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Blue Mussel Protein Concentrate Versus Prime Fish Meal Protein as a Dietary Attractant for Turbot (*Psetta maxima* L.) Given Rapeseed Protein-based Diets

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Abstract

A feeding experiment was conducted to test the attractant potential of a processed protein concentrate of the blue mussel (BMPC) as replacer for fish meal (FM) protein in rapeseed protein concentrate (RPC)-based diets for turbot. Triplicate fish groups received isonitrogenous and isoenergetic diets with a 50% or 75% replacement of FM protein by a RPC (RPC 50, RPC 75), and further FM protein substitution with 0, 2, 4 or 8% of BMPC. The application of RPC 50/0 provided no significant impact on fish performance compared to FM-reference diet, while daily feed intake (DFI), specific growth rate (SGR) and feed conversion were significantly impaired when fish fed with RPC 75/0 ($P<0.05$). Incorporation of BMPC failed to significantly improve DFI, SGR and FCR in RPC 50 and RPC 75 treatments ($P>0.05$). Protein efficiency ratio and protein productive values remained unaffected among all treatments ($P>0.05$). Excepting crude ash content, no changes in crude protein, crude lipid, dry matter and energy content were obtained. Hepatosomatic index tended to increased ($P>0.05$) in accordance with slightly hypertrophic hepatocytes of fish fed diets with a BMPC incorporation of 40-80 g kg^{-1}. Neither inflammatory nor degenerative alterations were detected in the intestine. In summary, we demonstrated that BMPC failed to stimulate the feed intake of turbot when dietary FM protein was substituted consecutively, but it was found to maintain performance level of turbot within the test diet groups. This indicates nutritional properties of BMPC comparable to prime FM protein, functional to further reduce the FM protein content in aquafeeds for carnivorous fish.

Keywords: Stimulant; Palatability; Feed intake; Specific growth rate; Nutrient utilization; *Mytilus edulis*

Introduction

Global supply of fishmeal (FM) and fish oil is unlikely to increase beyond current levels due to limited natural resources. In addition, the increasing concern of economic and ecological sustainability are creating a paramount pressure on the aquaculture industry to reduce levels of FM and fish oil in aqua feeds [1] and to seek for alternative sources of protein. It is already apparent that plant protein sources play an important role, especially in the efforts to move the formulation of fish feed "down the food chain". However, the consecutive replacement of FM by plant proteins is still challenging in nutrition of carnivorous fish, such as turbot (*Psetta maxima*), common sole (*Solea solea*) or Atlantic halibut (*Hippoglossus hippoglossus*). Complete FM replacement by plant proteins has been achieved in different species [2-5], but failed to keep fish performance unaffected [6-10]. Diet formulations with increasing levels of plant proteins such as rapeseed meal resulted in significant alterations in digestible nutrient content and levels of anti-nutritional factors (ANFs) [11].

Commercially, these in turn may affect palatability, feed intake and nutrient utilization as well as growth of carnivorous fish, [1,11,12]. Purified rapeseed products are generally characterised by high protein content and a balanced amino acid profile [12]. Furthermore, technical processing have been found to reduce the content of substantial ANFs, inluding glucosinolates, phytic acid, sinapinic acid and tannins. Purification protocols strengthen their application as feed ingredient for some species [4,5], while maintaining restrictions for other feeds and species [9,13-16]. Juvenile turbot showed reduced feed intake and growth performance when FM protein was replaced by 30% to 75% (corresponding to <300 g FM kg^{-1} diet) with rapeseed products [10,17-19], suggesting rapeseed-derived palatability and digestibility impairments of the diets.

A plurality of studies have attempted to stimulate feed intake and improve palatability of aquafeeds using attractants of various origins including chemical mixtures, natural compounds and tissue extracts [20-25]. Among the resources of marine origin, blue mussels (*Mytilus edulis*) are indicted by sufficient availability. Their farming is relatively simple and has expanded in several regions [26]. Since mussels smaller than 5 cm are of minor interest for human nutrition they are sorted out of harvested biomass [27]. Huge production volumes, high protein content and an amino acid pattern similar to fish meal have predestined blue mussels (or derived meals/extracts) for aquafeeds [27,28]. The amino acids glycine and alanine as well as the non-amino acid betaine have been identified as gustatory stimulants and have been detected in high amounts in muscle tissue extracts [27,29]. Mussel meat (either freshly or as freeze-dried meal) has been successfully applied as protein source in diets for rainbow trout (*Oncorhynchus mykiss*) [27,30],

