New species for EU aquaculture

Deliverable Report

Deliverable No:	D13.3	**Delivery Month:**	60
Deliverable Title	Determine the effects of pigments, essential fatty acids and Tau in grey mullet broodstock diets on egg quality, fecundity, hatching success, larval first feeding and vitellogenin expression and accumulation		
WP No:	13	**WP Lead beneficiary:**	P4. IOLR
WP Title:	Larval Husbandry – grey mullet		
Task No:		**Task Lead beneficiary:**	P4. IOLR
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Objective: Determine the effects of essential fatty acids in grey mullet broodstock diets on egg quality, fecundity, hatching success and larval first feeding

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1. Introduction

Fish reared in captivity may exhibit reproductive dysfunction, such as the inhibition of final oocyte maturation and spawning for females and the production of poor quality sperm by males (Mylonas and Zohar, 2001). These dysfunctions can be overcome by hormonal therapies and modulation of environmental parameters (Mylonas and Zohar, 2001) as well as providing an effective broodstock diet which should mimic body composition of brooders in the wild (Izquierdo et al., 2001; Rodríguez-Barreto et al., 2014).

During vitellogenesis significant quantities of proteins must be made available for transfer to the developing oocytes as well as lipids and carotenoids. Lipids that are mobilized are particularly rich in both saturated and monounsaturated fatty acids for energy provision as well as long chain polyunsaturated fatty acids

(LCPUFA), which are primarily represented by the essential fatty acids (EFA) eicosapentaenoic (EPA; 20:5n-3), arachidonic (ARA; 20:4n-6) and docosahexaenoic (DHA; 22:6n-3) acids (Sargent et al. 2002). EPA and ARA are precursors of eicosanoids, a group of active compounds with very important physiological functions such as reproduction (Tocher 2003). ARA derived eicosanoids have also shown to be involved in pheromone attraction, steroidogenesis, and oocyte maturation (Henrotte et al., 2011; Sorbera et al., 2001). Thus, supplying proper levels and ratios of EFA in broodstock diets is vital not only to produce eggs with the suitable contents of these fatty acids to ensure embryo and larvae development, but also in the regulation of reproductive physiology (Henrotte et al., 2011; Sorbera et al., 2001). N−3 PUFA derived directly from the dietary input of broodstock as well as from body reserves in the period of gonadogenesis are crucial to female fecundity and to embryo and early larval development, growth and survival (Rodríguez-Barreto et al., 2014).

The supplementation of fish oil and the essential n-3 LCPUFA to broodstock diets and its benefit to egg and larval quality has been well established in a number of commercially farmed teleosts (Watanabe et al., 1984a, b; Watanabe et al., 1985 a, b; Fernandez-Palacios et al., 1995; Navas et al., 1997). Tandler et al. (1995) reported that growth, survival and swimbladder inflation in gilthead seabream (*Sparus aurata*) larvae were improved when fish oil was used instead of soybean oil in broodstock diets. On the other hand, the herbivorous Nile tilapia, *Oreochromis niloticus*, demonstrated higher fry per spawning as well as total fry production when fed a diet supplemented with soybean oil, which is relatively rich in n-6 PUFA (Watanabe, 1982). Nevertheless, the most effective level of n-3 LCPUFA in brood stock diets is species specific and an excess can be detrimental (Fernandez-Palacios et al., 1995). In the case of sparids, the n-3 LCPUFA requirement reportedly ranged from 1.5 to 2.0% n-3 LCPUFA DW diet (Izquierdo, 1996). Levels above this caused yolk sac hypertrophy and a decrease in larval survival (Fernandez-Palacios et al., 1995). In contrast to the carnivorous gilthead sea bream and European sea bass (*Dicentrarchus labrax*), the grey mullet is omnivorous following metamorphosis, which suggests a diet relatively low in n-3 PUFA. However, grey mullet bottarga or intact roe is a highly prized delicacy in Japan and around the Mediterranean and is a rich source of n-3 LCPUFA (Scano et al., 2010). This suggests that grow out diets may not be suitable and that there is a dietary requirement for n-3 LCPUFA in the brood stock feed.

