New species for EU aquaculture

Deliverable Report

Deliverable No:	D13.4	**Delivery Month:**	62
Deliverable Title	Determine the effects of essential fatty acids and Tau in non fish-meal feeds on flesh and bottarga quality in grey mullet.		
WP No:	13	**WP Lead beneficiary:**	P4
WP Title:	Nutrition – grey mullet		
Task No:	13.3	**Task Lead beneficiary:**	P4
Task Title:	Determining grey mullet nutritional needs for a more cost-effective production.		
Other beneficiaries:	P.13 UNIBA	P. 15 ULL	P.18 CTAQUA	P.3 IRTA
Status:	Delivered	**Expected month:**	36
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Objective: To improve grow out and broodstock diets and bottarga quality

Subtask 13.3.1 Effect of DHA/EPA/ARA ration in non-fish meal grow out diets on fish performance (IOLR, Bill Koven).

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1. Introduction

The seawater servicing the 200 l V-tank experimental system is only filtered and not as yet UV treated as originally planned and is prone to bacterial infection. As a result the DHA study was performed in the 400 l v-tank hatchery system which has filtered (10

µm) and UV treated sea water that is also temperature and salinity controlled (Gavish, Israel).

Although, in general, the DHA requirement during the juvenile stage generally decreases compared to larval development, the necessity for this essential fatty acid is still considerable, particularly if the fish remains strictly carnivorous. However, the DHA requirement may be influenced further if the fish become omnivorous/herbivorous after metamorphosis and/or inhabit freshwater or brackish water. This is the case in the grey mullet, which appears to have some capability to produce Δ5 and Δ 6 desaturases and elongases to synthesize DHA, EPA and ArA from shorter chain 18 carbon n-3 and n-6 polyunsaturated fatty acid precursors. An indigenous production of essential LCPUFAs may result in a reduced dietary requirement for them. In fact, Zouiten et al., (2008) reported that mullet have desaturase activity as they begin to feed on plant and detritus as juveniles seek out less saline environments. The question remains what is the dietary DHA requirement and the contribution of this essential fatty acid to growth and body composition in juvenile mullet.

2. Methods and Materials

A study on the effect of dietary DHA on juvenile mullet performance, in terms of growth and survival was carried out in the late spring of 2016 on fish reared from hatching (26/1/16) at the ARDAG hatchery in Eilat, Israel and delivered to the IOLR when fish were 70 dph (2.7 ± 0.15 g) and maintained until the beginning of the experiment. The experimental system consisted of fifteen, 400 l V-tanks where UV treated, filtered (10 µm), ambient sea water (40 ‰) at 25 ^{o}C entered the bottom of the tanks and exited near the top through a 500 µm filter. Fish aged 138 dph (3.61 g ± 0.01 g) were stocked (45 fish tank^{-1}) in each of the tanks allowing the testing of three DHA treatments (0.4, 0.8 and 1.2 % DW diet) in replicates of 5 tanks per treatment until 222 dph (12 week experiment). Before stocking, 5 fish were sacrificed as a representative example at time 0 (T0) and samples of the liver, muscle and eyes taken for fatty acid analysis. The fish were fed 1 mm pellets produced by Sparos Ltd (Faro, Portugal) using the IOLR mullet closed formula at 5% of estimated tank biomass distributed over 5 rations per day.

At approximately monthly intervals a subsample of fish were individually live weighed and returned to the tank. The study continued until the fish had grown at least 200%. At the end of this part of the experiment and at the end of the study, the fish were weighed and fish samples taken from each tank for fatty acid analysis of the liver, eyes and muscle. The analysis of these samples has been delayed due to technical problems with the gas chromatograph and has recently resumed.

3. Results

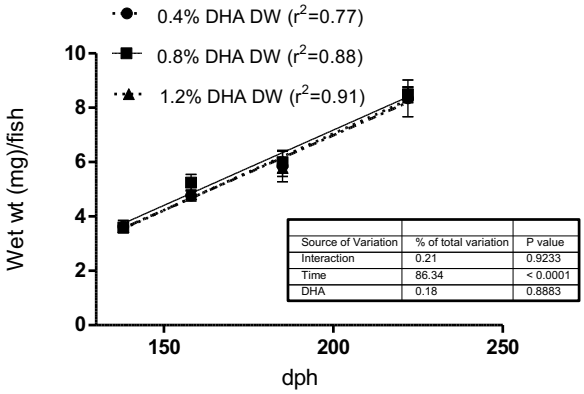

Figure 13.3.1.1. The effect of dietary DHA on wet weight with days post hatching (dph) (n=4). Two way ANOVA found that wet weight significantly increased with time (dph) (P<0.0001) which was not markedly affected by dietary DHA (P>0.05).

In **Fig. 13.3.1.1** wet weight gain was almost exclusively (86.34 % of variation) the result of age and not dietary DHA. All diets showed linear growth (linear was compared with non-linear growth using AIC analysis) with relatively high r^2 values while the DHA effect on size distribution was negligible (0.18%). In addition, all treatment fish exhibited generally similar size distribution in their respective populations (**Fig. 13.3.1.2**). All DHA treatments demonstrated very high percent survival which were 92.4, 88.8 and 97.6% in the 0.4, 0.8 and 1.2% DHA DW diets, respectively (**Fig. 13.3.1.3**).

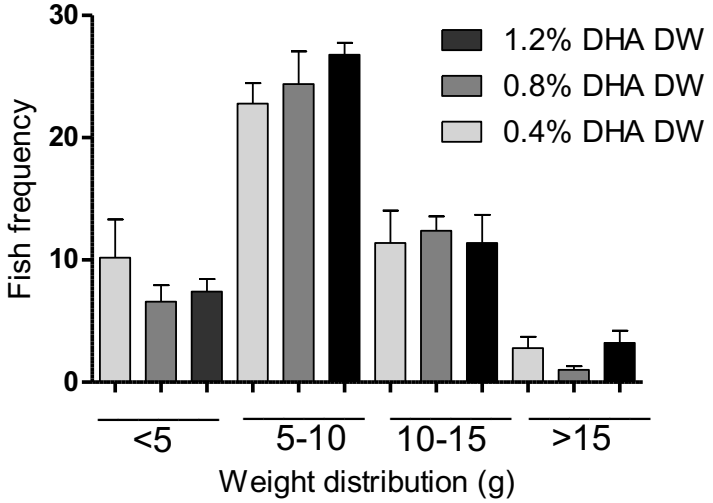

Figure 13.3.1.2 The effect of dietary DHA on weight distribution in 222 dph fish fed the 0.4, 0.8 and 1.2% dietary DHA treatments. (n=4). ANOVA was not significant (P>0.05).

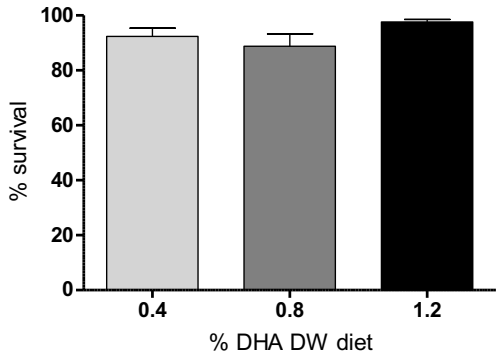

Figure 13.3.1.3 The effect of DHA diets (0.4, 0.8 and 1.2 % DHA DW diet) on percent (%) survival at the end of the experiment (n=4). ANOVA was not significant (P>0.05).

4. Discussion

The DHA requirement for juvenile fish, although generally less than during the larval stages, can still be considerably high. However, fish living in fresh water and/or are omnivorous or herbivorous after metamorphosis may have varying capability to produce sufficient Δ6 and Δ5 desaturases and elongases. Consequently, they may be able to synthesize DHA, EPA and ArA from their shorter chain precursors linolenic (18:3n-3) and linoleic (18:2n-6) fatty acids if they are present in the diet (Yu and Sinhuber, 1975). In contrast, marine species have very limited elongase and desaturase capability, requiring the ingestion of fully formed EFA for good growth and survival (Watanabe, 1982; Sargent and Henderson, 1995; Izquierdo, 1996). Grey mullet larvae, as in all teleosts at this developmental stage, are strict carnivores but incrementally transit to an omnivorous/herbivorous mode of feeding after metamorphosis. As they search out the less saline waters of estuaries, their DHA requirement as well as their capability to elongate and desaturate shorter chain precursors to LCPUFAs may change. Indeed, preliminary results from Deliverable 13.1 show an increasing gene expression of Δ6 desaturase and elongases that are salinity dependent.

5. References

Izquierdo, M.S., 1996. Review article: essential fatty acid requirements of cultured marine fish larvae. Aquac. Nutr. 2, 183–191.

Sargent, J.R., Henderson, R.J., 1995. Marine (n -3) polyunsaturated fatty acids. In: Hamilton, R.J. (Ed.), Developments in Oils and Fats. Blackie Academic and Professional, London, pp. 32-65.

Watanabe, T., 1982. Lipid nutrition in fish. Comp. Biochem. Physiol. 73, 3-15.

Yu, T.C., Sinnhuber, R.O., 1975. Effect of dietary linolenic and linoleic acids upon growth and lipid metabolism of rainbow trout (Salmo gairdneri). Lipids 10, 63-66.

Zouiten, D., Khemis, I. Ben, Besbes, R., Cahu, C., 2008. Ontogeny of the digestive tract of thick lipped grey mullet (Chelon labrosus) larvae reared in "mesocosms." Aquaculture 279, 166–172.

Subtask 13.3.2 Effect of four levels of Tau supplementation to best performing DHA/EPA/ARA non-fish meal grow-out diet from 13.3.1 on fish performance (IOLR, Bill Koven).

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1. Introduction

The β-amino sulfonic acid taurine plays an array of critical roles that include involvement in bile salt synthesis, anti-oxidative defense, cellular osmoregulation, as well as contributing to visual, neural and muscular function (Fang et al., 2002; Omura and Inagaki, 2000). Taurine is not found in terrestrial plant protein sources but is well represented in the natural diet of many marine species where fish meal is particularly taurine rich (5–7 mg g^{-1} DM) (Yamamoto et al., 1998). The taurine requirement in juvenile fish varies widely and is species dependent. For example the taurine requirements in juvenile Florida pompano (*Tranchinotus carolinus*) was 0.54-0.65% (Salze et al., 2014), 0.32-1.5% in California yellowtail (*Seriola lalandi*) (Jirsa et al., 2014), 1.15% in turbot (Qi et al.. 2012), 0.5% in cobia *Rachycentron canadum* (Lunger et al. 2007), 0.5% in dentex (*Dentex dentex*) (Chatzifotis et al. 2008), 0.6-1.6% in Japanese flounder, *Paralichthys olivaceus* (Kim et al 2007) and 0.5% in red sea bream, *Pagrus major* (Matsunari et al. 2008a,b). On the other hand, there was no reported taurine requirement in herbivores/omnivores such as the Red hybrid tilapia (Divakaran et al. 1992), channel catfish, *Ictalurus punctatus* (Robinson et al. 1978) and common carp, *Cyprinus carpio*, (Kim et al. 2008). As the grey mullet transits from carnivorous larvae in the sea to omnivorous juveniles in lower saline estuarine waters, the aim of the present study was to determine the taurine requirement in juvenile fish of this species that may have the capacity for taurine synthesis.

2. Methods and Materials

The experimental system consisted of sixteen, 400 l V-tanks where UV treated, filtered (10 µm), ambient sea water (40 ‰) at 25°C (computer controlled; Gavish, Israel) entered the bottom of the tanks and exited near the top through a 500 µm filter at a rate of 7 tank exchanges per day. This allowed the testing of four taurine 1 mm pelleted diets (0, 0.5, 1.0 and 2.0% DW diet) (Sparos Inc., Faro, Portugal) in replicates of 4 tanks per treatment. The experimental system was exposed to a light intensity of 500 lx with a photoperiod of 11 L/13 D. Each tank was stocked with 38 fish (126 dph) and fed their respective diets at

4% of tank biomass per day distributed over 5 rations. The experiment continued for 58 days (184 dph) where the fish had grown at least 100%. At the end of this period the weight of each fish and length was measured while 4 fish from each tank were sampled for fatty acid and taurine analyses of their eyes, muscle and liver. The digestive tract (DT) from 16 fish per tank was dissected out. Eight DTs were frozen at -80 ºC for RNA extraction (Pept1 gene expression) while the other 8 fish were placed in buffered formalin for histology. Five fish from each tank were frozen at -20 ºC for proximate analysis.

3. Results and discussion

The results showed that grey mullet juveniles have a minimum 0.5% requirement for dietary taurine **(Fig. 13.3.2.1),** which is within the range of taurine requirements measured in a variety of marine species such as the Florida pompano (Salze et al., 2014), California yellowtail (Jirsa et al., 2014b), cobia *Rachycentron canadum* (Lunger et al. 2007), dentex, *Dentex dentex*), (Chatzifotis et al. 2008), Japanese flounder, *Paralichthys olivaceus* (Kim et al 2007) and red sea bream, *Pagrus major* (Matsunari et al. 2008a,b). Although these fish were grown in 40 ‰ and showed a taurine requirement, they still exhibited taurine synthesis capability (see preliminary results in **Sub-task 13.2.1**), which increased in both the CSD and ADO pathways up to 1% taurine DW diet but then decreased substantially in the highest taurine diet (2% DW diet). This suggests that the overall taurine requirement might be higher than 0.5% as part of the taurine requirement is satisfied by endogenous synthesis of this nutrient. On the other hand, fish fed the 2% taurine diet may be ingesting excessive levels of taurine resulting in decreased production of endogenous taurine.