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Variables	FM	RPC	BMPC
Nutrient composition			
Dry matter (g kg^{-1})	934	944	893
Crude protein (g kg^{-1}DM)	706	691	728
Crude fat (g kg^{-1}DM)	122	16	30
NfE + crude fibre (g kg^{-1}DM)	0	195	141
Crude ash (g kg^{-1} DM)	172	98	101
Gross energy (MJ kg^{-1} DM)	21.2	20.6	21.3
Essential amino acids (g 100g^{-1} CP)			
Arginine	6.6	7.6	7.2
Histidine	2.2	3.0	2.1
Isoleucine	4.1	4.4	4.2
Leucine	6.8	8.0	6.7
Lysine	6.8	5.8	7.0
Methionine + Cystine	3.5	4.3	3.2
Phenylalanine	3.7	4.5	3.7
Threonine	4.1	4.4	4.8
Valine	4.8	5.3	6.7
Anti-nutritional factors			
Glucosinolates (µmol g^{-1})	-	< 0.1	-
Sinapinic acid and sinapinic acid ester (g kg^{-1})	-	0.024	-
Phytic acid (g kg^{-1})	-	22.6	-
Tannins (g 100 g^{-1})	-	< 0.1	-
Mustard seed oil (g kg^{-1})	-	< 0.1	

Table 1: Nutrient composition, amino acid profile and antinutritional factors (glucosinolates, sinapinic acid, phytic acid, tannins and mustard seed oil) of fish meal (FM), rapeseed protein concentrate (RPC) and blue mussel protein concentrate (BMPC).

red sea bream (*Pagrus major*) [31], Japanese flounder (*Paralichthys olivaceus*) [32,33] and common sole (*Solea solea*) [20,34]. Furthermore, mussel meat or solvent extracts have been found to be efficient feed attractants in diets for common sole [29] or Japanese flounder [35,36] and tiger puffer (*Takifugu rubripes*) [28,33]. For turbot, it was demonstrated recently, that low inclusion levels of crude blue mussel meal (substituted with wheat gluten) were found to diminish rapeseed-derived palatability impairments by increasing daily feed intake significantly [37]. However, studies utilizing technologically processed blue mussel protein concentrate (BMPC) as attractant in alternative diets have been hardly investigated. Therefore, the aim of the present study was to evaluate the attractant potential of a BMPC substituting high quality FM protein in a rapeseed protein concentrate-based diet for juvenile turbot.

Materials and Methods

The experiments were conducted following the Animal Welfare Legislation (§ 8 Section 1) of the Ministry of Energy, Agriculture, the Environment and Rural Areas Schleswig-Holstein (Germany).

Ingredients and experimental diets

Nutrient composition, amino acid profiles and ANF's of dietary ingredients are shown in Table 1. The experimental diets were formulated by replacing FM protein in standard control feed to 50% (RPC 50) and to 75% (RPC 75) with rapeseed protein concentrate (RPC). Remaining FM protein of RPC 50 and RPC 75 diets were further substituted by BMPC (20-80 g kg^{-1}). Accordingly, diets were designated as RD, RPC 50/0, RPC 50/2, RPC 50/4, RPC 50/8, RPC 75/0, RPC 75/2, RPC 75/4, RPC 75/8, (Table 2). BMPC was processed as by-product of CO_2 extraction of mussel oil by Flavex Naturextrakte GmbH (Rehlingen, Germany). Nutritional demands of turbot are fulfilled using diets formulated according to amino acid requirements [38,39]. The diets were pressed at a temperature of 60°C to pellets of 4 mm in diameter (L 14-175, Amandus Kahl, Reinbek, Germany) and were isonitrogenous (55.6 ± 0.85% CP kg^{-1} DM) and isoenergetic (22.51 ± 0.33 MJ kg^{-1} DM) in their composition.

