Deliverable Report

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Objective: Recommended essential fatty acids contents in diets to promote meagre growth, welfare and health: The deliverable will be in the form of a report to present the recommendation of the optimum levels of essential fatty acids in on-growing diets for meagre. The deliverable will include the main methodology employed, followed by the results that leaded to the recommendation of the essential fatty acid levels. The deliverable will describe the effects of essential fatty acids and will include: a) growth performance, b) feed utilization, c) welfare, d) health status, e) fish behavior and f) digestion processes.

Description: A feeding trial was conducted to determine the requirement for n-3 long-chain polyunsaturated fatty acids in diets for meagre fingerlings, followed by a stress challenge trial.

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1. -Introduction

A series of limitations constrain the commercial production of meagre (*Argyrosomus regius,* Asso 1801) juveniles. To achieve successful large-scale production, the development of commercial high quality species specific diets is required (Saavedra et al., 2016), but knowledge on the nutritional requirements of emerging

aquaculture species, such as meagre, is still scarce (Chatzifotis et al., 2010; El-Kertaoui et al., 2015). This results in inadequate feeds that lead to low fish survival and poor growth.

Fatty acids (FAs) play key biological roles in animals, with long-chain polyunsaturated fatty acids (LC-PUFAs) being involved in several metabolic pathways including energy production, membrane structure and function, eicosanoids production and control of lipid homeostasis (Watanabe, 1982; Tocher, 2003). The docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3), are the main n-3 LC-PUFAs presented in fish meal and oils, and are of pivotal importance for fish, particularly marine species (Tocher, 2003; Izquierdo and Koven, 2011). Unlike freshwater species, marine fish have restricted ability or are unable to synthetize *de novo* n-3 and n-6 LC-PUFAs from their precursors, alpha linolenic acid (ALA, 18:3n-3) and linoleic acid (LA, 18:2n-6), respectively (Tocher, 2003; Izquierdo and Koven, 2011; Oliva-Teles, 2012). This is due to a low expression or deficiency of the enzymes required for the desaturation and elongation pathways, specifically the Δ5 or Δ6 desaturases and the elongases (Sargent et al., 1995; Izquierdo, 2005; Tocher, 2010). Therefore, EPA, DHA and ARA are considered essential fatty acids (EFAs) for marine fish and must be included in the diet in adequate levels to fulfil fish requirements for growth, survival and development (Sargent et al., 1995; Glencross, 2009). The n-3 LC-PUFA requirements have been studied for juveniles of many marine fish species (Tocher, 2010; NRC, 2011), however specific EFA requirements for meagre are still undetermined.

EFA-deficiency is characterised by different symptomatology, such as reduced growth and survival, swimming disorders, fin erosion, and severe lipid infiltration, particularly in lipid storage tissue, such as liver (Izquierdo, 1996). Liver is recognized as a key organ in intermediary metabolism, playing a central role in the regulation of lipid metabolism, particularly in the synthesis and β-oxidation of FAs (Caballero et al., 1999; Caballero et al., 2004). Since the regulatory enzymes of lipid metabolism pathways present different affinities for different FAs, an imbalance of the dietary FAs could affect liver physiology and functionality (Caballero et al., 2004). Moreover, in many fish species, the liver is the main organ for lipid storage, which is the case of gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) (Peres and Oliva-Teles, 1999; Roberts, 2002). Therefore, when lipid infiltration in the liver surpasses its capacity to store them, liver functioning may be affected (Spisni et al., 1998; Roberts, 2002).

Granulomatosis has been considered a constraint for meagre aquaculture development (Ghittino et al., 2004; Kružić et al., 2016). This pathology is characterised by an extensive development of granulomas in internal organs, including liver (Paperna et al., 1980; Paperna, 1987). Meagre granulomatosis aetiology is unknown, being hypothesised to be an immunological response to different stimuli, including pathogenic infections (especially *Mycobacterium spp.* and *Nocardia sp*), heavy metals or nutritional deficiencies (Good et al., 2016). The specific causes of non-infectious granulomatosis remain unclear, but water quality and nutritional imbalances such as calcium, phosphorus, magnesium, ascorbic acid or nutritional inadequacy (plant ingredients, tyrosine, etc.) have been pointed out (Herman, 1996).

It is known that dietary composition modulates digestive enzyme activities (Castro et al., 2016). However, very limited information is available on the effect of lipid sources and, consequently, of FAs on digestive enzymes function (Castro et al., 2016).

Under this perspective, the overall objective of the present task was to determine the nutritional requirements and optimum levels of n-3 LC-PUFA for meagre fingerlings, evaluating its effects on survival, growth performance, feed utilization and fish composition. Additionally, the present study aimed to improve the understanding of the modulation action of dietary n-3 LC-PUFA on hepatic lipid profile and its possible role on the development of liver steatosis and granulomatosis in meagre. Besides, the effects on elongase and desaturase gene expression, digestive enzymes and stress resistance were also evaluated. Results of this study are relevant to properly design well-balanced grow-out diets for this species.

For this purpose, one feeding trial was conducted in order to determine the n-3 LC-PUFA requirements for meagre fingerlings optimum performance by using different lipid sources, followed by a stress challenge trial with the objective to evaluate the effect of increasing dietary n-3 LC-PUFA levels on meagre stress resistance. Both trials were conducted within the frame of Task 8.2 (leaded by FCPCT).

2.- Materials and Methods

Experimental fish and rearing conditions

The feeding trial was conducted with meagre fingerlings with an initial body weight of 2.80±0.23 g (mean±SD) and an initial total length of 6.37±0.20 cm (mean±SD). Triplicate groups of meagre fingerlings, produced at FCPCT facilities, were randomly distributed in 15 experimental tanks (200 l fibreglass cylinder tanks with conical bottom and painted with light grey colour) at a density of 45 fish per tank and fed manually one of the experimental diets until visual apparent satiety, three times a day, 6 days per week, during 30 days. Daily feed intake was calculated by recording diet uptake and subtracting uneaten pellets. The tanks were installed in open system and supplied with filtered seawater (37 mg l^{-1} salinity). Water was continuously aerated and dissolved oxygen was maintained above 6.0± 0.2 mg l^{-1} during the trial. Average water temperature along the trial was 23.0±0.2 ºC. The experiment was run under natural photoperiod between September and October 2016.

Experimental diets

Five isoproteic and isolipidic experimental diets were formulated containing fish oil (FO) and vegetable oils (VO; linseed, palm and rapeseed oils) as lipid sources. Five dietary increasing levels of n-3 LC-PUFAs, namely eicosatrienoic acid (20:3n-3, ETE), eicosatetraenoic acid (20:4n-3, ETA), docosapentaenoic acid (22:5n-3, DPA), EPA and DHA were defined: 0.8, 1.4, 2.0, 2.6 and 3.6% of dry matter (DM), where DHA and EPA accounted for 93% of total n-3 LC-PUFA. The desired n-3 LC-PUFA content of each experimental diet was achieved by successively replacing VO by FO. Diet composition and proximate analysis are shown in **Table 1** and dietary fatty acid composition in **Table 2**. The experimental diets were manufactured by Skretting ARC Feed Technology Plant (Stavanger, Norway) with a pellet size of 2 mm, analysed for proximate and fatty acid composition at FCPCT laboratories and kept in a cold room at 10ºC until use.

Table 1. Composition (%) and proximate analysis of the experimental diets for meagre fingerlings

	Dietary n-3 LC-PUFA level (% DM)				
	0.8	1.4	2.0	2.6	3.6
Ingredients (%)					
Fish meal, N. Atlantic[1]	15.0	15.0	15.0	15.0	15.0
Corn gluten[2]	10.0	10.0	10.0	10.0	10.0
Faba beans[1]	10.0	10.0	10.0	10.0	10.0
Wheat[1]	8.0	8.0	8.0	8.0	8.0
Wheat gluten[1]	18.4	18.4	18.4	18.4	18.4
Soy protein concentrate[1]	25.0	25.0	25.0	25.0	25.0
Fish oil, S. American[1]	0.0	2.7	5.4	8.2	10.9
Linseed oil[3]	1.6	1.2	0.8	0.4	0.0
Palm oil[3]	3.3	2.5	1.7	0.8	0.0
Rapeseed oil[1]	6.0	4.5	3.0	1.5	0.0
Premix[4]	2.8	2.8	2.8	2.8	2.8
Proximate analysis (% DM)					
Protein	56.5	54.5	54.5	56.0	54.3
Lipids	16.2	17.0	16.5	16.9	16.2
Ash	4.9	5.0	5.1	5.2	5.0
Moisture	8.7	8.5	8.5	8.2	7.9

1: Skretting, Stavanger, Norway;
2: Cargill Nordic AS, Charlottenlund, Denmark;
3: AAK AB, Karlshamn, Sweden;
4: Trouw Nutrition, Boxmeer, the Netherlands. Proprietary composition Skretting ARC, including vitamins and minerals; Vitamin and mineral supplementation as estimated to cover requirements according to NRC (2011).

Table 2. Fatty acid composition of the experimental diets (% of total identified fatty acids)

Fatty acid	Dietary n-3 LC-PUFA level (% DM*)				
	0.8	1.4	2.0	2.6	3.6
Σ SFA	29.3	30.9	32.2	37.9	36.8
14:0	1.3	2.0	3.0	3.8	5.1
15:0	0.1	0.2	0.2	0.3	0.4
16:0	14.8	15.1	15.4	15.2	16.2
17:0	0.1	0.2	0.4	0.5	0.7
18:0	2.8	2.8	2.8	2.9	3.1
20:0	0.5	0.4	0.3	0.3	0.3
Σ MUFA	36.1	33.4	29.7	11.6	20.8
14:1n-7	0.1	0.1	0.1	0.1	0.1
14:1n-5	0.1	0.1	0.2	0.2	0.2
16:1n-7	1.2	2.1	3.2	4.1	5.6
16:1n-5	0.1	0.1	0.2	0.2	0.3
18:1n-7	1.9	1.8	2.0	2.2	2.3
18:1n-5	0.1	0.1	0.1	0.1	0.1
20:1n-7	1.4	1.4	1.5	1.7	1.7
20:1n-5	0.1	0.1	0.1	0.5	0.2
22:1n-11	1.4	1.4	1.8	2.0	2.2
Σ n-9	31.4	27.8	22.4	15.4	10.7
18:1n-9	31.0	27.4	21.9	17.8	9.8
18:2n-9	0.0	0.0	0.0	0.0	0.1
20:1n-9	0.1	0.1	0.1	0.2	0.2
20:2n-9	0.0	0.0	0.0	0.1	0.1
20:3n-9	0.0	0.0	0.0	0.0	0.1
22:1n-9	0.2	0.2	0.3	0.3	0.3
Σ n-3	14.3	16.4	19.4	21.3	26.5
Σ n-3 LC-PUFA	5.2	8.0	12.2	15.1	21.9
16:3n-3	0.1	0.1	0.1	0.4	0.3
16:4n-3	0.2	0.4	0.6	1.4	1.1
18:3n-3	8.3	7.3	5.4	3.5	1.3
18:4n-3	0.5	0.7	1.1	1.4	1.9
20:3n-3	0.0	0.0	0.0	0.0	0.1
20:4n-3	0.1	0.2	0.3	0.3	0.5
20:5n-3	2.1	3.4	5.4	6.8	9.2
22:5n-3	0.2	0.4	0.6	0.8	1.1
22:6n-3	2.8	4.0	6.0	7.2	10.5
Σn-6	18.3	17.1	15.9	12.1	11.8
Σn-6 LC-PUFA	0.3	0.5	0.7	0.9	1.2
18:2n-6	18.0	16.6	15.1	12.6	10.4
18:3n-6	0.0	0.1	0.1	0.1	0.1
20:2n-6	0.1	0.1	0.1	0.1	0.2
20:3n-6	0.0	0.0	0.1	0.1	0.1
20:4n-6	0.1	0.2	0.4	0.5	0.7
22:4n-6	0.0	0.0	0.0	0.1	0.1
22:5n-6	0.0	0.1	0.1	0.2	0.2
EPA/DHA	0.7	0.8	0.9	0.9	0.9
EPA/ARA	14.0	13.7	13.6	13.7	13.9
EPA+DHA	4.9	7.9	10.9	16.5	22.0
Other FAs					
16:2n-4	0.1	0.2	0.4	0.5	0.7
16:3n-4	0.1	0.1	0.1	0.2	0.2
16:3n-1	0.0	0.0	0.1	0.1	0.1
18:2n-4	0.0	0.1	0.1	0.2	0.2
18:3n-4	0.1	0.1	0.1	0.1	0.1
18:4n-1	0.0	0.1	0.1	0.1	0.1

At the beginning and the end of the trial, fish were anesthetized and individually weighed and sized (total length) after being unfed for 24 h. The following formulas were used for calculating survival, growth and feed utilisation parameters: Survival (%): (number of final fish- number of initial fish)/ number of initial fish x 100; Weight gain, WG (g): final weight- initial weight; Specific growth rate, SGR (% day^{-1}): (ln final mean weight – ln initial mean weight) /number of days x 100; Thermal growth coefficient, TGC: (final $weight^{1/3}$- initial $weight^{1/3}$)/(Temperature x days); Feed intake, FI (g feed $fish^{-1}$ day^{-1}): feed intake (g) / days of experiment/ number of fish; Feed conversion ratio, FCR: feed intake (g) / weight gain (g); Condition factor, K (%): body weight / $total\ length^3$ x 100; Protein efficiency ratio, PER: weight gain (g) / protein intake (g) (dry matter); Lipid efficiency ratio, LER: weight gain (g) / lipid intake (g) (dry matter). Besides, retention efficiency of the most relevant dietary fatty acids was calculated as following: Fatty acid retention (% FA intake): final weight x FA in final whole body x final whole body lipids – initial weight x FA in initial whole body x initial whole body lipids / FI x dietary lipids x dietary FA x 100. Resulting data are presented as means ± standard deviation (SD).

