Deliverable Report

Deliverable No:	D6.7	**Delivery Month:**		58
Deliverable Title	Spawning induction method for spontaneous spawning of wreckfish in large tanks			
WP No:	6	**WP Lead beneficiary:**		P8. IEO
WP Title:	Reproduction and genetics-wreckfish			
Task No:	6.7	**Task Lead beneficiary:**		P8. IEO
Task Title:	Development of spawning induction procedures.			
Other beneficiaries:	P1. HCMR	P3. IRTA	P15.ULL	P19. CMRM
	P32. MC2			
Status:	Delivered		**Expected month:**	54
……..				

Lead Scientist preparing the Deliverable: Fakriadis, I. (HCMR), Mylonas, C.C (HCMR),

Other Scientists participating: Blanca Álvarez-Blázquez (IEO), Peleteiro, J.B. (IEO), Pérez Rial, E. (IEO), Lluch, N. (IEO), Pérez, M. (IEO), Rodríguez Villanueva, J.L. (CMRM), Linares, F. (CMRM), Vilar, A. (MC2), Rodríguez, C. (ULL), Perez, J. (ULL), Duncan, N. (IRTA)

Objective: A method for the induction of spawning of wreckfish in large tanks will be developed. The deliverable will present the methodology to (a) manipulate the broodstock in the tank to apply GnRHa implants, (b) procedures and doses for hormone application, (c) control photothermal conditions in large tanks and (d) methods to actually retrieve the eggs from the water surface. In addition, the deliverable will include the results from repeated trials and refining methodologies including the following data: standard parameters maintained between the different stocks, number of eggs obtained per kilo of female, egg quality parameters, timing of application of hormones in relation to egg collection.

TABLE OF CONTENTS

Introduction

Aquaculture will definitely be the most important seafood production technology in the years to come due to the increasing demand for a variable range of fish products. The diversification of aquaculture production could result both from the expansion of aquaculture production to new/emerging species and the diversification of the derived fish products. The wreckfish *Polyprion americanus* is one of the most prominent species for the above-mentioned diversification due to its fast growth (Machias et al., 2003; Papandroulakis et al., 2004), late reproductive maturation (Peres and Klippel, 2003), high market price and limited fisheries landings (Peres and Haimovici, 2004). Although the wreckfish seems an excellent candidate for aquaculture production, the lack of reproduction control and larval rearing protocols are considered major bottlenecks preventing the establishment of the wreckfish aquaculture.

The wreckfish is a gonochoristic species and information regarding its reproductive biology is limited (Peres and Klippel, 2003; Roberts, 1989). Recently, a more detailed study for the reproductive cycle of the species in captivity in different environmental conditions was held incorporating four different wild caught broodstock in Greece and Spain (Deliverable 6.5). It was mentioned that fish can adapt to captivity, mature and produce eggs both under fluctuating natural and under constant low temperatures. However, the fertilization success was generally low, even though males were fully spermiating good quality sperm and was correlated with a failing in the male breeding behavior. Reproductive dysfunctions have been recorded in different fish species in captivity, such as the greater amberjack *Seriola dumerili* (Zupa et al., 2017), meagre *Argyrosomus regius* (Mylonas et al., 2016) and sharpsnout seabream *Diplodus puntazzo* (Papadaki et al., 2018).

To overcome the observed reproductive dysfunctions different spawning induction protocols involving GnRHa implants and injections were tested combined with simulated photo-thermal conditions. The volume of the spawning tanks was also tested, since it has been suggested that groupers in large tanks have overcome the lack of spawning after ovulation (Marino et al., 2003). The experiments took place in Greece and Spain, incorporating four different broodstock facilities.

Materials and methods

Broodstock maintenance

P1. HCMR Stock

A stock of 5 wreckfish (captured from the wild as juveniles) has been maintained at P1.HCMR in Heraklion, Crete, Greece, in two 15-m^3 tanks, under simulated natural photoperiod and constant temperature (16°C). Unfortunately, two of the fish stopped eating in the summer of 2013 and eventually died prior to the reproductive season in 2014. On June 12, 2016 one of the males (6.0 kg) died, leaving the broodstock of HCMR with just one female (13.4 kg) and one male (9.5 kg). The fish were fed 3 times a week with raw fish (mackerel). Oxygen, temperature and water quality (NH3 –N and NO2-N) were measured once per week. For spawning induction experiments two different volumes of tanks were used, either 40 m^3 or 15 m^3.

P8. IEO Stock. A stock of 9 wreckfish (4 females, 3 males and 2 undetermined), weighing between 9.5 and 18.9 kg, were maintained during the years 2014- 2018 in a 130 m^3 tank with natural temperature (12-21ºC) and photoperiod. Animals were separated in two tanks and were fed 3 times a week, in one tank fishes were fed with semi-moist pellets elaborated at our research facility based on special fish paste normally used for parental diets, and in another tank fishes were fed with a specially designed wreckfish broodstock diet formulated and produced by SPAROS Lda. and P2. FCPCT. Each stock was sampled monthly during the period February-July (breeding season) and bimonthly during the period of resting August - January (**Fig. 1**).

Figure 1. Periodic samplings from the P8. IEO stock to monitor stage of reproductive development.

P19. CMRM Stock. A stock of 7 females with an average weight of 14.64±2.94 kg, 2 males with an average weight of 13.10± 0.57 kg and another undetermined fish of 14.8 kg, were reared in concrete tanks of 40 m^3 in open flow. The following parameters: oxygen, temperature and salinity, where monitored daily. The broodstock was fed during 2014 with Vitalis Repro/Vitalis Cal from Skretting and the food was changed at the end of this year, when the fish were observed to have too much fat. Subsequently, during 2015 to 2018 the broodstock were fed a mixture of hake and squid (1:1). The fish were fed three times a week with the dried feed and twice a week with the squid-feeding phase. They were fed until satiation. Samplings of weight and size for the fish were carried out regularly. Sex identification was conducted using ovarian biopsies in the case of females, and abdominal massage to obtain sperm, in the case of males. Prior to its manipulation, the fish were anesthetized with phenoxyethanol (0.3 mL/L).

P32. MC2 Stock. A stock of 27 wreckfish (11 females, 12 males and 4 unknown), weighing between 10.7 and 30.3 kg, were maintained in a 3500 m^3 exhibition tank (Nautilus) with natural temperature and photoperiod, and fed sliced fish and squid on a daily basis. When the first external evidence of reproductive maturation was detected (abdominal swelling), animals were transferred to a 50 m^3 tank for closer monitoring. Five females with evident abdominal dilatation were isolated, as well as three spermiating males. These males were replaced in two occasions due to aggressiveness between them. During the breeding season, the stock was sampled on a weekly basis to monitor the maturity stage of the females. Ovarian biopsies were taken from 9 females, to determine oocyte stage and diameter.

Evaluation of reproductive stage

Fish were starved for two days, before samplings for spawning induction experiments. Fish were initially tranquilized in their tank with the use of clove oil (0.01ml l^{-1}) and then transferred to an anesthetic bath for complete sedation with a higher respective concentration of clove oil (0.03ml 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) and photos were taken. 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.

To obtain sperm for evaluation, the genital pore was rinsed, blot dried and after gentle pressure the sperm was collected using a pipette. 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 urine or other somatic fluids. Spermiation index was reported on a subjective scale from 0 to 3, with S0 = no milt released, S1 = only a drop of milt released after multiple stripping attempts, S2 = milt was released easily after the first stripping attempt and S3 = milt was fluently released even without abdominal pressure.

Evaluation of sperm quality

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 fold dilution with 0.9% saline 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

Tanks were fitted with outflow egg collectors. 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 10 ml, after vigorous agitation. Fertilization success was evaluated at the same time by examining each of the eggs in this 10 ml for the presence of a viable embryo using a stereoscope. Photos of the eggs were taken to follow their development.

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 sperm quality parameters (for the time having two males) 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 significance was set at $P<0.05$. Statistical analysis was performed with JMP 12.

Results

P1. HCMR Stock

Sperm quality was fairly high during the whole 2014 reproductive season and though some significant variations were observed in different parameters (ANOVA, $P\leq0.05$), there was no trend indicating either a reduction during the season or improvement in response to the GnRHa implantation (data not shown) that was given to the fish at the start of the spawning induction experiment. The two males were in full spermiation (S3) during the next reproductive seasons as well, even in the summer and fall when the female was regressed. This was not the case at March – April 2016, where the produced sperm of one of the males was reduced (S0 or S1), probably because of an infection, which was treated with injectable antibiotic once a month for two months. Sperm quality parameters were evaluated and was fairly high and again no significant variations were observed in different parameters (ANOVA, $P\leq0.05$) (**Fig. 2**), even though the sperm from one male seemed to be a little yellowish and containing clumps (probably due to the before mentioned infection). Sperm at the time of each evaluation, after the death of one male on June 2016, was of good quality for both remaining 2016 and 2017 reproductive season (data not shown), even if only one male was left.

