New species for EU aquaculture

Deliverable Report

Deliverable No:	D6.5		**Delivery Month:**	60
Deliverable Title	Describe the reproductive cycle of wreckfish			
WP No:	6		**WP Lead beneficiary:**	P8. IEO
WP Title:	Reproduction and genetics-wreckfish			
Task No:	6.2		**Task Lead beneficiary:**	P8. IEO
Task Title:	Describe reproductive cycle			
Other beneficiaries:	P1. HCMR	P3. IRTA	P4. IOLR	P15.ULL
P19. CMRM	P32. MC2			
Status:	Delivered		**Expected month:**	48
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Objective: The objective of this Deliverable is to describe the reproductive cycle of wreckfish under captivity.

Description: Description of the reproductive cycle of wreckfish: The reproductive cycle of the wreckfish has not yet been completely described and understood, and therefore this deliverable will describe 1) reproductive season and behaviour, based on data from wild caught specimens as well as captive ones, for which research on gonad histology has been performed, 2) Blood samples of captive broodstock have been analyzed to determine sexual steroid levels, and ovarian biopsies have been used to determine the maturity stage of oocytes, 3) Samples of sperm have been analyzed and characterized, 4) Nutritional status of wreckfish broodstock was also assessed through comparisons of specific nutrients from gonads of wild matured specimens and eggs batches from wild-captive reared fish. This deliverable includes information on the reproductive behaviour of the wreckfish and definitions on the quality criteria of the sexual products (oocytes and sperm).

Introduction

The wreckfish *Polyprion americanus* 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 that spawns at the continental slope at depths of 300-500 m, with the formation of spawning aggregations (Peres & Klippel, 2003). Its long life and late maturation, good adaptation to captivity and fast growth (Machias et al., 2003; Papandroulakis et al., 2004), together with its high flesh quality and market value, make the wreckfish a good candidate for the diversification of aquaculture production. Viable eggs and larvae have been already obtained from wild broodstocks of the southern hemisphere congener of the wreckfish, the hapuku *Polyprion oxygeneios*, in New Zealand (Anderson et al., 2012). More recently, juvenile production by F1 generation hapuku has also been achieved (Symonds et al., 2014). In the wreckfish, however, although attempts have been made,

spawning is inconsistent, produced eggs exhibit low fertilization (Fauvel et al., 2008) and larvae don't manage to survive more than 25 days after hatching (unpublished data).

The establishment of methods for the control of spawning and the production of good quality eggs are essential for the culture of any given species. However, there are a number of reproductive dysfunctions observed in fish maintained in captivity, especially females, which result in lack of spawning. Examples of reproductive dysfunctions observed in fish held in captivity are lack of maturation in the European eel *Anguilla anguilla* (Perez et al., 2011), lack of maturation or ovulation in meagre *Argyrosomus regius* (Mylonas et al., 2013) and greater amberjack *Seriola dumerili* (Mylonas et al., 2004b; Zupa et al., 2017) and lack of spawning of the F1 generation of the sharpsnout seabream *Diplodus puntazzo* (Micale et al., 1996; Papadaki et al., 2017) and the Senegalese sole *Solea senegalensis* (Guzmán et al., 2008). In males, reproductive dysfunctions involve limited or no sperm production and lack of synchronization of sperm production and ovulation (Mylonas et al., 2017), with the most pronounced example being the European and the Japanese eel (*Anguilla japonica*), which remain immature and fail to produce sperm under captivity (Peñaranda et al., 2010). 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 a large number of viable eggs (Mylonas et al., 2013).

An adequate broodstock nutrition is also essential to obtain success in fish intensive culture. In marine fish, dietary lipids and in particular polyunsaturated fatty acids (PUFAs), carotenoids and some antioxidant related vitamins (vitamins C, E and A) play critical roles in the successful production of high quality gametes and eggs (Izquierdo et al., 2001; Sargent et al., 2002; Valdebenito et al., 2013). Research about wreckfish nutrition is very scarce and only some information was available from studies from feeding habits of wild population (Brick Peres & Haimovi, 2003), feeding rates (Papandroulakis et al., 2004), and biochemical composition of tissues from wild fish (Linares et al., 2015). More recently a study about proximate composition, fatty acid profile and cholesterol content of wild Mediterranean wreckfish was performed (Roncarati et al., 2014). Due to the scarce information about wreckfish nutrition and with the objective of knowing their nutritional status, and to identify potential nutritional deficiencies and requirements, a study performed in DIVERSIFY (see Deliverable 12.2), on the proximate and fatty acid composition of different tissues (muscle, liver and gonad) from wreckfish wild fish was done as preliminary studies to get some basic information of this species. Based on this information, some nutritional studies were further focused on broodstock feeds for enhancing fecundity and spawn quality. Basic information on carotenoids and other related antioxidant essential vitamins was not available yet in that study.

The aim of the present study was to describe the reproductive cycle of wreckfish both in the wild and in captivity. Wild fish were either found in the fish market (2014) or were fished (2014 and 2015) and their biometric parameters were measured. Captive wreckfish were also monitored, following oocyte growth and maturation stage, sperm quality variations and sex steroid plasma concentrations in four different broodstocks maintained in captivity in Spain and Greece for almost two consecutive years. To the same purpose, some new analysis of carotenoids and vitamins performed for the present Deliverable and some related available information of LC-PUFA from Deliverable 12.2 are brought together for assessment on nutritional status of these broodstock wreckfish specimens.

Materials and methods

Capture of wild wreckfish

A total of 60 wild fish were sampled between January and October of 2014 in the fish market in order to obtain information on this species. For each animal sampled, total length (cm), total and eviscerated weight (kg), % of the peri-visceral fat, as well as the gonadosomatic ((gonad weight/body weight) x 100) and hepatosomatic index ((liver weight/body weight) x 100) was determined. Samples from the stomach, liver, gonads, muscle and fins were taken for biochemical (P19. CMRM, P15. ULL) and histological studies.

During 2015 (on 7 and 14 of August) two wreckfish were captured using a hand net in a fishing area located 5 miles West of Corrubedo Cape, A Coruña (**Fig. 1**). Fish were transported by sea on a ship with flow-

through water until O Grove Aquarium facilities, where the fish were transferred to a quarantine tank. A sample from the fin was also taken for genetic analysis. These fish were transported to P14. IEO facilities in Vigo in March 2016. These two juveniles (4.86 and 0.94 kg in body weight) were maintained separated from the existent stock at the P14. IEO, until they become adults. Simultaneously, we are following the growth and development of the three juvenile specimens captured during 2014, two held at the P14. IEO, and the third at the Acuario de O Grove. Furthermore, we are monitoring the development of the Acuario O Grove wreckfish broodstock, which is constituted by 7 fish: 2 females, 3 males and 2 undetermined with an average weight of 11.57±1.86 Kg.

Figure 1. *Wreckfish* captured in 2015 in the fishing area 5 miles to the West of Corrubedo Cape, La Coruña.

Although increasing effort has been made to contact Galician fishermen, it is important to note that the decline in catches of wreckfish in Galicia makes it difficult to obtain specimens of wild wreckfish to establish new wreckfish broodstocks.

Captive wreckfish maintenance

Four different broodstocks at different research institutes in Greece and Spain were used; at the Hellenic Center for Marine Research (HCMR, n=3) in Heraklion, Crete, Greece, the Instituto Español de Oceanografía (IEO, n=13) in Vigo, Spain, the Aquarium Finisterrae (AF, n=21) in A Coruña, Spain and the Conselleria do Medio Rural e Mariño (IGAFA, CMRM, n=11) in Pontevedra, Spain. One female and 2 males of mean weight ± S.D. 13.1 and 9.08±2.66 kg, respectively, were kept at HCMR in a 15-m^3 tank, under simulated natural photoperiod and constant temperature of around 16°C. Fish were fed twice per week with frozen fish. On June 12, 2016 one of the males died, leaving the broodstock of HCMR with one female and one male fish. Ten females and 3 males of 16.24 ± 4.05 and 11.58±1.59 kg, respectively, were kept at IEO in two 110-m^3 tanks (S1 and S2) under natural photoperiod and water temperature. Twelve females and 9 males of 23.14±6.79 and 17.36±3.25 kg, respectively, were kept at MC2 (AF) in a large exhibition tank of 3500 m^3 and in the breeders' tank of 33 m^3 under simulated natural photoperiod and natural water temperature. Finally, 8 females and 3 males of 14.43±2.8 and 13.29±0.55 kg, respectively, were kept at CMRM (IGAFA) in two tanks of 120 and 180 m^3 under natural photoperiod and water temperature. On August 15, 2016 one female fish died from the CMRM broodstock. The temperature conditions at the four different locations are shown in **Fig. 2**.

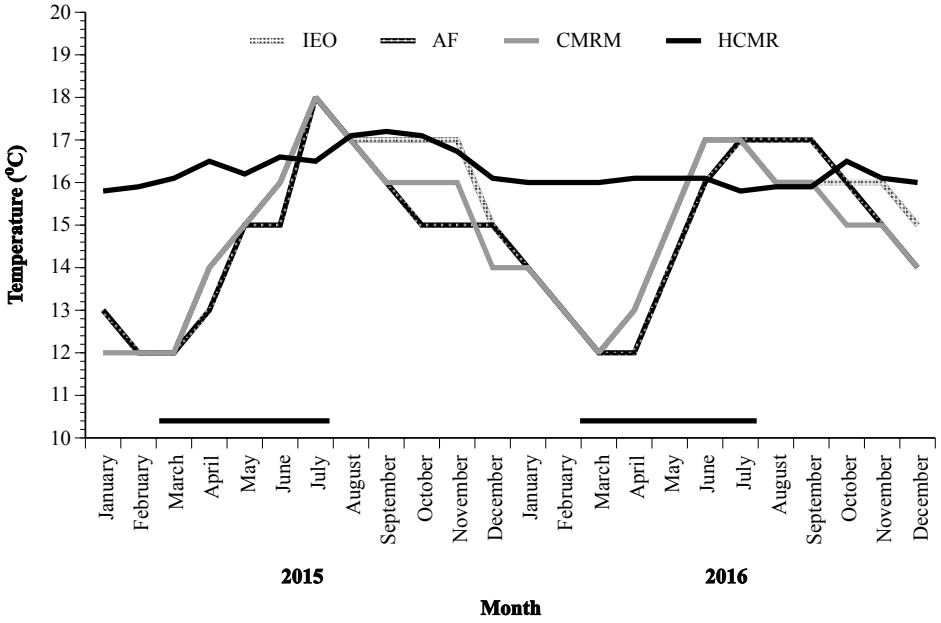

Figure 2. Tank water temperature (°C) of wreckfish broodstocks at four different sites in Greece (Hellenic Center for Marine Research, HCMR) and Spain (Instituto Español de Oceanografía (IEO), Aquarium Finisterrae (AF) and the Conselleria do Medio Rural e Mariño (CMRM) from January 2015 until December 2016.

