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Fishmeal replacement by mixed plant proteins and maggot meal on growth performance, target of rapamycin signalling and metabolism in juvenile turbot (*Scophthalmus maximus L.*)

Q. WANG, G. HE, K. MAI, W. XU & H. ZHOU

Key Laboratory of Aquaculture Nutrition and Feed (Ministry of Agriculture), Key Laboratory of Mariculture (Ministry of Education), Ocean University of China, Qingdao, China

Abstract

Fishmeal could only be replaced by plant proteins at limited levels in aquafeeds, especially for carnivorous fish. In this study, an experiment was designed to evaluate the possibility of improving the utilization of plant proteins by maggot meal supplementation in turbot diet. Five diets were formulated: a reference diet (FM) containing 63% fishmeal and four experimental diets (35(0%), 35(3%), 40 (0%), 40(3%)) in which fishmeal was substituted at different levels by plant proteins with 0–3% maggot meal. Turbot (4.90 ± 0.03 g) was fed with these diets for 9 weeks. Fishmeal was successfully replaced by plant proteins in turbot diet without growth reduction at 35% but not 40%. However, maggot supplementation (3%) at 40% plant protein replacement level achieved comparable growth performance with that of fishmeal. Maggot meal supplementation improved apparent digestibility coefficients, plasma hydroxyproline levels, intestine trypsin activities and activated target of rapamycin (TOR) signalling, all of which were decreased or down-regulated after high plant protein replacement. Therefore, this study demonstrated maggot meal, a potential valuable protein source for turbot.

KEY WORDS: fishmeal replacement, maggot meal, nutrient sensing, turbot

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Correspondence: *G. He, Ocean University of China, 5th Yushan Rd, Qingdao 266003, China. E-mail: hegen@ouc.edu.cn*

Introduction

With the increasing demand for fishmeal by the fast-growing aquaculture industry, searching for fishmeal substitutes has been a major challenge (Hardy 2010). Many efforts have been conducted to reduce fishmeal consumption and utilization of alternative protein sources in diets (Gatlin *et al*. 2007).

As a carnivorous species, turbot requires high protein in diet (Lee *et al*. 2003). Previous studies on fishmeal replacement in turbot diet have focused on corn gluten meal (Regost *et al*. 1999), lupin (Burel *et al*. 2000), soya bean protein concentrate (Day & Gonzalez 2000) and plant protein mixture (Fournier *et al*. 2004). However, fishmeal substitution levels in turbot diet remained very low (Hardy 2010): less than 250 g kg^{-1} soya protein concentrate (Day & Gonzalez 2000) and 200 g kg^{-1} corn gluten meal (Regost *et al*. 1999) in diet could be tolerated by turbot. The fishmeal replacement levels may also be influenced by fish size and developmental stages due to different nutrient requirements (NRC 2011). Plant protein sources are low or even absent in some bioactive molecules, such as taurine and hydroxyproline, which are important for animal growth (Wang *et al*. 2014). Inclusion of animal by-products in plant protein-based diet can help to overcome these problems (Webster *et al*. 1999). Maggot meal is a high-quality protein source that was reported to replace fishmeal at high levels in rainbow trout (St-Hilaire *et al*. 2007), African catfish (Aneibo *et al*. 2009), Nile tilapia (Ogunji *et al*. 2008) and clariid catfish (Fasakin *et al*. 2003). No experiments were conducted to examine the performances of maggot meal on turbot before. This research was therefore conducted to evaluate the performances of fishmeal replacement in turbot diet with plant protein mixtures and maggot meal. In addition, many experiments have been carried out to evaluate the effect of fishmeal replacement. However, the underlying mechanism that limits fishmeal replacement remains largely unknown. It is known in multiple species that the activation of target of rapamycin (TOR) signalling is required for

protein synthesis and postprandial anabolism (Laplante & Sabatini 2012). Therefore, the TOR signalling activities under different protein sources were also studied.

