-day larval survival after successive GnRHa treatment. Regression analysis showed a significant correlation between fertilization success and number of daily fecundity (Figure 7a,b). However, one-day embryo survival correlated significantly with daily egg production (Figure 7a) and not with the number of spawns (data not shown), while hatching success showed the opposite trend (Figure 7b).
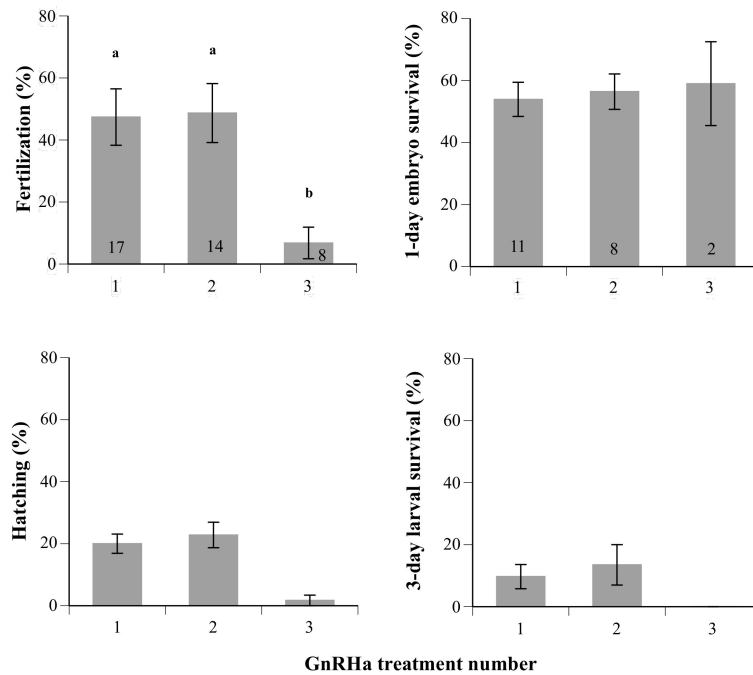
Figure 6. Mean (±SEM) egg quality parameters of F1 greater amberjack after three GnRHa implantations (May–July). Numbers in bars indicate the number of samples that constitute each mean. The number of samples of each mean for hatching (%) and three-day larval survival (%) are the same as for one-day embryo survival. Statistically significant differences among GnRHa implantations are indicated by different lower-case letters (a, b) (*p* = 0.05).


Figure 7. *Cont.*

$$Y = -0.71 + 5.77\,X - 0.11\,X^2 \qquad Y = -2.53 + 2.11\,X - 0.04\,X^2$$
$$r = 0.678;\ p < 0.001 \qquad\qquad r = 0.514;\ p = 0.046$$
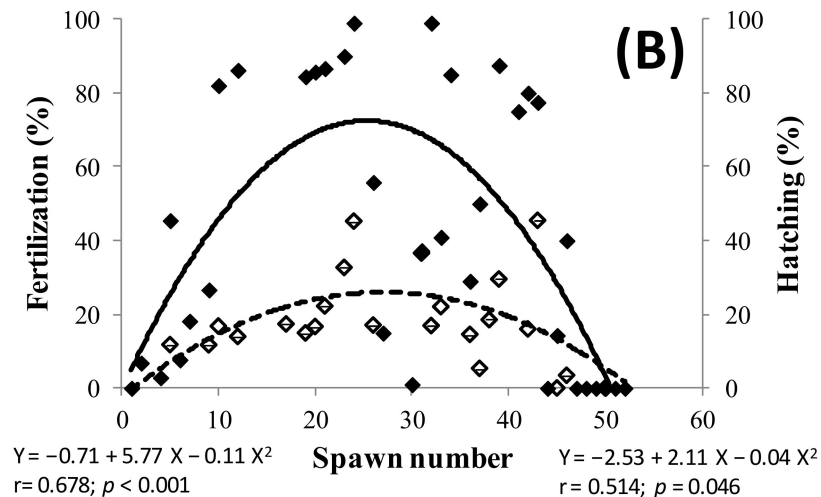
Figure 7. Fertilization (solid rhombus) and one-day embryo survival (open rhombus) in relation to daily egg released (**A**), and fertilization (solid rhombus) and hatching success (open rhombus) in relation to number of spawn (**B**).