2. Materials and methods

Sampling

Eight hatchery-produced 6-year old grey mullet stock were sampled by P4. IOLR during early October (3 females) and November (3 females and 2 males) 2016. In parallel, 16 wild specimens (10 females and 6 males), caught by traditional trap nets (lavoriera) in the Schiapparo Channel (Apulia, Italy) during their migration from the Lesina Lagoon to the spawning grounds of the Adriatic Sea, were sampled by P13. UNIBA in early September 2016. The age of wild grey mullets sampled by UNIBA was estimated through the analysis of the scales (Meunier, 2002). Gonads from wild and captive males and females individuals were taken, immediately frozen and kept at -80ºC, until analysis.

Proximal and main fatty acids composition analyses of diets and gonads from muture grey mullet (P15. ULL)

To evaluate gonad biochemical composition, sections of ovaries and testes were cut and kept on dry ice until 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 (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 (1982) and stored in chloroform/methanol (2:1), containing 0.01%

butylated hydroxytoluene (BHT). Analysis of lipid class (LC) composition was performed (Olsen and Henderson, 1989) 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). 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.).

Fish holding and experimental design

Based on the results of proximal and fatty acid (FA) composition in gonads from wild and domesticated mullets (Table 1), the present study to improve the broodstock diet for grey mullet focused on the increased supplementation of the n-3 LCPUFA through the addition of more fish oil to the diet. 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), which is relatively rich in n-3 LCPUFA. 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 (Aizen et al. 2005) with some modifications elaborated in WP7.

Larval rearing trials

Fertilized and spawned eggs from each of the broodstock groups (SO and FO) were stocked in eight 400 l V-tanks (200 eggs/l) or four tanks per treatment for the food deprivation experiment. SO and FO eggs were simultaneously stocked in 3 plastic plates per treatment where each plate contained 12 five ml wells where one egg was stocked per well. Hatching rate was determined 24 h after stocking and survival of the hatched larvae at the end of 0 dph. Tanks in the food deprivation experiment were supplied with temperature controlled (Gavish, Israel; 24-25 ^{o}C), filtered (10 µm) and UV treated sea water (40 ‰) that entered the tanks from the bottom and exited through a 500 µm filter at an exchange rate of 300% per day. In two tanks from each broodstock treatment set, the salinity was decreased to 25 ‰ at 2 dph over the course of one day so that 3-7 dph larvae were exposed to only 25 ‰ in these tanks. This means duplicate tanks were used for each of the four treatments (SO-25, SO-40, FO-25, FO-40). All larvae were not exposed to direct lighting during the course of the food deprivation experiment in order to avoid stimulating feeding activity. Larval samples from each of the tanks (average of 220 larvae/sample) were taken daily for DW determination and fatty acid analysis (not shown). However, fatty acid analyses of the gonads of females and males of both wild and captive broodstock fish were carried out (P15.ULL).

Statistics

One way ANOVA and regression analyses were carried out using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Regression data sets employed Akaike's Information Criteria (AIC) to compare linear, second order polynomial and other models to determine which most likely generated the data. ANOVA analyses and Barlett's test for equal variances were carried out simultaneously. If significance ($P < 0.05$) was found for ANOVA while Barlett's test was not significant ($P > 0.05$), then testing differences between groups was carried out by Newman-Keuls Multiple Comparison test. In cases where ANOVA and Barlett's test were both significant ($P < 0.05$), then the non-parametric Kruskal Wallis Test was applied followed by Dunn's multiple Comparison test to determine significant ($P < 0.05$) differences among treatments. Percentage values were first arcsine transformed before analyses. All data are presented as mean ± SEM.

3. Results

Results are given as mean ±SD for a variable number of samples (n=10 for wild females and n=6 for wild males; n=2 for regressed females, n=3 for fully mature females and n=1 for captive male). nd, non-detected. **Table 1** summarizes the reproductive performance of the FO and VO broodstocks during the natural spawning season (September-October 2017). All tested parameters including the percentages of fully mature specimens (spermiating males and post-vitellogenic females), successful spawns and fecundity show no significant variations between the treatment groups.

Table 1 Summary of grey mullet broodstock body weight (BW) and reproductive performance in two different diets distinguished by their oil source: fish oil (FO) and soybean oil (SO).