Experimental setup

The feeding trial was conducted at the experimental facilities of the Gesellschaft für Marine Aquakultur mbH (Büsum, Germany). A total of 496 juvenile turbot (obtained from MAXIMUS A/S, Gudnaesstrandvej 17, 7755 Bedsted Thy, Denmark) were stocked in 27 tanks (175 L each, bottom surface 0.27 m^2) in a recirculation system (3.5 m^3 water volume) equipped with mechanical and biological filters and a disinfection unit (UV-filter). Water exchange was approximately 540 L kg^{-1} feed and photoperiod was maintained at a 12L:12D-cycle. Water parameters were determined per day (salinity: 22.3 ± 1.2 g l^{-1} HI 96822 Seawater Refractometer, Hanna Instruments Inc., Woonsocket-RI-USA; temperature: 18.7 ± 0.4°C; dissolved oxygen: 7.8 ± 0.3 mg l^{-1}, Handy Polaris, Oxy Guard International A/S, Birkerod, Denmark; NH^{4+}: 0.2 ± 0.1 mg l^{-1}, NO^{2-}: 0.7 ± 0.3 mg l^{-1}, Microquant test kit, Merck KGaA, Darmstadt, Germany; pH-value: 7.7 ± 0.2, GMH 3530, Greisinger electronic GmbH, Regenstauf, Germany). For a 14 d acclimatization period, fish were fed with control diet once a day by hand until apparent satiation. After this period of acclimatization, fish were starved for 2 days and initial weight was determined individually (average initial body weight was 30.4 ± 0.2 g; stocking density: 1.68 kg m^2). A total of 10 fish were sacrificed and stored at -20°C for analysis of initial whole body composition. The remaining 486 fish were randomly restocked in triplicates to the tanks and fed twice a day by hand until apparent satiation over a period of 56 days. Unfed pellets were collected and re-counted for determination of daily feed intake.

Sampling

At the beginning and end of the experimental trial, biomass of each tank as well as individual weight and length were recorded. Condition factor (CF) and hepatosomatic index (HSI) were calculated. Daily feed intake (DFI, % BW day^{-1}) was recorded to evaluate the potential of

Variables	RD	RPC 50/0	RPC 50/2	RPC 50/4	RPC 50/8	RPC 75/0	RPC 75/2	RPC 75/4	RPC 75/8
Ingredients (g kg^{-1}diet)									
Herring meal [1]	340	170	149	128	87	85	65	44	0
RPC [2]	0	171	171	171	171	256	256	256	256
Soyprotein concentrate [3]	150	150	150	150	150	150	150	150	150
Wheat starch [4]	128	108	107	106	103	99	97	96	95
Wheat gluten [4]	120	120	120	120	120	120	120	120	120
Corn gluten [3]	50	50	50	50	50	50	50	50	50
Blood meal [5]	60	60	60	60	60	60	60	60	60
Mussel protein [6]	0	0	20	40	80	0	20	40	80
Shrimp meal [1]	40	40	40	40	40	40	40	40	40
Fish oil [1]	62	81	83	85	89	90	92	94	99
Linseed oil [7]	20	20	20	20	20	20	20	20	20
Vitamin/mineral mixture[8]	10	10	10	10	10	10	10	10	10
Ca_2PO_4 [9]	10	10	10	10	10	10	10	10	10
Titianium dioxide[10]	10	10	10	10	10	10	10	10	10
Nutrient composition									
Dry matter (g kg^{-1})	931	936	931	938	933	944	942	939	941
Crude protein (g kg^{-1}DM)	556	552	565	558	569	567	546	543	562
Crude lipid (g kg^{-1}DM)	150	153	159	157	162	157	158	159	169
Crude ash (g kg^{-1}DM)	116	100	98	97	94	92	90	89	86
Calcium (g kg^{-1}DM)	24	19	19	18	15	16	15	14	12
Phosphorus (g kg^{-1}DM)	15	14	14	13	12	13	13	12	11
NfE + crude fibre (g kg^{-1}DM)[11]	178	195	178	188	175	184	206	209	183
Gross energy (MJ kg^{-1} DM)	22.3	22.6	22.7	22.7	22.8	22.7	21.6	22.4	22.8
Essential amino acids (g 100g^{-1} CP)									
Arginine	4.0	4.2	4.2	4.2	4.2	4.3	4.3	4.3	4.3
Histidine	1.9	2.0	2.0	2.0	2.0	2.1	2.1	2.1	2.1
Isoleucine	2.7	2.8	2.8	2.8	2.7	2.8	2.8	2.8	2.8
Leucine	5.7	5.9	5.8	5.8	5.8	5.9	5.9	5.9	5.9
Lysine	3.9	3.8	3.8	3.7	3.7	3.7	3.7	3.7	3.6
Methionine + Cystine	2.3	2.4	2.4	2.4	2.4	2.4	2.5	2.5	2.5
Phenylalanine	3.3	3.4	3.4	3.4	3.4	3.5	3.5	3.5	3.5
Threonine	2.6	2.6	2.6	2.6	2.6	2.6	2.6	2.7	2.7
Valine	3.5	3.6	3.5	3.5	3.5	3.6	3.6	3.6	3.5
Anti-nutritional factors									
Glucosinolates (µmol g^{-1})	0.03	0.07	0.07	0.07	0.07	0.08	0.08	0.08	0.08
Phytic acid (g kg^{-1})	3.54	7.4	7.4	7.4	7.4	9.3	9.3	9.3	9.3
Sinapinic acid and sinapinic acid ester (g kg^{-1})	0.00	4.1	4.1	4.1	4.1	6.15	6.15	6.15	6.15