The diameter of the eggs ranged from 1.035 to 1.165 mm, with a mean of 1.111 ± 0.031 mm, with a lipid droplet diameter of 0.249 ± 0.013 mm (Figure 8). A significant correlation between egg and oil droplet diameters ($p = 0.019$) was observed. The egg diameter decreased significantly with the spawn number ($p = 0.050$) (data not shown), as well as with the daily fecundity, the latter correlation being more significant ($p = 0.004$) (Figure 8). However, no significant correlation was found between lipid droplet diameter and spawn number (data not shown) or daily fecundity (Figure 8). Regression analysis indicated that the egg and oil droplet diameter did not correlate with either of the egg quality parameters studied (data not shown).

$$Y = 1.139 - 7.75 \times 10^{-8}\,X$$
$$r = 0.496;\ p = 0.004$$
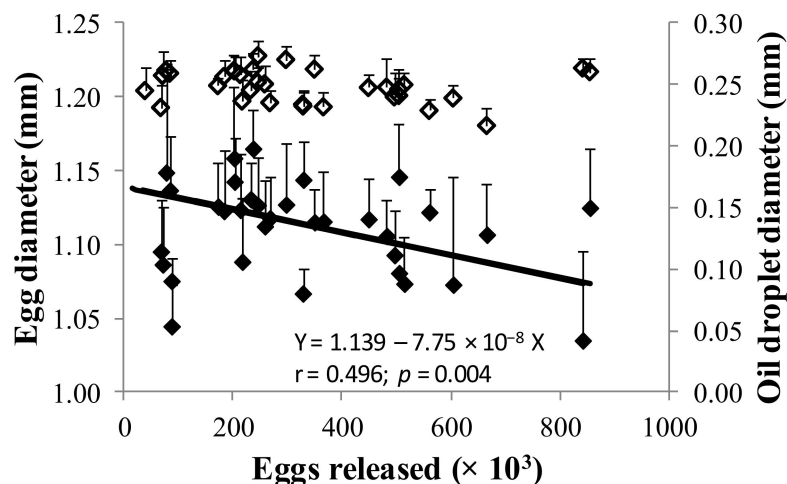
Figure 8. Egg diameter (black rhombus) and oil droplet diameter (white rhombus) in relation to number of eggs produced at different days of the monitoring period. Data show mean $\pm$ SEM.

2.3. Plasma Sex Steroid, Hematological and Biochemical Parameters

Female plasma 17β-estradiol (E2) levels were high at the beginning of the spawning period (May), although with elevated individual variability, as indicated by the high values of SEM, and showed a declining trend in the following months (Figure 9). Testosterone in the females was low throughout 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 testosterone (T) and

