New species for EU aquaculture

Deliverable Report

Deliverable No:	D6.6		**Delivery Month:**	60
Deliverable Title	An *in vitro* fertilization protocol to be employed by the industry to spawn wreckfish			
WP No:	6		**WP Lead beneficiary:**	P8. IEO
WP Title:	Reproduction and Genetics - Wreckfish			
Task No:	6.3		**Task Lead beneficiary:**	P8. IEO
Task Title:	Task 6.3: Development of spawning induction procedures.			
Other beneficiaries:	P1. HCMR	P2. FCPCT	P3. IRTA	P14. IFREMER
P15. ULL	P19. CMRM	P32. MC2		
Status:	Delivered		**Expected month:**	48
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Objective: The objective of this deliverable is to describe a method to perform in vitro fertilization for commercial use, setting a protocol to obtain fertilized eggs and produce larvae based on: 1) Determination of maturity stage of males and females based on the techniques described in D6.4. 2) Utilization of oocytes and sperm fresh or cryopreserved (D6.3), to perform artificial fertilization, relation sperm/oocyte, percentage of fertilization/hatching. This protocol will be published for its use by the sector.

Deliverable description from the DOW: An in vitro fertilization protocol to be employed by the industry to spawn wreckfish: A method to perform in vitro fertilization for commercial use will be described. The deliverable will include a protocol on how to obtain fertilized eggs to produce larvae based on: 1) Determination of maturity stage of males and females based on the techniques described in D6.4, 2) utilization of oocytes and sperm fresh or cryopreserved (D6.3), to perform artificial fertilization, relation sperm/oocyte, percentage of fecundation/hatching. This protocol will be published for its use by the sector.

Introduction

Wreckfish as a new species suitable for aquaculture in the EU was one of the six chosen in the DIVERSIFY project based on the following selection criteria:

Economic criteria, wreckfish have an important economic potential for the aquaculture industry, with a high market price, high consumer demand in large finfishes market and from producers due to the possibility to add value to the fish. The wreckfish market prices in Galicia (NW of Spain) varied in the last ten years between 13 and 22 €/Kg (**Fig. 1**) and nowadays catches mainly come from Azores (see D6.4).

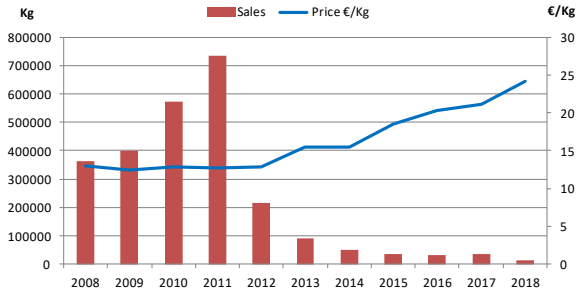

Figure 1. Price evolution of wreckfish in the Galician market during the last ten years.

According with the ***biological criteria***, wreckfish is a fast growing, large size species that accepts inert food readily. Despite its large size, the species acclimatizes easily to captivity and no significant mortalities have been reported due to handling. In a study of wild-caught individuals it was shown that fish grew from 1 kg to 5 kg in a period of 10 months (Papandroulakis et al., 2004; Rodriguez-Villanueva et al., 2011) (**Fig. 2**).

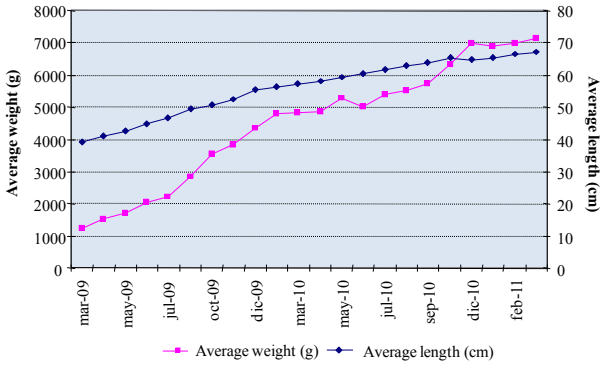

Figure 2. Average weight and length of 10 individuals captured in Galician coast with 1.215± 0.165 Kg of weight and kept in tanks en IGAFA (CMRM) during 418 days. Final weight was 7119.16± 0.769 kg.

Its long juvenile stage is a great advantage from the aquaculture viewpoint, allowing for commercialization before sexual maturity, and thus avoiding problems linked to maturation, such as reduction in growth, or loss of flesh quality and organoleptic properties. It has been demonstrated that growth is strongly influenced by sex and that wreckfish females grow faster than males, as observed in many other marine fish species (Rodríguez et al., 2017).

Regarding ***sustainable criteria***, wreckfish is a species with high nutritional value, with a high protein content in the muscle, 84% in wild fish and 78% in captive-reared fish (Linares et al., 2015). The possibility of artificial culture and grown from juveniles is a real choice due to limited fisheries and the scarcity of wild individual being landed. The extended pelagic juvenile phase and the easy adaptation to captivity could be an incentive to stimulate different aquaculture types, as cages, ponds, etc. The scarcity of broodstock from the fishery is a disadvantage for establishing aquaculture. Sales in Galicia decreased from 62 tm (2009) to 18 tm (2017) (**Fig. 1**).

The clear biological and economical potential of this species justified the allocated effort that the project DIVERSIFY has achieved by bringing together almost all partners involved with wreckfish in Europe for the species domestication in order to produce appropriate numbers of juveniles to launch commercial production (Mylonas et al., 2017). The establishment of methods for the management of spawning and production of good quality eggs are essential for the culture of any animal species.

