



The TOR pathway participates in the regulation of growth development in juvenile spotted drum (*Nibea diacanthus*) under different dietary hydroxyproline supplementation

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Abstract Commonly used aquatic feed naturally contains low-level or no hydroxyproline (Hyp). This study was conducted to evaluate the effects of dietary Hyp inclusion on growth performance, body composition, amino acid profiles, blood biochemistry, and the expression of target of rapamycin (TOR) pathway-related genes in juvenile *Nibea diacanthus*. Fish with similar size (initial body weight, 133.00 ± 2.14 g) were fed six isonitrogenous and isolipidic practical diets supplemented with graded levels of Hyp (0, 5, 10, 15, 20, and 25 g kg^{-1} of dry matter) for 8 weeks. The results indicated that growth performance and feed utilization were improved with increased levels of dietary Hyp ($P < 0.05$), and the optimum amount of dietary Hyp estimated from SGR as 16.6 g kg^{-1} . The crude protein of whole body

and swim bladder and the amino acid composition of muscle and swim bladder were significantly ($P < 0.05$) affected by the addition of dietary Hyp, which reflects the important role of feed composition in animal body composition. In addition, the expression levels of mammalian target of rapamycin (TOR) and ribosomal protein S6 kinase1 (S6K1) genes in the liver, muscle, and swim bladder increased with increasing Hyp content of diets, while the mRNA expression level of eukaryotic translation initiation factor 4E-binding protein (4E-BP) gene in these tissues decreased. These results indicated that Hyp improved fish growth and the ability to synthesize proteins, most likely through the TOR pathway. It is suggested that dietary Hyp supplementation is particularly necessary for application in aquatic feed.

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Introduction

Hydroxyproline (Hyp), which is mainly found in collagen, is not a natural free amino acid (Li and Wu 2018). All animals generate Hyp from proline (Pro), as it is a post-translational metabolite from proline-containing collagen (Li et al. 2009). Although Hyp can be generated from Pro in collagen by vitamin C-dependent prolyl hydroxylase, this metabolic process requires large energy input (6 mol ATP/mol Hyp, i.e., 4 mol ATP for collagen synthesis and 2 mol ATP for collagen

degradation) (Wu 2013; Li and Wu 2018). Thus, it is speculated that direct provision of Hyp in diets could save the huge energy required for promoting the growth of animals. Previous studies have shown that the addition of Hyp to feed has beneficial effects in animals. For instance, a plant-based protein diet supplemented with crystalline Hyp enhanced weight gain of salmon (*Salmo salar* L.) (Aksnes et al. 2008). Hou and Wu (2017) reported that adding an appropriate amount of Hyp to the diet can affect animal growth, muscle structure, and collagen content. It has also been reported that Hyp can scavenge oxidants and regulate the redox state of cells (Phang et al. 2008). Considering that the Hyp content in the feed is low, and there is energy waste in the biosynthesis of Hyp, it is therefore valuable to investigate the effect of dietary Hyp and to determine the possible impact of Hyp on growth when formulating diets.

Nibeia diacanthus is a large predatory fish that grows fast and lives long due to its disease resistance, as well as its strong adaptability, for which reasons that it is easy to breed. Due to these excellent characteristics, *N. diacanthus* has been well received by farmers in the south of China (Chen et al. 2011). In recent year, we found that *N. diacanthus* is regarded as an excellent food fish, especially, its large swim bladder, which is considered a tonic with a high market value and sales volume in Southeast Asia, particularly in Hong Kong and Southern China (Rong et al. 2019, 2020). However, there are few studies on *N. diacanthus*, especially the effects of dietary Hyp on growth performance of juvenile *N. diacanthus*, have not been well explored. Similarly, little is known about the effect of dietary Hyp on cell signaling, amino acid-sensing pathways, nutritional regulation, and the pathways for Hyp metabolism in fish. It is known that the mammalian target of rapamycin pathway (mTOR) has an important amino acid-sensing mechanism, and affects cell growth and function by integrating signals from nutrients (glucose and amino acid) (Holz et al. 2005; Wullschleger et al. 2006). Many studies have shown that various amino acids, e.g., tryptophan (Tang et al. 2013), arginine (Chen et al. 2012), proline (Liao et al. 2008), leucine (Zhou et al. 2019), lysine (Li et al. 2019), and methionine (He et al. 2019), improve growth and enhance protein synthesis of fish by activating the TOR signaling pathway. However, the effects of dietary Hyp on growth performance of *N. diacanthus* and whether this is through the activation of TOR signaling have not been studied. Therefore, this study sought to investigate the effects of Hyp on growth,

biochemical composition, and the expression of TOR-related genes in juvenile *N. diacanthus*-fed diets with different levels of Hyp. It is hoped that the findings here would give more insight on the functional properties of Hyp and therefore provide theoretical basis for the development of aquatic feed.

Materials and methods

Experimental diets

The basal practical diet (42.34% crude protein, 8.35% crude lipid, and 10.94% crude ash) was formulated according to our previous study (Rong et al. 2019), and Hyp was considered as the limiting factor to support the optimum growth of *N. diacanthus*. Six isonitrogenous and isolipidic diets with graded levels of extra addition of Hyp (0, 5, 10, 15, 20, and 25 g kg⁻¹ diet) were formulated (Table 1). Crude Hyp powder (a mixture of 4-hydroxyprolyl and 3-hydroxyprolyl, with the main components being 4-hydroxyproline) was purchased from Hebei Runying Biotechnology (Hebei, China). As shown in Table 1, fish meal, mixed amino acids, and crystalline amino acids were used as the main dietary protein sources, while fish oil was the main dietary lipid source. The quantity of Hyp was increased at the expense of glutamic acid to make the diets isonitrogenous, as glutamate levels not affect the parameters being measured. The levels of other nutrients met the requirements for *N. diacanthus* according to the National Research Council (NRC 2011). The levels of dietary amino acids are shown in Table 2. Feedstuffs were sifted through a 0.3-mm sieve and mixed thoroughly in ratios before being puffed with a 2.5-mm diameter extruder, and then air-dried in a natural ventilated environment. Dried pellets were sealed in plastic bags and stored at -20 °C until use.

Experimental systems and feeding

Experiments were carried out in floating pens at Nan'ao Marine Biology Station (NAMBS), Shantou University, Shantou, China. Fish (*N. diacanthus*) were obtained from a local marine fish hatchery (Raoping, Guangdong, China). Fish were reared in floating net pens (3 × 3 × 2 m, L:W:H) and fed with a commercial diet (40.0% crude protein, 10.0% crude lipid, Tongwei, Jieyang, China) for 2 weeks to allow them acclimatize

to the new environment. Before being used for the experiments, fish were fasted for 24 h after which they were weighed and anesthetized with 40 mg/L eugenol solution (Aladdin reagent Co., Ltd., China) to avoid being stressed. Next, 450 fish (133 ± 2.14 g) were randomly distributed into 18 pens ($1 \times 1 \times 1.5$ m, L:W:H) with 25 fish per pen. Three pens were randomly assigned to one of the six dietary treatments. Fish were hand-fed to apparent satiation twice (07:00 and 16:30 h) daily for 8 weeks. Satiation of fish was considered when they did not show feeding behavior towards the pellets. The amount of food (pellets) consumed in each pen was recorded. During the experiments, the temperature ranged from 23 to 30 °C, pH 7.8 to 8.1, ammonia nitrogen lower than 0.05 mg L⁻¹, salinity 31 to 33 g L⁻¹, and dissolved oxygen 5.2 to 6 mg L⁻¹. All the animal experiments were conducted according to the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of the People's Republic of China on October 31, 1988.

