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Effect of fish meal replacement by plant protein blend on amino acid concentration, transportation and metabolism in juvenile turbot (*Scophthalmus maximus* L.)

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Abstract

The objective of the study was to evaluate the effects of incorporating plant protein blend in juvenile turbot (*Scophthalmus maximus* L.) diet on free amino acid (AA) concentration and the expression of genes related to peptide and AA transporters, key enzymes of AA metabolism and AA response (AAR) pathway. Fish were fed diets with fish meal (FM), or 40% FM replacement by plant protein blend for 9 weeks. Compared with the FM diet, PP40 diet did not affect plasma essential amino acid (EAA) concentration or AA metabolic enzymes gene in intestine, while it significantly upregulated all the detected peptide and neutral AA transporters gene. Results in muscle indicated that PP40 diet led to a great reduction of EAA concentrations and mRNA abundance of two kinds of AA transporters (SNAT2 and $b^{0,+}$AT), while it greatly increased the gene expression of L-type and T-type AA transporters (LAT2 and TAT1) and the enzymes of AA catabolism (BCKDH-E2) and anabolism (asparagine synthetase). In addition, the expression of genes related to AAR pathway was all greatly stimulated by PP40 diet in muscle. Our results provide a molecular explanation for the change of tissues' AA concentrations caused by plant protein in turbot, which maybe applicable for general carnivorous fish.

KEYWORDS

amino acid, amino acid response pathway, fish meal, metabolism, plan protein, transporter

1 | INTRODUCTION

In the last few decades, limited fish meal (FM) availability, together with increased costs, has encouraged the successful use of some protein sources in aquafeeds (Hardy, 2010; Naylor et al., 2009). However, probably due to the inferior protein quality and quantity, replacement of FM with massive plant proteins led to many problems, especially for carnivorous species (Drew, Borgeson, & Thiessen, 2007; Hardy, 2010). Reports indicated that FM replaced by terrestrial plant sources could affect tissue free amino acid (AA) homoeostasis, which is closely related to growth performance (Regost, Arzel, & Kaushik, 1999; Wu et al., 2014; Xu et al., 2016b). However, the regulation of AA availability in different tissues is still not clear in fish.

Recent findings highlight that AA transporters, as a selective barrier to AAs, have an integral role in responding to changes in both intracellular and extracellular AAs providing a crucial link between the availability of AAs and protein anabolism (Dickinson & Rasmussen, 2013; Taylor, 2014). After the digestion of dietary proteins in the intestine, released oligopeptides and free AAs are absorbed by enterocytes and subsequently utilized by the other organs (Frühbeck, 1998; Pennings et al., 2012). Di- and tripeptides are largely absorbed by peptide transporter 1 (PepT1) into the gut mucosa in terrestrial animals (Gilbert, Wong, & Webb, 2008) and teleost (Verri, Terova, Dabrowski, & Saroglia, 2011). AAs uptake and transport requires multiple AA transporters, the binding sites of them generally recognize a range of structurally similar AAs as substrate for transport (Bröer, 2008). Although it is well demonstrated that PepT1 was regulated by dietary protein sources in fish (Bakke et al., 2010; Ostaszewska et al., 2010), little information is available for AA transporters. Furthermore, transport of a specific AA may depend on the integrated activity of

several parallel functioning AA transporters (Bröer, 2008; Poncet & Taylor, 2013). In addition, the expression of AA transporters shows tissue specific in fish (Rimoldi et al., 2015; Yang et al., 2014). Thus, we hypothesized that the majority of AA transporters at the plasma membrane of muscle cells might be broadly distinct from those in epithelial cells of intestine in teleost fish.

Amino acid transporters have a role in the delivery of AAs to their intracellular sensors. An increase of intracellular AAs regulates protein translation largely through the stimulation of the target of rapamycin (TOR) signalling pathway, a master regulator for cellular anabolism and cell growth (Dai et al., 2013; Jewell, Russell, & Guan, 2013; Librán-Pérez et al., 2015). Gene-silencing or inhibitor studies of AA transporters demonstrate that the reduced activation of TOR pathway is mainly due to the lack of AA uptake (Bröer et al., 2011; Nicklin et al., 2009). In contrast, a lack of intracellular AAs is sensed by an AA response (AAR) signalling pathway, leading to a reduction of general protein synthesis but an increase of specific gene expression like activating transcription factor (ATF4; Kilberg, Shan, & Su, 2009). Yet, the relationship between AA availability and the above regulation mechanisms still requires clarification in fish.

Our previous study (Wang, He, Mai, Xu, & Zhou, 2015) indicated that the growth performance and the activation of TOR pathway were downregulated after 40% FM replacement by plant protein blend in turbot (*Scophthalmus maximus* L.). However, their correlation with tissue AA concentrations, transporter response, and AA metabolism was still not clear. We hypothesized that high level of dietary plant protein blend replacement dietary FM would likely change the tissue AA concentrations, the expression of AA transporters genes and AA metabolic status. In this study, turbot was chosen to examine its free AA concentration, the expression of genes related to AA transporters, key enzymes of AA metabolism and AAR pathway after ingestion of FM-based diet, or plant protein blend-incorporated diet.

2 | MATERIALS AND METHODS

2.1 | Experimental diets and fish

Two isonitrogenous and isoenergetic diets were designed based on a reference diet containing 63% FM and a plant protein blend-incorporated diet formulated as 40% FM replacement by a mixture of plant protein sources (soybean meal, corn gluten meal, wheat gluten meal and peanut meal) balanced with essential amino acids (EAA) to match the AA requirements of juvenile turbot (PP40; Kaushik, 1998).

Their AA profiles are shown in Table 2. Ingredients and compositions of experimental diets were described as before (Wang et al., 2015) and are shown in Table 1. Phytase was used to reduce the phytate in plant proteins. Additional mineral compounds were used to abolish the chelation by antinutritional factors.