Description

Four different broodstocks with variable number of breeders collected as juveniles from the wild were maintained in research facilities in Greece and Spain. These facilities were the Hellenic Center for Marine Research (P1.HCMR, n=2) in Heraklion, Crete, Greece; the Instituto Español de Oceanografía (P8.IEO, n=14) in Vigo, Spain; the Aquarium Finisterrae (P32.MC2, n=19) in A Coruña, Spain; and the Conselleria do Medio Rural e Mariño (P19.CMRM, n=11) in Pontevedra, Spain.

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 checked 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 1**).

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 a dry pellet specifically formulated by SPAROS according to biochemical profile (see deliverable D12.2, Recommendations for wreckfish broodstock feeds).

The same environmental conditions were applied to 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 and the food was changed at the end of this year, because 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 with a mixture of hake and squid.

Finally a stock of 19 wreckfish with a sex ratio of 0.6:1 male/female was maintained at the MC2 facilities in a 3500 m^3 exhibition tank (Nautilus) with natural temperature and real simulation photoperiod, and fed daily sliced fish, hake and mackerel, and squid during the first stage, but then trained to feed on Fish breeders –M Inve® dry-food from year 2105 to late 2016 and since 2017 specifically formulated dry pellets food by SPAROS. When the first external evidence of reproductive maturation was detected (abdominal swelling), animals were transferred to a 33 m^3 tank for closer monitoring. Sadly in 2016, an accident in the pumping system of this tank caused the death of three females that were yearly naturally spawning.

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	

These fish were kept in diverse environmental conditions according to tank size and photothermal regime. That includes indoor and outdoor tanks with natural like photothermal conditions, and indoor tanks with simulated natural photothermal conditions or constant temperature. As an exception, the HCMR broodstock was kept at constant water temperature (15-16°C) throughout the year. Three Galician stocks were kept in relatively similar temperature conditions, ranging between 12 and 18°C (**Fig. 3**). Fish were periodically

monitored to check their stage of reproductive development between March 2015 and October 2016, encompassing two spawning seasons and one complete reproductive season (see deliverable D6.5, Description of the reproductive cycle of wreckfish).

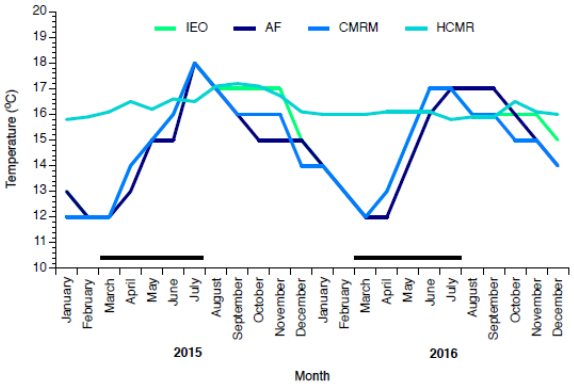

Figure 3. Tank water temperature (˚C) provided for wreckfish broodstocks at four different places: Hellenic Center for Marine Research (P1.HCMR), Greece, Instituto Español de Oceanografía (P8.IEO), Aquarium Finisterrae (P32.MC2) and the Conselleria do Medio Rural e Mariño (P19.CMRM) Spain from January 2015 until December 2016.

Female maturity was assessed by examining a wet mount of the biopsy under stereoscopic magnifying glass (40 and 100x) to evaluate the oogenesis stage and measure mean diameter of largest most advanced oocytes (n = 10) (**Fig. 4**) (Papadaki et al, 2018). Male fertility was examined by the release of sperm upon application of gentle abdominal pressure. Spermiation index was evaluated based on the presence and ease of milt release upon the application of gentle abdominal pressure (Mylonas et al., 2003). Spermiation index was reported on a subjective scale from 0 to 3 with S0 = no sperm released, S1 = only a drop of sperm released after multiple stripping attempts, S2 = milt was released easily after the first stripping attempt and S3= sperm was fluently released even without abdominal pressure. Sperm quality parameters that were evaluated included sperm concentration (number of spermatozoa.ml−1 of milt), percentage of spermatozoa showing forward motility right after activation (initial sperm motility, %) and duration of forward sperm motility of at least 10% of the spermatozoa in the field of view (motility duration, min).

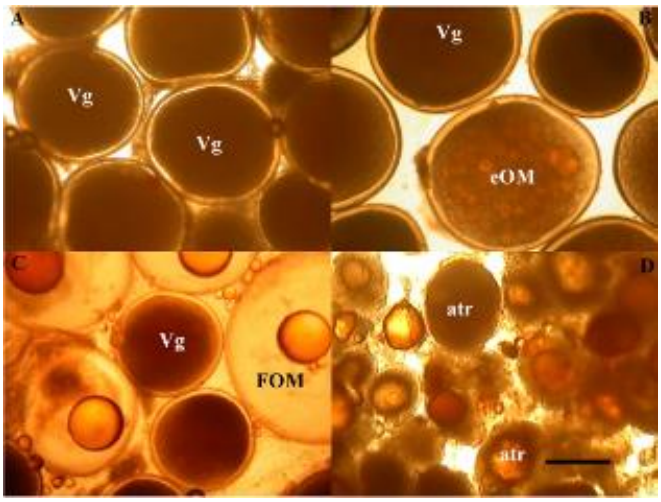

Figure 4. Photomicrographs of wreckfish biopsies, showing oocytes at successive stages of development: vitelogenesis (Vg, A), early oocyte maturation (eOM) with lipid droplet coalescence (B), final oocyte maturation (FOM, C) and atresia at the end of the reproductive season (D). The bar represents 500 µm.

