Deliverable Report

Deliverable No:	D3.9	**Delivery Month:**		59
Deliverable Title	Development of a spawning induction therapy for captive reared broodstock in the Mediterranean Sea based on the use of GnRHa in the correct mode of administration (hormone/implant), dose and timing.			
WP No:	3	**WP Lead beneficiary:**		P13. UNIBA
WP Title:	Reproduction and Genetics – greater amberjack			
Task No:	3.2	**Task Lead beneficiary:**		P1. HCMR
Task Title:	Development of an optimized spawning induction protocol for captive greater amberjack in the Mediterranean			
Other beneficiaries:	P23. ARGO	P40. GMF		
Status:	Delivered		**Expected month:**	54
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Objective: An optimized spawning induction protocol for captive greater amberjack broodstock in the Mediterranean Sea was produced. The deliverable includes the methodologies to (a) recruit wild fish and acclimate them in the rearing tanks and cages; (b) assess proper way (e.g. multiple injection or sustained delivery system) as well as proper dose for GnRHa treatment; (c) identify the right time to induce spawning based on histological observation of fish reproductive state as well as on previous years data. The spawning induction protocol is able to be transferred directly to and be implemented by the industry, in order to establish captive broodstocks and induce them to spawn reliably.

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Introduction

Aquaculture must bridge the growing gap between supplies of aquatic food, and demand from a growing and wealthier global population (FAO, 2018). This extra quantity could result either from the already established aquaculture species or from the diversification with new species that offer certain biological or market advantages. The greater amberjack *Seriola dumerili* is one of the most promising species, due to its cosmopolitan distribution (Paxton, et al., 1989) and acceptability, high growth rates and large size (Crespo, et al., 1994; Grau, et al., 1996; Jover, et al., 1999; Lazzari, 1991; Lazzari, et al., 2000; Mazzola, et al., 2000), and late maturation (Micale, et al., 1999; Zupa, et al., 2017b), which allows for the marketing of the fish before growth is affected by reproductive maturation.

As with many other newly cultured species, reproduction control has been one of the main bottlenecks for the commercialization of greater amberjack (Mylonas, et al., 2016a). The main reproductive dysfunctions observed in fishes is the absence or unreliable oocyte maturation, ovulation and spawning (Mylonas & Zohar, 2001) caused by inadequate pituitary luteinizing hormone (LH) synthesis and release at the end of vitellogenesis (Zohar & Mylonas, 2001). Failure of spawning can also result from the lack of the proper environmental conditions or by captivity-induced stress (Mylonas, et al., 2010). This reproductive dysfunction has been well known in cultured greater amberjack (Kozul, et al., 2001; Micale, et al., 1999; Mylonas, et al., 2004) and has so far prevented its large scale production in Europe, even if natural spawning has been reported in the Canary islands (Jerez, et al., 2006) and Japan (Kawabe, et al., 1996), but not in the Mediterranean (Grau, et al., 1996).

Different hormonal therapies have been used in the past in order to induce oocyte maturation, ovulation and spawning, via the administration of LH, gonadotropin preparations (GtH), pituitary extracts (PE), human chorionic gonadotropin (hCG) or gonadotropin releasing hormone agonist (GnRHa)(Mañanos, et al., 2009;

Mylonas, et al., 2010; Zohar & Mylonas, 2001). Of these therapies, GnRHa is more widely used due to its advantages related to a lower species-specificity and to its action at a higher level of the reproductive axis, stimulating the pituitary release of the endogenous LH (Mylonas, et al., 2010). In addition, GnRHa may be administered in the form of a bolus (liquid injection) or in a sustained-release delivery system (Mylonas & Zohar, 2001), each method having important advantages in different species. For example in meagre *Argyrosomus regius*, multiple injections of GnRHa was considered more advantageous compared to the GnRHa implants due to better egg production control and repeatability of response (Mylonas, et al., 2015). In greater amberjack, spawning induction experiments have been done with both GnRHa implants (Mylonas, et al., 2004) and multiple GnRHa injections (Fernández-Palacios, et al., 2015a), but a proper study comparing the two methods in greater amberjack in the Mediterranean has not be carried out so far.

The aim of the present study was to examine: a) the two methods of spawning induction using GnRHa either in the form of implants (sustained release) or injections (acute release), b) the proper dose for GnRHa treatment and c) the right time to induce spawning, in terms of spawning kinetics, egg production and quality, with the objective of delivering a sound and efficient protocol to the aquaculture industry.

Materials and methods

Broodstock maintenance

Live wild juveniles were fished in the Ionian and Aegean Sea, Greece, with a commercial purse seine fishing vessel. Broodstocks were maintained in different locations (ARGO: Argosaronikos Fishfarms SA, Salamina, Greece; GMF: Galaxidi Marine Farms SA, Galaxidi, Greece; AQUALABS: Hellenic Centre for Marine Research, Gournes, Herakleion, Crete, Greece; FORKYS: Forkys Aquacultures SA, Siteia, Crete, Greece) and reared under two different conditions, either in sea-cages or in land-based tanks (**Table 1**). Broodstocks were fed with live fish or raw fish or squid or moist pellet or dry pellet (Skretting Vitalis CAL, 22 mm), or a combination of the above. Feed was given 3 to 5 times a week to apparent satiation. Fish in sea were maintained in cages of at least 8x8x8 m size. Fish in land-based tanks were maintained in a single 35 (AQUALABS) or 25-m^3 (ARGO, FORKYS) tank under simulated natural (AQUALABS) or natural (ARGO, FORKYS) photoperiod regime, provided with well seawater of ambient temperature (ARGO, FORKYS) or simulated natural temperature profile (AQUALABS) under a semi-recirculation system (**Fig. 1**). At HCMR (AQUALABS), measurements of water quality were conducted once a week (dissolved oxygen, NH_3-N, NO_2-N) while temperature and dissolved oxygen parameters in different facilities were measured from 1 to 7 times a week.

Evaluation of reproductive stage and broodstock selection

Broodstock selection for spawning induction experiments was done after a 2-days starvation period. Fish were initially tranquilized in their tank (in case of land-based rearing conditions) or in a bounded sack (in case of sea-cages) with the use of either clove oil (0.01ml l^{-1}) or 2-phenoxyethanol (0.15 ml l^{-1}) and then transferred to an anesthetic bath for complete sedation with a higher respective concentration of clove oil (0.03ml l^{-1}) or 2-phenoxyethanol (0.4 ml l^{-1}) (Mylonas, et al., 2005). Ovarian biopsies for the evaluation of oocyte development were obtained by inserting a plastic catheter (Pipelle de Cornier, Laboratorie CCD, France) and applying gentle aspiration. A wet mount of the biopsy was first examined under a compound microscope (40 and 100x) to evaluate the stage of oogenesis and measure the most advanced batch of vitellogenic oocytes (n=10). A portion of some biopsies was fixed in a solution of 4% formaldehyde-1% glutaraldehyde for further histological processing. Females were considered eligible for spawning induction if they contained fully vitellogenic oocytes. Because of the hard musculature surrounding of the abdominal cavity and the limited quantity of sperm produced by captive greater amberjack, milt samples were obtained by cannulation as described above for the females. Milt was kept on ice until further quality evaluation.

Table 1. Description of the various broodstocks maintained in different rearing systems for the present study.

2014

Stock	Rearing method	Number of individuals	Size at sampling (kg)	Feeding
ARGO	sea-cages	49	7.1-16.0	live, raw fish
GMF	sea-cages	28	6.3-15-6	live fish
AQUALABS	land-based	27	6.5-23.8	raw fish, squid
ARGO	land-based	9	8.1-11.1	live, raw fish
FORKYS	land-based	22	7.7-10.3	raw fish, squid

2015

Stock	Rearing method	Number of individuals	Size at sampling (range in kg)	Feeding
ARGO	sea-cages	28	10.7-19.5	moist pellet, raw fish
GMF	sea-cages	28	9.0-18.0	live fish
AQUALABS	land-based	27	8.6-23.8	moist pellet, raw fish
FORKYS	land-based	21	9.4-15.9	raw fish, squid

2016

Stock	Rearing method	Number of individuals	Size at sampling (range in kg)	Feeding
ARGO	sea-cages	29	11.2-23.6	pellet
GMF	sea-cages	28	11.8-21.5	live fish
AQUALABS	land-based	26	9.8-18.5	pellet
FORKYS	land-based	19	12.6-20.3	raw fish, squid

2017

Stock	Rearing method	Number of individuals	Size at sampling (range in kg)	Feeding
ARGO	sea-cages	28	11.8-26.0	pellet
GMF	sea-cages	26	11.5-22.5	moist pellet
AQUALABS	land-based	19	13.5-22.7	pellet
FORKYS	land-based	15	12.7-20.5	raw fish, squid

2018

Stock	Rearing method	Number of individuals	Size at sampling (range in kg)	Feeding
ARGO	sea-cages	33	7.3-26.6	pellet
GMF	sea-cages	23	12.5-22.6	pellet
AQUALABS	land-based	19	12.5-24.5	pellet

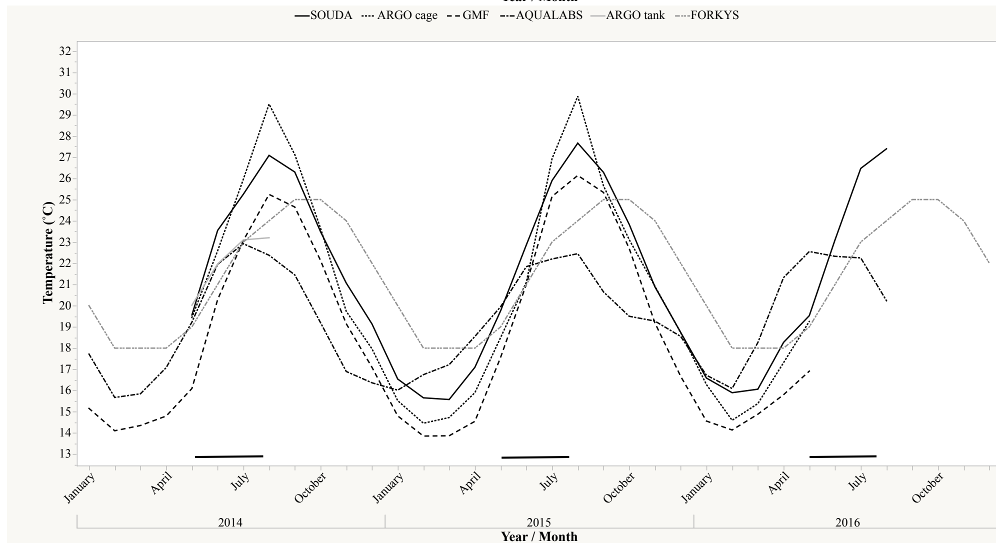

Figure 1. Representative water temperature profile for greater amberjack broodstocks, maintained in sea-cages or land-based facilities during the 2014-2016 period. Bars indicate the estimated breeding period.

