Deliverable Report

Deliverable No:	D3.10		**Delivery Month:**	58
Deliverable Title	Method for inducing spawning and collecting greater amberjack eggs in sea cages.			
WP No:	3		**WP Lead beneficiary:**	P13. UNIBA
WP Title:	Reproduction and Genetics – greater amberjack			
Task No:	3.5		**Task Lead beneficiary:**	P1. HCMR
Task Title:	Spawning induction of greater amberjack and egg collection in cages.			
Other beneficiaries:	P23. ARGO	P40. GMF		
Status:	Delivered		**Expected month:**	54
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Objective: A method was developed for the induction of spawning and collection of eggs of greater amberjacks in sea cages. The deliverable presents the methodology to (a) manipulate the large broodstock in the sea cage to apply hormones, (b) procedures and doses for hormone application, (c) cage set up for egg collection, i.e. clear descriptions of curtain deployed around the perimeter of the cage and (d) methods to actually retrieve the eggs from the water surface of the cage. In addition, the deliverable includes the results from repeated trails using and refining the methodologies including the following data: number of eggs obtained per kg female body weight, egg quality parameters, timing of application of hormones in relation to egg collection.

Introduction

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., 2017), which allows for the marketing of the fish before growth is affected by reproductive maturation. The latter could be a disadvantage in broodstock management of this species since fish of large size are needed for the egg production making the handling of them a more difficult task in the aquaculture facilities.

The first step for the establishment of a sustainable greater amberjack aquaculture industry is the reliable reproduction control of the species. It is well known that many fish species exhibit different reproductive dysfunctions when reared in captivity (Mylonas et al., 2010). The most commonly observed reproductive

dysfunction in fish maintained in aquaculture facilities is the failure to undergo oocyte maturation (OM) after vitellogenesis is completed in females, and the production of lower quantity of sperm in males (Mañanos, et al., 2009; Mylonas & Zohar, 2001; 2007; Zohar & Mylonas, 2001). Although greater amberjack reproduction in captivity has been achieved after hormonal treatments (Mylonas et al., 2004) and the occurrence of spontaneous spawning has been reported in Europe (Jerez et al., 2006) and in Japan (Kawabe, et al., 1998; Kawabe, et al., 1996), a reliable technology for reproduction control in aquaculture has not been achieved for this species.

Multiple GnRHa injections every 10 days have succeeded in producing a high number of eggs in the Canary islands (Fernández-Palacios, et al., 2015). However, such repetitive handling may be stressful and damaging to the brood fish and in situations where the broodfish are very large (e.g., groupers, amberjacks or tunas) or kept outdoors—in ponds or cages—it is very time consuming and labor intensive to crowd, capture, anaesthetize and inject the fish with hormones (Mylonas, et al., 2010). As a result, a variety of hormone-delivery systems have been developed during the last 20 years for use in cultured fishes (Mylonas & Zohar, 2001). Additionally, it was shown that providing more space to large fish, as the Pacific northern bluefin tuna *Thunnus orientalis*, can result in higher fecundity (Masuma, et al., 2011), so a cage spawning option could be beneficial over the tank spawning option, where usually the volume of the tanks is limited.

In the present study we examined the potential of greater amberjack broodstock to spawn in sea cages and developed methods to collect eggs after spawning adapted to the cage facility, as it was shown with other large farmed species like Atlantic bluefin tuna *Thunnus thynnus* (De Metrio, et al., 2010; Mylonas, et al., 2007) and Pacific northern bluefin tuna (Masuma, et al., 2011).

Materials and methods

Broodstock maintenance

Fish were wild collected either from Ionian or Aegean Sea, Greece as juveniles. Broodstock maintained in different locations (ARGO: Argosaronikos Fishfarms SA, Salamina, Greece; GMF: Galaxidi Marine Farms SA, Galaxidi, Greece; HCMR: Hellenic Centre for Marine Research, pilot sea-cage farm, Souda, Chania, Crete, Greece) and reared in sea-cages. Eighty-nine fish were utilized in 2014 of 6.3-14.8 kg, 69 fish in 2015 of 9.0-19.5 kg, 35 fish in 2016 of 11.8-21.5 kg and 7 fish in 2017-2018 of 14.2-22.1 kg, respectively (**Table 1**). Broodstock 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. In Souda fish were kept in a cage of 40 m circumference and 12 m depth. Measurements of temperature and dissolved oxygen in different facilities were measured from 1 to 7 times a week.

Table 1. Description of the various broodstocks maintained for this study.

2014

Stock	Number of individuals	Size at sampling (range in kg)	Feeding
ARGO	49	7.1-16.0	live, raw fish
GMF	28	6.3-15-6	live fish
SOUDA	12	7.4-14.8	moist pellet

2015

Stock	Number of individuals	Size at sampling (range in kg)	Feeding
ARGO	28	10.7-19.5	moist pellet, raw fish

GMF	28	9.0-18.0	live fish
SOUDA	13	9.9-18.4	moist pellet

2016

Stock	Number of individuals	Size at sampling (range in kg)	Feeding
GMF	28	11.8-21.5	live fish
SOUDA	7	14.0-20.7	moist pellet

2017-2018

Stock	Number of individuals	Size at sampling (range in kg)	Feeding
SOUDA	7	14.2-22.1*	moist pellet