When some individuals were difficult to biopsy due to gonad or gonopore blockage, ultrasounds were used (Martin et al., 2001). A first sex identification of the broodstock by identifying female gonads of breeders in MC2 and CMRM was performed and females were identified (**Fig.5**).

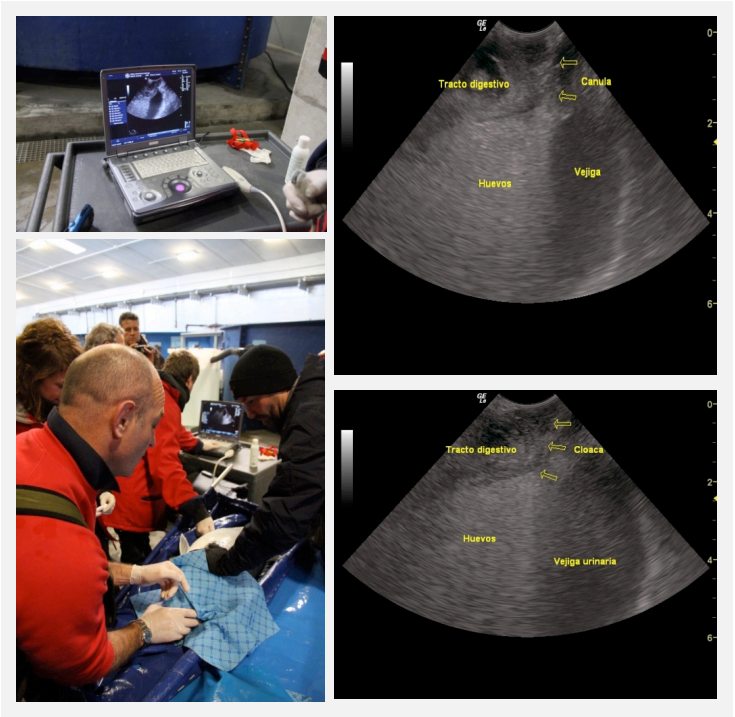

Figure 5. Gonad ultrasounds of two wreckfish to identify sex externally.

On the other hand, nutritional analyse of eggs and gonads of females, as well as several samples of muscle, kidney and liver from wild caught individuals were analyzed (Linares et al., 2015) (CIMA-CMRM) in order to collect some basic necessary data to perform the ideal pellet food for this species (see deliverable D12.2, Recommendations for wreckfish broodstock feeds). Additionally essential chemical components (carotenoids and vitamins) between wild mature female gonads and those from eggs produced under different rearing conditions and feeding regimes were investigated (P15.ULL).

During the project, three procedures to obtain viable eggs from wreckfish in captivity were tested (**Fig. 6**).

1) Natural and spontaneous spawns in large tanks (>30 m^3), eggs were naturally fertilized by the males and floating eggs collected using a surface egg collector device. (**Fig 6a**)
2) Spawning induction procedures, with gonadotropin releasing hormone analogues (GnRHa). Depending to de outcome the approach was conducted in large tanks (>30 m^3) under controlled photothermal conditions and allowed to spawn spontaneously or by stripping conducted in smaller tanks (< 15 m3), where fish were monitored for ovulation. Ovulated eggs were obtained by stripping and inseminated *in vitro* using sperm from spermiating males or eggs were collected from the tank after hormone induction and subsequent spontaneous spawning, where the induced spawning of eggs were naturally fertilized by a male. (**Fig. 6b**)
3) Naturally, ovulated eggs were stripped and the ovulated eggs were fertilized *in vitro*. Gametes were stripped from the mature females and males that were held in smaller tanks. Ovulated eggs were inseminated in vitro using sperm collected by stripping from spermiating males (**Fig. 6c)**

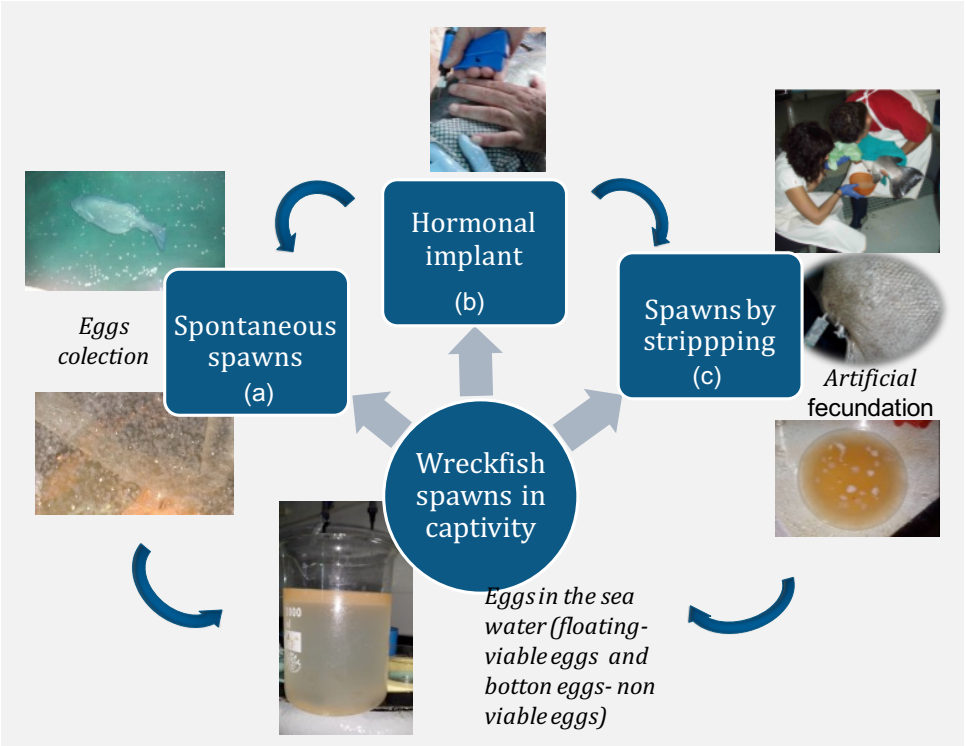

Figure 6. Different procedures to obtain wreckfish spawns in captivity: by spontaneous spawns (a), by hormonal implants (b) and artificial by stripping (c).

