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Comparative study on the effects of L-methionine or 2-hydroxy-4-(methylthio) butanoic acid as dietary methionine source on growth performance and anti-oxidative responses of turbot (*Psetta maxima*)

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ABSTRACT

A growth trial was conducted to evaluate the effects of L-methionine (L-Met) or 2-hydroxy-4-(methylthio) butanoic acid (HMTBa) as dietary methionine source on growth, whole-body composition, and ascorbic acid concentrations in serum and liver of turbot (*Psetta maxima*). Five levels (0.3, 0.6, 0.9, 1.2 and 1.5% dry matter) of L-Met or HMTBa were added to a practical basal diet, which was limiting in methionine (0.59%) and cystine (0.42%). The basal control diet and 10 experimental diets were fed to groups ($n = 5$) of juvenile turbot (initial weight: 5.6 g), which were reared in a flow-through seawater system. Fish were fed twice daily to satiation for 75 days. Fish fed the basal diet displayed significantly ($P < 0.05$) lower specific growth rate (SGR), feed efficiency (FE), feed intake (FI), protein efficiency ratio (PER), productive protein value (PPV), whole-body protein and lipid contents, but higher whole-body moisture and ash contents compared to those fed with L-Met or HMTBa supplemented diets. Ascorbic acid concentrations in serum increased significantly with dietary L-Met or HMTBa levels ($P < 0.05$), but not in liver ($P > 0.05$). On the basis of SGR or FI, the dietary total methionine requirement of juvenile turbot was estimated to be 1.58 and 1.59% (3.31 and 3.27% of dietary protein) based on L-Met or 1.56% and 1.49% (3.25% and 3.19% of dietary protein) based on HMTBa, respectively, using second-order polynomial regression analysis. Furthermore, fish fed HMTBa, in addition, showed higher PER and ascorbic acid concentrations in serum than those fed L-Met. In conclusion, turbot can use HMTBa as effectively as or better than L-Met to achieve a higher maximum performance.

1. Introduction

Turbot (*Psetta maxima*) is a flatfish species in Europe, which is increasingly being cultured in China since its introduction in 1992 (FAO, 2010). It is a fast-growing fish that is much sought after by consumers (Regost et al., 2001). There are several studies on protein and lipid requirement of this species (Cho et al., 2005; Lee et al., 2003; Li et al., 2011; Regost et al., 2001). However, there is little published data on the quantitative requirement of essential amino acids for turbot (Fournier et al., 2002; Kaushik, 1998; Peres and Oliva-Teles, 2008).

The turbot is a highly carnivorous species with a protein requirement of 49.4% (Lee et al., 2003), and feed formulations for this species have typically incorporated high levels of fish meal. Due to increasing costs, there has been a trend towards decreasing fish meal levels in dietary formulations and replacing it with plant ingredients. Methionine is an essential amino acid required by terrestrial vertebrates as well as various fish species for normal growth and metabolic functions (Luo et al., 2005). In many fish diet, methionine is usually the first limiting amino acid, especially those containing high levels of plant protein feedstuffs such as soybean meal, peanut meal and copra meal (Mai et al., 2006). It is suggested from the previous studies that requirements of dietary methionine for fish range from 1.8 to 4.0% of dietary protein (Wilson, 2002). Methionine is produced commercially by chemical processes and most commonly available in the DL-form. L-methionine is the natural isomer. And D-methionine form is converted to L-methionine in animals. Previous studies showed that fish can use DL-methionine as effectively as L-methionine (Goff and Gatlin, 2004; Robinson et al., 1978). The 2-hydroxy-4-(methylthio) butanoic acid (HMTBa) is an organic acid, which bears a hydroxyl group on the α-carbon instead of the amino group found in methionine. Because HMTBa can be converted to L-methionine within the body of the animal through broadly distributed enzymatic systems (Dibner, 2003), it has been widely used in diets of poultry, swine and ruminant. The use of HMTBa in aquatic animals has been reported in several fish and shrimp species (Cheng et al., 2003; Huai et al., 2010; Zhao et al., 2010), but not in turbot.

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Studies with terrestrial animals (e.g., broiler chicks, laying hens and pigs) showed variable results with regard to the relative biological efficiency (RBE) of HMTBa to L-methionine (L-Met) or DL-methionine (DL-Met) (Harms and Russell, 1994; Kim et al., 2006; Lemme et al., 2002; Liu et al., 2004; Yi et al., 2006). Varied responses have also been observed in aquatic animals, such as channel catfish (Robinson et al., 1978), hybrid striped bass (Keembiyehetty and Gatlin, 1995; Kelly et al., 2006; Li et al., 2009), red drum (Goff and Gatlin, 2004) and Pacific white shrimp (Forster and Dominy, 2006). Some studies have considered HMTBa as a dilution of methionine with the same form of dose–response and same plateau, and have used slope-ratio model or nonlinear common plateau asymptotic regression models to compare the different sources (Littell et al., 1997). However, other research has suggested that relative efficacies vary with dose (Kratzer and Littell, 2006), and that different dose responses and plateau responses occur for HMTBa and DL-Met as seen in broilers (Vázquez-Añón et al., 2006a) and turkeys (González-Esquerra et al., 2007). However, there is little published information on the response to doses of different methionine sources in aquatic animals.

Levine et al. (1999) showed that methionine residues in a wide variety of proteins play important roles in anti-oxidative defense activity from *Escherichia coli* as an in vitro model system. HMTBa increased plasma ascorbic acid concentrations and decreased thiobarbituric acid reactive substance (TBARs) concentrations in liver of hybrid striped bass (Li et al., 2009). Meanwhile, it improved anti-oxidative status and depressed lipid and protein oxidation in intestine, hepatopancreas, serum and muscle of juvenile Jian carp (Feng et al., 2011; Xiao et al., 2012).

