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Effects of dietary methionine on growth performance, muscle nutritive deposition, muscle fibre growth and type I collagen synthesis of on-growing grass carp (*Ctenopharyngodon idella*)

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Abstract

In the current research, a 60-d experiment was conducted with the purpose of exploring the impacts of methionine (Met) on growth performance, muscle nutritive deposition, muscle fibre growth and type I collagen synthesis as well as the related signalling pathway. Six diets (iso-nitrogenous) differing in Met concentrations (2·54, 4·85, 7·43, 10·12, 12·40 and 15·11 g/kg diets) were fed to 540 grass carp (178·47 (SD 0·36) g). Results showed ($P < 0·05$) that compared with Met deficiency, optimal level of dietary Met (1) increased feed intake, feed efficiency, specific growth rate and percentage weight gain (PWG); (2) increased fish muscle protein, lipid and free amino acid contents and improved fish muscle fatty acid profile as well as increased protein content in part associated with the target of rapamycin complex 1 (TORC1)/S6K1 signalling pathway; (3) increased the frequency distribution of muscle fibre with >50 μm of diameter; (4) increased type I collagen synthesis partly related to the transforming growth factor-β1/Smads and CK2/TORC1 signalling pathways. In conclusion, dietary Met improved muscle growth, which might be due to the regulation of muscle nutritive deposition, muscle fibre growth and type I collagen synthesis-related signal molecules. Finally, according to PWG and muscle collagen content, the Met requirements for on-growing grass carp (178–626 g) were estimated to be 9·56 g/kg diet (33·26 g/kg protein of diet) and 9·28 g/kg diet (32·29 g/kg of dietary protein), respectively.

Key words: **Methionine: Nutritive deposition: Muscle fibre growth: Collagen synthesis**

Methionine (Met) is an essential amino acid for grass carp *Ctenopharyngodon idella*[1]. Our previous study observed that Met deficiency caused growth retardation and feed utilisation reduction of sub-adult grass carp[2]. Fish growth is mainly dependent on the growth of muscle[3], which is closely associated with the deposition of nutrients, the growth of muscle fibre and the formation of intramuscular connective tissue[4–6]. However, to date, there has been only sporadic reports about the effect of Met on nutritive deposition, muscle fibre growth and the formation of intramuscular connective tissue in the muscle of fish. For example, in fish muscle, it has been reported that Met increased protein and lipid deposition in juvenile grouper *Epinephelus coioides*[7] and promoted muscle fibre hypertrophy in juvenile rainbow trout *Oncorhynchus mykiss*[3]. However, these studies lack systematicity and rarely explore involved mechanisms. Hence, systematically investigating the relationship between Met and muscle growth and deeply exploring the molecule mechanisms in fish are necessary.

Abbreviations: FAS, fatty acid synthase; Hyp, hydroxyproline; Met, methionine; PWG, percentage weight gain; TGF-β1, transforming growth factor-β1.

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It is well known that muscle growth primarily included the growth of muscle fibre and surrounding intramuscular connective tissue[8]. The recruitment of new muscle fibre (hyperplasia) and growth of existing fibre (hypertrophy) for muscle growth are primary contributors to the ultimate size of a fish species[9]. To our knowledge, only one study (as mentioned above) demonstrated that Met improved muscle growth by an increase in fibre hypertrophy in juvenile rainbow trout (carnivorous fish)[3]. However, the contribution of hyperplasia and hypertrophy to muscle growth affected by nutrients depends on fish species. For example, previous studies reported that lysine could accelerate muscle growth by promoting fibre hypertrophy for blunt snout bream *Megalobrama amblycephala* (herbivorous fish)[10], but had no impact on muscle fibre growth for Nile tilapia *Oreochromis niloticus* (L.) (omnivorous fish)[11]. Besides, collagen is the major component of intramuscular connective tissue influencing the functional and structural properties of muscle[12], the synthesis of which inhibits the differentiation of myogenesis of C2C12 muscle cells *in vitro*[13]. In grass carp, the main collagen in intramuscular connective tissue is type I collagen[14], the expression of which is regulated by the transforming growth factor-β1 (TGF-*β*1)/Smads signalling pathway at the transcriptional level[15]. Type I collagen consists of *α*1 and *α*2 peptide chains (called collagen type 1 α1 (Col1*α*1) and collagen type 1 α2 (Col1*α*2)), respectively[14]. It has been reported that Met could up-regulate the gene expression of *Col1α2* in the muscle of rainbow trout[3]. However, whether Met affects the Col1*α*1 and the molecular mechanism by which Met regulates type I collagen in animal muscle are unknown. In rainbow trout, Met could decrease the gene expression of TNF-α in the muscle[3]. Inagaki *et al.*[16] demonstrated that TNF-*α* was an antagonist of TGF-*β* signalling in the study of primary human fetal fibroblasts. Additionally, previous studies reported that Met could stimulate the target of rapamycin complex 1 (TORC1) signalling pathway in the muscle of rainbow trout[17]. It has been reported that mTORC1 played an important regulatory role in catalysing type I collagen synthesis in human dermal fibroblasts[18]. Thus, there may be some relationship between Met and muscle growth associated with muscle fibre and type I collagen as well as potential TGF-*β*1/Smads and TORC1 signalling in fish, which deserves research.

Taken together, based on our previous study demonstrating that Met deficiency resulted in growth retardation and feed utilisation reduction, the present research was for the first time conducted systematically with the primary objective of investigating the effect of Met on the deposition of muscle nutrients, the hyperplasia and hypertrophy of muscle fibre and type I collagen synthesis as well as related signalling pathways (such as TGF-*β*1/Smads and TORC1 signalling), which might provide partial theoretical evidence for the mechanisms of Met-regulated muscle growth in fish. In addition, grass carp, with rapid growth and high yield, is one of the most abundant freshwater fish species[19]. In our previous study for on-growing grass carp, the optimal Met requirement was 10·41 g/kg of the diet only based on specific growth rate[20]. However, amino acid requirement might vary with different indices[21]. Therefore, the optimal Met requirement for on-growing grass carp with different indices was also evaluated, which might possess important production significance for improving fish muscle growth.

Materials and methods

Experimental design and diets

The ingredients and nutrient levels in the six experimental diets are given in Table 1. The main protein sources used in the present experiment included fishmeal, soyabean protein concentrate and gelatin, and soyabean oil and fish oil were chosen to formulate the main lipid sources. According to Xu *et al.*[22], 286·8 g/kg of dietary protein level, optimum for the growth of on-growing grass carp, was chosen to fix experimental diets. Crystalline amino acid mixture without Met was used to meet essential amino acid requirements for on-growing grass carp reported by our laboratory in the last few years. Six experimental diets with graded Met concentrations of 2·5 (unsupplemented diet), 5·0, 7·5, 10·0, 12·5 and 15·0 g/kg diet were formed by adding different amounts of DL-Met to the diets. All diets were made iso-nitrogenous with graded L-glycine instead of incremental Met, which was in line with the method used in our previous studies[23,24]. The cysteine and Met concentrations in the six experimental diets, which were determined consistent with Wu *et al.*[2], were 1·55, 1·52, 1·58, 1·50, 1·53 and 1·53 g/kg diet and 2·54, 4·85, 7·43, 10·12, 12·40 and 15·11 g/kg diet. In line with the method described by Hong *et al.*[25], at room temperature, we used an electrical fan to air dry the pellets, and then they were stored at −20°C until being used.

Fish management and feeding

All protocols were approved by the University of Sichuan Agricultural Animal Care Advisory Committee, Sichuan, China, under permit no. PXR-S20163729. The on-growing grass carp were obtained from fishery, which were able to feed and swim normally, and were observed no diseases and parasites on microscopic examination. First, we kept the on-growing grass carp rearing in the outdoor tanks, which experienced natural light–dark cycle for 2 weeks to make them acclimatise to the experimental environment. Then, we randomly put 540 healthy on-growing grass carp (approximately 178·47 (SD 0·36) g) into eighteen cages ($140 \times 140 \times 140$ cm^3), which eventually led to three cages in each treatment and thirty fish in each cage, as described in our previous study[26]. In the bottom of each cage, there was a disc (diameter 100 cm) to collect the uneaten feed, as described by Jiang *et al.*[26]. During the growing trial, we fed fish with their six corresponding diets (three replicates) to apparent satiation four times per d for 60 d, primarily on the basic of the great differences in feed intake among six treatments and the study of Met in fish by our laboratory[2,27]. The daily feed supplied per cages was recorded, and after 30 min of feeding, the uneaten feed on the disc was collected, followed by drying using an electrical fan at room temperature and weighing to calculate the feed intake according to Cai *et al.*[28]. During the period of feed trial, the dissolved oxygen was at least 6·0 mg/l, and using the measurement method mentioned by Li *et al.*[29], the water temperature and pH were 28·6 (SD 3·1)°C and 7·4 (SD 0·3), respectively. Also, fish were reared under natural light–dark cycle throughout the trial.

Table 1. Composition and nutrient levels of experimental diets (Percentages)

	Met levels (g/kg diet)					
	2·54	4·85	7·43	10·12	12·40	15·11
Ingredients (%)						
Fishmeal	6·80	6·80	6·80	6·80	6·80	6·80
Gelatin	4·00	4·00	4·00	4·00	4·00	4·00
Soyabean protein concentrate	11·00	11·00	11·00	11·00	11·00	11·00
Crystal amino acid mix*	14·28	14·28	14·28	14·28	14·28	14·28
DL-Methionine (97·7 %)	0·0000	0·2559	0·5118	0·7677	1·0235	1·2794
L-Glycine (99·0 %)	0·6356	0·5084	0·3813	0·2542	0·1271	0·0000
α-Starch	22·00	22·00	22·00	22·00	22·00	22·00
Maize starch	26·5544	26·4257	26·2969	26·1681	26·0394	25·9106
Cellulose	5·00	5·00	5·00	5·00	5·00	5·00
Fish oil	2·48	2·48.	2·48	2·48	2·48	2·48
Soyabean oil	1·80	1·80	1·80	1·80	1·80	1·80
$Ca(H_2PO_4)_2$	1·40	1·40	1·40	1·40	1·40	1·40
Vitamin premix†	2·00	2·00	2·00	2·00	2·00	2·00
Mineral premix‡	1·00	1·00	1·00	1·00	1·00	1·00
Choline chloride (50 %)	1·00	1·00	1·00	1·00	1·00	1·00
Ethoxyquin (30 %)	0·05	0·05	0·05	0·05	0·05	0·05
Total	100·00	100·00	100·00	100·00	100·00	100·00
Nutrient levels (%)						
Crude protein§	28·74	28·52	28·84	28·78	28·56	28·86
Crude lipid§	4·82	4·75	4·95	4·66	4·76	4·92
n-3‖	1·04	1·04	1·04	1·04	1·04	1·04
n-6‖	0·96	0·96	0·96	0·96	0·96	0·96
Available P¶	0·40	0·40	0·40	0·40	0·40	0·40
Methionine	0·254	0·485	0·743	1·012	1·240	1·511
Cysteine	0·155	0·152	0·158	0·150	0·153	0·153

* Crystal amino acid mix (g/kg diet): arginine, 1·86; histidine, 4·90; isoleucine, 5·29; leucine, 1·92; lysine, 5·12; phenylalanine, 3·64; threonine, 6·53; tryptophan, 2·17; valine, 7·00; tyrosine, 4·11; glutamic acid, 54·12; glycine, 48·15, respectively.