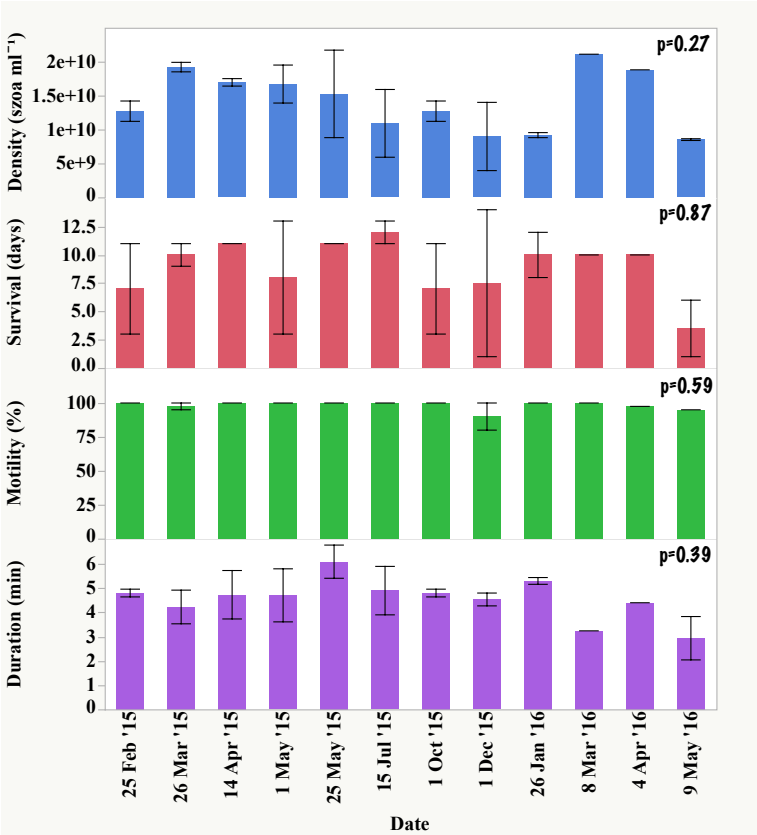

Figure 2. Sperm quality parameters of the wreckfish at P1. HCMR during the 2015-2016 reproductive season. No significant differences were observed. (ANOVA, $P < 0.05$).

On 28 April 2014, the female was undergoing vitellogenesis, having oocytes of 1325 µm in diameter (**Fig. 3**). On 12 May 2014, the female was examined again and at this time the ovaries contained not only vitellogenic oocytes (1250 µm), but also oocytes in oocyte maturation (1450 µm), while some eggs (25,000)

were also released in the tank, but were not fertilized (**Fig. 3**). The female was given a GnRHa implant (500 µg) and was placed together with one of the males (also given a 400 µg GnRHa implant), in a larger tank (40 m^3) for spawning. The first spawn was obtained on 16 May (52,000 eggs) and another spawn was obtained on 19 May (58,000), but in both cases a very small number of eggs were fertilized <<1%. A day later (20 May) the fish were sampled for maturity and the female contained post-ovulated eggs and many vitellogenic oocytes, some in atresia/apoptosis (**Fig. 3**), while the male was still in full spermiation. Both fish were implanted again with GnRHa and were returned to the tank for spawning. Another spawn was obtained on 23 May (3,000 eggs), but again no fertilization was observed.

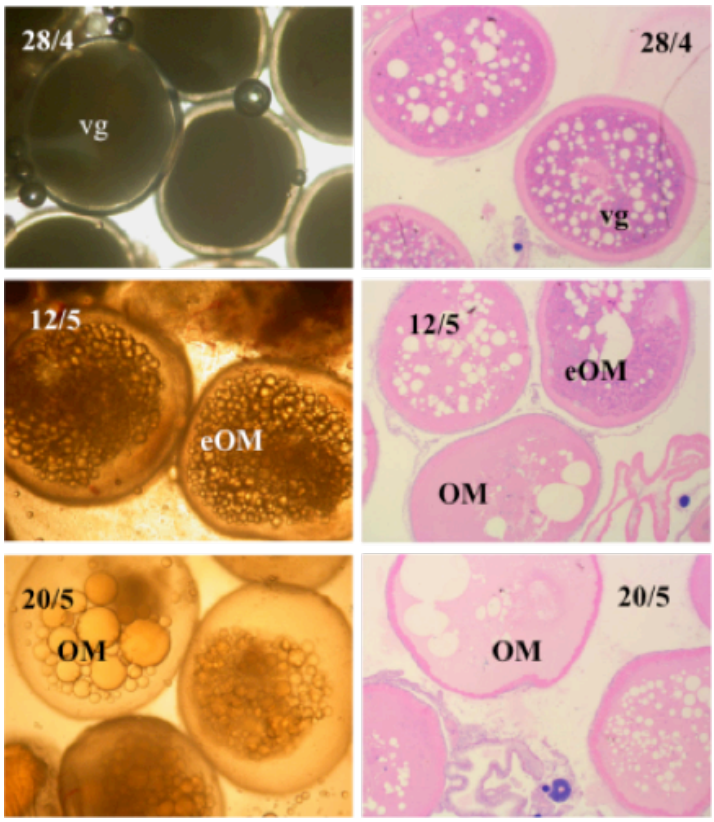

Figure 3. Wet mount and histological sections of biopsies from wreckfish during the 2014 reproductive season (dates on each photo). eOM = early oocyte maturation, OM = oocyte maturation, Vg = vitellogenic

A final effort to induce spawning was undertaken on 3 June, giving a higher dose of GnRHa (750 µg). At this time the female contained both post-ovulated eggs and vitellogenic oocytes, but with a high occurrence of atresia. The fish did not spawn again, and all three fish were removed from the tank on 6 June and the experiment was concluded for the first year of spawning induction trials. At that time, it was decided for the following years not to transfer the fish in a large tank (40 m^3) after spawning induction, since only two males and one female fish were available and the depth of the large tank, the parameter that was considered critical (Marino et al., 2003) was the same to the one (15 m^3) that maintained during the year and was also used after spawning induction trials.

In February 2015, the female was undergoing vitellogenesis (Vg), having oocytes of 1,025 µm in diameter (**Fig. 4.A**). In March, most of the oocytes were in Vg, with the maximum oocyte diameters between 950 and 1075 µm. There were some more developed oocytes with clear lipid coalescence (lc), which were between 1125 and 1250 µm in diameter. In addition, there were very few oocytes that looked much clearer, were

larger (1,450 µm) and they looked at Oocyte Maturation (OM) (**Fig. 4.B**). The same situation was observed during April, but the number of Vg oocytes had increased. On 24/4/2015, the female started spawning spontaneously a small number of dead eggs. On the morning of 1/5/2015, the female had spawned spontaneously 82,000 eggs with 56% fertilization success, while 35,000 eggs were stripped manually from the fish and were inseminated artificially (data for stripping trials will be presented in Deliverable 6.7). At this time (1/5/2015), the female fish also contained many Vg and early OM oocytes (**Fig. 4C**) and was induced to spawn with a GnRHa implant of 600 µg and was placed together with one of the males, which was also given a GnRHa implant of 300 µg. On 4/5/2015, the female contained oocytes in OM (1,525 µm in diameter), as well as ovulated eggs (2,250 µm) (**Fig. 5**). On 8/5/2015, 190,000 eggs were spawned spontaneously, having 12% fertilization success, but the embryos survived for only 5 days. The fish spawned up to 22/5/2015 and the biopsy taken on 25/5/2015 showed the presence of apoptotic oocytes and overripe eggs, while no Vg oocytes were found (**Fig. 5**).