Feeding regimes of wreckfish stocked at the three Spanish centers. Taken from Table 1 of D.12.2.

	2014	2015	2016	2017
Stock IEO Tank S1 n-5	Semi-moist diet	Semi-moist diet	Semi-moist diet	Semi-moist diet
Stock IEO Tank S2 n-6	Semi-moist diet	Dry food 1	Dry food 2	Dry food 2
Stock IGAFA n=10	Vitalis Repro/ Vitalis Cal	Squid	Squid	Hake/Squid
Stock AF n=17	Semi-moist diet & Fish breeders-M	Semi-moist diet & Fish breeders-M	Semi-moist diet & Fish breeders-M	Semi-moist diet & Fish breeders-M

For egg collection, a passive egg collector was placed in the outflow of the tank, in order to verify the occurrence of any spawning and collect the spawned eggs. At HCMR, eggs were collected into a 10-l bucket and their number (fecundity) was estimated by counting the total number of eggs in a sub-sample of 10 ml, after vigorous agitation. Fertilization success was evaluated at the same time by calculating the number of viable eggs in respect to the total number of eggs spawned. At IEO, CMRM (IGAFA) and MC2 (AF), eggs were collected in a graduated cylinder and the volume (ml) of the total number of eggs spawned and of floating eggs was determined (**Fig. 3**). Then, fertilization success was estimated in a sub-sample of the floating eggs using a stereoscope. The total number of eggs spawned was estimated by multiplying the observed egg volume by 150, which is the number of wreckfish eggs found in 1 ml of seawater.

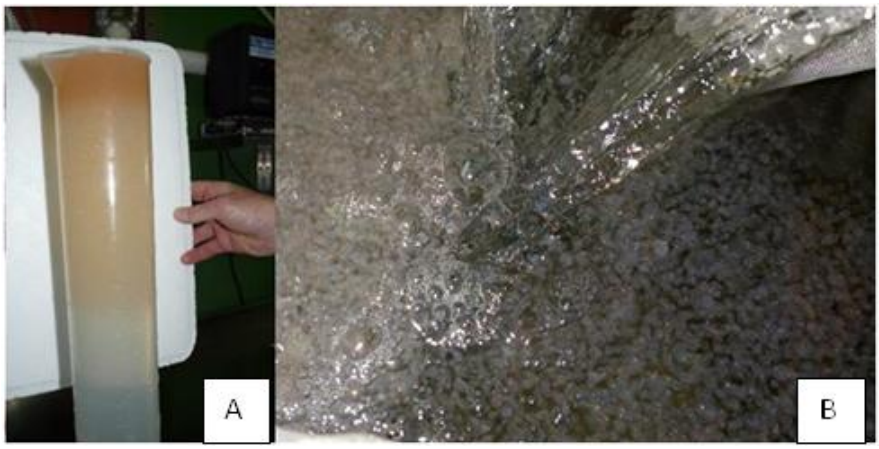

Figure 3. Spontaneous spawn (A) counted volumetrically from the egg collector and (B) in the egg collector of tank S-2 at the P14. IEO.

Samplings and histology

Fish reproductive cycle was monitored from March 2015 to October 2016. Samplings were conducted monthly from February until June, and bimonthly from July until January **(Fig. 4)**. To determine the sex of the specimens from which biopsy could not be obtained, as the gonopore was completely closed, ultrasound was used (**Fig. 5**).

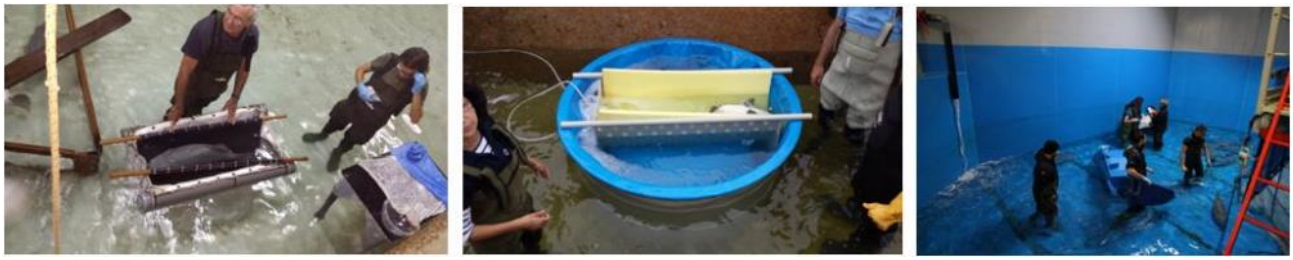

Figure 4. Broodstock sampling at P14. IEO, P19. CMRM and P32. MC2.

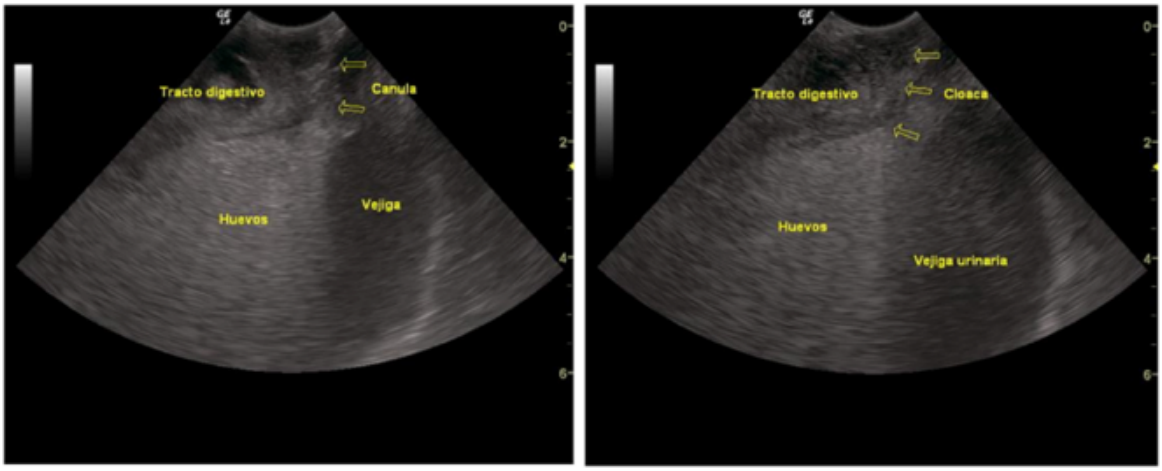

Figure 5. Gonad echography pictures of two wreckfish, to identify sex of breeders held at P32. MC2 and P19. CMRM.

At each sampling, the weight of the fish was measured and biopsies were collected from female fish. Fish were tranquilized initially in their tank with the use of clove oil (0.01 ml l^{-1}) and then transferred to an anesthetic bath for complete sedation with a higher concentration of clove oil (0.03 ml l^{-1}) (Mylonas et al., 2005). Ovarian biopsies for the evaluation of oocyte development were obtained with the use of a Pipelle de Cornier catheter. A wet mount of the biopsy was examined under a compound microscope (40 and 100x) to evaluate the stage of oogenesis and measure the mean diameter of the largest, most advanced oocytes (n = 10). A portion of the biopsy was fixed in a solution of 4% formaldehyde-1% glutaraldehyde for further histological processing. Females that did not show any sign of oocyte maturation were excluded from the analysis. Maturation of the males was examined by the release of sperm upon application of gentle abdominal pressure. Sperm was collected after rinsing the fish with clean seawater and blot drying the genital pore. Small volumes of sperm (50–100 µl) were collected in order to avoid influencing the quantity and/or quality of sperm during subsequent collections. The collected sperm was stored on ice and then transferred to a 4 °C refrigerator until evaluation. 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 2, 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. Sperm quality parameters that were evaluated included sperm concentration (number of spermatozoa ml^{-1} of milt), percentage of spermatozoa showing forward motility immediately 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). Sperm concentration was estimated after a 2121-fold dilution with seawater using a Neubauer haemocytometer under 200x magnification (in duplicate) in a compound light microscope (Nikon, Eclipse 50i). Sperm motility and motility duration were evaluated on a microscope slide (400x magnification) after mixing 1 µl of sperm with a drop of about 50 µl of saltwater (in duplicate). Males that did not show any sign of maturation (i.e. did not produce sperm for the duration of the study) were excluded from the analysis. Blood was collected from all fish at each sampling, in order to measure sex steroid hormone concentrations. Blood was centrifuged at 6000 rpm for 15 min and plasma was collected and stored at −80 °C until analysis. Due to a mistake during the March 2015 sampling in IEO, CMRM and MC2, samples of this month were excluded from the plasma sex steroid analysis.

Histological processing

Before embedding in methacrylate resin (Technovit 7100®, Heraeus Kulzer, Germany) ovarian biopsies were dehydrated in gradually increasing ethanol solutions (70–96%). Serial sections of 3 µm were obtained with a microtome (Reichert Jung, Biocut 2035, Germany). Sections were stained with Methylene Blue (Sigma, Germany)/Azure II (Sigma, Germany)/Basic Fuchsin (Polysciences, USA) according to (Bennett et al., 1976). Sections were examined under a light compound microscope (Nikon, Eclipse 50i) and photographed with a digital camera (Jenoptik progress C12 plus).

Hormone measurements

For the quantification of T, E2, 11-KT and 17, 20β-P in the plasma, already established and well described enzyme-linked immunoassays (ELISA) were used (Cuisset et al., 1994; Nash et al., 2000; Rodríguez et al., 2000) with some modifications, and using reagents from SpiBio (France). For steroid extraction, 200 µl of plasma were extracted twice with 2 ml diethyl ether. Extraction was done by vigorous vortexing (Vibramax 110, Heidolph, Germany) for 3 min. After decanting of the organic phase, drying of the supernatant was done under a stream of nitrogen (Reacti-vap III, Pierce, USA). Samples were reconstituted in 250 µl of reaction buffer for running in the ELISA.

Plasma LH levels were measured using the heterologous ELISA developed for striped bass (*Morone saxatilis*) LH (Mañanós et al., 1997) and validated for werckfish. Ninety-six well polystyrene plates were coated with recombinant LH (r-LH; 2.4 ng per well) and incubated overnight at 4°C. The plates were then

washed with PBST and blocked with BSA (2% in PBST; 100 µl per well) for 0.5 h at 37□. The primary antibody (anti-striped bass LH) was diluted 1:80,000 in PBST containing 2% normal goat serum (NGS). Samples and standards were serially diluted in PBST, mixed with the primary antibody solution (v:v in 1.5 ml tubes) and incubated overnight at 4□. Then the content in each tube was dispensed into the antigen-coated wells (100 µl per well in duplicate). Following an incubation (overnight at 4□), AffiniPure Goat anti-Rabbit IgG (H+L) (Jackson Immunoresearch laboratories, inc.) in 1% NGS-PBS T was added (100µl per well) for 0.5 h at 37□. The wells were washed and SureBlueTM TMB-microwell peroxidase substrate (1-component) (KPL, MD, USA) was added (100 µl per well). The reaction was stopped after 20 to 40 min at RT by the addition of 100µl of 1N phosphoric acid and the absorbance was read at 450 nm.