Spawning induction experiments

When the fish were in appropriate reproductive maturational stage, implants or injections of GnRHa were administered. Then two modes for spawning were selected for sea-cage broodstock, either moved back to their sea-cage (cage-spawning), which was equipped with an egg collection device that consisted of a two-piece curtain deployed around the perimeter of the cage or transferred to land-based tanks (tank-spawning), which were connected with overflow egg collectors. Fish reared in land-based facilities remained at the same location for spawning. After the cessation of spawning, evaluation of reproductive stage of the fish was done and, if they still were in an appropriate maturational stage, a 2nd treatment was given. Females (n=119) were implanted with 64 ± 17 μg kg^{-1} body weight GnRHa and males (n=118) with 48±12 μg kg^{-1}, respectively. At the end of the experiment, fish were transferred to their original location.

The spawning induction trial for the comparison of GnRHa injections and implants was conducted between 7 June and 28 June 2016 in Argosaronikos SA facilities. The female fish were treated either with GnRHa injection (20-25 μg GnRHa kg^{-1} body weight) or with EVAc GnRHa implant (Mylonas and Zohar, 2001), loaded with 750 or 1000 μg of Des-Gly^{10},D-Ala^{6}-Pro-NEth^{9}-mGnRHa (H-4070, Bachem, Switzerland), for an effective dose of 49-69 μg GnRHa kg^{-1} body weight. The selected doses were those used successfully in previous experiments using GnRHa implants (50 μg GnRHa kg^{-1} body weight) (Mylonas, et al., 2004) or GnRHa injections (20 μg GnRHa kg^{-1} body weight) (Fernández-Palacios, et al., 2015a). In order to enhance spermiation and ensure adequate sperm production, males were treated at the start of the experiment with a GnRHa implant at a dose of 45-70 μg GnRHa kg^{-1} and allocated randomly to the four tanks. For the first hormonal treatment the GnRHa dose was calculated according to an estimated body growth of about 30% from the previous weighing (1 year before, on 15 June 2015). As the fish did not have a uniform growth during this period, the actual GnRHa dose applied to the fish had a certain degree of variability. For the GnRHa implant treatments, variations were also caused by the fact that implants are loaded with fixed amounts of GnRHa. Even though implants loaded with different amounts of GnRHa were used, it was still not possible to adjust the dose exactly to the different body weights of the fish. During the initial treatment, females were allocated to four tanks in order to have two duplicates per treatment method (3 and 4 fish per duplicate). Females were treated weekly in the injected group, and biweekly in the implanted stocks (a total

of 3 injections and 2 implants). The treatments and samplings in the facility were implemented with the same procedure used for the first sampling. The water level was reduced in the tanks, the fish were anaesthetized slightly and were then moved to an anesthetic bath for complete sedation. Three weeks after the start of the experiment, on 28 June 2016, the final sampling was conducted and the fish were returned to the sea cage.

The spawning induction trial for the comparison of GnRHa doses was conducted between 7 June and 5 July 2017 in Argosaronikos SA facilities. Female fish were treated either with EVAc GnRHa implant of ~25 μg GnRHa kg^{-1} body weight (group named as "LOW") or EVAc GnRHa implant of ~75 μg GnRHa kg^{-1} body weight (group named as "HIGH") (Mylonas and Zohar, 2001), using implants loaded with 500–1000 μg of Des-Gly^{10},D-Ala^{6}-Pro-$NEth^{9}$-mGnRHa (H-4070, Bachem, Switzerland). In order to enhance spermiation and ensure the adequate sperm production, males were treated at the start of the experiment with EVAc GnRHa implant at a dose of 58.3±17.7 μg GnRHa kg^{-1} body weight and divided randomly between the stocks. First hormonal treatment with GnRHa was given calculating the dose accordingly to an estimated 20 % growth rate from the previous year (7 June 2016). As above explained, a certain degree of variability in the actual GnRHa dose applied to the fish occurred. Even though combinations of two 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 weight of the fish. The actual dose was 22.4 ±2.4 μg GnRHa kg^{-1} for LOW group and 74.4±4.5 μg GnRHa kg^{-1} for HIGH group, respectively. During the initial treatment, females were divided in four experimental groups in order to obtain two duplicates per treatment dose (2 and 3 fish per duplicate, respectively). Fish were divided equally between the two treatment methods according to their gonadal maturation stage. Before transferring the broodstock to experimental tanks, weight of each individual was recorded in order to calculate further doses accordingly to the actual fish weight. Females and males were treated again two weeks after (21 June 2017) with EVAc GnRHa implants of the same effective dose, since the actual dose was very close to the scheduled one. Four weeks after the start of the experiment, on 5 July 2017, final sampling was conducted and the fish were returned to the cage.

The spawning induction trial for the timing of GnRHa application was conducted between 30 May - 18 July 2017 and 15 June – 26 July 2018 at GMF. Female fish were treated with EVAc GnRHa implant (Mylonas and Zohar, 2001), loaded with 750–1000 μg of Des-Gly^{10}, D-Ala^{6}-Pro-$NEth^{9}$-mGnRHa (H-4070, Bachem, Switzerland), for an effective dose of 58 ± 9 μg GnRHa kg^{-1} body weight. In order to enhance spermiation and ensure the adequate sperm production, males were also treated with EVAc GnRHa implant at a dose of 67 ± 6 μg GnRHa kg^{-1} body weight. In order to estimate the best timing for GnRHa application, fish were split in four spawning induction groups. On 30 may 2017, three females and three males from cage A after reproductive evaluation were treated with GnRHa and transferred to the land-based tank for spawning (1st period). A week later, on 7 June 2017 the rest fish from cage A, were treated with GnRHa and transferred to a different land-based tank for spawning (2nd period). Similarly, three females and three males from cage B, after reproductive evaluation on 20 June 2017 and GnRHa treatment, fish were transferred to a land-based tank (3rd period). The same number of fish, following the same procedure and using six from the remaining fish in cage B were transferred for spawning in land based tank on 4 July 2017 (4th period). Each group remained in land-based facilities for 14 days and then were transferred back in the sea cage. Prior to transfer back to the sea cage, evaluation of the reproductive stage of the fish was done. The same trial was repeated in 2018 breeding season and the respective days of sampling were 15 June, 27 June and 12 July. In 2018, the fish were not divided in two cages prior the reproductive period since we had not observed any negative effect of the handling to the maturational stage in 2017 breeding season.

Evaluation of sperm quality

To obtain sperm for evaluation, the genital pore was rinsed, blot dried and a catheter was inserted as described above. Small volumes of sperm were stored in a 1.5 ml micro-centrifuge tube placed on ice and

then transferred to a 4°C refrigerator until evaluation. Care was taken to avoid contamination of sperm with blood or other somatic fluids.

Sperm quality parameters that were evaluated included (a) sperm concentration (number of spermatozoa ml^{-1} of sperm), (b) initial percentage of spermatozoa showing forward motility immediately after activation (sperm motility, %), (c) duration of forward sperm motility of ≥5% of the spermatozoa in the field of view (motility duration, min) and (d) survival of sperm during storage at 4°C (sperm survival, days). Sperm density was estimated after a 2121-2626 fold dilution with 0.9% saline buffer using a Neubauer haemocytometer under 200X magnification (in duplicate) under a compound light microscope. Sperm motility (% spermatozoa showing forward motility) and motility duration (min) were evaluated on a microscope slide (400X magnification) after mixing 1 µl of sperm with a drop of about 50 µl of saltwater (in duplicate). Activated sperm samples were observed under the compound light microscope for the first time 10 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.

Evaluation of egg quality

Egg collectors were examined three times a day (8:00 a.m., 3:00 p.m., 8:00 p.m.). For each spawn the date, collection time and developmental stage (Tachihara, et al., 1993) were recorded, in order to identify different spawns and estimate an approximate spawning time. The eggs were collected and transferred into a 10-L bucket. Their number (fecundity) was estimated by counting the total number of eggs in a sub-sample of 10 mL, collected with a pipette after vigorous agitation. The fertilization percentage was evaluated at the same time by examining each egg in the subsample. After collecting the sub-sample, the eggs were transferred into a 125-500 L conical tank-incubator fitted with an overflow filter (250 µm mesh size), and supplied with surface seawater (~90% h^{-1} renewal) and mild aeration.

To monitor embryo and larval survival, eggs from each spawn were collected from the tank incubators and placed individually in 96-well microtiter (mct) plates (in duplicates) according to the procedure of Panini et al., (2001) with some modifications. Briefly, a sample of floating (~100% fertilized) eggs were taken from the tank incubators with a 250 µm mesh sieve, rinsed with seawater and poured in 2 L beakers filled with seawater. Using the sieve, 100-200 floating eggs were scooped from the beaker and placed in a Petri dish. Together with 200 µL of seawater, the fertilized eggs were aspirated with a micropipette one by one and transferred individually to the 96-wells of a mct plate. The plates were checked under a stereoscope and any dead eggs were replaced. Once loaded, the mct plates were covered with a plastic lid, placed in a controlled-temperature incubator and maintained for 7 days at temperatures ranging between 21.0 and 23.5°C, according to the spawning temperature of each batch. Using a stereoscope, embryonic and early larval development was evaluated daily, recording the number of live embryos 24 hours after egg collection (or ~30 hrs after spawning), hatched larvae (examined ~55 hrs after spawning) and viable larvae on day 5 after hatching (near the time of yolk sack absorption). The 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 mct plates. The hatching success was calculated as the number of hatched larvae/24hr embryos, and the 5d larval survival was calculated as the number of live larvae 5d after hatching. Estimating survival percentage (%) by using as denominator the number of individuals that survived to the previous developmental stage was considered as a more accurate evaluation of survival within specific developmental stages, without the potential of a distortion effect of the previous stage.