† Per kg of vitamin premix (g/kg): retinyl acetate (344 mg/g), 0·19; cholecalciferol (12·5 mg/g), 0·20; DL-α-tocopheryl acetate (50 %), 23·23; menadione (96 %), 1·98; cyanocobalamin (1 %), 0·94; D-biotin (2 %), 0·75; folic acid (95 %), 0·17; thiamine nitrate (98 %), 0·09; ascorhyl acetate (95 %), 9·77; niacin (99 %), 3·44; meso-inositol (97 %), 28·53; calcium-D-pantothenate (90 %), 4·19; riboflavin (80 %), 0·73; pyridoxine hydrochloride (98 %), 0·45. All ingredients were diluted with maize starch to 1 kg.

‡ Per kg of mineral premix (g/kg): $MnSO_4{\cdot}H_2O$ (31·8 % Mn), 2·6590; $MgSO_4{\cdot}H_2O$ (15·0 % Mg), 256·7933; $FeSO_4{\cdot}H_2O$ (30·0 % Fe), 12·6083; $ZnSO_4{\cdot}H_2O$ (34·5 % Zn), 8·8700; $CuSO_4{\cdot}5H_2O$ (25·0 % Cu), 0·9560; CaI_2 (3·2 % iodine), 1·5625; Na_2SeO_3 (44·7 % Se), 0·0611. All ingredients were diluted with maize starch to 1 kg.

§ Crude protein and lipid contents were measured values.

‖ *n*-3 and *n*-6 were referred to Zeng *et al.*[89] and were calculated according to the National Research Council[90].

¶ Available P was referred to Wen *et al.*[91] and was calculated according to the National Research Council[90].

Sample collection and analysis

At the termination of the 60 d growth trial, we weighed and counted the fish in each cage. Six hours after the last feeding, six fish in each treatment were selected, anaesthetised in a benzocaine (50 mg/l) bath with the introduction mentioned by Geraylou *et al.*[30], after that, to determine the plasma ammonia content, we collected the blood samples from fish caudal vein, removed and stored the plasma, which was same as the method described in previous study[31]. Meanwhile, twelve fish conducted similar to above from each treatment were quickly caught and dissected, and the whole hepatopancreas and the left dorsal muscle above the lateral line and behind the head of body were quickly removed on ice, snap-frozen in liquid N_2 and stored at –80°C for later assay of enzyme activity, RNA extraction and quantification as well as Western blot analysis. The muscle located on the same position in the right side of the same fish was also sampled for other biochemical analysis. As described in the previous study[32], we analysed the muscle crude protein, crude lipid and ash. To determine collagen content, we measured the amout of hydroxyproline (Hyp) using the method as mentioned by Periago *et al.*[33]. The collagen content was calculated by multiplying the Hyp content by eight as provided by AOAC method 990.26[34], due to that content of Hyp in collagen was 12·5 % if the nitrogen-to-protein factor was 6·25. Using the method in line with Yu *et al.*[35], we measured the free amino acid contents by employing an automatic amino acid analyser. Using the gas chromatographic method determines the fatty acid constitutes[36]. Muscle and hepatopancreas samples were homogenised in ice-cold physiological saline solution (ten volumes, w/v) and centrifuged at 6000 ***g*** and 4°C for 20 min, and then, the supernatant was collected for enzyme activity analysis. According to the description of Jiang *et al.*[37] and Yang *et al.*[38], we measured plasma ammonia content, the activity of glutamate-oxaloacetate transaminase and glutamate-pyruvate transaminase in the hepatopancreas and muscle and fatty acid activity (fatty acid synthase; FAS) in the muscle of fish, respectively.

Morphometric analysis

The muscle tissue was fixed in 4 % paraformaldehyde, dehydrated in ethanol/methanol and embedded in paraffin. Then tissue was sectioned to 5–7 μm. Sections were stained using standard haematoxylin–eosin and examined by a Nikon TS100

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light microscope according to Kokou *et al.*[39]. Morphometric analysis was performed using Image Pro Plus® 4·5 image analysis software (Media Cybernetics). According to the description by Dubovitz *et al.*[40], the smallest diameter of 200 muscle fibres of per sample of three grass carp from each treatment was measured and distributed into three diameter classes (<20, 20–50 or >50 μm), as described by de Almeida *et al.*[41].

Real-time PCR reaction analysis

We used the RNAiso Plus Kit (TaKaRa) to extract total RNA from muscle samples to quantitatively analyse the differentiation of related target genes of collagen-related signalling molecules affected by different Met concentrations. We employed spectrophotometric analysis to verify the purity and concentration of total RNA, ensuring that the OD_{260}:OD_{280} ratio was from 1·9 to 2·0 and the amounts of total RNA for inverse transcription were at most 1 μg as requested by the manufacturer's instructions of reverse transcription kit (TaKaRa Biotechnology Co. Ltd), respectively. We used agarose gel electrophoresis (1 %) to determine the quality of total RNA (28S:18S rRNA bands were approximately 2:1). The cDNA was synthesised from RNA using PrimeScript™ RT Reagent Kit (TaKaRa), which was conducted under the guidance of the manufacturer's instructions. Online Supplementary Table S1 shows the primer sequences used in the present study. To normalise cDNA loading, according to the evaluation of reference gene conducted previously by our laboratory (data not shown), our selected reference gene was *β*-actin. On the basic of the specific gene standard curves, which generated from the 10-fold serial dilutions, we calculated the amplification efficiency involved with all genes (*n* 6). After confirming that the amplification efficiency of the primer was about 100 %, we used the $2^{-\Delta\Delta CT}$ method to calculate the transcript expression data according to Livak *et al.*[42].

Western blotting

The procedures used to conduct Western blotting analysis were the same as those mentioned in our previous reports[43,44]. We used the BCA protein assay kit (Beyotime Biotechnology Inc.) to determine the protein concentration in supernatant. Subsequently, we loaded equal amounts of protein samples (40 μg per lane) obtained from respective treatments to each lane and electrophoresed on an SDS-glycine polyacrylamide gel, and then, the protein samples were transferred to a polyvinylidene difluoride membrane for the Western blot analysis. The membrane was blocked for 1 h with 0·5 % BSA at room temperature and then incubated with primary antibody overnight at 4°C. The appropriate antibodies used in the current experiment are as follows: anti-t-TOR, p-$TOR^{Ser2448}$, t-S6K1, p-$S6K1^{Ser389}$, t-4E-BP1, p-$4E\text{-}BP1^{Thr37/46}$, t-Smad4, t-Smad2, p-$Smad2^{Ser467}$ and *β*-actin. In our previous study, we have proved that the antibodies of *β*-actin (AF7018, 1:3000 dilution), anti-total TOR (AF6308, 1:1000 dilution) and p-$TOR^{Ser2448}$ (AF3308, 1:1000 dilution) (Affinity BioReagents) chosen in the present research could successfully react with grass carp proteins of interest. The antibodies of anti-Smad4 (AF5247, 1:1000 dilution), t-Smad2 (AF6449, 1:1000 dilution) and p-$Smad2^{Ser467}$ (AF3449, 1:1000 dilution) were also purchased from the same company mentioned above (Affinity BioReagents), and t-S6K1 (9202, 1:1000 dilution), p-$S6K1^{Ser389}$ (9234, 1:1000 dilution), t-4E-BP1 (9452, 1:1000, dilution) and p-$4E\text{-}BP1^{Thr37/46}$ (9459, 1:1000 dilution) were purchased from Cell Signaling Technology and used in grass carp according to Shi *et al.*[45]. Immediately after, the polyvinylidene difluoride membranes were washed three times with tris-buffered saline with Tween, followed by an incubation with horseradish peroxidase-conjugated secondary antibody for 1·5 h. We visualised and quantified the bands using an ECL kit (Millipore) and an NIH Image 1.63 software, respectively. The protein levels for Met-supplemented groups were expressed as a relative value to that in Met-unsupplemented group. The experiment was conducted at least three times, and similar results were obtained each time.

Data analysis

Growth performance parameters were calculated as shown in Table 2.

We confirmed the normality and homoscedasticity assumptions prior to the statistical analysis for all data. Results are presented as mean values and standard deviations. Statistical analysis of all data was conducted using one-way ANOVA followed by the Duncan's multiple-range test to evaluate significant differences between the treatment groups at the level of $P < 0·05$ with SPSS 18.0 (SPSS Inc.). On the basis of the means and standard deviations of growth and muscle growth-related parameters, the minimum effect size was calculated to be 0·65 according to the method of Searcy-Bernal[46]. With the effect size of 0·65, a significance level of 0·05 and the six replicates in each treatment, the statistical power was calculated to be 0·80 using the R pwr package according to Grey *et al.*[47]. Quadratic regression mode was employed to determine the optimal dietary Met levels of on-growing grass carp according to different indices.

Results

Growth performance, feed utilisation and amino acid metabolism-related parameters of on-growing grass carp

All experimental diets were well accepted by the fish, and the mortality rates were zero for all grass carp during and after the 60- d trial. As shown in Table 3, there was no significant difference on initial body weight of fish ($P > 0·05$). With Met levels from 2·54 to 7·43 g/kg of the diet, the growth performance

Table 2. Index formula for percentage weight gain (PWG), specific growth rate (SGR), feed efficiency (FE) and feed intake (FI) of on-growing grass carp (*Ctenopharyngodon idella*)

Items	Formulas
PWG	PWG (%) = 100 × (FBW (g/fish) – IBW (g/fish))/IBW (g/fish)
SGR	SGR (%/d) = 100 × (ln (mean final weight) – ln (mean initial weight))/d
FE	FE (%) = 100 × (FBW (g/fish) – IBW (g/fish))/FI (g/fish)
FI	FI (g/fish) = total feed consumption$_{(dry)}$ g/fish – total uneaten feed$_{(dry)}$ g/fish

IBW, initial body weight.

