New species for EU aquaculture

Deliverable Report

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Deliverable Title	Spawning induction methods with in vitro fertilization of wreckfish			
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WP Title:	Spawning induction methods with in vitro fertilization of wreckfish			
Task No:	6.3		**Task Lead beneficiary:**	P8. IEO
Task Title:	Development of spawning induction procedures			
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P14. IFREMER	P15. ULL			
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Objective: The objective of this Deliverable was to develop a method for the induction of maturation of wreckfish (*Polyprion americanus*) with *in vitro* fertilization of the obtained eggs.

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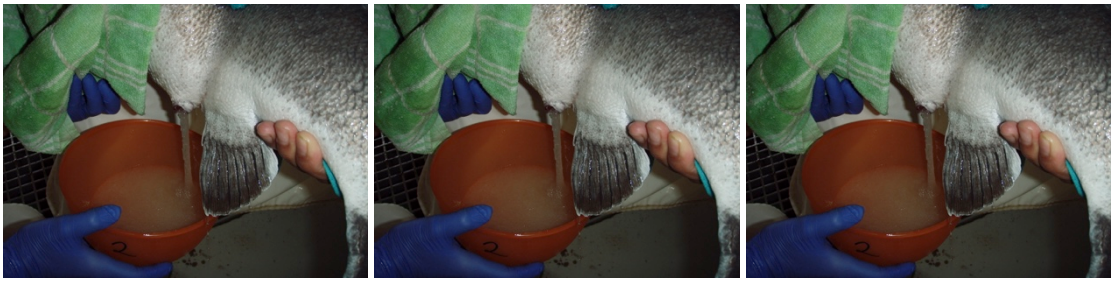

Description

During the years 2015 to 2018, four wreckfish broodstocks in different locations, one in the Mediterranean Sea (Crete, Greece) and another three on the NW Atlantic coast (Galicia, Spain) were followed in order to know all about the reproduction, larvae husbandry and nutrition, to address the study of this species as a candidate for European aquaculture. These fish were maintained in a variety of environmental conditions in regards to tank size and photothermal regime, including indoor and outdoor tanks with natural photothermal conditions, and indoor tanks with simulated natural photothermal conditions or constant temperature.

Background

One of the most important challenges once a species has been selected as a strong candidate to be part of industrial production of aquaculture is to know the reproductive potential in captivity. The bibliographical sources related with the biology and fisheries of wreckfish (Peres et al., 2003; Roberts et al., 1989; Sedberry et al., 1999) and the previous knowledge about the species acquired by different facilities (Fauvel et al., 2008; Papandroulakis et al., 2004, 2008; Peleteiro et al., 2010, 2011; Rodríguez Villanueva et al., 2011) laid the foundations of the investigation of this species under the DIVERSIFY project.

The knowledge of the reproductive biology and physiology derived from the exhaustive work carried out for ***Deliverable D6.5 Description of the reproductive cycle of wreckfish*** with wild animals (Álvarez-Blázquez et al., 2015, 2017; Martínez et al., 2015, 2016; Papadaki et al., 2017, 2018; Pérez Rial et al., 2017; Rodríguez et al. 2017), and in ***D12.2 Recommendations for wreckfish broodstock feeds*** (Linares et al., 2015, 2016, 2018) were essential to continue with the different challenges with wreckfish reproduction set out in the DIVERSIFY project.

Material and methods

A stock of 5 wreckfish (captured from the wild as juveniles) has been maintained at P1.HCMR in Crete, Mediterranean Sea, in two 15-m^3 tanks, under simulated natural photoperiod and constant temperature (16°C). The fish were fed 3 times a week with raw fish (mackerel) in simulated natural photoperiod and constant temperature (16ºC). Unfortunately two of the fish stopped eating in the summer of 2013 and eventually died prior to the reproductive season in 2014. On June 12th, 2016 one of the males died, leaving the broodstock of HCMR with just one female and one male. The remaining two fish (one male of 11.2 kg, and a single female of 11.8 kg) were followed during the rest of the time of the project.

Another three stocks have been maintained in different facilities and environmental conditions in NW Spain, Atlantic Ocean, at P8 (IEO), P19 (CMRM) and P32 (MC2) (**Table I**). The IEO stock was formed by 14 wreckfish maintained in two tanks with 120 m^3 of seawater with a ratio male/female of 0.4:1, with natural temperature and photoperiod. One batch was fed with semi-moist pellets based on special fish paste normally used for parental diets, and another batch was fed with dry food specially formulated for wreckfish.

The same environmental conditions were applied to the CMRM stock that consisted of 11 wreckfish maintained in two tanks of 40 m^3 with a ratio male/female of 0.8:1. The broodstock was fed during 2014 with Vitalis Repro/Vitalis Cal from Skretting (Norway) and the food was changed at the end of the year, because the fish had a large amount of fat. In the following years (2015 and 2016) the fish were fed with squid and in 2017 and 2018 a mixture of hake and squid (half and half).

Finally a stock of 19 wreckfish with a sex ratio of 0.6:1 male/female were maintained in a 3500 m^3 exhibition tank (Nautilus, MC2, A Coruna, Spain) 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. In 2017, three of the best spawning females died because of a failure in the flow of oxygenated water and this lose had an effect on later results.

Table 1. Different wreckfish broodstocks of four different facilities.

	HCMR	**MC2** AQUARIUM FINISTERRAE	**CMRM IGAFA/CIMA**	**IEO** Centro Oceanográfico de Vigo	TOTAL
MALES	1	6	4	3	14
FEMALES	1	10	5	8	24
UNDETERMINED		3	2	3	8
TOTAL	2	19	11	14	**46**
MALE/FEMALE	1:1	0.6:1	0.8:1	0.4:1	

During the first two years of the project, the different stocks were sampled for oocytes by canulation of the ovary and sperm by applying gentle abdominal pressure (**Fig. 1**). This sampling was conducted twice a month during the spawning season from March to July and once a month during the rest of the year (August to February) in order to describe the reproductive cycle and sexual maturation of the males and females. Weight and length were measured each 6 months.

