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Hydroxyproline supplementation on the performances of high plant protein source based diets in turbot (*Scophthalmus maximus* L.)

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ABSTRACT

Hydroxyproline (hyp) is one of the bioactive molecules rich in fishmeal but low in plant protein sources. With increased utilization of plant proteins in aquafeeds, a better understanding is warranted for the necessity of hyp supplementation in high plant protein based diets. In the present study, isonitrogenous and isoenergetic turbot diets were formulated with 40%, 50% and 60% fishmeal substituted by plant proteins, with (or without) addition of 0.6% hyp. After an 8-week feeding trial in juvenile turbot, hyp supplementation significantly improved specific growth rate (SGR) and feed efficiency ratio (FER) in fish fed diets with 50% or higher fishmeal replaced, but not in the group with 40% fishmeal replaced by plant proteins. Hyp levels in plasma and tissues were reduced after plant protein substitutions and replenished by dietary supplementation. Dietary hyp significantly increased muscle hardness, springiness and chewiness of fish ($P < 0.05$). These results suggested that dietary hyp supplementation is particularly necessary for fishmeal-replaced diet exceeding a certain high level.

1. Introduction

Fishmeal is the most commonly used protein source in aquaculture and indispensable for many farmed fish species. For the sustainability of aquaculture industry and lower cost, many aquafeed manufacturers have turned their focus on plant proteins for fishmeal replacement (Hardy, 2010). However, there are still major challenges remaining for fishmeal replacement especially in diets for carnivorous fish, such as Atlantic salmon (Carter and Hauler, 2000; Storebakken et al., 1998), rainbow trout (Gomes et al., 1995; Kaushik et al., 1995), gilthead sea bream (Gómez-Requeni et al., 2004), Japanese flounder (Kikuchi, 1999), Korean rockfish (Lim et al., 2004) and Atlantic cod (Hansen et al., 2007). Understanding the differences in composition and utilization of fishmeal and plant protein sources is necessary for the development of new protein sources for aquaculture.

Hydroxyproline (hyp) is one of several bioactive factors (such as taurine) identified to be rich in fishmeal but low or none in plant protein sources (Aksnes et al., 2006; Cheng and Hardy, 2004; Gaylord et al., 2007; Lunger et al., 2007). Hyp is crucial for collagen synthesis. The helical region of collagen comprises the repeat of Gly–X–Y, where hyp occurs in the Y position. Hyp is also necessary in many physiological processes. It is a substrate for the synthesis of glycine, pyruvate, and glucose (Wu et al., 2011) and may also scavenge oxidants and regulate the redox state of cells (Phang et al., 2008, 2010). In addition, hyp has been proved to be the only free amino acid in tissues that was positively correlated with the growth rate of juvenile salmon (Kousoulaki et al., 2012; Sunde et al., 2001).

Hydroxyproline is produced by hydroxylation of the amino acid proline by the enzyme prolyl hydroxylase following protein synthesis (Gorres and Raines, 2010). However, it is largely unknown for the ability of hyp biosynthesis in fish. In recent years, scientists have found that hyp might be a conditionally indispensible amino acid for fish (Li et al., 2009). It was reported that dietary hyp inclusion promoted growth in Atlantic salmon fed with high plant protein diets (Aksnes et al., 2008), but not in turbot (Zhang et al., 2013). The application of dietary hyp supplementation, especially to high plant protein based diets, requires further examinations. The present study was conducted to evaluate effects of hyp addition to diets with serial levels of plant proteins on growth performance, muscle texture, hyp content in tissues of juvenile turbot.

2. Materials and methods

2.1. Feeding ingredients and diet formulation

L-hydroxyproline (>99% pure) was obtained from Hengyuan Biotech (Shanghai, China) Co., LTD. Fishmeal, soybean meal, corn gluten meal, wheat gluten meal, peanut meal, and beer yeast were used as the primary protein sources. Fish oil and soybean lecithin were used as the lipid sources. Wheat flour was used as the carbohydrate sources. Seven isonitrogenous and isoenergetic diets were formulated: a reference diet (FM) containing 60% fish meal and three other diets (40I, 50I and 60I) in which 40%, 50% and 60% fish meal was substituted. Based on the supplementation levels used in reports (Asknes et al., 2008; Zhang

