New species for EU aquaculture

Deliverable Report

Deliverable No:	D7.7	**Delivery Month:**	60
Deliverable Title	Development of a breeding protocol for captive reared grey mullet broodstock based on optimized hormonal treatment, group structure and photo-thermal regime		
WP No:	7	**WP Lead beneficiary:**	P4. IOLR
WP Title:	Reproduction and Genetics – grey mullet		
Task No:	7.3	**Task Lead beneficiary:**	P4. IOLR
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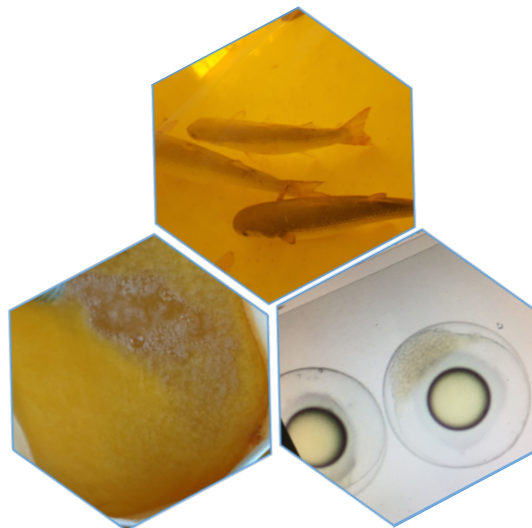
OBJECTIVES

The current study aimed to develop a comprehensive and effective breeding management for captive reared grey mullet broodstocks. For that purpose, following the optimization of hormone based treatments for induced gametogenesis and spawning (D7.3) additional parameters that could affect spawning success and reproductive outputs were addressed, including: (i) extension of spawning season through the use of manipulated photo-thermal regime, (ii) group structure, (iii) broodstock origin and (iv) broodstock diet.

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INTRODUCTION

The grey mullet (*Mugil cephalus*) is part of an ubiquitous teleost family occurring in most temperate, sub-tropical and tropical coastal waters in both hemispheres (Crosetti and Blabber, 2016). These species occupy a wide variety of marine, estuarine and freshwater environments but their spawning occurs in the sea (Thomson, 1955; Ibáñez and Gutiérrez-Benítez, 2004). They are gonochoristic fish (González-Castro and Minos, 2016), although they can sometime exhibit non-functional hermaphroditism (McDonough et al., 2005). Grey mullets show a group-synchronous ovarian development (Bartulović et al., 2011) and spawning in the Mediterranean occurs in late summer-early autumn (Assem et al., 2008; Bartulović et al., 2011). Mullet, in particular *Mugil cephalus*, which are fished and farmed around the world, have many favorable culture characteristics. They are herbivorous, euryhaline, fast growing, disease resistant and a popular eating fish. Nonetheless, the culture of grey mullet is mostly dependent on the availability of wild fry as breeding in captive condition is not standardized (Kumar et al., 2015). Among the major bottlenecks for the incorporation of a new species in the aquaculture industry, reproductive dysfunctions affect frequently fish in captivity, hindering the production of viable eggs. Reproductive dysfunctions commonly involve an inadequate pituitary GtHs synthesis and/or release (Zohar and Mylonas, 2001; Mylonas et al., 2010), which has been attributed to captivity-induced stress, lack of suitable environmental conditions (Mylonas et al., 2010) and/or nutritional deficiencies (Izquierdo et al., 2001).

When reared in captivity, grey mullets display severe reproductive dysfunctions (Yashouv, 1969; De Monbrison et al., 1997): spermiating males are rarely observed and, in most cases, the produced milt is highly viscous and fails to fertilize the eggs; females are unable to finalize vitellogenesis or fail to undergo oocyte maturation once vitellogenesis is completed (De Monbrison et al., 1997). The observed failure in the attainment of vitellogenesis not only prevents ovulation and spawning. Previous studies suggested that dopaminergic inhibition is a major barrier along the reproductive axis that arrests spontaneous spawning in captivity (Aizen et al., 2005). Consequently, a practical technique using dopamine antagonists and gonadotroping releasing hormone agonists (GnRHa) was developed. Nonetheless, the latter hormone-based treatment was found to enhance vitellogenesis in females, but fell short in stimulating spermatogenesis in males. To augment testicular development, grey mullet males were treated early on in the reproductive season with methyl testosterone (MT) administered *via* Ethylene-Vinyl acetate (EVAc) slow-release implants (Aizen et al., 2005). Despite the reported success, in most cases the treated males produced a very small volume of semen, which was highly viscous and failed to fertilize the eggs.

In mammals, luteinizing hormone (LH) regulates Leydig cell sex steroid production, while follicle stimulating hormone (FSH) regulates Sertoli cell activities, including structural, nutritional, and regulatory (paracrine) support of germ cell development (Huhtaniemi and Themmen, 2005). In fish, the relative roles of FSH and LH have not been fully described. In various species, plasma LH levels are very low or undetectable during the onset of testis development, become detectable when germ cells enter meiosis, and peak close to the spawning season.

Conversely, several lines of evidence indicate that FSH-mediated steroidogenesis stimulates early stages of spermatogenesis. Plasma FSH levels, show a transient increase in association with spermatogonial proliferation, then re-increase with spermiation, and decrease before the spawning season starts. Campbell et al. (2003) reported that elevated circulating levels of androgens and FSH coincided in male chinook salmon (*Oncorhynchus tshawytscha*) with active spermatogonial proliferation and Ohta et al. (2007) demonstrated that suppressing the steroidogenic activity of FSH abolished the stimulatory effect of FSH on spermatogonial proliferation in Japanese eel (*Anguilla japonica*).

Reproduction is a very complex process that can be affected by several factors such as genetic background, environmental conditions including nutrition. In this task, therefore, we found it crucial to further improve the reproductive potential of captive grey mullet broodstocks fed experimental diets and to identify possible reproductive/metabolic dysfunctions through the characterization of essential nutrients including essential fatty acids (EFA), lipid and carotenoid profiles of captive broodstock gonads and eggs reared under different dietary regimes.