Therefore, this study was conducted to comparatively evaluate the effects of L-methionine or HMTBa as dietary methionine sources on growth, feed utilization and anti-oxidative response in turbot (*P. maxima*).

2. Materials and methods

2.1. Experimental diets

A basal practical diet (Table 1) was formulated to contain 48% (dry matter) crude protein and 12% crude lipid. A crystalline L-amino acid premix was supplemented to diet according to the whole body amino acid pattern of turbot (Kaushik, 1998) except for methionine and cystine (Table 2). The basal diet contained 0.59% methionine and 0.42% cystine as determined by amino acid analyzer (S7130, Sykam, Munich, Germany). Graded levels (0.3, 0.6, 0.9, 1.2 and 1.5%) of either L-Met or HMTBa on an equivalent basis were added to the basal diet, respectively. Crystalline L-Met was used as the source of dietary L-Met. HMTBa was supplemented in the form of Mera™ Met (an 84% Ca salt of HMTBa, Novus International Inc., St. Charles, MO, USA). Final L-methionine concentrations in the five L-Met supplemented diets (Diet 2–6) were 0.91, 1.15, 1.49, 1.70 and 2.02%, respectively. And final HMTBa contents in the five Mera™ Met supplemented diets (Diet 7–11) were 0.31, 0.61, 0.97, 1.23 and 1.39%, respectively as determined by the method of Ontiveros et al. (1987). The reverse-phase high-pressure liquid chromatography (HPLC; HP 1100, HP, Palo Alto, USA) with a Zobar C18 column (4.6 mm × 250 mm) was used. Mobile

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

Ingredients	Diet number (% of methionine source added)										
	Diet1	Diet2	Diet3	Diet4	Diet5	Diet6	Diet7	Diet8	Diet9	Diet10	Diet11
	(0)	L-Met (0.3)	L-Met (0.6)	L-Met (0.9)	L-Met (1.2)	L-Met (1.5)	HMTBa (0.3)	HMTBa (0.6)	HMTBa (0.9)	HMTBa (1.2)	HMTBa (1.5)
Fish meal[a]	23.6	23.6	23.6	23.6	23.6	23.6	23.6	23.6	23.6	23.6	23.6
Soybean meal[a]	22.6	22.6	22.6	22.6	22.6	22.6	22.6	22.6	22.6	22.6	22.6
Beer yeast[a]	5	5	5	5	5	5	5	5	5	5	5
Amino acid mixture[b]	14.5	14.5	14.5	14.5	14.5	14.5	14.5	14.5	14.5	14.5	14.5
Wheat meal[a]	19.31	19.31	19.31	19.31	19.31	19.31	19.31	19.31	19.31	19.31	19.31
Fish oil	7	7	7	7	7	7	7	7	7	7	7
Lecithin	1	1	1	1	1	1	1	1	1	1	1
Mineral premix[c]	2	2	2	2	2	2	2	2	2	2	2
Vitamin premix[d]	2	2	2	2	2	2	2	2	2	2	2
Choline chloride	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Ca $(H_2PO_4)_2$•H_2O	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Attractant	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Mold inhibitor	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Antioxidant	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
L-methionine[e]	0	0.3	0.6	0.9	1.2	1.5	0	0	0	0	0
Mera™ Met[f]	0	0	0	0	0	0	0.36	0.71	1.07	1.43	1.79
Glutamic acid[e]	1.79	1.49	1.19	0.89	0.59	0.29	1.43	1.08	0.72	0.36	0
Proximate composition (n = 3)											
L-methionine (%)	0.59	0.91	1.15	1.49	1.70	2.02	0.60	0.59	0.58	0.61	0.58
HMTBa (%)	0	0	0	0	0	0	0.31	0.61	0.97	1.23	1.39
Crude protein (%)	48.3	48.2	48.0	48.2	48.1	48.4	47.8	47.7	47.6	47.4	47.2
Crude lipid (%)	12.4	12.3	12.5	13.1	12.7	12.9	12.9	12.9	13.0	13.0	13.1
Moisture (%)	5.7	5.9	5.9	8.6	5.3	4.9	8.3	6.1	5.9	6.6	7.4
Ash (%)	6.8	6.7	6.7	6.7	6.7	6.7	6.8	6.9	7.0	7.0	7.2

[a] Fish meal, obtained from Liu He Group (Shandong, China), crude protein 68.3%, crude lipid 14.2%; soybean meal, obtained from Great Seven Bio-tech (Shandong, China), crude protein 48.4%, crude lipid 2.0%; beer yeast, obtained from Great Seven Bio-tech (Shandong, China), crude protein 53.4%, crude lipid 1.4%; wheat meal, obtained from Great Seven Bio-tech (Shandong, China), crude protein 17.0%, crude lipid 0.6%.

[b] Amino acid premix (g/100 g diet): arginine 1.52, histidine 0.57, isoleucine 0.60, leucine 0.90, lysine 1.20, phenylalanine 0.74, threonine 0.76, valine 0.54, alanine 2.03, aspartic acid 1.39, glycine 2.52, serine 0.96, tyrsine 0.62, proline 0.14.

[c] Mineral premix (g/kg diet): $MgSO_4$•H_2O, 1.200; $CuSO_4$•$5H_2O$, 0.010; $FeSO_4$•H_2O, 0.080; $ZnSO_4$•H_2O, 0.050; $MnSO_4$•H_2O, 0.045; $CoCl_2$•$6H_2O$, 0.050; $Ca(IO_3)_2$, 0.060; Na_2SeO_3, 0.020; zeolite, 18.485.

[d] Vitamin premix (g/kg diet): thiamin, 0.025; riboflavin, 0.045; pyridoxine HCl, 0.020; vitamin B12, 0.010; vitamin K3, 0.010; inositol,0.800; pantothenic acid, 0.060; niacin acid, 0.200; folic acid, 0.020; biotin, 0.060; retinal acetate, 0.032; cholecalciferol, 0.005; α-tocopherol,0.240; ascorbic acid, 2.000; ethoxyquin 0.003; microcrystalline cellulose, 16.470.