Sample collection

Samples were collected following similar procedures previously described (Rong et al. 2019). At the end of the feeding trial, fish were fasted for 24 h before being used for subsequent experiments. All experimental fish were anaesthetized with 1:10,000 eugenol (purity 99%, Shanghai Reagent, China) to avoid stress before being weighed and counted to calculate fish growth and feed utilization. Four randomly collected fish from each pen was stored at -20 °C for whole body composition analysis. Another six fish from each pen were collected for blood samples, dorsal muscle, and swim bladder tissues. First, blood was withdrawn from the caudal vein using syringes, centrifuged (4000g for 10 min) at 4 °C to obtain serum samples before being snap frozen in liquid nitrogen and stored at -80 °C until analysis. Next, the fish were slaughtered and the dorsal muscle and swim bladder tissues sampled for amino acids analysis, while individual body weight, liver weight, and visceral weight were recorded for the calculation of hepatosomatic index and viserosomatic index. Four fish from each pen were sampled to obtain liver, muscle, intestinal, and swim bladder tissues. The obtained tissues were snap frozen in liquid nitrogen before being stored at -80 °C for later RNA extraction and real-time quantitative PCR analysis.

Calculation of growth parameters

Fish body weight (BW), initial body weight (IBW), final body weight (FBW), body length (BL), feed consumed (FC), protein intake (PI), viscera weight (VW), and liver weight (LW) were measured and recorded at the feeding trial. Growth performance was assessed in terms of percentage and efficiency ratio of feed and protein, while the body weight and body length were used to calculate the following parameters:

Specific growth rate (SGR, %day⁻¹)

$$= [(LnFBW (g) - LnIBW (g)) / \text{days}] \times 100$$

Weight gain (WG, %)

$$= (FBW (g) - IBW (g)) / IBW (g) \times 100$$

Feed conversion ratio (FCR)

$$= FC (g) / (FBW (g) - IBW (g))$$

Protein efficiency ratio (PER)

$$= (FBW (g) - IBW (g)) / PI (g)$$

Hepatosomatic index (HSI, %)

$$= 100 \times LW (g) / BW (g)$$

Viscerosomatic index (VSI, %)

$$= 100 \times VW (g) / BW (g)$$

Survival rate (SR, %) = 100

$$\times \text{final fish number} / \text{initial fish number.}$$

Proximate composition analysis

Proximate composition of diets and whole body expressed on a wet-based mass was determined in triplicates according to standard methods of the Association

Table 1 Formulation and proximate composition of the experimental diets

Ingredient composition (g kg ⁻¹)	Diets					
	0 Hyp	5 Hyp	10 Hyp	15 Hyp	20 Hyp	25 Hyp
¹ Fish meal	345	345	345	345	345	345
Fish oil	56	56	56	56	56	56
Starch	222.5	222.5	222.5	222.5	222.5	222.5
² Mixed amino acids	177	177	177	177	177	177
Choline chloride	10	10	10	10	10	10
Cellulose microcrystalline	134.5	134.5	134.5	134.5	134.5	134.5
Monocalcium phosphate	10	10	10	10	10	10
³ Hydroxyproline	0	5	10	15	20	25
Glutamic	25	20	15	10	5	0
⁴ Mineral premix	10	10	10	10	10	10
⁵ Vitamin premix	10	10	10	10	10	10
Proximate composition (%)						
Crude protein	42.34	42.38	42.45	42.19	42.06	42.22
Crude lipid	8.45	8.39	8.40	8.38	8.47	8.41
Crude ash	10.94	10.87	10.93	12.00	12.27	11.35
Hydroxyproline (% dry matter)	0.03	0.51	1.05	1.48	2.06	2.54

¹ The white fish meal of the USA from arctic cod: crude protein content of 64.7%, crude fat content of 10%

² Mixed crystalline amino acid (g/kg diet): glycine, 132.9; alanine, 36.3; arginine, 2.8; phenylalanine, 3.1; threonine, 1.8

³ The Hyp used in this study was derived from biological collagen lysates, which is made up of a mixture of 4-hydroxylprolyl and 3-hydroxylprolyl, with 4-hydroxyproline being the main component

⁴ Mineral premix (mg/kg diet): NaF, 1; KI, 0.4; CoCl₂·6H₂O, 25; CuSO₄·5H₂O, 5.0; FeSO₄·H₂O, 40; ZnSO₄·H₂O, 25; MnSO₄·H₂O, 30; MgSO₄·7H₂O, 600; Ca(H₂PO₄)₂·H₂O, 1500; NaCl, 50; zeolite, 7725

⁵ Vitamin premix (mg/kg diet): thiamin, 25; riboflavin, 45; pyridoxineHCl, 20; vitamin B12, 0.1; vitamin K3, 10; inositol, 800; pantothenic acid, 60; niacin acid, 200; folic acid, 20; biotin, 1.20; retinal acetate, 32; cholecalciferol, 5; α-tocopherol, 120; ascorbic acid, 2000; ethoxyquin, 150

of Official Analytical Chemists (AOAC 1995). Analysis of dry matter (105 °C, 24 h), crude protein (Kjeldahl nitrogen N × 6.25), crude lipid (Soxhlet method), and crude ash (550 °C, 6 h) was performed following standard laboratory procedures.

Amino acid content determination

The amino acid composition of samples was analyzed using an HPLC-Ultimate 3000 (Thermo Scientific Dionex, USA) based on a national standard method (GB/T 18246-2000) and method used in our previous work (Rong et al. 2020). Briefly, freeze-dried samples were hydrolyzed at 110 °C for 24 h with 15 mL of 6 mol L⁻¹ HCl. Next, the protein hydrolysate was filtered through a 0.45-μm filter with a sterile syringe, and 10 μL of filtered solution was injected into the HPLC system with a sodium cation-exchange column (3.0 ×

250.0 mm; Pickering, CA, USA) for the chromatographic separation at 48 °C. Ninhydrin was used as the post-column derivatization reagent, and pump flow rate was 0.3 mL min⁻¹ at 130 °C. The absorbance was recorded by ultraviolet detection at a wavelength of 440 nm (for proline) and 570 nm (for other amino acids). The identity and quantity of the amino acids were assessed by comparing their retention times and peak areas with that of amino acid standards. The tryptophan content was determined by the colorimetric method after alkaline hydrolysis of each sample, because tryptophan could not be detected after acid hydrolysis.

Determination of biochemical indices in serum and liver

Serum and liver triglyceride (TG), total cholesterol (T-CH), and serum calcium (SC) levels were all measured using a Diagnostic Reagent Kit (Art. No. A110-2,

Table 2 Amino acid contents of experimental diets (g kg⁻¹ protein)

Amino acid contents	Diets					
	0 Hyp	5 Hyp	10 Hyp	15 Hyp	20 Hyp	25 Hyp
¹ EAA						
Arginine Arg	68.99	67.09	67.14	71.13	71.75	67.82
Histidine His	23.35	22.50	22.87	28.37	23.36	26.67
Valine Val	25.87	24.61	23.50	23.99	22.32	23.94
Phenylalanine Phe	5.05	5.26	5.04	5.01	5.63	5.25
Leucine Leu	11.78	11.99	13.22	12.10	12.72	12.39
Isoleucine Ile	24.40	26.50	25.18	25.66	25.86	24.15
Threonine Thr	57.84	59.31	58.96	60.91	59.03	59.84
Methionine Met	14.93	13.88	13.64	13.77	13.98	13.65
Lysine Lys	23.56	23.55	23.50	23.36	23.36	23.31
² NEAA						
Aspartic Asp	35.34	34.91	35.04	34.62	34.42	34.02
Serine Ser	24.61	24.40	24.13	24.61	23.36	24.99
Glutamic Glu	68.78	63.51	57.07	54.02	51.10	45.98
Alanine Ala	85.61	79.29	77.42	76.34	84.27	73.91
Glycine Gly	273.66	259.31	252.63	246.54	255.31	254.70
Tyrosine Tyr	43.75	47.32	45.74	44.84	45.47	44.72
Proline Pro	33.66	31.55	31.05	29.62	36.29	35.28