Materials and methods

Diet formulation

Five isonitrogenous (500 g kg^{-1}) and isolipidic (125 g kg^{-1}) diets with fishmeal (FM) replaced at gradients by plant protein sources (soya bean meal, corn gluten meal, wheat gluten and peanut meal) as described before (Liu *et al*. 2014) with or without maggot meal addition (Table 1). Maggot meal was obtained from Guangzhou Xintai BioPro Co., and its composition was listed in Table 3. Crystalline amino acids were added to meet the essential amino acid requirement profile of turbot (Kaushik 1998). Composite attractant (betaine: dimethyl-β-propiothetin: threonine: glycine: inosine-5′-diphosphate trisodium salt = 3 : 4 : 1 : 1 : 1) and taurine were added to improve the diet palatability. Phytase was added to degrade the phytate in plant proteins and elevate protein digestibility. Additional mineral compounds were added to abolish the chelation by antinutritional factors. Y_2O_3 was added to evaluate the feed and nutrient digestibility.

Growth trial

Juvenile turbot were obtained from a fish-rearing farm (Laizhou, China). Fish (4.90 ± 0.03 g) were acclimated for

Table 1 Ingredient and nutrient composition of the experimental diets

	Diet 1 FM	Diet 2 35(0%)	Diet 3 35(3%)	Diet 4 40(0%)	Diet 5 40(3%)
Fish meal	63	40.95	40.95	37.8	37.8
Whole wheat meal	22.3	12.69	10.99	10.34	10.04
Soya bean meal	0	8	7.6	9.6	8.8
Corn gluten meal	0	8	7.6	9.6	8.8
Wheat gluten	0	7	6.7	8.4	7.7
Peanut meal	0	4	3.8	4.8	4.4
Maggot	0	0	3	0	3
Beer yeast	2.5	2.5	2.5	2.5	2.5
Vitamin premix[1]	1	1	1	1	1
Mineral premix[2]	2	2	2	2	2
Composite attractants[3]	1	1	1	1	1
Taurine	0	1	1	1	1
Binder (Na alginate)	0	1	1	1	1
Amino acid premix[4]	0	1.08	1.08	1.08	1.08
Fish oil	5.2	6.2	6.2	6.2	6.2
Soya bean lecithin	2.5	2.5	2.5	2.5	2.5
Choline chloride	0.25	0.25	0.25	0.25	0.25
Monocalcium phosphate	0	0.3	0.3	0.4	0.4
Phytase	0	0.2	0.2	0.2	0.2
Y_2O_3	0.1	0.1	0.1	0.1	0.1
Calcium propionate	0.1	0.1	0.1	0.1	0.1
Ethoxy quinoline	0.05	0.05	0.05	0.05	0.05
$FeSO_4.H_2O$	0	0.05	0.05	0.05	0.05
$ZnSO_4.H_2O$	0	0.03	0.03	0.03	0.03
Total	100	100	100	100	100
Proximate composition					
Crude protein (% DM)	50.26	51.13	51.74	51.95	52.06
Crude fat (% DM)	12.57	12.24	12.80	12.05	12.62
Ash (%DM)	12.44	12.21	12.56	12.13	12.15
Total energy(KJ g^{-1})	17.50	16.96	17.77	17.82	17.86

[1] Supplied the following(mg kg^{-1} diet):retinyl acetate, 32; cholecalciferol, 5; all-rac-a-tocopheryl acetate, 240; menadione sodium bisulphite, 10; ascorbic acid, 120; cyanocobalamin, 10; biotin, 60; choline dihydrogen citrate, 7 g; folic acid, 20; inositol, 800; niacin, 200; D-Ca-pantothenate, 60; pyridoxine HCl, 20; riboflavin, 45; thiamin HCl, 25,microcrystalline cellulose, 16473.
[2] Supplied the following (mg kg^{-1} diet): $MgSO_4$-$7H_2O$, 1200; $CuSO_4$-$7H_2O$, 10; $FeSO_4$-$7H_2O$, 80; $ZnSO_4$-H_2O, 50; $MnSO_4$-H_2O, 45; $COCl_2$, 5; Na_2SeO_3, 20; Calcium iodate, 60; Zeolite powder, 8485.
[3] Supplied the following (% diet): betaine, 0.4; DMPT, 0.2; threonine, 0.2; glycine, 0.1; inosine-5′-diphosphate trisodium salt, 0.1.
[4] Supplied (as L-racemer) the following (% diet): methionine (HCL): 0.48; threonine: 0.3; histidine: 0.17; lysine (HCL):0.13.