All experimental protocols were approved by the Animal Care Committee of Ocean University of China. Juvenile turbot were obtained from a fish-rearing farm (Laizhou, China). Fish were acclimated to the system and fed with a commercial diet (Great Seven Biotech, Qingdao, China) for 2 weeks before the trial. To start the experiment, juvenile turbot (4.90 ± 0.03 g) were randomly distributed into six 400-L fibreglass tanks in a circulating water system. Three replicates tanks were randomly assigned to each diet, and 30 initial fish were weighed and stocked in each tank. Fish were fed twice per day (07:00 and 19:00) till apparent satiation for 9 weeks. Feeding situation and management were identical to a previous study (Wang et al., 2015).

TABLE 1 Formulations of the experimental diets (g/kg dry matter)

Ingredient	FM	PP40
Fish meal	630	378
Whole wheat meal	223	103.4
Soybean meal	0	96
Corn gluten meal	0	96
Wheat gluten	0	84
Peanut meal	0	48
Fish oil	52	62
Soybean lecithin	25	25
Vitamin premix[a]	10	10
Mineral premix[b]	20	20
Amino acid mixture[c]	0	10.8
Taurine	0	10
$Ca(H_2PO_4)_2$	0	4
Sodium alginate	0	10
Attractanct[d]	10	10
Phytase	0	2
$FeSO_4{\cdot}H_2O$	0	0.5
$ZnSO_4{\cdot}H_2O$	0	0.3
Others[e]	30	30
Analytical composition		
Crude protein (g/kg)	502.6	519.5
Crude fat (g/kg)	125.7	120.5
Ash (g/kg)	124.4	121.3
Total energy (kJ/g)	175	178.2

FM, fish meal diet; PP40, 40% fish meal replacement by plant protein blend diet.

[a]Vitamin premix (mg/kg diet): retinyl acetate, 32; cholecalciferol, 5; α-tocopheryl acetate, 240; menadione sodium bisulphite, 10; ascorbic acid, 120; cyanocobalamin, 10; biotin, 60; choline dihydrogen citrate, 7 000; folic acid, 20; inositol, 800; niacin, 200; D-calcium pantothenate, 60; pyridoxine HCl, 20; riboflavin, 45; thiamine HCl, 25; microcrystalline cellulose, 16,473.

[b]Mineral premix (mg/kg diet): $MgSO_4{\cdot}7H_2O$, 1,200; $CuSO_4{\cdot}5H_2O$, 10; $FeSO_4{\cdot}7H_2O$, 80; $ZnSO4{\cdot}H_2O$, 50; $MnSO_4{\cdot}H_2O$, 45; $CoCl_2$, 5; Na_2SeO_3, 20; calcium iodine, 60; zeolite powder, 8,485.

[c]Amino acid mixture (% diet): methionine·HCl, 0.48; histidine, 0.17; L-threonine 0.3; lysine·HCl, 0.13.

[d]Attract (g/kg diet): betaine, 4; DMPT, 2; threonine, 2; glycine, 1; inosine-5′-diphosphate trisodium salt, 1.

[e]Others (g/kg diet): beer yeast, 25; choline chloride, 2.5; calcium propionate, 1; ethoxy quinoline, 0.5; Y_2O_3, 1.

2.2 | Sample collection

At the end of the feeding trial, fish were fasted for 24 hr. Then, four fish from each tank were anesthetized with MS222. Blood was

TABLE 2 Amino acid composition of the experimental diets (g/kg dry matter)

Amino acid	FM	PP40
Essential amino acids		
Threonine	20.5	21.0
Isoleucine	18.7	18.2
Leucine	34.9	39.4
Valine	21.3	21.1
Methionine	12.8	13.0
Phenylalanine	18.3	20.8
Lysine	34.4	34.2
Histidine	9.9	10.4
Arginine	29.3	29.3
TEAA	200.1	207.4
Non-essential amino acid		
Tyrosine	14.6	15.5
Serine	22.3	22.8
Glycine	31.1	25.7
Alanine	28.1	26.5
Proline	23.6	31.0
Aspartic acid	43.0	39.4
Glutamic acid	71.4	89.3
Cysteine	9.0	10.4
TNEAA	243.1	260.6
EAA/NEAA	0.82	0.80

TEAA, total essential amino acids; TNEAA, total non-essential amino acids.

removed from the caudal vein into heparinized tubes and centrifuged at 4,000 *g* for 10 min; the recovered plasma was immediately frozen and kept at −20°C. Six fish (two fish per tank) were randomly taken from every treatment ($n = 6$). Fish were anaesthetized and killed by cervical section. Intestine (remove intestinal contents) and dorsolateral muscle were dissected and immediately frozen in liquid N_2 and then stored at −80°C for further analysis.

2.3 | Analysis of free amino acids in plasma and muscle

Free AA in the plasma and muscle were detected as previously described (Xu et al., 2016b) and analysed by an ion-exchange AA analyzer (L8900; Hitachi, Tokyo, Japan). Briefly, 500 μl of plasma sample and 1.5 ml of 10% sulfosalicylic acid solution were mixed thoroughly and centrifuged at 13,000 rpm for 15 min; the supernatant fluid was collected and filtered for AA analysis. Similarly, 500 mg of dorsolateral muscle was homogenized in 1.5 ml 10% sulfosalicylic acid solution. After centrifuging at 13,000 rpm for 15 min, the supernatant fluid was collected and filtered for AA analysis.

