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Role of creatine supplementation on the myofibre characteristics and muscle protein synthesis of grass carp (*Ctenopharyngodon idellus*)

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Abstract

To assess the role of dietary creatine on myofibre characteristics and protein synthesis in muscle, we fed grass carp (*Ctenopharyngodon idellus*, initial body weight: 88·47 ± 1·44 g) creatine-supplemented diets (1·84, 5·91, 8·48 and 15·44 g/kg diet) for 8 weeks. Creatine supplementation did not affect growth performance, but significantly increased creatine contents in muscle and liver. At 8·48 g/kg, creatine decreased the activities of alanine transaminase and aspartate aminotransferase in serum and improved hardness and chewiness of muscle due to shorter myofibre mean diameter, higher myofibre density and the frequencies of the diameters of classes I and III and collagen content, longer sarcomere length and upregulated mRNA levels of slow myosin heavy chains. Creatine supplementation upregulated the mRNA expressions of myogenic regulatory factors. The 8·48 g/kg creatine-supplemented diet significantly increased the contents of protein, total amino acids (AA), essential AA and free flavour AAs in muscle, the protein levels of insulin-like growth factor I, myogenic differentiation antigen and PPAR-γ coactlvator-1α in muscle and stimulated the phosphorylation of target of rapamycin (TOR) pathway in muscle. In summary, 8·48 mg/kg creatine improved fish health and skeletal muscle growth and increased hardness and protein synthesis in muscle of grass carp by affecting myofibre characteristics and the TOR signalling pathway. A second-order regression model revealed that the optimal dietary creatine supplementation of grass carp ranges between 8·48 and 12·04 g/kg.

Key words: **Creatine: Grass carp: Flesh quality: Myofibre: TOR**

Aquatic products are sources of PUFA and essential amino acids (EAAs)[1]. Aquaculture products, which account for >50 % of the global fish consumption and provide ~30 % of the daily animal protein consumption in developing nations, have reduced our dependence on fishing[2]. China has led the global aquaculture production in the past decades, especially freshwater aquaculture. Grass carp (*Ctenopharyngodon idellus*), which is one of the predominant farmed freshwater fish in the world, had a total production of 5·57 million tons in 2020[3]. However, compared with wild grass carp, cultured grass carp has a lower content of protein and total amino acids in muscle and poor taste[4]. Flesh quality is closely associated with human health and consumer acceptance; therefore, high-quality fish is an important target in aquaculture.

Flesh quality is evaluated by traditional physicochemical texture and sensory characteristics, which in a great degree is determined by structural properties such as collagen, characteristics and types of myofibre[5] and nutrients levels in muscle[6]. In cultured fish, feed contributes to flesh quality due to dietary nutrients or special ingredients retained in a controlled manner[7]. For instance, the hardness and muscle collagen content of grass carp are significantly enhanced by arginine supplementation[8]. In white muscle of rainbow trout (*Oncorhynchus mykiss*), diets rich in soybean meal decrease the mean and median diameters of myofibres and the expression of *myogenic differentiation antigen* (*MyoD*) and increase the expression of *fast myosin heavy chain* (*fast-MyHC*)[9].

Creatine is a nitrogenous organic acid that is metabolised from arginine, methionine and glycine in the liver, kidneys and pancreas[10]. Dietary creatine especially from meat and fish is transported to skeletal muscles, brain and testes and plays an important role in energy production during muscle contraction[11]. Therefore, creatine supplementation has been used to improve exercise capacity in healthy individuals and athletes.

Abbreviations: AA, amino acids; EAA, essential amino acids; Hyp, hydroxyproline; IGF-I, insulin-like growth factor I; MRF, myogenic regulatory factors; TOR, target of rapamycin.

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In fish, creatine supplementation promotes flesh quality. At 12·5 g/kg, creatine increases muscle endurance in juvenile rainbow trout[12]. Our previous study on *Litopenaeus vannamei* showed that diets supplemented with 8·28 g/kg creatine enhanced the hardness and chewiness of muscle by improving the diameter and density of myofibres and increasing collagen content. Furthermore, creatine supplementation improves flesh nutrition and flavour by increasing levels of protein, EAA, and flavour free amino acids in muscle[13]. In mice, creatine increases muscle protein content by phosphorylating serine/threonine kinase (protein kinase B, Akt), the target of rapamycin (TOR) and ribosome S6 protein kinase ($P70^{S6K}$)[14]. In rats, creatine transforms MyHCII myofibres to MyHCI[15] myofibres by regulating myogenic regulatory factors (MRF)[10]. The creatine-induced myofibre transformation is mediated by the AMPK/PGC-1α (AMP-activated protein kinase/peroxisome proliferator-activated receptor-γ coactlvator-1α)[16] and TOR pathways[17]. Therefore, creatine plays a central role in growth and nutrients levels of muscle by inducing gene expressions and participate in the TOR activation response. However, the effects of dietary creatine on fish flesh quality have not been systematically evaluated except for few studies that have evaluated the associations between creatine supplementation and growth performance[12,13,18,19,20].

The continuous high aquaculture production of grass carp is due to intensive farming based on the high utilisation of artificial formulated feed[21]. In general, the creatine content in grass carp feed is low because animal source feedstuffs are rarely used. Therefore, in the current study, the effect of dietary supplementation of four graded levels of creatine (1·84, 5·91, 8·48 & 15·44 g/kg) was evaluated on growth performance, feed utilisation, serum chemistry, flesh texture, proximate and amino acid composition, myofibre characteristics and metabolic pathways in grass carp. The study's findings will provide a deeper understanding of dietary creatine on the flesh quality and creatine metabolism of fish, and thus eventually provide a novel technology to produce high-quality aquatic products.

Materials and methods

Ethics statement

The Institutional Animal Care and Use Committee of Yangtze River Fisheries Research Institute approved our study (YFI 2018-40).

Experimental diets

We designed a control diet based on the nutritional requirements of grass carp[22] and formulated three-creatine supplemented diets[12,20]: 0 (T1), 3 (T2), 6 (T3) and 12 (T4) g/kg creatine monohydrate (Shanghai yuanye Bio-Technology Co., Ltd). The measured creatine contents in four diets were 1·84, 5·91, 8·48 and 15·44 g/kg. First, we pulverised the ingredients and mixed them uniformly with soybean oil. Second, we added different volumes of a creatine monohydrate solution (50 g/l water) based on the treatment. Third, we added water (300 ml/kg dry ingredients) and passed the mixture though a pelletiser with a dye (diameter: 2 mm). The diets were dried at 60°C for 4 h, broken into small pellets and stored at −20°C. Table 1 shows the ingredients and proximate compositions, and Table 2 shows the amino acid contents. Because of the determination method, even though the crude protein contents were different, the TAA contents were similar among the four diets.

Table 1. The ingredients and proximate composition of diets with creatine supplement

	Dietary creatine levels (g/kg diet)			
Ingredients	1·84 (T1)	5·91 (T2)	8·48 (T3)	15·44 (T4)
Fish meal	30·00	30·00	30·00	30·00
Soybean meal	320·00	320·00	320·00	320·00
Rapeseed meal	280·00	280·00	280·00	280·00
Cellulose	91·00	88·00	85·00	79·00
Rice bran	30·00	30·00	30·00	30·00
Wheat flour	160·00	160·00	160·00	160·00
Soybean oil	40·00	40·00	40·00	40·00
$Ca(H_2PO_4)_2$	20·00	20·00	20·00	20·00
Choline chloride	2·50	2·50	2·50	2·50
Vitamin C	1·00	1·00	1·00	1·00
NaCl	2·50	2·50	2·50	2·50
Lysine	2·00	2·00	2·00	2·00
Methionine	1·00	1·00	1·00	1·00
Vitamin premix*	10·00	10·00	10·00	10·00
Mineral premix†	10·00	10·00	10·00	10·00
Creatine monohydrate	0·00	3·00	6·00	12·00
Proximate composition (g/kg diet, DM)				
Dry matter	962·61	953·72	945·76	950·54
Crude protein	291·65	296·93	302·21	312·77
Crude lipid	61·73	62·37	60·84	62·95
Ash	65·10	68·36	66·07	67·35

* Vitamin premix consisted of (mg/kg diet): vitamin A 4500 mg; vitamin D 1000 mg; vitamin E 100; vitamin K 5; thiamine 10; riboflavin 20; pyridoxine 10; cyanocobalamin 0·05; vitamin C 400; calcium pantothenate 100; folic acid 5;biotin 1; inositol 500; nicotinic acid 150.

† Mineral premix consisted of (g/kg premix): KH_2PO_4, 321; NaCl, 101; $MgSO_4{\cdot}7H_2O$, 150; $Ca(H_2PO_4)_2{\cdot}H_2O$, 353; $FeSO_4{\cdot}7H_2O$, 19·9; $ZnSO_4{\cdot}7H_2O$, 3·56; MnSO4·4H_2O, 1·62; CuSO4 5H_2O, 0·31; $CoCl_2{\cdot}6H_2O$, 0·01; KIO_3, 0·03;$AlCl_3$ 6H_2O, 0·25; Na_2SeO_3, 0·04.