[1]VFC GmbH, Cuxhaven, Germany, [2]HELM AG, Business Unit Animal Nutrition, Hamburg, Germany, [3]Cargill Deutschland GmbH, Krefeld, Germany, [4]Kröner Stärke GmbH, Ibbenbüren, Germany, [5]Euroduna-Technologies GmbH, Barmstedt, Germany, [6]Frozen blue mussel meat from Royal Frysk Muscheln GmbH, Emmelsbüll-Horsbüll, Germany; freeze-dried meal manufactured by the Costal Research & Management, Kiel, Germany, Processing of blue mussel protein concentrate (by-product of CO_2-oil extraction) by Flavex Naturextrakte GmbH, Rehlingen, Germany, [7]Makana Produktion und Vertrieb GmbH, Offenbach a.d. Queich, Germany, [8]Vitamin and mineral mixture, Vitfoss, Gråsten, Denmark, [9]Lehmann & Voss & Co, Hamburg, Germany, [10]Kronos Titan GmbH, Leverkusen, Germany,[11]Nitrogen-free extracts (NfE, g kg^{-1} DM) = 1000 - (crude protein + crude lipid + crude ash + crude fiber)

Table 2: Ingredients, nutrient composition and dietary amino acid profile of experimental diets (RD, Reference diet; RPC 50 or 75 indicate 50% or 75% fish meal protein replacement with rapeseed protein concentrate; 0-8 indicate inclusion of blue mussel protein concentrate with 0-80 g kg^{-1} diet).

BMPC as feed attractant. Furthermore, for each treatment, mortalities, specific growth rate (SGR, % BW day^{-1}) and feed conversion ratio (FCR) were calculated as average of the triplicates according to formulae given in Table 3. A representative sample of three fish per each tank was taken for analysis of final whole body composition. Protein efficiency ratio (PER) and protein productive value (PPV) were calculated according to formulae given in Table 3.

Dietary and whole fish body nutrient composition

Diets and homogenised fish body samples were analyzed for dry matter (DM), crude ash, crude protein (CP), crude lipid (CL) and gross energy according to EU guideline (EC/152/2009) [40]. DM was determined after drying at 105°C until weight remained constant and ash content after 4 h incineration at 550°C with a combustion oven (P300; Nabertherm, Lilienthal, Germany). CP content (N x 6.25) was analyzed by the Kjeldahl method (InKjel 1225 M, WD 30; Behr, Düsseldorf, Germany), CL content after hydrolysis with hydrochloric acid (for experimental diets but not for whole body composition) followed by a petroleum ether extraction with a Soxleth extraction system (R 106 S; Behr). Gross energy was measured in a bomb calorimeter (C 200; IKA, Staufen, Germany). Sum of nitrogen free extracts (NfE) and crude fibre were calculated on DM basis by 100 – (% CP +% CL +% ash). ANFs have been analyzed as follows: Glucosinolates and phytic acid were determined by the ÖHMI Analytic GmbH (Magdeburg, Germany) in accordance to the methods registered in the Gazette of the EC (1864/90 Nr. L 170/28 and SAA A 006). Mustard seed oil was determined by