*Weight from June 2017

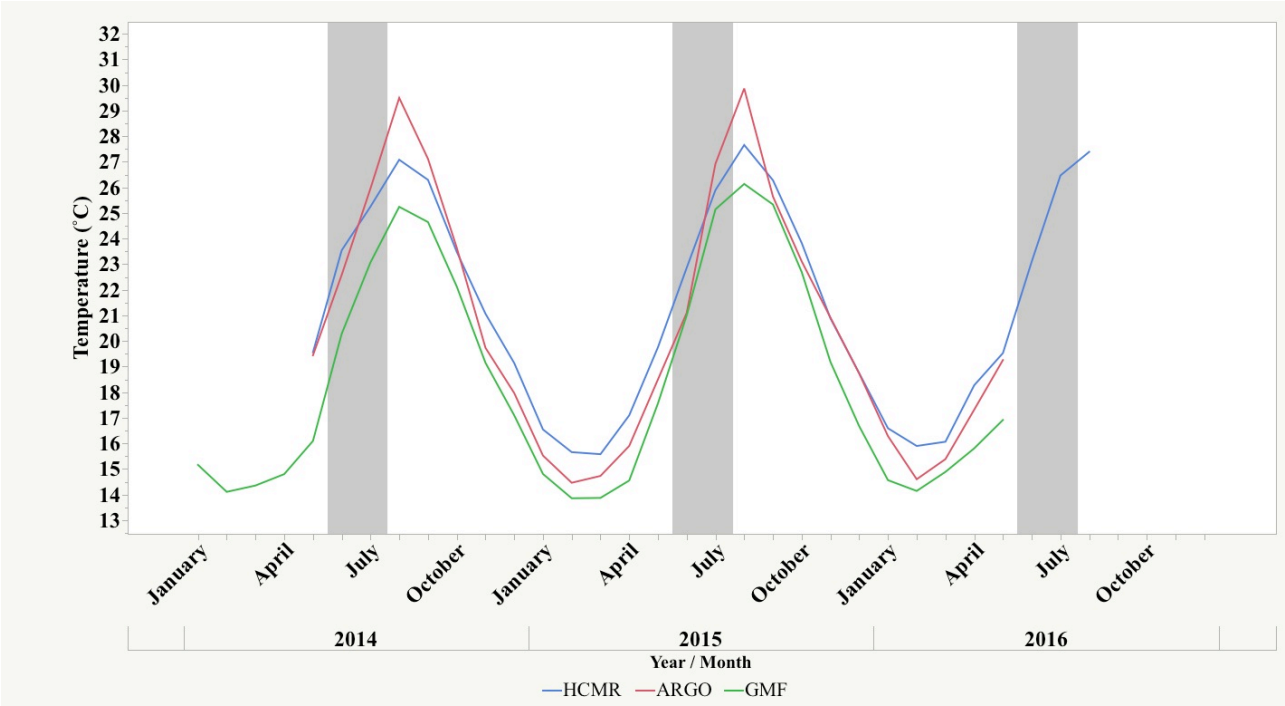

Figure 1. Representative water temperature profile for the greater amberjack *Seriola dumerili* broodstocks for the period 2014-2016, maintained in sea-cages. Bars indicate the estimated breeding period.

Evaluation of reproductive stage and broodstock selection

Broodstock selection for spawning induction experiments was done after 2 days starvation period. Fish were initially tranquilized in a bounded sack 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 produced sperm quantity of the captive greater amberjack, milt samples were obtained by cannulation as described above for the females. Milt was kept on ice until further quality evaluation.

Spawning induction experiments

When the fish were in appropriate reproductive maturational stage implants of GnRHa were administered. Broodstock moved to a sea-cage which was equipped with an egg collection device consisted of a two-piece curtain deployed around the perimeter of the cage. The designed egg collector is a passive trapping device, which restricts the movements of floating eggs within the cage, on the water surface. Eggs of pelagic fish containing a lipid droplet rise to the water surface in calm weather. Egg collectors limit the movements of eggs inside the cage because is mounted on the net of the cage, like a "curtain" and does not allow water movements. The egg collector consists of two sections. The "lower" section is secured on the net of the cage throughout its perimeter through portholes in the tarpaulin every 30 cm (**Fig. 2**). This section starts at about 30 cm above the water line and goes down to about 3.5 m in depth. The "upper" section is hanging from the rails of the cage using ropes every 30 cm along the perimeter of the cage, and drapes down the cage over the lower section, overlapping with the top 1.5 m below the water surface (**Fig. 2**). The objective of this two-piece design is to allow wind pressure to be relieved by allowing the upper section on the windward side to lift above the water, while the leeward side is push tightly against the net and the lower section, thus preventing any eggs from "jumping" over the cage and being lost (**Fig. 3**).

Figure 2. The "lower" section (left photo) together with part of the "upper" section (right photo) of the egg collector in Souda Bay, during installation. The lower section is attached to the cage net using cable ties, while the upper section is hanging from the rail using ropes.

Figure 3. The function of the two-piece design of the egg collector is to allow wind pressure to be relieved by allowing the upper section on the windward side to lift above the water (left photo), while the leeward side (right photo) is pushed tightly against the net and the lower section, thus preventing any eggs from "jumping" over the cage and being lost.

After the cessation of spawning, evaluation of reproductive stage of the fish was done and if in appropriate maturational stage a 2nd treatment was given. At the end of the experiment fish were transferred to their original position.

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 using a Neubauer haemocytometer under 200X magnification (in duplicate) in 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

Eggs were collected every morning into a 10-l bucket and their number (fecundity) was estimated by counting the total number of eggs in a sub-sample of 5 or 10 ml (depending on the total number of eggs), after vigorous agitation. Fertilization success was evaluated at the same time by examining each of the eggs in this 5 or 10 ml for the presence of a viable embryo using a stereoscope. When eggs were found multiple egg collection trials were done during the day, until no eggs were found anymore.