Sampling

At the beginning of the feeding trial 10 fish were euthanized with excess of clove oil, and frozen at -80ºC to determine the initial whole-body composition. At the end of the experiment, 5 fish per tank were collected for the same purpose. Livers from 15 fish were collected and weighed to calculate hepatosomatic index (HSI). Five of those livers were used for biochemical and FA composition analysis, 5 for histological studies, and 3 for bacteriological analysis. Additionally, livers from 5 fish per tank were collected and conserved in RNA later (Sigma-Aldrich, Madrid, Spain) for gene expression studies. The next day, RNA later was removed and liver samples stored at -80ºC until analysis.

Biochemical analysis

Prior to biochemical analysis, samples were homogenized (T25 Digital Ultra-turrax, IKA®, Germany) to obtain one pooled sample from each tank, which was analysed in technical triplicates. The same method was applied for initial fish sample. Moisture, ash and protein were determined according to A.O.A.C. (2000). Total lipid content was extracted with chloroform/methanol (2:1 v/v) (Folch et al., 1957). Fatty acid methyl esters (FAMES) were obtained by transmethylation of total lipids as described by Christie et al. (1989). FAMES were separated by gas liquid chromatography under the conditions described by Izquierdo et al. (1990), quantified by a flame ionizator detector (Finnigan Focus SG, Thermo electron Corporation, Milan, Italy) and internal standards (19:0) and identified by comparison with previously characterized standards. For livers, due to the small size of livers only moisture and lipid analysis could be performed. Neutral and polar lipid fractions from liver lipid content were separated according to Juaneda and Rocquelin (1985). Polar lipids were eluted with methanol whereas neutral lipids by chloroform and chloroform:methanol (49:1 v/v). Fatty acid methyl esters (FAMES) from liver total lipid and polar fraction were obtained by transmethylation following the above-mentioned conditions.

Gene expression

Total RNA was extracted from meagre livers using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Samples were homogenized with the TissueLyzer-II (Qiagen) with TRI Reagent (Sigma-Aldrich, Sant Louis, MO, USA) and centrifuged with chloroform at 12 000 g for 15 min, at 4°C. The upper aqueous phase containing RNA was mixed with 75% ethanol and transferred into an RNeasy spin column, where RW1 and RPE buffers (Qiagen) were used to purify RNA bonded to a membrane. Purified RNA was then eluted with 25 µL of RNase-free water. The quantity of RNA was analysed using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and RNA integrity using Gel Red™ staining (Biotium Inc., Hayward, CA) on a 1.4% agarose electrophoresis gel. Synthesis of cDNA was run with iScriptcDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions in an iCycler thermal cycler (Bio-Rad). Gene expression of fatty acyl desaturase (*fads2)* and fatty acyl elongase 5 (*elovl5)* genes were determined by Real-Time PCR (RT-PCR) in an iQ5 Multicolour Real-Time PCR detection system (Bio-Rad) using *β-actin* as housekeeping gene, with the following conditions: a first step of 3 min 30 s at 95°C followed by 40 cycles of 15 s at 95ºC, 30 s at 60.5ºC, 30 s at 72ºC, 1 min at 95ºC, and a final 81 cycles of 10s from 55°C to 95°C. *β-actin*, *fads2* and *elovl5* primer sequences used were the same

described in Monroig et al. (2013) and are shown in **Table 3**, together with their annealing temperatures. All PCR reactions were carried out in in a final volume of 20 µl, with 7.5 µl of Brillant SYBR Green QPCR Master Mix (Bio-Rad Hercules, CA, USA), 0.6 µl of each primer (10 mM), 5 µl of cDNA (1:10 dilution) and 1.3 µl of MiliQ water. MiliQ water also replaced cDNA in blank control reactions. Each run was ended with an analysis of melting curve leading to a melting peak specific for the amplified target DNA.

Table 3. Sequences of primers used for running RT-PCR analysis of fads2 and elovl5 gene expression of meagre hepatic tissue

Gene	Primer Sequence (5'-3')	Concentration	Temperature	Accession nos*
β-actin	F: 5′-CCATCGAGCACGGTATTGT-3′	0.4 µM	60.5ºC	GU584189
	R: 5′-CAGCTTCTCCTTGATGTCACG-3′			
Fads2	F: 5′-TGACTGGGTGACAATGCAGT-3′	0.4 µM	60.5ºC	KC261978
	R: 5′-TGGTGCTAACTTTGTGCCCT-3′			
Elovl5	F: 5′-CATCACACAGTTACAGCTGGTC-3′	0.4 µM	60.5ºC	KC261977
	R: 5′-GAATTGTGTGCACGGTTTCT-3′			

*GenBank: http://www.ncbi.nlm.nih.gov/.

Histological studies

Immediately after collection, livers were fixed in 4% buffered formalin, dehydrated in a graded ethanol series and embedded in paraffin wax. Samples were processed to obtain paraffin blocks that were cut with a Leica microtome to form sections of 4 µm. The sections were placed in slides, dried, stained with haematoxylin and eosin (H&E) (Martoja and Martoja-Pierson, 1970) and mounted with xylol. Additional sections of liver tissue were stained with acid-fast (Ziehl-Neesen) for searching the presence of *Mycobacterium sp.* and *Nocardia sp.* All slides were examined under light microscopy (BX51TF, Olympus, Tokyo, Japan) and blind evaluated by three different investigators to define visual differences among treatments. A semi-quantitative score evaluation of lipid infiltration level was used, ranging from 0 to 3. Score 0-1 was defined as normal liver morphology, score 1-2 was considered as moderate steatotic alterations in hepatic tissue with moderate lipid infiltration and score 2-3 severe steatotic alterations in hepatic tissue with high lipid infiltration.

Bacteriological analysis

At the end of the trial, three liver samples from each experimental treatment were seeded using blood (sheep) agar, supplemented with 1.5% sodium chloride (AS-1.5% NaCl), brain-heart infusion agar supplemented with salt (BHIA-1.5% NaCl) and YEME as culture media to detect the presence of *Nocardia spp*. and *Streptomyces sp*. Cultures were incubated at 25ºC for 4 weeks with daily check for bacterial growth.

Digestive enzymes activity

Samples of tissue (intestine and stomach) and their inner content were collected and homogenized in 5 volumes (v/w) of ice-cold Milli-Q water, centrifuged at 3300 x g for 3 min at 4ºC, the supernatant removed for enzyme quantification and kept at -80ºC until further analysis. Enzymatic determinations for total amylase, lipase, alkaline protease and pepsin activities were based on methods performed and described by Gisbert et al. (2009). In brief, alpha-amylase (E.C. 3.2.1.1) activity was determined according to Métais and Bieth (1968) using 0.3% soluble starch. Amylase activity was defined as the amount of starch (mg)

hydrolyzed during 30 min at 37ºC per mg of protein read at 580 nm. Total alkaline proteases were measured using azocasein (0.5%) as substrate in Tris-HCl 50 nmol l^{-1} at pH 9. Alkaline proteases activity is expressed in nmoles azodye per minute and per mg protein at 366 nm. Bile salt-activated lipase (BALT, E.C. 3.1.1) activity was assayed for 30 min at 30ºC using p-nitrophenyl myristate as substrate. The reaction was stopped with a mixture of acetone: n-heptane (5:2), the extract centrifuged for 2 min at 6080 x g and 4ºC and the absorbance read at 405 nm. Bile salt-activated lipase activity was defined as the amount (nmol) of myristate produced per min per mg of protein (Iijima_et al., 1998). Finally, pepsin activity was defined as the µmol of tyrosine liberated per min at 37ºC per ml of tissue homogenate at 280 nm (Worthington, 1972).

All enzymatic activities were expressed as specific activity defined as units per milligram of protein. Soluble protein of crude enzyme extracts was quantified by means of the Bradford's method (Bradford, 1979) using bovine serum albumin as standard. Absorbance was read using a spectrophotometer (Beckman Coulter DU800, Fullerton, CA).

Stress challenge trial

Triplicate groups of meagre juveniles fed 0.8, 1.4 and 2.6% n-3 LC-PUFA from the previous feeding trial (initial body weight ~25 g), were randomly distributed in 9 experimental tanks (working volume 100 l $tank^{-1}$) at a density of 10 fish $tank^{-1}$. Fish were manually fed at a water temperature of 23ºC. All tanks (200 l light grey color cylinder fibre-glass tanks) were supplied with filtered seawater (37 g l^{-1} salinity) at an increasing rate of 100% h^{-1} along the feeding trials. Water entered the tank from the bottom and went out from the top; water quality was daily tested. Water was continuously aerated (125 ml min^{-1}, attaining 5–8 g l^{-1} dissolved O2, and saturation ranged between 60% and 80%). The flow exchange rate was maintained at 70 % h^{-1}. Light: natural + fluorescent light (minimum intensity, 12:12 photoperiod, light from 7 AM). Temperature was measured daily in each tank. At the last day of the trial (4 days after allocation of the fish), fish were exposed to a mild stressor (episodic water level reduction). Samples from 4 fish per tank were taken at 0 (pre-stress), 1h, and 5 hours for biochemical analysis at the end of the trial. Plasma stress markers (cortisol, glucose, lactate), as well as brain serotonergic activation (in the telencephalon), were analyzed.

Additionally, 8 fish from the three dietary treatments mentioned above (0.8, 1.4 and 2.6%) were tested in terms of fast escape response. The trial was conducted in a white semi-translucent polyethylene circular tank with a diameter of 38 cm and a water depth of 5 cm, using a slight modification of previously described methods (Marras et al., 2011). Fish were allowed to acclimatize to the tank for a period of 1 hour. The experimental setup was covered in black opaque plastic to prevent visual disturbance of the fish. Video recordings were made at a rate of 250 frames per second (fps) using a Casio high-speed camera (EX-FH100) mounted 80 cm above the water surface. The setup was illuminated from below using a 28 W fluorescent light. The escape response was triggered by mechanical stimulation by releasing an iron rod (ø 10mm, l 15 mm) manually from a height of 90 cm above the water surface. To avoid visually stimulating the test subject, the iron rod fell inside a vertical PVC pipe suspended approximately 1 cm above the water surface. Fast escape was determined for single fish with two repetitions performed with 30 minutes of recovery between tests. There was no water replacement or water current during experiments. Fish were subsequently anaesthetized and measured for standard and total length to the nearest half mm, blotted dry and weighed to the nearest mg or g. Escape responses were analysed using Tracker (v. 4.72, www.cabrillo.edu/~dbrown/tracker). Time 0 was set as the nearest 4 ms interval at which the stimulus broke the water surface. The centre of mass was plotted every 4 ms from stimulus and 20 frames forward. These x,y coordinates were used to calculate escape latency (defined as the time elapsed between stimulus breaking the water surface and the first detectable escape motion of the fish), peak velocity during the escape response (bl s^{-1}), distance covered during the first 80 ms of the escape response, and peak acceleration (m s^{-2}).

Statistical analysis

All data were tested for normality and homogeneity of variances using Shapiro–Wilk and Levene's tests, respectively, and analysed by one-way ANOVA. Relative gene expression data were normalized according to the Livak method and required a logarithmic transformation before being analysed by one-way ANOVA. When p-values were significant ($P<0.05$), means were compared with Tukey's multiple range test (Tukey, 1949). When appropriated, response data were also subjected to regression analysis (linear or exponential),

where dietary FA level (analysed) served as the independent variable. A broken-line model was applied to estimate meagre n-3 LC-PUFA requirements. All statistical analyses were done using the SPSS 21.0 software package for Windows.

3. - Results

Growth performance

During the feeding trial, no external damage or abnormal behaviour were observed. Survival was high in all treatments and was not affected by diet composition (**Table 4**). After 30 days of feeding, meagre final weight ranged from 9.5 (in fish fed 0.8 n-3 LC-PUFA diet) to 10.7 g (in those fed 2.6 n-3 LC-PUFA diet), achieving a 3-fold increase from initial body weight. Fish fed the 0.8% n-3 LC-PUFA diet showed the lowest values for total length, body weight, WG, SGR and TGC. Increase in dietary n-3 LC-PUFA, significantly ($P<0.05$) increased final total length and body weight. Thus, the highest total length was found in fish fed 2.6% n-3 LC-PUFA diet, being significantly higher than fish fed 0.8%, 1.4% or 3.6% n-3 LC-PUFA. The highest body weight was also found in fish fed 2.6% n-3 LC-PUFA, being significantly higher than those fed 0.8% n-3 LC-PUFA. Dietary n-3 LC-PUFA increase was significantly correlated to final body weight ($P=0.09$, $r^2=0.82$), WG ($P=0.05$, $r^2=0.86$), SGR ($P=0.01$, $r^2=0.94$) or TGC ($P=0.01$, $r^2=0.94$). Feed intake was not affected by the dietary treatment, and fish fed the 2.6% n-3 LC-PUFA diet showed the best K, but not significantly different from fish fed other n-3 LC-PUFA levels. The broken-line linear model fitted best to the dietary n-3 LC-PUFA relation to final length, final weight, WG, SGR or TGC and pointed out a requirement of 2.1% n-3 LC-PUFA for maximum growth of meagre fingerlings (i.e. WG showed in **Fig. 1**).