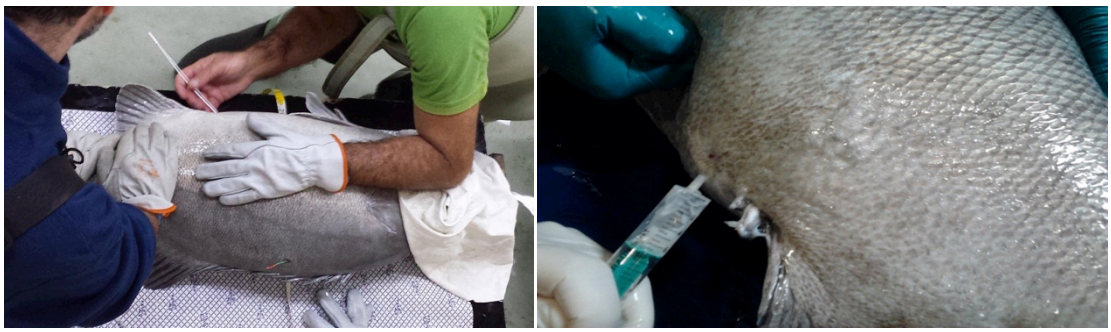

Figure 1. Sampling wreckfish broodstock. Gonadal biopsy (left) and sperm collecting (right).

During sampling, fish were anaesthetised to reduce the stress of the procedure. The individual data of sex, growth, female ovarian stage, presence and quality of sperm were determined and registered. The stage of oogenesis was determined by morphology and oocyte size (µm diameter) and sperm quality parameters were evaluated: sperm concentration, mobility and motility in individual samples (see ***D6.5 Description of the reproductive cycle of wreckfish***), reporting spermiation index 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.

During the spawning season, egg quality of 15 different spawns was measured by a Leica Microsoft image analyser choosing the following parameters: egg size (cm), mayor droplet size (mm), and number of droplets (Yair et al., 2012), fertilisation success (%) and hatching success (%). Three types of spawns depending on the number of egg droplets were classified: I >60% eggs with ≤25 droplets; II >60% of eggs with ≥ 25 droplets; I-II 50-60% of eggs with both type of droplets and III: >60 % of eggs with one droplet (**Fig. 2**).

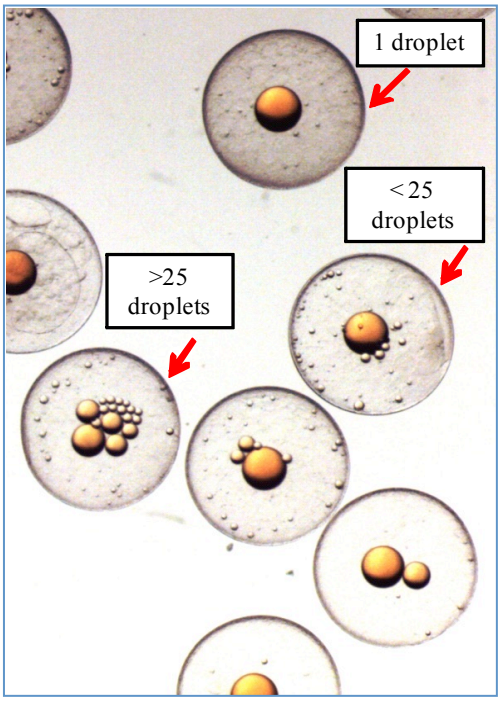

Figure 2. Different egg types depending of the number of droplets.

To determine the sex of the specimens, from which a biopsy could not be obtained due to the gonopore being closed, ultrasound was used (Martin et al., 2001). Using ultrasound the female gonads of breeders were observed to identify females in MC2 and CMRM.

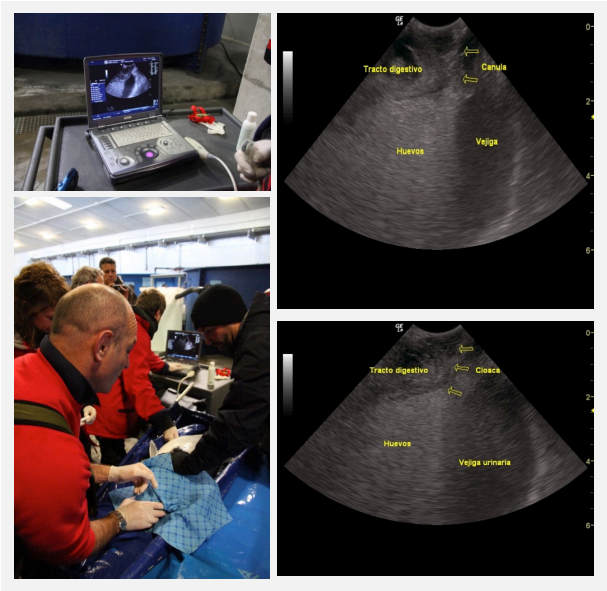

Figure 3. Gonad ecographies of two wreckfish, to identify sex externally.

During 2014, two spawns by stripping were done at MC2 facilities (**Table 2)**, with a result of 8000 and 100 cc respectively of floating eggs, but without any fertilization after insemination with a male sperm at a ratio of 1000 cc oocytes ml^{-1}, sperm density $17.5x10^{9}$ spzoa ml^{-1}. No results in terms of egg fertility were achieved.

