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Weaning wild flathead grey mullet (*Mugil cephalus*) fry with diets with different levels of fish meal substitution

Enric Gisbert [a,*], Mansour Torfi Mozanzadeh [b], Yannis Kotzamanis [c], Alicia Estévez [a]

[a] IRTA, Centre de Sant Carles de la Ràpita (IRTA-SCR), Unitat de Cultius Experimentals, Crta. del Poble Nou Km 5.5, 43540 Sant Carles de la Rápita, Spain
[b] Fisheries Department, Faculty of Marine Natural Resources, Khoramshahr Marine Science and Technology University, Iran
[c] Institute of Marine Biology, Biotechnology and Aquaculture, Athens, Greece

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ABSTRACT

The culture of flathead grey mullet (*Mugil cephalus*) is based on wild fry captured during their migration into estuarine environments and consequently, optimizing weaning diets is of special importance for this species at this particular stage of development. Thus, authors have tested a weaning protocol for wild flathead grey mullet fry (202 mg initial body weight) during 60 days (18.1 ± 0.3 °C, salinity, 1.2 ± 0.2‰) using compound diets (36% crude protein, 16% crude fat) with different levels of fish meal (FM) substitution by plant protein (PP) sources (50% and 75% of FM replacement by a blend of corn gluten, wheat gluten, soy bean meal and soy protein concentrate). Fry were progressively weaned onto experimental diets during the first 20 days of the trial (25% *Artemia* metanauplii replacement each 5 days), whereas compound diets were offered at a feed ratio of 5% of stocked biomass until the end of the trial (day 60). A blend of PP sources (corn gluten, wheat gluten and soy protein concentrate) as the main dietary protein sources in combination with crystalline L-lysine and DL-methionine dietary supplementation were as good as a FM-based diet in terms of growth performance, digestive physiology and fish condition (*i.e.* proximate composition, oxidative stress status). As the cost-benefit proxy analysis of the tested weaning diets indicated, the inclusion of alternative PP was a satisfactory strategy in terms of feed price reduction, since PP50 and PP75 diets were 15.5 and 23.6% cheaper than the FM diet. Although wild flathead grey mullet fry were satisfactorily weaned onto diets containing 75% FM substitution by PP sources, present results indicated that complete FM replacement in weaning diets for this species might also be feasible.

Statement of relevance: In this study, authors have tested a weaning protocol for wild flathead grey mullet (*Mugil cephalus*) fry based on diets with different levels of fish meal substitution by alternative plant protein sources. This is of special importance due to the importance of the aquaculture of this species in several regions of the word, as well as for the use of wild animals for on-growing purposes. Results showed that diets with 75% of fish meal substitution can be successfully used for weaning and on-growing wild fry without any detrimental effect of fry performance and condition.

1. Introduction

The flathead grey mullet (*Mugil cephalus*) is an economically important euryhaline and eurythermal species contributing to sizable fisheries of estuarine and coastal regions in many countries (Saleh, 2006; Whitfield et al., 2012). This fish species has been recognized as a potential species for aquaculture diversification in the Mediterranean region, as well as in other regions of the world (Republic of Korea, Taiwan Province of China, South Africa), because of its good adaptation to captivity, rapid growth, omnivorous feeding habits and high market price of its salt-cured and dried eggs named "bottarga" (Whitfield et al., 2012). Grey mullet 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 (Italy, Israel, Egypt, Tunisia), Asia and South Africa (Whitfield et al., 2012), the intensive monoculture of this species has to be developed. This fact implies the development of a breeding technology and the development of a suitable and economical grow-out diet. However, before the juveniles could be provided by the aquaculture industry, the culture of this species is still based on wild fry (Whitfield et al., 2012; Biswas et al., 2012; El-Dahhar et al., 2014). In countries where collection for aquaculture of wild flathead grey mullet fry is practiced, social problems usually result from the competition for resources between fish farmers and fishermen (Saleh, 2006).