Biochemical analysis of nutrients

Analysis of carotenoids and vitamins of available samples from two wild female mature gonads and batches of eggs from the 3 Spanish centers, sent by CMRM, were performed at ULL facilities (P.15).

Carotenoids were extracted following the method described by Barua et al. (1993). Approximately 200-300 mg sample were homogenized with ethyl acetate/ethanol containing 0.01% BHT (10mL, 1:1 v/v), ethyl acetate (5 ml), and hexane containing 0.01% BHT (10 ml) as extracting solvents, respectively, in the presence of darkness and cold atmosphere. Afterwards, the solvent was evaporated and the extract re-dissolved in 1.5 ml hexane and maintained in the presence of nitrogen and darkness until their quantification by spectrophotometry (Beckman Coulter DU-800, IN, USA) at 470 nm.

Sample preparation and quantification of vitamins

Water-soluble vitamin C was extracted by homogenization of 100-200 mg sample in 2.5 mL metaphosphoric acid 0.5% containing 0.2% dithiothreitol (DTT). Homogenates were centrifuged at 1500 rpm, 4 ^{o}C for 5 min and the supernatant diluted 1:10 using 0.5% metaphosphoric acid before injection into HPLC system (FAO, 1997).

Preparation of fat-soluble vitamins (FSVs), more specifically vitamins A, D and E, was carried out by hot saponification at 100 ^{o}C for 20 min of approx. 100 mg sample, in a mixture of ethanol and 20% (w/v) aqueous KOH solution (8:1, v:v) in the presence of BHT as antioxidant. After a cooling period, FSVs were extracted with 3 mL hexane over 3 times, and centrifuged at 1000 rpm for 5 min. Finally, the solvent was evaporated to dryness with a gentle steam of N_2 and the residue reconstituted with 1 mL methanol and vigorously mixed for 5 min (Ball, 2006; Blake, 2007). Vitamin analysis were performed using a Thermo-Scientific ultra-high performance liquid chromatograph (Thermo-Fisher Scientific, San José, CA, USA) equipped with a Hypersil GOLD (100 x 2.1 mm, particle size: 1.9 µm, Thermo Scientific) column. An isocratic mobile phase composed of buffer acetate 0.2% DTT, pH 3.6: Milli-Q water: MeOH (1.5:94.5:4) or MeOH: Milli-Q water (91:9) was used for water-soluble and fat-soluble vitamins determinations, respectively. The injection volume was 5 µL and the flow rate 400 µL min^{-1}. All extracts were filtered through a 0.20 µm pore size polyester membrane filter prior to injection. The eluate was detected using an Accela photodiode array (PDA) detector (Thermo-Fisher Scientific) set at 245 nm (vitamin C), 265 nm (vitamin D), 292 nm (vitamin E) and 325 nm (vitamin A).

The concentration of vitamins in the samples was determined using an external standard method. A seven point calibration curves (n=3) were prepared with standard stock solutions of vitamins diluted in appropriate solvent mixtures at concentrations spanning those present in samples.

Statistical analysis

Differences in mean oocyte diameter, sperm motility duration, motility percentage, density and survival within months, were assessed with the use of Student's t test, at a minimum significance of $P<0.05$. Differences in oocyte diameter and steroid hormone concentrations in relation to oocyte stage and spermiation index were assessed with the use of one-way ANOVA, followed by Tukey's HSD test, at a minimum significance of $P<0.05$. Unless otherwise mentioned, results are presented as mean ± SEM.

Statistical analyses were performed using the statistical software JMP (SAS Institute Inc., Cary, NC).

Results

Biometric parameters of wild wreckfish captured in 2014 are shown in **Table 1**. Total weight of wild wreckfish varied between 3.6 and 18 kg, and total length varied between 56 and 98 cm (**Fig. 6**). A relation between weight and length was established, both for males and females (**Fig. 7**).

Table 1. Biometric parameters and indexes of the 60 animals sampled in the Azores fisheries.

BIOMETRIC PARAMETER (60 WILD WRECKFISH)	MEAN	STD
TOTAL LENGHT	76,09	6,788
ST LENGHT	66,38	7,629
PERÍMETER	55,68	5,986
WEIGHT (Kg)	7,52	2,169
EVIS. WEIGHT (Kg)	6,99	1,967
GONAD WEIGHT (g)	17,10	20,831
LIVER WEIGHT (g)	95,70	71,671
FAT PERIVIS. WEIGHT (g)	76,25	72,233
STOMACH WEIGHT (g)	125,90	56,183
INTESTINE LENGHT (cm)	94,53	15,555
INTESTINE WEIGHT (g)	99,27	62,688
GSI FEMALES	0,30	0,184
GSI MALES	0,13	0,126
SHI	1,21	0,497
VSI	10,31	17,233

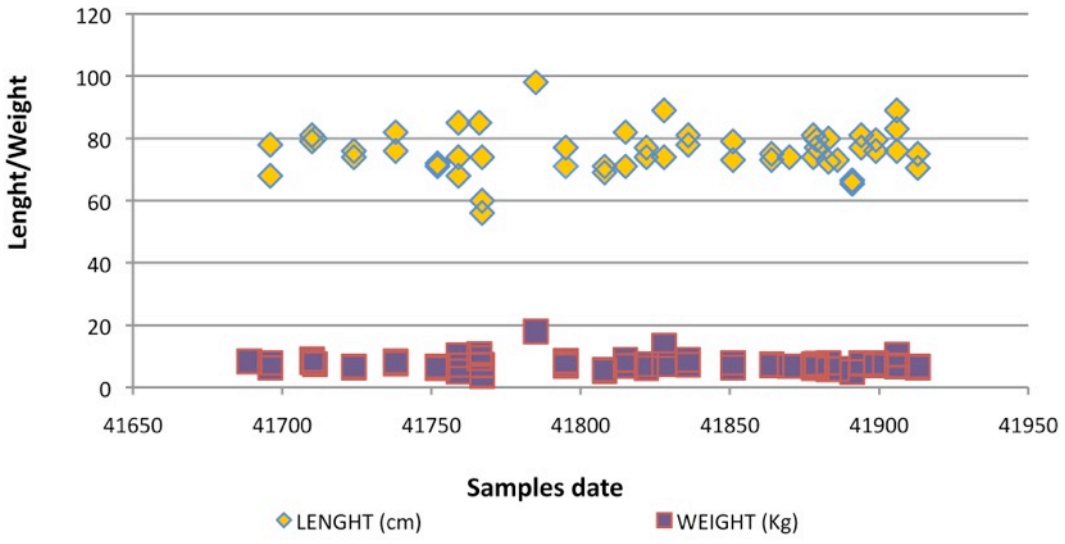

Fig. 6. Total length and weight of the 60 animals sampled in the Azores fisheries.

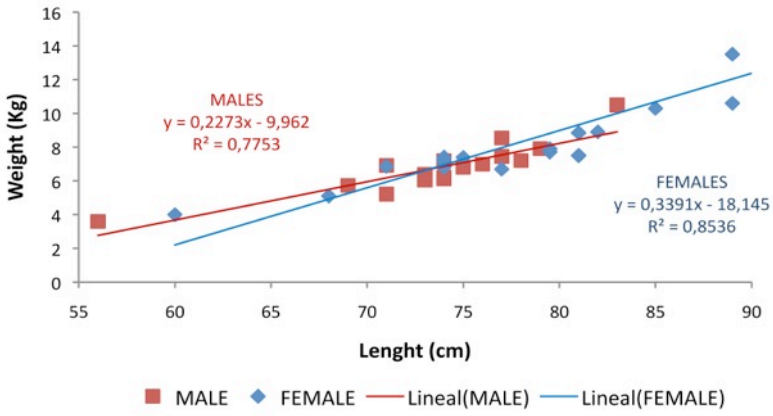

Figure 7. Weight/length relationship from males and females sampled in the Azores fisheries.

No relation was observed between weight, sex and perivisceral fat from the 60 fish sampled in the fish market (**Fig. 8**). No relation was observed between perivisceral fat % and date of capture from the 60 dead wild fish sampled (**Fig. 9**). This information would be important for nutrition studies and future elaboration of artificial feeds for this species (WP12 Nutrition - wreckfish).

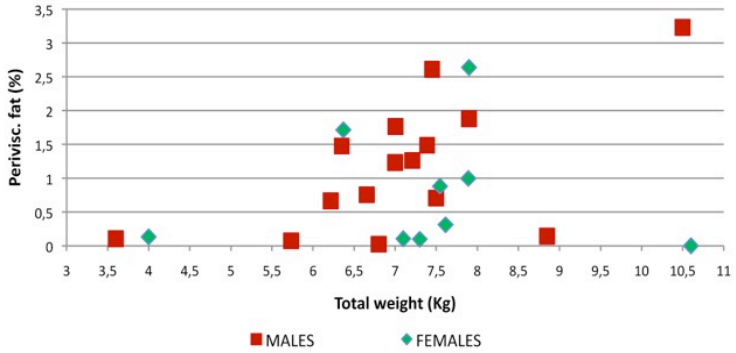

Figure 8. Relation between weight, sex and perivisceral fat (%) of the 60 animals sampled in the Azores fisheries.

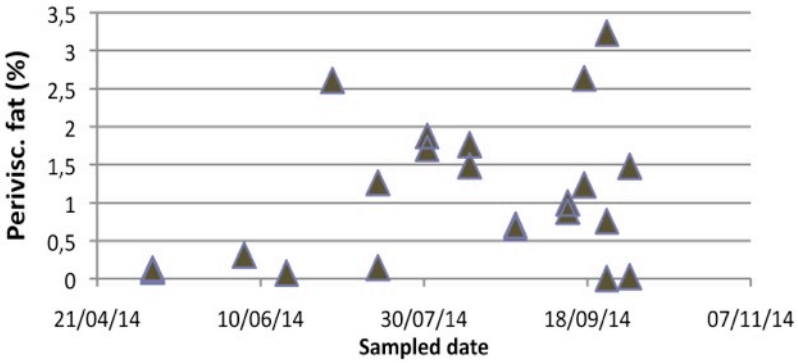

Figure 9. Relation between perivisceral fat % and time of capture of the 60 animals sampled in the Azores fisheries.

Wreckfish reproductive period begun with oocytes reaching vitellogenesis (**Fig. 10A, 11A**) and proceeded with oocyte maturation after lipid droplets coalescence (**Fig. 10B, 11B**). During final oocyte maturation, yolk coalescence was completed and the germinal vesicle was located in the periphery of the oocyte **(Fig. 10C, 11C)**, whereas after ovulation small primary, lipid vesicle and atretic oocytes could be observed in female fish gonads, together with post-ovulatory follicles if spawning had occurred (**Fig. 10D, 11D**).

