

Research Article

Dietary Creatine Reduces Lipid Accumulation by Improving Lipid Catabolism in the Herbivorous Grass Carp, *Ctenopharyngodon idella*

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Herein, our goal was to explore the creatine role in the fat suppression of grass carp (GC). *In vivo*, four semipurified diets containing 5% lipid (normal diet, ND), 8% lipid (high-fat diet, HFD), 8%lipid + 1%creatine (HFD + CR1), and 8%lipid + 2% creatine (HFD + CR2) were designed and formulated, which were fed to juvenile GC (18.51 ± 0.04 g) for 56 days. HFD significantly increased the intraperitoneal fat index as well as the serum and hepatic triglyceride (TG), total cholesterol (TC), and nonesterified fatty acid (NEFA) levels compared to ND; these effects were all attenuated following the addition of creatine in HFD ($p < 0.05$). Relative to HFD, HFD + CR2 markedly enhanced lipid catabolic gene expressions in the hepatopancreas. This included *peroxisome proliferator-activated receptor α* , *adipose TG lipase (atgl)*, *hormone-sensitive lipase*, and *carnitine palmitoyltransferase (cpt-1)*. The protein concentrations of mitochondrial respiratory chain complexes I/II/III/IV/V in the hepatopancreas were upregulated in fish fed HFD + CR1 and HFD + CR2 diets, relative to the HFD-fed fish diet ($p < 0.05$). *In vitro*, hepatocytes (L8824) preincubated with oleic acid for 24 h were exposed to varying concentrations of creatine (0, 10, 50, 100, 200, and 400 μ M) for another 24 h. The number of lipid droplets in each cell remarkably decreased as the concentration of creatine increased in the medium ($p < 0.05$). Creatine also markedly enhanced the relative transcript levels of genes related to lipolysis (such as *atgl*), β -oxidation (such as *cpt-1* and *acyl-CoA dehydrogenase very long chain*), and the mitochondrial respiratory chain (such as *succinate dehydrogenase complex subunit A* and *cytochrome c-1*). Collectively, the study demonstrates that dietary creatine can suppress lipid accumulation and increase lipid catabolic capacity in GC.

1. Introduction

Creatine (N-methylguanidine acetic acid) is a small organic molecule that is mainly found in meat and fish [1]. Creatine can also be synthesized endogenously in a two-step process using three amino acids, namely, arginine, glycine, and methionine, and enzymes L-arginine:glycine amidinotransferase (AGAT), and guanidinoacetate N-methyltransferase (GAMT) [2]. In tissues, creatine strongly facilitates adenosine triphosphate (ATP) turnover, which is the energy source of the cell [3]. Thus, the level of creatine may influence the tissue's physiological or pathological status linked

to energy homeostasis. It is widely taken by many athletes as an ergogenic aid [4]. Moreover, growing evidence supports creatine's role in cognitive enhancement and in abrogating the impact of mild sleep deprivation [5]. More recently, scientists reported that dietary creatine is critical for lowering hepatic fat accumulation in mice [6]. Mechanistically, increasing fatty acid oxidation and lipoprotein secretion are responsible for this process [7, 8]. Moreover, creatine could also enhance thermogenesis in the adipocytes of mice [9, 10].

Although discovered over a century ago, creatine was rarely used or mentioned in aquaculture until recent years,

when researchers started to focus on this molecule. A study in gilthead seabream (*Sparus aurata*) shows that dietary creatine produces no improvements in muscle performance as it does in mammals but alters physiological protein expression (such as in lipid metabolism, metabolic pathways, and in cell processes) in the liver [11]. Interestingly, appropriate amounts of creatine in the diet improved growth and feeding efficiency of carnivorous red drum (*Sciaenops ocellatus*) [12, 13], whereas there were no obvious differences in regard to the lipid accumulation in the whole body or muscle in this fish species. Dietary creatine can reduce the serum triglyceride (TG) and total cholesterol (TC) contents in red tilapia (*Oreochromis* sp.) and also improved robustness against stress [14].

Grass carp (*Ctenopharyngodon idella*, GC) is a highly prevalent and economically productive fish in China, carrying a production rate of 5.57 million tons per year, which accounts for one-fifth of the cultured freshwater fishes in 2020 [15]. In the aquaculture industry, nonprotein sources with high energy (such as lipids) are increasingly used for growth promotion in aquafeed [16]. However, GC easily accumulate excessive lipid in the mesentery and hepatopancreas during farming when eating relatively high-energy commercial feeds [17]. In commercial GC feeds, fish meal (as a source-deficient and highly priced feedstuff) has largely been replaced by plant proteins [18]. Because creatine exists in the fish meal and other animal proteins sources, it is speculated that GC cannot obtain sufficient creatine from a plant protein-based diet [12]. Moreover, the plant protein also lacks the two main precursors of creatine, arginine, and methionine, which may further reduce the production of self-synthesized creatine [2, 19, 20]. Thus, the relatively low level of creatine in GC feed may be one of the factors in its performance in the industry. In this study, we investigated whether increasing the creatine level could suppress lipid aggregation in the GC, as it does in mammals. To address this, we carried out both *in vivo* and *in vitro* experiments after treatment with creatine, and lipid accumulation indices and lipid metabolism were measured.

2. Material and Methods

2.1. *In Vivo* Experimentation

2.1.1. Experimental Diets. Based on the formulation of Lavell [21], four purified, isonitrogenous experimental diets (36.0% crude protein) were designed to satisfy the protein and energy demands of juvenile GC with some modifications [22]: normal diet (5.0% lipid, ND), high-fat diet (8.0% lipid, HFD), HFD (8.0% lipid) with 1% creatine (Sigma-Aldrich St. Louis, MO, USA) (HFD + CR1), and HFD (8.0% lipid) with 2% creatine (HFD + CR2) (Table 1). Casein and gelatin were added as the main protein sources, and soybean oil (YiHai Kerry Co., Shanghai, China) and fish oil (Guanchen Biotechnology Co., Xi'an, China) were used as the oil sources in a ratio of 1:1. All raw materials were pulverized into fine powder and filtered using a 60-mesh size. They were weighed and mixed from small to large portions step by step based on the formulation. Afterward, oils were added

to the ingredients and fully mixed by hand. 0.1% butylated hydroxytoluene (Sigma-Aldrich, St. Louis, MO, USA) was introduced to the feed to serve as an antioxidant. All raw materials were blended with water (added at 700 ml kg^{-1}) to form a dough, which was then prepared into noodle-like pellets carrying a diameter of 2 mm using a feed processing machine, model, and producer (LETSFIND, Beijing, China), which were then forced air-dried at room temperature ($25\text{--}28^\circ\text{C}$, RT) for 24 h prior to storage at -20°C until additional analysis.