Fish and feeding trial

The feeding trial was conducted at Yangtze River Fisheries Research Institute (Wuhan, China). We obtained grass carp from a commercial farm (Wuhan, China). Prior to the feeding trial, we maintained the fish in an indoor recirculation aquarium system for 2 weeks and fed them the control diet. The recirculation aquarium system was equipped with heating and cooling refrigeration and temperature control switches, which enabled the water temperature keep at the set value. After fasting for 24 h, we anaesthetised the fish with 80 mg MS-222/l water to minimise suffering. A total of 120 healthy fish of similar size (initial body weight: 88·47 ± 1·44) were selected, weighed and randomly distributed into 12 tanks (400 l water volume). Each diet was randomly assigned to triplicate tanks. Fish were hand-fed with the corresponding diet at 2 % to 3 % of their average body weight until apparent satiation[23]. The fish were fed 3 times daily at 08:30, 12:30 and 16:30. The trial was carried out with a natural photoperiod in recirculation aquarium system and lasted 8 weeks. The filtered freshwater was used as cultured water. Approximately 30 % of the wastewater was drained and replenished in each tank every 2 d. During the feeding period, we

Table 2. The amino acids compositions of diets with creatine supplement

	Dietary creatine levels (g/kg diet)			
Amino acid	1·84 (T1)	5·91 (T2)	8·48 (T3)	15·44 (T4)
Arg	16·18	15·96	15·97	16·07
His	7·89	8·04	8·39	8·15
Ile	10·68	10·72	10·84	10·83
Leu	18·81	19·29	20·02	19·74
Lys	21·99	22·10	22·65	22·31
Met	7·29	7·27	7·47	7·40
Phe	20·06	20·17	20·13	20·04
Thr	10·98	11·17	11·05	11·05
Val	6·41	6·35	6·56	6·47
Ala	11·57	11·64	11·47	11·75
Asp	24·15	24·45	24·50	24·24
Cys	8·77	8·71	8·40	8·59
Gly	12·61	12·46	12·80	12·70
Glu	52·34	52·15	51·09	51·79
Pro	18·61	18·38	18·21	18·49
Ser	13·42	13·50	13·93	13·85
Tyr	21·65	21·38	21·66	21·61
ΣEAA	120·29	121·06	123·08	122·06
ΣAA	283·41	283·73	285·14	285·07

ΣEAA, total essential amino acids; ΣAA, total amino acids.

monitored the water quality daily at 8:00. Dissolved oxygen was >5·0 mg/l, water temperature was maintained at 27·0 ± 2·0°C, pH was 7·5 ± 0·1, NH_4^+-N did not exceed 0·2 mg/l and NO_2–N was <0·05 mg/l.

Sampling procedure

At the end of feeding trial, we counted the number of fish per tank and weighed them after a 24-h fasting period for the calculation of weight gain rate, specific growth rate, feeding rate and feed conversion ratio. After being anaesthetised with 200 mg MS-222/l water, the body weight and length of three fish per tank were measured, and blood samples were collected from vertebra vein with a 2-ml injector and stored at 4°C for 4 h. We obtained sera following centrifugation (3200 g/min, 10 min) and stored the serum at –80°C for free amino acid and blood chemistry analysis. We dissected the fish and weighed the viscera and liver to calculate condition factor, viscerosomatic index and hepatosomatic index. We cut the dorsal muscle was into a rectangle (1·0 cm × 1·0 cm × 0·5 cm) for texture analysis and collagen content determination. Additionally, we placed cuboidal muscles (0·2 cm × 0·2 cm × 0·2 cm) in fixative fluid containing 2·5 % glutaraldehyde and stored them at 4°C for transmission electron microscopy analysis. Other cuboidal muscle samples (0·5 cm × 0·5 cm × 0·5 cm) were dipped in PBS containing 4 % paraformaldehyde for 24 h and stored in 70 % alcohol for paraffin section analysis. The muscle samples for histology and transmission electron microscopy were taken from the same region from different fish across treatment.

Following euthanasia, we disinfected another three fish per tank using 75 % alcohol. We transferred muscle (0·2 g) from each fish into a 2-ml microcentrifuge tube, which was immediately frozen in liquid nitrogen and stored at –80°C for mRNA expression and Western blotting. Additionally, muscle (0·1 g) and liver (0·1 g) samples per fish were used for the measurement of creatine, glycocyamine and creatinine contents. We stored the remaining muscle samples at –40°C for amino acid profile and proximate composition analysis. Finally, three fish per tank were stored at –20°C for whole-body composition analysis.

Serum chemistry

The serum triacylglycerol and glucose levels were measured using commercial kits based on the GK-GPO-POD method and hexokinase method, respectively. The serum total protein was estimated using commercial kits based on the bicinchoninic acid assay. The serum enzymatic activities of alanine transaminase and aspartate aminotransferase were estimated using commercial kits based on the LDH-UV method and MDH-UV method, respectively. All the above parameters were quantified in an automatic biochemistry analyser (CHEMIX-800, Sysmex Corporation). All kits were purchased from Sysmex Corporation.

Creatine and metabolite analysis

The levels of creatine and its metabolites in diets, muscle and liver were analysed as previously reported[13]. Briefly, the samples were homogenised and centrifuged (0·1 g), the supernatants were passed through a 0·45-μm filtration membrane and analysed using ultrahigh-performance liquid chromatography coupled with a triple quadrupole-mass spectrometry (UPLC-QqQ-MS) in multiple reaction monitoring mode with an Acquity UPLC system (Waters Corp.). To quantify and identify the peaks, the standards of creatine, creatinine and glycocyamine were added to the samples. We normalised and converted the peak areas into a two-dimensional data matrix using Excel 2010 software (Microsoft).

Proximate composition and collagen determination in muscle

The contents of crude protein, crude lipid and ash, respectively, in the diets, muscle and whole body were detected using Micro-Kjeldahl, Soxhlet and combustion methods[24]. For the determination of moisture, we placed the samples in a vacuum freeze dryer (Christ Beta 2–4 LD plus LT, Marin Christ Corporation) for 48 h. The crude protein content was determined using the Kjeldahl method ($n \times 6{\cdot}25$). The crude lipid content was measured using the extraction method of ether-methanol. The ash content was determined by incinerating samples at 550°C for 24 h in a muffle furnace (PCD-E3000 Serials, Peaks, Japan). To estimate collagen content, we measured hydroxyproline (Hyp) using UV–vis spectrophotometry[25]. Briefly, muscle samples (1·0 g) were homogenised in cold water (9 ml, 4°C) for 1 min and mixed with ice cold sodium hydroxide (0·2 M, 10 ml) at 4°C on a wheel roller for 4 h. Following centrifugation at 10 000 ***g*** for 30 min at 4°C, we collected and stored the supernatant (1 ml) at 4°C for Hyp content determination.

Texture analysis

The texture profile analysis was performed within 2 h of sample collection as previously reported[26]. Texture indexes including hardness, springiness, gumminess, chewiness, cohesiveness and resilience were measured using a Perten Ruihua instrument (model TVT-300XP) equipped with a cylindrical stainless-steel

probe of 50 mm diameter and analysed using a texture analyser program (version 3.42, Perten Instruments Inc.).

Microscopy evaluation

The muscle samples were dehydrated in a series of xylene and alcohol solutions, embedded in paraffin, sliced and stained with haematoxylin-eosin to evaluate the morphology. We captured images (15 images/sample) using a microscope (Olympus BX53).

The transmission electron microscopy analysis of muscle samples was conducted in the Electron Microscope Center of Renmin Hospital of Wuhan University (Wuhan, China). Briefly, the muscle samples were washed 3 times with phosphate buffer (0·1 mmol/l, pH = 7·4) for 15 min each time and fixed with 1 % OsO_4 in phosphate buffer (0·1 mmol/l, pH = 7·4) for 2 h at room temperature. Following the removal of OsO_4, the samples were rinsed 3 times (15 min each) in phosphate buffer (0·1 mmol/l, pH = 7·4). Subsequently, the samples were dehydrated in a series of alcohol solutions, embedded in epoxy resin for 6 h at 37°C and moved the embedding models with resin and samples into 65°C oven to polymerise for more than 48 h. And then the resin blocks were taken out from the embedding models for standby application at room temperature. The resin blocks were cut to 70 nm thin on the Leica UC7 ultra-microtome. Finally, the ultramicrocuts were stained with 2 % uranium acetate saturated alcohol solution avoid light for 8 min. The images were took using Hitachi HT7800 TEM.

The myofibre characteristics including myofibre diameter (a minimum of fifty myofibres width per image), density (myofibre number per unit area), length of sarcomere and I-band and A-band (a minimum of twenty length/image) were analysed using the Image J Launcher software. Myofibre diameter was calculated as follow: one myofibre area was measured and then the mean diameter was calculated according to πR^2 formula (at least tested fifty myofibres per image). Then the myofibre fibres were divided into three classes according to the calculated diameter (d, μm). Classes I, II and III were categorised according to $d \leq 60$, $60 < d \leq 100$ and $d > 100$, respectively. Class I muscle fibres were categorised as hyperplasia fibres. Class III fibres were categorised as hypertrophic fibres. Myofibre density was a rate of the total number of myofibres in the unit area. The length of the sarcomere is obtained from the shortest distance between two adjacent Z-disk. I-band length was calculated by two deepest points that were perpendicular to the same Z-disk (at least tested 20 length/image). A-band length was analysed by the distance between two boundaries that belong to the same A band in the longitudinal direction (at least tested twenty length per image).

Analysis of composition and content of amino acids

For combined amino acid analysis, we transferred the freeze-dried diets (0·2 g) and muscle (0·1 g) to sealed tubes. The samples were hydrolysed with 6 M hydrochloric acid (15 ml) at 110°C for 24 h. After filtering the hydrolysed solutions with medium speed filter paper, their volumes were adjusted to 100 ml with distilled water. Using a vacuum dryer, the filtrates (1·5 ml) were dried twice in vacuum dryer for 24 h to avoid hydrochloric acid corroding separation column. For free amino acids analysis, the serum (400 μl) and fresh muscle (1·0 g) were mixed with 3 ml of 10 % sulfosalicylic acid. Following incubation at room temperature, the samples were centrifuged at 18 000 g/min for 15 min at 4°C. After pretreatment, approximately 1 ml of samples was passed through a 0·22-μm Millipore membrane and analysed using an amino acid analyser (HITICHI L-8900).