Figure 4. Fertilization *in vitro*: a) stripping mature female and male, b) *in vitro* insemination, c) sperm activation with seawater after mixing egg and sperm, d) and egg incubation of fertilized eggs.

During the spawning season of 2015, a total of 10 spawns were obtained from one female of the IEO broodstock between March and June, and 14 from three females of MC2 broodstock (**Table 2**). The majority of spawns were spontaneous, except one casual by stripping from IEO at the moment of sampling, with a volume of 150 cc of eggs, 93 cc of floating eggs and it was fertilized with a pool (1.0 ml sperm /1000 cc of oocytes) of stripped sperm of two males with a mean density of $19x10^9$ spzoa ml^{-1}, obtaining 62% of egg fertilization success (**Fig. 4**). Hatching was only 0.4% with larval survival only to 17 days post hatching (dph). Two spawns by stripping were occurred in the MC2 facilities with 190 and 1000 cc of total egg volume, without fertilization success after *in vitro* insemination with sperm from two males (1 ml sperm/1000 cc oocytes) having a sperm density of 17.5 and 13.3 spzoa.$ml^{-1.}$

Results

Stocks in the Atlantic (Spain)

In summary, during the first years of the project, between 2014 and 2017, trials with *in vitro* fertilization were very few as the females in the IEO and CMRM stock did not reach advanced stages of maturity to enable work with *in vitro* techniques (**Table 2**). Trials were focused on GnRHa implanted trials (see **D6.7** Spawning induction method for spontaneous spawning of wreckfish in large tanks) in an attempt to induce reproductive development. During the period 2014-2017, in the IEO only two females matured, in MC2 three females matured and in CMRM no females matured until the year 2017. Two spawns by stripping were achieved in the MC2 facilities, with 125 ml of total eggs obtained from each attempt, but the eggs remained unfertilized after sperm was added. Most of the females were initiated vitellogenesis, but did not reach advanced stages of oocyte maturation and, therefore, it was not possible to work with *in vitro* techniques. The males were fluent most of the year with a good quantities and quality of sperm.

During 2017, two females in the stock of the IEO matured spontaneously, sequential spawns were collected every 3-4 days that had been naturally and spontaneously fertilized by males in the tank, with an average of 10 spawns/female. In addition, two females from the stock of the CMRM reached advanced stages of maturation for first time, but the other females remained in the early stages of maturation. Work with the stock of the MC2, in spite of the loss of female stock (mortality caused by an error), achieved two spawns by stripping. The females were induced to ovulate with GnRHa, but the stripped eggs were not fertilized by the sperm and did not develop. Stripping was done with one induced female, without success with the *in vitro* fertilization. On the second stripping, the fish died during the trial (see ***D6.7 Spawning induction method for spontaneous spawning of wreckfish in large tanks***).

Table 2. Total spawns by stripping from females in all of the three Atlantic wreckfish broodstock along the years 2014-2018.

STOCK	DATE	FEMALE	Tª	TOTAL EGG (CC)	FLOATING EGG (CC)	FEC (%)	HATCH (%)	LARVAE Nº	LARVAE DENSITY (nº larv/l)	MEAN Tª	FEED	SURVIVAL (dph)
MC2	27-05-14	1678	15,4	800	...	0	...	...	...	...	...	...
MC2	04-06-14	5554	15,8	100	...	0	...	...	...	...	...	...
IEO	10-04-15	9703	13,75	350	330	62-24	30	110	0,2	17,4	Enriched rot.	14
MC2	07-05-15	5679	16,1	1000	...	0	...	...	...	...	...	...
MC2	28-05-15	5679	14,2	1000	...	0	...	...	...	...	...	...
MC2	12-05-16	5679	14,5	125	...	0	...	...	...	...	...	...
MC2	19-05-16	5679	14,3	125	...	0	...	...	...	...	...	...
CMRM (IEO)	18-07-16	7B19	16,2	750	350	27	NO	...	...	...	...	...
MC2	20-06-17	7B78	15,9	3500	...	0	...	...	...	...	...	...

In 2018 natural and spontaneous spawns in all stocks increased considerably. No invasive stripping trials were attempted due to the success with spontaneous spawns and the effect that handling the fish would have on this success. In the MC2 facilities, females were induced with GnRHa, but these fish were left to spawn spontaneously.

EGG CHARACTERISTICS

Spawned wreckfish eggs have a diameter of 2.12±0.064 mm. This large egg size compared with the smaller egg size of other cultured marine fish species such as the blackspot sea bream (*Pagellus bogaraveo*) (**Fig. 7**), led to some modifications in the wreckfish egg incubation methods (see ***D6.6 An in vitro fertilization protocol to be employed by the industry to spawn wreckfish***).

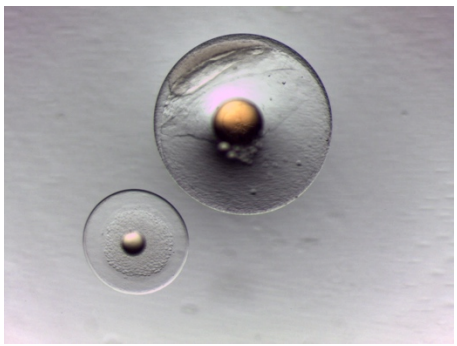

Figure 5. Morphological differences between a blackspot sea bream (*Pagellus bogaraveo*) egg and a wreckfish (*Polyprion americanus*) egg.