[e] 99.56%, Jizhoucity Huayang Chemical Co., LTD., China

[f] 84% (Calcium salt of HMTBa; Novus International Inc., St. Charles, MO, USA).

Table 2
Amino acid composition of dietary protein sources (g/100 g diet dry matter).

Amino acids	Amount in					Total	50% whole body protein
	23.6 g FM	22.6 g SBM	5 g Yeast	19.3 g WM	AAP		
EAA							
Arginine	0.77	0.78	0.11	0.08	1.52	3.26	3.26
Histidine	0.42	0.25	0.04	0.06	0.57	1.34	1.34
Isoleucine	0.54	0.37	0.09	0.08	0.60	1.68	1.68
Leucine	0.96	0.75	0.16	0.18	0.90	2.96	2.96
Lysine	1.10	0.61	0.15	0.05	1.20	3.11	3.11
Methionine	0.38	0.15	0.04	0.05	Variable	Variable	1.58
Phenylalanine	0.51	0.49	0.08	0.12	0.74	1.94	1.94
Threonine	0.63	0.38	0.10	0.07	0.76	1.93	1.93
Valine	0.72	0.45	0.11	0.10	0.54	1.92	1.92
NEAA							
Alanine	0.31	0.46	0.13	0.08	2.03	3.01	3.01
Aspartic acid	1.34	1.12	0.20	0.10	1.39	4.14	4.14
Cystine	0.14	0.18	0.03	0.08	0.00	0.42	0.49
Glutamic acid	2.10	2.15	0.32	1.16	Variable	Variable	6.28
Glycine	0.96	0.45	0.10	0.10	2.52	4.12	4.12
Serine	0.53	0.47	0.10	0.11	0.96	2.18	2.18
Tyrsine	0.38	0.34	0.18	0.07	0.62	1.60	1.60
Proline	1.58	0.21	0.11	0.29	0.14	2.33	2.33

FM, fish meal; SBM, soybean meal; WM, wheat meal; AAP, crystalline amino acid premix; EAA, essential amino acid; NEAA, non-essential amino acid.

phase was 0.05% trifluoroacetic acid in water, and the effluent was monitored by a UV detector (wave length 210 nm).

2.2. Feeding trial

Juvenile turbot were obtained from a commercial farm in Haiyang, Shandong, China. Prior to the start of the experiment, fish were acclimated to a commercial diet for two weeks. Eleven groups of fish (initial weight 5.59 ± 0.02 g) were randomly assigned the basal control diet or one of 10 experimental diets. There were five replicates in each group. Each tank (300 L) was used as a replicate, and 30 fish were reared in each tank. Fish were fed twice daily to apparent satiation at 7:30 and 19:30, respectively. The 75-day growth trail was carried out in a flow-through seawater system. During the growth trail, the water temperature ranged from 19 to 22 °C, salinity from 27 to 29‰, and dissolved oxygen was higher than 5 mg/L.

2.3. Sample collection and chemical analysis

Before the growth trial, 20 fish from the same population were randomly selected for determination of initial whole-body proximate composition. At the termination of the growth trial, fish were fasted for 24 h, and then counted and weighed. Three fish per tank were randomly selected and frozen at −20 °C prior to determination of whole-body composition. Ten fish per tank were anesthetized with eugenol (1:10000) (Shanghai Reagent Corp, China). Blood samples were collected from the caudal vein with 1 ml syringes, stored in 4 °C for 5 h and then centrifuged at 4000 *g* for 10 min, and the serum was separated and stored at −20 °C. Liver samples were initially frozen in liquid nitrogen, and then stored at −80 °C before analysis.

Feed ingredients, experimental diets and fish whole-bodies were analyzed for dry matter, crude protein, crude lipid and ash using standard methods of AOAC (1995). Samples of diets and fish were dried to a constant weight at 105 °C to determine moisture. Crude protein was determined by measuring nitrogen (N × 6.25) using the Kjeldahl method (2300-Auto-analyzer, FOSS, Hillerød, Denmark), crude lipid by ether extraction using Soxhlet method (36680-analyer, BUCHI, Flawil, Switzerland), ash by combustion at 550 °C. Except for methionine and cysteine, amino acid compositions of dietary ingredients and the experimental diets were determined by automatic amino acid analyzer (Biochrom 30 Ltd®, Cambridge, UK) after acid hydrolysis in 6 N HCl for 24 h at 110 °C. Contents of ascorbic acid in serum and liver samples were assayed using a commercial assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). QuantiChrom

Table 3
Effects of the two dietary methionine sources on growth and feed utilization in turbot (n = 5).