2.4 | RNA isolation and quantitative real-time PCR

Total RNA was extracted from frozen intestine or muscle using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Quantification and RNA quality assessment were performed on a NanoDrop 2000 Spectrophotometer (Thermo, Waltham, MA, USA). The integrity of the RNA was confirmed with 1.2% denaturing agarose gel electrophoresis and stained with ethidium bromide. Subsequently, one microgram of the resulting total RNA was reverse-transcribed into cDNA using a PrimeScript® RT reagent Kit With gDNA Eraser (Perfect Real Time; TAKALA, Shiga, Japan). Primer sequences of target genes were as described before (Xu et al., 2016a,b) and are listed in Table 3. Quantitative PCR analyses were carried out using a real-time PCR kit with an SYBR Green fluorophore (TAKALA) according to the manufacturer's instruction. Quantitative RT-PCR analyses were concentrated on gene expression of peptide and AA transporters, which were peptide transporters 1 and 2 (PepT1 and PepT2) mediating the absorption of all di- and tripeptides (Gilbert et al., 2008); the neutral AA transporters including B^0-type AA transporter 1 (B^0AT1), L-type AA transporter 2 (LAT2), system ASC AA transporter 2 (ASCT2), sodium-coupled neutral AA transporter 2 (SNAT2), T-type AA transporter 1 (TAT1) and proton-coupled AA transporter 1 (PAT1); and the cationic AA transporters including $b^{0,+}$-type AA transporter ($b^{0,+}AT$), system y^+L AA transporters 1 and 2 (y^+LAT1 and y^+LAT2) and cationic AA transporter 2 (CAT2; Bröer, 2008; Fotiadis, Kanai, & Palacín, 2013). Moreover, we examined the mRNA abundance of key enzymes of AA metabolism, which were shown as follows: branch-chain α-keto acid dehydrogenase E2 subunit (BCKDH-E2) and serine dehydratase (SD) for AA catabolism and asparagine synthetase (ASNS) for AA anabolism. Thirdly, ATF4 and its possible upstream and downstream genes were also involved in this study, which were general control non-derepressible 2 (GCN2) activated by cellular AA depletion or deprivation and ATF4-dependent genes including ATF3, eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) and regulated in development and DNA damage responses 1 (REDD1; Kilberg et al., 2009). Results were normalized to reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for gene quantitation. No expression change of GAPDH gene was observed between two treatments (data were not shown). The quantity of target mRNAs were calculated by the comparative cycle threshold (C_t) values method expressed as $2^{-(\Delta\Delta C_t)}$.

2.5 | Statistical analyses

Results were expressed as means ± *SEM*. SPSS 16.0 was used for all statistical analysis. Comparison of data from two treatments was performed by independent samples *t* tests. Other data were subjected to one-way analysis of variance (ANOVA) followed by Tukey's multiple range test. The level of significance was chosen at $p < .05$.

3 | RESULTS

3.1 | Free amino acid concentrations in plasma

Data on plasma free AA concentrations are shown in Table 4. Compared with the FM diet, PP40 diet resulted in the great reduction of glycine concentration ($p < .05$). The concentrations of the other individual AAs, total EAA, total non-essential AA (NEAA) and total free AA were not affected by the two diets after 9 weeks of feeding trial ($p > .05$).

TABLE 3 Primer sequences used for real-time quantitative PCR

Gene	Forward prime	Reverse primer	Product size, bp
Peptide and amino acid transporters			
PepT1	GCATCCACACCCAGCAGAAG	GTCCTCAGCCCAGTCCATCC	232
PepT2	CAAGGAGAATGTCAGCGAGAGG	AACAACCAGAGCCACGACCAT	184
B^0AT1	AGACTCTCAACACCTCCGAAGC	AGCCTTTCCTGTGGTCTCAATCC	131
LAT2	TGCCTCGTGCCATCTTCATC	GCTCCAGCAAAGAACAATCTCG	241
SNAT2	TGCTGCTGGTGACGCTCTTC	CAGGTGTCCTCGCTGTAGTCC	249
ASCT2	ACCTTGATCGCCTCGTCCATC	CATCTGTGCCGTTCCTTGTAACC	232
TAT1	TCTCCCATCGTCAGCGTCTTC	CTGCCAGCCGTCACAATGC	245
PAT1	TCAGTGACAACATCAAGCAGGTG	GAAGGCGGGCAGGAAGAAGAG	140
$b^{0,+}AT$	GGGCTTTGGGCTTATGATGGATG	TGGAGACAACAGCAGTTCAGTGG	180
y^+LAT1	TGTGACGTTTGCGGACCAG	GACGGGAGTGTAGCGGAAGAC	186
y^+LAT2	TGCCCATCGTCACCATCAT	AGCACGACAAAGCCACAGC	162
CAT2	TGCTGCTGTTCGTGACCATCTC	AGGTTCCAGAAATGCCATAAGGG	174
Amino acid response pathway-related genes			
GCN2	ACAGACGGCGATCAACCTC	CCTAAACAGCCTCCATAACC	135
ATF4	GGTTCGTATTCTCCCGTATTG	GACTTGACTTTGACACTGGTGG	220
ATF3	TCGCCATCCAGACCAAGC	ACTCCTTCTGCAAATCCTCC	234
4E-BP1	CCGCAAGTTCCTACTGGAC	AGGCTTGCCATCGTGGTTGT	154
REDD1	TGGAGCACATCGGACAGGAG	GACGAGGTAGGGGTCCACAG	140
Key enzymes of amino acid metabolism			
BCKDH-E2	CTGCTCAGCGTGTTGGATG	GAGGGAGAATCACTGGTTTGG	157
SD	CCCGCAATTACCAGTGTTG	GCTTTCACGGCCTCTTGGTC	122
ASNS	TCACATTGGCAGCGAACACC	CATCGGAACCCTCACCAGAG	194
Reference gene			
GAPDH	AGCAGCAGCCATGTCAGACC	TTGGGAGACCTCACCGTTGTAAC	193

Abbreviations and GenBank Accession Nos.: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), DQ848904.1. Partial sequences of target genes in turbot were obtained through a degenerate PCR strategy in this study, including peptide transporters 1 and 2 (PepT1 and PepT2); B^0AT1, B^0-type amino acid transporter 1; LAT2, L-type amino acid transporter 2; SNAT2, sodium-coupled neutral amino acid transporter 2; ASCT2, system ASC amino acid transporter 2; TAT1, T-type amino acid transporter 1; PAT1, proton-coupled amino acid transporter 1; $b^{0,+}AT$, $b^{0,+}$-type amino acid transporter; y^+LAT1 and y^+LAT1, system y^+L amino acid transporter 1 and 2; CAT2, cationic amino acid transporter 2; GCN2, general control non-derepressible 2; ATF3 and ATF4, activating transcription factor 3 and 4; 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; REDD1, regulated in development and DNA damage responses 1; BCKDH-E2, branch-chain α-keto acid dehydrogenase E2 subunit; SD, serine dehydratase; ASNS, asparagine synthetase.