The wreckfish eggs exhibited differences in the distribution and number of oil droplets. These differences were used to classify the eggs and were related to egg quality parameters, such as egg size, droplet size, fertilisation and hatching (**Table 3**)**.** The spawns and eggs were classified as follows: egg type 1 (>60 % of eggs with nº droplets/EGG ≤25) represented 56,2% of spawned eggs; egg type 2 (>60% of eggs with nº droplets/EGG ≥25) represented 37,3% of spawned eggs and egg type 1-2 (50-60% of both) represented 6,5% of spawned eggs. No spawns were obtained with ≥60% off eggs with only one droplet (type 3). The morphological parameters of the eggs exhibited no significant differences in egg fertilisation (%) and hatching (%) between different spawn types obtained (**Table 3**)**.**

Table 3. Egg and droplet mean size (mm), appearance (%), fertility (%) and hatching (%) in different spawns classes.

SPAWN CLASS	EGG SIZE mm	DROPLET SIZE (mm the bigger)	APPEARANCE %	FERTILITY %	HATCHING %
TYPE 1	2,12±0,035	0,51 ± 0,053	56,2	96,5±1,86	28,01±12,21
TYPE 2	2,15±0,087	0,46±0,096	37,2	89,5±10,47	32,77±23,14
TYPE 1-2	both	both	6,25	84	25,1
TYPE 3	2.10±0.070	0.56±0.059	-	-	-

SPERM CHARACTERISTICS

Sperm quality parameters were evaluated in the four wreckfish broodstocks. During 2015 sperm concentration and motility was registered (**Fig**. **5**). In the Atlantic broodstocks (IEO, MC2 and CMRM) motility and duration of active sperm was also registered during 2016 (**Fig**. **6**). The males in all stocks produced large volumes of good quality sperm for a very long period of time in captivity. Sperm concentration during spawning season was between 11.2 and 24.0x10^9 spzoa.ml^{-1} for the males from IEO stock, and 13.3 and 30.4 x10^9 spzoa.ml^{-1} from MC2 stock **(**NW Atlantic Stocks**).**

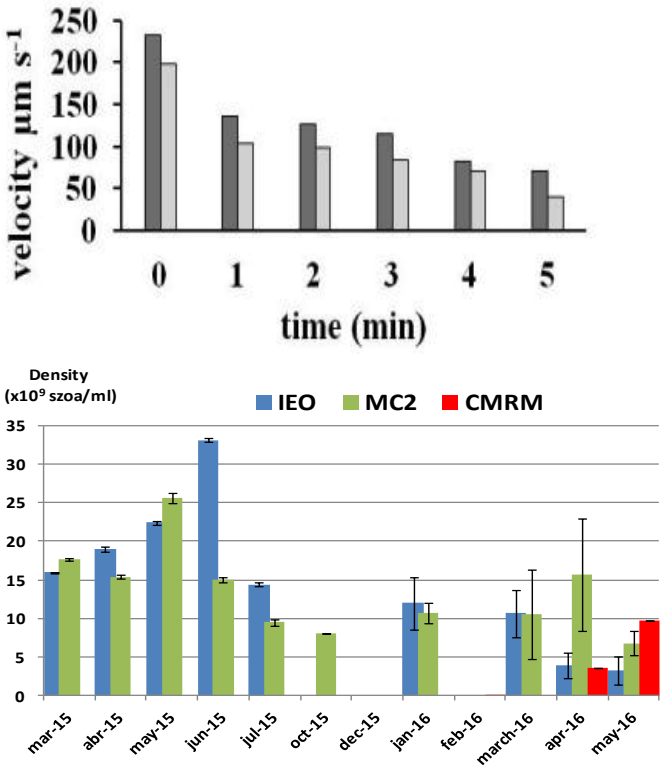

Figure 6. Variations with time of average path velocity along smoothed trajectory (dark grey) and straight line velocity picturing the progressive movement (left) and sperm concentration during reproductive season (2015 and part of 2016) in the male broodstock of IEO and MC2 (right).

Spermiating males were found all-year round both in the Atlantic stocks (**Fig. 7**) and in the Mediterranean (D.6.5 Reproductive cycle of wreckfish), with the percentages of S0, S1, S2 and S3 stage fish varying between months. The highest percentage of non-spermiating fish (S0 spermiation index) was found from September until December, whereas high percentages of spermiating fish (S2 and S3 spermiation index) were found just before and during the reproductive season of females, from January until July, during both years of the study (**Fig. 8**). Sperm motility duration and motility percentage exhibited high and almost unchanged values during both years of the study (**Fig. 8**), whereas sperm density exhibited high values during the whole year, with the highest values observed in March of both years. As far as sperm survival at 4°C is concerned, constant values were observed during the whole year, with lower values only in March, June and September 2016..

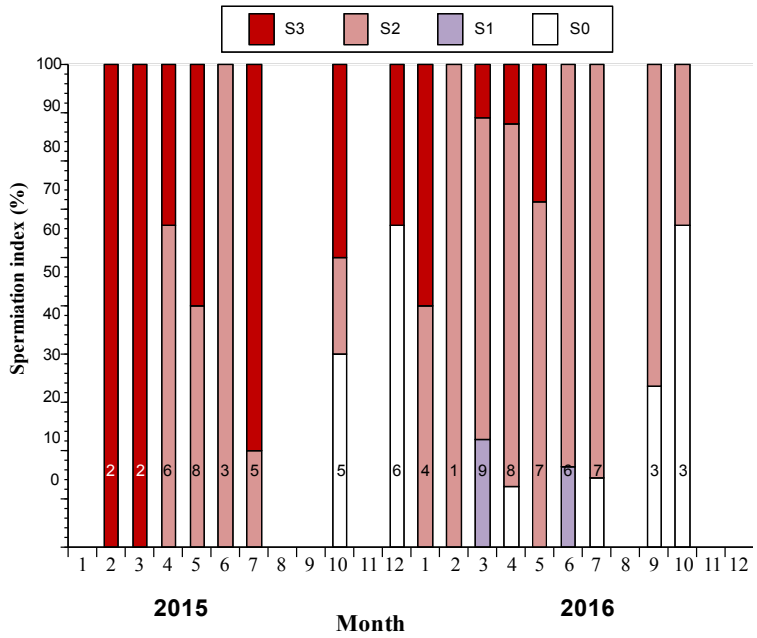

Figure 7. Density, percentage motility and duration of sperm from MC2, IEO and CMRM (Atlantic stocks) during 2016.

