Deliverable Report

Deliverable No:	D23.1	**Delivery Month:**	22
Deliverable Title	Cost-effective weaning strategies for wild-caught grey mullet grow out and their effect on growth and health status		
WP No:	23	**WP Lead beneficiary:**	P4
WP Title:	Grow out husbandry – grey mullet		
Task No:	23.1	**Task Lead beneficiary:**	P3
Task Title:	Determine the cost-benefit of different weaning diets on the performance and health status of wild juveniles.		
Other beneficiaries:	P3. IRTA		
Status:	Completed	**Expected month:**	18
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Objective: The objective of this Deliverable was to determine the cost-benefit of different weaning diets on the performance and health status of grey mullet fry.

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1. Introduction

The diversification of the aquaculture industry, which is based on social, economic and ecological considerations, is an important tool for the sustainability of this fast-growing industry. The grey mullet (*Mugil cephalus*) is an economically important euryhaline and eurythermal species contributing to sizable fisheries of estuarine and coastal regions in many countries. This species has been recognized as a potential species for aquaculture diversification in the Mediterranean region, because of its good adaptation to captivity, rapid growth, omnivourous feeding habits and high market price of its salt-cured and dried eggs named "bottarga", in addition to its flesh (Whitfield et al., 2012).

This species is generally reared extensively in mono- or polyculture systems (Oren, 1981; Biswas et al., 2012), but in order to supply an established market in the North of Africa and the growing demand in the Mediterranean area, Asia (China, Taiwan) and South Africa (Whitfield et al., 2012), the intensive monoculture of this species has to be developed. This fact implies the development of a technology for the breeding of this species under aquaculture conditions and the development of a suitable and economical grow-out diet. However, before the seed could be provided by the aquaculture industry for on-growing purposes, the culture of this species is still based on wild fry captured during their migration into estuarine environments (Whitfield et al., 2012; Biswas et al., 2012; El-Dahhar et al., 2014). Thus, optimizing weaning diets for this species at this particular stage of development is of special importance.

Mullets are best described as omnivorous, very opportunistic feeders, thriving on all available food (Oren, 1981). However, information on the formulation of practical feeds for cultured mullets is relatively scarce (Wassef et al., 2001). The diet of large juvenile (>10 cm in standard length) and adults of grey mullet is mainly based on detritus and benthic microalgae together with foraminiferans, filamentous algae, protists, meiofauna and small invertebrates (see review in Whitfield et al., 2012), which seems to indicate that on-growing diets for this species may be formulated with high levels of fish meal (FM) substitution by alternative protein sources (Wassef et al., 2001; Kalla et al., 2003; Jana et al., 2012; El-Dahhar et al., 2014). This is relevant as the global FM supply remains relatively static; resulting in high production costs and a reduction in its availability for the large-scale utilization in high-quality fish FM based diets. Moreover, the use of the plant products in aquafeeds is more economical and environmentally sustainable; suggesting that a focus on omnivorous and detritivorous fish species is needed. Consequently, FM replacement by a blend of plant protein (PP) sources in fish feeds is presently a major trend in aquaculture (Gatlin et al., 2007).

In this task, we aimed to evaluate a weaning protocol for wild grey mullet fry based on the transition from live prey to inert diets with different levels of fish meal substitution (0, 50 and 75%) in diets, in terms of growth performance, survival, proximate composition and digestive processes.

2. Materials and methods

2.1 Biological material and experimental design

Three thousand five hundred wild grey mullet fry measuring 24.2 ± 0.8 mm in standard length (SL) and weighting 202 ± 5 mg in body weight (BW) were obtained by a local dealer (Pescados y Mariscos Roset SL, Deltebre, Spain). Fish were captured at night during their migration from coastal waters into estuarine and freshwater environments, and in the following morning transported by road (1 hour trip) to the IRTA facilities, were they were acclimated in two 400-L quadrangular tanks to 17ºC for 7 days before the onset of the experiment. At their arrival, fish were treated with a bath of oxytetracycline (20 mg/L) for 12 hours in order to prevent the outbreak of pathogenic bacteria, and two days after, fish were kept in brackish water (salinity = 14‰) during 14 h in order to remove external parasites. Grey mullet fry were identified according to the number of pyloric caeca in their gut and external pigmentation patterns as shown in **Figure 1** (Cambrony, 1984).

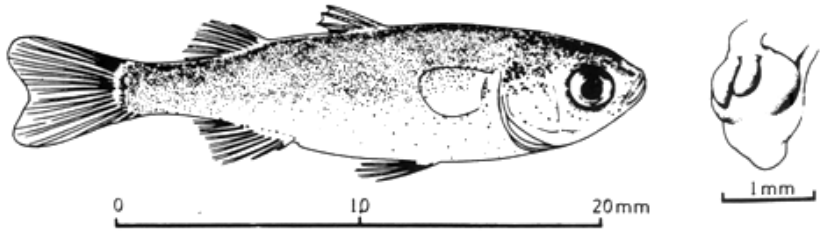

Figure 1. Drawing of a grey mullet fry (*Mugil cephalus*) showing the number of pyloric caeca in their stomach and external pigmentation patterns used for its proper identification from other grey mullet species recruiting in the same area and season of the year.

During their acclimation, fish were fed *ad-libitum* with *Artemia* metanauplii and dead fish were removed from the tanks on a daily basis. *Artemia* metanauplii used for feeding grey mullet fry during the acclimation period and weaning trial were obtained after 6 days feeding of a mixture of microalgae (*Tetraselmis suecica*, *Isochrysis galbana*). After the acclimation period, fry were distributed among 12 cylindroconical 100-L tanks (**Figure 2**) (n = 200/tank) connected to a recirculation system IRTAmar® in order to maintain water quality and temperature during the trial (60 days). Water quality conditions were as follows: temperature, 18.1 ± 0.3ºC (mean ± standard deviation, SD); salinity, 1.2 ± 0.2 ‰; dissolved oxygen, 6.5 ± 0.4 mg/L (~90% saturation); $NH4^+$, 0.20-0.29 ppm; NO_2, 0.001 ppm, and the photoperiod was 10L:14D (light : darkness).

Grey mullet fry were weaned onto each of the three experimental diets with different levels of fish meal substitution (FM, no fish meal substitution; PP50, 50% substitution of fish meal with plant protein sources; PP75, 75% substitution of fish meal with plant protein sources) according to the following protocol:

i) days 0-5: 100% live 6 days-old *Artemia* metanauplii (15-20 metanauplii/mL);
ii) days 6-10: 75% *Artemia* metanauplii + 25% inert feed (FM, PP50, PP75);
iii) days 11-15: 50% *Artemia* metanauplii + 50% inert feed (FM, PP50, PP75);
iv) days 16-20: 25% *Artemia* metanauplii + 75% inert feed (FM, PP50, PP75);
v) days 21-60: 100% inert feed (FM, PP50, PP75).

Figure 2. General view of the research facility used in this study. Each black fiberglass tank has a functional volume of 100 L and is connected to a recirculation water system (IRTAmar®). The upper image shows grey mullet fry in a tank (200 fish/tank).

2.2 Diets

The experimental diets used in this trial were formulated and manufactured by Sparos Lda. (Portugal). Diets (pellet size: 0.8 mm) were formulated to be isoproteic, isolipid and isoenergetic and contain *ca*. 36% proteins and 16.0% lipids (**Table 1**). The purpose of diet formulation was to evaluate the effects of different levels of partial fish meal substitution (50 and 75%) on the weaning of grey mullet fry. Fish meal (FM) was partially substituted at 50 and 75% by means of plant protein sources (corn gluten, wheat gluten and soy protein concentrate; **Table 1**) and they were named according to the level of fish meal substitution: FM (control, 0% substitution), PP50 (50% of fish meal substitution with plant proteins) and PP75 (75% of fish meal substitution with plant proteins). Diets PP50 and PP75 contained L-lysine and DL-methionine in order to balance their respective amino acid profile.

The fatty acid composition and the amino acid profile of experimental diets are shown in **Tables 2 and 3**, respectively. Although this species has an omnivorous diet based on grazing on plant detritus and microflora (Whitfield et al., 2012), a diet based on the complete substitution of fish meal by plant protein sources was discarded since this species is carnivorous at the fry stage (Gisbert et al., 1995).

Table 1. Ingredient list and proximate chemical composition of experimental diets tested to evaluate the effects on weaning and performance in grey mullet (*Mugil cephalus*) fed experimental diets with different levels of fish meal substitution (FM, no fish meal substitution; PP50, 50% substitution of fish meal with plant protein sources; PP75, 75% substitution of fish meal with plant protein sources).