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. (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 egg and sperm quality parameters were tested using one-way Analysis of Variance (ANOVA) followed by Tukey's HSD post hoc test. Percentage data were Arcsine transformed prior to statistical analyses to normalize variances. Results are presented as mean ± standard error (SEM), unless mentioned otherwise. A level of $P<0.05$ was set as statistically significant. Statistical analysis was performed with JMP 12.

Results

2014 Spawning season

The egg collectors (**Fig. 2, 3**) were placed in each site after the initial sampling for reproductive evaluation, *i.e.* on 23/6/2014 in P1.HCMR, 25/6/2014 in P23.ARGO, 26/6/2014 in P40.GMF. The lower section of the egg collector went down to 3.5 m in depth, while the cage was 6-m deep in P1. HCMR and 8-m deep in P23.ARGO and P40.GMF.

The initial sampling for evaluation of reproductive maturation was done in P23.ARGO facilities on 13th of May 2014 in one of the two cages (cage A). All sampled males produced sperm, which was accessible only with a catheter. The sperm was motile upon activation with seawater, having initial motility of 10-80%. Almost all females were in vitellogenesis (Vg) with oocytes of 450-650 µm in diameter. On 25th of June 2014 the sampling was done using fish of the second cage (Cage B). Males had IT sperm, while females were in all stages of development including po, eVg, Vg, as well as one female having ovulated oocytes in its ovary, indicating that it ovulated spontaneously. However, in almost all females there was a high occurrence of AT. Sperm motility ranged between 0-100%. Five males and females, respectively, were given a GnRHa treatment but no eggs were collected the following days.

The sampled females from HCMR stock on 23th of June 2014 were in Vg with a significant number of oocytes in early oocyte maturation (OM) with oocytes of 680-700 µm in diameter (**Fig. 4A and B, 5**). All sampled males produced IT sperm with initial motility of 70-100%, motility duration was 2.4 – 4.5 min and density was 10 - 31 x 10^9 szoa ml^{-1} (**Fig. 6**). Four males and three females were treated with GnRHa implants. Fish started spawning after 48 h, and they were spawning for 6 days (3 spawns) after implantation (**Fig. 7**). The fertilization success ranged between 67-90%. Upon re-examination of the fish when spawning appeared to stop, it was found that the females sampled continued to contain post-Vg oocytes of a diameter of 630-700 µm, together with oocytes in early OM as well as post ovulated oocytes (from the spawning of two days before). So, it was decided to treat the fish with another GnRHa implants. Females received the same dose of GnRHa implants as in the 1st treatment, while the males were not given any hormonal treatment, as they appeared to be spermiating well. At this time it was much easier than the first time to obtain the sperm using the catheter, as the genital pore was enlarged and sperm could be taken with the slightest aspiration. At this time, sperm motility was 70-90%, motility duration 2.0-6.2 min and density 18-28 x 10^9 szoa ml^{-1} (**Fig. 6**). In response to this second GnRHa implantation of the females, a single spawning was collected (**Fig. 7**).

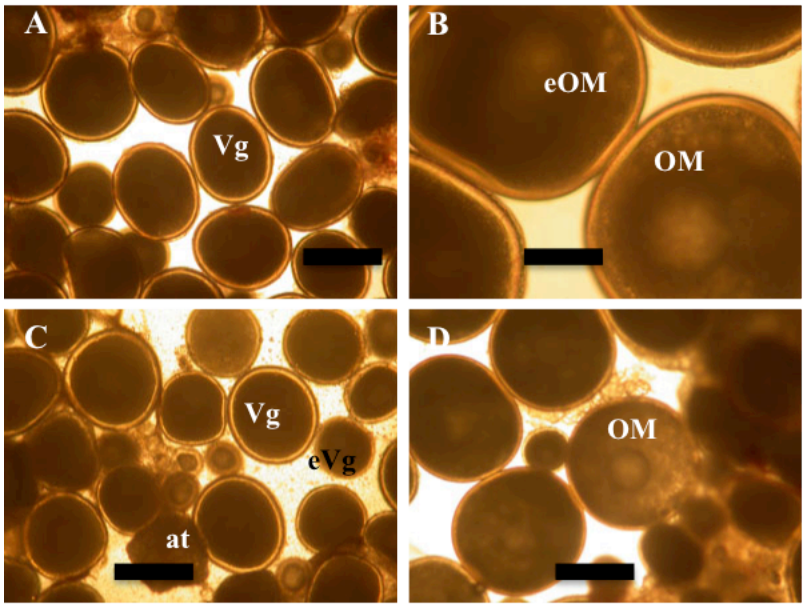

Figure 4. Wet mount photographs of greater amberjack oocytes obtained from the P1.HCMR and GALAXIDI broodstocks maintained in sea cages during 2014 spawning season. (A and B) Females on 23/6/2014 at the P1.HCMR Souda Bay cages, being in full vitellogenesis or in oocyte maturation. (C and D) Females on 26/6/2014 at the P40.GALAXIDI cage, being in full vitellogenesis with some females in oocyte maturation, and some signs of apoptosis/atresia. at = atresia/apoptosis, eOM = early OM, OM = oocyte maturation, Vg = vitellogenic oocytes. Bar = 200 µm.