11-keto testosterone (11-KT) levels followed a decreasing trend from May to July, while they increased significantly in September ($p < 0.05$). Also, 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) followed almost the same pattern throughout the spawning season, with the highest detected levels in September.
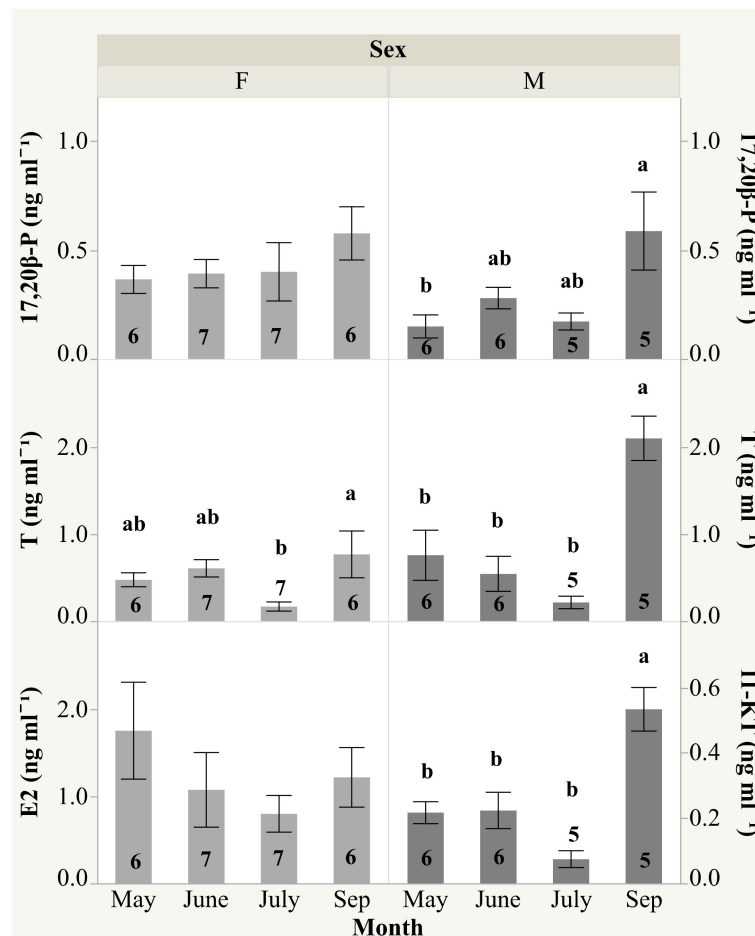
Figure 9. Mean (±SEM) plasma levels of 17β-estradiol (E2), T and 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) in greater amberjack females (F) and of 11-keto testosterone (11-KT), testosterone (T) and 17,20β-P in males (M) at different times during the reproductive season. Different letters indicate significant differences ($p < 0.05$).

Most of the studied blood and biochemical parameters both for females (Table 4) and males (Table 5) remained unchanged during the study, but the number of erythrocytes and plasma levels of protein, cholesterol, alkaline phosphatase and amylase changed during the experimental period. In both sexes, a significant gradual decrease ($p < 0.05$) in the number of erythrocytes was observed throughout the spawning season, reaching the lowest levels in September. On the contrary, alkaline phosphatase increased gradually until peaking in September. In females, the plasma levels of protein and amylase showed slight changes during the experimental period, being lower in September in respect to the previous samplings ($p < 0.05$). The male plasma levels of cholesterol were high in June and July and decreased significantly in September ($p < 0.05$).

Table 4. Erythrocytes (10^4 mm^{-3}), leucocytes (10^3 mm^{-3}), hematocrit (%), triglycerides (mg dL^{-1}), cholesterol (mg dL^{-1}), protein (g L^{-1}), glucose (mg dL^{-1}), alanine aminotransferase (ALT/GPT) (U L^{-1}), aspartate aminotransferase (AST/GOT) (U L^{-1}), alkaline phosphatase (U L^{-1}), cholinesterase (U L^{-1}), amylase (U L^{-1}), cortisol (ng mL^{-1}), lactate (mg dL^{-1}), sodium (mg dL^{-1}), potassium (mg dL^{-1}) in blood from female amberjack during experimental spawning period.