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et al., 2013), experimental diets with plant protein substitutions were either supplemented with 0.6% hyp or not. All ingredients were grounded into fine powder through 178 μm mesh. Ingredients were blended with oil and water then added. Pellets (3 mm × 4 mm) were made automatically by pellet-making machine and dried for 12 h in a ventilated oven at 40 °C. The hyp concentrations after diet preparation were quantitated as 0.23% (FM), 0.16%(40I), 0.58%(40I HYP), 0.18% (50I), 0.62% (50I HYP), 0.14% (60I) and 0.57% (60I HYP). DL-Methionine, L-Threonine, L-Histidine, and Lys-H_2SO_4 (Crystalline amino acids) were supplemented to meet the essential amino acid (EAA) requirements of juvenile turbot based on the amino composition of FM diet. All the compositions of experiment diets were shown in Table 1.

Table 1
Formulation and proximate chemical composition of the tested diets (% dry matter).

Ingredients	Treatments						
	FM	40I	40I HYP	50I	50I HYP	60I	60I HYP
Fish meal[a]	60.00	36.00	36.00	30.00	30.00	24.00	24.00
Wheat flour[a]	27.50	12.95	12.35	10.23	9.63	7.51	6.91
Soybean meal[a]	0.00	15.68	15.68	19.60	19.60	23.52	23.52
Corn gluten meal[a]	0.00	8.00	8.00	10.00	10.00	12.00	12.00
Wheat gluten meal[a]	0.00	5.12	5.12	6.40	6.40	7.68	7.68
Peanut meal[a]	0.00	3.20	3.20	4.00	4.00	4.80	4.80
Beer yeast[a]	2.50	2.50	2.50	2.50	2.50	2.50	2.50
Vitamin premix[b]	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Mineral premix[c]	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Attranct[d]	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Taurine	0.00	1.00	1.00	1.00	1.00	1.00	1.00
Sodium alginate	0.00	1.00	1.00	1.00	1.00	1.00	1.00
DL-Methionine	0.00	0.26	0.26	0.32	0.32	0.38	0.38
L-Threonine	0.00	0.18	0.18	0.22	0.22	0.26	0.26
L-Histidine	0.00	0.19	0.19	0.23	0.23	0.27	0.27
Lys-H_2SO_4	0.00	0.74	0.74	0.92	0.92	1.10	1.10
Fish oil	3.00	5.50	5.50	5.90	5.90	6.30	6.30
Soybean lecithin	2.50	2.50	2.50	2.50	2.50	2.50	2.50
Choline chloride	0.25	0.25	0.25	0.25	0.25	0.25	0.25
$Ca(H_2PO_4)_2$	0.00	0.40	0.40	0.40	0.40	0.40	0.40
Phytase	0.00	0.20	0.20	0.20	0.20	0.20	0.20
Y_2O_3	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Calcium propionate	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Ethoxyquin	0.05	0.05	0.05	0.05	0.05	0.05	0.05
$FeSO_4 \cdot H_2O$	0.00	0.05	0.05	0.05	0.05	0.05	0.05
$ZnSO_4 \cdot H_2O$	0.00	0.03	0.03	0.03	0.03	0.03	0.03
L-Hydroxyproline (% dry matter)	0.00	0.00	0.60	0.00	0.60	0.00	0.60
Moisture	3.90	4.23	3.84	5.00	4.37	3.94	4.97
Crude protein	50.75	50.97	51.75	51.39	51.51	52.17	51.83
Crude lipid	10.95	11.48	11.14	11.56	11.10	11.75	11.35
Ash	11.64	10.08	9.77	9.44	9.15	8.77	8.60
Gross energy (KJ g^{-1})	20.23	20.57	20.67	20.60	20.66	20.99	20.73

Note: FM, diet fish meal; 40I, replacement of 40% fish meal by plant protein mixture; 40I HYP, replacement of 40% fish meal by plant protein mixture with addition of 0.6% hydroxyproline; 50I, replacement of50% fish meal by plant protein mixture; 50I HYP, replacement of 50% fish meal by plant protein mixture with addition of 0.6% hydroxyproline; 60I, replacement of 60% fish meal by plant protein mixture; 60I HYP, replacement of 60% fish meal by plant protein mixture with addition of 0.6% hydroxyproline.