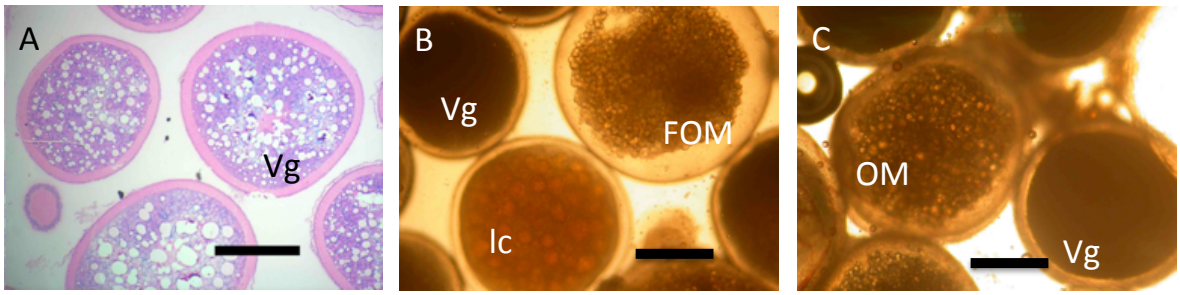

Figure 4. Microphotographs of histological sections (A) and wet mounts (B,C) from the ovary of the wreckfish maintained in P1 HCMR. A: In February 2015, in vitellogenesis (Vg). B: In March – mid April 2015, with oocytes in Vg, lipid coalescence (lc) and Final Oocyte Maturation (FOM). C: Female on 1/5/2015, with Vg, OM. Bar = 500 µm.

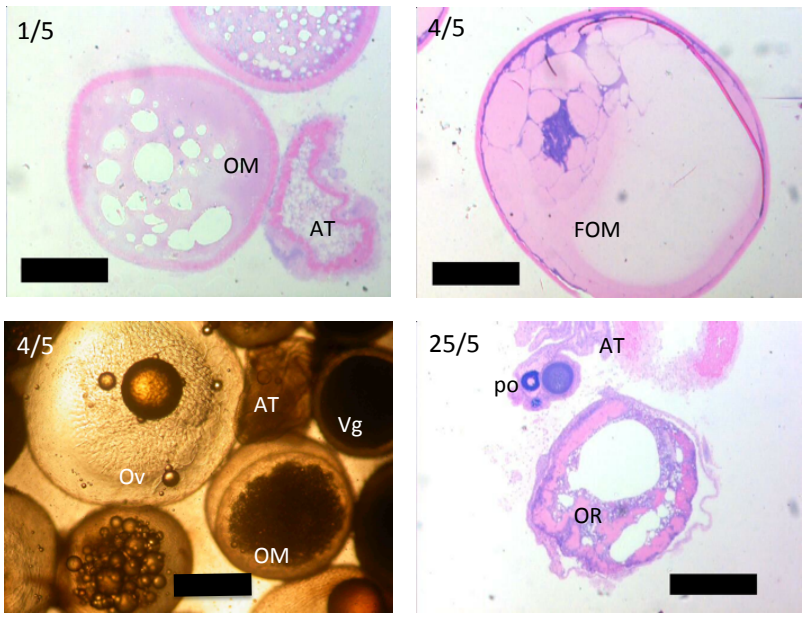

Figure 5. Histological sections or wet mount of ovarian biopsies from wreckfish during the 2015 reproductive season (dates on each photo). AT = atresia, Vg = vitellogenic, OM = early Oocyte Maturation, FOM = Final OM, Ov = Ovulated, po = primary oocyte OR = Overripe. Bar = 500 µm.

In April 2016, when the Vg oocyte diameters were at 1,150 µm while a few AT oocytes were present (**Fig. 6**), a first GnRHa treatment was given both to the female and males. The female was almost in the same situation after 4 days, with a small increase of the diameter of the Vg oocytes (1,250 µm) (**Fig. 6**). Some signs of early maturation were observed on 2/5/2016, with oocytes undergoing lipid coalescence (lc) stage with no further change in oocyte diameters (**Fig. 6**). At that time, a second GnRHa treatment was given to the female. After 7 days (9/5/2016) the fish spawned spontaneously ~1,000 eggs, but due to their small number, unfortunately the eggs were discarded by mistake before being evaluated.

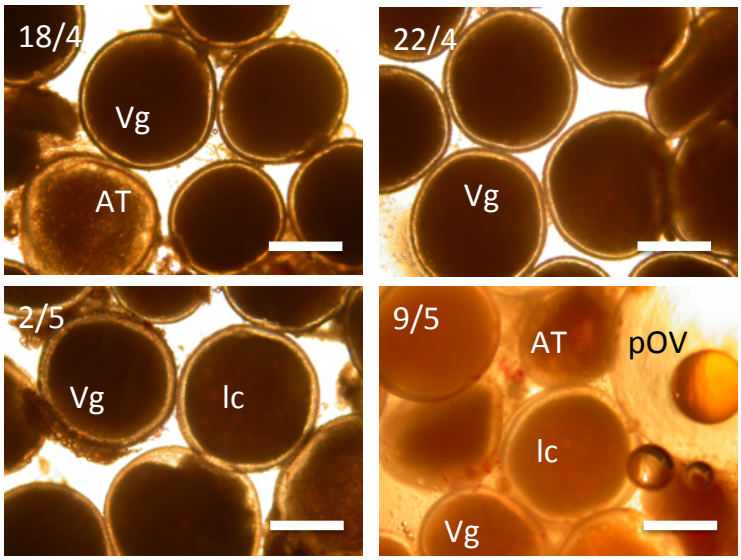

Figure 6. Wet mount of ovarian biopsies from wreckfish during 2016 (dates on each photo). AT = atresia, Vg = vitellogenic, lc = lipid coalescence, pOV = post Ovulated eggs. Bar = 500 µm.

After the first spawning (9/5/2016), fish were handled again on 23/5/2016, when the female was in post ovulation stage, having at the same time some oocytes in early OM of 1500 µm diameter (**Fig. 7**). The female was given a GnRHa implant of 1000 mg to promote the maturation and male an implant of 500 µg to ensure sperm production. Three days (26/5/2016) and 5 days later (28/5/2016) the female was stripped of the eggs (data will be presented in Deliverable 6.3). The female also contained Vg, OM and overripe oocytes, of 1225, 1550 and 1750 µm diameter, respectively (**Fig. 7**). A small number of eggs was released daily in early June, but were not fertilized.

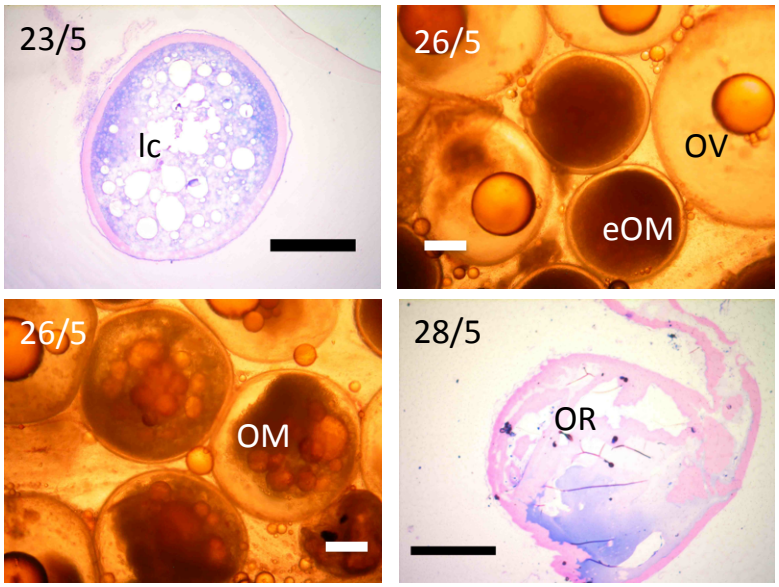

Figure 7. Histological sections or wet mount of ovarian biopsies from wreckfish during the 2016 reproductive season (dates on each photo). lc = lipid coalescence, eOM = early Oocyte Maturation, OM = Oocyte Maturation, OV = Ovulated, OR = Overripe. Bar = 500 µm

In the 2017 spawning season, the female was treated with a 600 µg GnRHa implant on 8/5/2017 and the male with 400 µg. At that time, the female was at the stage of vitellogenesis with oocytes of 1225 µm in diameter (**Fig. 8**), while the male was producing fluent sperm. Four days later (12/5/2017) the female had progressed since oocytes at early OM of 1500 µm, concomitantly with Vg oocytes of 1250-1300 µm in diameter were present, and a liquid GnRHa injection of 20 µg/kg was given. Two days later (14/5/2017) the female was at the same situation as before, in terms of ovarian maturation condition. On 16/5/2017 at noon, the female had oocytes in OM of 1750 µm in diameter, and after 11 hours, it had oocytes at pre-ovulation stage of 2125 µm (**Fig. 8**). We again injected the fish with the same dose to induce the maturation of the second batch of oocytes (1500 µm) and let the fish to spawn spontaneously. Two days later, 114,000 eggs were collected and 46% were fertilized (**Fig. 9, Fig. 10**). The next day (19/5/2017), oocytes at the OM stage of 1550 µm were present in the ovary of the female, with some overripe eggs (**Fig. 8**). Spawning events of variable fecundity and fertilization took place the next days (**Fig. 9**). The fish were checked again on 1/6/2017 and the female had early OM oocytes of 1375 µm in diameter (**Fig. 8**). At the same time, a 3rd GnRHa injection was given to the female, while a GnRHa implant was given to the male. The last treatment provoked six spawns the next days, however with lower fecundity and fertilization (**Fig. 9**).

