Aquaculture 354–355 (2012) 68–74

Contents lists available at SciVerse ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Mixtures of lupin and pea protein concentrates can efficiently replace high-quality fish meal in extruded diets for juvenile black sea bream (*Acanthopagrus schlegeli*)

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ARTICLE INFO

Article history:
Received 12 October 2011
Received in revised form 28 March 2012
Accepted 29 March 2012
Available online 6 April 2012

Keywords:
Lupin
Pea
Nutrient utilization
Maltase
Black sea bream

ABSTRACT

A 60-day feeding experiment was carried out to investigate the effects of including lupin protein concentrate (LPC) and pea protein concentrate (PPC) in multiple essential amino acid-supplemented extruded diets for black sea bream (*Acanthopagrus schlegeli*). Nine diets, including eight diets formulated to contain four mixtures of LPC and PPC (L/P ratio, 3:0, 2:1, 1:2 and 0:3) with two dietary inclusion levels (300 or 500 g plant protein kg^{-1} dietary protein) and one diet with high-quality fish meal as the sole protein source (FM diet) were fed to 18 tanks of 13-g black sea bream. Growth performance, nutrient utilization, and brush-border membrane bound maltase activities were evaluated. An average weight gain (WG) of 32.7 g $fish^{-1}$ and an average feed conversion ratio (FCR) of 1.13 g ingested dry matter (g gain)$^{-1}$ were obtained. Neither plant protein inclusion level nor L/P ratio significantly affected body composition (except ash), fish somatic indices or plasma parameters. The high inclusion of 500 g kg^{-1} resulted in significantly higher FCR than what was obtained with 300 g kg^{-1} inclusion. The WG, whole body ash content, and nitrogen (N) and energy retentions of these fish were, however, significantly lower than that of the fish fed diets with low plant protein inclusion (300 g kg^{-1}). The highest LPC inclusion (L/P ratio = 3:0) resulted in significantly higher feed intake and FCR, and lower N retention than the treatments with less LPC, but did not affect the growth rates or energy retentions. The diet with the highest PPC inclusion resulted in significantly reduced maltase activity in distal intestine. Any combination of LPC and PPC in essential amino acid-supplemented extruded diets, accounting for up to half of dietary protein, can be used without impairing fish growth. At high inclusion, combinations with more PPC are preferred, due to less efficient feed conversion caused by the LPC.

1. Introduction

Aquaculture of black sea bream (*Acanthopagrus schlegeli*) is increasing in East Asia. The main traditional source of feed for this species is 'trash fish', leading to a series of problems such as unbalanced and incomplete nutrient composition, contamination by unsafe transport and handling, water pollution, and growth of pathogenic bacteria. Published nutritional studies on black sea bream have up to now mainly focused on the basic nutrient requirements, including protein, essential amino acids, vitamins and phosphorous (Shao et al., 2008; Peng et al., 2009). There is, however, limited published information concerning the nutritional value of different feed protein ingredients in the diet for black sea bream.

Lupin and pea are legumes with high potentials as sources of protein in diets for salmonid fish (Burel et al., 1998; Carter and Hauler, 2000; Glencross et al., 2002, 2003, 2004a, 2004b, 2008; Refstie et al., 2006). These plant protein sources have also demonstrated their potentials in diets for temperate and warm water fish such as European sea bass (*Dicentrarchus labrax*) (Gouveia and Davies, 1998, 2000; Adamidou et al., 2009), milkfish (*Chanos chanos Forsskal*) (Borlongan et al., 2003), and silver perch (*Bidyanus bidyanus* (Mitchell)) (Booth et al., 2001). In gilthead sea bream, dietary inclusion of unprocessed lupin and pea meals is limited to 20% and 30% of the dietary protein, respectively (Pereira and Oliva-Teles, 2002, 2004). The low dietary inclusion level can be related to the low protein content, imbalanced amino acid composition and presence of anti-nutritional factors (ANF) in the lupin and pea.

Removing the indigestible carbohydrates by extraction from lupin and the starch by air classification from peas, results in lupin (LPC) and pea protein concentrates (PPC) with high nutrient digestibilities, that can be efficiently used in diets for salmonids (Carter and Hauler, 2000; Thiessen et al., 2003; Glencross et al., 2006; Øverland et al., 2009). PPC can provide 40% protein in the diet for gilthead sea bream diet without

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doi:10.1016/j.aquaculture.2012.03.038

any negative effects on growth and feed utilization when replacing fish meal as a source of protein (Sánchez-Lozano et al., 2011). Combining PPC and rice protein concentrate rather than using PPC alone, allowed an increase of PPC up to 60% of the protein in diets for gilthead sea bream (Sánchez-Lozano et al., 2009). Zhang et al. (2012) showed that any combination of LPC and PPC with multiple essential amino acid-supplementation can be efficiently used when total plant protein inclusion is limited to 300 g kg^{-1} crude protein in extruded diets for rainbow trout (*Oncorhynchus mykiss*). At higher inclusion (500 g kg^{-1}) PPC appeared to be a preferable source of protein.