Trials with hormonal induction (GnRHa) were described in the deliverable D6.7 (Spawning induction method for spontaneous spawning of wreckfish in large tanks), which reported irregular results in the egg quality obtained. Regarding achieving spawns by stripping, some results were obtained. Wreckfish stripping when mature individual weigh between 15-30 kg made the procedure risky. Best results were obtained with spontaneous spawns by females in the mature season with natural fertilization by males in tanks that had natural conditions of sea water temperature and photoperiod (see deliverable D6.3, Spawning induction methods with *in vitro* fertilization of wreckfish).

Regarding wreckfish commercial production, at present mature wreckfish stock seems to be the easiest way to get spawning and when female is not mature enough, hormonal induction at a dose over 80 µg/kg when oocyte is at the correct stage ≥ 1200 µm can cause or accelerate spawns. However stripping to get eggs for *in vitro* fertilization remains unachieved. Protocols to be employed by the fish farming industry are focused on natural maturation of males and females, which result in spontaneous spawns and natural fertilization of oocytes by the sperm of mature males in the tanks. In years 2017 and 2018, GnRHa implantations of two MC2 female with doses > 80 µg/kg led to natural spawns.

RESULTS

Regarding food intake of the broodstocks along the year, low feeding rates were recorded during the spawning season (from March to July) and high during autumn (October-November) **(Fig. 7)**. Ingestion rate varied between 0.2 and 0.5 % for fish fed with dry pellets and between 1 and 1.8 % those fed with semi-moist diet.

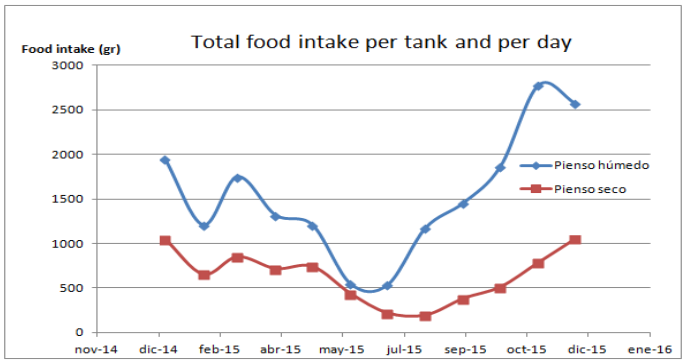

Figure 7. Total food intake/tank/day during the year 2015 the two wreckfish broodstock from IEO.

During these last years, the number of spontaneous spawns has increased and the number of induced and artificial spawns has decreased. The reason is due to the adaptation of females to the natural maturation cycle, resulting in the spontaneous completion of vitellogenesis and oocyte maturation (**Fig. 8**).

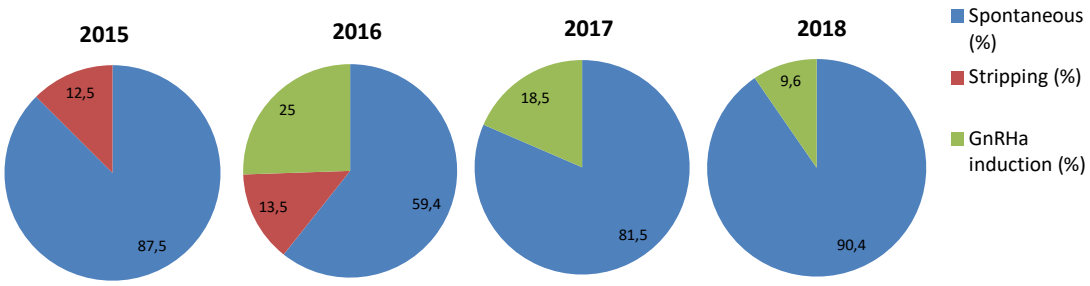

Figure 8. Number (%) of spontaneous, artificial (by stripping) and induction (with GnRHa implants) along 2015-2018 in the three Galician wreckfish broodstock.

The natural spawning behaviour was characterized by mature males chasing the mature females with liberation of eggs, which were immediately fertilized with the sperm released into the water by the male. Spawning mainly takes place during the night or very early in the morning. In 2017 and 2018, spontaneous spawning in the IEO, MC2 and MCMR stocks produced a large number of fertilized eggs and achieved satisfactory fertilization success, establishing clearly a gap between one female spawns of 3-5 days in all stocks and verifying a time of spawns in early morning between 5-8 h, except for some that took place at mid day. The total number of annual spawns of all the Spanish stocks in the Diversify project increased substantially in 2018 (**Table 2**).

Table 2. Total spawning events from 2015 to 2018 at IEO, MC2 and CMRM stocks.