Histological analysis

Before embedding in methacrylate resin (Technovit 7100®, Heraeus Kulzer, Germany) ovarian biopsies were dehydrated in gradually increasing ethanol solutions (70-96%). Serial sections of 3 µm were obtained with a

microtome (Leica RM 2245, Germany). Sections were stained with Methylene Blue (Sigma, Germany)/Azure II (Sigma, Germany)/Basic Fuchsin (Polysciences, USA) according to Bennett et al. (1976). Sections were examined under a light compound microscope (Nikon, Eclipse 50i) and photographed with a digital camera (Jenoptik progress C12 plus).

Statistical analysis

Differences in mean oocyte diameter, relative and total fecundity, egg/larval performance parameters (fertilization success, 24 hr embryo survival, hatching, and 5 d larval survival) between the two GnRHa treatments and the different doses were examined using Student's t-test at a $P \leq 0.05$ significance level. Within GnRHa treatment, differences among sampling times were examined using one-way ANOVA followed by the post-hoc test of Tukey HSD at a $P \leq 0.05$ significance level. Data were examined for normality in the distribution of variances, in order to comply with the prerequisites of the ANOVA. Percentages were arcsin transformed before statistical analysis. All analyses were performed with a statistics software (JMP 12, SAS Institute Inc., Cary, NC, USA). Results are presented as mean ± SEM, unless otherwise stated.

Results

Different spawning induction trials were conducted in the various facilities to implement the proposed work as written in DOW. Unfortunately, the fish in the land-based facilities were mostly immature (as described in Periodic Reports 1, 2 and 3) and the egg collection in sea cages was ineffective. It was decided to implement the proposed experiments following the "tank spawning" mode, as described earlier, to overcome the observed reproductive dysfunctions in the land-based facilities and the egg collection technical problems in the sea cages.

Multiple GnRHa injections vs implants

There was no significant difference in the mean oocyte diameters between the females from the two GnRHa treatments (t-test, $P \leq 0.05$), since the samples represented only females eligible for induction of spawning (**Fig. 2**). However, the number of females eligible for induction of spawning (*i.e.* having fully vitellogenic oocytes >600 μm) decreased from the initial to the final sampling, from 7 to 6 females for the GnRHa implant treatment and from 7 to 3 females for the GnRHa injection treatment. In addition, in the females of the GnRHa injection treatment, there was a significant decrease in the diameter of the largest vitellogenic oocytes among consecutive sampling times (ANOVA, Tukey HSD, $P \leq 0.05$), from 790±78μm at the time of the 1st treatment to 628±30μm at the time of the 3rd treatment (**Fig. 2**).

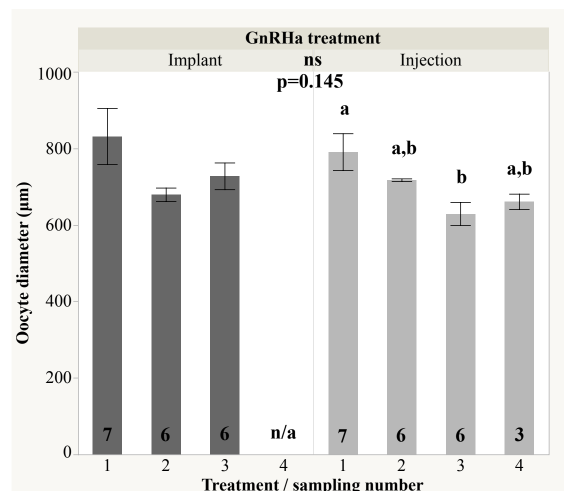

Figure 2. Mean diameter (± SEM) of the largest oocytes from the biopsies of female greater amberjack treated with multiple GnRHa implant or GnRHa injection treatments. Sampling No 3 for the GnRHa implant and No 4 for the GnRHa injection group was the final sampling of the study, and it did not include a GnRHa treatment. Statistically significant difference between the two GnRHa treatments is indicated by the P value of the t-test on the GnRHa treatment legend (ns = no significance). Lowercase letters above the Treatment/sampling means indicate significant differences within GnRHa treatments (ANOVA, Tukey HSD, $P < 0.05$). n/a: not applicable. The number in the bar indicates the number "n" of females that could be induced with GnRHa to spawn.

At the time of the 1st GnRHa treatment, females had mainly vitellogenic oocytes in their ovaries, while 4 out of the 14 were in oocyte maturation (**Fig. 3**, **first row**) and only a small number of apoptotic oocytes was observed. During the 2nd sampling, only the injected fish were biopsied. Vitellogenic oocytes were still present, except in one female where only post ovulated eggs and apoptotic oocytes were observed (**Fig. 3**, **second row**), but also ovulated eggs and atretic oocytes were observed in almost all the biopsies taken. At the 3rd sampling, vitellogenic oocytes were visible in both treatment groups (**Fig. 3**, **third row**), except from one fish from each treatment that had only apoptotic oocytes and ovulated eggs, while the number of primary oocytes was increased. At the 4th sampling, the implanted fish still had vitellogenic oocytes and some of them were in oocyte maturation, and the proportion of atretic oocytes was minimal (**Fig. 3**, **fourth row**). On the other hand, 4 of 7 injected fish had concluded their reproductive period (*i.e.* they were spent), since only primary and early vitellogenic oocytes were present in the ovaries, together with advanced apoptotic oocytes and unreleased, overripe oocytes (not shown in Fig. 3). The other 3 females from the injected treatment still had some vitellogenic oocytes, but with increased number of apoptotic oocytes.

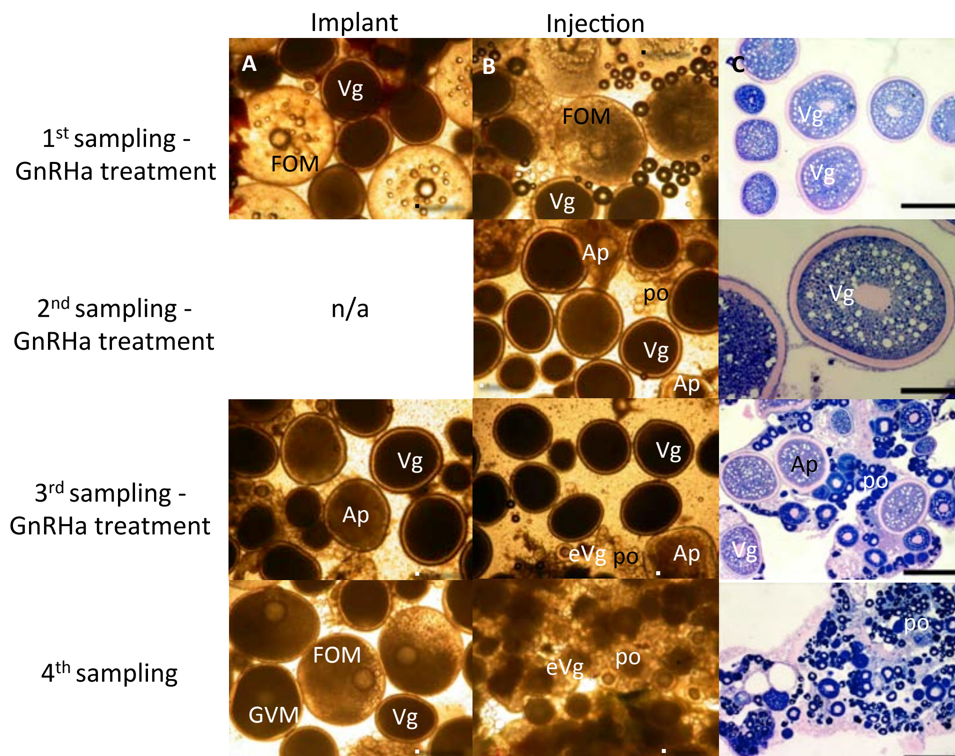

Figure 3. Microphotographs of representative ovarian biopsies from greater amberjack at different treatment/sampling times, presented as wet mounts or after histological processing. Fish were treated either with GnRHa implants or injections. po=primary oocyte, Vg=vitellogenic oocyte, eVg=early Vg, GVM=Germinal Vesicle Migration, FOM=Final Oocyte Maturation, Ap=apoptotic oocyte. Bars=500µm.

In response to both GnRHa treatments, spawning started 1 day after the 1st application, because of the existence of maturing oocytes, whereas after the following GnRHa administrations spawning commenced after 2 days (**Fig. 4**). Implanted fish spawned for up to ten times after the 1st treatment and only four times after the 2nd treatment. Injected fish spawned for seven times after the 1st treatment, three to five times after the 2nd treatment and one to three times after the 3rd treatment.