Table 4. Growth performance of meagre fingerlings fed the experimental diets for 30 days[1]

	Dietary n-3 LC-PUFA level (% DM*)				
	0.8	1.4	2.0	2.6	3.6
Survival (%)	93.3±0.7	97.8±1.3	99.3±0.7	94.8±1.5	97.8±2.2
Initial total length (cm)	6.4±0.0	6.3±0.1	6.3±0.0	6.4±0.0	6.2±0.0
Final total length (cm)	9.0±0.1^{c}	9.4±0.1^{b}	9.3±0.1^{ab}	9.6±0.1^{a}	9.3±0.1^{bc}
Initial body weight (g)	2.8±0.1	2.8±0.1	2.7±0.1	2.7±0.1	2.6±0.1
Final body weight (g)	9.5±0.3^{b}	10.4±0.3^{a}	10.2±0.4^{ab}	10.7±0.3^{a}	10.4±0.3^{a}
WG (g)	6.7±0.4	7.5±0.4	7.6±0.3	8.0±0.3	7.8±0.2
SGR (% day^{-1})	4.1±0.1	4.3±0.2	4.5±0.1	4.5±0.1	4.6±0.1
TGC	1.0±0.0	1.1±0.1	1.1±0.0	1.2±0.0	1.2±0.0
FI (g feed fish^{-1} day^{-1})	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0
FCR	0.8±0.1	0.7±0.0	0.7±0.0	0.7±0.0	0.7±0.0
K (%)	1.3±0.0	1.3±0.0	1.3±0.1	1.2±0.1	1.3±0.0
PER	2.4±0.2	2.6±0.1	2.5±0.1	2.6±0.1	2.6±0.1

[1]Values (mean ± SE) with different superscript letters in the same row are significantly different ($P<0.05$).
* n-3 LC-PUFA (% total FA in DM diet)

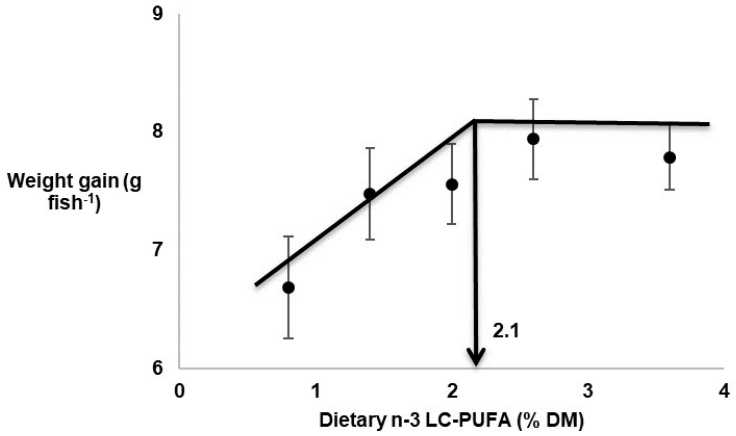

Figure 1. Broken-line linear model fitting dietary n-3 LC-PUFA levels to weight gain of meagre fingerlings fed the experimental diets for 30 days; the arrow indicates the requirement for dietary n-3 LC-PUFA (% DM).

Whole-body biochemical and fatty acid composition

Compared to the initial values, final body composition of meagre was higher in lipid and lower in ash contents (**Table 5**). At the end of the trial, no significant differences were observed in protein, lipids, ash, and water content in whole-body composition among the dietary treatments.

Table 5. Whole-body composition (% wet weight) of meagre fingerlings fed the experimental diets for 30 days

	Dietary n-3 LC-PUFA level (% DM*)					
	Initial	0.8	1.4	2.0	2.6	3.6
Protein	15.6±0.5	15.8±0.2	16.1±0.1	15.6±0.3	16.3±0.5	16.0±0.6
Lipid	2.2±0.1	4.1±0.5	4.1±0.3	3.8±0.2	3.8±0.3	3.9±0.3
Ash	3.9±0.3	2.7±0.2	2.7±0.1	2.8±0.1	2.8±0.0	2.6±0.3
Moisture	78.6±0.3	78.1±0.8	77.7±0.0	78.0±0.6	78.5±0.1	78.7±0.2

* n-3 LC-PUFA (% total FA in DM diet)

Regarding the whole-body fatty acid composition (**Table 6**), SFA linearly increased and MUFA linearly decreased ($r^{2=}$0.72 and r^2=0.96, respectively) with the increase of dietary n-3 LC-PUFA content, both following the dietary pattern. LA and ALA also reflected the dietary trend, decreasing linearly (r^2=0.86 and 0.93, respectively) with the increase of dietary n-3 LC-PUFA levels. DHA content linearly increased (r^2=0.84) while total n-3 LC-PUFA, EPA and ARA exponentially increased (r^2=0.89, 0.92 and 0.93, respectively) with the increase of dietary n-3 LC-PUFA levels, also similarly to the tendency observed in diets. Furthermore, fish fed 0.8% n-3 LC-PUFA had a 3-fold reduction in ARA level in whole-body compared to the initial fish, while the reduction of EPA compared to the initial fish was only 2-fold and for

DHA there was a slight reduction. Additionally, fish fed the 0.8% and the 1.4% n-3 LC-PUFA diets presented the highest content in eicosadienoic acid (20:2n-6) and ETE (20:3n-3), despite their lowest inclusion levels in the diets ($P<0.05$).

Table 6. Fatty acid composition (% of total identified fatty acids) of total lipids of whole-body of meagre fingerlings fed the experimental diets for 30 days[1]

Fatty acid	Dietary n-3 LC-PUFA level (% DM*)					
	Initial	0.8	1.4	2.0	2.6	3.6
Σ SFA	27.2±0.7	24.8±0.7^{cb}	23.8±0.3^{c}	30.0±1.2^{abc}	32.1±2.4^{b}	30.1±1.1^{ab}
14:0	2.7±0.2	1.2±0.1	1.4±0.1	2.46±0.1	3.8±0.2	3.8±0.2
15:0	0.4±0.0	0.2±0.0	0.2±0.0	0.4±0.0	0.4±0.0	0.5±0.0
16:0	18.4±0.5	16.6±0.9	15.9±0.3	20.2±0.8	21.4±1.6	19.8±0.9
17:0	0.2±0.0	0.2±0.0	0.1±0.0	0.2±0.0	0.2±0.0	0.4±0.0
18:0	5.9±0.5	6.3±0.7	5.9±0.1	6.4±0.5	6.3±0.7	5.3±0.1
20:0	0.3±0.0	0.4±0.0	0.3±0.0	0.4±0.0	0.4±0.0	0.3±0.0
Σ MUFA	40.2±0.8	44.0±0.1^{a}	42.1±0.2^{ab}	40.2±0.8^{bc}	37.8±0.8^{c}	32.4±0.7^{d}
14:1n-5	0.1±0.0	0.1±0.0	0.1±0.00	0.1±±0.01	0.2±0.0	0.2±0.0
15:1n-5	0.0±0.0	0.0±0.0	0.0±0.00	0.0±0.0	0.0±0.0	0.1±0.0
16:1n-7	4.8±0.2	2.3±0.1	2.8±0.1	4.0±0.2	5.3±0.1	6.6±0.1
16:1n-5	0.2±0.0	0.1±0.0	0.1±0.0	0.2±0.0	0.2±0.0	0.3±0.0
18:1n-7	3.5±0.1	2.0±0.1	2.5±0.0	2.8±0.1	3.1±0.1	3.2±0.1
18:1n-5	0.2±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.2±0.0
20:1n-7	3.0±0.1	2.1±0.0	2.0±0.0	2.2±0.1	2.2±0.1	2.1±0.1
20:1n-5	0.2±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.2±0.0	0.3±0.0
22:1n-11	2.1±0.1	1.3±0.1	1.3±0.0	1.6±0.1	2.0±0.0	2.0±0.1
Σ n-9	29.3±0.7	35.8±0.1^{a}	33.1±0.2^{b}	28.9±0.9^{c}	24.3±0.55^{d}	17.6±0.4^{e}
18:1n-9	28.5±0.7	35.2±0.2^{a}	32.6±0.22^{b}	28.3±0.9^{c}	23.7±0.5^{d}	17.0±0.4^{e}
18:2n-9	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0
20:1n-9	0.4±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.3±0.0	0.3±0.0
20:2n-9	0.1±0.0	0.0±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0
20:3n-9	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.1±0.0
22:1n-9	0.8±0.1	0.5±0.0	0.5±0.0	0.5±0.0	0.5±0.0	0.5±0.0
Σ n-3	13.6±1.0	11.8±0.5^{b}	13.6±0.1^{b}	12.7±1.1^{b}	13.1±2.1^{b}	20.3±1.8^{a}
Σ n-3 LC-PUFA	10.4±1.0	6.4±0.5^{b}	7.4±0.1^{b}	8.8±0.9^{b}	9.8±1.6^{b}	17.1±1.6^{a}
16:3n-3	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.2±0.0	0.2±0.0
16:4n-3	0.2±0.0	0.2±0.1	0.1±0.0	0.2±0.1	0.2±0.0	0.3±0.0
18:3n-3	2.0±0.4	4.9±0.3^{a}	5.6±0.0^{a}	3.1±0.4^{b}	2.4±0.4^{bc}	1.6±0.1^{c}
18:4n-3	0.5±0.0	0.3±0.0^{b}	0.4±0.0^{b}	0.4±0.1^{b}	0.6±0.1^{b}	1.2±0.1^{a}
20:3n-3	0.2±0.0	0.2±0.0^{a}	0.1±0.0^{a}	0.1±0.0^{ab}	0.1±0.0^{b}	0.1±0.0^{b}
20:4n-3	0.4±0.0	0.2±0.0^{b}	0.3±0.0^{b}	0.2±0.0^{b}	0.3±0.1^{b}	0.5±0.1^{a}
20:5n-3	2.7±0.6	1.5±0.1^{b}	2.4±0.1^{b}	1.9±0.5^{b}	2.7±0.8^{b}	6.2±0.7^{a}
22:5n-3	1.0±0.1	0.5±0.0^{b}	0.7±0.0^{b}	0.5±0.1^{b}	0.7±0.2^{b}	1.4±0.2^{a}
22:6n-3	5.6±0.5	4.2±0.5^{b}	3.9±0.1^{b}	6.1±0.8^{ab}	6.0±0.7^{ab}	8.9±0.8^{a}
Σ n-6	13.1±0.3	18.1±0.7^{ab}	19.3±0.1^{a}	15.5±0.9^{bc}	15.1±0.9^{bc}	14.9±0.1^{c}
Σ n-6 LC-PUFA	1.7±0.1	0.8±0.0^{b}	0.9±0.1^{b}	0.9±0.1^{b}	1.0±0.1^{b}	1.5±0.1^{a}
16:2n-6	0.0±0.0	0.0±0.0	0.0±0.0	0.1±0.0	0.1±0.0	0.0±0.0
18:2n-6	11.4±0.3	17.3±0.6^{ab}	18.4±0.1^{a}	14.6±0.9^{bc}	14.0±0.8^{c}	13.4±0.0^{c}
18:3n-6	0.1±0.0	0.1±0.01^{b}	0.1±0.0^{ab}	0.1±0.0^{b}	0.1±0.0^{ab}	0.2±0.0^{a}
20:2n-6	0.6±0.0	0.4±0.0^{a}	0.3±0.0^{ab}	0.3±0.0^{b}	0.3±0.0^{b}	0.3±0.0^{ab}
20:3n-6	0.1±0.0	0.1±0.0^{b}	0.1±0.0^{b}	0.1±0.0^{b}	0.1±0.0^{b}	0.1±0.0^{a}
20:4n-6	0.8±0.1	0.3±0.0^{b}	0.4±0.0^{b}	0.3±0.1^{b}	0.4±0.1^{b}	0.8±0.0^{a}
22:4n-6	0.1±0.0	0.1±0.0^{c}	0.1±0.0^{bc}	0.1±0.0^{bc}	0.1±0.0^{b}	0.1±0.0^{a}
22:5n-6	0.0±0.0	0.1±0.0	0.0±0.0	0.1±0.0	0.1±0.0	0.1±0.0
EPA/DHA	0.6±0.6	0.4±0.1^{b}	0.6±0.0^{ab}	0.3±0.1^{b}	0.5±0.1^{ab}	0.7±0.0^{a}
EPA/ARA	4.3±0.1	5.6±0.3	6.7±0.6	5.6±0.5	6.1±0.8	7.5±0.4
EPA+DHA	8.8±0.9	5.7±0.5^{b}	5.2±1.3^{b}	8.0±0.9^{b}	8.8±1.4^{b}	15.0±1.3^{a}
Other FAs						
16:2n-4	0.3±0.0	0.2±0.0	0.2±0.0	0.3±0.1	0.4±0.0	0.6±0.0
16:3n-4	0.3±0.0	0.2±0.0	0.2±0.01	0.2±0.0	0.2±0.0	0.3±0.0
16:3n-1	0.3±0.1	0.3±0.0	0.3±0.0	0.3±0.0	0.3±0.0	0.3±0.0
16:4n-1	0.1±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.1±0.0
18:2n-4	0.2±0.0	0.1±0.0	0.1±0.0	0.2±0.0	0.2±0.0	0.2±0.1
18:3n-4	0.1±0.0	0.1±0.1	0.1±0.1	0.1±0.0	0.1±0.0	0.2±0.0
18:4n-1	0.1±0.0	0.1±0.0	0.1±0.0	0.2±0.0	0.2±0.1	0.2±0.0

[1]Values (mean ± SE) with different superscript letters in the same row are significantly different ($P<0.05$).
* n-3 LC-PUFA (% total FA in DM diet)

Whole-body FA retention efficiency, expressed as percentage of FA intake, is presented in **Table 7**. In general, fish presented retentions below 100% for most FA, indicating a net reduction. However, retention of Δ6 desaturase activity products from OA and LA, 18:2n-9 and 18:3n-6, was over 100% in fish fed the 0.8% and 1.4% n-3 LC-PUFA diets ($P<0.05$), indicating a net accumulation. In contrast, the retention of Δ6 desaturase activity products from ALA, 18:4n-3, was lower compared to those originated from LA or OA. Furthermore, elovl5 products from ALA and LA, 20:3n-3 and 20:2n-6, respectively were also over 100% in fish fed the 0.8% and 1.4% n-3 LC-PUFA diets ($P<0.05$). Retention of EPA was unaffected by diet composition. In contrast, DHA, as well as ARA, retention was the highest in fish fed 0.8% n-3 LC-PUFA, ($P=0.18$). Furthermore, both DHA and ARA retention values were higher than EPA retentions. Additionally, retention of ALA was highest in fish fed 3.6% n-3 LC-PUFA ($P<0.05$).

