

# Postprandial nutrient-sensing and metabolic responses after partial dietary fishmeal replacement by soyabean meal in turbot (*Scophthalmus maximus* L.)

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## Abstract

In this study, we chose a carnivorous fish, turbot (*Scophthalmus maximus* L.), to examine its nutrient-sensing and metabolic responses after ingestion of diets with fishmeal (FM), or 45% of FM replaced by soyabean meal (34.6% dry diet) balanced with or without essential amino acids (EAA) to match the amino acid profile of FM diet for 30 d. After a 1-month feeding trial, fish growth, feed efficiency and nutrient retention were markedly reduced by soyabean meal-incorporated (SMI) diets. Compared with the FM diet, SMI led to a reduction of postprandial influx of free amino acids, hypoactivated target of rapamycin signalling and a hyperactivated amino acid response pathway after refeeding, a status associated with reduced protein synthesis, impaired postprandial glycolysis and lipogenesis. These differential effects were not ameliorated by matching an EAA profile of soyabean meal to that of the FM diet through dietary amino acid supplementation. Therefore, this study demonstrated that the FM diet and SMI diets led to distinct nutrient-sensing responses, which in turn modulated metabolism and determined the utilisation efficiency of diets. Our results provide a new molecular explanation for the role of nutrient sensing in the inferior performance of aquafeeds in which FM is replaced by soyabean meal.

**Key words:** Fishmeal: Soyabean meal: Turbot: Nutrient sensing: Metabolism

With the rapid growth of aquaculture, demand for aquafeed with less fishmeal (FM) has increased because of the cost of this protein source and its limited supply<sup>(1)</sup>. During the past decade, considerable progress has been made towards replacing portions of FM in aquafeeds with alternative protein sources<sup>(1,2)</sup>. Currently, FM is becoming a minor protein source in the feed for omnivorous species. However, it continues to be the primary protein source in aquafeed for marine species and other species during the fry or fingerling stage<sup>(2)</sup>. The over substitution of FM has generally led to reduced growth performance in marine carnivorous species<sup>(1,2)</sup>. The inferior performance of non-FM protein sources (plant proteins, in particular) has been attributed to nutritional limitations, such as the imbalanced amino acid profiles, presence of anti-nutritional compounds and other factors<sup>(2)</sup>. However, little is known regarding the nutrient-sensing and metabolic changes after FM replacement in aquatic animals.

Postprandial responses represent a critical step towards defining the utilisation efficiency of dietary protein sources<sup>(3,4)</sup>. Feeding-induced stimulation of anabolic protein synthesis depends on postprandial repletion of amino acid pools in plasma and other tissues<sup>(5)</sup>. Mediated by amino acid transporters, amino

acid availability in turn mediates the activation of nutrient-sensing cascades, including target of rapamycin (TOR) and amino acid response (AAR) pathways, both of which control protein synthesis and downstream metabolism<sup>(6)</sup>. The activated TOR signalling pathway promotes the translation of many anabolic enzymes and other proteins involved in diverse cellular functions<sup>(7–9)</sup>. However, any individual amino acid limitation can activate the AAR pathway, which triggers global protein synthesis repression and induces translation of rate-limiting enzymes related to amino acid and lipid metabolism<sup>(10,11)</sup>. The counter-regulatory mechanisms of amino acid sensing exist to coordinate the action of TOR and AAR pathways and their downstream effects on translation, which provide the molecular basis for nutritional responses<sup>(12)</sup>.

Most nutrient-sensing studies in fish focus on cellular responses *in vitro*<sup>(13–15)</sup>. However, such information may not provide important explanations concerning the physiological responses generated by a certain protein source in animals. We hypothesised that dietary FM replacement would probably change the postprandial nutritional response of fish, which, in turn, would have an impact on their metabolism and phenotypic performance. To test this hypothesis, an economically valuable marine carnivorous fish species, turbot (*Scophthalmus maximus* L.),

**Abbreviations:** AAR; amino acid response; *B<sup>0</sup>ATI*; B<sup>0</sup>-type amino acid transporter 1; EAA; essential amino acids; eIF2 $\alpha$ ; eukaryotic initiation factor 2  $\alpha$ ; FM; fishmeal; *PepT1*; peptide transporter 1; S6; ribosomal protein S6; SMI; soyabean meal-incorporated diet; SMI + AA; SMI diet with dietary EAA supplementation; *SNAT2*; sodium-coupled neutral amino acid transporter 2; TOR; target of rapamycin; *y<sup>+</sup>LAT1*; *y<sup>+</sup>L*-type amino acid transporter 1.

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was chosen as the model species because of its high dietary protein requirement<sup>(16)</sup>. Fish were fed either an FM diet or partial FM replacement by soyabean meal with or without essential amino acid (EAA) supplementation diets, which represented the most frequently used strategy for improving the performance of non-FM proteins<sup>(17)</sup>. After the feeding trial, a comprehensive characterisation of the postprandial dynamics of the expression of amino acid transporters, free amino acid pools, the activation of nutrient-sensing molecules and the regulated expression of key metabolic enzymes was evaluated. Our results could contribute to mechanistic explanations on the performance changes after FM replacement in mariculture.

## Methods

### Diets

The ingredients and composition of the experimental diets are shown in Table 1. Their amino acid profiles are shown in online Supplementary Table S1. Turbot were fed with three iso-nitrogenous (50.1% crude protein) and isoenergetic (20.8 kJ/g) diets comprising different protein sources (Table 1): 60% FM and 33% FM+34.6% soyabean meal (45% FM replaced with soyabean meal) incorporated (SMI) without or with (SMI diet with dietary EAA supplementation (SMI+AA)) amino acids supplemented to match the EAA profile of the FM diet. The ingredients were ground into a fine powder through a 320- $\mu$ m mesh. All of the ingredients were thoroughly mixed and extruded as pellets, dried at 45°C for 12 h, sieved and refrigerated at -20°C before feeding.

### Feeding trial and sampling

All experimental protocols were approved by the Animal Care Committee of Ocean University of China. Juvenile turbot were obtained from Haiyang fish farm (Haiyang, China). During the acclimatisation period, fish were fed a commercial diet (Great Seven Bio-tech) twice per day, for 2 weeks. To start the experiment, the juvenile turbot with an initial weight of 9.19 (SEM 0.01) g were randomly distributed into tanks filled with 500 litres of seawater, with forty fish in each tank. Diets were randomly allocated in triplicate to the tanks. Fish were fed twice per day, until apparent satiation, for 30 d. During the experimental period, the water temperature was 16–20°C. The uneaten feed was collected 1 h after each meal, dried to a constant weight and weighed to allow calculation of food intake.

Before the feeding trial, twenty fish from the same population were randomly collected for initial biochemical analysis. At the end of the feeding trial, fish were fasted for 48 h to obtain the basal levels of metabolites in fish<sup>(18)</sup>. After 24 h of fasting, fish in each tank were weighed and counted, and four fish per tank were randomly selected for biochemical analysis. At the end of the 48-h fasting period, the sampled fish were designated as the 0-h sample (fasted fish). The fish were then fed a single meal of their allocated diet until visible satiation. Samples were collected at 2-, 8- and 24-h intervals after refeeding. At each interval, six fish (two fish per tank) were randomly taken from every treatment (*n* 6). Fish were anaesthetised with benzocaine

**Table 1.** Formulations of experimental diets

Ingredients (%)	Diets (% dry weight)		
	FM	SMI	SMI + AA
Fishmeal*	60.0	33.0	33.0
Soyabean meal†	0	34.6	34.6
Wheat gluten meal	3.0	5.0	3.5
Wheat meal‡	23.6	12.0	12.0
Fish oil	3.0	5.0	5.0
Palm oil	1.5	0.5	0.5
Lecithin	2.5	2.5	2.5
Mineral premix§	1.5	1.5	1.5
Vitamin premix	1.5	1.5	1.5
Ca(H <sub>2</sub> PO <sub>4</sub> ) <sub>2</sub> ·H <sub>2</sub> O	0	1.0	1.0
Amino acid mixture¶	0	0	1.5
Others**	3.4	3.4	3.4
Analytical composition (DM basis)			
DM (%)	95.0	94.8	95.1
Crude protein (%)	50.1	50.3	49.9
Crude lipid (%)	11.6	11.4	11.2
Ash (%)	13.4	11.4	11.2
Gross energy (kJ/g)	20.7	20.8	21.0

FM, fishmeal; SMI, soyabean meal-incorporated diet; SMI + AA, SMI diet with dietary essential amino acid supplementation.

