New species for EU aquaculture

Technical Manual – Wreckfish (*Polyprion americanus*)

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DIVERSIFY 2018

Table of Contents

Introduction

Wreckfish is one of the largest Serranid species, **reaching a size of 100 Kg**. It is a deep-water fish found **almost throughout the world** and is characterized by an extended pelagic juvenile phase (Sedberry et al., 1999; Ball et al., 2000; Deudero et al., 2000). Wreckfish is one of the most interesting new species for aquaculture, due to its **fast growth** (Suquet & La Pomélie, 2002; Rodriguez-Villanueva et al., 2011), **late reproductive maturation** (Sedberry et al., 1999), **high market price and limited fisheries landings** --quotas have been reduced by 90% in 2012 in the U.S.A. (NOOA, www.fishwatch.com)-- and ease of manipulation in captivity (Papandroulakis et al., 2008) Rodriguez-Villanueva et al., 2011). Its large size lends itself to **processing and development of value added products**, and its **cosmopolitan distribution may enable** EU exports.

Wreckfish acclimatizes easily to captivity and, despite its large size, no mortalities have been reported due to handling. It accepts inert food easily, being a very voracious carnivore. 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** (Rodriguez-Villanueva et al., 2011). The slow reproductive maturation of wreckfish, which occurs at an age of 5-10 y in captivity, may be a problem for broodstock development and management. On the contrary, 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 influence by sex and that female wreckfish are significantly heavier than males, as observed in many other marine fish species (Rodríguez, J.L.2017).

The wreckfish is a globally distributed, anti-tropical species that inhabits continental coasts and oceanic islands at depths of 100-1000 m (Roberts, 1989), forming three genetically distinct stocks, in the North Atlantic and the Mediterranean Sea, in Brazil and in the South Pacific (Ball *et* al., 2000). It is a gonochoristic species with no sexual dimorphism and spawns at the continental slope at depths of 300-500 m, with the formation of spawning aggregations (Peres & Klippel, 2003).

The establishment of methods for the control of spawning and the production of good quality eggs are essential for the culture of any animal species. The description of the reproductive cycle, except for allowing for the identification of the spawning period and spawning preferences of each species (temperature and photoperiod), enables the recognition of possible reproductive dysfunctions and leads to the development of protocols for spawning induction and production of viable eggs (Mylonas et al., 2013).

Lack of reproduction control and of established larval rearing protocols have been the major bottlenecks preventing wreckfish aquaculture so far. Limited egg collection has been achieved from captive spawners using hormonal induction (Papandroulakis et al., 2008) or stripping of naturally maturing fish (Peleteiro et al., 2011). Embryonic development and the early life stages have been described (Papandroulakis et al., 2008; Peleteiro et al., 2011), indicating that the **large egg size of this fish (~2 mm in diameter) may offer significant advantages for its larval rearing**. Reproduction and larval rearing of a very close relative, the hapuku (*Polyprion oxygeneios*) has been achieved recently in New Zealand (Anderson et al., 2012; Symonds et al., 2014; Wylie et al., 2018a; Wylie et al., 2018b). The **scarcity of broodstock is a disadvantage for this fish**, but the **clear biological and economical potential of this species justified allocation of part of the effort of DIVERSIFY** in bringing together almost all partners involved so far in Europe in wreckfish domestication, to **overcome its documented bottlenecks --*i.e.*, reproduction and larval rearing--** in order to produce appropriate numbers of juveniles to launch commercial production.

In the present Technical Manual, we provide all the information obtained during the 5-years of the DIVERSIFY project, relevant to the acquisition of new broodstock, broodstock management and control of reproduction, nutrition and larval rearing. This information is targeted towards commercial and research organizations, that are interested in investigating the potential of wreckfish for aquaculture. As the work in wreckfish is still on-going at this time, not all data have been evaluated thoroughly, and thus are not presented here. They will become available, nevertheless, in the near future in the website of the project (www.diversifyfish.eu).

Reproduction and Genetics

Acquisition of wild fish and establishment of captive broodstock

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Wreckfish is a deep-water fish found around the world. Demersal wreckfish individuals inhabit rocky and muddy bottoms, at depths of 40-200 m; however, individuals are frequently found in waters deeper than 300 m, with a maximum-recorded depth of 1000 m (Fischer *et al*., 1987). The first part of their life (from hatching to a body length about 60cm) is pelagic and juveniles live associated with floating debris near the coast. In Galicia, the cities of Vigo and A Coruña are the two main important ports for wreckfish sales (**Fig. 1**). The sales decreased from 60,5 mt (2007) to 10 mt (2017) in A Coruña and from 102 mt (2007) to 10 mt (2017) in Vigo. The price varied in the last ten years between 13-22 €/Kg (**Fig. 2**). Most of the catches came from Azores fishery.

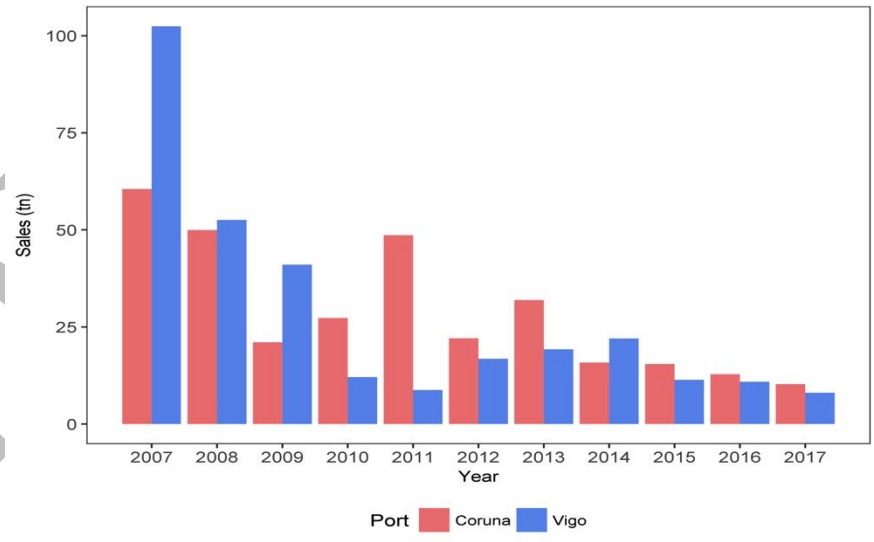

Figure 1. Evolution of wreckfish sales in the Galician ports (2007-2017) A Coruña (red) and Vigo (blue).

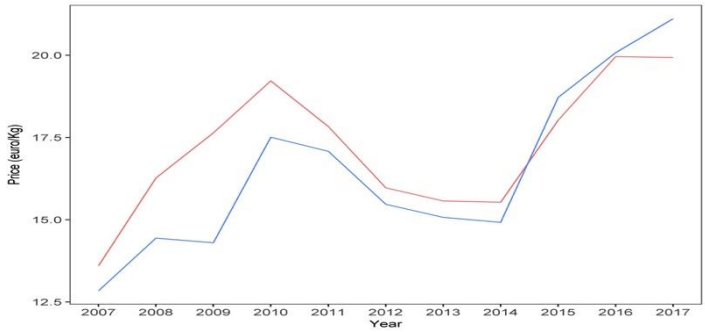

Figure 2. Evolution of prices of wreckfish in Galicia (2007-2017).

There are two main methods of catch: by a net (similar to a purse seine) that surrounds a floating object or the juveniles (weight < 3 Kg) and by a long-line for adults (weight > 3kg) (**Fig. 3**). The fishing season of wreckfish is between April and July.

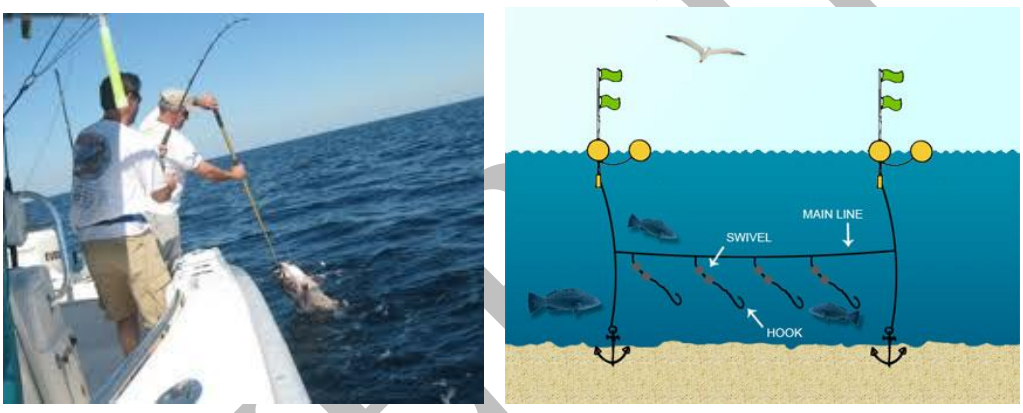

Figure 3. Fishing with hook (left) and fFishing with demersal long line (right).

The decline in wreckfish catches in Galicia makes it difficult to obtain individuals to increase the number of captive wreckfish broodstock. A small number of fish were captured in the Atlantic coast (NW of Spain) and were maintained in quarantine. Prior to sampling, animals were anesthetized, and length, perimeter and weight were determined. A sample from the fin was also taken for genetic analysis. The capture of adults (>3 Kg) becomes difficult because the ascent to the surface must be done very slowly to avoid problems of decompression that can cause the fish death.

Death in fish is mainly due to barotrauma, an overexpansion of the swimming bladder gas. When the fish is lifted to the surface too rapidly, gas expands and results in the rupture of the swim bladder wall producing haemorrhage and collapse of internal organs, resulting in the death of the animal in the following hours. So, a slow lift must be carried out giving the fish enough time to evacuate the expanded gas. As soon as fish is hooked, the fishing line must be lifted slowly. Once the fish is on the surface of the water, hanging fish up from the line out of the water must be avoided. It is advisable to collect the animal from in the water with the help of a non-abrasive mesh stretcher. Fish should be transferred into a tank with water and anaesthetic. Once the individuals are under sedation, it is possible to proceed with hook extraction. Then transfer the fish to a new tank with oxygen and clean water. Upon arrival at the harbour, the fishes are transferred to a truck equipped with a tank provided with oxygen. After that, the individuals are moved to the facilities and maintained in quarantine.