The aims of the present experiment were to 1) determine the effect of including LPC and PPC in extruded high energy diets on the growth, nutrient utilization and intestinal enzyme activities of juvenile black sea bream, 2) investigate if the combination of these ingredients allowed a higher dietary inclusion rate than when applied separately, and 3) determine the optimal combination of LPC and PPC in extruded diets for juvenile black sea bream.

2. Materials and methods

2.1. Ingredients and diets

The LPC was derived from white lupine (*Lupinus albus*), produced by dehulling, milling, aqueous extraction of lupine seeds to remove sugars and soluble non-starch polysaccharides (NSP), heating and spray-drying. The PPC was produced from yellow field pea (*Pisum sativum* L.) by dehulling, fine grinding and air-classification. The chemical composition of these two plant concentrates and LT fish meal has been previously reported by Zhang et al. (2012). The LPC and PPC were each supplemented with the first-three limiting essential amino acids to balance the essential amino acid profile to that of LT fish meal. A 2×4 factorial design was used in the present experiment, where the factors were inclusion level of plant protein concentrate (300 or 500 g protein kg^{-1} dietary protein), and ratio between essential amino acid-supplemented LPC and PPC in the diets (L/P ratio at 3:0, 2:1, 1:2 and 0:3). The diets were isonitrogenous (530 g crude protein (CP) kg^{-1}) and isolipidic (160 g crude lipid (CL) kg^{-1}). In addition, a diet with LT fish meal as the sole source of protein (FM diet) was produced with 570 g CP and 180 g CL kg^{-1}, and formulated to keep the same ratio between protein and lipid ratio as the 8 diets with plant protein sources. Yttrium oxide was used as a marker for digestibility measurement (Austreng et al., 2000). Feed formulation and chemical composition are shown in Table 1. Feed processing and equipment are described in detail by Zhang et al. (2012). All dry ingredients were ground, mixed, preconditioned and extruded in a twin screw extruder with 2.0 mm dies and the pellets were dried and coated with fish oil in a vacuum coater. Yttrium oxide was added to the diets for determination of nutrient digestibilities, but fecal collection by stripping was not successful, and digestibility results are not presented.

2.2. Fish and feeding

The experiment was conducted at the Joint Laboratory of Nutrition and Feed for Marine Fish, Marine Fisheries Research Institute of Zhejiang Province (Putuo, Zhoushan, China). The black sea bream juveniles were obtained from a hatchery in Fodu (Putuo), acclimated in an indoor concrete pond for three weeks, and fed a commercial diet (52% CP, 8% fat). Before the start of the experiment, 900 bream with an initial weigh of 13 g were depleted of feed for 24 h, anaesthetized with MS-222 (90 mg l^{-1}), batch-weighed, then randomly assigned to 18 circular 500-l tanks, fifty fish per tank. Each tank was supplied with sand-filtered seawater at a flow rate of 1.5 l min^{-1} and additional aeration via air stone. A natural photoperiod (13 h light, 11 h

Table 1
Diet formulation and analyzed chemical composition (based on dry matter).

Diets	FM	LLP1	LLP2	LLP3	LLP4	HLP1	HLP2	HLP3	HLP4
Ingredients, g kg^{-1}									
Fish meal[a]	706.0	436.0	436.0	436.0	436.0	309.0	309.0	309.0	309.0
LPC[b]	–	280.0	186.0	94.0	–	467.0	311.0	155.0	–
PPC[c]	–	–	86.0	173.0	260.0	–	145.0	289.0	433.0
Fish oil[d]	121.0	98.0	103.0	107.0	112.0	94.0	102.0	110.0	118.0
Wheat	160.9	165.0	168.0	170.0	173.0	103.0	108.0	112.0	116.0
Premix[e]	4.3	4.3	4.3	4.3	4.3	4.3	4.3	4.3	4.3
Y_2O_3[f]	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
L-Lys[g]	–	6.1	5.0	3.9	2.7	10.2	8.3	6.4	4.6
DL-Met[h]	–	3.4	3.3	3.2	3.1	5.7	5.6	5.4	5.2
L-Trp[i]	–	0.6	0.4	0.2	–	1.0	0.7	0.3	–
L-Thr[j]	–	–	0.5	1.0	1.6	–	0.9	1.7	2.6
Analyzed content, kg^{-1}									
Dry matter, g	939	943	934	936	945	936	950	933	940
In dry matter									
Crude protein, g	575	529	534	534	533	522	522	531	528
Crude fat, g	174	163	164	152	143	161	157	152	149
Starch, g	110	100	107	110	121	69	84	98	107
Ash, g	107	78	81	83	89	70	76	78	77
Gross energy, MJ	23.5	23.1	23.1	23.0	22.2	23.0	22.5	22.8	22.1
Phosphorous, g	16.3	11.0	11.3	11.6	12.7	8.9	9.8	10.6	11.2
Phytic acid, IP6, g	1.47	3.02	3.49	4.83	5.61	4.19	4.47	6.07	8.40