2.1.2. Experimental Feeding Protocol. Juvenile GCs were acquired from Tongwei Aquaculture Co., Ltd. (Foshan, Guangdong, China) and grown in aquaria ($0.73 \times 0.46 \times 1.0 \text{ m}$; with 0.27 m^3 water), with ND for 2 w to acclimatize them to the upcoming experiment. Prior to experimentation, all fish went without food for 24 h and received anesthesia via tricaine methanesulfonate (MS222, Sigma-Aldrich St. Louis, MO, USA). Overall, 264 healthy fish of similar size ($18.51 \pm 0.04 \text{ g}$) were arbitrarily separated into 12 aquaria (22 individuals/aquarium). Each diet was arbitrarily provided to three aquaria. Fish received twice daily (at 08:30 and 16:30) hand-feeding till apparent satiation for 8 w. During this process, food consumption was monitored, and dead fish were weighed and eliminated. In addition, routinely filtered water was circulated; with the daily renewal of one-third water to maintain appropriate quality. The O_2 content in water was $6.6 \pm 0.8 \text{ mg/L}$, the pH was 7.2 ± 0.5 , the water temperature was $29.6 \pm 1.6^\circ\text{C}$, the ammonia content was $\leq 0.2 \text{ mg/L}$, and the room was set to a 12/12 h light/dark setting, with a light intensity of 550 lx on average.

2.1.3. Sampling Procedures. Following the 8 w feeding experiment, all fish were kept without food for 24 h and anaesthetized using 0.06 g/L MS-222 to reduce discomfort in the fish. The body weights (BW) and lengths of all fish were measured. Next, we arbitrarily picked six fish per aquarium for caudal vein blood extraction with a syringe. Blood samples were allowed to clot at 4°C for 6 h, the serum was obtained following 10 min of spinning ($825 \times \text{g}$, 4°C), and frozen via liquid N_2 prior to storage at -80°C until further examination. Nine fish were sacrificed, and we obtained the weights of the hepatopancreas, intraperitoneal fat (IPF), and intestine. Hepatopancreatic and adipose tissue samples from three fish per aquarium underwent fixation in 4% paraformaldehyde for histological analysis. Hepatopancreatic samples from six fish per aquarium were frozen in liquid N_2 prior to storage at -80°C for lipid content, gene expression, enzyme activity, and biochemistry analysis. The remaining fish were frozen in -80°C for whole body proximate composition analysis. All animal protocols followed the national and institutional guidelines on the ethical use and care of experimental animals. Our experimental procedure also received ethical approval from the Chinese Academy of Fishery Sciences. The viscerosomatic (VI), hepatopancreatic (HI), and intraperitoneal fat (IPFI) indices were computed as follows:

TABLE 1: The chemical configuration of experimental diets (g/kg air-dry basis).

Components	ND	HFD	HFD + CR1%	HFD + CR2%
Casein	320	320	320	320
Gelatin	80	80	80	80
Dextrin	280	280	280	280
Cellulose	219	189	179	169
Fish oil	25	40	40	40
Soybean oil	25	40	40	40
Carboxymethylcellulose	30	30	30	30
Butylated hydroxytoluene	1	1	1	1
Mineral and vitamin mix ¹	20	20	20	20
Creatine	0	0	10	20
Total	1000	1000	1000	1000
Chemical composition (g kg ⁻¹)				
Moisture	96.8	98.5	98.6	97.4
Crude protein (N% × 6.25)	358.8	360.5	359.9	361.2
Crude fat	48.8	78.9	79.5	79.1
Ash	55.6	54.8	56.7	54.0
Gross energy (kJ g ⁻¹) ²	15.38	15.97	15.95	16.01

¹The mineral mix was composed of (g/100 g of the total mineral): KAl (SO₄) 0.159, CaCO₃ 18.101, Ca (H₂PO₄)₂ 44.601, CoCl 0.070, MgSO₄ 5.216, MnSO₄·H₂O 0.070, KCl 16.553, KI 0.014, ZnCO₃ 0.192, NaH₂PO₄ 13.605, Na₂SeO₃ 0.006, CuSO₄·5H₂O 0.075, ferric citrate 1.338. The vitamin mix was composed of (mg/1000 g of diet): vitamin C, 200; thiamine, 10; riboflavin, 20; vitamin A, 3000 IU; vitamin E, 50 IU; vitamin D3, 1500 IU; menadione, 10; pyridoxine HCl, 10; cyanocobalamin, 0.02; biotin, 1.0; calcipantothenate, 40; folic acid, 5; niacin, 20; inositol, 400; choline chloride, 2000; and cellulose was employed as a carrier. ²The gross energy was calculated based on the formula: gross energy (kJ g⁻¹) = crude protein (%) × 17 + crude fat (%) × 37 + carbohydrate (%) × 17 ND, normal diet; HFD: high-fat diet; HFD + CR1: HFD supplemented with 1% creatine; HFD + CR2: HFD supplemented with 2% creatine.

$$\text{VI (\%)} = \text{viscerosomatic weight (g)} \times 100/\text{BW (g)}, \quad (1)$$

$$\text{HI (\%)} = \text{hepatopancreas weight (g)} \times 100/\text{BW (g)}, \quad (2)$$

$$\text{IPFI (\%)} = \text{IPF weight (g)} \times 100/\text{BW (g)}, \quad (3)$$

$$\text{Feed conversion ratio (FCR)} = \text{amount of feed given (g)}/\text{weight gain (g)}, \quad (4)$$

$$\text{Condition factor (CF)} (\text{gcm}^{-3}) = \text{BW} \times 100/\text{body length}^3, \quad (5)$$

$$\text{Survival rate (\%)} = \text{final fish quantity} \times 100/\text{initial fish quantity}. \quad (6)$$

2.1.4. Proximate Composition. Approximate composition of diet and fish (three fish per tank) was established based on the Association of Official Analytical Chemists (AOAC) protocols, 1995 [23]. In short, samples were dried at 105°C till a constant weight, at which point their moisture content was measured. The crude protein concentration was measured via nitrogen content (N × 6.25) employing the Kjeldahl technique. The crude fat amount was established by ether extraction employing the Soxhlet technique. The ash amount was measured via combustion at 550°C using a muffle furnace.

2.1.5. TG, TC, and Nonesterified Fatty Acid (NEFA) Analysis. Serum and hepatopancreatic TG, TC, and NEFA were assessed with corresponding assays, following kit directions

(Jiancheng Biotech Co., Nanjing, China). Each assay was conducted thrice (three individual samples) per aquarium sample.