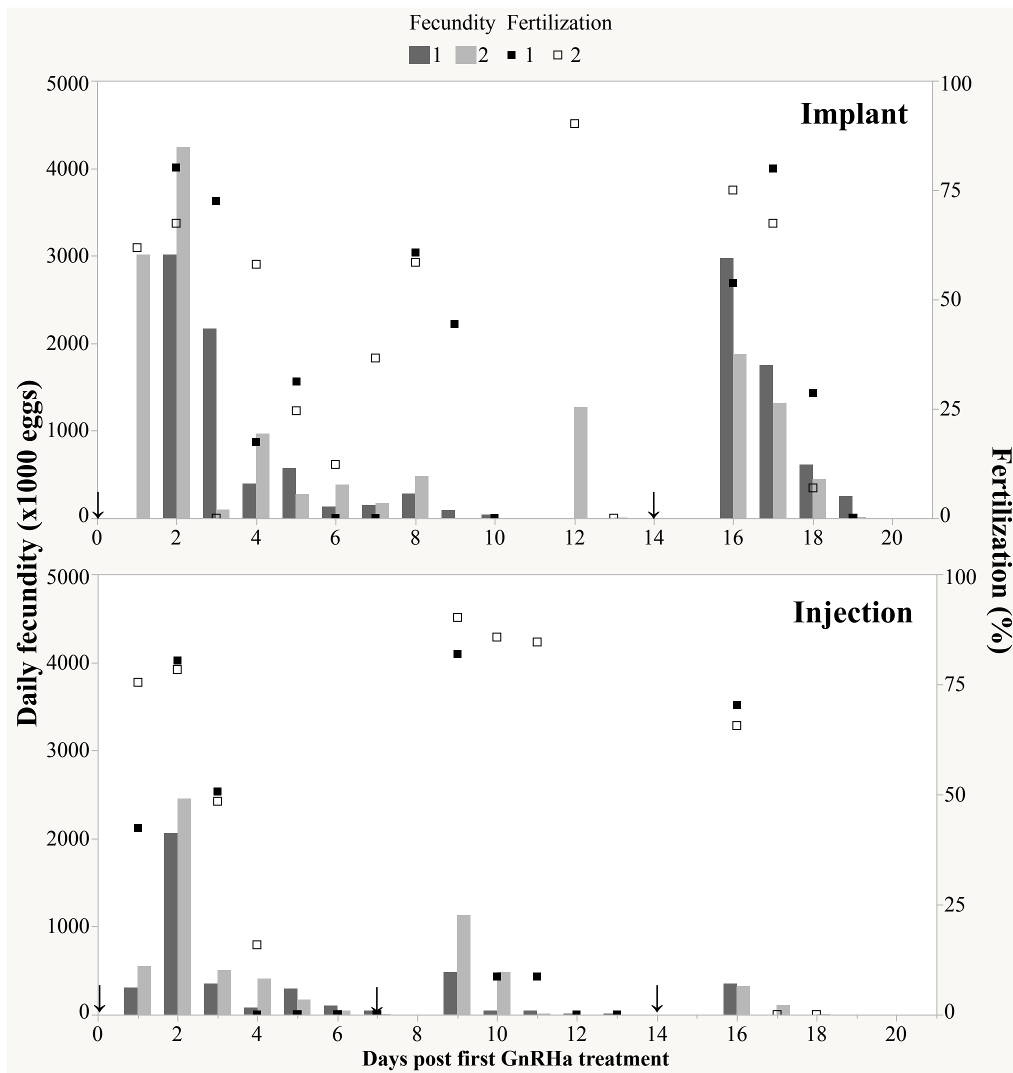

Figure 4. Daily fecundity (bars, x1000 eggs) and fertilization success (boxes, %) of GnRHa implanted or injected greater amberjack (n=2 stocks for each method, numbered 1 and 2). Arrows (n=2 for GnRHa implanted and n=3 for injected fish) indicate the time of treatment. The first application was done on 7 June 2016.

Mean daily relative fecundity was significantly higher ($P = 0.012$) in the implanted fish (15,170 ± 2,738 eggs $kg^{-1}day^{-1}$) compared to the injected fish (6,119 ± 2,790 eggs $kg^{-1}day^{-1}$) (**Fig. 5**). There was no reduction in the daily relative fecundity over time in either of the GnRHa treatments. Total relative fecundity was also significantly higher (, $P = 0.003$) in the implanted fish (102,402 ± 20,337 eggs kg^{-1}) compared to the injected ones (26,517 ± 9,938 eggs kg^{-1}) (**Fig. 6**). Total egg production decreased significantly ($P = 0.05$) over time in fish from the injected treatment, but not in the GnRHa implanted treatment. The highest daily egg production was observed in implanted fish, with 4,242,000 eggs $tank^{-1}$ two days after the 1st treatment, while in injected fish maximum daily egg production was 2,454,000 eggs $tank^{-1}$ at the same time.

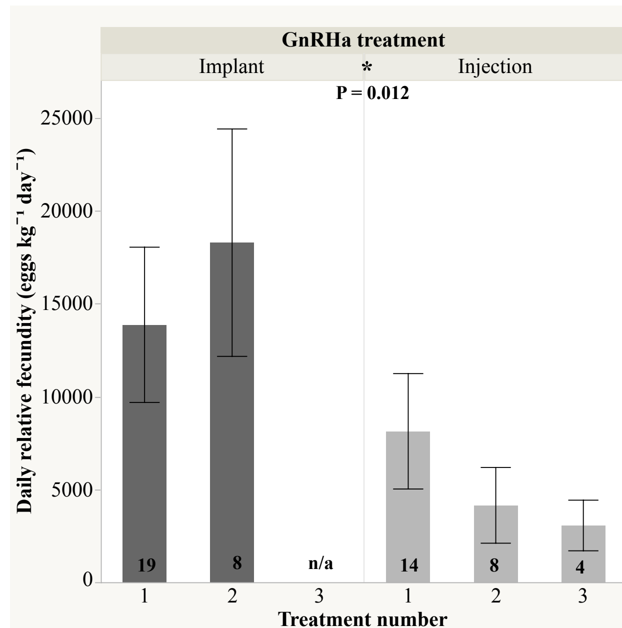

Figure 5. Mean daily relative fecundity (± SEM) of GnRHa implanted or injected greater amberjack. Numbers inside the bars are the spawns constituting each mean. Statistically significant difference between the two GnRHa treatments is indicated by an "*" and the P value of the t-test on the GnRHa treatment legend. There were no significant differences between treatment times within GnRHa treatments (ANOVA, $P \geq 0.05$). n/a: not applicable.

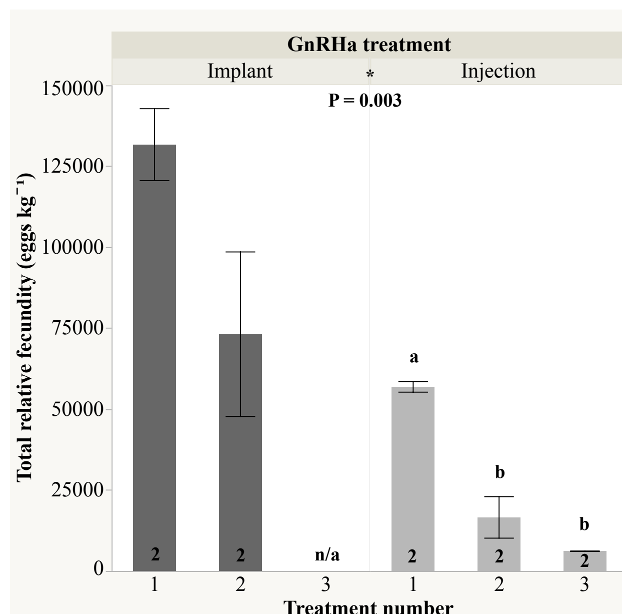

Figure 6. Mean total relative fecundity (± SEM) of GnRHa implanted or injected greater amberjack. Numbers inside the bars are the number of replicated tanks constituting each mean. Statistically significant difference between the two GnRHa treatments is indicated by an "*" and the P value of the t-test on the GnRHa treatment legend. Lowercase letters above the treatment means indicate significant differences within GnRHa treatments (ANOVA, Tukey HSD, $P < 0.05$). n/a: not applicable.

Fertilization success, 24 h embryo survival, hatching and 5d larval survival was similar between eggs obtained with the two GnRHa treatments, and there were also no differences over the course of the study (**Fig. 7**). Overall, mean fertilization success was 36 ± 5%, 24 h embryo survival was 53 ± 7%, hatching was 70 ± 4% and 5d larval survival was 20 ± 4%.

Figure 7. Mean (±SEM) fertilization, 24-h embryo survival, hatching and 5-d larval survival after consecutive treatments with GnRHa implants or injections. Numbers inside the bars are the number of spawns constituting each mean. No statistical differences were observed between different treatment methods (t-test, P value on the graph) or among treatment means within GnRHa treatments (ANOVA, $P \geq 0.05$). n/a: not applicable.

Comparison of two GnRHa doses

Before the first treatment, females had mainly vitellogenic (Vg) oocytes, while 2 fish out of 10 were in advanced maturational stage. Some percentage of atresia was observed in almost all ovarian biopsies (**Fig. 8**, **first row)**. At the same time, males had IT sperm. During the second sampling, three fish from LOW group had still Vg oocytes, but also apoptotic oocytes and ovulated eggs, while in one female ovarian biopsy was not feasible (**Fig. 8**, **second row)**. The last female from LOW group had mostly primary oocytes, and apoptotic oocytes as well. The 4 females from the HIGH group had Vg oocytes, with apoptotic oocytes and post ovulated eggs in their ovarian biopsies. One female was found with ovulated eggs and oocytes in maturation stage. The last female from this group had mostly primary oocytes with increased percentage of atresia. Males were still having IT sperm. On the last sampling, one female from each group had Vg oocytes, with increased percentage of POs and early signs of atresia. The rest were devoid of Vg oocytes, having mostly POs, ovulated eggs, signaling the end of the reproductive season for the specific females (**Fig. 8**, **third row)**.