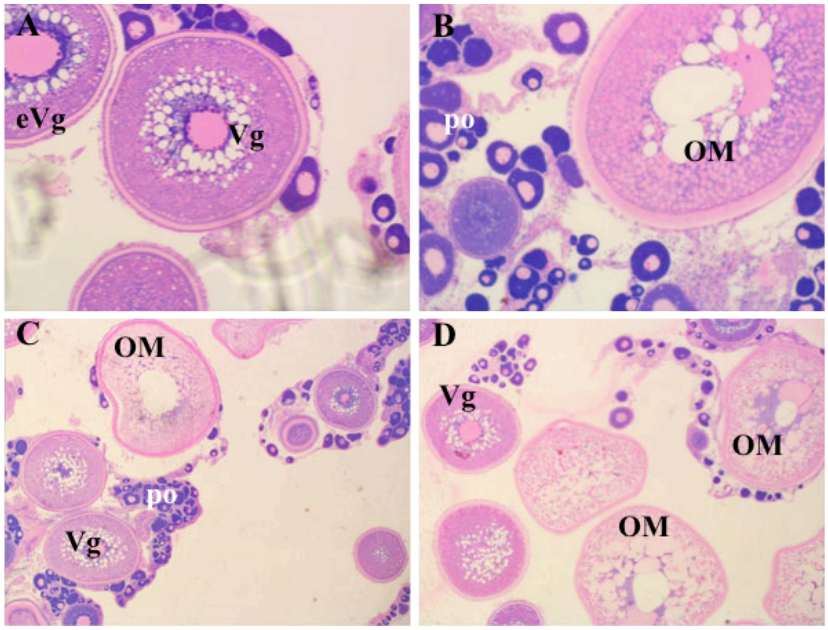

Figure 5. Histological sections of greater amberjack oocytes obtained from the P1.HCMR and GALAXIDI broodstocks maintained in sea cages during the study, showing the various stages of oogenesis and oocyte maturation. at = atresia/apoptosis, eVg = early vitellogenic oocytes OM = oocyte maturation, po = primary oocyte, Vg = vitellogenic oocytes.

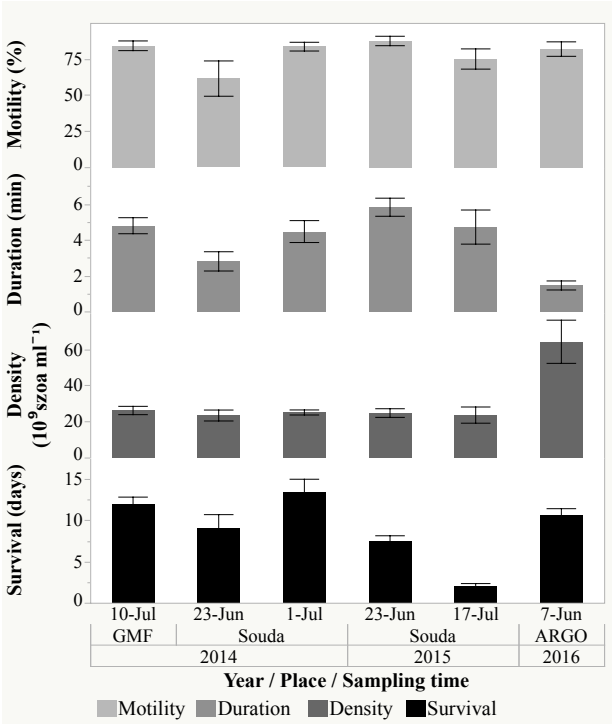

Figure 6. Representative sperm quality parameters from various stocks of greater amberjack sampled during the 2014-2016 reproductive seasons.

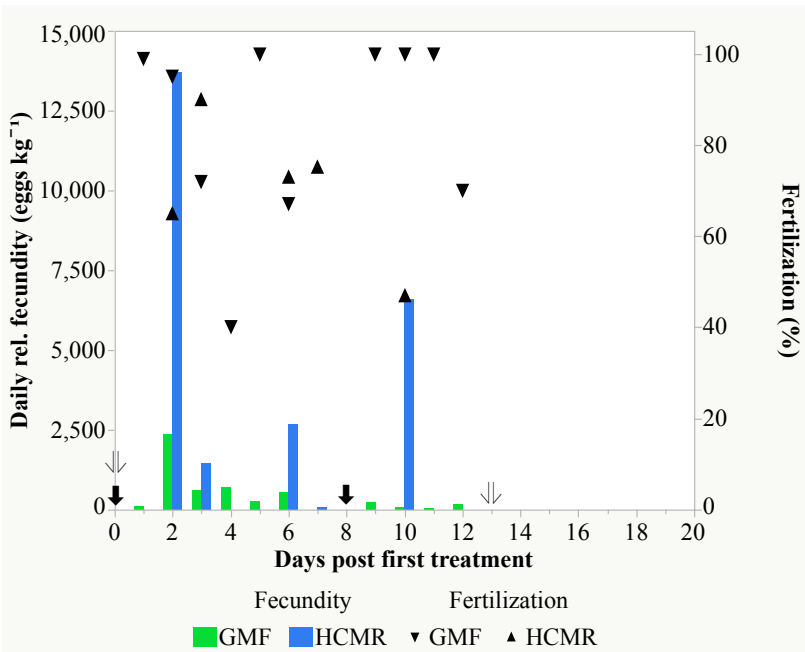

Figure 7. Daily relative fecundity (bars, eggs kg^{-1} female BW) and fertilization success (marks, %) of greater amberjack reared in P1.HCMR or P40.GMF sea-cages in 2014 breeding season (Day 0 was 23 June for P1.HCMR and 26 June for P40.GMF, respectively). Arrows indicate the time of treatment (thick for P1.HCMR, dashed for P40.GMF).

