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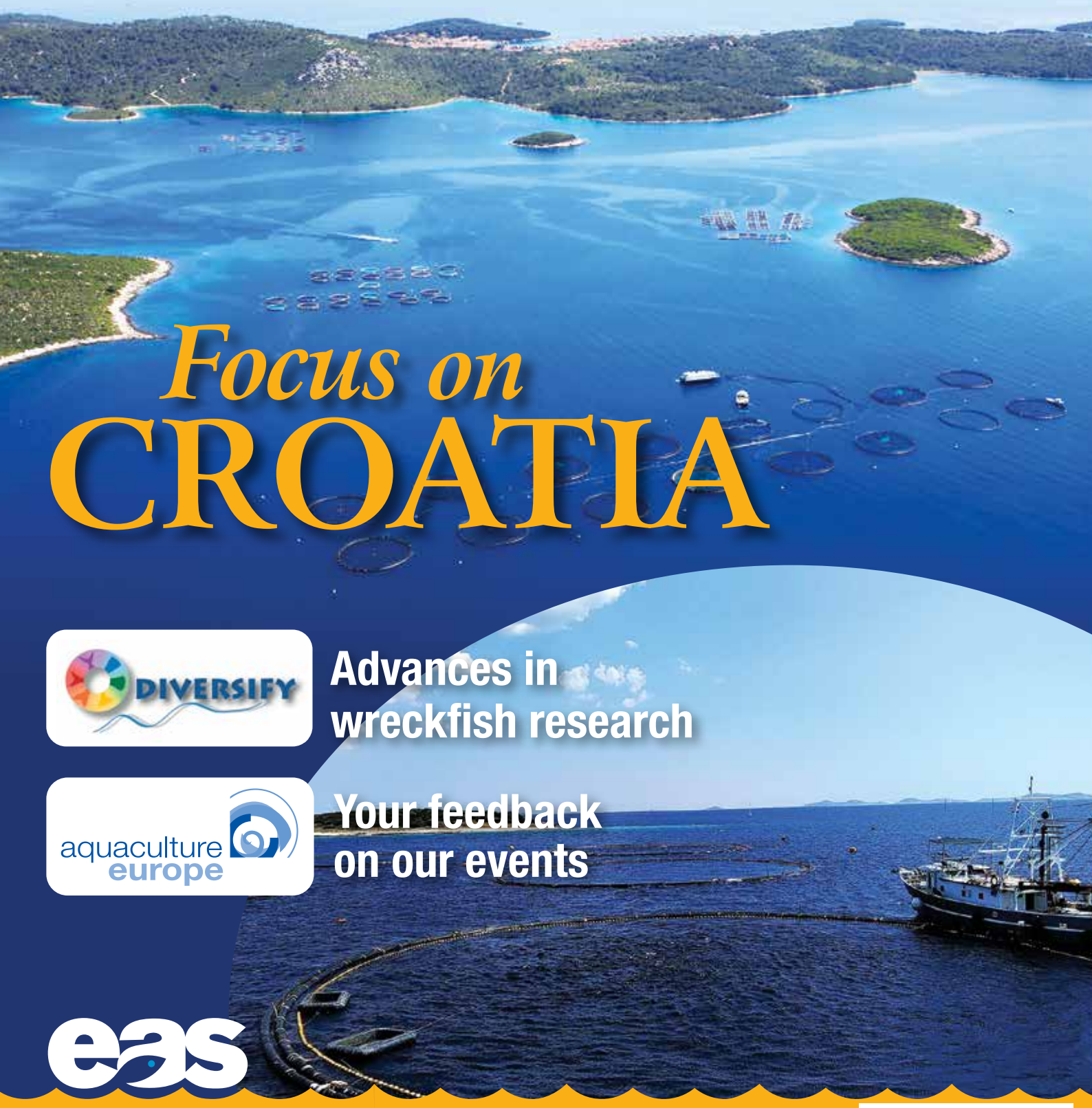

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Advances in wreckfish (*Polyprion americanus*) research: the DIVERSIFY project

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Why wreckfish?