STOCK	**YEAR**			
	2015	2016	2017	2018
IEO	10	9	13	43
MC2	14	23	5	19
CMRM	0	4	9	30
Total	24	36	27	92

Spawns at the three Galician broodstock started at different times of the year due to their different latitude location and hence temperature profile of water. The duration of the reproduction time in the different stocks

was also different, with a period of January to May 2018 in IEO, from mid-March to July in MC2 and from February to June in the CMRM stock. The sea water temperature was similar in centers further South, 12-20ºC, for the CMRM-IGAFA stock, in Ría de Arousa and for the IEO stock in the Ría de Vigo, whilst temperatures were and lower in the north, 12-18ºC, for the MC2 stock (**Fig.9**).

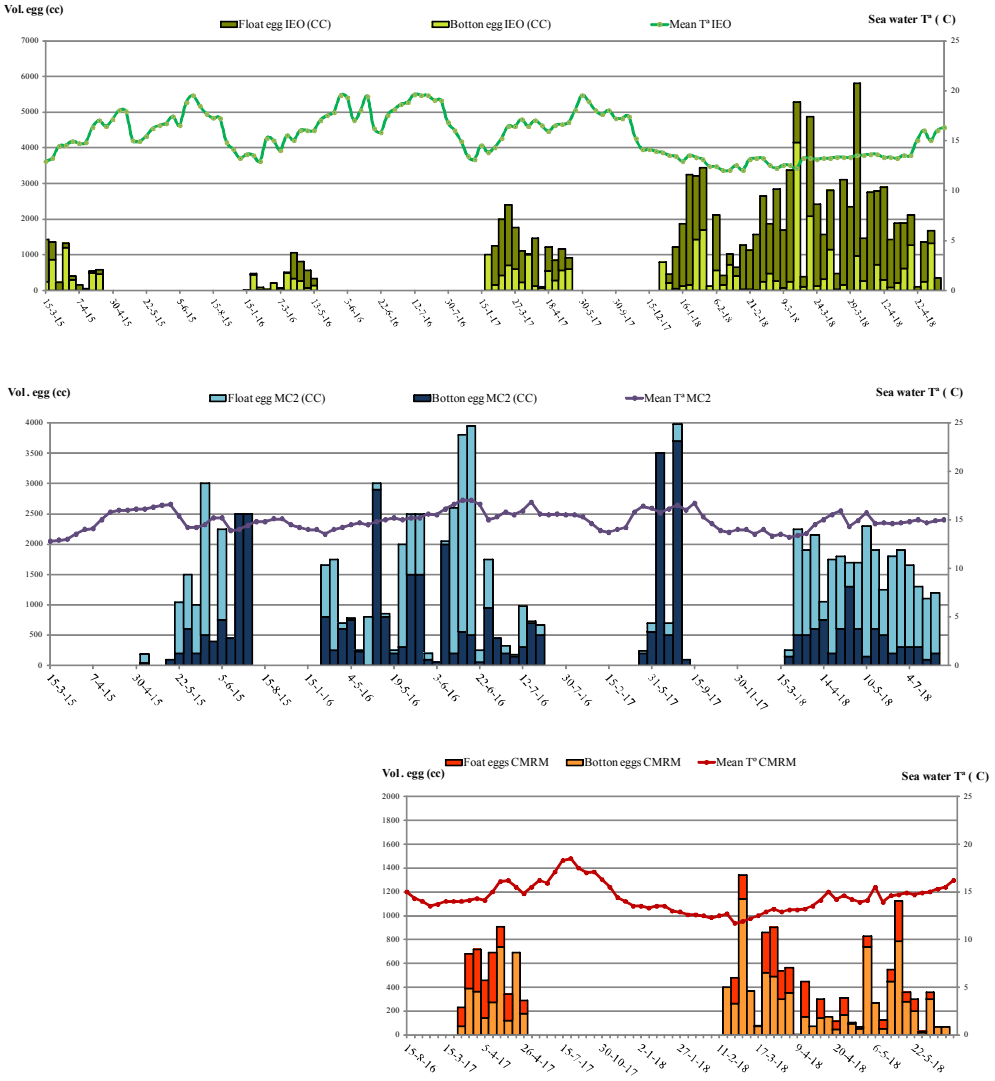

Figure 9. Volume of viable floating and non-viable sunk eggs (cc) of wreckfish obtained from spawns at the IEO (upper), MC2 (medium) and CMRM facilities (lower) and seawater temperatures at the different tanks since May 2015 to July 2018.

Percentage of egg fertility was between 50 and 100 % with better quality eggs at the medium-final part of the spawning period. It has been found that females were able to spawn an average of 10 times per breeding season.

With respect to males stock, sexual maturation covers the same period of females, with maximums in April and June, with peak concentrations of 25-35 x 10^9 spzoa/ml of sperm. The duration of motility was high, with mean values between 2-3.5 minutes. The mean survival time of sperm, conserved refrigerated at 4ºC, was 4 days. However, in some cases it reached 18 days of survival after collection. In the IEO facilities one male had the capacity to fertilise 30 spawn in a period of 150 days.

Egg characteristics

Spawned wreckfish eggs have a diameter of 2.12±0.064 mm (Papadaki, 2018). This large egg size compared with the smaller egg size of other cultured marine fish species such as blackspot sea bream (*Pagellus bogaraveo*) (**Fig. 10** left**)**, led to some modifications in the egg incubation methods (see below, Spontaneous spawn management).

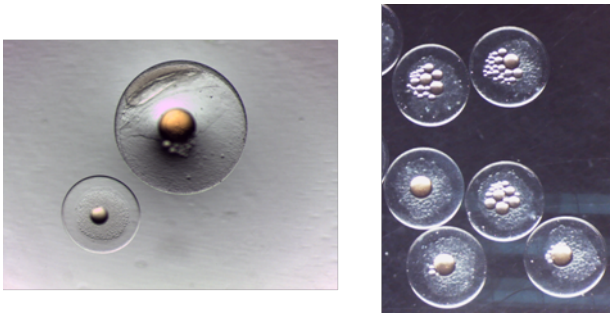

Figure 10. Morphological differences between blackspot sea bream (*Pagellus bogaraveo*) eff and wreckfish *(Polyprion americanus*) egg (**a**). Fertilized eggs (morula) with different number of lipid droplets (**b**).