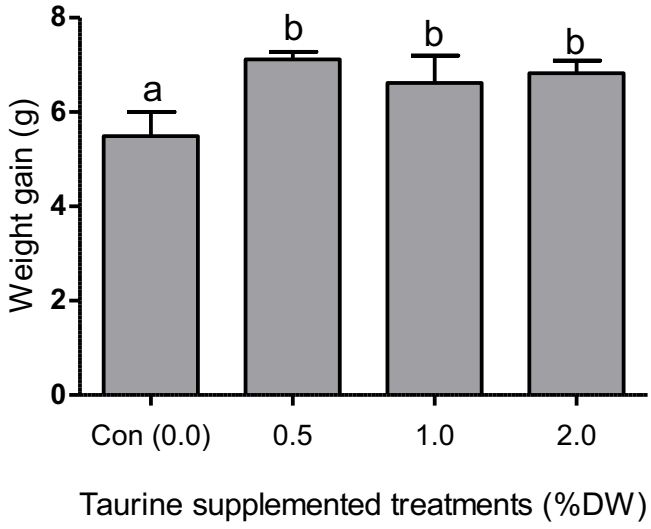

Figure 13.3.2.1 The effect of the taurine diets (control-0, 0.5, 1.0 and 2.0% DW diet) on average weight gain per fish per treatment (n=4). ANOVA was significant (P<0.05) where fish fed all diets containing taurine exhibited superior weight gain compared to the control fish.

Although there was a tendency for diets from 0 to 1% taurine to produce less smaller and more larger fish, overall there was no significant (P>0.05) dietary taurine effect on size distribution in the population. Once all the analyses are completed, the results should demonstrate a more comprehensive picture of the effect of dietary taurine on juvenile grey mullet.

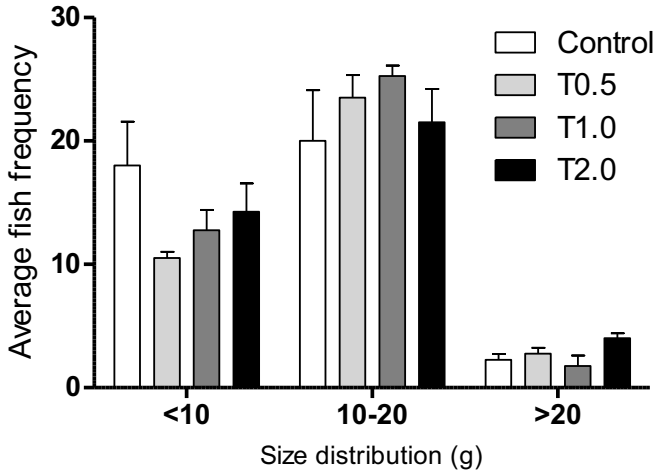

Figure 13.3.2.2 The effect of the taurine diets (control-0, 0.5, 1.0 and 2.0% DW diet) on size distribution. ANOVA analysis was not significant.

References

Chatzifotis, S., Polemitou, I., Divanach, P., Antonopoulou, E., 2008. Effect of dietary taurine supplementation on growth performance and bile salt activated lipase activity of common dentex, *Dentex dentex*, fed a fish meal/soy protein concentrate-based diet. Aquaculture 275, 201–208.

Divakaran, S., Ramanathan, S., Ostrowski, A.C., 1992. Endogenous production of taurine in two teleost fish: *Coryphaena hippurus* and red hybrid tilapia. Comp. Biochem. Physiol. B Comp. Biochem. 101 (3), 321–322.

Fang, Y., Yang, S., Wu, G., 2002. Free radicals, antioxidants, and nutrition. Nutrition 18, 872–879.

Jirsa, D.O., Stuart, K.R., Salze, G.P., Rhodes, M.A., Davis, D.A., Drawbridge, M.A., 2014. Limiting amino acids in practical diets for California yellowtail *Seriola lalandi*. J. World Aquacult. Soc. 45, 681–690.

Kim, S.K., Matsunari, H., Takeuchi, T., Yokoyama, M., Furuita, H.,Murata, Y., Goto, T., 2008. Comparison of taurine biosynthesis ability between juveniles of Japanese flounder and common carp. Amino Acids 35, 161–168.

Kim, S.-K., Matsunari, H., Takeuchi, T., Yokoyama,M., Murata, Y., Ishihara, K., 2007. Effect of different dietary taurine levels on the conjugated bile acid composition and

growth performance of juvenile and fingerling Japanese flounder *Paralichthys olivaceus*. Aquaculture 273, 595–601

Lunger, A.N., McLean, E., Gaylord, T.G., Kuhn, D., Craig, S.R., 2007. Taurine supplementation to alternative dietary proteins used in fish meal replacement enhances growth of juvenile cobia (*Rachycentron canadum*). Aquaculture 271, 401–410.

Matsunari, H., Furuita, H., Yamamoto, T., Kim, S.-K., Sakakura, Y., Takeuchi, T., 2008a. Effect of dietary taurine and cystine on growth performance of juvenile red sea bream *Pagrus major.* Aquaculture 274,142–147.

Matsunari, H., Yamamoto, T., Kim, S.-K., Goto, T., Takeuchi, T., 2008b. Optimum dietary taurine level in casein-based diet for juvenile red sea bream *Pagrus major.* Fish. Sci. 74, 347–353.

Omura, Y., Inagaki,M., 2000. Immunocytochemical localization of taurine in the fish retina under light and dark adaptations. Amino Acids 19, 593–604.

Qi, G., Ai, Q., Mai, K., Xu, W., Liufu, Z., Yun, B., Zhou,H., 2012. Effects of dietary taurine sup- plementation to a casein-based diet on growth performance and taurine distribution in two sizes of juvenile turbot (*Scophthalmus maximus* L.). Aquaculture 358–359, 122–128.

Robinson, E.H., Allen, O.W., Poe, W.E., Wilson, R.P., 1978. Utilization of dietary sulfur com- pounds by fingerling channel catfish: L-methionine, DL-methionine, methionine hydroxy analogue, taurine and inorganic sulfate. J. Nutr. 108, 1932–1936.

Salze, G.P., Davis, A.D., Rhodes, M.A., 2014. Quantitative requirement of dietary taurine in Florida pompano *Trachinotus calorinus*. Aquaculture America 2014, Seattle, WA.

Yamamoto, T., Akimoto, A., Kishi, S., Unuma, T., Akiyama, T., 1998. Apparent and true availabilities of amino acids from several protein sources for fingerling rainbow trout, common carp and red sea bream. Fish. Sci. 64, 448–458.

Sub-task 13.3.4 (IOLR, Bill Koven) The selected feed from Sub-tasks 13.3.1, 13.3.2, 13.3.3 will be compared to the current feed on the market used for mullet culture and fed to adult mullet until gonadal maturation.

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1.0 Introduction

Carotenoids in fish, such as astaxanthin and β carotene have a range of functions that include protection against ultraviolet rays, production of provitamin A, tolerance against hypoxia, better growth performance, enhancement of the immune system, and as antioxidants (Torrissen and Christiansen, 1995). Carotenoid synthesis occurs only in plants algae, bacteria and fungi while all animals are incapable of producing these compounds (Moller et al. 2000). Grey mullet can ingest carotenoids through the consumption of micro and macroalgae, epiphytes, small invertebrates and detritus. Once these compounds are absorbed in the middle and terminal parts of the digestive tract, they accumulate in the muscles, liver and skin (Chatzifotis et al. 2005). During female gonad development, carotenoid is released into the circulatory system from liver and muscle tissues before transferring to the ovaries. Carotenoids can directly affect the rate of glycogen storage in the liver (Barber et al. 2000) and improve egg maturation (Kerfeld et al., 2003) and are critical scavengers of free radicals (Izquierdo et al. 2001). A highly absorbed carotenoid, astaxanthin, improves egg buoyancy, hatching rate and larval vitality and health (Sawanboonchun et al., 2008) as well as contributing to the desired color of bottarga. In a previous deliverable (D13.3), we suggested that it was the carotenoids in the fish oil diet that improved hatching rate as well as larval swim bladder inflation and not n-3 long chain polyunsaturated acids (LCPUFA). In fact, the results from D13.1 suggested that grey mullet has EPA and DHA synthesis capability, which may explain why long chain polyunsaturated fatty acids were very similar in the female gonads of broodstock fed either a fish oil (FO) or soybean oil (SO) based diet, but differed markedly in their n-3 and n-6 PUFA content. On the other hand, the biosynthesis of LCPUFA in our studies demonstrated that this pathway is influenced by lower salinity and it is unknown if broodstock mullet retain this capability during spawning in seawater.

In this experiment we have taken these results one step further and tested if dietary carotenoid supplementation also enhance egg roe size and coloration as an initial stage for controlled bottarga (karasumi) production.

2.0 Materials and methods

Experimental design.

Grey mullet captive broodstocks consisting of 3-year old (G2 domesticated) females and males, were maintained in outdoor 4 m^3 tanks supplied with ambient (Red Sea) seawater at 40 ‰ salinity which were exposed to natural fluctuations of light and temperature. Fish were fed daily at the rate of 1-1.5% of their body weight using the IRIDA extruded diet (based on the IOLR formula) and contained 35% protein and 7.2% lipid. During early June, concomitant with the onset of gametogenesis, the fish were divided into two groups and fed over 3 months with the IOLR pelleted diet containing either fish oil (FO) or soybean oil (SO) as the main neutral lipid (see D13.3). However, the FO pelleted diet was also supplemented with Marigold petal meal (MgM; 3 mg kg^{-1} feed) as another carotenoid source, apart from the fish oil and 3% dry Ulva (produced at the IOLR). 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}. During mid-September, coinciding with advanced stages of gametogenesis, 10 to 12 fish were sampled from each dietary group and the following parameters were measured (**Table 13.3.4.1**); total length (cm), body weight (BW;g), gonadal weight [GW;g), liver weight [LW;g] and viscera weight (g). The gonado-somatic and hepato-somatic indices were calculated as GSI = 100xGWxBW^{-1} and HSI = 100xLWxBW^{-1}, respectively.

Gonadal histology and reproductive status assessment.

For histological evaluations of maturational stage, fixed gonad samples were dehydrated in increasing ethanol concentrations, clarified in xylene and embedded in paraffin wax. Five µm thick sections were cut and stained with hematoxylin-eosin. For the classification of the reproductive status of females, the most advanced oocyte stage was recorded for each specimen,

according to the scheme used by Corriero et al. (2007). For the classification of the reproductive status of males, the type of spermatogenic cysts was recorded and the quantity of spermatozoa in the lumen of seminiferous lobules was evaluated subjectively, as in Corriero et al. (2007).

In order to determine oocyte yolk accumulation in wild, the largest vitellogenic oocytes, which had a large and centrally located nucleus, were selected. Oocyte diameter (µm) and surface occupied by yolk granules (μm^2) were measured from microphotographs taken with a digital camera (DFC 420; Leica, Cambridge, UK) connected to a light microscope (DIAPLAN; Leitz, Wetzlar, Germany), using an image analysis software (Leica Application Suite, version 3.3.0; Cambridge, UK).

Gonad proximate composition, lipid classes and fatty acid profiles

To evaluate gonad biochemical composition, samples of ovaries and testes 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 (1982) 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 only from gonads at advanced gametogenesis and spawning periods, 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.

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 ºC for 5 min and the supernatant diluted 1:10 using 0.5% metaphosphoric acid before injection into HPLC system (FAO, 1997).

Fat-soluble vitamins A, D and E analyses were carried out by hot saponification at 100 ºC 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).

Quantification of vitamins

Vitamin analysis were performed using a ThermoScientific ultra high performance liquid chromatograph (ThermoFisher 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 (ThermoFisher 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.

Vitellogenin reverse transcription and real-time PCR

Total RNA was obtained from gonad and liver using the RNeasy Mini Kit (Qiagen) as described by the manufacturer. The RNA was re-suspended in 50 µl of RNase free water and stored at –80°C. The cDNA was prepared from 1.2 µg total RNA. Random hexamer primers were used for the cDNA synthesis using SuperScript III Reverse Transcriptase (Invitrogen).

Vitellogenin (VgA, VgB and VgC) primers were designed (**Table 13.3.4.2**) against the relevant grey mullet Vgs sequences reported in GenBank (accession number AB288932, AB288933, AB288934) using the Primer3 software (Rozen & Skaletsky, 2000) and their specificity was checked with both in silico (the UCSC "In-Silico PCR" and the NCBI Primer-BLAST tools) and by means of agarose gel electrophoresis. It was confirmed that the VgA primer set did not produce any amplified fragments using as a template VgB or VgC cDNA, and vice versa. Total RNA was obtained from liver using the RNeasy Mini Kit (Qiagen) as described by the manufacturer. Reverse transcription of 1000 ng of total RNA was performed using SuperScript III Reverse Transcriptase (Invitrogen®) and diluted cDNA (1:10) was used in all following qPCR reactions. For gene expression analysis qRT-PCR experiments were carried out in triplicate using the QuantStudio™ 7 Flex System (Applied Biosystems®, Thermo Fisher SCIENTIFIC) using 1µl of diluted cDNA as template for each reaction with SYBR Green PCR Master Mix (Bio-Rad). No template controls were included as negative controls for each primer pair. The quantification of the β-actin gene was used as the endogenous control. Amplification parameters were as follows: hot start at 95°C for 15 min; 40 amplification cycles (95°C for 15 sec, 60°C for 30 sec, 72°C for 30 sec). Fluorescence raw data were exported from the QuantStudio Real Time PCR software (Applied Biosystems®, Thermo Fisher SCIENTIFIC) and analyzed with the DART-PCR Excel workbook (Peirson et al., 2003). Actual amplification efficiency values (E) for each amplicon were used to correct Cq values before analyzing these data by the ΔCq method to compare relative expression results. Gene expression levels were calculated by: relative expression = $2^{-\Delta Ct}$ (Livak & Schmittgen, 2001).

Ovarian Vgs transcription levels were expressed as mean ± SD and differences between wild and hatchery-produced fish were assessed through the Student's t-test.