Ingredients	FM	PP50	PP75
	%	%	%
Fishmeal 70 LT	32.0	16.0	8.0
Hydrolized fish protein concentrate	5.0	5.0	5.0
Soy protein concentrate	0.0	5.0	7.0
Wheat Gluten	0.0	6.9	10.5
Corn gluten	0.0	5.0	7.0
Soybean meal 48	6.0	6.0	6.0
Rapeseed meal	5.3	5.3	5.3
Sunflower meal	5.3	5.3	5.3
Wheat meal	16.5	12.6	11.0
Pea starch	12.5	12.5	12.5
Fish oil	11.3	12.5	13.1
Vit & Min Premix PV01	1.5	1.5	1.5
Soy lecithin	1.0	1.0	1.0
Binder	1.5	1.5	1.5
Antioxidant	0.2	0.2	0.2
Dicalcium phosphate	1.7	3.0	4.0
L-Lysine	0.0	0.4	0.7
DL-Methionine	0.2	0.3	0.4
Total	100.0	100.0	100.0

Proximate composition	FM	PP50	PP75
Crude protein	36.0	35.9	35.9
Crude fat	15.8	15.9	15.9
Fiber	2.5	2.7	2.8
Starch	14.8	14.2	13.8
Gross Energy	19.3	19.4	19.3

The fatty acid profile of the experimental diets is shown in **Table 2**. Most differences in fatty acid composition between diets were due to FM substitution by alternative plant protein (soy, corn and wheat) sources, as well as the increase in fish oil in both PP diets. In particular, saturated fatty acids in the FM diet were 7.5% and 12.3% higher than in PP50 and PP75, respectively. In addition, PP50 and PP75 diets differed from the FM diet in their levels of total n-6 PUFA that were higher in levels with high levels of FM substitution, as a consequence of higher levels of linoleic acid (C18:2n-6) in those diets. Regardless of the absence of differences in terms of total n-3 PUFA between experimental diets and particularly in DHA and EPA, the FM diet had lower levels of stearidonic acid (C18:4n-3) in comparison to PP50 and PP75 diets.

Table 2. Fatty acid composition (mg/g lipid) of experimental diets tested to evaluate the effects on weaning and performance in grey mullet (*Mugil cephalus*) fed experimental diets with different levels of fish meal substitution (FM, no fish meal substitution; PP50, 50% substitution of fish meal with plant protein sources; PP75, 75% substitution of fish meal with plant protein sources).

	Experimental diets		
	FM	PP50	PP75
14:0	25.0 ± 2.6	21.4 ± 1.2	20.1 ± 2.2
15:0	2.6 ± 0.1	2.4 ± 0.1	2.3 ± 0.2
16:0	119.4 ± 2.4	115.6 ± 2.3	114.2 ± 3.0
18:0	18.9 ± 1.4	18.3 ± 1.4	17.3 ± 0.2
SFA	172.6 ± 6.5 a	159.6 ± 4.9 b	151.2 ± 5.9 b
16:1	38.7 ± 2.1	37.1 ± 0.6	36.2 ± 1.6
18:1n-9	79.7 ± 1.3	82.3 ± 1.9	79.4 ± 1.2
18:1n-7	17.1 ± 0.4	16.6 ± 0.2	16.0 ± 1.1
20:1	74.0 ± 1.6 b	78.0 ± 0.8 a	79.4 ± 0.4 a
22:1	108.5 ± 1.1	110.8 ± 0.8	107.9 ± 2.5
MUFA	318.0 ± 2.2	324.9 ± 4.2	312.9 ± 2.2
18:2n-6	42.9 ± 1.1 b	58.1 ± 0.5 a	61.4 ± 1.3 a
18:3n-6	0.7 ± 0.1	0.6 ± 0.2	1.3 ± 0.1
20:4n-6, ARA	2.7 ± 0.3	3.0 ± 0.1	2.8 ± 0.3
22:5n-6	nd	nd	0.9 ± 0.1
n-6 PUFA	46.3 ± 1.0 b	61.7 ± 0.3 a	66.3 ± 1.2 a
18:3n-3	10.7 ± 0.5	12.3 ± 0.2	11.6 ± 0.5
18:4n-3	26.8 ± 0.3 b	28.2 ± 0.2 a	28.1 ± 0.1 a
20:4n-3	4.0 ± 0.2	4.2 ± 0.3	3.9 ± 0.2
20:5n-3, EPA	75.2 ± 1.2	79.8 ± 0.8	77.3 ± 1.9
21:5n-3	2.5 ± 0.3	3.4 ± 0.1	3.1 ± 0.2
22:5n-3	5.5 ± 0.4	5.9 ± 0.2	5.9 ± 0.1
22:6n-3, DHA	75.5 ± 1.1	78.1 ± 1.6	73.0 ± 1.9
n-3 PUFA	200.1 ± 3.4	211.9 ± 2.7	202.9 ± 4.7
Total PUFA	246.4 ± 4.1 b	276.7 ± 2.4 a	269.2 ± 6.2 a

Differences in fatty acid composition between experimental diets are indicated by different letters (ANOVA, $P < 0.05$, n = 3). *Abbreviations*: SFA; saturated fatty acids; MUFA: monounsaturated fatty acids; ARA; arachidonic acid; PUFA: polyunsaturated fatty acids; EPA; eicosapentaenoic acid; DHA; docosahexaenoic acid.

The amino acid composition of experimental diets is shown in **Table 3**. Most of the amino acid analyzed differed in terms of their content between diets with different levels of fishmeal substitution with plant protein sources, as a result of their different composition in terms of amino acids. However these differences were always of reduced magnitude (5-10%) with the exception of glutamic acid content that was *ca.* 30% lower in the FM diet in comparison to PP50 and PP75 diets. Regardless of this fact, diets met in excess the nutritional requirements for fish in terms of essential amino acid requirements (NRC, 2011).

Table 3. Amino acid profile (mg/100 mg sample) of experimental diets tested to evaluate the effects on weaning and performance in grey mullet (*Mugil cephalus*) fed experimental diets with different levels of fish meal substitution (FM, no fish meal substitution; PP50, 50% substitution of fish meal with plant protein sources; PP75, 75% substitution of fish meal with plant protein sources).

	Experimental diets		
	FM	PP50	PP75
HyPro	0.43 ± 0.01 a	0.30 ± 0.02 b	0.28 ± 0.01 b
His	0.81 ± 0.01	0.76 ± 0.03	0.76 ± 0.01
Tau	0.25 ± 0.01 a	0.15 ± 0.03 b	0.10 ± 0.00 c
Ser	1.53 ± 0.01	1.63 ± 0.01	1.71 ± 0.04
Arg	2.32 ± 0.02	2.08 ± 0.05	2.08 ± 0.07
Gly	2.47 ± 0.02 a	2.13 ± 0.06 b	2.05 ± 0.04 b
Asp	3.16 ± 0.01 a	2.88 ± 0.03 b	2.72 ± 0.01 b
Glu	5.61 ± 0.07 c	7.51 ± 0.08 b	8.30 ± 0.07 a
Thr	1.49 ± 0.00 a	1.35 ± 0.02 b	1.31 ± 0.02 b
Ala	2.19 ± 0.02 a	2.07 ± 0.01 b	1.90 ± 0.01 c
Pro	1.82 ± 0.01 c	2.36 ± 0.03 b	2.64 ± 0.03 a
Cys	0.14 ± 0.01 c	0.20 ± 0.01 b	0.24 ± 0.01 a
Lys	2.58 ± 0.02 a	2.54 ± 0.04 a	2.34 ± 0.02 b
Tyr	0.96 ± 0.02	1.00 ± 0.05	1.12 ± 0.03
Met	0.84 ± 0.02	0.77 ± 0.01	0.83 ± 0.03
Val	1.74 ± 0.02 a	1.60 ± 0.03 b	1.57 ± 0.01 b
Ile	1.40 ± 0.02	1.37 ± 0.01	1.35 ± 0.03
Leu	2.59 ± 0.03 b	2.88 ± 0.01 a	2.97 ± 0.05 a
Phe	1.41 ± 0.01 b	1.53 ± 0.06 ab	1.76 ± 0.06 a

Differences in amino acid composition between experimental diets are indicated by different letters (ANOVA, $P < 0.05$, n = 2).

2.3 Survival and growth performance

Grey mullet fry were sampled for growth by measuring body weigth (BW) and standard length (SL) at 30 and 60 days after the beginning of the weaning trial. In both cases, all fish (fastened overnight) were taken from tanks with a dip net, gently anesthetized with 100 mg MS-222/L, and then their wet BW (g) and SL (mm) determined to the nearest mg and mm, respectively. In addition, 50 specimens (fastened overnight) per tank (replicate) were sacrificed with an overdose of anesthetic for assessing the histological organization of the liver and intestine (n = 10), the activity of pancreatic and intestinal digestive enzymes and (n = 10), proximate biochemical composition, fatty acid and amino acid analyses (n = 20), and assessment of the activity of antioxidative stress enzymes and levels of lipid peroxidation (n = 10).

The following formulae were used to calculate the specific growth rate in BW (SGR_{BW}, %) = [(ln BWf - ln BWi) x 100] / time (days) and the Fulton's condition factor (K) = (BWf / SL^3) x 100. The K factor is a morphometric index that estimates fish's body condition, which is determined by measuring the weight and length of individual fish. This approach assumes that heavier fish of a given length are more robust. Survival was determined by counting the number of surviving fish at 30 and 60 days and subtracting it from the number of specimens at the onset of the trial.

2.4 Biochemical analyses

2.4.1 Proximate composition, amino acid and fatty acid analyses

For determining the body proximate composition of grey mullet fry, sacrificed fish were homogenized, and small aliquots were dried (120ºC for 24 h) to estimate water content. The total fat content from fish was quantified gravimetrically after extraction in chloroform/methanol (2:1) and evaporation of the solvent under a stream of N followed by vacuum desiccation overnight (Folch et al., 1957). Protein content was determined according to Lowry et al. (1951). Ash contents were determined by keeping the sample at 500 to 600ºC for 24 h in a muffle furnace (AOAC, 1990). All chemical analyses were performed in triplicate per groups of ten fish per tank (methodological replicate) and feed samples.