Variables			Experimental diet						
	RD	RPC 50/0	RPC 50/2	RPC 50/4	RPC 50/8	RPC 75/0	RPC 75/2	RPC 75/4	RPC 75/8
IBW [1]	30.2 ± 0.1	30.3 ± 0.6	30.6 ± 0.5	30.6 ± 0.3	30.4 ± 0.6	30.0 ± 0.4	30.7 ± 0.3	30.6 ± 0.5	30.5 ± 0.4
FBW [2]	95.6 ± 11.1^{A}	84.6 ± 11.0^{A}	87.2 ± 12.9	91.9 ± 12.1	94.4 ± 11.8	66.0 ± 11.4^{B}	73.1 ± 9.0	71.7 ± 10.2	73.9 ± 11.4
DFI [%BW day^{-1}] [3]	1.65 ± 0.02^{A}	1.51 ± 0.11^{A}	1.49 ± 0.07	1.51 ± 0.09	1.53 ± 0.07	1.25 ± 0.04^{B}	1.32 ± 0.02	1.31 ± 0.05	1.32 ± 0.02
SGR [% BW day^{-1}] [4]	2.15 ± 0.07^{A}	1.92 ± 0.09^{A}	1.95 ± 0.09	2.04 ± 0.14	2.11 ± 0.14	1.49 ± 0.09^{B}	1.63 ± 0.06	1.61 ± 0.06	1.68 ± 0.09
FCR[5]	0.77 ± 0.02^{A}	0.78 ± 0.05^{A}	0.77 ± 0.01	0.74 ± 0.02	0.73 ± 0.01	0.84 ± 0.02^{B}	0.81 ± 0.02	0.82 ± 0.01	0.79 ± 0.03

[1]Initial body weight (g); [2]Final body weight (g); [3]Daily feed intake (% BW day^{-1}); [4]Specific growth rate (% BW day^{-1}) = [ln (FBW) - ln (IBW)]/feeding days × 100; [5] Feed conversion ratio = Feed intake (g DM)/ weight gain (g)

Table 3: Growth performance of juvenile turbot fed experimental diets for 56 days. Values (mean ± S.D.) with different superscript letters indicate significant differences of fish meal protein replacement by rapeseed protein concentrate (comparison of reference diet (RD) with RPC 50/0 and RPC 75/0 diets). No significance differences were detected within the RPC 50 or RPC 75 group when BMPC was included (one-way ANOVA Tukey's HSD post hoc test; P>0.05).