Table 7. Retention efficiency (net accumulation or reduction) of specific fatty acids in whole-body of meagre fingerlings fed the experimental diets for 30 days[1]

Fatty acid	Dietary n-3 LC-PUFA level (% DM*)				
	0.8	1.4	2.0	2.6	3.6
18:2n-9	251.9±43.0^{a}	125.7±16.5^{b}	55.1±16.2^{b}	47.2±1.2^{b}	37.7±1.4^{b}
20:2n-6	227.8±57.4^{a}	165.4±11.4^{ab}	114.2±12.8^{ab}	82.2±110.7^{b}	86.5±10.3^{ab}
18:3n-6	190.8±44.8	107.8±14.7	48.2±13.7	40.9±2.4	65.5±12.6
20:4n-6	55.0±18.2	57.2±2.8	28.7±8.9	32.9±3.0	53.1±6.7
18:3n-3	25.7±5.3^{b}	38.7±4.1^{ab}	24.5±4.3^{b}	31.7±2.9^{b}	56.8±5.2^{a}
20:3n-3	315.0±88.6^{a}	256.1±24.6^{ab}	136.6±12.9^{abc}	50.8±0.6^{b}	68.2±10.7^{b}
18:4n-3	23.3±3.6	26.9±4.7	14.6±4.3	18.9±3.3	30.0±3.2
20:5n-3	25.0±6.7	31.3±4.4	13.3±4.7	17.9±4.1	30.3±4.3
22:6n-3	56.1±5.8^{a}	41.4±3.^{b}	41.5±4.7^{b}	37.5±0.7^{b}	39.9±3.7^{b}

[1]Values (mean ± SE) with different superscript letters, in the same row, are significantly different ($P<0.05$);
* n-3 LC-PUFA (% total FA in DM diet)

Fads2 and elovl5 gene expression

The results of the RT-PCR showed that the relative gene expression of *fads2* was significantly higher for fish fed the lowest n-3 LC-PUFA level (0.8%) than for fish fed the highest n-3 LC-PUFA level (3.6%; $P<0.05$; **Fig. 2**). Although no significant differences were observed, in fish fed the lowest n-3 LC-PUFA level, gene expression of *elovl5* was 2 to 14-fold higher than those fed the other n-3 LC-PUFA levels (**Fig. 3**).

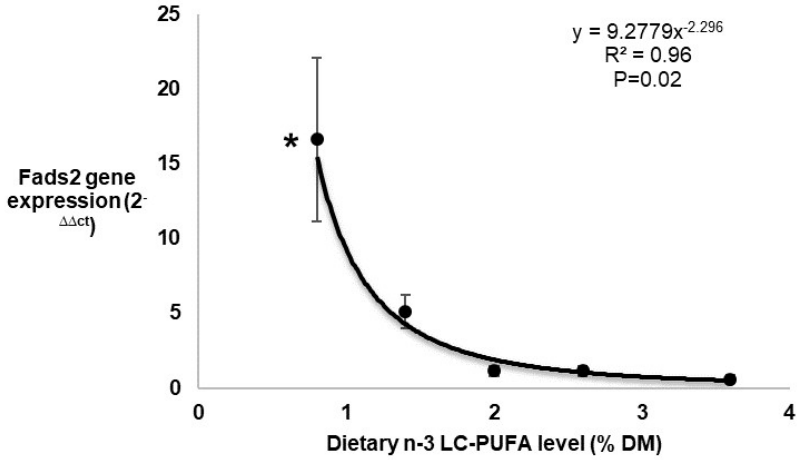

Figure 2. Fatty acyl desaturase (*fads2*) gene expression ($2^{-\Delta\Delta ct}$) of meagre hepatic tissue fed the experimental diets for 30 days; * indicates significant difference (p=0.04) compared to diet 3.6% n-3 LC-PUFA.

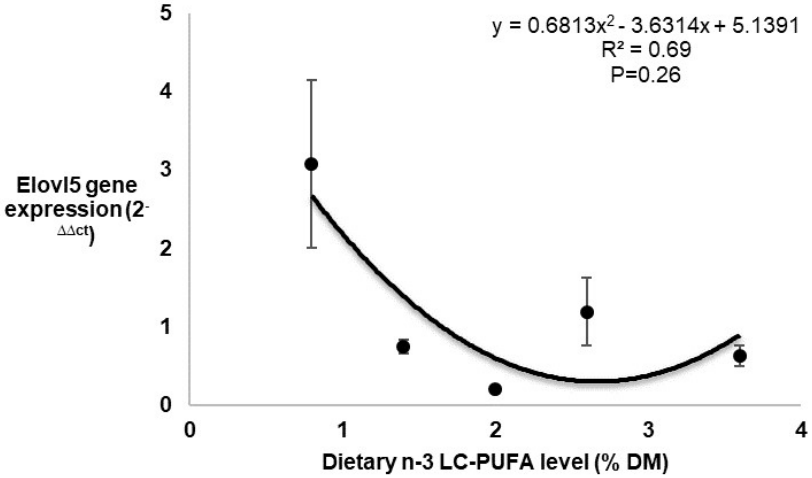

Figure 3. Fatty acyl elongase 5 (*elovl5*) gene expression ($2^{-\Delta\Delta ct}$) of meagre hepatic tissue fed the experimental diets for 30 days.

Liver indexes and composition

At the end of the feeding trial, HSI of fish fed 0.8% n-3 LC-PUFA was significantly higher than that of fish fed ≥2.0 % n-3 LC-PUFA (**Table 8**). Thus, HSI decreased linearly with the increase of dietary n-3 LC-PUFA levels (r^2=0.96, P=0.01). Similarly, hepatic lipid content was significantly higher in fish fed 0.8 or 1.4% n-3 LC-PUFA (**Table 8**) and it was linearly decreased with the increase of dietary n-3 LC-PUFA levels (r^2=0.93, P=0.02). The reverse was true for hepatic moisture content (r^2=0.91, P=0.03; **Table 8**).

Table 8. Hepatosomatic index (%) and biochemical composition (% wet weight) of meagre liver fed the experimental diets for 30 days[1]

	Dietary n-3 LC-PUFA level (% DM*)				
	0.8	1.4	2.0	2.6	3.6
Hepatosomatic index (%)[2]	3.08^{a}±0.22	2.97^{ab}±0.19	2.57^{bc}±0.18	2.23^{cd}±0.17	2.14^{d}±0.16
Liver composition (% wet weight)					
Crude lipids	16.67^{a}±1.08	16.34^{a}±1.06	11.67^{ab}±1.94	10.14^{b}±1.02	8.93^{b}±1.21
Moisture	60.91^{c}±2.04	62.45^{bc}±1.24	68.24^{ab}±0.90	68.03^{ab}±1.30	70.98^{a}±1.50

[1]Values (mean ±SE) with different superscript letters in the same row are significantly different (P<0.05)
* n-3 LC-PUFA (% total FA) x dietary lipids (%DM).

In general, FA composition of liver total lipids reflected the dietary composition (**Table 9**). Saturated fatty acids (SFA) increased linearly (r^2=0.88, P=0.02), while monounsaturated fatty acids (MUFA) decreased linearly (r^2=0.99, P=0.00) with the increase of dietary n-3 LC-PUFA. N-3 and n-6 LC-PUFAs increased exponentially (r^2=0.99, P=0.00 and 0.96, P=0.00, respectively), as well as DHA (r^2=0.99, P=0.00), EPA (r^2=0.99, P=0.01) and ARA (r^2=0.98; P=0.00) with the increase of dietary n-3 LC-PUFA levels. In contrast, N-9 fatty acids decreased linearly (r^2=0.99; P=0.00), reflecting the dietary pattern. Consequently, the ratio oleic acid (OLA)/n-3 LC-PUFA also decreased linearly (r^2=0.90; P=0.00), being significantly higher in fish fed 0.8 and 1.4% n-3 LC-PUFA than in fish fed levels above 2.0% (P<0.05). Interestingly, fish fed the two lowest n-3 HUFA levels (0.8 and 1.4%) presented the highest content in eicosadienoic acid (20:2n-6) and eicosatrienoic acid (20:3n-3, ETE), despite their lowest inclusion levels in the diets (P<0.05).

FA composition of liver polar lipids was less affected by the n-3 LC-PUFA level of the experimental diets (**Table 10**). Although for few FA the same tend was observed as in liver total lipids, for most FA no significant differences were observed among treatments. MUFA content of polar lipids was higher in meagre fed the lowest n-3 LC-PUFA level (0.8%) than in those fed 2.0-3.6% n-3 LC-PUFA (P<0.05), reflecting the dietary composition. Moreover, meagre fed 0.8% n-3 LC-PUFA showed significantly higher content in LA and ALA than fish fed n-3 LC-PUFA levels above 2.0% (P<0.05). Meagre fed the 0.8% n-3 LC-PUFA also showed the highest content in OLA in hepatic polar lipid fraction (P<0.05); consequently, a tendency to decrease the OLA/n-3 LC-PUFA ratio in meagre fed the two lowest n-3 LC-PUFA levels (0.8 and 1.4%) was observed.

Table 9. Fatty acid composition (% total identified fatty acid) of total lipids of liver of meagre fed the experimental diets for 30 days[1]

Fatty acid	Dietary n-3 LC-PUFA level (% DM*)				
	0.8	1.4	2.0	2.6	3.6
Σ SFA	23.61±0.90^{c}	25.70± 1.16^{c}	27.33±0.96^{bc}	30.81±0.58^{a}	30.66±0.19^{ab}
14:0	0.64±0.06	0.87±0.02	1.39±0.28	1.95±0.40	2.58±0.16
15:0	0.13±0.02	0.15±0.00	0.27±0.03	0.32±0.04	0.41±0.01
16:0	13.28±0.22	14.64±0.62	17.36±1.03	19.66±0.62	19.71±0.11
17:0	0.05±0.01	0.07±0.00	0.13±0.01	0.01±0.01	0.27±0.01
18:0	9.28±1.05	9.71±0.53	7.61±0.44	8.45±0.69	7.47±0.18
20:0	0.23±0.01	0.25±0.01	0.26±0.03	0.23±0.04	0.22±0.00
Σ MUFA	45.87±0.46^{a}	44.15± 0.13^{a}	40.94±0.11^{b}	35.77±1.28^{c}	31.27±0.46^{d}
14:1n-7	0.00±0.00	0.00±0.00	0.01±0.00	0.12±0.00	0.01±0.00
14:1n-5	0.04±0.00	0.05±0.00	0.09±0.01	0.01±0.01	0.15±0.00
15:1n-5	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.01	0.03±0.00
16:1n-7	2.25±0.08	2.95±0.06	4.10±0.14	5.91±0.35	7.45±0.29
16:1n-5	0.07±0.01	0.12±0.00	0.17±0.00	0.21±0.01	0.29±0.01
18:1n-7	2.24±0.08	2.44±0.04	2.85±0.03	3.08±0.06	3.45±0.06
18:1n-5	0.08±0.01	0.09±0.00	0.11±0.00	0.13±0.00	0.15±0.00
20:1n-7	2.03±0.03	2.16±0.08	2.04±0.04	1.94±0.09	1.78±0.01
20:1n-5	0.09±0.01	0.12±0.00	0.16±0.01	0.18±0.01	0.24±0.01
22:1n-11	0.67±0.01	0.69±0.02	1.05±0.24	10.93±0.31	1.02±0.03
Σ n-9	38.32±0.63^{a}	35.42± 0.13^{a}	30.21±0.10^{b}	23.08±1.34^{c}	16.54±0.04^{d}
18:1n-9	37.78±0.63^{a}	34.89±0.13^{a}	29.68±0.11^{b}	22.56±1.33^{c}	15.98±0.04^{d}
18:2n-9	0.13±0.02	0.11±0.02	0.07±0.01	0.08±0.01	0.06±0.00
20:1n-9	0.13±0.00	0.15±0.01	0.21±0.01	0.25±0.02	0.28±0.01
20:2n-9	0.05±0.01	0.05±0.01	0.05±0.00	0.06±0.00	0.07±0.01
20:3n-9	0.01±0.00	0.02±0.00	0.03±0.00	0.04±0.01	0.06±0.00
22:1n-9	0.47±0.01	0.47±0.03	0.45±0.01	0.42±0.02	0.44±0.01
Σ n-3	10.22±0.63^{b}	11.37± 0.48^{b}	13.03±0.78^{b}	17.01±2.29^{b}	21.89±0.93^{a}
Σ n-3 LC-PUFA	4.19±0.60^{c}	5.93±0.21^{c}	8.43±0.57^{bc}	13.67±2.32^{b}	19.57±1.03^{a}
16:3n-3	0.06±0.01	0.08±0.00	0.11±0.00	0.27±0.03	0.21±0.00
16:4n-3	0.04±0.01	0.05±0.01	0.12±0.06	0.03±0.04	0.14±0.01
18:3n-3	5.72±0.13^{a}	5.04± 0.27^{a}	3.92±0.25^{b}	2.33±0.16^{c}	1.04±0.05^{d}
18:4n-3	0.20±0.02	0.27±0.01	0.45±0.03	0.69±0.07	0.93±0.05
20:3n-3	0.23±0.01^{a}	0.21±0.01^{a}	0.15±0.01^{b}	0.11^{b}±0.00^{c}	0.08±0.01^{c}
20:4n-3	0.18±0.01^{c}	0.25±0.01^{bc}	0.31±0.04^{bc}	0.43±0.08^{ab}	0.62±0.02^{a}
20:5n-3	1.08±0.17^{c}	1.62± 0.08^{c}	2.46±0.27^{bc}	4.23±0.75^{b}	6.04±0.03^{a}
22:5n-3	0.39±0.05^{c}	0.60±0.03^{bc}	0.81±0.13^{bc}	1.42±0.30^{b}	2.01±0.08^{a}
22:6n-3	2.31±0.38^{c}	3.25± 0.12^{bc}	5.19±0.12^{bc}	7.43±1.22^{b}	10.80±0.98^{a}
Σ n-6	19.46±0.64^{a}	17.82± 0.79^{a}	17.36±0.39^{a}	14.86±0.39^{b}	14.23±0.38^{b}
Σ n-6 LC-PUFA	0.83±0.04^{c}	0.91±0.01^{bc}	1.01±0.05^{bc}	1.31±0.13^{b}	1.73±0.09^{a}
16:2n-6	0.00±0.00	0.00±0.00	0.02±0.02	0.01±0.02	0.01±0.00
18:2n-6	18.62±0.62^{a}	16.91±0.79^{a}	16.32±0.35^{a}	13.60±0.50^{b}	12.48±0.46^{b}
18:3n-6	0.21±0.02	0.18±0.01	0.15±0.02	0.22±0.02	0.21±0.01
20:2n-6	0.52±0.00^{a}	0.50±0.00^{a}	0.41±0.04^{b}	0.38±0.02^{b}	0.41±0.01^{b}
20:3n-6	**0.08±0.01^{b}**	**0.08±0.01^{b}**	**0.08±0.01^{b}**	**0.10±0.01^{ab}**	**0.13±0.00^{a}**
20:4n-6	0.18±0.04^{c}	0.26±0.02^{c}	0.42±0.03^{bc}	0.68±0.11^{b}	0.99±0.07^{a}
22:4n-6	0.04±0.00^{c}	0.04±0.00^{c}	0.06±0.00^{c}	0.10±0.01^{b}	0.14±0.01^{a}
22:5n-6	0.02±0.00^{b}	0.02±0.00^{b}	0.04±0.01^{ab}	0.05±0.01^{ab}	0.07±0.00^{a}
OLA/n-3 LC-PUFA	7.67±0.99^{a}	5.07± 0.15^{ab}	3.15±0.21^{bc}	1.54±0.42^{c}	0.75±0.04^{d}
EPA/DHA	0.47±0.02	0.50±0.02	0.52±0.05	0.57±0.03	0.59±0.05
EPA/ARA	6.19±0.57	6.30±0.69	5.84±0.31	6.28±0.29	6.17±0.43
EPA+DHA	3.39±0.54^{c}	4.88± 0.17^{c}	7.16±0.39^{bc}	11.66±1.97^{b}	16.85±0.95^{a}
Other FAs					
16:2n-4	0.07±0.01	0.11±0.01	0.24±0.03	0.30±0.03	0.42±0.03
16:3n-4	0.16±0.01	0.19±0.01	0.21±0.00	0.20±0.01	0.31±0.01
16:3n-1	0.02±0.00	0.02±0.01	0.13±0.10	0.21±0.10	0.06±0.02
16:4n-1	0.00±0.00	0.01±0.00	0.02±0.01	0.10±0.01	0.02±0.00
18:2n-4	0.07±0.11	0.11±0.00	0.17±0.01	0.16±0.01	0.31±0.01
18:3n-4	0.06±0.01	0.10±0.00	0.10±0.02	0.17±0.02	0.20±0.00
18:3n-1	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
18:4n-1	0.03±0.00	0.04±0.00	0.09±0.02	0.10±0.02	0.12±0.01