Acid catalyzed transmethylation was carried out using the method of Christie (1982) in order to determine the fatty acid profile of grey mullet fry. Methyl esters were extracted twice using isohexane: diethyl ether (1:1, v:v), purified on TLC plates (Silica gel 60, VWR, Lutterworth, UK) and analyzed by gas-liquid chromatography on a Thermo ElectronTrace GC (Winsford, UK) instrument fitted with a BPX70 capillary column (30 m x 0,25 mm id), using a two-stage thermal gradient from 50 ºC (injection temperature) to 150ºC after ramping at 40 ºC min-1 and holding at 250ºC after ramping at 2ºC/min, helium (1.2 ml/min constant flow rate) as the carrier gas and on-column injection and flame ionization detection at 250ºC. Peaks of each fatty acid were identified by comparison with known standards (Supelco Inc., Spain) and a well characterized fish oil, and quantified by means of the response factor to the internal standard, 21:0 fatty acid, added prior to transmethylation, using a Chrom-card for Windows (TraceGC, Thermo Finnigan, Italy). Results of fatty acid content are expressed as a percentage of total fatty acids (TFA). Data on fatty acid content were expressed as µg/mg lipid.

Amino acids (AA) were analyzed by Dr. Y. Kotzamanis in the HCMR (P1). The AA profile was determined after acid hydrolysis (6 N, 110ºC, 24 h), and derivatization by AccQ-Tag according to the amino acid analysis application solution (Waters, USA). DL-Norvaline (2.5 mM) was used as an internal standard. UPLC was performed on an Acquity system (Waters) equipped with PDA detector set at 260 nm. The column used was BEH C18 column (100 x 2.1 mm i.d., 1.7 µm) from Waters. The flow rate was 0.7 ml/min and the column temperature was kept at 55°C. Peak identification and integration was performed by the software Waters Empower 2 (Milford, MA) using an Amino Acid Standard H (Pierce, USA) as an external standard. Cysteine and tryptophan were not quantified due to their susceptibility to acid hydrolysis. All chemical analyses were run in triplicate for fish samples and duplicate for diets (methodological replicates).

2.4.2 Quantification of digestive enzymes

Samples were homogenized and sonicated for 1-2 min in ice in 30 volumes (v/w) of cold 50 mM mannitol, 2 mM Tris–HCl buffer (pH 7.0) as described by Gisbert at al. (2009). After homogenization, samples were centrifuged at 3,300×g for 3 min at 4°C and the supernatant was collected for the determination of pancreatic enzymes (total alkaline proteases, trypsin, chymotrypsin, α-amylase and non-specific lipase) and intestinal enzymes (alkaline phosphatase, aminopeptidase N, maltase and leucine-alanine peptidase). The above-mentioned supernatant was divided into several aliquots and frozen at -80ºC in order to avoid freezing and thawing cycles that may affect enzyme activity. Intestinal brush border membranes were purified according to the method developed for intestinal scrapping (Crane et al., 1979). Briefly, sample homogenates were

centrifuged at 9,000 g for 10 min and their supernatants transferred to new tubes and centrifuged at 24,000 g for 30 min in order to recover the precipitate (brush border) that was diluted in 1 mL of 1 mM DTT solution in 5 mM Tris-HEPES, 0.1 M KCl buffer and frozen at -80ºC.

Total alkaline proteases were assayed by the azo-casein method described by Walter (1984) using 50 mM Tris-HCl buffer (pH 9.0). The reaction was conducted at 37ºC during 30 min, stopped by adding 20% TCA, and the absorbance measured at 366 nm. Trypsin (E.C. 3.4.21.4) activity was assayed at 25 ºC using BAPNA as substrate in 50 mM Tris-HCl, 20 mM $CaCl_2$ buffer, pH 8.2. One unit of trypsin per ml (U) was defined as 1 µmol BAPNA hydrolyzed per min per ml of enzyme extract at 407 nm (Holm et al., 1988). Chymotrypsin (EC. 3.4.21.1) activity was quantified at 25ºC using BTEE as substrate in 80 mM Tris-HCl, 100 mM $CaCl_2$ buffer, pH 7.2. Chymotrypsin activity (U) corresponded to the µmol BTEE hydrolyzed per min per ml of enzyme extract at 256 nm (Worthington, 1991).

Alpha-amylase (E.C. 3.2.1.1) activity was measured according to Métais and Bieth (1968), using 0.3% soluble starch dissolved in Na_2HPO_4 buffer pH 7.4 as substrate. Amylase activity (U) was defined as the mg of starch hydrolyzed during 30 min per ml of tissue homogenate at 37ºC at 580 nm. Bile salt-activated lipase (E.C. 3.1.1) activity was assayed for 30 min at 30ºC using p-nitrophenyl myristate as substrate dissolved in 0.25 mM Tris-HCl, pH 9.0, 0.25 mM 2-methoxyethanol and 5 mM sodium cholate buffer. The reaction was stopped with a mixture of acetone: n-heptane (5:2), the extract centrifuged for 2 min at 6,080 x g and 4ºC and the increase in absorbance of the supernatant read at 405 nm. Lipase activity (U/ml) was defined as the µmol of substrate hydrolyzed per min per ml of enzyme extract (Iijima et al., 1998).

Alkaline phosphatase (E.C. 3.1.3.1) was quantified at 37ºC using 4-nitrophenyl phosphate (PNPP) as substrate in 30 mM Na_2CO_3 buffer (pH 9.8). One unit (U) was defined as 1 µg BTEE released per min per ml of brush border homogenate at 407 nm (Bessey et al., 1946). Aminopeptidase N (E.C.3.4.11.2) was determined at 25ºC according to Maroux et al. (1973), using 80 mM sodium phosphate buffer (pH 7.0) and L-leucine p-nitroanilide as substrate (in 0.1 mM DMSO). One unit of enzyme activity (U) was defined as 1 µg nitroanilide released per min per ml of brush border homogenate at 410 nm. Maltase (E.C.3.2.1.20) activity was determined at 37ºC using D(+)-maltose as substrate in 100 mM sodium maleate buffer, pH 6.0 (Dahkqvist, 1970). One unit of maltase (U) was defined as µmol of glucose liberated per min per ml of homogenate at 420 nm.

The assay of a cytosolic peptidase, leucine–alanine peptidase (E.C. 3.4.11) was performed on intestinal homogenates using the method described by Nicholson and Kim (1975), using leucine-alanine as substrate in 50 mM Tris-HCl buffer (pH 8.0). One unit of enzyme activity (U) was defined as 1 nmol of the hydrolyzed substrate per min per ml of tissue homogenate at 37ºC at 530 nm.

Enzymatic activities were expressed as the total activity defined as milli-units per larval fish (mU/larva) based on the whole fish or intestinal segment homogenate. The specific activity was expressed as milli-units per milligram of protein (mU/mg protein). Soluble protein of crude enzyme extracts was quantified by means of the Bradford's method (Bradford, 1976) using bovine serum albumin as standard. All the assays were made in triplicate.

2.4.3 Lipid peroxidation levels and activity of antioxidative stress enzymes

Levels of lipid peroxidation and activity of antioxidative stress enzymes were assayed in the liver and muscular tissue in order to evaluate the health condition of the organism. In particular, the liver was chosen as the main metabolic organ of the organism, and the muscle as the main target tissue reflecting somatic growth. Quantification of lipid peroxidation in grey mullet fry (liver and muscle) was conducted by means of the acid reactive substances (TBARs) method described in Solé et al. (2004). In brief, lipid peroxidation was measured using 200 µl of the homogenate mixed with 650 µl of methanol, 1-methyl-2-phenylindole (solution stock of 10.3 mM) in acetonitrile: methanol (1:3; v/v) and 150 µl of 37% HCl. This mixture was incubated for 40 min at 45°C, cooled on ice for 10 min and centrifuged at 13,000 rpm for 10 min to remove protein precipitates. Absorbance was read at 586 nm, and the amount of peroxidized lipids (in nmol

malondialdehyde (MDA) g 1; w/w) was evaluated by means of a calibration curve made of a standard solution of 1,1,3,3-tetramethoxypropane (10 mM).

Homogenized samples (liver and muscle), prepared for the determination of the levels of lipid peroxidation, were used to measure antioxidant enzyme activities in fry. Catalase (CAT, E.C. 1.11.1.6) activity was measured in larvae by the decrease in absorbance at 240 nm (e = 40 M/cm) using 50 mM H_2O_2 as substrate (Aebi, 1974). Glutathione reductase (GR, E.C. 1.8.1.7.) activity was determined by measuring the oxidation of NADPH at 340 nm (e = 6.22 mM/cm), using 20 mM glutathione disulphide and 2 mM NADPH as substrates (Carlberg and Mannervik, 1975). Superoxide dismutase (SOD, E.C. 1.15.1.1) activity was measured at 550 nm as the degree of inhibition of cytochrome c reduction by O_2 generated by the xanthine oxidase/hypoxanthine system, according to McCord and Fridovich (1969). Total glutathione peroxidase (GPX, EC 1.11.1.9) was determined according to Günzler and Flohé (1985) by measuring the consumption of NADPH at 340 nm (e = 6.22 mM/cm), using 75 mM glutathione and 8.75 mM NADPH as substrates. Soluble protein of crude enzyme extracts was quantified by Bradford's method using bovine serum albumin as the standard. Enzymatic activities were expressed as specific enzyme activity, in nmol of catalysed substrate per milligram of protein (nmol/mg protein). All assays were carried out in triplicate at 25°C, and absorbance was read using a spectrophotometer (Tecan™ Infinite M200).