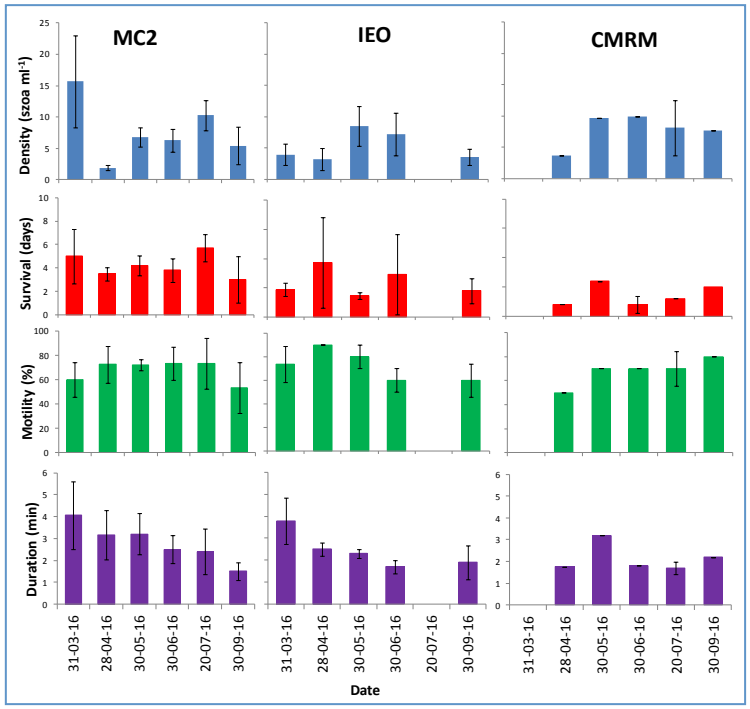

Figure 8. Percentage of male wreckfish at different spermiation index stages, in respect to month from February 2015 until October 2016. Spermiation index was reported on a subjective scale, with S0 = no milt released, S1 = only a drop of milt released after multiple stripping attempts, S2 = milt was released easily after the first stripping attempt and S3= milt was fluently released even without abdominal pressure. The numbers inside the bars indicate the number of wreckfish males examined each month.

Mediterranean stock

On 24/4/2015, the female started spawning spontaneously a small number of dead eggs (**Fig. 9**). On the morning of 1/5/2015, the female had spawned spontaneously 82,000 eggs with 56% fertilization success, while approximately 35,000 eggs were stripped manually from the fish and were inseminated artificially. Eggs were transferred to incubators and photos were taken from floating eggs every day (**Fig. 10**). Eggs from the spontaneous spawn were floating for 4 days, while from stripped spawning were floating/viable for 2 days. At this time (1/5/2015), the female fish also contained many Vg and early OM oocytes (**Fig. 11. A,B,C**) and was induced to spawn using GnRHa implants. The female was given 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. In response to this therapy, the fish spawned up to 22/5/2015 (**Fig. 1**). On 4/5/2015, the female contained oocytes in OM (1,525 µm in diameter), as well as ovulated eggs (2,250 µm) (**Fig. 11 D,E**). The female was stripped –although the ovipore of the fish seemed to be blocked- and 10,000 eggs were artificially inseminated with 2 ml of freshly obtained sperm from the males. Eggs were kept in the incubator for 3 days and later the floating, viable eggs (around 6,000 eggs) were transferred to the larval rearing facility (**Fig. 12**). On 8/5/2015, 190,000 eggs were spawned spontaneously, having 12% fertilization success but the embryos survived for only 5 days (**Fig. 12**). The biopsy taken on 25/5/2015 showed the presence of apoptotic oocytes and overripe eggs, while no Vg oocytes were found (**Fig. 11 F**).

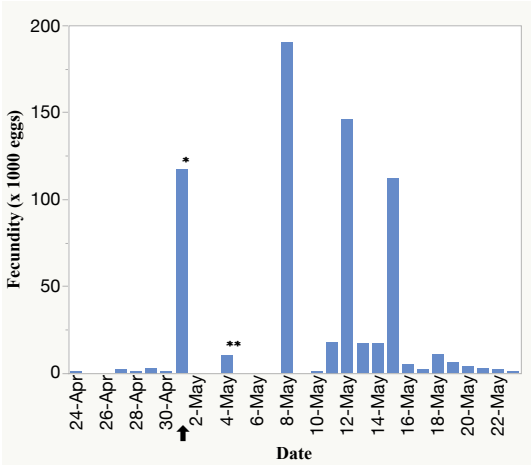

Figure 9. Fecundity from a wreckfish maintained by P1. HCMR. The black arrow indicates the date of spawning induction during 2015. * Fecundity on 1/5/2015 was the sum of spontaneous and stripped spawns. ** eggs on 4/5/2015 were obtained with stripping.