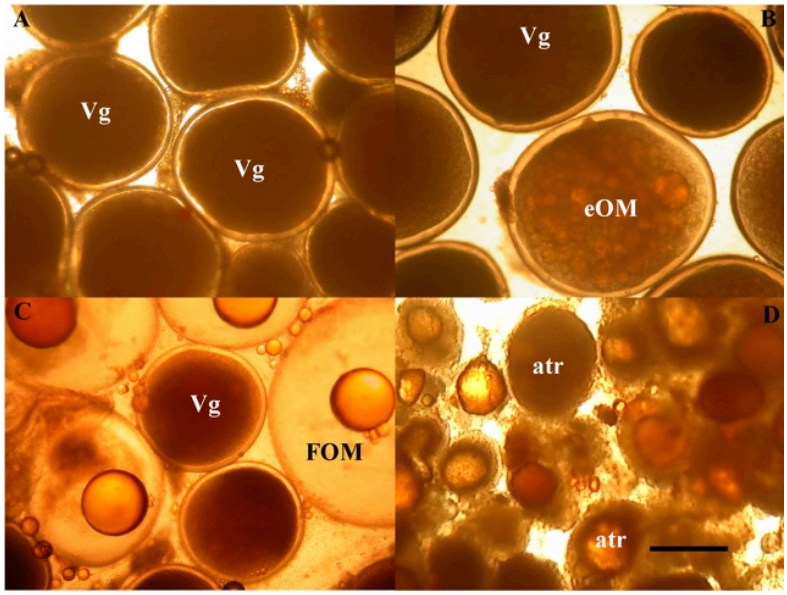

Figure 10. Photomicrographs of wreckfish biopsies, showing oocytes at successive stages of development: vitellogenesis (Vg, A), early oocyte maturation (eOM) with lipid droplet coalescence (B), final oocyte maturation (FOM, C) and atresia at the end of the reproductive season (D). The bar represents 500 µm.

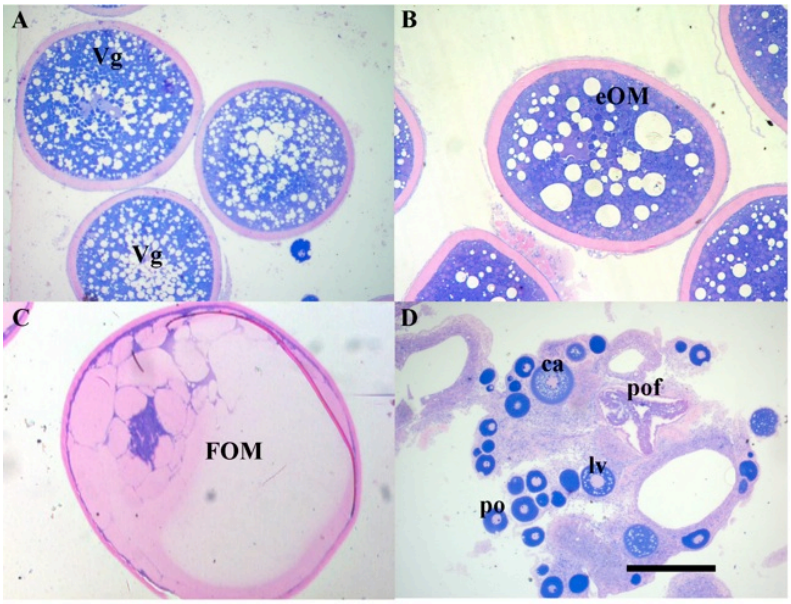

Figure 11. Photomicrographs of histological sections of wreckfish biopsies, showing vitellogenic oocytes (Vg, A), early maturing oocytes with lipid droplet coalescence (eOM, B), final oocyte maturation (FOM, C) and gonad at the end of the reproductive season with primary oocytes (po), lipid vesicle oocytes (lv), cortical alveoli oocytes (ca) and post-ovulatory follicles (pof). The bar represents 500 µm.

Oocyte development of some of the wreckfish females did not seem to advance beyond the cortical alveoli stage, and their oocytes did not reach more than 350 μm in diameter during the whole year. When excluding these fish (4 females from IEO and 2 females from CMRM), wreckfish oocyte diameter was found to reach its highest values from March until June or July and its lowest values from September until December during both years of the study (**Fig. 12**).

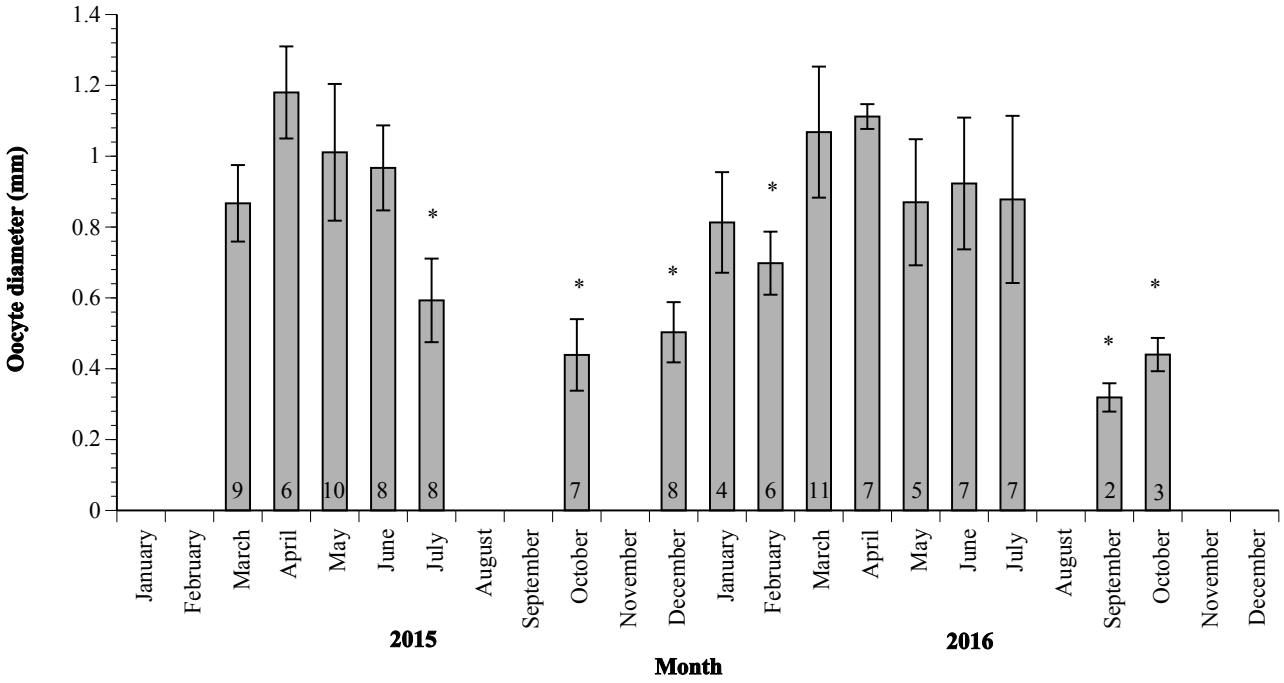

Figure 12. Mean (±SEM) oocyte diameter of wreckfish broodstocks at four different sites in Greece and Spain during the annual reproductive cycles from March 2015 until October 2016. The numbers inside the bars indicate the number of females biopsied at each month. Asterisks (*) denote significantly lower values than maximums observed (April 2015 and 2016).

The first significant increase of oocyte diameter was observed at vitellogenesis, with oocytes reaching around 1 mm diameter. Oocytes grew significantly during oocyte maturation, and remained at the same size at ovulation, at a mean diameter of around 1.4 mm (**Fig.13A**). Due to the differences in the environmental conditions under which the fish were held at the different locations, sex steroid hormone levels were expressed in the present study not versus time, but versus the reproductive stage of both females and males. Moreover, due to the high variation between samples, female wreckfish E2 concentration did not show statistically significant changes with oocyte stage, although a trend of increasing values until vitellogenesis and decreasing values thereafter was visible (**Fig.13B**). As far as 17, 20-P concentrations are concerned, its values did not seem to change in respect to oocyte stage (**Fig. 13C**), whereas T remained at low levels at the lipid vesicle and cortical alveoli stage and increased during vitellogenesis. A second increase was observed during oocyte maturation and a significant decrease at ovulation (**Fig. 13D**). The plasma LH levels, consistently increased in a stage dependent manner reaching their maximum concomitant with oocyte maturation and ovulation (**Fig. 13E**).