[a] Norse LT-94®, low-temperature dried fish meal, Norsildmel, Bergen, Norway.
[b] NaProLup PO54®, Lupin protein concentrate, derived from white lupins (*Lupinus albus*), NaProFood, Bruckberg, Germany.
[c] PPC 55 PELLET, Pea protein concentrate, derived from yellow field pea (*Pisum sativum* L.), AgriMarin AS, Stavanger, Norway.
[d] Silfas, Karmsund, Norway.
[e] Per kg diet: vitamin A: 2000 IU, vitamin D_3: 1200 IU; vitamin E: 160 mg; vitamin K_3: 8 mg; vitamin B_1: 12 mg; vitamin B_2: 20 mg; vitamin B_3: 60 mg; vitamin B_5: 24 mg; vitamin B_6: 12 mg; vitamin B_9: 4 mg; vitamin B_{12}: 0.016 mg; vitamin C: 100 mg; Biotin: 0.2 mg; Ca: 876 mg; Cu: 4 mg; Co: 0.8 mg; I: 2.4 mg; Mn:12 mg; Zn: 96 mg.
[f] Metal Rare Earth Limited, Shenzhen, China.
[g] *L*-Lysine-HCl, 99% feed-grade, CJ Indonesia, Jakarta, Indonesia.
[h] Rhodimet® NP 99, *DL*-methionine, 99% feed-grade, Adisseo Brasil Nutricao Animal Ltda, Sao Paulo, Brazil.
[i] TrypAMINO®, *L*-tryptophan, 98% feed-grade, Evonik Fermas S.R.O., Slovenska Lupca, Slovakia.
[j] *L*-Threonine, 98.5% feed-grade, Ajinomoto Eurolysine S.A.S., Paris, France.

dark) was applied throughout the feeding period. The average water temperature and salinity were 25.0 °C and 29 g l^{-1}, respectively. Each diet was fed to fish in duplicate tanks and all the fish were fed three times per day, at 06:30, 11:30, and 17:30. Before each feeding, the water flow was stopped, while continuous aeration was maintained. The fish were fed by hand for 1 h. After each feeding, all uneaten feed particles remained intact, and the number of uneaten pellets in the bottom of each tank was counted and siphoned out immediately. The amount of uneaten feed was set by multiplying the number of uneaten pellets with the average pellet weight for each feed (counting 4×100 pellets). The daily feeding rate was tentatively set 10% in excess based on the average feed intake over the last 3-day feeding, but the fish received more feed if they showed signs of feed intake at the end of the one hour meals. The feeding experiment lasted for 60 days.

2.3. Sampling

Before the start of the experiment, 2×8 fish (depleted of feed for 24 h) from the acclimation pond were killed by overdose of MS-222, and kept at −20 °C for whole body analysis. Fish were anaesthetized with MS-222 (90 mg l^{-1}) and batch-weighed in the beginning (Day 0) of the experiment. At the end of the feeding experiment, five fish were randomly sampled from each tank for blood samples. The fish were weighed individually, blood was collected from the caudal vein by a 1-ml disposable syringe with a 27-gauge needle, and kept on ice until centrifuged at 3000×*g* for 10 min. The plasma was aliquoted into two Eppendorf (EP) tubes, frozen in liquid N_2, and kept at −80 °C until analysis. Another three fish were taken from each tank, individually measured for weight and length, and then killed by a blow to the head. The sea breams were cut open to remove the intestinal contents, the whole viscera, liver and carcass were weighed separately, and then put together and stored at −20 °C for whole body analysis. Ten fish were taken from each tank, weighed individually, then the intact gastrointestinal tracts were gently removed and divided into 3 regions as follows: stomach, mid intestine (MI, from distal side of the stomach region to distal intestine) and distal intestine (DI, from the start of the last fold of intestine until the anus). Surface fat and connective tissue were carefully removed. The intestinal tissue walls of MI and DI were placed in pre-weighed EP tubes, frozen in liquid N_2 and kept at −80 °C for the determination of brush border maltase activity. The remaining fish in each tank were weighed.