Females	May		June		July		September	
Erythrocytes	341.87 ± 29.00	a	283.84 ± 34.71	ab	171.87 ± 30.35	bc	99.25 ± 17.1	c
Leucocytes	93.5 ± 21.65		53.14 ± 11.65		62.54 ± 9.48		82.2 ± 12.01	
Hematocrit	51.00 ± 2.66		46.14 ± 4.85		41.83 ± 5.52		39.00 ± 4.96	
Triglycerides	161.25 ± 25.48		204.23 ± 58.28		188.05 ± 26.07		193.95 ± 36.23	
Cholesterol	264.15 ± 26.02		275.28 ± 26.59		278.46 ± 43.10		229.89 ± 60.84	
Protein	46.39 ± 4.58	ab	46.98 ± 6.00	ab	52.54 ± 5.66	a	26.65 ± 2.14	b
Glucose	98.48 ± 14.77		83.68 ± 11.24		95.98 ± 13.40		127.54 ± 31.97	
ALT/GPT	13.05 ± 0.95		17.78 ± 5.03		13.33 ± 2.64		23.03 ± 9.04	
AST/GOT	35.00 ± 7.93		35.83 ± 9.63		23.79 ± 6.60		14.30 ± 2.49	
Alkaline phosphatase	61.04 ± 7.88	c	78.93 ± 11.16	bc	104.51 ± 5.17	b	142.12 ± 6.23	a
Cholinesterase	186.44 ± 10.97		153.54 ± 21.93		296.12 ± 65.25		235.80 ± 16.45	
Amylase	12.44 ± 2.04	a	14.81 ± 2.74	a	8.34 ± 0.81	ab	1.83 ± 0.73	b
Cortisol	44.00 ± 27.82		24.46 ± 18.55		31.34 ± 12.31		13.56 ± 7.21	
Lactate	35.61 ± 4.38		33.88 ± 1.90		41.53 ± 7.61		40.35 ± 5.42	
Sodium	423.28 ± 9.45		416.34 ± 11.67		496.45 ± 72.57		376.26 ± 5.26	
Potassium	19.52 ± 3.98		16.28 ± 1.68		24.41 ± 3.97		15.40 ± 1.08	

Values are means ± SEM. Different letters indicate significant differences (Analysis of Variance (ANOVA), *p* < 0.05).

No significant differences (*p* < 0.05) in the cortisol levels were observed throughout the spawning season, although a diminishing trend was observed at the end of the experimental period (September) in both sexes. Regarding other secondary responses to stress, no differences were found in glucose and lactate, although sodium showed lower values at the end of the spawning season in males (*p* < 0.05).

Table 5. Erythrocytes (10^4 mm^{-3}), leucocytes (10^3 mm^{-3}), hematocrit (%), triglycerides (mg dL^{-1}), cholesterol (mg dL^{-1}), protein (g L^{-1}), glucose (mg dL^{-1}), ALT/GPT (U L^{-1}), AST/GOT (U L^{-1}), alkaline phosphatase (U L^{-1}), cholinesterase (U L^{-1}), amylase (U L^{-1}), cortisol (ng mL^{-1}), lactate (mg dL^{-1}), sodium (mg dL^{-1}), potassium (mg dL^{-1}) in blood from male amberjack during experimental spawning period.

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 ± 1.11	
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	

Values are means ± SEM. Different letters indicate significant differences (ANOVA, *p* < 0.05).

Partial correlation analysis between steroids and plasma parameter levels showed that plasma cholesterol was significantly correlated with E2 (*p* = 0.046) in females and with T (*p* = 0.03) and 11-KT in males (*p* = 0.049). Also, triglycerides (*p* = 0.008) and aspartate aminotransferase (AST/GOT) (*p* = 0.028) were correlated with E2 in females, whereas cholinesterase was correlated with T (*p* = 0.003) and 11-KT (*p* = 0.001) in males. Furthermore, regression analysis between plasma parameters and

spawning performance indicated that plasma protein was correlated with oocyte diameter ($p = 0.034$), while plasma potassium was correlated with duration of sperm motility ($p = 0.017$).

3. Discussion

In the Mediterranean, 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 [39], 400–600 μm at the beginning of the spawning season in May [15], and the largest diameter (650–750 μm) in June [33,34]. Even after reaching appropriate oocyte sizes, a considerable number of females fail to undergo OM and do not ovulate, and hormonal treatments are necessary to induce OM and 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 spawned successfully after having been induced with hCG injections when females had vitellogenic oocytes of 550–600 μm in diameter [14,33], and after injection [31] or implantation [34] with GnRHa 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 at all sampling times from May to September. This demonstrates that under these rearing conditions and hormonal treatments, F1 greater amberjack undergo normal oogenesis and maintain their vitellogenic production for an extended period of time, as is customary for this species in the subtropical area of the Canary Islands [31,35]. This is the first study demonstrating that F1 generation greater amberjack in Europe do undergo gametogenesis in captivity and are capable of producing fertilized eggs, albeit after exogenous hormonal therapy for the induction of OM, ovulation and spawning. The only other report of F1 greater amberjack broodstock reproducing spontaneously in captivity comes from Japan [37].