¹EAA essential amino acid

²NEAA nonessential amino acid

A111-2, C004-2, respectively; Nanjing Jiancheng Bio-engineering Institute, Nanjing, China) according to the manufacturer's instructions. Briefly, liver was homogenized in nine volumes (w/v) of chilled phosphate buffer (0.1 mol/L pH 7.4) or 0.9% saline solution and then centrifuged (2500g) at 4 °C for 10 min. The floating lipid layer was then removed, and the supernatant was divided into aliquots in 1.5-mL tubes. All the tubes were then stored at -80 °C until analysis. Aliquots of 10- μ L sample (serum or liver), 10- μ L ultra-pure water, or 10- μ L standard sample were mixed with 1-mL working liquid (provided by each kit) and incubated for 10 min at 37 °C, and then the absorbance of TG and T-CH was determined at 510 nm. The absorbance of SC was determined at 610 nm, while the concentration was determined from each standard curve.

Analysis of metabolism-related gene expression

The total RNA of each tissue (liver, muscle, intestines, and swim bladder) was isolated using Trizol (InvitrogenTM) according to manufacturer's instructions,

while genomic DNA was eliminated by DNase I digestion (Takara, Dalian, China). The concentration and quality of RNA were determined by measuring the absorbance at 260 nm/280 nm using a NanoDrop 2000 spectrophotometer (Thermo Scientific Nanodrop, USA). The integrity of purified RNA was determined using agarose gel electrophoresis. cDNA was synthesized using TransScript[®] One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (TransGen Biotech, Beijing, China). Specific primers for TOR-related genes were designed by the Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA) based on the sequences of *Larimichthys crocea* (Genbank accession nos. KC510145, XM_010732552, XM_010748995, XM_010736764, and XM_019275101) (Table 3). The polymerase chain reaction (PCR) was carried out on an Applied Biosystems VeritiTM Thermal Cycler (Thermo Fisher Scientific) to obtain the amplified cDNA fragment of target gene. The PCR conditions were as follows: initial denaturation step at 95 °C for to 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, primer annealing at 58 °C

for 30 s, primer extension 72 °C for 1 min, final, extended at 72 °C for 10 min. The PCR fragments were subjected to electrophoresis on a 1.2% agarose gel to confirm the product size, and then sequenced (The Beijing Genomics Institute, Shenzhen city). The cloned sequences were verified by comparison with those on NCBI.

For the qRT-PCR analysis, the total reaction volume was 20 µL including 1 µL (10 mM) primer (housekeeping or target gene), 2 µL cDNA, 7 µL ultra-pure water, and 10 µL SYBR® Premix Ex Taq™ II (Applied Takara). The qRT-PCR program was as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 20 s. After the amplification phase, a melting curve of 0.5 °C increments from 65 to 95 °C was performed to confirm only single PCR product in each reaction. The housekeeping gene was used for normalization. Each sample was run in triplicate, and samples without templates used as negative controls. The qRT-PCR reaction was run on a Roche Light Cycler® 480 System and the relative quantification calculated using the $\Delta\Delta C_T$ method (Livak and Schmittgen 2001).

Statistical analysis

The results were expressed as means \pm SEM (standard error of the mean). All data were tested for normality by using the Kolmogorov–Smirnov test and homogeneity of variances by using Levene's test before statistical

Table 3 Real-time PCR primer sequences

Name	Sequences of primers	Product length
m-TOR	Forward 5'-CCCATCATTGATAT TGCTCCTA-3'	245
	Reverse 5'-CGAATGGACACTCG TAGAATCA-3'	
4E-BP	Forward 5'-CCAGAAGACCACTG CCAAGG-3'	165
	Reverse 5'-CGACACTCCAGCAG GAACTTT-3'	
S6K1	Forward 5'-ACGAGGATGTGAGC CAGTTTG-3'	134
	Reverse 5'-TTCTAGGACCGATG GAGCAAC-3'	
β -actin	Forward 5'-GGTACTCCTTCAC CACCACAG-3'	147
	Reverse 5'-TCCGTCGGGCAGCT CATA-3'	

analyses. One-way ANOVA was used to determine the significance of each parameter among different treatments using the SPSS 20.0 (SPSS Inc., Chicago, IL, USA) followed by Tukey's test. The optimum dietary Hyp supplementation for juvenile *Nibea diacanthus* based on SGR was estimated by polynomial regression analysis. Significance levels were set at $P < 0.05$. Data are presented as means \pm standard error.

Results

Growth performance

The results of growth performance, survival rate, and feed utilization including SGR, WG, FCR, PER, SR, HSI, and VSI of juvenile *N. diacanthus* fed different experimental diets are shown in Table 4. There were no pathological differences observed during the trial. Survival of each group was over 90%, and there were no significant differences among the dietary treatments. The PER of fish fed on diets containing 10–15 g kg⁻¹ dietary Hyp increased significantly compared with those on the basal diet (0 g kg⁻¹ Hyp). WG and SGR increased significantly with Hyp content up to 10 g kg⁻¹ in diets. The polynomial regression analysis of SGR ($y = -1857.1x^2 + 61.743x + 0.8671$ $R^2 = 0.9222$) against dietary Hyp levels in diets is presented in Fig. 1. The amount of dietary Hyp added, which was estimated from SGR, was 16.6 g kg⁻¹. PER, HSI, and VSI of juvenile *N. diacanthus* increased initially and decreased subsequently with increasing Hyp content of diets, while FCR had a reverse trend ($P < 0.05$).

Proximate composition of whole body, muscle, and swim bladder

The proximate compositions of whole body, muscle, and swim bladder of *N. diacanthus* are presented in Table 5. In the dietary treatment groups, all proximate compositions for swim bladder and muscle had the same trend (Table 5). The moisture content of the different tissues followed the same trend with increasing Hyp content of diets, with no significant differences between any two dietary treatment groups. Similarly, there were no significant differences in the crude lipid and crude ash contents among the dietary treatments. For fish fed with 10 g kg⁻¹ Hyp, the crude protein content in whole body and swim bladder was significantly higher than

that of the control group (0 g kg⁻¹ Hyp) ($P < 0.05$). There were no significant differences in all proximate compositions of muscle among the dietary treatments.

Amino acid content of muscle and swim bladder

The amino acid profile of muscle and swim bladder is presented in Tables 6 and 7, respectively. Apart from leucine, the essential amino acid (EAA) content of muscle initially increased (almost reaching maximum in diet (10 g kg⁻¹ Hyp)) followed by a decrease with increasing Hyp content of diets. Similarly, the non-essential amino acid (NEAA) content of muscle generally increased first and then decreased with increasing dietary Hyp content (Table 6). For the EAA of swim bladder, there were no significant differences in the retention of histidine, valine, phenylalanine, isoleucine, and methionine among the dietary treatments. On the other hand, the retention of EAA (arginine, leucine threonine, and lysine) in swim bladder generally increased initially followed by a decrease with increasing Hyp content of diets (Table 7). Generally, the proline content of muscle and swim bladder varied similarly with increasing Hyp content of diets, reaching the peak value with diet (15 g kg⁻¹ Hyp), and then slowly decreased with increasing Hyp content of diets.

Biochemical indices in serum and liver

As shown in Table 8, there were no significant differences in the SC, TG, and T-CH contents of serum and liver among the dietary treatments with increasing dietary Hyp levels.