Comparisons of proximate and lipid classes and fatty acid compositions from tissues of wild fish and their cultivated counterparts can provide a good estimation of the suitability of the diet offered to the broodstock (Cejas et al., 2004; Rodríguez-Barreto et al., 2012, 2014; Zupa et al., 2017). The efficacy of dietary ingredients for broodstock has been ascribed to its superior protein quality, as well as its higher phospholipid and cholesterol content. In fact, two thirds of the lipid fraction in vitellogenin (Vg) is made of phospahatidylcholine (PC) that is also the main phospholipid in mature ovaries and fertilised eggs (Watanabe & Vasallo-Agius, 2003). Similarly, sperm is particularly rich in phosphatidylserine (PS) and phosphatidylethanolamine (PE). Regarding nutrition, dietary essential fatty acids (EFA) such as ARA, EPA and DHA have also proven to be particularly important in the reproduction of several species, since they determine gonad composition and function, affecting not only sperm and egg quality (Izquierdo et al., 2001; Tocher, 2010), but also being involved in the synthesis of eicosanoids, autocrine mediators in the reproductive process (Mercure & Van Der Kraak, 1996; Sorbera et al., 2001; Patiño et al., 2003; Stacey & Sorensen, 2005; Henrotte et al., 2011). The formulation of a broodstock diet which approximates the ovary lipid composition of cultured fish to that from wild specimens, has demonstrated some improvement of reproduction success under captive conditions of greater amberjack (Rodríguez-Barreto et al., 2012, 2014) and many other species, and it is also intended for grey mullet females and males in the present research.

In view of the above, to further improve spawning success and reproductive outputs, the basic hormonal treatments (D7.3) have been used to test (i) the feasibility of extending the natural spawning period by the implantation of manipulated photo-thermal regime, (ii) the best performing brooder unit giving rise to maximal spawning success, and (iii) dietary effects on captive grey mullet reproductive performance and productivity. In addition, the latter hormonal treatments were implemented at both the IOLR and IRTA facilities, which enabled to compare treatments' effectiveness at different locations and on different broodstock origins, i.e., hatchery produced G1 vs. wild- caught captive-acclimated.

MATERIALS AND METHODS

Broodstock source and conditioning

IOLR broodstocks. Grey mullet breeders, consisted of 4-6 year old hatchery-produced (G1; first generation) fish that were individually tagged and maintained in 4-m^3 tanks supplied with ambient seawater at 40-ppt salinity (Gulf of Eilat, Red Sea) and subjected to either natural fluctuations of light and temperature conditions (elevation to 25°C in June, 28°C in August). In parallel, 4-year old G1 grey mullet females (n=18; av. BW= 1868 ± 74 g) and males (n=18; av. BW= 1154 ± 46 g) were acclimatized to a 4-month shifted photo-thermal regime. The experimental fish were fed daily at the rate of 1-1.5% of their body weight using a 30% crude protein and 4% lipid commercial feed (Raanan, Israel). Sex was predicted according to vitellogenin dotblot immunoassay as described in Aizen et al. (2005), and then validated during gametogenesis when gonadal biopsies were performed.

IRTA broodstocks. Grey mullet breeders were randomly assigned to the experiment from the two groups, wild caught from the Ebro Delta and from extensive culture in Seville (Spain), which had been acclimated and reared in IRTA Sant Carles de la Ràpita, for a period of over a year and 3 months, respectively. Three months or more before the experiment started, all fish were PIT tagged for identification and sexed. Fish were sexed depending on the presence of oocytes in the gonads obtained by cannulation. If an ovarian biopsy was obtained the fish were classified as female and fish that could not be biopsied were considered males. Fish were maintained in a 17 m^3 tank with seawater at 34-ppt salinity in recirculation (IRTAMAR®). Conditions before the experiment were natural temperature and photoperiod. During the experiment, temperature was controlled and maintained at 24ºC, whilst photoperiod and light conditions were natural. Breeders were fed daily at the rate of 1.5 % body weight with a broodstock commercial feed (Sole broodstock diet SPAROS, Portugal) and two days a week with frozen mussels (Sariego Intermares, Spain) and polychaetes (TOPSY Bait, Netherlands).

Hormonal acceleration of gonadal development

IOLR. To accelerate gonadal development mullet females and males were injected at the onset of gametogenesis (natural season: mid-July; shifted season: mid-October) with metoclopramide (15mg/KgBW) combined with r-FSH (5 µg per kg BW). One month later the males received MT-EVAc implant (5 mg/kgBW). Gonadal biopsies were timed with the advanced stages of gametogenesis (natural season: September-October; shifted season: December-January). The relative abundance of fully mature females, and spermiating males were recorded. Females were considered fully mature once their oocytes reached an average diameter greater than 550 µm and more than 50% of sampled oocytes exhibit germinal vesicle migration. Sperm quality was classified into one of four categories based on its quantity, fluidity and ability to spread in the water.

IRTA. Nine grey mullet females and six males with a mean weight of $1,116.69 \pm 395.78$ g and 640.87 ± 293.90 g, respectively, were hormonally treated. The control group was formed with five females and two males with a mean weight of 909.76 ± 392.20 g and 532.60 ± 2.47 g, respectively. All fish were feeding well and in good condition with a condition factor that ranged between 1.18 and 1.86, with a mean of 1.6.

On the 24th July 2018, both males and females in the treated group received intramuscular injections of rFSH 5 µg/kg (provided by P4. IOLR), and 15 mg/Kg Metoclopramide (Metoc) (Sigma, Spain). On the same date, control fish were injected with a similar volume (0.5 mL) of saline solution. The fish were anaesthetized with 80 mg/L of MS222 for the application of treatments. No other sampling, maturity status or blood, was made on the fish. However, fish from the same stock that were not used in this experiment were sampled for maturity and blood. Two weeks (7th August) following the first injection to males and females, males received 17alpha-methyltestosterone (MT) loaded on EVAc slow-release implants at 5 mg/kg BW (provided by P1. HCMR). Males under 1 kg received one implant of 5 mg MT and males over 1 kg weight received two implants. Actual MT implant doses were higher than planned and ranged from 6.7 to 11.6 mg/kg. Males in the control group were injected with 0.5 mL of saline solution and a large bore syringe as used for the implants. At the beginning of September (4th), males received a second implant of MT following the same procedure. On the 1st October, the maturity status of all males and females were revised and determined. Ovarian biopsies were taken and the diameter of the 20 largest and most advanced oocytes were recorded. Maturation of the males was examined by the release of sperm upon application of gentle abdominal pressure and spermiation stage was determined on a scale from 0 to 3 (0 = not fluent, 1 = fluent but little sample can be obtained, 2 = fluent, 3 = very fluent). Total volume of sperm produced and sperm density was recorded. Percentage of sperm motility and duration of motility was checked in situ (a drop of sperm activated with 20 µL of clean seawater) with the fresh samples using light microscope (x10) and an hour later through videos recorded and analyzed with Computer Assisted Sperm Analysis (CASA). Statistical comparisons were made between rFSH treated and control fish with a Student T-test.