[1]Values (mean ±SE) with different superscript letters in the same row are significantly different (P<0.05)
[2]HSI: (liver weight/body weight) x100.
* n-3 LC-PUFA (% total FA) x dietary lipids (%DM).

Table 10. Fatty acid composition (% total identified fatty acids) of polar lipids of liver of meagre fed the experimental diets for 30 days[1]

Fatty acid	Dietary n-3 LC-PUFA level (% DM*)				
	0.8	1.4	2.0	2.6	3.6
Σ SFA	41.83±3.93	40.89±4.27	44.80±5.59	49.32±5.97	51.12±2.67
14:0	0.85±0.23	0.87±0.16	1.20±0.33	1.40±0.21	1.83±0.27
15:0	0.27±0.07	0.28±0.04	0.38±0.05	0.47±0.07	0.60±0.06
16:0	22.32±2.28	23.49±2.49	28.71±4.27	30.64±4.41	33.02±1.97
17:0	0.09±0.02	0.08±0.01	0.10±0.01	0.05±0.03	0.09±0.01
18:0	16.73±0.93	15.79±1.58	14.05±1.11	16.15±1.65	15.18±0.42
20:0	0.39±0.03	0.37±0.04	0.36±0.03	0.40±0.03	0.40±0.03
Σ MUFA	28.95±1.82^{a}	24.94±0.73^{ab}	23.20±0.48^{b}	21.18±0.60^{b}	22.42±1.17^{b}
14:1n-7	0.08±0.04	0.10±0.07	0.06±0.03	0.06±0.03	0.14±0.06
14:1n-5	0.09±0.05	0.09±0.05	0.10±0.04	0.09±0.01	0.18±0.05
15:1n-5	0.02±0.01	0.03±0.01	0.03±0.01	0.03±0.01	0.02±0.00
16:1n-7	1.25±0.23	1.14±0.02	1.34±0.04	1.66±0.13	2.25±0.27
16:1n-5	0.09±0.02	0.12±0.02	0.20±0.04	0.23±0.04	0.31±0.01
18:1n-7	2.64±0.16	2.52±0.05	2.98±0.16	2.95±0.07	3.32±0.09
18:1n-5	0.08±0.01	0.08±0.01	0.09±0.01	0.10±0.01	0.11±0.01
20:1n-7	1.85±0.12	1.62±0.04	1.67±0.11	1.67±0.04	1.58±0.04
20:1n-5	0.10±0.01	0.08±0.01	0.12±0.02	0.12±0.01	0.18±0.02
22:1n-11	0.55±0.09	0.45±0.04	0.49±0.09	0.58±0.04	0.64±0.04
Σ n-9	16.37±6.13	17.49±0.19	16.07±0.80	9.99±3.01	13.62±0.88
18:1n-9	21.94±1.32^{a}	17.09±0.16^{b}	15.78±0.81^{b}	13.31±0.63^{b}	13.32±0.86^{b}
18:2n-9	0.04±0.00	0.03±0.00	0.05±0.01	0.03±0.01	0.04±0.00
20:1n-9	0.10±0.03	0.07±0.02	0.09±0.03	0.08±0.02	0.12±0.02
20:2n-9	0.03±0.01	0.01±0.00	0.02±0.00	0.01±0.00	0.03±0.00
20:3n-9	0.01±0.01	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00
22:1n-9	0.33±0.02	0.28±0.02	0.26±0.00	0.28±0.01	0.26±0.02
Σ n-3	10.40±2.24	10.10±4.46	16.00±3.66	16.13±4.36	13.93±3.33
Σ n-3 LC-PUFA	7.42±1.88	7.28±4.52	13.68±3.38	14.20±4.16	12.03±3.56
16:3n-3	0.10±0.02	0.10±0.01	0.21±0.06	0.26±0.04	0.33±0.02
16:4n-3	0.49±0.07	0.77±0.04	0.54±0.15	0.77±0.10	0.48±0.05
18:3n-3	2.29^{a}±0.44	1.86^{ab}±0.18	1.45^{ab}±0.24	0.81^{b}±0.13	0.96^{b}±0.28
18:4n-3	0.11±0.04	0.09±0.01	0.13±0.02	0.10±0.03	0.13±0.01
20:3n-3	0.21±0.04	0.23±0.01	0.22±0.03	0.17±0.02	0.15±0.02
20:4n-3	0.13±0.02	0.14±0.02	0.18±0.03	0.15±0.03	0.16±0.04
20:5n-3	1.53±0.40	2.36±0.67	2.47±0.54	2.33±0.65	2.12±0.59
22:5n-3	0.57±0.18	0.96±0.29	0.98±0.30	0.90±0.31	0.84±0.34
22:6n-3	4.98±1.26	10.46±1.91	9.83±2.48	10.66±3.17	8.77±2.61
Σ n-6	14.36±5.52	16.99±1.88	13.98±2.02	8.60±2.56	10.52±0.54
Σ n-6 LC-PUFA	2.33±0.69	2.47±0.20	2.32±0.34	2.71±0.55	2.49±0.57
16:2n-6	0.10±0.05	0.04±0.02	0.06±0.02	0.09±0.04	0.14±0.07
18:2n-6	16.81±1.75^{a}	14.48±1.65^{ab}	11.60±1.70^{abc}	9.20±1.13^{bc}	7.89±0.19^{c}
18:3n-6	0.07±0.01^{c}	0.08±0.02^{bc}	0.13±0.01^{abc}	0.14±0.01^{ab}	0.17±0.03^{a}
20:2n-6	0.68±0.12	0.63±0.08	0.64±0.07	0.60±0.08	0.58±0.04
20:3n-6	**0.16±0.05**	**0.16±0.02**	**0.13±0.02**	**0.12±0.01**	**0.13±0.02**
20:4n-6	0.60±0.13	1.26±0.07	1.21±0.19	1.45±0.25	1.44±0.41
22:4n-6	0.05±0.01	0.09±0.02	0.10±0.02	0.11±0.03	0.11±0.04
22:5n-6	0.20±0.04	0.33±0.03	0.24±0.09	0.31±0.09	0.22±0.08
OLA/n-3 LC-PUFA	3.42±0.92	4.74±2.10	1.31±0.33	1.20±0.45	1.33±0.39
EPA/DHA	0.31±0.02^{a}	0.19±0.04^{b}	0.26±0.01^{ab}	0.22±0.01^{ab}	0.25±0.02^{ab}
EPA/ARA	3.77±0.59^{a}	1.19±0.44^{b}	2.04±0.36^{ab}	1.90±0.51^{ab}	1.47±0.05^{b}
EPA+DHA	6.51±1.65	12.82±2.56	12.31±3.02	12.99±3.80	10.88±3.20
Other FAs					
16:2n-4	0.10±0.06	0.03±0.00	0.03±0.02	0.14±0.08	0.07±0.05
16:3n-4	0.12±0.01	0.12±0.01	0.16±0.03	0.16±0.01	0.21±0.00
16:3n-1	0.30±0.08	0.36±0.03	0.25±0.07	0.42±0.07	0.49±0.18
16:4n-1	0.04±0.02	0.06±0.02	0.05±0.02	0.09±0.02	0.10±0.04
18:2n-4	0.22±0.06	0.34±0.15	0.36±0.05	0.27±0.04	0.28±0.06
18:3n-4	0.12±0.01	0.12±0.01	0.16±0.03	0.16±0.01	0.21±0.00
18:3n-1	0.02±0.01	0.03±0.02	0.02±0.00	0.02±0.00	0.04±0.03
18:4n-1	0.20±0.08	0.32±0.21	0.26±0.05	0.21±0.03	0.28±0.10

[1]Values (mean ±SE) with different superscript letters in the same row are significantly different (P<0.05).
* n-3 LC-PUFA (% total FA) x dietary lipids (%DM).

Liver histopathology

Histological examination of cross-sections of hepatic tissue showed that no necrotic tissue was found in meagre fed different dietary n-3 LC-PUFA levels. However, liver of fish fed 0.8% n-3 LC-PUFA showed a significantly higher degree of steatosis than those fed >2% n-3 LC-PUFA (**Table 11**). Liver of fish fed 0.8 and 1.4% n-3 LC-PUFA presented a severe steatosis, reflected by the hypertrophy of the hepatocytes (**Fig. 4A & 4B**). Furthermore, in the same fish, an extensive infiltration of lipid vacuolization in hepatocytes was observed and consequently, nuclei were displaced from the central position to the periphery of the cell. Contrarily, liver of fish fed 2.0-3.6% n-3 LC-PUFA showed smaller hepatocytes with spherical nuclei located at a central position of the cell although some lipid infiltration was also observed at a lower extension (**Fig. 4C, 4D & 4E**). Thus, hepatic steatotic alterations decreased linearly with the increase of the dietary n-3 LC-PUFA levels (r^2=0.88, P=0.19).

Additionally, despite the fact that gross examination did not reveal the presence of granulomatous lesions in any organ of any fish, those fed the lowest dietary n-3 LC-PUFA level (0.8%) presented higher (P<0.05) number of hepatic granulomas than fish fed ≥0.2 %n-3 LC-PUFA (**Table 11**). Regardless the dietary n-3 LC-PUFA level, two main developmental stages were observed: an early stage characterized by concentric layers of macrophages and inflammatory cells (**Fig. 5A & 5B**); and a more developed stage characterized by a necrotic center surrounded by an external fibroblast layer and inflammatory cells (**Fig. 5C & 5D**).

Table 11. Histomorphological evaluation of hepatic tissue of meagre fed the experimental diets for 30 days[1]

	Dietary n-3 LC-PUFA level (% DM*)				
	0.8	1.4	2.0	2.6	3.6
Steatosis[2]	$2.6^{a}\pm0.2$	$2.4^{ab}\pm0.2$	$1.7^{ab}\pm0.2$	$1.2^{b}\pm0.0$	$1.2^{b}\pm0.2$
Granulomas[3]	$5.3^{a}\pm1.6$	$2.1^{ab}\pm1.1$	$1.4^{b}\pm1.0$	$0.1^{b}\pm0.1$	$0.7^{b}\pm0.4$

[1]Means with different superscript letters in the same row are significantly different (P<0.05)
[2]Mean score value: score 0-1: normal liver histomorphology, score 1-2: moderate lipid infiltration; and score 2-3 high lipid infiltration.
[3]Granulomas: measured in number of granulomas observed in each sample.
* n-3 LC-PUFA (% total FA) x dietary lipids (%DM).

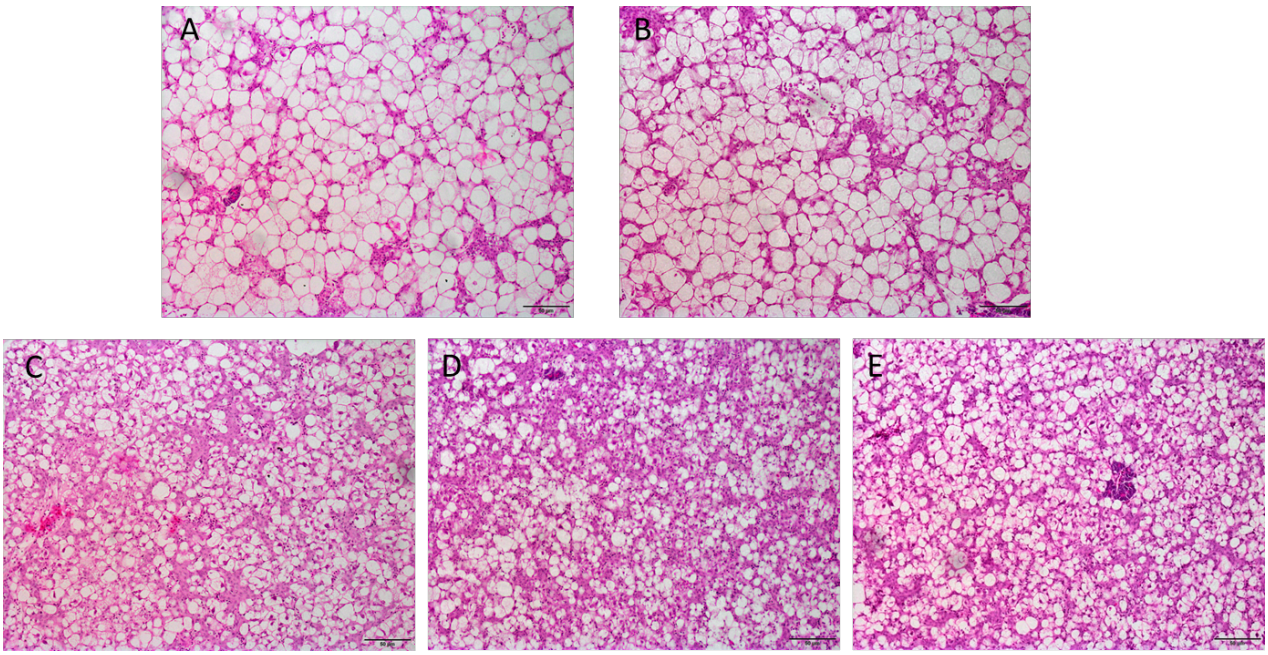

Figure 4: Liver sections from meagre fed different n-3 LC-PUFA levels stained with H&E, Bars 50µm: (A) 0.8% n-3 LC-PUFA; (B) 1.4% n-3 LC-PUFA; (C) 2.0% n-3 LC-PUFA; (D) 2.6 % n-3 LC-PUFA; (E) 3.6% n-3 LC-PUFA.