Providing adequate nutrition and fulfilling the nutritional requirements of the species are key-factors to successful growth, development and survival of fish. Mullets are described as omnivorous, opportunistic

* Corresponding author.
E-mail address: enric.gisbert@irta.cat (E. Gisbert).

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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 for large juveniles (>10 cm in standard length) and adults of flathead grey mullet is mainly based on detritus and benthic microalgae together with foraminiferans, filamentous algae, protists, meiofauna and small invertebrates (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 of vegetal origin (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 FM based diets (Tacon and Metian, 2008). Moreover, the use of the plant products is more economical and environmentally sustainable, which suggests that a focus on omnivorous and detritivorous fish species is needed. However, several disadvantages of using plant-based ingredients in fish diets in comparison to fish-based ingredients were reported (Gatlin et al., 2007). There have been many recent advances in the field of fish nutrition of farmed fish raised on plant feeds, including improvements of dietary manipulations, feed supplementation with additives and processing technologies of raw vegetable material to enhance growth and feed efficiency (Klinger and Naylor, 2012). 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; Naylor et al., 2009).

This study aimed to test a weaning protocol for wild flathead grey mullet fry using compound diets with different levels of FM substitution by plant protein sources (50% and 75% of FM replacement by a blend of corn gluten, wheat gluten, soy bean meal and soy protein concentrate).

2. Material and methods

2.1. Biological material, experimental design and diets

Wild flathead grey mullet fry (24.2 ± 0.8 mm in standard length, SL; 202 ± 5 mg in wet body weight, BW; $n = 3500$) were caught as described in Gisbert and López (2008) and obtained from Pescados y Mariscos Roset S.L. (Deltebre, Spain). Fish were acclimated to 17 °C in two 400-L quadrangular tanks for 7 days, and bathed in oxytetracycline (20 mg l^{-1}; 12 h), and thereafter kept in brackish water (14‰; 14 h). Fish were identified to species according to the number of pyloric caeca ($n = 2$) in their gut and external pigmentation patterns (Cambrony, 1984).

During the acclimation period, fish were fed *ad-libitum* with 6 days-old *Artemia* metanauplii. *Artemia* metanauplii were fed a mixture of microalgae (*Tetraselmis suecica* and *Isochrysis galbana*). Fish were distributed among 12 cylindroconical 100-L tanks ($n = 200$ per tank) connected to a recirculation system IRTAmar®. Water quality conditions during the acclimation and experimental periods were as follows: temperature, 18.1 ± 0.3 °C (mean ± standard deviation, SD); salinity, 1.2 ± 0.2‰; dissolved oxygen, 6.5 ± 0.4 mg l^{-1} (~90% saturation); NH_4^+, 0.20–0.29 mg l^{-1}; NO_2^-, 0.001 mg l^{-1}, and the photoperiod was 10L:14D (light:darkness).

Wild 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^{-1}); 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). The inert diet was offered at 5% of the stocked biomass (apparent satiation). Although this is an omnivorous species (Whitfield et al., 2012), a diet based on the complete substitution of fish meal by plant protein sources was not taken into consideration since this species is carnivorous during the fry stage (Gisbert et al., 1995).

Extruded diets (pellet size: 0.8 mm) were formulated and manufactured by Sparos Lda. (Portugal). The FM dietary component was partially substituted at 50 and 75% by PP (corn gluten, wheat gluten soy bean meal and soy protein concentrate; Table 1), whereas PP50 and PP75 diets were supplemented with L-lysine and DL-methionine in order to balance their respective amino acid profiles (NRC, 2011). The fatty acid composition and the amino acid profile of diets are shown in Tables 2 and 3, respectively.