Oocyte yolk accumulation was compared in oocytes of similar diameter from wild and hatchery-produced grey mullet. In total, 42 oocytes from hatchery-produced individuals and 23 oocytes from wild individuals (mean oocyte diameter: 395.2 ± 18.5 µm *vs* 414.4 ± 3.3 µm; P = 0.06) were selected from haematoxylin-eosin stained sections obtained from ovary samples fixed in Bouin's solution and embedded in paraffin wax (D. 7.5). All the analysed oocytes were at late stage of vitellogenesis with a large and centrally located nucleus. Oocyte diameter (µm) and surface occupied by yolk granules (μm^2) were measured from microphotographs taken with a digital camera (DFC 420; Leica, Cambridge, UK) connected to a light microscope (DIAPLAN; Leitz, Wetzlar, Germany), using an image analysis software (Leica Application Suite, version 3.3.0; Cambridge, UK). Differences in mean values of oocyte diameter and oocyte surface occupied by yolk granules among hatchery-produced and wild grey mullet were assessed by Student's t-test. The statistical probability significance was established at the $P < 0.05$ level.

3.0 Results

In **Table 13.3.4.3,** the fatty acid profiles of the female gonads from broodstock fed the FO+MgM (marigold meal) or SO diets demonstrated no marked differences between fatty acid groups while the polar lipids, particularly phosphatidylcholine, were lower in the SO gonads compared to the FO+MgM gonads (**Table 13.3.4.4**). Conversely, in **Table 13.3.4.4** the FO+MgM gonads were lower in neutral lipids than the SO gonads, particularly wax and sterol esters.

Although the FO+MgM dietary treatment demonstrated significantly ($P<0.05$) higher carotenoids than the SO diet (**Fig. 13.3.4.1**), no dietary effects on body or gonad weights were observed (**Table 13.3.4.1**). Interestingly, in both treatment groups only half of the females had fully developed gonads (GSI: 14.56 ± 1.47 %) with oocytes (mean diameter 527 ± 8.4 µm) at the late vitellogenic stage (**Fig. 13.3.4.2C**). The gonads of all other females were undeveloped (GSI: 0.26 ± 0.02%) and showed cortical alveoli as the most advanced oocyte stage (**Fig. 13.3.4.2A**). A similar bipolar gonadal development could be seen in males of the two treatment groups (**Fig. 13.3.4.2 B, D**). Nonetheless, the GSI values of the most advanced (1.69 ± 0.42%) and undeveloped (0.013 ± 0.01%) males were an order of magnitude less than the females (**Table 13.3.4.1**).

On the other hand, **Fig. 13.3.4.3** clearly shows that fish receiving the (A) FO+MgM had distinctly yellow gonads compared to (B) the pale, colourless gonads from females fed the SO diet and this is supported by the significantly ($P<0.05$) higher carotenoid level in the FO+MgM female gonads compared to those feeding on the FO diet (**Fig. 13.3.4.4).**

Table 13.3.4.1 Morphometric parameters (i.e., total length, weight of the body, gonads, liver and viscera) and body indices (GSI and HSI) of 3-year old mullet fed a fish oil based diet (FO) or a vegetable oil based diet (SO).

	sex	Body weight (g)	Total length (cm)	Viscera weight (g)	Liver weight (g)	Gonadal weight (g)	GSI (%)	HIS (%)	Oocyte diameter (µm)
so	F	240	30	18.93	2.56	0.55	0.23	1.06	
	F	390	35	33.73	5.83	1.00	0.26	1.49	
	F	470	35	41.80	6.15	1.55	0.33	1.31	
	F	580	37	18.0	4.68	102.0	17.59	0.81	≥590
	F	1100	46	48.79	10.15	169.29	15.39	0.92	≥600
	F	780	44	34.77	6.84	122.78	15.68	0.88	≥600
	F	525	39	38.94	9.56	51.43	9.80	1.82	≥470
	F	480	37	41.22	6.03	1.27	0.26	1.26	
	M	340	33	38.79	4.76	0.15	0.04	1.40	
	M	580	40	21.36	5.40	15.96	2.75	0.93	
	M	440	36	17.01	6.10	7.87	1.79	1.39	
	M	240	25	24.17	2.15	0.0	0.00	0.89	
FO	F	1143	48	59.09	11.87	114.0	9.97	1.04	≥500
	F	780	42	20.65	4.59	159.22	20.41	0.59	≥600
	F	530	37	29.60	5.67	69.49	13.11	1.07	≥500
	F	180	29	13.56	1.50	0.45	0.25	0.83	
	F	370	24	31.05	4.01	1.08	0.29	1.08	
	F	360	25	35.06	3.17	0.67	0.19	0.88	
	M	670	41	38.12	17.96	9.85	1.47	2.68	
	M	230	29	19.54	1.92	0.03	0.01	0.83	
	M	430	36	26.16	5.19	3.16	0.73	1.21	
	M	210	24	8.77	0.9	0.0	0.00	0.43	

Table 13.3.4.2 Primers for greater amberjack real-time PCR

VgA FOR	GCAGTAGACTCAGCTCTTCAG
VgA REV	CAGCCTGGGAGGAGTGAGC
VgB FOR	ATCCCCGCTGACCTGTCAAG
VgB REV	TGACTGGTCCAGCTGGGGC
VgC FOR	CCACAGTGAGATGTGTTTACAC
VgC REV	TCTCCATTGGCCCGAACGTG
β-actin FOR	CCTTCTACAACGAGCTGAGAG
β-actin rev	CGTCATGGACTCCGGTGATG

Table 13.3.4.3 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)

	FO+MgM		SO	
	Female	Male	Female	Male
Total FA	90.24 ± 4.62	16.09 ± 2.65	81.12 ±14.86	11.33 ± 2.03
14:0	0.46 ± 0.02	0.85 ± 0.02	0.61 ± 0.09	0.66 ± 0.11
16:0	7.57 ± 0.63	19.56 ± 0.20	8.24 ± 1.11	21.51 ± 0.05
18:0	2.36 ± 0.18	5.00 ± 0.16	2.53 ± 0.17	5.62 ± 0.28
Total SFA	10.70 ± 0.75	26.80 ± 0.23	11.87 ± 1.34	29.79 ± 0.24
16:1[1]	6.13 ± 0.45	2.27 ± 0.31	6.45 ± 0.96	1.80 ± 0.22
18:1[2]	38.41 ± 1.15	16.91 ± 0.99	37.43 ± 0.32	14.55 ± 1.01
20:1[2]	7.35 ± 0.84	3.73 ± 0.47	6.25 ± 1.14	3.09 ± 0.01
22:1[2]	0.44 ± 0.04	1.80 ± 0.19	0.33 ± 0.00	2.21 ± 0.49
Total MUFA	47.39 ± 1.95	25.75 ± 0.80	47.01 ± 0.48	20.26 ± 0.25
18:2n-6	16.61 ± 1.96	9.63 ± 0.60	16.82 ± 1.42	8.32 ± 0.48
20:2n-6	0.90 ± 0.06	2.09 ± 0.14	0.93 ± 0.04	1.97 ± 0.03
20:4n-6	0.54 ± 0.06	2.29 ± 0.49	0.55 ± 0.07	2.42 ± 0.10
Total n-6 PUFA	19.74 ± 1.87	15.45 ± 1.45	19.91 ± 1.22	13.92 ± 0.29
18:3n-3	1.64 ± 0.27	0.74 ± 0.08	1.50 ± 0.29	0.54 ± 0.13
18:4n-3	0.22 ± 0.19	nd	0.31 ± 0.08	nd
20:5n-3	1.46 ± 0.15	2.21 ± 0.22	1.41 ± 0.30	1.69 ± 0.09
22:5n-3	2.60 ± 0.21	3.79 ± 0.29	2.64 ± 0.40	2.96 ± 0.09
22:6n-3	10.91 ± 0.88	23.53 ± 3.06	10.84 ± 1.85	28.27 ± 1.74
Total n-3 PUFA	18.27 ± 1.26	31.19 ± 2.43	18.15 ± 2.89	34.18 ± 1.58
DHA/EPA	7.53 ± 0.90	10.78 ± 2.47	7.75 ± 0.85	16.79 ± 1.93
ARA/EPA	0.37 ± 0.01	1.03 ± 0.12	0.39 ± 0.04	1.44 ± 0.13
n-3/n-6	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; MgM, marigold meal; DHA, docosahexaenoic acid, 22:6n-3; EPA, eicosapentaenoic acid, 20:5n-3; ARA, arachidonic acid, 20:4n-6. nd, not detected.

Table 13.3.4.4 Lipid content (% dry matter) and lipid class composition (% total lipid) of gonads from 3 years old mullet fed a fish oil based diet with marigold petal meal (FO+MgM) or a soybean oil based diet (SO).

	FO+MgM		SO	
	Female	Male	Female	Male
Total lipid	30.63±5.98	13.58±1.52	35.87±3.43	15.63±1.70
Lipid class				
Lysophosphatidylcholine	0.47±0.15	1.12±0.09	nd	nd
Sphingomyelin	0.57±0.24	0.82±0.02	0.65±0.42	1.47±0.26
Phosphatidylcholine	9.66±0.11	12.37±0.5	6.80±0.38	13.18±0.43
PS + PI *	1.25±0.22	9.16±0.96	0.34±0.12	8.08±0.20
Phosphatidylglycerol	nd	nd	0.23±0.28	2.41±0.70
Phosphatidylethanolamine	2.08±0.87	15.58±1.2	1.78±0.14	14.94±0.91
Total Polar Lipids	**14.03±0.94**	**39.04±0.11**	**9.81±0.67**	**40.08±1.99**
Diacylglycerols	nd	nd	1.42±0.35	5.66±1.12
Cholesterol	10.41±0.30	45.08±1.03	7.38±0.34	37.54±0.99
Free fatty acids	2.10±0.67	4.36±0.21	0.80±0.42	1.17±0.21
Triacylglycerols	14.54±2.68	7.72±1.90	11.43±2.16	10.72±0.44
Wax + Sterol esters	57.10±3.04	1.43±0.33	68.59±3.00	4.83±1.62
Unknown	1.82±0.31	2.37±0.89	0.56±0.23	nd
Total Neutral Lipids	**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. MgM= marigold meal, PS=Phosphatidylserine, PI= Phosphatidylinositol.* mainly PS

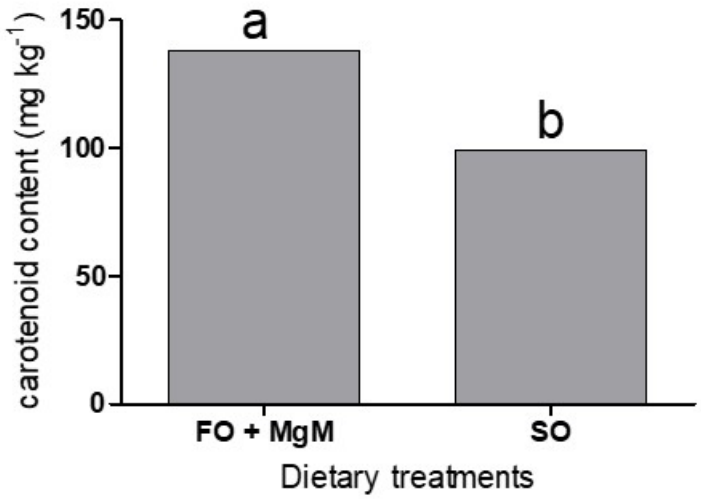

Figure 13.3.4.1 Carotenoid content of the different diets. The fish oil (FO) + marigold petal meal (MgM) and soybean oil (SO) treatments were based on the IOLR formula that were pelleted in a California pelleting mill.

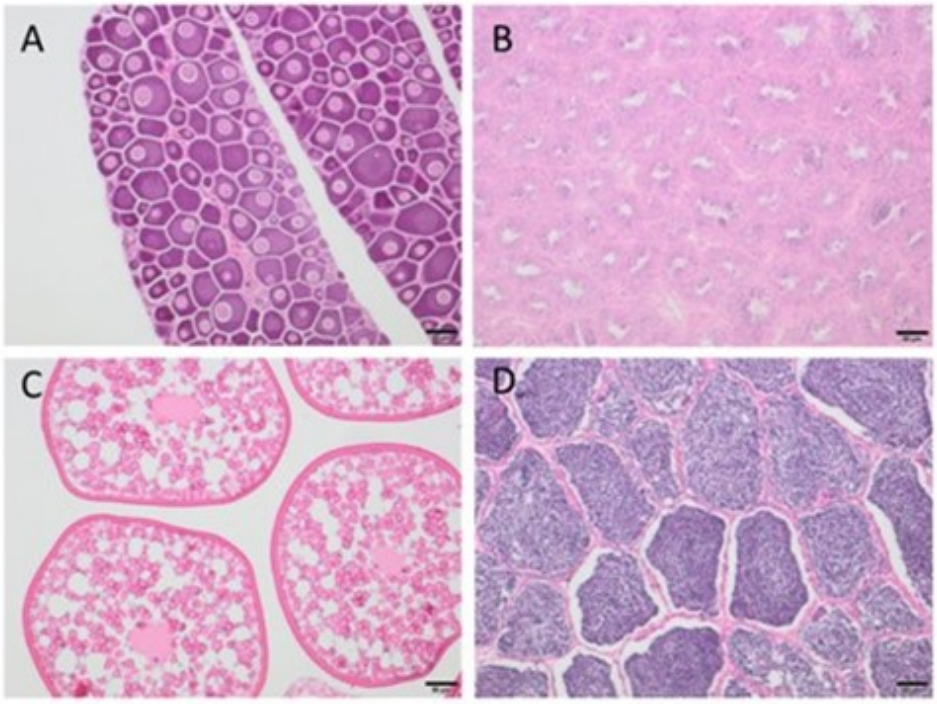

Figure 13.3.4.2 Micrographs of 3-year old captive grey mullet gonad sections. Ovaries from sexually immature and mature specimens exhibiting oocytes at cortical alveoli (A) and late vitellogenic stage (C), respectively. Testis from sexually immature and mature specimens showing germinal epithelium constituted mainly by spermatogonia (B) and luminal spermatozoa (D), respectively. Haematoxylin-eosin staining. Magnification bars = 50 µm.