Real-time qPCR

Using TRIzol reagent (Life Technologies), we extracted total RNA from muscle tissue and reversed-transcribed 4 μg RNA to cDNA using PrimeScript® RT reagent kit (Takara). The quantitative real-time PCR (qRT-PCR) was performed using a quantitative thermal cycler (Light 217 Cycler 480II, Roche) with SYBR® Premix Ex Taq™ (Takara). Table 3 shows the sequences of target genes and housekeeping genes (*18S*). These primers were successfully used in previous studies in grass carp[21,27]. The volume of qRT-PCR reaction was 20 μl including 10 μl SYBR® Premix Ex Taq, 2 μl cDNA sample, 6 μl nuclease-free water, 0·8 μl forward/reverse primers (10 μM) and 0·4 μl ROX reference dye II. The qRT-PCR conditions were the following, 95°C for 5 min, followed by forty cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s, and one step at 72°C for 5 min. Each sample was measured three times. We calculated mRNA expression levels using the $2^{-\Delta\Delta CT}$ method.

Western blotting

Western blotting was performed as previously reported[28,29]. The antibodies used in this study were successfully used in previous studies in grass carp[28,29]; the antibodies against these antigenic regions are conserved in fish. We obtained antibodies against phospho-TOR (Ser2448, P-TOR; Cat. no. 2971), TOR (Cat. no. 2972), phospho-ribosomal protein S6 kinase (Thr 389; P-S6K1; Cat. no. 9205), S6K1 (Cat. no. 9206), phospho-4E-binding protein 1 (Thr37/46; P- 4E-BP1; Cat. no. 9459), 4E-BP1 (Cat. no. 9452) and *β*-tubulin (Cat. no. 2146) from Cell Signaling Technology Inc. Wuhan ABclonal Biotechnology Co. Ltd supplied the antibodies against IGF-I (insulin-like growth factor I; Cat. no. A11985), MyoD1 (Cat. no. A16218) and PGC1α (Cat. no. A11971). We used Image J Launcher software to quantify band intensity.

Statistical analysis

The data were expressed as mean ± SD. The homogeneity of variances and normality of the data were evaluated prior to statistical analysis. Using SPSS 20.0 statistical software (SPSS Inc.), we performed one-way ANOVA and Tukey's multiple comparison tests. $P < 0{\cdot}05$ represented statistical significance.

Results

Growth performance

After an 8-week feeding trial, the survival rate of grass carp was 100 % in all treatments (Table 4). Feed conversion ratio, feeding rate, condition factor, hepatosomatic index and viscerosomatic index were not different among the four treatments ($P > 0{\cdot}05$).

Table 3. Nucleotide sequences of primers and cycling conditions used for PCR amplification

Gene	Accession no.		Primer sequence (5′ to 3′)	Amplication size	Ta	Amplification efficiency
Reference gene						
18S	EU047719·1	Forward	GGAATGAGCGTATCCTAAACCC	137	59	0·95
		Reverse	CTCCCGAGATCCAACTACAAGC			
Fast fibre gene						
fMyHCs	EU367966·1	Forward	CCCAGAGTCAAGGTCGGAAAT	178	60	0·97
		Reverse	CCAGCACGCCAATGTAGAAA			
fMyHCc	AB167710·1	Forward	TCCAGGGTGCGTGAGTTAGA	218	60	0·98
		Reverse	GCCTGCTCCTCAGATTCTTCA			
Slow fibre gene						
fMyHCs$_{30}$	AB104626·1	Forward	ATGAAGGGAGGCAAGAAGCA	195	60	1·02
		Reverse	GTCCACCAGATCCTGAAGACG			
fMyHCs$_{10}$	AB104625·1	Forward	CGCATCAAAGAACTCACCTACCAG	124	58	0·97
		Reverse	CTTCAGCCTCTTCAGCAACTCTC			
Myogenic regulatory factors						
MyoG	JQ793897	Forward	AGAGGAGGTTGAAGAAAGTC	159	56	0·98
		Reverse	GTTCCTGCTGGTTGAGAGA			
MyoD	GU218462·1	Forward	CCCTTGCTTCAACACCAACG	229	60	1·03
		Reverse	TCTCCTCTCCCTCATGGTGG			

18s, ribosomal protein 18; *fMyHC*$_S$, s-myosin heavy chain; *fMyHC*$_c$, c-myosin heavy chain; *fMyHCs*$_{30}$, S30-myosin heavy chain; *fMyHCs*$_{10}$, S10-myosin heavy chain; *MyoG*, myogenic determination gene; *MyoD*, myogenic differentiation antigen.

Table 4. Effects of creatine-supplemented diets on growth performance of grass carp (Mean values and standard deviations)

	Dietary creatine levels (g/kg diet)							
	1·84 (T1)		5·91 (T2)		8·48 (T3)		15·44 (T4)	
Index	Mean	SD	Mean	SD	Mean	SD	Mean	SD
IBW (g)	88·47	1·44	88·67	1·95	89·23	1·70	89·53	1·86
FBW (g)	316·76	12·79	324·28	19·80	348·16	20·43	329·96	23·36
WGR (%)*	258·18	10·24	266·03	18·43	290·54	22·25	267·93	21·10
SGR(%/d)†	2·28	0·09	2·31	0·13	2·43	0·14	2·32	0·22
FCR‡	1·27	0·06	1·18	0·08	1·12	0·11	1·20	0·05
Feeding rate (%/d)§	2·79	0·23	2·88	0·19	2·71	0·13	2·60	0·16
CF‖	1·90	0·04	1·92	0·03	1·92	0·09	1·95	0·05
HSI (%)¶	1·76	0·05	1·74	0·11	1·78	0·02	1·83	0·09
VSI (%)**	8·74	0·32	8·79	0·32	8·67	0·30	8·96	0·30

* Weight gain rate (WGR, %) = 100 × (final body weight – initial body weight)/initial body weight.
† Specific growth rate (SGR, %/d) = 100 × ln (final weight/initial weight)/d.
‡ Feed conversion ratio (FCR) = dry feed consumed/(final biomass – initial biomass + dead fish).
§ Feeding rate (FR) = 100 × dry feed consumed × 2/(final body weight + initial body weight)/d.
‖ Condition factor (CF) =100 × (body weight/body length^3).
¶ Hepatosomatic index (HSI, %) =100 × (hepatosomatic weight/whole-body weight).
** Viscerosomatic index (VSI, %) = 100 × (viscerosomatic weight/whole-body weight).Data are presented as means ± SD (*n* 3 tanks) and were analysed by one-way ANOVA followed by Tukey's multiple comparison test.
Different letters indicate the effect was significantly different between treatments ($P < 0·05$).

Fish fed 8·48 g/kg creatine (T3) had the highest weight gain rate and specific growth rate; no significant differences were obtained among the other diets ($P > 0·05$).

Haematological data

Table 5 shows that the contents of glucose and total protein in serum were not different among the four treatments ($P > 0·05$). The activities of alanine transaminase and aspartate aminotransferase in serum decreased with increasing dietary creatine supplemented levels, mainly in T3 and T4 ($P < 0·05$). The triacylglycerol concentrations in serum significantly decreased in T3 than in the control ($P < 0·05$).

Creatine metabolism analysis

Table 6 shows that muscle creatine contents increased with dietary creatine, mainly in T3 and T4 ($P < 0·05$). The concentrations of creatine and creatinine in liver increased with >5·91 g/kg creatine ($P < 0·05$). There were no differences in glycocyamine contents in muscle and liver among the four treatments ($P > 0·05$).

Muscle textural properties

Table 7 shows that creatine supplementation increased muscle hardness, chewiness and gumminess. These indicators were significantly higher in T3 than in the control ($P < 0·05$). Muscle

Table 5. Effects of creatine-supplemented diets on haematological data of grass carp (Mean values and standard deviations)

	Dietary creatine levels (g/kg diet)							
	1·84 (T1)		5·91 (T2)		8·48 (T3)		15·44 (T4)	
Index	Mean	SD	Mean	SD	Mean	SD	Mean	SD
AST (U/l)	50·67	2·52^{b}	47·33	3·06^{b}	39·67	1·15^{a}	37·67	2·52^{a}
ALT (U/l)	119·33	7·02^{b}	114·00	2·00^{b}	107·33	5·13^{a}	98·00	7·00^{a}
TG (mmol/l)	3·89	0·14^{b}	3·66	0·11^{ab}	3·18	0·42^{a}	3·49	0·18^{ab}
GLU (mmol/l)	8·82	0·38^{a}	9·25	0·10^{a}	9·01	0·60^{a}	9·17	0·85^{a}
TP (g/l)	33·67	1·77^{a}	32·00	2·00^{a}	35·67	2·31^{a}	37·33	2·08^{a}

TG, triacylglycerol; GLU, glucose; TP, total protein; ALT, alanine transaminase; AST, aspartate aminotransferase.
Data are presented as means ± SD (n 3 tanks) and were analysed by one-way ANOVA followed by Turkey's multiple comparison test.
Different letters indicate the effect was significantly different between treatments ($P < 0·05$).

Table 6. Effects of creatine-supplemented diets on the contents of creatine and metabolites in the muscle and liver of grass carp (Mean values and standard deviations)

		Dietary creatine levels (g/kg diet)							
		1·84 (T1)		5·91 (T2)		8·48 (T3)		15·44 (T4)	
Tissue	Metabolites	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Muscle	Creatine (g/kg)	5·75	0·18^{a}	6·08	0·18^{ab}	6·27	0·17^{b}	6·76	0·25^{b}
	Creatinine (mg/kg)	0·70	0·03	0·75	0·04	0·74	0·02	0·76	0·03
	Glycocyamine (mg/kg)	0·26	0·02	0·27	0·02	0·24	0·02	0·25	0·03
Liver	Creatine (g/kg)	0·23	0·02^{a}	0·25	0·01^{a}	0·30	0·02^{b}	0·36	0·02^{c}
	Creatinine (mg/kg)	0·06	0·02^{a}	0·09	0·02^{ab}	0·11	0·02^{b}	0·12	0·01^{b}
	Glycocyamine (mg/kg)	0·70	0·10	0·66	0·09	0·67	0·10	0·68	0·05

Data are presented as means ± SD (n 3 tanks) and were analysed by one-way ANOVA followed by Tukey's multiple comparison test.
Different letters indicate the effect was significantly different between treatments ($P < 0·05$).