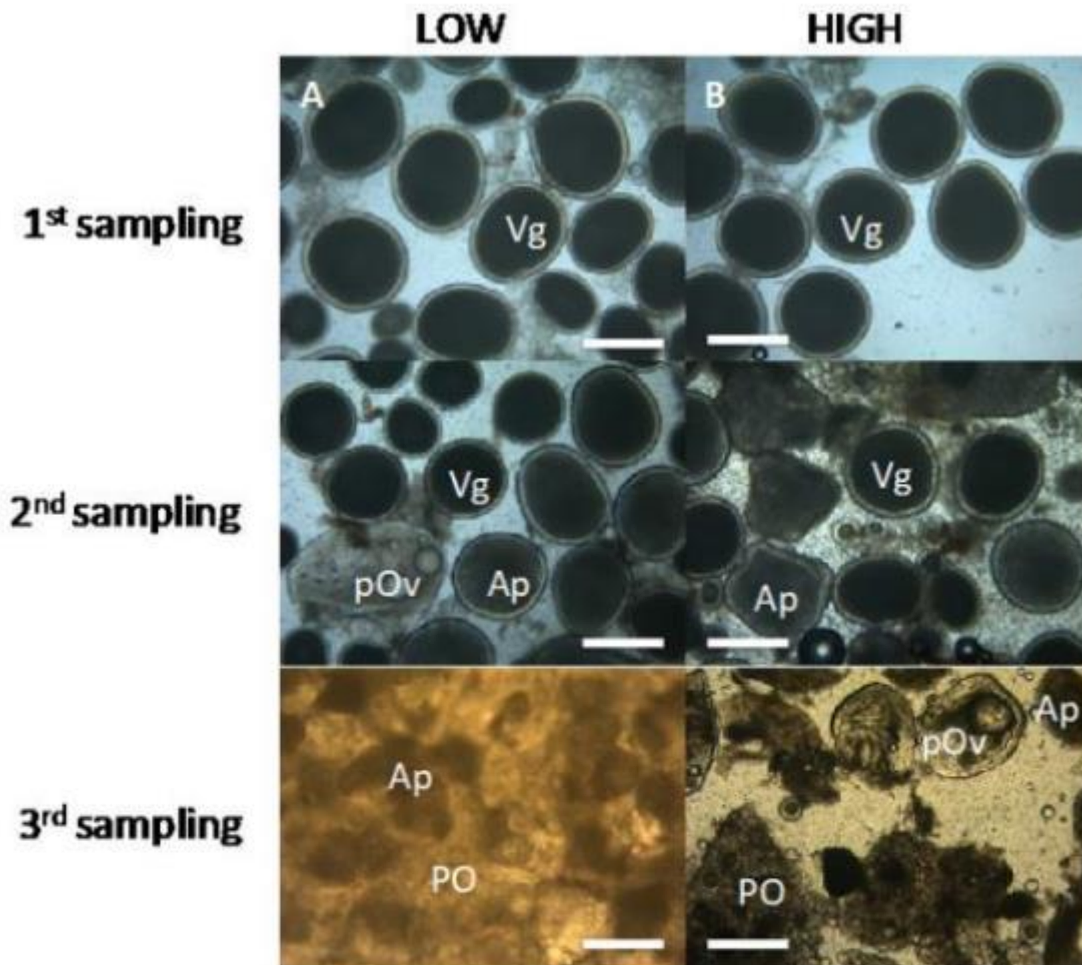

Figure 8. Microphotographs of representative ovarian biopsies from greater amberjack at different sampling times, presented as wet mounts. Fish were treated either with (A) LOW (~25 μg kg^{-1}) or (B) HIGH (~75 μg kg^{-1}) GnRHa dose using EVAc implants. PO=primary oocyte, Vg=vitellogenic oocyte, pOv=post Ovulated egg, Ap=apoptotic oocyte. Bars=500μm.

In both treatment doses, spawning started one day after 1st application because of the existence of oocytes in maturation stage. Fish spawned for 7-9 times after 1st treatment, while only 5 times after 2nd treatment. The highest egg production was observed in the LOW group with 33,826 eggs kg^{-1} two days after 1st treatment (**Fig. 9**).

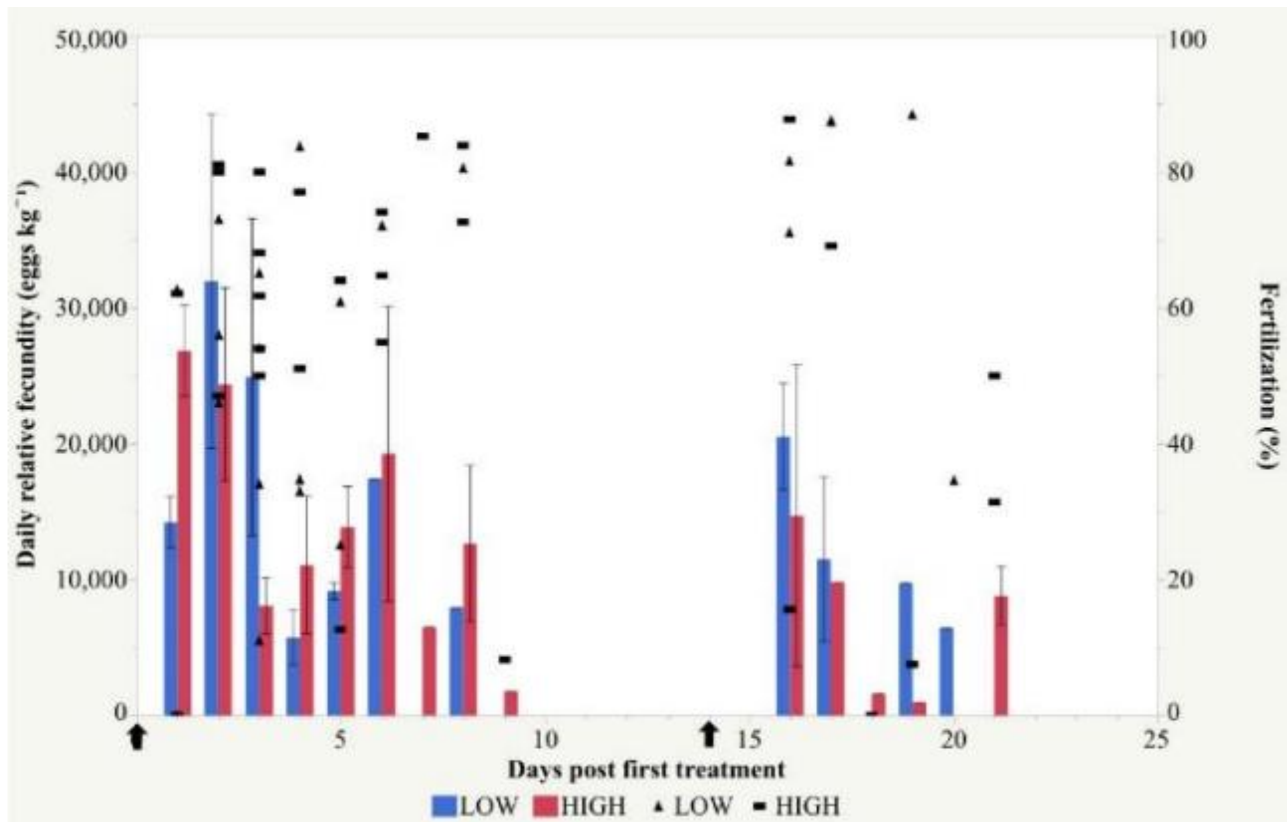

Figure 9. Daily relative fecundity (bars, eggs kg^{-1}) and fertilization success (marks, %) of LOW (blue bars) and HIGH (red bars) groups of greater amberjack implanted with different doses of GnRHa. Arrows indicate the time of treatment. First application was done on 7 June 2017.

Mean daily relative fecundity was not significantly different among the LOW and HIGH dose groups. LOW group produced 17,801±4,127 eggs $kg^{-1}day^{-1}$ and HIGH group 14,648±2,285 eggs $kg^{-1}day^{-1}$ after the 1st treatment, respectively (**Fig. 10**). After the 2nd treatment, LOW group spawned 13,373±3,022 eggs $kg^{-1}day^{-1}$ and HIGH group 8,484±3,228 eggs $kg^{-1}day^{-1}$. Fertilization success was different between the two doses at the 2nd treatment, with the LOW group having significantly higher success of fertilization compared to the HIGH group. On the other hand, fertilization success after the 1st treatment, 24 h embryo survival, hatching and 5d larval survival was similar among treatment methods, while no statistical differences were observed among different treatment number (**Fig. 10**).

Figure 10. Mean daily relative fecundity, fertilization, 24h embryo survival, hatching and 5-day larval survival (± SEM) of LOW (blue) or HIGH (red) GnRHa dose group of greater amberjacks. Numbers in bars indicate the number of spawns that constitute each mean. Lowercase letters indicate significant differences between different doses groups in 2nd treatment for fertilization percentage (t test, p=0.015). No other

statistical differences were observed between different treatment methods or different treatment numbers (t test, $P < 0.05$).

Timing of GnRHa treatment application

In 2017 before the first treatment, the females had mainly vitellogenic (Vg) oocytes of 740-760 µm in diameter. Atresia was present in one female, while signs of a past spawning event was visible in a second female fish (**Fig. 11**, **first row)**. Three weeks later, on 20/6/2017, there was variability in ovarian biopsies, since one was found to be fully apoptotic and primary oocytes were visible (PO), the second had Vg oocytes of 700 µm and no biopsy was obtained from the 3rd female. The reproductive evaluation on 6/6/2017 showed that the females were mostly in Vg stage of 730-760 µm in diameter and one of them had ovulated eggs (**Fig. 11**, **second row)**. Two weeks later that fish had still some Vg oocytes of 650-700 in diameter, and post ovulated eggs and atresia were present. In one fish oocytes in maturation stage were found. At the beginning of the 3rd period the females were mostly in Vg stage and early signs of atresia were present. (**Fig. 11**, **third row).** One female was more progressed and had oocytes in early maturation of 800 µm in diameter. At the end of this period the females had apoptotic oocytes and post ovulated eggs. One female had only POs (spent) and Vg oocytes of lower diameter were present in another one. The last period started with the reproductive evaluation of the fish on 4/7/2017, where the females had again Vg oocytes of 650-680 µm in diameter (**Fig. 11**, **fourth row)**. At the end of this period, two weeks later, the females were spent, having POs, apoptotic oocytes or ovulated eggs. Males had IT sperm from the first sampling to the last one.