Table 3. Growth performance, feed utilisation, amino acid metabolism-related parameters and regular nutritional compositions in the muscle of on-growing grass carp (*Ctenopharyngodon idella*) fed diets with graded levels of methionine (Met) (g/kg) for 60 d (Mean values and standard deviations)

Met level (g/kg diet)...	2·54		4·85		7·43		10·12		12·40		15·11		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	SEM
IBW*	178·76^{a}	1·01	178·10^{a}	0·19	178·54^{a}	0·70	177·99^{a}	0·88	178·87^{a}	0·51	178·54^{a}	0·19	
FBW*	373·56^{a}	7·43	508·89^{c}	15·64	626·67^{f}	18·33	592·89^{e}	6·19	557·56^{d}	5·05	483·33^{b}	18·58	20·20
FI*	395·90^{a}	0·53	559·48^{c}	0·50	646·16^{f}	2·55	622·97^{e}	0·37	585·61^{d}	0·91	522·48^{b}	0·53	19·87
FE*	0·49^{a}	0·02	0·59^{b}	0·03	0·69^{d}	0·03	0·67^{c,d}	0·01	0·65^{c}	0·01	0·58^{b}	0·03	0·02
PWG*	108·99^{a}	5·35	185·73^{c}	8·85	251·01^{f}	11·09	233·13^{e}	5·12	211·70^{d}	2·90	170·70^{b}	10·20	11·37
SGR*	1·23^{a}	0·04	1·75^{c}	0·05	2·09^{f}	0·05	2·01^{e}	0·03	1·89^{d}	0·02	1·66^{b}	0·06	0·07
Hepatopancreas													
GOT†	3074·08^{a}	283·02	3868·19^{b}	338·15	4978·25^{c}	489·98	5266·86^{c}	390·98	4835·01^{c}	224·71	3985·84^{b}	373·94	139·90
GPT†	974·55^{a}	76·79	1257·19^{b}	93·69	1561·10^{d}	141·57	1695·92^{e}	74·76	1405·23^{c}	129·61	1240·00^{b}	98·79	42·79
Muscle													
GOT†	1835·34^{a}	115·83	2217·40^{b}	121·69	2555·54^{c}	192·67	2596·50^{c}	140·12	2385·99^{b}	96·91	2212·64^{b}	164·05	48·40
GPT†	629·27^{a}	48·08	786·76^{b}	51·66	1005·25^{c}	83·06	1116·40^{d}	53·43	985·02^{c}	32·98	837·47^{b}	57·02	28·52
PAC†	327·29^{c}	15·76	287·06^{b}	18·79	226·74^{a}	12·37	232·53^{a}	16·07	280·28^{b}	11·35	319·08^{c}	16·69	6·93
Muscle													
Moisture‡	78·63^{c}	1·18	77·89^{b,c}	0·62	76·54^{a}	0·26	76·76^{a}	0·72	77·13^{a,b}	0·32	78·71^{c}	0·54	0·18
Crude protein‡	16·81^{a}	0·55	17·79^{b,c}	0·64	18·59^{d}	0·63	18·34^{c,d}	0·97	18·03^{b,c,d}	0·21	17·40^{a,b}	0·44	0·14
Crude lipid‡	2·20^{a}	0·14	2·42^{b}	0·09	2·73^{c}	0·19	2·72^{c}	0·11	2·44^{b}	0·07	2·30^{a,b}	0·09	0·04
Ash‡	1·48^{b}	0·09	1·49^{b}	0·04	1·32^{a}	0·08	1·35^{a}	0·09	1·37^{a}	0·09	1·42^{a,b}	0·05	0·02
Hyp‡	0·56^{a}	0·04	0·64^{b,c}	0·04	0·71^{d}	0·06	0·67^{c,d}	0·04	0·67^{c,d}	0·05	0·60^{a,b}	0·03	0·01
Collagen‡	4·47^{a}	0·29	5·14^{b,c}	0·34	5·64^{d}	0·48	5·39^{c,d}	0·30	5·33^{c,d}	0·38	4·83^{a,b}	0·23	0·08
Regression													
$Y_{FBW} = -4{\cdot}708X^2 + 90{\cdot}089X + 183{\cdot}334$									$X = 9{\cdot}57$; R^2 0·947; $P < 0{\cdot}05$				
$Y_{PWG} = -2{\cdot}652X^2 + 50{\cdot}701X + 2{\cdot}219$									$X = 9{\cdot}56$; R^2 0·948; $P < 0{\cdot}05$				
$Y_{SGR} = -0{\cdot}016X^2 + 0{\cdot}306X + 0{\cdot}598$									$X = 9{\cdot}56$; R^2 0·950; $P < 0{\cdot}05$				
$Y_{FI} = -4{\cdot}599X^2 + 88{\cdot}458X + 218{\cdot}825$									$X = 9{\cdot}62$; R^2 0·938; $P < 0{\cdot}05$				
$Y_{FE} = -0{\cdot}004X^2 + 0{\cdot}073X + 0{\cdot}330$									$X = 9{\cdot}13$; R^2 0·966; $P < 0{\cdot}01$				
$Y_{GOT\ in\ the\ muscle} = -14{\cdot}103X^2 + 275{\cdot}151X + 1234{\cdot}275$									$X = 9{\cdot}76$; R^2 0·964; $P < 0{\cdot}01$				
$Y_{GPT\ in\ the\ muscle} = -8{\cdot}384X^2 + 167{\cdot}066X + 228{\cdot}920$									$X = 9{\cdot}96$; R^2 0·950; $P < 0{\cdot}05$				
$Y_{Crude\ protein\ in\ the\ muscle} = -0{\cdot}035X^2 + 0{\cdot}660X + 15{\cdot}421$									$X = 9{\cdot}43$; R^2 0·955; $P < 0{\cdot}01$				
$Y_{Collagen\ in\ the\ muscle} = -0{\cdot}023X^2 + 0{\cdot}427X + 3{\cdot}584$									$X = 9{\cdot}28$; R^2 0·939; $P < 0{\cdot}05$				

IBW, initial body weight (g/fish); FBW, final body weight (g/fish); FI, feed intake (g/fish); FE, feed efficiency; PWG, percentage weight gain (%); SGR, specific growth rate (%/d); GOT, glutamate-oxaloacetate transaminase (U/g tissue); GPT, glutamate-pyruvate transaminase (U/g tissue); PAC, plasma ammonia contents (μmol/l).

^{a,b,c,d,e,f} Mean values in a row with unlike superscript letters are significantly different ($P < 0{\cdot}05$; ANOVA and Duncan's multiple-range tests).

* Mean values and standard deviations for three replicate groups, with thirty fish in each group.

† Mean values and standard deviations for six replicate groups.

‡ Mean values and standard deviations for six replicate groups. Moisture (%); crude protein (%); crude lipid (%); ash (%); Hyp, hydroxyproline (μg/mg tissue); collagen (μg/mg tissue).

and feed utilisation parameters (specific growth rate, feed efficiency and feed intake) were elevated significantly individually ($P < 0{\cdot}05$) and then decreased significantly for feed intake and specific growth rate ($P < 0{\cdot}05$) and gradually for feed efficiency with higher Met levels in the diet. From Table 3, we found that the Met requirement for on-growing grass carp suggested to be 7·43 g/kg of the diet as growth was maximised at this point and showed a decline with further increase in Met supplementation. However, when we used quadratic regression analysis to determine the optimal dietary Met level for on-growing grass carp based on percentage weight gain (PWG), it was 9·54 g/kg diet, corresponding to 33·19 g/kg protein of diet, as shown in Fig. 1. When Met compositions increased from 2·54 to 10·12 g/kg of the diet, the activities of glutamate-oxaloacetate transaminase in the hepatopancreas and muscle increased gradually and then decreased gradually. The activities of glutamate-pyruvate

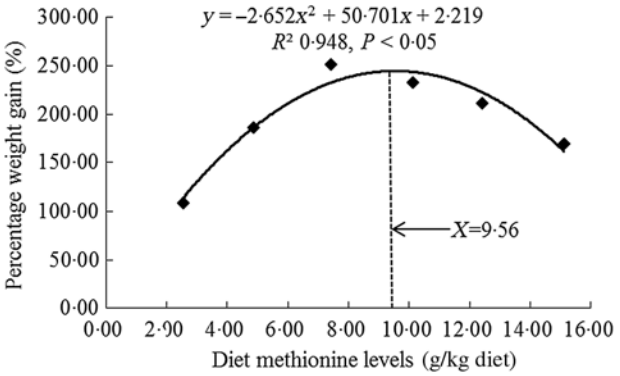

Fig. 1. Quadratic regression analysis of percentage weight gain for on-growing grass carp (*Ctenopharyngodon idella*) fed diets with graded levels of methionine (g/kg) for 60 d.

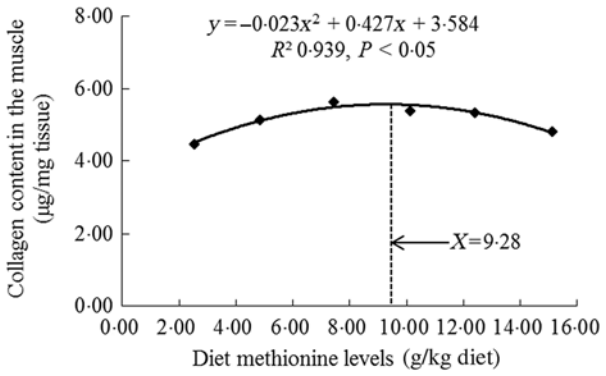

Fig. 2. Quadratic regression analysis of collagen in the muscle for on-growing grass carp (*Ctenopharyngodon idella*) fed diets with graded levels of methionine (g/kg) for 60 d.

transaminase in the hepatopancreas and muscle prominently advanced with Met compositions range 2·54 to 10·12 g/kg of the diet ($P < 0·05$) and then reduced significantly ($P < 0·05$). With Met compositions not higher than 7·43 g/kg of the diet, the plasma ammonia content was markly decreased ($P < 0·05$) and then gradually increased when Met was at higher concentrations.