2.4. Chemical analyses

The initial and final fish samples were autoclaved at 120 °C for 20 min, homogenized and oven-dried at 70 °C. The dried whole fish samples and feed samples were analyzed for dry matter, crude protein, lipid, ash, and energy. Dry matter was determined by drying at 105 °C to constant weight. Crude protein content was measured using a 2300 Kjeltec Analyzer Unit (Foss, Tecator, Sweden). Lipid was determined by petroleum ether extraction using a Soxtec system (Soxtec 2055, Foss Analytical, Denmark), ash by combustion at 550 °C, and gross energy by bomb calorimetry (Phillipson Microbomb Calorimeter; Gentry Instruments Inc., Aiken, SC, USA). Minerals in feed samples were determined by inductively coupled plasma mass spectroscopy (ICP-MS) after complete digestion of the homogenized and dried samples in HNO_3 after cooking in a microwave oven for 1 h. For each measurement, duplicate samples were analyzed. Phytic acid was determined according to the method described by Carlsson et al. (2001). Plasma cholesterol and triacylglycerols were analyzed by RSBIO® kits (Shanghai Rongshen Biotech Co., Ltd. Shanghai, China) and spectrophotometer micro-plate reader (PowerWave XS, BioTek Instruments Inc., Winooski, VT, USA). Activities of brush-border membrane bound maltase in MI and DI were analyzed as described by Krogdahl et al. (2003). Only the samples from the fish fed the FM diet and the diets with 500 g plant protein kg^{-1} crude protein inclusion were measured for maltase activity.

2.5. Calculations and statistical analysis

Feed intake (FI) was calculated by subtracting uneaten feed from feed fed on a dry matter basis. Specific growth rate (SGR) was calculated as: $100\times(\ln(FBW)-\ln(IBW))\times d^{-1}$, where ln(IBW) and ln(FBW) are the natural logarithms of initial and final body weight of individual fish (tank mean), and d is feeding days. Feed conversion ratio (FCR) was calculated as: $FI\times(FBW-IBW)^{-1}$. Nitrogen or energy retentions (%) were calculated as: $100\times(N_1\times FBW-N_0\times IBW)\times(N_d\times FI)^{-1}$, where N_0 and N_1 represent the nitrogen or energy content in the initial and final whole fish samples (pooled samples of 3 fish per tank), respectively. Hepatosomatic index (HSI, %) or viscerosomatic index was calculated as: $100\times(\text{weight of organ})\times(\text{total fish weight})^{-1}$. Condition factor (CF) was calculated as: $100\times(\text{fish weight})\times(\text{body length})^{-3}$, where weight is expressed in g and length is in cm.

Each tank was considered an experimental unit. The results were analysed using the GLM procedure of SAS statistical software (SAS, 1990). One-way analysis of variance (ANOVA) was used to compare effects of the FM diet with those of the diets with plant protein. Factorial ANOVA was used to analyze the effects of plant protein inclusion level and L/P ratio. Significant ($P<0.05$) interactions between inclusion level and L/P ratio were rationalized by regression analysis of L/(L+P) within inclusion level, provided that at least one of the main effects were significant (Snedecor and Cochran, 1967). The results were expressed as means and pooled standard errors of means (S.E.M). Duncan's multiple-range test was used to rank significant differences among diets in the one way ANOVA and main effects in the factorial ANOVA.

3. Results

3.1. Growth and feed utilization

All fish had good appetite and grew well on all diets. The fish weights were more than tripled after the 60-day feeding period. Only one fish died during the experimental period; one fed the HLP3 diet on day 14. An average feed conversion ratio (FCR) of 1.13 g DM ingested (g gain)$^{-1}$ was achieved (Table 2). The feed intake (FI) of the fish fed the FM diet was significantly lower than that of the fish fed the HLP1 diet, and did not differ significantly from those fed the other diets. No significant difference was found among diets for growth (WG). The FCR for the LLP1, LLP3, LLP4 and HLP4 diets did not differ from that of FM diet, while the others were significantly higher.

The diets with most LPC (L/P ratio = 3:0) resulted in significantly higher FI than the diets with less LPC. Diets with 500 g plant protein kg^{-1} resulted in significantly lower WG than the diet with 300 g kg^{-1}. No significant effect of L/P ratio on WG was found. Higher dietary inclusion of plant protein resulted in significantly higher FCR during both feeding periods, and diets with the highest LPC ratio (L/P ratio = 3:0) resulted in significantly higher FCR compared to diets with less LPC during both feeding periods. The FCR for the diets with L/P ratios of 2:1, 1:2 and 0:3 did not differ significantly from each other. Significant interaction was seen between inclusion level and L/P ratio for FCR (Fig. 1). This was due to a steep and significant increase in FCR with increasing dietary LPC at 500 g plant protein kg^{-1} inclusion, while, this effect was not significant for 300 g kg^{-1}.

3.2. Body composition and nutrient retention

No significant difference was found in whole body composition among diets except for ash (Table 3). The ash content in the fish fed the FM diet was significantly higher than those fed the dietary plant protein based diets, except for the LLP3 and LLP4 diets. Neither

Table 2
Growth performance and feed utilization of black sea bream fed the experimental diets.