Description of the reproductive cycle in captivity

Constantinos C. Mylonas, Hellenic Center for Marine Research, Greece

Four different broodstock batches with variable number of breeders (collected as juveniles from the wild) were maintained in research facilities in Greece and Spain: the Hellenic Center for Marine Research (HCMR, n=3) in Heraklion, Crete, Greece and in 3 facilities in Spain, the Instituto Español de Oceanografía (IEO, n=13) in Vigo; the Aquarium Finisterrae (MC2, n=21) in A Coruña; and the Conselleria do Medio Rural e Mariño (CMRM, n=11) in Pontevedra. Except from the HCMR broodstock that was maintained at constant water temperature (15-16°C) throughout the year, the other three stocks were maintained in relatively similar temperature conditions, ranging between 12 and 18°C. Fish were monitored for their stage of reproductive development between March 2015 and October 2016, encompassing two spawning seasons (2015 and 2016) and one complete reproductive season. Samplings were conducted monthly from February until June and bimonthly from July until January.

Vitellogenesis in wreckfish is a long process and it starts in October (**Fig. 4**), many months before the first spawns could be obtained. During the expected spawning season, vitellogenic or early oocyte maturation, the oocyte diameters reach a maximum ranging between 1200-1600 μm. Males could be in spermiation condition throughout the year, as was observed in all four facilities (**Fig. 5**).

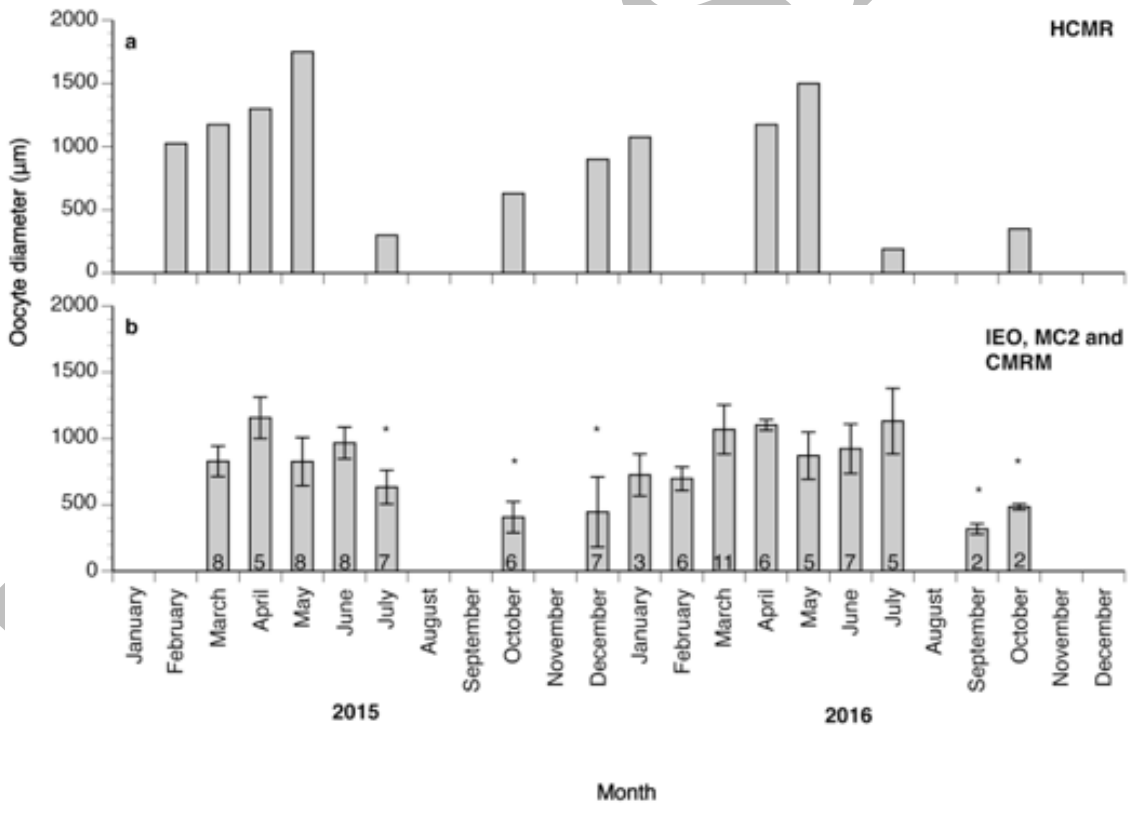

Figure 4. Mean (±SEM) oocyte diameters of wreckfish (*Polyprion americanus*) from HCMR (a) and from three different broodstock in Spain (b) during the annual reproductive cycles in 2015 and in 2016. The numbers inside the bars indicate the number of females biopsied at each month. Asterisks (*) denote significantly lower values than maximum observed (April 2015).

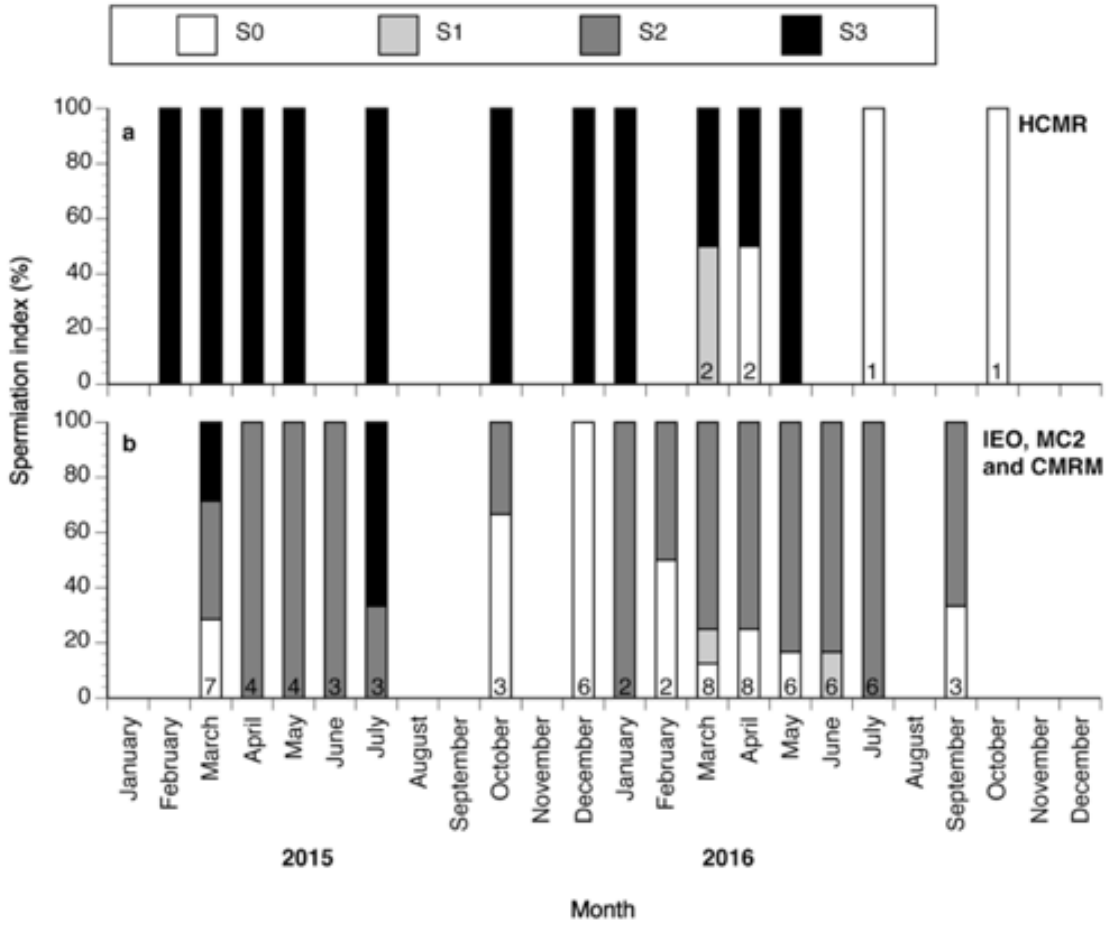

Figure 5. Percentage of male wreckfish at different spermiation index stages, in respect to month at HCMR (a) and at three different broodstock in Spain (b) from February 2015 until October 2016. Spermiation index was reported on a subjective scale, with S0 = no milt released, S1 = only a drop of milt 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.

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 spawning was obtained in late spring in all monitored years, but fertilization success was generally low and variable (**Fig. 6**).

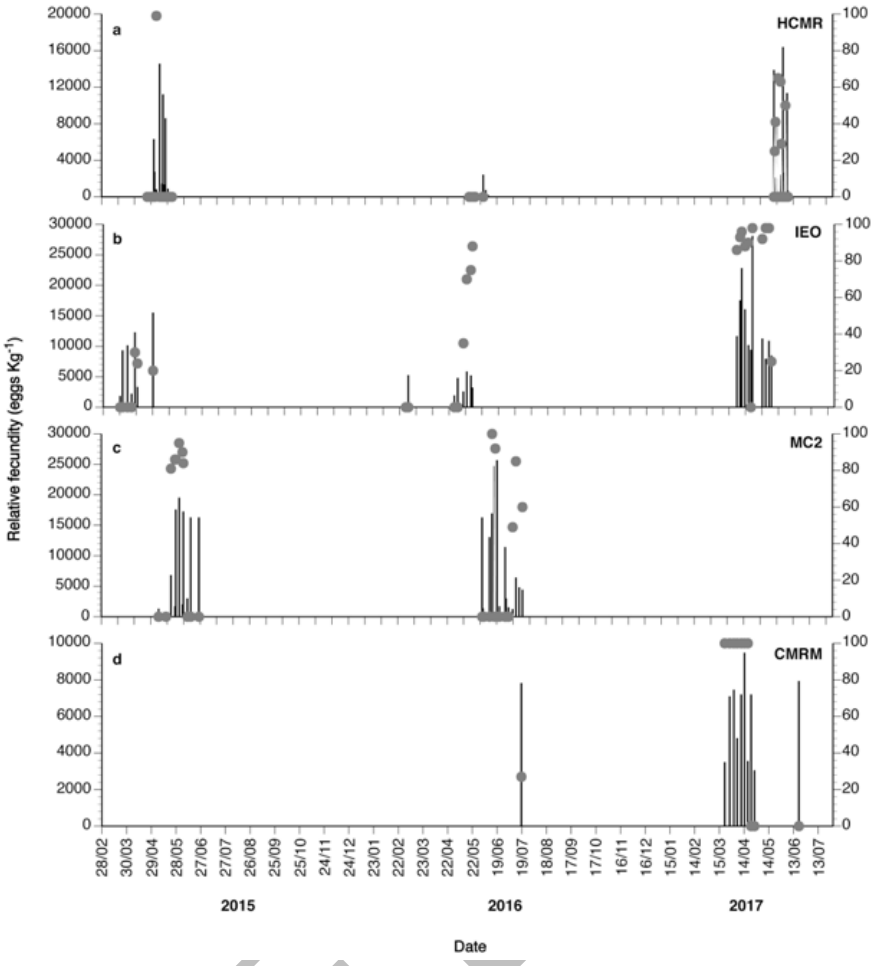

Figure 6. Mean relative fecundity and fertilization (%) of the spontaneous spawns of wreckfish broodstock

obtained at HCMR (a), IEO (b), MC2 (c) and CMRM (d) during 2015, 2016 and 2017.