Sources	Level (%)	Initial weight (g)	Final weight (g)	SGR[a] (%/day)	FI[b] (%/day)	FE[c]	PER[d]	PPV[e] (%)	Survival (%)
Control	0	5.59 ± 0.02	12.51 ± 0.97	1.07 ± 0.10	1.42 ± 0.04	0.71 ± 0.05	1.47 ± 0.10	23.68 ± 1.36	98.67 ± 2.98
L-Met	0.3	5.59 ± 0.02	20.08 ± 0.88	1.70 ± 0.06	1.58 ± 0.02	0.95 ± 0.02	1.97 ± 0.05	33.21 ± 0.68	99.33 ± 1.49
	0.6	5.59 ± 0.02	22.14 ± 1.20	1.83 ± 0.08	1.68 ± 0.03	0.94 ± 0.03	1.95 ± 0.07	32.45 ± 0.90	97.33 ± 4.35
	0.9	5.58 ± 0.02	21.97 ± 1.28	1.82 ± 0.08	1.74 ± 0.05	0.90 ± 0.05	1.87 ± 0.11	31.46 ± 1.58	97.33 ± 2.79
	1.2	5.59 ± 0.02	24.34 ± 2.44	1.96 ± 0.13	1.73 ± 0.06	0.96 ± 0.04	1.99 ± 0.08	33.12 ± 1.20	97.33 ± 4.35
	1.5	5.59 ± 0.02	22.28 ± 2.11	1.84 ± 0.13	1.67 ± 0.03	0.95 ± 0.06	1.96 ± 0.12	33.22 ± 1.66	97.33 ± 4.35
HMTBa	0.3	5.60 ± 0.02	18.68 ± 2.38	1.60 ± 0.17	1.58 ± 0.04	0.89 ± 0.08	1.87 ± 0.17	30.69 ± 2.27	98.00 ± 1.83
	0.6	5.59 ± 0.00	23.85 ± 3.27	1.92 ± 0.18	1.69 ± 0.03	0.97 ± 0.08	2.04 ± 0.17	33.58 ± 2.42	99.33 ± 1.49
	0.9	5.57 ± 0.01	25.26 ± 0.76	2.01 ± 0.04	1.73 ± 0.04	0.98 ± 0.03	2.06 ± 0.05	34.25 ± 0.87	98.67 ± 1.83
	1.2	5.58 ± 0.02	24.09 ± 1.96	1.95 ± 0.11	1.68 ± 0.04	0.97 ± 0.04	2.05 ± 0.08	34.10 ± 1.09	94.67 ± 2.98
	1.5	5.59 ± 0.02	23.21 ± 3.74	1.88 ± 0.22	1.62 ± 0.04	0.99 ± 0.07	2.09 ± 0.15	34.95 ± 1.97	98.00 ± 2.98
Methionine source	L-Met		20.55 ± 4.13	1.70 ± 0.31	1.64 ± 0.12	0.90 ± 0.10	1.87 ± 0.20	31.19 ± 3.66	97.89 ± 3.33
	HMTBa		21.27 ± 5.02	1.74 ± 0.36	1.62 ± 0.11	0.92 ± 0.11	1.93 ± 0.25	31.87 ± 4.29	97.89 ± 2.70
Methionine level	0		12.51 ± 0.97[c]	1.07 ± 0.10[c]	1.42 ± 0.04[e]	0.71 ± 0.05[b]	1.47 ± 0.10[b]	23.68 ± 1.36[c]	98.67 ± 2.98
	0.3		19.38 ± 1.85[b]	1.65 ± 0.14[b]	1.58 ± 0.03[d]	0.92 ± 0.06[a]	1.92 ± 0.13[a]	31.95 ± 2.07[b]	98.67 ± 1.72
	0.6		22.99 ± 2.49[a]	1.88 ± 0.14[a]	1.69 ± 0.03[b]	0.95 ± 0.06[a]	1.99 ± 0.13[a]	33.02 ± 1.82[ab]	98.33 ± 3.24
	0.9		23.61 ± 2.00[a]	1.92 ± 0.12[a]	1.74 ± 0.05[a]	0.94 ± 0.06[a]	1.97 ± 0.13[a]	32.85 ± 1.90[ab]	98.00 ± 2.33
	1.2		24.21 ± 2.09[a]	1.95 ± 0.12[a]	1.70 ± 0.06[ab]	0.96 ± 0.04[a]	2.02 ± 0.08[a]	33.61 ± 1.20[a]	96.00 ± 3.78
	1.5		22.75 ± 2.91[a]	1.86 ± 0.17[a]	1.65 ± 0.04[c]	0.97 ± 0.06[a]	2.03 ± 0.14[a]	34.09 ± 1.95[a]	97.67 ± 3.53
Source		0.514	0.186	0.288	0.106	0.289	0.038	0.091	1.000
Level		0.183	0.000	0.000	0.000	0.000	0.000	0.000	0.379
Source × Level		0.728	0.178	0.182	0.360	0.185	0.084	0.009	0.545

Values (means ± S.D.) in the same column sharing a common superscript letter were not significantly different ($P > 0.05$).

[a] SGR (Specific growth rate, %/day) = 100 × ln (final body weight / initial body weight) / days;

[b] FI (Feed intake, %/day) = 100 × feed fed / [days × (initial body weight + final body weight) / 2];

[c] FE (Feed efficiency) = (body weight gain) / (feed fed);

[d] PER (Protein efficiency ratio) = body weight gain / protein intake;

[e] PPV (Productive protein value, %) = 100 × (body protein gain / protein intake).

™ TBARs assay kit (Bas-biotech, Inc, Chengdu, China) was used for determination of TBARs contents in liver.

2.4. Calculations and statistical analysis

The growth and feed utilization were expressed as follows:

$$\text{Survival}(\%) = 100 \times (\text{final amount of fish})/(\text{initial amount of fish})$$

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

$$\text{Feed efficiency}(\text{FE}) = (\text{body weight gain})/(\text{feed fed})$$

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

$$\text{Protein efficiency ratio}(\text{PER}) = \text{body weight gain}/\text{protein intake}$$

$$\text{Productive protein value}(\text{PPV}, \%) = 100 \times (\text{body protein gain}/\text{protein intake}).$$

The interaction between the main effect factors (dietary methionine source and level) was evaluated by a factorial design (2 × 6). Two-way ANOVA was conducted for all the data using SPSS 16.0 for windows. Duncan's multiple range test was used to compare differences among treatments, where significant differences ($P < 0.05$) were observed. Second-order polynomial regression analysis was used to analyze the correlation between SGR (or FI) and dietary L-Met or HMTBa levels.

3. Results

3.1. Growth and feed utilization

There were no significant differences in survival rate which ranged from 97.33% to 99.33% for the different treatments (Table 3). Fish fed the basal diet showed the significantly lowest SGR ($P < 0.05$). Then SGR significantly increased as each methionine source supplemented level increased from 0.3 to 0.6% ($P < 0.05$), and thereafter not further increased ($P > 0.05$).