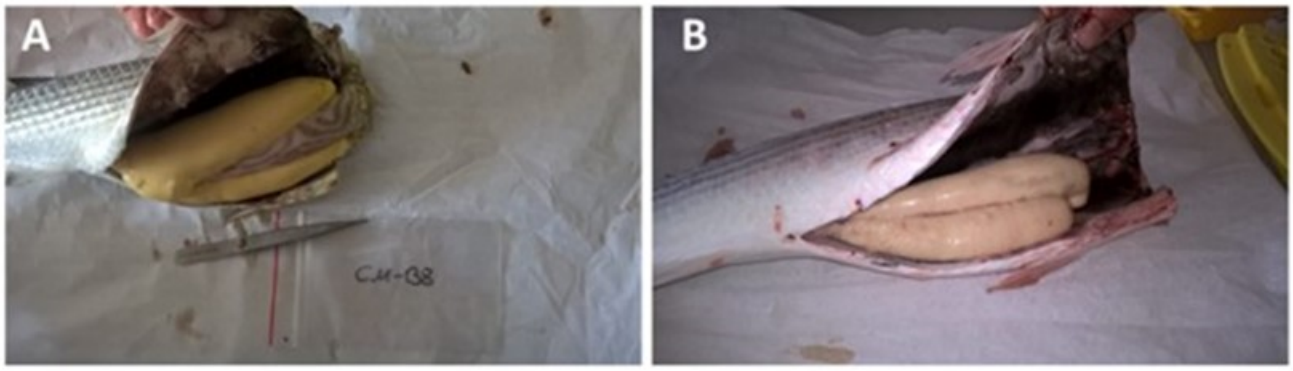

Figure 13.3.4.3 Roe derived of 3-year old captive mullet fed (A) a fish oil based diet (FO) demonstrating a yellow colour and (B) a vegetable oil based diet (SO) showing a lack of colour.

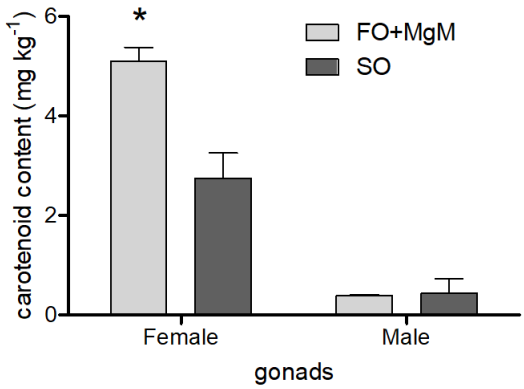

Figure 13.3.4.4 The effect of the fish oil +marigold petal meal (FO+Mgp) and soybean oil (SO) on carotenoid (mg kg^{-1}) levels in female and male gonads. The asterisk (*) represents a significant ($P<0.05$) difference between bar values in female gonads.

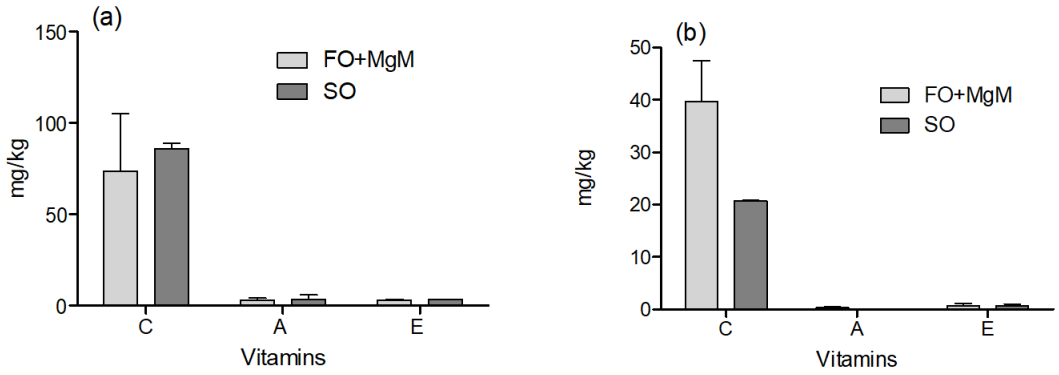

Figure 13.3.4.5 The gonadal vitamins C, C and E in (a) female and (b) male gonads

There was markedly more gonadal vitamin C than vitamins A and E in both female and male gonads while no dietary effect was observed on these nutrients in the female gonads. On the other hand, male gonads from fish fed the FO+MgM diet demonstrated higher vitamin C than male gonads from fish fed the SO diet (**Fig. 13.3.4.5**).

Mean values of Vgs mRNA expression levels relative to β-actin of grey mullet individuals showing vitellogenic oocytes are reported in **Fig. 13.3.4.6**. Liver VgA and VgB transcript levels of hatchery-produced individuals were significantly lower than wild breeders, whereas no difference in VgC transcript levels was found between the two groups (**Fig. 13.3.4.6**).

Wild grey mullet sampled during their migration from the Lesina Lagoon to the open sea spawning grounds showed significantly larger oocytes at late vitellogenesis stage than hatchery-produced individuals (487.4 ± 10.0 *vs* 428.5 ± 7.9; $P<0.05$). However, the yolk content of oocytes from hatchery-produced grey mullet did not differ from that of oocytes having similar diameter from wild adults (**Fig. 13.3.4.7**).

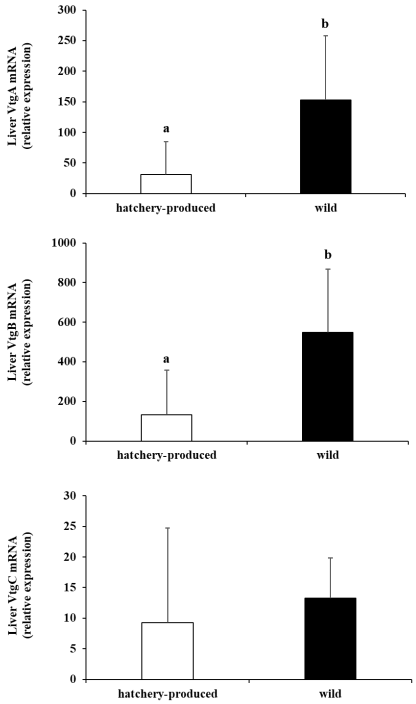

Figure 13.3.4.6 Mean vitellogenin A (VgA), vitellogenin B (VgB) and vitellogenin C (VgC) expression levels relative to β-actin in wild and hatchery-produced grey mullet.

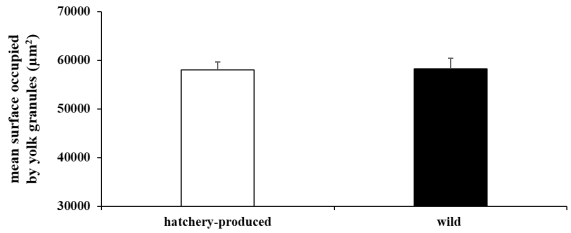

Figure 13.3.4.7 Mean surface occupied by yolk granules in late vitellogenic oocytes having similar diameter from hatchery-produced and wild grey mullet.

4.0 Discussion

In the Mediterranean, captive reared grey mullet brooders fail to proceed with gametogenesis. Males with running milt are rarely observed (De Monbrison et al., 1997; Yashouv, 1969), and female dysfunctions were confined to two critical phases, i.e., the early stages of vitellogenesis, and final oocyte maturation and ovulation (De Monbrison et al., 1997). Furthermore, Aizen et al. (2005) reported that certain female mullets (approximately 20%) in untreated groups manage to go through the processes of vitellogenesis, while most females do not. It was suggested that low percentages of fully mature females, surrounded by undeveloped females (of the same age and size), typify a state of social hierarchy, in which the dominant female(s) suppresses sexual maturation of conspecifics. Interestingly, the current study points to an increased abundance (50%) of spontaneously developed females that have managed to complete vitellogenesis. The main difference between the two studies was the origin of the fish: wild-caught captive reared (Aizen et al., 2005) vs. G2 domesticated broodstocks, suggesting potential effects of

domestication. Similar effects were demonstrated in the study of oogenesis in captive-reared vs. hatchery-produced grey mullet (D7.5). In the latter, analysis of GSI and oocyte diameter clearly confirmed that age 3 hatchery-produced specimens attained a more advanced ovarian development than fish caught from the wild and reared in captivity. Nonetheless, the 3-year old hatchery-produced grey mullets had less advanced oocytes than 4-6 year old wild breeders. About 50% of the hatchery-produced fish had late vitellogenic oocytes whose mean diameter was lower than that of late vitellogenic oocytes from wild individuals. VgA and Vg B liver gene expression was significantly lower in hatchery-produced compared to wild fish, whereas VgC expression was similar. Oocytes from hatchery-produced individuals had the same capacity to uptake vitellogenin and accumulate yolk proteins compared with oocytes of similar size from wild breeders. It was concluded that (i) the rearing condition established at IOLR allows a growth rate equivalent to that of the wild grey mullet population from the Mediterranean Sea, and (ii) hatchery-produced grey mullet have a good potential to develop ovaries spontaneously up to a condition useful for bottarga production (advanced vitellogenesis).

Grey mullet ovaries described 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. This pattern can be related to the role of neutral lipids as metabolic energy resources for oocyte formation (Zudaire et al., 2014) and embryo development. In fact, wax esters (WE) have been described as a particularly monounsaturated fatty acid (MUFA)-rich lipid fraction. It can be seen in the fatty acid profiles of all female gonads that the MUFA group is the most abundant one (45-50%).

In spite of 18:2n-6 being higher in the SO diet, homeostatic mechanisms seem to allow the broodstock to maintain a balance and similar profile of polyunsaturated fatty acids (PUFA) in the gonads, independently of the sex or diet, displaying also a similar pattern to that of the wild counterparts. Higher levels of 18:2n-6 and lower levels of EPA and DHA are normally present in herbivorous fish compared to carnivorous ones. Finally, it is also worthwhile to highlight that, independently of the diet, the testes always displayed lower contents of total fat and lower proportions of 18:2n-6 but much higher ones of DHA and ARA than the ovaries.

Unpredictable and variable reproductive performance is an important limiting factor for the successful mass production of juveniles. An improvement in broodstock nutrition and feeding has been shown to greatly improve not only egg and sperm quality but also seed production. Gonadal development and fecundity are affected by certain essential dietary nutrients, whereas early development in fish is also dependent on the essential nutrients present in the egg (Izquierdo et al., 2001). 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 reported. Certain types of vitamin D, such as cholecalciferols, are essential for bone formation and integrity whereas 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 and its deficiency affects reproductive performance, causing immature gonads and lower hatching rate and survival of offspring. 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.

Carotenoids including astaxanthin, 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. There is also evidence that carotenoids protection against oxidative damage is directly related to sperm functional fertility.

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 were investigated in D13.3 in order to collect necessary basic data for the future development of a quality diet for this species.

As shown in **Fig. 13.3.4.4** the carotenoid contents in female gonads of the FO+MgP diet was significantly ($P<0.05$) higher than those in females fed the SO diet females and similar to wild fish gonads while carotenoids present in male testes were lower and similar between the FO+MgM and the SO individuals.

Fig. 13.3.4.5 shows that the vitamin contents in gonads and eggs and confirms the importance quantitatively of vitamin C for reproduction and embryo development while vitamin A and E are at considerably lower levels but nonetheless are potent antioxidants in the female gonads and the eggs. These preliminary results suggest that further studies are necessary to establish if the quality of eggs of grey mullet are related to the carotenoid and vitamins availability.

Taken altogether, the supplementation of carotenoids to broodstock diets not only appears to improve larval performance (**D13.3**) but also contributes to bottarga coloration and a platform for further dietary and bottarga product development.

5.0 References

Aizen, J., Meiri; I., Tzchori; I., Levavi-Sivan, B., Rosenfeld, H., 2005. Enhancing spawning in the grey mullet (*Mugil cephalus*) by removal of dopaminergic inhibition. Gen. Comp. Endocrinol. 142, 212-221.

AOAC. (2012). Official Methods of Analysis of the Association of Analytical Chemistry. (AOAC) International, Gaithersburg, MD, USA. 1766 pp.

Ball, G.F.M. (2006) Vitamins in Foods. Analysis, Bioavailability, and Stability. CRC Press, Boca Raton. 824 pp.

Barber, I., S.A. Arnott, V.A. Braithwaite, J. Andrew, W. Mullen & F.A. Huntingford. 2000. Carotenoid-based sexual coloration and body condition in nesting male sticklebacks. J. Fish Biol., 57: 777-790.

Barua, A.B., Kostic, D., and Olson, J.A. (1993). New simplified procedures for the extraction and simultaneous high-performance liquid chromatographic analysis of retinol, tocopherols and carotenoids in human serum. J. Chromatogr. *617B*, 257–264.

Blake, C.J. (2007) Status of methodology for the determination of fat-soluble vitamins in foods, dietary supplements, and vitamin premixes. *Journal of AOAC Internatinal*, **90(4),** 897-910.

Chatzifotis S, Pavlidis M, Jimeno CD, Ardanis GV, Sterioti A, Divanach P. 2005. The effect of different carotenoid sources on skin coloration of cultured red porgy (Pagrus pagrus). Aquaculture Research 36:1517–1525.

Christie, W.W. (1982). Lipid analysis (2nd edn). Pergamon Press, Oxford.

Corriero, A., Medina, A., Mylonas, C.C., Abascal, F.J., Deflorio, M., Aragón, L., Bridges, C.R., Santamaria, N., Heinisch, G., Vassallo-Agius, R. , Belmonte, A., Fauvel, C., Garcia, A., Gordin, H., De Metrio, G. 2007. Histological study of the effects of treatment with gonadotropin-releasing hormone agonist (GnRHa) on the reproductive maturation of captive-reared Atlantic bluefin tuna (*Thunnus thynnus* L.). Aquaculture 272, 675-686.

De Monbrison, D., Tzchori, I., Holland, M.C., Zohar, Y., Yaron, Z., Elizur, A., 1997. Acceleration of gonadal development and spawning induction in the Mediterranean grey mullet, *Mugil cephalus*: Preliminary studies. Isr. J. Aquacult. Bamid. 49, 214–221

FAO (1997) Producción y manejo de datos de composición química de alimentos en nutrición. Capítulo 17. www.fao.org/docrep/010/ah833s/AH833S19.htm.