Wreckfish is one of the largest Serranid species, reaching a size of 100 kg (**Fig. 1)**. It is a deep-water fish found almost throughout the world and is characterized by an extended pelagic juvenile phase (Ball et al., 2000). Wreckfish is one of the most interesting new species for aquaculture diversification, due to its fast growth, late reproductive maturation, high market price, limited fisheries landings and easy manipulation in captivity (Suquet et al., 2001). Its large size makes this fish suitable for processing and development of value added products. However, there are major bottlenecks for its incorporation into the aquaculture industry, such as the difficulty in acquiring wild fish for initial broodstock formation, the lack of reproduction control in captivity and the lack of any larval rearing protocols (Fauvel et al., 2008; Papandroulakis et al., 2004; Papandroulakis et al., 2008). Reproduction and larval rearing of a very close relative, the hapuku (*Polyprion oxygeneios*) has been achieved recently in New Zealand (Anderson et al., 2012) providing some information that may be relevant to the wreckfish rearing efforts.

Figure 1. Wreckfish *(Polyprion americanus)* broodstock at the Aquarium Finisterrae, A Coruña Spain.

The EU FP7-funded DIVERSIFY project (www.diversifyfish.eu) begun in December 2013 in order to acquire the necessary knowledge for the diversification of the European Aquaculture production with some new/emerging finfish species. The project has a total budget of 11.8 million € for its 5 year duration (2013-2018), making it one of the largest research projects in the area of aquaculture funded by the

European Commission. In the case of wreckfish, DIVERSIFY examines the potential for wreckfish aquaculture, bringing together almost all partners involved so far in Europe in wreckfish domestication (**Fig. 2**), in order to acquire the necessary knowledge and develop the required procedures for the production of fertilized eggs and high quality juveniles to launch commercial production of this species. This article provides some highlights from the first 3 years of the DIVERSIFY project.

Reproduction

The research activities of DIVERSIFY regarding wreckfish reproduction focus on four objectives:

- Increase the availability of broodstocks,
- Describe the reproductive cycle in captivity,
- Develop spawning induction protocols for tank spawning, as well as artificial fertilization,
- Develop protocols for Computer Assisted Sperm Analysis (CASA) and sperm cryopreservation.

Table 1. Biometric data of wild wreckfish captured by the commercial fishery in the Azores Islands (Atlantic Ocean, Portugal).

	Min-Max	Mean	std
Total length (cm)	54-98	75.36	7.39
Standard length (cm)	48-99	65.99	7.66
Perimeter (cm)	40-81	55.13	6.14
Body weight (kg)	2.6-18.0	7.25	2.22
Eviscereated weight (kg)	2.4 – 16.0	6.73	2.00
Perivisceral fat (g)	0-339.3	70.42	71.93
Stomach weight (g)	54.2-457.2	147.98	72.12
Intestine length (cm)	61-144	96.48	13.96
Intestine weight (g)	34.2-274.0	94.48	61.57
GSI females (%)	0.05-0.65	0.29	0.17
GSI males (%)	0.01-0.54	0.11	0.11
Viscerosomatic index (VSI)	2.40-16.02	7.26	2.11

The collection of wild fish to establish new broodstocks has been carried out along the Galician coast of Spain (**Fig. 3**). It has been hindered by the scarcity of wild wreckfish and unfortunately until now only a limited number of fish (n = 5, 1-4 kg body weight) have been collected. However, this small number of fish adapted easily in captivity and showed resistance to handling, which is very encouraging for the future development of wreckfish aquaculture. Biometric data were obtained from a large number of fish captured by the commercial fishery in the Azores Islands (Atlantic Ocean), and sold fresh at the market in Vigo, Spain (**Table 1**). There was a sexual dimorphism in body size (**Fig. 4**) and fish were mostly immature.

During the years 2014, 2015 and 2016, broodstocks in different locations were followed in order to describe the reproductive cycle in captivity. These fish were maintained in a variety of environmental conditions in regards to tank size and photothermal regime, including indoor and outdoor tanks with natural photothermal conditions, and indoor tanks with simulated natural photothermal conditions or constant temperature. Maintaining these fish for a long period of time (starting well before the beginning of the project), it was quite apparent that this species exhibits a fast rate of growth (**Fig. 5**), and easy adaptation to the captive environment and handling procedures.