Regular nutritive compositions in the muscle of on-growing grass carp

As shown in Table 3, with Met compositions in the diet not higher than 7·43 g/kg of the diet, the crude protein, crude lipid, Hyp and collagen contents were significantly heightened ($P < 0·05$) and then diminished smoothly. When the increase in Met compositions in the diet was from 2·54 to 7·43 g/kg of the diet, we also found that the moisture content gradually decreased, and with Met compositions from 7·43 to 15·11 g/kg of the diet, that increased sustainably. The ash decreased as Met levels increased, with plateauing at 7·43 g/kg diet ($P > 0·05$). With using quadratic regression analysis to estimate the optimal Met requirement for on-growing grass carp according to the muscle collagen content, we found that it was 9·28 g/kg of the diet, corresponding to 32·29 g/kg protein of diet (Fig. 2).

Free amino acid compositions in the muscle of on-growing grass carp

As shown in Table 4, the contents of total essential amino acid, total amino acid, Asp and Ser were all maximum at point of Met concentration of 10·12 g/kg diet, and in the 7·43 g Met/kg diet group, the contents of Ile, Lys, Gly, Glu, Leu and Arg were highest. The Met content was lowest in the group fed with Met-unsupplemented diet, while there were no significant change when dietary Met levels were higher than 2·54 g/kg of the diet. We found that the Thr content did not significantly change in Met-supplemented groups, but all were slightly higher than that in 2·54 g Met/kg of the diet group. In comparison with Met-unsupplemented group, the content of Phe obviously elevated with Met compositions reached to 10·12 g/kg diet ($P < 0·05$) and then showed flat ($P > 0·05$). The cysteine content gradually increased as increasing Met levels even though there were no significant difference within the range of 7·43–15·11 g Met/kg diet groups ($P > 0·05$). We also found that the differences of the contents of Ala,Val, His, Tyr and Pro were not significant among all treats ($P > 0·05$).

Fatty acid profile and fatty acid synthase activity in the muscle of on-growing grass carp

The fatty acid profile and FAS of on-growing grass carp muscle affected by graded Met levels are shown in Table 5. With Met compositions in the diet increased up to 7·43 g/kg of the diet, the content of C14:0 gradually decreased and then increased gradually. The contents of C16 : 1, C20 : 2, C20 : 3*n*-3, C18 : 3*n*-6, C18 : 1*n*-9t, C18 : 1*n*-9c, C20 : 5*n*-3, ∑MUFA, ∑PUFA and ∑*n*-3 (PUFA) were all highest at point of Met concentrations of 10·12, 12·40, 12·40, 7·43, 15·11, 7·43, 10·12, 7·43, 12·40 and 10·12 g/kg diet, respectively. The contents of C17:1 in the 2·54, 4·83 and 15·11 g/kg of the diet groups were obviously lower than that in groups with 7·43, 10·12 and 12·40 g Met/kg diet ($P < 0·05$). We found in the diet with 7·43 g/kg of Met that the C16:0 content was lowest ($P < 0·05$), and the differences among remaining groups for that were not significant ($P > 0·05$). With Met concentrations reached to 12·40 and 7·43 g/kg of the diet, the contents of C17 : 0 and C20 : 3*n*-6 showed significantly higher in comparison with control group ($P < 0·05$), respectively, and then evidently reduced for C17 : 0 ($P < 0·05$) and plateaued for C20 : 3*n*-6 ($P > 0·05$). In the diet with Met level of 15·11 g/kg diet, the content of C18:0, which showed no significant difference with 12·40 g Met/kg diet group ($P > 0·05$), was highest, and that was not statistically significant in remaining groups ($P > 0·05$). In the muscle of fish fed a diet containing 12·40 g/kg of Met, the C20 : 0 content was highest, but showed no significant difference with 4·85 g Met/kg diet group ($P > 0·05$), while in groups with 2·54 and 10·12 g Met/kg diet, the C14 : 1 content was highest. The C23 : 0 content decreased as Met levels increased to 7·43 g/kg diet, and there was no significant difference in remaining groups ($P > 0·05$). The content of ∑UFA was highest in 7·43 g Met/kg diet group ($P < 0·05$), followed by 10·12 g Met/kg diet group, and in remaining groups, that showed no significant difference ($P > 0·05$). For fish fed a diet with 7·43 g/kg of Met, the content of C22 : 1*n*-9 was lowest ($P < 0·05$). The ∑SFA content decreased with methionine compositions in the diet reached to 7·43 g/kg diet, and when Met compositions were above 7·43 g/kg of the diet, that increased gradually. However, we found that Met compositions in the diet had no impact on the contents of C15 : 0, C21 : 0, C22 : 0, C20 : 1*n*-9, C24 : 1*n*-9, C22 : 6*n*-3, C18 : 3*n*-3, C18 : 2*n*-6t, C18 : 2*n*-6c, C22 : 2 and ∑*n*-6 (PUFA) as well as the ratio of ∑*n*-3:∑*n*-6 ($P > 0·05$). The activity of FAS gradually increased when Met concentrations reached to 10·12 g/kg of the diet and then smoothly reduced.

Histological analysis of muscle of on-growing grass carp

Figure 3 shows the muscle morphology of on-growing grass carp affected by graded Met compositions in the diet, and the statistical results related to frequency of distribution (%) of muscle fibres in diameter classes are shown in Table 6. Haematoxylin–eosin stain showed that muscle fibres of all groups were characterised by mosaic appearance with different diameters. With increasing Met in the diet up to 7·43 g/kg diet,

Table 4. Free amino acid compositions (mg/100 g tissue) in the muscle of on-growing grass carp (*Ctenopharyngodon idella*) fed diets with graded levels of methione (Met) (g/kg) for 60 d
(Mean values and standard deviations; six replicate groups)

Met level (g/kg diet)...	2·54		4·85		7·43		10·12		12·40		15·11	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Asp	2·64^{a}	0·08	2·86^{a,b}	0·03	2·99^{b,c}	0·15	3·12^{c}	0·11	2·84^{a,b}	0·21	2·94^{b,c}	0·12
Glu	9·25^{a}	0·67	9·39^{a,b}	0·48	10·31^{b,c}	0·60	9·85^{a,b}	0·70	9·22^{a}	0·21	8·86^{a}	0·25
Ser	6·07^{a}	0·41	6·39^{a,b}	0·20	7·02^{b,c}	0·66	7·30^{c}	0·26	6·72^{a,b,c}	0·43	6·59^{a,b,c}	0·32
Gly	67·79^{a}	3·47	72·34^{a,b}	2·50	81·25^{c}	4·80	79·41^{b,c}	7·53	74·81^{a,b,c}	3·28	69·33^{a}	4·15
Ala	14·73^{a}	0·10	14·48^{a}	0·42	14·93^{a}	0·45	14·79^{a}	0·79	14·52^{a}	1·25	14·58^{a}	1·38
Met	3·88^{a}	0·32	3·97^{a,b}	0·21	4·36^{a,b}	0·38	4·47^{b}	0·38	4·52^{b}	0·23	4·33^{a,b}	0·18
Lys	29·50^{a}	1·76	32·89^{b,c}	0·82	35·58^{d}	1·44	34·64^{c,d}	0·60	31·32^{a,b}	0·63	30·80^{a,b}	1·07
Val	4·14^{a}	0·31	4·12^{a}	0·34	4·46^{a}	0·15	4·42^{a}	0·07	4·36^{a}	0·25	4·09^{a}	0·14
Ile	3·17^{a}	0·18	3·21^{a}	0·14	3·58^{b}	0·05	3·50^{a,b}	0·23	3·27^{a,b}	0·22	3·27^{a,b}	0·19
Phe	3·65^{a}	0·17	3·92^{a,b}	0·26	4·15^{a,b,c}	0·36	4·33^{b,c}	0·20	4·58^{c}	0·39	4·36^{b,c}	0·21
Leu	3·39^{a}	0·10	3·50^{a,b}	0·28	3·90^{c}	0·07	3·84^{b,c}	0·35	3·71^{a,b,c}	0·11	3·53^{a,b,c}	0·10
Thr	10·12^{a}	0·15	10·61^{a,b}	0·16	10·70^{b}	0·29	10·95^{b}	0·30	10·98^{b}	0·34	10·55^{a,b}	0·36
Arg	22·03^{a}	1·75	23·85^{a,b}	1·42	25·39^{b}	1·55	24·39^{a,b}	1·75	23·84^{a,b}	2·31	22·34^{a,b}	0·78
His	184·53^{a}	17·85	190·65^{a}	11·40	198·31^{a}	10·77	204·03^{a}	15·27	202·21^{a}	15·23	181·95^{a}	7·34
Cys	0·94^{a}	0·05	1·02^{a,b}	0·03	1·11^{b,c}	0·07	1·13^{c}	0·05	1·14^{c}	0·06	1·19^{c}	0·05
Tyr	1·90^{a}	0·15	2·00^{a}	0·14	1·97^{a}	0·17	2·02^{a}	0·18	2·03^{a}	0·09	1·93^{a}	0·03
Pro	6·76^{a}	0·16	6·56^{a}	0·23	6·61^{a}	0·21	6·72^{a}	0·15	6·45^{a}	0·49	6·35^{a}	0·33
TAA	374·50^{a}	16·30	391·78^{a,b}	8·01	416·63^{c}	12·12	418·85^{c}	8·94	406·52^{b,c}	17·23	376·98^{a}	10·66
TEAA	264·42^{a}	20·01	276·73^{a,b}	11·02	290·43^{a,b}	8·23	294·52^{b}	15·50	288·79^{a,b}	14·49	265·22^{a}	6·41

TAA, total amino acids; TEAA, total essential amino acids.
^{a,b,c,d} Mean values in a row with unlike superscript letters are significantly different ($P < 0·05$; ANOVA and Duncan's multiple-range tests).