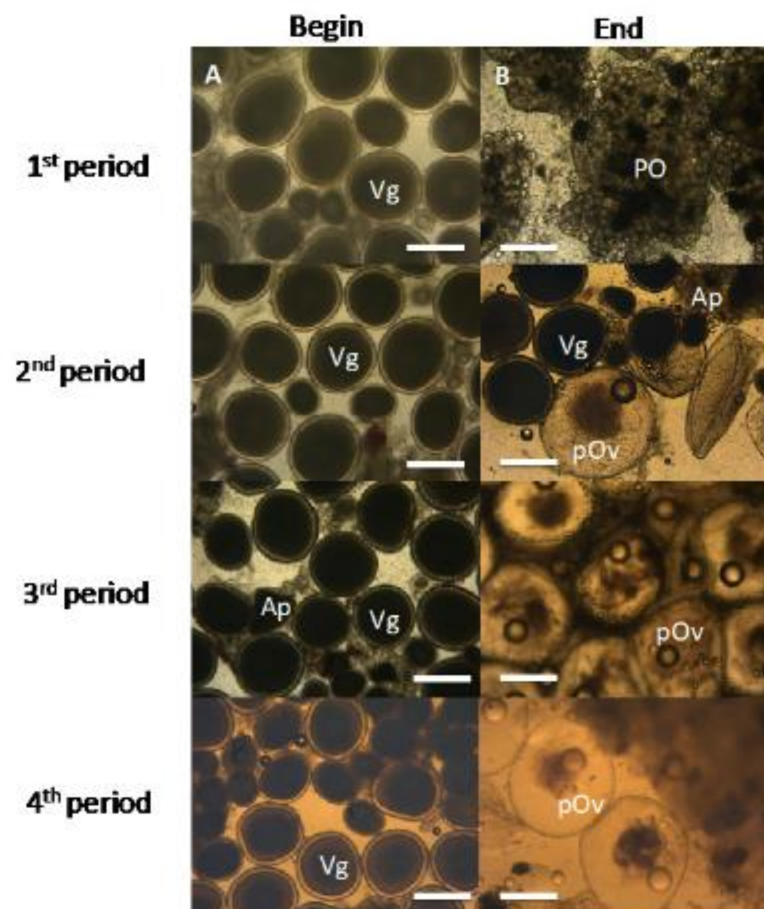

Figure 11. Microphotographs of representative ovarian biopsies from greater amberjack at different sampling times, presented as wet mounts. Fish were treated with GnRHa implants for spawning at 1st period (30/5/2017), 2nd period (6/6/2017), 3rd period (20/6/2017) and 4th period (4/7/2017). Column (A) presents pictures during the ovarian evaluation before each treatment and (B) after the end of spawning period in land-based tank. PO=primary oocyte, Vg=vitellogenic oocyte, pOv=post Ovulated egg, Ap=apoptotic oocyte. Bars=500µm.

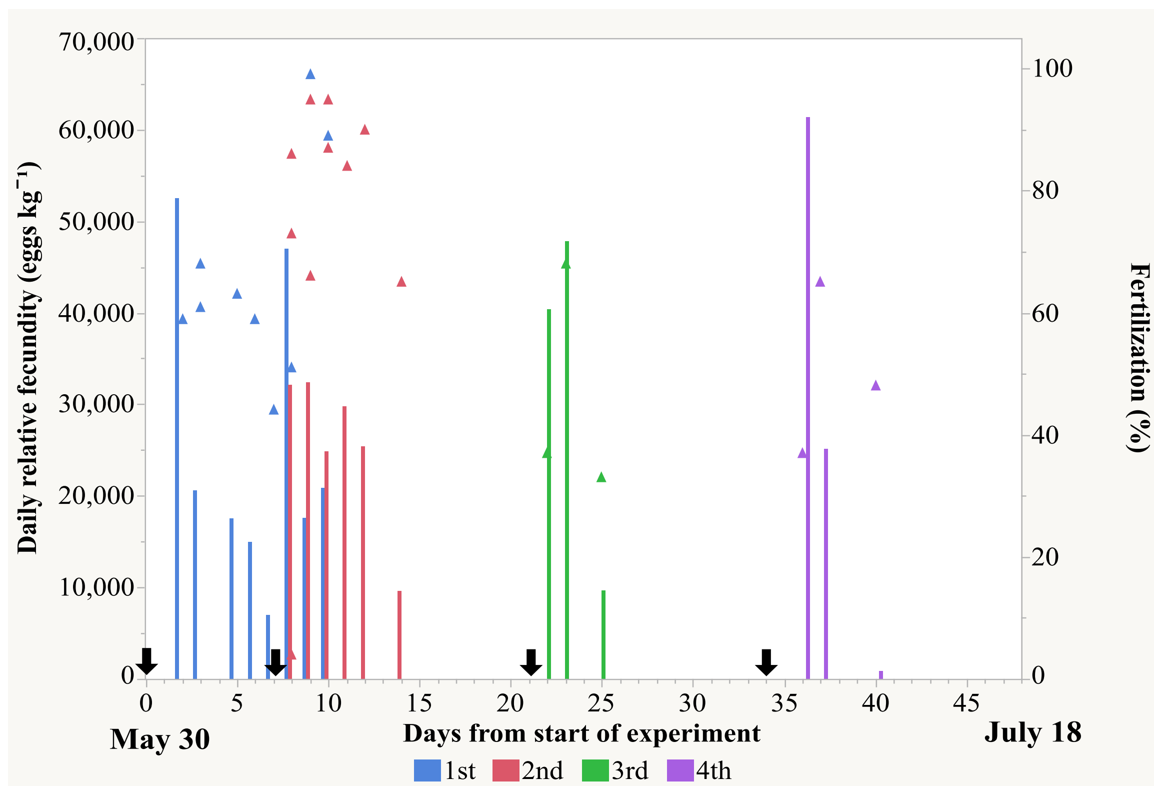

Figure 12. Daily relative fecundity (bars, eggs kg^{-1}) and fertilization success (marks, %) of 1st (blue bars), 2nd (red bars), 3rd (green bars) and 4th (purple bars) period of GnRHa treatment of greater amberjack. Arrows indicate the time of treatment. First application was done on 30 May 2017.

Mean daily relative fecundity was not significantly different between the four periods of GnRHa treatment and was 27,173±3,144 eggs kg^{-1}day^{-1} (**Fig. 12 and Fig. 13**). The first two periods the number of spawns was higher (9 and 10 for the 1st and 2nd period, respectively) compared to the last two periods (3 for 3rd and 4th period, respectively). Mean fertilization was 65±4%, mean 24h embryo survival 79±5%, mean hatching 60±8% and 5-days larval survival 23±5% (**Fig. 13**).

In 2018 the situation was almost the same with the previous reproductive season. The females had mainly vitellogenic (Vg) oocytes of 680-730 μm in diameter. One female, apart from the Vg oocytes, was in post ovulation stage (pOV). Two weeks later, on 27/6/2018, females had Vg oocytes of 680-700μμ in diameter but the number of PO was increased. The reproductive evaluation on 12/7/2018 showed that the females still were in Vg with oocyte diameter of 650-750 μm with some signs of early AT. One female was found at early oocyte maturation stage. Males had IT sperm from the first sampling to the last one.

Mean daily relative fecundity was not significantly different between the three periods of GnRHa treatment and was 26,505±5,846 eggs kg^{-1}day^{-1} (**Fig. 14 and Fig. 15**). The first two periods the number of spawns was five and the last one was three. Mean fertilization was 43±7%, mean 24h embryo survival 36±9%, mean hatching 73±12% and 5-days larval survival 7±5%, respectively (**Fig. 15**).

Figure 13. Mean daily relative fecundity, fertilization, 24h embryo survival, hatching and 5-day larval survival (± SEM) of 1st (blue bars), 2nd (red bars), 3rd (green bars) and 4th (purple bars) period of GnRHa treatment of greater amberjack. Numbers in bars indicate the number of spawns that constitute each mean. No statistical differences were observed between different periods of GnRHa application (ANOVA, $P < 0.05$).

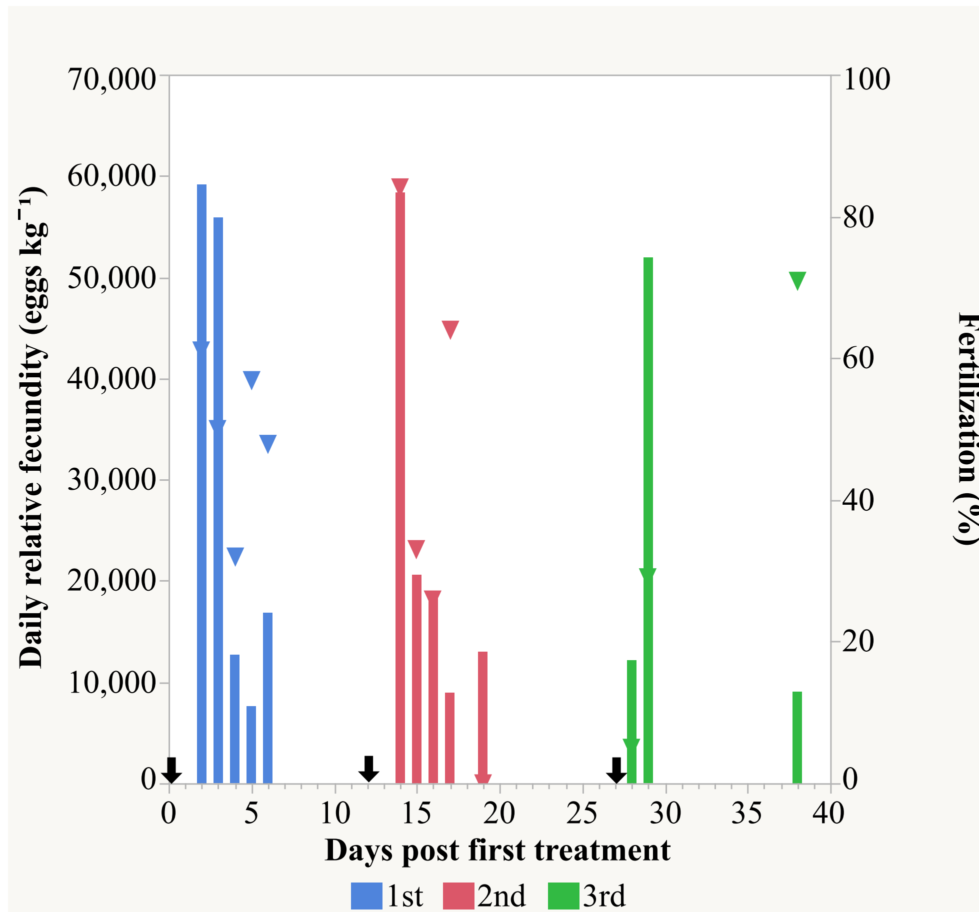

Figure 14. Daily relative fecundity (bars, eggs kg^{-1}) and fertilization success (marks, %) of 1st (blue bars), 2nd (red bars) and 3rd (green bars) period of GnRHa treatment of greater amberjack. Arrows indicate the time of treatment. First application was done on 15 June 2018.