2.5 Histological analyses

For histological purposes, the visceral mass of 30 fish per dietary treatment was dissected and fixed in 4% buffered formaldehyde (pH = 7.4), dehydrated in a graded series of ethanol, cleared with xylene, embedded in paraffin, and cut in serial sections (3 to 5 µm thick). Histological sections of the liver and intestine were observed under a light microscope (Leica DM LB; Leica Microsystems, Wetzlar, Germany) and photographed (Olympus DP70 Digital Camera; Olympus Imaging Europa GmbH, Hamburg, Germany) in order to evaluate the health and condition of the intestine and liver as described in Boglino et al. (2012).

2.6 Statistics

Data presented in this Deliverable is expressed as the mean ± standard error of the mean. Values for different parameters were compared among them by means of One-way ANOVA at a reliability level of 5%. Data were checked for normality (Kolmogorov–Smirnov test) and homogeneity of variances (Bartlett's test) prior to their comparison. When statistical differences were found among data with the ANOVA, the Duncan's Multiple Range test was applied in order to detect which treatments/groups differed among each others.

2.7 Ethics statement

All animal experimental procedures were conducted in compliance with the Guidelines of the European Union Council (86/609/EU) for the use of laboratory animals.

3. Results

3.1 Growth performance and survival

In all experimental groups, diets were actively ingested by grey mullet fry. Data on growth performance, Fulton's condition factor and survival of fry at 30 and 60 days are shown in **Table 4**. The analysis of the above-mentioned data revealed that diets with different levels of FM substitution with a blend of different plant protein sources (corn gluten, wheat gluten and soy protein concentrate) did not significantly affect the weaning (days 0-30) and the early on-growing period (days 30-60) for grey mullet fry, as there were no statistically significant differences among different treatments. In addition, the weaning protocol tested in

wild grey mullet may be considered as appropriate, since the average survival of fry at day 30 ranged between 71.1 and 78.6%. Once weaning was completed, fry survival until the end of the trial (day 60) was between 96.3 to 98.7% (survival values expressed with regards to the number of surviving fish at the beginning of the second part of the trial, days 30-60). It is remarkable that values of SGR_{BW} were similar between both parts of the study (days 0-30 and 30-60), which seems to confirm that inert diets were well accepted and digested by fish, as visual observations indicated. Consequently, the weaning schedule performed based on a progressive substitution of live prey by the inert diet, had no detrimental effects on fish performance and condition.

Table 4. Final body wet weight (BW, mg), standard length (SL, mm), Fulton's condition factor, specific growth rate in BW (%/day) and survival (%) of wild grey mullet (*Mugil cephalus*) fry weaned onto diets with different levels of fish meal substitution (FM, no fish meal substitution; PP50, 50% substitution of fish meal with plant protein sources; PP75, 75% substitution of fish meal with plant protein sources). Data is presented for the two distinct phases in which the study was divided: weaning of fry with the above-mentioned diets (days 0-30) and the early on-growing phase fed the above-mentioned diets (days 30-60).

Day 30 (end of the weaning)					
	BW (mg)	**SL (mm)**	**K**	**SGR_{BW} (%/day)**	**S (%)**
FM diet	390 ± 15	2.5 ± 0.01	2.64 ± 0.06	1.9 ± 0.1	78.6 ± 5.1
PP50 diet	385 ± 12	2.4 ± 0.02	2.72 ± 0.05	1.8 ± 0.1	73.4 ± 3.1
PP75 diet	375 ± 18	2.4 ± 0.02	2.72 ± 0.05	1.9 ± 0.1	71.1 ± 4.2
Day 60 (end of the trial)					
	BW (mg)	**SL (mm)**	**K**	**SGR_{BW} (%/day)**	**S (%)**
FM diet	707 ± 17	3.2 ± 0.02	2.17 ± 0.05	2.1 ± 0.05	74.6 ± 3.1
PP50 diet	661 ± 10	3.1 ± 0.02	2.18 ± 0.04	1.9 ± 0.07	70.4 ± 4.2
PP75 diet	681 ± 20	3.2 ± 0.02	2.17 ± 0.03	1.8 ± 0.09	69.1 ± 3.1

The absence of different letters between columns indicated similar values between dietary groups (ANOVA, $P > 0.05$)

The analysis of the final BW distribution of grey mullet fry fed different diets revealed that even though all groups of fish showed a normal distribution in their BW, fish fed the PP50 and PP75 diets showed a more homogeneous distribution than that of fish fed the FM diet (**Figure 3**). Similar results were obtained with regards to SL (data not showed). In this sense, fish fed PP50 and PP75 diets showed higher Kurtosis values (0.79-0.44) than those fed the FM diet (0.14), whereas skewness values were quite similar among the three of the tested diets (0.36, 0.48 and 0.35 for FM, PP50 and PP75 diets, respectively). These results may be of practical significance since this may reduce the task of fish grading during the rearing process, as production lots will be more homogenous. The link between the experimental diets with different levels of FM substitution and a more homogenous distribution in body weight at the end of the trial remains unclear.

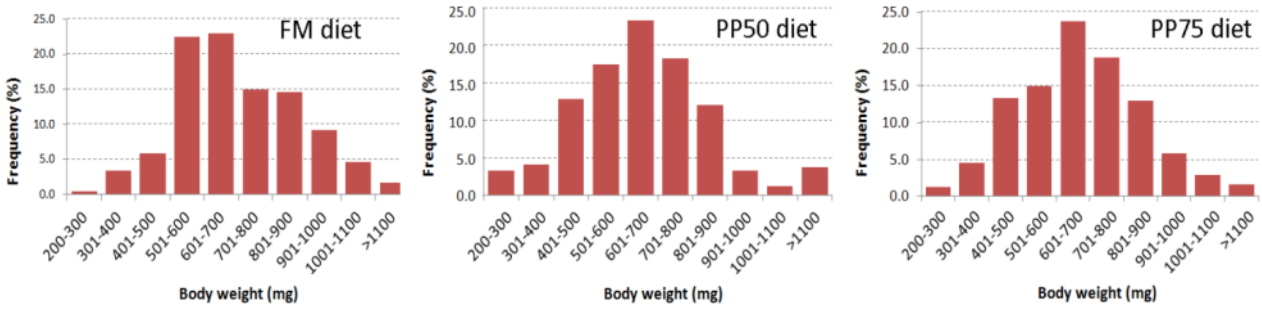

Figure 3. Frequency of body weight distribution of wild grey mullet (*Mugil cephalus*) fry weaned onto experimental diets with different levels of fish meal substitution (FM, no fish meal substitution; PP50, 50% substitution of fish meal with plant protein sources; PP75, 75% substitution of fish meal with plant protein sources).

3.2 Proximate composition, and fatty acid and amino acid profiles

Data on the proximate composition of grey mullet fry fed diets with different levels of FM substitution is shown in **Table 5**. At both of the chosen sampling dates (day 30 and 60), there were no differences in the proximate composition of fry (ANOVA, $P > 0.05$), and even though there was a change in protein and lipid content between fry sampled at days 30 and 60 (ANOVA, $P < 0.05$). In particular, we found a reduction in the total content of proteins between fry sampled at days 30 and 60 (56.8-56.3% *vs*. 46.6-45.9%), coupled with an increase in the total levels of lipids (26.0-26.5% *vs*. 32.8-34.5%), whereas carbohydrate and ash content remained stable during this period. These changes in total protein and lipid fry contents between days 30 and 60 may be attributed to the transition from live prey (*Artemia* metanauplii) to inert diets and fry growth, which in fact was visually evidenced by the accumulation of perivisceral fat in older fry (data not calculated due to the difficulties in clearly separating fat from viscera, but revealed by histology).

Table 5. Proximate composition in dry weight of wild grey mullet (*Mugil cephalus*) fry weaned onto diets with different levels of fish meal substitution (FM, no fish meal substitution; PP50, 50% substitution of FM with plant protein sources; PP75, 75% substitution of FM with plant protein sources).