2 weeks before being randomly distributed into eighteen 400-L fibreglass tanks with 30 fish per tank in a circulating water system. The water flow was at 0.5 L min^{-1} and oxygen content of outlet water at higher than 85% saturation. Experiment was carried out in National Oceanographic Center, Qingdao. Day length and temperature increased over the course of the trial (July 12–September 13) following natural changes, while water temperature was maintained at 19 ± 2 °C. Each diet was randomly allocated in triplicate to fish for 9 weeks. Feed was offered by hand to apparent satiety in two meals per day (7 am and 7 pm), and feed intake was recorded daily to examine its palatability. Every 2 weeks, fish were counted and weighed under moderate anaesthesia (3-aminobenzoic acid ethyl ester, MS 222; 100 μg mL^{-1}). All works were performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Sample analysis

At the end of the growth trial, three fish per tank were randomly chosen and sacrificed after anesthetization with MS222 3 h after the final feeding and liver was harvested for Western blot analysis. Rest fish were sacrificed 24 h after feeding. All fish were weighed in bulk from each tank. Blood samples were collected from the caudal vein of six fish randomly selected from each tank. Plasma was obtained after centrifugation at 3000 ***g*** for 20 min at 4 °C and stored at −80 °C until further analyses. Muscle, liver and intestinal tract were collected and then stored at −80 °C for further assays.

Growth parameters The following variables were calculated:

Survival (%) = 100 × final number of fish/(initial number of fish);

Specific growth rate (SGR) (%/day) = (LnWt−LnW0)/t × 100;

Feed efficiency ratio (FER) = (Wt−W0)/dry feed intake;

Protein retention ratio (PRR) = 100 × (final weight× protein content in final fish)/(Initial weight × protein content in initial fish);

Feed intake (FI) (%/day) =dry feed intake × 2/((Wt+W0) × t);

Condition factor (%) = 100 × (body weight)/(body length^3);

Hepatosomatic index (HSI) (%) = 100 × (liver weight)/(body weight);

Viscerosomatic index (VSI) (%) = 100 × (visceral weight)/(body weight); and

Wt and W0 mean the final and initial weight of turbot, while t means the rearing days.

Feed and Body composition analysis Moisture, crude protein, crude lipid, ash and energy were analysed for ingredients, experimental diets and fish samples using AOAC (2012) methods. Dry matter was analysed by drying the samples to constant weight at 105 °C. Crude protein was determined by using the Kjeldahl method (Kjeltec TM 8400, FOSS, Sweden) and estimated by multiplying nitrogen by 6.25. Crude lipid was measured after diethyl ether extraction using Soxhlet method (Buchi 36680, Switzerland). Ash was examined after combustion in a muffle furnace at 550 °C for 16 h. Gross energy was determined with Parr1281 Automatic Bomb Calorimeter (Parr, Moline, IL, USA).

Plasma hydroxyproline content was determined using a procedure as described by Reddy & Enwemeka (1996) with modifications. Aliquots of 100 μL plasma samples were mixed with 2 mL buffered chloramines T reagent (1.4 g chloramines T dissolved in 20 mL water and then diluted with 30 mL n-propanol and 50 mL acetate–citrate buffer (pH 6.5); made fresh daily) and incubated for 20 min at room temperature. Then, 2 mL perchloric acid (27 mL 70% perchloric acid diluted into 100 mL volumetric flasks) was added and the mixture was incubated for a further 5 min at room temperature before addition of 2 mL P-DMAB solution (10% w/v P-DMAB in n-propanol). The mixture was heated for 20 min at 60 °C and then cooled immediately. The absorbance was measured at 560 nm and the unit was expressed as μg per mL.