3.2 | Free amino acid concentrations in muscle

As shown in Table 5, incorporated plant protein blend in turbot diet significantly decreased the concentrations of several individual EAA including isoleucine, arginine and lysine ($p < .05$), while the levels of leucine, valine, methionine, threonine, histidine, tryptophan and phenylalanine were not different ($p > .05$) in dorsolateral muscle. Moreover, PP40 diet reduced the concentration of total EAA 16.57% below that of FM diet ($p < .05$). Individual NEAA including glycine, proline and tyrosine ($p < .05$) were significant lower than that in the FM diet ($p < .05$). Finally, the total free AA concentration was reduced by 23.89% in PP40 diet compared with the FM diet in turbot muscle ($p < .05$).

3.3 | Expression pattern of peptide and amino acid transporters in intestine and muscle

Expression pattern of variety of peptide and AA transporters in intestine is shown in Fig. 1a. PepT1 expressed at a high level in intestinal tract. Its mRNA level was approximately four times higher than that of neutral AA transporter B^0AT1 and 12 times higher than that of cationic AA transporter CAT2 ($p < .05$). Among twelve kinds of peptide and AA transporters, mRNA abundance of these genes were summarized as PepT1 > B^0AT1 > CAT2 > LAT2 > y^+LAT1 > $b^{0,+}AT$ > TAT 1 > PAT1 > PepT2 > SNAT2 > y^+LAT2 > ASCT2.

Expression pattern of variety of peptide and AA transporters in muscle was shown in Fig. 1b. Gene expression of PepT1 was not detected in turbot muscle. LAT2 expressed at a high level in muscle. Its mRNA level was approximately two times higher than that of cationic AA transporter $b^{0,+}AT$ ($p < .05$). Among eleven kinds of peptide and AA transporters, mRNA abundance of these genes were summarized as LAT2 > $b^{0,+}AT$ > TAT1 > B^0AT1 > SNAT2 > y^+LAT1 > ASCT2 > PepT2 > PAT1 > CAT2 > y^+LAT2.

3.4 | Relative expression of peptide and amino acid transporters in intestine between two diets

The data for relative expression of peptide and AA transporters genes are shown in Fig. 2. Compared with the FM diet, PP40 diet significantly upregulated gene expression of all detected intestinal peptide and neutral AA transporters including PepT1, PepT2, B^0AT1, LAT2, SNAT2, ASCT2, TAT1 and PAT1 ($p < .05$). As for intestinal cationic

TABLE 4 Free amino acid concentrations in plasma of juvenile turbot fed different diets for 24 hr, μg/mL

Amino acid	FM	PP40	*p* Values
Essential amino acid (EAA)			
Isoleucine	53.57 ± 7.58	62.67 ± 5.79	.39
Leucine	127.58 ± 16.65	118.33 ± 8.36	.65
Valine	124.56 ± 6.56	148.55 ± 9.20	.10
Methionine	66.56 ± 2.62	66.87 ± 4.04	.95
Threonine	149.95 ± 8.44	149.50 ± 3.14	.96
Arginine	78.77 ± 9.16	75.38 ± 3.73	.75
Lysine	164.12 ± 15.21	160.61 ± 4.21	.83
Histidine	41.92 ± 1.84	35.47 ± 2.76	.12
Tryptophan	206.73 ± 25.67	218.55 ± 18.90	.73
Phenylalanine	64.70 ± 7.25	75.68 ± 2.34	.22
Sum of EAA	1078.46 ± 57.13	1111.61 ± 17.65	.61
Non-essential amino acid (NEAA)			
Serine	150.11 ± 10.41	129.49 ± 8.10	.19
Aspartic acid	17.26 ± 0.46	17.73 ± 0.76	.62
Asparagine	14.36 ± 0.85	18.92 ± 1.44	.05
Glutamic acid	50.64 ± 5.65	43.00 ± 4.10	.34
Glycine	215.98 ± 7.06	161.18 ± 11.54*	.02
Alamine	410.80 ± 31.80	406.19 ± 33.43	.93
Proline	54.78 ± 4.55	64.98 ± 8.60	.35
Tyrosine	55.54 ± 7.01	45.39 ± 7.22	.37
Sum of NEAA	969.48 ± 48.96	886.89 ± 31.32	.23
Total AA	2047.93 ± 105.79	1998.50 ± 47.73	.69

FM, fish meal diet; PP40, 40% fish meal replacement by plant protein blend diet. Values are means ± *SEM* (n = 3) and were analysed by independent samples t tests.
*p < .05 different from FM diet.

TABLE 5 Free amino acid concentrations in muscle of juvenile turbot fed different diets for 24 hr, μg/g wet weight

Amino acid	FM	PP40	*p* Values
Essential amino acid (EAA)			
Isoleucine	21.40 ± 1.89	9.43 ± 1.00**	.005
Leucine	12.17 ± 1.26	14.27 ± 2.57	.50
Valine	20.88 ± 2.38	19.79 ± 1.68	.73
Methionine	4.28 ± 0.91	7.87 ± 0.81	.05
Threonine	43.21 ± 4.49	51.43 ± 2.23	.18
Arginine	53.50 ± 7.46	22.58 ± 2.67*	.02
Lysine	97.23 ± 5.15	42.86 ± 2.86**	.001
Histidine	8.94 ± 0.33	8.96 ± 0.07	.94
Tryptophan	415.53 ± 9.59	380.74 ± 31.95	.36
Phenylalanine	41.35 ± 4.03	41.29 ± 0.61	.99
Sum of EAA	718.48 ± 3.08	599.22 ± 32.67*	.02
Non-essential amino acid (NEAA)			
Serine	93.35 ± 6.78	102.86 ± 5.63	.34
Aspartic acid	25.69 ± 4.23	22.68 ± 1.62	.54
Glutamic acid	151.80 ± 18.08	127.02 ± 7.71	.28
Glycine	958.64 ± 121.04	521.99 ± 27.36*	.02
Alamine	238.82 ± 19.70	225.96 ± 4.74	.56
Proline	55.55 ± 5.06	113.69 ± 10.44**	.007
Tyrosine	23.44 ± 2.45	10.99 ± 0.33**	.007
Sum of NEAA	1547.30 ± 162.04	1125.18 ± 45.26	.07
Total AA	2265.79 ± 160.99	1724.40 ± 74.53*	.04

FM, fish meal diet; PP40, 40% fish meal replacement by plant protein blend diet. Values are means ± *SEM* (n = 3) and were analysed by independent samples t tests.
*p < .05; **p < .01 different from FM diet.