2.1.6. Malondialdehyde (MDA) Content, Superoxide Dismutase (SOD), Alanine Aminotransferase (ALT), and Aspartate Aminotransferase (AST) Activities. Serum MDA content, SOD, ALT, and AST activities were assessed with corresponding assays (Jiancheng Biotech Co., Nanjing, China). The aforementioned four indices were determined according to the kit directions. Each assay was conducted thrice (three individual samples) per aquarium sample.

2.1.7. Histological Analysis of Hepatopancreas and Adipose Tissue. Fixed hepatopancreases were used for Oil Red O (ORO) staining. Samples were maintained in 30% sucrose at 4°C for 3 d, prior to embedding at an optimal sections temperature (Leica, Solms, Germany), followed by slicing into 6–10 μm section, rinsing with distilled water (dH₂O), permeation with 60% isopropanol for 20–30 s, and staining with ORO (Sigma-Aldrich St. Louis, MO, USA) for 10 min. We instantly destained the slides with 60% isopropanol for 3 min, rinsed with dH₂O to clear up the background, followed by counterstaining with Mayer's hematoxylin for 1 min, rinsed again with dH₂O for 10 min, followed by seal-capping with glycerogelatin, and finally imaging with a light microscope (Olympus BX41, Olympus Corporation).

The fixed adipose tissue samples were rinsed with tap water for 12 h, before twice standard dehydration using

ethanol gradient (30, 50, 70, 80, 90, 95, and 100%), with subsequent equilibration in xylene, standard embedding in paraffin, slicing into 5 μm sections with the use of a rotary microtome (RM2235, Leica, Germany), mounting on glass slides, staining with hematoxylin and eosin (H&E), and finally observing with an upright fluorescence microscope (Leica Biosystems, Germany).

2.1.8. Mitochondrial Respiratory Chain Complex Assay. The hepatopancreas was homogenized for determination of the mitochondrial respiratory chain complex contents in each treatment, including complex (COX) I, COX II, COX III, COX IV, and COX V. These indices were assessed via fish enzyme-linked immunosorbent assay (ELISA) kits (MEIMIAN, Wuhan, China). Each assay was conducted thrice (three individual samples) per aquarium sample.

2.1.9. Real-Time Quantitative RT-PCR. Total RNA was recovered from hepatopancreas (three individual samples per aquarium) with TRIzol (TaKaRa, Dalian, China), following kit directions, and quantified by an Implen NanoPhotometer (Implen, Inc., Westlake Village, CA, USA), and the integrity of the RNA was confirmed with the use of 1.2% denaturing agarose gel electrophoresis. The copy DNA (cDNA) was generated via a PrimeScript[®] RT reagent kit (TaKaRa), as per kit directions. The primer sequences from our study are summarized in Table 2. According to a 6-step series, we demonstrated that a 10-fold dilution of our target template yielded primer amplification efficiencies between 94.44% and 102.64% after quantitative real-time polymerase chain reaction (qRT-PCR). The determination coefficient (R^2) values were > 0.99 . Additionally, following the recommendations of the geNorm (version 3.5) and NormFinder algorithms, β -actin was employed as the internal control. qRT-PCR assays were conducted in triplicate (three times in each sample) using the LightCycler[®] 96 RT-PCR apparatus (Roche, Switzerland), using 20 μL reaction mixture with 2.0 μL primer (2.5 μM), 2.0 μL diluted first-strand cDNA product, 10 μL 2 \times Power SYBR[™] Green PCR Master Mix (Thermo Scientific, USA), and 6.0 μL sterile double- dH_2O . The following parameters were used in the qRT-PCR reaction: 95°C for 5 min, 40 cycles at 95°C for 15 s, and 60°C for 1 min. Once this was completed, the melting curve was monitored from 72–95°C (in 1°C/20 s steps) to validate the formation of a single product. Negative references, namely, zero-cDNA and a DNase-digested, non-cDNA converted RNA sample, were employed to verify only cDNA quantification in all reactions. Relative gene expression was computed with the CT ($2^{-\Delta\Delta C_t}$) formula, as previously reported [24, 25].

2.2. In Vitro Experiments

2.2.1. Cell Culture. The GC hepatocyte line (L8824) was obtained from the China Center for Type Culture Collection (CCTCC, Wuhan, China). Cell media were composed of M199 medium (GIBCO, USA), 10% fetal bovine serum (FBS, GIBCO, USA), 100 U/mL penicillin (GIBCO, USA), and 100 U/mL streptomycin (GIBCO, USA). Cells were grown in 28°C in ambient air in a cell incubator (Thermo

Scientific, USA). The medium was refreshed every 2 d. Once the cells became confluent, 200 μM oleic acid (OA, coated in bovine serum albumin; a regular fatty acid used in setting up lipid droplets in cells) was introduced to the medium, and the cells were placed back in the incubator for 24 h. Afterwards, the cells were placed back in the incubator in media supplemented with 10, 50, 100, 200, and 400 μM of creatine (Sigma-Aldrich St. Louis, MO, USA) for 24 h.

2.2.2. BODIPY Staining and Lipid Droplets Quantification. GC hepatocytes were seeded at 1.2×10^6 /well in six-well plates. Following treatment, the medium was suctioned, and cells were phosphate-buffered saline- (PBS-) rinsed twice, before a 30-min fixation in 10% formalin, followed by two more PBS rinses, staining with BODIPY (10 $\mu\text{g}/\text{mL}$; Invitrogen, USA) for 30 min, three more PBS rinses, followed by DAPI (10 $\mu\text{g}/\text{mL}$; Invitrogen, USA) nuclear staining (10 min), then more washes with PBS, and finally observation under an inverted fluorescence microscope (Nikon TS2, Japan). Nine cells (three cells per well/picture) in each group were analyzed, and particle numbers were quantified using ImageJ software 1.52A (<https://imagej.nih.gov/ij/download.html>) in three-dimensional stacks [26].

2.2.3. Real-Time qRT-PCR. We plated 1.2×10^6 L8824 cells/well in 6-well plates (three replicates in each treatment). Following treatment, cells were twice rinsed with PBS, prior to total RNA extraction with TRIzol (Life Technologies Inc., USA), following kit directions. Agarose gel electrophoresis and spectrophotometry (Implen NanoPhotometer; Implen, Inc., Westlake Village, CA, USA) were employed for RNA integrity assessment. The cDNA conversion was done with a PrimeScript[®] RT reagent kit (TaKaRa, Otsu, Japan). Real-time qRT-PCR was carried out as reported in Section 2.1.8.