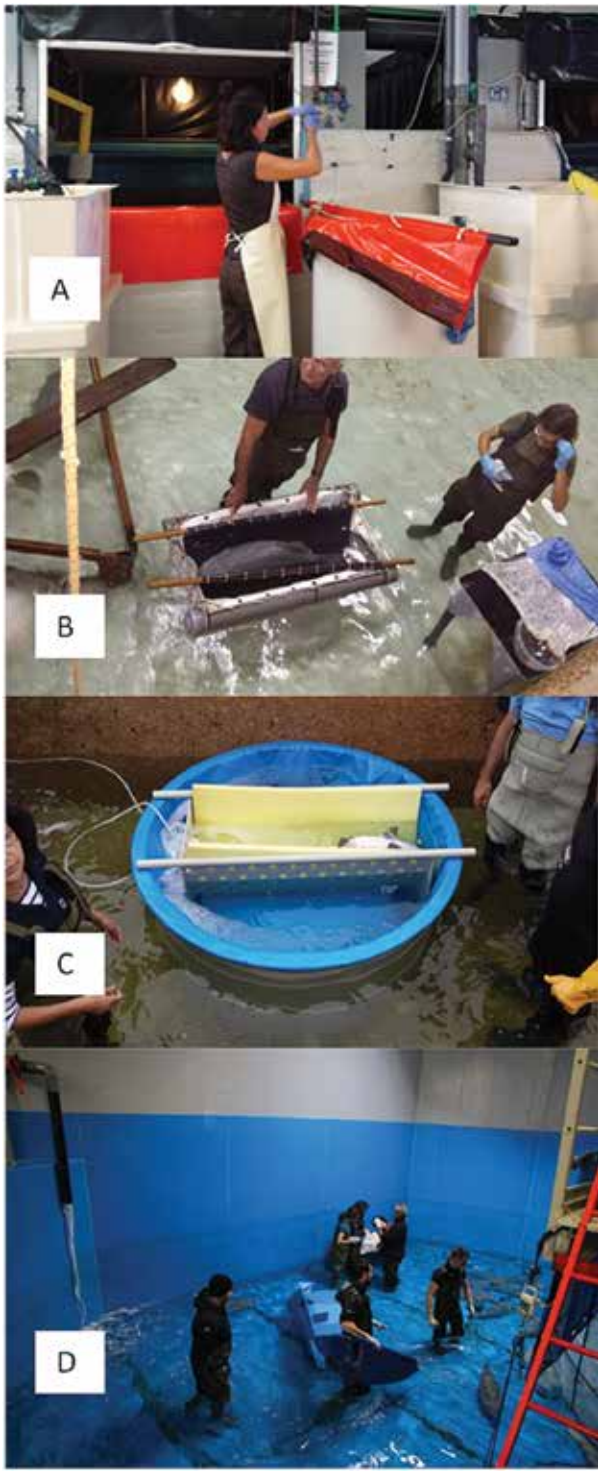

Figure 2. Different facilities working with wreckfish broodstock. (A) Hellenic Center for Marine Research (HCMR) Greece, (B) Institute of Oceanography, (IEO) Spain, (C) Xunta de Galicia (CMRM, IGAFA), Spain, (D) Aquarium Finisterrae, (MC2) Spain.

Monthly or bimonthly samplings were performed and blood, ovarian biopsies and sperm samples were

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Figure 3. Wreckfish captured in the West of Corrubedo Cape, La Coruña, Spain.

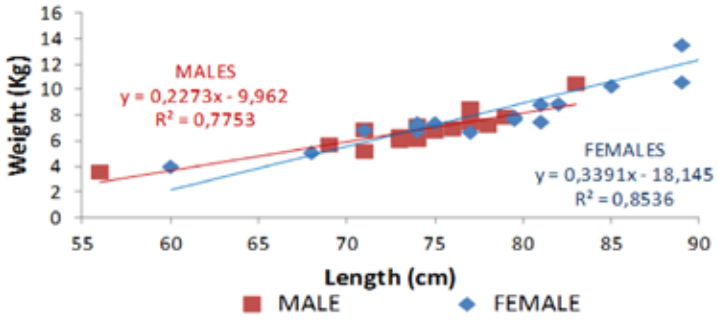

Figure 4. Length-weight relationship of wild wreckfish showing a sexual dimorphism.

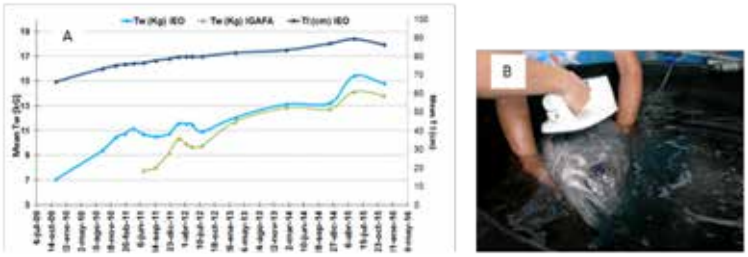

Figure 5. A) Mean total length (cm) and body weight (kg) of two wreckfish broodstocks maintained in captivity since July 2009 at IEO and CMRM (IGAFA), Spain. B) All breeders were individual tagged with PIT tags.