[a] Supplied by Qihao Biotech. Co., Ltd. (Qingdao, Shandong); red fish meal, crude protein, 73.38%, crude lipid, 10.42%; wheat flour, crude protein, 17.05%, crude lipid, 2.29%; soybean meal, crude protein, 55.04%, crude lipid 2.02%; corn gluten meal, crude protein, 70.45%, crude lipid, 1.67%; wheat gluten meal, crude protein, 80.27%, crude lipid, 1.24%; peanut meal, crude protein, 50.82%, crude lipid, 2.90%; beer yeast, crude protein, 49.78%, crude lipid, 1.61%.

[b] Vitamin premix (mg kg^{-1} diet): retinal palmitate, 32; cholecalciferol, 5; DL- -to-copherol acetate, 240; menadione, 10; thiamin-HCl, 25; riboflavin, 45; pyridoxine-HCl, 20; cyanocobalamin, 10; D-calcium pantothenate, 60; amine nicotinic acid, 200; folic acid, 20; biotin, 60; mesoinositol, 800; ascorbyl polyphosphate (contained 35% ascorbic acid), 2000; microcrystalline cellulose, 16473.

[c] Mineral premix (mg kg^{-1} diet): $MgSO_4 \cdot 7H_2O$, 1200; $CuSO_4 \cdot 5H_2O$, 10; $FeSO_4 \cdot H_2O$, 80; ZnSO4 · H_2O, 50; $MnSO_4 \cdot H_2O$, 45; $CoCl_2 \cdot 6H_2O$ (1%), 50; Na_2SeO_3 (1%), 20; calcium iodine, 60; zoelite, 8485.

[d] Attractant: betaine: dimethyl-propiothetin: glycine: alanine: 5-phosphate inosine = 4:2:2:1:1.

2.2. Fish and experimental procedure

Juvenile turbot were obtained from Haiyang fish farm (Haiyang, Shandong, China). Fish were acclimated to the system and fed with commercial diet for 2 weeks before the trials. Juvenile turbot (initial body weight: 8.63 ± 0.03 g) were randomly distributed into 28 tanks (60 cm * 60 cm * 60 cm), and each of the 7 experimental diets was assigned to 4 tanks with 30 fish per tank. Seawater, continuously pumped from the coast adjacent to the experimental station, passed through sand filters into each tank at approximately 1.5 l/min. The experimental fish were fed to apparent satiety twice a day at 7:00 and 19:00 for 9 weeks. The consumption of each tank was recorded every day. Any uneaten feed was collected 1 h after each meal, dried to constant weight at 70 °C and reweighed. During the whole experimental period, water temperature was ranged from 19.0 to 22.0 °C; pH from 7.5 to 8.0; salinity from 30‰ to 33‰; ammonia nitrogen was lower than 0.1 mg/l; nitrite was lower than 0.1 mg/l; dissolved oxygen was higher than 6.0 mg/l.

2.3. Sample collection

Before the feeding, 20 fish from the same population were randomly selected for determination of initial whole-body proximate composition. At the end of the feeding trial, the experimental fish in each tank were anesthetized with eugenol (1:10,000) (99% purity, Shanghai Reagent, China). Total number and mean body weight of fish in each tank were measured. Five fish were randomly sampled from each tank and stored frozen at −20 °C for whole body composition analysis. Six fish from each tank were sampled to measure individual body weight, body length, liver weight and visceral weight so as to calculate condition factor, hepatosomatic index and viserosomatic index. Meanwhile, the liver and back muscle were sampled and frozen in liquid nitrogen. Blood samples were taken from the caudal vein of another five fish from each tank using heparinized syringes to obtain plasma samples after centrifugation (4000 *g* for 10 min) at 4 °C and immediately stored at −20 °C until analysis.

2.4. Chemical analyses

2.4.1. Body composition

Dry matter, crude protein, crude lipid, ash and energy were analyzed for ingredients, experimental diets and fish samples using standard Association of Official Analytical Chemist (AOAC) methods (1995). Dry matter was analyzed 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).

2.4.2. Digestibility determination

Y_2O_3 (0.1%) was supplemented as the indicator for the dry matter and crude protein digestibility determination following previous studies (Glencross et al., 2007; Regost et al., 2003). Fecal samples were collected for 2 weeks from each tank, using an automatic fecal collector by siphoning 1 h after feeding. Collected fecal samples were stored at −20 °C. Yttrium oxide and phosphorus content in the diet and feces were determined by inductively coupled plasma-atomic emission spectrophotometer (ICP-OES, VISTA-MPX).