The significantly lowest FE (0.71), FI (1.42%/day), PER (1.47) and PPV (23.68%) were found for fish fed the basal diet, compared with those in the other experimental treatments ($P < 0.05$). Graded levels (from 0.3 to 0.9%) of dietary L-Met or HMTBa to the basal diet resulted in significantly increased FI, and thereafter declined. Based on the two-way ANOVA, the HMTBa diets showed the higher PER than L-Met diets ($P = 0.038$). Significant interaction between methionine source and added level was shown for the PPV. Diet supplemented with 0.3% L-Met resulted in significantly higher PPV than that with 0.3%HMTBa ($P < 0.05$). But diet supplemented with 0.9% HMTBa had significantly higher PPV than that with 0.9% L-Met ($P < 0.05$) (Fig. 1a).

Second-order polynomial regression analysis of SGR data indicated that the dietary methionine requirement of juvenile turbot was estimated to be 1.58% (3.31 of dietary protein) based on L-Met or 1.56% (3.25% of dietary protein) based on HMTBa (Fig. 2). When FI was plotted against dietary total methionine, the dietary total methionine requirement was to be 1.59% (3.27% of dietary protein) based on L-Met or 1.49% (3.19% dietary protein) based on HMTBa (Fig. 3).

3.2. Relative responses of fish fed HMTBa or L-Met

Based on SGR, the turbot fed HMTBa and L-methionine exhibited different dose–response curves. Responses varied with dietary methionine levels, and each of the dose–response curves had a separate plateau (Fig. 2). L-Met-fed fish showed better growth than HMTBa-fed fish when dietary methionine level increased from 0.59 to 0.83%, whereas

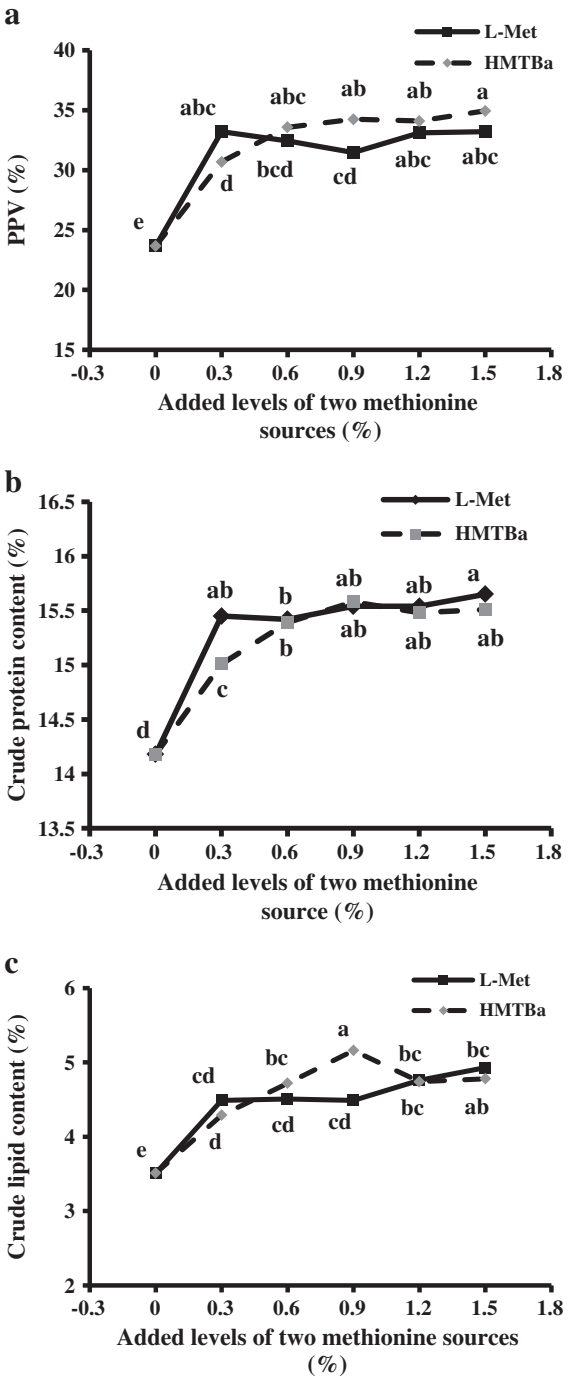

Fig. 1. Means of the Productive protein value (PPV, a), Crude protein (b) and lipid content (c) of fish body composition for the 11 experimental diets show the effects of interaction between methionine source and added level. Diamonds represent data from fish fed HMTBa; squares represent data from fish fed L-methionine (L-Met). Values denoted with different letters are significantly different ($P < 0.05$).

HMTBa-fed fish outperformed L-Met-fed fish when dietary methionine levels ranged from 0.83 to 2.01%. Furthermore, fish fed HMTBa displayed a higher maximum SGR response (2.03%/day) than fish fed L-Met (1.96%/day).