At P40.GMF stock on 26th of June 2014 all females were at Vg with oocyte diameters of 500-700 µm and very little occurrence of AT (**Fig. 4C**). In addition, some females were found to contain oocytes in advanced OM (**Fig. 5**) and some that had already ovulated spontaneously (**Fig. 4D**). Male fish produced IT sperm with initial motility of 60-100%. Ten males and 14 females received GnRHa implants and were allowed to spawn. Small numbers of fertilized eggs were collected the following days (**Fig. 7**). Upon re-examination of the fish when spawning appeared to decrease, it was found that the females sampled continued to contain post-Vg oocytes of a diameter of 650-800 µm, together with oocytes in early OM or advanced OM, as well as post ovulated oocytes. So, it was decided to treat the fish with another GnRHa implant (**Table 3.2.3**). Male fish produced IT sperm and were implanted again with GnRHa. Six females received GnRHa implants of 750 mg and 8 males of 450 mg GnRHa. Sperm motility was 60-100%, motility duration 2.6–7.3 min and spermatozoa density 5.7-35.7 x 10^9 szoa ml^{-1} (**Fig. 6**). Fish have not spawned after the 2nd treatment (**Fig. 7**).

2015 spawning season

The egg collectors were placed in each site after the initial sampling for reproductive evaluation, *i.e.* on 9/6/2015 in P23.ARGO, 10/6/2015 in P40.GMF and 23/6/2015 in P1.HCMR. At that time, the egg collectors were placed to an increased depth of 5 m compared to 3.5 m of the 2014 spawning season. Also, the cage depth was reduced to avoid loss of eggs from the bottom of the cage.

On 9 June 2015 in P23.ARGO facilities male fish produced IT sperm, which was motile, having initial motility of 45-80%. Almost all females were in Vg with oocytes of 660-690 µm in diameter, with little occurrence of AT in one fish. Two females were found in Oocyte Maturation (OM) or just prior to Ovulation (Ov) stage with oocytes at 1000 µm in diameter (**Fig. 8A,B**). Eleven female and nine male fish were treated with GnRHa implants and were left in the cage to spawn. Only 16,000 eggs were collected once, two days after treatment (**Fig. 10**). On 2 July 2015 male fish had IT sperm of 30-85% initial motility and motility duration of 0.85 - 4.05 min. Females were in Vg stage of 600-680 µm with some occurrence of AT. Some females were in post ovulation stage with po and occurrence of AT (**Fig. 9A**). Since just a few eggs (16,000) were collected from the cage during the previous sampling, no fish were induced to spawn in the cage this time.

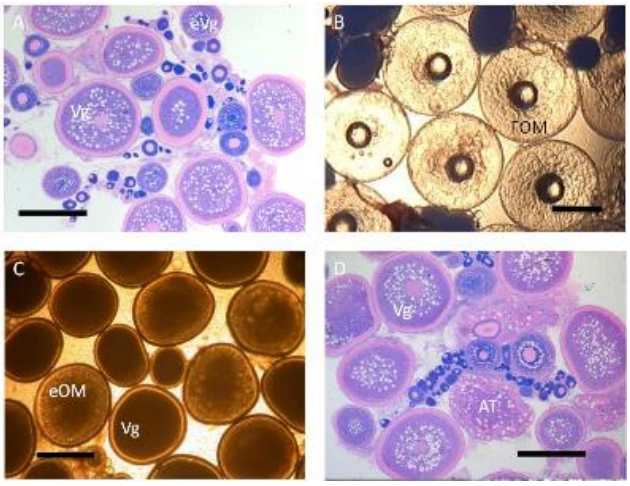

Figure 8. Female greater amberjack maintained in sea cages (1st sampling, June 2015). Histological sections (A,D) and wet mount photographs (B,C) of greater amberjack oocytes obtained from the P23.ARGO (A,B), P1.HCMR (C), and P40.GMF broodstocks (D). A: Female on 9/6/2015 in vitellogenesis (Vg) having also early Vg oocytes. B: Female on 9/6/2015 in Final Oocyte Maturation (FOM). C: Females on 23/6/2015 in vitellogenesis (Vg) and some oocytes in early Oocyte Maturation (eOM). D: Females on 10/6/2015 in Vg and occurrence of AT. Bar = 500 µm.

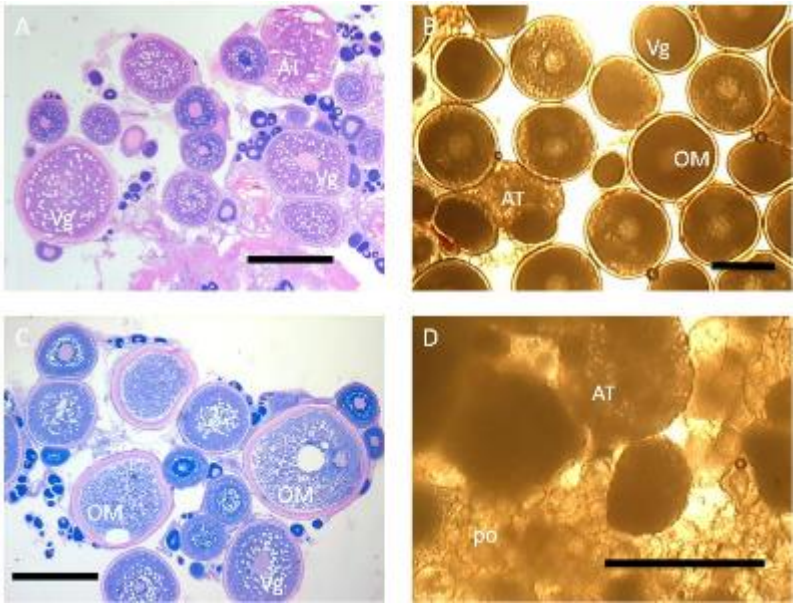

Figure 9. Female greater amberjack maintained in sea cages (2nd sampling, July 2015). Histological sections (A,C) and wet mount photographs (B, D) of greater amberjack oocytes obtained from the P23.ARGO (A), P40.GMF (B,C) and P1.HCMR (D) at the second sampling of the broodstocks, after an initial induction of spawning with GnRHa implants. A: Female on 2/7/2015 in vitellogenesis (Vg) having also occurrence of atresia (AT). B,C: Female on 1/7/2015 in Oocyte Maturation (OM) having also Vg oocytes. D: Female on 17/7/2015 with primary oocytes (po) and occurrence of AT. Bar = 500 µm.