AA transporters, plant protein blend incorporated in diet significantly increased gene expression of y^+LAT2 (p < .05), while it showed no significant impact on mRNA abundance of y^+LAT1, $b^{0,+}$AT and CAT2 between two diets (p > .05).

3.5 | Relative expression of peptide and amino acid transporters and AAR pathway-related genes in muscle between two diets

The data for relative expression of peptide and AA transporters genes are shown in Fig. 3a. Gene expression of PepT2 was markedly increased by PP40 diet (p < .05). Compared with the FM diet, PP40 diet significantly upregulated the gene expression of LAT2 and TAT1, while it downregulated the gene expression of SNAT2 in muscle (p < .05). However, no significant differences were found for gene expression of B^0AT1, ASCT2, and PAT1 in muscle (p > .05). As for muscle cationic AA transporters, plant protein blend incorporated in diet significantly reduced the gene expression of $b^{0,+}$AT (p < .05), but it showed no significant impact on mRNA abundance of y^+LAT1, y^+LAT2 and CAT2 between two diets (p > .05).

As shown in Fig. 3b, the gene expression of GCN2 and ATF4 was markedly increased in PP40 diet compared with the FM diet in muscle (p < .05). The same increased results were also found for ATF4-dependent genes, including ATF3, 4E-BP1 and REDD1 (p < .05).

3.6 | Relative expression of key enzymes of amino acid metabolism in intestine and muscle between two diets

As shown in Fig. 4, there were no significant difference between two diets for the gene expression of BCKDH-E2, SD and ASNS (p > .05) in intestine (Fig. 4a). However, fish fed with PP40 diet showed a significant increase in mRNA abundance of BCKDH-E2 and ASNS compared with the FM diet (p < .05) in muscle, while the gene expression of SD was not changed between two diets (p > .05; Fig. 4b).

4 | DISCUSSION

Growth and feed utilization observed in this trial have been published by Wang et al. (2015) in our team, who found that 40% FM replacement by plant protein blend in juvenile turbot diet led to the reduced growth and reduction of TOR signalling pathway. In the present study, we further studied that there was no detectable differences between dietary protein types in any of the individual EAA concentrations in turbot plasma, which is similar as previous investigations in turbot after partial FM replacement by soybean meal (Xu et al., 2016b) or maize gluten meal (Regost et al., 1999). However, PP40 diet greatly reduced muscle's free AA levels much more pronounced than FM diet, indicating that the quality of dietary proteins greatly affected postprandial muscle AA pool. As the activation of TOR signalling pathway is mainly regulated by the availability of AAs (Dai, Panserat, Plagnes-Juan, Seiliez, & Skiba-Cassy, 2015; Lansard et al., 2011), this

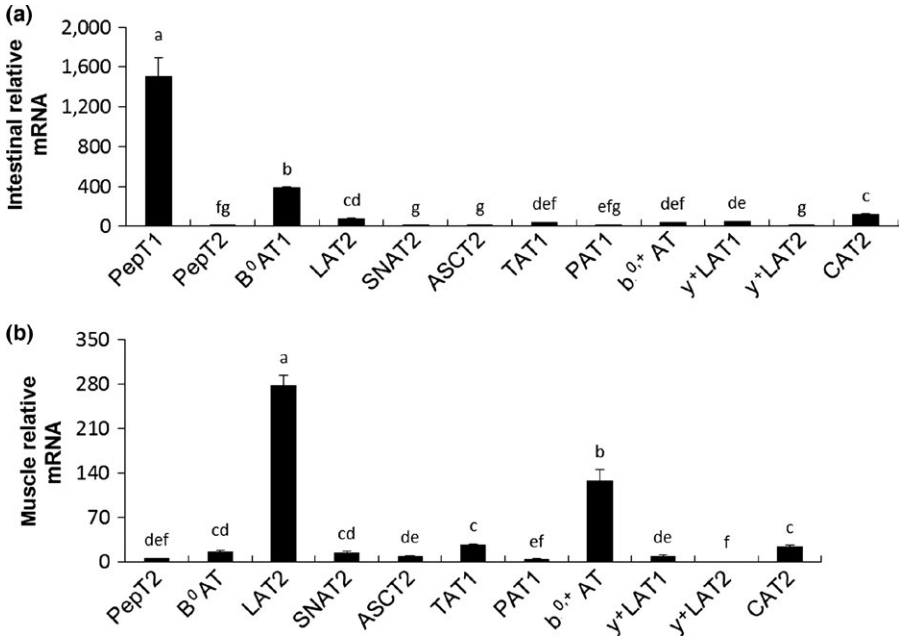

FIGURE 1 Expression pattern of peptide and amino acid transporters in intestine (a) and muscle (b). All results were analysed by the data of fish meal diet. Transcriptional levels were normalized by the reference gene of *GAPDH*. Data were showed as the ratio of the target gene mRNA to y^+*LAT2* mRNA which showed the lowest mRNA abundance. Values are means ± *SEM* (n = 6) and were analysed by one-way ANOVA followed by Tukey's multiple range test. Before analysis, all the data were transformed to square roots to ensure homoscedastic and meet ANOVA criteria. [a,b,c]Means without a common letter differ significantly (p < .05). ASCT2, system ASC amino acid transporter-2; $b^{0,+}$AT, $b^{0,+}$-type amino acid transporter; B^0AT1, B^0-type amino acid transporter 1; CAT2, cationic amino acid transporter; LAT2, L-type amino acid transporter 2; PAT1, proton-coupled amino acid transporter 1; PepT, peptide transporter; SNAT2, sodium-coupled neutral amino acid transporter 2; TAT1, T-type amino acid transporter 1; y^+LAT, system y^+L amino acid transporter