2.2. Sampling, and survival and growth performance determinations

Experimental procedures were conducted in compliance with the Guidelines of the European Union Council (86/609/EU) for the use of laboratory animals. Growth in BW and SL was determined at 30 and 60 days after the beginning of the trial. All fish (fastened overnight) were taken from tanks with a dip net, gently anesthetized with 100 mg MS-222 l^{-1}, and then their wet BW (g) and SL (mm) determined to the nearest 1 mg and 1 mm, respectively. In addition, 50 specimens (fastened overnight) per tank (replicate) were sacrificed with an overdose of anaesthetic for assessing the histological organization of the liver and intestine ($n = 10$), the activity of pancreatic and intestinal digestive enzymes ($n = 10$), proximate composition, fatty acid and amino acid analyses ($n = 20$), and assessment of the activity of antioxidative stress enzymes and lipid peroxidation levels ($n = 10$).

The following formulae were used to calculate the specific growth rate in BW (SGR_{BW}, %) = [(ln BW_f − ln BW_i) × 100]/time (days) and the Fulton's condition factor (K) = (BW_f/SL^3) × 100. 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 beginning of the trial then multiplying the result by 100.

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).

	Experimental diets		
	FM	PP50	PP75
Ingredient			
Fish meal 70 LT	32.0	16.0	8.0
CPSP90	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
Vitamin and mineral 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	0.04	0.7
DL-Methione	0.2	0.3	0.4
Total	100.0	100.0	100.0
Proximate composition			
Crude protein (%)	36.0 ± 0.2	35.8 ± 0.1	35.9 ± 0.2
Crude fat (%)	15.9 ± 0.1	15.8 ± 0.2	15.9 ± 0.1
Fiber (%)	2.5	2.7	2.8
Starch (%)	14.8	14.2	13.8
Gross energy (J kg^{-1})[a]	1771.7	1755.8	1757.3

[a] Gross energy content was estimated as: total carbohydrate × 17.2 J kg^{-1}; fat × 39.5 J kg^{-1}; and protein × 23.5 J kg^{-1}.

Table 2
Fatty acid composition (mg g lipid^{-1}) 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.

2.3. Proximate composition, amino acid (AA) and fatty acid (FA) analyses

Fish ($n = 10$ per tank) and diets ($n = 2$) were homogenized and aliquots dried (120 °C for 24 h) to estimate gravimetrically their water content; total fat and protein levels were determined according to Folch et al. (1957) and Lowry et al. (1951), respectively; and ash determined by heating the sample at 500 to 600 °C for 24 h in a muffle furnace (AOAC, 1990). All analyses were conducted in triplicate (methodological replicates).

Acid catalyzed transmethylation was carried out in order to determine the FA profile (Christie, 1982). 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 a gas-liquid chromatography instrument (Thermo Electron Trace GC, Winsford, UK) fitted with a BPX70 capillary column (30 m × 0.250 mm ID × 0.25 μm film thickness, USA) as detailed in Boglino et al. (2012a). Peaks of each FA 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 (Trace GC, Thermo Finnigan, Italy). Results of FA content were expressed as μg mg^{-1} lipid.

The AA profile of the muscle of flathead grey mullet fry weaned onto the experimental diets was determined after acid hydrolysis (6 N, 110 °C, 24 h) and derivatization by AccQ-Tag according to the AA 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 × 2.1 mm ID × 1.7 μm, USA) from Waters. The flow rate was 0.7 ml min^{-1} 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 AA 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).

Table 3
Amino acid profile (mg 100 mg sample^{-1}) 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, $P < 0.05$, $n = 2$).

2.4. Quantification of digestive enzymes

The activity of digestive pancreatic and intestinal enzymes was conducted at the end of the trial (60 days). Fish were dissected ($n = 10$ fish per tank) on a glass plate at 0–4 °C, and their digestive tracts pooled, homogenized (1–2 min at 0–4 °C; 30 volumes v/w of 50 mM mannitol, 2 mM Tris–HCl buffer, pH 7.0) and sonicated (Gisbert et al., 2009). Samples were centrifuged (3300*g*, 3 min at 4 °C) and the supernatant was collected, aliquoted and frozen at −80 °C for the determination of pancreatic and intestinal enzymes. For the quantification of intestinal brush border (BB) enzymes, intestinal BB membranes were purified according to Crane et al. (1979).