The results from DIVERSIFY show that wreckfish females can adapt to captivity, mature and produce eggs both under fluctuating natural and under constant low temperatures. Plasma sex steroid hormones (data not shown here) in females correlate well with the maturity stages of females. Some females, however, cease oocyte development at the cortical alveoli stage and their oocytes do not grow bigger than 350 μm, or stop at the vitellogenesis stage and cannot reach maturation.

Males, on the other hand, produce sperm of good quantity and quality, capable to fertilize the eggs produced. Moreover, wreckfish males can produce sperm all-year round, making it available to fish farmers for artificial fertilization whenever it is needed. Plasma sex steroid hormones in males (data not shown here) rise when fish are fully spermiating, suggesting that except for 17,20β-P, they correlate well with the maturity stages of males. A reproductive dysfunction that could be attributed to male wreckfish is the very low fertilization percentage found in a big number of spontaneous spawns. In some cases, although females spawned large numbers of eggs, these eggs were unfertilized, a fact that could be attributed to a failure in the male breeding behaviour.

During the last year of the project, better results have been obtained in term of spontaneous spawning and the production of fertilizable egg, resulting in an increase number of viable larvae, and in one occasion, two males fertilized 40 spawns, with 80-100% fertilization success. The results are

currently under evaluation and were not included in this Manual, but will be made available in the website in a future time.

Spontaneous and induced spawning procedures

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During the years 2014 to 2018, the four wreckfish broodstock in the different facilities: HCMR, MCA, IGAFA AND IEO (**Fig. 7**) were followed in order to know all about the reproduction, larvae husbandry and nutrition, thinking in the best conditions of this specie to form part as a new species candidate for the European aquaculture.

Fig 7. Different wreckfish broodstock at the fourth facilities: from left to right: HCMR (Crete). MC2 (A Coruña), IGAFA (Illa de Arousa) and IEO (Vigo).

These fish were maintained in a variety of environmental conditions with regards to tank size and photothermal regime, including indoor and outdoor tanks with natural conditions, and indoor tanks with simulated natural photothermal conditions or constant temperature. This species exhibits a high growth rate and easy adaptation to the captive environment and to the handling procedures. Low feeding rates were recorded during the spawning season (from March to July) and high feeding rates occurred during autumn **(Fig. 8)**. Ingestion rate varied between 0.2 and 0.5 % for fish fed with semi-moist diet, and between 1 and 1.8 % those fed dry pellets.

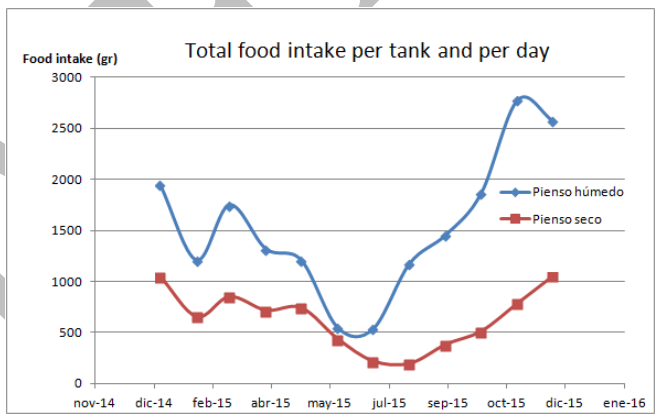

Figure 8. Total food intake/tank/day during a year in the two IEO wreckfish broodstock.

On the other hand, the nutritional analysis of eggs and gonads of females was investigated in order to develope an appropriate broodstock diet for this species. Most results are shown in the Nutritional section of this Manual (see later). Some differences were found in carotenoids and vitamins content between wild mature female gonads and the ones from eggs produced under different rearing conditions and feeding regimes. These data are currently being evaluated and will be published in the DIVERSFY web in the very near future.

There are three procedures to obtain wreckfish spawns in captivity (**Fig. 9)**:

1. Natural and spontaneous spawns in large tanks (>40 m3), collecting the eggs as they exit the outflow of the tank (**Fig. 9a**)
2. Spawning induction with exogenous hormones (GnRHa) (**Fig. 9b**). This approach can be conducted in large tanks (>40 m3) under controlled photothermal conditions, allowing the fish to spawn spontaneously.
3. Spawning induction followed by *in vitro* fertilization by stripping of the mature females and males, maintained in smaller tanks (**Fig. 9c)**.

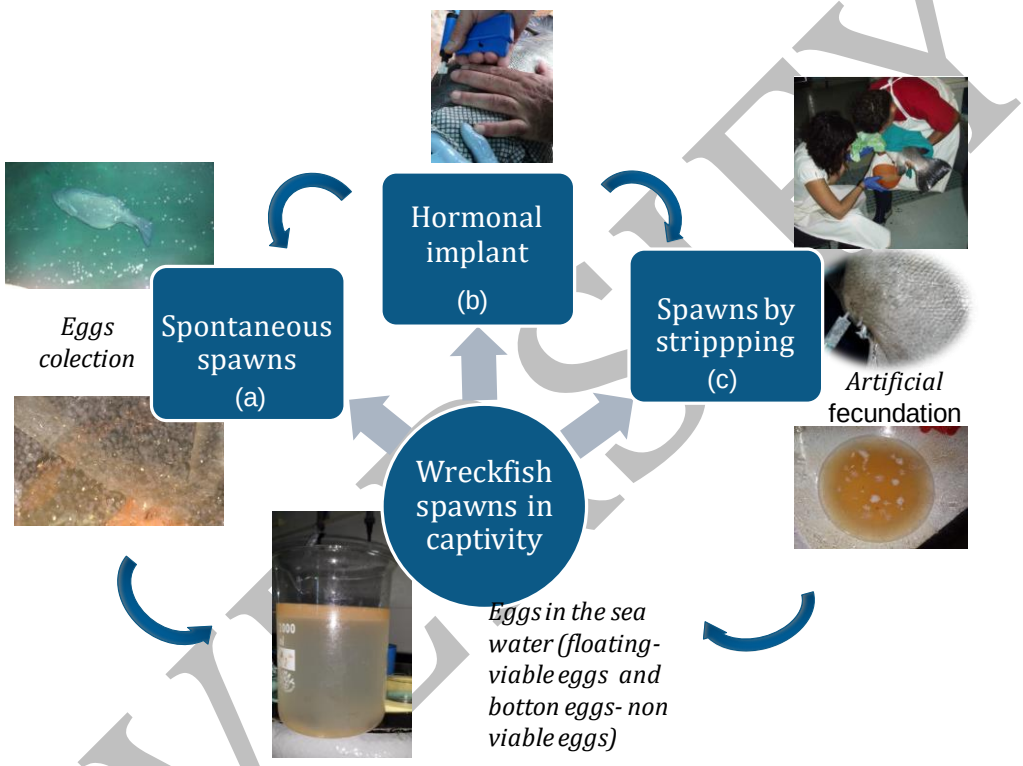

Figure 9. Different procedures to obtain wreckfish spawns in captivity.

Females do undergo vitellogenesis and oocyte maturation spontaneously in captivity, reaching spontaneous spawns mainly in the three stocks maintained in Spain, with increasing regularity and fertilisation rates as the project progressed and more experience was acquired (**Fig. 10**).

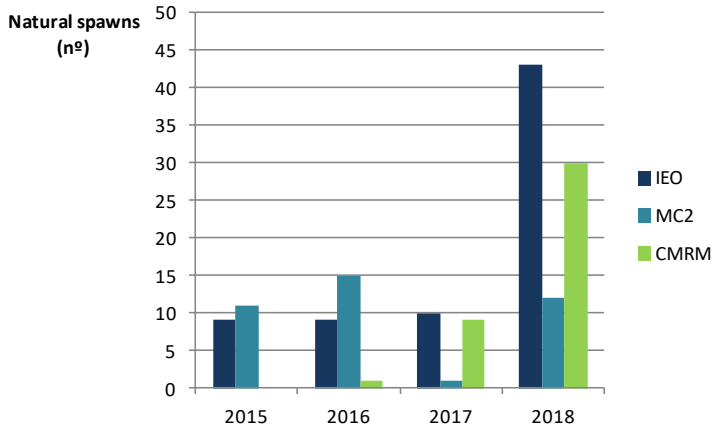

Figure 10. Total number of natural spawns of the three Galician broodstock (IEO, MC2 and CMRM) during 2015-2018.

During these last years, the number of spontaneous spawns has increased, and the number of induced spawns has been reduced. The reason is probably a better adaptation of the females to the captive conditions and the promotion of the natural maturation cycle, resulting in not only vitellogenesis, but also spontaneously oocyte maturation (**Fig. 11**).

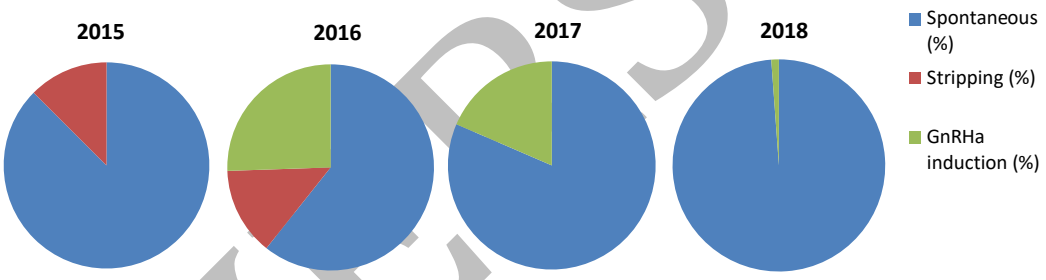

Figure 11. Percentage (%) of spontaneous, artificial (by stripping) and GnRHa-induced spawns during the last three years in the three Galician wreckfish broodstock.

The natural spawning behaviour is characterized by males chasing the females followed by the release of the gametes. Spawning takes place during the night or very early in the morning. In 2017 and 2018, spontaneous spawning in the IEO, MC2 and CMRM stocks produced a large number of fertilized eggs and achieved satisfactory fertilization success. Spawning periodicity was 3-5 days in all stocks and the time of spawning was mainly between 05:00 and 08:00 h, with some exceptions that took place at mid day. Fertilization success was between 50 and 100% with better quality eggs towards the mid or end of the spawning season for each female. It has been found that a female is able to spawn an average of 10 times per breeding season.