Expression of TOR signaling pathway-related genes

The qRT-PCR analysis of juvenile *N. diacanthus* tissues revealed that swim bladder and muscle had high mRNA expression levels of TOR, which were significantly higher than liver and intestines ($P < 0.05$), with no significant difference between liver and intestines. For the basal diet, swim bladder had significantly higher TOR mRNA levels compared with muscle ($P < 0.05$, Fig. 2). On the other hand, in the same tissues, the TOR mRNA levels in liver and muscle increased gradually with increasing levels of Hyp ($P < 0.05$), but there was no significant difference in intestines. The mRNA levels of TOR in swim bladder initially increased and decreased subsequently with increasing Hyp content of

diets, with the highest observed for fish fed with diets containing 15 g kg⁻¹ Hyp ($P < 0.05$). In terms of 4E-BP gene expression in fish (juvenile *N. diacanthus*) fed with diets containing graded levels of Hyp (Fig. 3), it was observed that for different tissues, muscle had the highest level of 4E-BP, which was significantly higher than that of the liver, intestines, and swim bladder ($P < 0.05$). In muscle, there was no significant difference in 4E-BP gene expression with increasing levels of Hyp. However, there was an initial increase in 4E-BP expression followed by a decrease in the liver, with the highest levels found in fish fed with diets containing 10 g kg⁻¹ supplemented Hyp ($P < 0.05$). In swim bladder and intestines, the relative expression of 4E-BP gene decreased significantly with increasing Hyp ($P < 0.05$). The expression of the S6K1 gene in swim bladder of fish fed with diets containing graded levels of Hyp showed a significant ($P < 0.05$) initial increase followed by a decrease (Fig. 4). The highest S6K1 gene expression in swim bladder was found in fish fed with diets supplemented with 10 g kg⁻¹ Hyp. However, there was no significant difference in S6K1 gene expression in intestines with increasing levels of Hyp. In the muscle and liver, the S6K1 gene expression increased gradually with increasing levels of Hyp, while the S6K1 gene expression of fish on diets containing 15–25 g kg⁻¹ Hyp increased significantly compared with those on the basal diet (0 g kg⁻¹ Hyp) ($P < 0.05$). For different tissues, the S6K1 gene expression of fish fed basal diets was significantly different, with the transcript level of S6K1 in the liver being the highest among all tested tissues.

Discussion

Dietary hydroxyproline supplementation promote growth of juvenile *Niber diacanthus*

There has been growing interest in research on Hyp metabolism and nutrition as well as dietary supplementation of Hyp in fish diets (Liu et al. 2014; Zhang et al. 2013, 2015; Wei et al. 2016). Some studies have revealed that the appropriate level dietary Hyp supplementation has an effect on growth, muscle texture, and collagen content in fish (Aksnes et al. 2008; Zhang et al. 2013). In the current study, significant growth promotion was observed in juvenile *N. diacanthus* when supplemented with dietary Hyp. This observation is similar

Table 4 Growth performance of juvenile *Nibea diacanthus* fed diets with different levels of hydroxyproline

Growth performance	Diets					
	0 Hyp	5 Hyp	10 Hyp	15 Hyp	20 Hyp	25 Hyp
Survival rate (SR, %)	91.11 ± 4.44	95.56 ± 2.22	93.33 ± 3.85	97.78 ± 2.22	95.56 ± 2.22	97.78 ± 2.22
Weight gain (WG, %)	162.5 ± 6.74 ^a	184.7 ± 2.55 ^b	202.7 ± 5.95 ^c	214.85 ± 4.51 ^c	205.23 ± 8.29 ^c	207.24 ± 8.02 ^c
Specific growth rate (SGR, % day ⁻¹)	0.87 ± 0.07 ^a	1.09 ± 0.03 ^b	1.36 ± 0.05 ^c	1.40 ± 0.04 ^c	1.27 ± 0.07 ^c	1.29 ± 0.07 ^c
Protein efficiency ratio (PER)	1.47 ± 0.14 ^a	1.65 ± 0.19 ^{ab}	2.11 ± 0.36 ^b	2.25 ± 0.32 ^b	1.76 ± 0.36 ^{ab}	2.02 ± 0.42 ^{ab}
Feed conversion ratio (FCR)	1.56 ± 0.04 ^b	1.26 ± 0.12 ^{ab}	1.23 ± 0.07 ^a	1.33 ± 0.13 ^{ab}	1.23 ± 0.07 ^a	1.46 ± 0.09 ^{ab}
Hepatosomatic index (HSI, %)	1.25 ± 0.13 ^{ab}	1.72 ± 0.01 ^c	1.25 ± 0.09 ^{ab}	1.35 ± 0.12 ^b	1.06 ± 0.03 ^a	1.08 ± 0.06 ^a
Viscerosomatic index (VSI, %)	6.03 ± 0.22 ^a	6.47 ± 0.07 ^a	5.82 ± 0.12 ^a	5.95 ± 0.18 ^a	7.34 ± 0.39 ^b	6.23 ± 0.15 ^a

Values are presented as means ± SEM, $n = 3$. Means in the same row with different letters are significantly different from each other ($P < 0.05$)

to a previous report (Wei et al. 2016), where the growth of large yellow croaker was significantly improved by optimum supplemented levels of dietary Hyp. However, fish fed with excess or insufficient dietary Hyp experienced nutritional stress, which resulted in decreased fish growth and protein deposition. On the other hand, Albrektsen et al. (2010) reported that there was no significant effect of dietary Hyp supplementation on growth performance of Atlantic salmon (*Salmo salar* L.). Similarly, Zhang et al. (2015, 2013) found that growth performance of juvenile turbot (*Scophthalmus maximus* L.) was not significantly affected by dietary Hyp level. These reports on dietary Hyp supplementation on fish growth indicate that moderate levels of Hyp are important for growth promotion. Nonetheless, large

differences existed in fish sizes (among species), experimental diets, and aquaculture conditions in these previous studies, which suggest that the effect of dietary Hyp on fish growth could partly depend on size, dietary amino acid composition, and duration of supplementation.

Dietary Hyp supplementation affects protein-related body composition and amino acid contents

Proteins are the main components of tissue structure. In this study, dietary Hyp levels significantly affected the crude protein contents of the whole body and swim bladder of *N. diacanthus*; however, muscle protein was not affected (Table 5). The crude protein contents

Fig. 1 Quadratic regression analysis of specific growth rate (SGR, %/d) according to dietary hydroxyproline levels. Each point represents the mean of six groups of *Nibea diacanthus* with twenty-five fish per group. Addition amount of dietary hydroxyproline estimated from SGR was 16.6 g kg⁻¹ ($y = -1857.1x^2 + 61.743x + 0.8671$ $R^2 = 0.9222$)

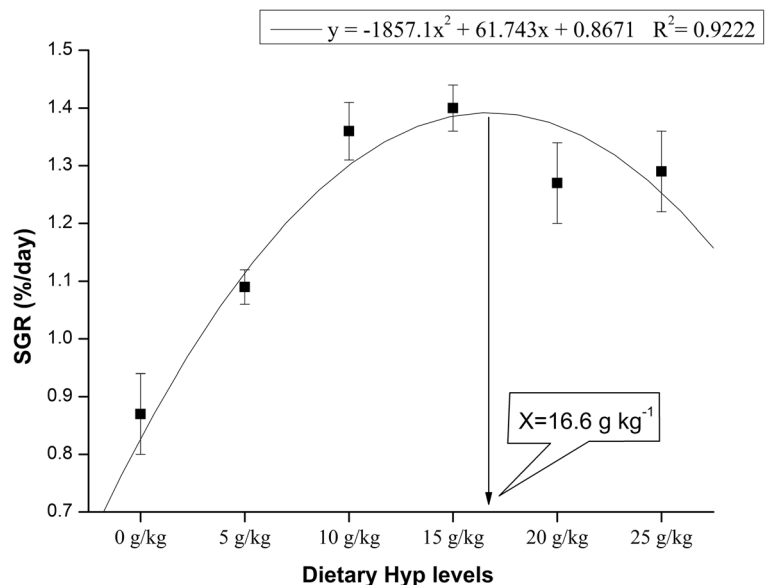


Table 5 Whole-body proximate composition (% wet matter basis) of juvenile *Nibea diacanthus* fed with experimental diets containing different levels of hydroxyproline