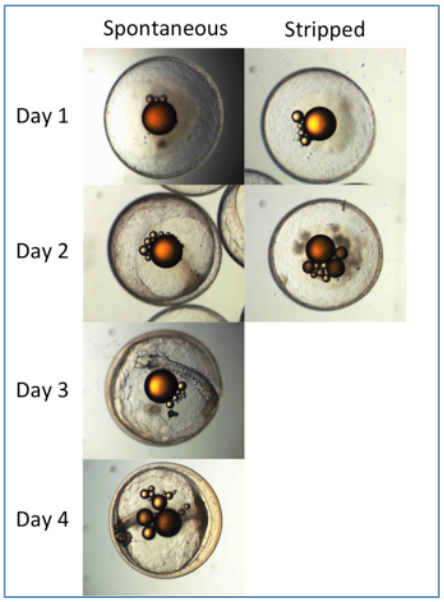

Figure 10. Development of wreckfish eggs from spontaneous or stripped spawning on 1/5/2015 at P1. HCMR.

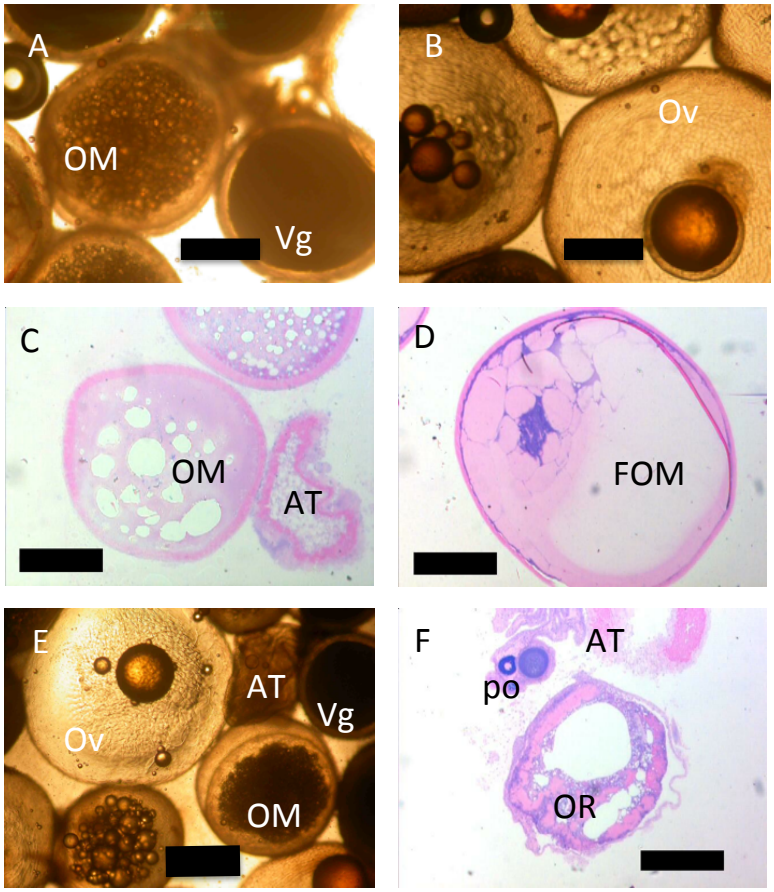

Figure 11. Microphotographs of wet mounts (A,B,E) and histological sections (C,D,E) from the ovary of the wreckfish maintained in P1 HCMR. A,B,C: On 1/5/2015, with oocytes in vitellogenesis (Vg), early Oocyte Maturation (OM), Ovulated eggs (Ov) and some atretic oocytes (AT). D,E: On 4/5/2015, with oocytes in Vg, OM and Final Oocyte Maturation (FOM). F: Primary oocytes and Overripe eggs. Bar = 500 µm.

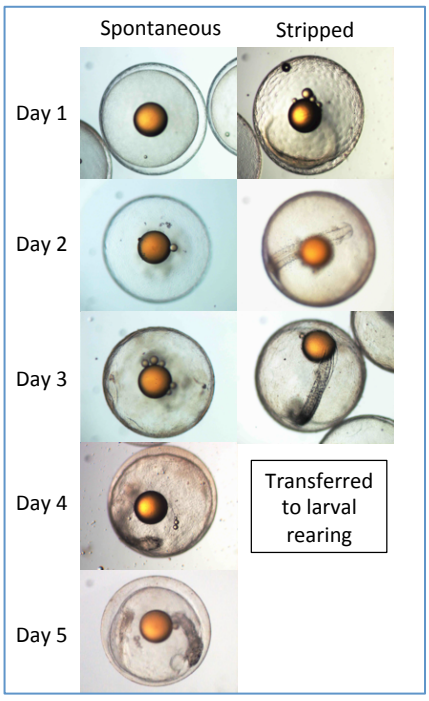

Figure 12. Floating wreckfish eggs from spontaneous or stripped spawning on 8/5/2015 or 4/5/2015, respectively, after spawning induction with GnRHa.

In 2016, a first GnRHa treatment was given on 18/4/2016 both to the female and males. At that time Vg oocytes (1,150 µm) were observed while few oocytes were in AT. 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). 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. At that time, a second GnRHa treatment was given to the female. In 9/5/2016, the fish was observed to spawn spontaneously after a GnRHa treatment on 2/5/2016, and the

number of eggs was estimated to be ~1,000 (**Fig. 13**). The female had Vg and post Ovulated (pOV) eggs, but an effort to strip her did not result in any egg release. On 23/5/2016 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 later (26/5/2016) the female was stripped of the eggs (5,000 eggs, **Fig. 13**), which were inseminated artificially. The female also contained Vg, OM and overripe oocytes, of 1225, 1550 and 1750 µm diameter, respectively (**Fig. 14**). On 28/5/2016, a batch of 1,293 g of eggs (approximately 400,000 eggs) was stripped off the female and again artificial inseminated with sperm. However, both artificial insemination trials were not successful, since no viable eggs were observed the following days.