	Treatment groups	
	FO	SO
Av. BW females (g)	1660.36±67.8	1753.67±75.5
Av. BW males (g)	987.86±46.06	905.77±48.11
Post vitellogenic females (%)	71.86±5.9	69.86±9.4
Spermiating males (%)	28.97±16.8	13.09 ±7.2
Fertilized spawns (%)	50	66
Fecundity	2.12±0.1	2.89±0.9

Tables 2 and **3** present the proximate and fatty acid composition, as well as the lipid class distribution of the 2 experimental diets, respectively. The FO diet was higher in monounsaturated fatty acids (MUFA) and n-3 polyunsaturated fatty acids (PUFA) (48.7 and 13.1, respectively) than the SO diet (39.4 and 10.0 %, respectively). On the other hand, the SO diet was considerably higher in n-6 PUFA (31.8%) than the FO diet (19.3%). The FO diet had higher total phospholipids (PL) and triacylglycerols (1.8 and 34.5%, respectively) than the SO diet (1.6 and 25.6%, respectively). The FO diet was also higher in wax and sterol esters (9.4%) compared to the SO diet (5.2%).

Table 2. Proximate composition and main fatty acid profile (% total fatty acids) of experimental diets.

	FO	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		
14:0	1.89 ± 0.01	1.06 ± 0.01
16:0	11.79 ± 0.06	11.54 ± 0.18
18:0	3.00 ± 0.00	3.34 ± 0.09
Total SFA	17.82 ± 0.01	17.23 ± 0.24
16:1[1]	3.54 ± 0.02	2.14 ± 0.13
18:1[2]	39.46 ± 0.41	34.05 ± 0.32
20:1[2]	3.22 ± 0.02	1.82 ± 0.29
22:1[3]	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
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

Data are means ± SD (n=2). [1] mainly n-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. FO = fish oil based diet; SO = vegetable oil based diet.

Tables 4 to **9** correspond to the lipid classes and fatty acid profiles of female gonads of 6 years old captive broodstocks, female and male gonads from 3 years old wild and captive specimens and of the resultant fertilized and unfertilized eggs from fish fed FO or SO diets. **Tables 4** and **5** showed no marked differences between the lipid classes and fatty acid composition in 6 year old captive female gonads fed the FO or SO diets.

Table 3. Lipid class composition (% total lipid) of **experimental diets**.

	FO	SO
Sphingomyelin	0.40	0.20
Phosphatidylcholine	0.87	0.74
Phosphatidylserine + Phosphatidylinositol	0.21	0.17
Phosphatidylethanolamine	0.06	0.09
Phosphatidylglycerol	0.28	0.36
Unknown	0.00	0.00
Total Polar Lipids	**1.82**	**1.57**
Diacylglycerols	8.72	10.11
Cholesterol	6.13	6.06
Free fatty acids	39.50	51.44
Triacylglycerols	34.45	25.62
Wax + Sterol esters	9.38	5.21
Total Neutral Lipids	**98.18**	**98.43**

Table 4. Lipid content (% dry matter) and lipid class composition (% total lipid) of **gonads from 6 years old captive female** mullet fed a fish oil based diet (FO) or a vegetable oil based diet (SO).

	FO	SO
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 5 Fatty acid content (mg g^{-1}) and main fatty acid composition (% total fatty acids) of **gonads from 6 years old female** mullet fed a fish oil based diet (FO) or a vegetable oil based diet (SO).

	FO	SO
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.

In 3 year old wild and F2 females fed the FO or SO diets (**Table 6**), there are no distinct differences in total lipid (TL) (28.5 and 30.6%, respectively) although the SO fish exhibited an increase in their gonads to 35.9%. The FO wild and captive females demonstrated no large gonadal differences in PL (16.5 and 14.0%, respectively) and were both considerably higher in the PL lipid classes compared to the SO group (9.8%), particularly phosphatidylcholine (10.1, 9.7 and 6.8%, respectively). In the neutral lipids, there was a drop in cholesterol from the FO wild and captive females when compared to the SO fed broodstock (10.4, 11.2 and 7.4%, respectively). Conversely, there was a marked increase in waxes and colesterol esters in the FO wild and captive groups compared to the SO fish (55.4, 57.1 and 68.6%, respectively). However, the most dramatic difference was in total lipid between the FO wild and captive female (28.5 and 30.6%, respectively)