Different number of lipid droplets were found (**Fig**. **10** right), but evaluating morphological parameters of eggs, no significant differences in egg fertility % and hatching % between different spawn types were found (see deliverable D6.3, Spawning induction methods with *in vitro* fertilization of wreckfish).

Sperm characteristics

In DIVERSIFY, we established a Computer Assisted Sperm Analysis (CASA) for the evaluation of wreckfish sperm, and the method is available as a movie describing the procedure of sperm activation and CASA analysis on the website of the project (www.diversifyfish.eu) and in the deliverable D6.1, Computer Assisted Sperm Analysis (CASA) for wreckfish sperm and D6.2, Cryopreservation method for wreckfish. The analyses demonstrated that sperm of captive wreckfish shares a common pattern of motility with both marine and freshwater fish, based on a general activation of all the sperm at the same time of ejaculation in activating environment, then a decrease with time down to zero in a rapid lapse of time from 30 sec to more than 20 min due to exhaustion of energetic stores badly compensated by respiration.

The mean concentration of wreckfish sperm was 2.41 $x10^{10}$ (sd: 0.4 $x10^{10}$, n=9) spermatozoa ml^{-1} in Galicia in January, while it remained around $1x10^{10}$ from April to September with no significant variation between sampling dates in Crete, Greece. The spermatozoa concentration in wreckfish stripped semen was 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 (Fauvel et al., 2008).

The high speed of the wreckfish sperm was associated with a long swimming duration compared to other marine fish (**Fig. 11**). The long duration exhibited a double trajectory shape. The first trajectory was straight (associated with the search of target eggs) and then the trajectory began bending, which was interpreted as a phase of searching for the micropyle on the egg surface. Moreover, the results obtained by CASA are in agreement with field observations obtained by human inspection under the microscope, and complement them by objective data that can be more easily statistically analyzed (see deliverables D6.1 and D6.2).

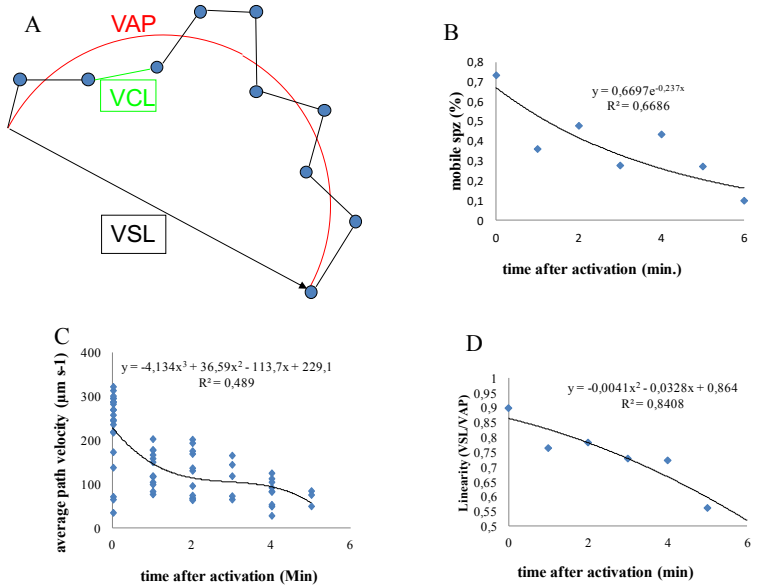

Figure 11. Mobility parameters of wreckfish sperm: A) schematic representation of spermatozoa movement illustrating the three parameters of velocity generated by CASA; B) variations of the percentage of swimming spermatozoa (spz) with time; C) average path velocity decrease with time after activation; D) decrease of linearity of spermatozoa trajectories after active.

Wreckfish sperm exhibits a high percentage of motile cells at activation and one of the highest initial speeds recorded for fish sperm. Sperm motility percentage exhibited high and almost unchanged values during both years of the study (60-90%), sperm motility duration ranged between 2-6 min, and was longer than most marine fish common in Mediterranean aquaculture. Sperm density exhibited high values during the whole year (4-18 x10^9 szoa ml^{-1}), and sperm survival at 4°C ranged between 3-14 days.

Spontaneous spawns: egg fertility and females fecundity

The mean fertility and the percentage of spontaneous spawns were good indicators of egg quality, maintaining values around 80% through the year (**Fig. 12**). Floating egg (%) per spawn increased along the years from mean values of 43% in 2015 to 83% in 2018.

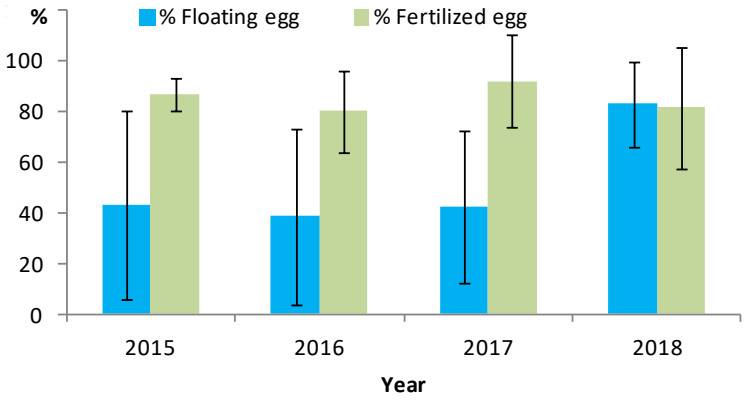

Figure 12. Total floating egg (% floating egg of the total egg spawned) and total fertility egg (% fertility egg in the total egg spawned) per year from the three Galician broodstock.