In respect to the males, sexual maturation covers the same period of females, reaching its peak in the months of April and June, with peak sperm concentrations of 25-35 x 10^9 szoa/ml. The spermatozoa motility is high, with mean values between 2-3 min, and the mean survival time of sperm maintained at 4ºC is 4 days. However, in some cases sperm may reach 18 days of survival after collection. At the IEO facilities, we have checked that one male has the capability to fertilize at least 30 spawns in a period of 150 days.

The characteristics of wreckfish eggs and embryogenesis have been monitored too . Wreckfish eggs have large diameter (1.996±0.034 mm), with a large lipid droplet. Hatching takes place after five days of incubation at 16±0.8ºC of water temperature (**Fig. 12**). Trials of incubation of the eggs are described in the next section of larval husbandry.

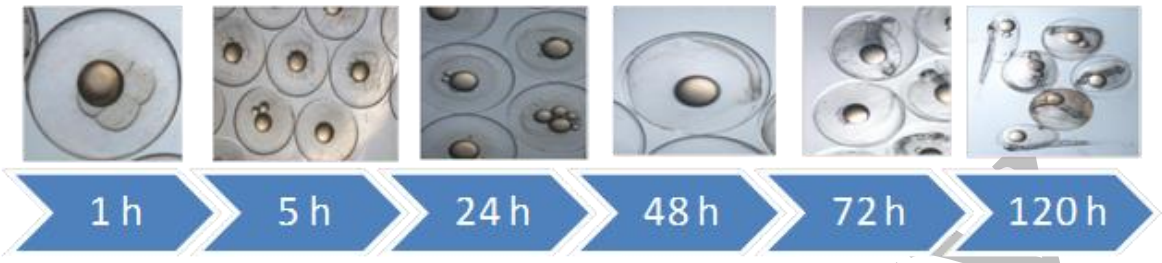

Figure 12. Embryonic development of wreckfish at 16±0.8°C.

During the hormonal induction trials, females in three of the four stocks were implanted with 500 µg of GnRHa. The response of the females to the GnRHa implants varied, with no response in 2015, spontaneous or stripped spawns with non-viable eggs in 2016, or with good results in fertilization, but not in hatching in 2017. There was only one spawn that resulted in a successful culture of larvae until 25-day post hatching in 2016.

The need of more information on this subject required using more females. Therefore, we used the stock from the company Isidro de la Cal (Spain), and worked with two of their females, based on an agreement made for that purpose. The experiments were carried out during the months of June and July of 2017. As a result of these trials, we obtained more information on the ovulation time after induction with the GnRHa hormone (**Fig. 13**). Results suggest that the correct oocyte size for GnRHa implantation should be >1200 µm, but it could be better at >1300-1400 µm. Also, it was found that GnRHa injections are more effective than GnRHa implants, and provide a faster response. A problem is the risk of gonadal plugs, if the hormonal dose is not adequate. Results showed a time of response of about six days after the injection.

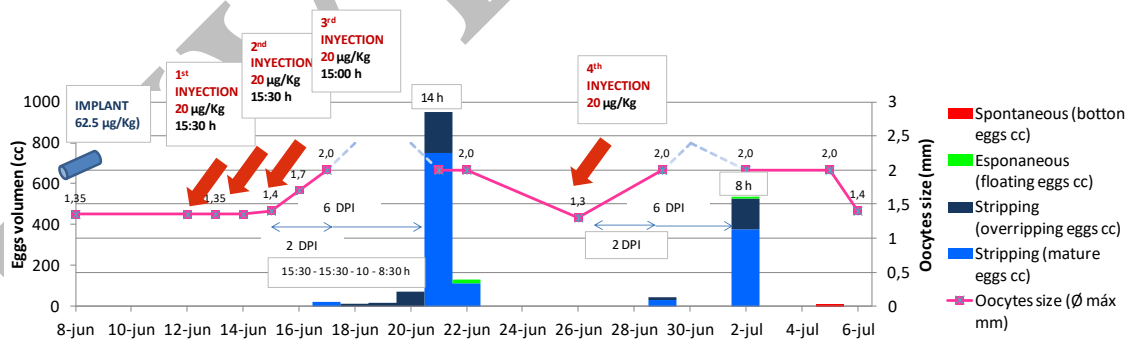

Figure 13. Spawning induction trials with a female from Isidro de la Cal company (Spain).

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 a 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.

On the other hand, the maturation and spawning induction methods using gonadotrophic hormones (FSH and LH from Rara Avis, Spain) have proven to be quite efficient to enable the complete oocyte

development in some wreckfish specimens that failed to do so by themselves it in captivity. The results of this work showed that the use of FSH and LH stimulate gonadal development, but it is necessary to implement such a trial with a larger stock of individuals and different hormonal doses.

Regarding the method of artificial spawning by stripping in wreckfish, it could be possible with mature females that exhibit problems of spontaneous spawns after GnRHa induction, but not for females that undergo oocyte maturation naturally (without exogenous hormones), because the adult individuals are heavy and the stress caused by striping could result in problems with egg quality and fertilization success.

Sperm characteristics and cryopreservation

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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). The best adapted CASA parameters for wreckfish sperm analyses were determined and reported to optimize their abilities to check fertility potential of the semen in the course of their future spawning induction experiments. 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 energy stores, that are not replenished due to poor 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. In 2015, the concentration reached higher values of up to $2x10^{10}$. The standard deviation was very high between and within males of the different locations, and it is not possible to conclude that there was a significant difference in sperm concentration between wreckfish in Crete and Galicia. These concentrations levels do not differ from earlier data (from 1.5 to 2.71 10^{10} spz ml^{-1}) from the HCMR broodstock. Finally, 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.

The fresh sperm showed a high initial percentage of mobile spermatozoa at activation, which had regularly decreased with time for 5 minutes. The mean initial VAP or mean velocity along smoothed trajectory was around 230µm per second, which progressively decreased to 0 after 5 minutes (**Fig. 14**). The velocity of spermatozoa was one of the highest reported for marine fish and the trajectories vary from straight forward at activation to progressive circling as the speed decreased. This is corroborated by the decrease of linearity of the trajectories calculated by the ratio between the average path velocity and straight-line velocity (**Fig. 14**).

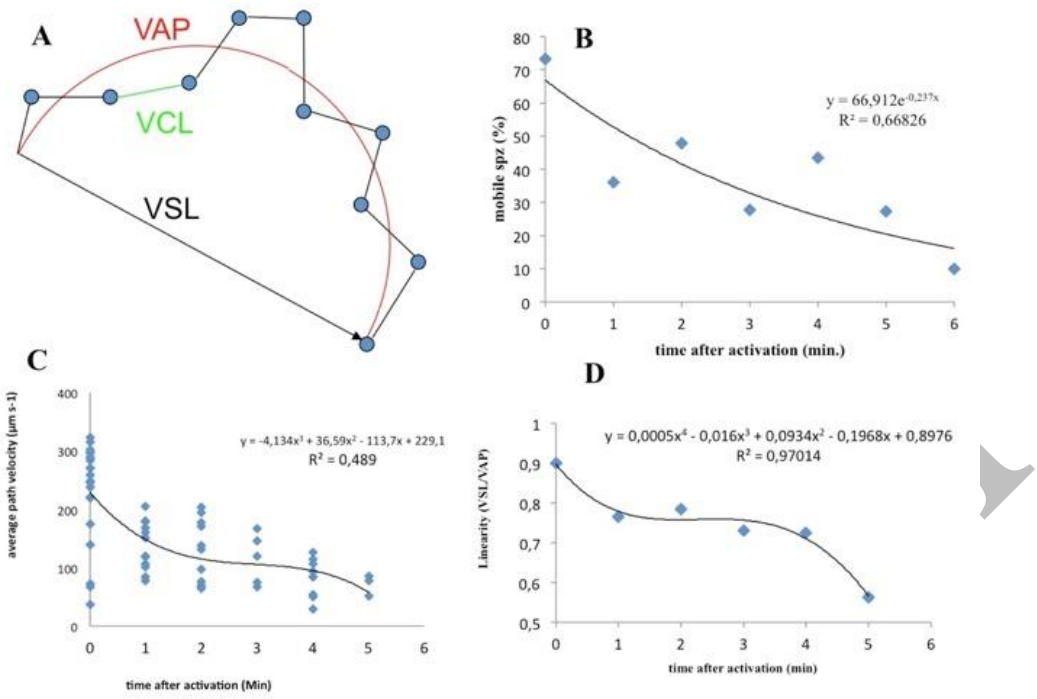

Figure 14. Motility parameters of wreckfish sperm: A) schematic representation of spermatozoa movement illustrating the three parameters of velocity generated by CASA; B) Variations of the percentage of swimming spermatozoa (spz) with time; C) average path velocity decrease with time after activation; D) decrease of linearity of spermatozoa trajectories after active

Wreckfish males produced a high volume of easily expressible milt with a concentration considered as medium range for marine fish and of course much higher than that of flatfish. On the top of those general features, the setup of a CASA protocol adapted to wreckfish sperm demonstrated that wreckfish 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. 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 analysed.

Nutrition

Effectiveness of live prey and influence of enrichments

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Some new enrichment products for live food were developed. Data of biochemical analyses performed at CMRM of gonads from wild wreckfish females, and eggs and larvae obtained from reared fish were sent to the University of Las Palmas de Gran Canaria (FCPCT) to develop some live food enrichment products for larval wreckfish. Three experimental enrichment products were formulated during 2017 to meet the EPA, DHA and ARA levels obtained from tissues of wild-cached wreckfish. For experimental enrichment preparation, a combination of different products based on microalgae were used : Chlorella meal powder (>1% Fat, > 12% Protein; Shaanxi Pioneer Biotech Co., Ltd, Xi'an, China), Microalgae DHA Powder (>40% DHA; Shaanxi Pioneer Biotech Co., Ltd,

Xi'an, China), and ARA Powder (>40% ARA); Shaanxi Pioneer Biotech Co., Ltd, Xi'an, China)(**Table 1**).

Table 1. Experimental enrichment products

Experimental Enrichment	*DHA- Rot*	*ARA-Rot*	*ARA-Art*
	Ingredients (g kg^{-1}diet)		
Chlorella powder	*500*	*500*	*400*
Microalgae DHA Powder	*400*	*400*	*500*
ARA Powder	*20*	*100*	*100*

The experimental enrichments for rotifer were formulated using two different levels of ARA (3 and 10% of total fatty acids) and one level of ARA (9% was used for *Artemia*). The composition of these products is shown in **Table 2**. The effect of the enrichment of the new enrichment products on the biochemical composition of rotifers and *Artemia* was evaluated and the results showed an efficient enrichment in both preys. The influence of these enrichment products for live food on wreckfish larvae could not be tested, because in 2017 the amount and the survival of larvae obtained was not sufficient to perform the experiments.