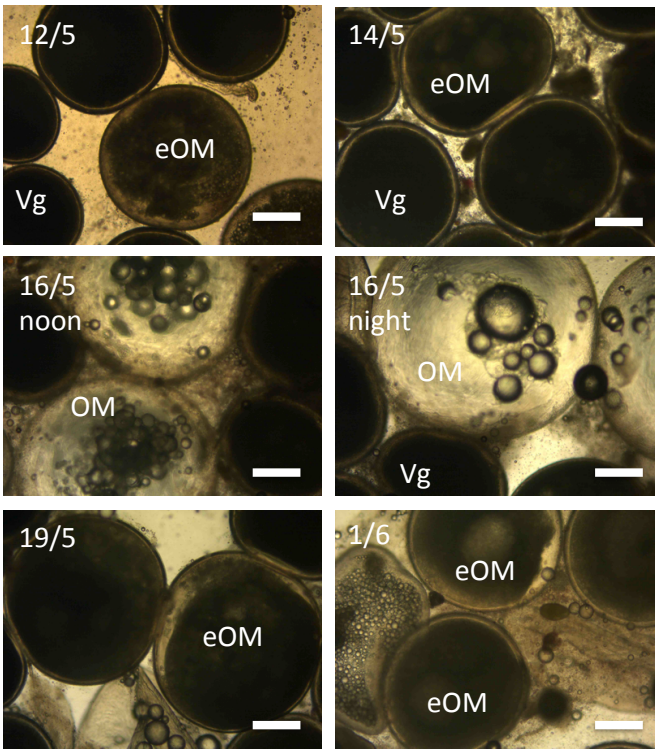

Figure 8. Wet mount of ovarian biopsies from wreckfish during the 2017 reproductive season (dates on each photo). Vg = vittelogenic oocyte, eOM = early Oocyte Maturation, OM = Oocyte Maturation. Bar = 500 µm

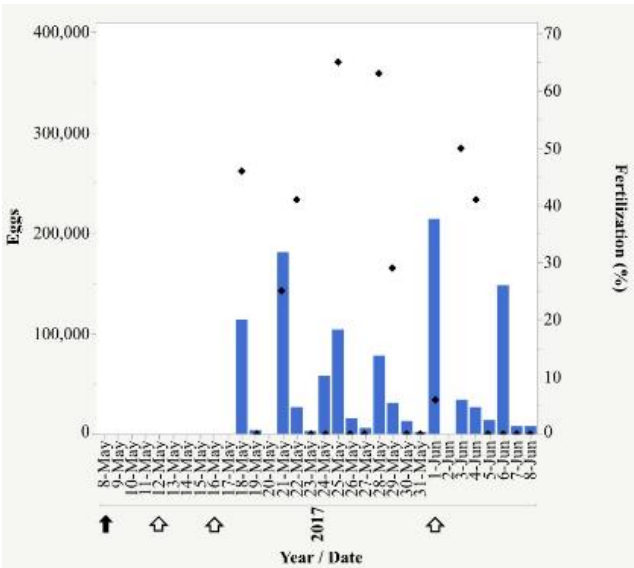

Figure 9. Fecundity (eggs, bars) and fertilization (%, marks) of induced spawning of wreckfish with GnRHa treatment in 2017 spawning period. Filled arrows indicate induction with GnRHa implants while empty arrows indicate induction with GnRHa injections.

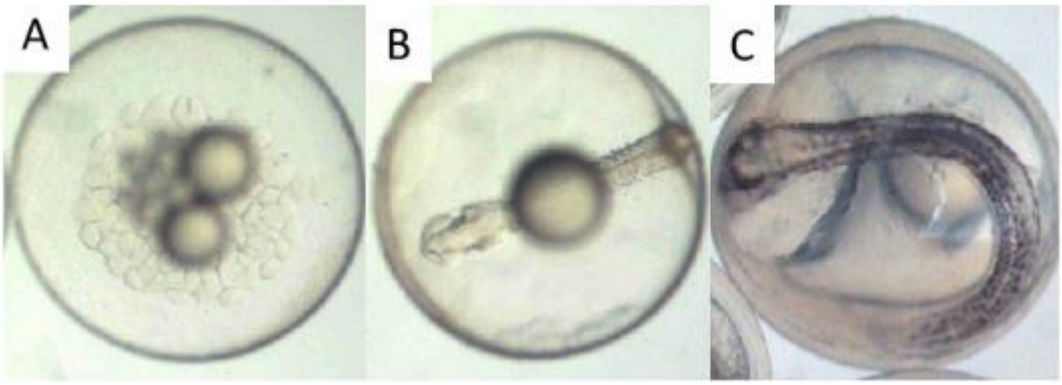

Figure 10. Wreckfish eggs at different developmental stages after fertilization.

P8. IEO Stock P19. CMRM Stock P32. MC2 Stock

In 2015, fluent sperm was present in 7 males in MC2, and in 2 males in IEO. In 2016 the same quantity of fluent males are present in the MC2. One male was moved from MC2 to CMRM. In both broodstocks the male behaved as fluent. The IEO stock had three mature males, two were fluent as the previous year. Sperm quality parameters were evaluated: density, motility, mobility and duration of active sperm were calculated. Results demonstrated that males produce large volumes of good quality sperm for a very long period of time in captivity. Sperm concentration during spawning season was between 2.64 and 16.5 x 10^9 szoa ml^{-1} for the IEO males stock (**Fig. 11**).

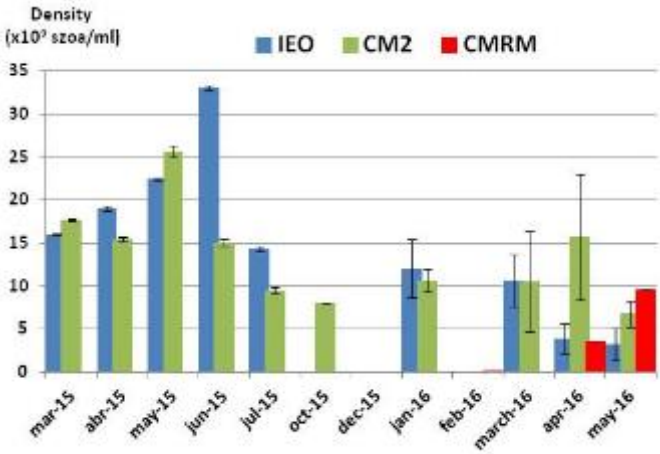

Figure 11. Sperm concentration during reproductive season (2015 and part of 2016) in the broodstocks of IEO and CM2. The CMRM male was transferred from MC2 in January 2016.

The sexual maturation of males covers the same period of females, reaching its peak in the months of April and June. The duration of motility was high, with mean values between 2 - 3.5 min. The mean survival time of the sperm, conserved refrigerated at 4ºC was 4 days. However, in some cases it reaches the 18 days of survival after the collection (**Fig. 12**).

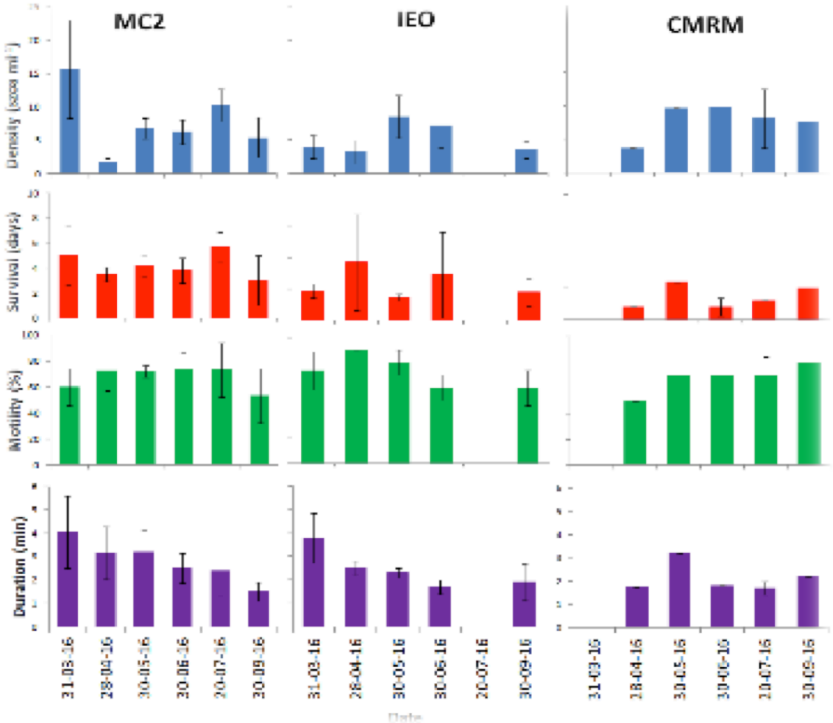

Figure 12. Sperm quality of the males of different stocks (MC2, IEO and CMRM). Density, survival, motility and duration.