and male gonads in both the FO and SO groups (13.6 and 15.6%, respectively). In contrast, PL classes increased markedly in the male gonads compared to the female gonads in both the FO and the SO groups (11.4 to 39.0% and 9.8 to 40.1%, respectively) as well as the neutral lipid, cholesterol (10.4 to 45.1% and 7.4 to 37.5%, respectively). The drop in total lipid is mostly explained from the marked reduction in waxes and cholesterol esters comparing female to male gonads in the FO and SO groups (57.1 to 1.43% and 68.6 to 4.8%, respectively).

In **Table 7**, there was a total fatty acid increase in FO captive female gonads compared to FO wild female gonads (90.2 vs 81.3%) while male gonads contained only 16.1 and 11.3% in the FO and SO groups, respectively. Although female gonads had less saturated fatty acids (SFA) than male gonads in both FO and SO groups (10.7 vs 26.8% and 11.9 vs 29.8%, respectively), they had more MUFA than male gonads in the FO and SO groups (47.4 vs 25.8 and 47.0 vs 20.3%, respectively). However, FO captive female gonads did not differ markedly in their n-3 PUFA content from FO wild female gonads but contained considerably less n-3 PUFA than male gonads in both FO and SO (18.3 vs 31.2 and 18.2% vs 34.2%, respectively). The main fatty acid to explain this difference was DHA which in the female and male gonads of the FO and SO groups were 10.9 vs 23.5% and 10.8 vs 28.3%, respectively. Interestingly, the male gonads from the SO group were higher in DHA than the FO group despite the fact that soybean oil does not contain this essential fatty acid.

Table 6 Lipid content (% dry matter) and lipid class composition (% total lipid) of **gonads from 3 years old** mullet fed a fish oil based diet (FO) or a soybean oil based diet (SO).

	Wild	Captive G2			
	FO	FO		SO	
	Female	Female	Male	Female	Male
Total lipid	28.54±2.04	30.63±5.98	13.58±1.52	35.87±3.43	15.63±1.70
Lipid class					
Lysophosphatidylcholine	0.93±0.11	0.47±0.15	1.12±0.09	nd	nd
Sphingomyelin	1.47±0.10	0.57±0.24	0.82±0.02	0.65±0.42	1.47±0.26
Phosphatidylcholine	10.12±0.06	9.66±0.11	12.37±0.50	6.80±0.38	13.18±0.43
PS + PI *	2.11±0.15	1.25±0.22	9.16±0.96	0.34±0.12	8.08±0.20
Phosphatidylglycerol	nd	nd	nd	0.23±0.28	2.41±0.70
Phosphatidylethanolamine	1.86±0.31	2.08±0.87	15.58±1.28	1.78±0.14	14.94±0.91
Total Polar Lipids	**16.49±0.32**	**14.03±0.94**	**39.04±0.11**	**9.81±0.67**	**40.08±1.99**
Diacylglycerols	nd	nd	nd	1.42±0.35	5.66±1.12
Cholesterol	11.22±1.73	10.41±0.30	45.08±1.03	7.38±0.34	37.54±0.99
Free fatty acids	1.09±0.30	2.10±0.67	4.36±0.21	0.80±0.42	1.17±0.21
Triacylglycerols	13.46±0.16	14.54±2.68	7.72±1.90	11.43±2.16	10.72±0.44
Wax + Sterol esters	55.37±1.11	57.10±3.04	1.43±0.33	68.59±3.00	4.83±1.62
Unknown	2.38±0.77	1.82±0.31	2.37±0.89	0.56±0.23	nd
Total Neutral Lipids	**83.51±0.32**	**85.97±0.94**	**60.96±0.11**	**90.19±0.67**	**59.92±1.99**

Data are means ± SD (n=3 for females; n=2 for males). nd, not detected. PS=Phosphatidylserine, PI= Phosphatidylinositol.* mainly PS

Table 7 Fatty acid content (mg g^{-1}) and main fatty acid composition (% total fatty acids) of **gonads from 3 years old** mullet fed a fish oil based diet (FO) or a vegetable oil based diet (SO). nd – not detected.