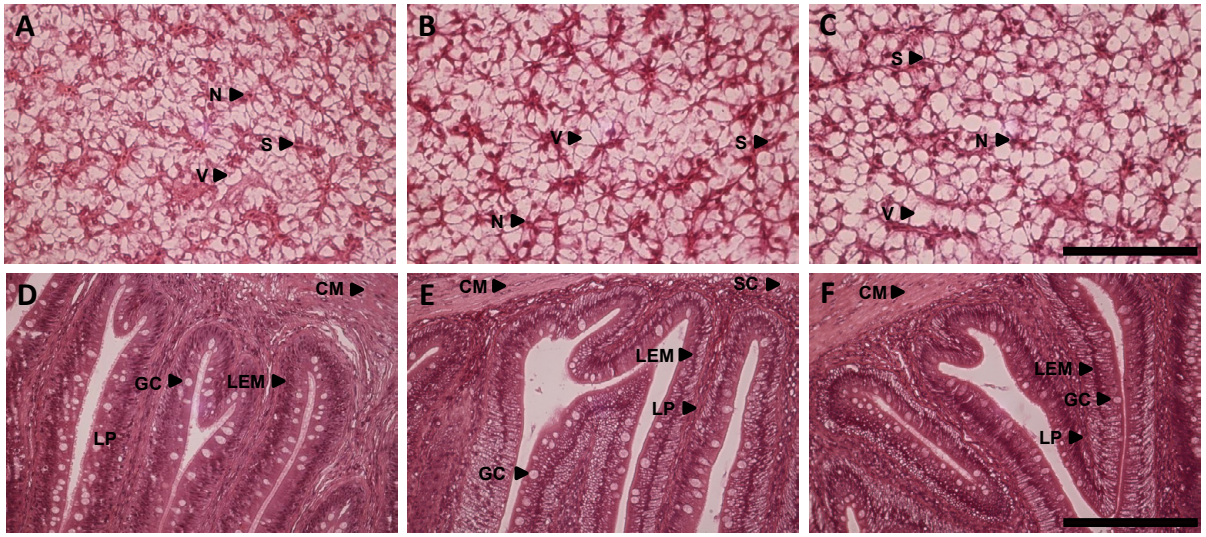

Figure 1: Histological sections (Hematoxylin and eosin staining) of the liver (A-C) and midgut (D-F) of juvenile turbot fed experimental diets. Images are representative for fish fed reference diet (A,D), RPC 50 and RPC 75 diets with 0-20 g kg^{-1} blue mussel protein concentrate (B,E) and RPC 50 and RPC 75 diets with 40-80 g kg^{-1} blue mussel protein concentrate (C,F). Black arrowheads point to S: Sinusoid; V: Vacuole; N: Nucleus; GC: Goblet cell; LEM: Lamina epithelialis mucosae; LP: Lamina propria; SC: Stratum compactum; CM: Tunica muscularis. Scale bar = 100 µm (A-C), 200 µm (D-F).

the LUFA-ITL GmbH (Kiel, Germany; analogue VDLUFA III 16.6.1) and tannin concentration was detected by Phytolab GmbH & Co Kg. (Vestenbergsgreuth, Germany; Ph. Eur. 5.0 2.8.14 v). Sinapinic acid and sinapinic acid ester were determined by HPLC analysis at the A.C.T. FOODs GmbH (Bad Fallingbostel, Germany). Detailed ANF analysis of RPC is shown in Table 1. Dietary concentration of the most prominent ANF phytic acid was then calculated to be 2.0 g kg^{-1} feed (control group) - 13.0 g kg^{-1} feed (RPC 75/0; Table 2).

Histology

Sections of liver and mid gut were randomly sampled from three fish of each tank. After fixation in 4% paraformaldehyde, the samples were dehydrated in a graded ethanol series and embedded in paraffin. Slices were cut at 3 µm and stained with Haematoxylin-eosin. Labelling was visualized by light microscopy using an Olympus CKX 41 microscope (Olympus Deutschland GmbH, Hamburg, Germany) equipped with a Canon EOS 500D digital camera.

Statistical analysis

Statistical analyses were performed using SPSS 17.0 for Windows (SPSS Inc., Chicago, USA). Data were checked for normal distribution by the Kolmogorov Smirnov test. Effects of fish meal replacement by RPC (comparison of reference diet, RPC 50/0 and RPC 75/0) or impact of BMPC application (within the RPC 50 or RPC 75 treatments) on growth performance, nutrient utilization and health parameters were analysed by one-way analysis of variance (ANOVA) followed by Tukey's HSD post hoc-test for homogeneous variances (Levene's test). Significance was attributed to P<0.05.

Results

During the experiment no mortality of fish was recorded. 50% of dietary FM replacement (by RPC, inclusion 171 g kg^{-1}) did not caused significant reduction in performance of juvenile turbot (P>0.05). However, DFI, SGR and FCR were significantly affected by FM substitution of 75% (RPC inclusion 256 g kg^{-1}; P<0.05, Table 3). Incorporation of blue mussle protein concentrate (BMPC) into both, RPC 50 and RPC 75 diets did not enhance DFI or SGR of fish (P>0.05, one-way ANOVA, Table 3). With BMPC incorporation (20-80 g kg^{-1}), FCR showed a slight improvement without reaching statistical significance (P>0.05).

Hepatosomatic Index (HSI) was unaffected by RPC and BMPC incorporation (P>0.05). However, a tendency towards increased liver size was observed for fish fed 40-80 g BMPC kg^{-1} diets. Histopathological investigation of this liver tissue showed slighty hypervacuolated hepatocytes (Figures 1A-1C). For the other treatments a normal vacuolization was observed (Figures 1A and 1B). Among the groups neither inflammatory nor degenerative changes were determined for the intestine (Figures 1D-1F).