Day 30 (end of the weaning)				
	Proteins (%)	Lipids (%)	Carbohydrates (%)	Ash (%)
FM diet	56.6 ± 1.1	26.2 ± 0.6	3.6 ± 0.1	4.8 ± 0.1
PP50 diet	56.8 ± 1.7	26.5 ± 0.5	3.6 ± 0.1	3.9 ± 0.1
PP75 diet	56.3 ± 1.1	26.0 ± 0.8	3.5 ± 0.1	4.2 ± 0.1
Day 60 (end of the trial)				
	Proteins (%)	Lipids (%)	Carbohydrates (%)	Ashes (%)
FM diet	44.4 ± 0.9	34.5 ± 0.6	5.2 ± 0.05	3.0 ± 0.03
PP50 diet	46.6 ± 1.0	32.8 ± 1.0	5.0 ± 0.13	3.2 ± 0.08
PP75 diet	45.9 ± 1.1	32.5 ± 1.2	4.8 ± 0.13	3.2 ± 0.10

The absence of different letters between columns indicated similar values between dietary groups (ANOVA, $P > 0.05$)

The fatty acid profile of the body of grey mullet fry weaned onto experimental diets with different levels of FM substitution was quite similar (**Table 6**). Although fish oil, the main source of fatty acids in experimental diets, did not change very much between experimental diets, we decided to analyze the fatty acid profile in fish as diets differed in their levels of saturated fatty acids, n-6 PUFA and total PUFA as a consequence of using non-defatted alternative plant protein (soy, corn and wheat) sources. In general terms, the fatty acid profile of fish reflected that of the diet, but FM replacement by plant protein sources did not affect the fatty acid profile of fish; information that may be of relevance for further studies in this species covering the on-growing phase. In particular, fish from different dietary groups only differed in terms of their levels in n-6 PUFA, which were 26% higher in fish fed PP50 and PP75 diets in comparison to fish fed the FM diet (ANOVA, $P < 0.05$). The above-mentioned differences were attributed to the higher levels of n-6 PUFA in PP50 and PP75 diets, especially to the higher content of linoleic acid (C18:2n-6) in those diets (Table 2). In addition, although the experimental diets differed in their levels of total saturated fatty acids, there were no differences in their content in fry, as a consequence of their use for energetic purposes by the organism. This is of especial relevance, since the content of SFA in PP50 and PP75 diets was 8% and 12.4% lower than in the FM diet, but this issue did not affect the growth performance of fish fed different diets. The absence of differences in terms of body content of stearidonic acid (C18:4n-3) regardless of its higher content in PP50 and PP75 diets, reflected that there is not a differential accumulation of this fatty acid.

Table 6. Fatty acid (FA) profile (mg/g lipid) of wild grey mullet (*Mugil cephalus*) fry weaned onto diets with different levels of fish meal substitution (FM, no fish meal substitution; PP50, 50% substitution of FM with plant protein sources; PP75, 75% substitution of FM with plant protein sources).

	Day 60 (end of the trial)		
	FM diet	PP50 diet	PP75 diet
14:0	27.4 ± 1.4	29.1 ± 2.7	27.2 ± 1.1
15:0	2.6 ± 0.2	1.8 ± 0.8	2.5 ± 0.2
16:0	110.5 ± 4.7	116.8 ± 4.5	109.1 ± 2.0
SFA	154.0 ± 6.3	161.9 ± 6.4	152.6 ± 3.0
16:1	43.7 ± 2.2	48.1 ± 3.0	46.3 ± 2.2
18:1n-9	77.3 ± 4.3	96.6 ± 5.8	102.0 ± 4.4
18:1n-7	17.0 ± 3.6	11.0 ± 4.9	10.1 ± 3.3
20:1	48.6 ± 2.5	55.1 ± 4.1	54.2 ± 1.1
22:1	40.5 ± 2.5	53.1 ± 5.6	52.9 ± 5.4
MUFA	237.1 ± 10.5	263.9 ± 18.9	260.3 ± 9.4
18:2n-6	46.6 ± 2.2 b	66.6 ± 3.1 a	67.6 ± 1.0 a
18:3n-6	0.4 ± 0.1	0.2 ± 0.1	0.6 ± 0.2
20:4n-6, ARA	2.8 ± 0.2	2.4 ± 0.4	2.3 ± 0.3
22:5n-6	1.1 ± 0.3	0.5 ± 0.2	0.8 ± 0.3
n-6 PUFA	51.6 ± 3.3 b	69.9 ± 4.1 a	72.0 ± 1.0 a
18:3n-3	10.3 ± 0.5	13.0 ± 1.1	12.3 ± 0.5
18:4n-3	13.6 ± 0.7	15.8 ± 0.8	15.6 ± 0.6
20:4n-3	4.4 ± 0.2	3.5 ± 1.1	3.7 ± 1.2
20:5n-3, EPA	47.6 ± 1.8	44.4 ± 2.1	44.8 ± 3.1
21:5n-3	2.4 ± 0.4	2.1 ± 0.2	1.8 ± 0.3
22:5n-3	13.4 ± 0.4	16.7 ± 1.1	15.6 ± 0.2
22:6n-3, DHA	71.7 ± 0.4	78.0 ± 3.3	77.3 ± 0.2
n-3 PUFA	163.4 ± 4.4	179.3 ± 10.2	171.1 ± 10.6
Total PUFA	215.0 ± 8.0	229.2 ± 13.1	231.1 ± 11.7

Abbreviations: SFA; saturated fatty acids; MUFA: monounsaturated fatty acids; ARA; arachidonic acid; PUFA: polyunsaturated fatty acids; EPA; eicosapentaenoic acid; DHA; docosahexaenoic acid. Different letters between columns indicate significant differences between groups (ANOVA, $P < 0.05$).

The analysis of the amino acid profile of the muscle in wild grey mullet weaned onto diets containing different levels of plant proteins revealed no significant difference between them (ANOVA, $P > 0.05$; **Table 7**). The conserved amino acid compositions between treatments were expected, as long as the experimental diets satisfy fish nutritional requirements in terms of essential amino acids (NRC, 2011).

Table 7. Amino acid profile (mg/100 mg) of the muscle of wild grey mullet (*Mugil cephalus*) fry weaned onto diets with different levels of fish meal substitution (FM, no fish meal substitution; PP50, 50% substitution of FM with plant protein sources; PP75, 75% substitution of FM with plant protein sources).

	Day 60 (end of the trial)		
	FM diet	PP50 diet	PP75 diet
HyPro	0.08 ± 0.01	0.10 ± 0.01	0.09 ± 0.00
His	0.47 ± 0.01	0.46 ± 0.03	0.42 ± 0.01
Tau	0.37 ± 0.01	0.38 ± 0.02	0.37 ± 0.01
Ser	0.69 ± 0.01	0.70 ± 0.01	0.69 ± 0.00
Arg	0.98 ± 0.01	0.99 ± 0.03	0.98 ± 0.01
Gly	0.83 ± 0.01	0.86 ± 0.01	0.84 ± 0.01
Asp	1.66 ± 0.04	1.62 ± 0.01	1.63 ± 0.01
Glu	2.41 ± 0.03	2.35 ± 0.03	2.37 ± 0.03
Thr	0.72 ± 0.01	0.72 ± 0.01	0.71 ± 0.01
Ala	1.04 ± 0.02	1.03 ± 0.02	1.03 ± 0.01
Pro	0.63 ± 0.00	0.66 ± 0.01	0.65 ± 0.01
Cys	0.07 ± 0.00	0.07 ± 0.00	0.07 ± 0.01
Lys	1.54 ± 0.03	1.48 ± 0.04	1.48 ± 0.03
Tyr	0.49 ± 0.01	0.49 ± 0.02	0.48 ± 0.00
Met	0.45 ± 0.01	0.43 ± 0.01	0.43 ± 0.01
Val	0.80 ± 0.01	0.81 ± 0.01	0.80 ± 0.02
Ile	0.68 ± 0.01	0.68 ± 0.01	0.68 ± 0.02
Leu	1.26 ± 0.02	1.26 ± 0.02	1.25 ± 0.02
Phe	0.65 ± 0.01	0.67 ± 0.03	0.65 ± 0.01

The absence of different letters between columns indicated similar values between dietary groups (ANOVA, $P > 0.05$)

Protein synthesis requires that all amino acids needed are present in the cell, and if one amino acid is absent or present in lower concentrations than required for the synthesis to occur in any particular tissue, the protein cannot be synthesized and the constituent amino acids will be metabolized and used as fuel due to the simple fact that free amino acids are never stored in tissues. Thus, the delivery of adequate amounts of amino acids with a balanced profile is essential for maximal protein accretion and growth in fish (El-Mowafi et al., 2010). Generally, animal derived protein ingredients have a balanced indispensable amino acid profile providing what is required by the fish. In contrast, plant protein sources may be lacking in specific indispensable amino acids, which need to be supplemented to the diet (NRC, 2011). In the current study, the formulation of diets with FM replacement by a mixture of plant protein sources (soy, corn and wheat) was conducted considering the amino acid profile of FM as a reference; consequently, PP50 and PP75 diets were supplemented with the inclusion of L-lysine and DL-methionine. This strategy was satisfactory in terms of growth performance and the overall condition of grey mullet fry fed different experimental diets, as it was also reflected by their similar protein content and amino acid profile of the muscle, the main amino acid depot in the organism.

3.3 Activity of digestive enzymes

The activity of pancreatic (total alkaline proteases, trypsin, lipase and α-amylase) digestive enzymes reflected the feeding habits of this species and it was not affected by the levels of FM substitution by PP in diets (ANOVA, $P > 0.05$; Table 8). Regarding the activity of total alkaline proteases and trypsin, results indicated that plant protein ingredients did not reduce the activity of those proteolytic enzymes, as it has been reported in other fish species due to the presence of protease inhibitors in plant protein sources (Santigosa et al., 2008). These results indicated that the inclusion of vegetal protein sources in grey mullet did not affect their digestive capacities with regard to proteolytic, lipolytic and amylolytic enzymes, which would be in agreement with the similar growth performances found between FM, PP50 and PP75 groups.

Table 8. Specific activity (mU/mg protein) of pancreatic and intestinal digestive enzymes in wild grey mullet (*Mugil cephalus*) fry weaned onto diets with different levels of fish meal substitution (FM, no fish meal substitution; PP50, 50% substitution of FM with plant protein sources; PP75, 75% substitution of FM with plant protein sources).