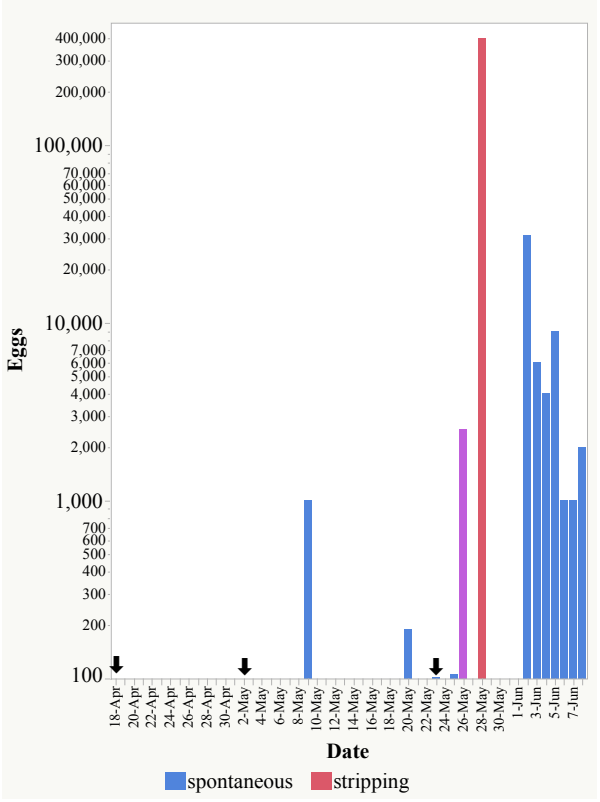

Figure 13. Fecundity from a wreckfish maintained by P1. HCMR in 2016. The black arrow indicates the dates of spawning induction.

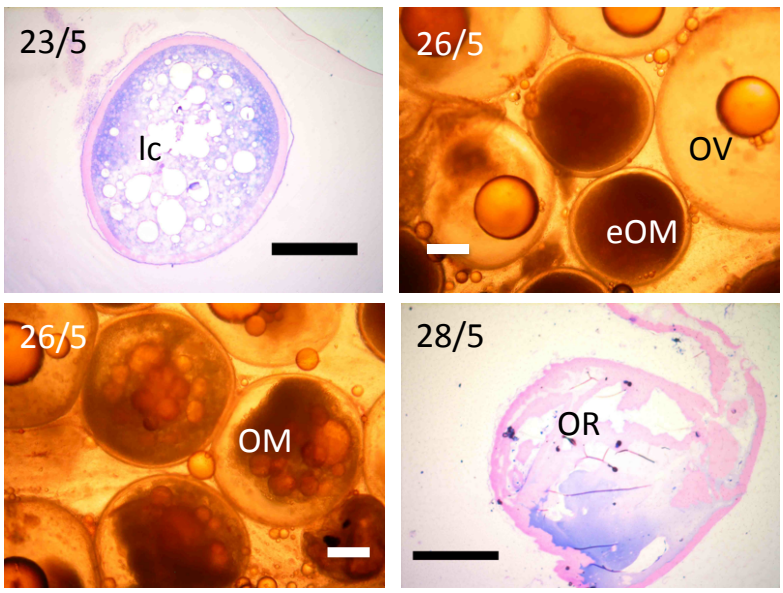

Figure 14. Histological sections or wet mount of ovarian biopsies from wreckfish during the 2016 reproductive season (dates on each photo). lc = lipid coalescence, eOM = early Oocyte Maturation, OM = Oocyte Maturation, OV = Ovulated, OR = Overripe. Bar = 500 µm

The two males were in full spermiation (Spermiation Index = 3, copious sperm released with very gentle abdominal pressure) during the two reproductive seasons. Sperm quality was fairly high during the whole season and no significant variations were observed in different parameters except the motility duration (ANOVA, Tukey's HSD, P≤0.05) (**Fig. 15**).