One way ANOVA model			
	Feed intake, g DM fish^{-1}	Weight gain, g fish^{-1}	Feed conversion ratio, g DM ingested (g gain)$^{-1}$
Diet[1]			
LLP1	37.2^{bc}	33.8	1.10^{bcde}
LLP2	37.5^{bc}	33.7	1.11^{bcd}
LLP3	36.0^{bc}	34.9	1.03^{de}
LLP4	35.9^{bc}	32.8	1.10^{cde}
HLP1	40.5^{a}	29.4	1.38^{a}
HLP2	38.0^{ab}	31.4	1.21^{b}
HLP3	35.5^{bc}	30.8	1.16^{bc}
HLP4	34.5^{c}	31.3	1.10^{bcde}
FM	36.2^{bc}	36.5	1.00^{e}
Pooled SEM	0.9	1.4	0.03
ANOVA P<F	0.025	0.097	0.046
Factorial ANOVA model			
	Feed intake	Weight gain	Feed conversion ratio
Factor			
Inclusion[2]			
300	36.6	33.8^{x}	1.08^{y}
500	37.1	30.7^{y}	1.21^{x}
L/P ratio[3]			
3:0	38.8^{r}	31.6	1.24^{r}
2:1	37.7^{r}	32.5	1.16^{s}
1:2	35.7^{s}	32.8	1.09^{s}
0:3	35.2^{s}	32.1	1.09^{s}
Pooled SEM	0.8	1.2	0.03
ANOVA P<F			
Inclusion[2]	0.40	0.008	< 0.001
L/P ratio[3]	0.005	0.76	0.007
Inclusion × L/P ratio	0.072	0.60	0.020

[1] For diet codes see Table 1. Different superscript letters [a, b, c, d, e] within a column indicate significant (P<0.05) difference among diets.
[2] Inclusion level of plant protein sources (g protein kg^{-1} dietary protein). Different superscript letters [x, y] within a column indicate significant (P<0.05) difference between inclusion levels.
[3] Mixing ratio between essential amino acid-supplemented LPC and PPC in diets. Different superscript letters [r, s, t] within a column indicate significant (P<0.05) differences among L/P ratios.

Table 3
Whole body composition[1] of black sea bream fed the experimental diets (g kg^{-1}).

One way ANOVA model					
	Moisture	Crude protein	Lipid	Ash	Energy (MJ kg^{-1})
Diet[2]					
LLP1	667	176	113	46.6^{bc}	8.74
LLP2	664	176	115	46.3^{bc}	8.02
LLP3	669	181	101	49.6^{ab}	8.11
LLP4	663	176	113	49.6^{ab}	8.22
HLP1	670	174	112	40.9^{c}	8.06
HLP2	668	177	113	45.4^{bc}	8.05
HLP3	676	177	102	45.0^{bc}	7.91
HLP4	682	173	104	46.0^{bc}	7.99
FM	676	181	92	55.3^{a}	7.36
Pooled S.E.M.	7	2	5	2.2	0.35
ANOVA P<F	0.57	0.17	0.13	0.043	0.49
Factorial ANOVA model					
	Moisture	Crude protein	Lipid	Ash	Energy
Factor					
Inclusion[3]					
300	666	177	110	48.0 ^{x}	8.27
500	674	175	108	44.3 ^{y}	8.00
L/P ratio[4]					
3:0	669	175	113	43.8	8.40
2:1	666	176	114	45.9	8.04
1:2	672	179	101	47.3	8.01
0:3	673	175	108	47.8	8.11
Pooled S.E.M.	7	2	6	1.7	0.37
ANOVA P<F					
Inclusion[3]	0.13	0.27	0.55	0.015	0.33
L/P ratio[4]	0.71	0.16	0.18	0.16	0.70
Inclusion × L/P ratio	0.61	0.71	0.79	0.56	0.81

[1] Initial values, kg^{-1}: moisture 733 g, crude protein 174 g, lipid 34 g, ash 55.3 g, energy 5.08 MJ.
[2] For diet codes see Table 1. Different superscript letters [a, b, c] within a column indicate significant (P<0.05) difference among diets.
[3] Inclusion level of plant protein sources (g protein kg^{-1} dietary protein). Different superscript letters [x, y] within a column indicate significant (P<0.05) difference between inclusion levels.
[4] Mixing ratio between essential amino acid-supplemented LPC and PPC in diets.

inclusion level nor the L/P ratio resulted in significant differences in whole body composition, except for ash, which was significantly lower in fish fed the diets with 500 g kg^{-1} plant protein inclusion than with 300 g kg^{-1} inclusion.