Digestibility Faeces were drawn 3 h after feeding from the bottom of tanks, which is the only feasible method for a small size fish. However, the digestibility might be influenced by leaching in water. Dry matter, protein and energy contents were determined as described above. Yttrium oxide in the diet and faeces was determined according to the method described by Mai & Tan (2000) with modifications. Briefly, samples were digested in perchloric acid at a ratio of 1 : 50 (w/v) and then diluted to 100 mL. The apparent digestibility coefficients of nutrients and energy were calculated as follows: ADC (100%)=(1−(Y % in diet)/(Y % in faeces))*(%nutrient or energy in faeces/% nutrient or energy in diet)*(100%). For dry matter, the apparent digestibility coefficients were as follows: ADC (100%) = (1− (Y% in diet)/(Y% in faeces))*100%.

Enzyme assays Intestinal samples were homogenized in ice-cold water in the proportion of 1:9 (w/v). Following centrifugation (1800 × ***g***, 30 min, 4 °C), the supernatants were removed and kept at 4 °C for analysis. Alpha-amylase (E.C. 3.2.1.1) activity was measured according to Worthington (1993), and the enzyme activity was expressed as U mg/protein, with 1U indicating 10 mg starch hydrolysed per 30 min. Lipase activity was assayed based on measurements of fatty acids released due to enzymatic hydrolysis of triglycerides in a stabilized emulsion of olive oil according to Borlongan (1990), and the enzyme activity was expressed as U mg/protein, with 1U indicating 1 μmol triglycerides hydrolysed per minute. Trypsin activity was assayed by specific kits and the enzyme activity was expressed as U mg/protein, with 1U indicating 1 μmol substrate hydrolysed per minute. Enzymatic kits for amylase, lipase and trypsin were provided by Jiancheng Bioengineering Institute (Nanjing, China).

Western blot

Proteins from tissue homogenates were separated on SDS-PAGE. Proteins were transferred to PVDF membranes (Pall Corporation) for Western blot analysis. Primary antibodies for TOR (total and phosphor-Ser2448) and S6 (total and phosphor-Ser235) were from Cell Signaling. Antibody for GAPDH was from Hangzhou Goodhere Biotechnology.

Statistical analysis

Results were analysed by one-way ANOVA for the effects of fishmeal replacement by other mixed proteins. Homogeneity of variance test was conducted to ensure that variance is homogeneous. Turkey's test was utilized to compare individual means. Differences were considered significant at $P < 0.05$.

Results

Survival rate and growth performance

During the experimental period, survival rate (100%) was not affected by treatments. As shown in Table 2, compared to FM, 35(0%) diet did not influence growth performance of turbot. However, 40(0%) showed significant decrease in SGR and FBW. After maggot meal supplementation, 40 (3%) showed similar growth performances to fishmeal diet. FER and PPR of plant protein replacement at both 35 (0%) and 40(0%) were significantly lower than that of FM ($P < 0.05$). However, maggot meal supplemented groups, 35(3%) and 40(3%), showed similar FER to FM and increased PPR compared to 35(0%) and 40(0%) (Table 2). No significant differences in CF, VSI and HSI values were found among all treatments (Table 2).

Body composition

No significant changes in whole body protein, lipid, moisture and ash content were found in dietary treatments, compared to FM (Table 3). After plant protein replacement at both 35% and 40% levels, plasma hydroxyproline concentration was reduced significantly compared to that of turbot fed with fishmeal (separately 32.60 ± 5.38 and 28.54 ± 0.92 μg mL^{-1}). Maggot meal supplementation

Table 2 Final body weight (FBW), specific growth rate (SGR), feed intake (FI), feed efficiency ratio (FER), protein retention ratio (PPR), condition factor (CF), hepatosomatic index (HIS) and Viscerosomatic index (VSI) of turbot fed diets substituting fishmeal with mixed plant proteins with (or without) maggot meal for 9 weeks (means of triplicate±SE)