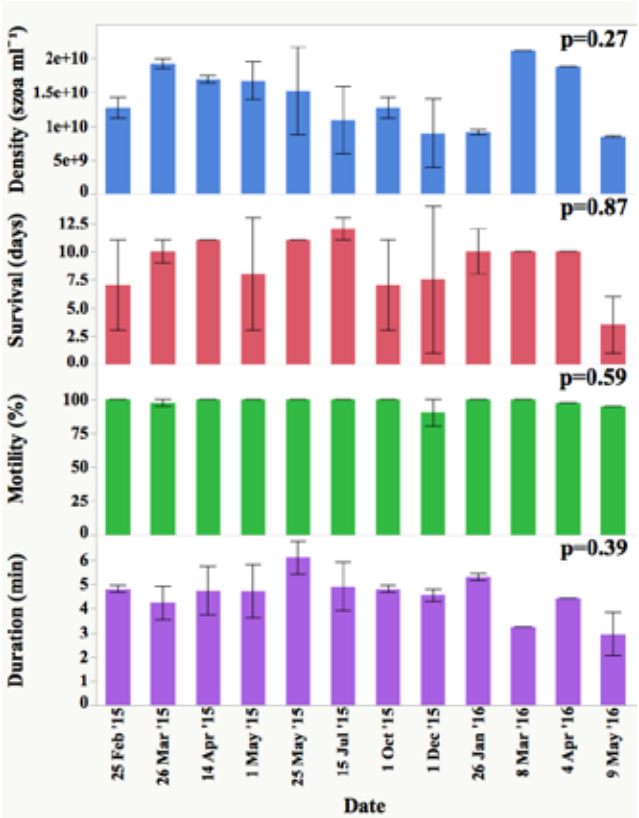

Figure 6. Sperm quality parameters of captive wreckfish that were in spermiating condition throughout the year. The fish were maintained under constant temperature (15-16°C), but simulated natural photoperiod at HCMR (Greece).

obtained. The ovarian biopsies were examined on site, as well as after histological processing, and the blood samples are currently being analyzed for their sex steroid content (testosterone, 11-ketotestosterone, 17β-estradiol, and 17,20β-dihydroxyporgesterone), which are involved in the process of gametogenesis and maturation. Based on the results obtained from the various broodstocks over the 3-year period, it has been demonstrated that males exhibit good sperm quality with large amounts of expressible sperm during an extended reproductive period (April-July), while a proportion of males were shown to be spermiating throughout the year (**Fig. 6**). Gonadal recrudescence in females begins in the fall, but the main part of vitellogenesis takes place in the Winter (Dec-Feb), and oocyte maturation in captivity starts in March and peaks between April and June. Vitellogenesis continues until the oocytes reach a size of ~1200-1400 µm in diameter, at which time oocyte maturation may take place (**Fig. 7**). The vitellogenic process is long, probably related to the low water temperatures that this species can be found and also to the relatively large egg size for a marine fish.

Spontaneous spawning was observed, even though in an unpredictable pattern, mostly at the IEO and Aquarium Finisterrae facilities. At the HCMR facilities, fish that were in the appropriate reproductive maturation were treated with ethylene-vinyl acetate (EVAc) implants loaded with gonadotropin releasing hormone agonist (GnRHa), and were induced to undergo oocyte maturation and ovulation successfully. Both spontaneous spawning in different tank conditions and artificial fertilization were tried as different methods of viable egg production, since batches of eggs with low fecundity and fertilization rates were produced if the fish were allowed to spawn spontaneously in the tank. This may be indicative of a breeding behavior dysfunction –similar to what has been reported in Senegalese sole (*Solea senegalensis*) (Guzmán et al., 2009)- since the males always produced large volumes of high quality sperm. Using artificial insemination at the HCMR, a number of fertilized eggs were delivered to the hatchery for larval rearing (**Fig. 8**). Unfortunately, the exact timing of ovulation after the hormonal treatment and the post-ovulation survival of the eggs are currently not known and might be the reasons for the low fertilization of the artificially inseminated eggs.

On the other hand, spontaneous spawning in the IEO and Aquarium Finisterrae stocks produced a large number of fertilized eggs and achieved satisfactory

fertilization success (**Fig. 9**). During the spawning season of 2015, a total of 10 spawns were obtained from the IEO broodstock between March and June. The majority of spawns were spontaneous, except for one artificial stripping from the IEO and two from the Aquarium Finisterrae broodstock. During 2016, from April to the end of May, 7 spontaneous spawns were obtained from the IEO, and 12 spontaneous spawns and two by stripping from the Aquarium Finisterrae broodstocks.