2.3. Statistical Analysis. All data analyses were done in PASW Statistics 20 (SPSS, Chicago, IL, USA) and are shown as mean \pm standard deviation (S.D.). To elucidate differences in indices, one-way analysis of variance, then *post hoc* least significant difference (LSD) test was employed. Different letters represent statistically significant differences ($p < 0.05$).

3. Results

3.1. Growth Performance, Feed Utilization, and Biological Features of Juvenile GC. The BW of fish that consumed the four diets rose from 18.43 ± 0.07 g to 39.39 ± 0.55 g, but no statistical significance was reached among treatments ($p > 0.05$; Table 3). In fact, the four treatments had comparable feed conversion ratios ($p > 0.05$). Fish fed HFD had a higher IPFI than ND-fed fish ($p < 0.05$), whereas this index decreased when 1% and 2% creatine were added to HFD diets ($p < 0.05$). No obvious changes were seen in other biological features like the condition factor, VI, and HI among the four groups ($p > 0.05$).

3.2. Serum Biochemistry Properties of Juvenile GC. Serum biochemistry indices are shown in Table 4. Expectedly, HFD-fed fish had elevated TG, TC, and NEFA levels than

TABLE 2: Primers used in real-time quantitative PCR.

Target genes	Forward (5'-3')	Reverse (5'-3')	Product length
Ppara	CGCTGAGGTTCCGATATTT	ACGTCACCTGGTCATTTAAG	122
Atgl	TCGTGCAAGCGTGTATATG	GCTCGTACTGAGGCAAATTA	136
Hsl	TGGAACGTTACTGAGTCTGG	AAGCGCACGTTGACTGG	188
Cpt-1	GCATCCATGACACGTTTATTC	GAAGTTTCTTCTCTCGTCTC	118
Acadvl	GGCTCCCATCAGCCAATCAT	GGCTCCCATCAGCCAATCAT	129
Acadm	GACCAGCCCTCAAACACTGA	ACAGCATCTTCTCGGCATGT	81
Acads	AGACGTGTAGGGACTACGCT	TCTTGTACCTGCTTGGCTGG	91
Hadha	AGCTGGCGATGACATCAGAG	GCGCCCAAAGTGGTCTTTT	83
Hadhb	CCATTGCCCTCAGGTTTGCC	CGGTCTGACTTTGGACGACA	83
Mt-nd1	AGTCATGGTGGGCATGAGTG	GACATGTTTCGTTTCAGCGGG	77
Sdha	CCAAAGCTGCTTCCTTGACAC	AGGGCCGGGAAAATAATACG	92
Cycl	CATACTCCACGGGCCAACAT	AGCACTCCAATCGTGGTCAG	77
Mt-co1	CAGCTCCAGCAGTGTCAAGA	TCCAGACGGTCCCAGTACAT	93
Mt-atp	TGAACTGGGTCGAACAGAGC	TCCGCAATGCACTTGGAGAT	79
β -Actin	TCCACCTTCCAGCAGATGTGGATT	AGTTTGAGTCGGCGTGAAGTGGTA	236

TABLE 3: Outcome of high-fat diet supplemented with creatine on growth, feed utilization, survival, and biological characteristics of juvenile grass carp¹.

	ND	HFD	HFD + CR1	HFD + CR2
Initial weight (g)	18.50 ± 0.02	18.50 ± 0.11	18.26 ± 0.15	18.37 ± 0.67
Final weight (g)	39.84 ± 0.93	38.46 ± 0.14	39.52 ± 0.36	39.72 ± 0.50
Feed conversion ratio	2.56 ± 0.14	2.72 ± 0.16	2.59 ± 0.09	2.54 ± 0.06
Condition factor (g cm ⁻³)	2.07 ± 0.03	2.09 ± 0.18	2.08 ± 0.12	1.96 ± 0.04
Viscerosomatic index (%)	9.94 ± 0.81	10.10 ± 0.80	9.00 ± 1.12	8.99 ± 0.23
Hepatopancreas index (%)	2.17 ± 0.07	1.91 ± 0.25	2.09 ± 0.47	2.15 ± 0.19
Intraperitoneal fat index (%)	1.98 ± 0.14 ^c	2.71 ± 0.10 ^a	2.33 ± 0.20 ^b	2.28 ± 0.15 ^b
Survival rate (%)	90.91 ± 4.55	86.36 ± 4.55	84.85 ± 2.62	86.36 ± 4.55

¹Distinct symbols represent marked differences ($p < 0.05$; ANOVA); data shown as means ± standard deviation, $n = 3$ per group. ND: normal diet; HFD: high-fat diet; HFD + CR1: HFD supplemented with 1% creatine; HFD + CR2: HFD supplemented with 2% creatine.

fish fed ND, whereas these lipid indices were suppressed when the HFD diets were supplemented with 1% and 2% creatine ($p < 0.05$). Similar trends were also found in SOD activity, MDA content, ALT, and AST activities ($p < 0.05$).

3.3. Whole Body Proximate Composition and Lipid Content of the Hepatopancreas in Juvenile GC. The whole-body composition of GC is summarized in Table 5. No marked alterations were observed in the crude protein and ash amounts among treatments ($p > 0.05$). Nevertheless, HFD-fed fish exhibited elevated crude fat, compared to ND-fed fish ($p < 0.05$); this was attenuated when the fish were fed HFD + CR2 ($p < 0.05$). Similarly, in the hepatopancreas, HFD increased the crude fat content over the ND fish, whereas dietary creatine decreased lipid content in the hepatopancreas; the HFD + CR2 group exhibited a marked alteration from the HFD group ($p < 0.05$).

3.4. Hepatopancreatic and Adipose Lipid Accumulation in Juvenile GC. The GC-fed HFD had more lipid droplets than the fish fed ND, which decreased in the fish-fed creatine-enriched diets, especially at 2% creatine (Figure 1(a)). Quantitatively, TG, TC, and NEFA concentrations rose in the hepatopancreas after feeding HFD, however, all these indices were decreased when creatine was added in the HFD ($p < 0.05$; Table 4). In adipose tissue, the HFD-fed fish displayed a larger adipocyte size than the fish fed ND, whereas a smaller adipocyte size was observed in the HFD + creatine-fed fish (Figure 1(b)).

3.5. Relative Lipid Catabolism-Related Gene Transcript Levels in the Hepatopancreas of Juvenile GC. There were no marked alterations in the relative expression of peroxisome proliferator-activated receptor α (*ppara*), adipose TG lipase (*atgl*), hormone-sensitive lipase (*hsl*), and carnitine palmitoyltransferase (*cpt-1*) genes between HFD and ND groups

TABLE 4: Outcome of a high-fat diet supplemented with creatine on the serum and hepatopancreas biochemistry parameters of juvenile grass carp.