3.3. Body composition

The whole-body composition of turbot fed experimental diets is presented in Table 4. The lowest crude protein and crude lipid contents,

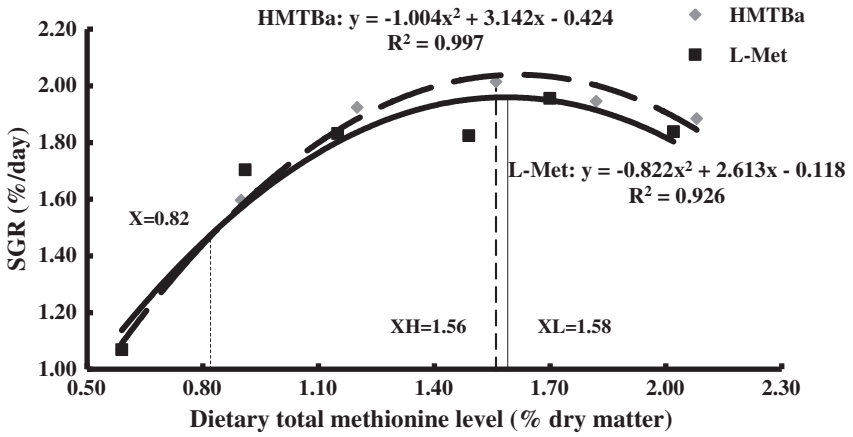

Fig. 2. Relationship between dietary total methionine level and specific growth rate (SGR) of turbot fed diets containing various levels of two methionine sources for 75 days. Diamonds represent data from fish fed HMTBa; squares represent data from fish fed L-methionine (L-Met); XH represents the dietary methionine requirement of turbot fed HMTBa; and XL represents the dietary methionine requirement of turbot fed L-Met.

and the highest moisture and ash contents in body were found in the control group. Compared with those in the control group, these parameters improved significantly with each methionine source supplementation ($P < 0.05$). Significant interaction between methionine source and added level was found in the body crude protein or crude lipid contents. Diet supplemented with 0.3% L-Met resulted in significantly higher crude protein contents than that with 0.3% HMTBa ($P < 0.05$) (Fig. 1b). But diet supplemented with 0.9% HMTBa had significantly higher crude lipid content than that with 0.9% L-Met ($P < 0.05$) (Fig. 1c).

3.4. Ascorbic acid in serum and liver and liver TBARs

Serum ascorbic acid concentration of turbot significantly increased as supplementation of each dietary methionine source ranging from 0 to 0.6%, and did not further increase when supplemented levels increased from 0.6 to 1.5% (Table 5). Two-way ANOVA showed a significant effect due to the methionine source ($P = 0.002$) for serum ascorbic acid concentration. Generally, fish fed the HMTBa diets had higher serum ascorbic acid concentrations than that fed the L-Met diets. There were no significant differences in liver ascorbic acid concentrations and liver TBARs among the all treatments.

4. Discussion

In the present study, growth response and feed utilization were improved by dietary L-Met or HMTBa supplementation to a methionine-deficient diet. Results suggest that the turbot is able to effectively utilize either L-methionine or HMTBa as dietary methionine source. Positive effects of HMTBa on growth were also observed in rainbow trout (Cheng et al., 2003), hybrid striped bass (Li et al., 2009) and Jian carp (Xiao et al., 2011). In the present study, black body color, low appetite and growth performance were observed in fish fed the methionine deficient diet (basal diet). However, unlike in the hybrid striped bass (Keembiyehetty and Gatlin, 1993), yellowtail (Ruchimat et al., 1997) or Arctic charr (Simmons et al., 1999), no cataract was found.

In the present study, second-order polynomial regression analysis of SGR data estimated the dietary total methionine requirement of juvenile turbot to be 1.58% based on L-Met or 1.56% based on HMTBa. These results are similar to those in some other fish species, such as Chinook salmon (1.60%) (Halver et al., 1959), Japanese flounder (1.49%) (Alam et al., 2001) and large yellow croaker (1.42%) (Mai et al., 2006). It is higher than those for channel catfish (0.56%) (Harding et al., 1977), rainbow trout (0.52%) (Kim et al., 1992), hybrid striped bass (0.87%) (Keembiyehetty and Gatlin, 1993) and Cobia (1.19%) (Zhou

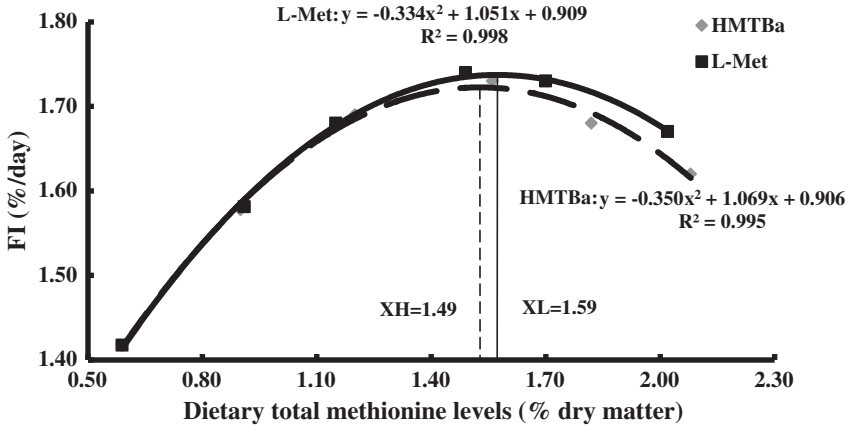

Fig. 3. Relationship between dietary total methionine level and feed intake (FI) of turbot fed diets containing various levels of two methionine sources for 75 days. Diamonds represent data from fish fed HMTBa; squares represent data from fish fed L-methionine (L-Met); XH represents the dietary methionine requirement of turbot fed HMTBa; and XL represents the dietary methionine requirement of turbot fed L-Met.

Table 4
Effects of the two dietary methionine sources on the whole body compositions of turbot (% on a wet weight; n = 5).