\* FM: steam-dried FM (Copeinca Group), with crude protein: 70.10%, crude lipid: 7.58%.

† Soyabean meal: dehulled, solvent-extracted soyabean meal (Great Seven Bio-tech), with crude protein: 54.61%, crude lipid: 2.33%.

‡ Wheat meal and wheat gluten meal act as a carbohydrate source and as a filler (Great Seven Bio-tech).

§ Mineral premix (mg/kg diet): CoCl<sub>2</sub>·6H<sub>2</sub>O (1%), 50; CuSO<sub>4</sub>·5H<sub>2</sub>O (25%), 10; FeSO<sub>4</sub>·H<sub>2</sub>O (30%), 80; ZnSO<sub>4</sub>·H<sub>2</sub>O (34.50%), 50; MnSO<sub>4</sub>·H<sub>2</sub>O (31.80%), 45; MgSO<sub>4</sub>·7H<sub>2</sub>O (15%), 1200; sodium selenite (1%), 20; calcium iodine (1%) 60; zeolite, 11 470.

|| Vitamin premix (mg/kg diet): thiamin (98%), 25; riboflavin (80%), 45; pyridoxine-HCl (99%), 20; vitamin B<sub>12</sub> (1%), 10; vitamin K<sub>3</sub> (51%), 10; inositol (98%), 800; pantothenic acid (98%), 60; niacin acid (99%), 200; folic acid (98%), 20; biotin (2%), 60; retinyl acetate (500 000 IU/g), 32; cholecalciferol (500 000 IU/g), 5;  $\alpha$ -tocopherol (50%), 240; ascorbic acid (35%), 2000; antioxidants (oxygen ling grams, 100%), 3; rice husk powder (100%), 11 470.

¶ Amino acid mixture: L-lysine (coated amino acid, 60%) 0.6%, L-methionine (coated amino acid obtained, 90%) 0.32%, L-isoleucine (crystalline amino acid, 99.4%) 0.12%, L-leucine (crystalline amino acid, 99.4) 0.13%, L-threonine (crystalline amino acid, 99.9%) 0.13%, L-valine (crystalline amino acid, 99.1%) 0.16%. Coated amino acid was obtained from Beijing XingHuo Yuan Science and Technology Co. Ltd and crystalline amino acid was obtained from Jizhou City Huayang Chemical Co. Ltd.

\*\* Others: beer yeast 2%, choline chloride 0.25%, mould inhibitor 0.1%, antioxidant 0.05%, attractant (betaine-dimethylpropiothetin-glycine-alanine-inosine 5'-phosphate = 4:2:2:1:1) 1.0%.

(30 mg/l) and killed by cervical section. The stomach and gut contents of each fish were checked to ensure that the fish had effectively consumed the diet. The liver, intestine and dorso-lateral white muscle were immediately dissected and frozen in liquid N<sub>2</sub> and kept at -80°C. The other six fish in each treatment were selected, anaesthetised, visually checked as to whether or not the belly was bulging greatly because of a full diet in the digestive tract and blood was extracted from the caudal vein into heparin anti-coagulation tubes and centrifuged at 3000 **g** for 5 min. The recovered plasma was kept at -80°C until analysis.

### Biochemical analysis

DM (105°C for 24 h), crude protein (N×6.25, Kjeltac nitrogen analyser<sup>TM</sup> 8400; FOSS), crude lipid (ether extraction, Soxhlet method) and ash (combustion in a muffle furnace at 550°C for 12 h)

**Q3** **Table 2.** Primer sequences used for real-time quantitative PCR\*

Genes	Forward prime	Reverse primer	Product size (bp)
Peptide and amino acid transporters			
<i>PepT1</i>	GCATCCACACCCAGCAGAAG	GTCCTCAGCCCAGTCCATCC	232
<i>B<sup>0</sup>AT1</i>	AGACTCTCAACACCTCCGAAGC	AGCCTTTCCTGTGGTCTCAATCC	131
<i>y<sup>+</sup>LAT1</i>	TGTGACGTTTGCGGACCAG	GACGGGAGTGTAGCGGAAGAC	186
<i>SNAT2</i>	TGCTGCTGGTGACGCTCTTC	CAGGTGTCCTCGCTGTAGTCC	249
Key enzymes of nutrient metabolism			
<i>GK</i>	CGACACGAGGACATTGACAAG	CCAAACATCATCCCGACTTCAC	218
<i>PK</i>	TGGATACGCTGAAGGAGATG	ACGCACGTTCTTGATGGTC	236
<i>FBPase</i>	CAGGAAGGCTGGGATCGCTAAC	CTCATCTTCCCTCCGACACAAG	157
<i>G6Pase</i>	CACGAGACGGCTCATTATGC	CTTTGCTGCTGGATTTCTTGC	193
<i>SREBP1</i>	GCCATTGACTACATCCGTTAC	CATCAGCCTGTCCATCTACTTC	136
<i>FAS</i>	GGCAACAACACGGATGGATAC	CTCGCTTTGATTGACAGAACAC	195
<i>DGAT1</i>	ATACTCGTGTCCTCTGTGTCTC	AGTCGTCTCATCAGGAACCTTAC	177
<i>DGAT2</i>	TGCTGTGGTCATCGTTATC	CTTGTAGCGCTCGTTCTC	163
<i>CPT1A</i>	ATGGGAAGAGTGGACTGAATG	GCTGGAAGGCATCTGTGG	96
<i>ACO1</i>	AGTCTCGCCAGCTTTACT	GGCTTCACATAGTTCCGTCT	240
Reference genes			
<i>RPSD</i>	CTGCTGTTCCCTAAAGAGTTCG	GAGCCGTGTAGTTCAGGGTCT	151
<i>EF1<math>\alpha</math></i>	TCATTGGCCATGTCGACTCC	ACGTAGTACTTGGCGGTCTC	226

\* Abbreviations and GenBank accession nos: *ACO1*, acyl-CoA oxidase 1, KC189925; *CPT1A*, carnitine palmitoyltransferase 1 isoforms A, KC189926; *DGAT1*, diacylglycerol *O*-acyltransferase homolog 1, KC189938; *DGAT2*, diacylglycerol *O*-acyltransferase homolog 1, KC189939; *EF1 $\alpha$* , elongation factor-1  $\alpha$ , AF467776.1; *FAS*, fatty acid synthase, KC189927; *FBPase*, fructose 1,6-bisphosphatase, KC184130; *G6Pase*, glucose 6 phosphatase, KC184131; *GK*, glucokinase, JX678944; *PK*, pyruvate kinase, DQ848903; *RPSD*, RNA polymerase II subunit D, DQ848899.1; partial sequences of some target genes in turbot were obtained through a degenerate PCR strategy in this study, including *B<sup>0</sup>AT1*, B<sup>0</sup>-type amino acid transporter 1; *PepT1*, peptide transporter 1; *SNAT2*, sodium-coupled neutral amino acid transporter 2; *SREBP1*, sterol regulatory element-binding protein 1; *y<sup>+</sup>LAT1*, y<sup>+</sup>L-type amino acid transporter 1.