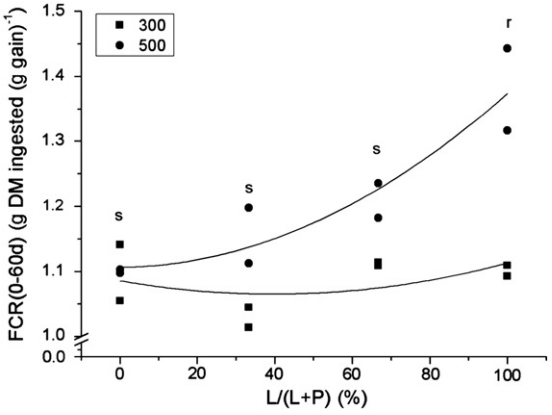

Fig. 1. Feed conversion ratio (FCR) in black sea bream during the whole 60 days of feeding diets with 300 and 500 g kg^{-1} of total crude protein from LPC and PPC, and with different ratios of protein from LPC and PPC (LPR, expressed by L/(L+P)). $FCR_{300} = (1.31\ E-5)\ LPR^2 - 0.00104\ LPR + 1.09$, $P_{model} = 0.57$, $R^2 = 0$; $FCR_{500} = (2.61\ E-5)\ LPR^2 + (6.06\ E-5)\ LPR + 1.11$, $P_{model} = 0.008$, $R^2 = 0.80$. Different superscript letters [r, s] indicate significant (P<0.05) differences in main effects among the 4 different L/P ratios.

The nitrogen (N) retention of fish fed the FM diet was significantly higher than those fed the HLP1 and HLP2 diets, but did not differ from those fed the other diets (Table 4). Both N and energy retentions of fish fed diets with inclusion of 500 g plant protein kg^{-1} crude protein were significantly lower than those fed diets with 300 g kg^{-1} inclusion. The diets with lower LPC levels (L/P ratio = 1:2 or 0:3) resulted in significantly higher N retention than diets with the highest LPC level (L/P ratio = 3:0).

3.3. Fish somatic indices, plasma parameters and intestinal maltase activity

No significant difference was found for the somatic indices, HIS, VSI, and CF, or the plasma parameters, total plasma cholesterol and triacylglycerol levels among the different diets (Table 5). Also, none of these parameters was significantly affected by plant protein inclusion level or L/P ratio. No significant differences were seen for maltase activity in MI, but the activity in DI was significantly lower in fish fed the HLP4 diet (Table 6).

4. Discussion

The growth rates obtained in the present experiment, corresponding to SGR ranging from1.4 to 1.7) were consistent with previous findings in this species with comparable fish size and rearing conditions (Shao et al., 2008, SGR ranging from 1.8 to 2.3 for fish with 13 g start weight; Peng et al., 2009, SGR ranging from 1.7 to 2.1 for

Table 4
Nitrogen and energy retentions (%) of black sea bream fed the experimental diets.

One way ANOVA model	Nitrogen	Energy
Diet[1]		
LLP1	30.3^{abc}	39.9
LLP2	29.8^{bc}	35.7
LLP3	33.3^{a}	39.1
LLP4	30.3^{abc}	38.9
HLP1	24.2^{d}	29.6
HLP2	28.2^{c}	34.1
HLP3	29.2^{bc}	34.6
HLP4	29.7^{bc}	37.8
FM	32.1^{ab}	35.0
Pooled S.E.M.	0.3	0.9
ANOVA P<F	0.004	0.11
Factorial ANOVA model	Nitrogen	Energy
Factor		
Inclusion[2]		
300	30.9^{x}	38.4^{x}
500	27.8^{y}	34.0^{y}
L/P ratio[3]		
3:0	27.2^{s}	34.8
2:1	29.0^{rs}	34.9
1:2	31.3^{r}	36.8
0:3	30.0^{r}	38.3
Pooled S.E.M.	1.0	2.2
ANOVA P<F		
Inclusion[2]	0.002	0.025
L/P ratio[3]	0.023	0.38
Inclusion × L/P ratio	0.089	0.23

[1] For diet codes see Table 1. Different superscript letters [a, b, c, d] within a column indicate significant (P<0.05) difference among diets.
[2] Inclusion level of plant protein sources (g protein kg^{-1} dietary protein). Different superscript letters [x, y] within a column indicate significant (P<0.05) difference between inclusion levels.
[3] Mixing ratio between essential amino acid-supplemented LPC and PPC in diets. Different superscript letters [r, s] within a column indicate significant (P<0.05) differences among L/P ratios.

Table 5
Somatic indices, and total plasma cholesterol (TC) and triacylglycerol (TG) concentrations of black sea bream fed the experimental diets.