	Wild	Captive G2			
	FO	FO		SO	
	Female	Female	Male	Female	Male
Total FA	81.29 ± 5.59	90.24 ± 4.62	16.09 ± 2.65	81.12 ±14.86	11.33 ± 2.03
14:0	0.60 ± 0.02	0.46 ± 0.02	0.85 ± 0.02	0.61 ± 0.09	0.66 ± 0.11
16:0	8.18 ± 0.13	7.57 ± 0.63	19.56 ± 0.20	8.24 ± 1.11	21.51 ± 0.05
18:0	2.42 ± 0.01	2.36 ± 0.18	5.00 ± 0.16	2.53 ± 0.17	5.62 ± 0.28
Total SFA	11.60 ± 0.03	10.70 ± 0.75	26.80 ± 0.23	11.87 ± 1.34	29.79 ± 0.24
16:1[1]	7.99 ± 0.19	6.13 ± 0.45	2.27 ± 0.31	6.45 ± 0.96	1.80 ± 0.22
18:1[2]	38.57 ± 1.19	38.41 ± 1.15	16.91 ± 0.99	37.43 ± 0.32	14.55 ± 1.01
20:1[2]	7.76 ± 0.59	7.35 ± 0.84	3.73 ± 0.47	6.25 ± 1.14	3.09 ± 0.01
22:1[2]	0.42 ± 0.01	0.44 ± 0.04	1.80 ± 0.19	0.33 ± 0.00	2.21 ± 0.49
Total MUFA	49.49 ± 1.65	47.39 ± 1.95	25.75 ± 0.80	47.01 ± 0.48	20.26 ± 0.25
18:2n-6	15.83 ± 0.15	16.61 ± 1.96	9.63 ± 0.60	16.82 ± 1.42	8.32 ± 0.48
20:2n-6	0.82 ± 0.01	0.90 ± 0.06	2.09 ± 0.14	0.93 ± 0.04	1.97 ± 0.03
20:4n-6	0.62 ± 0.04	0.54 ± 0.06	2.29 ± 0.49	0.55 ± 0.07	2.42 ± 0.10
Total n-6 PUFA	19.09 ± 0.09	19.74 ± 1.87	15.45 ± 1.45	19.91 ± 1.22	13.92 ± 0.29
18:3n-3	1.41 ± 0.01	1.64 ± 0.27	0.74 ± 0.08	1.50 ± 0.29	0.54 ± 0.13
18:4n-3	0.27 ± 0.03	0.22 ± 0.19	nd	0.31 ± 0.08	nd
20:5n-3	1.36 ± 0.05	1.46 ± 0.15	2.21 ± 0.22	1.41 ± 0.30	1.69 ± 0.09
22:5n-3	2.02 ± 0.14	2.60 ± 0.21	3.79 ± 0.29	2.64 ± 0.40	2.96 ± 0.09
22:6n-3	9.18 ± 0.72	10.91 ± 0.88	23.53 ± 3.06	10.84 ± 1.85	28.27 ± 1.74
Total n-3 PUFA	15.53 ± 0.95	18.27 ± 1.26	31.19 ± 2.43	18.15 ± 2.89	34.18 ± 1.58
DHA/EPA	6.75 ± 0.27	7.53 ± 0.90	10.78 ± 2.47	7.75 ± 0.85	16.79 ± 1.93
ARA/EPA	0.46 ± 0.01	0.37 ± 0.01	1.03 ± 0.12	0.39 ± 0.04	1.44 ± 0.13
n-3/n-6	0.81 ± 0.05	0.93 ± 0.10	2.04 ± 0.35	0.92 ± 0.19	2.46 ± 0.17

Data are means ± SD (n=3 for females; n=2 for males). [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.