130 of the experimental diets and fish samples were analysed as  
131 described elsewhere<sup>(19)</sup>. The gross energies of feed and fish sam-  
132 ples were measured using an adiabatic bomb calorimeter (C2000;  
133 Ika Werke). Amino acids in ingredients and diets were analysed  
134 using an amino acid analyzer (L-8900; Hitachi). Plasma glucose  
135 (GLU) and TAG concentrations were measured using commercial  
136 kits according to the manufacturer's instructions (Sysmex).

### 137 *Free amino acid analysis*

138 The postprandial free amino acid concentrations in the plasma  
139 and muscle were analysed by an automated amino acid analyser  
140 (L-8900) with a lithium high-performance column<sup>(20)</sup>. Briefly,  
141 plasma (400  $\mu$ l) was deproteinised by mixing it with 1.2 ml of  
142 10 % sulfosalicylic acid solution and incubating it at 4°C for 5 min.  
143 After centrifugation at 13 000 rpm for 15 min, 1 ml of supernatant  
144 was filtered through 0.22- $\mu$ m filters for free amino acid mea-  
145 surement; in addition, white muscle samples (400 mg) were  
146 homogenised in 1.2 ml of 10 % sulfosalicylic acid solution. After  
147 centrifugation at 13 000 rpm for 15 min, the supernatant was  
148 filtered through 0.22- $\mu$ m filters for free amino acid measurement.

### 149 *Quantitative real-time PCR*

150 Total RNA was extracted from intestine, liver or muscle  
151 (approximately 50 mg) using Trizol reagent (Invitrogen) accord-  
152 ing to the manufacturer's recommendations, quantified by a  
153 Nanodrop 2000 spectrophotometer (Thermo) and electro-  
154 phoresed on a 1.2 % denaturing agarose gel to test the integrity.  
155 complementary DNA synthesis and quantitative real-time PCR  
156 (qRT-PCR) reactions were conducted as described previously<sup>(21)</sup>.  
157 Specific primer sequences of target genes for qRT-PCR are listed

158 in Table 2. qRT-PCR analyses were focused on the postprandial  
159 kinetics of peptide and amino acid transporters, which were  
160 peptide transporter 1 (*PepT1*) mediating the uptake of essentially  
161 all dipeptides and tripeptides<sup>(22)</sup>; B<sup>0</sup>-type amino acid transporter 1  
162 (*B<sup>0</sup>AT1*) transporting all neutral amino acids; sodium-coupled  
163 neutral amino acid transporter 2 (*SNAT2*) preferring alanine and  
164 other small and polar neutral amino acids; and y<sup>+</sup>L-type amino  
165 acid transporter 1 (*y<sup>+</sup>LAT1*) mediating the transport of cationic  
166 amino acids<sup>(23)</sup>. We also examined the gene expression of several  
167 key enzymes of hepatic metabolism, which were as follows:  
168 glucokinase (*GK*) and pyruvate kinase (*PK*) for glycolysis; fruc-  
169 tose 1,6-bisphosphatase (*FBPase*) and glucose 6 phosphatase  
170 (*G6Pase*) for gluconeogenesis; transcription factor sterol  
171 regulatory element-binding protein 1 (*SREBP1*) and fatty acid  
172 synthase (*FAS*) for fatty acid synthesis; diacylglycerol *O*-acyl-  
173 transferase homolog (*DGAT1*) and *DGAT2* for TAG synthesis; and  
174 carnitine palmitoyltransferase 1 isoforms A (*CPT1A*) and acyl-CoA  
175 oxidase 1 (*ACO1*) for fatty acid oxidation<sup>(13,24)</sup>. Results were  
176 normalised to reference genes RNA polymerase II subunit D  
177 (*RPSD*) for intestine and muscle samples, and elongation factor 1 $\alpha$   
178 (*EF1 $\alpha$* ) for liver samples. No expression changes of *RPSD* and  
179 *EF1 $\alpha$*  were observed in the corresponding tissues among treat-  
180 ments (online Supplementary Fig. S1). The expression levels of  
181 target mRNA were calculated using the comparative cycle  
182 threshold (*C<sub>t</sub>*) values expressed as 2<sup>(- $\Delta\Delta C<sub>t</sub>$ )</sup>. Transcription levels  
183 were normalised by the reference gene. Gene expression was  
184 represented as fold change to the control (T 0 h FM).

### 185 *Protein extraction and Western blot analysis*

186 Tissues (approximately 40 mg) were homogenised with Glass  
187 Tenbroeck Tissue Grinders (Kimble Chase) on ice and lysed in

**Table 3.** Growth performance and nutrient utilisation of turbot after 30-d diet feeding trial (Mean values with their standard errors; *n* 3)

	FM		SMI		SMI + AA		<i>P</i>
	Mean	SE	Mean	SE	Mean	SE	
Initial body weight (g)	9.2	0.01	9.2	0.01	9.2	0.01	0.95
Final body weight (g)	28.8 <sup>a</sup>	1.01	21.4 <sup>b</sup>	0.13	23.3 <sup>b</sup>	0.22	<0.001
Specific growth rate (%/d)*	3.3 <sup>a</sup>	0.10	2.4 <sup>b</sup>	0.02	2.6 <sup>b</sup>	0.03	<0.001
Feed intake (%/d)†	2.3	0.13	2.2	0.05	2.2	0.05	0.64
Feed efficiency‡	1.5 <sup>a</sup>	0.06	1.2 <sup>b</sup>	0.04	1.3 <sup>b</sup>	0.02	0.01
Protein retention (%/§)	48.1 <sup>a</sup>	1.94	39.6 <sup>b</sup>	0.64	40.5 <sup>b</sup>	0.40	0.004
Fat retention (%/§)	53.8 <sup>a</sup>	2.30	34.4 <sup>b</sup>	1.34	29.4 <sup>b</sup>	1.42	0.001
Energy retention (%/§)	36.6 <sup>a</sup>	1.45	27.7 <sup>b</sup>	1.21	27.1 <sup>b</sup>	1.44	0.004
Whole-body protein content (% wet weight)	15.4	0.14	15.4	0.28	14.9	0.18	0.21
Whole-body fat content (% wet weight)	3.5 <sup>a</sup>	0.17	2.7 <sup>b</sup>	0.04	2.3 <sup>b</sup>	0.09	0.001
Percentage of survival	100		100		98.3	2.89	0.42

FM, fishmeal diet; SMI, soyabean meal-incorporated diet; SMI + AA, SMI diet with dietary essential amino acids supplementation.

<sup>a,b</sup> Mean values within a row with unlike superscript letters were significantly different ( $P < 0.05$ ).

\* Specific growth rate (%/d) =  $100 \times (\ln \text{ final body weight} - \ln \text{ initial body weight}) / \text{d}$ .

† Feed intake (% body weight/d) =  $100 \times \text{feed consumption} / (30 \text{ d} \times (\text{initial body weight} + \text{final body weight}) / 2)$ .

‡ Feed efficiency = wet weight gain (g)/total feed consumed (g).

§ Nutrient retention =  $(100 \times (\text{final body weight} \times \text{final carcass nutrient content}) - (\text{initial body weight} \times \text{initial carcass nutrient content})) / \text{nutrient intake}$ , where nutrient refers to protein, lipid and energy.

|| Percentage survival =  $100 \times (\text{final fish number} / \text{initial fish number})$ .