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 [7,8,40,41]. 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 [42,43]. In addition, in captive-reared fish, it is very common for males to produce lower amounts of sperm, often of high sperm density [28,44], 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 [45,46]. Sustained-release GnRHa-delivery systems have been used successfully to induce an overall increase both in the expressible sperm and spermatozoa production in various cultured fishes (reviewed by [28]), enhancing the quality and the quantity of sperm produced [46–50], including wild-caught greater amberjack [34]. 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 [34]. A gradual reduction in the sperm motility duration during the reproductive season was observed in another study with wild-caught broodstock in the eastern Mediterranean Sea [7]. However, as water temperatures do not rise to the same levels in the summer in the Canary Islands, greater amberjack apparently remain in spermiating condition for a much longer period of time, reportedly from May to October [31,35]. The sperm density in GnRHa-treated F1 males showed an increasing trend over the course of the spawning period, although it was always lower than the one obtained in wild-caught males treated with GnRHa implants [34]; however, it was in the same range as a study using untreated males [7]. 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, while sperm density and motility did not change [51], while in Atlantic halibut *Hippoglossus hippoglossus* [52,53] and common dentex *Dentex dentex* [54], the sperm motility was enhanced slightly and the sperm density decreased. In contrast, GnRHa implants were ineffective in enhancing sperm production in the flatfishes Southern flounder *Paralichthys lethostigma* and summer flounder *Paralichthys dentatus* [55,56]. 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 [13].

Spontaneous and induced spawning of wild-caught greater amberjack has been achieved from May to October in the Canary Islands [31,35], but only from May to July in the Mediterranean Sea [57,58], where summer temperatures are much higher, and from May to June in Japan, either using reared or wild-caught fish [37,59]. In the present study, a total of 52 spawns were obtained from GnRHa-treated F1 greater amberjack, which is higher from than the number reported previously [59], with the number obtained after each consecutive treatment decreasing gradually. The use of GnRHa for inducing and increasing spawning frequency is well known [12,30], and it has also been reported in wild-caught greater amberjack [34,60], longfin yellowtail *Seriola rivoliana* [61] and F1 yellowtail kingfish [38]. 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 potentially to a determinant type of fecundity characteristic belonging 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 [34,40,60], perhaps due to lower stress inflicted on the broodstock and a longer period of hormonal stimulation by the controlled-release GnRHa delivery systems [62].

The total number of eggs obtained from GnRHa-implanted F1 females (14.95×10^6 eggs) was less than that obtained from natural spawning of hatchery-reared broodstock in Japan (23.33×10^6 eggs) [59], but similar to that of wild-caught greater amberjack reported previously [35], and three times higher than that obtained from wild-caught females treated with multiple GnRHa injections in the Canary Islands [31]. 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 that obtained from wild-caught females in the Canary Islands treated with successive injections of GnRHa [31] and six times higher than the eggs obtained naturally from wild-caught females of greater amberjack [35]. 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 [32,34,63]. Therefore, our results show that implants of GnRHa can induce a higher number of spawns and higher fecundity in the greater amberjack, than produced spontaneously or in response to simple hormonal injections. Unfortunately, no comparison of relative fecundity is possible with the other studies with hatchery-reared greater amberjack, since this information was not provided [37,59].