One way ANOVA model	Somatic indices			In plasma	
	HSI[a]	VSI[b]	CF[c]	TC (mM)	TG (mM)
Diet[d]					
LLP1	1.41	9.35	3.15	6.84	15.0
LLP2	1.48	8.31	3.83	6.89	20.7
LLP3	1.35	7.44	3.82	7.03	16.8
LLP4	1.49	8.37	3.84	6.33	10.5
HLP1	1.59	8.23	3.72	7.04	19.8
HLP2	1.40	8.10	3.88	7.58	19.3
HLP3	1.42	7.64	3.92	7.70	15.7
HLP4	1.35	8.22	3.71	7.60	13.3
FM	1.23	7.46	3.75	7.70	17.7
Pooled S.E.M.	0.08	0.53	0.32	0.66	0.6
ANOVA P<F	0.22	0.38	0.82	0.80	0.96
Factorial ANOVA model					
	HSI	VSI	CF	TC	TG
Factor					
Inclusion[e]					
300	1.43	8.38	3.66	6.77	15.7
500	1.44	8.05	3.81	7.48	17.0
L/P ratio[f]					
3:0	1.50	8.79	3.43	6.94	17.4
2:1	1.44	8.20	3.86	7.24	20.0
1:2	1.39	7.54	3.87	7.37	16.3
0:3	1.42	8.30	3.78	6.97	11.9
Pooled S.E.M.	0.08	0.56	0.34	0.68	4.2
ANOVA P<F					
Inclusion[e]	0.84	0.44	0.55	0.18	0.67
L/P ratio[f]	0.52	0.25	0.56	0.91	0.34
Inclusion × L/P ratio	0.21	0.69	0.77	0.88	0.85

[a] Hepatosomatic index.
[b] Viscerosomatic index.
[c] Condition factor.
[d] For diet codes see Table 2.
[e] Inclusion level of plant protein sources (g protein kg^{-1} dietary protein).
[f] Mixing ratio between essential amino acid-supplemented LPC and PPC in diets.

fish with 18–19 g start weight). The similar growth rates among fish fed the FM diet and those fed the other plant protein based diets, showed that using multiple amino acid supplemented diets with LPC or PPC alone or in combination could provide 50% of dietary protein for black sea bream without impairing growth.

Adequate feed intake is a precondition to guarantee a precise nutritional evaluation of plant proteins in fish feed. A feed intake reduction has been observed when including high levels of plant protein concentrates in the diet for gilthead sea bream (Kissil et al., 2000). This may be related to the dilution or removal of palatable constituents derived from FM and the presence of detractive compounds in plant-derived ingredients. The high feed intake in the present experiment was consistent with previous findings with LPC and PPC in rainbow trout fed the same diets, but with higher content of lipid (Zhang et al., 2012). The current results, thus, show that the concentration of detractive components, such as alkaloids from lupin and saponins from pea were not sufficient to impair feed intake, even at a dietary inclusion level of 467 and 433 g kg^{-1} LPC and PPC respectively.

The FCR values in the present experiment suggest efficient utilization of the feed by black sea bream. One main explanation is the removal of indigestible material from the plant protein ingredients, since the aqueous extraction and air-classification mainly remove the soluble NSP from the lupin seed meal and carbohydrates from the pea meal. However, other ANF may still exist. Extrusion is known both to improve protein utilization by inactivating heat labile ANF, and by unfolding globular storage proteins to facilitate access of the digestive enzymes. In addition, extrusion results in gelatinization of the starch, which is necessary for efficient digestion (Sørensen, 2003). Thus, the high performance of sea bream obtained in the present experiment can both be related to the nutritional qualities of the LPC and PPC (Carter and Hauler, 2000; Refstie et al., 2006; Øverland et al., 2009) and the use of extrusion to produce the diets.

The main advantage of PPC over LPC as a dietary protein source seems to be the lower content of NSP. This is in keeping with previous observations (Carter and Hauler, 2000; Zhang et al., 2012). NSP are almost indigestible for fish due to the absence of α-galactosidase and β-xylanase in the digestive tract (Kuz'mina, 1996; Bansleben et al., 2008). In addition, the undigested NSP in digesta can negatively affect the digestion and absorption of other nutrients (Sinha et al., 2011). Soluble NSP have a viscous nature and can bind to the intestinal brush border and form a thick unstirred water layer adjacent to the mucosa to block the access of substrates to brush border enzymes, and reduce nutrient digestibilities by increasing the intestinal viscosity. A major reason for processing lupin into LPC was to remove the soluble carbohydrate fraction, thus soluble NSP was not the major reason for the preference of PPC over LPC at high inclusion rate.

The total phosphorus concentration of all the diets exceeded the levels sufficient for maximum for growth of black sea bream (Shao et al., 2008). The lack of successful collection of feces made it impossible to estimate phosphorus availabilities in the current experiment. The whole body composition of the black sea bream was similar to that reported by Peng et al. (2009) for fish fed a fish oil control diet. Whole-body ash content (WBA) significantly increased with increasing dietary total P concentration (DPc) ($WBA = -0.10\ DPc^2 + 4.27\ DPc + 11.2$; $R^2 = 0.66$, $P = 0.0001$). Whole-body ash concentration was not significantly related to dietary phytate concentration. This indicates that the total dietary P concentration was limited for bone mineralization. The growth rates of the sea bream were, however, not significantly affected by dietary P-concentration. This is in

Table 6
Maltase activities in intestinal sections of black sea bream fed the experimental diets with 500 g plant protein kg^{-1} crude protein inclusion and the FM diet (n = 2).