RIPA buffer (50 mM-Tris, 150 mM-NaCl, 0.5 % Nonidet P-40, 0.1 % SDS, 1 mM-EDTA, pH 7.5) with protease and phosphatase inhibitors (Roche). Lysates were centrifuged at 12 000 *g* for 20 min at 4°C. Protein concentrations were determined using an Enhanced BCA Protein Assay Kit (Beyotime Biotechnology) with BSA as standard. Lysates (10 µg of total protein/lane) were separated by SDS-PAGE and transferred to 0.45-µm PVDF membranes (Millipore) for Western blot analysis. Primary antibodies against phospho-Akt (Ser473) (Cat. no. 9271), Akt (Cat. no. 9272), phospho-TOR (Ser2448) (Cat. no. 2971), TOR (Cat. no. 2972), phospho-S6 (Ser235/236) (Cat. no. 4856), S6 (Cat. no. 2217), phospho-4E-BP1 (Thr37/46) (Cat. no. 9459), 4E-BP1 (Cat. no. 9452), phospho-eIF2α (Ser51) (Cat. no. 3597) and eIF2α (Cat. no. 9722) were purchased from Cell Signaling Technology Inc. Anti-ATF4 antibody (Cat. no. sc-200) was purchased from Santa Cruz Biotechnology Inc. All antigenic regions of these antibodies have been reported as conserved in turbot<sup>(25)</sup>, and also successfully used in rainbow trout in previous studies<sup>(18,26)</sup>. Next, membranes were incubated with HRP-labelled goat anti-rabbit IgG (H + L) second antibody (Beyotime Biotechnology), and developed using a Beyo ECL Plus kit (Beyotime Biotechnology). The density of protein bands was quantified using the NIH Image 1.63 software (*n* 6).

### Statistical analysis

The SPSS 16.0 software was used for all statistical analysis. The data of growth performance were subjected to one-way ANOVA, followed by Tukey's multiple range tests. The data for the time course and different diets were analysed by two-way ANOVA, testing the main effects of time (T) and diet (D), and their full-factorial interaction. Tukey's multiple range tests were conducted in order to detect treatment differences among the interactions. In cases in which data were nonparametric or not

homoscedastic, data transformations (such as logarithms, square roots and reciprocals) were used to meet ANOVA criteria. Normality was assessed using the Shapiro-Wilks' test, whereas homoscedasticity was determined using Levene's test. For all statistical analyses, the level of significance was set at  $P < 0.05$ .

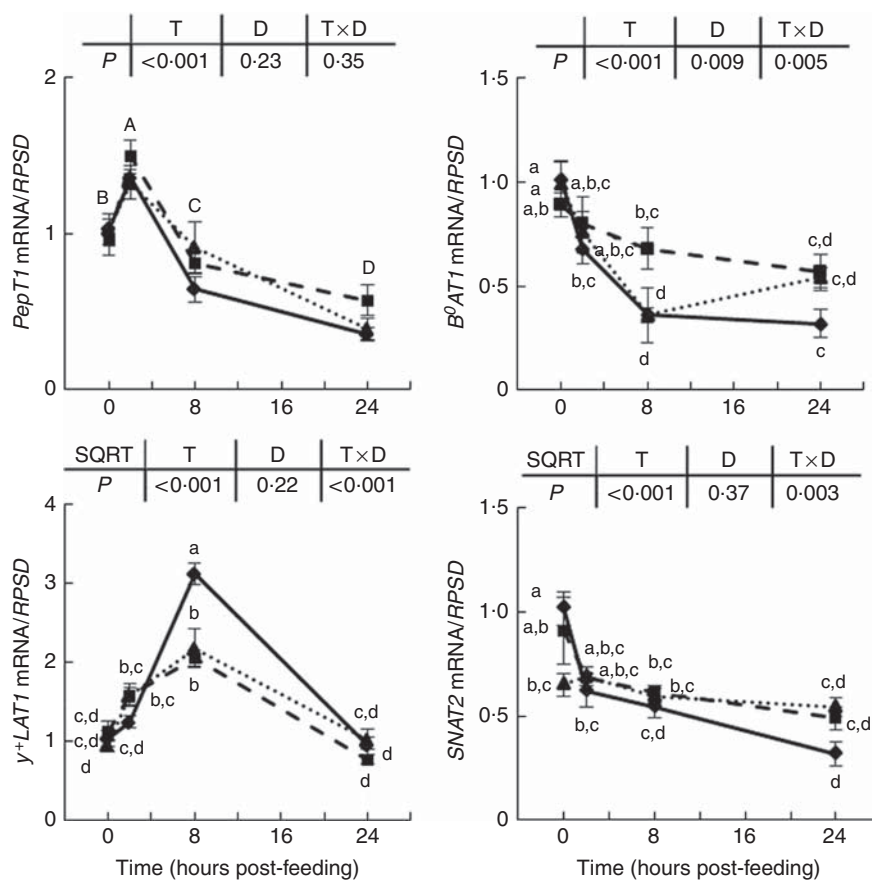
## Results

### Phenotypic characterisation of dietary performance

After the 1-month feeding trial, no significant differences were found for feed intake, whole-body protein content or percentage survival for all treatments (Table 3). Compared with the FM diet, two SMI diets (SMI and SMI + AA) resulted in decreased specific growth rate ( $P < 0.001$ ), feed efficiency ( $P = 0.01$ ), protein ( $P = 0.004$ ), fat ( $P = 0.001$ ) and energy ( $P = 0.004$ ) retention, and whole-body fat content ( $P = 0.001$ ) (Table 3). No significant differences were found between the SMI and SMI + AA diets for any of these measures.

### Postprandial modulations of amino acid transporters by dietary proteins

Postprandial gene expressions of major amino acid transporters in the intestine were measured (Fig. 1). After refeeding, the expression levels of intestinal *PepT1* and  $\gamma^+$ *LAT1* were significantly increased and peaked at 2 and 8 h, respectively, whereas the mRNA levels of *B<sup>0</sup>AT1* and *SNAT2* were markedly decreased (Fig. 1). Compared with the FM diet, SMI and SMI + AA diets did not significantly influence the postprandial gene expressions of *PepT1* or *SNAT2*. However, the SMI diet significantly decreased the peak level of  $\gamma^+$ *LAT1* and reduction of *B<sup>0</sup>AT1* after refeeding in the intestine (Fig. 1). Supplementation of EAA to the SMI diet significantly shifted the postprandial gene expression pattern of



**Q5** **Fig. 1.** Postprandial expressions of peptide and amino acid transporters in intestine of juvenile turbot. Values are means (*n* 6), with their standard errors and were analysed by two-way ANOVA followed by Tukey's multiple range test. <sup>a,b,c,d</sup> Mean values among all treatments with unlike letters are significantly different when the interaction was significant (*P* < 0.05). <sup>A,B,C,D</sup> Mean values among four time points with unlike letters were significantly different (*P* < 0.05). <sup>x,y,z</sup> Mean values among three diets with unlike letters are significantly different (*P* < 0.05). T, time points; D, diets; T × D, interaction between T and D; fishmeal diet (—■—); SMI, soyabean meal-incorporated diet (---▲---); SQRT indicates that data were transformed and statistically analysed with square roots; B<sup>0</sup>AT1, B<sup>0</sup>-type amino acid transporter 1; PepT1, peptide transporter 1; SNAT2, sodium-coupled neutral amino acid transporter 2; y<sup>+</sup>LAT1, y<sup>+</sup>L-type amino acid transporter 1; RPSD, RNA polymerase II subunit D.

249 B<sup>0</sup>AT1 to that of the FM diet, but it showed no significant impact  
250 on postprandial peak values of y<sup>+</sup>LAT1 (Fig. 1).

251 *Postprandial free amino acid influx was modulated by*  
252 *dietary proteins*

253 The postprandial kinetics of the free amino acid concentrations  
254 in plasma and muscle are shown in Tables 4 and 5, and in  
255 online Supplementary Tables S2 and S3. Plasma free amino acid  
256 concentrations were markedly increased and peaked 2–8 h after  
257 refeeding, before returning to basal levels. Compared with the  
258 FM diet, the SMI diet provided significantly lower free  
259 amino acids entering into plasma, whereas dietary EAA  
260 supplementation fully compensated for these deficits (Table 4  
261 and online Supplementary Table S2). Postprandial free amino  
262 acid concentrations in muscle were markedly increased and  
263 peaked 8 h after refeeding. The SMI diet led to significantly  
264 reduced postprandial peak values of free amino acids and  
265 shorter duration of total EAA concentrations in muscle. EAA  
266 supplementation in the SMI diet did not significantly affect  
267 postprandial peak values of the majority of free amino acids in  
268 muscle (Table 5 and online Supplementary Table S3).