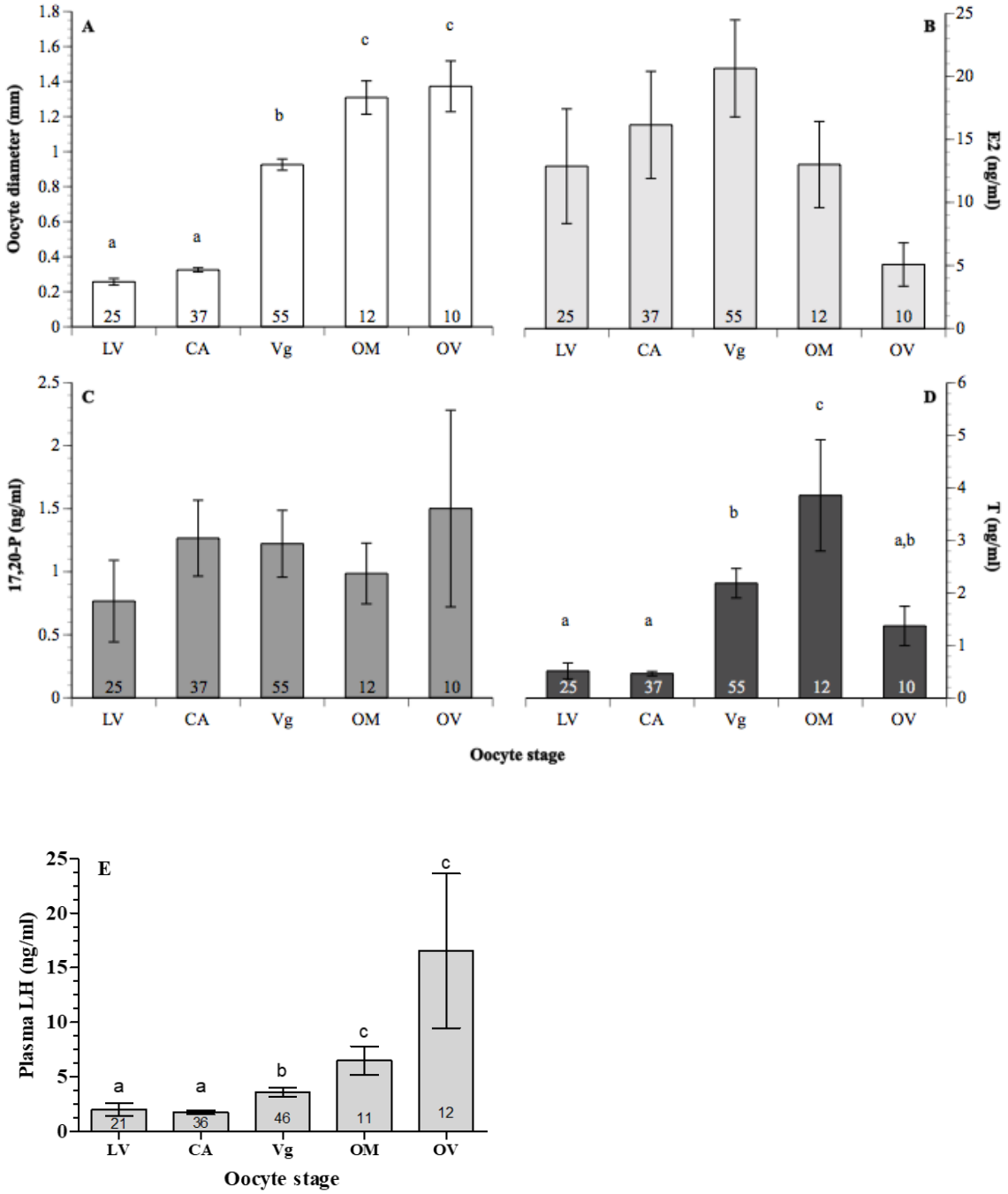

Figure 13. Mean (±SEM) oocyte diameter (A), concentrations of 17β-estradiol (E2, B), 17, 20-dihydroxy-pregnenolone (17,20-P, C), testosterone (T, D) and LH (E) at different stages of oocyte development (LV: lipid vesicle, CA: cortical alveoli, Vg: vitellogenesis, OM: oocyte maturation, OV: ovulation). Different letter superscripts indicate statistically significant differences in oocyte diameter and T between different oocyte stages. The numbers inside the bars indicate the number of samples at each oocyte stage.

Spermiating males could be found all-year round, with the percentages of S0, S1, S2 and S3 stage fish varying between months (**Fig. 14**). The highest percentage of non-spermiating fish (S0 spermiation index)

was found from September until December, whereas high percentages of spermiating fish (S2 and S3 spermiation index) were found just before and during the reproductive season of females, from January until July, during both years of the study (**Fig.14**).

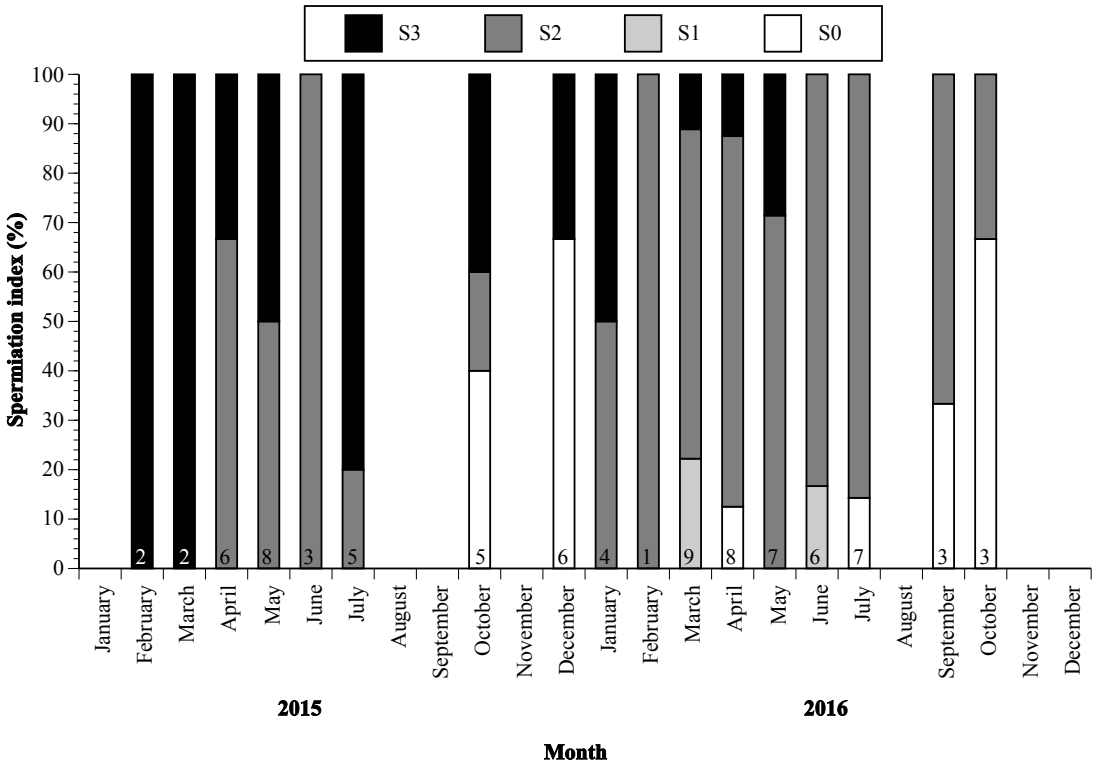

Figure 14. Percentage of male wreckfish at different spermiation index stages, in respect to month from February 2015 until October 2016. Spermiation index was reported on a subjective scale, with S0 = no milt released, S1 = only a drop of milt released after multiple stripping attempts, S2 = milt was released easily after the first stripping attempt and S3= milt was fluently released even without abdominal pressure. The numbers inside the bars indicate the number of wreckfish males examined each month.

Sperm motility duration and motility percentage exhibited high and almost unchanged values during both years of the study (**Fig. 15A and B**), whereas sperm density exhibited high values during the whole year, with the highest values observed in March of both years (**Fig. 16A**). As far as sperm survival at 4°C is concerned, it exhibited constant values during the whole year, with lower values only in March, June and September 2016 (**Fig. 16B**).

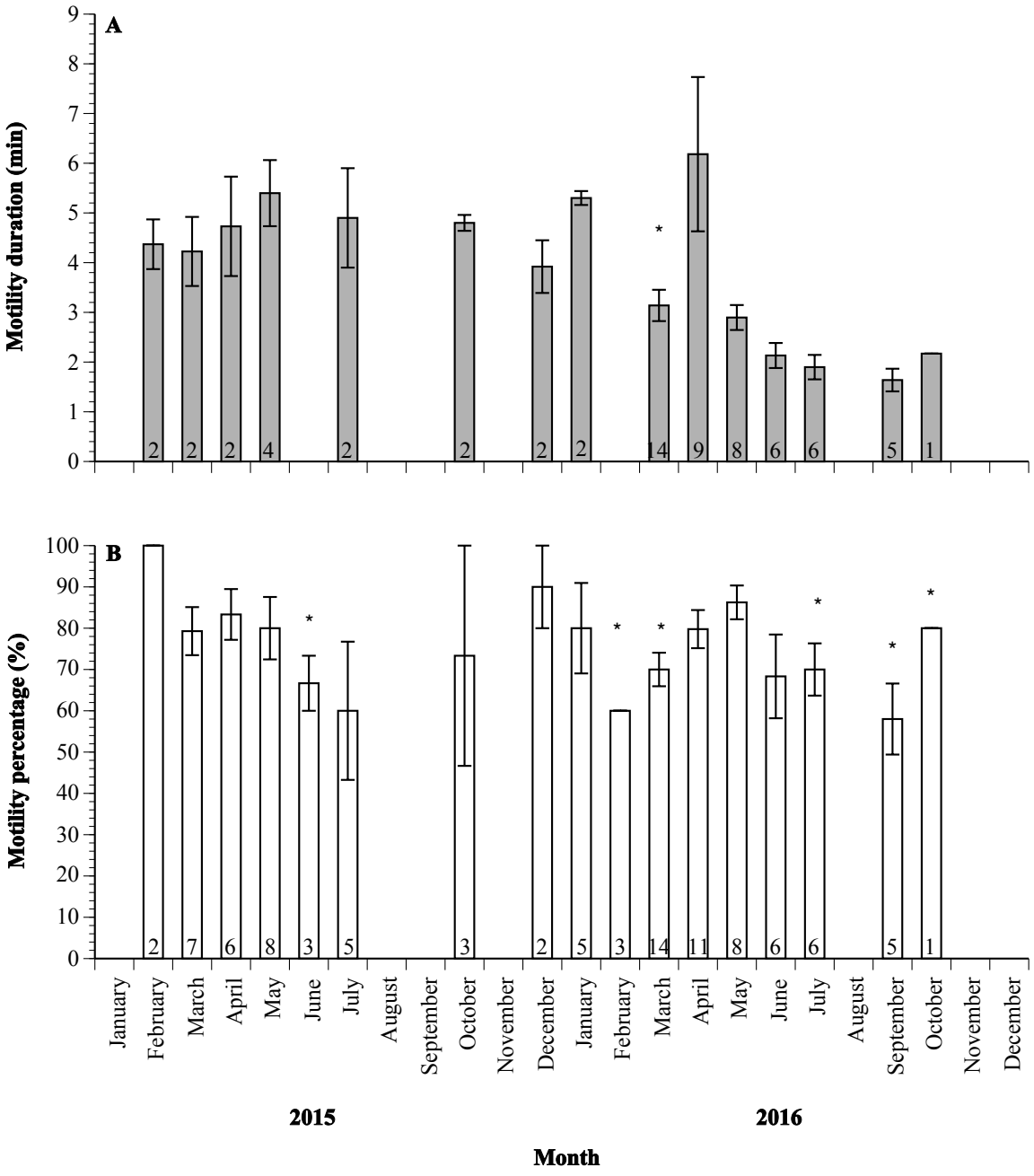

Figure 15. Mean (±SEM) sperm motility duration (A) and sperm motility percentage (B) of sperm collected by wreckfish of four different broostocks in Greece and Spain at different months of the year from February 2015 until October 2016. Asterisks indicate statistically significant differences from the maximums (April 2016 for motility duration and February 2015 for motility percentage). Different numbers inside the bars indicate the number of sperm samples used for each sperm quality parameter.