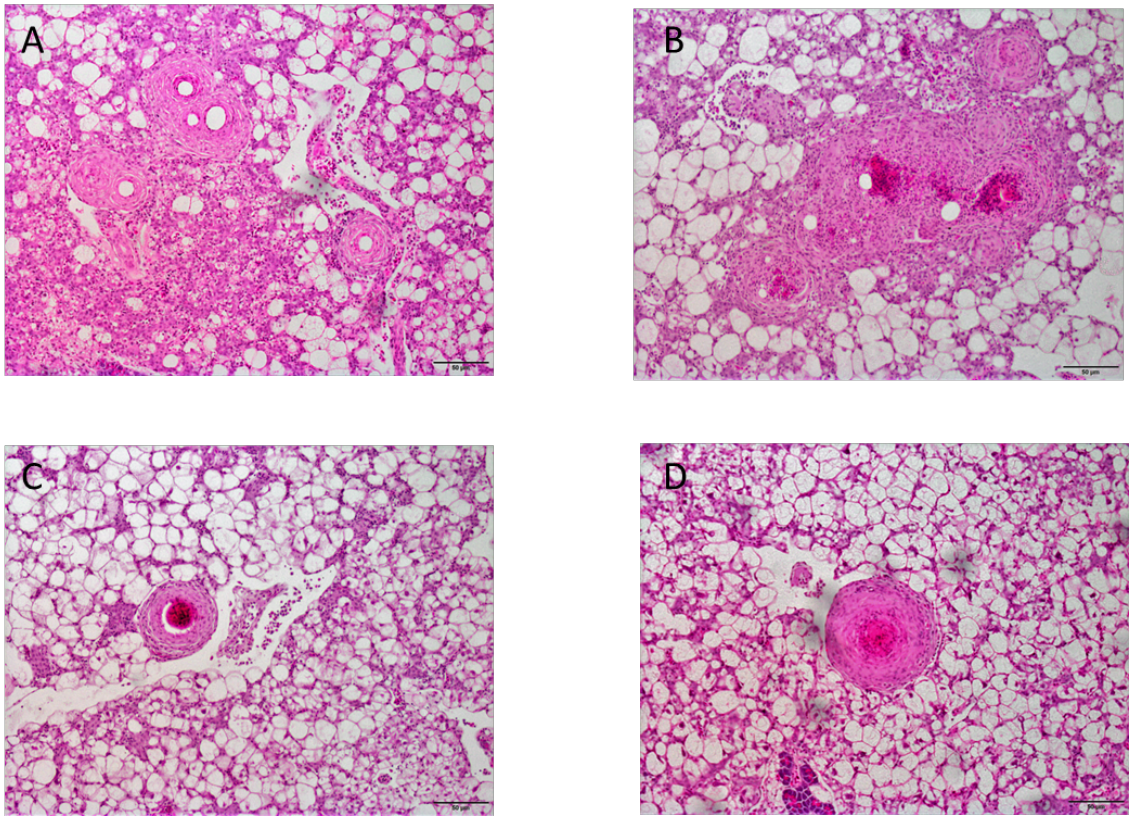

Figure 5: Liver sections of meagre with presence of granulomas at different stages of development: A and B showing concentric layers of macrophages and inflammatory cells around them and C and D showing necrotic centre with external fibroblast layer and inflammatory cells (Bars 50 µm).

Bacteriological results

Liver sections stained with acid-fast (Ziehl-Neelsen) led to negative results for the presence of *Mycobacterium sp*. and *Nocardia spp.*, and no colony forming units (CFU) of *Nocardia spp*. and *Streptomyces sp*. grew in any culture media utilised.

Digestive enzymes activity

As shown in **Table 12**, in general terms and except for the pepsin, activities measured in the gut content (inner) were clearly higher than those of the empty tissue. This is particularly evident for the pancreatic alkaline protease, which, in addition, was very active at the sampled fingerlings age, for all treatments.

Only inner amylase and inner pepsin displayed some significant differences among treatments, with amylase activity being higher in fish fed 2.6% n-3 LC-PUFA compared to 3.6% and 1.4% n-3 LC-PUFA.

Table 12. Specific enzymatic activity (U/mg protein) in meagre juveniles fed the experimental diets for 30 days[1]

	AMYLASE		PROTEASES	
Dietary n-3 LC-PUFA level (%DM*)	Tissue	Inner	Tissue	Inner
0.8	1.1 ± 0.5	5.5 ± 1.6 ab	310.9 ± 169.5	1350.3 ± 316.9
1.4	1.6 ± 0.2	3.6 ± 1.1 b	274.8 ± 131.1	1445.6 ± 495.9
2.0	1.4 ± 0.3	4.8 ± 2.3 ab	155.6 ± 36.8	930.2 ± 162.2
2.6	1.8 ± 0.2	8.1 ± 1.5 a	262.3 ± 46.4	1205.6 ± 116.6
3.6	1.3 ± 0.3	3.6 ± 0.9 b	252.7 ± 102.6	1010.6 ± 225.1

[1]Means with different superscript letters in the same row are significantly different (P<0.05). * n-3 LC-PUFA (% total FA) x dietary lipids (%DM).

Stress challenge trial

No mortality in fish was observed during and after the stress challenge. Fish fed the 2.6% n-3 LC-PUFA diet showed the highest growth in both body weight and fork length (**Fig. 6 and 7**).

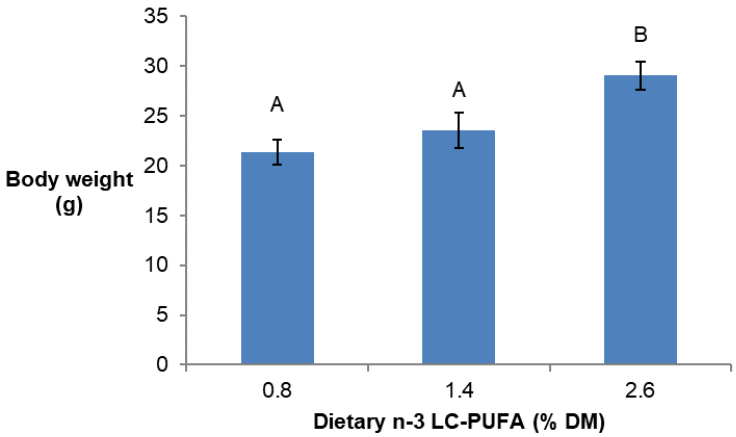

Figure 6. Final body weight of meagre fed the experimental diets at the end of the trial; bars with different letters are significant different (P<0.05).

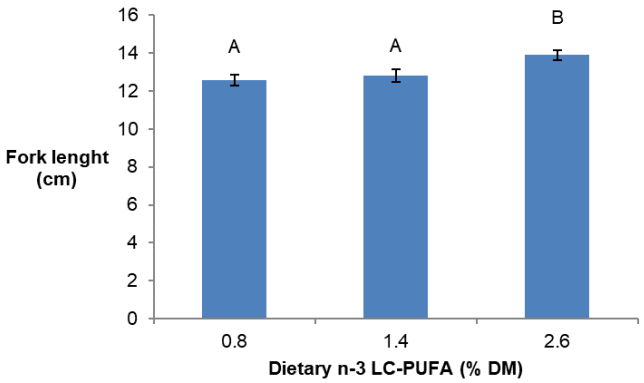

Figure 7. Final fork length of meagre fed the experimental diets at the end of the trial; bars with different letters are significant different (P<0.05).

In general, the results of the stress challenge showed nice activation and recovery of all markers induced by the stressor. Regarding plasma cortisol, 1h after inducing the stress all treatments presented higher plasma cortisol levels than respective control levels (pre-stress; **Fig. 8**). However, no significant differences were detected 1h and 5h post-stress between fish fed different n-3 LC-PUFA levels (**Fig. 8**). For glucose levels, 1h after inducing the stress, fish fed 1.4% showed significantly lower levels of plasma glucose than fish fed 0.8% n-3 LC-PUFA, however no significant differences were observed between different sampling times (**Fig. 9**). Furthermore, fish fed 0.8% n-3 LC-PUFA showed significantly higher plasma lactate concentration 1h after inducing stress than fish fed 1.4% and 2.6% n-3 LC-PUFA. After 5h, all treatments showed a recovery to basal levels (**Fig. 10**).

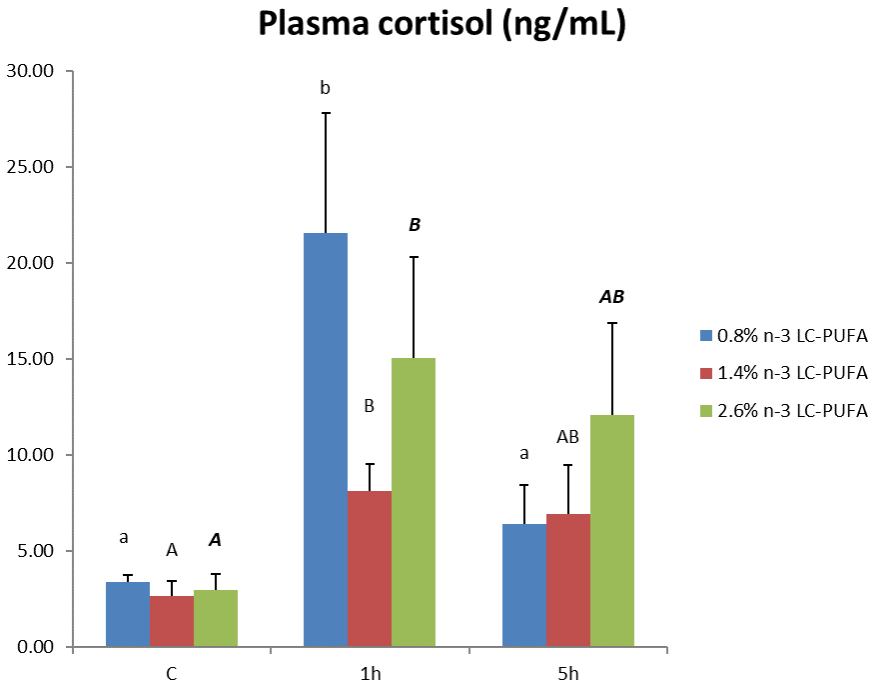

Figure 8. Plasma cortisol levels (ng/ml) in meagre fed the experimental diets before, 1h and 5h after the stressor; at specific sampling times, bars with different letters are significantly different (P<0.05).

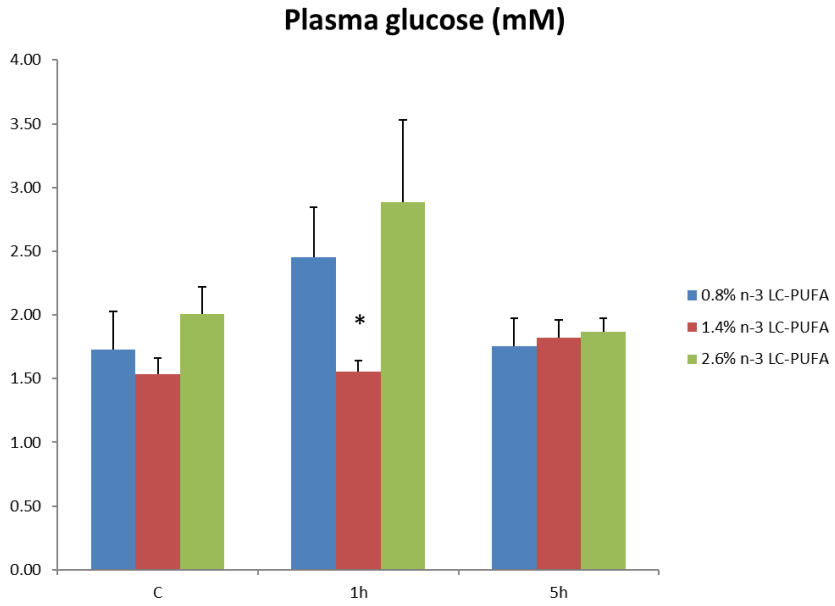

Figure 9. Plasma glucose levels (mM) in meagre fed the experimental diets before, 1h and 5h after the stressor; Asterisk denotes significant differences with respect to diet 0.8% n-3 LC-PUFA (P<0.05).