Folch, J., Lees, M., and Sloane-Stanley, G.H. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497-509.

Guerin, M., Huntley, M.E., Olaizola, M., 2003. Haematococcus astaxanthin: Applications for human health and nutrition. Trends in Biotechnology 21, 210–216.

Izquierdo, M.S., Fernandez-Palacios, H., Tacon, a G.J., 2001. Effect of broodtsock nutrition on reproductive performance of fish. Aquaculture 197, 25–42.

Kerfeld, C.A., M.R. Sawaya, V. Brahmandam, D. Cascio, K.K. Ho, C.C. Trevithick-Sutton, D.W. Krogmann and T.O. Yeates. 2003. The crystal structure of a cyanobacterial water-soluble carotenoid binding protein. Structure, 11: 55-65.

Miki, W. (1991) Biological Functions and Activities of Animal Carotenoids. Pure and Applied Chemistry, 63, 141- 146.

Moller AP, Biard C, Blount JD, Houston DC, Ninni P, Saino N, Surai PF. 2000. Carotenoid-dependent signals: Indicators of foraging efficiency, immune competence or detoxification ability? Poultry and Avian Biology Reviews 11:137–159.

Olsen, R.E., Henderson, R.J. 1989. The rapid analysis of neutral and polar marine lipids using double-development HPTLC and scanning densitometry. J. Exp. Mar. Biol. Ecol. 129, 189–197.

Sawanboonchun, J., W.J. Roy, D.A. Robertson & G. Bell. 2008. The impact of dietary supplementation with astaxanthin on egg quality in Atlantic cod broodstock (Gadus morhua, L.). Aquaculture, 283: 97-101.

Torrissen, O.J., Christiansen, R., 1995. Requirements for carotenoids in fish diets. Journal of Applied Ichthyology 11, 225–230.

Yashouv, A., 1969. Preliminary report on induced spawning of M. cephalus (L.) reared in captivity in fresh water ponds. Bamidgeh 21, 19–24

Zudaire, I., Murua. H., Grande , M., Pernet, F. , Bodin. N. 2014 Accumulation and mobilization of lipids in relation to reproduction of yellowfin tuna (*Thunnus albacares*) in the Western Indian Ocean. Fisheries Research 160, Pages 50-59.

Sub-task 13.3.5 (CTAQUA, Rocio Robles) Comparison of vegetable oil-no fish meal grow out diet with a n-3 HUFA rich fish meal finishing diet on the nutritional and organoleptic values of fish flesh and bottarga quality

Table of Contents

1. Introduction

As already stated in **D28.7**, fish have long been recognized as a valuable source of high quality protein in the human diet. In recent years, fish lipids have also assumed great nutritional significance owing to their protective role against the development of many different health disorders (Zárate *et al.*, 2017). Fish content is comprised of 60 to 85% water, around 20% protein, between 0.1 to 10% of fat and less than 2% inorganic matter including minerals. In some cases, a very small percentage of carbohydrates is also present. Fish are easily digested due to low levels of collagen content and constitutes a healthy food suitable for all ages, due to the presence of

essential amino acids, minerals and vitamins that the body cannot synthesize. Fish are also providing a low percentage of very high quality fat (CECOPESCA, 2012).

Fish are the primary dietary source of long chain n-3 (omega-3; w3) polyunsaturated fatty acids (LC-PUFA), particularly, eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3), that exert a range of health benefits through all life stages by their molecular, cellular and physiological actions. Farmed fish were traditionally fed a diet containing high levels of marine ingredients, such as fish oil and fishmeal, which are derived from pelagic fisheries. However, the continued pressures on wild fish stocks as aquaculture production grows along with greater competition for LC-PUFA sources from the nutraceutical and pharmaceutical industries have resulted in changes in aquaculture feed formulations. Terrestrial ingredients such as plant sources of meal and fats, mainly of oilseed origin, are increasingly used in fish feeds without detriment to fish health or growth. Like humans, most marine species of fish are inefficient at converting the shorter-chain fatty acid, α -linolenic acid (ALA; 18:3n-3), into EPA and DHA, and must therefore obtain the n-3 LC-PUFA through the diet. Nevertheless, the fatty acid profiles of vegetable oils differ from those of fish oil, being richer in n-6 and n-3 18C PUFA (18:2n-6 and 18:3n-3) and devoid of n-3 LC-PUFA, resulting in changes of the fatty acid composition of farmed fish (Sprague *et al*., 2016). Consequently, a rational use of these terrestrial ingredients as well as sufficient knowledge of new targeted species for aquaculture diversification, is needed to ensure the nutritional benefit of aquatic products to the consumer, as well as the growth and health of the fish.

The grey mullet, an euryhaline fast growing omnivore, provides high dress-out and fillet yield, and is suitable for product diversification and development of value-added products (see D28.3. 28.5, 28.6 and 28.7). Among these products is not only the flesh fillets but also the salted and dried roe (bottarga) from gravid females. This is considered a highly prized delicacy in the southern Mediterranean and an added value product from the culture of this species (Mylonas *et al.,* 2017). A highly nutritious value product (>100 € kg^{-1}), rich in ω3 LC-PUFA and carotenoids, whose markets are expanding around the Mediterranean.

At present, one of the major challenges for the aquaculture industry is the environmental sustainability of its activity. New sustainable and environmentally friendly ingredients for aqua feeds that do not compromise the nutritional benefits of fish consumption for humans are needed. As fish oil production is currently declining, other sources of ω3 LC-PUFAs are currently also being evaluated. Micro and macroalgae, where some have valuable nutritional fatty acid and antioxidants profiles, are already being harvested industrially for human consumption or as fish feed ingredients to optimize fish culture (Zárate *et al.*, 2016). In this way, an herbivorous fish such as the grey mullet would be more acceptable to an increasingly aware consumer public that demands product sustainability that has lower environmental impact. An important aspect that affects quality of the final fish product refers to the fillet proximate composition and total content of ω3 polyunsaturated fatty acids (ω3 LC-PUFA) (due to their numerous health benefits). All these aspects have been evaluated in deliverables **D28.7** for a few batches of mullet specimens grown in earthen ponds. The present deliverable adds to the evaluation of some new fish batches reared in tanks within the framework of DIVERSIFY´s nutrition tasks included in **WP13**. As controversial issues in aquaculture regarding food safety, nutrition, and sustainability are directly related to the nutrition and feeds for farmed fish, the proximate and fatty acid composition of the extruded diets and their correlation with the fillets and female gonad (bottarga) ω3 LC-PUFA can be expressed in relative and absolute terms per 100 g serving portions.

In order to evaluate the difference between two feeds with complete different formulations, a lab trial was conducted with adult specimens of grey mullet. The fish were harvested from a farm located in the South Spain, (not part of the Diversify consortium) producing seabass in earthen ponds and having also few ponds with grey mullet in monoculture (personal communication). A batch of adult grey mullet of 550-600 g average body weight were received at the facilities of Ctaqua. The fish were maintained in a recirculation aquaculture system (RAS) system for 6

months. The two feeds that were tested were: an experimental diet (specifically formulated diet for the grey mullet by **P4. IOLR**) and a feed with less than 5 % fish meal, which was the commercial diet in use at the farm (commercial carp diet).

2. Materials and Methods

Farmed grey mullet adults specimens were obtained at the farm and transported to the premises of CTAQUA facilities. The transport of adult fish is always a challenge but the grey mullet arrived in fairly good conditions at CTAQUA. Upon arrival, they were evenly distributed among the tanks of the RAS and left undisturbed during 24 h. Only after that period, did they receive a preventive formalin treatment.

The fish arrived at the facilities of **P18. CTAQUA** on the 25th of January 2018. A total of 130 adult grey mullet, were distributed in a RAS with 6 tanks of 1200 l each. The RAS is comprised of units for mechanical filtration (drum filter), biofiltration, protein skimmer and UV treatment (**Fig. 13.3.5.1)**.

Figure 13.3.5.1. RAS system used for the trial with the adult grey mullet at Ctaqua facilities.

Water quality parameters were controlled twice per week, except temperature and dissolved oxygen which were checked daily as well as mortality and fish welfare.

Grey mullet were reared under farm earthen ponds (natural temperature and photoperiod as well as lower salinity than the culture water of **P18. CTAQUA**). They arrived to the facility in good condition but their adaptation to the captive conditions in the RAS was not easy.

During the first week, progressive water change was applied to the RAS in order to adapt the fish from the 12 psu salinity of the ponds to the 36 psu of the culture water in the RAS.

The two diets were tested in triplicates:

- Experimental diet: tanks 1, 3 and 5. This diet was specifically formulated for the species by **P4.IOLR**. Total protein content of this diet is 35% and total fat content is 15%. The experimental diet was prepared by Sparos (Portugal).
- Commercial diet: tanks 2, 4 and 6. This diet was the commercial diet used in the farm for this species. It is a commercially available diet for carp. According to the label information, total protein content is 32% and total fat content is 9%.

Feed samples have been sent to **P.16 ULL** for further analyses of proximate composition and fatty acid profile.

Experimental fish were hand fed twice per day to apparent satiation during week days. However, during the first weeks of acclimation, fish were feeding poorly. In order to test the influence of the people around the tank on the feeding behavior, automatic feeders were also used.

Stocking of the fish, which established the initial number of fish per tank, was done on February 21, 2018. The trial was done from February to August. Fish were individually weighted and measured. Fish from the initial batch that presented small wounds or any abnormality were not used for the stocking.

Since grey mullet are known to be good "jumpers", tanks had to be cover with rigid plastic nets to avoid losing fish during the hours that the staff was not around the tanks (**Fig. 13.3.5. 2**). Fish were stocked at a ratio of 15 individuals per tank, at initial density of 7.01±0.38 kg/m^3. Natural photoperiod was established for the trial; the artificial lights of the system were used only during staff working hours, keeping the fish in low light conditions to avoid any source of stress. Working hours for the daily maintenance of the system were reduced to the minimum (1 hour per day) until the fish started to eat normally. Then lights were on during the standard working hours from 8.00 h to 15.00 h. Temperature was kept at 19ºC during the first two weeks of the trial and then gradually increased during a 5 days period to 22 ±1 ºC. The temperature of 22±1 ºC was maintained for the rest of the experimental period. Trial duration was 180 days of feeding the two diets.

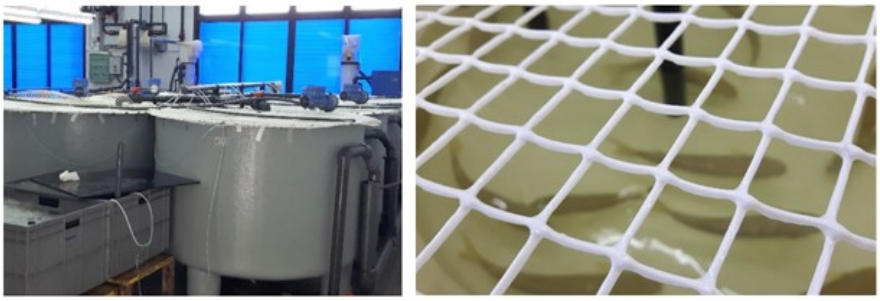

Figure 13.3.5.2. View of the tanks covered with plastic nets to avoid the jumping of the grey mullet (left) and a close-up of the net (right).

Six samplings were performed: at stocking (February 2018) and four intermediate samplings with the final sampling in August 2018. In the final sampling all the fish were individually weighed and measured and dissected to collect the data for the somatometric measurements and to collect the different tissues for further analyses. From 4 individuals per tank, samples of filet and gonads have were taken to be analyzed for proximate composition and fatty acid profile (following the protocol developed with **P16. ULL**).

For all the samplings, fish were previously fasted during 24 h. On the sampling day, the fish were harvested from the tanks and transferred immediately to a tank with 2-phenoxyethanol in order to tranquilize them for the weight and measure sampling (**Fig. 13.3.5.3).**

Figure 13.3.5.3. Sampling at the facilities of **P18.Ctaqua**. Note that the nets had to be strongly fixed to the tanks to absorb the powerful jump of the grey mullet, which could open the nets.

Somatometric measurements and production parameters

Grey mullet were weighed and measured individually at each sampling. At the end of the trial, four fish per tank (8 fish for Experimental diet and 12 fish for Commercial diet) were gutted and the body weighed while the liver, viscera and gonad weights were determined separately. It was not possible to separate out the visceral fat to be weighed. The following somatometric indexes were calculated individually:

- Condition index (CI) = 100× body weight (g) / body length^3 (cm^3),
- Dressing yield (DY) = 100× (gutted body weight / body weight),
- Filleting yield (FY) = 100 × (fillet weight / body weight),
- Hepatosomatic index (HSI) = 100 × (liver weight / body weight),
- Gonadosomatic index (GSI) = 100 × (gonad weight / body weight) and
- Viscerosomatic index (VSI) = 100 × (total viscera weight / body weight).

During the sampling, a 15 g sample was taken from the right side fillet of each sampled fish, labelled and stored at -80ºC, to be sent to **P18. ULL** for proximate analysis and fatty acid profile determination. Similarly, when the fish had gonads, they were weighed, labelled and immediately frozen at -80ºC to be sent also to **P18. ULL** for proximate analysis and fatty acid profile analyses. The rest of the right side fillet of each sampled fish, was packed and stored at -80 °C to be further processed for sensory analysis (**P3.IRTA**), while the second fillet was stored also at -80ºC as a back up sample.

The following production parameters were calculated per tank for the 180 days of the trial:

- Survival= 100×final number of fish per tank/ initial number of fish per tank; (%)
- Specific growth rate (SGR)= 100×ln(final weight (g)/initial weight(g))/days of feeding); (%/day)
- Total feed per individual = total feed intake per tank (g)/number of fish per tank; (g)
- Feed intake expressed as percentage of the initial and final average body weight per tank per day (Feed intake %ABW/d) = 100×feed intake per tank in the period (g) ×number of days of the period/ (final weight (g)+initial weight(g))/2); (%ABW/d)
- Food conversion ratio (FCR)= (average feed intake (g)/average wet weight gain (g))

Proximate and fatty acid composition analyses

Proximate composition analysis (protein, fat, moisture and ash) of extruded diets were conducted following the AOAC (2005) methods. Specifically, moisture was calculated gravimetrically after complete drying of fish tissue and total inorganic content (ash %) after total burn of organic matter. Total protein content was determined by the Kjeldahl method, calculated as % Nitrogen x 6.25. Crude fat from lyophilized samples of fillets and gonads as well as from samples of the extruded diets were determined after chloroform/methanol (2:1; v:v) cold extraction (Folch *et al*., 1957).