2.4.3. Texture profile analysis

The flesh of back was obtained from the same position of each fish with similar size (40.10 ± 2.51 g). The texture profile analysis (TPA) was carried out with a texture analyzer immediately after the flesh

samples were collected. TPA was made using a metal detector with 8 mm diameter. Samples were compressed at three dots anterior to posteriorly. Three relative parameters, including hardness, chewiness, and springiness were obtained from the TPA.

2.4.4. Hyp and collagen content determination

Hyp concentration in tissues was determined using the method described by Reddy and Enwemeka (1996). Aliquots of 1 ml standard hyp (1–30 μg/ml in 1 mM HCl) or 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.

Before the hyp determination of liver and muscle, the samples was hydrolyzed using 1 ml 6 M hydrochloric acid under 130 °C for 3 h. Samples were then diluted into 10 ml volumetric flasks, and filtered through 0.20 μm filter. One milliliter of the solution was used to determine the hyp concentration using the same method as for plasma. The collagen content was estimated by multiplying the hyp content (% of sample) by 8.

2.4.5. Calculations and statistical methods

Growth parameters were calculated as follows:

$$\text{Survival rate}(SR\%) = 100 \times (\text{final fish number}/\text{initial fish number})$$

$$\text{Weight gain rate}(WGR\%) = 100 \times (\text{final body weight} - \text{initial body weight})/\text{initial body weight}$$

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

$$\text{Feed intake}(FI\%/d) = 100 \times \text{dry feed intake}(g)/[(\text{final body weight} + \text{initial body weight})/2]/\text{days}$$

$$\text{Feed efficiency rate}(FER) = \text{wet weight gain}(g)/\text{dry feed intake}(g)$$

$$\text{Protein efficiency ratio}(PER) = \text{wet weight gain}(g)/\text{protein ingested}(g)$$

$$\text{Condition factor}(K\%) = 100 \times \text{final body weight}/\text{body length}^3$$

$$\text{Hepatosomatic index}(HIS\%) = 100 \times \text{liver wet weight}/\text{final body weight}$$

$$\text{Viscerosomatic index}(VSI\%) = 100 \times \text{viscera wet weight}/\text{final body weight}$$

$$\text{Apparent digestibility coefficients}(ADC) = 100 \times [1 - (Y_2O_3\text{diet} \times \text{nutrient feces})/(Y_2O_3\text{feces} \times \text{nutrient diet})]$$

The Software SPSS, 17.0 was used for all statistical evaluations. All data were subjected to one-way analysis of variance (ANOVA) followed by Tukey's test. Differences were regarded as significant when $P < 0.05$. Data are presented as means ± standard error.

3. Results

3.1. Growth performance

As shown in Table 2, both survival rate (SR) and feed intake (FI) showed no differences among groups. When 40% of fishmeal was replaced by plant proteins in diet (40I), no significant difference was found in weight gain rate (WGR) and specific growth rate (SGR) of turbot, compared to FM. However, 50% (50I) and 60% (60I) levels of fishmeal replacement significantly reduced WGR and SGR without hyp. Supplementation of hydroxyproline to 40I (40I HYP) did not significantly affect growth parameters, while hydroxyproline supplementation significantly improved growth parameters at both 50% (50I HYP) and 60% (60I HYP) fishmeal-replacing levels. Noticeably, turbot fed 60I HYP even showed a similar value in SGR and WGR to those fed with FM.

3.2. Body composition

No significant differences were found in the crude protein (app. 16.2%) and lipid (app. 3.5%) content of fish among all dietary treatments (Table 3). No significant differences were found in moisture (app. 77.0%) and ash (4.1%) content of turbot. No significant differences were found among dietary treatments in condition factor, hepatosomatic index, and viscerosomatic index (Table 4). The apparent digestibility coefficients (ADC) of dry matter and crude protein decreased significantly with increasing fishmeal-replacing levels in diets (Table 5). Supplementation of hydroxyproline did not show any effect on ADC of dry matter and crude protein at all replacing levels.

3.3. Texture

As shown in Table 6, muscle springiness was not affected at 40% fishmeal-replacing level with (40I HYP) or without (40I) hyp. However, muscle texture (including hardness, chewiness and springiness) performances were reduced when fishmeal was replaced at 50% and 60% (Table 6). Fish fed diets with 50I HYP and 60I HYP showed significantly

Table 2
Growth parameters of juvenile turbot fed the experimental diets*.