Spawning induction trials

Spawning induction trial were carried out during 2016 and 2017. Once identified, a reproductively mature female was stocked with either two or three spermiating males (unless specified otherwise) in a 1-m³ tank supplied with seawater at 24-27°C. The selected fish were treated with GnRHa combined with Metoc. Each treatment consisted of priming (GnRHa 10 µg/kg; Metoc 15mg/kg) and resolving injections (GnRHa 20 µg/kg; Metoc 15 mg/kg) given 22.5 h apart.
During the natural spawning season (2016) three breeding units varying in ratios of female to male and tank size, were tested. These include: **A)** 1 female and 3 males, in 1 m³ tank, **B)** 2 females and 3 males, in 1 m³ tank and **C)** 3 females and 6 males, in 3 m³ tank. The rates of spawns per induction trails, attaining fertilized eggs per spawning event and hatching success were recorded and summarized in **Table 4.**

Broodstock diet experiment

Based on the results of proximal and fatty acid (FA) composition in gonads from wild and domesticated mullets (see D13.3), the present study was aimed to improve the broodstock diet for grey mullet, through the: (i) replacement of soy oil (SO) with fish oil (FO) for increased supplementation of the n-3 LCPUFA, and (ii) addition Marigold petal meal (MgM; 3 mg kg^{-1} feed) as another carotenoid source, apart from that provided by the 3% dry Ulva to the diet. This meant that the total carotenoid level in the FO+MgM diet was *ca.*138 mg kg^{-1} while the SO diet was *ca.* 99 mg kg^{-1}. **Tables 1 and 2** summarizes the respective proximate composition, lipid classes, and fatty acids profiles in the FO+MgM and SO diets.

During the onset of the reproductive season (early July 2017), 6-year old captive grey mullet broodstocks were divided into two groups, that were fed with either a mullet grow out diet containing soybean oil (SO) that was previously developed by P4. IOLR, or with diet containing fish oil (FO+MgM), which is relatively rich in n-3 LCPUFA and carotenoids. The experiment was conducted in triplicates. Fish were maintained in 4 m^3 tanks supplied with ambient (Gulf of Eilat, Red Sea) seawater at 40 ‰ salinity and subjected to natural fluctuations of light and temperature. Food was provided at the rate of 1-1.5% of their body weight. Fish were conditioned for spawning using protocols developed by P4. IOLR (as above) and the spawning data obtained from the two broodstocks is summarized in **Table10**.

In parallel, from each dietary group samples of ovaries, fertilized and unfertilized eggs were subjected to proximate composition, lipid classes, fatty acids and carotenoid analyses. Of note, the ovarian samples were taken from fully vitellogenic females that accidently died during the experiment (FO+MgM, n=1; SO, n=2).

Gonad proximate composition, lipid classes and fatty acid profiles

To evaluate gonad biochemical composition, samples of ovaries were removed and kept on dry ice until they were transported to the laboratory, where they were immediately stored at -80ºC until analysis. Dry matter and protein contents were calculated using the methods of analysis of the Association of Official Analytical Chemists (AOAC; 2012). Moisture content was determined in 500 mg samples by thermal drying in an oven at 110ºC, until constant weight. Protein was determined by sample digestion according to the Kjeldahl method. Total lipid (TL) was extracted by sample homogenization in chloroform/methanol (2:1, v/v) according to the method of Folch et al. (1957). The organic solvent was evaporated under a stream of nitrogen and the lipid content was determined gravimetrically and stored in chloroform/methanol (2:1), containing 0.01% butylated hydroxytoluene (BHT). Analysis of lipid class (LC) composition was performed by one-dimensional double development high performance thin layer chromatography (HPTLC; Merk, Darmstadt, Germany), and methyl acetate/isopropanol/chloroform/ methanol/0.25% (w/v) KCl (5: 5: 5: 2: 1.8, by volume) used as developing solvent system for the polar lipid classes and isohexane/diethyl ether/acetic acid (22.5: 2.5: 0.25, by volume), for the neutral lipid separation. Lipid classes were visualized by charring at 160ºC for 15 min after spraying with 3% (w/v) aqueous cupric acetate containing 8% (v/v) phosphoric acid, and quantified by scanning densitometry using a dual-wavelength flying spot scanner Shimadzu CS-9001PC (Shimadzu, Duisburg, Germany) (Olsen and Henderson, 1989). To determine the fatty acid profiles, TL extracts were subjected to acid-catalysed transmethylation with 1% sulphuric acid (v/v) in methanol. The resultant fatty acid methyl esters (FAME) and dimethyl acetals (DMA) were extracted using isohexane: diethylether (1:1 by volume) and purified by TLC using isohexane/ diethyl ether/acetic acid (90:10:1, by volume) as developing system (Christie, 1982). Fatty acid methyl esters were separated and quantified using a TRACE-GC Ultra gas chromatograph (Thermo Electron Corp., Waltham, MA, USA) equipped with an on-column injector, a flame ionization detector and a fused silica capillary column, Supelcowax TM 10 (30 m x 0.32 mm I. D. x 0.25 μm; Sigma-Aldrich, Madrid, Spain). Helium was used as carrier gas and temperature programming was 50±150ºC at 40ºC min-1 slope, then from 150 to 200ºC at 2ºC min-1, to 214ºC at 1ºC min-1 and, finally, to 230ºC at 40ºC min-1. Individual FAME and DMA were identified by reference to authentic standards, and further confirmation of FAMEs and DMAs identity was carried out by GC-MS (DSQ II; Thermo Electron Corp.).

Carotenoids were obtained from gonads at advanced gametogenesis and from the spawned eggs, according to the method of Barua et al. (1993), using ethyl acetate/ethanol (10mL, 1:1 v/v), ethyl acetate (5 ml) and

hexane (10 ml) as extracting solvents. Afterwards, carotenoids contents were quantified by spectrophotometry at 470 nm.

Table 1. Proximate composition and main fatty acid profile (% total fatty acids) of experimental diets

		FO+MgM	SO
Moisture (%)		9.76 ± 0.03	9.96 ± 0.22
Lipid (% dry matter)		15.29 ± 1.09	14.08 ± 0.15
Protein (% dry matter)		32.75 ± 2.01	32.70 ± 0.52
Ash (% dry matter)		7.79 ± 0.04	6.76 ± 0.03
Fatty acids			
	14:00	1.89 ± 0.01	1.06 ± 0.01
	16:00	11.79 ± 0.06	11.54 ± 0.18
	18:00	3.00 ± 0.00	3.34 ± 0.09
Total SFA		**17.82 ± 0.01**	**17.23 ± 0.24**
	16:11	3.54 ± 0.02	2.14 ± 0.13
	18:12	39.46 ± 0.41	34.05 ± 0.32
	20:12	3.22 ± 0.02	1.82 ± 0.29
	22:13	2.20 ± 0.05	1.18 ± 0.05
Total MUFA		**48.68 ± 0.36**	**39.46 ± 0.54**
18:2n-6		17.44 ± 0.20	30.98 ± 0.05
20:2n-6		0.99 ± 0.01	0.58 ± 0.19
20:4n-6		0.46 ± 0.04	0.24 ± 0.13
Total n-6 PUFA		**19.32 ± 0.32**	**31.84 ± 0.64**
18:3n-3		4.42 ± 0.01	5.02 ± 0.18
18:4n-3		0.72 ± 0.03	0.54 ± 0.13
20:5n-3		2.02 ± 0.12	0.99 ± 0.00
22:5n-3		1.15 ± 0.06	0.70 ± 0.07
22:6n-3		3.50 ± 0.10	2.11 ± 0.17
Total n-3 PUFA		**13.09 ± 0.48**	**9.97 ± 0.35**
Ratios			
DHA/EPA		1.74 ± 0.05	2.13 ± 0.17
ARA/EPA		0.23 ± 0.01	0.19 ± 0.03
n-3/n-6		0.68 ± 0.04	0.31 ± 0.02