Total alkaline proteases were assayed by the azo-casein method described by Walter (1984) using 50 mM Tris-HCl buffer (pH 9.0). Trypsin (E.C. 3.4.21.4) was assayed at 25 °C 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 min^{-1} ml^{-1} of extract at 407 nm (Holm et al., 1988). Chymotrypsin (EC. 3.4.21.1) activity was quantified at 25 °C 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 min^{-1} ml^{-1} of extract at 256 nm (Worthington, 1991). Alpha-amylase (E.C. 3.2.1.1) was measured using 0.3% soluble starch dissolved in Na_2HPO_4 buffer (pH 7.4) as substrate (Métais and Bieth, 1968) and its activity (U) was defined as the mg of starch hydrolyzed during 30 min/ml of extract at 37 °C at 580 nm. Bile salt-activated lipase (E.C. 3.1.1) activity was assayed for 30 min at 30 °C 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 (6080*g*, 2 min at 4 °C) and the absorbance of the supernatant read at 405 nm. Lipase activity (U) was defined as the μmol of substrate hydrolyzed min^{-1} ml^{-1} of extract (Iijima et al., 1998). Alkaline phosphatase (E.C. 3.1.3.1) was

quantified at 37 °C 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 min^{-1} ml^{-1} of BB homogenate at 407 nm (Bessey et al., 1946). Aminopeptidase N (E.C.3.4.11.2) was determined at 25 °C 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 min^{-1} ml^{-1} of BB homogenate at 410 nm. Maltase (E.C.3.2.1.20) activity was measured at 37 °C 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. Leucine–alanine peptidase (E.C. 3.4.11) was performed 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 min^{-1} ml^{-1} of extract at 37 °C at 530 nm (Nicholson and Kim, 1975). Activities were expressed as specific activity (mU mg^{-1} protein), and 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 (methodological replicates).

2.5. 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 muscle tissue of flathead grey mullet fry in order to evaluate the fry's health condition. The liver was chosen as the main metabolic organ, and the muscle as the main tissue reflecting somatic growth. Quantification of lipid peroxidation 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 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, centrifuged (15,000*g*, for 10 min at 4 °C) to remove protein precipitates and the absorbance read at 586 nm. The amount of peroxidized lipids (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). Catalase (CAT, E.C. 1.11.1.6) activity was measured 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.) 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 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 (Günzler and Flohé, 1985). Soluble protein of crude enzyme extracts was quantified by Bradford's method. Enzymatic activities were expressed as specific enzyme activity, in nmol mg^{-1} protein, with the exception of SOD that was expressed as percentage of inhibitory activity. All assays were carried out in triplicate at 25 °C, and the absorbance was read using a spectrophotometer (Tecan™ Infinite M200, Switzerland).

2.6. Histological analyses

The visceral mass of 30 fish per dietary treatment at 60 days 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 (3–5 μm thick). Sections of the liver and intestine were observed under a light microscope (Leica DM LB; Leica Microsystems, Germany) and photographed (Olympus DP70 Digital Camera; Olympus Imaging Europa GmbH, Germany) in order to evaluate their condition as described in Boglino et al. (2012b).

2.7. Statistics

Data are presented as the mean ± standard error of the mean. Values for different parameters were compared between them by means of one-way ANOVA at a reliability level of 5%. Data on proximal composition was compared between groups at day 30 and 60 with a two-way ANOVA. 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 groups differed among each other.

3. Results

3.1. Survival and growth performance

There were no significant differences in survival, growth performance and K of wild fry weaned onto diets at the end of the weaning (day 30) and early on-growing (day 60) periods (Table 4; $P > 0.05$). Values for SGR_{BW} were similar among experimental groups in both parts of the study (days 0–30 and 30–60), which confirmed that inert diets were well accepted and digested by fish, as visual observations indicated (data not shown).