In **Table 8**, there are no outstanding differences in phospholipid and neutral lipid classes between the FO and SO fertilized eggs. On the other hand, FO unfertilized eggs show higher PLs and somewhat lower NLs (11.5 and 88.5, respectively) than SO unfertilized eggs (9.8 and 90.2%, respectively). The fatty acid profiles of fertilized and unfertilized eggs are shown in **Table 9**. Although fertilized and unfertilized eggs from the FO and SO groups demonstrated similar SFA levels, the unfertilized FO eggs exhibited higher MUFA than the SO eggs (48.6 and 41.5%, respectively) while these fatty acids were similar among the treatments in fertilized eggs. However, SO eggs, independent of fertilzation success, exhibited higher n-6 PUFA (30.1 and 28.9%, respectively) than levels in FO eggs (28.9 and 21.5%, respectively).

Table 8. Lipid content (% dry matter) and lipid of **eggs** from mullet fed a fish oil based diet (FO) or a vegetable oil based diet (SO).

	Unfertilized eggs			Fertilized eggs		
	FO	SO	B9UF	FO	SO	Grey tank
Total lipid	28.49±5.85	23.15±9.71	25.71	25.88±2.75	25.85±5.07	29.92±4.63
Lipid class						
Sphingomyelin	0.21±0.30	0.42±0.14	nd	nd	nd	0.38±0.24
Phosphatidylcholine	8.28±0.27	6.51±0.34	8.35	6.45±0.20	7.14±1.32	9.00±0.71
PS + PI *	0.68±0.13	0.76±0.07	0.83	1.11±0.61	1.02±0.21	1.09±0.19
Phosphatidylglycerol	0.60±0.17	0.35±0.28	0.81	0.53±0.12	0.59±0.41	0.60±0.01
Phosphatidylethanolamine	1.72±0.10	1.39±0.46	1.68	2.03±0.31	1.77±0.19	2.37±0.39
Total Polar Lipids	**11.48±0.11**	**9.81±0.94**	**11.67**	**10.13±0.91**	**10.72±1.34**	**13.43±0.76**
Diacylglycerols	nd	1.90±0.63	0.27	0.25±0.39	0.21±0.12	0.25±0.08
Cholesterol	5.01±0.38	5.69±0.06	4.96	5.38±0.29	5.19±0.71	5.77±0.52
Free fatty acids	2.35±1.01	0.64±0.05	2.37	nd	0.83±0.44	3.34±0.24
Triacylglycerols	11.37±0.55	9.93±0.75	13.06	12.35±1.89	10.24±1.47	9.02±0.86
Wax + Sterol esters	69.78±1.83	71.04±0.94	67.68	71.89±1.34	72.50±1.34	68.18±0.30
Total Neutral Lipids	**88.52±0.11**	**90.19±0.94**	**88.33**	**89.87±0.91**	**89.28±1.34**	**86.57±0.76**

Data are means ± SD (n=2 for unfertilized eggs and grey tank; n=4 for fertilized eggs). nd, not detected. PS=Phosphatidylserine, PI= Phosphatidylinositol. * mainly PS

In **Figure 1** the rates of decline of weight loss with age (dph) during food deprivation in the four treatments were not significant (P>0.05) from each other. However, there was an observed pattern where the highest rate of dry weight (DW) decline was in the SO larvae where all fish were dead at 5 dph or 3 days after the capability of exogenous feeding. In contrast, larvae from the FO broodstock demonstrated a slower DW decline, particularly in the 25 ‰ treatment, and were still alive at 7 dph, which was 5 days after the onset of the ability to consume live prey. Interestingly, the 2 dph larvae from the FO treatment were larger than the 2 dph fish from the SO treatment, although not significantly (P>0.05). Nevertheless, the percent hatching of eggs from the FO broodstock was significantly (P<0.05) higher than eggs from the SO broodstock (**Figure 2**), while survival in larvae in the two treatments at the end of 0 dph were not significantly different (P>0.05) from each other, although the FO was higher (**Figure 2**). There was a very significant effect (P<0.0001) of broodstock treatment on swimbladder inflation in the food deprivation experiment (**Figure 3**). Larvae from the FO broodstock, regardless of salinity exposure, demonstrated 100% swim bladder inflation by 5 dph, where there was no swim bladder inflation at all in fish from the SO broodstock during the course of the food deprivation study.

Table 9. 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) or a vegetable oil based diet (SO).