Table 2. Lipid class composition (% total lipid) of experimental diets

	FO+MgM	SO
Sphingomyelin	0.4	0.2
Phosphatidylcholine	0.87	0.74
Phosphatidylserine + Phosphatidylinositol	0.21	0.17
Phosphatidylethanolamine	0.06	0.09
Phosphatidylglycerol	0.28	0.36
Unknown	0	0
Total Polar Lipids	**1.82**	**1.57**
Diacylglycerols	8.72	10.11
Cholesterol	6.13	6.06
Free fatty acids	39.5	51.44
Triacylglycerols	34.45	25.62
Wax + Sterol esters	9.38	5.21
Total Neutral Lipids	**98.18**	**98.43**

RESULTS

Captive grey mullet reproductive performance during a shifted spawning season (IOLR)

Four year old grey mullet females (n=18; av. BW= 1868 ± 74 g) and males (n=18; av. BW= 1154 ± 46 g) were acclimatized to 4-month shifted photo-thermal regime. Fish were conditioned for spawning and their reproductive performance was monitored as described above. Results demonstrate the fish responsiveness to the shifted reproductive season. During December 2016 through January 2017, females (45%) gradually reached the post-vitellogenic stage with oocyte diameter exceeding 500 μm (**Fig. 1**), whereas males (40-50%) were steadily spermiating (**Fig. 2**). Following hormonal treatment spawning has occurred in 3 out of 4 trials (**Table 3**). Fecundity in the shifted (2.22 ± 0.19 million eggs / kg BW) and the natural (2.5 ± 0.1 million eggs / kg BW) spawning season are comparable.
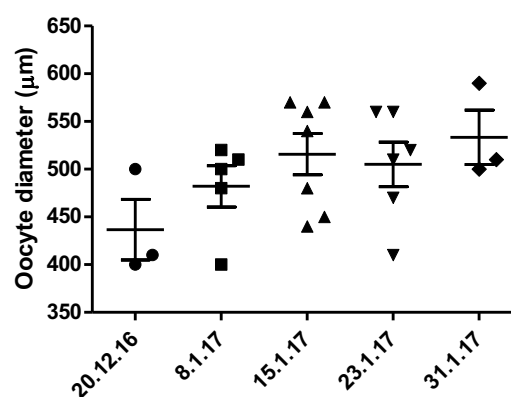
Figure 1. Oocyte growth in hormonally-treated grey mullet females subjected to shifted photo-thermal regime.
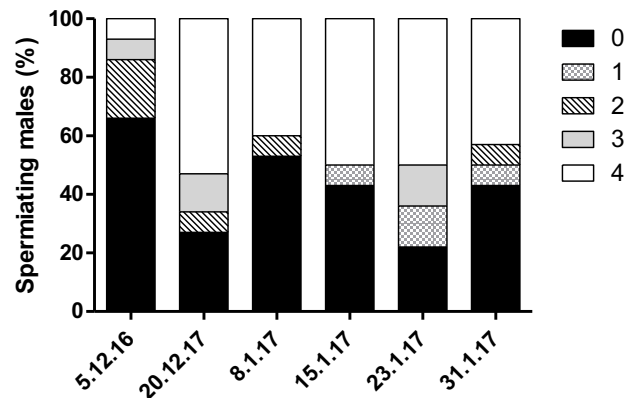
Figure 2. The abundance of spermiating males during a shifted spawning season (December 2016-January 2017). 0- no milt, 1- traces of viscous milt, 2-relatively small amounts of white milt, 3- fluid milt 4- flowing fluid milt, easily spread in the water.

Table 3. Summary of the spawning data obtained from hormonally induced females during a shifted spawning season (December 2016-January 2017).

Date	Female BW (g)	Floating eggs (ml)	Sinking eggs (ml)	Fecundity (10^6 eggs/Kg BW)
20.12.2016	1660	272	710	2.1296
15.1.2017	2310	1070	180	1.9481
15.1.2017	2470			
23.1.2017	1930	1310	70	2.5741

Breeding unit effects on spawning success (IOLR)

In previous spawning induction trials (see D7.3) fertilization rates were inconsistent and ranged between 0 to 98%, pointing to impaired breeding behavior, either in terms of courtship or spawning synchronization between the sexes. Moreover, on some occasions hormonally-conditioned females went through ovulation but failed to release the eggs and ultimately have died (**Fig. 3**).