Regarding females, the development of oocytes showed that the average size throughout the year reached the expected values, being higher during the spawning season (**Fig. 13**). Biopsies of immature female oocytes indicated that these females did not reach vitellogenesis.

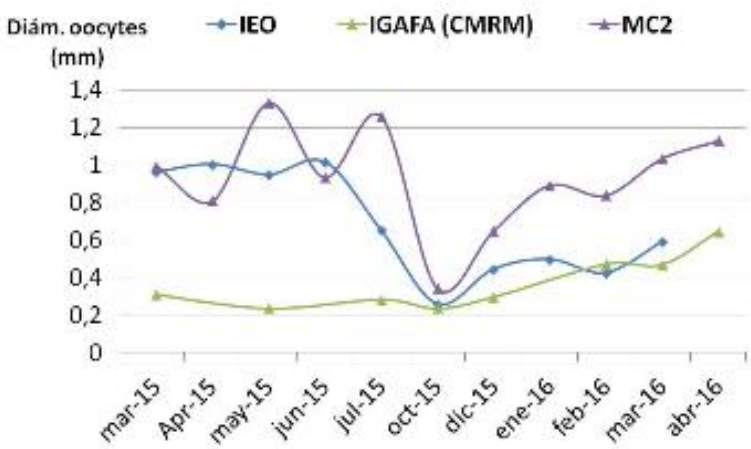

Figure 13. Oocyte size variation from females of the three stocks throughout the years 2015-2016 (IEO, IGAFA-CMRM and MC2).

Oogenesis sequence from primary oocytes to post-maturation was identified, both in fresh samples examined under the microscope and through histology **(Fig. 14)**.

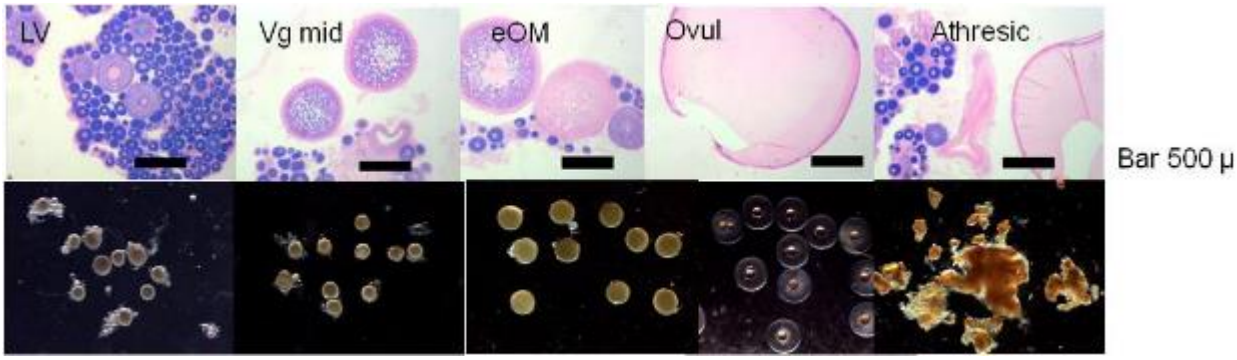

Figure 14. Oogenesis sequence: LV=lipid vesicle, Vg (mid)= mid vitelogénesis, eOM=lipid coalescence, Ovul=ovulación, Athresic=atresia.

A female of 16.5 Kg from the IEO with oocyte size of 1.3 mm was implanted on 9 of June 2015 with 500 µg of GnRHa. No response was obtained from this treatment, due to possible wrong induction time. On June 11 2015, two females (27.35 and 33.15 kg) were implanted with 500 µg of GnRHa at MC2 facilities. Oocyte sizes were 1.121 and 1.092 mm, respectively. None of these implanted females spawned (**Fig. 15**).

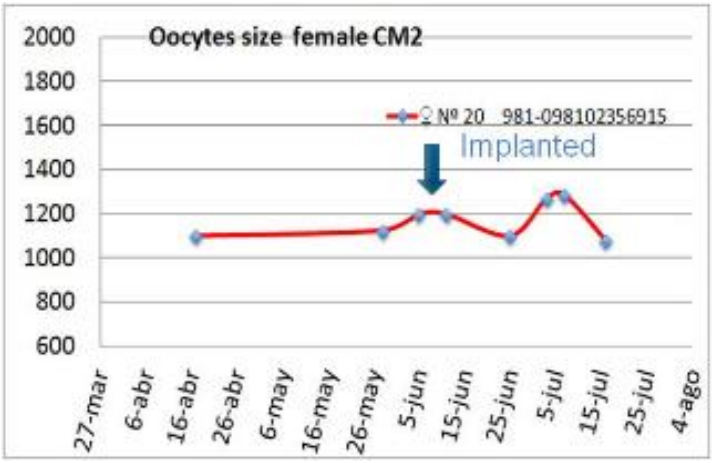

Figure 15. Oocyte size evolution for a treated female from MC2 stock in 2015.

The experiments were continued in order to achieve the objectives along the years 2015-2018. Some fish of the two stocks, both at the CMRM (2016 and 2017) and the MC2 (2017 and 2018) facilities were selected to test different GnRHa doses to induce spawning (**Table 1**).

The females response to the GnRHa implants varied, from no response (2015), to spontaneous or stripping spawns with non viable eggs (2016) up to good results in the fecundity but not in hatching (2017 and 2018). There were only two spawns from MC2 stock that were successfully reared until 25 days post hatching (DPH) in 2016 and 30 DPH in 2018.

Table 1. GnRHa implant data of the different trials during 2015, 2016, 2017 and 2018 in some females of the three broodstocks in Galicia: IEO, MC2 and CMRM.

YEAR	STOCK	FISH	WEIGHT (KG)	DATE IMPLANT	OOCYTES SIZE	Imp. GnRH (µg)	Dosis (µg/kg)	SPAWNING DATE	TOTAL EGGS (ml)	FECUNDATION (%)	OBSERV.
2015	IEO	7938	16,5	09/06/2015	0,95	500	30,3				NO SPAWNS
	MC2	6915	27,35	11/06/2015	1,121	900	32,9				
		7438	33,15		1,092	900	27,1				
2016	MC2	5853	18,2	23/06/2016	1,197	1000	54,9	29/06, 3/07, 8/07, 12/07, 16/07 and 20/07/2016	2200, 325, 180, 980, 730 and 670	86, 78, 49, 85, 75 and 60	ALL SPONTANEOUS
		5544	16		1,119	750	46,9				NO SPAWNS
	CMRM	3FF2	14,3	28/06/2016	1,388	750	52,4	11/07 and 12/07/2016	100 y 150		SPONTANEOUS AND STRIPPING. NON VIABLE EGGS
		7B19	13,5	28/06/2016	1,255	750	55,6				NO SPAWNS
			13,5	12/07/2016	1,36	750	55,6	18/07/2016	750	47	Stripping. NON VIABLE EGGS
			13,7	26/07/2016	2,23	750	54,7				NO SPAWNS
2017	MC2	7B78	23	1 ST:07/06/2017	1,161	1750	76,1	20/06/2017	3500	0	STRIPPING. DIED BECAUSE IT DOESN'T STRIPPING IN ADECUATE TIME
				2ND:15/06/2017	1,161	2000	87,0				
		2B47	18,5	1st: 11/05/2017	1,135	1000	54,0	27/05/2017	240	65,6	NO HATCHING
				2nd: 18/05/2017	1,035	500	27,0	30/05/2017	700	3,5	LARVAE ALIVE UNTIL 25 DPH
		5853	21,3	1st: 30/06/2017	1,200	1750	82,2	16/07/2017	900	0	SPONTANEOUS. NON VIABLE EGGS
				2nd:04/08/2017	1,800	1500	70,4	09/08/2017	3750	0	SPONTANEOUS. NON VIABLE EGGS
	CMRM	499A	17,6	1st: 30/05/2017	1,29	1200	69,4				NO SPAWNS
				2nd:16/06/2017	1,74	INJECT. 359	20	19/06/2017		0	SPONTANEOUS. NON VIABLE EGGS
2018	MC2	2B47	20,1	20/06/2018	1,172	1750	87,0	26/06, 30/06, 4/07, 8/07, 12/07, 16/07, 19/07, 23/07 and 26/07/2018	1800, 1900, 1650, 1300, 1100, 1200, 1000, 1600 and 1600	97, 94, 97, 82, 77, 91, 83, 95, 36 and 76	ALL SPONTANEOUS

During 2017, spawning induction trials were completed with a female from CMRM facilities. On May 30th, a female with 1.29 mm maximum oocyte diameter was administrated a GnRHa implant of 69.4 µg/kg. As no response was obtained, on June 16th when the female had a mean oocyte size of 1.75 mm a single injection of 20 µg/kg of GnRHa was administered. A spontaneous spawn was collected on June 19th, but the eggs were overriped (**Fig 16**).