Overall, spontaneous spawns were achieved in two of the four stocks (IEO, Aquarium Finisterrae) while both spawning after GnRHa therapy and by artificial stripping and fertilization was achieved in three of the four stocks (HCMR, IEO and Aquarium Finisterrae), with a fertilization success ranging between 49 and 100 % (**Fig. 10**). The wreckfish eggs have a large diameter (1.996±0.034 mm), with a large lipid droplet allowing them to float. Hatching takes place after 5 d of incubation at 16±0.8°C (**Fig. 11**)

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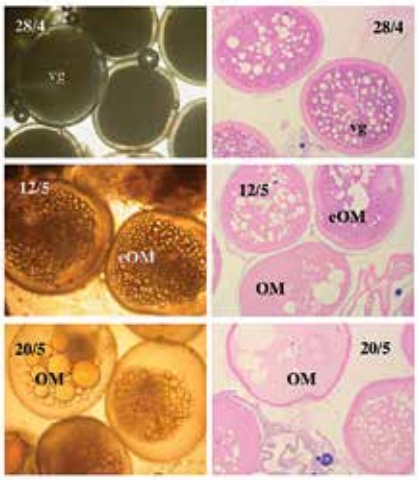

Figure 7. Wet mount and histological sections of biopsies from wreckfish during the 2014 reproductive season (dates on each photo). eOM = early oocyte maturation, OM = oocyte maturation, Vg = vitellogenic oocytes

Figure 8. Artificial insemination of wreckfish at the HCMR facilities.

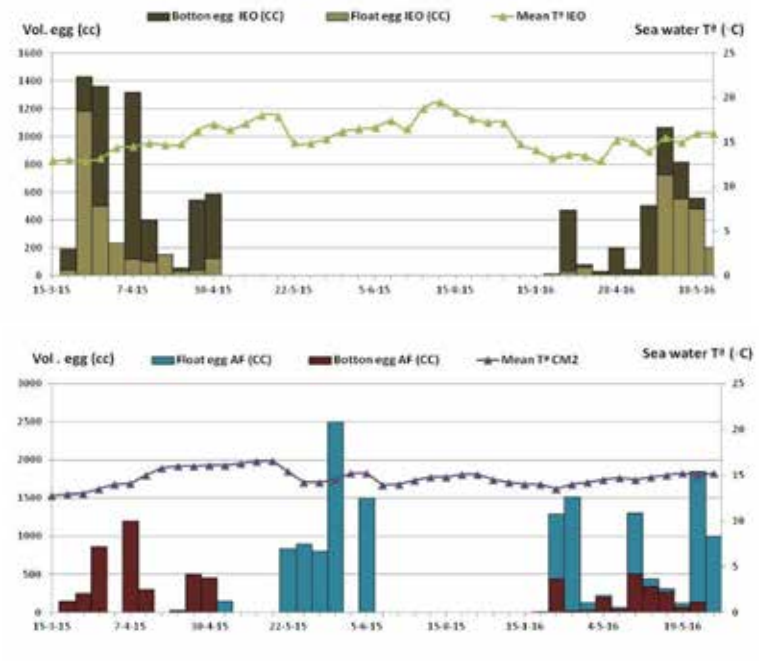

Figure 9. The volume (cubic centimetres cc) of viable floating and non-viable sinking eggs of wreckfish obtained from spawns at the IEO (upper) and Aquarium Finisterrae (lower) facilities between March 2015 and May 2016. A number of spawns were incubated and larvae were obtained.