Table 7. The muscle textural properties of grass carp fed diets with creatine supplement (Mean values and standard deviations)

	Dietary creatine levels (g/kg diet)							
	1·84 (T1)		5·91 (T2)		8·48 (T3)		15·44 (T4)	
Index	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Hardness (g)	1323·25	38·44^{a}	1410·10	60·34^{ab}	1539·21	49·73^{b}	1385·36	43·43^{ab}
Chewiness (g)	145·90	6·86^{a}	157·85	6·96^{a}	203·94	6·32^{b}	167·24	10·53^{a}
Gumminess (g)	410·41	8·12^{a}	477·63	14·85^{b}	471·35	12·15^{b}	376·07	12·28^{a}
Springiness	0·36	0·01	0·36	0·01	0·40	0·01	0·38	0·01
Resilience	0·15	0·01	0·14	0·01	0·15	0·01	0·12	0·01
Cohesiveness	0·33	0·01^{c}	0·32	0·01^{bc}	0·29	0·01^{ab}	0·25	0·01^{a}

Data are presented as means ± SD (n 3 tanks) and were analysed by one-way ANOVA followed by Turkey's multiple comparison test.
Different letters indicate the effect was significantly different between treatments ($P < 0·05$).

cohesiveness showed a decreasing tendency with increasing dietary creatine levels; T4 (15·44 g/kg) had the lowest cohesiveness value ($P < 0·05$). Springiness and resilience of muscle were unaffected by creatine levels ($P > 0·05$). Second-order polynomial regression analyses based on flesh hardness revealed that the optimum creatine requirement supplementation for grass carp was 9·56 g/kg (Fig. 1).

Morphology of myofibre

Fish in T3 and T4 exhibited tighter myofibre arrangement (Fig. 2(a)) and smaller myofibre mean diameter (Fig. 2(b)) than those in the control group. Statistical analysis (Fig. 2(c)) revealed that fish fed 8·48 g/kg creatine had the smallest myofibre mean diameter and the highest myofibre density, which were different to those in control fish ($P < 0·05$). The frequency distribution of the myofibres diameters is presented in Fig. 2(c) in regard to the muscle fibre frequency distribution, class II was higher than class I and class III in all treatment. Diameter classes I (hyperplasia fibres) was significantly higher in T3 and T4 than that in the control ($P < 0·05$). Frequencies of class III (hyperplastic fibres) were significantly higher in T2 and T3 than that in the control ($P < 0·05$).

The transmission electron microscopy images of fish myofibril in T1 and T3 are shown in Fig. 3. Complete mitochondrion and sarcoplasmic reticulum were evident in T1 and T3, and

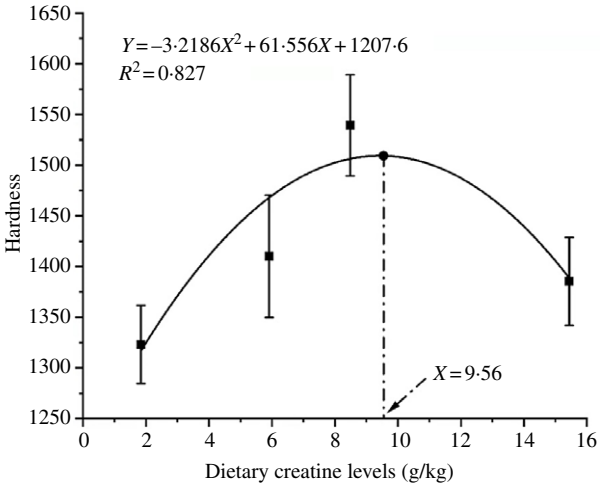

Fig. 1. Second-order polynomial regression analyses between flesh hardness and dietary creatine levels for grass carp.

myofibrils were made of regular sarcomeres. The Z line, M line, I-band and A-band appeared (Fig. 3(a)). Compared with the control fish, fish fed 8·48 g/kg creatine had longer sarcomere and I-band length ($P < 0·05$; Fig. 3(b)).

Proximate composition and collagen content

Table 8 shows that dietary creatine levels had no significant impact on whole-body composition ($P > 0·05$). The contents of moisture, ash and crude fat in muscle were not affected by dietary creatine levels ($P > 0·05$). Muscle crude protein content significantly increased with 8·48 g/kg creatine ($P < 0·05$).

Fish fed creatine had higher contents of total Hyp and alkaline-insoluble Hyp than the control fish ($P < 0·05$). The alkaline-soluble Hyp content was unaffected by dietary creatine levels ($P > 0·05$).

We used second-order polynomial regression analyses to assess the optimum creatine requirements for maximum Hyp levels. The results revealed that the optimum creatine supplementation was 12·04 g/kg (Fig. 4).

Amino acid composition in muscle

Table 9 shows the amino acid composition and contents in muscle. The contents of total amino acids and EAA increased with dietary creatine levels and were significantly higher in T3 than in the control ($P < 0·05$). The contents of aspartic acid, glutamic acid, leucine and lysine in muscle shared a similar increasing trend and were significantly higher in T3 ($P < 0·05$) than in the control. The contents of methionine and phenylalanine gradually increased in response to dietary creatine levels and achieved the maximum levels in T4 ($P < 0·05$). Glycine content was higher in creatine-supplemented diets than in the control ($P < 0·05$). Fish fed 8·48 g/kg creatine had lower proline content compared with fish fed 15·44 g/kg creatine ($P < 0·05$). There were no significant effects on the contents of other amino acids ($P > 0·05$).

Free amino acid composition in muscle

The results of free amino acids in muscle are shown in Table 10. Free flavour free amino acids including aspartic acid, glycine, glutamic acid and alanine increased with ≥ 8·48 g/kg creatine ($P < 0·05$). The concentrations of free total amino acids and free EAA gradually increased in response to dietary creatine supplementation and were significantly higher in T4 than in other treatments ($P < 0·05$). Furthermore, the contents of free serine, threonine, valine, isoleucine, leucine and phenylalanine increased, reaching maximum values in T4 ($P < 0·05$). Free arginine content was higher in T3 than in other treatments ($P < 0·05$). Compared with the control, free hydroxylysine content was lower in T4 ($P < 0·05$). No significant differences were obtained with other free amino acids ($P > 0·05$).

Free amino acid composition in serum

Free TAA content in serum was higher in T4 than in other treatments (Table 11; $P < 0·05$). The free EAA contents were higher in creatine-supplemented diets than in the control ($P < 0·05$). The contents of free threonine, valine and phenylalanine in serum significantly increased in T4 ($P < 0·05$). T2 had the highest values of free isoleucine, leucine and lysine ($P < 0·05$), and T4 had the highest values of free arginine and glutamic acid in serum ($P < 0·05$).

mRNA expression levels

Fig. 5 shows that supplemental creatine significantly downregulated the mRNA levels of fast fibre genes including *fMyHCs* and *fMyHCc*, which reached the lowest values in T3 ($P < 0·05$). Conversely, supplemental creatine increased the mRNA levels of slow fibre genes including $fMyHC_{S10}$ and $fMyHC_{S30}$, mainly in T3 ($P < 0·05$). Creatine supplementation enhanced the expression levels of myogenic regulator factors (MRF) including *MyoG* and *MyoD* in muscle, which reached their highest values in T4 ($P < 0·05$).

Effects of dietary creatine on nutrient sensing networks

Diets supplemented with 5·91 and 8·48 g/kg creatine significantly increased the protein expressions of IGF-I, MyoD1 and PGC-1α in muscle (Fig. 6; $P < 0·05$). Additionally, 8·48 g/kg creatine increased the phosphorylation of TOR, S6K1 and 4E-BP1 in muscle ($P < 0·05$).

Discussion

In this study, grass carp fed 8·48 g/kg creatine had the highest weight gain rate; however, it was not significantly to that of control fish. Similar results have been reported in juvenile rainbow trout[12], *L. vannamei*[13] and gilthead seabream (*Sparus aurata*)[18,19]. In red drum, 19·8 g/kg creatine improved weight gain and feed efficiency[20]. The possible explanation for this inconsistency may be attributed to differences in fish species and feeding environments such as surrounding temperature[30]. Further research should be conducted to draw more definitive conclusions.

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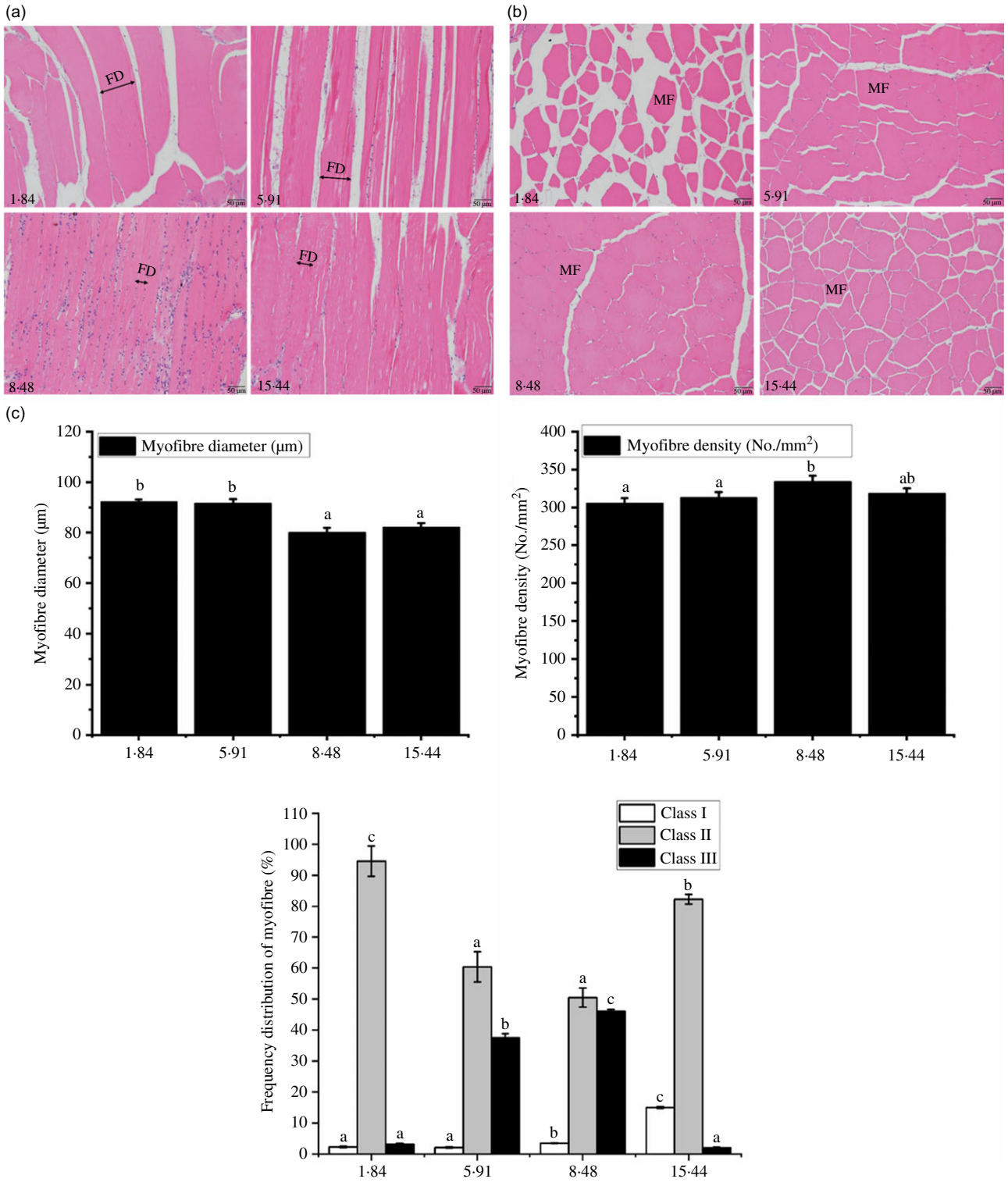