Table 5. Fatty acid (FA) profile (% of total FA methyl esters) and fatty acid synthase (FAS) activity in the muscle of on-growing grass carp (*Ctenopharyngodon idella*) fed diets with graded levels of methionine (Met) (g/kg) for 60 d
(Mean values and standard deviations; six replicate groups)

Met level (g/kg diet)...	2·54		4·85		7·43		10·12		12·40		15·11	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
C14 : 0	2·20^{b,c,d}	0·12	2·07^{b}	0·09	1·71^{a}	0·13	2·16^{b,c}	0·03	2·31^{c,d}	0·13	2·40^{d}	0·13
C15 : 0	0·180^{a}	0·018	0·183^{a}	0·007	0·186^{a}	0·005	0·182^{a}	0·003	0·176^{a}	0·013	0·182^{a}	0·009
C16 : 0	22·2^{b}	0·19	21·97^{b}	0·57	19·05^{a}	1·85	21·21^{b}	0·50	21·33^{b}	0·27	22·48^{b}	1·24
C17 : 0	0·26^{a}	0·02	0·25^{a}	0·01	0·26^{a}	0·01	0·27^{a,b}	0·02	0·31^{c}	0·01	0·29^{b}	0·01
C18 : 0	4·18^{a,b}	0·27	4·31^{a,b,c}	0·24	4·17^{a,b}	0·31	3·87^{a}	0·20	4·67^{b,c}	0·33	4·83^{c}	0·49
C20 : 0	0·167^{a,b}	0·015	0·196^{c,d}	0·008	0·154^{a}	0·011	0·167^{a,b}	0·012	0·212^{d}	0·008	0·184^{b,c}	0·011
C21 : 0	0·022^{a}	0·003	0·018^{a}	0·001	0·016^{a}	0·001	0·019^{a}	0·002	0·031^{a}	0·001	0·018^{a}	0·001
C22 : 0	0·51^{a}	0·04	0·52^{a}	0·02	0·53^{a}	0·04	0·56^{a}	0·03	0·55^{a}	0·04	0·51^{a}	0·05
C23 : 0	1·52^{b,c}	0·07	1·40^{a,b}	0·10	1·37^{a}	0·05	1·57^{c}	0·04	1·56^{c}	0·12	1·59^{c}	0·08
C14 : 1	0·10^{c}	0·01	0·08^{a,b}	0·00	0·07^{a}	0·00	0·10^{c}	0·01	0·09^{b,c}	0·01	0·08^{a,b}	0·00
C16 : 1	9·69^{a,b}	0·86	9·45^{a,b}	0·67	8·83^{a}	0·84	10·13^{b}	0·35	10·06^{b}	0·36	9·50^{a,b}	0·46
C17 : 1	0·24^{a}	0·02	0·21^{a}	0·02	0·30^{b}	0·03	0·29^{b}	0·02	0·32^{b}	0·01	0·25^{a}	0·01
C18 : 1*n*-9t	0·22^{a}	0·01	0·24^{a}	0·01	0·25^{a,b}	0·02	0·23^{a}	0·00	0·27^{a,b,c}	0·02	0·28^{c}	0·01
C18 : 1*n*-9c	37·32^{a}	0·63	37·58^{a}	1·37	41·00^{b}	3·29	36·87^{a}	1·11	35·50^{a}	0·41	36·26^{a}	0·97
C20 : 1*n*-9	1·57^{a}	0·04	1·78^{a}	0·08	1·67^{a}	0·12	1·68^{a}	0·06	1·66^{a}	0·17	1·57^{a}	0·24
C22 : 1*n*-9	0·128^{c}	0·011	0·131^{c}	0·003	0·096^{a}	0·006	0·112^{b}	0·004	0·126^{c}	0·001	0·109^{b}	0·004
C24 : 1*n*-9	0·0210^{a}	0·0016	0·0213^{a}	0·0021	0·0206^{a}	0·0018	0·0253^{a}	0·0024	0·0191^{a}	0·0007	0·0232^{a}	0·0015
C18 : 3*n*-3	1·15^{a}	0·07	1·20^{a}	0·04	1·26^{a}	0·07	1·17^{a}	0·05	1·12^{a}	0·04	1·12^{a}	0·22
C20 : 3*n*-3	0·079^{a,b}	0·008	0·082^{a,b}	0·002	0·075^{a}	0·005	0·075^{a}	0·005	0·087^{b}	0·008	0·080^{a,b}	0·002
C20 : 5*n*-3	2·46^{a,b}	0·21	2·49^{a,b}	0·10	2·69^{b}	0·19	2·95^{c}	0·15	2·61^{a,b}	0·01	2·37^{a}	0·05
C22 : 6*n*-3	5·15^{a}	0·32	5·17^{a}	0·25	5·43^{a}	0·20	5·50^{a}	0·30	5·57^{a}	0·34	5·25^{a}	0·07
C18 : 2*n*-6t	0·017^{a}	0·002	0·018^{a}	0·002	0·019^{a}	0·001	0·019^{a}	0·002	0·017^{a}	0·001	0·028^{a}	0·001
C18 : 2*n*-6c	9·44^{a}	0·79	9·47^{a}	0·21	9·57^{a}	0·39	9·58^{a}	0·62	10·04^{a}	0·83	9·32^{a}	0·34
C18 : 3*n*-6	0·201^{a,b}	0·012	0·212^{a,b}	0·014	0·222^{b}	0·009	0·189^{a}	0·009	0·211^{a,b}	0·022	0·186^{a}	0·020
C20 : 3*n*-6	0·46^{a}	0·02	0·47^{a,b}	0·01	0·55^{c}	0·05	0·53^{b,c}	0·05	0·53^{b,c}	0·04	0·57^{c}	0·02
C20 : 2	0·45^{a}	0·04	0·45^{a}	0·04	0·51^{b}	0·02	0·52^{b}	0·03	0·53^{b}	0·04	0·45^{a}	0·02
C22 : 2	0·028^{a}	0·003	0·028^{a}	0·002	0·028^{a}	0·003	0·030^{a}	0·003	0·031^{a}	0·003	0·030^{a}	0·003
∑SFA	31·28^{b,c}	0·17	30·93^{b,c}	0·55	27·47^{a}	2·27	30·00^{b}	0·65	31·18^{b,c}	0·13	32·48^{c}	1·08
∑UFA	68·72^{a,b}	0·17	69·09^{a,b}	0·55	72·56^{c}	2·27	69·99^{b}	0·65	68·79^{a,b}	0·13	67·48^{a}	1·08
∑MUFA	49·29^{a}	1·36	49·53^{a}	0·75	52·28^{b}	2·49	49·44^{a}	1·46	48·07^{a}	0·41	48·09^{a}	1·07
∑PUFA	19·43^{a}	1·22	19·54^{a,b}	0·29	20·25^{a,b}	0·21	20·55^{a,b}	0·89	20·75^{b}	0·52	19·43^{a}	0·07
∑*n*-6 (PUFA)	10·12^{a}	0·81	10·18^{a}	0·20	10·36^{a}	0·36	10·32^{a}	0·67	10·80^{a}	0·84	10·10^{a}	0·28
∑*n*-3 (PUFA)	8·84^{a}	0·55	8·89^{a}	0·31	9·35^{a,b}	0·42	9·69^{b}	0·48	9·40^{a,b}	0·30	8·85^{a}	0·21
∑*n*-3:∑*n*-6	0·88^{a}	0·07	0·87^{a}	0·04	0·90^{a}	0·07	0·94^{a}	0·07	0·87^{a}	0·10	0·88^{a}	0·05
FAS (U/mg protein)	8·96^{a}	0·58	12·29^{b}	0·65	15·93^{d}	0·69	16·61^{d}	0·76	13·37^{c}	0·76	12·56^{b,c}	0·89

UFA, unsaturated fatty acids.
^{a,b,c,d} Mean values in a row with unlike superscript letters are significantly different ($P < 0·05$; ANOVA and Duncan's multiple-range tests).

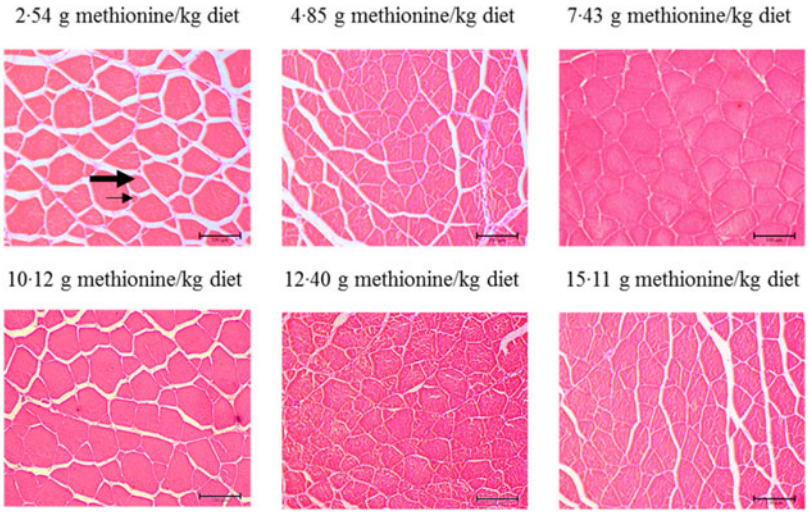

Fig. 3. Cross section of muscle of on-growing grass carp (*Ctenopharyngodon idella*) fed diets with graded levels of methionine (g/kg) for 60 d using haematoxylin–eosin stain. Different muscle fibre diameters are composed of small (arrows) to large (thick arrowhead) fibres.

Table 6. Frequency of distribution (%) of muscle fibres in diameter classes of on-growing grass carp (*Ctenopharyngodon idella*) fed diets with graded levels of methionine (Met) (g/kg) for 60 d
(Mean values and standard deviations; three replicates)

Met level (g/kg diet)...	2·54		4·85		7·43		10·12		12·40		15·11	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
<20 μm	26·44^{d}	1·82	25·58^{d}	1·38	15·09^{a}	1·09	21·68^{b}	1·46	22·95^{b,c}	1·33	24·90^{c,d}	0·58
20–50 μm	46·01^{a}	2·34	47·99^{a}	0·62	45·67^{a}	1·74	45·08^{a}	3·90	49·30^{a}	2·74	49·62^{a}	2·36
>50 μm	27·55^{a}	0·67	26·43^{a}	1·24	39·25^{c}	1·40	33·24^{b}	2·59	27·75^{a}	2·47	25·48^{a}	1·91

^{a,b,c,d} Mean values in a row with unlike superscript letters are significantly different ($P < 0·05$; ANOVA and Duncan's multiple-range tests).

the frequency of <20 μm diameter gradually decreased and then increased gradually. The frequency distribution of >50 μm diameter in 7·43 and 10·12 g Met/kg diet groups showed higher than that in remaining four groups ($P < 0·05$). However, the frequency distribution of 20–50 μm diameters showed no significance among all treats ($P > 0·05$).