3.2. Proximate composition, and FA and AA profiles

There were no differences in the proximate composition of fry at both of sampling dates (Two-way ANOVA, $P > 0.05$; Table 5), whereas protein and lipid content within the same experimental group varied between day 30 and 60 ($P < 0.05$). There was a reduction in protein content between fry sampled at day 30 and 60 (56.8–56.3% *vs.* 46.6–45.9%), coupled with an increase in lipid levels (26.0–26.5% *vs.* 32.8–34.5%), whereas carbohydrate and ash content remained stable during this period ($P > 0.05$).

At the end of the study (day 60), the fatty acid profile of fry weaned onto experimental diets with different levels of FM substitution was quite similar, regardless the fact that diets differed in their levels of saturated fatty acids, n–6 polyunsaturated fatty acids (PUFA) and total PUFA (Table 2). Fish only differed in terms of their levels in linoleic acid (LA, 18:2n-6) and n–6 PUFA, which were 26% higher in fish fed PP50 and PP75 diets in comparison to those fed the FM diet (Table 6; $P < 0.05$). There were no differences in the AA profile of the muscle of fish fed different diets (Table 7; $P > 0.05$).

Table 4
Final body wet weight (BW, mg), standard length (SL, mm), Fulton's condition factor, specific growth rate in BW (% day^{-1}) 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^{-1})	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^{-1})	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

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). 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)			
	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

3.3. Organization and functionality of the digestive system

No major differences in the histological organization of the liver were found between different experimental groups. In general terms, fry showed a healthy liver with central or moderately displaced nuclei towards the periphery of hepatocytes, and a moderate accumulation of fat deposits and glycogen within them. 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; $P > 0.05$). The level of lipid accumulation in the intestinal mucosa was negligible, which indicated that the dietary lipid levels did not compromise the absorptive and transport capacities of the intestine. The activity of pancreatic digestive enzymes was not significantly affected by the levels of FM substitution by PP in diets (Table 8; $P > 0.05$).

Table 6
Fatty acid (FA) profile (mg g lipid^{-1}) 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

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

Table 7
Amino acid profile (mg 100 mg^{-1}) 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

Regarding intestinal enzymes, the activities of leucine-alanine peptidase, alkaline phosphatase, aminopeptidase-N and maltase were similar among experimental groups, although maltase tended to be higher in flathead grey mullet fry weaned onto PP50 and PP75 diets in comparison to those fed the FM diet ($P > 0.05$).

3.4. Levels of lipid peroxidation and activity of antioxidative stress enzymes

Table 9 shows the levels of lipid peroxidation and activity of antioxidative stress enzymes in the liver and muscle of wild fry weaned onto diets with different levels of FM substitution. There were no differences in the content of TBARs and activities of SOD, CAT, GPX, GR and GST between experimental groups ($P > 0.05$) regardless of the tissue considered.

4. Discussion

The progressive replacement of live prey with an inert diet has been reported as a good weaning strategy in different fish species, resulting in better fish (Hamre et al., 2013). Thus, a long co-feeding weaning

Table 8
Specific activity (mU mg protein^{-1}) 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
-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			
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