	FM	35 (0%)	35 (3%)	40 (0%)	40 (3%)
FBW	37.84 ± 0.79^{b}	35.68 ± 0.99^{ab}	36.03 ± 1.07^{ab}	33.43 ± 0.59^{a}	35.06 ± 0.47^{ab}
SGR[1]	3.24 ± 0.06^{b}	3.17 ± 0.08^{ab}	3.16 ± 0.08^{ab}	3.05 ± 0.05^{a}	3.12 ± 0.04^{ab}
FI[2]	1.75 ± 0.03^{a}	1.96 ± 0.03^{b}	1.88 ± 0.02^{ab}	1.87 ± 0.07^{ab}	1.82 ± 0.08^{ab}
FER[3]	1.46 ± 0.03^{b}	1.33 ± 0.02^{a}	1.35 ± 0.02^{ab}	1.33 ± 0.01^{a}	1.38 ± 0.06^{ab}
PPR[4]	47.65 ± 0.44^{c}	41.97 ± 0.34^{a}	43.20 ± 0.35^{ab}	43.51 ± 0.17^{ab}	44.86 ± 0.88^{b}
CF[5]	4.13 ± 0.22	4.11 ± 0.23	4.19 ± 0.37	3.96 ± 0.34	4.04 ± 0.16
HSI[6]	1.03 ± 0.24	0.97 ± 0.10	0.95 ± 0.14	1.06 ± 0.13	0.92 ± 0.02
VSI[7]	5.37 ± 0.20	5.21 ± 0.40	5.26 ± 0.36	5.31 ± 0.37	5.02 ± 0.38

[1] Specific growth rate (SGR) (%/day) = (LnWt−LnW0) /t× 100;
[2] Feed intake (FI) (%/day) = dry feed intake × 2 /((Wt + W0) × t);
[3] Feed efficiency ratio (FER) = Weight gain of fish/dry feed intake;
[4] Protein retention ratio (PRR) = 100 × (final weight× protein content in final fish)/ (Initial weight × protein content in initial fish);
[5] Condition factor (%) = 100 × (body weight)/(body length^{3});
[6] Hepatosomatic index (HSI) (%) = 100 × (liver weight)/ (body weight);
[7] Viscerosomatic index (VSI) (%) = 100 × (visceral weight)/ (body weight);
Mean values not sharing a common letter were considered significantly different ($P < 0.05$).

Table 3 Proximate moisture (% of wet weight) and protein, lipid and ash (% of dry weight) composition of maggot meal and turbot at the end of the 9-week feeding trial (means of triplicate±SE)

	Maggot meal	FM	35(0%)	35(3%)	40(0%)	40(3%)
Moisture	6.65%	76.48 ± 0.79%	76.73 ± 0.52%	76.86 ± 0.66%	76.60 ± 0.30%	76.52 ± 0.42%
Crude protein	58.51%	68.34 ± 0.31%^{abc}	67.49 ± 0.59%^{a}	68.69 ± 0.18%^{bc}	67.67 ± 0.35%^{ab}	69.28 ± 0.35%^{c}
Crude lipid	20.73%	13.35 ± 0.28%^{a}	14.47 ± 0.49%^{ab}	13.78 ± 0.93%^{ab}	13.78 ± 0.66%^{ab}	14.93 ± 1.02%^{b}
Ash	5.78%	15.43 ± 0.45%	15.29 ± 0.21%	15.43 ± 0.40%	15.34 ± 0.17%	15.24 ± 0.24%

Mean values not sharing a common letter were considered significantly different ($P < 0.05$).