Protin and type I collagen synthesis-related mRNA and protein levels in the muscle of on-growing grass carp

As shown in Fig. 4, with Met supplements in the diet added to 10·12 and 7·43 g/kg diet, the mRNA abundances of *TOR* and *CK2α* were advanced individually, and in further dietary Met concentrations, all were gradually plateaued ($P < 0·05$), respectively. When dietary Met compositions changed from 2·54 to 7·43 g/kg diet, the mRNA abundance of *CK2β* was significantly up-regulated ($P < 0·05$), and in higher Met compositions in the diet, there showed gradually downward trend for *CK2β*.

As shown in Fig. 5, when Met compositions in the diet increased in the range 2·54 to 10·12 g/kg diet, we found that the protein abundance of p-TOR^{Ser2448} increased and then decreased gradually. Also, we found that the protein level of t-TOR increased gradually with methioinine supplements added to 10·12 g/kg of the diet and then decreased gradually. The ratio of p-TOR^{Ser2448}: t-TOR was lowest in 4·85, 7·43 and 10·12 g Met/kg diet groups and showed no significant change in remaining groups ($P > 0·05$). The protein level of p-S6K1^{Ser389} and the ratio of p-S6K1^{Ser389}: t-S6K1 were highest in 7·43, 10·12 and 12·40 g Met/kg diet groups ($P < 0·05$), and the changes among remaining groups were not significant ($P > 0·05$). The levels of dietary Met had no impact on the protein abundances of t-S6K1, p-4E-BP1$^{Thr37/46}$ and t-4E-BP1 as well as the ratio of p-4E-BP1$^{Thr37/46}$: t-4E-BP1 ($P > 0·05$).

As shown in Fig. 6, in the muscle, as Met supplements added to 7·43, 10·12, 7·43 and 7·43 g/kg of the diet, the mRNA abundances of *Col1α1*, *Col1α2*, *TGF-β1* and *Smad4* were smoothly increased and then gradually declined, respectively. With Met compositions in the diet from 2·54 to 7·43 g/kg of the diet, the *Smad2* mRNA abundance increased markedly ($P < 0·05$), and when Met compositions were higher than 7·43 g/kg diet, that trended flat ($P > 0·05$). The mRNA abundance of *TNF-α* was highest in Met-unsupplemented group ($P < 0·05$); however, there was no marked difference in remaining groups ($P > 0·05$).

As shown in Fig. 7, when dietary Met supplements added to 7·43 g/kg diet, the protein level of Smad4 significantly elevated ($P < 0·05$), and as Met compositions in the diet were higher than

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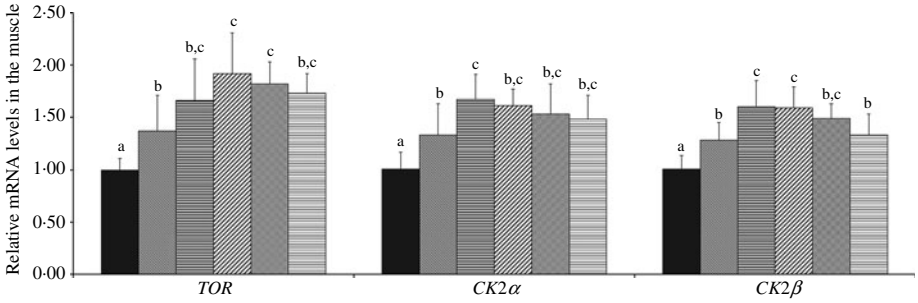

Fig. 4. Effects of dietary methionine levels on *TOR*, *CK2α* and *CK2β* gene expressions in the muscle of on-growing grass carp (*Ctenopharyngodon idella*). Data represent means of six fish in each group, error bars indicate standard deviations. ^{a,b,c} Mean values with unlike letters are significantly different ($P < 0·05$; ANOVA and Duncan's multiple-range tests). Dietary methionine levels (g/kg diet): ■, 2·54; ▧, 4·85; ▤, 7·43; ▨, 10·12; ▩, 12·40; ▤, 15·11.

7·43 g/kg diet, that decreased gradually. With Met compositions in the diet at 10·12 and 12·40 g/kg diet, the protein abundance of t-Smad2 was higher than that in remaining four groups ($P < 0·05$). The p-Smad2^{Ser467} protein level was highest in the 10·12 g Met/kg diet group ($P < 0·05$) and lowest in the control group ($P < 0·05$), and the remaining four Met levels had no effect on that ($P > 0·05$). The ratio of p-Smad2:t-Smad2 was highest in the 7·43 and 10·12 g Met/kg diet groups.

Discussion

Methionine improved fish growth performance

In this study, we discovered that the optimal level of Met enhanced the feed intake, feed efficiency, PWG and the activities of glutamate-oxaloacetate transaminase and glutamate-pyruvate transaminase in the hepatopancreas and muscle as well as decreased the plasma ammonia content of on-growing grass carp, indicating that Met improved amino acid utilisation and growth performance of fish. Meanwhile, the optimal dietary Met requirement of on-growing grass carp (178–626 g) was 9·56 g/kg of the diet (corresponding to 33·26 g/kg protein of diet, Fig. 1) for maximum PWG. This value was slightly different with our previous research for on-growing grass carp, which was 10·33 g/kg of the diet (34·43 g/kg protein of diet, calculated PWG by their results)[20]. That slight difference in Met requirements between our two studies might be partially relevant to diverse types and amounts of protein sources (fishmeal: 6·80 %, gelatin: 4·00 %, soyabean protein concentrate: 11·00 % in current experiment *v.* fishmeal: 7·80 %, gelatin: 3·99 %, casein: 3·00 % in our previous experiment) and the variation in concentrations of crystal amino acid mix (14·28 % in current experiment *v.* 20·96 % in our previous experiment) in these two experiments. Additionally, fish growth is mainly due to muscle growth, which is closely relevant to the deposition of nutrients and the growth of muscle fibre and surrounding intramuscular connective tissue in the muscle[4,9]. Therefore, we next investigated the effects of dietary Met on these processes in the muscle of on-growing grass carp.

Methionine promoted the deposition of nutrients in the muscle of fish

To our knowledge, in fish, muscle is the main site of nutritive deposition[48], while muscle protein and lipid are the main DM[49]. First, our current result in on-growing grass carp displayed that compared with Met insufficiency, optimal level of Met increased muscle protein content, as the same results for juvenile grouper[7] and *Pseudobagrus ussuriensis*[50]. The increased protein content in the muscle was potentially in part contacted to the TORC1 signalling pathway. As the core component of mTORC1, phosphorylation of mTOR at Ser2448 is stimulated by amino acids, which is an reasonable indicator for the activation status of mTOR[51]. In addition, ribosomal S6 kinase (S6K1) is a key signalling molecule that regulates protein synthesis, which can activate 40S ribosomal protein S6 through phosphorylation to improve the translation efficiency of some mRNA[52,53], while eukaryotic translation initiation factor 4E binding protein 1(4E-BP1), as a negative regulator of translation, can inhibit the initiation of translation by combining with the eukaryotic translation initiation factor 4E's mRNA cap binding subunit[54]. It has been reported that mTORC1 can modify S6K1 and 4E-BP1 by direct phosphorylation to promote protein translation process[55]. Our current observations indicated that optimal Met level elevated muscle *TOR* mRNA abundance, the total and phosphorylation of TOR protein levels of on-growing grass carp. These results were consistent with reports in cobia *Rachycentron canadum*[56], *songpu* mirror carp[57] and broiler[58] demonstrating that Met could increase *TOR* (*mTOR*) mRNA abundance in the muscle, as well as in grass carp[59], showing that Met could advance the protein levels of total and phosphorylated TOR in the intestine. Also, we found that optimal Met level reduced the ratio of p-TOR^{Ser2448}/t-TOR in the current experiment, which was inconsistent with that in cobia[56] and turbot *Scophthalmus maximus* L.[60]. That result might be due to the fact that the effect of Met on translation was greater than activation to TOR in our result, and similar result could be found in the study of bovine mammary epithelial cells[61]. In addition, optimal Met level enhanced the protein level of p-S6K1^{Ser389} and the ratio of p-S6K1^{Ser389}: t-S6K1, while we failed to test