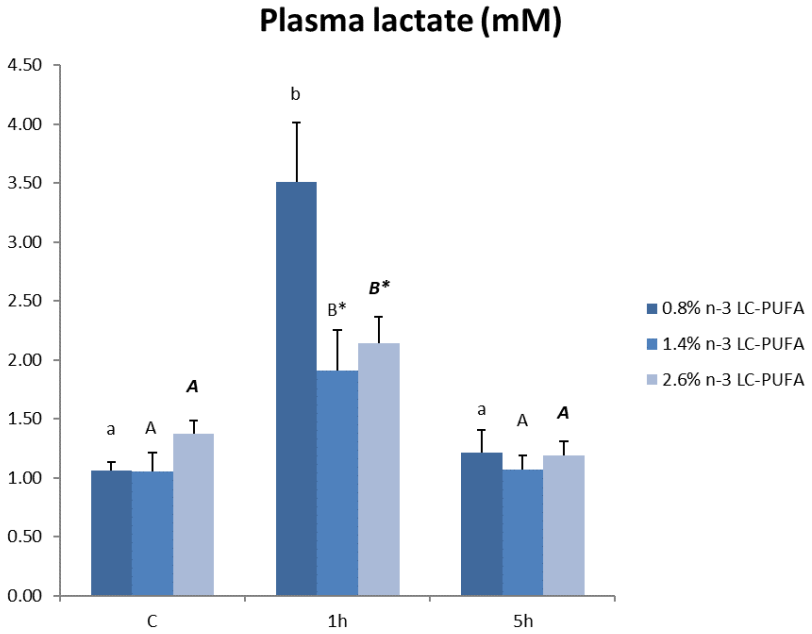

Figure 10. Plasma lactate levels (mM) in meagre fed the experimental diets before, 1h and 5h after the stressor; asterisk denotes significant differences with respect to diet 0.8% n-3 LC-PUFA (P<0.05).

The results of telencephalic serotonergic system showed that fish exhibited maximum 5-hydroxyindoleacetic acid (5-HIAA) levels 1h after inducing the stress, recovering at its basal levels 5h after inducing the stress, with no differences observed between fish fed different n-3 LC-PUFA levels (**Fig. 11**). In contrast, serotonin (5-HT) levels were unaffected by experimental diets (**Fig. 12**). The 5-HIAA/5-HT ratio, an indicator of serotonergic activity, increased significantly 1h after inducing the stress in meagre fed 0.8 and 1.4% n-3 LC-PUFA, returning to values even lower than basal values 5h after inducing the stress (**Fig. 13**). However, this ratio was unaffected by stress in meagre fed 2.6% n-3 LC-PUFA.

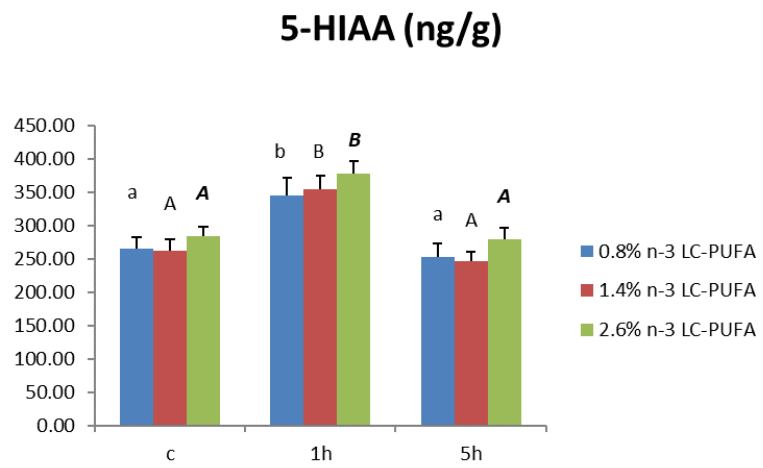

Figure 11. 5-hydroxyindoleacetic acid (5-HIAA) levels in brain of meagre fed the experimental diets before, 1h and 5h after the stressor. Different letters denote significant differences (P<0.05).

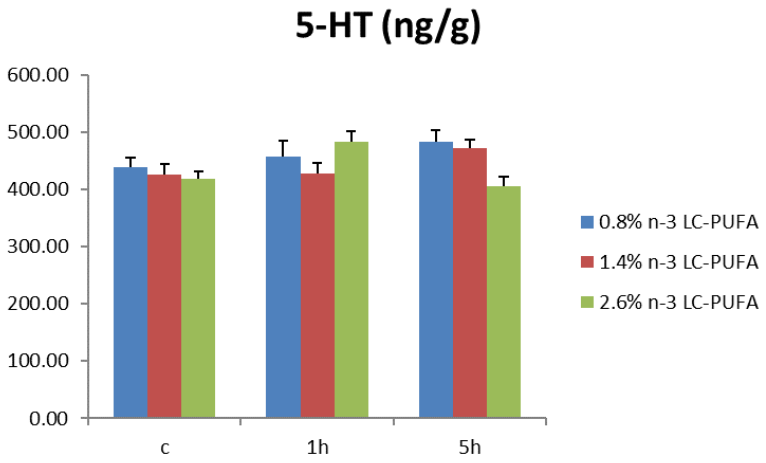

Figure 12. Serotonin (5-HT) levels in brain of meagre fed the experimental diets before, 1h and 5h after the stressor; no significant differences were observed.

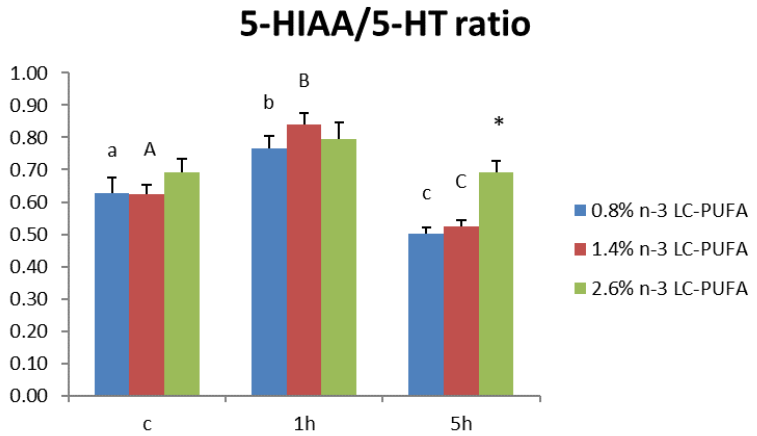

Figure 13. 5-hydroxyindoleacetic acid (5-HIAA)/ serotonin (5-HT) ratio values in brain of meagre fed the experimental diets before, 1h and 5h after the stressor; at specific sampling times, bars with different letters are significantly different, and asterisk denotes significant differences with respect to diet 0.8% n-3 LC-PUFA ($P<0.05$).

4.-Discussion and conclusions

Meagre is one of the best candidates for Mediterranean aquaculture production. Nevertheless, little information is available regarding its specific nutritional requirements. To our knowledge, this is the first study aiming to determine the n-3 LC-PUFA requirements for meagre juveniles.

Reduced survival, bacterial diseases, fin erosion or shock syndrome are some of the usual effects observed in fish with EFA-deficiency (Izquierdo, 2005; Glencross, 2009; Tocher, 2010). However, in the present study none of the above mentioned EFA deficiency signs was detected, possibly as the EFA dietary levels were not extremely low. In the present study, growth increased as dietary n-3 LC-PUFA levels increased up to 2.0-2.6%. However, no further improvement of growth occurred by the inclusion of higher dietary levels. Based on these results and the broken-line model, dietary n-3 LC-PUFA levels of, at least, 2.1% n-3 LC-PUFA were required for maximum growth performance of meagre fingerlings. This conclusion is in agreement with the concomitant higher ($P<0.05$) growth of meagre fed n-3 LC-PUFA higher than 2% obtained by the fish of the present study, at the moment of stress challenge trial, even after 78 days of feeding.

Diet is the most important factor influencing fish fatty acid composition (Cowey and Sargent, 1972). In general, the whole-body FA composition of meagre reflected the FA composition of the diets. The increase of SFA and ARA, EPA and DHA, present in FO in higher levels than in VO, was expected with the increase in dietary n-3 LC-PUFA in relation to the dietary substitution of VO by FO, as well as the decrease in MUFA, LA and ALA. Marine fish have a restricted physiological capacity to produce LC-PUFA from C_{18}-PUFA precursors due to a limited enzymatic capacity for elongation and desaturation. Therefore, ARA, EPA and DHA are EFA for marine species (Izquierdo, 1996, Kanazawa, 1997). In the present study, the high retention of 18:2n-9 and 18:3n-6 in fish fed the two diets with the lowest n-3 LC-PUFA level (0.8% and 1.4%) suggests an endogenous synthesis of these PUFA through Δ6 desaturases. Accordingly, fish fed 0.8% n-3 LC-PUFA confirmed a higher relative gene expression of *fads2* than fish fed the highest n-3 LC-PUFA level (3.6%).

Similarly, the high retention of chain-elongation products of LA and ALA, namely 20:2n-6 and 20:3n-3, in meagre fed the lowest n-3 LC-PUFA levels (0.8% and 1.4%) also suggests an endogenous biosynthesis of these PUFA through Elovl5. Furthermore, the results of relative gene expression of *elovl5* showed a tendency for up-regulation in meagre fed the lowest n-3 LC-PUFA level. These results indicate that meagre has some *in vivo* capacity to elongate and desaturate C_{18}-PUFA. However, synthesis of DHA and EPA also requires Δ5 and, possibly, Δ4 desaturases, besides Δ6 desaturase and Elovl5. Thus, the insufficient capacity

of meagre to satisfy EFA requirements from PUFA precursors may be due to the lack of these enzymes or to the insufficient activity of one or several elongases or desaturases.

The production of n-9 FA, a product of Δ6 desaturase activity, is a biochemical indicator of EFA-deficiency (Watanabe, 1982). In the present study, the highest retention of 18:2n-9, was observed in fish fed the 0.8% n-3 LC-PUFA diet, suggesting that these fish are EFA-deficient. This result agrees well with other studies, which reported the production of 18:2n-9 in tissues of sea bream and turbot fed insufficient EFA levels (Tocher et al., 1988; Kalogeropoulos et al., 1992; Tocher, 2010).

All the three main LC-PUFAs ARA, EPA and DHA have been found to be essential for marine fish (Izquierdo and Koven, 2011), and to be preferentially retained over other fatty acids during periods of starvation (Tandler et al., 1989) or low dietary levels (Izquierdo et al., 2000). In the present study, retentions of DHA and ARA were higher than that of EPA, particularly in fish fed 0.8% n-3 LC-PUFA diet, suggesting that EPA was preferably catabolised, for energy or for eicosanoids production rather than DHA and ARA (Mourente and Bell, 2006). The mechanisms justifying this selective retention of DHA comprise the higher specificity of fatty acyl transferases to be incorporated into lipid membranes and the lower functionality as substrate for β-oxidation of DHA, in such way that EPA and other FAs are preferably catabolised for energy production and DHA conserved in membrane phospholipids (Froyland et al., 1997). Besides, despite the fact that dietary ARA plays an important role in fish health, it is not as efficient as DHA or even EPA in improving fish growth (Izquierdo et al., 2000). Thus, increase in dietary DHA or EPA in the present study seems to be more responsible for the growth improvement in fingerling meagre than ARA increase.

Organ indexes, such as HSI, have been frequently used to evaluate the effect of dietary components, such as lipids and carbohydrates (Peres and Oliva-Teles, 1999; Montero et al., 2001; Bolla et al., 2011; Castro et al., 2015). Increase in dietary n-3 LC-PUFA increases β-oxidation, phospholipid synthesis and, consequently, lipoprotein formation avoiding hepatic lipid accumulation (Watanabe, 1982; Montero et al., 2001). Therefore, increased HSI, liver lipid content and hepatic steatosis have been frequently used as indicators of EFA deficiency (Watanabe, 1982; Verreth et al., 1994; Montero et al., 2001). In the present study, the high HSI, liver lipid content and hepatic steatosis degree found in meagre fed 0.8 and 1.4% n-3 LC-PUFA denote symptoms of EFA deficiency in these fish.

Total lipids FA composition of liver showed the same tendency as whole-body FA composition. Furthermore, the ratio OLA/n-3 LC-PUFA is recognized as biochemical indicator of EFA-deficiency (Baweja and Babbar, 2015; Torrecillas et al., 2017). In the present study, meagre fed n-3 LC-PUFA levels below 2% showed significantly higher values of OLA/n-3 LC-PUFA ratio than fish fed levels around or above the EFA requirement, suggesting that these fish are EFA-deficient. Contrarily to the FA profile of level total lipids, the FA profile of liver polar lipids was less affected by diet composition, with most of the FA levels being constant among meagre fish fed different n-3 LC-PUFA levels. Since n-3 LC-PUFA are required for sustaining the structure and functionality of cell membranes, the FA profile of polar lipids is more constant than neutral or total lipid (Sargent et al., 1989).

EFA deficient meagre in the present study also showed a higher incidence of granulomas than fish fed ≥2% n-3 HUFA. Granulomatosis is one of the pathological features more common in cultured meagre (Ghittino et al., 2004; Pantelis Katharios, pers.com.) and affects a variety of internal organs, particularly liver and kidney.

Despite the fact that bacteria were not specifically studied in the meagre of the present study, the analysis conducted led to negative results for the presence of *Nocardia sp*. and *Mycobacterium spp*. Furthermore, no signs of bacterial disease were found, including external lesions, liver necrosis, reduced appetite and feed intake, melanosis, mortality, etc., a fact that further supported the lack of a bacterial infectious disease origin in the formation of granulomas. Thus, these results suggest that hepatic granulomas of meagre fingerlings are probably associated to the dietary n-3 LC-PUFA deficiency, as higher incidence of this disease was observed in fish fed n-3 LC-PUFA levels below the requirement estimated for the species.

Cortisol magnitude and duration depends on the species, and also intensity, duration and type of stress (Iwama et al., 2006). The effect of certain fatty acids in the physiological response to stress in the alteration of HPI is studied (Montero et al., 2015). In the present research, cortisol levels reached their highest levels

1h after inducing the stress, and were followed by a decrease after 5h, suggesting acclimatization. Similarly, in most fish species, the maximum cortisol levels are obtained after 30 minutes and 1 hour, with a recovery of the basal levels after 2-48 hours after submitting them to stress (Rotllant et al., 2001). In the same way, lactate plasma levels were the highest 1h after inducing stress, returning to basal levels 5h after inducing stress. Contrary to cortisol levels, plasma lactate was the highest in fish fed 0.8% n-3 LC-PUFA, suggesting a stronger effect of a mild stressor in these fish.