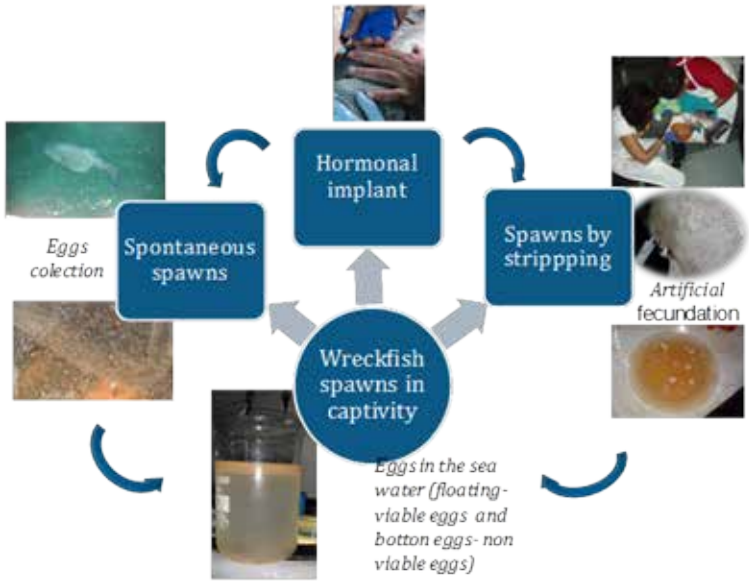

Figure 10. Wreckfish spawned spontaneously in captivity, and after a hormonal therapy with GnRHa implants eggs could be obtained either after tank spawning or using artificial fertilization. In all cases the eggs were collected for evaluation and subsequent incubation and larval rearing.

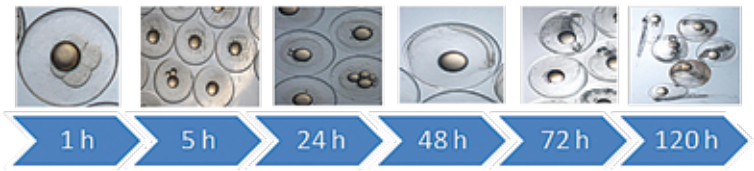

Figure 11. Embryonic development of wreckfish using eggs obtained from captive breeders.

As far as the development of the CASA (**Fig. 12**), wreckfish sperm exhibited 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. Cryopreservation of wreckfish sperm was achieved, while chilled storage does not seem to be a good solution for the management of sperm for artificial fertilization. The performance of frozen/thawed wreckfish sperm was half compared to samples of fresh sperm in terms of percentage of motile sperm and duration of swimming, while the velocity in modified Leibovitz medium was similar to that of fresh sperm. Since wreckfish produce large volumes of high quality sperm in terms of concentration, velocity and duration of motility, the loss of sperm quality due to freezing may be compensated by increasing the number of spermatozoa used per egg, as is usually practiced in other species.

Larval husbandry

The main objectives of DIVERSIFY are to understand the larval requirements in order to establish a rearing protocol. In particular, the effect of rearing temperature was studied and the description of the ontogeny of the digestive system was considered as a prerequisite for the development of an appropriate feeding protocol.

Progress during the last year was made towards the optimization of the environmental parameters. Taking advantage of the improved spawns and the availability of eggs, which allowed us to perform several trials, testing different incubation temperatures, it was shown that the optimal incubation temperature is 16±0.8°C. At this temperature range we obtained the best results regarding normal embryonic development and hatching rate of the eggs that reached 65%.

Regarding larval rearing, the results were not satisfactory, as the maximum period that the larvae survived never exceeded 27 days post hatching (dph). Several larval rearing trials were performed during the project's life. In all cases, similar rearing results were obtained by both the HMCR and IEO (**Fig. 13**). Larval total length was 4.70±0.27 mm at 1 dph. Yolk sac was consumed by 11 dph at 14-17°C and by 8 dph at 17-20°C seawater temperature. Mouth opening occurred at 7 and 4 dph at 14-17°C and 17-20°C, respectively. Following mouth opening, larvae were fed with enriched rotifers and *Artemia* nauplii, using different enrichment protocols.

During rearing, some malformed individuals were observed (**Fig. 14**). This problem could be related to inadequate nutrition, environmental conditions, oxidative stress, and husbandry conditions. We expect that in the coming year further studies will produce better results, towards the development of an efficient larval rearing protocol for this species.