269 *Postprandial modulations of amino acid-sensing pathways*  
270 *by dietary proteins*

271 Postprandial activation of amino acid-sensing pathways was  
272 examined in muscle (Fig. 2(A)) and liver (Fig. 2(B)). Two-way  
273 ANOVA statistical analysis showed that the phosphorylation of  
274 TOR and AAR pathways were both significantly affected by  
275 refeeding time courses and different diets (*P* < 0.01). The TOR  
276 pathway was markedly activated by refeeding both in muscle  
277 and liver, characterised by the phosphorylation of protein  
278 kinase B (Akt) on Ser473, TOR on Ser2448, ribosomal protein S6  
279 (S6) on Ser235/236 and eukaryotic initiation factor 4E-binding  
280 protein 1 (4E-BP1) on Thr37/46 (Fig. 2). Contrarily, the  
281 AAR-related molecule eukaryotic initiation factor 2 α (eIF2α) on  
282 Ser51 was markedly activated during starvation (time 0 h) and  
283 inhibited after refeeding in all three diets. Compared with the  
284 FM diet, the SMI diet led to significantly lower levels and shorter  
285 duration of phosphorylation of Akt, TOR, S6 and 4E-BP1 in both  
286 muscle and liver tissues (Fig. 2). However, the SMI diet  
287 significantly induced stress-responsive eIF2α phosphorylation  
288 and activating transcription factor 4 (ATF4) (Fig. 2).  
289 Supplementation of EAA to the soyabean diet did not have a

**Table 4.** Changes of plasma individual free essential amino acid (EAA) concentrations in turbot after refeeding ( $\mu\text{g}/\mu\text{l}$ ) (Pooled standard errors)

Diets	T (h)	Leu	Ile	Val	Thr	Met	Phe	Lys	His	Arg	EAA	TAA
Individual treatment means*												
FM	0	13.3	8.3 <sup>f</sup>	9.3 <sup>d</sup>	10.0 <sup>e</sup>	5.6 <sup>f</sup>	5.4	14.2	4.1	5.7	75.9 <sup>f</sup>	161.8
FM	2	58.9	28.6 <sup>a,b</sup>	42.2 <sup>a,b</sup>	25.5 <sup>a</sup>	18.6 <sup>c,d</sup>	23.7	30.1	9.0	36.8	273.4 <sup>a,b</sup>	469.4
FM	8	69.0	31.1 <sup>a</sup>	53.9 <sup>a</sup>	27.4 <sup>a</sup>	27.4 <sup>b</sup>	35.2	23.9	8.7	28.6	305.2 <sup>a</sup>	485.9
FM	24	33.0	18.5 <sup>c,d,e</sup>	20.7 <sup>c,d</sup>	19.9 <sup>a,b,c</sup>	9.8 <sup>e,f,g</sup>	19.6	18.9	6.2	12.6	159.2 <sup>c,d,e</sup>	248.2
SMI	0	17.3	10.4 <sup>d,e,f</sup>	14.7 <sup>d</sup>	9.6 <sup>e</sup>	4.9 <sup>h</sup>	8.0	16.6	3.2	7.6	93.6 <sup>e,f</sup>	162.4
SMI	2	38.1	19.5 <sup>b,c,d</sup>	23.0 <sup>c,d</sup>	15.2 <sup>c,d,e</sup>	10.4 <sup>e,f</sup>	22.8	20.8	7.5	33.7	190.8 <sup>b,c,d</sup>	349.1
SMI	8	49.4	26.0 <sup>a,b,c</sup>	31.9 <sup>b,c</sup>	17.1 <sup>b,c,d</sup>	13.3 <sup>d,e</sup>	37.2	19.5	9.0	30.2	233.7 <sup>a,b,c</sup>	369.5
SMI	24	15.4	9.3 <sup>e,f</sup>	12.2 <sup>d</sup>	10.6 <sup>d,e</sup>	5.8 <sup>f,g,h</sup>	11.1	10.5	5.9	10.8	91.6 <sup>e,f</sup>	166.4
SMI + AA	0	17.2	10.8 <sup>d,e,f</sup>	14.5 <sup>d</sup>	11.5 <sup>d,e</sup>	7.2 <sup>f,g,h</sup>	9.0	16.1	4.1	9.6	99.9 <sup>e,f</sup>	173.5
SMI + AA	2	51.7	28.2 <sup>a,b,c</sup>	41.8 <sup>a,b</sup>	22.4 <sup>a,b,c</sup>	24.6 <sup>b,c</sup>	28.1	30.3	8.8	43.6	279.3 <sup>a,b</sup>	457.5
SMI + AA	8	59.6	32.0 <sup>a</sup>	55.5 <sup>a</sup>	19.9 <sup>a,b,c</sup>	47.4 <sup>a</sup>	43.1	29.6	8.3	37.0	332.5 <sup>a</sup>	489.5
SMI + AA	24	20.3	11.6 <sup>d,e,f</sup>	15.6 <sup>c,d</sup>	12.0 <sup>d,e</sup>	13.4 <sup>d,e</sup>	15.5	15.0	6.8	11.6	121.7 <sup>d,e,f</sup>	200.2
Pooled SEM		3.4	1.6	2.8	1.1	2.0	2.1	1.2	0.4	2.3	15.7	23.3
ANOVA: P					LG10	SQRT				SQRT	SQRT	LG10
D		<0.001	0.002	<0.001	<0.001	<0.001	0.05	0.003	0.37	0.04	<0.001	<0.001
T		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
D × T		0.07	0.04	0.002	0.02	<0.001	0.14	0.10	0.79	0.69	0.03	0.15

T, time points; TAA, total free amino acids; FM, fishmeal diet; SMI, soyabean meal-incorporated diet; SMI + AA, SMI diet with dietary EAA supplementation; D, diet; LG10 indicates that data were transformed and statistically analysed with log transforms; SQRT indicates that data were transformed and statistically analysed with square roots. a,b,c,d,e,f,g,h Mean values among all treatments within a row with unlike superscript letters were significantly different ( $P < 0.05$ ).

\* Treatment means represent the average values for three tanks per treatment and were analysed by two-way ANOVA ( $n 3$ ). Tukey's test was conducted for individual means only if there was a significant interaction ( $P < 0.05$ ).

**Table 5.** Changes of muscle individual free essential amino acid (EAA) concentrations in turbot after refeeding ( $\mu\text{g}/\text{g}$ ) (Pooled standard errors)