Treatments	Initial body weight (g)/IBW	Final body weight (g)/FBW	Weight gain rate (%)/WGR	Specific growth rate (%)/SGR	Feed intake (%/d)/FI	Feed efficiency ratio/FER	Protein efficiency ratio/PER	Survival rate (%)/SR
FM	8.60 ± 0.03	38.97 ± 0.99^{a}	3.53 ± 0.13^{a}	2.70 ± 0.05^{a}	2.15 ± 0.06	0.98 ± 0.01^{a}	51.21 ± 1.12	92.5 ± 4.20
40I	8.63 ± 0.04	37.89 ± 0.88^{ab}	3.40 ± 0.10^{a}	2.64 ± 0.04^{ab}	2.11 ± 0.04	0.93 ± 0.03^{bcd}	50.49 ± 1.86	93.3 ± 3.85
40I HYP	8.62 ± 0.01	36.82 ± 1.08^{bc}	3.27 ± 0.13^{ab}	2.59 ± 0.05^{bc}	2.14 ± 0.09	0.95 ± 0.04^{abc}	50.31 ± 1.99	92.2 ± 6.94
50I	8.66 ± 0.04	35.24 ± 0.29^{cd}	3.08 ± 0.02^{bc}	2.51 ± 0.02^{cd}	2.13 ± 0.05	0.91 ± 0.04^{cd}	48.79 ± 1.00	88.9 ± 3.85
50I HYP	8.63 ± 0.02	36.96 ± 0.77^{b}	3.29 ± 0.08^{ab}	2.60 ± 0.04^{b}	2.07 ± 0.09	0.97 ± 0.01^{ab}	49.73 ± 1.12	94.2 ± 3.85
60I	8.61 ± 0.05	33.94 ± 1.47^{d}	2.93 ± 0.16^{c}	2.45 ± 0.08^{d}	2.11 ± 0.01	0.89 ± 0.01^{d}	48.28 ± 0.11	94.4 ± 3.85
60I HYP	8.63 ± 0.01	37.79 ± 0.98^{ab}	3.37 ± 0.11^{a}	2.64 ± 0.05^{ab}	2.10 ± 0.04	0.92 ± 0.01^{cd}	48.94 ± 0.57	91.7 ± 6.38
ANOVA								
F	1.43	9.82	10.49	9.03	0.68	5.20	2.21	0.50
P	0.25	0.00	0.00	0.00	0.67	0.004	0.10	0.80

Note: * Values show mean ± standard error, n = 4; values in the same column with different superscripted small letters mean significant difference ($P < 0.05$).

Table 3
Whole body composition of juvenile turbot fed the experimental diets*.

Treatments	Moisture (%)	Crude protein (%)	Crude lipid (%)	Ash (%)
FM	77.31 ± 0.81	16.24 ± 0.28	3.52 ± 0.18	4.13 ± 0.18
40I	76.74 ± 0.59	16.38 ± 0.30	3.47 ± 0.01	4.07 ± 0.04
40I HYP	76.26 ± 0.22	16.40 ± 0.22	3.38 ± 0.17	4.10 ± 0.20
50I	77.38 ± 0.77	16.28 ± 0.27	3.33 ± 0.09	3.98 ± 0.19
50I HYP	77.45 ± 0.95	16.23 ± 0.18	3.44 ± 0.30	4.06 ± 0.14
60I	76.27 ± 0.60	16.18 ± 0.18	3.68 ± 0.07	4.00 ± 0.13
60I HYP	77.11 ± 0.79	16.38 ± 0.15	3.66 ± 0.07	3.94 ± 0.21
ANOVA				
F	2.02	0.72	1.39	0.56
P	0.11	0.64	0.26	0.76

Note: * Values show mean ± standard error, n = 4; values in the same column with different superscripted small letters mean significant difference ($P < 0.05$).

Table 5
Apparent digestibility coefficients (%, ADC) for dry matter and crude protein of the experimental diets*.