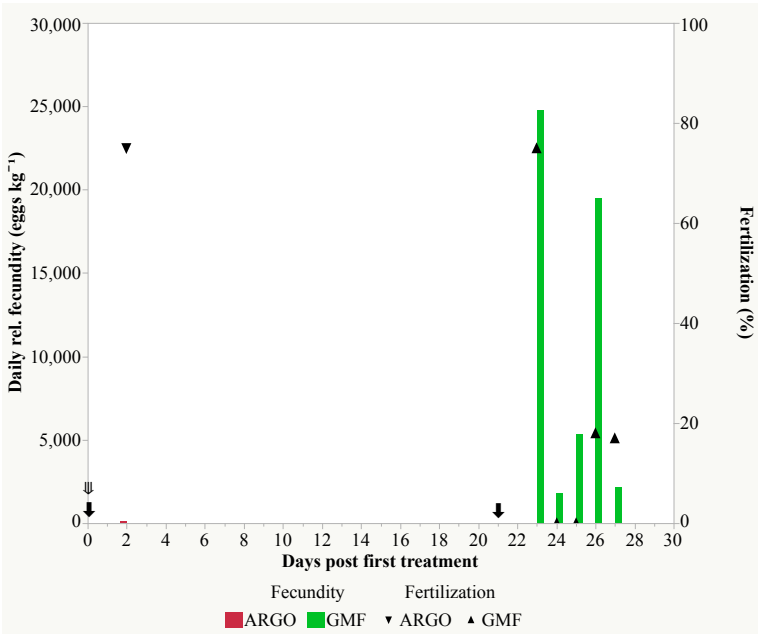

Figure 10. Daily relative fecundity (bars, eggs kg^{-1} female BW) and fertilization success (marks, %) of greater amberjack reared in P23.ARGO or P40.GMF sea-cages in 2015 breeding season (Day 0 was 9 June for P23.ARGO and 10 June for P40.GMF, respectively). Arrows indicate the time of treatment (dashed for P23.ARGO, thick for P40.GMF). After the 2nd treatment in P40.GMF fish were maintained in a plastic sack.

The five sampled females of P1.HCMR stock were in Vg with a significant number of oocytes in early OM (eOM) with oocytes of 640-780 µm in diameter (**Fig. 8C**). All sampled males produced IT sperm with initial motility of 75-100%, motility duration was 3.4 – 8.1 min and density was 1.56 – 3.04 x 10^{10} szoa ml^{-1} (**Fig. 6**). Thirteen fish were treated with GnRHa implants but no eggs were collected the following days. During the 2nd sampling male fish had IT sperm. Initial motility of spermatozoa was 35-90% and motility duration 3.85 - 9.86 min, while density was 1.92 – 4.48 x 10^{10} szoa ml^{-1} (**Fig.6**). Females were immature having only po, while one fish had occurrence of AT (**Fig. 9D**).

The females of P40.GMF stock were at Vg on 10 June 2014 with oocyte diameters of 650-700 µm and occurrence of limited AT in 30% of the female fish (**Fig. 8D**). Male fish produced IT sperm with initial motility of 50-85%. Sixteen fish were treated with GnRHa implants and were left in the cage to spawn. In the sea cage no eggs were collected. On 1 July 2015 females were again in Vg with oocytes of 550-780 µm, while some of them were in different stages of OM. One female found to be in post ovulation stage with increased occurrence of AT, and still Vg oocytes (**Fig. 9B,C**). Males had IT sperm of 40-80% initial motility and motility duration of 4.07 - 8.97 min. Eight fish were treated with GnRHa implants for the second and were transferred in a plastic sack into the cage (which is used for anesthetizing fish), filled with seawater (**Fig. 11**). In the anaesthetic bag, a total of 3,020,000 eggs were produced with fertilization success 0-75% (**Fig. 10**).

Figure 11. The anaesthetic bag (plastic sack) used for sampling the fish at P40.GMF. At the conclusion of the sampling and GnRHa treatment, a small number of fish were placed in this bag and were left there for 6 days to verify that they would spawn.

2016 spawning season

The egg collectors were mounted again to a depth of 5-m deep, as in 2015. However, this year the bottom of the cages was lifted even more, so that all the vertical sides of the cage were covered with the egg collector. At both the GMF and HCMR site, the bottom of the cage was covered with an extra fine mesh to reduce the possible currents that remove the eggs from the egg collector, and also prevent the eggs from passing

through (**Fig. 12**). At HCMR, the fish were allowed to spawn spontaneously without any hormonal treatment, as we saw in the previous year that at any time we sampled the fish to induce them to spawn, some females contained oocytes at OM, suggesting that some spontaneous spawning does take place without any hormonal therapies. At GMF, the fish were induced to spawn and remained in the cage for spawning.

Figure 12. Underwater photo of the broodstock cage of HCMR, Souda Bay, at a depth of 5 m showing the new modified egg collection system. The blue tarpaulin (egg collector) is covering the side of the cage until 0.5 m from the bottom, while the green shading mesh is covering the bottom of the cage. A side section of about 0.5 m of the side of the cage is left unblocked to allow for water exchange in the cage.