Fig. 2. Effects of creatine-supplemented diets on microstructure of muscle of grass carp. (a) Longitudinal sections of muscle. FD: myofibre diameter. (b) Cross-sections of muscle. MF: muscle fibre. Magnification: 200×. (c) The myofibre diameter and density of muscle in grass carp (n 3). Classes I, II and III were categorised according to diameter = d ≤ 60, 60 < d ≤ 100 and d > 100, respectively. Class I myofibres were categorised as hyperplastic/hyperplasia fibres. Class III myofibres were categorised as hypertrophic fibres.

Blood biochemical parameters are usually used to determine the general health status of fish[31]. Aspartate aminotransferase and alanine transaminase are amino transferases related to amino acid metabolism. When organs such as liver are damaged, their activities in serum will be increased[32]. We found for the first time in fish that 8·48–15·44 g/kg creatine decreased the activities of

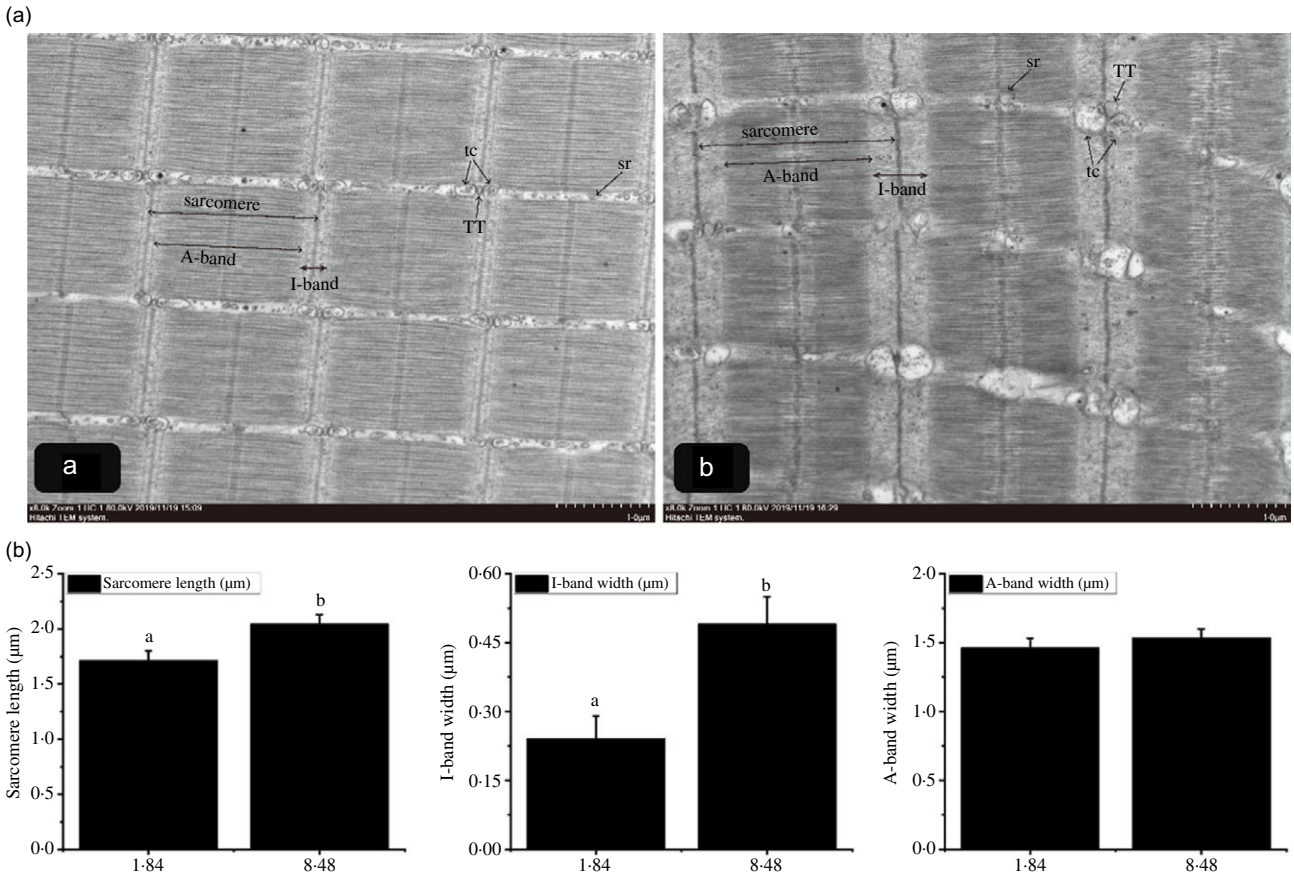

Fig. 3. Effects of creatine-supplemented diets on morphology of myocytes of grass carp. (a) Transmission electron microscope of fish sarcomere; a: Myofibrillar structure in control group, 8000×magnification; b: Myofibrillar structure in 8·48 g/kg group, 8000×agnification. sr, sarcoplasmic reticulum; tc, terminal cisternae; TT, transverse tubules. (b) Myofibrillar sarcomere length, I-band and A-band width in control group and 8·48 g/kg group (*n* 3).

Table 8. Effects of creatine-supplemented diets on proximate composition and collagen contents in the muscle of grass carp (Mean values and standard deviations)

	Dietary creatine levels (g/kg diet)							
	1·84 (T1)		5·91 (T2)		8·48 (T3)		15·44 (T4)	
Index (g/kg wet weight)	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Muscle								
Moisture	791·11	2·93	787·52	2·25	786·37	1·49	785·97	2·33
Crude protein	175·72	1·74^{a}	177·95	1·72^{ab}	182·13	1·90^{b}	175·72	1·43^{a}
Crude lipid	13·89	0·79	14·11	0·87	13·98	0·66	13·64	0·88
Ash	11·86	0·19	12·09	0·26	12·08	0·09	12·02	0·15
Alkaline-soluble Hyp	0·10	0·01	0·10	0·00	0·10	0·01	0·10	0·01
Alkaline-insoluble Hyp	0·85	0·03^{a}	1·08	0·07^{b}	1·18	0·02^{b}	1·18	0·04^{b}
Total Hyp	0·95	0·03^{a}	1·18	0·07^{b}	1·27	0·02^{b}	1·27	0·04^{b}
Whole body								
Moisture	724·17	3·69	726·10	3·27	720·30	2·59	721·78	2·52
Crude protein	147·63	1·66	144·75	1·00	146·96	1·57	145·57	3·06
Crude lipid	66·28	1·54	64·42	1·46	70·02	3·46	67·15	2·04
Ash	37·92	3·31	40·87	3·57	37·27	2·52	46·17	4·04

Hyp means hydroxyproline.
Data are presented as means ± SD (*n* 3 tanks) and were analysed by one-way ANOVA followed by Turkey's multiple comparison test.
Different letters indicate the effect was significantly different between treatments ($P < 0·05$).

aspartate aminotransferase and ALT in serum, and 8·48 creatine decreased serum triacylglycerol content, which were consistent with the results reported in rats[33]. These results suggested creatine supplementation had a positive effect on fish health.

It is important to understand the metabolic process of feed additives in fish. In general, creatine synthesis is initiated in the kidneys with the formation of guanidinoacetate from arginine and glycine, a reaction catalysed by glycine

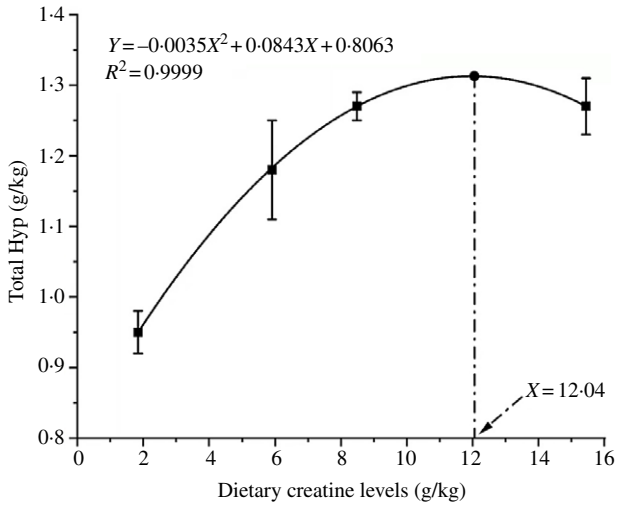

Fig. 4. Second-order polynomial regression analyses between flesh Hyp contents and dietary creatine levels for grass carp.