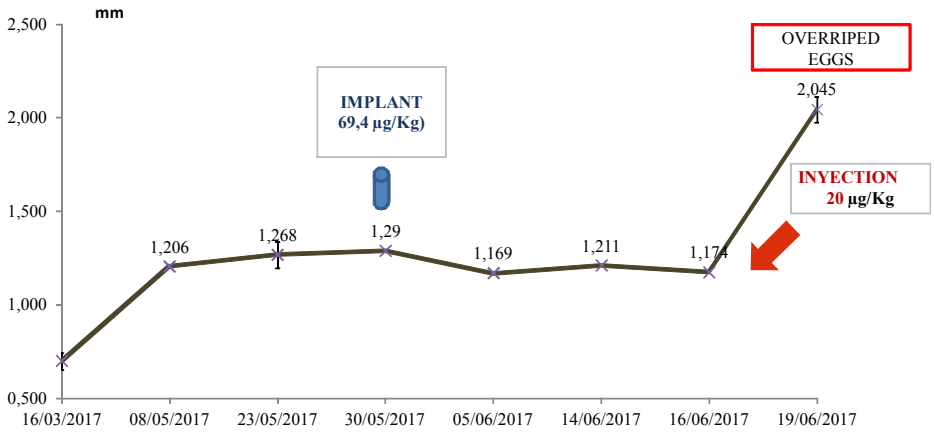

Figure 16. Spawning induction trial with a female from CMRM facilities.

In MC2 facilities three females were implanted during 2017, with a variety of results in terms of spawns (**Table 1**). Gonadal biopsies to control the oocytes maturation were done twice a week (**Fig. 17).**

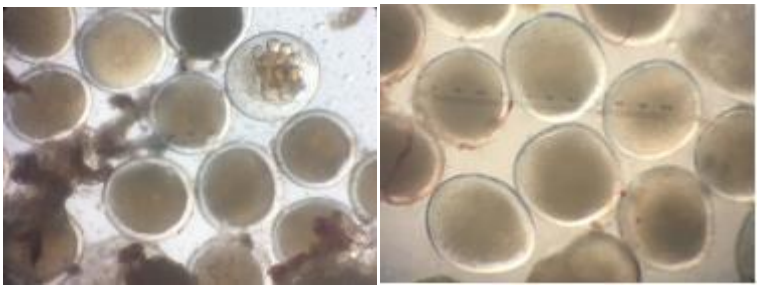

Figure 17. Gonadal biopsies: **A** ♀ 5549 1097 µm, **B** ♀ 2B47 1115µm diameter.

One female (Ref. 7B78) died because of over-maturation. This female was implanted twice, on June 7th (76 µg/kg GnRHa) and on June 15th (87µg/kg GnRHa) when oocyte size was 1.161 mm. Consequently maturation developed, although this female was not able to spawn naturally. On June 20th stripping was carried out, 3500 ml of overripe ova (or eggs) where collected. On June 24th, the night before second stripping was scheduled the female died.

Another female (2B47) was implanted on May 11th with 54 µg/kg GnRHa with no results and a second implant was administered on May 18th, and the female spawned spontaneously on the 27th and 30th May, with viable eggs and poor fertilization (3.5%). Larval survival was good until the 30 DPH (**Fig. 18**).

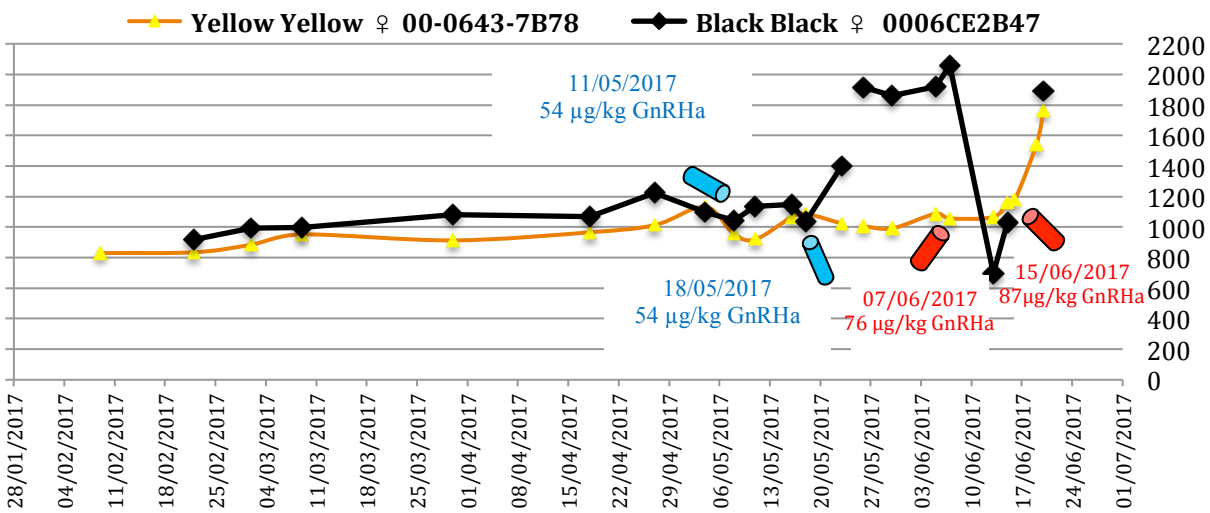

Figure 18. Response (increase oocyte size) to GnRHa treatment in two MC2 females: 7B78 and 2B47.

The necessity for more information on this subject required more females. Therefore, we used the stock from Isidro de la Cal Company, and worked with two of their females, based on an agreement made for that purpose. The experiments were carried out during the June and July 2017.

On the 8/06/2017, a 16 Kg female with oocytes size of 1.35mm was implanted at first with a 62.5 µg / kg of GnRHa, with no response. On the 12th of June the first GnRHa liquid intramuscular injection was administrated (**Fig. 19**). The female was checked every 12 h after injection (**Fig. 20**). Three days after, there were no changes in the oocytes development, and a second injection was administrated with a 20 µg/kg of

GnRHa (Fernández Palacios et al, 2014). As there were no changes, on June 15th a third injection was given, of the same hormone dose. Two days later, a small quantity of eggs was obtained by stripping. Every 12 h the female was checked and stripped, and a few overriped eggs were obtained. Six days after the third injection, 950 ml of eggs were obtained by stripping. From these, 750 ml of floating eggs were fertilized. One day after the stripping, a spontaneous spawn of 110 ml was obtained, with only 20 ml of floating eggs. On the 26th June, a forth injection with the same dose of GnRHa (20 µg/Kg of female) was given. During the successive days, the female was checked and a small amount of eggs was obtained by stripping until the 2nd July with a spontaneous spawn, as well as a high volume of eggs was obtained by stripping (525 ml). From the floating eggs (375 ml), only 10% were fertilized and all eggs died before hatching.