	ND	HFD	HFD + CR1	HFD + CR2
Serum				
TG (mmolL ⁻¹)	1.90 ± 0.34 ^b	2.85 ± 0.44 ^a	2.16 ± 0.22 ^b	2.05 ± 0.42 ^b
TC (mmolL ⁻¹)	4.66 ± 0.33 ^b	6.24 ± 0.26 ^a	5.40 ± 0.16 ^b	5.28 ± 0.65 ^b
NEFA (mmolL ⁻¹)	0.49 ± 0.07 ^b	0.79 ± 0.07 ^a	0.56 ± 0.14 ^b	0.54 ± 0.12 ^b
SOD (Uml ⁻¹)	22.31 ± 0.26 ^b	23.51 ± 0.33 ^a	22.86 ± 0.38 ^b	22.34 ± 0.32 ^b
MDA (μmolL ⁻¹)	13.47 ± 0.49 ^c	18.54 ± 0.63 ^a	16.81 ± 0.59 ^b	15.71 ± 0.68 ^b
ALT (UL ⁻¹)	27.83 ± 1.44 ^b	40.00 ± 1.00 ^a	30.00 ± 4.36 ^b	28.50 ± 4.77 ^b
AST (UL ⁻¹)	65.67 ± 10.69 ^b	86.17 ± 8.81 ^a	70.00 ± 3.12 ^b	68.43 ± 3.11 ^b
Hepatopancreas				
TG (mmol g prot ⁻¹)	0.63 ± 0.10 ^b	0.88 ± 0.03 ^a	0.70 ± 0.08 ^b	0.69 ± 0.02 ^b
TC (mmol g prot ⁻¹)	1.93 ± 0.14 ^b	3.27 ± 0.38 ^a	2.03 ± 0.78 ^b	1.84 ± 0.83 ^b
NEFA (mmol g prot ⁻¹)	1.04 ± 0.08 ^b	1.90 ± 0.05 ^a	0.96 ± 0.25 ^b	0.95 ± 0.09 ^b

¹Distinct symbols in each row represent marked differences ($p < 0.05$; ANOVA); data expressed are means ± standard deviation, $n = 3$ per group. ND: normal diet; HFD: high-fat diet; HFD + CR1: HFD supplemented with 1% creatine; HFD + CR2: HFD supplemented with 2% creatine; TG: triglyceride; TC: total cholesterol; NEFA: non-esterified fatty acid; SOD: superoxide dismutase; MDA: malondialdehyde; ALT: alanine aminotransferase; AST: aspartate aminotransferase.

TABLE 5: Outcomes of high fat diet supplemented with creatine on the proximate composition of the whole body and lipid content in the hepatopancreas of grass carp¹.

	ND	HFD	HFD + CR1	HFD + CR2
Whole body (g kg⁻¹)				
Moisture	743.54 ± 3.65 ^{ab}	718.79 ± 6.89 ^b	725.00 ± 9.91 ^{ab}	748.49 ± 11.77 ^a
Crude protein ($N \times 6.25$)	145.54 ± 8.13	153.99 ± 4.66	150.93 ± 4.46	140.09 ± 17.07
Crude fat	64.92 ± 3.17 ^b	84.47 ± 2.71 ^a	73.83 ± 4.79 ^{ab}	71.21 ± 5.33 ^b
Ash	32.22 ± 1.46	28.75 ± 1.93	31.33 ± 11.81	26.94 ± 1.59
Hepatopancreas (g kg⁻¹)				
Crude fat (%)	157.81 ± 2.40 ^b	221.14 ± 25.24 ^a	176.31 ± 17.76 ^{ab}	162.69 ± 18.31 ^b

¹Distinct symbols in a row represent marked differences ($p < 0.05$; ANOVA); *data expressed are means ± standard deviation, $n = 3$ per group. ND: normal diet; HFD: high-fat diet; HFD + CR1: HFD supplemented with 1% creatine; HFD + CR2: HFD supplemented with 2% creatine.

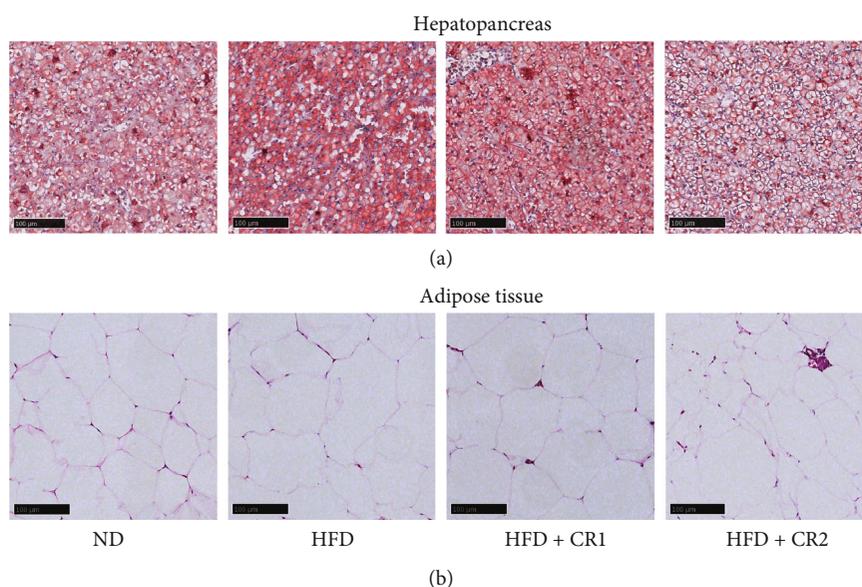


FIGURE 1: The outcome of a creatine-supplemented high-fat diet on hepatopancreatic and adipocytic histologies in juvenile grass carp. (a) Hepatopancreas stained with Oil Red O. (b) Adipose tissue stained with hematoxylin and eosin. ND: normal diet; HFD: high-fat diet; HFD + CR1: HFD supplemented with 1% creatine; HFD + CR2: HFD supplemented with 2% creatine.

(Figure 2(a)). However, these genes were increased when the HFD diet was supplemented with creatine. We also observed marked alterations in *ppara*, *atgl*, and *cpt-1* levels between the HFD- and HFD + CR2-fed fish ($p < 0.05$).