	Unfertilized eggs			Fertilized eggs		
	FO	SO	B9UF	FO	SO	Grey tank
Total FA	16.27±4.65	16.43±4.95	20.49	19.89±2.91	18.57±2.59	18.64±1.38
14:0	0.34±0.01	0.26±0.01	0.35	0.38±0.04	0.30±0.02	0.40±0.01
16:0	8.78±0.48	8.11±0.17	7.93	8.72±0.73	8.37±0.48	8.65±0.18
18:0	2.80±0.10	2.77±0.14	2.57	2.84±0.16	2.87±0.17	3.28±0.06
Total SFA	12.55±0.59	11.83±0.39	11.51	12.60±0.93	12.20±0.69	13.09±0.22
16:1[1]	7.61±0.25	5.39±0.03	5.95	5.80±0.15	6.61±0.77	8.86±0.18
18:1[2]	38.95±2.30	34.36±0.23	37.87	39.77±2.75	34.45±0.74	38.28±0.27
20:1[2]	1.21±0.08	1.22±0.02	1.10	1.12±0.18	0.80±0.05	1.09±0.04
22:1[3]	0.12±0.17	nd	nd	0.14±0.16	0.10±0.12	nd
Total MUFA	48.55±2.87	41.49±0.18	45.34	47.32±3.14	42.46±0.64	48.81±0.65
18:2n-6	17.40±0.65	25.00±0.77	19.40	17.19±1.06	23.59±0.88	15.35±0.06
20:2n-6	0.79±0.04	1.03±0.00	0.91	0.79±0.04	0.90±0.07	0.75±0.03
20:4n-6	0.46±0.08	0.44±0.01	0.45	0.40±0.04	0.45±0.06	0.55±0.01
Total n-6 PUFA	21.80±1.11	30.13±0.29	24.19	21.49±1.64	28.98±0.73	20.77±0.06
18:3n-3	1.07±0.09	1.57±0.01	1.40	1.46±0.08	1.35±0.22	0.57±0.08
18:4n-3	0.10±0.14	0.26±0.01	0.29	0.27±0.04	0.28±0.03	nd
20:5n-3	0.62±0.09	0.51±0.02	0.68	0.61±0.05	0.44±0.05	0.36±0.00
22:5n-3	2.02±0.14	2.60±0.21	1.55	3.79±0.29	2.64±0.40	2.96±0.09
22:6n-3	8.18±1.58	7.28±0.20	8.09	7.45±1.14	6.35±1.63	7.17±0.09
Total n-3 PUFA	12.30±2.28	11.82±0.18	12.90	12.21±1.48	10.29±1.42	9.69±0.03
DHA/EPA	13.07±0.65	14.20±0.88	11.95	12.13±1.17	14.42±2.83	19.82±0.30
ARA/EPA	0.74±0.03	0.86±0.04	0.66	0.65±0.04	1.02±0.09	1.52±0.04
n-3/n-6	0.56±0.08	0.39±0.00	0.53	0.57±0.04	0.36±0.05	0.47±0.00

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.

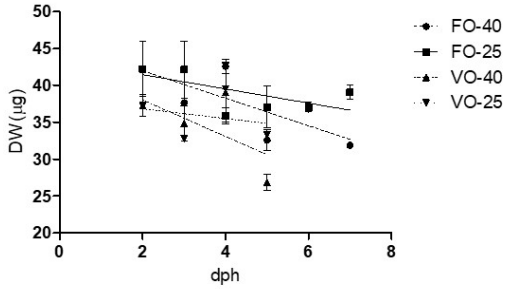

Figure 1 The rate of decline in 2-7 dph larval dry weight (DW) during food deprivation. Akaike's Information Criteria (AIC) found the linear model best represented the regressed DW data of larvae maintained in 25 or 40 ‰ from brood stock fed fish oil (FO) or vegetable oil (VO) diet treatments. Slopes of lines were not significantly different from each other (P=0.5754)

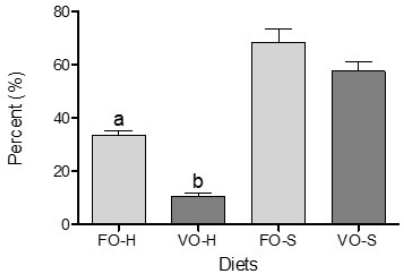

Figure 2 The effect of broodstock diets FO (Fish oil) and VO (vegetable oil) on percent hatching (H) and survival (S) at the end of the day of hatching (T0). Percent values having different letters were significantly different (P<0.05).