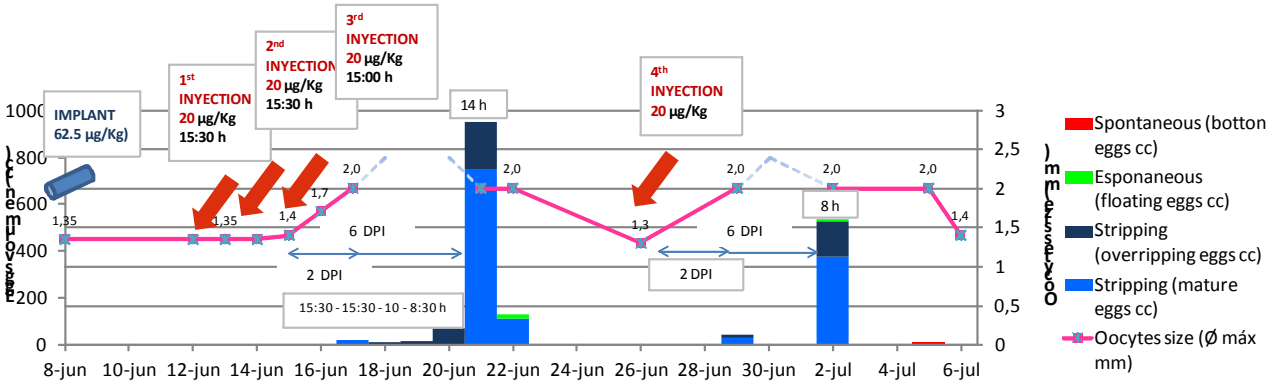

Figure 19. Trial of spawning induction using GnRHa with a 16 kg female from Isidro de la Cal facilities.

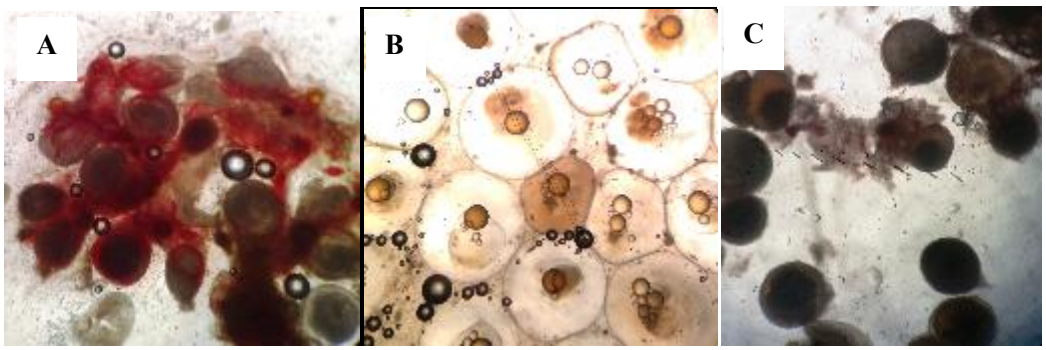

Figure 20. Gonadal biopsies of the 16 kg female, the 17th (A), 18th (B) and 19th (C) June 2017.

Another Isidro de la Cal's female was treated with a GnRHa injection with the same protocol (**Fig. 21**). On the 23rd of June the first dose was administrated with oocytes size of 1.3 mm. Three days later, the second injection was given and one day after the female was very swollen and a plug was extracted. The next day, 315 ml of eggs were obtained with stripping and were fertilized. Every three days after the stripping, batches of overripped eggs were obtained.

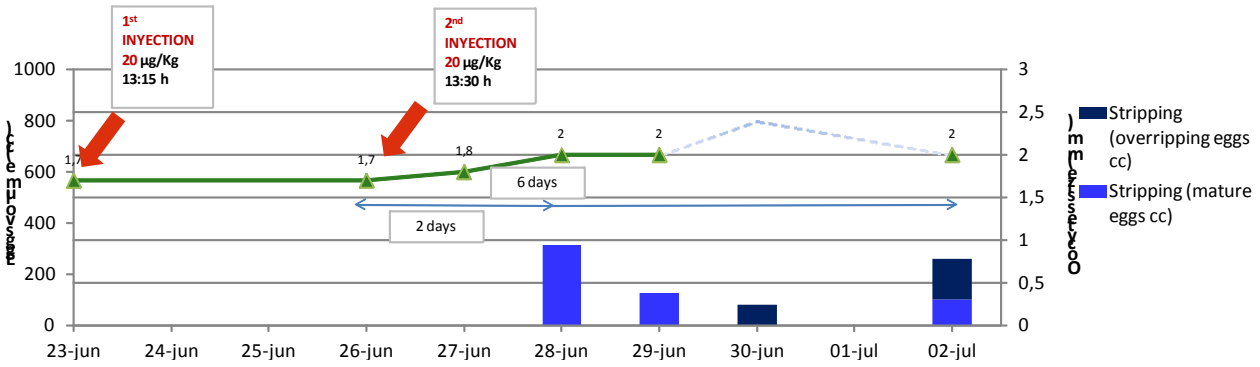

Figure 21. Trial of hormonal induction (Injection with GnRHa) with a 18 Kg female from Isidro de la Cal facilities.

As a result of these trials, information on the ovulation time after induction with the GnRHa hormone was obtained. Results suggest that the injections are more effective than the implants, with a faster response. The problem is the risk of gonadal plugs if the hormonal dose is not adequate. Results show a time of response of about six days after the injection.

In 2018, another experiment with a female from MC2 with an induction with a higher dose of GnRHa implant was performed, with good results and spawns with good egg fertility, after 6 days from the hormonal induction. The same results were achieved with the injections trials a year before at Isidro de la Cal facility (**Table 1**). On June 20th, the 20 kg female 2B47 (that was implanted also in 2017), was implanted with a dose of (87 µg/kg GnRHa) when oocyte size was 1.172 mm. Six days later, the female started natural spawning and spawned seven times every 4 days. High volume of spawns between 1900 ml and 1000 ml of eggs with fertilization rates between 94 and 97 % and hatching percentage between 39% and 5% were obtained. Several larval cultures were successfully carried out with those eggs until 30 DPH.

Induction with gonadotropin hormones (FSH and LH)

Differentially and out of the diversify project, experiments with luteinizing and follicle stimulating (LH and FSH) hormones were performed (Giménez et al, 2015). Several trials of induction were carried out during 2015-2018 period at IEO and CMRM facilities. Immature males (n=2) and females (n=2) and a mature female with vitellogenic oocytes were treated between three and four months. Gonadal biopsies and blood samples were taken weekly to evaluate the effect of hormone dose on gonadal development. Only the vitellogenic female showed a positive response and its oocytes developed from 0.48 to 1.24 mm (Pérez Rial et al., 2017).

Discussion

P1. HCMR Stock

This experiment demonstrated a few important aspects about wreckfish broodstocks in captivity. Males produce large volumes of good quality sperm for a very long period of time under constant (16°C) water temperature. In previous studies under constant temperature of 15°C, fish sperm was collected but no sperm quality data were presented (Fauvel et al., 2008; Papandroulakis et al., 2008). We can assume that sperm quantity and quality was good since the unfertilized eggs were not correlated with diminished sperm characteristics, but with males' behaviour that were not releasing adequate sperm quantities when the female was spawning (Papandroulakis et al., 2008) since an *in vitro* fertilization trial resulted in 86% fertilization success (Fauvel et al., 2008). The failure of the males to release sperm when females spawn was also hypothesized in D6.5 where unfertilized eggs were also collected. In the present study males produced copious amount of sperm almost during the whole year without decreased quality characteristics.

Treatments of GnRHa did not enhance sperm production or quality, but treatments may be maintained the capacity of the males having adequate sperm quantity. Unfortunately, there are no sperm data available even for the congener hapuku *Polyprion oxygeneios* for comparison (Symonds et al., 2014).

Similarly, females do undergo vitellogenesis -- and were also observed to undergo oocyte maturation spontaneously in captivity—and remain in fully vitellogenic stage for at least 3 months (under constant water temperature). Treatment of females with GnRHa implants or combination of GnRHa implants and injections can induce oocyte maturation and ovulation consistently, but spontaneous spawning is somehow impaired and the resulting eggs were not always fertilized. It cannot be determined at this stage if this problem is because of the males were not releasing sperm at the time the female liberated eggs due to behavioral reproductive dysfunctions, as mentioned above, or if the female does not release eggs soon after ovulation, thus undergoing over-ripening. Successful spawning in wreckfish after GnRHa treatments was also observed in previous studies, but the fertilization percentage was either very low (Papandroulakis et al., 2008) or 86% in one spawn (Fauvel et al., 2008). In the present study, the fertilization success of the eggs spawned was variable, being almost zero in 2014 or zero in 2016, up to 56% in 2015 and 0-65% in 2017. The hapuku were also induced after treatment with GnRHa implants of 25 or 50 µg kg^{-1}, but more detailed data are not available (Symonds et al., 2014).