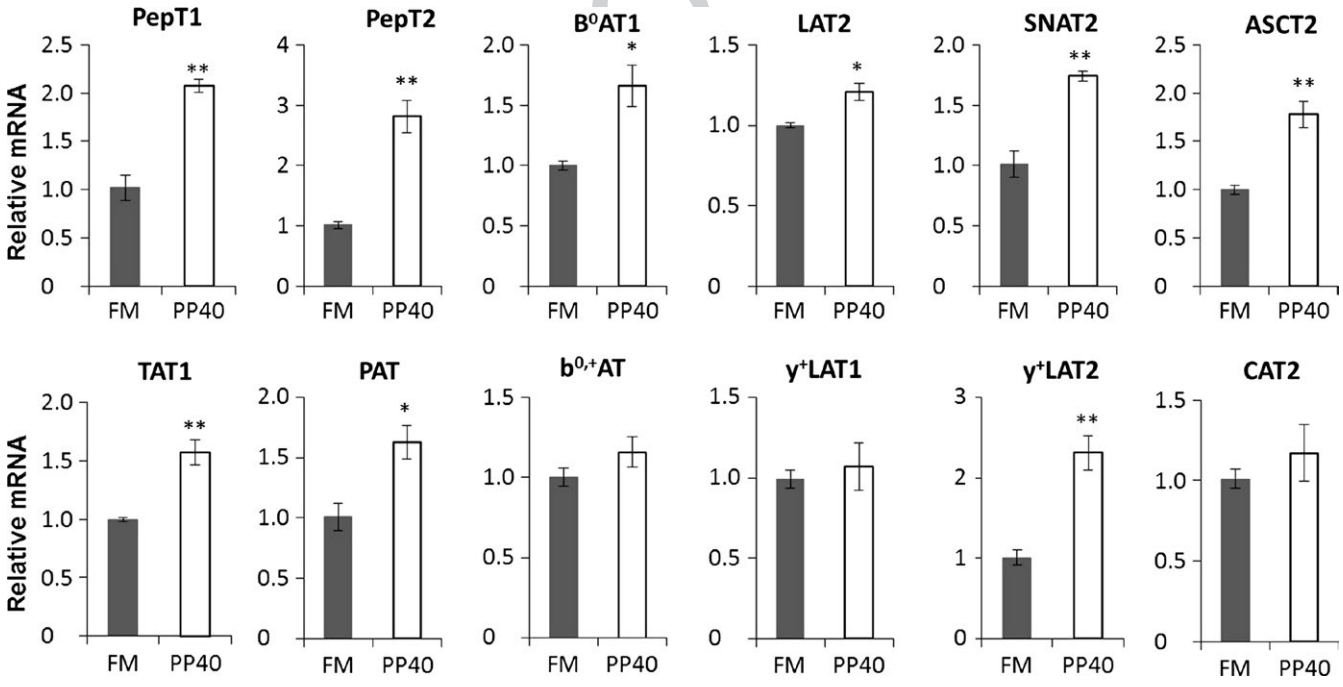

FIGURE 2 Relative expression of peptide and amino acid transporters in intestine of juvenile turbot fed different diets. Transcriptional levels were normalized by the reference gene of *GAPDH*. Values are means ± *SEM* (n = 6) and were analysed by independent samples t tests. *p < .05, **p < .01 versus FM diet. FM, fish meal diet; PP40, 40% fish meal replacement by plant protein blend diet. ASCT2, system ASC amino acid transporter-2; $b^{0,+}$AT, $b^{0,+}$-type amino acid transporter; B^0AT1, B^0-type amino acid transporter 1; CAT2, cationic amino acid transporter; LAT2, L-type amino acid transporter 2; PAT1, proton-coupled amino acid transporter 1; PepT, peptide transporter; SNAT2, sodium-coupled neutral amino acid transporter 2; TAT1, T-type amino acid transporter 1; y^+LAT, system y^+L amino acid transporter