On the basis of the results obtained in 2017, three new enrichment products (two of them for rotifer and one for *Artemia*) were formulated in 2018 and the experiments on the effect of these enrichment products for live food on wreckfish larvae are still on-going at this time. The results will become available, nevertheless, in the near future in the website of the project.

Table 2. Proximate (% DM) and fatty acid composition (% of TFA) of experimental enrichments

HUFA. highly unsaturated fatty acid; ARA. arachidonic acid; DHA. docosahexaenoic acid; EPA. eicosapentaenoic acid.

	DHA- Rot	*ARA-Rot*	*ARA-Art*
	Proximate analysis (% dry matte		
Lipids	9.34±0.14	9.34±0.24	9.23±0.34
Proteins	25.58±0.21	30.08±0.13	22.54±0.09
	Fatty acid content (%TFA)		
Saturated	19.39	17.61	21.93
Monoenoics	8.30	8.76	8.35
n-3	48.82	45.72	45.14
n-6	20.87	25.43	24.26
n-9	4.55	5.65	4.88
Total n-3HUFA	44.20	41.42	44.62
20:4n-6 (ARA)	3.10	9.86	9.03
20:5n-3 (EPA)	6.33	5.87	6.40
22:6n-3 (DHA)	33.06	31.16	33.55
EPA/ARA	2.04	0.60	0.71
DHA/EPA	5.22	5.31	5.24
n-3/n-6	2.34	1.80	1.86

Influence of broodstock feeds on fecundity and spawning quality

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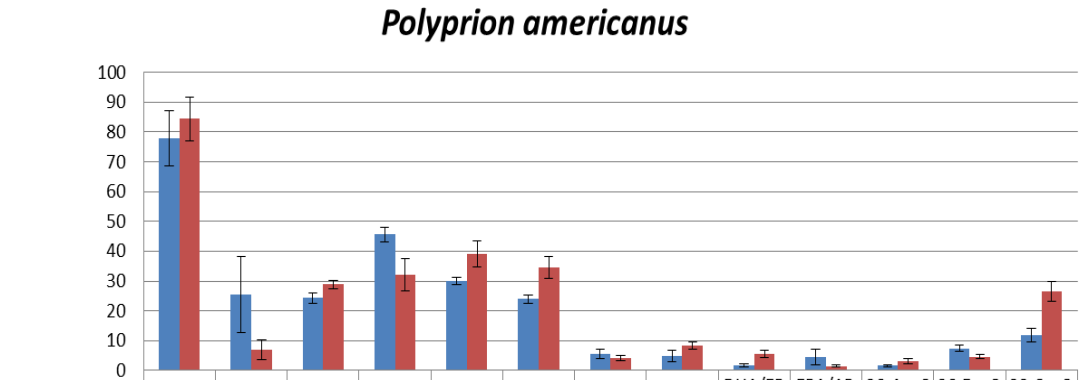
The nutritional requirements of wreckfish are unknown so far and there are only a few references related to feeding habitats from commercial captures (Brick Peres & Haimovi, 2003) and feeding rates in captivity (Papandroulakis et al., 2004). Recently, some studies were done on the composition of wild wreckfish (Roncarati et al., 2014; Linares et al., 2015). The optimum development of broodstock diets for wreckfish is essential for the future of the aquaculture of this species. Dietary lipids and especially fatty acids play a critical role in the successful production of high quality gametes and eggs of marine fish (Izquierdo et al., 2001; Sargent et al., 2002).

The experiments carried out in DIVERSIFY included:

- ✓ A preliminary study on the biochemical composition of some tissues of wild wreckfish and a comparison with the biochemical composition of tissues of captive-reared wreckfish.
- ✓ The biochemical composition of different broodstock feeds with special attention to the fatty acid contents.
- ✓ The effect of different feeding regimes based on fresh and commercial dry feeds on oocyte and egg fatty acid composition.
- ✓ The effect of feeding regimes on fecundity, and egg and sperm quality

For the preliminary experiment, 91 fish were sampled from the Azores from February 2014 to April 2015. The objectives of this experiment were to get some basic information about this species, to advance in the knowledge of wreckfish nutritional requirements, identify potential nutritional deficiencies and finally formulate suitable diets for wreckfish broodstock.

Samples of muscle, liver and gonads were taken out to be analysed and compared with tissues composition of captive-reared fish (**Fig. 15**).



	Proteins	Lipids	SAFAs	MUFAs	PUFAs	Σn-3	Σn-6	n-3/n-6	DHA/EPA	EPA/ARA	20:4 n-6 (ARA)	20:5 n-3 (EPA)	22:6 n-3 (DHA)
Reared muscle	77,78	25,41	24,34	45,62	30,04	23,95	5,45	4,91	1,67	4,61	1,58	7,45	11,94
Wild muscle	84,41	6,92	28,83	32,09	39,08	34,51	4,08	8,5	5,69	1,54	3,11	4,55	26,38

Figure 15. Proteins, lipids and fatty acid of muscle from wild and captive-reared wreckfish.

The results show that wreckfish have a very high amount of proteins in the muscle (84% in wild fish and 78% in reared fish) and the level of lipids is lower in muscle from wild fish (7% DW) than in

reared fish (25%). Concerning fatty acid composition in muscle, values of PUFA and Σn-3 are higher in wild wreckfish (39 and 34% of total fatty acids respectively) than in reared fish (30 and 24%). DHA values represent 12% in cultured fish and 26% in wild fish. EPA content represents 7% in reared fish and 4% in wild fish and ARA 1.6% and 3.1% in reared and wild fish respectively. The EPA/ARA ratio had values of 4.6 from reared fish and 1.5 from wild fish.

Additionally, some samples of mature gonads from females and males of wild wreckfish were analysed showing high values of proteins (60% in females and 44% in males). Lipid content represents (21% in females and 13% in males). The n-3 PUFA values reached more than 35% in females and male gonads having a big amount of DHA (25-31%) and ARA content represented the 7 and 10% of total fatty acids in females and males gonads respectively.

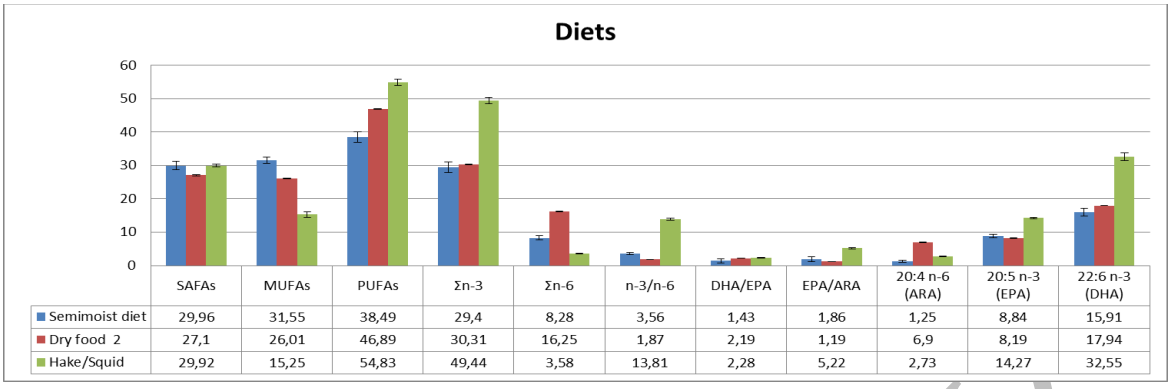
Different diets were used for wreckfish broodstock feeding in DIVERSIFY. In 2015 the first dry food was formulated by FCPCT and Sparos SA (50% of fish meal, 12.5% of squid meal, 6% of krill). This diet had 60% of proteins and 16% of lipids. An experiment was carried out at IEO facilities comparing the effect of fatty acid composition of this dry food and semi-moist diet on oocyte fatty acid composition from females fed with both diets. Based on the first results obtained in 2015 from data obtained of wild fish preliminary experiments and the analyses of samples of eggs from captured fish, a new dry food (25% of fish meal, 34.2% of squid meal, 4.5% of krill) was formulated. The ingredients of this dry food are shown in **Table 3.**

Table 3. Ingredients of dry food

Ingredients	*Dry food %*
Fishmeal 70 LT FF Skagen	**25.000**
CPSP 90	10.000
Squid meal	**34.200**
Krill meal (Aker Biomarine)	**7.500**
Wheat Gluten	7.000
Wheat Meal	7.250
Tuna oil	1.000
Algatrium 70% DHA	0.200
Incromega DHA 500TG	1.000
VEVODAR	1.300
Vit & Min Premix PV01	2.000
Lutavit E50	0.050
Soy lecithin – Powder	1.500
Macroalgae mix	1.000
Antioxidant powder (Paramega)	0.200
Antioxidant liquid (Naturax)	0.200
SelPlex - Se yeast	0.020
Carophyll Pink 10% - astaxanthin	0.050
Nucleotides (Nucleoforce)	0.030
L – Taurine	0.500
Total	100.000

The effect of different feeding regimes: semi-moist diet, dry food supplied to two broodstock batches at the IEO facilities and a mixture of hake and squid (half and half) supplied to IGAFA wreckfish broodstock on the fatty acid composition of oocytes and eggs from females fed with these diets was checked. The experiments were carried out during 2016 and 2017.

The semi moisture diet was a mixture of 14.8% of white fish, 14.8% of oily fish, 18% mussels, 17.6% squid and 34.8% fishmeal. The semi-moist diet had 65% of proteins and 17% of lipids, the dry food had 68% of proteins and 12.5% of lipids and the hake/squid diet had 63% of proteins and 8% of lipids. The fatty acid profile of these diets is shown in **Fig. 16**.



	SAFAs	MUFAs	PUFAs	Σn-3	Σn-6	n-3/n-6	DHA/EPA	EPA/ARA	20:4 n-6 (ARA)	20:5 n-3 (EPA)	22:6 n-3 (DHA)
Semimoist diet	29,96	31,55	38,49	29,4	8,28	3,56	1,43	1,86	1,25	8,84	15,91
Dry food 2	27,1	26,01	46,89	30,31	16,25	1,87	2,19	1,19	6,9	8,19	17,94
Hake/Squid	29,92	15,25	54,83	49,44	3,58	13,81	2,28	5,22	2,73	14,27	32,55

Figure 16. Fatty acid profile of diets: Semi-moist, dry food and hake/squid.