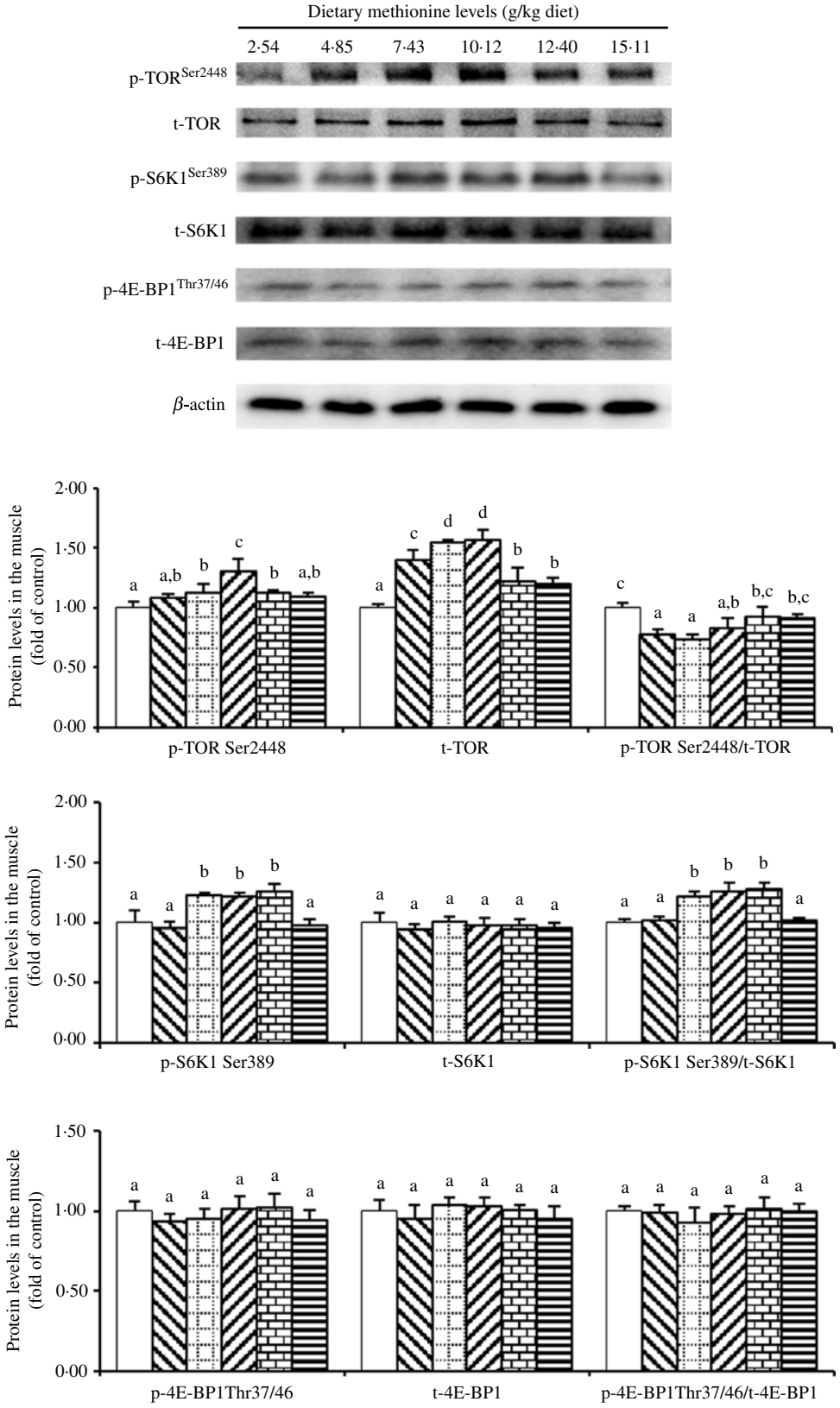

Fig. 5. Western blot analysis of protein expression of genes involved in protein metabolism in the muscle of on-growing grass carp (*Ctenopharyngodon idella*) fed diets with graded levels of methionine (g/kg) for 60 d. Data represent means of three fish in each group, error bars indicate standard deviations. [a,b,c,d] Mean values with unlike letters are significantly different ($P < 0·05$; ANOVA and Duncan's multiple-range tests). Dietary methionine levels (g/kg diet): □, 2·54; ▨, 4·85; ■, 7·43; ◪, 10·12; ▣, 12·40; ▤, 15·11.

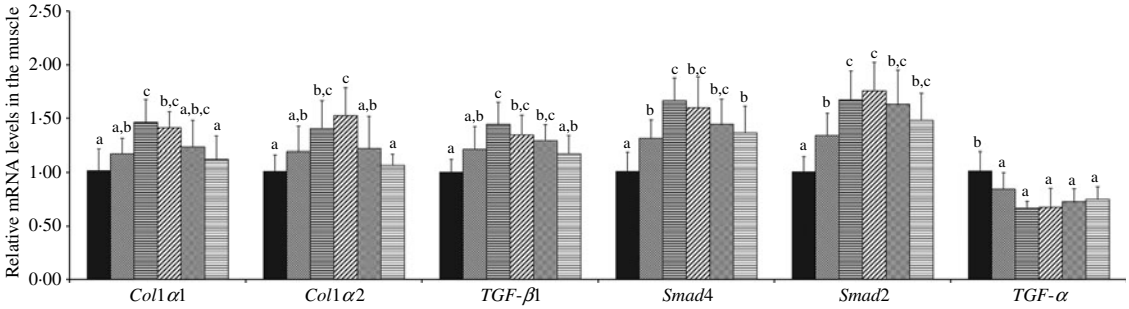

Fig. 6. Effects of dietary methionine levels on *Col1α1*, *Col1α2*, *TGF-β1*, *Smad4*, *Smad2* and *TNF-α* gene expressions in the muscle of on-growing grass carp (*Ctenopharyngodon idella*). Data represent means of six fish in each group, and error bars indicate standard deviations. [a,b,c] Mean values with unlike letters are significantly different ($P < 0{\cdot}05$; ANOVA and Duncan's multiple-range tests). Dietary methionine levels (g/kg diet): ■, 2·54; ▨, 4·85; ▤, 7·43; ▨, 10·12; ■, 12·40; ▤, 15·11.

any obvious change in the level of p-4E-BP1$^{Thr37/46}$ and the ratio of p-4E-BP1$^{Thr37/46}$: t-4E-BP1, as the similar results in cobia[56]. Such a discrepancy between the phosphorylation status of these two proteins (S6 and 4E-BP1) has already been discovered in the muscle of rainbow trout[17] and in the liver of rats[62] in response to dietary Met intake and highlights the complexity of the signalling network associated with the regulation of the activation of these translation initiation factors. By correlation analysis, we found positive correlations between the content of muscle protein and the protein level of p-TOR^{Ser2448} and p-S6K1^{Ser389} (online Supplementary Table S2). These data indicated that optimal Met level promoting muscle protein content might be in part associated to elevate muscle protein synthesis via the TORC1/S6K1 (not 4E-BP1) signalling pathway in fish. We envisaged that the regulating effect of Met on TORC1 signalling might be partially relevant to incremental muscle free amino acid contents, particularly Met, arginine, threonine and leucine. Therefore, we next investigated the effects of dietary Met on free amino acid content in the muscle of on-growing grass carp.

In the muscle, previous studies reported that Met in rainbow trout[17], arginine in Jian carp *Cyprinus carpio* var. Jian[31] and threonine and leucine in hybrid catfish *Pelteobagrus vachelli* × *Leiocassis longirostris*[63,64] could stimulate protein synthesis via the TORC1 signalling pathway. In the present study, we found that compared with the control group, optimal Met level increased muscle-free Met, arginine, threonine and leucine contents of on-growing grass carp, supporting our hypothesis. Meanwhile, in the present study, we also found that optimal level of Met enhanced muscle-free total amino acid and total essential amino acid contents of on-growing grass carp, which might be to some extent associated with increased amino acids absorption. In fish, most AA absorption required an extracellular Na ion concentration gradient[65]. It has been reported that Na^+, K^+-ATPase was located on the basolateral membrane and could carry out the uptake of Na^+ in most higher eukaryotes[66]. Our previous study in on-growing grass carp reported that optimal Met level increased the activity of Na^+, K^+-ATPase in the intestine (proximal intestine and mid intestine)[20], supporting our assumption.

On the other hand, from current study, we found that in comparison with Met deficiency, optimal Met level also increased on-growing grass carp muscle lipid content, which was similar to the report by Luo *et al.*[7]. Meanwhile, it has been reported that the nutritive value of lipid mainly depended on the contents and species of its fatty acid, and decreased SFA and increased unsaturated fatty acids in the muscle could improve the nutritive value of on-growing grass carp[4]. In the present research, we for the first time found that optimal Met level decreased the SFA (C14 : 0, C16 : 0, C18 : 0, C20 : 0, C23 : 0) concentrations and increased the unsaturated fatty acids (MUFA, such as C16 : 1, C17 : 1 and C18 : 1*n*-9c and PUFA, such as C20 : 5 *n*-3, C18 : 3 *n*-6 and C20 : 3 *n*-6) concentrations in on-growing grass carp muscle. We speculated that these results might be partially related to FAS. Ganguly *et al.* demonstrated that FAS played a crucial role in catalysing the synthesis of PUFA in the muscle of Indian shad hilsa *Tenualosa ilisha*[67]. Our present data observed that optimal Met level elevated the activity of FAS in the muscle of on-growing grass carp, supporting our assumption.

In addition, muscle growth was also relevant to the growth of muscle fibre and the formation of intramuscular connective tissue[8], which were primarily involved with the hyperplastic and hypertrophic growth of muscle fibre and the synthesis of type I collagen, respectively. Therefore, we next examined the effects of Met on these processes as well as related molecular mechanisms (TGF-*β*1/Smads and TORC1 signalling) in the muscle of on-growing grass carp.

Methionine promoted muscle fibre hypertrophy and type I collagen synthesis in fish

Generally, it was well known that muscle morphology was an important auxiliary mean in fish nutrition research[11]. Previous study reported that the processes of hyperplasia and hypertrophy would maintain active for a long time to muscle growth in large-sized fish with rapid growth[68]. It has been reported that a new fibre arised by hyperplasia was relatively small[69], of which the diameter of fibres <20 μm always represented the fibres recruited by hyperplasia, and those exceeding this diameter represented fibres that have been subsequently grown by hypertrophy[70]. Our current observations in on-growing grass carp displayed that fibres <20 μm in diameter occurred in all treatments, and compared with Met unsufficient, optimal level of Met significantly decreased the frequency of fibres <20 μm in diameter and increased the frequency distribution of muscle fibres with >50 μm of diameter. These data indicated