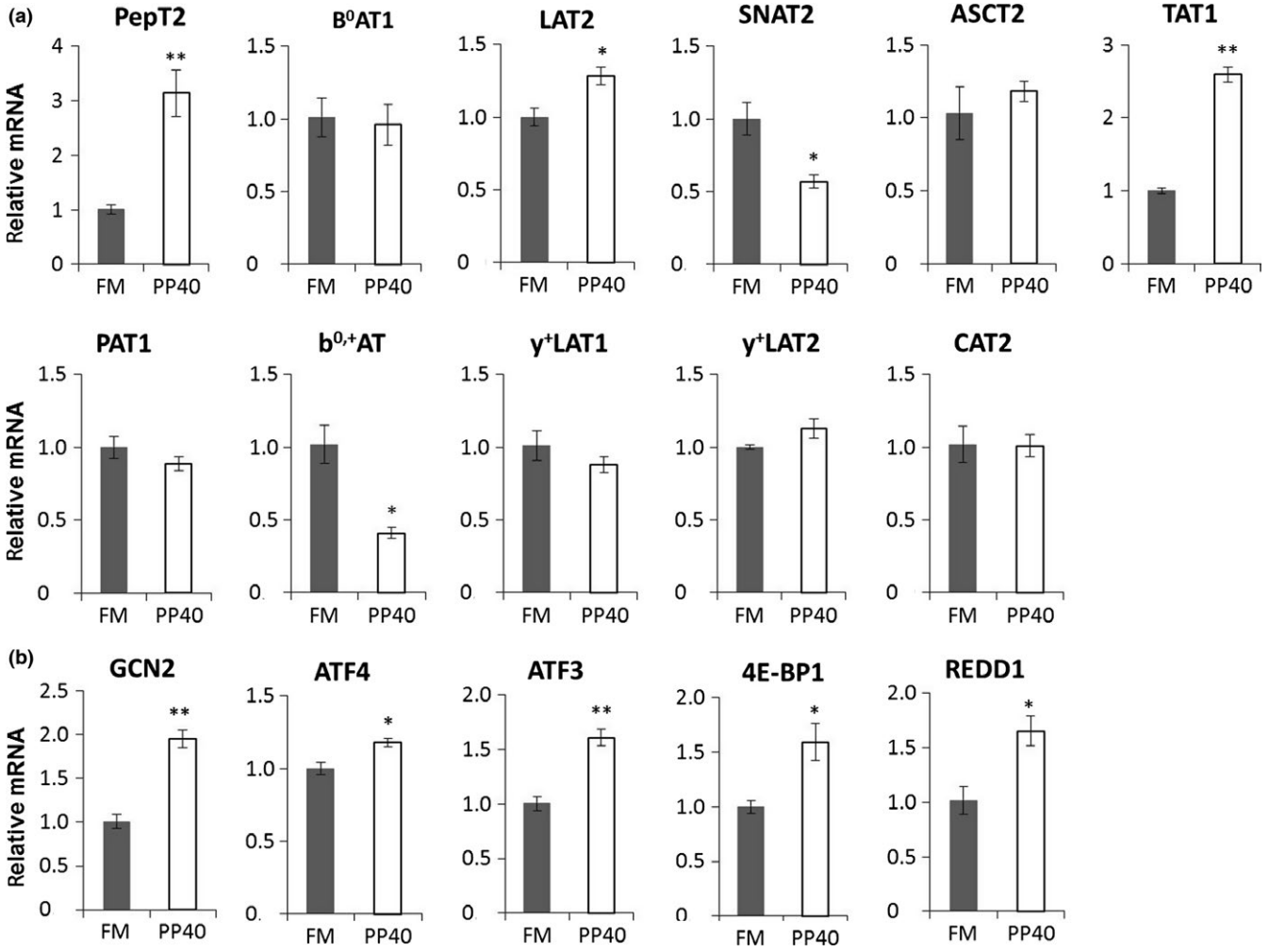

FIGURE 3 Relative expression of peptide and amino acid transporters (a) and AAR pathway-related genes (b) in muscle of juvenile turbot fed different diets. Transcriptional levels were normalized by the reference gene of GAPDH. Values are means ± *SEM* ($n = 6$) and were analysed by independent samples *t* tests. $^{*}p < .05$, $^{**}p < .01$ versus FM diet. FM, fish meal diet; PP40, 40% fish meal replacement by plant protein blend diet. ASCT2, system ASC amino acid transporter 2; $b^{0,+}$AT, $b^{0,+}$-type amino acid transporter; B^{0}AT1, B^{0}-type amino acid transporter 1; CAT2, cationic amino acid transporter; LAT2, L-type amino acid transporter 2; PAT1, proton-coupled amino acid transporter 1; PepT2, peptide transporter 2; SNAT2, sodium-coupled neutral amino acid transporter 2; TAT1, T-type amino acid transporter 1; y^{+}LAT, system y^{+}L amino acid transporter; ATF, activating transcription factor; GCN2, general control non-derepressible 2; REDD1, regulated in development and DNA damage responses 1; 4E-BP1, eukaryotic initiation factor 4E-binding protein 1

decreased EAA concentration in turbot muscle may contribute to the reduction of TOR signalling in plant protein-incorporated diet as previously reported (Wang et al., 2015). Given that AA transporters serve as a selective protein for the tissue AA availability, thereby mediating relevant intracellular AA signalling pathways (Poncet & Taylor, 2013; Taylor, 2014), we focused on the impact of dietary proteins on the regulation of genes related to AA transporters, key enzymes of AA metabolism and AAR pathway in turbot.

In culture media, cells use transporters of different affinities to regulate AA influx. Low-affinity transporters are helpful when concentrations of nutrients are high. When nutrients are depleted, low-affinity transporters are replaced by high-affinity ones (Levy, Kafri, Carmi, & Barkai, 2011). Similar to those in vitro conditions, enterocytes are exposed to digestive tract filled with dietary nutrients. We found that all the detected low-affinity AA transporters were highly expressed in intestine, which were PepT1, B^{0}AT1, LAT2, PAT1, TAT1 and CAT2, while high-affinity AA transporters such as PepT2, SNAT2, ASCT2 and y^{+}LAT2 showed low mRNA abundance. Therefore, we speculate that those highly expressed low-affinity transporters in intestine were responsible for effective AA absorption. Meanwhile, those low expressed high-affinity transporters were worked when fish were being in starvation condition (Levy et al., 2011). Dual AA transporter systems in intestine are essential for optimal growth and maintenance in turbot.

On the other hand, relative expression pattern of various AA transporters in muscle differed largely from those in intestine. Similar as other fish species, the gene expression of PepT1 was not or low expressed in muscle (Rønnestad et al., 2010; Terova et al., 2013), while mRNA abundance of LAT2 showed higher level than the other transporters. In mammals, the flux of AAs in non-epithelial cells is mainly

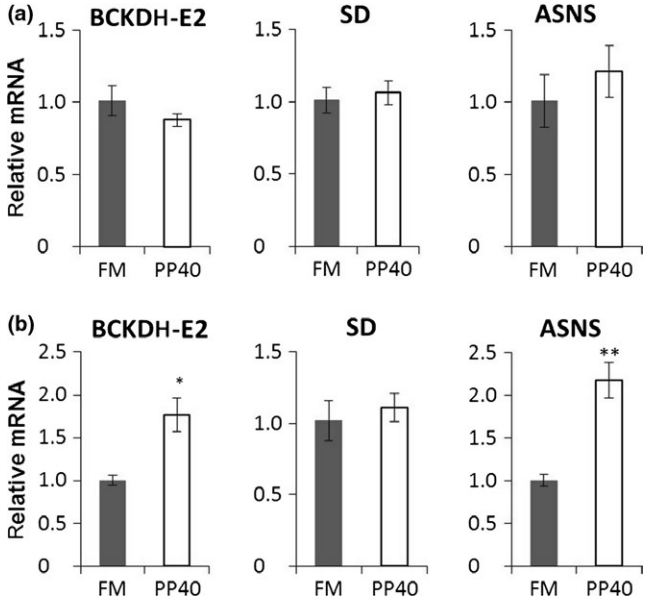

FIGURE 4 Relative expression of key enzymes of amino acid metabolism in intestine (a) and muscle (b) of juvenile turbot fed different diets. Transcriptional levels were normalized by the reference gene of GAPDH. Values are means ± *SEM* ($n = 6$) and were analysed by independent samples *t* tests. $^{*}p < .05$, $^{**}p < .01$ versus FM diet. FM, fish meal diet; PP40, 40% fish meal replacement by plant protein blend diet. ASNS, asparagine synthetase; BCKDH-E2, branch-chain α-keto acid dehydrogenase E2 subunit; SD, serine dehydratase