Diets	T (h)	Leu	Ile	Val	Thr	Met	Phe	Lys	His	Arg	EAA	TAA
Individual treatment means*												
FM	0	19.0 <sup>e</sup>	13.2 <sup>c</sup>	16.9 <sup>c</sup>	103.4 <sup>b,c</sup>	8.9 <sup>c</sup>	9.9	62.8	10.7 <sup>c,d</sup>	38.3 <sup>c,d,e,f</sup>	279.6 <sup>b,c</sup>	2506.4 <sup>b,c</sup>
FM	2	34.8 <sup>b</sup>	16.5 <sup>a,b,c</sup>	25.5 <sup>a,b,c</sup>	80.4 <sup>c,d</sup>	13.7 <sup>c</sup>	18.8	30.7	14.7 <sup>c</sup>	31.5 <sup>d,e,f</sup>	266.5 <sup>c</sup>	2446.3 <sup>b,c</sup>
FM	8	48.9 <sup>a</sup>	20.6 <sup>b</sup>	37.0 <sup>a</sup>	128.6 <sup>b</sup>	23.4 <sup>b</sup>	28.2	62.7	35.4 <sup>a</sup>	106.3 <sup>a</sup>	491.1 <sup>a</sup>	3166.7 <sup>a</sup>
FM	24	36.6 <sup>a,b</sup>	21.1 <sup>a</sup>	32.8 <sup>a,b</sup>	178.5 <sup>a</sup>	12.3 <sup>c</sup>	23.1	126.2	35.9 <sup>a</sup>	110.9 <sup>a</sup>	577.4 <sup>a</sup>	2919.4 <sup>a,b</sup>
SMI	0	20.5 <sup>d,e</sup>	13.8 <sup>b,c</sup>	21.1 <sup>b,c</sup>	61.9 <sup>d</sup>	11.9 <sup>c</sup>	10.3	49.1	7.8 <sup>d</sup>	15.8 <sup>f</sup>	212.0 <sup>c</sup>	1830.6 <sup>e</sup>
SMI	2	31.4 <sup>b,c,d,e</sup>	16.9 <sup>a,b,c</sup>	21.3 <sup>b,c</sup>	49.7 <sup>d</sup>	8.9 <sup>c</sup>	17.8	21.2	11.9 <sup>c,d</sup>	22.5 <sup>e,f</sup>	201.5 <sup>c</sup>	2125.7 <sup>c,d,e</sup>
SMI	8	33.8 <sup>b,c</sup>	16.7 <sup>a,b,c</sup>	23.5 <sup>b,c</sup>	78.7 <sup>c,d</sup>	11.2 <sup>c</sup>	28.7	26.6	28.3 <sup>a,b</sup>	60.3 <sup>b,c</sup>	307.9 <sup>b,c</sup>	2403.0 <sup>c,d</sup>
SMI	24	20.7 <sup>d,e</sup>	11.6 <sup>a,b,c</sup>	15.1 <sup>c</sup>	83.5 <sup>c,d</sup>	8.1 <sup>c</sup>	15.9	49.4	14.7 <sup>c</sup>	52.3 <sup>b,c,d</sup>	269.5 <sup>c</sup>	2476.7 <sup>b,c</sup>
SMI + AA	0	21.2 <sup>c,d,e</sup>	15.7 <sup>a,b,c</sup>	23.8 <sup>b,c</sup>	80.0 <sup>c,d</sup>	20.4 <sup>c</sup>	5.3	34.3	10.7 <sup>c,d</sup>	22.9 <sup>e,f</sup>	234.2 <sup>c</sup>	1704.1 <sup>e</sup>
SMI + AA	2	32.9 <sup>a,b,c</sup>	20.4 <sup>a,b</sup>	29.4 <sup>a,b</sup>	71.6 <sup>c,d</sup>	23.8 <sup>b</sup>	12.6	14.2	14.9 <sup>c</sup>	32.0 <sup>d,e,f</sup>	251.9 <sup>c</sup>	1686.2 <sup>e</sup>
SMI + AA	8	36.7 <sup>a,b</sup>	20.2 <sup>a,b</sup>	31.0 <sup>a,b</sup>	103.9 <sup>b,c</sup>	34.5 <sup>a</sup>	24.1	29.6	32.8 <sup>a</sup>	70.2 <sup>b</sup>	382.8 <sup>b</sup>	2158.3 <sup>c,d,e</sup>
SMI + AA	24	25.0 <sup>b,c,d,e</sup>	17.1 <sup>a,b,c</sup>	25.6 <sup>a,b,c</sup>	88.0 <sup>b,c,d</sup>	22.0 <sup>b</sup>	10.1	61.6	22.7 <sup>b</sup>	42.6 <sup>c,d,e</sup>	308.1 <sup>b,c</sup>	1908.8 <sup>d,e</sup>
Pooled SEM		1.6	0.6	1.2	5.8	1.4	1.3	5.1	1.7	5.2	19.2	79.2
ANOVA: P							LG10	SQRT				
D		<0.001	0.004	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
T		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
D × T		0.01	0.03	0.002	0.001	0.000	0.23	0.20	0.001	<0.001	<0.001	0.02

T, time points; TAA, total free amino acids; FM, fishmeal diet; SMI, soyabean meal-incorporated diet; SMI + AA, SMI diet with dietary EAA supplementation; LG10 indicates that data were transformed and statistically analysed with log transforms; SQRT indicates that data were transformed and statistically analysed with square roots; D, diet. a,b,c,d,e,f,g,h Mean values among all treatments within a row with unlike superscript letters were significantly different ( $P < 0.05$ ).

\* Treatment means represent the average values for three tanks per treatment and were analysed by two-way ANOVA ( $n 3$ ). Tukey's test was conducted for individual means only if there was a significant interaction ( $P < 0.05$ ).

290 significant effect on the activation of these nutrient-sensing  
291 molecules except for phosphor-eIF2 $\alpha$  in liver and muscle, and  
292 ATF4 levels in liver (Fig. 2).

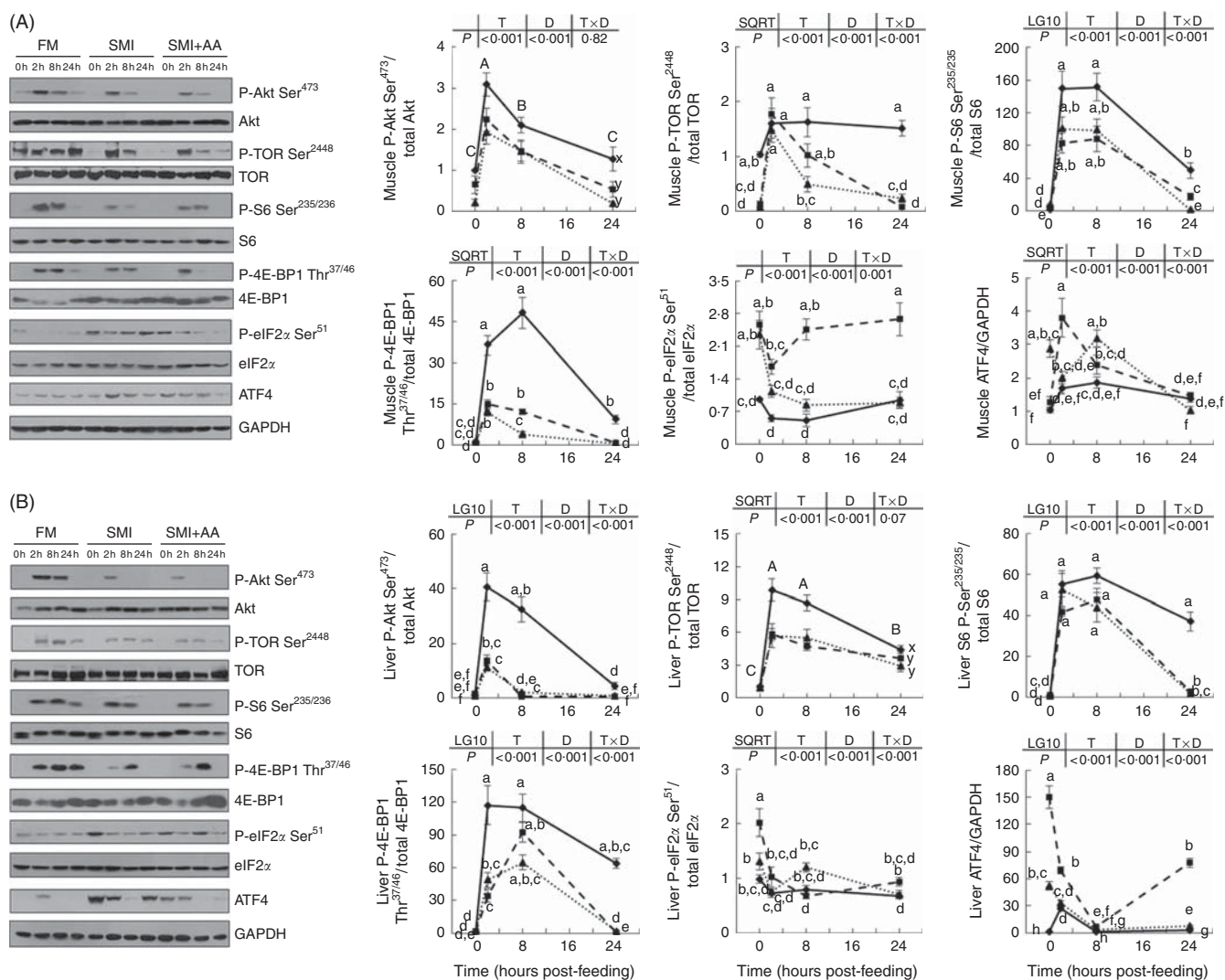
### 293 *Postprandial modulations of the metabolism* 294 *by dietary proteins*

295 Postprandial glucose and TAG reached peak concentration at  
296 2 and 8 h, respectively, after refeeding. However, the peak

values of both GLU and TAG were lower in SMI diets (SMI and  
SMI + AA), compared with those in the FM group (Fig. 3(A)).