In mid-June males in GMF stock did not release any sperm quantity after abdominal pressure, but IT sperm collection was possible. Sperm motility was 0-90%. At the same time, females were in Vg stage with oocytes of 670-740 μm in diameter (**Fig. 13A**). Only one female was found to contain atretic oocytes in its ovarian biopsy. On 16/6 seven fish were treated with GnRHa implants and were left in the cage to spawn. Fish started spawning two days later and eggs were collected from the sea cage once. Only 200,000 floating eggs were possible to be collected in cage (**Table 2**).

In HCMR, early July males had IT sperm of low sperm motility (20-35%). Females on the other hand had mostly PO in their ovarian biopsies (**Fig. 13B**). At the same time, AT was present at 75% of the females, while in one female there were signs of possible previous ovulation. Although no eggs were collected, during the spawning period a significant number of wild juveniles (probably *Trachurus* sp) were found in several cases (and then removed) in the experimental cage indicating a possible foraging activity.

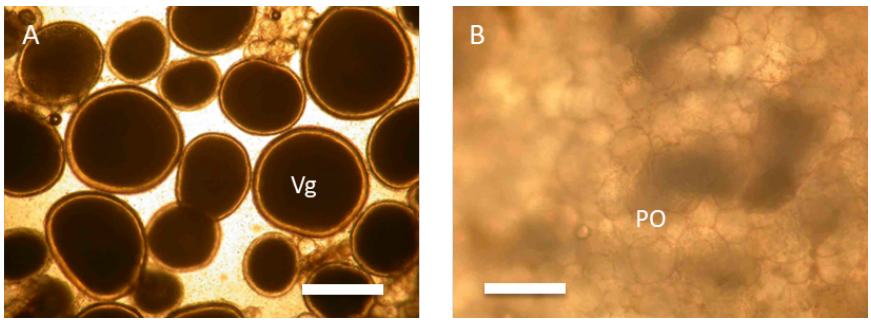

Figure 13. Female greater amberjack maintained in sea cages during 2016. Wet mount photographs (A) from GMF and (B) HCMR sea cages broodstock. A: Female on 16/6/2016, in vitellogenesis (Vg). B: Female on 6/7/2016 having only primary oocytes PO. Bar = 500 µm.

Table 2. Egg collection in sea cages after or without hormonal treatment with GnRHa implants of greater amberjack broodstock.

2016

Stock	Number of GnRHa treated individuals	Spawn number	Eggs (number or g)
GMF	3	1	200.000
HCMR Souda	0	1	eggs*
	0	2	500 g
	0	3	3 g
	0	4	5 g
	0	5	10 g
	0	6	2 g

*eggs were present but not collected

2017 spawning season

The stock that was used for the purposes of Task 3.5 in 2017 was the one of HCMR. In the HCMR sea cage the same trial with 2016 was repeated leaving the fish to spawn spontaneously, without any hormonal treatment. However, instead of not examining the fish for maturity status until the end of the reproductive season in July, we planned to intervene in the middle of the season in June. This was done in order to (a) document how many fish were spawning in the days before and (b) to induce all the fish to spawn so that we could determine what percentage of the spawned eggs we would collect. At the end, a very small number of eggs (a few 1000s) was collected at three times (**Table 3)** and when the fish were evaluated towards the end of June, females had mostly POs and AT oocytes, with two of them having signs of possible previous ovulations and males had IT sperm with sperm motility 35-75% and motility duration 1.77-2.12 min.. So, unfortunately again we were not able to conclusively confirm that the egg collecting method used, with complete covering of the cage walls and extensive covering of the bottom of the cage is capable of being

used for the large scale collection of fertilized eggs from greater amberjack, since we do not know (a) how many fish spawned and (b) how many times. Again in 2017 during the spawning period significant numbers of wild juveniles (probably *Trachurus* sp) were found and removed in several cases in the experimental cage.

Table 3. Egg collection in HCMR sea cages without hormonal treatment with GnRHa implants of greater amberjack broodstock.

2017

Stock	Number of GnRHa treated individuals	Spawn number	Eggs (number or g)
HCMR Souda	0	1	15 g
	0	2	10 g
	0	3	4 g

2018 spawning season

In 2018 the stock that was used for the purposes of Task 3.5 was again the one of HCMR. The same trial with 2016 was repeated leaving the fish to spawn spontaneously, without any hormonal treatment. This year however the net of the cage was changed. Instead of using the standard net with openings of 22 mm, it was replaced with one for fry with openings of 5 mm. This resulted in avoiding the entry of any wild juvenile in the cage minimizing any potential foraging activity on the newly spawned eggs

The fish were not sampled for the whole reproductive period. The total egg production was 1,195 – 1,265 g in 8 spawns (**Table 4**).

Table 4. Egg collection in HCMR sea cages without hormonal treatment with GnRHa implants of greater amberjack broodstock.

2018

Stock	Number of GnRHa treated individuals	Spawn number	Eggs (number or g)
HCMR Souda	0	1	80-100 g
	0	2	750 g
	0	3	20 g
	0	4	20 g
	0	5	200-250 g
	0	6	100 g
	0	7	10 g
	0	8	15 g

Statistical analysis

No statistical differences were found for egg production both between the different facilities and spawning periods. The fertilization success was increased in 2014 spawning season compared to 2015 spawning season (ANOVA, Tukey's HSD, P<0.05) (**Table 5**). Statistical differences were found in sperm quality characteristics between the different spawning periods. Specifically, motility duration was lower in 2016, compared to 2014-2015 spawning seasons, while the opposite was found for the sperm density (ANOVA, Tukey's HSD, P<0.05) (**Table 6**). Sperm survival in 4°C was higher in 2014 and 2016 compared to 2015 spawning season (ANOVA, Tukey's HSD, P<0.05).