Discussion

Greater amberjack in the present study were induced successfully to spawn using either GnRHa implants of ~25 μg kg^{-1} fish, ~50 μg kg^{-1} fish or ~75 μg kg^{-1} fish or GnRHa injections of 20 μg kg^{-1} fish. The induced fish, spawned many times over the course of 3 weeks, and producing fertilized eggs of adequate quality for large scale larval rearing trials (Mylonas et al., 2016a). These results confirmed previous studies where GnRHa implants (Mylonas et al., 2004) or injections (Fernández-Palacios et al., 2015a) induced multiple spawning of viable eggs, overcoming the observed reproductive dysfunctions of the species in captivity. The GnRHa therapy was effective in all fish treated, and resulted in many more spawns and a longer reproductive season than reported previously (Mylonas et al., 2004), starting from mid-May to end of July. Developing a reliable method for the production of eggs from a large number of breeders is important for the development of a sustainable industry of greater amberjack, because in Europe (and especially in the Mediterranean) spontaneous reproduction of the species has been very rare (Jerez et al., 2006; Lazzari et al., 2000; Mylonas et al., 2004; Rodríguez-Barreto, et al., 2014). In addition, synchronizing spawning of a large group of breeders could mitigate the problem of disproportionate parental contribution and inbreeding, when eggs are produced from only a limited number of breeders, as it was shown for the congeners yellowtail kingfish *Seriola lalandi* (Setiawan et al., 2016) and longfin yellowtail *Seriola rivoliana* (Fernández-Palacios et al., 2015b).

Figure 15. Mean daily relative fecundity, fertilization, 24h embryo survival, hatching and 5-day larval survival (± SEM) of 1st (blue bars), 2nd (red bars) and 3rd (green bars) period of GnRHa treatment of greater amberjack. Numbers in bars indicate the number of spawns that constitute each mean. No statistical differences were observed between different periods of GnRHa application (ANOVA, $P < 0.05$).

Early studies on the induction of spawning of greater amberjack utilizing chum salmon *Oncorhynchus keta* pituitary extract resulted in the production of 1.4 and 7 million eggs in two different reproductive seasons (Tachihara, et al., 1993), whereas the use of hCG injection produced mostly unfertilized eggs (Kozul, et al.,

2001). The recent use of GnRHa seems to be the most efficacious method in greater amberjack (Fernández-Palacios, et al., 2015a; Jerez, et al., 2017; Jerez, et al., 2018; Mylonas, et al., 2004), as it was shown in other species as well (Mylonas, et al., 2010). However, the differences in response (*e.g.* spawning kinetics, egg production and quality) between the methods of administration of GnRHa, the doses and the timing of application, have not been systematically evaluated so far. In the present study, GnRHa implanted females produced significantly more eggs of the same quality in the same number of spawns compared to the GnRHa injected females, with less handling. In addition, most of the GnRHa implanted females still contained a significant number of vitellogenic oocytes at the end of the experiment, when spawning ceased, and presumably had the potential to produce more eggs if treated with another GnRHa implant. In a similar comparison in the Canary Islands (Spain), GnRHa implants were shown to be more reliable in inducing spawning than GnRHa injections, since fish responded always after a GnRHa implant administration, spawning an average of 2.2±1.2 spawns per implantation (Sarih, et al., 2016). On the contrary, after GnRHa injection fish spawned only 0.8±0.5 spawns per injection, suggesting that only 80% of the injections resulted in spawning. If fish spawned more than once after some injections (this information is not provided in the article), then the success of that study was even less than 80%. Fecundity was similar in the two methods in that study, even if the number of spawns obtained after GnRHa implantation was more than 2x higher. Based on the higher fertilization success of eggs obtained from the GnRHa-injected group, however, it was reported that this was a preferred method of spawning induction (Sarih, et al., 2016). Greater amberjack in the Canary Islands exhibit somewhat different reproductive characteristics with a much more prolonged reproductive period --lasting from April to October (Jerez, et al., 2006; Sarih, et al., 2016)-- presumably due to the more stable environmental conditions (photoperiod and temperature) that exist in sub-tropical regions. Perhaps the use of a bolus GnRHa treatment once every ~10 days in the study of Sarih et al. (2016) was physiologically a more appropriate method under these conditions, as it was shown in meagre maintained under constant temperatures from the beginning of the spawning season and for the next 4 months (Mylonas, et al., 2016b). In the present study, two administrations of GnRHa implants spaced 14 days apart produced significantly more eggs of the same quality compared to three weekly treatments with GnRHa injections, with the GnRHa implantation approach involving less handling.

The greater amberjack is characterized as group synchronous or asynchronous regarding its ovarian development, with a multiple spawning reproductive strategy (Marino, et al., 1995). The GnRHa implant is considered as the most appropriate method of treatment for this group of fishes, since it can both induce multiple cycles of oocyte maturation and ovulation, and it can support also the final stages of vitellogenesis in fish that are not quite ready to undergo oocyte maturation (Mylonas, et al., 2004). In the multiple batch spawner Senegalese sole *Solea senegalensis*, two different GnRHa sustained-release delivery systems were proven to be the most effective for spawning induction when compared with a single (Guzmán, et al., 2009) or multiple injections of GnRHa (Agulleiro, et al., 2006). However, in meagre, a species which is also characterized by asynchronous oocyte development (Gil, et al., 2013; Mylonas, et al., 2013a), multiple GnRHa injections were reported to be a more appropriate method of spawning induction compared to GnRHa implants, even if the overall results of egg production and quality did not differ significantly between the two methods (Mylonas, et al., 2015). In meagre, GnRHa implants induce daily spawns for up to 3 weeks, but the majority of the eggs are produced during the first 2-3 spawns after treatment, while the following spawns consist of small batchers of eggs that cannot be utilized efficiently by commercial hatcheries (Mylonas, et al., 2013b), and eventually the gonad is depleted of vitellogenic oocytes and further GnRHa implantation does not produce consistent results (Mylonas, et al., 2015). On the contrary, weekly GnRHa injections induced only two high-fecundity spawns after each injection (day 2 and 3 after treatment) -- presumably due to the short-lived elevation in plasma LH-- and the treatment produced consistently spawns of high fecundity and egg quality for up to 17 weeks (Mylonas, et al., 2016b). So, the choice between GnRHa injection(s) or sustained-release delivery systems for the induction of spawning must be examined in each species of interest, as well as in relation to the specific environmental conditions and genetic origin of the broodstock in question. Recent genetic studies suggested that greater amberjack may be separated into an Atlantic and Mediterranean population (Šegvić-Bubić, et al., 2016), and this may explain –

at least in part-- the differences in response to the GnRHa injections and implants of the fish between the present study and the one in the Canary Islands (Sarih, et al., 2016).

Group synchronous oocyte development is exhibited also by the congeners of greater amberjack such as the yellowtail kingfish (Poortenaar, et al., 2001), the Japanese yellowtail *Seriola quinqueradiata* (Kagawa, 1989) and the longfin yellowtail (Fernández-Palacios, et al., 2015b), and all have been induced to spawn successfully with various hormonal therapies (Chuda, et al., 2002; Fernández-Palacios, et al., 2015a; Roo, et al., 2014; Setiawan, et al., 2016). In the Japanese yellowtail a comparison among three different hormonal therapies was examined, using a single or double hCG injection, or a single cholesterol implant with GnRHa. Even though the latter treatment produced the highest egg quality, a single hCG injection was considered as the most efficient method to induce oocyte maturation and ovulation, based on the production of eggs of better quality (Chuda, et al., 2001). However, when hCG treatment is used repeatedly in subsequent years, the fish may develop an immune response and the injected preparation is immune-neutralized (Zohar & Mylonas, 2001). This is an important disadvantage of the hCG over the GnRHa preparations for inducing spawning of fishes for aquaculture production. This is especially true in species such as the greater amberjack that are mainly of wild-caught origin, are difficult to acquire and they mature after >3-4 years.

Eighty-six % of the females from the GnRHa implanted group and 43% of the injected group still had a large number of fully vitellogenic oocytes 3 weeks after the initial hormonal treatment and after spawning multiple times. We expect that these fish would produce more eggs if given another hormonal therapy. This observation demonstrates the capacity of GnRHa, primarily in a sustained-release delivery system, to not only induce maturation of the available post-vitellogenic oocytes, but also to further support vitellogenesis of the smaller oocytes, as it was shown in the dusky grouper *Epinephelus marginatus* (Marino, et al., 2003), eventually resulting in multiple cycles of oocyte maturation, ovulation and spawning. Unfortunately, oocyte diameter data at the time of consecutive GnRHa administrations are not available from some other spawning induction studies of greater amberjack (Fernández-Palacios, et al., 2015a; Sarih, et al., 2016). In a recent study in the Canary Islands, F1 greater amberjack broodstock treated with GnRHa implants every 30-40 days maintained their maximum oocyte diameter above 650 μm for a period of 5 months (May - September) (Jerez, et al., 2017; Jerez, et al., 2018). The fact that the fish in these studies spawned for a period of many months after multiple injections every 10 days in the Canary Islands underlines the capacity of GnRHa to induce vitellogenesis, in addition to oocyte maturation, probably through the induction of follicle stimulating hormone (FSH) release, in addition to LH release from the pituitary (Zohar, et al., 2010). The same capacity was evident in greater amberjack in the present study, albeit to a lesser extent, since in the Mediterranean (Zupa, et al., 2017b) and Japan (Kawabe, et al., 1998; Kawabe, et al., 1996) the rapid elevation of water temperature in the summer most likely causes a cessation of reproductive function. The effectiveness of GnRHa in sustained release delivery systems to promote the process of vitellogenesis has been demonstrated in only limited situations, such as in the ayu *Plecoglossus altivelis* (Aida, 1983), bitterling *Acheilognathus rhombea* (Shimizu, 1996), winter flounder *Pleuronectes americanus* (Harmin, et al., 1995) and the congener yellowtail kingfish (Setiawan, et al., 2016).