On the other hand, the increase in the individual relative fecundity (nº eggs/kg female) along these five years, as well as the quantity of fecund females was observed (**Fig. 13)**, with maximum values of relative fecundity of 174466 eggs/kg female in 2018 spawning season (MC2 stock). The high quantity of spontaneous spawns is another good indicator of egg quality.

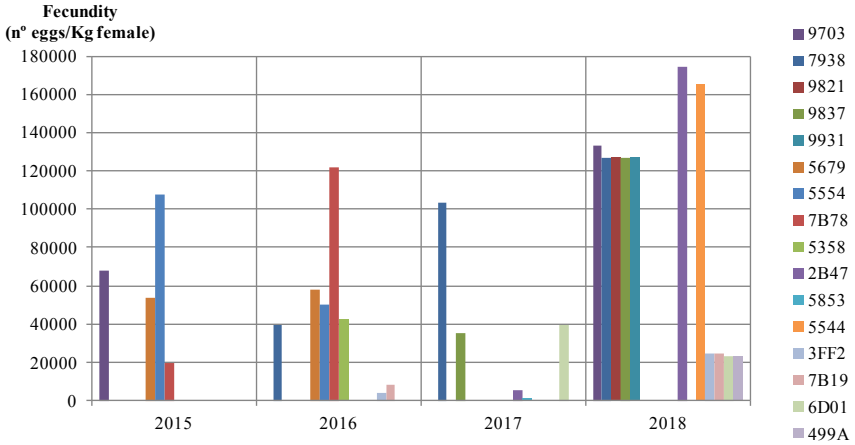

Figure 13. Relative fecundity (nº eggs/kg female) during 2015-2018 from the three Galician stock. The numbers in legend of the graph indicate the identification number tag of each female, as well as the bar color.

Another indicator of egg quality such as floating eggs (%), fertility (%) or hatching rate (%) was evaluated in 27 spawns. Egg development stage at the time of collection ("developing") was identified and annotated following the criteria: 1 = first division did not occur, 2 = 2 or 4 cells were observed and 3 = a more advanced state of those described above was detected. This scale allowed us to infer how long the eggs remained in the collectors after spawning before being collected (**Fig. 14**).

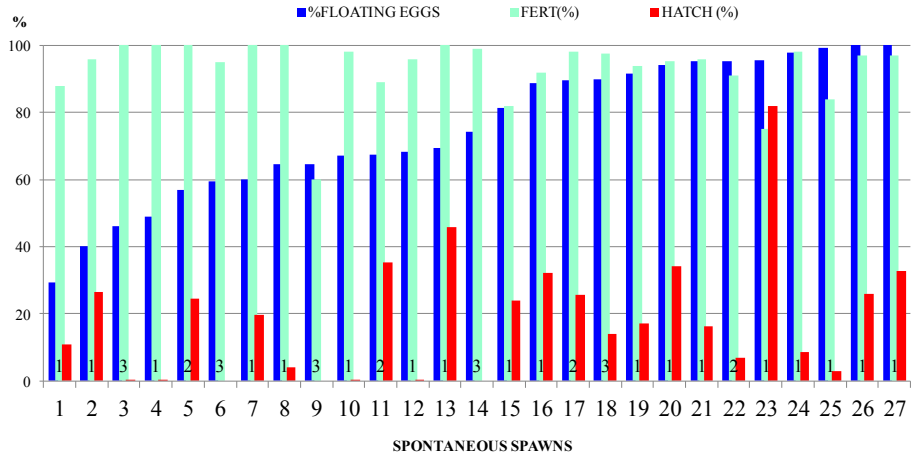

Figure 14. Floating eggs (%), fertility eggs (%) and hatching (%) from the three Galician stocks. The number in the bar indicates the development status at the moment of egg collecting: 1 = collected before first, 2 = before third, and 3 = after third cellular division respectively.

A statistical analysis (ANOVA and multiple linear regression) was carried out to determine the effect of the three parameters on the hatching rate. No significant differences ($p > 0.05$) between floating eggs (%),

fertilization (%) and the state of development of the eggs on the hatching percentage were observed, nor was it possible to obtain a regression curve due to the high error and low resolution of the generated models. However, the stage of egg development or "developing" factor when eggs were collected has been the factor that has explained the variability observed in the rate of hatching.

Spontaneous spawn management

During spawning, a passive egg collector was placed in the outflow of the tank, in order to verify the occurrence of any spawning. Once a spawn was collected (**Fig. 15** ***a***), it was transferred to a container with a sufficient volume of water to be able to separate the fraction of sinking eggs from floating eggs (**Fig. 15** ***b*** and **Fig.16**).

Figure 15. Spontaneous spawning management. Collecting eggs (***a***), separating viable eggs by floating (***b***), incubating at first phase (***c***), the second phase (***d***) and finally transfer to the larval culture (***e***).