Table 5. Mean (± SEM) daily relative fecundity (10^3 eggs kg^{-1}fish) and fertilization success (%) for spawns (n value in parenthesis) under cage spawning conditions. Lowercase letters indicate statistical differences between the years (ANOVA, Tukey's HSD, P<0.05).

Spawning condition	Mean		2014		2015		2016	
Cage spawning	Rel. fec. (10^3eggs kg^{-1} fish)	Fer. (%)	Rel. fec. (10^3eggs kg^{-1} fish)	Fer. (%)	Rel. fec. (10^3eggs kg^{-1} fish)	Fer. (%)	Rel. fec. (10^3eggs kg^{-1} fish)	Fer. (%)
ARGO	0.1	75			0.1 (1)	75 (1)		
GMF	3.8±1.8	66±10	0.5±0.2 (10)	84±7 (10)	10.7±4.8 (5)	22±14 (5)	2.7 (1)	100 (1)
HCMR	5.5±2.1	65±8	4.9±2.5 (5)	70±7 (5)			8.5 (1)	39 (1)
Mean	4.1±1.4	66±7	2.0±0.9	80±5[a]	8.9±4.3	31±14[b]	5.6±2.9	70±31[ab]

Table 6. Mean (± SEM) sperm motility (%), motility duration (min), sperm density (10^9 spermatozoa ml^{-1}) and survival (days) from greater amberjack *Seriola dumerili* males reared in sea-cages for the period 2014-2016. Lowercase letters indicate statistical differences between the years for the same rearing condition (ANOVA, Tukey's HSD, P<0.05).

Cage spawning					
Year	N	Motility (%)	Duration (min)	Density (10^9 szoa ml^{-1})	Survival (days)
2014	26	78±4	4.16±0.33[a]	25.1±13.6[b]	12±1[a]
2015	16	81±4	5.26±0.54[a]	24.1±2.5[b]	5±1[b]
2016	7	82±5	1.45±0.25[b]	64.2±12.0[a]	11±1[a]

Mean	49	80±3	4.13±0.30	30.3±2.8	9±1

Discussion

Spawning induction and egg collection of greater amberjack in cages was successful, but rather inefficient if we consider the number of eggs collected from the cages compared to the tanks (Fakriadis, et al., 2017). Although the cage sites were in areas protected from the wind and no strong wave action was observed, the failure to collect eggs in sea cages is perhaps due to low buoyancy of eggs immediately upon spawning (and until full hydration is completed) or strong currents in the cage facilities. In order to confirm that the fish left in the sea cages did actually spawn in response to the GnRHa therapy, a test was held with the anaesthetic plastic bag in GMF and the amount of eggs collected using this method was comparable to the produced amount of the tank, confirming that the lack of egg collection from the cages was not because of failure of the fish to spawn, but of failure to collect the spawned eggs, as they were carried away by the current. The same difficulty to collect fertilized eggs from the cages in a study with the Atlantic bluefin tuna was correlated with the high current, but also due to some moderate wave action (Mylonas, et al., 2007). In that study, it was considered the fertilized eggs were quickly dispersed throughout the water column and were carried outside the cage by the current and wave action contrary to what happened in Japan, where Pacific bluefin tuna spawning takes place in similar cages, the egg collection was reported to be significantly more efficient (Kumai, 1998; Masuma, 2006; Sawada, et al., 2005) obviously because the location of the cages in weather protected natural coves limits the effects of strong current and wave action on the dispersion of the eggs in the water column. In the present study, the location of the cages was inside protected natural bays but still the egg collection was difficult. The development of land-based breeding facilities will ensure the proper collection of all fertilized eggs produced, either in response to GnRHa-induced or spontaneous spawning.

Spontaneous natural spawning (without any GnRHa therapy) also occurred in all sites confirmed by the ovarian biopsies during the reproductive evaluation of the fish prior to spawning induction. However, collection of eggs after natural spawning was achieved only at the HCMR site, where the fish were left to spawn without any hormonal treatment in the 2016-2017 breeding seasons, suggesting that it is possible for some eggs to be collected from the cages, without any hormonal stimulation. However, the very small amount that was collected suggests that (a) only a very small percentage of the females spawned and (b) only for a limited number of spawns, contrary to what has been achieved in response to a hormonal therapy with GnRHa. Unfortunately, we were not able to conclusively confirm that the egg collecting method used is capable of being used for the large-scale collection of fertilized eggs from greater amberjack, since we do not know (a) how many fish spawned and (b) how many times, even if we sampled the broodstock at two different times in the spawning seasons of 2016 and 2017. Greater amberjack is known to reproduce in captivity spontaneously (Jerez, et al., 2006), but egg production is scarce and unpredictable or with unfertilized eggs (Rodríguez-Barreto, et al., 2014).

Apart from the low number of eggs produced in cage spawning, even if different setups were tested, it was observed in the hatchery that had been collected eggs from other species also, that were naturally spawning in the same area the same period, which created problems in the greater amberjack larval rearing procedures. That fish had higher growth rate at this stage and fed with the greater amberjack newly hatched larvae. The incidental collection of other fish species eggs can not be avoided in sea cages, unless the sea cage is totally closed/protected from the entrance of outside seawater.

In conclusion, egg collection in the sea cages is feasible in greater amberjack after induced (using GnRHa implants) or spontaneous spawning. Unfortunately, it is impossible to collect similar number of eggs with the tanks unless the cage is totally closed from the outside seawater, as was tested using the anesthetic bag for spawning. It seems that tank spawning with cage rearing during the year is preferable for this species

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