A total lipid fraction (1-2 mg) from each sample was subjected to direct acid-catalysed trans methylation during 16 h at 50 °C to obtain fatty acid methyl esters (FAME). FAME were purified by TLC with hexane/diethyl ether/acetic acid (90:10:1 by volume) and then separated and analysed using a TRACE-GC Ultra gas chromatograph (Thermo-Fisher Scientific Inc., Waltham, Massachusetts, USA) equipped with an on-column injector, a flame ionization detector (240 °C) and a fused silica capillary column (Supelcowax™ 10; Sigma- Aldrich Co., St. Louis, Missouri, USA). The column temperature was programmed with four different ramps of temperature for an increase from 50 to 230 °C. FAME were identified by comparison with retention times of a standard FAME mixture consisting of C4-C24 (Supelco 18,919-

1AMP), PUFA No. 3 from menhaden oil (Supelco 47085-U) and a commercial cod roe FAME. When necessary, identification of individual FAME was confirmed by GC–MS chromatography (DSQ II, Thermo Fisher Scientific Inc. Waltham, Massachusetts, USA).

Methodology for sensory analyses

This task has been done by **P3.IRTA** . The fillets from grey mullet were sensory characterized in four modalities: odour, appearance, flavour and texture, as described in Deliverable **D28.3**. To make this characterization, a list of sensory attributes previously generated was used. Panellists were specifically trained to be familiarized with the descriptors and their intensity scales. Eight panellists with previous experience in the sensory profiling of food products were recruited for this training before evaluating the samples.

Sample's analysis was performed in three session of five samples each. Samples from the different diets and tanks were tasted in each session. Panellists assessed samples in the subsequent order: odour modality first then appearance followed by flavour and finally texture.

In each tasting session, the order of sample presentation and the first order and carry-over effects (Macfie et al., 1989) were blocked. In all cases, the evaluation was carried out in isolated sensory testing booths (ISO, 2007). All assessors were provided with mineral water to cleanse their palates between samples.

Samples were cooked in a convection oven at 115ºC for 20 minutes in individual transparent glass jars designed to make samples easy to visualize. Jar lids were used to keep the samples' odour from disappearing (Model B-250, Juvasa, Spain). Jars were then placed inside electrical heaters at 60ºC to keep them warm while being tasted.

Data were analysed by means of an ANOVA. The selected model included the diet, the growing tank, the interaction diet x growing tank, the taster and the tasting session as fixed effects.

3. Results and Discussion

With regard to feeding, fish did not show any interest for the feed at any time during the first 5 weeks after arrival. After this period and once they started to show some interest for the feed, both diets (experimental and commercial diet) were provided to the corresponding tanks

Experimental diet (formulated by **P4.IOLR** and prepared by Sparos) was not accepted by the fish at any time (tanks 1, 3 and 5). As mentioned before, the experimental diet was provided during 5 consecutive days without success. Since the fish could not continue without eating, it was decided to provide the also formulated by **P4.IOLR** and prepared by **P.31 IRIDA**. This diet was used in **WP23**, for the farm trial performed by **P18.Ctaqua** in grow out of grey mullet in earthen ponds (**Task 23.4**, Deliverable **D23.3**).

Grey mullet did eat the IRIDA diet so it was decided to use this diet as Experimental diet for the rest of the trial. **IRIDA** diet is a complete diet including *Ulva lactuca* in its formulation. The details of the **IRIDA** formulation are protected by the IPR of **P4. IOLR**.

After the diet change, the two diets used for the trial have been:

- **IRIDA** diet: tanks 1, 3 and 5.
- Commercial diet: tanks 2, 4 and 6.

Commercial diet (carp) was well accepted by the grey mullet from the first moment they started to eat actively. This diet is currently used by the farm for the ponds where they culture grey mullet. No problem was encountered with this diet in the RAS at **P18.Ctaqua** facilities.

Grey mullet have shown to be powerful jumpers against the nets during the whole trial duration. It seems the interactions among the individuals within the tanks provoked the fish to jump and in several occasions, were found dead on the floor or even in the tank besides the tank where they belonged. Some of these individuals could be recuperated but others were just found dead.

After the stocking of the fish to start the trial, the nets were firmly fixed to the tank to avoid losing fish. Probably as a consequence of the poor adaptation and as result of the jumping out of the tank by pushing the nets and damaging their skin, several specimens were found in the tanks with heavy wounds. Peroxide treatments were applied locally and some specimens were cured. Four specimens (2 from tank 5 and 2 from tank 2) were too badly damaged and were euthanized (**Fig**. **13.3.5.4)**. In the month of May, during one the peroxide treatments, all the fish from tank 3, (Irida diet) died accidentally due to a technical failure.

Although the results from tank 3 are included in **Table13.3.5.1**, they have not been taken into account for the average calculation of production parameters per dietary treatment. The growth and production results from this tank correspond to a trial period of only 84 days.

Figure 13.3.5.4. Grey mullet specimens heavily wounded that were euthanized during the trial.

The growth results obtained during the trial reflect the difficulty of the adult grey mullet to cope with the captivity conditions. The fact that they did not eat during 5 weeks and the problem of diet acceptance have heavily affected the trial results. Only at the end of the trial did the fish start to be adapted to the RAS tank conditions. Thus survival during the trial has been influenced by the inadaptability of adult grey mullet (the batch used for this trial coming from earthen ponds) to the captive conditions. As it is presented in **Fig. 13.3.5.5,** survival ranged from 100% in tank 6 to 53% in tank 2. Tank 3 was lost accidentally.

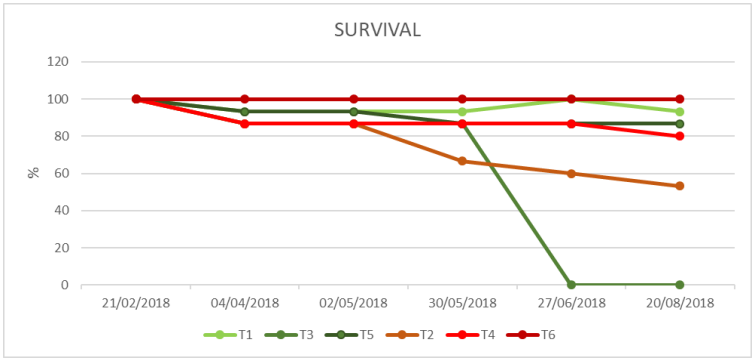

Figure 13.3.5.5. Survival (expressed as percentage per tank) of grey mullet during the trial.

With regard to the growth curve, the data are very different from tank to tank, indicating a strong tank effect. This could be observed also during the feeding. Grey mullet from tanks 1 and 2 were always reluctant to eat and it took longer time to feed those fish. As it can be observed in the growth curve (**Fig. 13.3.5.6**) fish of tank 2 hardly increased the individual average body weight during the 6 months of trial. On the contrary, the fish from tank 5 had the best growth of all. Average data of the productive parameters have not been calculated as the mean of the corresponding replicates per dietary treatment due to the high standard deviation values and the out of range variation coefficient values. Data is presented per tank.

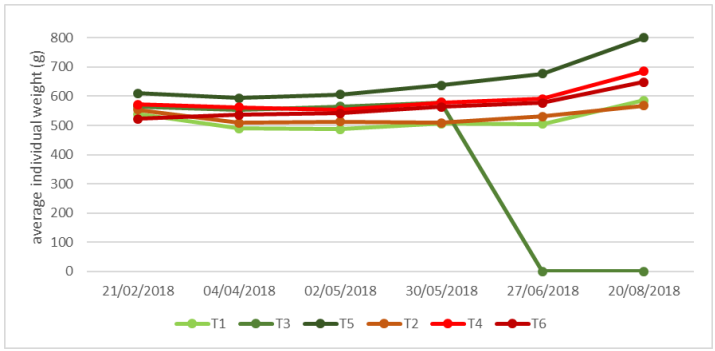

Figure 13.3.5. 6. Growth curve of the grey mullet (per tank) during the trial (fish from tank 3 were accidentally lost).

Growth within each tank fish group was very irregular with a high variation in individual weight increase during each period of the trial (**Fig. 13.3.5.7**). Specific growth rate (SGR) was rather low and food conversion ratio (FCR) values demonstrated that the feed were not well used by the fish. In **Tables 13.3.5.2** and **13.3.5.3** production results per tank are shown. SGR data from the five tanks after 180 days of trial ranged from 0.01 % day^{-1} in tank 2 to 0.15 % day^{-1} in tank 5. In the case of tank 3, data shown correspond to the results of 84 days of trial. FCR data are very different from tank to tank, also due to the strong tank effect observed in this trial and reflecting the growth data. The FCR for tank 2 was 30.81 and for tank 5, FCR was 2.63, clearly reflecting the strong tank effect. Results of somatic indexes, CI, HSI and VSI are presented in **Fig. 13.3.5.8.** One-way ANOVA analyses of the CI, HSI and VSI data did not show significant differences ($P>0.05$) for any of the indexes. However, the CI index is quite low which is just the reflection of the poor feeding during the trial.

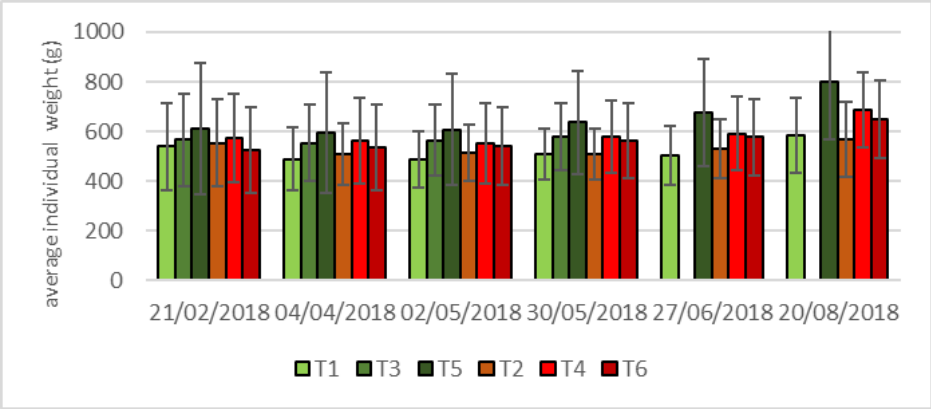

Figure 13.3.5. 7. Evolution of the average individual weight per tank during the trial. Tanks 1 and 5 were fed with Irida diet; tanks 2,4 and 6 were fed with Commercial diet for carp.

Feeds provided during the trial have been analyzed by **P16.ULL** and the results are shown in **Table 13.3.5.1**. Irida diet is an improved extruded diet. The Commercial diet is formulated for carp and it is commercially available. Proximate composition of the two diets used in the trial differ mainly in total fat content. The Irida diet was 15.09 ± 0.68 % compared with the 8.79 ± 0.42% of the Commercial diet. Irida diet also displayed higher contents of EPA and DHA.

The proximate analyses of the diets show that regarding total protein content, both dietary treatments were very similar (35.10 ± 2.46% for Irida and 35.80 ± 1.95% for Commercial diet). Another remarkable difference is the total n-3 PUFA value of both diets, 15.02 ± 0.16 % total fatty acids for Irida diet compared with 8.37 ± 0.37% total fatty acids for the Commercial diet. Condition index, HSI and VSI are presented in **Fig. 13.3.5.8.**

Table 13.3.5.2. Proximate composition (% wet matter) and main fatty acid profile (% total fatty acids) of Irida diet and Commercial diet used in the trial.

	Irida	Commercial
Moisture	6.47 ± 0.02	6.67 ± 0.06
Fat	15.09 ± 0.68	8.79 ± 0.42
Protein	35.10 ± 2.46	35.80 ± 1.95
Ash	7.01 ± 0.07	9.02 ± 0.20
16:0	11.99 ± 0.05	18.56 ± 0.05
18:0	2.50 ± 0.04	5.38 ± 0.09
Total SFA	19.16 ± 0.22	27.05 ± 0.01
16:1[1]	4.19 ± 0.05	3.75 ± 0.17
18:1[2]	32.89 ± 0.32	32.61 ± 0.16
Total MUFA	47.02 ± 0.29	39.94 ± 0.12
18:2n-6	15.57 ± 0.00	22.50 ± 0.02
20:4n-6	0.37 ± 0.01	0.54 ± 0.01
Total n-6 PUFA	16.62 ± 0.01	23.50 ± 0.18
18:3n-3	3.35 ± 0.02	2.47 ± 0.13

20:5n-3	3.45 ± 0.06	1.52 ± 0.03
22:6n-3	4.98 ± 0.09	3.06 ± 0.10
Total n-3 PUFA	15.02 ± 0.16	8.37 ± 0.37
DHA/EPA	1.45 ± 0.00	2.02 ± 0.03
ARA/EPA	0.11 ± 0.00	0.35 ± 0.01
n-3/n-6	0.90 ± 0.01	0.36 ± 0.01

Data correspond to average ± standard deviation (n=2)

Table13.3.5.3. Production data from tanks 1, 3 and 5 (fish fed Irida diet during 180 days). In the case of tank 3, data correspond to only 84 days of trial.