	Day 60 (end of the trial)		
	FM diet	PP50 diet	PP75 diet
Exocrine pancreas			
Total alkaline proteases	3.70 ± 0.32	3.70 ± 0.19	3.51 ± 0.17
Trypsin	0.71 ± 0.13	0.81 ± 0.05	0.81 ± 0.07
Lipase	31.6 ± 8.97	26.6 ± 4.31	26.2 ± 3.43
α-amylase	23.6 ± 6.83	17.0 ± 3.51	20.5 ± 2.39
Intestine			
Alkaline phosphatase (AP)	2.9 ± 0.65	2.3 ± 0.86	4.1 ± 0.68
Aminopeptidase-N (AN)	0.05 ± 0.004	0.06 ± 0.009	0.04 ± 0.006
Maltase	1.56 ± 0.50	2.16 ± 0.31	2.04 ± 0.17
Leucine alanine peptidase (LAP)	717 ± 131	849 ± 104	845 ± 141
Intestinal maturation indexes		0	
AP/LAP	4.4 ± 1.1	2.9 ± 0.8	5.1 ± 1.4
AN/LAP	0.07 ± 0.01	0.06 ± 0.01	0.08 ± 0.02

The absence of different letters between columns indicated similar values between dietary groups (ANOVA, $P > 0.05$)

Alkaline phosphatase is found primarily in cell membranes where active transport takes place and it is considered as a general marker of nutrient absorption. The activity of this enzyme is often used to evaluate intestinal membrane function and integrity, in contrast to many other enzymes of the intestinal microvillus membrane such as maltase, aminopeptidase-N, which are extrinsic enzymes, the function of which does not seem to be intimately related to the membrane structure. The activity of all these enzymes is greatly modulated by the intraluminal presence of their dietary substrates (Silva et al., 2010). When considering the activity of brush border (alkaline phosphatase, aminopeptidase-N and maltase) and cytosolic (leucine alanine peptidase) intestinal enzymes in grey mullet fry weaned onto diets with different levels of fish meal substitution, no differences were found between different dietary treatments, nor in the different intestinal maturation indexes calculated (ANOVA, $P > 0.05$; Table 8).

These results were in agreement with those reported in the omnivorous goldfish (*Carassius auratus*) and gibel carp (*C. auratus gibelio*), which confirms that omnivorous species are less sensitive to the introduction of vegetable feedstuffs in diets, as it was reported by Silva et al. (2010) and Xie et al. (2001). Similarly, as it was previously discussed with regards to the impact of experimental diets on the function of the exocrine pancreas, partial levels of FM substitution by plant protein sources did not affect the integrity, function and maturation level of the intestinal mucosa, which was in concordance with the similar growth performance and fish condition values found between different groups.

3.4 Lipid peroxidation levels and activity of antioxidative stress enzymes

The levels of lipid peroxidation and activity of antioxidative stress enzymes in wild grey mullet fry weaned onto diets with different levels of fish meal substitution is shown in **Table 7**. The inclusion of plant protein sources in the diets implies the addition of a certain amount of vegetable oils, which might be susceptive of oxidation and affect the cellular redox status and cause some oxidative damage in tissues. In addition, redox status in tissues may be informative about the health and condition of the cell (Solé et al., 2004). Under present experimental conditions, we did not find any statistical difference between the levels of lipid peroxidation (TBARs) and different levels of FM substitution in diets (ANOVA, $P > 0.05$; Table 9).

Table 9. Lipid peroxidation levels (TBARs in nmol MDA/g) and activity of antioxidative stress enzymes (SOD, CAT, GR, GPX and GST) in the liver and muscle of wild grey mullet (*Mugil cephalus*) fry weaned onto diets with different levels of fish meal substitution (FM, no fish meal substitution; PP50, 50% substitution of FM with plant protein sources; PP75, 75% substitution of FM with plant protein sources).

	Day 60 (end of the trial)		
	FM diet	PP50 diet	PP75 diet
Liver			
TBARs	253.7 ± 61.4	204.2 ± 36.8	254.9 ± 20.7
SOD	85.7 ± 2.28	83.4 ± 1.98	81.6 ± 1.27
CAT	0.679 ± 0.065	0.550 ± 0.134	0.620 ± 0.255
GR	2.02 ± 0.26	1.52 ± 0.11	1.81 ± 0.20
GPX	8.24 ± 0.39	7.43 ± 0.20	8.05 ± 0.73
GST	2.96 ± 0.33	2.88 ± 0.20	3.30 ± 0.15
Muscle			
TBARs	243.4 ± 22.8	231.9 ± 20.4	262.7 ± 14.2
SOD	88.2 ± 1.28	89.2 ± 1.91	88.6 ± 0.80
CAT	0.535 ± 0.101	0.473 ± 0.024	0.511 ± 0.061
GR	2.02 ± 0.93	2.58 ± 0.69	1.82 ± 0.87
GPX	6.52 ± 0.42	5.87 ± 0.79	7.14 ± 0.72
GST	5.47 ± 0.57	4.75 ± 0.50	5.41 ± 0.15

The absence of different letters between columns indicated similar values between dietary groups (ANOVA, $P > 0.05$)

Antioxidant defenses in fish are affected by nutritional factors; however, there is little known about how the nature of dietary ingredients affects the oxidative status of fish. Several plants contain phenolic and flavonoid compounds with potent antioxidant activities, which have been associated with beneficial health effects in humans (Adom and Liu, 2002); thus, we decided to assess the activity of antioxidative stress enzymes in grey mullet fed diets with different levels of FM substitution. In this context, Sitjà-Bobadilla et al. (2005) and Koukou et al. (2015) have reported that PP replacement enhanced the glutathione metabolism and therefore the antioxidant defenses in gilthead sea bream (*Sparus aurata*). The former authors associated those changes in the activity of antioxidant stress enzymes in gilthead sea bream to some of the plants constituents like flavonoids present or phenolic compounds. In contrast, we did not find any statistical difference in the activity of antioxidative stress enzymes (SOD, CAT, GPX, GST and GR) between fish diets with higher PP content in comparison to those fed a FM diet (ANOVA, $P > 0.05$; Table 9). Such differences between those studies might be linked to the different levels of protein plant sources and processing procedures between studies, as well as a potential species-specific response to plant protein with regard to grey mullet and gilthead sea bream.

3.5 Histological organization of the liver and intestine

The intestine and the liver are considered reliable nutritional and physiological biomarkers, because their histological organization is very sensitive to dietary changes. The histological organization of the liver, notably the fat deposition in hepatocytes, accurately reflects any physiological disorder originated from nutritionally unbalanced diet or unsuitable feeding conditions (Gisbert et al., 2008).

Under the present experimental conditions, the general histological organization of the liver in grey mullet fry consisted of polyhedral hepatocytes with central nuclei and arranged in tightly packed anastomosed laminae around veins. The hepatic parenchyma was surrounded by a thin capsule of fibroconnective tissue. No melanomacrophage centers were found during the observation of different histological fields under optical microscope of the hepatic parenchyma.

Regarding the effects of experimental diets on the level of fat accumulation and organization of the hepatic parenchyma, no major changes in the histological organization were found in the liver of fish weaned onto diets with different levels of FM substitution (**Figure 4**). In general terms, grey mullets fed the experimental diets showed a healthy liver with a moderate accumulation of fat deposits and glycogen within hepatocytes, and no signs of hepatic steatosis. This fact was also reflected by the average size of fat deposits within hepatocytes that was similar between groups (220.7 ± 15.8 μm^2 in fish fed the FM diet, 230.4 ± 7.8 μm^2 in fish fed the PP50 diet and 197.4 ± 11.0 μm^2 in fish fed the PP75 diet; ANOVA, $P > 0.05$). These results were supported by the fact that in all groups hepatocytes showed a central and round nucleus or a moderately displaced round nucleus towards the periphery of the cell, but not deformation of nuclei were observed as a consequence of a large accumulation of fat stores within hepatocytes. These results regarding lipid accumulation in the hepatic parenchyma revealed by optical microscopy are in agreement with lipid peroxidation values and activity of selected antioxidative stress enzymes that were similar among different experimental groups (**Table 9**). In this sense, most of lipids were stored as perivisceral fat and not in the liver or intestine (**Figure 5**).

The intestine is the longest portion of the digestive tract in grey mullet, especially considering the omnivorous-detritivorous feeding habits of this species during the juvenile period. Regarding the intestine, no major changes were observed among different dietary groups. In this sense, different regions of the gut in grey mullet showed prominent villi with a moderate number of goblet cells in their epithelium, whereas lipid accumulation within enterocytes was negligible (**Figure 6**). No signs of epithelial abrasion or microvilli disarrangement were observed along different intestinal regions.