Samples of oocytes (by biopsies of females) and viable eggs from females fed with the three diets were taken out to examine the influence of fatty acid composition of diets on oocytes and eggs. The total amount of lipids was higher in eggs from females of the IEO broodstock fed with semi-moist diet, and with dry food, (25 and 19% DW respectively) than in eggs from females from the IGAFA broodstock fed with hake and squid with 9% of lipids, which is clearly influenced by the food. The highest content of n-3 PUFA was observed in oocytes and eggs from females fed with hake/squid and n-6 PUFA values are much higher in oocytes (12%) and eggs (10%) from females fed with dry food than oocytes and eggs from the other two diets. In the case of ARA, the highest values were found in oocytes and eggs from females fed with dry food, 7 and 5% respectively. A correlation was found between the fatty acid profile of wreckfish broodstock feeding and oocytes and eggs fatty acid profile.

Additionally, the first data of sperm fatty acid profile from males of different wreckfish broodstock were obtained, showing there are not significant differences between the main groups of fatty acids in the different broodstock, with values of SAFA`s between 32-33%, MUFA`s 9-11% and PUFA´s 56-58% of the total fatty acids. The n-3 PUFA content varies between 49-51% of the TFA and the n-6 PUFA between 5.5 and 9%TFA. The first results obtained about the relative fecundity (eggs/Kg of female) and number of spawns of females fed with different diets show that those fed with dry food have the highest fecundity and the largest number of spawns per female. These values have been increasing from 2015 to 2018. However, these results need to be confirmed in the future with a higher number of data.

Concluding remarks of nutrition results in wreckfish:

- ✓ Enrichment products for live prey (rotifers and *Artemia*) were designed. Two levels of ARA were used for enrichment products for rotifer and one level for *Artemia*, and the effect of the new enrichment products on the biochemical composition of rotifers and *Artemia* was evaluated,
- ✓ Wreckfish has a large amount of proteins in the muscle, 84% in wild fish and 78% in captive-reared fish,
- ✓ Wild wreckfish have less lipids in muscle and liver (7 and 39%) than in muscle and liver from culture (25 and 43%), and some differences were observed in the fatty acid profile between wild and reared wreckfish, with PUFA and n-3 (%TFA) being higher in wild than in reared fish,
- ✓ A clear relationship between fatty acid profile of broodstock diet (semi-moisture, dry food and a mixture of hake and squid) and fatty acid profile of oocytes and eggs from females fed with the different diet was found,

- ✓ Results obtained with dry food demonstrated that the wreckfish diet must contain a large amount of proteins, low level of lipids, a high amount of n-3 PUFA, and the EPA/ARA ratio must be similar to the one obtained in wild female gonads,
- ✓ First data of fatty acid profile of sperm from wreckfish males of different broodstock were obtained,
- ✓ Relative fecundity (nº of eggs/Kg of female) and number of spawns per female have been increasing in females fed with dry feed over the years, from 2015 to 2018.

Larval husbandry

Development of the digestive system in wreckfish

Ioannis Papadakis, Hellenic Center for Marine Research, Greece

Larval rearing of wreckfish is currently the major bottleneck for the successful culture of this species, due to the low survival rates observed during this period. One of the main scientific goals for wreckfish larval rearing is the development of protocols according to the specific requirements of the larvae during the early developmental stages. The study of the development of the organs related with larval feeding behaviour offers part of the necessary information for the optimization of the larval rearing protocols. During larval stages, the systems that are closely related with the feeding behaviour are the vision system, by which the fish perceive the different food items in the rearing environment, and the digestive system, which enables fish larvae to capture, ingest, digest and absorb nutrients from the food. These two systems and the structures of which they are composed are related with the larval rearing feeding protocols. The vision system (*i.e.* the eye) determines the ability of larvae to identify the prey under the light conditions that exist in the rearing environment, whereas the digestive system is also determined by the qualitative and quantitative composition of the feeding protocol that is used during rearing.

Most of the organs (except for the maxillary teeth at the upper jaw that became visible at 19 dph) appeared by 8 dph **(Fig. 17)**.

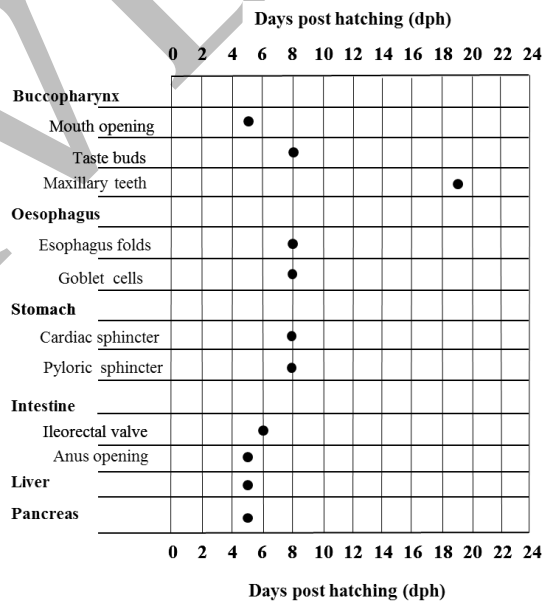


Figure 17. Schematic representation of the main structures of the digestive system that were studied. The time of appearance of each structure is presented with a black solid circle as a function of days post hatching (dph, horizontal axis).

From the ontogenetical point of view, the digestive system ontogeny up to 23 dph can be categorized into two distinct periods.

1: 0-5 dph. This period refers to the the prelarval stages, during which larval feeding was based exclusively on lecithotrophic reserves. During this period, the digestive tract appeared as a closed straight tube located dorsal to the yolk sac and consisted of a single-layer epithelium of simple cuboidal and columnar cells. At 5 dph mouth and annus opening occurred (**Fig. 18**). At the same time, the liver and the pancreas also appeared. The early hepatic cells appeared at around 5 dph and were located initially behind the yolk sac under the anterior intestine; later they surrounded the anterior part of the intestine (**Fig. 18**). The pancreas appeared also at 5 dph around the first part of the digestive canal (**Fig. 18**).

Period 2: 5-23 dph. The mouth opening at 5 dph, initiates the transition from period 1 to period 2 or from the prelarval to the larval stage. Until 23 dph endogenous yolk sack material was visible inside the lecithotrophic sack. The ileorectal valve that separates the midgut from the hindgut appeared at 6 dph (**Fig. 18**). At 8 dph the formation of the esophagus folds and the goblet cels on the esophagical epithelium was visible (**Fig. 18**). The pyloric and cardiac sphincter at the intestine of wreckfish larvae also appeared at 8 dph (**Fig. 18**) indicating the area that the stomach will be formed. This area is defined between the cardiac and the pyloric sphincter. The first taste buds were formed around the buccopharynx at 8 dph (**Fig. 18**). The last structures related with the digestive system ontogeny were the maxillary teeth at the upper jaw that became visible at 19 dph (**Fig. 18**).

In wreckfish, the ontogenesis of the digestive system is considered as a slow procedure in comparison with other species. The development of the digestive system is controlled by endogenous factors and generally it is genetically programmed, but the time of appearance of the digestive system structures can be influenced by a number of exogenous factors, with temperature being one of the most important (Kamler, 2002). The ontogenesis of the organs related to the digestive and the vision system was not completed until 23 dph. Major structures like the gastric glands or the pyloric caeca, the appearance of which characterizes the time when the development of the digestive system is completed, were not identified in this study. However, the ontogenetic events that occurred in the digestive system, such as the opening of the mouth at 5 dph, the appearance of goblet cells on the esophageal epithelium, the creation of the esophageal folds and the appearance of the pyloric and the cardiac sphincter, suggest that the digestive system of the wreckfish had already developed the ability to manage zooplanktonic organisms at 23 dph. The appearance of the maxillary teeth at 19 dph indicates that the larvae were able to successfully capture a zooplanktonic organism by this age. Moreover, the above indication, that the larvae could be successfully fed, is strengthened by the fact that the visual system of the wreckfish larvae after 6 dph had been developed to such an extent that it could distinguish objects in the rearing environment with the presence of light, since only the cones had developed.

The ontogeny of the retina of the wreckfish (not shown here) was found to be similar to the general pattern shown in most fish species. At hatching, the retina was an undifferentiated and non-functional tissue, as occurs in most marine fishes with pelagic early life stages. Cone cells were the first photoreceptors that appeared. This fact indicates that at this developmental stage wreckfish larvae were able to see different items in the rearing environment only during daylight hours. Thus, it is necessary to provide light in the rearing tanks of wreckfish after 5 dph. Wreckfish visual acuity - the distance the eye can differentiate between two points - improved over time (not shown here). Although the density of cones (number per 100 μm length) decreased over time in the retina, the radius of the eye lens increased, which contributes to an overall increase in the distance that the fish are able to see food items like rotifers and *Artemia* nauplii. Therefore, the density of the rotifers which are considered the smallest food particle provided in the rearing tank, could be theoretically calculated according the visual abilities of the larvae of wreckfish.