Treatments	Dry matter	Crude protein
FM	59.14 ± 0.58^{a}	91.05 ± 0.34^{a}
40I	56.84 ± 0.68^{b}	89.41 ± 0.35^{b}
40I HYP	55.72 ± 0.34^{bc}	89.43 ± 0.35^{b}
50I	54.61 ± 1.47^{cd}	87.59 ± 0.45^{bc}
50I HYP	53.95 ± 0.50^{d}	88.43 ± 0.47^{c}
60I	51.56 ± 0.45^{e}	85.68 ± 0.69^{d}
60I HYP	51.89 ± 2.62^{e}	85.61 ± 0.52^{d}
ANOVA		
F	56.44	74.54
P	0.00	0.00

Note: * Values show mean ± standard error, n = 4; values in the same column with different superscripted small letters mean significant difference ($P < 0.05$).

higher hardness and chewiness than those fed with diet 50I and 60I and showed no significant difference in muscle texture to those in control group.

3.4. Free Hyp content in plasma, total Hyp and collagen in muscle

As shown in Table 7, plasma free hyp showed significant decreases in fish fed with 40I, and 50I, and 60I diets after fishmeal replacement. Similar decrease in muscle total hyp and collagen was found in fish fed with 60I diet compared to control. Supplementation of hyp significantly increased hyp levels in plasma and muscle, and muscle collagen level. Indeed, these parameters showed higher values in groups with hyp supplementation than those in control group, which was with fishmeal diet (Table 7).

4. Discussion

The benefits of dietary hydroxyproline supplementation have been studied in Atlantic salmon fed with high plant protein diets (Aksnes et al., 2008). In that experiment, different levels of hyp were added to diet with a designated level of plant proteins and the results showed stimulated growth performances in salmon. A similar design was also carried out in a study on turbot, but no apparent effects of dietary hyp supplementation were observed on growth performances. However, the necessity of hydroxyproline supplementation might be directly correlated with the substitution levels of fishmeal by plant proteins and the physiological responses of the culture animals toward the plant protein diets. In this study, no growth stimulation was observed in turbot fed with 40% fishmeal replaced diet by hyp addition, a result similar to what was reported (Zhang et al., 2013). However, growth stimulation was observed in groups fed with 50% and 60% fishmeal fed diets by hyp addition, a result coincided with what was observed in salmon (Aksnes et al., 2008). These results suggested that hydroxyproline was particularly necessary for fish fed diets exceeding a certain level of plant proteins. In fact, fishmeal substitution significantly reduced plasma free hyp levels ($P < 0.05$). This indicated that dietary hydroxyproline is necessary for its homeostasis in turbot, supporting the assumption that hydroxyproline is a conditional indispensible amino acid (Li et al., 2009). On the other hand, hyp addition in diets significantly increased hyp levels in plasma and muscle. It is very likely that hyp is directly absorbed and transported in tissues without dehydroxylation (Pinilla-Tenas et al., 2003). To date, there is no information on the hydroxyproline biosynthesis in fish. However, from what was reported and our study, it is likely the capacity of proline hydroxylation in fish is limited, which makes dietary hyp intake necessary.

Hydroxyproline is necessary for collagen biosynthesis. Collagen level in muscle was decreased in 60I group, likely as a consequence of hydroxyproline shortage. On the other hand, hyp addition significantly increased muscle collagen content. This result indicated that hydroxyproline levels could be a limiting factor for collagen biosynthesis in turbot. Under the condition of plant protein based diet, shortage of dietary hyp supply reduced hyp levels in plasma and muscle, and the collagen synthesis rate was likely to be influenced. Furthermore, collagen content is directly related to muscle textures (Hagen et al., 2007; Li et al., 2005). In the present study, fishmeal replacement reduced muscle hardness, springiness and chewiness while hyp supplementation significantly improved these parameters, which can be important for the quality of farmed fish species. Periago et al. (2005) found wild specimens of sea bass showed higher collagen and hyp content. Collagen concentration and collagen crosslinks influence muscle texture in Atlantic halibut and in Atlantic salmon (Hagen et al., 2007; Li et al., 2005). From our study and others (Aksnes et al., 2008; Kousoulaki et al., 2012; Zhang et al., 2013), dietary free hyp resulted in significantly increased collagen and crosslink concentrations, which could improve muscle firmness. Therefore, hydroxyproline is beneficial for flesh quality improvement under aquaculture.

Table 4
Condition factor (CF), hepatosomatic index (HSI) and viscerosomatic index (VSI) of juvenile turbot fed the experimental diets*.