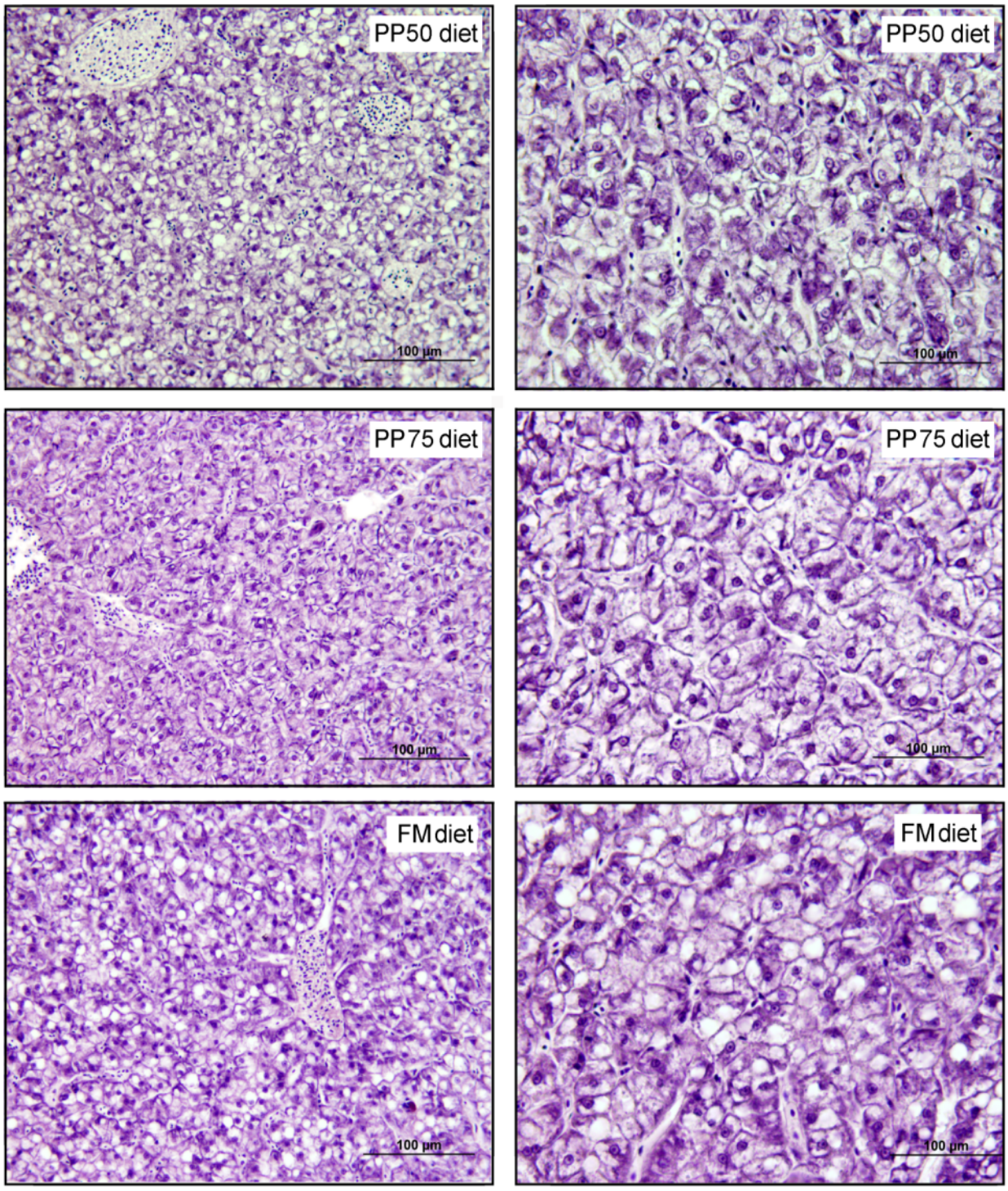

Figure 4. General view (left column; 200X) and detail (right column; 400X) of the hepatic parenchyma of grey mullet (*Mugil cephalus*) fry weaned onto diets with different levels of fish meal substitution (FM, no fish meal substitution; PP50, 50% substitution of FM with plant protein sources; PP75, 75% substitution of FM with plant protein sources). Lipid accumulation within hepatocytes corresponds to whitish areas, since lipids are removed from tissues during the paraffin embedding and staining process. Staining: hematoxylin-eosin.

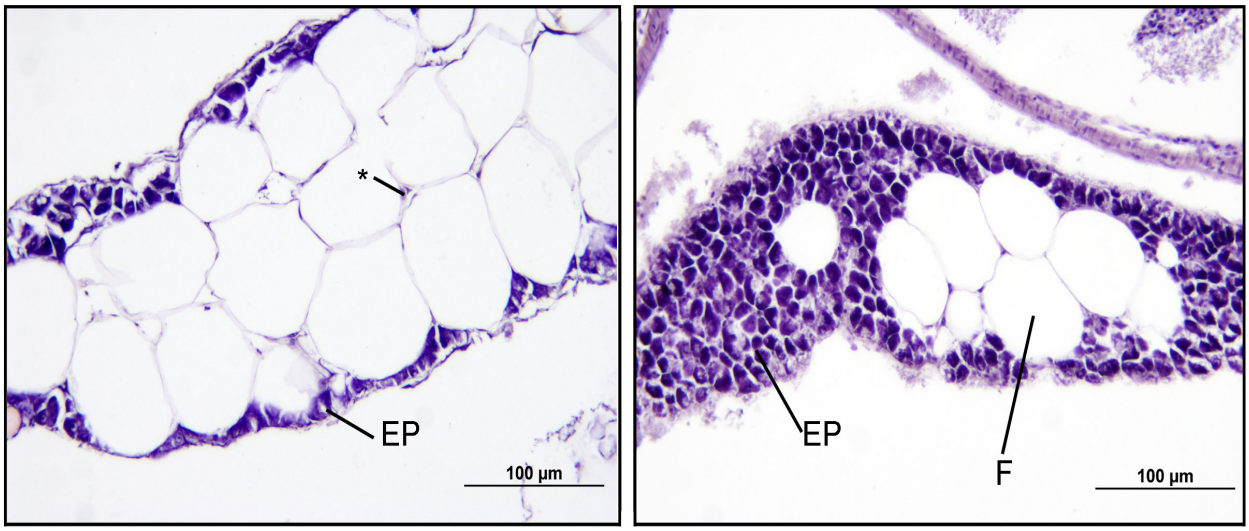

Figure 5. Detail of perivisceral fat deposits surrounded by pancreatic tissue in grey mullet (*Mugil cephalus*) fry weaned onto diets with different levels of fish meal substitution (FM, no fish meal substitution; PP50, 50% substitution of FM with plant protein sources; PP75, 75% substitution of FM with plant protein sources). As there were no obvious differences between groups, images are not labeled according to a specific dietary group. Lipid accumulation corresponds to whitish areas in adipocytes, since lipids are removed from tissues during the paraffin embedding and staining process. *Abbreviations*: EP, exocrine pancreas, F, fat accumulation within adipocytes; the asterisk indicate adipocytes nucleus. Staining: hematoxylin-eosin.

The accumulation of lipid deposits in the intestinal mucosa has been generally considered as a temporary storage form of re-esterified fatty acids in cases when the rate of lipid absorption exceeds the rate of lipoprotein synthesis, or because of an inability to metabolize lipids (Gisbert et al., 1998). Consequently, the accumulation of lipids in this part of the digestive system has been indicative of potential problems in lipid physiology and metabolism originated from dietary lipid imbalances (Deplano et al., 1989; Mobin et al., 2000), that may be responsible for a large accumulation of lipids in the enterocytes, the so-called intestinal steatosis, may cause some pathological damage since large lipid inclusions produce epithelial abrasion, cellular necrosis, and/or inflammatory reactions along the intestinal mucosa. Under the present experimental conditions, the level of lipid accumulation in the intestinal mucosa was negligible, which indicated that the lipid content of the tested diets did not compromise the absorptive and transport capacity of the intestine and consequently, lipids were not accumulated in the intestinal mucosa.