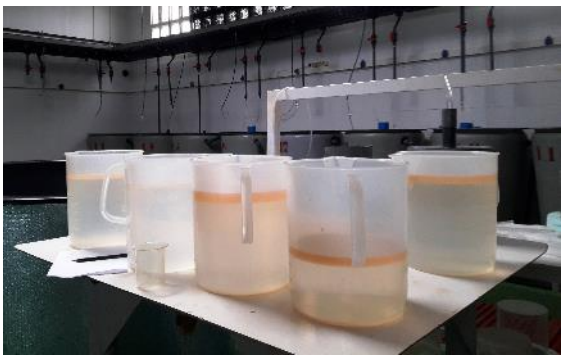

Figure 16. Total eggs of one spontaneous spawn separated in jars to measure the egg volume (floating and sinking) before incubation transfer to incubation tank.

The total egg volume was also be measured, as well as the volume of floating and sinking eggs. Sinking eggs were subsequently discarded. The floating eggs, were examined using a stereoscopic microscope to

record egg diameter, the fertilization (%) and the stage of embryonic development. The total number of eggs spawned was estimated by multiplying the observed egg volume by 150, which is the number of wreckfish eggs contained in 1 mL.

The floating eggs were transferred into a cylindrical incubator tank with a conical bottom (**Fig. 16 *c*)** and a water flow at 16ºC (see D.18.2), with enough air bubbles coming from the bottom to gently allow eggs distribution throughout the water column. On each of the first three days, one purge was made to collect dead eggs from the bottom of the tank, pulling out air and water inlet for few minutes so viable eggs raise to surface and dead eggs sunk to bottom. On the third day of incubation (at 16ºC), when the embryogenesis began, the air and water were removed and the floating egg collected and transferred to another tank with different circulation and aeration system and similar water temperature (**Fig. 17**) until hatching. During this last stage of embryogenesis, eggs density increase respect to water and the eggs lost buoyancy and tended to settle at the bottom of the incubator, so they remained in the water column with a water flow from the bottom and the entire base of the tank. The air was arranged around the containment mesh to prevent the eggs sticking. Once larvae hatched (6dpf), they were transferred to the larval culture tank (**Fig. 16 *e***), where husbandry continued (see deliverables D18.2, Determine optimum temperature conditions for rearing wreckfish larvae; D18.3, Develop a feeding protocol for wreckfish larvae and D18.4, Determine the most effective culture system (RAS vs) flow-through) for wreckfish larvae.

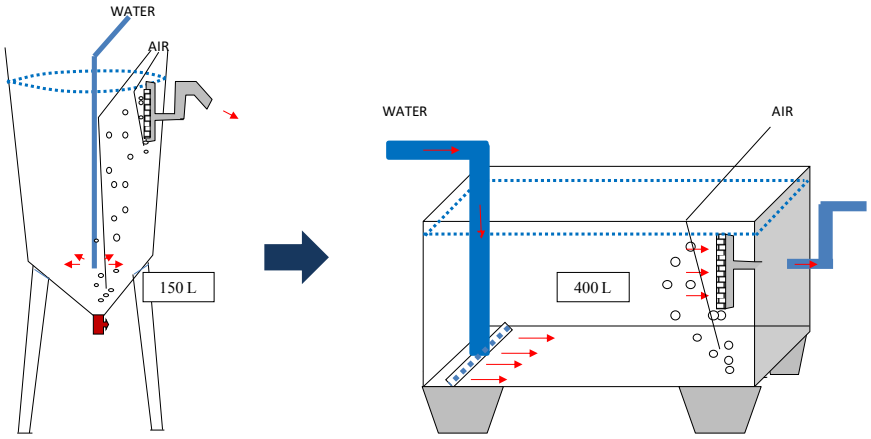

Figure 17. Scheme of incubation and hatching tanks for wreckfish eggs.

CONCLUSSIONS

In summary, knowledge acquired during the development of this project (D6.1, D6.2, D6.3 and D6.5) regarding wreckfish reproduction was:

- The spawning season covers the months of January to July, and occurs sequentially in batches. Spawning takes place mainly during the night or early in the morning, between 05:00 and 08:00 h, with some exceptions that took place at midday.
- All wreckfish broodstocks produce a large number of fertilized eggs and achieve satisfactory fertilization success.
- Spawning periodicity is 3-5 days and fertilization success is between 50 and 100% with better quality eggs towards the mid or end of the spawning season for each female.
- Males chasing the females followed by the release of the gametes and produces large volume of good quality sperm for a very long period of time, covering the same period of females, reaching maximum values in the months of April and June. The mean concentration of wreckfish sperm is 2.41 $x10^{10}$ (sd: 0.4 $x10^{10}$, n=9) spermatozoa ml^{-1} in Galicia in January, while it remains around $1x10^{10}$ from April to September with no significant variation between sampling dates in Crete, Greece.

- Sperm shows 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. The mean survival time of sperm maintained at 4ºC is 4 days. However, in some cases (male from IEO) sperm may reach 18 days of survival after collection.
- The spermatozoa concentration in wreckfish stripped semen has similar magnitude compared to pelagic fish such European seabass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*) or meagre (*Argyrosomus regius*) and it is higher than sole (*Solea solea*) and Turbot (*Scophthalmus maximus*).
- It has been found that one female is able to spawn an average of 10 times per breeding season even 12 times registered in one female at MC2 in 2018.
- One male has the capability to fertilize at least 30 spawns in a period of 150 days (during 5 months in 2018 at IEO broodstock).
- No significant differences ($p > 0.05$) between effect of floating eggs (%), fertilization (%) and the state of development of the eggs on the hatching percentage has been observed. However, due to the large size and vulnerability, the delay in the collection of newly spawned eggs may affect their embryonic development.
- The adaptation and development of a specific incubation system for the proper development of egg embryogenesis of this species (described above) has been essential to achieve very good results in the hatching rate, reaching values up to 80%.

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