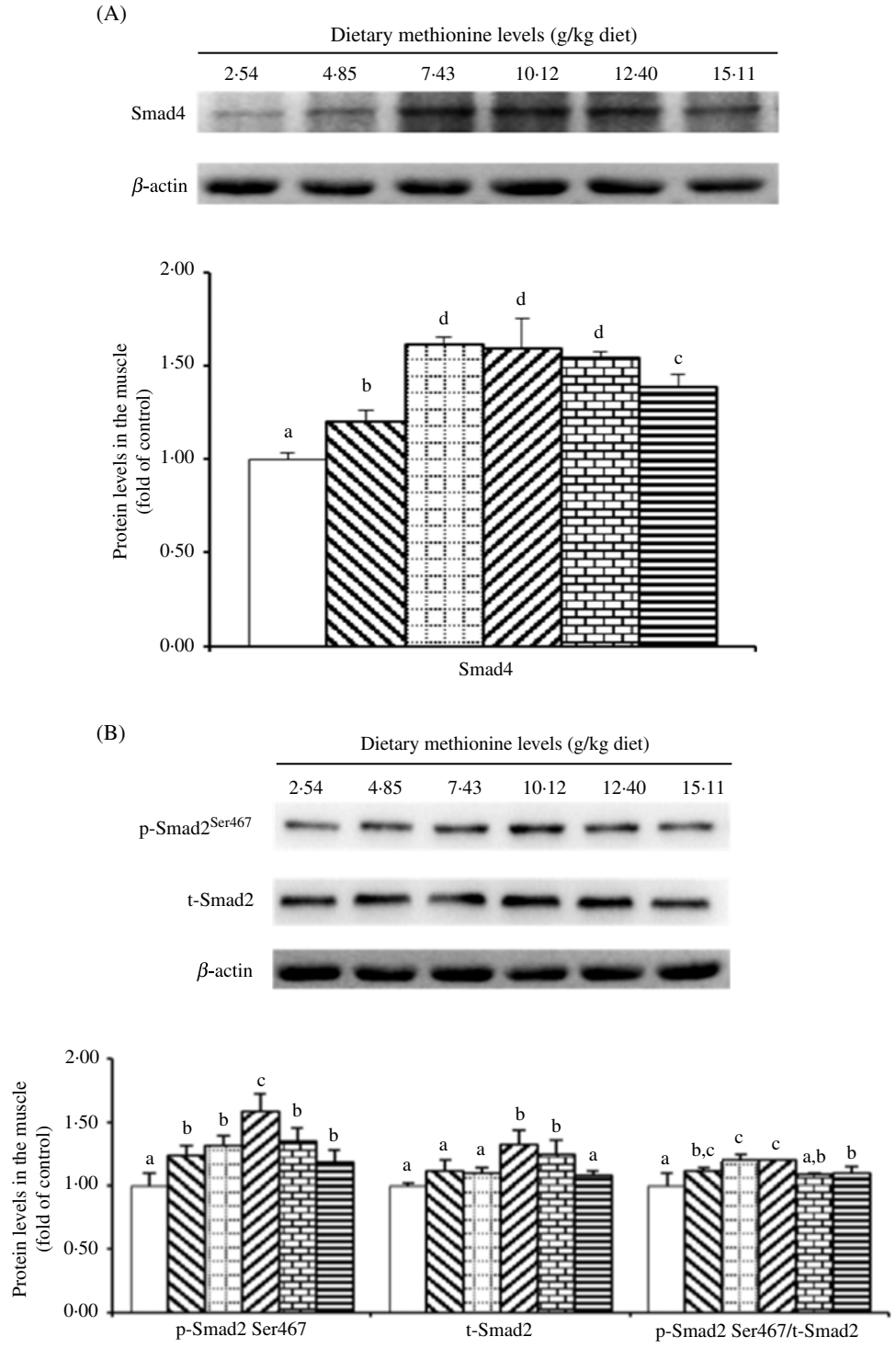

Fig. 7. Western blot analysis of protein expression of genes involved in type I collagen metabolism in the muscle of on-growing grass carp (*Ctenopharyngodon idella*) fed diets with graded levels of methionine (g/kg) for 60 d. Data represent means of three fish in each group, and error bars indicate standard deviations. [a,b,c,d] Mean values with unlike letters are significantly different ($P < 0·05$; ANOVA and Duncan's multiple-range tests). Dietary methionine levels (g/kg diet): ■, 2·54; ▧, 4·85; ▤, 7·43; ◪, 10·12; ■, 12·40; ▤, 15·11.

that first, hyperplasia occurred in all treatment groups, and this process was also affected by the dietary Met concentrations and second, optimal level of Met promoted muscle fibre growth mostly by hypertrophy in fish.

Meanwhile, except for muscle fibre, the collagen was an important protein that constituted muscle connective tissue and had the characteristics of maintaining structural stability and integrity of fibre in previous study of sea bass *Dicentrarchus labrax* L.[33].

Its content increase in fish muscle could result in higher mechanical strength[71]. Johnston *et al.*[72] reported that measuring the content of Hyp could relatively quantify the collagen amount in fish. Our present research in on-growing grass carp displayed that compared with Met deficiency leading to decreased muscle collagen content, optimal Met level significantly enhanced the content of collagen in the muscle. We supposed that this result might be partially linked to de novo synthesis of collagen. In grass carp, the main collagen in the muscle intramuscular connective tissue was type I collagen (included Col1α1 and Col1α2 peptide chains)[14]. As demonstrated in this study, optimal Met level advanced the mRNA abundances of *Col1α1* and *Col1α2* in the muscle of on-growing grass carp. The elevated mRNA abundance of *Col1α2* was in line with the report for rainbow trout[3]. In addition, we also found significant positive correlations via correlation analysis on the basic of muscle collagen content and Col1α1, Col1α2 mRNA abundances (online Supplementary Table S2). These data suggested that optimal Met level increased muscle collagen content, which was partially due to up-regulated type I collagen *de novo* synthesis in the muscle of fish. On the basic of muscle collagen content, the Met requirement was recommended to be 9·28 g/kg diet (32·29 g/kg protein of diet, Fig. 2), which was close to or slightly lower than the optimal Met requirement for growth (9·54 g/kg diet). Similar observation could be found in the study of tryptophan for on-growing grass carp[26]. Furthermore, recent research has displayed that type I collagen expression in the muscle could be modulated by the TGF-β1/Smads signalling pathway in grass carp[15]. Therefore, we next explored the impact of Met on TGF-β1/Smads in the muscle of on-growing grass carp.

As the TGF-β1/Smads signalling pathway, it should be noted that TGF-β1 bound to the TGF-β type II and type I receptor in turn, and the activated TGF-β type I receptor interacted with Smad2 to phosphorylate it, and then, phosphorylated Smad2 bound to Smad4 forming the Smad2/4 complex, and finally, the complex was transferred to the nuclear to work[73]. In addition, previous studies showed that Smad2 and Smad4 played important regulatory role in type I collagen expression in mammals and grass carp[15,74–76]. For the first time, our present results for on-growing grass carp displayed that in comparison with control group, optimal level of Met significantly elevated the mRNA abundances of *TGF-β1*, *Smad4*, *Smad2* and the protein levels of Smad4, t-Smad2 and p-Smad2^{Ser467} as well as the ratio of p-Smad2^{Ser467}: t-Smad2 in the muscle. We also found positive correlations between Col1α1 mRNA abundance and TGF-β1 mRNA abundance, t-Smad4, p-Smad2^{Ser467} protein levels via correlation analysis and Col1α2 possessed the similar trend (as shown in online Supplementary Table S2). The results studied above manifested that optimal Met level increased the gene expression of type I collagen via regulating the TGF-β1/(Smad4 and Smad2) signalling pathway. We speculated that there were two pieces of evidence for the Met-regulated TGF-β1/(Smad2 and Smad4) signalling pathway. First, Met acting as a functional signalling molecule directly up-regulated the expression of TGF-β1, and similar result could be found in the study by Pan *et al.*[77], further activated the downstream signalling pathway involved with Smads. However, this speculation needs deeper exploration. Second, that potentially related to TNF-α. Previous scholars have demonstrated that TNF-α could depress the protein expression of TGF-β type II receptor in human dermal fibroblasts[78] and prevent the phosphorylation and nuclear translocation of Smads in mice fibroblast cell lines[79]. Our present research with on-growing grass carp revealed that optimal level of Met increased muscle *TNF-α* mRNA abundance, which supports our assumption.

Furthermore, type I collagen synthesis was also regulated by mTORC1 signalling in human dermal fibroblasts[18]. As described in the current study, optimal level of Met increased the mRNA level, phosphorylation and total protein levels of TOR in the muscle of on-growing grass carp. Also, we discovered that there were positive correlations between Col1α1, Col1α2 mRNA abundances and p-TOR^{Ser2448} protein level (as shown in online Supplementary Table S2), indicating that optimal Met level promoted type I collagen synthesis might in part by regulating the TORC1 signalling pathway. The regulation of TORC1 by Met might be partially in connection with protein kinase casein kinase 2 (CK2). CK2 is a constitutively active and highly conserved serine/threonine protein kinase, which includes two catalytic (α and/or α') and two regulatory (β) subunits[80]. CK2 is commonly expressed in subcellular compartments of all eukaryotes and phosphorylates over 300 substrates and regulates several different metabolic events as a result[81]. Study reported that CK2 inhibition down-regulated the expression of type I collagen in NIH-3T3 fibroblasts[82]. Also, in human glioblastoma cells, CK2 depletion led to the reduction of phosphorylation level of mTOR[83]. Our data found that compared with Met deficiency, optimal level of Met elevated muscle *CK2α* and *CK2β* mRNA abundances of on-growing grass carp. We also discovered that there were positive correlations between p-TOR^{Ser2448} protein level and CK2α, CK2β mRNA abundances (as shown in online Supplementary Table S2), supporting our assumption.

Methionine excess decreased fish growth

Met was a sulphur-containing amino acid, the excessive intake of which could result in poor growth performance and feed utilisation for juvenile Chinese sucker *Myxocyprinus asiaticus*[84]. Our present data displayed that compared with Met-insufficiency (2·54 g/kg diet), although excess Met level (15·11 g/kg diet) led to better growth performance, feed utilisation and muscle growth, while in comparison with optimal Met level (7·43 g/kg diet), these indicators significantly decreased. That consequence could be explained by the following possibility that excessive Met intake might (1) reduce the palatability of feed[85]; (2) reduce the ability of digestion and absorption in fish[2,27]; (3) occupy a large number of amino acid transporters, affecting the transportation and absorption of other amino acids[86,87] and (4) increase the accumulation of oxidised Met, leading to oxidative stress and reducing the antioxidant capacity[88], which ultimately reduced the growth performance of fish. However, this speculation needs further research.

Conclusion

In short, similar to our previous study, our current research showed that optimal Met level also improved the growth performance and feed ulitisation of on-growing grass carp. Meanwhile,

we for the first time systematically revealed that optimal Met level promoted the growth of muscle in fish by regulating the deposition of nutrients, muscle fibre growth and type I collagen synthesis as well as related signalling molecules, as displayed in the following aspects: (1) optimal Met level promoted the deposition of muscle nutrients such as protein, lipid, total free amino acid and unsaturated fatty acid contents, and elevated muscle protein content partly linked to the TORC1/S6K1 signalling pathway; (2) optimal Met level accelerated muscle growth by muscle fibre hypertrophy; (3) optimal Met level enhanced muscle collagen content by increasing type I collagen synthesis in part relevant to (TGF-β1/(Smad4 and Smad2)) and (CK2α, β/TORC1) signalling pathways. Additionally, on the basic of growth indicator (PWG) and muscle collagen content, the Met requirements for on-growing grass carp (178–626 g) were estimated to be 9·56 g/kg of the diet (33·26 g/kg protein of diet) and 9·28 g/kg of the diet (32·29 g/kg protein of diet), respectively.

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X.-Q. Z. and L. F. designed the study; C.-C. F., W.-D. J. and L. F. conducted the study and analysed the data; Y. L., P. W., S.-Y. K., L. T. and X.-A. L. participated in the interpretation of the results; C.-C. F. and W.-D. J. wrote the manuscript; X.-Q. Z. had primary responsibility for the final content of the manuscript. All authors read and approved the final manuscript.

The authors declare that there are no conflicts of interest.

Supplementary material

For supplementary material referred to in this article, please visit https://doi.org/10.1017/S0007114520002998

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