Diet	Irida Tank 1	Irida Tank 3(†)	Irida Tank 5
Survival (%)(*)	93.33	86.67	86.67
Average initial weight (g)	538.41±177.08	565.19±186.09	610.49±262.95
Average final weight (g)	585.46±150.06	578.28±137.99	800.68±233.327
Weight gain (g)	47,04	26.42	190.19
SGR (%/d)/ind	0.05	0.06	0.15
Total feed/ind (g)	402.12	146.83	500.41
Feed intake (%ABW/d)	0.40	0.31	0.39
FCR	8.55	5.56	2.63
CI (**)	1.16±0.11	1.16±0.09	1.20±0.07
HIS(**)	0.98±0.14	1.32±0.28	1,03±0,21
VSI(**)	5.36±0.88	6.34±1.24	6.08±0.96
GSI(***)	0.61±0.14	0.31±0.21	0.64±0.18

*180 days trial period; (†) all data from tank 3 correspond to 84 days of trial (fish lost accidentally)

** data correspond to average and standard deviation of 14, 14 and 13 individual for tanks 1, 3 and 5 respectively.

*** data correspond to average and standard deviation of 6, 9 and 9 individuals for tanks 1, 3 and 5.

Table 13.3.5.4. Productive data from tanks 2, 4 and 6 (fish fed Commercial diet during 180 days).

Diet	Commercial Tank 2	Commercial Tank 4	Commercial Tank 6
Survival (%)(*)	53.33	80	100
Initial weight (g)	554.14±137.89	572.35±178.51	522.41±172.33
Final weight (g)	567.46±148.75	684.85±151.49	648.53±158.78
Weight gain (g)	13.31	112.50	81.32
SGR (%/d)/ind	0.01	0.10	0.07
Total feed/ind (g)	410.17	496.54	460.58
Feed intake (%ABW/d)	0.41	0.44	0.42
FCR	30.81	4.41	5.66
CI (**)	1.20±0.10	1.13±0.07	1.18±0.09
HIS(**)	1.18±0.21	0.98±0.20	1,02±0,19
VSI(**)	5.22±1.35	5.02±0.59	5.36±0.96
GSI (***)	0.48±0.15	0.63±0.21	0.52±0.19

*180 days trial period

** data correspond to average and standard deviation of 8, 12 and 15 individual for tanks 2, 4 and 6 respectively.

*** data correspond to average and standard deviation of 6, 7 and 11 individual for tanks 2, 4 and 6 respectively

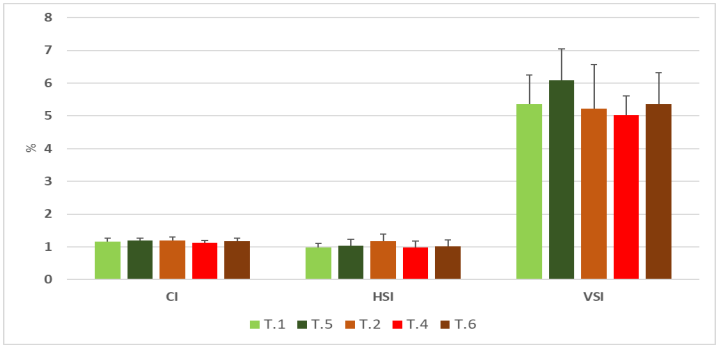

Figure 13.3.5.8. Average values of condition index (CI), hepatosomatic index (HSI) and viscerosomatic index (VSI) per tank. Tanks 1 and 5 were fed with Irida diet; tanks 2, 4 and 6 were fed with Commercial diet for carp.

Concerning the gonadal development, as it could be expected due to the poor adaptation of the fish, only some of the fish had visible gonads, and the GSI was in all cases lower than 1% (**Fig. 13.3.5.9).** One way ANOVA analyses of the GSI data for both diets, did not show significant difference (P>0.05). It is likely that these fish would have needed a much longer cultivation period to exhibit adequate gonadal development.

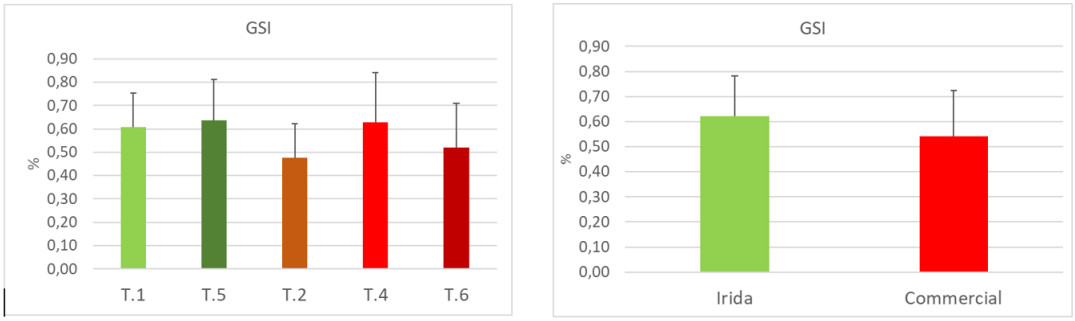

Figure 13.3.5. 9. GSI values per tank (left) and per diet (right) of the grey mullet that presented a incipient gonadal development.

At the final sampling after 180 days of culture, the grey mullet did not display any wound or lesion on the skin nor in the fins (**Fig. 13.3.5.10)**. Although no statistically significant difference was found between the HSI of the grey mullet fed the Irida diet and the commercial diet for carp, there was a marked effect on the liver appearance of fish fed the commercial carp diet. As it can be observed in **Fig. 13.3.5.11**, the livers presented dark green spots on the external surface of the organ. This problem might be caused by a deficiency in taurine, which would provoke an imbalance on the release of bile acids from the liver and they would accumulate in the liver causing the green coloration. Grey mullet fed the Irida diet did not present any green spot or abnormal coloration on the liver of any of 27 dissected specimens. In the case of the grey mullet fed the commercial diet, 46% of the livers had green spots, indicating that such a diet is causing a hepatic disorder. Further analyses of the liver could help to elucidate these findings. Unfortunately no liver samples were taken at the end of trial.

Figure 13.3.5.10. Healthy grey mullet specimens during final sampling showing the typical blueish spot on the base of the pectoral fin of *Múgil cephalus*.

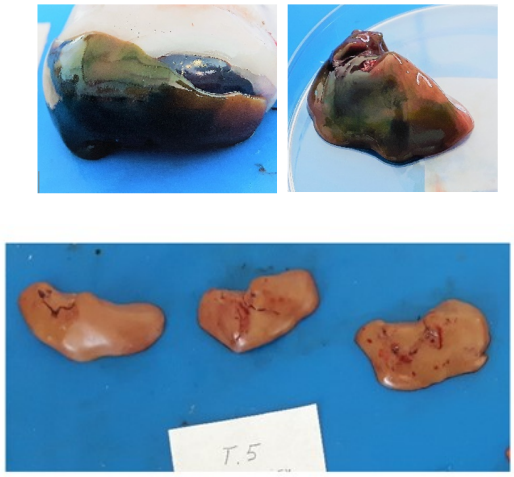

Figure 13.3.5.11. External appearance of the livers from fish fed the commercial diet with evident green dark spots on the surface of the organ (top images) compared with the liver coloration of grey mullet fed Irida diet.

Proximate composition and fatty acid profile of the filet

Data on filleting yield and dressing yield of the filet samples taken at the end of the trial are included in **Table 13.3.5.4.** Filleting yield of the reared fish was very good and, as it was concluded in the **D 23.3**, indicate the potential of using the species for elaborating processed fish products such as smoked fish fillet or fillet preserved in olive oil.

Table 13.3.5. 5. Average technical yields of grey mullet (*Mugil cephalus*) reared in the RAS at Ctaqua facilities and fed extruded diets for the trial: Irida diet and Commercial diet.

	Irida(*)	Commercial
Body weight (g)	575.51 ± 139.66	687.28 ± 113.23
Dressing yield (%)	93.68 ± 0.68	93.32 ± 1.05
Filleting yield (%)	53.82 ± 1.77	54.08 ± 1.10

(*) Fish from tank 3 have not been taken into account for these calculations.

Data are means ± SD (n=4).

Accordingly to the diet composition the fat contents of the fillets and the gonads were also significantly higher for the Irida fed fish (**Table 13.3.5.6** and **Table 13.3.5.6**) which also displayed higher contents of EPA+DHA when data are given in absolute terms (mg 100 g tissue^{-1}). Grey mullet is supposed to contain high fat contents in fillet, and has been characterized as a medium- to high-fat species (El-Sebaiy *et al.*, 1987; Özogul & Özogul, 2007; Özogul *et al.*, 2009; Kumaran *et al*., 2012). A recent study to evaluate fatty acid profiles of 77 commercially available finfish fillets in the United States, also confirmed this higher fat contents expected for wild specimens (2.75 ± 2.07 g per 100 g) (Cladis *et al*., 2014), however 1.31-1.87 g per 100 g is in between reported levels for wild and reared specimens.

In spite of the improved and more sustainable formula used by Irida diet, the fish seemed not to easily get use to the assayed diets, starving for a while (CTAQUA, personal communication). Therefore, the above mentioned difficulties to accept the diets, may explain the lower flesh and gonad fat contents and the low GSI found in these individuals.

The fatty acids profiles of the tissues generally resemble those of the diets. As shown in **Table 13.3.5.7** and **Table 13.3.5.6**, the assayed extruded diets contributed with high contents of MUFA and SFA, followed by n-6 and then n-3 PUFA. Compared to the Irida-experimental diet, the commercial diet provided higher contents of SFA and 18:2n-6 and lower of EPA and DHA, denoting a higher degree of dietary substitution of marine origin ingredients. Therefore higher absolute contents of EPA+DHA were present in the Irida fish but also lower relative percentages of saturates and 18:2n-6. As a contrary Irida fish displayed higher levels of monounsaturated fatty acids and 20:4n-6.

Sustainability of aquaculture extruded diets has obliged feed manufactures to use alternative sources of plant meals and oils in the last few years, a fact that is clear from the higher fish fat contents and fatty acid profiles. The relative contents of n-3 LC PUFA in farmed fish is generally lower than that in wild fish because commercial feeds usually contain high proportions of lipids from vegetable sources that are richer in SFA, MUFA and n-6 PUFA, but poorer in n-3 PUFA (Grigorakis, 2007). Therefore, changes in lipid quality and quantity will be expected with the replacement of marine oils by vegetable oils, which will particularly increase the levels of linoleic acid, 18:.2n-6 in farmed fish profiles (Rodríguez *et al*., 2004; Grigorakis 2007; O´Neill *et al*., 2015; Chaguri *et al*., 2017). In this sense, Irida fish displayed a more balanced lipid profile than fish fed the PIMSA diet. For instance, the fillets from Irida diet are poorer in 18:2n-6 but also exhibit a higher absolute content of n-3 LC PUFA (EPA+DHA).

Table 13.3.5.8. Total lipid, total fatty acid and EPA+DHA contents, and main fatty acid composition (% total fatty acids) of fillets of grey mullet (*Mugil cephalus*) reared in tanks with different extruded diets

	IRIDA	COMMERCIAL
Total lipid (% ww)	1.87 ± 0.39	1.31 ± 0.07 *
Total lipid (% dw)	7.50 ± 3.17	5.25 ± 0.30
Total FA (mg 100 g fillet^{-1})	1559.0 ± 319.4	994.6 ± 89.3 *
Total EPA+DHA (mg 100 g fillet^{-1})	95.3 ± 15.0	72.4 ± 7.5 *
16:0	20.97 ± 0.85	22.45 ± 0.42 *
18:0	3.81 ± 0.24	4.20 ± 0.46
Total SFA	27.82 ± 1.38	29.08 ± 0.67
16:1^{1}	8.84 ± 1.53	9.48 ± 0.89
18:1^{2}	29.73 ± 0.53	26.36 ± 0.82 *
Total MUFA	42.68 ± 1.43	37.67 ± 1.80 *
18:2n-6	13.94 ± 0.20	15.29 ± 0.10 *
20:4n-6	0.92 ± 0.29	1.33 ± 0.15
Total n-6 PUFA	16.42 ± 0.74	19.00 ± 0.22 *
18:3n-3	1.48 ± 0.05	0.98 ± 0.09 *
20:5n-3	1.38 ± 0.41	1.02 ± 0.19

22:6n-3	6.09 ± 1.32	7.20 ± 0.74
Total n-3 PUFA	11.40 ± 2.25	10.91 ± 0.95
DHA/EPA	4.59 ± 0.47	7.24 ± 0.66 *
ARA/EPA	0.70 ± 0.01	1.33 ± 0.13 *
n-3/n-6	0.70 ± 0.10	0.57 ± 0.05 *

Data are means ± SD (n = 3). [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. * Indicate significant differences among groups (P<0.05).

Table 13.3.5. 9. Total lipid, total fatty acid and EPA+DHA contents, and main fatty acid composition (% total fatty acids) of gonads of grey mullet (*Mugil cephalus*) reared in tanks with different extruded diets

	IRIDA	COMMERCIAL
Total lipid (% ww)	0.72 ± 0.02	0.64 ± 0.06 *
Total lipid (% dw)	3.81 ± 0.12	3.38 ± 0.33 *
Total FA (mg 100 g fillet^{-1})	425.8 ± 48.3	364.4 ± 44.6
Total EPA+DHA (mg 100 g tissue^{-1}))	64.0 ± 13.0	40.9 ± 4.8 *
16:0	19.93 ± 1.26	20.65 ± 0.66
18:0	7.14 ± 0.20	8.36 ± 0.57 *
Total SFA	29.56 ± 1.54	31.12 ± 0.64
16:1[1]	4.41 ± 0.31	4.83 ± 0.71
18:1[2]	21.62 ± 1.07	19.17 ± 1.00 *
Total MUFA	30.23 ± 1.45	25.96 ± 1.79 *
18:2n-6	9.53 ± 0.39	12.67 ± 0.35 *
20:4n-6	3.72 ± 0.16	4.61 ± 0.62 *
Total n-6 PUFA	16.72 ± 0.63	23.46 ± 1.25 *
18:3n-3	0.86 ± 0.19	0.52 ± 0.04 *
20:5n-3	3.52 ± 0.78	1.44 ± 0.06 *
22:6n-3	11.63 ± 1.22	9.76 ± 0.45 *
Total n-3 PUFA	19.13 ± 2.97	13.50 ± 0.65 *
DHA/EPA	3.27 ± 0.31	6.82 ± 0.27 *
ARA/EPA	1.08 ± 0.20	3.19 ± 0.27 *
n-3/n-6	1.13 ± 0.14	0.58 ± 0.01 *

Data are means ± SD (n = 3). [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. * Indicate significant differences among groups (P<0.05).