The overall daily and total relative fecundity after the two GnRHa implantations in the GnRHa treatment method comparison experiment were 2.5 and 3.8 times higher, respectively, than the three GnRHa injections. Considering only the first GnRHa implantation (9.5±0.7 spawns) and injection (7±0 spawns), the overall daily and total relative fecundity were 1.7 and 2.4 times higher, respectively. Multiple GnRHa injections produced less than 45,000 eggs kg^{-1} fish per treatment (Fernández-Palacios, et al., 2015a), slightly lower from the respective maximum fecundity in response to GnRHa injections in the present study. In studies using GnRHa implants in the Canary Islands (Jerez, et al., 2017; Jerez, et al., 2018) and Greece (Mylonas, et al., 2004), the respective daily relative fecundity was less than 6,000 eggs kg^{-1} and 30,000 eggs kg^{-1}, respectively, which is 9 and 2 times lower compared to the maximum observed in the present study after GnRHa implantation. Wild-caught fish spawning spontaneously in the Canary Islands produced 114,490 eggs kg^{-1} (or a total of 14,311,200 eggs) during the extended spawning period (April to October) (Jerez, et al., 2006). In the present study, the overall mean (± SD) total fecundity of the GnRHa implanted fish was

204,805 ± 20,214 eggs kg^{-1} (or a total of 13,489,000 ± 1,507,000 eggs), in a period of only 21 days. The mean total fecundity per female was 3,889,000 ± 355,00 eggs female^{-1}, which is higher than another study in the Canary Islands using multiple GnRHa injections (2,480,000 eggs female^{-1})(Fernández-Palacios, et al., 2015a).

Fertilization success was similar in the eggs obtained using either GnRHa induction method, suggesting that in both treatments the process of oocyte maturation, ovulation and spawning was equally successful, if we assume that the performance of the males was similar in both groups. To ensure the latter, males in the two GnRHa treatment groups were treated in a similar way (GnRHa implants) and the sperm quality was found to be the same at the beginning of the experiment. Same values of fertilization success were found in a spawning induction experiment of greater amberjack using GnRHa implants in Greece (Mylonas, et al., 2004) or in the Canary Islands using F1 broodstock (Jerez, et al., 2017; Jerez, et al., 2018). Significantly higher values, however, were reported in a multiple GnRHa injections experiment (96.0 ± 6.5%) with the same species (Fernández-Palacios, et al., 2015a). In the latter study, the sex ratio was higher in favor of the males (1:2 ♀ : ♂) than the present study (which was 1:1 ♀:♂), and this could have resulted in better fertilization success. In a different study where natural (*i.e.* no hormonal treatment), GnRHa injected and GnRHa implanted groups were compared, fertilization success was 84.4 ± 21.6%, 58.8 ± 26.8% and 32.5 ± 34.6%, respectively, being significantly different among the groups (Sarih, et al., 2016). Again, in the latter study the sex ratio of the most successful group (natural) was higher in favor of the males (2:5 ♀: ♂). Based also on reports from greater amberjack in Japan, it seems clear that an increased male to female ratio is preferable for this species, since more than one male appears to fertilize the eggs of one female (Tachihara, et al., 1993). In the congener yellowtail kingfish, it has been shown that in a communal tank with 14 breeders, in 50% of the recorded spawning events two males fertilized the eggs of one female, resulting in >99% fertilization success, suggesting also that a higher male to female ratio is preferable for that congener as well (Moran, et al., 2007). In the same species, a high male contribution (60% in all egg batches) in the fertilized eggs was found after parentage analysis of the eggs spawned in a communal tank with 14 females and 10 males (Setiawan, et al., 2016).

The quality of the eggs obtained in the present study --in terms of embryonic development, hatching and larval survival until yolk sack absorption—did not exhibit any significant differences between the two GnRHa treatments, different doses and timing of application, but was lower than other studies. One day after spawning, only 53 ± 7% (GnRHa treatment method), 54 ± 5% (GnRHa doses comparison), 79 ± 5% in 2017 and 36 ± 9% in 2018 (timing of GnRHa application), respectively, of embryos were still alive compared to 92.2 ± 9.4%, 86.4 ± 25.4% and 77.6 ± 34.0% for eggs obtained from natural, GnRHa injected or GnRHa implanted females, respectively, in a study in the Canary Islands (Sarih, et al., 2016). Hatching was also lower in the present study compared to the above-mentioned study, being 96.6 ± 6.6% for eggs from the natural, 91.1 ± 25.4% from the injected and 78.0 ± 34.9% from the implanted females. However, 5d larval survival was higher (GnRHa treatment method and doses comparison) or lower (timing of GnRHa application 2017, 2018), compared to the 10.8±14.7% for eggs from the natural, 5.50± 7.2% from the injected and 8.0 ± 12.5% from the implanted group, respectively (Sarih, et al., 2016). Although there is room for improvement in terms of the quality of the eggs produced in response to the spawning induction therapy, the eggs produced were of adequate fecundity and quality to implement a number of larval rearing trials, for the development of commercial production protocols (Mylonas, et al., 2016a). Improvements in egg quality are expected to result from a number of actions, such as better selection of female breeders according to their production characteristics (Symonds, et al., 2014), optimized sex ratios, tank size and perhaps above all, broodstock nutrition (Izquierdo, et al., 2001; Roo, et al., 2015; Valdebenito, et al., 2013). The latter is expected to gain more importance once a growing greater amberjack aquaculture industry is established, making it worthwhile for feed companies to invest in both specialized grow out and broodstock feeds for this species.

For the GnRHa dose comparison no significant differences were found between the LOW and the HIGH groups in terms of daily relative fecundity, fertilization, 24h embryo survival, hatching and 5d larval

survival. The only significant statistical difference was found within the 2nd treatment comparison of the two used doses, where the LOW dose group showed higher fertilization success compared to the HIGH group. In the walking catfish *Clarias batrachus* a GnRHa dose comparison experiment using four different doses of Ovatide (sGnRHa) showed that the medium dose was the most effective in terms of breeding performance and egg quality (Sahoo, et al., 2005). In the present study, the MEDIUM dose (~50 µg/kg) seemed to be the most effective based on the 2016 experiment results for the comparison of GnRHa treatment methods. Even if the year of the experiment was different, the used broodstock was the same, as well as the facilities (Argosaronikos SA) that the experiment was held, so a comparison between the different years -even if scientifically biased – is meaningful. On the other hand, the minimum effective dose of sGnRHa in channel catfish *Ictalurus punctatus* was shown to be 6.7 times lower than the one commonly used in spawning inductions of that fish (Chatakondi, 2017). In the greater amberjack the low dose, even if it was not the most effective, could be used for production purposes, spending half the cost compared to the commonly medium used dose.

The greater amberjack seems to be able to produce eggs of the same quality in the Mediterranean for almost 2.5 months, from mid-May to the end of July. As fish were shown not to proceed properly with their gametogenesis in the land-based facilities, the tank spawning mode (*i.e.* keep the broodstock in sea cages during the year and transfer them to the land based tanks for spawning) was proven to be effective in egg production with this species, since egg collection in cages was problematic (see Deliverable 3.10 for details). Fortunately, this mode of spawning could supply "on demand" greater amberjack eggs to the hatchery for an extended period, since the fish in sea cages seems to preserve their proper maturational stage if they remained unhandled before their reproductive period, meaning being able to be induced for spawning. On the contrary, it was shown that greater amberjack kept in sea cages had severe gametogenesis impairment when the fish were handled before the beginning of their reproductive season, possibly related to this broodstock management procedures (Pousis, et al., 2018; Zupa, et al., 2017a; Zupa, et al., 2017b).

In conclusion,

- Spawning induction of captive-reared greater amberjack was **more effective using GnRHa implants than injections**. More eggs were produced using GnRHa implants compared to injections, without altering the quality of eggs in terms of fertilization, 24-h embryo survival, hatching and 5-d larval survival. In addition to apparently promoting the proper endocrine pathways leading to multiple cycles of oocyte maturation, ovulation and spawning, the use of GnRHa implants was effective also in supporting the vitellogenesis process. This method may be more effective in greater amberjack than multiple injections, also because of the less handling it involves (*i.e.* one handling every two weeks as opposed to one handling every week). It has recently been shown that handling greater amberjack during the reproductive season may induce significant reductions in spermatogenesis and oogenesis (Pousis, et al., 2018; Zupa, et al., 2017a; Zupa, et al., 2017b), so it may be important to minimize handling as much as possible when implementing spawning induction methods, until spontaneous spawning can be achieved reliably in greater amberjack aquaculture.
- No significant differences were observed between the LOW and HIGH GnRHa doses, while **the MEDIUM GnRHa seemed to be the most effective**.
- The greater amberjack in the Mediterranean **can produce eggs "on demand" after spawning induction with GnRHa for a period of 2.5 months** when keeping the broodstock in sea cages and transferring them in land based tanks for spawning. We expect that the spawning induction method presented here would be a valuable tool for the efforts to incorporate this species in the aquaculture industry in Europe.

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Deviations:

The deliverable is submitted a few months later than planned, in order to utilize one more reproductive season and carry out an additional experiment (P40. GMF).