Table 9. Effects of creatine-supplemented diets on muscle amino acid composition of grass carp (Mean values and standard deviations)

	Dietary creatine levels (g/kg diet)							
	1·84 (T1)		5·91 (T2)		8·48 (T3)		15·44 (T4)	
Amino acid (g/kg wet weight)	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Arg	7·90	0·30^{a}	7·87	0·50^{a}	8·49	0·29^{a}	8·04	0·49^{a}
His	4·85	0·40^{a}	4·67	0·41^{a}	5·23	0·21^{a}	4·83	0·35^{a}
Ile	6·59	1·03^{a}	6·62	0·54^{a}	7·06	0·69^{a}	6·56	0·76^{a}
Leu	11·73	0·13^{a}	11·39	0·22^{a}	13·05	0·14^{b}	11·28	0·31^{a}
Lys	12·18	0·46^{a}	12·86	0·56^{a}	15·20	0·60^{b}	13·07	0·29^{a}
Met	4·67	0·25^{a}	4·86	0·44^{ab}	5·34	0·36^{ab}	5·57	0·20^{b}
Phe	7·74	0·52^{a}	8·19	0·37^{ab}	8·84	0·19^{b}	9·14	0·30^{b}
Thr	5·92	0·26^{a}	5·34	0·32^{a}	6·05	0·41^{a}	5·81	0·77^{a}
Val	0·80	0·06^{a}	0·88	0·08^{a}	0·89	0·05^{a}	0·85	0·08^{a}
Ala	7·32	0·31^{a}	7·65	0·43^{a}	8·11	0·34^{a}	7·44	0·19^{a}
Asp	12·59	0·33^{a}	12·83	0·36^{a}	14·48	0·49^{b}	12·56	0·46^{a}
Cys	2·52	0·12^{a}	2·59	0·22^{a}	2·54	0·18^{a}	2·50	0·23^{a}
Gly	4·82	0·38^{a}	5·63	0·19^{b}	6·44	0·23^{c}	6·13	0·12^{bc}
Glu	18·18	0·46^{a}	18·96	0·78^{a}	21·38	0·41^{b}	19·45	0·64^{a}
Pro	5·59	0·41^{ab}	5·52	0·41^{ab}	5·37	0·18^{a}	6·32	0·31^{b}
Ser	5·58	0·37^{a}	5·23	0·30^{a}	5·96	0·19^{a}	5·94	0·49^{a}
Tyr	6·93	0·30^{a}	7·15	0·81^{a}	7·68	0·34^{a}	7·03	0·56^{a}
ΣEAA	49·63	1·61^{a}	50·14	1·34^{a}	56·45	1·98^{b}	52·28	1·65^{ab}
ΣAA	125·91	2·37^{a}	128·24	1·77^{a}	142·12	3·73^{b}	132·53	2·58^{a}

ΣEAA, total essential amino acids; ΣAA, total amino acids.
Data are presented as means ± SD (n 3 tanks) and were analysed by one-way ANOVA followed by Turkey's multiple comparison test.
Different letters indicate the effect was significantly different between treatments ($P<0{\cdot}05$).

amidinotransferase. Guanidinoacetate is released by the kidneys and methylated in the liver to produce creatine, which mainly accumulates in the muscle (95 %) and to a lesser extent in the liver, brain, kidneys and testes. Finally, creatine triggers a series of reactions to improve muscle performance and is primarily broken down into creatinine[34]. The major function of exogenous creatine is to maximise the intracellular pool of total creatine[20]; however, the effect of creatine supplementation on the content of glycocyamine and creatinine in fish is unknown. In this study, we measured the contents of creatine, creatinine and glycocyamine in muscle and liver of fish. Creatine supplementation increased creatine levels but did not affect the contents of creatinine and glycocyamine in muscle. Furthermore, creatine supplementation increased the contents of creatine and creatinine in the liver. Extensive retention of creatinine in liver may cause hepatic traumatic necrosis and high-grade nephrosis[35], suggesting that dietary creatine should be appropriate in grass carp. The content of glycocyamine in muscle and liver was unaffected by dietary creatine levels. Therefore, exogenous creatine levels between 1·84 and 15·44 g/kg had no impact on endogenous creatine synthesis of grass carp in this study.

Creatine supplementation induced several biochemical reactions that affected meat quality[34,36]. Textural characteristics including chewiness, hardness (firmness), cohesiveness,

Table 10. Effects of creatine-supplemented diets on free amino acid composition in the muscle of grass carp (Mean values and standard deviations)

	Dietary creatine levels (g/kg diet)							
	1·84 (T1)		5·91 (T2)		8·48 (T3)		15·44 (T4)	
Amino acid (mg/kg wet weight)	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Arg	136·18	11·68^{a}	148·58	11·78^{a}	204·11	20·09^{b}	147·01	19·06^{a}
His	1452·64	123·83^{a}	1435·01	134·71^{a}	1424·99	131·67^{a}	1642·56	143·75^{a}
Ile	15·40	1·01^{a}	16·20	0·72^{a}	18·48	1·05^{ab}	20·44	2·09^{b}
Leu	19·09	1·02^{a}	19·39	1·41^{a}	24·16	0·88^{b}	28·38	1·04^{c}
Lys	214·13	26·66^{a}	214·42	21·08^{a}	224·97	16·73^{a}	256·71	22·71^{a}
Met	11·34	1·31^{a}	11·26	0·93^{a}	11·45	1·22^{a}	11·95	1·20^{a}
Phe	8·73	0·65^{a}	9·21	0·70^{a}	13·87	1·08^{b}	20·82	1·53^{c}
Thr	57·55	3·39^{a}	65·02	4·09^{a}	65·39	3·57^{a}	82·69	1·60^{b}
Val	20·88	1·95^{a}	20·28	2·00^{a}	23·89	1·68^{ab}	27·91	2·07^{b}
Ala	149·41	11·08^{a}	147·62	10·47^{a}	163·78	12·22^{ab}	180·81	9·61^{b}
Asp	51·23	2·52^{a}	50·68	1·92^{a}	57·67	3·89^{a}	58·72	4·20^{a}
Gly	167·87	3·87^{a}	164·35	4·87^{a}	173·38	4·08^{ab}	178·29	2·73^{b}
Glu	66·61	3·50^{a}	70·87	3·62^{ab}	82·03	5·24^{b}	94·79	4·66^{c}
Pro	72·87	6·70^{a}	69·49	5·79^{a}	66·23	5·88^{a}	69·29	5·09^{a}
Ser	20·08	1·67^{a}	19·44	2·49^{a}	23·29	1·44^{ab}	26·66	1·95^{b}
Tyr	23·77	1·90^{ab}	22·02	1·31^{a}	23·04	1·51^{ab}	26·76	1·34^{b}
ΣFAA	435·12	13·96^{a}	433·53	14·21^{a}	476·86	12·13^{b}	512·62	17·17^{b}
ΣEAA	347·12	20·46^{a}	355·76	18·28^{a}	382·22	16·18^{a}	448·90	18·31^{b}
ΣAA	3263·89	96·77^{a}	3295·69	89·45^{a}	3437·90	61·17^{a}	3738·93	94·91^{b}

ΣFAA, total flavour amino acids; ΣEAA, total essential amino acids; ΣAA, total amino acids.
Data are presented as means ± SD (*n* 3 tanks) and were analysed by one-way ANOVA followed by Turkey's multiple comparison test.
Different letters indicate the effect was significantly different between treatments ($P < 0·05$).

Table 11. Effects of creatine-supplemented diets on free amino acid composition in the serum of grass carp (Mean values and standard deviations)

	Dietary creatine levels (g/kg diet)							
	1·84 (T1)		5·91 (T2)		8·48 (T3)		15·44 (T4)	
Amino acid (mg/l)	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Arg	35·45	1·28^{ab}	38·06	1·34^{b}	33·71	1·12^{a}	45·03	2·11^{c}
His	7·69	0·32^{a}	7·84	0·24^{a}	7·95	0·15^{a}	7·36	0·21^{a}
Ile	20·11	1·75^{a}	22·37	0·84^{ab}	24·38	1·00^{bc}	26·90	0·65^{c}
Leu	31·10	2·87^{a}	35·11	1·94^{ab}	37·37	1·79^{bc}	42·11	1·45^{c}
Lys	21·76	1·55^{a}	26·79	2·46^{ab}	29·31	2·24^{b}	29·80	2·27^{b}
Met	14·73	0·86^{a}	15·90	1·50^{a}	16·40	0·87^{a}	16·59	0·67^{a}
Phe	32·20	1·08^{a}	36·81	2·73^{a}	36·45	1·35^{a}	46·27	2·64^{b}
Thr	18·75	1·49^{a}	20·65	1·94^{a}	22·51	1·96^{ab}	26·39	2·18^{b}
Val	30·22	2·25^{a}	33·69	1·08^{a}	34·78	1·44^{a}	41·58	2·25^{b}
Ala	43·46	3·11^{a}	40·46	3·71^{a}	39·31	1·73^{a}	56·89	3·47^{b}
Asp	1·68	0·01^{c}	1·46	0·07^{ab}	1·58	0·05^{bc}	1·41	0·07^{a}
Cys	10·54	0·65^{a}	11·13	0·48^{a}	10·75	0·72^{a}	10·02	0·57^{a}
Gly	16·25	1·58^{a}	16·65	1·35^{a}	17·72	1·25^{a}	18·52	1·38^{a}
Glu	12·79	1·00^{a}	15·39	1·02^{b}	16·95	0·86^{b}	19·38	0·37^{c}
Pro	10·68	0·43^{ab}	10·99	0·31^{b}	10·17	0·22^{a}	10·25	0·17^{ab}
Ser	7·73	0·62^{a}	7·29	0·32^{a}	7·25	0·58^{a}	7·94	0·37^{a}
ΣEAA	212·01	9·23^{a}	237·21	9·40^{b}	242·86	6·74^{b}	282·01	6·68^{c}
ΣAA	408·36	16·71^{a}	427·92	14·64^{a}	430·24	8·71^{a}	501·20	11·87^{b}