The rearing conditions and diet may affect the pattern of energy usage and reserves that are mobilized towards reproduction. Interestingly, the selective retention of the three so called essential fatty acids for marine fish EPA (20:5n-3), DHA (22:6n-3) and ARA (20:4n-6) was particularly evident for the gonads compared to the flesh, independently of the dietary regime. The surprisingly high levels of ARA in the tissues compared to the poor amount supplied by the diets highlights the physiological relevance of this fatty acid in this species particularly for reproductive purposes and suggests the potential capacity for its endogenous production from the 18:2n-6 precursor.

The use of fish oil rich in DHA and EPA in broodstock diets of mullet leading to better hatchability, tolerance of food deprivation and improved swim bladder inflation, was described in **D13.3**, and 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.

EPA and arachidonic acid (ARA, 20:4n-6) are precursors of a group of highly biologically active compounds known as eicosanoids. ARA eicosanoids have a wide range of functions in fish reproduction, including steroidogenesis (Henrotte et al., 2001; Mercure and Van der Kraak, 2008; Patiño et al., 2003; Sorbera et al., 2001). Since EPA and ARA compete for the same enzymatic complex to generate different series of prostanoids with different biological activities, the relative proportions of these two fatty acids are even more important than the level of each fatty acid in broostock specific diets. In fact, imbalances in the EPA/ARA ratio could lead to unfavorable production of different mediators involved in reproduction.

Sensorial analysis

Tables 13.3.5.7 and **13.3.5.8** show respectively the mean values obtained for each diet and growing tank. No significant differences were detected in any case. The absence of differences can be explained by the high variability observed within each treatment. This high variability has affected the trial in all the measured parameters.

Table 13.3.5.10. Mean values and standard deviation (SD) for the sensory parameters (O, Odour; F, Flavour; T, Texture) from each diet.

Sensory descriptor	IRIDA		Commercial	
	Mean	SD	Mean	SD
O_Sardine	1,8	1,617	1,7	1,435
O_Ammonia	0,9	1,328	0,9	1,500
O_Earthy	1,9	1,574	1,2	1,554
O_Butter	3,3	2,322	2,1	2,201
O_Sea food	0,9	0,984	0,9	1,125
O_Acid	0,5	1,015	0,4	0,770
O_Boiled vegetables	0,6	1,391	0,7	1,290
Colour white to brown	2,3	1,079	2,9	1,370
Colour uniformity	8,2	1,001	7,8	1,160
White spots	3,4	1,248	2,3	1,218
Laminar structure	2,1	1,298	2,8	1,322
Exudates quantity	4,4	1,716	2,9	1,770
Exudates turbidity	5,5	2,116	3,6	2,057

Fat droplets	2,4	1,614	0,9	1,681
Exudate particles	4,3	2,567	4,2	2,605
Exudate proteins	1,2	1,743	2,1	1,693
Black lines in the flesh	0,1	0,373	0,1	0,405
Brightness	6,0	2,919	5,0	2,740
F_Sweet	4,0	2,119	2,9	2,068
F_Acid	2,8	2,165	1,0	2,129
F_Bitter	1,7	2,283	3,9	2,416
F_Earthy	1,4	2,205	2,2	1,980
F_Sardine	1,4	2,066	1,9	1,792
F_Butter	1,7	1,492	1,8	1,354
F_Sea food	1,0	1,436	0,9	1,286
F_Boiled vegetables	1,1	1,263	1,2	1,381
T_Firmness	5,1	1,599	5,0	1,595
T_Crumbliness	4,5	1,987	5,8	1,889
T_Juiciness	5,0	1,326	4,4	1,312
T_Chewiness	5,9	1,963	5,4	1,718
T_Pastiness	3,4	1,628	4,0	1,713
T_Teeth adherence	4,2	1,860	5,2	1,499

Table 13.3.5. 11. Means values and standard deviation (SD) for the sensory parameters (O, Odour; F, Flavour; T, Texture) from each tank. Irida diet: tanks 1 and 5; Commercial diet: tanks 2, 4 and 6.

Sensory descriptor	T1		T2		T4		T5		T6	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
O_Sardine	2,1	1,873	1,4	1,132	2,3	1,833	1,7	1,363	1,4	1,096
O_Ammonia	1,0	1,225	0,7	1,565	1,0	1,572	1,1	1,504	0,8	1,384
O_Earthy	0,6	1,056	1,9	1,602	1,8	1,654	1,5	1,863	1,7	1,517
O_Butter	1,9	2,266	3,5	2,253	2,8	2,432	2,1	2,376	3,3	2,029
O_Sea food	0,8	0,926	0,9	0,988	0,8	1,260	1,0	1,056	1,1	1,161
O_Acid	0,5	1,007	0,5	0,922	0,4	0,698	0,5	1,029	0,4	0,733
O_Boiled vegetables	0,8	1,487	0,7	1,296	0,7	1,309	0,6	1,294	0,6	1,363
Colour white to brown	2,9	1,199	1,9	1,127	2,9	1,495	2,5	0,927	2,7	1,372
Colour uniformity	7,8	0,970	8,4	1,191	7,9	1,228	8,1	1,059	7,8	0,979
White spots	1,8	1,138	3,0	1,141	3,5	1,123	2,0	1,425	4,0	1,145
Laminar structure	2,7	1,132	2,3	1,372	2,4	1,375	2,7	1,461	2,3	1,301
Exudates quantity	1,6	1,073	4,3	1,917	4,2	1,753	3,5	1,730	4,5	1,776
Exudates turbidity	2,9	1,937	5,4	2,148	5,1	2,057	3,9	2,211	5,2	2,109
Fat droplets	0,1	1,031	2,4	1,549	2,2	2,097	1,5	1,802	2,1	1,423
Exudate particles	3,7	2,636	4,5	2,756	4,2	2,383	4,2	2,653	4,5	2,733
Exudate proteins	2,0	1,761	1,2	1,535	1,3	1,784	2,0	1,726	1,5	1,878
Black lines in the flesh	0,1	0,471	0,2	0,518	0,1	0,471	0,0	0,236	0,1	0,118
Brightness	4,2	3,010	6,1	2,793	6,3	2,935	5,1	2,850	6,0	2,694
F_Sweet	2,6	2,222	3,6	2,182	4,0	1,912	2,9	2,011	4,3	2,211

F_Acid	0,9	2,321	2,5	2,036	2,7	2,218	0,8	2,012	2,6	2,283
F_Bitter	4,9	2,503	1,7	2,432	2,2	2,396	3,8	2,228	1,3	2,210
F_Earthy	2,4	2,157	1,8	2,480	1,2	1,713	2,3	2,265	1,3	1,767
F_Sardine	1,9	2,184	1,6	1,857	1,6	2,013	1,8	1,984	1,2	1,549
F_Butter	1,4	1,437	1,7	1,574	1,9	1,342	2,1	1,491	1,5	1,163
F_Sea food	0,6	1,165	1,0	1,396	0,9	0,987	1,1	1,650	1,1	1,505
F_Boiled vegetables	1,1	1,156	1,2	1,468	1,2	1,507	1,0	1,366	1,1	1,263
T_Firmness	4,7	1,437	5,1	1,638	5,2	1,634	4,7	1,775	5,6	1,604
T_Crumbliness	6,3	1,871	4,8	1,886	4,6	1,971	6,2	2,099	3,8	1,815
T_Juiciness	4,2	1,419	5,1	1,226	4,9	1,312	4,5	1,215	4,9	1,478
T_Chewiness	4,8	1,838	6,0	1,646	6,2	1,517	5,4	2,042	5,9	2,079
T_Pastiness	4,7	1,445	3,1	1,841	3,5	1,350	3,7	1,730	3,6	1,908
T_Teeth adherence	5,3	1,699	4,4	1,625	4,2	1,396	5,3	2,079	4,4	1,504

4. References

AOAC (Association of Official Analytical Chemists) (2005) Official Methods of Analysis of

AOAC INTERNATIONAL, 18th Edition.CECOPESCA (Centro Técnico Nacional de Conservación de Productos de la Pesca y la Acuicultura) (2012) Guía de las cualidades nutricionales de los productos procedentes de la pesca extractiva y de la acuicultura: binomio riesgo-beneficio. Ministerio de Agricultura, Alimentación y Medio Ambiente. 64pp.

Chaguri, M.P., Maulvault, A.L., Costa, S., Gonçalves, A., Nunes, M.L., Carvalho, M.L., Sant'ana, L.S., Bandarra, N. & Marques, A., 2017. Chemometrics tools to distinguish wild and farmed meagre (*Argyrosomus regius*). Journal of Food Processing and Preservation, 41(6**),** e13312.

Cladis, C.D., Kleiner, A.C., Freiser, H.H. & Santerre, C.R., 2014. Fatty acid profiles of commercially available finfish fillets in the United States. Lipids 49(10), 1005-18.

El-Sebaiy, L.A., Metwalli, S.M., Khalil, M.E., 1987. Phospholipid changes in muscles of plathead grey mullet (*Mugil cephalus*) during frozen storage. Food Chemistry 26, 85-96.

Folch J., Lees M. & Sloane-Stanley G.H., 1957. A simple method for the isolation and purification of total lipids from animal tissues. The Journal of Biological Chemistry 226, 497–509.

Grigorakis, K., 2007. Compositional and organoleptic quality of farmed and wild gilthead sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) and factors affecting it: A review. Aquaculture 272, 55-75.

Henrotte, E., Milla, S., Mandiki, S.N.M., and Kestemont, P., 2011. Arachidonic acid induces production of 17,20b-Dihydroxy-4-pregnen-3-one (DHP) via a putative PGE2 receptor in fish follicles from the Eurasian perch. Lipids 46,179-187.

ISO Standard 8589 Sensory analysis -- General guidance for the design of test rooms.

Kumaran, R., Ravi, V., Gunalan, B., Murugan, S. & Sundramanickam, A., 2012. Estimation of proximate, amino acids, fatty acids and mineral composition of mullet (*Mugil cephalus)* of Parangipettai, Southeast Coast of India. Advances in Applied Science Research 3(4), 2015-2019.

Macfie, H., Bratchell, N., Greenhoff, K. & Valiis, L.V., 1989. Designs to balance the effect of order of presentation and first-order carry-over effects in hall tests. Journal of Sensory Studies 4, 129-148.

Mercure, F., and Van Der Kraak, G., 1996. Mechanisms of action of free arachidonic acid on ovarian steroid production in the goldfish. Gen. Comp. Endocr. 102, 130-140.

Mylonas, C.C., Robles, R., Estévez, A., Papandroulakis, N., Fontaine, P., Norberg, B., Álvarez-Blázquez, B., Koven, B. & Tacken, G. (2017) Exploring the biological and socio-economic potential of new/ emerging candidate fish species for the expansion of the European aquaculture industry. Aquaculture Europe Magazine 42(2), 7-28.

Navas, J.M., Bruce, M., Trush, M., Farndale, B.M., Bromage, N., Zanuy, S., Carrillo, M., Bell, J.G., Ramos, J., 1997. The impact of seasonal alteration in the lipid composition of broodstock diets on egg quality in the European sea bass. J. Fish Biol. 51, 760–773

Patiño ,R., Yoshizaki, G., Bolamba, D., and Thomas, P., 2003. Role of arachidonic acid and protein kinase C during maturation-inducing hormone-dependent meiotic resumption and ovulation in ovarian follicles of Atlantic croaker. Biol. Reprod. 68, 516–523.

O'Neill, B., Le Roux, A. & Hoffman, L.C., 2015. Comparative study of the nutritional composition of wild versus farmed yellowtail (*Seriola lalandi*). Aquaculture 448, 169–175.

Özogul, Y. & Özogul, F., 2007. Fatty acid profiles of commercially important fish species from the Mediterranean, Aegean and Black Seas. Food Chemistry 100, 1634-1638.

Özogul, Y., Özogul, F., Çiçek, E., Polat, A. & Kuley, E., 2009. Fat content and fatty acid compositions of 34 marine water fish species from the Mediterranean Sea. International Journal of Food Sciences and Nutrition 60, 464-475.

Rodríguez, C., Acosta, C., Badía, P., Cejas, J.R., Santamaría, F.J. & Lorenzo, A., 2004. Assessment of lipid and essential fatty acids requirements of black seabream (*Spondyliosoma cantharus*) by comparison of lipid composition in muscle and liver of wild and captive adult fish. Comparative Biochemistry and Physiology B 139, 619–629.

Sorbera, L.A., Asturiano, J.F., Carrillo, M. and Zanuy, S., 2001. Effects of polyunsaturated fatty acids and prostaglandins on oocyte maturation in a marine teleost, the European sea bass (*Dicentrarchus labrax*). Biol. Reprod. 64, 382-389.

Sprague, M., Dick, J.R. & Tocher, D.R., 2016. Impact of sustainable feeds on omega-3 long-chain fatty acid levels in farmed Atlantic salmon, 2006–2015. Scientific Reports 6, 21892.

Tufan, B., Balcık Mısır, G. & Kose, S., 2018. Comparison of seasonal fatty acid composition in relation to nutritional value of three commercial fish species caught from different zones of Eastern Black Sea. Aquatic Sciences and Engineering 33(1), 11-19.

Zárate R., el Jaber-Vazdekis N. & Ramírez-Moreno R., 2016. Importance of polyunsaturated fatty acids from marine algae. In: Hegde M., Zanwar A., Adekar S. (eds) Springer International Publishing, Cham, pp 101–126.

Zárate, R., el Jaber-Vazdekis, N., Tejera, N., Pérez, J.A. & Rodríguez, C., 2017. Significance of long chain polyunsaturated fatty acids in human health. Clinical and Translational Medicine 6 (1), 2-25.

Deviations from the DOW

This deliverable was delayed due to technical problems with experimental systems, fish food delivery, transfer of samples to partner laboratories for analysis and availability of fish.

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