Therefore, during the natural spawning season (2016) three breeding units varying in ratios of female to male and tank size, were tested and the rates of spawns per induction trails, attaining fertilized eggs per spawning event and hatching success were recorded and summarized in **Table 2.** While the bigger breeding unit, consisting of 3 females and 6 males in 3 m³ tank, was found to be successful in terms of spawning events (100% of the induction trials), the fertilization rate was relatively low (50%). In contrast, the small breeding unit, consisting of a single female and 3 males in 1 m³ (**Fig. 4**), demonstrated reduced spawning events (50% of the induction trials) yet, with higher rates of fertilization and hatching successes (**Table 4**).
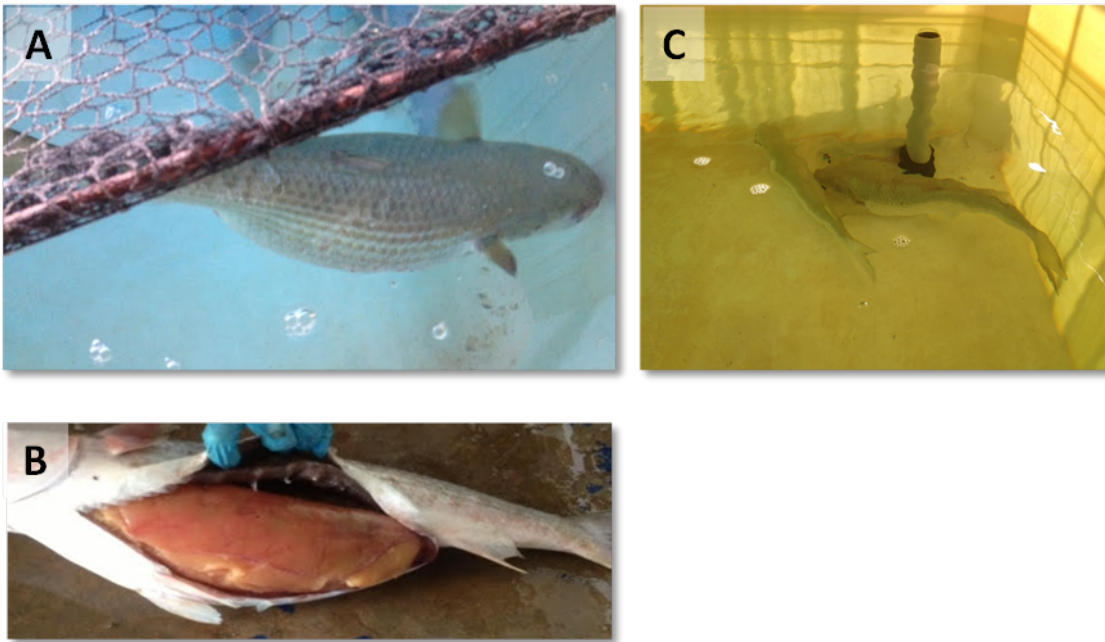
Figure 3. Spawning of captive grey mullet. (A) Hormonally- stimulated grey mullet female exhibiting swollen belly prior to egg release. (B) Hormonally-stimulated female that failed to release the eggs and died. (C) Grey mullet female and male (big and small specimens, respectively) courtship prior to spawning.

Table 4. Variable breeding units of captive grey mullet and their outputs in terms of spawning, fertilization and hatching success

Breeding unit	A (n=10)	B (n=5)	C (n=2)
Spawning rate (%)	50	80	100
Fertilization rate (%)	80	75	75
Hatching success (%)	92.6 ± 3.5	72.9 ± 11.6	73.4 ± 7.3

A= 1 female X 3 males, 1 m^3 tank; **B**= 2 females X 3 males, 1 m^3 tank; **C**= 3 females X 6 males, 3 m^3 tank

Figure 4. (A) Grey mullet experimental tanks consisting of 4 m^3 and 1 m^3 tanks for broodstock holding and spawning induction trials, respectively. (B) Brooder unit consisting of a single female and 3 males in a 1 m^3 tank, and (C) An egg collector.

Hormonal treatment to enhance gametogenesis in wild-caught grey mullet (IRTA)

Administration of rFSH produced in *Pichia* and METOC did not led to the development of vitellogenesis of wild grey mullet females acclimated to captivity. On the 1st October when maturity was assessed, both rFSH treated and control females, had respective mean oocyte diameters of 182.86 ± 19.27 µm and 92.60 ± 5.46 µm, which represented oocytes in pre-vitellogenesis ovarian stages.

Sperm was produced by four out of the six males (66.6%) that were hormonally treated with rFSH and METOC. Two of the males had a spermiation stage of 1 (a viscous drop of sperm - 10-20 µL) and two had stage 2, with fluid sperm and 100 and 200 µL were collected. Mean spermatozoa density in sperm samples from these two males was $1.76 \cdot 10^{10}$ spzoa/mL (n=2, stage 2 spermiation males). The mean values of percentage of motility and duration of motility obtained in situ were 83.3 ± 7.69 % and 102 ± 24 sec (n=4). Once analysed with CASA, the values obtained were 68.78 ± 6.79 % (n=3) for sperm motility and 63.20 ± 1.99 sec (n=3) for motility duration. The two hormonally treated males that did not produce sperm were examined by inserting a cannula into the gonopore. No oocytes were obtained, however it cannot be discounted that the two fish were actually females. No sperm or oocytes were obtained from the males in the control group.

Broodstock diet (IOLR)

As expected, the FO+MgM diet exhibited higher triacylglycerols (TAG) and wax and sterol esters (WE-SE) levels and 2-folf higher n-3 to n-6 PUFA ratio than in the SO diet (**Tables 1** and **2**). Similar analyses of proximate composition, lipid classes and fatty acid profiles of the 6-year old G1 grey mullet gonads and eggs indicated a predominance of neutral lipids, mainly TAG and WE-SE, over the polar lipids, comprising around 70% of the total lipids in ovaries and eggs (**Tables 5** and **7**, respectively).

Table 5. Lipid content (% dry matter) and lipid class composition (% total lipid) of **gonads from 6-year old mullet females** fed a fish oil based diet (FO+MgM) or a soy oil based diet (SO)

	FO+MgM (B8F4)	SO (9A85 & 9F67)
Total lipid	31.44	32.87 ± 0.67
Lipid class		
Sphingomyelin	0.91	0.85 ± 0.21
Phosphatidylcholine	10.39	10.98 ± 0.45
Phosphatidylserine + Phosphatidylinositol	1.29	1.50 ± 0.04
Phosphatidylethanolamine	2.95	2.10 ± 1.21
Total Polar Lipids	**15.53**	**15.43 ± 1.41**
Diacylglycerols	4.68	4.48 ± 0.25
Cholesterol	9.00	9.20 ± 0.23
Free fatty acids	1.91	1.66 ± 0.01
Triacylglycerols	13.58	10.53 ± 0.48
Wax + Sterol esters	54.19	57.24 ± 1.91
Unknown	1.11	1.46 ± 0.04
Total Neutral Lipids	**84.47**	**84.57 ± 1.41**

Data are means ± SD (n=2) for SO.

Table 6. Fatty acid content (mg g^{-1}) and main fatty acid composition (% total fatty acids) of **gonads from 6-year old mullet females** fed a fish oil based diet (FO+MgM) or a soy oil based diet (SO)

	FO+MgM (B8F4)	SO (9A85 & 9F67)
Total FA	**82.27**	**85.80 ± 0.42**
14:0	0.38	0.35 ± 0.01
16:0	8.35	8.96 ± 0.19
18:0	2.71	2.94 ± 0.02
Total SFA	**11.98**	**12.81 ± 0.20**
16:1[1]	7.23	8.16 ± 0.56
18:1[2]	39.71	38.69 ± 1.32
20:1[2]	7.51	8.27 ± 0.06
22:1[2]	0.25	0.22 ± 0.03
Total MUFA	**49.00**	**48.95 ± 1.97**
18:2n-6	18.53	18.13 ± 2.98
20:2n-6	0.88	0.82 ± 0.07
20:4n-6	0.41	0.44 ± 0.01
Total n-6 PUFA	**22.14**	**21.98 ± 2.90**
18:3n-3	1.65	1.01 ± 0.17
18:4n-3	0.20	nd
20:5n-3	0.73	0.55 ± 0.02
22:5n-3	1.74	1.33 ± 0.16
22:6n-3	7.69	8.62 ± 0.96
Total n-3 PUFA	**13.06**	**12.28 ± 1.03**
DHA/EPA	10.48	15.67 ± 1.30
ARA/EPA	0.55	0.81 ± 0.01
n-3/n-6	0.59	0.57 ± 0.12

Data are means ± SD (n=2) for SO. [1] mainly n-7 isomer; [2] mainly n-9 isomer; DHA, docosahexaenoic acid, 22:6n-3; EPA, eicosapentaenoic acid, 20:5n-3; ARA, arachidonic acid, 20:4n-6. nd, not detected.