ΣEAA, total essential amino acids; ΣAA, total amino acids.
Data are presented as means ± SD (*n* 3 tanks) and were analysed by one-way ANOVA followed by Turkey's multiple comparison test.
Different letters indicate the effect was significantly different between treatments ($P < 0·05$).

springiness, gumminess and resilience are important qualities in aquatic products and are key attributes in the mechanical processing of fillets[37]. In gilthead seabream, diets supplemented with 20 g/kg creatine improved textural properties including hardness and chewiness[19]. In this study, 8·48 g/kg creatine improved muscular hardness and chewiness. In raw fish texture, flesh quality varies with collagen amount, myofibre density and myofibre diameter[38,39]. Collagen is positively associated with muscular hardness in grass carp and contributes to tensile strength[40]. Our findings revealed that creatine improved flesh hardness by increasing alkaline-insoluble collagen content. Similar results were reported in *L. vannamei*[13]. Moderate dietary creatine levels promote muscular hardness and collagen deposition, and the optimum creatine supplementation for grass

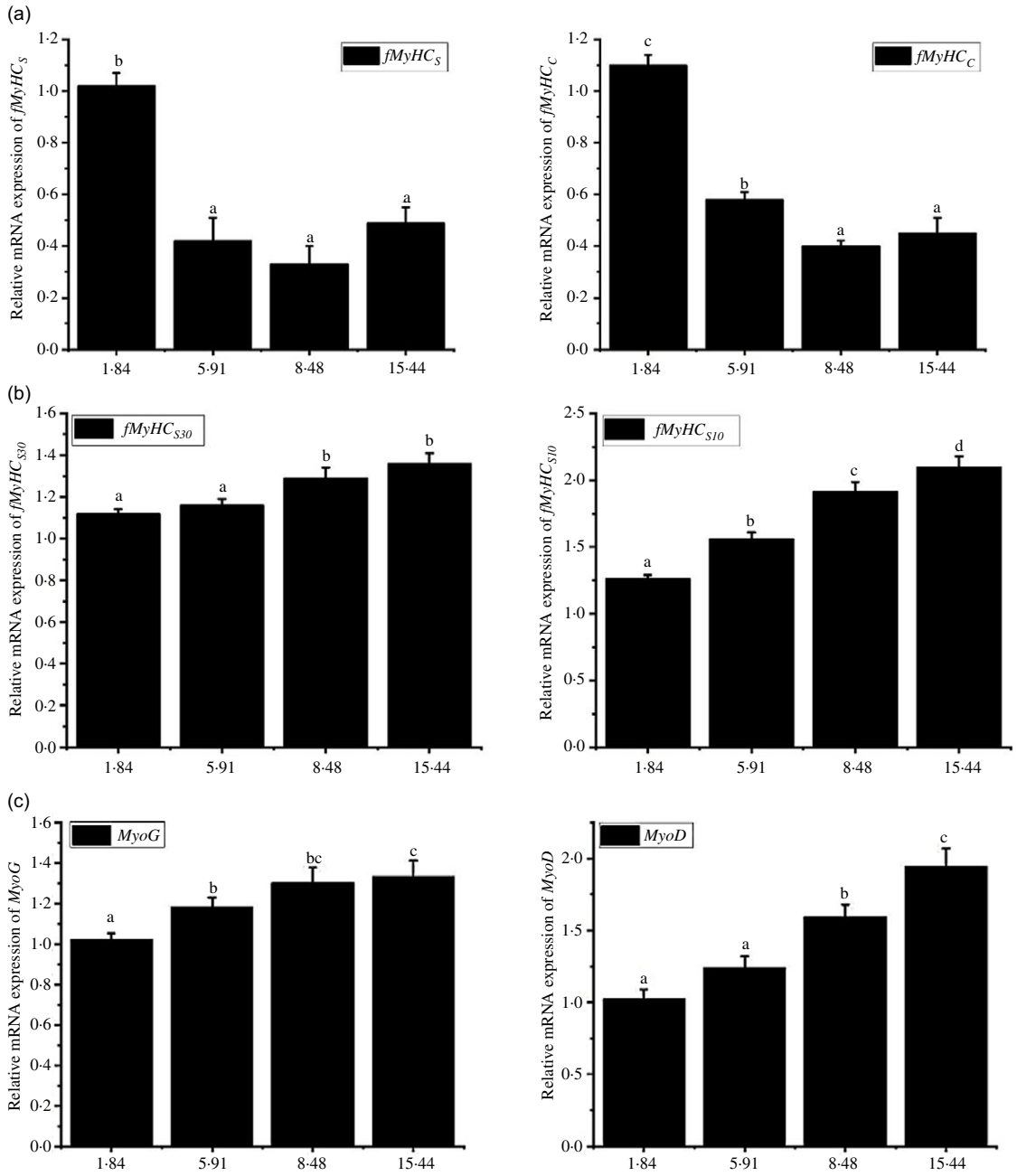

Fig. 5. Effects of creatine-supplemented diets on relative mRNA expression of genes in muscle of grass carp. (a) *fMyHCs* and *fMyHCc* expressed in fast skeletal muscle. (b) $fMyHC_{S10}$ and $fMyHC_{S30}$ expressed in slow skeletal muscle. (c) Myogenic regulator factors (*MyoG* and *MyoD*) expressed in skeletal muscle of grass carp (n 3).

carp ranges between 9·56 and 12·04 g/kg. High flesh quality is characterised by a small myofibre diameter and a high myofibre density, which increase firmness[41]. Until now, little was known about the effects of dietary creatine on fish fibre cellularity. In this study, 8·48 g/kg creatine significantly increased fibre density and the frequencies of the diameters of classes I and III, which was evident by the improvement in myofibre hyperplasia and hypertrophic, indicating creatine supplementation at 8·48 g/kg promotes the growth of skeletal muscle in grass carp. However, 15·44 g/kg creatine decreased muscular cohesiveness and the frequencies of class I, which may negatively affect flesh texture and muscle growth[42], suggesting the level of dietary creatine should be <15·44 g/kg.

Sarcomere length is a predictor of muscle function and flesh quality[43,44]. Sarcomeres are precisely aligned in fibres and consist of alternating light (I-band) and dark (A-band) bands. The postmortem sarcomere length affects textural characteristics, water-holding capacity of raw muscle, flesh taste and colour. For example, short sarcomeres after rigor increase hardness but reduce water-holding capacity in pork muscle[45]. In this study, 8·48 g/kg creatine significantly increased sarcomere length by increasing the I-band length. Therefore, creatine

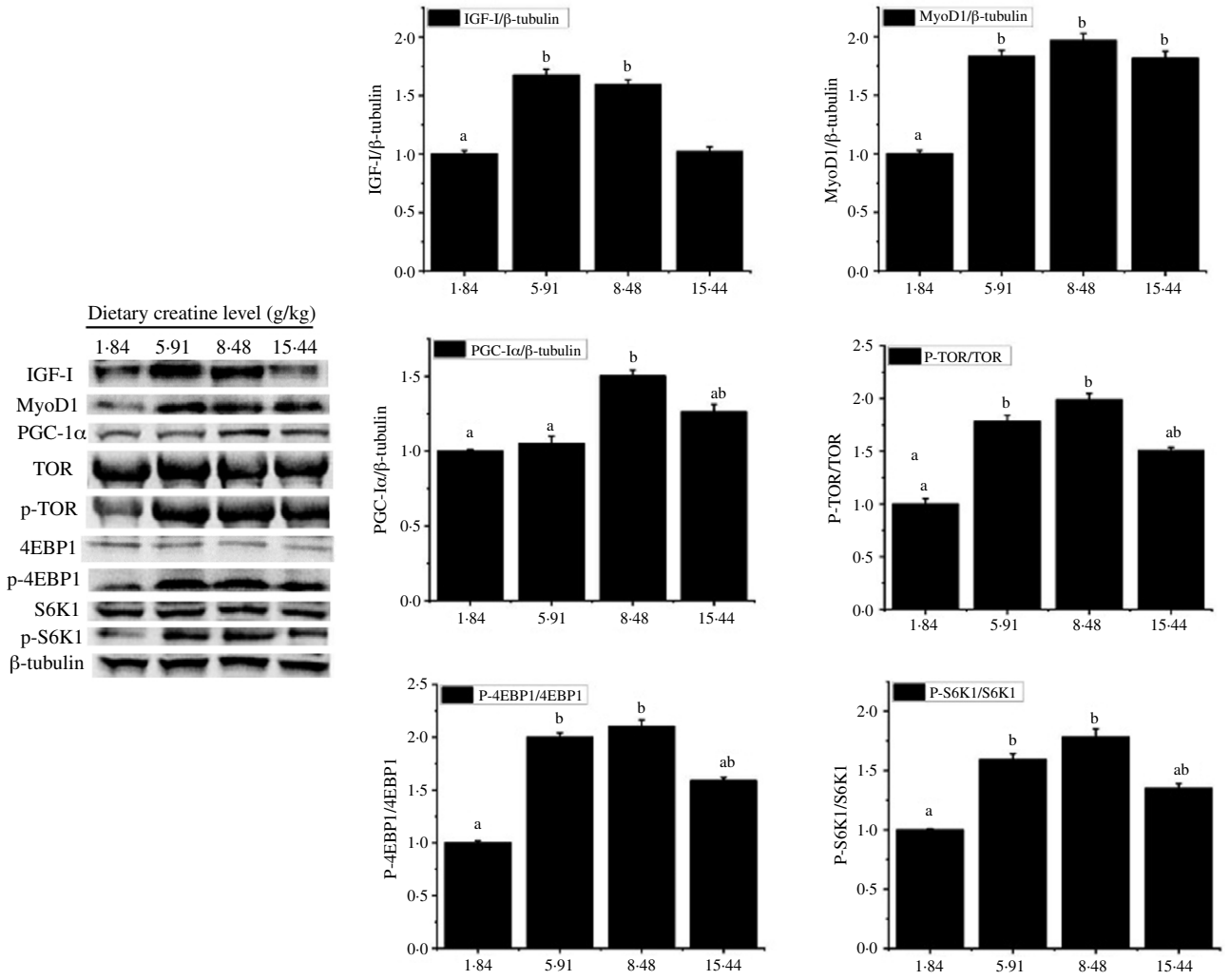

Fig. 6. Effects of creatine-supplemented diets on nutrient-sensing signalling pathways in muscle of grass carp (n 3). IGF-I, insulin-like growth factor I; MyoD1, myogenic differentiation antigen; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; TOR, target of rapamycin; p-TOR, phosphorylated TOR; 4EBP1, eukaryotic translation initiation factor 4E-binding protein 1; p-4EBP1, phosphorylated 4EBP1; S6K1, ribosome S6 protein kinase 1; p-S6K1, phosphorylated S6K1.

increased flesh hardness in fish by affecting the long sarcomeres. The different results between pigs and fish may be attributed to differences in living surroundings and movement patterns.