Treatments	CF (%)	HIS (%)	VSI (%)
FM	3.43 ± 0.16	1.05 ± 0.18	4.70 ± 0.22
40I	3.35 ± 0.15	1.12 ± 0.21	4.76 ± 0.15
40I HYP	3.44 ± 0.13	1.09 ± 0.17	4.74 ± 0.25
50I	3.43 ± 0.25	1.02 ± 0.15	4.59 ± 0.35
50I HYP	3.37 ± 0.17	1.12 ± 0.26	4.97 ± 0.13
60I	3.30 ± 0.11	1.08 ± 0.16	4.99 ± 0.11
60I HYP	3.29 ± 0.11	1.15 ± 0.09	5.00 ± 0.46
ANOVA			
F	0.45	0.44	1.00
P	0.83	0.85	0.46

Note: * Values show mean ± standard error, n = 4; values in the same column with different superscripted small letters mean significant difference ($P < 0.05$).

Table 6
Muscle texture analysis of juvenile turbot fed the experimental diets*.

Treatments	Hardness (gf)	Chewiness (mJ)	Springiness (mm)
FM	552.9 ± 34.7^{a}	187.8 ± 18.1^{a}	0.98 ± 0.06^{ab}
40I	471.2 ± 12.5^{ab}	153.1 ± 8.8^{ab}	0.92 ± 0.04^{bc}
40I HYP	576.6 ± 33.3^{a}	194.9 ± 27.1^{a}	1.05 ± 0.01^{a}
50I	445.2 ± 11.7^{b}	129.9 ± 6.4^{b}	0.82 ± 0.02^{cd}
50I HYP	567.8 ± 31.8^{a}	186.2 ± 14.5^{a}	1.06 ± 0.05^{a}
60I	442.0 ± 41.4^{b}	129.3 ± 16.3^{b}	0.75 ± 0.05^{d}
60I HYP	587.5 ± 51.0^{a}	189.9 ± 9.4^{a}	1.07 ± 0.04^{a}
ANOVA			
F	5.32	3.42	26.65
P	0.00	0.02	0.00

Note: * Values show mean ± standard error, n = 4; values in the same column with different superscripted small letters mean significant difference ($P < 0.05$).

Table 7
Plasma free hyp, muscle total hyp contents, and muscle total collagen of juvenile turbot fed the experimental diets*.

Treatments	Plasma free Hyp (μg/ml)	Muscle total Hyp (g/kg wet basis)	Muscle total collagen (% wet basis)
FM	50.36 ± 2.78^{b}	0.403 ± 0.023^{b}	0.322 ± 0.019^{b}
40I	46.10 ± 3.14^{c}	0.320 ± 0.007^{bc}	0.256 ± 0.005^{bc}
40I HYP	68.88 ± 3.58^{a}	0.654 ± 0.074^{a}	0.523 ± 0.059^{a}
50I	43.83 ± 2.87^{c}	0.324 ± 0.068^{bc}	0.260 ± 0.054^{bc}
50I HYP	75.83 ± 4.23^{a}	0.675 ± 0.080^{a}	0.540 ± 0.064^{a}
60I	40.53 ± 2.00^{c}	0.281 ± 0.031^{c}	0.225 ± 0.025^{c}
60I HYP	70.40 ± 2.63^{a}	0.713 ± 0.081^{a}	0.571 ± 0.065^{a}
ANOVA			
F	22.19	36.15	36.15
P	0.00	0.00	0.00

Note: * Values show mean ± standard error, n = 4; values in the same column with different superscripted small letters mean significant difference ($P < 0.05$).

Fishmeal replacement remains a major challenge for aquaculture researches. Many factors were listed as limiting factors, including differences in amino acid profiles and palatability, the presence of anti-nutritional factors in plant proteins, low digestibility of non-fishmeal proteins etc. However, it should also be noted the importance of bioactive molecules enriched only in fishmeal. So far, taurine (Wang et al., 2014) and hydroxyproline have been shown to be necessary for optimal growth and health of fish. In the present study, without hydroxyproline inclusion, the plant protein mixture could only substitute 40% of fishmeal without affecting growth in turbot. However, hydroxyproline supplementation improved the replacement ratio to 60%. Therefore, hydroxyproline could be a limiting factor when high levels of plant protein are used in diet, which makes dietary supplementation necessary.

Conflict of interest

The authors declare no conflict of interest.

Authorship

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

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