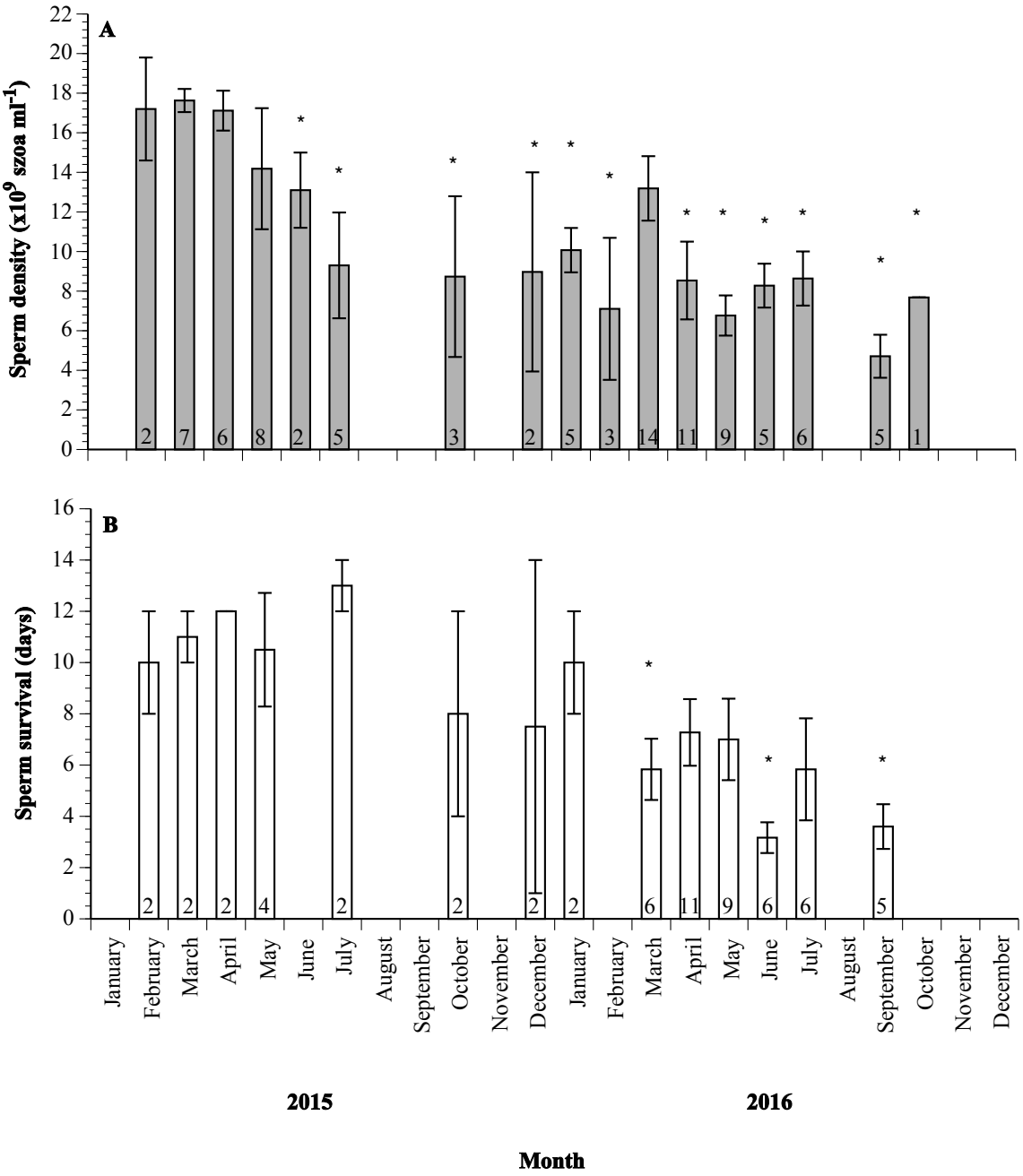

Figure 16. Mean (±SEM) sperm density (A) and survival at 4°C (B) of sperm collected by wreckfish of four different broostocks in Greece and Spain at different months of the year from February 2015 until October 2016. Asterisks indicate statistically significant differences from the maximums (March 2015 for sperm density and July 2015 for survival). Different numbers inside the bars indicate the number of sperm samples used for each sperm quality parameter.

Testosterone (**Fig. 17A**) and 11-KT (**Fig. 17C**) had low values at the S0 and reached their highest values at the S3 spermiation stage. The levels of both androgens correlated well with the levels of plasma LH (**Fig. 17D**). Nonetheless, the 17, 20β-P did not change significantly neither with fish spermiation index (**Fig. 17B**) nor in accordance with the LH levels (**Fig. 17D**).

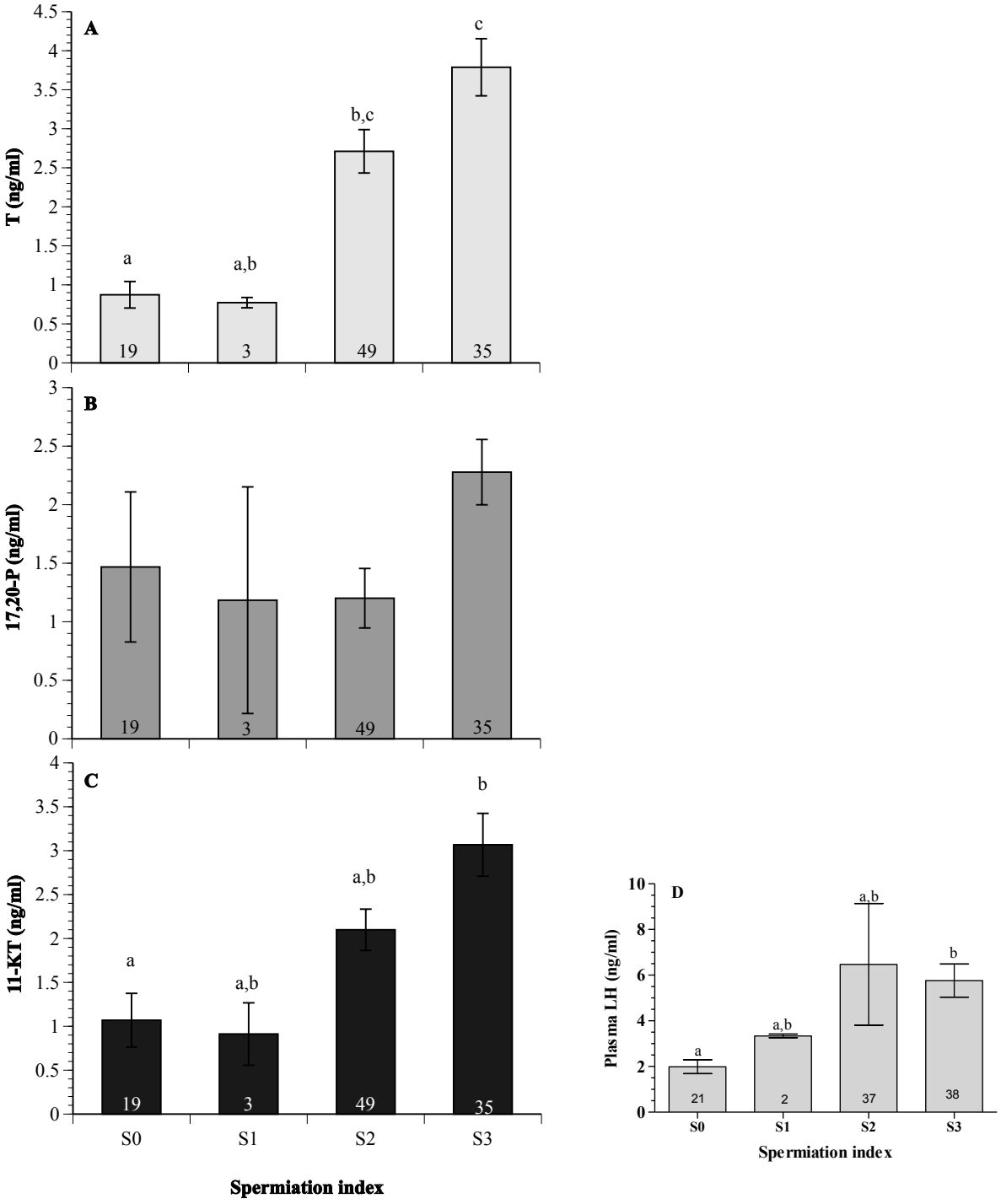

Figure 17. Mean (±SEM) plasma concentrations of testosterone (A), 17, 20β-dihydroxy-pregnenolone (B) and 11-ketotestosteone (C) and LH (D) of four different broodstocks of wreckfish in Greece and Spain at different spermiation index stages. Spermiation index was reported on a subjective scale, with S0 = 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. Different letter superscripts indicate statistically significant differences in T and 11-KT at different spermiation index stages, whereas numbers inside the bars indicate the number of male wreckfish found at each spermiation index stage.

Spontaneous spawns of captive wreckfish broodstocks were observed during 2015, 2016 and 2017 **(Fig. 18)**. In 2015 and 2016 spawning lasted from March until July, with most of the spawns coming from the IEO and the MC2 broodstocks. In 2017, spawning lasted from March until May and spontaneous spawns were also achieved at the CMRM broodstock, showing very high fertilization percentages (**Fig. 18C**). Relative fecundity and fertilization varied a lot, with fertilization percentage exhibiting values from 0% until 100% (**Fig. 18**). Mostly in 2015 and 2016, even when fecundity values were high, fertilization percentage was often zero or close to zero. Individual female wreckfish were observed to spawn every 3-4 days (not shown).

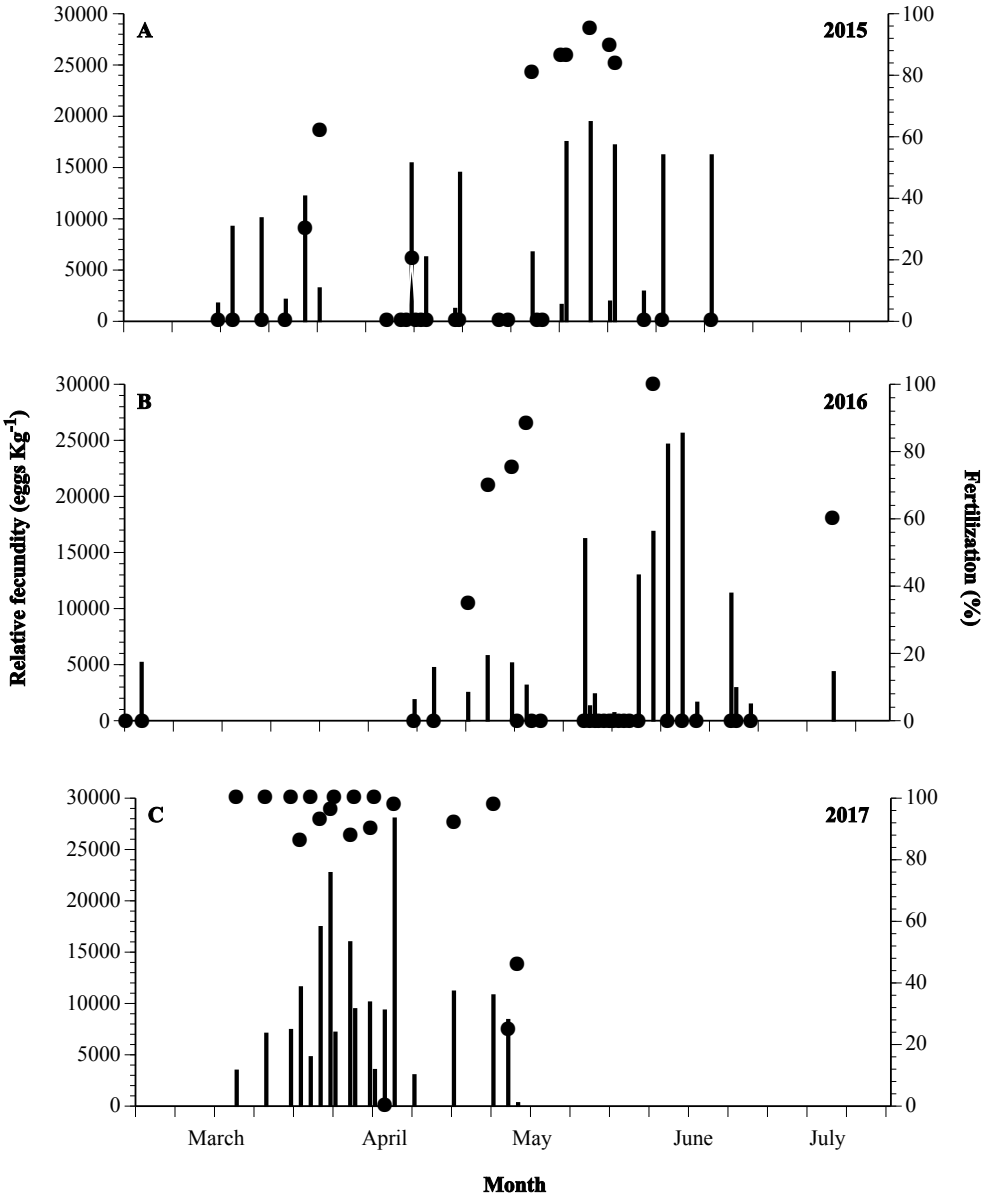

Figure 18. Mean relative fecundity and fertilization percentage (%) of the spawning events of the four different wreckfish broodstocks during 2015 (A), 2016 (B) and 2017 (C).