The gene expressions of key metabolic enzymes in the liver  
were determined. In the FM group, the expressions of enzymes  
for glycolysis (*GK* and *PK*) (Fig. 3(B)), fatty acid synthesis  
(*SREBP1* and *FAS*) (Fig. 3(D)) and TAG synthesis (*DGAT1* and  
*DGAT2*) (Fig. 3(E)) were significantly up-regulated and peaked  
at 2–8 h after refeeding. The gene expressions of gluconeogenic  
enzymes (*FBPase* and *G6Pase*) were markedly down-regulated

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**Q6** **Fig. 2.** Dietary modulations of nutrient-sensing responses involving the total and phosphorylation levels of proteins related to target of rapamycin (TOR) and amino acid response signalling pathways in muscle (A) and liver (B). A representative blot is shown from replicated examinations (*n* 6). Values are means with their standard errors and were analysed by two-way ANOVA followed by Tukey's multiple range test. Values with unlike letters are significantly different (*P* < 0.05). FM, fishmeal diet (—●—); SMI, soyabean meal-incorporated diet (---■---); SMI+AA, SMI diet with dietary essential amino acids supplementation (---▲---); T, time points; D, diets; T × D, interaction between T and D; LG10 indicates that data were transformed and statistically analysed with log transforms; Akt, protein kinase B; S6, ribosomal protein S6; 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; eIF2 $\alpha$ , eukaryotic initiation factor 2 $\alpha$ ; ATF4, activating transcription factor 4; GAPDH, glyceraldehyde-3-phosphate dehydrogenase (see Fig. 1 legend for details).

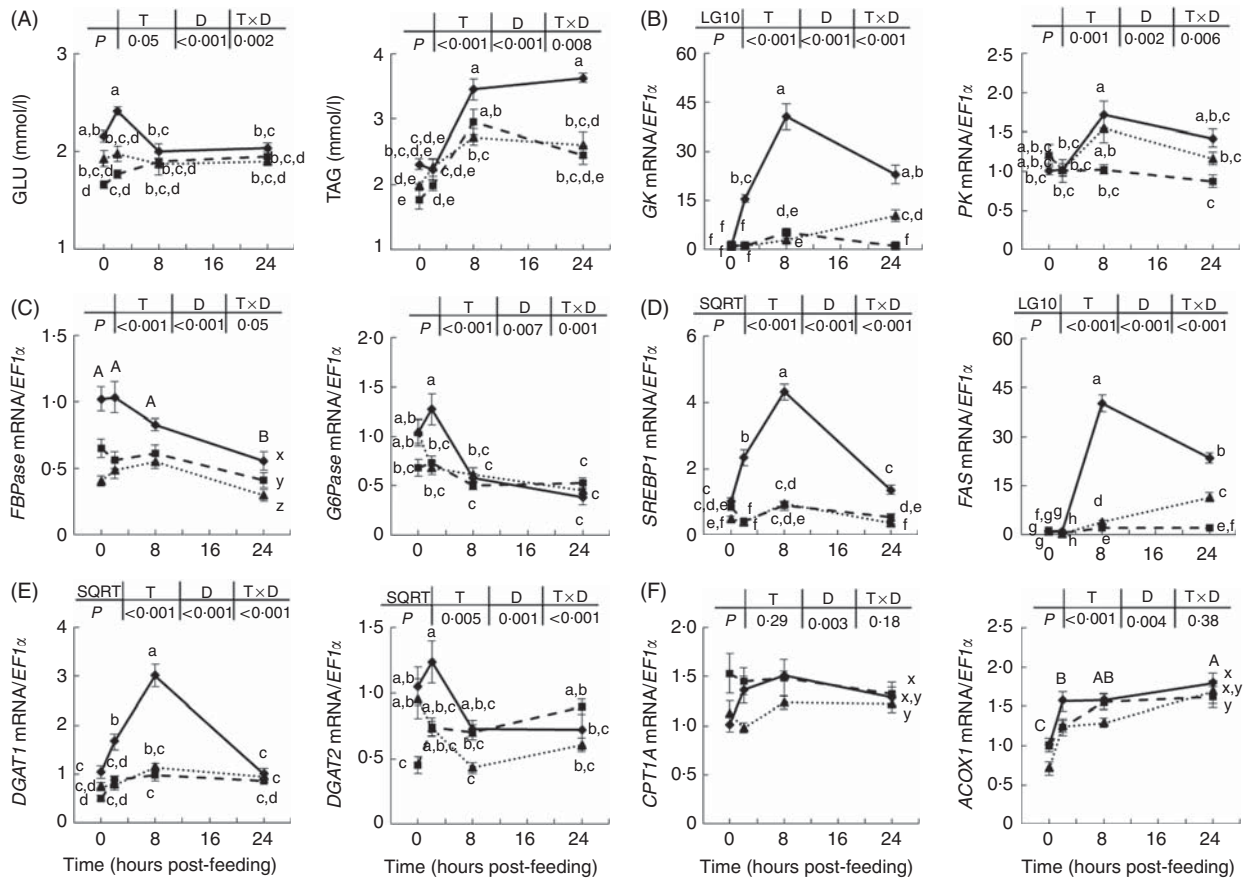
306 (Fig. 3(C)). In contrast, these postprandial modulations were not  
 307 obvious in the two SMI diets with lower transcription levels  
 308 than those of the FM diet. No significant difference was  
 309 observed for the gene expression involved in fatty acid  
 310 oxidation (*CPT1A* and *ACO11*) among different treatments  
 311 (Fig. 3(F)).

### 312 Discussion

313 Turbot is an aquaculture species that is highly sensitive to the  
 314 replacement of dietary FM by plant sources of protein. In pre-  
 315 vious reports, FM replacements over 20% using maize gluten  
 316 meal<sup>(27)</sup> and over 25% using soyabean meal concentrate<sup>(28)</sup>  
 317 were found to reduce growth rates in turbot. In accordance with  
 318 these studies, we observed that 45% dietary FM replacement by

319 soyabean meal reduced growth, feed utilisation and nutrient  
 320 retention, irrespective of EAA supplementation.

321 Cellular uptake and transport of peptide and amino acids in  
 322 the intestine represent a critical step for protein absorption.  
 323 *PepT1* is the major peptide transporter across the intestine brush  
 324 border membrane in fish<sup>(29)</sup>. We observed that *PepT1* expres-  
 325 sion peaked at 2 h after refeeding, which is consistent with  
 326 previous reports stating that the expression of *PepT1* was  
 327 up-regulated by refeeding and down-regulated by fasting in sea  
 328 bass<sup>(30)</sup>. *B<sup>0</sup>ATI* and *y<sup>+</sup>LAT1* were also identified in fish<sup>(31,32)</sup>  
 329 and had a major role in mediating EAA transport across the  
 330 apical and basolateral sides of the brush border membrane,  
 331 respectively<sup>(23,33)</sup>. Intestinal *B<sup>0</sup>ATI* was down-regulated,  
 332 whereas *y<sup>+</sup>LAT1* peaked at 8 h after refeeding, highlighting  
 333 their different mechanisms in response to feed ingestion.