Sources	Level (%)	Crude protein	Crude lipid	Moisture	Ash
Control	0	14.18 ± 0.12	3.51 ± 0.55	78.20 ± 1.04	4.32 ± 0.26
L-Met	0.3	15.45 ± 0.14	4.49 ± 0.24	76.37 ± 0.94	3.77 ± 0.13
	0.6	15.42 ± 0.31	4.51 ± 0.46	76.30 ± 0.63	3.82 ± 0.13
	0.9	15.54 ± 0.17	4.49 ± 0.24	76.23 ± 0.83	3.76 ± 0.11
	1.2	15.54 ± 0.15	4.76 ± 0.25	76.15 ± 0.93	3.66 ± 0.14
	1.5	15.65 ± 0.17	4.93 ± 0.19	75.75 ± 0.53	3.68 ± 0.15
HMTBa	0.3	15.01 ± 0.21	4.29 ± 0.39	76.93 ± 1.53	3.85 ± 0.18
	0.6	15.39 ± 0.19	4.72 ± 0.16	76.41 ± 0.71	3.64 ± 0.14
	0.9	15.58 ± 0.11	5.16 ± 0.24	75.70 ± 0.49	3.58 ± 0.13
	1.2	15.48 ± 0.28	4.74 ± 0.37	76.34 ± 0.87	3.50 ± 0.14
	1.5	15.51 ± 0.29	4.78 ± 0.47	76.05 ± 0.71	3.63 ± 0.20
Methionine source	L-Met	15.30 ± 0.54	4.54 ± 0.48	76.50 ± 1.10	3.79 ± 0.23
	HMTBa	15.19 ± 0.53	4.63 ± 0.56	76.61 ± 1.19	3.70 ± 0.28
Methionine level	0	14.18 ± 0.12^{d}	3.51 ± 0.55^{c}	78.20 ± 1.04^{a}	4.32 ± 0.26^{a}
	0.3	15.23 ± 0.28^{c}	4.39 ± 0.33^{b}	76.65 ± 1.23^{b}	3.81 ± 0.16^{b}
	0.6	15.41 ± 0.25^{b}	4.61 ± 0.35^{ab}	76.36 ± 0.64^{b}	3.73 ± 0.17^{bc}
	0.9	15.56 ± 0.14^{a}	4.83 ± 0.42^{a}	75.97 ± 0.70^{b}	3.67 ± 0.15^{cd}
	1.2	15.51 ± 0.21^{ab}	4.77 ± 0.31^{a}	76.25 ± 0.86^{b}	3.58 ± 0.16^{d}
	1.5	15.58 ± 0.24^{a}	4.86 ± 0.36^{a}	75.90 ± 0.61^{b}	3.65 ± 0.17^{cd}
Source		0.005	0.187	0.653	0.011
Level		0.000	0.000	0.000	0.000
Source × Level		0.003	0.002	0.841	0.054

Values (means ± S.D.) in the same column sharing a common superscript letter were not significantly different ($P > 0.05$).

et al., 2006). The differences in methionine requirement may be due to fish species and size, feed ingredients and dietary cystine content, feeding regime, environmental conditions, and method of data analysis (Goff and Gatlin, 2004; Kim et al., 1992; Shearer, 2000; Tacon and Cowey, 1985).

In fish, it has been suggested that the absorption rate of crystalline amino acids is faster than intact protein, which needed to be digested into peptides or amino acids before absorption (Rønnestad et al., 2000; Schuhmacher et al., 1997), therefore giving rise to an asynchronous absorption of crystalline amino acids. Consequently, fish do not appear to utilize dietary crystalline amino acids as effectively as intact protein (Luo et al., 2005). HMTBa is converted to L-methionine in body following absorption and this could result in a synchronous availability for efficient protein synthesis and feed utilization in turbot (unpublished data). Fish fed HMTBa diet had higher SGR, PER, PPV but lower FI than those fed L-Met diet when the supplementation level ranged from 0.6 to 0.9% in the present study. It is likely that methionine from HMTBa was available for protein synthesis along with that from intact protein. However, the specific mechanism needs to be investigated in greater detail.

There has been considerable debate in the literature on the availability of HMTBa to aquatic animals. Some of the earlier studies have suggested that HMTBa is not as available as L-methionine, DL-methionine or other sources of methionine in channel catfish (Robinson et al., 1978), rainbow trout (Poston, 1986) and hybrid striped bass (Keembiyehetty and Gatlin, 1995, 1997; Li et al., 2009). A recent review based on these earlier studies concluded that HMTBa is 75–80% as efficacious as other sources of methionine (NRC, 2011). However, more recent studies conducted in shrimp (Forster and Dominy, 2006) and fish (Goff and Gatlin, 2004; Xiao et al., 2011; Yang et al., 2010; Zhao et al., 2010) including turbot in the present study observed no significant differences in performance between animal fed HMTBa or other sources of methionine. Some of these studies indicated that HMTBa was ≥98% as effective as L-methionine or DL-methionine in promoting growth (Forster and Dominy, 2006; Goff and Gatlin, 2004; Xiao et al., 2011; Yang et al., 2010). Studies with practical diets have demonstrated that HMTBa can be supplemented to methionine-deficient low fish meal diets for fish and shrimp and can promote growth, which is similar to that seen in animals fed fish meal based control diets (Boonyoung et al., 2012; Browdy et al., 2012; Cheng et al., 2003; Hu et al., 2008; Huai et al., 2010; Shen et al., 2007). Similarly, there have been a wide range of availability estimates for HMTBa and DL-methionine in the terrestrial animal literature (Vázquez-Añón et al., 2006b). Both HMTBa and DL-methionine are sources of methionine activity, but differ in chemical structure, mode and sites of absorption (Knight and Dibner, 1984), transport (Lobley et al., 2006; Wester et al., 2006) and conversion to L-methionine in the body (Dibner, 2003). Due to these differences, animals fed different methionine sources exhibit different dose–response relationships. Different statistical models have been fit to data from experimental studies and have arrived at different conclusions on the availability of different methionine sources. Many studies have made comparisons in the deficient

Table 5
Effects of the two dietary methionine sources on ascorbic acid concentration in serum and liver and TBARs contents in liver of turbot (n = 5).