Table 4 Apparent digestibility coefficients of dry matter, protein and energy in turbot fed experimental diets at the end of the 9-week feeding trial (means of triplicate±SE)

ADC (%)	FM	35(0%)	35(3%)	40(0%)	40(3%)
Dry diet[1]	55.92 ± 1.43%^{b}	54.70 ± 6.24%^{ab}	59.90 ± 3.83%^{ab}	48.02 ± 2.83%^{a}	60.65 ± 1.78%^{b}
Protein[2]	64.01 ± 1.31%^{c}	63.48 ± 0.46%^{c}	58.94 ± 1.62%^{ab}	56.12 ± 1.88%^{a}	62.44 ± 1.82%^{bc}
Energy[3]	76.08 ± 0.88%^{c}	72.20 ± 0.34%^{b}	80.40 ± 0.78%^{d}	64.08 ± 1.56%^{a}	78.71 ± 1.05%^{cd}

[1] Apparent digestibility coefficients of dry diet = (1−(Y% in diet)/(Y% in faeces))*100%.
[2] Apparent digestibility coefficients of dietary protein= (1−(Y % in diet)/(Y % in faeces))*(% protein in faeces/% protein in diet)*(100%).
[3] Apparent digestibility coefficients of dietary energy = (1− (Y % in diet)/(Y % in faeces))*(% energy in faeces/ % energy in diet)*(100%).
Mean values not sharing a common letter were considered significantly different ($P < 0.05$).

Table 5 Alpha-amylase, lipase and trypsin in the intestine of turbot fed diets substituting fishmeal with mixed plant proteins with (or without) maggot meal for 9 weeks (means of triplicate±SE)

	FM	35(0%)	35(3%)	40(0%)	40(3%)
Alpha-amylase (U mg/protein)	0.90 ± 0.08^{b}	1.11 ± 0.26^{b}	0.86 ± 0.22^{b}	1.85 ± 0.27^{a}	1.02 ± 0.42^{b}
Lipase (U mg/protein)	6.37 ± 1.26	6.25 ± 0.65	4.88 ± 0.29	7.97 ± 1.59	6.31 ± 0.48
Trypsin (U mg/protein)	31.33 ± 7.03^{b}	11.81 ± 5.40^{ab}	21.55 ± 0.97^{ab}	4.60 ± 3.38^{a}	30.34 ± 2.86^{b}

Mean values not sharing a common letter were considered significantly different ($P < 0.05$).

significantly increased plasma hydroxyproline levels (separately 42.67 ± 3.06 and 42.62 ± 1.61 μg mL^{-1}), although it was still lower than that in fishmeal fed group (71.08 ± 4.07 μg mL^{-1}).

Digestibility

Compared to fishmeal group, 40(0%) diet reduced significantly the apparent digestibility coefficients of dry matter, protein and energy (Table 4). However, maggot meal supplementation improved these coefficients to levels comparable to those of fishmeal group.

Digestive enzymes

Alpha-amylases, lipases and trypsin in the intestine were assayed (Table 5). At 40% plant protein replacement level, the alpha-amylase activity increased but trypsin activity decreased significantly compared to those of fishmeal group, while maggot meal supplementation reversed these changes to activities similar to those observed in fishmeal group. To the contrary, lipase activity was not influenced among the groups.

TOR signalling

The levels of protein nutrient-sensing molecules, TOR and S6, were not influenced among the groups (Fig. 1). However, the phosphorylation of TOR and S6 levels, indicating their activation, were decreased dramatically in 40(0%) group while recovered after maggot meal supplementation.

Discussion

Plant proteins have intrinsic limitations for fishmeal replacement in aquafeeds because of shortage of multiple bioactive molecules including taurine (Wang *et al.* 2014) and hydroxyproline (Liu *et al.* 2014). Therefore, exploration of