Table 9
Lipid peroxidation levels (TBARs in nmol MDA g^{-1}) 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 (nmol MDA g^{-1})	253.7 ± 61.4	204.2 ± 36.8	254.9 ± 20.7
SOD (% inhibition)	85.7 ± 2.28	83.4 ± 1.98	81.6 ± 1.27
CAT (nmol mg $protein^{-1}$)	0.679 ± 0.065	0.550 ± 0.134	0.620 ± 0.255
GR (nmol mg $protein^{-1}$)	2.02 ± 0.26	1.52 ± 0.11	1.81 ± 0.20
GPX (nmol mg $protein^{-1}$)	8.24 ± 0.39	7.43 ± 0.20	8.05 ± 0.73
GST (nmol mg $protein^{-1}$)	2.96 ± 0.33	2.88 ± 0.20	3.30 ± 0.15
Muscle			
TBARs (nmol MDA g^{-1})	243.4 ± 22.8	231.9 ± 20.4	262.7 ± 14.2
SOD (% inhibition)	88.2 ± 1.28	89.2 ± 1.91	88.6 ± 0.80
CAT (nmol mg $protein^{-1}$)	0.535 ± 0.101	0.473 ± 0.024	0.511 ± 0.061
GR (nmol mg $protein^{-1}$)	2.02 ± 0.93	2.58 ± 0.69	1.82 ± 0.87
GPX (nmol mg $protein^{-1}$)	6.52 ± 0.42	5.87 ± 0.79	7.14 ± 0.72
GST (nmol mg $protein^{-1}$)	5.47 ± 0.57	4.75 ± 0.50	5.41 ± 0.15

protocol (20 days) was used in this study to improve and stabilize the nutritional condition of wild fry, as well as to pre-condition them to the inert diet (Parma and Bonaldo, 2013). In this sense, weaning flathead grey mullet fry with diets with partial substitution of FM with a blend of PP sources (corn gluten, wheat gluten, soy bean meal and soy protein concentrate) did not affect their growth, survival and condition when compared the control diet. These results were in agreement with those reported by other authors testing a mixture of microalgae and seaweed meal in diets for this species (Wassef et al., 2001; El-Dahhar et al., 2014). Wassef et al. (2001) reported that algal-meal based diets could be satisfactorily used for feeding flathead grey mullet fingerlings when 20% of FM was replaced by *Ulva* sp. meal. The inclusion of 21% of a mixture of the microalga *Nannochloropsis oculata* and *Ulva* sp. was advisable in terms of growth performance and feed conversion rate (El-Dahhar et al., 2014).

The reduction in diet palatability as a consequence of FM replacement with PP sources usually results in a decrease in feed intake, which may affect growth performance (Tibaldi et al., 2006; Li et al., 2012; Song et al., 2014). In this trial, feeds were offered *ad-libitum* and palatability was not adversely affected by the level of PP inclusion in diets, which might be because of the supplementation of crystalline AA. Similar results were observed in flathead grey mullet juveniles fed processed full-fat soybean diets supplemented with lysine and methionine (Jana et al., 2012). Body proximate composition of fry did not change in both sampling times; however, whole protein and lipid contents varied between 30 and 60 days. The above-mentioned changes may be partially attributed to fry weaning from live prey to the inert diet (Akbary et al., 2010; Pradhan et al., 2014), but also associated with fish growth and the accumulation of mesenteric fat (data not shown).

It is generally accepted that the whole body FA composition of fish reflects that of the diet (Bell et al., 2002). Fish fed PP50 and PP75 diets had higher n–6 PUFA content in comparison to those fed the FM diet. These differences were attributed to the higher levels of n–6 PUFA in PP50 and PP75 diets, especially to the higher levels of linoleic acid (18:2 n-6). Similar results have been reported in other marine fish species fed diets with high levels of PP sources (De Francesco et al., 2007; Valente et al., 2011; Ribeiro et al., 2015). Although diets differed in their levels of total saturated fatty acids, there were no differences in their content in fry, which may be attributed to their use for energetic purposes. The lower retention and the absence of differences in terms of body content of stearidonic acid (18:4 n-3) regardless of dietary content, suggests there is no selective accumulation of this FA. In addition, the levels of dietary total monounsaturated fatty acids that were higher than their concentrations in the whole body, indicated that these FA were mainly catabolised for energy purposes (Bell et al., 2003; Regost et al., 2003; Mozanzadeh et al., 2016). The lower retention of eicosapentaenoic acid (EPA, 20:5n-3) with regard to docosahexaenoic acid (DHA, 22:6n-3) might indicate that EPA was more efficiently catabolised than DHA, and/or that DHA tended to be deposited selectively in body tissues (Bell et al., 2002; Valente et al., 2011; Zuo et al., 2012; Mozanzadeh et al., 2015 among others). Amino acid profiles of whole-body of a given fish species resembles its dietary requirements; this profile depends on the dietary AA supply, digestibility of the protein source, the respective AA intakes and the various metabolic processes occurring in the organism. Generally, animal derived protein ingredients have a balanced essential AA profile; in contrast, PP sources may be lacking in specific essential AAs, which need to be provided as a supplement (NRC, 2011). In this study, PP diets were formulated considering the AA profile of the FM as a reference; and consequently, PP50 and PP75 diets were supplemented with crystalline L-lysine and DL-methionine. This strategy was satisfactory in terms of growth performance and the overall condition of fry fed experimental diets, which was reflected by their similar body protein content and AA profile.