Proximate composition (%)	Diets					
	0 Hyp	5 Hyp	10 Hyp	15 Hyp	20 Hyp	25 Hyp
Whole body						
Moisture	74.14 ± 1.00	73.79 ± 0.69	72.78 ± 0.38	73.52 ± 0.90	73.44 ± 1.10	73.67 ± 1.49
Crude protein	15.14 ± 0.16 ^a	15.38 ± 0.34 ^{ab}	15.98 ± 0.35 ^b	15.41 ± 0.29 ^{ab}	15.65 ± 0.35 ^{ab}	15.34 ± 0.25 ^{ab}
Crude lipid	3.16 ± 0.03	3.35 ± 0.14	3.40 ± 0.02	3.11 ± 0.13	3.21 ± 0.17	3.32 ± 0.27
Crude ash	7.03 ± 0.30	6.99 ± 0.34	7.04 ± 0.17	7.43 ± 0.21	7.26 ± 0.24	6.77 ± 0.17
Muscle						
Moisture	79.05 ± 0.77	78.80 ± 0.32	79.31 ± 0.18	78.53 ± 0.43	79.21 ± 0.18	79.24 ± 0.39
Crude protein	18.03 ± 0.30	17.99 ± 0.34	18.03 ± 0.87	17.63 ± 1.01	18.26 ± 1.24	17.77 ± 0.67
Crude lipid	0.96 ± 0.03	1.15 ± 0.04	1.00 ± 0.02	1.11 ± 0.03	1.21 ± 0.07	1.32 ± 0.07
Crude ash	1.44 ± 0.29	1.26 ± 0.41	1.33 ± 0.32	1.22 ± 0.12	1.13 ± 0.34	1.20 ± 0.39
Swim bladder						
Moisture	68.50 ± 0.36	68.15 ± 0.20	67.68 ± 0.01	67.30 ± 0.12	67.67 ± 0.07	66.99 ± 0.06
Crude protein	29.48 ± 0.10 ^a	29.71 ± 0.14 ^b	30.77 ± 0.27 ^c	29.63 ± 0.21 ^{ab}	30.81 ± 0.24 ^c	30.67 ± 0.17 ^c
Crude lipid	0.37 ± 0.01	0.40 ± 0.03	0.39 ± 0.04	0.40 ± 0.01	0.38 ± 0.01	0.42 ± 0.01
Crude ash	0.96 ± 0.03	1.15 ± 0.04	1.00 ± 0.02	1.11 ± 0.03	1.01 ± 0.07	1.12 ± 0.07

Values are presented as means ± SEM, $n = 3$. Means in the same row with different letters are significantly different from each other ($P < 0.05$)

Table 6 Amino acid profile in dorsal muscle of juvenile *Nibea diacanthus* fed with experimental diets containing different levels of hydroxyproline (% dry matter basis)

Muscle		Diets					
		0 Hyp	5 Hyp	10 Hyp	15 Hyp	20 Hyp	25 Hyp
¹ EAA							
Arginine	Arg	4.99 ± 0.07 ^{ab}	5.01 ± 0.10 ^{ab}	5.23 ± 0.10 ^b	4.95 ± 0.13 ^{ab}	4.56 ± 0.13 ^a	4.65 ± 0.11 ^{ab}
Histidine	His	2.48 ± 0.06 ^{bc}	2.65 ± 0.13 ^c	2.76 ± 0.10 ^c	2.22 ± 0.06 ^{ab}	2.08 ± 0.13 ^a	2.60 ± 0.07 ^c
Valine	Val	4.31 ± 0.23 ^b	4.42 ± 0.15 ^b	4.53 ± 0.16 ^b	4.25 ± 0.08 ^b	3.65 ± 0.13 ^a	4.12 ± 0.06 ^b
Phenylalanine	Phe	3.64 ± 0.16 ^{ab}	3.77 ± 0.07 ^b	4.24 ± 0.13 ^c	3.66 ± 0.11 ^{ab}	3.17 ± 0.15 ^a	3.62 ± 0.10 ^{ab}
Leucine	Leu	6.05 ± 0.12 ^{ab}	6.50 ± 0.14 ^{cd}	6.70 ± 0.06 ^d	6.36 ± 0.16 ^{bcd}	5.70 ± 0.15 ^a	6.07 ± 0.16 ^{abc}
Isoleucine	Ile	2.78 ± 0.26	2.81 ± 0.08	3.14 ± 0.13	2.74 ± 0.03	2.57 ± 0.26	2.64 ± 0.19
Threonine	Thr	3.38 ± 0.17 ^{ab}	3.40 ± 0.09 ^{ab}	3.38 ± 0.08 ^{ab}	3.48 ± 0.12 ^b	3.06 ± 0.05 ^a	3.16 ± 0.08 ^{ab}
Methionine	Met	2.52 ± 0.04 ^{ab}	2.69 ± 0.15 ^{ab}	2.81 ± 0.11 ^b	2.63 ± 0.01 ^{ab}	2.45 ± 0.02 ^a	2.61 ± 0.08 ^{ab}
Lysine	Lys	7.48 ± 0.01 ^{ab}	7.84 ± 0.07 ^b	8.20 ± 0.06 ^b	7.70 ± 0.09 ^{ab}	6.69 ± 0.19 ^a	7.67 ± 0.12 ^{ab}
² NEAA							
Aspartic	Asp	7.91 ± 0.24 ^a	8.05 ± 0.35 ^{ab}	8.24 ± 0.24 ^b	8.13 ± 0.17 ^{ab}	8.04 ± 0.33 ^{ab}	7.95 ± 0.35 ^{ab}
Serine	Ser	3.25 ± 0.17 ^{ab}	3.27 ± 0.09 ^{ab}	3.24 ± 0.08 ^{ab}	3.35 ± 0.12 ^b	2.93 ± 0.05 ^{ab}	3.03 ± 0.08 ^a
Glutamic	Glu	12.23 ± 0.10 ^c	11.88 ± 0.11 ^b	11.72 ± 0.57 ^{ab}	11.85 ± 0.21 ^b	11.81 ± 0.13 ^b	11.63 ± 1.03 ^a
Alanine	Ala	4.18 ± 0.26 ^{ab}	4.15 ± 0.13 ^{ab}	4.20 ± 0.27 ^{ab}	4.34 ± 0.04 ^b	4.18 ± 0.28 ^{ab}	4.09 ± 0.33 ^a
Glycine	Gly	3.63 ± 0.18	3.75 ± 0.16	3.65 ± 0.10	3.74 ± 0.01	3.88 ± 0.22	3.66 ± 0.19
Tyrosine	Tyr	1.98 ± 0.14 ^a	1.85 ± 0.06 ^a	2.61 ± 0.12 ^b	1.85 ± 0.09 ^a	2.10 ± 0.05 ^{ab}	1.96 ± 0.09 ^a
Proline	Pro	4.29 ± 0.26 ^{ab}	4.21 ± 0.13 ^{ab}	4.31 ± 0.27 ^{ab}	4.45 ± 0.04 ^b	4.29 ± 0.28 ^{ab}	4.20 ± 0.33 ^a