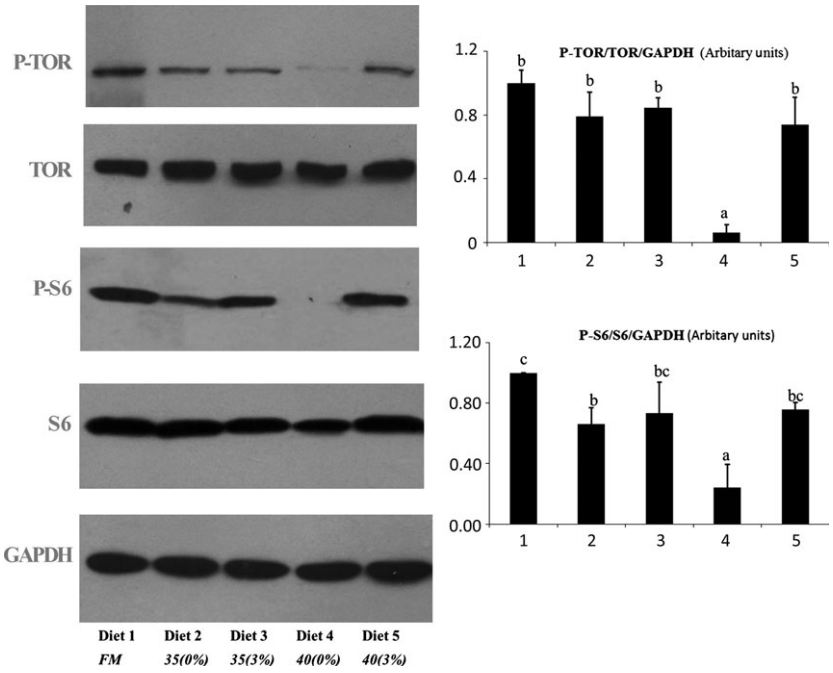

Figure 1 TOR, p-TOR, S6 and p-S6 relative protein level in liver of turbot fed diets substituting fishmeal with mixed plant proteins with (or without) maggot meal for 9 weeks (means of triplicate±SE). TOR and S6 protein levels did not change among all the treatments, but p-TOR and p-S6 in 40 (0%) showed a significant decrease compared to other treatments.

combined mixtures of plant and animal proteins for aquafeeds would be feasible for improvement of non-fishmeal diet utilization (Webster *et al*. 1999). In the present study, plant proteins could replace 35% fishmeal without growth reduction but not higher. However, 3% maggot meal supplementation would increase the replacement level at least to 40%. In this experiment, we assayed the whole composition of maggot meal, which has a protein level at 585 g kg^{-1} and a fat level at 207 g kg^{-1} (Table 3), and the same amount of methionine, lysine and arginine as fishmeal and higher content of histidine, tyrosine and phenylalanine than fishmeal (data not shown).

Maggot meal improved FER, protein retention, apparent digestibility coefficients and trypsin activity in intestine. Furthermore, the plasma-free hydroxyproline was significantly decreased after fishmeal replacement by plant proteins, which could potentially jeopardize collagen biosynthesis (Uitto *et al*. 1976; and Chojkier *et al*. 1983) and muscle growth. Supplementation of maggot significantly improved this deficit. These all suggest a role of maggot meal in promoting growth of turbot, which is similar to results of the combination of plant proteins and yeast (Muzinic *et al*. 2004; Trosvik *et al*. 2013) and plant protein and poultry by-product (Webster *et al*. 1999). In fact, maggot meal has been used to replace fishmeal in rainbow trout (St-Hilaire *et al*. 2007), African catfish (Aneibo *et al*. 2009), Nile tilapia (Ogunji *et al*. 2008) and clariid catfish (Fasakin *et al*. 2003). All those experiments showed that maggot could partially or totally replace fishmeal without affecting the growth performance and no antinutrients were found in maggot meal in those experiments.

Upon activation, TOR signal pathway provides the driving force for protein synthesis and anabolism (Ma & Blenis 2009 and Laplante & Sabatini 2012). In this experiment, after fishmeal replacement, the SGR of fish in 40(0%) showed a significant decrease compared to FM, However, when Western blots of TOR, p-TOR, S6 and p-S6 were conducted, p-TOR and p-S6 were significantly decreased in 40(0%). In 40(0%), when 40% of fishmeal was replaced by plant proteins without added maggot meal, the unbalanced amino acid profile in the diet was unable to activate TOR signalling pathway and protein synthesis was inhibited, which was reflected in the final growth performance.

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Conflict of interest

The authors declared that there is no conflict of interest.

Authorship

Gen He and Kangsen Mai designed the research. Qingchao Wang conducted the research. Wei Xu and Huihui Zhou provided the essential reagents and materials. Gen He and Qingchao Wang wrote the manuscript. All authors have read and approved the final manuscript.

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