It is generally accepted that one of the major drawbacks associated to PP based diets is an impaired digestive capacity (Krogdahl et al., 1999; Santigosa et al., 2008; Silva et al., 2010; Estévez et al., 2011), which in extreme cases may be associated to intestinal histopathological lesions (Uran et al., 2008; Bansemer et al., 2015). In this study, the activity of pancreatic and intestinal digestive enzymes was not affected by the levels of FM substitution in diets. Although flathead grey mullet is a zooplanktivorous species at the fry stage (Gisbert et al., 1996), adults forage at the base of the food web and are considered as detritivorous (Whitfield et al., 2012), which may explain the tolerance of early juveniles to PP sources. These results were in agreement with those reported in juveniles of this species (Jana et al., 2012), and in goldfish (*Carassius auratus*) and gibel carp (*C. auratus gibelio*), confirming that omnivorous species are less sensitive to PP sources in inert diets (Silva et al., 2010; Xie et al., 2001). In addition, the activity of brush border intestinal enzymes coupled with the histological observations of the intestinal mucosa and activity levels of antioxidative stress enzymes indicated that the inclusion of PP ingredients did not affect the integrity, health nor intestinal functionality.

Phenolic and flavonoid compounds in PP sources have potent antioxidant activities (Adom and Liu, 2002), which have been associated with beneficial health effects in humans; thus, we decided to assess the activity of antioxidative stress enzymes in fry fed experimental diets. In this context, Sitjà-Bobadilla et al. (2005) and Kokou et al. (2015) have reported that FM replacement by different PP sources enhanced the glutathione metabolism and cellular redox status, thereby increasing the antioxidant defences in gilthead sea bream (*Sparus aurata*); the activity of the antioxidant stress enzymes in that study were associated to flavonoids and/or phenolic compounds present in PP sources. In contrast, we did not find any difference in the activity of antioxidative stress enzymes associated with the glutathione metabolism (GPX, GST and GR) nor in others like SOD and CAT, between fish fed PP50 and PP75 diets and the control diet. Such differences between those studies might be linked to the different sources, levels and processing procedures of PP sources, as well as a potential species-specific response to PP with regard to both considered species.

The intestine and the liver are considered reliable nutritional and physiological biomarkers, because both tissues sensitively reflect any physiological disorder originating from a nutritionally imbalanced diet or unsuitable feeding conditions (Gisbert et al., 2008). Fry fed experimental diets were shown to have a healthy liver with a moderate accumulation of fat deposits and no signs of hepatic steatosis. In the other hand, no signs of intestinal steatosis, epithelial abrasion or microvilli disarrangement were observed along different intestinal regions. These results indicated that dietary lipid levels or composition (?) did not compromise the absorptive and transport capacity of the intestine