Table 7. Lipid content (% dry matter) and lipid of **eggs** from mullet fed a fish oil based diet (FO+MgM) or a soy oil based diet (SO)

	Unfertilized eggs		Fertilized eggs	
	FO+MgM	SO	FO+MgM	SO
Total lipid	28.49±5.85	23.15±9.71	25.88±2.75	25.85±5.07
Lipid class				
Sphingomyelin	0.21±0.30	0.42±0.14	nd	nd
Phosphatidylcholine	8.28±0.27	6.51±0.34	6.45±0.20	7.14±1.32
PS + PI *	0.68±0.13	0.76±0.07	1.11±0.61	1.02±0.21
Phosphatidylglycerol	0.60±0.17	0.35±0.28	0.53±0.12	0.59±0.41
Phosphatidylethanolamine	1.72±0.10	1.39±0.46	2.03±0.31	1.77±0.19
Total Polar Lipids	**11.48±0.11**	**9.81±0.94**	**10.13±0.91**	**10.72±1.34**
Diacylglycerols	nd	1.90±0.63	0.25±0.39	0.21±0.12
Cholesterol	5.01±0.38	5.69±0.06	5.38±0.29	5.19±0.71
Free fatty acids	2.35±1.01	0.64±0.05	nd	0.83±0.44
Triacylglycerols	11.37±0.55	9.93±0.75	12.35±1.89	10.24±1.47
Wax + Sterol esters	69.78±1.833	71.04±0.94	71.89±1.34	72.50±1.34
Total Neutral Lipids	**88.52±0.11**	**90.19±0.94**	**89.87±0.91**	**89.28±1.34**

Table 8. Fatty acid content (mg g^{-1}) and main fatty acid composition (% total fatty acids) of **eggs** from mullet fed a fish oil based diet (FO+MgM) or a vegetable oil based diet (SO)

		Unfertilized eggs		Fertilized eggs	
		FO+MgM	SO	FO+MgM	SO
Total FA		**16.27±4.65**	**16.43±4.95**	**19.89±2.91**	**18.57±2.59**
	14:00	0.34±0.01	0.26±0.01	0.38±0.04	0.30±0.02
	16:00	8.78±0.48	8.11±0.17	8.72±0.73	8.37±0.48
	18:00	2.80±0.10	2.77±0.14	2.84±0.16	2.87±0.17
Total SFA		**12.55±0.59**	**11.83±0.39**	**12.60±0.93**	**12.20±0.69**
	16:1[1]	7.61±0.25	5.39±0.03	5.80±0.15	6.61±0.77
	18:1[2]	38.95±2.30	34.36±0.23	39.77±2.75	34.45±0.74
	20:1[2]	1.21±0.08	1.22±0.02	1.12±0.18	0.80±0.05
	22:1[3]	0.12±0.17	nd	0.14±0.16	0.10±0.12
Total MUFA		**48.55±2.87**	**41.49±0.18**	**47.32±3.14**	**42.46±0.64**
18:2n-6		17.40±0.65	25.00±0.77	17.19±1.06	23.59±0.88
20:2n-6		0.79±0.04	1.03±0.00	0.79±0.04	0.90±0.07
20:4n-6		0.46±0.08	0.44±0.01	0.40±0.04	0.45±0.06
Total n-6 PUFA		**21.80±1.11**	**30.13±0.29**	**21.49±1.64**	**28.98±0.73**
18:3n-3		1.07±0.09	1.57±0.01	1.46±0.08	1.35±0.22
18:4n-3		0.10±0.14	0.26±0.01	0.27±0.04	0.28±0.03
20:5n-3		0.62±0.09	0.51±0.02	0.61±0.05	0.44±0.05
22:5n-3		2.02±0.14	2.60±0.21	3.79±0.29	2.64±0.40
22:6n-3		8.18±1.58	7.28±0.20	7.45±1.14	6.35±1.63
Total n-3 PUFA		**12.30±2.28**	**11.82±0.18**	**12.21±1.48**	**10.29±1.42**
DHA/EPA		13.07±0.65	14.20±0.88	12.13±1.17	14.42±2.83
ARA/EPA		0.74±0.03	0.86±0.04	0.65±0.04	1.02±0.09
n-3/n-6		0.56±0.08	0.39±0.00	0.57±0.04	0.36±0.05

Data are means ± SD (n=2 for unfertilized eggs and grey tank; n=4 for fertilized eggs). [1] mainly pn-7 isomer; [2] mainly n-9 isomer; [3] mainly n-11 isomer; DHA, docosahexaenoic acid, 22:6n-3; EPA, eicosapentaenoic acid, 20:5n-3; ARA, arachidonic acid, 20:4n-6. nd, not detected.

Interestingly, in spite of 18:2n-6 PUFA being higher in the SO diet (**Table 1**) and TAG being higher in the FO+MgM diet (**Table 2**), homeostatic mechanisms seem to allow the maintenance of a balanced and similar profiles of these components in the 6-year old captive mullets gonads (**Tables 5** and **6**) and eggs (**Tables 7** and **8**) regardless of the dietary treatment.

Comparison of proximate composition, lipid classes and fatty acid profiles of unfertilized and fertilized eggs revealed no significant change (**Tables 7** and **8**). The only exception was sphingomyelin (SM), which showed a markedly decrease to non-detected levels in fertilized eggs of both treatment groups (**Table 7**).

A dietary effect could be observed on egg coloration: bright-yellow vs. pale color in the FO+MgM and SO treatment groups, respectively (**Fig. 5**). Furthermore, gonads and eggs in the FO+MgM treatment group had to significantly (P < 0.01; P < 0.05, respectively) higher carotenoid levels than the SO equivalents (**Table 9**).