	RD	RPC 50/0	RPC 50/2	RPC 50/4	RPC 50/8	RPC 75/0	RPC 75/2	RPC 75/4	RPC 75/8
Proximate body composition (% wet weight)									
Dry matter	24.7 ± 0.9	25.1 ± 0.4	24.7 ± 0.7	25.2 ± 0.6	24.4 ± 2.7	23.9 ± 0.5	23.5 ± 0.9	24.2 ± 0.4	24.5 ± 0.5
Crude protein	15.8 ± 0.4	16.0 ± 0.1	15.8 ± 0.2	15.9 ± 0.2	15.3 ± 1.5	15.9 ± 0.1	15.6 ± 0.7	15.7 ± 0.1	15.7 ± 0.1
Crude lipid	5.5 ± 0.4	5.9 ± 0.3	5.7 ± 0.5	6.1 ± 0.6	6.3 ± 1.1	4.9 ± 0.6	4.9 ± 0.3	5.5 ± 0.3	5.9 ± 0.5
Ash	3.4 ± 0.1	3.3 ± 0.1^{a}	3.2 ± 0.1^{ab}	3.2 ± 0.0^{ab}	2.8 ± 0.2^{b}	3.1 ± 0.1^{a}	3.0 ± 0.1^{ab}	3.0 ± 0.1^{ab}	2.8 ± 0.0^{b}
Gross energy (MJ kg^{-1} DM)	23.3 ± 0.4	23.4 ± 0.2	23.4 ± 1.0	23.4 ± 0.3	24.1 ± 0.1	22.9 ± 0.2	23.2 ± 1.0	23.2 ± 0.3	23.8 ± 0.2
Biometric parameters									
CF [1]	2.23 ± 0.06	2.05 ± 0.11	2.17 ± 0.11	2.21 ± 0.11	2.25 ± 0.13	2.10 ± 0.06	2.14 ± 0.04	2.17 ± 0.07	2.27 ± 0.09
HSI [2]	1.97 ± 0.24	1.94 ± 0.28	2.18 ± 0.18	2.41 ± 0.49	2.44 ± 0.27	2.08 ± 0.27	2.18 ± 0.19	2.41 ± 0.17	2.45 ± 0.44

Table 4: Proximate whole body composition and biometric parameters of turbot fed experimental diets for 56 days. Values (mean ± S.D.) with different superscript letters indicate significant differences within the RPC 50 or the RPC 75 group with 0%, 2%, 4% or 8% BMPC inclusion (one way ANOVA, Tukey's HSD-post hoc test, P<0.05).

Variables	Experimental diet								
	RD	RPC 50/0	RPC 50/2	RPC 50/4	RPC 50/8	RPC 75/0	RPC 75/2	RPC 75/4	RPC 75/8
PER [1]	2.34 ± 0.05	2.31 ± 0.15	2.31 ± 0.02	2.42 ± 0.07	2.42 ± 0.05	2.24 ± 0.06	2.26 ± 0.05	2.26 ± 0.03	2.25 ± 0.08
PPV [2]	38.6 ± 0.7	39.1 ± 2.5	38.43 ± 1.1	40.4 ± 1.2	38.1 ± 5.5	36.0 ± 1.0	37.4 ± 3.3	37.8 ± 0.2	37.6 ± 1.0

[1]Protein efficiency ratio = Body weight gain (g)/protein intake (g); [2]Protein productive value = 100 × [(crude protein final fish (%) × biomass final tank weight (g)) – (crude protein initial fish (%) × biomass initial tank weight (g))]/(crude protein diet (g))

Table 5: Protein efficiency ratio (PER) and protein productive value (PPV) of turbot. No significant differences were detected among the groups (one way ANOVA, Tukey's post hoc test, P>0.05).

Proximate whole body composition of fish at the beginning and at the end of the feeding trial is presented in Table 4. Treatments showed no significant changes for most of the analyzed parameters compared to control with exception of crude ash content, which declined significantly within the RPC 50 and RPC 75 groups (P<0.05). Crude lipid content tended to increase with incorporation of BMPC (P>0.05). The protein efficiency ratio (PER) and protein productive value (PPV) remained unaffected among all treatments (P>0.05), suggesting no impact of diet composition on protein retention (Table 5).

Discussion

Previous findings with turbot showed that DFI and SGR decreased significantly when FM protein was substituted by RPC to levels below 300 g FM kg^{-1} diet [10,19,37]. In the present study, this concentration was further reduced below 170 g FM kg^{-1} feed (RPC 50 diets) expecting a reduced feed intake of turbot to investigate the potential of BMPC as feed stimulant. However, RPC 50 diets did not affect neither DFI nor FCR significantly; fish maintained on a good performance level compared with turbot of same size and origin at previous trials [10,37], which emphasises the nutritional quality of test diets, but restricted the validity of the RPC 50/0 diets to evaluate the full potential of BMPC as feed stimulant. However, FM substitution towards ≤85 g FM kg^{-1} feed (RPC 75 diets) triggered a significant decline in the general performance including DFI of juvenile turbot. Hence, it was suitable to utilize these diets to evaluate the BMPC-stimulating effect on turbot.