Values are presented as means ± SEM, $n = 3$. Means in the same row with different letters are significantly different from each other ($P < 0.05$)

¹EAA essential amino acid

²NEAA nonessential amino acid

Table 7 Amino acid profiles in swim bladder of juvenile *Nibea diacanthus* fed with experimental diets containing different levels of hydroxyproline (% dry matter basis)

Swim bladder	Diets	Diets					
		0 Hyp	5 Hyp	10 Hyp	15 Hyp	20 Hyp	25 Hyp
¹ EAA							
Arginine	Arg	4.89 ± 0.07 ^{ab}	5.04 ± 0.10 ^{ab}	5.25 ± 0.10 ^b	4.91 ± 0.13 ^{ab}	4.59 ± 0.13 ^a	4.68 ± 0.11 ^{ab}
Histidine	His	0.64 ± 0.06	0.67 ± 0.05	0.69 ± 0.02	0.68 ± 0.00	0.64 ± 0.03	0.70 ± 0.02
Valine	Val	3.00 ± 0.23	3.11 ± 0.15	3.22 ± 0.16	2.94 ± 0.08	3.34 ± 0.13	3.05 ± 0.06
Phenylalanine	Phe	2.92 ± 0.13	2.96 ± 0.07	3.02 ± 0.03	2.91 ± 0.06	2.93 ± 0.06	2.90 ± 0.01
Leucine	Leu	3.02 ± 0.12 ^a	3.47 ± 0.14 ^{ab}	3.67 ± 0.06 ^b	3.33 ± 0.16 ^{ab}	3.01 ± 0.19 ^a	3.04 ± 0.16 ^a
Isoleucine	Ile	2.03 ± 0.11	2.40 ± 0.08	2.19 ± 0.17	2.33 ± 0.03	2.16 ± 0.26	2.23 ± 0.19
Threonine	Thr	3.26 ± 0.17 ^{ab}	3.28 ± 0.09 ^{ab}	3.26 ± 0.08 ^{ab}	3.36 ± 0.12 ^b	2.94 ± 0.05 ^a	3.04 ± 0.08 ^{ab}
Methionine	Met	1.19 ± 0.03	1.22 ± 0.02	1.17 ± 0.01	1.23 ± 0.01	1.18 ± 0.02	1.18 ± 0.02
Lysine	Lys	4.04 ± 0.01 ^a	4.39 ± 0.07 ^c	4.22 ± 0.05 ^{bc}	4.26 ± 0.09 ^{bc}	4.14 ± 0.04 ^{ab}	4.33 ± 0.05 ^{bc}
² NEAA							
Aspartic	Asp	3.21 ± 0.12	3.51 ± 0.35	3.90 ± 0.24	3.79 ± 0.17	3.70 ± 0.33	3.44 ± 0.20
Serine	Ser	2.69 ± 0.15 ^{ab}	2.65 ± 0.06 ^{ab}	2.68 ± 0.09 ^{ab}	2.79 ± 0.07 ^b	2.59 ± 0.09 ^{ab}	2.37 ± 0.07 ^a
Glutamic	Glu	5.87 ± 0.10 ^{ab}	6.02 ± 0.11 ^{ab}	6.29 ± 0.12 ^b	6.06 ± 0.19 ^{ab}	5.85 ± 0.13 ^a	5.97 ± 0.10 ^{ab}
Alanine	Ala	10.71 ± 0.18 ^{bc}	10.69 ± 0.05 ^{abc}	10.54 ± 0.05 ^{abc}	10.81 ± 0.03 ^c	10.40 ± 0.12 ^{ab}	10.34 ± 0.11 ^a
Glycine	Gly	24.93 ± 0.18	25.05 ± 0.16	24.95 ± 0.10	25.04 ± 0.01	25.18 ± 0.22	24.96 ± 0.19
Tyrosine	Tyr	0.81 ± 0.05	0.75 ± 0.01	0.81 ± 0.02	0.81 ± 0.02	0.80 ± 0.01	0.79 ± 0.01
Proline	Pro	1.94 ± 0.10 ^a	2.08 ± 0.13 ^{ab}	2.19 ± 0.08 ^{ab}	2.26 ± 0.04 ^b	2.04 ± 0.06 ^{ab}	2.08 ± 0.04 ^{ab}

Values are presented as means ± SEM, $n = 3$. Means in the same row with different letters are significantly different from each other ($P < 0.05$)

¹ EAA essential amino acid

² NEAA nonessential amino acid

of whole body and swim bladder showed a positive correlation with dietary protein, while the moisture, ash, and lipid contents were not affected. In similar previous studies, Wei et al. (2016) revealed that muscle crude protein of large yellow croaker slowly increased with increasing dietary Hyp levels, reaching the highest level with 6.9 g kg⁻¹ Hyp supplementation. On the other hand, Liu et al. (2014) found no significant differences in the proximate composition (moisture, crude protein, crude lipid, ash) of the whole body of turbot (*Scophthalmus maximus* L.) among all dietary treatments with increasing Hyp levels. The differences observed in these studies could be due to specific function of Hyp, i.e., major amino acid in collagen proteins, and major extracellular components of connective tissues such as skin, tendon, cartilage, and swim bladder. Thus, the demand for Hyp would vary in different tissues or body parts. Protein deposition is actually the deposition of amino acids. Gunasekera et al. (1997)

reported that muscle amino acid composition could be affected by the nutritional quality of diets. The amounts and levels of EAA in diets must follow the amino acid balance. Whole-body EAA retentions are considered the most sensitive indicators of an inadequate supply of amino acids in diets (Bulbul et al. 2014), as excess EAA for amino acid balance in diets is channeled towards catabolism and energy production, thereby reducing the retention efficiency (Alam et al. 2008; Unnikrishnan and Paulraj 2010). In the present study, except leucine, significant differences were observed in EAA contents in the muscle of fish-fed diets with increasing Hyp content (Table 6). The EAA of swim bladder showed no significant differences in the retention of histidine, valine, phenylalanine, isoleucine, and methionine among the dietary treatments (Table 7). The maximum EAA retention in muscle and swim bladder were observed in fish fed with diet Hyp 10 g kg⁻¹. It has been reported that growth and efficient protein synthesis

Table 8 SC, TG, and T-CH contents in serum and liver of juvenile *Nibea diacanthus* fed with experimental diets containing different levels of hydroxyproline

	Diets					
	0 Hyp	5 Hyp	10 Hyp	15 Hyp	20 Hyp	25 Hyp
Serum (mmol L ⁻¹)						
SC	1.44 ± 0.01	1.46 ± 0.07	1.51 ± 0.01	1.47 ± 0.03	1.53 ± 0.04	1.47 ± 0.03
TG	0.25 ± 0.02	0.21 ± 0.01	0.28 ± 0.14	0.23 ± 0.04	0.23 ± 0.03	0.16 ± 0.01
T-CH	1.97 ± 0.04	1.35 ± 0.01	1.83 ± 0.14	1.61 ± 0.24	1.56 ± 0.01	1.87 ± 0.14
Liver (mmol gprot ⁻¹)						
TG	1.30 ± 0.26	1.13 ± 0.19	0.85 ± 0.34	0.82 ± 0.02	0.86 ± 0.11	1.08 ± 0.18
T-CH	0.25 ± 0.02	0.32 ± 0.11	0.35 ± 0.01	0.30 ± 0.07	0.28 ± 0.06	0.36 ± 0.04

Values are presented as means ± SEM, n = 3. Means in the same row with different letters are significantly different from each other (P < 0.05)

depend on the availability of sufficient levels of all EAA in diets (Wu 2014). In this study, it was observed that the appropriate Hyp level in the experimental diets improved the amino acid balance of diets, which

therefore promoted the growth and protein synthesis of fish. The optimum supplemented level of Hyp in the experimental diets was 10 g kg⁻¹. For growth and protein synthesis, it is necessary to consider both dietary