The textural improvements in muscle are not solely attributable to fibre characteristics, but rather to myofibre types. Dietary creatine increased *MyHC I* mRNA levels and decreased *MyHC II* mRNA levels in rat muscle[15] and *L. vannamei*[13]. In our study, $fMyHC_{s30}$ and $fMyHC_{s10}$ in the slow skeletal muscle increased while *fMyHCs* and *fMyHCc* in the fast skeletal muscle decreased with increasing creatine supplementation. Compared with fast fibres, slow fibres have a higher number of long sarcomeres[46], which correspond to the increased sarcomere length in our study.

The growth and differentiation of myofibres can be mediated by IGF-I and MRF[47], which can be induced by creatine[10,48,49]. Our study found that mRNA expression including MyoD and MyoG in skeletal muscle increased with increasing creatine supplementation levels. Similar results were reported in gilthead seabream[18]. Creatine supplementation increased the protein expression of IGF-I and MyoD1, suggesting that creatine affected the myofibre types of grass carp by regulating the expression of IGF-I and MRF. Additionally, the transformation of myofibres from fast myofibres to slow myofibres can be mediated by PGC-1α, which may be activated by energy stress[17] and increases the mRNA levels of genes involved in the regulation of the mitochondrial function and biogenesis[50]. Our study findings showed that creatine supplementation increased PGC-1α levels, consistent with the increased mRNA levels of $fMyHC_{s30}$ and $fMyHC_{s10}$ in the slow skeletal muscle. These results revealed that the transformation of fibre types by creatine supplementation is mediated by PGC-1α.

In grass carp, 8·48 g/kg creatine-induced muscle protein synthesis. Creatine increased the protein level of IGF-I and the phosphorylation levels of TOR, 4E-BP1 and S6K1. Creatine-induced hypertrophy of C2C12 cells with through partially mediated by overexpression of IGF-I and MRF[51]. Creatine increased muscle protein deposition by activating IGF-I-Akt/TOR pathways. Protein synthesis induced by feeding stimulation in fish depends on the adequacy of amino acid pools in plasma and tissues, which mediate the activities of nutrient-sensing pathways such

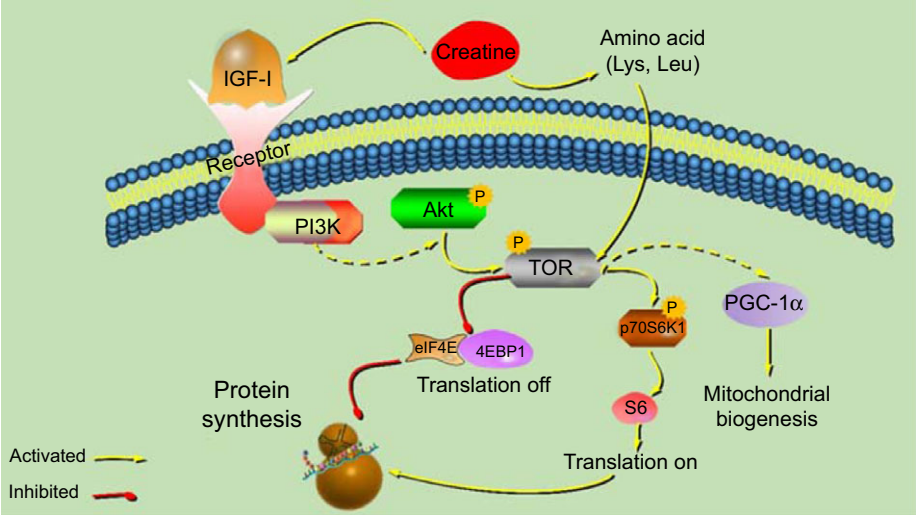

Fig. 7. The mechanism of dietary creatine supplementation on the muscular protein synthesis of grass carp.

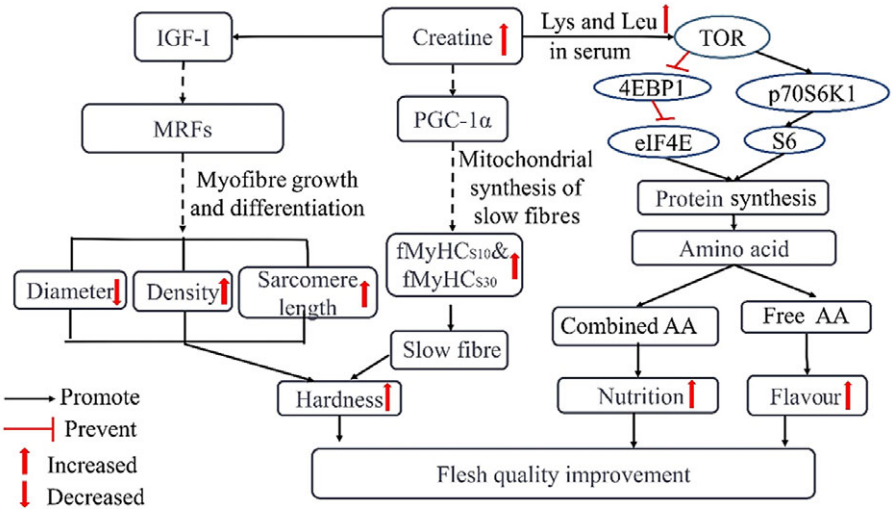

Fig. 8. The conclusion of the effects of dietary creatine supplementation on the flesh quality of grass carp.

as TOR. In blunt snout bream (*Megalobrama amblycephala*), arginine activates the TOR signalling by increasing the postprandial-free amino acid contents in plasma[52]. In this study, creatine-supplemented diets increased the contents of free EAA in serum compared with the control diet, especially the contents of lysine and leucine, which are effective in nutrient sensing[53]. TOR activation is the main driver of creatine-induced protein synthesis, which is in response to an increase in free EAA contents in serum. However, the highest level of dietary creatine did not contribute to the highest content of muscular protein. It is possible that excess creatine promoted insulin resistance through negative feedback and affected IGF-I expression, thereby reducing the stimulation of TOR[54]. Consistent with the above results, IGF-I in the presence of serum-free amino acids could activate TOR signalling pathways (Fig. 7), and the effect was most significant when creatine content was 8·48 g/kg.

High-quality proteins are readily digestible and contain EAA[55]. In this study, dietary creatine increased muscular EAA content, suggesting that the nutritional value of flesh increased. Lysine and leucine, required for protein synthesis in muscle of fish[56], increased in fish fed 8·48 g/kg creatine. Glycine, aspartic acid and glutamic acid, which provide several health benefits[57], increased with 8·48 g/kg creatine. Therefore, diets supplemented with 8·48 g/kg creatine improved muscle nutrition, which enhances the utility of fish as a protein source.

Flavour affects flesh quality and consumer acceptance. Free amino acids contribute to flesh flavour[58]. In fish, aspartic acid and glutamic acid are representatives of the umami taste, and glycine and alanine contribute to sweetness[59]. Dietary creatine at 8·48 g/kg increased FAA content, suggesting that creatine may improve flesh umami and sweetness in grass carp. Leucine and isoleucine generate aroma compounds through the Maillard

reaction[59]; therefore, creatine may promote the formation of aroma compounds in fish muscle.

In vivo, the phosphogen system, glycolysis system and aerobic oxidation system are activated successively to meet the energy requirements of muscles during exercise. The phosphogen system is mainly composed of creatine, phosphocreatine and creatine kinase, which is the earliest and most efficient energy supply pathway for muscle movement[60]. These results in present study suggested that exogenous creatine increased the creatine pool of body, reduced the consumption of other energy nutrients and enhanced other's nutrients deposition. Meanwhile, creatine transporter regulates the creatine pool through a negative feedback inhibition mechanism and is considered to be a regulatory switch to control creatine concentration *in vivo*[61]. Future experiments must address new questions, like the effects of creatine on regulation energy homeostasis and creatine transporters in fish.

In conclusion, dietary creatine effectively improved the flesh quality of grass carp (Fig. 8) by the following mechanisms. First, creatine supplementation increased flesh hardness and chewiness by improving myofibre characteristics, which is mediated by IGF-I, MRF and PGC-1α. Second, creatine supplementation increased the contents of EAA in serum, which further activate the TOR pathway and increase muscle protein synthesis (Fig. 7). Third, creatine supplementation improved flesh flavour by increasing the content of flavour free amino acids. Therefore, flesh quality of grass carp increased when dietary creatine content was 8·48–12·04 g/kg.

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J. T., W. Z. and H. W. designed the research; X. C., W. G., M. J. and X. L. conducted the experiments and analysed the data; X. C., J. T. and L. Y. wrote the paper; J. T. and W. Z. have primary responsibility for final content and all the authors have read and approved the final manuscript.

The authors declare no conflict of interest.

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