3.6. Hepatopancreatic Mitochondrial Respiratory Chain Complex Expression in Juvenile GC. Fish fed the HFD had higher COX I, COX II, COX IV, and COX V expression in the hepatopancreas than the ND-fed fish ($p < 0.05$; Figure 2(b)). Upon creatine supplementation of HFD, the COX expression level was substantially increased. Specifically, COX I, COX II, COX III, and COX V activities were elevated in both HFD + CR1- and HFD + CR2-fed fish. COX IV activity was increased in the HFD + CR2-fed fish, relative to the HFD-fed fish ($p < 0.05$).

3.7. Outcome of Creatine on Lipid Accumulation in GC Hepatocytes. After incubation with creatine, the accumulation of lipid droplets decreased with the increase in creatine concentration (Figure 3(a)). The number of lipid droplets in each cell was decreased 21.22%, 39.79%, 55.97%, 85.94%, and 86.74% in response to 10 μM , 50 μM , 100 μM , 200 μM , and 400 μM creatine, respectively ($p < 0.05$; Figure 3(b)).

3.8. Outcome of Creatine on Lipid Catabolism-Related Gene Expression in GC Hepatocytes. The key transcription factor of the lipid catabolic gene, *ppara*, showed an increased expression level, which was different when the creatine content reached 200 μM ($p < 0.05$; Figure 3(c)). Similarly, the relative lipolysis related gene, *atgl*, level had a higher expression from 100 μM to 400 μM ($p < 0.05$). No marked alteration was present in *hsl* levels ($p > 0.05$).

The mitochondrial β -oxidation-related gene expression showed an increase with the increase in creatine concentration (Figure 4(a)). To be specific, the relative expression of *cpt-1* was increased when the creatine level was higher than 100 μM ($p < 0.05$). During β -oxidation, acyl-CoA dehydrogenase very long chain (*acadvl*) and acyl-CoA dehydrogenase short chain (*acads*) gene expression increased when the incubated concentration of creatine was 200 μM ($p < 0.05$). The transcript expression of hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex b (*hadhb*), a key enzyme of β -oxidation, also increased at 50 μM creatine ($p < 0.05$). No marked change was observed in the acyl-CoA dehydrogenase medium chain (*acadm*) and hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex a (*hadha*) expression among groups ($p > 0.05$).

In the mitochondrial respiratory chain, two genes, succinate dehydrogenase complex subunit A (*sdha*) and cytochrome c-1 (*cyc1*), from complex II and complex III, respectively, increased in response to creatine incubation. Specifically, 100 and 200 μM creatine increased the mRNA level of *sdha* over the control; 200 and 400 μM creatine elevated *cyc1* transcript levels, as opposed to the 10 μM treatment ($p < 0.05$; Figure 4(b)). The levels of *cytochrome c oxidase I mitochondrial (mt-co1)* and *ATP synthase mitochondrial (mt-atp)* were comparable among all groups ($p > 0.05$).

4. Discussion

After a long period of evolution, the physiological features of fish reflect the food they consume. Studies have shown that fat accumulates in the viscera of herbivorous fish (GC) easier than in omnivorous (channel catfish, *Ictalurus punctatus*) and carnivorous (largemouth bass, *Micropterus salmoides*) fishes [27]. Because creatine and its some precursors are “carninutrients,” the herbivorous fish should consume less creatine than omnivorous or carnivorous fishes [2]. In this study, we hypothesized that an increase in dietary creatine in an herbivorous fish could reduce the fat deposition, since many scientists demonstrated that dietary creatine can reduce lipid accumulation in mice and have proposed it as a possible therapy for treating fatty liver disease and obesity in humans [6, 8, 28]. We found that dietary creatine reduces lipid accumulation in GC both *in vivo* and *in vitro*, which may be due to increased lipid catabolism. Overall, this is a novel research investigating the significance of creatine in lipid metabolism in this type of fish and provides a reference for its use in aquatic diets.

In the *in vivo* experiment, the serum and hepatic TG, TC, and NEFA were all suppressed when the diets were supplemented with 1% and 2% creatine, similar to the response in red tilapia [14]. Our study further proved that hepatic lipid droplets and adipocyte size were reduced in these two groups, demonstrating that dietary creatine could ameliorate the fat deposition induced by high-fat diets. However, the minimum dose of creatine that should be added to the diets is not clear. In cell culture, we showed that lipid droplets were decreased when the concentration of creatine exceeded 100 μM , suggesting that minute amounts of creatine can directly affect the lipid accumulation in hepatocytes. Reducing lipid accumulation using creatine in GC is consistent with the response in mammals [6, 28]. A previous study showed that fat accumulation increased when tissue creatine decreased in GC upon faba bean consumption [29], suggesting a negative correlation between fat accumulation and creatine. However, our results are not consistent with the red drum carnivorous fish [12, 13]. The difference between these two results may be (1) fish specific; GC are herbivorous fish that may not tolerate higher levels of creatine in the body; (2) feed differences; our study use semi-purified diets (creatine-free), whereas the red drum study used commercial diets which included 29% fish meal (containing a certain amount of creatine).

PPAR α transcriptionally regulates genes modulating the peroxisomal and mitochondrial β -oxidation and FA transport [30]. In this study, dietary creatine increased the transcripts of PPAR α in the hepatocytes of GC both *in vivo* and *in vitro*, suggesting that dietary creatine may increase the lipid catabolic ability in this fish specifically, which is consistent with the result in mice [8]. Our study demonstrated that dietary creatine can increase the lipid degradation rate in fish. First, the gene expression of the rate-limiting enzyme of lipolysis, *ATGL* [31], but not *HSL*, increased in the hepatocytes. Second, both *in vivo* and *in vitro* data showed that dietary creatine enhanced *CPT-1* transcripts, a rate-limiting event in mitochondrial β -