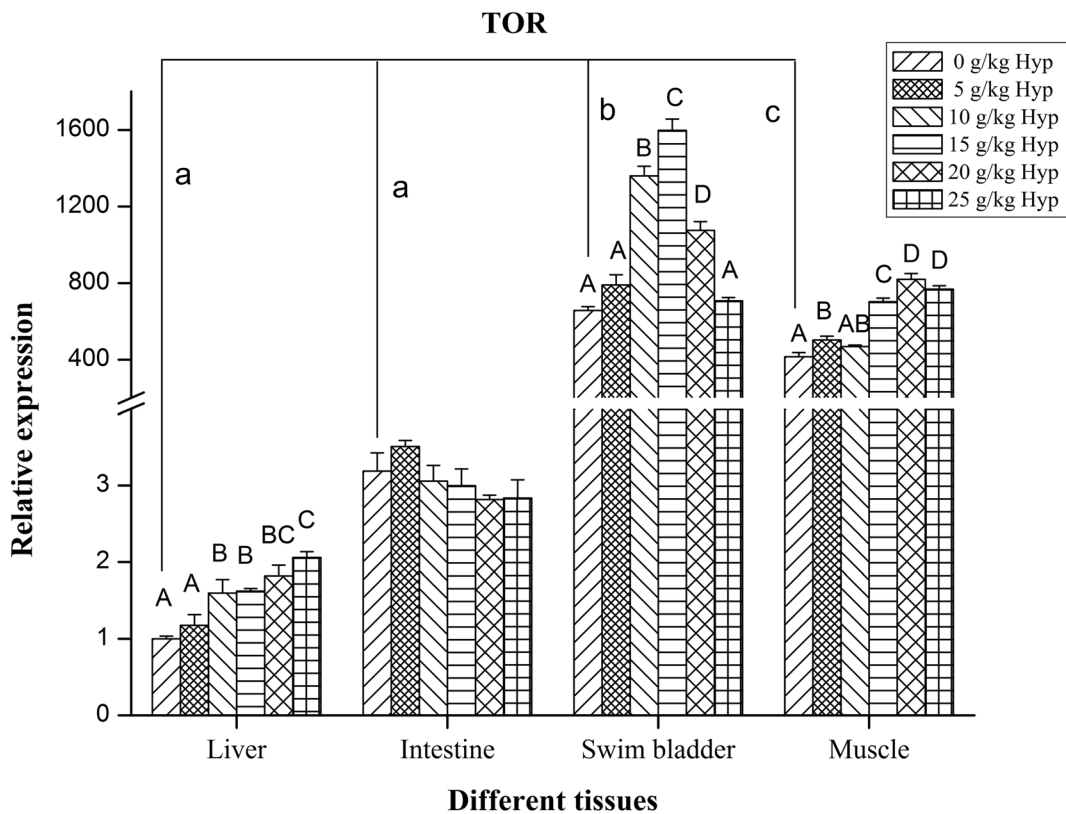


Fig. 2 Relative expression of TOR gene of juvenile *Nibea diacanthus* fed with diets containing graded levels of supplemented hydroxyproline. Values are means ± SEM (n = 3). Mean values with different capital letters are significantly different among the

dietary treatments in the same tissues (P < 0.05). Mean values with different lowercase letters are significantly different among the different tissues in the basal diet group (diets I) (P < 0.05)

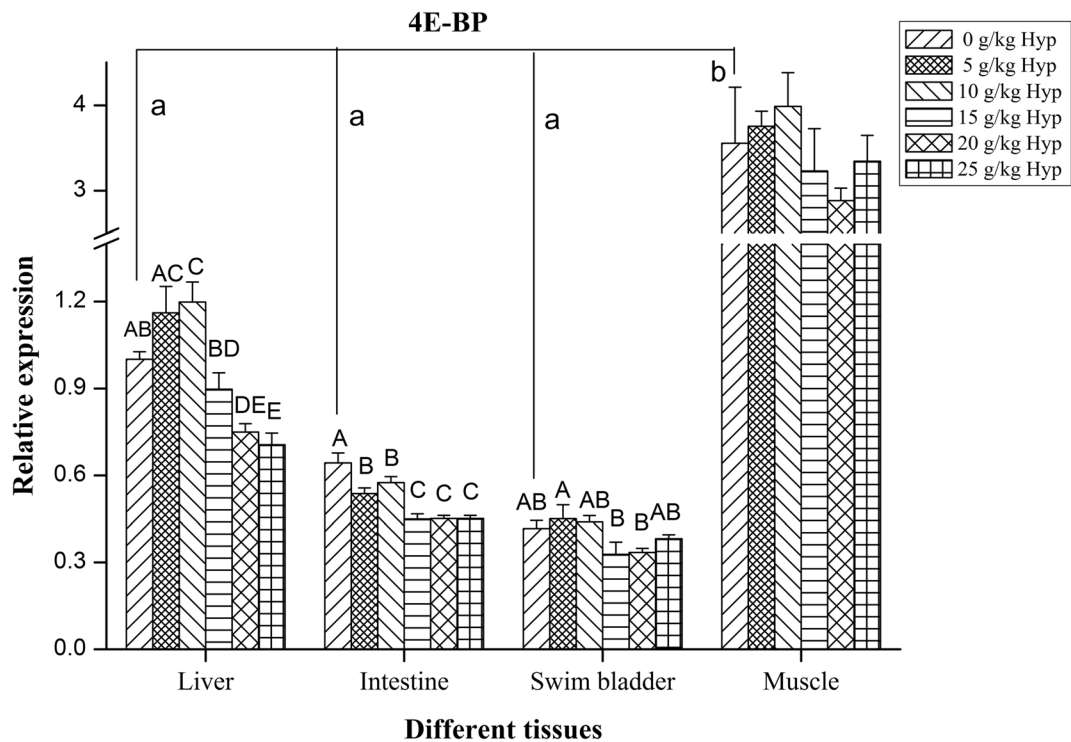


Fig. 3 Relative expression of 4E-BP gene of juvenile *Nibea diacanthus* fed with diets containing graded levels of supplemented hydroxyproline. Values are means \pm SEM ($n = 3$). Mean values with different capital letters are significantly different among the

dietary treatments in the same tissues ($P < 0.05$). Mean values with different lowercase letters are significantly different among the different tissues in the basal diet group (diets I) ($P < 0.05$)

protein content and amino acid content (EAA intake), in order to fulfill amino acid balance. We observed variations in the EAA of muscle and swim bladder with increasing Hyp levels among all dietary treatments. It has been suggested that the retention of EAA is greater when one of them is limited in the diet (Silvia et al. 2012). A lower retention could suggest an excess of EAA in diets and a higher retention by a deficiency (Sanchez-Lozano et al. 2011; Deng et al. 2017). Thus, the difference in retention of EAA in the different tissues (muscle and swim bladder) could be explained by the fact that the amino acid requirement of muscle and swim bladder is different. Fish bladders, for example, are rich in collagen, which is more Hyp-dependent than muscle because of the relationship between hydroxyproline and collagen. On the basis of this, it is plausible to state that the restrictive amino acids of various tissues are different. On the other hand, NEAA seem to be affected differently, as among the dietary treatments in this study, the variation of EAA contents in muscle and swim bladder correlated with body PER and WG. This observation seems to suggest that the addition of 10–15

g kg^{-1} dietary Hyp might be linked with EAA deposition in tissues and the changes in PER and WG.