	Diet						
	HLP1	HLP2	HLP3	HLP4	FM	Pooled S.E.M.	*ANOVA* P<F
Mid intestine							
μmol h^{-1} (g tissue)$^{-1}$	0.326	0.352	0.309	0.472	0.408	0.043	0.17
μmol h^{-1} (kg BW)$^{-1}$	19.9	18.2	17.1	13.0	16.8	1.9	0.26
μmol h^{-1} (mg protein)$^{-1}$	129	133	105	112	129	14	0.61
Distal intestine							
μmol h^{-1} (g tissue)$^{-1}$	0.305^{a}	0.331^{a}	0.322^{a}	0.163^{b}	0.312^{a}	0.027	0.033
μmol h^{-1} (kg BW)$^{-1}$	8.22^{a}	9.02^{a}	7.31^{a}	4.47^{b}	6.38^{ab}	0.74	0.044
μmol h^{-1} (mg protein)$^{-1}$	63.9^{bc}	74.3^{ab}	69.6^{ab}	51.6^{c}	86.3^{a}	4.5	0.020

Different superscript letters $^{a, b, c}$ indicate significant (P<0.05) difference among treatments.

keeping with the results obtained by Shao et al. (2008) who found that black sea bream of similar size had a requirement of available dietary P for growth at 0.55%, while the estimated requirement for vertebral mineralization was higher (0.87%). The whole-body ash concentrations in the sea bream fed diets with the lowest inclusion of plant protein concentrates or the fish meal diet, were also similar to or higher than the values obtained in fish fed diets with adequate P-supply by Shao et al. (2008). Thus, the results indicate that diets not supplemented with P and containing 500 g plant protein concentrates kg^{-1} resulted in mild deficiency of this element. No indications of short term phosphorus deficiency were detected in the fish fed diets with 300 g plant protein concentrates kg^{-1}. Other essential elements like Ca and Mg are highly abundant in and taken up by the fish from saltwater, while Cu, I, Mn and Zn were supplemented by the micromineral premix (Zhang et al., 2012).

There is no published information available on N and energy retentions for black sea bream. The significantly lower N retention in fish fed HLP1 and HLP2 compared with the other diets may have been related to the high inclusion of LPC. The design of the experiment does not, however, provide explanations to weather this was caused by the high NSP content, differences in nutrient digestibilities, or other differences between the two plant protein concentrates. The protein retention efficiencies indicate that essential amino acids were provided in excess by all diets. Thus, inadequate amino acid supply was not a plausible explanation to the differences in N retention.

Hughes (1991) and Lairon (1996) reported that NSP of legume seeds was an effective cholesterol-reducing agent. A clear hypocholesterolemic effect was also observed in our previous experiment with rainbow trout (Zhang et al., 2012). The absence of hypocholesterolemic effect in the present experiment indicates that such effect may be species specific.

The brush border enzymes are responsible for the final stages of hydrolysis of protein and starch. Their activities do not only indicate the capacity of digestion but also the integrity of intestinal structure, especially in the distal part. The reduction of maltase activity in DI is in keeping with our previous finding with rainbow trout (Zhang et al., 2012). The trout also had a slight decrease in mucosal fold height and a slight increase in fold fusion in DI of fish fed diet with the highest level of PPC. This may indicate mild changes associated with the mechanism that resulted in inflammation in DI at higher dietary PPC levels (Penn et al., 2011). Histological studies are, however, needed to find out if the reduced maltase activity is related to changes in the integrity of the distal intestine.

To conclude, both LPC and PPC are promising dietary protein sources for black sea bream. Any combination of LPC and PPC in essential amino acid-supplemented extruded diets, accounting for up to half of dietary protein, can be used without impairing fish growth. At higher inclusion, combinations with more PPC are preferred, while high inclusion of LPC resulted in less efficient feed conversion.

Acknowledgements

The authors would like to acknowledge Xiaoming Zhu, Yuhang Chen, Haokun Liu, and Bianzhi Liu of the Institute of Hydrobiology, Chinese Academy of Science, and Jianjun Xie of the Marine Fisheries Research Institute of Zhejiang for their assistance during sampling. Thanks to Marine Fisheries Research Institute of Zhejiang for providing the research facilities. This study was funded by the Open Fund of Key Laboratory for Mariculture in Zhejiang, National Key Basic Research Program (NKBRP) (2009CB118702), National Sci-Tech Support Program of China (2011BAD13B08), and the Research Council of Norway (Grant # 417091). Yuexing Zhang was supported by a PhD grant from the Norwegian University of Life Sciences, Ås, Norway.

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