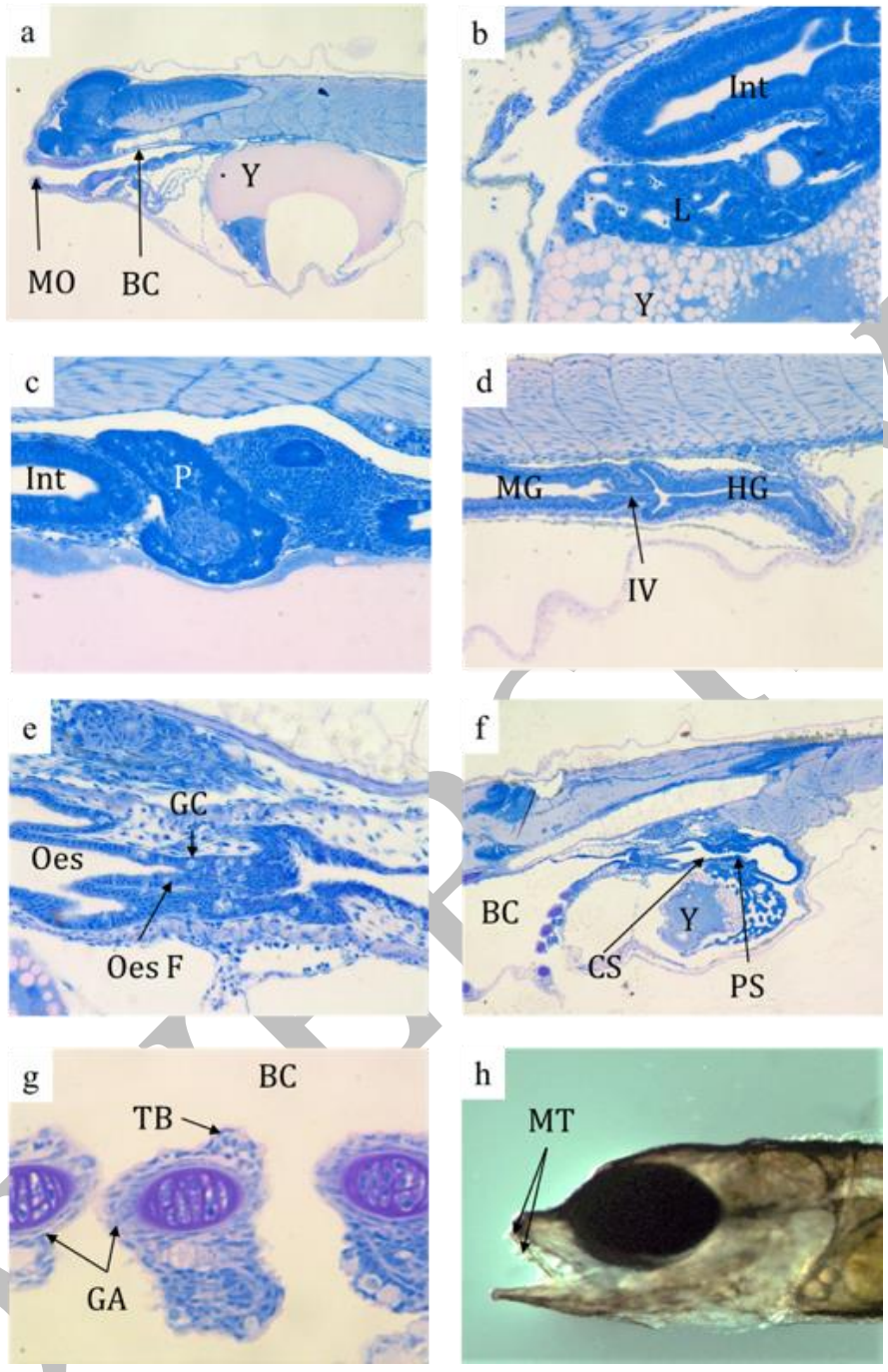


Figure 18. Microphotographs of histological sections from wreckfish larvae at different developmental stages.(a) At 5 dph showing the opened mouth, (b) At 5 dph when the liver appeared. (c) At 5 dph when the pancreas appeared. (d) At 6 dph when the ileo-rectal valve appeared. (e) At 8 dph showing the formation of folds and goblet cells at the oesopagus. (f) At 8 dph showing the formation of the stomach area from the cardiac and pyloric sphincter. (g) At 8 dph when the taste buds appeared. (h) At 19 dph showing the formation of the maxillary teeth at the upper jaw. BC = buccopharynx CS = cardiac sphincter, GA = gill arches, GC = goblet cells, HG = hindgut, Int = intestine, IV = ileo-rectal valve, L = liver, MO = mouth opening, MG = midgut, MT = maxillary teeth, Oes = oesophagus, Oes F = oesophageal folds, P= pancreas, PS= pyloric sphincter, TB = taste buds, Y = yolk.

Summarizing the results of this study, it appears that after the first 23 days of rearing, the digestive system and the eye of the larvae was developed to such a degree that by that time fish were, in principle, able to detect, capture and utilize the different types of zooplanktonic organisms. Wreckfish larvae were also characterized by the large size of the yolk sac, the absorption of which lasts until 20 dph at 17 ± 0.5°C. The presence of the large yolk sac and the large oil droplet, indicates the presence of a long autotrophic larval stage. From a hydrodynamic point of view, the large yolk sac and the large oil droplet increase the buoyancy of the wreckfish larvae. This phenomenon is inverted over time as the volume of the yolk sac is reduced by the procedure of yolk sac absorption by the larvae. The high buoyancy may affect negatively the velocity of the larval horizontal movement. The above explains the reason why wreckfish larvae are observed close to the surface of the rearing water during the early stages exhibiting a relatively small swimming performance (personal operation in previous larvar rearing experiments). However, during the autotrophic stage the digestive system and the vision system of wreck fish larvae were developed to such an extent that larvae were able to identify, capture and assimilate zooplanktonic organisms and this should be included to the feeding rearing protocol. As the main organs such as the gastric glands did not appear until the length of 5.5 mm, a combination of easily captured and more digestible preys as rotifers or different types in different developmental stages of copepods, have to be included in the larval rearing feeding protocol of wreckfish. The above, in combination with the optimization of the rearing conditions, such as the tank hydrodynamics, the temperature protocol during the rearing procedure and the photic conditions in the rearing water, is considered necessary for the development of the wreckfish larval rearing protocol.

Optimum conditions for larval rearing

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The main objectives during the first year of the project (2015) were to develop a culture protocol and study the influence of different sea water temperatures. The partners involved were HCMR, IEO, CMRM and MC2. A spawn from MC2 was incubated at 14.6ºC, with a hatching rate of 14%. The feeding protocol used normally in marine fish was applied and larvae survived until 20 days after hatching (dph). Two batches of eggs were used for the initial experiments, one from the HCMR broodstock and another one from MC2 by transporting 2000 larvae from Galicia to Crete in polystyrene boxes (**Fig. 19**).

Figure 19. Wreckfish eggs transported from Finisterrae Aquarium (Galicia) to HCMR (Crete).

Trials were performed in tanks connected to a closed water recirculating system. After incubation and during the autotrophic stage, temperature was maintained at 16ºC, and increased gradually afterwards to 17.5ºC. First feeding was at 10 dph, and was based on enriched rotifers, *Artemia* AF (since 13 dph) and *Artemia* EG (since 24 dph). Survival of the batch from HCMR was until 24 dph. During the rearing some malformed individuals were observed (**Fig. 20**).

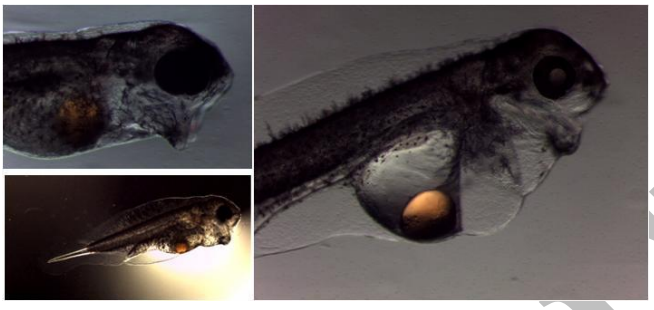

Figure 20. Malformed wreckfish larvae observed during rearing in the IEO.

The problem was identified as similar to a syndrome related to swollen yolk sac (SYSS) described in Murray cod (freshwater fish in Australia) that it is related to inadequate nutrition of the broodstock (Gunasekera et al., 1998). Furthermore, similar appearance has been also described in the Blue Sac Disease – (BSD) that is common in trout (Brzuzan et al., 2007). Several reasons suggested; most common toxicity from Nitrogen compounds such as ammonia, oxidative stress plays significant role. Although it seems that for the wreckfish the SYSS seems to be the case, further studies are required.

Larvae from natural spawns from Aquarium Finisterrae (MC2) were cultured in 50 l tanks in MC2 facilities (**Fig. 21**), with rotifers enriched with microalgae and copepods in flow-through circuit until 22 DPH.

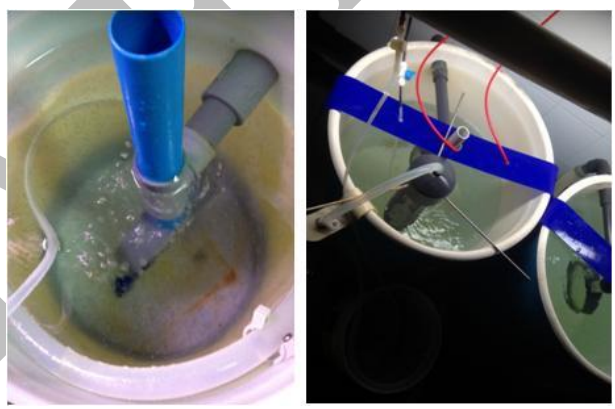

Figure 21. Culture tanks with recirculation (water renewal) eat MC2.

Larvae at the IEO from *in vitro* fertilization were cultured in 500 l tanks in closed circuit until 10 dph, using microalgae (green water), with rotifers enriched with T-Iso until 14 dph at low density in IEO (0.2 larvae/l) and at high density with larvae of MC2 natural spawns (52 larvae/l), with natural photoperiod during endogenous feeding. After opening the mouth and consumption of the yolk sac, artificial light (410 Lux) was used for 12 h per day until the end of the culture period. Larvae, yolk sac and lipid droplet length were monitored (**Fig. 22**) and photos were taken (**Fig. 23**).

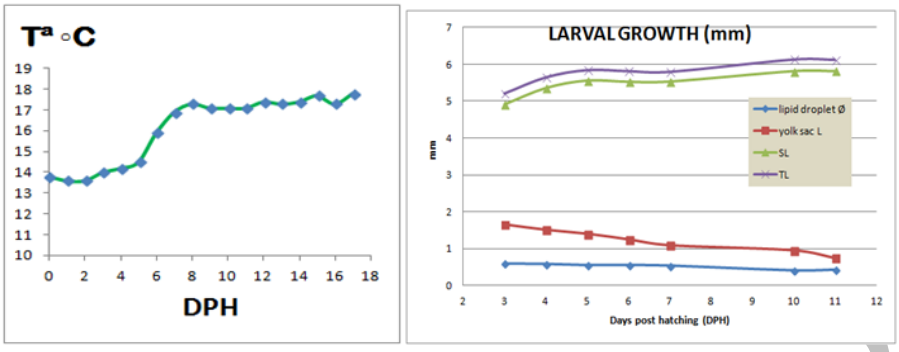

Figure 22. Water temperature during larvae culture (left). Larval growth, and droplet and yolk sac consumption (right).

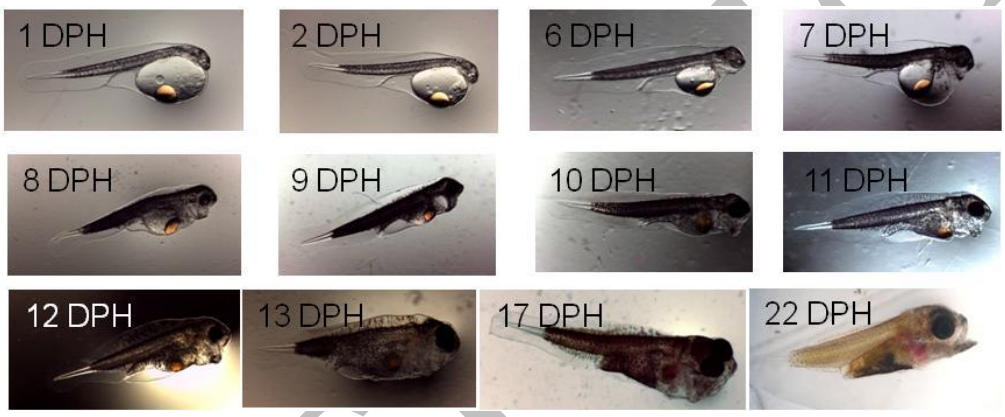

Figure 23. Morphometric development until 22 days post-hatching (DPH).