No dietary effects were observed on body weight or reproductive performance of the grey mullet broodstocks (**Table 10**). Nonetheless, the FO+MgM diet appears to increase the hatching success and survival rate of the larvae (**Table 10**).
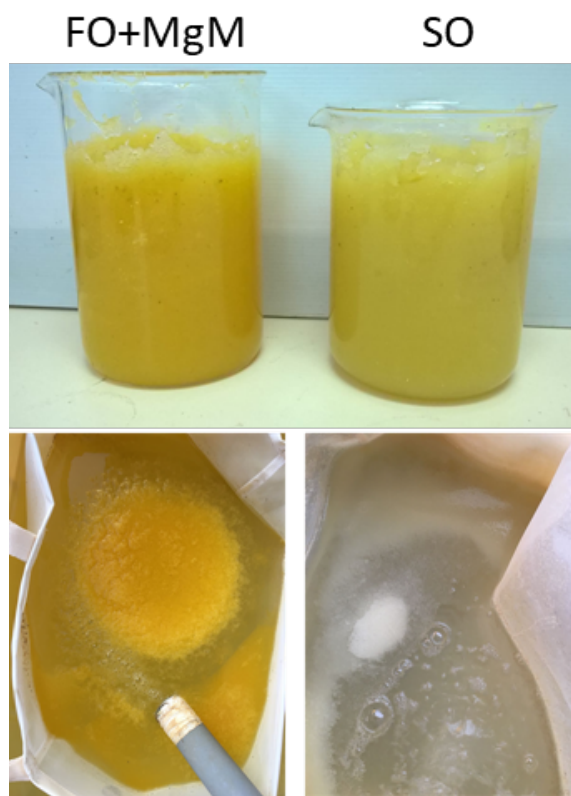
Figure 5. Effects of diets containing fish (FO+MgM) and soybean (SO) oil on grey mullet egg color.

Table 9. Total **carotenoid** content (mg kg^{-1}) of gonads and eggs from experimental mullets, and diets. Average values are expressed as mean ± SD. Different superscript letters indicate a significant (P<0.05) difference between mean values.

	Diets	Gonads	Fertilized eggs	Unfertilized eggs
FO+MgM	138.07	7.94	4.53 ± 0.50[a]	4.74 ± 0.36[a]
SO	99.25	5.68 ± 0.04	1.95 ± 0.48[b]	2.87 ± 0.50[b]

Table 10. Comparison of spawning data obtained from grey mullet broodstocks fed diets differing in their oil source: fish oil (FO) vs. soybean oil (VO).

	DIETS	
	FO+MgM	SO
Females BW (g)	1660.36 ± 67.8	1753.67 ± 75.5
Males BW (g)	987.86 ± 46.06	905.77 ± 48.11
Abundance of post vitellogenic females (>500 μm)	71.86 ± 5.9	69.86 ± 9.4
Abundance of spermiating males - September, 2017	41.67 ± 4.8	46.30 ± 13.0
Abundance of spermiating males - October, 2017	28.97 ± 16.8	13.09 ± 7.2
Fertilization rate (%)	50	66
Fecundity (million eggs/kg BW)	2.12 ± 0.1	2.89 ± 0.9
% hatching 0 DPH	37.25	32.2
% Survival 0 DPH	60.25	51.25

DISCUSSION AND CONCLUSIONS

The aim of the present work was to systematically describe an expanded tool box for successful breeding of captive grey mullet, which includes hormonal, social (brooder unit) photo-thermal, and dietary conditioning compounds.

During several spawning seasons vast progress was made in optimizing hormonal treatments for alleviating reproductive dysfunctions among captive grey mullet broodstocks. In males, the combination of r-FSH injection and EVAc implant for sustained release of Methyltestosterone (MT), was the treatment that both induced a further advance in spermatogenesis and a higher percentage of breeders to advance to spermiation among captive grey mullet males (see D7.3). A previous study demonstrated that sole MT-EVAc treatment stimulates circulating levels of 11-KT, however in most cases the volume of the produced milt was relatively low (Aizen et al., 2005). In this regard, the value-added of the r-FSH-priming treatment administered as a single and to a greater extent double injections, seem to be attributed to the hormone's unique capabilities to regulate Sertoli cell activities (Schulz et al., 2010).

Grey mullet, like the European eel *Anguilla anguilla* (Dufour et al., 1988), are prone to dopaminergic inhibition during early stages of gonadal growth. To release the latter inhibition in the grey mullet, a single injection of domperidone (an antagonist of dopamine D2 receptors) was sufficient (Aizen et al., 2005), whereas in the eel, a triple treatment consisting of GnRHa, testosterone, and pimozide (an antagonist of dopamine D2 receptors) was necessary (Vidal et al., 2004). Aiming to improve the onset and progression of vitellogenesis among captive grey mullet, broodstock were treated with Metoclopramide (Metoc; an antagonist of dopamine D2 receptors) and rFSH. Indeed, the Metoc+r-FSH treatment enhanced and synchronized ovarian development in captive grey mullet females, giving rise to 91% post-vitellogenic females within the treatment-group.

Interestingly, relatively higher abundance of fully mature females and males (50-70%) could be found in untreated groups, compared to the relatively low percentages (10-20%) that were reported previously (Aizen

et al., 2005). We tend to think that the enhanced spontaneous maturational process among captive grey mullet population also relates to the fact that all experimental fish at the IOLR (Israel) were hatchery-produced G1 broodstock, which have begun the process towards domestication. The fact that similar treatment was less successful in inducing vitellogenesis in wild-caught mullets at the IRTA (Spain) further emphasizes the discrepancy between the two broodstocks, i.e., hatchery produced vs. wild-caught. In that respect, a comparative study that evaluated pubertal development in hatchery-produced vs. wild-caught captive-reared grey mullet highlighted marked effects of domestication, with 3-year old hatchery-produced specimens exhibiting a more advanced ovarian development than fish caught from the wild and reared in captivity (see D7.5 and D7.6).

In grey mullet, as in many other teleosts (Dufour et al., 2003), dopamine is involved in the control of spermiation, ovulation, and spawning. So far, regardless of the broodstock origin (i.e., wild- caught or G1) no spontaneous spawning have been documented in captivity. It is well established that dopamine can inhibit basal and GnRH-induced LH release (Peter et al., 1986, 1991; De Leeuw et al., 1986; Yu and Peter, 1992, Yaron et al., 2003), and also may modulate pituitary sensitivity to GnRH by decreasing the number of GnRH-receptors (De Leeuw et al., 1986, 1988; Omeljaniuk et al., 1989; Levavi-Sivan et al., 2004). Therefore, to induce grey mullet spawning, fully mature females and males were treated with two consecutive injections consisting of GnRHa combination with Metoc. The rationale behind this treatment was to stop the strong dopaminergic inhibition with the dopamine antagonist, and promote LH release from the pituitary through a GnRHa injection (reviewed by Zohar and Mylonas, 2001). Results obtained so far, indicate up to 50% spawning successes following a treatment with priming (GnRHa 10 µg/kg; Met 15mg/kg) and resolving (GnRHa 20 µg/kg; Met 15mg/kg) injections given 22.5-h apart to selected fully mature females and males. Grey mullet seem to exhibit intricate social interplay in- and between- sexes, with dominant female(s) being capable of suppressing sexual maturation of conspecifics and enhancing males' spermiation (Aizen et al., 2005). Therefore, to promote the occurrence of successful spawning, several brooder group structure were tested. Our results indicate that the best performing unit consists of a single female and 3 males, which prevents female intrasexual competition.