**Fig. 3.** The dietary modulations of postprandial metabolism. (A) Plasma glucose (GLU) and TAG levels. Expression of selected enzymes involved in (B) glycolysis, (C) gluconeogenesis, (D) lipogenesis, (E) TAG synthesis and (F) fatty acid oxidation were analysed ( $n = 6$ ). Values are means with standard errors and were analysed by two-way ANOVA followed by Tukey's multiple range test.  $a, b, c, d, e, f, g, h; A, B, C; x, y, z$  Values with unlike letters are significantly different ( $P < 0.05$ ). Fishmeal diet (—◆—); soyabean meal-incorporated diet (—■—); soyabean meal-incorporated diet with dietary essential amino acid supplementation (---▲---); GK, glucokinase; *EF1 $\alpha$* , elongation factor 1 $\alpha$ ; PK, pyruvate kinase; *FBPase*, fructose 1,6-bisphosphatase; *G6Pase*, glucose 6 phosphatase; *FAS*, fatty acid synthase; *SREBP1*, sterol regulatory element-binding protein 1; *DGAT*, diacylglycerol *O*-acyltransferase homolog; *ACOX1*, acyl-CoA oxidase 1; *CPT1A*, carnitine palmitoyltransferase 1 isoforms A (see Fig. 1 legend for details).

334 *SNAT2*, a proven amino acid availability sensor in mammals<sup>(34)</sup>,  
 335 was down-regulated after refeeding, which is similar to obser-  
 336 vations from mammalian studies<sup>(35)</sup>. Among the transporters  
 337 characterised, gene expression of *Pept1* and *SNAT2* levels was  
 338 not influenced by different diets. However, *B<sup>0</sup>AT1* and *y<sup>+</sup>LAT1*  
 339 were less responsive to the SMI diet than the FM diet,  
 340 suggesting that different protein sources resulted in differential  
 341 responses of amino acid transporters, which in turn would  
 342 possibly have influenced amino acid absorption and transport  
 343 efficiency, accompanied by the change of plasma and muscle  
 344 free amino acid pools in the present study.

345 Our results demonstrated that postprandial peak values of  
 346 plasma free amino acids in circulation occurred 2 h after  
 347 refeeding and were transported to muscle in <8 h. This was  
 348 similar to observations of rainbow trout<sup>(36)</sup>, but slower than  
 349 those of rats<sup>(37)</sup>. Postprandial peak values of free amino acids in  
 350 the SMI group were much lower than in the FM group.  
 351 Supplementation of EAA to match the amino acid profile of the  
 352 FM diet in the SMI diets increased postprandial free amino acid  
 353 levels in plasma, but not in muscle. A similar result was found in  
 354 turbot after partial FM replacement by maize gluten meal<sup>(27)</sup>.  
 355 Crystalline amino acids were reported to have a lower retention

in muscle compared with protein-bound amino acids<sup>(38)</sup>. This  
 asynchronous amino acid retention may explain the inefficiency  
 of free amino acid supplementation in the SMI diet.

Intracellular sensing of amino acid availability is mediated  
 mainly by two distinct, yet complimentary, pathways – the AAR  
 and the TOR pathways – both of which regulate protein  
 synthesis and metabolism<sup>(8,39)</sup>. TOR activation is the primary  
 driving force for postprandial anabolism<sup>(6,7)</sup> and is required for  
 postprandial protein synthesis in response to amino acid  
 availability<sup>(40)</sup>. Consistent with findings in mammals<sup>(8)</sup> and  
 rainbow trout<sup>(41)</sup>, we observed that refeeding activated TOR  
 signalling. A previous study reported that increased amino acid  
 levels enhanced S6 kinase 1 and S6 phosphorylation in rainbow  
 trout primary hepatocytes<sup>(13)</sup>. In the present study, SMI diets  
 reduced the postprandial peak values of free amino acids  
 compared with the FM diet. Accordingly, it reduced the levels  
 and duration of postprandial phosphorylation of TOR, Akt, S6  
 and 4E-BP1, suggesting a hypoactivated TOR signalling status.  
 However, the SMI diet induced higher eIF2 $\alpha$  phosphorylation  
 and ATF4 levels, indicating hyperactivated AAR signalling and  
 inhibited cellular protein synthesis<sup>(39)</sup>. In particular, AAR  
 enhanced total 4E-BP1 in muscle during fasting and decreased

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its phosphorylation in FM replacement diets, further inhibiting protein translation in cells<sup>(42)</sup>. The combination of hypoactivated TOR signalling and hyperactivated AAR signalling would reduce postprandial protein synthesis<sup>(8,39)</sup>. Chronically, it would lead to lower protein accretion, evidenced by decreased protein retention after 1 month of SMI diet feeding.

TOR and AAR signalling not only control protein synthesis but also regulate metabolic gene expression, both in mammals and in fish<sup>(11,26,43,44)</sup>. In the present study, postprandial increased expressions of key enzymes involved in glycolysis and lipogenesis were associated with the activation of TOR signalling after refeeding, which is consistent with previous reports demonstrating that postprandial activation of hepatic *GK* and lipogenesis require TOR activation in rainbow trout<sup>(26)</sup>. Furthermore, we observed that SMI diets reduced tissue amino acid concentrations and TOR signalling activities, and suppressed the expression of genes involved in glycolysis and lipogenesis. As previous findings in trout hepatocytes showed that increased amino acid availability effectively up-regulated fatty acid synthetic and glycolytic genes expression in a TOR-dependent manner<sup>(13,45)</sup>, we presume that SMI diets reduced postprandial tissue amino acid concentration, which in turn suppressed TOR activation and subsequently down-regulated fatty acid synthetic and glycolytic gene expression. However, the soyabean meal-induced increase of eIF2 $\alpha$  phosphorylation and ATF4 levels was associated with down-regulation of hepatic lipogenic gene expression. These results were compatible with previous reports, concerning mice, that activation of the AAR signalling pathway reduced gene expression involved in fatty acid and TAG synthesis, alongside liver TAG and adipose tissue weight<sup>(11)</sup>. Thus, the reduced postprandial lipogenesis gene expression in SMI groups may be related to the combination effects of hypoactivated TOR signalling and hyperactivated AAR signalling. Chronically, it would lead to a lower body fat content of turbot. Therefore, our study provides a reasonable mechanistic explanation for reduced lipogenesis and fat deposition after FM replacement in many studies<sup>(46,47)</sup>.

It has been widely accepted that FM replacement in aquafeeds by plant proteins is limited by factors including low digestibility, imbalanced amino acid profile, anti-nutritional factors, presence or absence of other factors and so on, in plant proteins<sup>(1,2,17)</sup>. However, few studies have compared the responses by fish towards different protein sources at a molecular level, nor provided mechanistic explanations of the dietary effects. Our results, together with previous reports<sup>(18,26,41)</sup>, demonstrated that nutrient-sensing networks were conserved in fish, although varied in some aspects, such as the metabolism of glutamine in muscle<sup>(48)</sup>. The present study demonstrated a cascade of sophisticated, but mechanistically connected, responses towards partial FM replacement by soyabean meal in turbot: reduced postprandial influx of free amino acids led to hypoactivated TOR signalling, which in turn reduced protein synthesis and lipogenesis. In contrast, induced AAR responses further aggravated the postprandial anabolism. Such postprandial changes would lead to differences of growth and feed efficiency after long-term feeding. Further understanding the molecular responses of animals to different diets should pave the way for better feed utilisation.

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G. H. and K. M. designed the study. D. X., F. S., H. Z. and W. X. performed data acquisition and data analysis. G. H. and D. X. wrote the manuscript. All authors read and approved the final manuscript.

The authors declare no conflicts of interest.

## Supplementary material

For supplementary material/s referred to in this article, please visit <http://dx.doi.org/doi:10.1017/S0007114515004535>

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