Table 2. Concentration of vitamin C and fat-soluble vitamins (mg/100 g dry matter) and total carotenoids (µg/g dry matter) in wreckfish wild female mature gonads (WFG) and captive-reared wreckfish eggs from different culture facilities and batches.

	Vit C	Vit D	Vit A	Vit E	Carotenoids
Gonads					
WFG (25-05-16)	80.81	0.43	3.31	4.46	12.30
WFG (02-03-17)	34.39	0.72	3.77	0.12	4.46
Eggs					
IEO 1179 (20-03-15)	6.33	2.11	Nd	3.65	24.00
IEO 7938 (17-04-17)	21.85	1.58	0.70	8.08	9.41
IGAFA 3FF2 (14-06-16)	12.11	2.81	Nd	1.89	8.13
IGAFA 6D01 (22-03-17)	2.64	2.02	Nd	1.28	3.18
A. FINISTERRAE 7B78 (16-07-15)	38.15	17.99	Nd	2.26	68.26
A. FINISTERRAE 5853 (12-07-16)	Nd	1.04	Nd	3.19	8.17

As shown in table 2, the gonad and egg carotenoid and vitamin contents greatly differed according to their origin and sampling period.

WFG (25-05-16) and WFG (02-03-17) are two of four samples of wild female mature gonads sampled on 2016 and 2017, respectively from Vigo market, as have been described and analyzed for proximate and fatty acid composition in D12.2. With 59.8±10.5% of protein and 20.7±4.8% of lipids (DW) and 7.07±1.4; 5.35±1.5; and 25.1±7.4, of ARA, EPA and DHA, respectively (see Table 6, D12.2). Although consistent values of Vitamin D, and A were measured in these two samples contents of vitamin E and also vitamin C and carotenoids were more variable. Average values of total carotenoids were of around 8mg per 100g of dry gonad.

Available samples of eggs corresponded to two batches from IEO stocks, two from IGAFA and two from AF. IEO 1179 (20-03-15) eggs were sampled on 2015 from a S1 female fed a semi-moist diet consisting of a mixture of white and oily fish, fish meal and mussels, whereas IEO 7938 (17-04-17) were sampled from an S2 female fed for at least one year a dry formulated feed containing increased quantities of squid meal and also fish meal, krill meal, macroalgae mix and tuna oil among marine origin ingredients (D12.2, Table 3). Reported relative fecundity expressed in number of eggs per kilogram of female was of around 35000 in 2016, over 100000 in 2017 and over 120000 in 2018, for this second IEO female (D12.2). Compared to the mature wild gonads, the eggs from the S1 IEO female displayed marked lower contents of Vit C, nule of Vit A and particularly high levels of carotenoids, whereas those from the S2 IEO presented more comparable values of most of the nutrients, except for an apparent deficiency of Vit A and an excess of Vit E.

IGAFA 3FF2 (14-06-16) eggs came from a squid fed female with 5000-10000 eggs released per Kg in 2016, not reported spawning on 2017 and 40000 of relative fecundity on 2018. IGAFA 6D01 (22-03-17) eggs were sampled on 2017 from a squid and hake fed female who's reported relative fecundity was of around 40000 eggs on both 2016 and 2017 (D12.2). The two IGAFA egg batches lack of Vit A with the squid fed sample (3FF2) better resembling the average carotenoid and vitamins measured values in wild mature gonads.

Finally, as shown in Table 2, another two batches of eggs from AF facilities were available for the analysis. These eggs were sampled on 2015 and 2016, but since the feeding control became more variable (fresh and frozen marine origin food) and very difficult to control, this broodstock was not used for the feeding experiments described in D12.2. Therefore, no data is available on the specific fecundity of these two females. One more time, Vit A was not detectable in these eggs, with extremely high contents of Vit D and carotenoids in the A. FINISTERRAE 7B78 (16-07-15) compared to the wild gonads, and huge differences and much lower values of most nutrients measured with respect to the other AF batch (A. FINISTERRAE 5853 (12-07-16)).

Discussion

The spawning period of the southern hemisphere wreckfish occurs in the austral winter, from July until October (Peres & Klippel, 2003), and coincides with the spawning period of the hapuku (Wakefield et al., 2010). In the present study, increased oocyte diameter of wreckfish oocytes was found between March and July, defining this to be its reproductive period, in accordance with former studies, where attempts were made for spawning induction in May (Papandroulakis et al., 2008; Peleteiro et al., 2011). Oocytes were small during the lipid vesicle and the cortical alveoli stage and increased in size at vitellogenesis, when they reached 1 mm in size (Martínez-Vázquez et al., 2016), whereas spawned eggs were around 2 mm in diameter and had from one to multiple oil globules, as has been also shown for the hapuku (Anderson et al., 2012). Big egg size is considered essential for demersal fishes, as it is related to higher individual survival in a relatively constant environment, in contrast to pelagic small eggs that have to face a changing environment, where survival is more difficult (Duarte & Alcaraz, 1989). For comparison, in pelagic fishes, mean egg diameter is 1.02 mm in the red porgy *Pagrus pagrus* (Mylonas et al., 2004a), 1.03 mm in greater amberjack (Mylonas et al., 2004b), 1.15 mm in European sea bass *Dicentrarchus labrax* (Cerdá et al., 1994) and 0.96 mm in the Senegalese sole (Dinis et al., 1999). On the contrary, in demersal flatfishes egg diameter ranges between 1.84-2 mm in the plaice *Pleuronectes platessa* (Kennedy et al., 2007) and 2.9-3.3 mm in the Atlantic halibut *Hipoglossus hipoglossus* (Brown et al., 2006).

However, although some of the females managed to proceed with oocyte development and reach maturation, 4 females from IEO and 2 females from CMRM did not progress beyond the cortical alveoli stage and their oocytes did not increase in diameter more than 350 μm. This fact occurred during both years of the study in the same females. Normally, the cortical alveoli stage defines the early developing ovary (Lowerre-Barbieri et al., 2011). However, in some fish species, ovaries can maintain the cortical alveoli oocyte developmental stage for up to 9 years before maturation and first spawning, such as the common and the spotted wolffish *Anarhichas minorin* and *Anarhichas lupus* (Gunnarsson et al., 2006; 2008). Other species may be able to have cortical alveoli oocytes in their ovaries when immature, such as the Greenland halibut *Reinhardtius hippoglossoides* (Rideout et al., 1999). The specific wreckfish females of the present study were all captured in 2009, so it cannot be certain whether they were able to mature yet, as wreckfish first maturation is achieved at 10 years for females and 9 years for males in nature (Peres & Klippel, 2003). However, of a total of 31 females, these are the only ones that ceased oocyte development at the cortical alveoli stage and remained at this stage for almost two years. Considering the above, it can be inferred that the specific females exhibited a kind of reproductive dysfunction that led to arrest of oocyte development at a very early stage of vitellogenesis. Another option is that the specific females skipped spawning by arresting oocyte development at the cortical alveoli stage, a phenomenon which has received increased attention in recent years and has been demonstrated in a number of species (Rideout et al., 2005; Skjæraasen et al., 2012). Skipped spawning in nature has been mainly attributed to poor nutrition during the spawning period and it can occur mostly in smaller females that do not migrate to the spawning grounds but stay at the feeding grounds for energy reservation (Skjæraasen et al., 2012; Zupa et al., 2009).

Reproductive dysfunctions at later stages of vitellogenesis due to dysfunctional release of LH from the pituitary at the end of vitellogenesis are common in a number of species (Mylonas et al., 2010), a phenomenon that was also observed in a large number of females of the present study. In particular, 20 fish in the present study reached vitellogenesis, but did not seem to proceed to maturation. This reproductive

dysfunction could be attributed to the environmental conditions of the tanks where the fish were kept. However, constant temperature of 16°C did not seem to have a significant effect on the reproductive development of fish, since fish held at this temperature exhibited the same reproductive performance as fish under naturally fluctuating temperature, e.g. they developed vitellogenic oocytes and managed to give some sporadic spawning events, suggesting that environmental parameters other than photoperiod and temperature may affect wreckfish reproductive performance. It is known that low temperature is preferred by the wreckfish, as loss of appetite and growth cessation were observed at temperatures >20°C (Papandroulakis et al., 2004); the same holds true for the hapuku, as it was shown that fish over 1 kg grow better at 18 than at 22°C (Tromp et al., 2016). Moreover, hapuku broodstocks in New Zealand are maintained at fluctuating temperatures from 10-19°C during the non-reproductive season and at 10 or 13.5°C during the reproductive season (Symonds et al., 2014).

Endocrine control of fish oocyte maturation has been described as follows: during vitellogenesis, T is produced in the theca cells and converted to E2 in the granulosa cells (Nagahama, 1994); E2 then promotes vitellogenin synthesis in the liver. After vitellogenesis, E2 drops and T increases during germinal vesicle migration; at that time, 17,20β-P or 17,20β, 21-trihydroxy-pregnenolone (20β-S) is produced as the maturation inducing steroid (MIS) in order to induce final oocyte maturation (Nagahama, 1994). The pattern of sex steroid changes in relation to the stage of oocyte development in the present study followed the above-mentioned biochemical cascade. In particular, T was low until vitellogenesis and increased as maturation proceeded but decreased at ovulation; at the same time, E2 showed a pattern of increase until vitellogenesis and decrease thereafter, although not statistically significant. On the other hand, 17, 20β-P seemed to maintain low and unchanged levels during oocyte development. Recent studies in different fish species support the involvement of T in oocyte maturation and the low levels of both T and E2 at ovulation. For example, T induced oocyte maturation, in terms of germinal vesicle breakdown, but not ovulation when administered in vivo to zebrafish *Danio rerio* oocytes (Tokumoto et al., 2011). Moreover, estrogen-synthesizing genes were down regulated in the preovulatory period of the rainbow trout *Oncorhynchus mykiss* (Bobe et al., 2006). On the other hand, 17,20β-P remained relatively stable at maturation both in Atlantic cod *Gadus morhua* (Kjesbu et al., 1996) and in greater amberjack (Zupa et al., 2017). Low and relatively stable levels of 17,20β-P may reflect rapid catabolism of the hormone or transformation to conjugated forms (Zupa et al., 2017) or may indicate that this steroid is not the actual MIS for the specific fish species, as has been suggested for the Atlantic cod and the yellowfin porgy *Acanthopagrus latus* (Jeng et al., 2012; Kjesbu et al., 1996).