The grey mullet, like other temperate fish species are annual spawners and mainly rely on annually cycling cues (temperature and photoperiod) to synchronise their reproductive cycle (Wang et al., 2010). The results of this study indicated that captive mullet responded very well to the 4-month shifted photoperiod giving rise to successful spawning events. Moreover, similar fecundities were documented during the shifted (2.22 ± 0.19 million eggs / kg BW) and the natural (2.5 ± 0.1 million eggs / kg BW) spawning seasons. These results suggest that photoperiod manipulation has significant potential for extending the availability of eggs and larvae for grey mullet aquaculture.

One additional way to overcome reproductive dysfunctions and/or improve gamete quality of captive broodstock is by providing an effective broodstock diet which should mimic body composition of brooders in the wild (Izquierdo et al., 2001; Rodríguez-Barreto et al., 2014). Following the same rational, the current study examined the effect of two diets differing in their oil source and carotenoid levels, on the grey mullet reproductive outputs. The basic diets that were used for the pigment-enrichment trial, consisted of the IOLR formulated pelleted diet containing either fish oil (FO) or soybean oil (SO) as the main neutral lipid. Interestingly, despite of the difference of the PUFA in the diets (i.e., 18:2n-6 being higher in the SO diet), homeostatic mechanisms seem to allow the broodstock to maintain a balance resulting in a similar profile of polyunsaturated fatty acids (PUFA) in the gonads and eggs, independently of the diet, displaying also a similar pattern to that of the wild counterparts (see D13.3). Higher levels of 18:2n-6 and lower of EPA and DHA are normally present in herbivorous fish compared to carnivorous ones. In addition, the results of this study indicate that in both diets the ovaries described a predominance of neutral lipids (mainly triacylglycerol and wax-esters) over the polar lipids, comprising around 70% of the total lipids in ovaries and eggs. This unique lipid composition, rich in wax esters is considered to play a role as metabolic energy resources for oocyte formation (Zudaire et al., 2014).

A comparison of proximate composition, lipid classes and fatty acid profiles in fertilized and unfertilized eggs revealed no marked changes regardless of the broodstock dietary regime. The only exaction was the noticeable disappearance (non-detectable levels) of sphingomyelin (SM), one of the four

common phospholipids found in the plasma membrane of cells, in the viable fertilized eggs, independent of the treatment group. Lipid dynamic through the reproductive cycle is related to the functions of each lipid class during reproduction and a decrease in their quantity could limit fish productivity (Henderson et al., 1996; Marshall et al., 1999; Wiegand et al., 2007) and affect the viability of progeny (Rainuzzo et al., 1997). Indeed, similar decrease in SM levels was observed in *Xenopus laevis* fertilized eggs (Petcoff et al., 2008). In the latter study, it was found that during fertilization sphingomyelinase activation leads to SM hydrolysis and formation of ceramide, which can participate in a variety of cellular signaling, such as regulating differentiation, proliferation, and programmed cell death (PCD) of cells.

Carotenoids are widely present in fish gonads and eggs. They are precursors of vitamin A being involved in reproduction and embryonic development, as well as in the prevention of oxidative stress processes (Miki, 1991; Guerin et al., 2003) and to ensure larval visual function and adequate chromatophore responses. Carotenoids are actively mobilized into the gonads during sexual reproductive activity in aquatic animals. 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, we tested the effect of pigment-enriched diet on the reproductive outputs of captive mullet broodstock. Our results indicate that the dietary administration of marigold petal meal (MgM) pigment over 3 months, spanning the entire period of vitellogenesis, markedly contributed to the carotenoid levels in the ovaries and eggs giving rise to a bright yellowish color. The MgM is a concentrated source of lutein and zeaxanthin (xanthophylls) pigments used to enhance the color of chicken eggs. The xanthophyll content of marigold petal meal (7000 mg/kg of meal) exceeds that of commonly used feed ingredients like yellow corn, corn gluten, alfalfa and algae by a factor which ranges from over 300:1 in the case of yellow corn (22 mg/kg) down to 3.5:1 for dried algae (2000 mg/kg) (North, 1984).

No dietary effects were observed on body weight or reproductive performance of the grey mullet broodstocks. However, the FO+MgM diet appears to increase the hatching success and survival rate of the larvae. Furthermore, this diet contributed also to larvae tolerance of food deprivation and improved swim bladder inflation (see D13.3). In that respect, Navas et al. (1997) found that egg quality and hatching rates were improved in seabass (*Dicentrarchus labrax*) by feeding appropriate amounts of n-3 LCPUFA during the vitellogenin period. The importance of species specific effective levels of n-3 LCPUFA was also reported by Li et al. (2005) who determined that levels of these essential fatty acids below 2.40 or above 3.7% DW diet in crescent sweetlips, *Plectorhynchus cinctus*, had a negative effect, while between these values resulted in good egg quality and larval performance. Zakeri et al. (2011) showed that replacing soybean oil with increasing levels of fish oil in the broodstock of yellowfin sea bream, *Acanthopagrus latus,* improved relative fecundity, percentage of buoyant eggs, hatchability, survival rate of larvae at 3 dph and higher starvation tolerance. Fish oil components, such as vitamin A and E as well as carotenoids may also effect egg quality and larval performance (Izquierdo and Koven, 2011).

Conclusions

1) The basic breeding units consisting of a single female and three males, seems to improved synchronization and increase the fertilization rate.
2) Shifted spawning season can be easily achieved via photo-thermal manipulation.
3) The established breeding protocol for captive grey mullet can be applied effectively during natural as well as shifted spawning seasons. During the past three years, tens millions of quality eggs were produced giving rise to mass production of robust fingerlings.
4) Broodstock diet enriched with pigment (MgM) and fish oil (FO), which is relatively rich in n-3 LCPUFA, positively affected hatching success and larvae survival.

   Nevertheless, our results, which highlight episodic fertilization rates ranging between 0 to 98%, implicate the need to further fine tune and optimize the hormone-based and dietary regimes for successful captive breeding of grey mullet as a standardized technology for commercial venture.

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Deviations:

Aiming to describe an expanded tool box for successful captive breeding of grey mullet the deliverable includes additional work studying the effects of broodstock diet on gamete quality and reproductive outputs.

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