Dietary Hyp supplementation affects transcript levels of TOR but not 4E-BP and S6K1

Although hydroxyproline (Hyp) has been reported to be a signaling molecule that regulates cellular redox state and apoptosis (Phang et al. 2008), limited information is currently available on the effect of dietary Hyp level on biochemistry indices in serum and liver. In this study, increasing dietary levels of Hyp had no significant effect on SC, TG, and T-CH contents of serum and liver. While Hyp has limited effect on lipid metabolism, it seems to promote protein metabolism, but the pathways involved require further studies. Dietary amino acids are reported to play an important role in controlling gene expression (Zhou et al. 2019; He et al. 2019; Liang et al. 2016, 2019). In addition to their promotion of protein synthesis, recent studies have shown that amino acids regulate metabolism through the TOR pathway in different fish species (Liao et al. 2008; Marine et al. 2011;

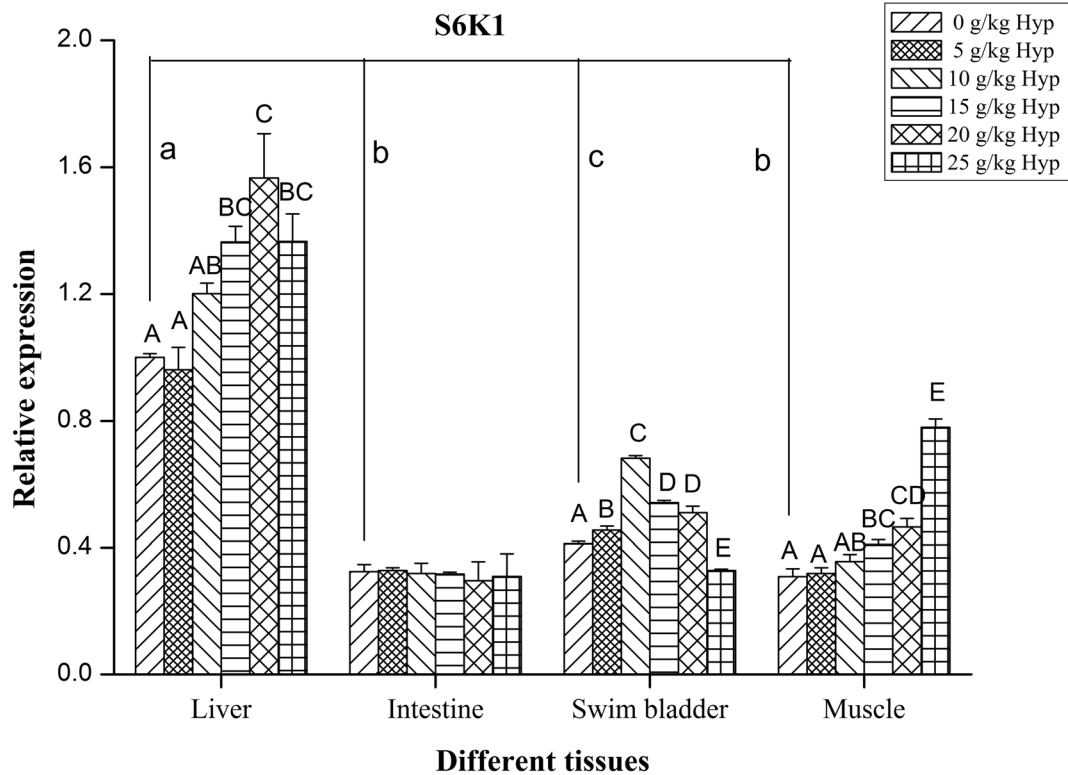


Fig. 4 Relative expression of S6K1 gene of juvenile *Nibea diacanthus* fed with diets containing graded levels of supplemented hydroxyproline. Values are means \pm SEM ($n = 3$). Mean values with different capital letters are significantly different among the

dietary treatments in the same tissues ($P < 0.05$). Mean values with different lowercase letters are significantly different among the different tissues in the basal diet group (diets I) ($P < 0.05$)

Chen et al. 2012; Tang et al. 2013; Liang et al. 2016; Li et al. 2019; Zhou et al. 2019; He et al. 2019). However, there is currently no information on the effect of Hyp on the major kinases involved in the TOR pathway in fish. In the present study, we revealed that in *N. diacanthus* transcript levels of TOR in the liver, muscle, and swim bladder increased with increasing levels of dietary Hyp up to an optimum level (diets containing 25 g kg⁻¹, 20 g kg⁻¹, and 15 g kg⁻¹ Hyp supplements, respectively). Moreover, increased dietary Hyp levels increased the SGR, PER, WG, and the crude protein of whole body, which suggest that dietary Hyp improves these parameters in fish via increased activation of TOR. The expression and activation of TOR are however tissue-specific, as transcript levels of TOR in intestines were not affected by the increase in dietary Hyp. These findings are consistent with Manor et al. (2015), who revealed that the TOR pathway might be involved in nutrient redistribution during maturation, and accounts for the differences in the expression of related genes. In terms of the TOR pathway, our results are in agreement

with studies on leucine (Zhou et al. 2019), arginine (Liang et al. 2016), and methionine (He et al. 2019), where it has been demonstrated that these amino acids promote protein synthesis of fish by altering the expression of TOR gene.

Eukaryotic translation initiation factor 4E-binding protein (4E-BP) and ribosomal protein S6 kinase1 (S6K1) are found downstream of TOR and have been implicated in the regulation of protein synthesis (Seiliez et al. 2008). In the Jian carp, the activity of 4E-BP is reported to be stimulated by amino acids (Chen et al. 2012; Tang et al. 2013). Similarly, leucine has been shown to regulate the phosphorylation of S6K1 and 4E-BP through the TOR signaling pathway (Liang et al. 2019), while dietary arginine is reported to regulate the phosphorylation of 4E-BP in the intestinal epithelia of rats through the TOR signaling pathway (Ban et al. 2004). In this study, the relative expression of 4E-BP decreased with higher dietary Hyp levels in the tissues examined, except muscle. Notably, 4E-BP and TOR had different expression patterns, with transcript levels

of TOR downregulated by increased dietary Hyp, while that of 4E-BP were upregulated. On the other hand, S6K1 expression was synonymous with TOR, increased initially followed by a decrease with increasing dietary Hyp level. This phenomenon was only observed in swim bladder. Increased transcript levels of S6K1 were observed in the liver and muscle, but not in intestine with increasing dietary levels of Hyp. These differences in the expression patterns of 4E-BP, S6K1, and TOR in the liver, muscle, and swim bladder suggest that Hyp improved protein synthesis by increasing TOR activity. This observation is however independent of the expression regulation of 4E-BP and S6K1 genes, as the mTOR pathway is regulated by phosphorylation of mTORC1 targets. Thus, the phosphorylation status of these genes in response to dietary Hyp supplementation would have to be further explored. In any case, the current study highlights the beneficial effects of Hyp on promoting *N. diacanthus* growth and the synthesis of proteins. Hyp seems to regulate the TOR signaling pathway through the phosphorylation of 4E-BP and S6K1, but the underlying mechanism requires further investigation.

Conclusion

The findings here revealed that appropriate Hyp supplementation in diet could improve fish growth, feed conversion ratio, and protein efficiency ratio. Regression analysis of the SGR data and dietary Hyp levels revealed that the optimum amount of Hyp to add to the basal diet was 16.6 g kg⁻¹. Proximate composition and amino acid composition of tissues were affected by addition of dietary Hyp, but physiology indices of serum and liver were not affected by dietary Hyp supplementation. Dietary Hyp was shown to affect transcript levels of TOR, 4E-BP, and S6K1 in different tissues, which suggest that Hyp might enhance fish growth by regulating fish growth through the TOR pathway.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

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