Nutritional status among the female broodstock was particularly variable among the different centers and during 2015 and 2016, and seemed to mostly vary accordingly to the existing variations of the dietary regimes. As stated in D12.2 the reduction of the amount of lipids in diets for wreckfish broodstock with values of 12.5 and 8% in dry food 2 (S2 IEO stock) and hake/squid (IGAFA) respectively, seemed to have a beneficial effects producing a greater number of spawns. The good quality of squid meal was also documented previously. Zohar et al., (1995) reported that squid meal contain nutritional components which are essential for successful spawning in gilthead seabream. PUFA and in particular n-3 content in oocytes and eggs from females of IGAFA broodstock fed with hake/squid showed that the values were higher than in oocytes and eggs from females fed with the other diets as it was said above. This diet could be used as a wreckfish broodstock feed but it is recommended to reinforce with dry food 2 at certain times of the year for example in the maturation and spawning phases of the broodstock. Some ingredients as raw krill often included in diets for sparids has a distinct quality of having and enhancing effect on feed intake compared wih fishmeal (Izquierdo et al., 2001). The spawning quality enhancement effect of raw krill has shown that both polar and nonpolar lipid fractions contain important nutritional components for red seabream stock (Watanabe et al., 1991 a, b). In wreckfish broodstocks dry food 2 that was specifically formulated for this species, included among other ingredients a big amount of squid meal (34%) and Krill meal (7.5%) and this diet exerts a beneficial effect in terms of eggs viability, biochemical composition of oocytes and eggs, fecundity.

Ascorbic acid (vitamin C) has been shown to play an important role in fish reproduction, where the dietary requirement of broodstock is higher than that of juveniles. High ascorbic acid concentrations have been

associated with gonad and brain tissues in teleost fishes. Data relating to gamete production and quality, as well as a possible protective role for ascorbate against cumulative genetic defects during gametogenesis and congenital malformation during gestation (embryonic development) have been also reported (Dabrowski & Ciereszko, 2001; Valdebenito et al., 2013). Vitamin C deficiency also leads to a reduction in spermatozoa concentration and motility during and after the spawning period (Sandnes, 1991). Although data from Table 2 must be taken with caution until more replicates are available, there seems to be some consistency with the these important roles and the high contents of Vit C found in the analysed wild mature female gonads and also with a better egg quality of the IEO 7938 female, which were also richer in this vitamin.

The steroid hormone vitamin D is historically recognized for its relevance to bone health and calcium homeostasis. Recent years have witnessed a shift in focus to non-skeletal benefits of vitamin D; in this latter context, an accruing body of literature attests to a relevance of vitamin D to reproductive physiology, suggesting that vitamin D deficiency may be detrimental to reproductive biology. However, and although the exact mechanisms whereby vitamin D may participate in the regulation of reproductive physiology remain far from clear even for humans (Luk et al., 2012), compared to the mature wild gonads none of the released eggs seemed to be Vit C-deficient.

Vitamins E and A are critical to the normal development of fish embryos. Vitamin E functions primarily as an antioxidant to protect unsaturated lipids from oxidative degradation. Vitamin E deficiency affects reproductive performance, causing immature gonads and lower hatching rate and survival of offspring (Verakunpiriya et al., 1996; Valdebenito et al., 2013). However, according to the wild mature ovaries Vit E requirements of developing embryos seemed to be covered. Vitamin A or its provitamin carotenoid forms support growth, are required for the differentiation and maintenance of epithelial tissue and can be converted to forms of the vitamin that are potent morphogens. In this sense, only the Dry feed supply some Vit A, but well below the levels found in the mature female gonads. However, carotenoids were present in all the eggs batches. The hake/squid diet seemed to provide lower contents of carotenoids than those attained with the squid diet at IGAFA whereas mussels and other marine components in the dietary regime of semi-moist fed S1 fish from IEO or AF eggs sampled in 2015, seemed to provide an excess of these antioxidant pigments, well above the average values found in the mature wild female gonads. Carotenoids, including astaxanthin, are widely present in fish gonads and eggs. They are precursors of vitamine A being involved in reproduction and embryonic development, as well as in the prevention of oxidative stress processes and to ensure larval visual function and adequate chromatophore responses (Guerin et al., 2003; Miki, 1991).

Since fish cannot synthesise either of the vitamins or carotenoids, the maternal dietary content of each prior to oogenesis is an important determinant of reproductive fitness and egg and larval quality. For this reason, the differences of these essential chemical components between wild mature female gonads and those from eggs produced under different rearing conditions and feeding regimes was investigated and some basic data collected necessary for the future development of a quality pellet for this species.

Spermiating wreckfish males were present in the captive broodstocks studied all-year round, even at small numbers from September until December. This fact can be very useful for aquaculturists, as wreckfish sperm seems to be available most of the year for artificial insemination practices. Spermiating males all –year round have been also found in the Senegalese sole, a species able to reproduce at least at two different periods during the year (García-López et al., 2006a). Most spermiating males could be found from March until July, covering the reproductive period of females.

Sperm quality parameters maintained high levels throughout the year. For example, mean sperm density ranged between 4.5-11.5 spermatozoa x 10^9 ml^{-1}, sperm motility was always higher than 60%, motility duration ranged between 1.5 and 6 min and survival of sperm at 4°C ranged between 3 and 10 days. To our knowledge, this is the first study reporting on sperm quality of wreckfish and no study has been conducted on its congener, the hapuku. However, the mean values observed fall within the values already reported for other marine fish species, for example mean density has been found to be 8.6 - 23.7 spermatozoa x 10^9 ml^{-1} in the red porgy (Mylonas et al., 2003), 12-27.1 spermatozoa x 10^9 ml^{-1} in the sharpsnout seabream (Papadaki et al., 2008), 18.9-31.5 spermatozoa x 10^9 ml^{-1} in the meagre (Mylonas et al., 2013) and 38 spermatozoa x 10^9 ml^{-1} in the Atlantic bluefin tuna *Thunnus thynnus* (Suquet et al., 2010). Similarly, motility duration ranged

between 2-4 min in the red porgy (Mylonas et al., 2003), 2-6 min in the sharpsnout seabream (Papadaki et al., 2008), 0.78-1.27 in the meagre (Mylonas et al., 2013) and 10-11 min in the Atlantic bluefin tuna (Suquet et al., 2010). In this sense, sperm quality of wreckfish males can be considered good, with no noticeable dysfunction involved, neither in the quality nor in the quantity of sperm produced.

The good nutritional status of these male wreckfish broodstock specimens in terms of sperm quality is also mentioned in D12.2. Wreckfish males produced a high volume of sperm with a high percentage of motile cells which is also consistent with the very high DHA contents (almost 37% of total fatty acids). ARA was also high, particularly in the IEO stock fed an ARA enriched dry food, which better resembled the LC-PUFA profiles of wild mature male gonads (D12.2). DHA is known to positively improve sperm motility and the efficiency of membrane fusion events such as those taking place between spermatozoa and oocytes, or during synapses. Since sperm fatty acid composition depends upon the essential fatty acid content of broodstock diet (Asturiano et al., 1999; Labbé et al., 1993), fertilization could be also affected by lipid profiles. Studies by Asturiano et al. (2001) showed that male European sea bass *Dicentrarchus labrax* fed commercial HUFA-enriched diets exhibited more successful reproductive performance in terms of length of spermiation, total milt production, milt spermatozoa density, and fertilization than fish fed with a non-enriched diet. Baeza et al., (2015) also pointed out the usefulness of the development of enriched diets that may improve sperm quality and reproductive abilities of European eel males, thus improving fertilization success and embryo development.

In male fish, spermatogenesis is controlled by the two gonadotrophic hormones, follicle –stimulating hormone (FSH) and luteining hormone (LH). Follicle-stimulating hormone acts on the Leydig cells to stimulate androgen production, whereas LH acts on the Sertoli cells to support germ cell survival and development (Schulz & Miura, 2002). Androgens then act on the gonads; T has been associated with spermatogenesis and 11-KT with spermiation, with 11-KT being the most effective androgen in teleosts (Borg, 1994). In the present study, both T and 11-KT were found to reach their highest values at late stages of spermiation (S3 spermiation index), like in cod, where higher T and 11-KT levels were observed concomitantly with the presence of free spermatozoa in the testis lumen (Dahle et al., 2003), but unlike the Senegalese sole, which was found to exhibit the highest T and 11-KT values at late spermatogenesis and not at functional maturation (García-López et al., 2006b). On the other hand, 17,20β-P seemed to keep constant low values at all stages of spermiation. These low and unchanged levels of 17,20β-P can be attributed to the reasons discussed above for females. Another possible explanation for the unchanged values of 17,20β-P may be that this hormone is produced constantly by fish that are able to produce sperm all-year round, as happens also with the Senegalese sole, which shows relatively constant 17,20β-P values, as well (García-López et al., 2006b). Moreover, 20β-S has been suggested to be the MIS in most males in marine fishes, in contrast to freshwater species, for which 17,20β-P is the effective MIS (Schulz et al., 2010).

In conclusion, the results of the present study show that wreckfish females can adapt to captivity, mature and produce eggs both under fluctuating natural and under constant low temperatures, as well as under different dietary regimes. Plasma sex steroid hormones in females correlate well with the maturity stages of females, except for 17,20β-P. Some females, however, cease oocyte development at the cortical alveoli stage and their oocytes don't seem to grow bigger than 350 μm, or 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 rise when fish are fully spermiating, suggesting that except for 17,20β-P, they correlate well with the maturity stages of males. One possible 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. Further studies should look into the lack of maturation in the females and conduct experiments on the environmental conditions that the fish are held, in order to hopefully increase the number of females that can mature and spawn. Moreover, hormonal induction of oocyte maturation and spawning, for a better control of spawning and egg production, as well as in vitro fertilization trials, could overcome the breeding behavioral problem of males and increase the current observed fertilization percentage.

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Deviations from the DOW

It was not possible to measure Follicle Stimulating Hormone (FSH) in the blood, as the available heterologous assay (striped bass *Morone saxatilis* ELISA) could not be validated to be used to measure this hormone, probably in combination with the fact that plasma levels were low in the blood of wreckfish.

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