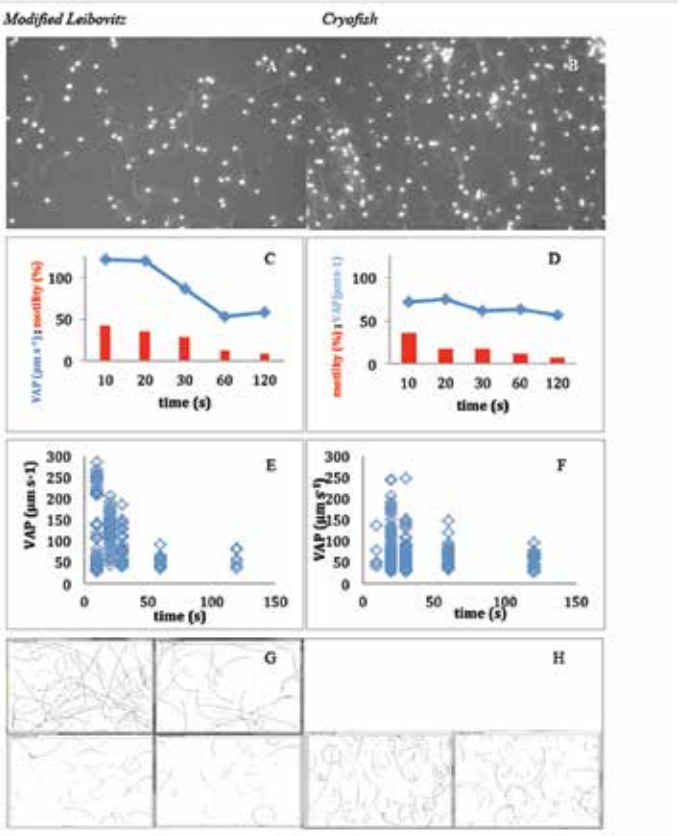

Figure 12. Wreckfish sperm status after cryopreservation in modified Leibovitz and Cryofish media. A and B: Pictures of the spermatozoa diluted in the different media (extracted from video records), Cryofish samples (B) show aggregations of spermatozoa unlike samples cryopreserved in modified Leibovitz (A). C and D: Mean velocity decrease and variations of the percentage of motile spermatozoa with time in the different media. E and F: individual velocities of spermatozoa recorded in the different media showing that modified Leibovitz (E) allows a high recovery of a larger number of spermatozoa compared to Cryofish (F). G and H: illustration of tracks generated by CASA for the spermatozoa stored in the two media: Leibovitz (G) and Cryofish (H).

Nutrition

Wreckfish nutritional requirements and optimum diets are missing. There is only limited information related to feeding habits and rates of wild-caught fish reared in captivity. The research in DIVERSIFY focuses on two main important aspects. Firstly, broodstock nutrition to determine the influence of broodstock feeds on fecundity and spawning quality. Secondly, larval nutrition to test the effectiveness of live prey and the influence of enrichment on wreckfish larval performance.

A comparative study on the composition of wild fish *vs* captive-reared wreckfish broodstocks was conducted. Analysis of tissues of wild and captive-reared wreckfish showed that cultured fish have more lipids in the muscle (27.5% DW) and liver (62%) than wild fish, which have 7% in their muscle and 40% in liver. In contrast, protein content is higher in the muscle of wild wreckfish than in captive-reared fish and some differences were also observed in the fatty acid profile with higher values of polyunsaturated fatty acids (PUFA) and n-3 PUFA in wild than in captive-reared wreckfish. The docosahexaenoic acid (DHA) values represent 11% in the muscle of captive-reared fish and 26% in wild fish **(Table 2)**.

Some commercial broodstock feeds were analyzed, showing that they have a high amount of fat for wreckfish broodstock and a new dry food was formulated on the basis of the data obtained from wild fish (**Table 3**). Our initial results showed that broodstock feed must contain high amounts of protein, low lipid content and a large amount of n-3 highly unsaturated fatty acids (HUFA), and the eicosapentaenoic acid (EPA) arachidonic acid (ARA) ratio must be around 1.5, similar to what has been observed previously in wild wreckfish. A comparison of feeding of broodstock with semi-moist diet and the new formulated diet was conducted, and a clear relationship between fatty acid profile of oocytes from the females and the two diets was found. Furthermore, some differences were observed in the fatty acid profile of oocytes from females of different wreckfish broodstock showing that there is a relationship between fatty acid content and oocyte development.

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Table 2. Biochemical composition of muscle, liver and gonad of wild and captive-reared wreckfish.

	Muscle		Liver	
	Wild fish	Captive-reared	Wild fish	Captive-reared
Proteins(%DW)	84.41±7.34	75.92±8.88	37.94±13.66	31.10±9.42
Lipids(%DW)	6.92±3.39	27.49±10.06	40.19±15.25	61.76±12.18
Fatty acids (% total)				
SAFA´s	28.83±1.28	24.46±1.25	26.11±3.51	22.44±2.27
MUFA´s	32.09±5.43	44.98±1.02	56.23±8.80	60.50±5.45
ARA	3.11±0.79	1.32±0.38	1.55±0.88	0.58±0.12
EPA	4.55±0.70	8.11±1.17	3.09±1.37	2.92±0.19
DHA	26.38±3.33	10.85±2.66	9.31±5.05	7.29±1.71
PUFA´s	39.08±4.41	30.57±0.58	17.66±8.19	17.07±3.32
Total n-3	34.51±3.75	23.78±1.68	14.93±7.01	13.44±2.00
Total n-6	4.08±0.81	6.02±1.85	2.55±1.23	3.48±1.99
n-3/n-6	8.50±1.18	4.51±2.39	5.79±1.42	4.97±3.31
DHA/EPA	5.69±1.23	1.38±0.49	2.99±0.91	2.48±0.47
EPA/ARA	1.54±0.37	6.58±2.15	2.13±0.60	5.16±1.00