During 2016, spawn quality at MC2 improved considerably, and the stock at the IEO had started to have good quality spawns as well. Larvae were fed on rotifers and *Artemia* enriched with T-Iso until 27 dph (Álvarez-Blázquez et al. 2016). Growth performance of the larvae until 24 dph was obtained, with similar results for the Mediterranean and Atlantic stocks (MC2, CMRM and IEO) (**Fig. 24**). Larval length was 4.70±0.27 at one day post hatching, yolk sac consumption was at 11dph at 14-17°C sea water temperature and 8 dph at 17-20°C. The moment of mouth opening was at 7 dph at 14-17°C and 4 dph at 17-20°C (Álvarez-Blázquez, 2017).

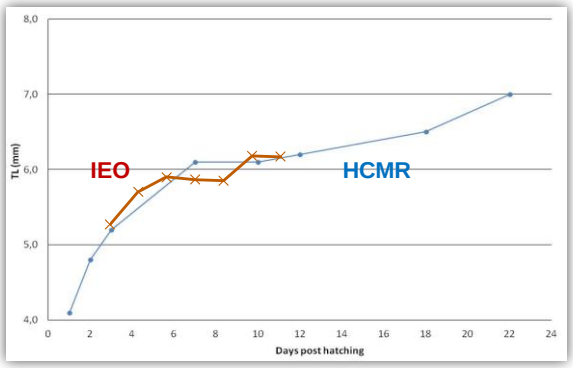

Figure 24. Larval growth in Mediterranean and Atlantic larval reraing trials.

During May of 2016, two trials testing different incubation temperatures with eggs from two different spawns from the IEO broodstock were made (**Fig. 25**). At 16±0.8ºC, the best results in terms of normal embryonic development and hatching rate were obtained. At 14±0.5ºC, 7.5% hatching occurred at 5 dpf. At 16±0.8ºC, hatching was done 1 day before (4 dpf) at a higher success 11.9% (**Fig. 26)**.

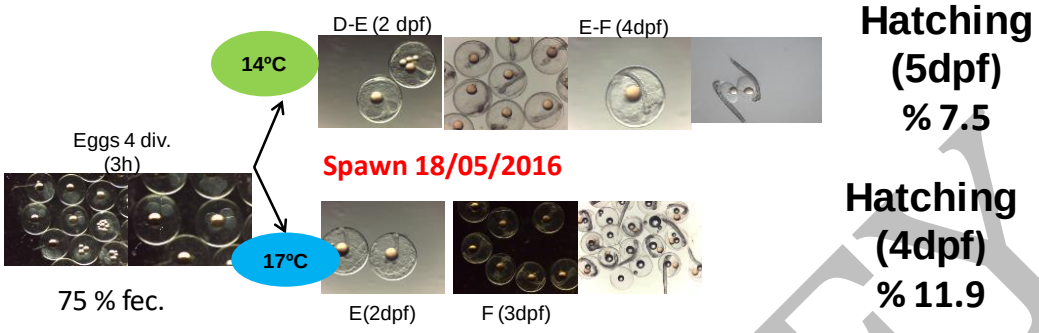

Figure 25. First incubation trials with two water temperatures.

During April 2017, an incubation experiment with larvae was carried out with three temperatures (13-14ºC, 16-17ºC an 19-20ºC) to extend and validate the results obtained during trials before **(Fig. 8).**

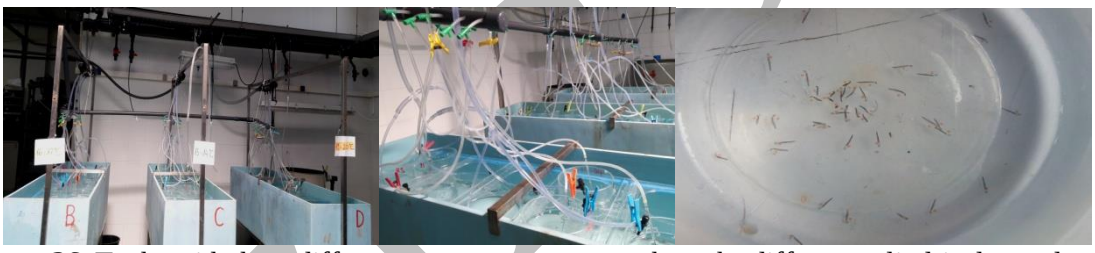

Figure 26. Tanks with three different water temperature, where the different cylindrical vessels with a mesh base were introduced to incubate the eggs.

Significant differences were observed ($p < 0.05$) in both hatching and the occurrence of deformed larvae between the temperatures of 13.7ºC and 19.5ºC, while the differences were not significant with respect to 16.6ºC. These results suggest that low temperature promote very low hatching and high larvae deformity (**Fig. 27**).

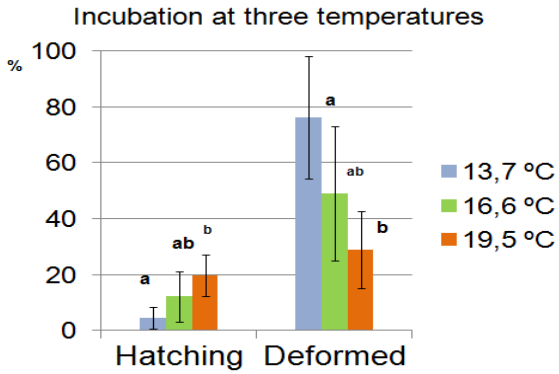

Figure 27. Hatching rate (%) and larval quality (deformed) during the trial with three water temperatures.

Hatching rate and deformity larvae for each temperature was calculated as follows:

Hatching (%) = N_L/N_H x 100

Deformities (%) = N_D/N_L x 100

Where N_L is the total number of hatched larvae, N_H the number of eggs hatched and N_D the number of deformed larvae. Embryonic development lasted for different period at each temperature, and was 4, 5 and 7 days at 19.5ºC, 16.6ºC and 13.7ºC, respectively. The quality and number of individuals hatched is shown in the **Table 3**.

Table 3. Percentage of eggs collected from the bottom (dead eggs) during the first three days of incubation with respect to the total of eggs that were incubated at first.

Tª	Days of incubation		
	1	2	3
13,7	24% ± 9,7%	30,2% ± 9,5%	29,5% ± 10,9%
16,6	35,4% ± 23,8%	40,8% ± 20,9%	4,7% ± 3,4%
19,5	56,8% ± 8%	18,2% ± 7,5%	-

At 13.7°C, egg mortality was around 30% day^{-1} during the first three days of incubation. At 16.6ºC, around 75% mortality occurred in the first two days. At 19.5ºC the majority of the mortality was during the first day of incubation. This fact indicates that during the first stages of egg development, vulnerability to external conditions is higher; therefore, the incubation parameters must be adjusted and the facilities and systems optimized in order to support proper embryogenesis and increase embryo survival and hatching.

In order to know the optimal temperature range for wreckfish larval husbandry, an experiment was carried out during April and May 2017, testing two different ranges: 15ºC-17ºC and 19ºC-21ºC, leaving aside the minimum temperature of 14ºC tested during 2016, under which poor results were obtained. Larvae at 3 dph were introduced, in triplicate, at a concentration of 7 larvae/l in 100-liter cylinder tanks, with continuous water flow and low renewal of filtered water at 1 µ and mild aeration (**Fig. 28**). Rotifers enriched with T-Iso were administered at a concentration of 3 rot/ml. The average temperatures obtained during the experiment were 16.4±0.6ºC and 19.7±0.6ºC for each of the ranges used. Data of total length (mm) and dry weight (mg) of 10 larvae/treatment were taken at 3 and 10 dph (**Table 4**).

Figure 28. Cylindrical tanks for larval rearing experiments under different water temperatures.

The mean length increased at 10 dph in the larvae at 16.4ºC, while it decreases in those at 19.7ºC. Dry weights also decrease in both treatments at 10 days of age. This may be due to the fact that from day 8 dph at 19.7ºC a large number of larvae with deformed mouth and operculum appeared, preventing from the proper development, movement and feeding of the larvae. This same effect was observed at 16.4ºC from day 10 of life (**Table 2**).

As the days passed, the deformities appeared in most of the larvae of both treatments causing massive mortalities. During the whole experiment no food was observed in the digestive system of any of the larvae sampled. For this reasons, it was not considered appropriate to carry out more samplings, since the data generated would not be representative of the study. Larvae at 19.7ºC survived to 24 days, while at 16.4ºC larvae reached 29 days.

Table 2. Average length (mm) and dry weight (mg) of larvae cultured in two different temperatures, during 3 and 10 days post hatching

	16,4 ºC		19,7 ºC	
Age (days)	Length (mm)	Dry weight (mg)	Length (mm)	Dry weight (mg)
3	5,582 ± 0,364	0,3553 ± 0,0261	5,582 ± 0,364	0,3553 ± 0,0261
10	5,92 ± 0,347	0,3044 ± 0,0164	5,49 ± 0,289	0,2492 ± 0,0331

During 2018, more advances in achieving natural spawns and in larval husbandry have been done in the three Galician wreckfish stocks resulting in very good larval hatching (42-82%) and in live larvae until 34-37 dph. At CMRM (IGAFA) two batches of larvae, one from IEO natural spawn and one from MC2, produced larvae surviving until 60-90 dph (**Fig. 29**). This was the first time in the project that we succeeded in producing juveniles weaned to inert food, and it signifies a milestone in the efforts to produce wreckfish under aquaculture conditions. This trial acquired important data on growth and increased our knowledge about the feeding protocol and the specific behavior and metamorphosis of wreckfish larvae.

Figure 29. Wreckfish juveniles cultured in IGAFA (CMRM) facilities.

To this end, changes are being made in incubation, embryogenesis and larval husbandry that can be decisive to avoid the problem of malformed larval and achieve greater survival. During the first stages of egg development, vulnerability to external conditions is higher; nowadays the incubation parameters were adjusted and the facilities and systems were optimized in order to increase the quality of the embryogenesis, and larvae in the best conditions, and increase survival.

It has also advanced in the knowledge of the optimal incubation temperature and larval culture. The study of the technical conditions and the adequate parameters regarding the aeration, the flow and form of creating an adequate circulation of water, as well as continue investigating the larval malformations that occur in a high percentage are needs for the immediate future. The achievements in animal husbandry and larval culture systems will be published on the DIVERSIFY website, as soon as they are finalized.

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