In some species, spawning induction using GnRHa has been reported to have a negative effect on egg quality [64]. For example, GnRHa implantation has been associated with decreased egg buoyancy, fertilization and number of viable eggs, as well as smaller oil globule diameters [65–69], a situation that was also observed using F1 yellowtail kingfish [38]. The fertilization success obtained from hormonally treated wild-caught greater amberjack has been highly variable, ranging between 16–50% [33] and 58–99% [70] for hCG-injected fish, and 22% to $96 \pm 6.5\%$ for GnRHa-implanted [34] and -injected [31] fish, respectively. In the latter study, the male:female sex ratio used was higher (2♂:1♀), and this might have improved the fertilization success [71]. In the present study, the GnRHa-implanted F1 greater amberjack produced eggs with mean fertilization success of $42 \pm 6.2\%$, a value that was slightly lower than that obtained from natural spawning of wild-caught greater amberjack maintained in the same facilities ($62 \pm 3.6\%$) at a similar sex ratio [35]. Furthermore, the hatching success ($35 \pm 4.8\%$) was lower than that obtained from hCG-injected (52–97%) [70] and GnRHa-injected (93%) [31] wild-caught greater amberjack, and also lower than that obtained in F1 broodstocks [37,59], but it was more than

double that obtained by natural spawning in the Canary islands [35]. 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, showing similar fertilization and higher hatching success to that obtained from natural spawning of a wild-caught broodstock in our facilities, but lower hatching success compared to the natural spawning of hatchery-reared greater amberjack in Japan, where the spawning season was shorter. 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 through breeding selection.

In some studies, lower embryo and larval survival were recorded at the first spawns after hormonal treatment [31,33]. In contrast, 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, in the same treatment period, the three-day larval survival was lower than the one recorded using wild-caught greater amberjack injected with GnRHa [31] or hCG [70]. A reduction in embryo and larval survival may occur towards the end of the spawning period in fishes with asynchronous ovarian development [72–74], perhaps due to depletion of the nutritional maternal stores, as fish often reduce their feed intake during the spawning period.

The consecutive treatments of F1 greater amberjack with GnRHa implants 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 [35]), but the oil droplet was smaller compared to eggs from natural spawning of other F1 broodstocks [37,59]. Interestingly, lower egg diameters were recorded when a higher number of eggs was released, in the present study. It has been suggested in F1 yellowtail kingfish [38] that the GnRHa treatment could result in the production of smaller eggs, due to the premature ovulation of oocytes before completion of vitellogenesis. However, in the present study, one-day embryo survival was positively correlated with fecundity, so apparently there were no negative effects of GnRHa treatment on egg quality.

GnRHa delivery systems have been tested in a wide variety of hatchery-produced and wild fish to induce the natural progression of plasma steroid increases associated with OM and spermiation through increases in circulating luteinizing hormone (LH) levels [28]. Plasma sex steroid levels provide indicative information on the reproductive performance of fish, and they have been studied in wild greater amberjack [8,75] and yellowtail kingfish [76]. In wild greater amberjack females, plasma T and E2 levels were positively correlated during reproductive season, increasing during the period of gametogenesis and peaking at the start of the spawning season [75]. Yellowtail kingfish females showed similar kinetics in plasma sex steroids, with T as a precursor for the synthesis of E2, and reaching the highest levels during vitellogenesis, while 17,20β-P peaked in females with ovaries undergoing OM [76]. In the present study, however, levels of plasma sex steroids in F1 greater amberjack females were considerably lower than those in wild females [8,75], but closer to those of captive females [8], and no significant changes after the successive GnRHa implants were observed. In fact, E2 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 E2, and between T and 17,20β-P. The unchanged levels of E2 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 E2 synthesis, as vitellogenesis was coming to an end. The E2 and T levels covariate during the spawning season and T levels in plasma increase when it is not used to obtain E2 by aromatization [77,78].

In wild greater amberjack males [75], 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 11-KT 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 [76]. In the present study, sex steroids 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. For 17,20β-P, there is evidence that in addition to the involvement of this sex steroid in spermiation, a second peak is observed in some species at the end of the spawning season [79]. Plasma levels of T were positively correlated with 11-KT and 17,20β-P. The absence of significant changes in sex steroids with the successive implants between May and July was in agreement with the absence of significant differences in sperm quality parameters during the experiment, with the exception of motility duration, which was lower in June compared to May.