Sources	Level (%)	Serum ascorbic acid (μg/ml)	Liver ascorbic acid (μg/mgprotein)	Liver TBARs (μMMDA equivalents)
Control	0	35.26 ± 12.82	2.41 ± 0.84	77.90 ± 13.75
L-Met	0.3	39.65 ± 6.93	2.79 ± 0.90	63.76 ± 32.04
	0.6	56.77 ± 7.19	2.25 ± 0.50	64.14 ± 25.59
	0.9	46.30 ± 7.98	1.69 ± 0.24	80.99 ± 3.34
	1.2	56.74 ± 14.94	2.21 ± 0.40	72.75 ± 9.30
	1.5	56.29 ± 13.53	2.08 ± 0.42	72.57 ± 7.65
HMTBa	0.3	52.25 ± 13.11	1.95 ± 0.41	85.49 ± 29.05
	0.6	69.16 ± 17.96	2.18 ± 0.63	65.92 ± 22.19
	0.9	65.72 ± 11.58	2.20 ± 0.73	82.68 ± 8.12
	1.2	65.81 ± 16.87	1.94 ± 0.49	74.91 ± 19.17
	1.5	65.02 ± 3.06	1.91 ± 0.26	70.13 ± 5.52
Methionine source	L-Met	48.52 ± 13.41	2.24 ± 0.64	72.02 ± 18.21
	HMTBa	58.87 ± 17.17	2.10 ± 0.57	76.17 ± 18.20
Methionine level	0	35.26 ± 12.09^{c}	2.41 ± 0.79	77.90 ± 13.75
	0.3	45.95 ± 11.91^{bc}	2.37 ± 0.79	74.63 ± 31.29
	0.6	62.97 ± 14.46^{a}	2.21 ± 0.54	65.03 ± 22.86
	0.9	56.01 ± 13.88^{ab}	1.94 ± 0.58	81.84 ± 5.99
	1.2	61.27 ± 15.77^{a}	2.08 ± 0.44	73.83 ± 14.41
	1.5	60.66 ± 10.33^{a}	2.00 ± 0.34	71.35 ± 6.49
Source		0.002	0.378	0.340
Level		0.000	0.383	0.330
Source × Level		0.649	0.474	0.633

Values (means ± S.D.) in the same column sharing a common superscript letter were not significantly different ($P > 0.05$).

part of the dose–response curve and have assumed these to hold good for the entire curve. Such analyses can result in the underestimation of the response of an animal to HMTBa (Kratzer and Littell, 2006; Littell et al., 1997). Studies with chickens (Vázquez-Añón et al., 2006a) and turkeys (González-Esquerra et al., 2007) have demonstrated different dose–response relationships when fed different dietary methionine sources. In the present study, juvenile turbot responded differently to the different methionine sources. At sub-optimal levels in the deficiency part of the curve, fish fed L-methionine performed better than those fed HMTBa, but HMTBa-fed fish performed better at and near the methionine requirement level. Moreover, fish fed HMTBa exhibited the highest growth rate during the study demonstrating that it is as efficacious a methionine source as L-methionine in fish.

The lowest crude protein, crude lipid contents and highest ash content in the whole body were observed for fish fed the basal diet in the present study, and these parameters improved significantly with L-Met or HMTBa supplementation. This is in agreement with reports on other fish, such as rainbow trout (Kim et al., 1992) and cobia (Zhou et al., 2006). At low supplementation levels, the whole-body protein content of turbot fed a diet supplemented with 0.3% L-Met was significantly higher than that in 0.3% HMTBa group. Thereafter, however, no significant differences were found. This was similar with PPV data. Fish fed a diet supplemented with 0.9% HMTBa had higher whole-body lipid content than those fed the other diets including with 0.9% L-Met. At the optimal supplementation level, HMTBa diet had higher body lipid content than L-Met diet, which was similar to the observation made in sunshine bass (Keembiyehetty and Gatlin, 1995) and red drum (Goff and Gatlin, 2004). The role of HMTBa supplementation in lipid deposition in turbot needs to be further studied.

In the present study, serum ascorbic acid concentration of turbot significantly increased with dietary methionine (and HMTBa) levels regardless of the methionine sources, and then remained constant when the supplemented levels were more than 0.6%. In juvenile hybrid striped bass, plasma ascorbic acid concentration significantly increased with dietary HMTBa but not DL-Met supplemented levels. And liver TBARs concentrations were negatively related to dietary inclusion level of DL-Met or HMTBa (Li et al., 2009). In the present study, however, liver ascorbic acid and liver TBARs concentrations did not respond to dietary supplement of L-Met or HMTBa. In previous studies on anti-oxidative response to methionine source in fish, it was shown that both dietary DL-Met and HMTBa increased glutathione contents in juvenile sunshine bass liver (Keembiyehetty and Gatlin, 1995), and dietary HMTBa improved anti-oxidative status and depressed lipid and protein oxidation in intestine, hepatopancreas, serum and muscle of juvenile Jian carp (Feng et al., 2011; Xiao et al., 2012). In the present study, in addition, serum ascorbic acid concentration of fish fed the diets containing HMTBa was higher than L-Met groups. Ascorbic acid has anti-oxidative activity and immune function in fish (Ai et al., 2006; Hamre et al., 1997). It also protects vitamin E and plays a role in vitamin E regeneration in fish (Hamre et al., 1997). This suggests that dietary methionine plays an important role in anti-oxidative defense and may spare other antioxidants such as ascorbic acid (Li et al., 2009). It appears that HMTBa has a higher anti-oxidative activity than L-Met and further study is needed to understand this.

5. Conclusion

In conclusion, on the basis of SGR or FI, the dietary total methionine requirement of juvenile turbot (*P. maxima*) was estimated to be 1.58 and 1.59% (3.31 and 3.27% of dietary protein) based on L-Met or 1.56% and 1.49% (3.25% and 3.19% of dietary protein) based on HMTBa, respectively. HMTBa was as or more efficacious as L-methionine in promoting growth in *P. maxima*. Fish fed HMTBa also showed higher serum ascorbic acid concentrations suggesting improved anti-oxidative activity and immune function.

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