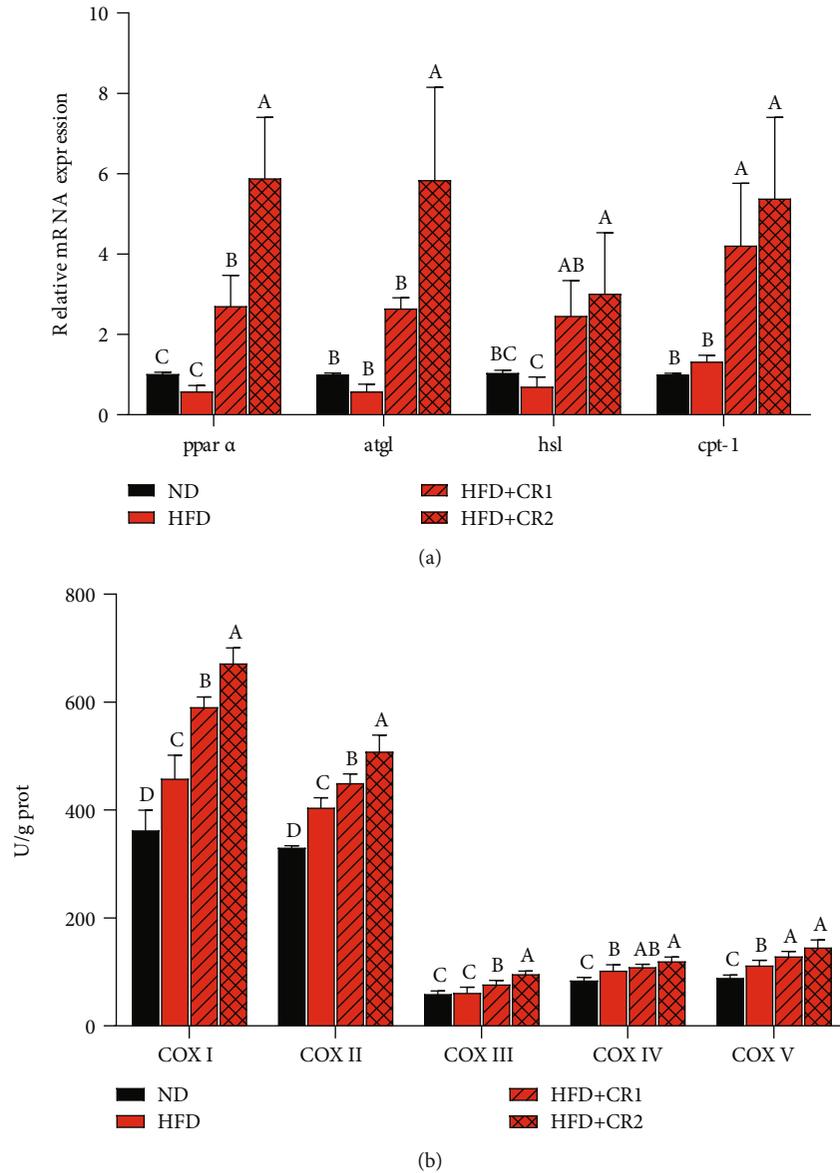
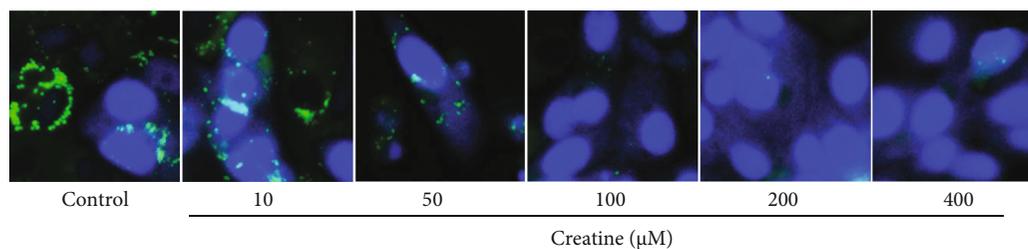


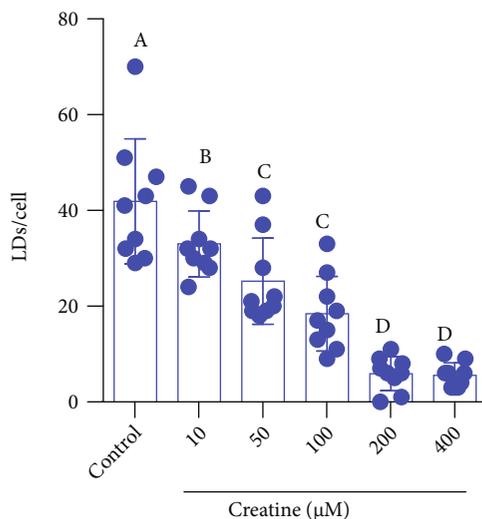
FIGURE 2: The outcome of a high-fat diet supplemented with creatine on the lipid catabolism-related gene expression (a) and mitochondrial respiratory chain complex expression (b) in the juvenile grass carp hepatopancreas. Data shown as means \pm standard deviation (shown as error bars; $n = 3$). The average in each panel with absent common letter is statistically significant ($p < 0.05$; ANOVA). ND: normal diet; HFD: high-fat diet; HFD + CR1: HFD supplemented with 1% creatine; HFD + CR2: HFD supplemented with 2% creatine; *pparα*: peroxisome proliferator-activated receptor α ; *atgl*: adipose triglyceride lipase; *hsl*: hormone-sensitive lipase; *cpt-1*: carnitine palmitoyltransferase 1; COX: mitochondrial complex.

oxidation [32]. The *in vitro* results also exhibited increased relative mRNA expression of *acadvl*, *acads*, and *hadhb*, which are the key enzymes of β -oxidation [33]. Third, it is known that the end product of β -oxidation, acetyl-CoA forms NADPH and FADH₂, as part of the tricarboxylic acid cycle. Moreover, NADPH and FADH₂ then enter the mitochondrial respiratory chain, whereby with the help of five enzymatic complexes ATP is produced [29]. Our *in vivo* study showed that the protein concentrations of complexes I–V were all increased after the fish were fed creatine, and the *in vitro* study showed that appropriate concentrations of creatine increased the relative transcript levels of several