Analysis of hematological and biochemical parameters in the blood is a valuable tool that can be used as an effective index to monitor fish health and pathological changes. Both hematological and biochemical parameters obtained in the present experiment were within the normal range for greater amberjack, compared to previous studies [80–82]. 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 throughout 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 hematological and biochemical parameters suggests that the physiological condition of F1 greater amberjack broodstock 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. 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 was 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 [34]. Despite the repetitive handling required to administer the GnRHa implants during the prolonged spawning season in the Canary Islands, the present study demonstrated that there was no negative effect on the welfare and reproductive performance of the fish, and that it seems to be an appropriate method for cultured fish in terms of welfare status.

4. Materials and Methods

4.1. Broodstock Maintenance

Rearing was undertaken in the facilities of the Centro Oceanográfico de Canarias, Instituto Español de Oceanografía, Tenerife, Spain. The broodstock consisted of 14 hatchery-produced F1 fish (seven males and seven females), individually identified with Passive Integrated Transponder tags, from eggs obtained from wild-caught broodstock between 2005 and 2009. Fish were maintained during the year in two outdoor 50 m^3 tanks covered with shading mesh, supplied with seawater from a well (10 renewals day^{-1}) at ambient water temperature until the beginning of the experiments on 13 May 2015 (Table 1). After the first GnRHa treatment, the selected fish (see later for selection criteria) were placed in an outdoor covered (shading mesh) raceway tank of 500 m^3 with flow-through water supply (6 renewals day^{-1}) under natural photoperiod. Fish were fed three times per week to apparent satiation with raw fish (mackerel, *Scomber colias*). Measurements of temperature and water

quality (Dissolved Oxygen, NH$_3$–N and NO$_2$–N) were conducted once per week throughout the year. All work and maintenance of broodstocks was in agreement with European regulations on animal welfare (Federation of Laboratory Animal Science Associations, FELASA, http://www.felasa.eu/).

4.2. Fish Sampling

The fish were sampled four times during the 2015 spawning season (May, June, July and September). Fish were starved for two days prior to sampling and were tranquilized initially with the use of chlorobutanol (0.1 mL L^{-1}) diluted in the tank 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, 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) into the ovary. A wet mount of the biopsy was examined first under a compound microscope (Nikon Eclipse, Tokyo, Japan) (40 and 100×) equipped with a Nikon Digital Sight DS-Fi1 camera (Nikon Instruments Europe BV, Amsterdam, Netherlands) to evaluate the stage of oogenesis and to measure the mean diameter of the ten largest, most advanced vitellogenic oocytes. 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 at the opening of the genital pore. The collected sperm was stored on ice and then transferred to a 4 °C refrigerator. Evaluation of sperm parameters was done immediately after the completion of the sampling, and spermatozoa survival under storage at 4 °C was done every other day thereafter, until cessation of movement. At each sampling, samples of blood (1–3 mL depending on how easy it was to collect the blood from the fish) was collected from all fish from the caudal vein 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.

All the experimentation on fish was in accordance with the Spanish (R.D. 53/2013) and European regulations (Directive 2010/63/UE) for the protection of animals used for experimentation and other scientific purposes and supervised and approved by the Ethical Committee CEIBA-IEO (Comité de Ética de la Investigación y Bienestar Animal del Instituto Español de Oceanografía).

4.3. Spawning Induction Therapies

Fish were treated with an ethylene–vinyl acetate implant [28] loaded with Des-Gly10, D-Ala6-Pro-NEth9-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 (Table 2) due to the fact that the implants were loaded with fixed amounts of GnRHa. At the time of GnRHa implantation, selected females were in advanced vitellogenesis and selected males were in spermiation, although sperm was collected with a catheter.

4.4. Evaluation of Sperm Quality

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 hemocytometer (Hirschmann, Eberstadt, Germany) under a compound light microscope at 400× magnification (in duplicate). Sperm motility and duration were evaluated on a microscope slide at 400× 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 s after activation. Sperm motility was determined subjectively using increments of 10% and sperm was

considered immotile when <5% of the spermatozoa were exhibiting forward motility. Sperm was stored at 4 °C for the following days, and was examined every other day for motility, until no forward motility was observed. The survival time (days) for each sample was considered as the day before the sample was found to have lost all its motility capacity. This last parameter was evaluated only for the last two samplings in June and July.