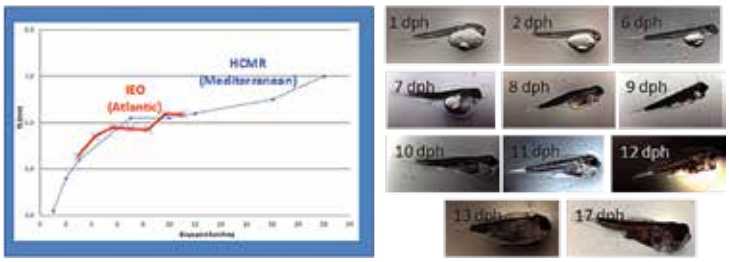

Figure 13. Wreckfish larval growth obtained in the trials that took place at HCMR and IEO (A). Development of larvae for the first 17 days post hatching (B).

Figure 14. Wreckfish larvae with malformations, similar to what has been referred to as "swollen yolk sac syndrome" or the "blue sack disease".

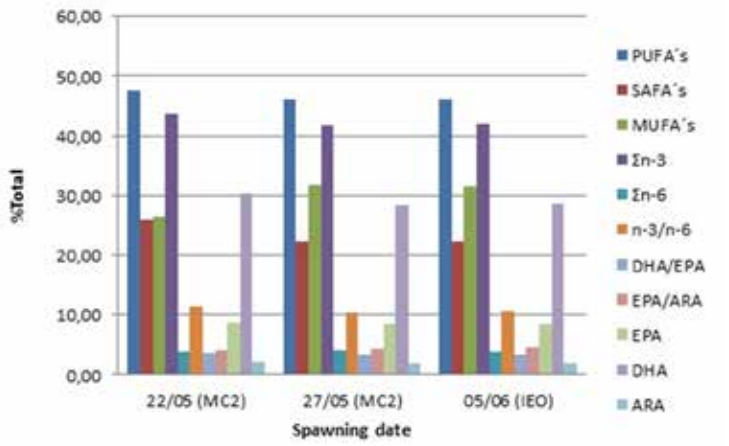

Figure 15. Fatty acid composition of wreckfish larvae at 10 dph, from three different spawnings (two from Aquarium Finisterrae broodstock and one from IEO broodstock).

Table 3. Composition of a new formulated dry feed from wreckfish broodstocks.

Ingredients	Dry food (%)
Fishmeal 70 LT FF Skagen	25.0
CPSP 90	10.0
Squid meal	34.2
Krill meal (Aker Biomarine)	7.5
Wheat Gluten	7.0
Wheat meal	7.25
Tuna oil	1.0
Algatrium 70% DHA	0.2
Incromega DHA 500TG	1.0
VEVODAR	1.3
Vit & Min Premix PV01	2.0
Lutavit E50	0.05
Soy lecithin - Powder	1.5
Macroalgae mix	1.0
Antioxidant powder (Paramega)	0.2
Antioxidant liquid (Naturox)	0.2
SelPlex - Se yeast	0.02
Carophyll Pink 10% - astaxanthin	0.05
Nucleotides (Nucleoforce)	0.03
L-Taurine	0.5
Total	100.0

Related to larval nutrition a new enrichment medium for larval wreckfish was designed on the basis of analyses of wreckfish eggs and gonads, and it will be tested in the next years. In addition, the fatty acid profile of larvae in the first days of life was described. The main fatty acids of wreckfish larvae at 10 dph are shown in **Fig. 15**. It appears that PUFA, saturated fatty acids (SAFA) and mono-unsaturated fatty acids (MUFA) values have a little variation in the first days of life.

The results obtained so far in DIVERSIFY in the studies of wreckfish indicate that acquisition of eggs is difficult but achievable in captivity, either by spontaneous spawns or hormonally induced protocols. However, juvenile production is not yet achieved, and in the following two years of the project it is expected to optimize the larval rearing methods, based on the use of new enrichment media for live food.

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