Under natural conditions, juvenile turbot of comparable size as in this study forage mainly on molluscs and annelids [41], which contain higher amounts of feeding stimulants [42,43]. The utilization of blue mussels either fresh, as freeze-dried meal or extracts in diets of several species provided conflicting results [27,28,32,34-37]. For example, the application of mussel meal failed to increase DFI in experimental trials with Japanese flounder [28,32,33], juvenile barramundi (*Lates calcarifer*) [44] or rainbow trout [8]. On the contrary, Kikuchi [35] and Nagel et al. [37] have observed increased feed intake of flounder and turbot when fed with mussel meal-supplemented diets, suggesting its potential use to alleviate plant protein-derived palatability impairments. In protein-extracts of the blue mussel glycine and alanine together with betaine and/or taurine have been identified to improve feeding behavior of common sole [29] and Japanese flounder [36]. Chemical analogue confirmed the potential of mussel amino acids acting as feed attractants [29,43]. In the present investigation, BMPC (with a comparable amino acid profile to FM protein) was utilized to further replace FM protein totally in the RPC 75/8 treatment. In contrast to increased DFI of turbot fed diets where wheat gluten [37] or soybean meal [35] were replaced by mussel meal, no DFI improvements were detected when turbot fed diets of BMPC-mediated FM protein reduction in this study. The latter has been confirmed by Kikuchi and Sakguchi [32], who utilized mussel meal as total FM replacer. This indicates that BMPC is comparable with high quality FM protein in term of palatability and feed acceptance. Furthermore, SGR showed comparable values and feed conversion tend to be improved for fish fed with BMPC supplemented diets. This FCR-trend is in accordance with findings by Anagnostidis et al. [45], who suggested a better nutrient utilization of mussel protein compared to FM protein. Although our PPVs (36.0% to 40.4%) are in line [6] or slightly above recently reported protein retention levels for juvenile turbot [46], indicating an efficient protein utilization of turbot [47], these values do not support the BMPC-driven trend in the FCR values for RPC 75 diets. Experimental diets were balanced in terms of crude protein and lipid content, which could not exclude varying values on the digestibility level. This issue could influence feed intake, protein digestion and absorption as well as intestinal evacuation rates [48].

In turbot, feed intake and growth performance were in accordance with unaffected CF and HSI. In contrast, investigations [21,49] showed decreased CF (but unaffected HSI) when FM protein was progressively substituted with soybean protein concentrate. Considering the HSI, Regost et al. [6] and Fournier et al. [50] had determined a significant decline in HSI when turbot have been fed with diets of high plant protein incorporation level. This might have been affected by reduced protein and energy intake and less efficient conversion and retention needed for growth [46]. Consequently, the lack of ingested energy could have been balanced by mobilization of liver-stored energy.

Histopathological investigations of gut and liver are rarely correlated with the dietary utilization of animal proteins. Recently, it has been reported that either a 30% incorporation of blood and feather meal or a 60% FM protein substitution with an animal protein blend (a mixture of poultry, meat, bone, blood and hydrolyzed feather meal)

induced hepatic steatosis in Japanese seabass *Lateolabrax japonicus* [51,52], which was indicated by an increased HSI and fat accumulation in the liver. Whether fat accumulation or increased density of intracytoplasmatic lipidic droplets [53] is caused by a deficiency of lipitrophic factors or elevated lipogenic enzymes was beyond the scope of the present study. However, liver of fish fed diets with 40-80 g BMPC kg^{-1} diet showed slight hypervacuolated hepatocytes. This might be an early indication for incipient hepatic steatosis [51,52].

Conclusion

BMPC failed to stimulate the feed intake of turbot when FM protein was substituted in already RPC-based diets. However, the maintenance of performance within the test diet treatments revealed a nutritional value comparable to prime FM protein. It indicates BMPC as ingredient with the potential to further reduce the FM protein content in aquafeeds of carnivorous fish.

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