4.5. Evaluation of Egg/Larval Quality

At the expected onset of the spawning season (May 2015), 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 3–5 sub-samples randomly 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 10 randomly collected eggs and their lipid droplet were measured using a binocular microscope equipped with a Nikon Digital Sight DS-Fi1 camera. Each spawn was incubated in a 90 L tank with gentle aeration and filtered (5 µm) 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. [83], 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 five days at 21 ± 0.5 °C. Using a stereoscope, embryonic and early larval development was evaluated once a day for five days. The number of (a) live embryos was recorded one day after egg collection (or ~36 h after spawning, day 1), (b) hatched larvae was recorded two and three days after egg collection (>60 h after spawning) and (c) viable larvae was recorded four and five days after egg collection (~yolk sack absorption).

Embryo survival was calculated as the proportion of live embryos one day after egg collection in the microtiter plates. Hatching success was calculated as the proportion of hatched larvae out of the number of live one day embryos, and five days larval survival was calculated as the proportion of live larvae five days after egg collection out of the number of hatched larvae. Estimating percentage survival (%) by using in the denominator the number of individuals that survived from 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 [84,85].

4.6. Histological Processing

Before embedding in methacrylate resin (Technovit 7100®, Heraeus Kulzer, Hanau, 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, Nussloch, Germany). Sections were stained with Methylene Blue (Sigma-Aldrich, Steinheim, Germany)/Azure II (Sigma)/Basic Fuchsin (Polysciences, Warrington, PA, USA) [86]. Sections were examined under a light compound microscope (Nikon, Eclipse 50i, Tokyo, Japan) and photographed with a digital camera (Jenoptik progress C12 plus, Jena, Germany).

4.7. Hormone Measurements

The enzyme-linked immunoassays (ELISA) used for the quantification of T, E2, 11-KT and 17,20β-P in the plasma of fish were performed according to established methods [87–89] that have been already applied to the greater amberjack [7,8], with some modifications and using reagents from Cayman Chemical Company (Ann Arbor, MI, 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, Rockford, IL, USA). Samples were reconstituted in different volumes of Reaction Buffer, depending on the hormone measured. The dilutions used were 1:20 for E2, 1:2 or 1:4 for T and 1:2 for KT and 17,20β-P.

4.8. Hematology and Blood Biochemical Analysis

Hematological 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 hemocytometer. Hematocrit count was carried out by capillary diffusion and centrifugation. Plasma levels of protein, triglycerides, cholesterol, glucose, lactate and enzymes Alanine Aminotransferase/Glutamic Piruvic Transaminase (ALT/GPT), Aspartate Aminotransferase/Glutamic Oxaloacetic Transaminase (AST/GOT), Alkaline phosphatase, Cholinesterase and amylase) were measured in duplicates by enzymatic colorimetric assays (Biosystems, Barcelona, Spain). Plasma concentrations of sodium (Mg-Uranylacetate Method), potassium (Sodium tetraphenylboron Method), and chloride (Thiocyanate-Hg. Colorimetric) were determined using standard spectrophotometric assays (Spinreact, Girona, Spain). Plasma cortisol level was analyzed by ELISA (Arbor Assay, Ann Arbor, MI, USA).

4.9. Statistical Analysis

Differences in spawning and egg and sperm quality parameters were tested using one-way Analysis of Variance (ANOVA) followed by Tukey's post hoc test. The data were checked for normal distribution with the one sample Kolmogorov–Smirnoff test, as well as for homogeneity of the variances with the Levene test, and percentage data were Arcsine transformed prior to statistical analysis to normalize variances. Pearson's correlation coefficients were used to assess the relationships between egg quality variables. Percentage data were Arcsine transformed prior to statistical analyses to normalize variances. Results are presented as mean $\pm$ standard deviation (SD) of the pre-transformed data, 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 SPSS statistics package, International Business Machines Corporation (IBM) version 20.0 for Windows (Armonk, NY, USA) and JMP 12 (SAS Institute Inc., Cary, NC, USA).

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