and consequently, lipids were not accumulated in the intestinal mucosa. Similar to our results, Ribeiro et al. (2015) reported that, the level 50 and 60% of FM and fish oil replacement by vegetal sources was well tolerated by meagre (*Argyrosomus regius*) juveniles, since no alterations were observed in the intestinal architecture, neither in the morphometrics of the intestinal epithelium nor in the number of mucous cells. The above-mentioned authors also reported that the activity of alkaline phosphatase was not affected by FM replacement with blends of different PP sources, whereas aminopeptidase-N activity was suppressed by PP, which indicated a high sensitivity of meagre to PP sources (Estévez et al., 2011; Ribeiro et al., 2015). In this sense, soy lecithin used in experimental diets is rich in phosphatidylcholine that is a natural component of lipoproteins required for lipoprotein synthesis, lipid mobilisation and digestibility (Tocher et al., 2008). Benedito-Palos et al. (2008) reported no signs of intestinal damage or steatosis in gilthead sea bream fed PP-rich diets with 1% lecithin, which contrasted to severe intestinal and hepatic steatosis found in juveniles fed plant protein based-diets without lecithin supplementation (Sitjà-Bobadilla et al., 2005).

Finally, a cost-benefit proxy analysis of the tested experimental diets for weaning flathead grey mullet fry has been conducted. As grey mullet showed similar growth performance values regardless of the experimental diet evaluated, the main variable considered by authors affecting yield production costs was the price of feed. Thus, we have evaluated the cost-benefit of the tested diets just considering the price of ingredients that varied between diets used for their manufacture (fishmeal 70 LT, wheat gluten, corn gluten, wheat meal, fish oil, L-lysine and DL-methionine), and consequently, their impact in the final diet cost. However, other potential variables or associated indirect costs like labour for tank maintenance and feed conservation have not been incorporated into the analyses, as we considered them as a fixed cost according to Lipton and Harrell (1990). Additionally, the feed conversion ratio (FCR) was not considered as a variable for cost analysis, since fish were fed *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 could not be accurately calculated. As fish were fed the same feed ratio for all the groups, this parameter should be similar among groups if we infer a similar ingestion of experimental diets, as indicated by growth performance and condition factors. Thus, considering that feed costs account for over 50% of the production costs in aquaculture facilities (Rana et al., 2009), the results shown 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. Thus, 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, nor its biological performance in terms of fish growth and condition.

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, %) was calculated with regard 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.

	Price (€/ton)	Level of inclusion (%)			Price (€/ton)		
		FM	PP50	PP75	FM	PP50	PP75
Fishmeal LT 70	2240	32.0	16.0	8.0	77,440	38,720	19,360
Wheat gluten	1710	0.0	6.9	10.5	0	11,799	17,955
Soy protein concentrate	1340	0.0	5.0	7.0	0	6700	9380
Corn gluten	720	0.0	5.0	7.0	0	3600	5040
Wheat meal	270	16.5	12.6	11.0	4455	3402	2970
Fish oil	1250	11.3	11.5	13.1	14,125	15,625	16,375
L-Lysine	1950	0.0	0.4	0.7	0	780	1365
DL-Methionine	3550	0.2	0.3	0.4	710	1065	1420
Total	–	–	–	–	96,730	81,691	73,865
Δ feed price (%)					0	**−15.5**	**−23.6**

Note: Prices of ingredients were provided by feed manufacturer (SPAROS Lda., Portugal), although changes in the price of ingredients may exist considering the volume of product transacted.

5. Conclusion

These results indicated that weaning diets for wild flathead grey mullet fry may be formulated with high levels of FM replacement (50 and 75%) by alternative PP sources. A blend of PP sources (corn gluten, wheat gluten and soy protein concentrate) as the main dietary protein sources in combination with crystalline L-lysine and DL-methionine dietary supplementation was equivalent to a FM-based diet in terms of growth performance, digestive physiology and fish condition, although a long term study is needed to confirm present results. As the cost-benefit proxy analysis of the tested weaning diets indicated, the inclusion of alternative plant proteins was a satisfactory strategy in terms of feed price reduction, since PP50 and PP75 diets were 15.5 and 23.6% cheaper than the FM diet. Although wild flathead grey mullet fry were satisfactorily weaned onto diets containing 75% FM substitution by PP sources, present results indicated that complete FM replacement in weaning diets for this species might be also feasible.

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