Large tanks (40 m^3) do not seem to be the most critical parameter in the case of wreckfish, since in the present study fertilized eggs were collected mainly in a smaller tank (15 m^3). However, the experiment was conducted with only one couple so the results cannot be used to draw more general conclusions. In hapuku, the tanks used in various studies were 40 to 70 m^3 and the fish spawn spontaneously (Anderson et al., 2012; Kohn and Symonds, 2012; Symonds et al., 2014), but there is no comparison with that species for the volume of the spawning tanks. On the other hand, wreckfish is forming spawning aggregations (Peres and Klippel, 2003) and may be this is the reason that in the present study fish failed to spawn fertilized eggs repeatedly. In other groupers, like the dusky grouper *Epinephelus marginatus*, it was shown that fish in nature need a depth of 7-12 m to spawn in repetitive observations (Zabala et al., 1997). The same spawning behavior was observed with a different grouper species red spotted grouper *Epinephelus akaara* in captive conditions even though the water depth in the tank was 1.7 (Okumura et al., 2002). In the latter study, low percentages of fertilization success were connected with the water depth, as it was different from the natural spawning grounds. The fertilization rates were improved two-fold when a larger tank was used of 3.5 m depth (Okumura et al., 2003).

P8. IEO Stock, P19. CMRM Stock, P32. MC2 Stock

In the Atlantic stocks, males had no problem with the natural spermiation at the same time of females spawn naturally. Like HCMR, males were mature more time than females, in the normal spawn season, starting to produce sperm before the females spawn and ending later than the females.

In different volume tanks males and females have the same behavior with induced spawns with irregular results in terms of egg fertility and viability. The females' response to the GnRHa implants varied, with no response (2015), with a spontaneous or stripping spawns with non-viable eggs (2016), with good results in fertilization, but no hatching (2017) and with viable eggs with a female spawned in MC2 during 2018.

Results suggest that the injections were more effective than the implants, with a faster response (Duncan et al. 2012). The problem is the risk of gonadal plugs if the hormonal dose is not adequate. Data show a time of response of about six days after the injection. For another hand, a higher dose of GnRHa implants gave better results (MC2) this last year, with a continued spawns with a latency for one female of six times between four-five days. The same results were achieved with the injections trials a year before at Isidro de la Cal facility.

The hormonal induction with GnRHa gave success for wreckfish and may be advisable in cases of problems with incomplete maturation that results in no natural spawning. However, naturally females and males wreckfish were observed to perform spawns spontaneously in captivity, with very good results in terms of fertilization and hatching.

References

Anderson, S.A., Salinas, I., Walker, S.P., Gublin, Y., Pether, S., Kohn, Y.Y., Symonds, J.E., 2012. Early development of New Zealand hapuku *Polyprion oxygeneios* eggs and larvae. Journal of Fish Biology 80, 555-571.

Bennett, H.S., Wyrick, A.D., Lee, S.W., McNeil, J.H., 1976. Science and art in preparing tissues embedded in plastic for light microscopy, with special reference to glycol methacrylate, glass knives and simple stains. Stain Technology 51, 71-97.

Fauvel, C., Suquet, M., Sévère, A., Mylonas, C.C., Papandroulakis, N., 2008. Slow-release GnRHa therapy prevented atresia during vitellogenesis and induced ovulation of captive wreckfish (*Polyprion americanus*). Cybium 32(2) suppl, 191.

Duncan, N.J., Estévez, A., Porta, J., Carazo, I., Norambuena, F., Aguilera, C., Gairin, I., Bucci, F., Valles, R., Mylonas,.C.C. 2012. Reproductive development, GnRHa-induced spawning and eff quality of wild meagre (Argyrosomus regius) acclimatissed to captivity. Fish Fishiology Biochem. 38(5): 1273-86

Giménez I., De La Gándara F., Arizcun M., Baixauli P., Ortega A., García-Alcázar A. 2015. Inducción de la vitelogénesis mediante gonadotropinas recombinantes en lubina (Dicentrarchus labrax). XV Congreso Nacional de Acuicultura. C. Oral. Actas XV CNA 173-174.

Kohn, Y.Y., Symonds, J.E., 2012. Evaluation of egg quality parameters as predictors of hatching success and early larval survival in hapuku (*Polyprion oxygeneios*). Aquaculture 342-343, 42-47.

Machias, A., Somarakis, S., Papandroulakis, N., Spedicato, M.-T., Suquet, M., Lembo, G., Divanach, P., 2003. Settlement of the wreckfish (*Polyprion americanus*). Marine Biology 142, 45-52.

Marino, G., Panini, E., Longobardi, A., Mandich, A., Finoia, M.G., Zohar, Y., Mylonas, C.C., 2003. Induction of ovulation in captive-reared dusky grouper, *Epinephelus marginatus* (Lowe, 1834) with a sustained-release GnRHa implant. Aquaculture 219, 841-858.

Mylonas, C.C., Cardinaletti, G., Sigelaki, I., Polzonetti-Magni, A., 2005. Comparative efficacy of clove oil and 2-phenoxyethanol as anesthetics in the aquaculture of European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) at different temperatures. Aquaculture 246, 467-481.

Mylonas, C.C., Salone, S., Biglino, T., de Mello, P.H., Fakriadis, I., Sigelaki, I., Duncan, N., 2016. Enhancement of oogenesis/spermatogenesis in meagre *Argyrosomus regius* using a combination of temperature control and GnRHa treatments. Aquaculture 464, 323-330.

Okumura, S., Okamoto, K., Oomori, R., Nakazono, A., 2002. Spawning behavior and artificial fertilization in captive reared red spotted grouper, *Epinephelus akaara*. Aquaculture 206, 165-173.

Okumura, S., Okamoto, K., Oomori, R., H., S., Nakazono, A., 2003. Improved fertilization rates by using a large volume spawning tank in red spotted grouper (*Epinephelus akaara*). Fish Physiology and Biochemistry 28, 515-516.Papadaki, M., Mazzella, D., Santinelli, V., Fakriadis, I., Sigelaki, I., Mylonas, C.C., 2018. Hermaphroditism and reproductive function of hatchery-produced sharpsnout seabream (*Diplodus puntazzo*) under attenuated annual thermal cycles. Aquaculture 482, 231-240.

Papandroulakis, N., Suquet, M., Spedicato, M.T., Machias, A., Fauvel, C., Divanach, P., 2004. Feeding rates, growth performance and gametogenesis of wreckfish (*Polyprion americanus*) kept in captivity. Aquaculture International 12, 395-407.

Papandroulakis, N., Mylonas, C.C., Syggelaki, E., Katharios, P., Divakaran, S., 2008. First reproduction of captive-reared wreckfish (*Polyprion americanus*) using GnRH implants, European Aquaculture 2008, Krakow, Poland.

Peres, M.B., Klippel, S., 2003. Reproductive biology of southwestern Atlantic wreckfish, *Polyprion americanus* (Teleostei: Polyprionidae). Environmental Biology of Fishes 68, 163-173.

Peres, M.B., Haimovici, M., 2004. Age and growth of southwestern Atlantic wreckfish *Polyprion americanus*. Fisheries Research 66, 157-169.

Pérez Rial, E., Giménez, I., Rodríguez Villanueva, J.L., Álvarez-Blázquez, B., Chaves-Pozo, E., Lluch, N., Pazos, G. and Linares, F. Primeras experiencias de inducción a la maduración con gonadotropinas recombinantes en reproductores de cherna (*Polyprion americanus*). National Aquaculture Congress 2017. Zaragoza. Spain.

Roberts, C.D., 1989. Reproductive mode in the percomorph fish genus *Polyprion* Oken. Journal of Fish Biology 34, 1-9.

Symonds, J.E., Walker, S.P., Pether, S., Gublin, Y., McQueen, D., King, A., Irvine, G.W., Setiawan, A.N., Forsythe, J.A., Bruce, M., 2014. Developing yellowtail kingfish (Seriola lalandi) and hāpuku (*Polyprion oxygeneios*) for New Zealand aquaculture. New Zealand Journal of Marine and Freshwater Research 48, 371-384.

Zabala, M., Garcia-Rubies, A., Louisy, P., Sala, E., 1997. Spawning behaviour of the Mediterranean dusky grouper *Epinephelus marginatus* (Lowe, 1834) (Pisces, Serranidae) in the Medes Islands Marine Reserve (NW Mediterranean, Spain). Scientia Marina 61: 65-77, Epinephelus, dusky grouper, spawning, behavior.

Zupa, R., Rodríguez, C., Mylonas, C.C., Rosenfeld, H., Fakriadis, I., Papadaki, M., Pérez, J.A., Pousis, C., Basilone, G., Corriero, A., 2017. Comparative study of reproductive development in wild and captive-reared greater amberjack *Seriola dumerili* (Risso, 1810). PLoS ONE 12, e0169645.

Deviations:

The deliverable is delivered with a four-month delay.

Co-funded by the Seventh Framework Programme of the European Union