Serotonin release and turnover is responsive to multiple stressors, for example, social interaction, nutritional status, and immune challenges. Stressful situations lead to a rapid elevation in plasma cortisol along with an activation of the brain 5-HT system, as indicated by elevated brain concentrations of 5-HIAA, a serotonin precursor, and 5-HIAA/5-HT ratios. In the present study, meagre showed the highest levels of 5-HIAA in brain 1h after inducing the stress, returning to basal levels 5h after inducing the stress, suggesting a recovery at this time. Interestingly, the 5-HIAA/5-HT ratio in fish fed 0.8 and 1.4% n-3 LC-PUFA increased 1h after inducing the stress, while in fish fed 2.6% n-3 LC-PUFA this ratio was unaffected, suggesting these fish were less affected by the stressor than meagre fed lower values of n-3 LC-PUFA.

In conclusion, meagre is a competitive candidate for Mediterranean aquaculture production and this fact was highlighted by its high growth rates and excellent feed conversion ratios in the present study. Meagre showed the ability to selectively conserve key FA, particularly DHA and ARA over other FA, in response to EFA-deficiency. Furthermore, meagre seems to have active Δ6 desaturases and Elovl5, but their activities were insufficient to produce DHA and EPA from PUFA precursors to sustain fast growth. Based in overall results the present study shows that the requirement for n-3 LC-PUFA for meagre fingerlings is at least 2.0% DM in diets containing 16.5% DM lipids, 0.9 EPA/DHA and 0.4% ARA of total FA contents.

5.-References

A.O.A.C. Association of Official Analytical Chemists, 2000. Official methods of analysis, 17th ed. Association of Official Analytical Chemists, Gaithersburg, MD, USA.

Baweja, S., Babbar, B. K., 2016. Restoration of fatty acid composition of common carp *Cyprinus carpio* (Linnaeus, 1758) fed terrestrial oil based diets using fish oil finishing diet. Indian Journal of Fisheries, *63*(1).

Bolla, S., Nicolaisen, O., Amin, A., 2011. Liver alterations induced by long term feeding on commercial diets in Atlantic halibut (*Hippoglossus hippoglossus L.)* females. Histological and biochemical aspects. Aquaculture. 312, 117-125.

Bradford M.M., 1979. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Analytical Biochemistry 72, 248-254.

Caballero, M.J., Izquierdo, M. S., KjØrsvik, E., Férnandez, A., Rosenlund, G., 2004. Histological alterations in the liver of sea bream, *Sparus aurata L.*, caused by short- or long-term feeding with vegetable oils. Recovery of normal morphology after feeding fish oil as the sole lipid source. Journal of Fish Diseases, 27, pp. 531-541.

Caballero, M.J., López-Calero, G., Socorro, J., Roo, F.J., Izquierdo, M.S., Férnandez, A.J., 1999. Combined effect of lipid level and fishmeal quality on liver histology of gilthead seabream (*Sparus aurata*). Aquaculture, 179, pp. 277-290.

Castro, C., Couto, A., Pérez-Jiménez, A., Serra, C. R., Díaz-Rosales, P., Fernandes, R., Corraze, G., Panserat, S., Oliva-Teles, A., 2016. Effects of fish oil replacement by vegetable oil blend on digestive enzymes and tissue histomorphology of European sea bass (*Dicentrarchus labrax*) juveniles. Fish physiology and biochemistry *42*(1), 203-217.

Castro, C., Corraze, G., Perez-Jimenez, A., Larroquet, L., Cluzeaud, M., Panserat, S., Oliva-Teles, A., 2015. Dietary carbohydrate and lipid source affect cholesterol metabolism of European sea bass (*Dicentrarchus labrax*) juveniles. The British journal of nutrition. 114, 1143-1156.

Chatzifotis, S., Panagiotidou, M., Papaioannou, N., Pavlidis, M., Nengas, I. y Mylonas, C.C., 2010. Effect of dietary lipid levels on growth, feed utilization, body composition and serum metabolites of meagre (*Argyrosomus regius*) juveniles. Aquaculture 307, 65-70.

Christie, W.W., 1989. Gas Chromatography and Lipids: A Practical Guide, The Oily Press, Glasgow, UK.

Cowey, C.B., Sargent, J.R, 1972. Fish nutrition. Mar. Biol. 10, 383-492.

El-Kertaoui, N., Hernández-Cruz, C. Montero, D., Caballero, M.J., Saleh, R., Alfonso, J.M., Izquierdo, M., 2015. The importance of dietary HUFA for meagre larvae (*Argyrosomus regius*; Asso, 1801) and its relation with antioxidant vitamins E and C. Aquacult. Res. 148(2), 419-433.

Folch, J., Lees, M., 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.

Froyland, L., Madsen, L., Vaagenes, H., Totland, G.K., Auwerx, J., Kryviv, H., Staels, B., Berge, R.K. 1997. Mitochondrion is the principal target for nutritional and pharmacological control of triglyceride metabolism. J. Lipid Res. 38 (9), 1851-1858.

Ghittino, C., Manuali, E., Latini, M., Agnetti, F., Rogato, F., Agonigi, R., Colussi, S., Prearo M. (2004): Caso di granulomatosis sistemica in ombrina boccadoro (*Argyrosomus regius*) e raffronto con le lesioni istologiche presenti nell'orata. Ittiopatologia, 1, 59-67.

Gisbert, E., Giménez, G., Fernández, I., Kotzamanis, Y and Estevez. A., 2009. Development of digestive enzymes in common dentex *Dentex dentex* during early ontogeny. Aquaculture 287 (3), 381-387.

Glencross, B., 2009. Exploring the nutritional demand for essential fatty acids by aquaculture species. Rev. Aquacult. 1, 71-124.

Good, C., Marancik, D.P., Welch, T.J., May, T., Davidson, J., Summerfelt, S., 2016. Systemic granuloma observed in Atlantic Salmon *Salmo salar* raised to market size in a freshwater recirculation aquaculture system. Aquaculture Research, pp. 1-5.

Herman, R., 1996. Systemic noninfectious granulomatoses of fishes. Fish Disease Leaflet, 79.

Iijima, N., Tanaka, S. and Oka, Y., 1998. Purification and characterization of bile-salt activated lipase from the heaptopancreas of red sea bream *Pagrus maj*or. Fish Physiol. Biochem. 18, 59-69.

Iwama, G.K., Afonso, L.O.B., and Vijayan, M., 2006. Stress in fish, En: Evans, D.H., Claiborne, J.B.(Eds.), The Physiology of Fishes, Third ed. CRC Press, Boca Raton, Florida, 319–342 pp.

Izquierdo, M., 1996. Essential fatty acid requirements of culture marine larvae. Aquaculture Nutrition 2, 183-191.

Izquierdo, M.S., 2005. Essential fatty acid requirements in Mediterranean fish species. Cah. Opt. Méd., 63, 91-102.

Izquierdo, M.S., Koven, W., 2011. Lipids. In: Larval Fish Nutrition, J. Holt (Ed.) Wiley-Blackwell, John Willey and Sons Publishers, pp. 47-82.

Izquierdo M.S., Socorro, J., Arantzamendi, L., Hernandez-Cruz, C.M. 2000. Recent advances in lipid nutrition in fish larvae. Fish Physiol. Biochem. 22, 97-107.

Izquierdo, M., Watanabe, T., Takeuchi, T., Arakawa, T., Kitajima, C., 1990. Optimum EFA levels in Artemia to meet the EFA requirements of red seabream (*Pagrus major*). In: Takeda, M., and Watanabe, T. (Eds.). The current Status of Fish Nutrition in Aquaculture. Tokyo Univ. Fisheries, Tokyo, pp. 221– 232.

Juaneda, P., Rocquelin , G., 1985. Rapid and convenient separation of phospholipids and non phosphorus lipids from rat heart using silica cartridges. Lipids, V.20, Issue 1, pp. 40-41.

Kalogeropoulos, N., Alexis, MN., Henderson, RJ., 1992. Effects of dietary soybean and cod-liver oil levels on growth and body composition of Gilthead Bream (*Sparus aurata*). Aquaculture 104, 293–308.

Kanazawa, A., 1997. Effects of docosahexaenoic acid and phospholipids on stress tolerance of fish. Aquaculture155, 129-134.

Kružić, N., Mustać, B., Župan, I., Čolak, S., 2016: Meagre (*Argyrosomus regius* Asso, 1801) aquaculture in Croatia. Journal of Fisheries 74, 14-19.

Marras, S., Killen, S. S., Claireaux, G., Domenici, P., McKenzie, D. J., 2011. Behavioural and kinematic components of the fast-start escape response in fish: individual variation and temporal repeatability. Journal of Experimental Biology 214(18), 3102-3110.

Martoja R., Martoja-Pierson M., 1970. Tecnicas de Histologıa Animal. Ed. Toray-Masson S.A., Barcelona, 350 pp.

Métais, P. and Bieth, J., 1968. Détermination de l´α-amylase. Ann. Biol. Clin. 26,133-142.

Montero, D., Robaina, L.E., Socorro, J., Vergara, J.M., Tort, L., Izquierdo, M.S., 2001. Alteration of liver and muscle fatty acid composition in gilthead sea bream (*Sparus aurata*) juveniles held at high stocking density and fed an essential fatty acid deficient diet. Fish Physiology and Biochemistry, 24, pp. 63–72.

Montero, D., Terova, G., Rimoldi, S., Tort, L., Negrin, D., Zamorano, M. J., Izquierdo, M., 2015. Modulation of adrenocorticotrophin hormone (ACTH)-induced expression of stress related genes by PUFA in inter-renal cells from European sea bass (*Dicentrarchus labrax*). Journal of nutritional science, 4 (e16): 1-13.

Monroig, O., Tocher, D.R., Hontoria, F., Navarro, J.C., 2013. Functional characterisation of a Fads2 fatty acyl desaturase with Δ6/Δ8 activity and an Elovl5 with C16, C18 and C20 elongase activity in the anadromous teleost meagre (*Argyrosomus regius*). Aquaculture 412-413, 14-22.

Mourente, G., Bell, J.G., 2006. Partial replacement of dietary fish oil with blends of vegetable oils (rapeseed, linseed and palm oils) in diets for European sea bass (*Dicentrarchus labrax L.*) over a long term growth study: Effects on muscle and liver fatty acid composition and effectiveness of a fish oil finishing diet. Comp. Biochem. Physiol. Part B, 145 389–399.

NRC, 2011. Lipids. In: Nutrient requirements of fish and shrimp, National Academies Press, Washington D. C., USA.

Oliva-Teles, A. 2012. Nutrition and health of aquaculture fish. J. Fish Dis. 35 (2), 83–108.

Paperna I., 1987. Systemic granuloma of spariid fish in culture. Aquaculture, 67, pp. 53–58.

Paperna, I., J.G., Harrison, G.W., Kissil., 1980. Pathology and histopathology of a systemic granuloma in *Sparus aurata (L.*) cultured in the Gulf of Aqaba. Journal Fish Disease, 3, pp. 213-221.

Peres, H., Oliva-Teles, A., 1999. Effect of dietary lipid level on growth performance and feed utilization by European sea bass juveniles (*Dicentrarchus labrax*). Aquaculture 179, 325-334.

Roberts, R.J., 2002. Nutritional pathology, Fish nutrition, 3rd Edition. Elsevier, pp. 453.

Rotllant, J., Balm, P. H. M., Perez-Sanchez, J., Wendelaar-Bonga, S. E., Tort, L., 2001. Pituitary and interrenal function in gilthead sea bream (*Sparus aurata L.,* Teleostei) after handling and confinement stress. General and comparative endocrinology, 121(3): 333-342.

Saavedra, M., Grade, A., Candeias-Mendes, A., Pereira, T.G., Teixeira, B., Yúfera, M., Conceição, L.E.C., Mendes, R., Pousão-Ferreira, P., 2016. Different dietary proteins levels affect meagre (*Argyrosomus regius*) larval survival and muscle cellularity. Aquaculture 450, 89-94.

Sargent, J. R. Bell, J. G, Bell, M. V., Henderson, R. J., Tocher, D.R., 1995. Requirement criteria for essential fatty acids. Symposium of European Inland Fisheries Advisory Commission. J. Appl. Ichthyol. 11, 183–198.

Sargent, J.R., Henderson, J., Tocher, D. R., 1989. The Lipids. 154-209 In: Fish Nutrition, 2da ed. Ed. Halver, J.E. Academic Press. London.

Spisni, E., Tugnoli, M., Ponticelli, A., Mordenti, T., Tomasi, V., 1998. Hepatic steatosis in artificially fed marine teleosts. Journal of Fish Diseases, 21, pp. 177-184.

Tandler, A., Watanabe, T., Satoh, S., Fukusho, K., 1989. The effect of food deprivation on the fatty acid and lipid profile of red seabream (*Pagrus major*) larvae. British Journal of Nutrition 62(2), 349-361.

Tocher, D.R., 2003. Metabolism and functions of lipids and fatty acids in teleost fish. Rev. Fish. Sci.11(2), 107-184.

Tocher, D.R., 2010. Fatty acid requirements in ontogeny of marine and freshwater fish. Aquac. Res. 41, 717-732.

Tocher, D.R., Sargent, J.R., Frerichs, G.N., 1988. The fatty acid compositions of established fish cell lines after long-term culture in mammalian sera. Fish Physiol. Biochem. 5, 219-227.

Torrecillas, S., Robaina, L., Caballero, M. J., Montero, D., Calandra, G., Mompel, D., Karalazos, V., Kaushik, S., Izquierdo, M.S., 2017. Combined replacement of fishmeal and fish oil in European sea bass (*Dicentrarchus labrax*): Production performance, tissue composition and liver morphology. Aquaculture 474, 101-112.

Tukey, J.W., 1949. Comparing individual means in the analysis of variance. Biometrics, 5, 99–114.

Watanabe, T., 1982. Lipid nutrition in fish. Comp. Biochem. Physiol. Part B 73, 3–15.

Worthington Biochemical Coorporation., 1972. Worthington Enzyme manual: Enzymes, Enzyme Reagents, Related Biochemicals. Worthington Biochemical Coorp., Freehold, N.J.

Verreth, J., Coppoolse, J., Segner, H., 1994. The effect of low HUFA- and high HUFA-enriched Artemia, fed at different feeding levels, on growth, survival, tissue fatty acids and liver histology of *Clarias gariepinus* larvae. Aquaculture, 126, pp. 137–150.

Deviations:

This deliverable was prepared according to the work described in Task 8.2. No deviations exist from DOW.

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