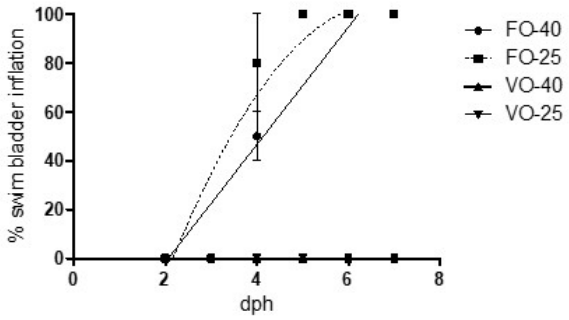

Figure 3 The rate of % swim bladder increase in in 2-6 dph larvae during food deprivation. Akaike's Information Criteria (AIC) analyzing regressed percent data of swim bladder inflation rate in larvae from broodstock fed the FO diet found the linear model (61.9%) best represented larvae in 40 ‰ sea water while a second order polynomial model (74.4%) best represented larvae in 25 ‰. There was no swim bladder inflation in larvae from the VO broodstock diet, irrespective of rearing salinity. The regression lines of swim bladder inflation in the FO larvae were highly significantly different (P<0.0001) than the regression lines of the VO larvae but not significantly (P>0.05) different from each other.

4. Discussion

The rearing conditions including diet may affect the pattern of energy usage and reserves that are mobilized towards reproduction. In this sense, artificial diets and confinement conditions have been reported to increase tissue fat composition (Rodríguez-Barreto et al., 2014). However, in this study the mobilization of energy reserves in terms of lipids and proteins is quite similar between wild and captive mature females (**Tables 6, 7**). Moreover, in fatty acids and fatty acid groups, there were no conspicuous differences, independent of age, between FO wild and captive FO broodstock and SO female broodstock gonads. Nevertheless, when comparing the fatty acid and lipid class profiles between female and male gonads, there were highly marked differences. Researchers have reported higher contents of total lipids, TAG and wax and sterol esters in female gonads compared to male gonads and higher quantities of phospholipids PC, PS and PE as well as cholesterol in male gonads compared to female gonads (Falch et al., 2006; Rodríguez-Barreto et al., 2014; Zuppa et al., 2017). These trends agree with the results in this study (**Tables 6 and 7**) and are independent of dietary regime (FO or SO). This suggests that another FO component, possibly carotenoids, are responsible for the observed benefit of the fish oil diet on larval performance. Carotenoids such as β-carotine or astaxanthin act as antioxidants and are involved in various physiological processes. Importantly, they are precursors for retinal and vitamin A synthesis that are critical in development.

Nevertheless, the use of fish oil in broodstock diets of mullet leading to better hatchability, tolerance of food deprivation and improved swim bladder inflation, which was found in this study, is largely supported in the literature. 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.

There are no definitive results tying n-3 LCPUFA in the broodstock diet with swim bladder inflation. In support of this, Koven et al. (1990) argued that there is no compelling evidence that n-3 LCPUFA markedly affected swim bladder inflation in gilthead sea bream. In contrast, Tandler et al. (1995) observed that a broodstock diet high in these essential fatty acids was associated with over 80% of the resulting larvae having a functional swim bladder compared to only 55% in the progeny of broodstock consuming a low n-3 PUFA diet. 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) and conceivably contributed to swim bladder development and inflation. In particular, a more functional rete mirabilis and pneumatic duct compared to the SO larvae, which are critical to successful swim bladder inflation.

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Deviation from DOW

It was not possible to carry out a separate broodstock experiment to determine the taurine requirement. As the juvenile requirement for taurine was determined to be 0.5% of DW diet, it was decided to include this level in the broodstock diet as well. Pigment and vitellogenin expression will be reported in D13.4.

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