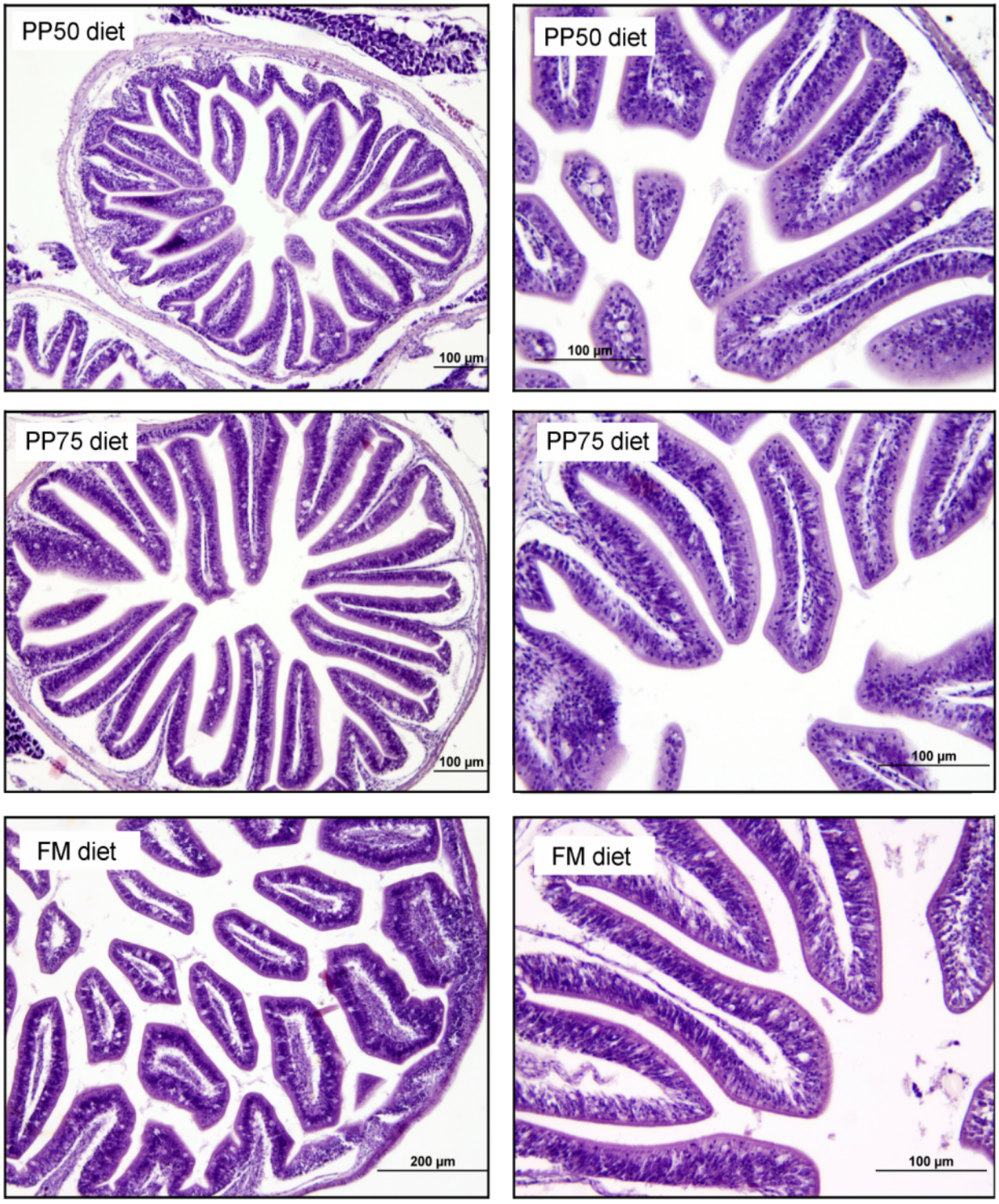

Figure 6. General view (left column; 200X) and detail (right column; 400X) of the intestinal mucosa of grey mullet (*Mugil cephalus*) fry weaned onto diets with different levels of fish meal substitution (FM, no fish meal substitution; PP50, 50% substitution of FM with plant protein sources; PP75, 75% substitution of FM with plant protein sources). The separation between the intestinal epithelium and the connective tissue in the intestinal submucosa is an artifact derived from tissue fixation. Staining: hematoxylin-eosin.

3.6 Cost-benefit proxy analysis of experimental diets

Production costs are typically divided between variable costs (or proportional) and fixed costs (or non-proportional). Variable costs depend on the production level and include seed and feed, whereas fixed costs must be paid regardless of the level of production and include such outlays as payroll and capital costs, interest and depreciation on the aquaculture facility. Although some labor may be considered as a variable cost, we have decided to include it as a fixed cost according to Lipton and Harrell (1990).

As grey mullet showed similar growth performance values regardless of the experimental diet used as shown in **Table 4**, the main variable affecting yield production costs is the price of feed. Thus, we have evaluated the cost-benefit of the tested diets just considering the price of ingredients (fishmeal 70 LT, wheat gluten, corn gluten, wheat meal, fish oil and L-Lysine and DL-methionine) used for their manufacture (**Table 10**), and consequently, the variable component in the final cost of the diet. However, other potential variables like labor associated with feeding and tank cleaning tasks or indirect costs associated with feed conservation have not been incorporated into the analyses, as we considered them as a fixed cost regardless of the inert diet considered. Additionally, the feed conversion ratio (FCR) was not considered as a variable for cost analysis, since fish were *ad libitum* and non-ingested feed particles were not recovered from the bottom of the tank due to their small size and, consequently, FCR values were not calculated. In this sense, fish were fed the same feed ratio for all the groups, so this parameter should be similar among groups if we infer a similar ingestion of experimental diets, as growth performance and condition factors indicated

Table 10. List of variable ingredients in FM, PP50 and PP75 diets, their price and level of inclusion in experimental diets. The difference in feed price (Δ feed price, %) were related to the FM diet. Those ingredients that did not differ between diets have not been included in the analysis, as they were considered as a fixed cost in diet price. Prices of ingredients were provided by feed manufacturer (SPAROS Lda.), although changes in the price of ingredients may exist considering the volume of product transacted.

		Level of inclusion (%)			Price (€/ton)		
	Price (€/ton)	FM	PP50	PP75	FM	PP50	PP75
Fishmeal LT 70	2,240	32.0	16.0	8.0	77,440	38,720	19,360
Wheat gluten	1,710	0.0	6.9	10.5	0	11,799	17,955
Soy protein concentrate	1,340	0.0	5.0	7.0	0	6,700	9,380
Corn gluten	720	0.0	5.0	7.0	0	3,600	5,040
Wheat meal	270	16.5	12.6	11.0	4,455	3,402	2,970
Fish oil	1,250	11.3	11.5	13.1	14,125	15,625	16,375
L-lysine	1,950	0.0	0.4	0.7	0	780	1,365
DL-methionine	3,550	0.2	0.3	0.4	710	1,065	1,420
Total	-	-	-	-	96,730	81,691	73,865
Δ feed price (%)					**0**	**-15.5**	**-23.6**

Considering that feed costs account for over 50 percent of the production costs in aquaculture facilities (Rana et al., 2009), the results showed in Table 10 indicated that PP50 and PP75 diets were 15.5 and 23.6% cheaper than the FM diet, which was mainly due to the lower inclusion of high quality fish meal (LT 70) in diets. In addition, the inclusion of alternative plant proteins was a satisfactory strategy in terms of feed price reduction, since the higher inclusion of fish oil and L-lysine and DL-methionine for balancing the dietary lipid and amino acid levels in PP50 and PP75 did not substantially affect the final price of the diet.

4. Conclusions

Fish meal substitution between 50 and 75% by a mixture of different plant protein sources (corn gluten, wheat gluten and soy protein concentrate) in wild grey mullet fry weaned onto compound diets did not affect their growth performance and survival, as fish fed diets with high levels of fish meal substitution grew similar to those fed the control diet with a high content of fish meal. Similarly, the implemented weaning strategy of wild specimens onto experimental compound diets resulted to be correct in terms of growth performance and survival rates.

The growth rates obtained at the end of the present study (day 60) were quite satisfactory and even slightly higher to those reported by Kalla et al. (2003) in grey mullet fed diets with different levels of fish meal substitution by soy protein, and those reported by other authors that fed this species with compound diets in which fish meal was substituted by seaweeds and marine algae (Wassef et al., 2001; El-Dahhar et al., 2014). These results are in agreement with other studies conducted on this species, confirming the capacity of this species to digest plant protein sources at early life stages, which might be attributed to the gradual change in feeding habits, observed from a zooplanktonic to detritivorous benthic foraging behavior observed at body sizes longer than 25 mm in SL (Oren, 1981).

No differences in the proximate composition, fatty acid and amino acid profiles were found among fish fed different experimental diets. Similarly, the digestive capacities of fish measured by the activity of different pancreatic and intestinal enzymes was not affected by diet composition and indicated that diets were successfully digested and nutrients absorbed by the organism, which was translated into similar growth performances between groups. It should be also mentioned that diets did not affect the organization of the liver and intestine, nor their antioxidative stress defenses, which also confirmed the capacity of this species at early life stages of development to use satisfactorily alternative plant protein sources without compromising its performance and overall condition.

These results indicated that weaning diets for wild grey mullet harvested for restocking aquaculture ponds and on-growing purposed may be formulated with a high level of fish meal replacement by alternative plant protein sources. In addition, considering the good performance and condition (proximate composition, activity of antioxidative stress enzymes, organization of the liver and intestine, and activity of pancreatic and intestinal digestive enzymes) of fish fed the experimental diet with a 75% of fish meal replacement by different plant protein sources (corn gluten, wheat gluten and soy protein concentrate), it seems plausible that fry of this species may accept and use satisfactorily compound diets with a complete fish meal substitution by plant protein sources.

Diets with a 50 and 75% of fish meal replacement by plant protein sources (corn gluten, wheat gluten and soy protein concentrate) were 15.5 and 23.6% cheaper than the FM diet, which is of special relevance considering that feed costs account for over 50 percent of the production costs in aquaculture facilities.

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Deviations: In the initial experimental design we had planned to use cladocerans (*Daphnia* sp.) as the main live prey for wild grey mullet fry instead of *Artemia* metanauplii. However, due to the difficulties in getting enough biomass of *Daphnia* sp. for supporting the first part of the trial (weaning period) during 30 days, we decided to change this zooplanktonic species and use *Artemia* instead of it, which was easier to get and maintained under controlled conditions. This change did not affect the experimental design nor the results obtained in this study. Additionally, in the DOW document it was planned to analyze the effects of experimental diets on non-innate immune response parameters analyzed from serum; however, it was not possible to obtain blood from grey mullet fry due to their small size at the end of the trial. In contrast, we conducted a deep analysis of the activity of antioxidative stress enzymes in different tissues (liver and muscle) to characterize the putative impact of experimental diets on the health condition of cells.

Co-funded by the Seventh Framework Programme of the European Union