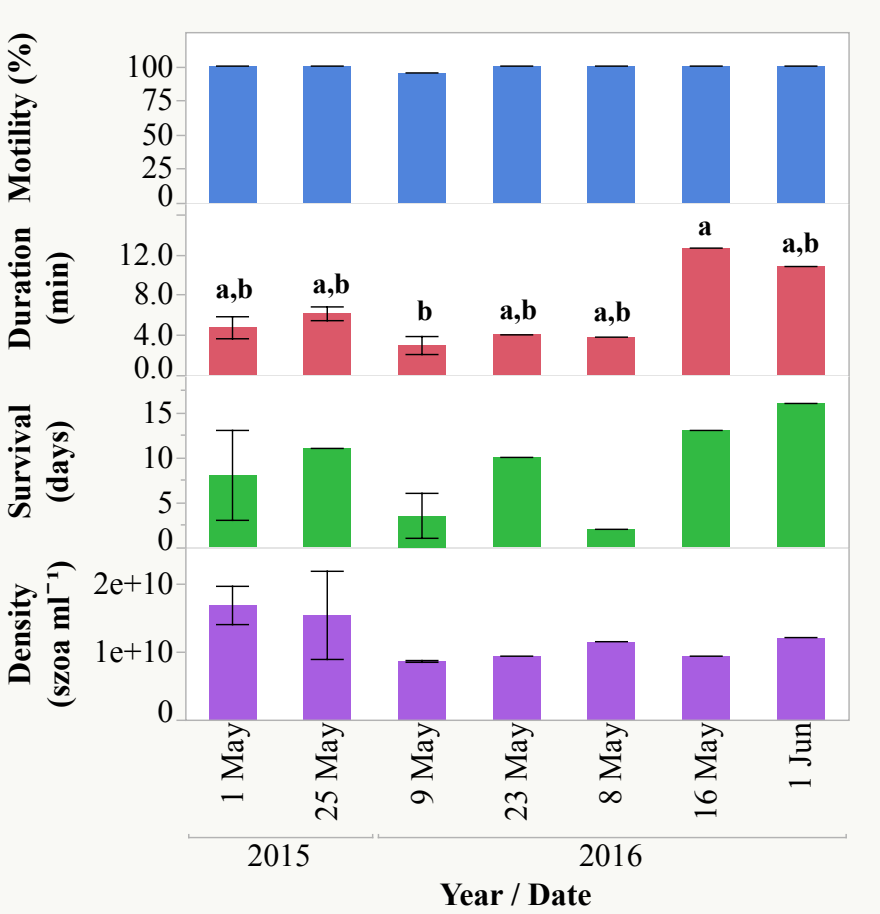

Figure 15. Sperm quality parameters of the wreckfish at P1. HCMR during the 2015-2016 reproductive season samplings for *in vitro* fertilization trials. Significant differences were observed only in motility duration (ANOVA, Tukey HSD, P < 0.05).

Conclusions

Overall, from the monitoring during the season 2016 – 2017 it was observed that the males produce large volumes of good quality sperm for a very long period of time, perhaps throughout the year, under constant 16°C or fluctuating natural water temperature, and was not a limiting factor for any *in vitro* fertilization trial. Sperm of most fish species can be stripped, however care must be taken to avoid contamination of sperm with water or urine as this would result in activation of spermatozoa and loss in fertilizing ability (Urbanyi, et al., 2009). In aquaculture practice in commercial hatcheries, usually the sperm is stripped directly onto the eggs to avoid any contamination (Urbanyi, et al., 2009). In the present study, sperm was either stripped

directly or using a syringe, but in both cases the quality of the sperm was found fairly high meaning that the sperm quality is not responsible for low fertilization success in most of the cases.

On the other hand females do undergo vitellogenesis --and they may even undergo oocyte maturation spontaneously in captivity—and remain in fully vitellogenic stage for at least 3 months (under constant 16°C water temperature). Fertilized eggs could be produced both from spontaneous and stripped spawning but spawned eggs were not of good quality, since their fertilization success was either 0 or very low in most of the cases. May be the lack of knowledge regarding the exact timing of the ovulation after the hormonal treatment and the post-ovulation survival of the eggs is the reason of low fertilization success and egg quality in the obtained eggs. In yellowtail (*Seriola quinqueradiata*) it was shown that the hatching rate of the eggs was decreasing with the time after ovulation (Chuda, et al., 2001).

The work completed on *in vitro* fertilisation demonstrated that this method is complicated to apply to large expensive broodstock and that the manipulation has negative effects and caused some mortality. In addition, to successfully apply *in vitro* techniques it is necessary that the females reach advanced stages of maturity. In the initial stages of the project very few females reached advanced stages and this complicated the work with *in vitro* fertilisation. Later in the project more females reached advanced stages of maturation and this coincided with these females spawning naturally and spontaneously. The over-all aim of the project for the reproduction of wreckfish was to achieve large quantities of fertilized eggs and this was achieved with the spontaneous spawning. The work on *in vitro* fertilisation was therefore limited due firstly to few mature females and secondly due to the success of spontaneous spawning reducing the importance to research methods for in vitro fertilisation.

During the last three years of the project (2014-2018), the number of spontaneous spawns increased. The reason was probably a better adaptation of females to captive conditions resulting in less dysfunctions in the maturation cycle. Females were not only able to complete the process of vitellogenesis, but also final oogenesis, achieving oocytes that were ovulated and spawned to be fertilized naturally by fluent males in captivity.

The spawning biology of wreckfish in captivity was characterized by the following characteristics:

- It has been found that a female is able to spawn an average of 10 times per breeding season.
- There are no differences in egg fertilization (%) and hatching (%) between different types of egg droplet distribution.
- Males produce large volumes of good quality sperm for a very long period of time, covering the same period that females mature, reaching a peak in the months of April and June. The mean concentration of wreckfish sperm was $2.41\pm0.4 \times 10^{10}$ (n=9) spermatozoa ml^{-1} in Galicia in January, while it remained around 1×10^{10} from April to September with no significant variation between sampling dates in Crete, Greece.
- The spermatozoa motility duration was high, with mean values between 2-3 min, and the mean survival time of sperm maintained at 4ºC was 4 days. However, in some cases sperm may reach 18 days of survival after collection.
- Sperm exhibits a high percentage of motile cells at activation and one of the highest initial speeds recorded for fish sperm. This high speed was associated with a long swimming duration compared to other marine fish. ((see ***D6.1 Computer Assisted Sperm Analysis (CASA) for wreckfish sperm*** and ***D6.2 Cryopreservation method for wreckfish***).
- The spermatozoa concentration in wreckfish stripped sperm is of the same order of magnitude as that of pelagic fish such as European seabass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*) or meagre (*Argyrosomus regius*) and it was higher than that of sole (*Solea solea*) and turbot (*Scophthalmus maximus*).
- Despite the easy handling of this species in captivity, its large size requires large volumes of seawater for its welfare in captivity and avoid stress that would affect gametogenesis and therefore

the achievement of maturation in captivity. The stripping method entails a regular handling of the breeding specimens at the time of spawning, which seems unfavorable in the case of the wreckfish, especially once it has been proven that the spontaneous spawns in tanks of large volumes (15-40 m^3) occurs at the scheduled time.

This study complements the previous observations on the biology of this species described for wild populations, and represents the most detailed record of their reproduction in captivity.

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Deviations

This deliverable was delayed, as we were trying to obtain as much experimental data as possible from all the years of the study, from different spawning seasons

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