regulated by LATs and, generally, LAT2 mainly contain in normal cell membranes (Oda et al., 2010). Thus, LAT2 might function as the main large neutral AA transporters in turbot muscle. Although the CATs are considered as the major entry pathway for cationic AAs in non-epithelial cells of mammals (Closs, Boissel, Habermeier, & Rotmann, 2006), we found that the gene expression of $b^{0,+}AT$ was approximately 30 times higher than CAT2 in muscle. Both LAT2 and $b^{0,+}LAT$ are bidirectional antiporter that regulates the exchange of intracellular low molecular AAs for extracellular large neutral and cationic AAs (Fotiadis et al., 2013). Meanwhile, low molecular AAs are the substrates of SNAT2 and ASCT2, which were also higher expressed in muscle and could motivate the net influx of EAA (Hundal & Taylor, 2009). Taken together, the different expression pattern of AA transporters in intestine and muscle endows these two tissues with distinct regulation mechanisms for AA available.

In the present study, both expression of PepT1 and PepT2 were significantly increased by plant proteins in turbot intestine. Similarly, peptide transporters were regulated by dietary proteins in other fish species (Bakke et al., 2010; Ostaszewska et al., 2010). Biophysical analyses of PepT1 and PepT2 reveal a specific interaction with the intestinal protease trypsin, which recognizes and cleaves the peptide chain at arginine and lysine residues (Beale et al., 2015). In this regard, we speculated that FM-based diet can release more basic AA-binding peptides than plant protein sources because of the high basic AAs content. Indeed, in vitro digestion by pepsin and pancreatin indicates that FM is tend to release more small molecular peptides than plant proteins (Savoie, Agudelo, Gauthier, Marin, & Pouliot, 2005). Therefore, compared with FM diet, the plant protein blend incorporation coupled with extra crystalline AAs in PP40 diet would release more free AAs after feeding which were absorbed by various AA transporters in the digestive tract. Studies in mammals indicated that an increase in EAA levels could upregulate the expression of AA transporters (Drummond et al., 2010). This could fully explain our results that most of the detected AA transporters were upregulated by PP40 diet in intestine. The upregulation of peptide and AA transporters in intestine serve as an important adaptive response to maximum delivery dietary AAs into intestine and blood in fish fed PP40 diet (Poncet & Taylor, 2013).

As for muscle AA transporters, PP40 diet significantly upregulated the expression of LAT2, while the gene expression of SNAT2 and $b^{0,+}AT$ was downregulated. We presumed that the decreased mRNA abundance of SNAT2 suppressed the transportation of small molecular AAs, which, in turn, blocked the uptake of large neutral AAs. Thus, this change affected the levels of muscle AAs such as isoleucine and tyrosine. The reduction of both SNAT2 and $b^{0,+}AT$ gene expression would reduce the transport of cationic AAs leading to the low levels of muscle cationic AAs such as arginine and lysine. Therefore, this decreased AA availability may explain the repression of TOR signalling in muscle (Wang et al., 2015).

In response to muscle EAA deprivation, the gene expression of GCN2, the AA sensor of AAR pathway, is greatly induced (Kilberg et al., 2009). Similarly, FM replacement by soybean meal results in a significantly decreased TOR pathway and increased AAR pathway, a status associated with reduced muscle AA concentration (Xu et al., 2016b). The activation of AAR pathway suppresses general protein synthesis (Kilberg et al., 2009), but promotes a paradoxical increase of selected genes like ATF4 (Kilberg et al., 2009; Ye et al., 2010). Our results indicate that both ATF4 and ATF4-dependent genes were increased in PP40 diet confirming the fact of AAR pathway activation. Taken together, cellular transporters in response to dietary proteins regulated muscle AA availability, which in turn sensed by intracellular AA signalling pathways and regulated tissue AA pool homoeostasis.

In energetic terms, a major function of AAs is converted to protein, while the remainder AAs serve as catabolic substrates to provide metabolic precursors (Wu et al., 2014). The branched-chain AAs (BCAA) are unique among AAs in that they are important for muscle protein synthesis and primarily metabolized in muscle (Platell, Kong, McCauley, & Hall, 2000). In the present study, we found that the gene expression of BCKDH-E2, a key irreversible regulatory enzyme in BCAA catabolism (Platell et al., 2000), was significantly increased by PP40 diet in muscle. The upregulated BCAA catabolism would produce a relative deficit of BCAA and further amplified the imbalance of muscle AA pool. In addition, gene expression of muscle ASNS was also greatly induced by PP40 diet. Previous studies have shown that ASNS gene expression is potently activated upon culture medium deprived of one or more EAAs and that this induction is GCN2 and ATF4 dependent (Harding et al., 2003; Siu, Bain, LeBlanc-Chaffin, Chen, & Kilberg, 2002), which were indeed elevated by PP40 diet in muscle. Therefore, low levels of AA sensed by AAR pathway led to the synthesis of NEAAs

to maintain muscle AA pool homoeostasis (Kilberg et al., 2009). But it led to the useless of energy consumption and impaired energy retention. Taken together, high levels of intracellular EAA catabolism and NEAA synthesis in muscle were also responsible for the reduction of muscle EAA concentration.

5 | CONCLUSIONS

In summary, the present study demonstrates a mechanism connecting response towards partial FM replacement by plant protein blend in turbot: the change of muscle AA transporters coupled with increased EAA catabolism and NEAA anabolism enzyme genes lead to the low EAA concentration in muscle, which in turn increase the activation of AAR signalling and depress the activation of TOR signalling, resulting in the repression of growth (Wang et al., 2015). The novel finding of this study is that different protein sources lead to distinct AA regulation response involving AA transportation, AA metabolism and AA signalling pathways, which transmit dietary AAs into intracellular nutrient signalling pathways and determine the utilization efficiency of diets. Collectively, our finding provides a molecular basis for the future development of effective nutritional strategies to increase nutrient utilization in fish farming.

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