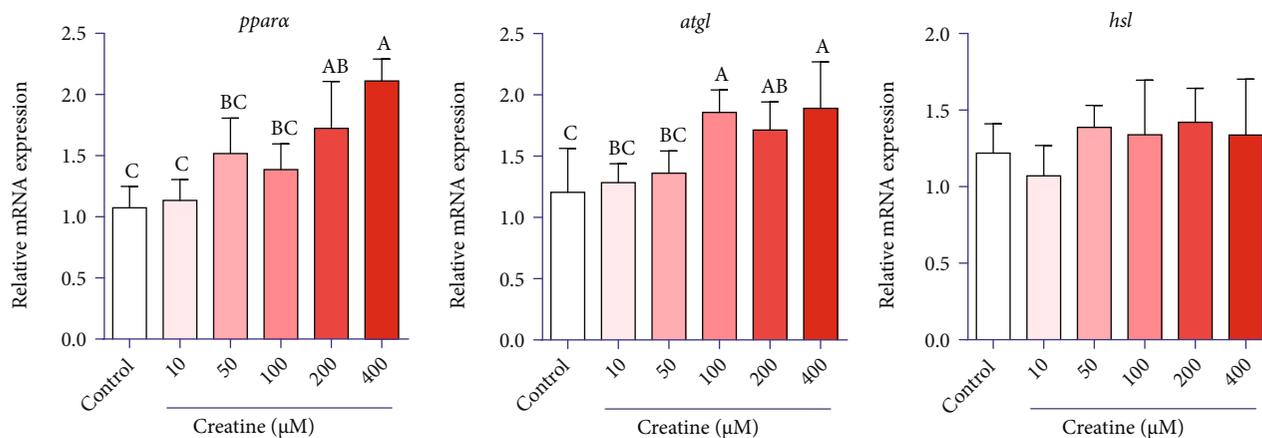
genes in complexes I–III. Collectively, these results demonstrate that dietary creatine can increase the rate of lipolysis, β -oxidation, and oxidative phosphorylation in GC hepatocytes. This may be one of the reasons why dietary creatine decreases lipid accumulation, as lipid catabolism is an important determinant in regulating lipid accumulation in fish [34]. The results in mammals have shown that dietary creatine induces fatty acid oxidation and lipoprotein secretion [7, 8] and enhances energy expenditure [9], which is consistent with our study. No study has shown that creatine can directly bind to the enzymes for lipid catabolism. However, creatine only accelerates one enzymatic reaction



(a)



(b)



(c)

FIGURE 3: Lipid droplet (LD) accumulation and lipolysis-linked gene expression in grass carp hepatocytes following treatment with different concentrations of creatine for 24 h. Data shown as means ± standard deviation (shown as error bars; $n = 3$). The average in each panel with absent common letter is statistically significant ($p < 0.05$; ANOVA). (a) BODIPY staining of grass carp hepatocytes, green dots represent LDs; blue dots represent the nucleus. (b) Number of LDs per cells. (c) Lipolysis-related gene expression. *pparα*: peroxisome proliferator-activated receptor α ; *atgl*: adipose triglyceride lipase; *hsl*: hormone-sensitive lipase.

(creatine kinase), to interconvert creatine and phosphocreatine in tissues that have an elevated need for ATP [35]. Thus, increased creatine concentration in cells may impact the AMP/ATP ratio, which further influences the AMPK activity, thereby increasing lipid degradation [36]. However, this remains to be confirmed.

A high lipid level in fish diets has been attracting increasing attention because it produces a protein-sparing effect in

numerous fish species like snout bream [37], sea bream [38], and GC [16]. However, this would induce excess lipid accumulation in the whole body and carcass of these fish species. Thus, improving lipid utilization via the lipid catabolism pathway may be a promising strategy not only to reduce excessive lipid accumulation but also to reduce protein degradation. In the current study, we showed that dietary creatine promotes lipid catabolism, which may have a potential role in the

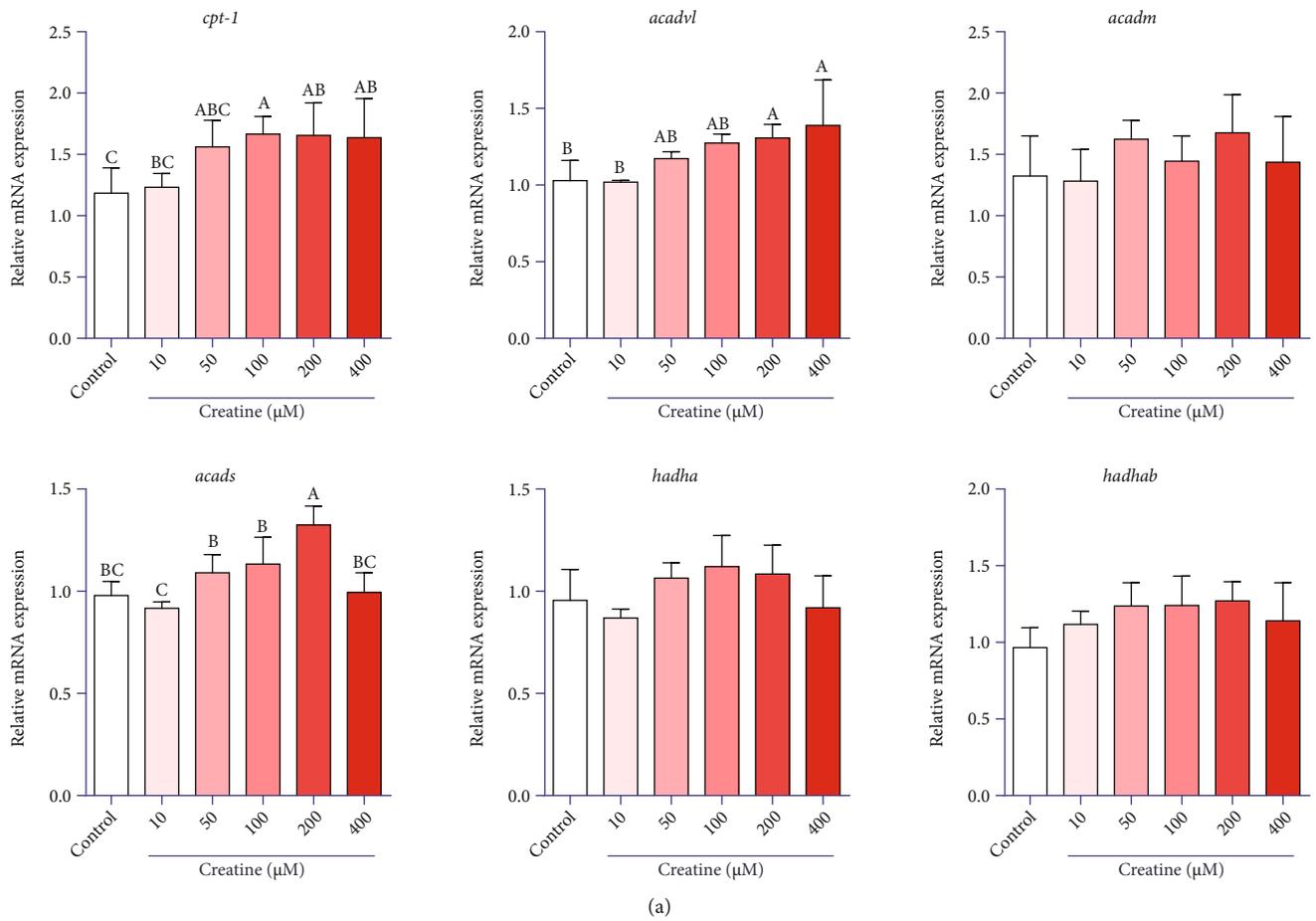


FIGURE 4: Continued.

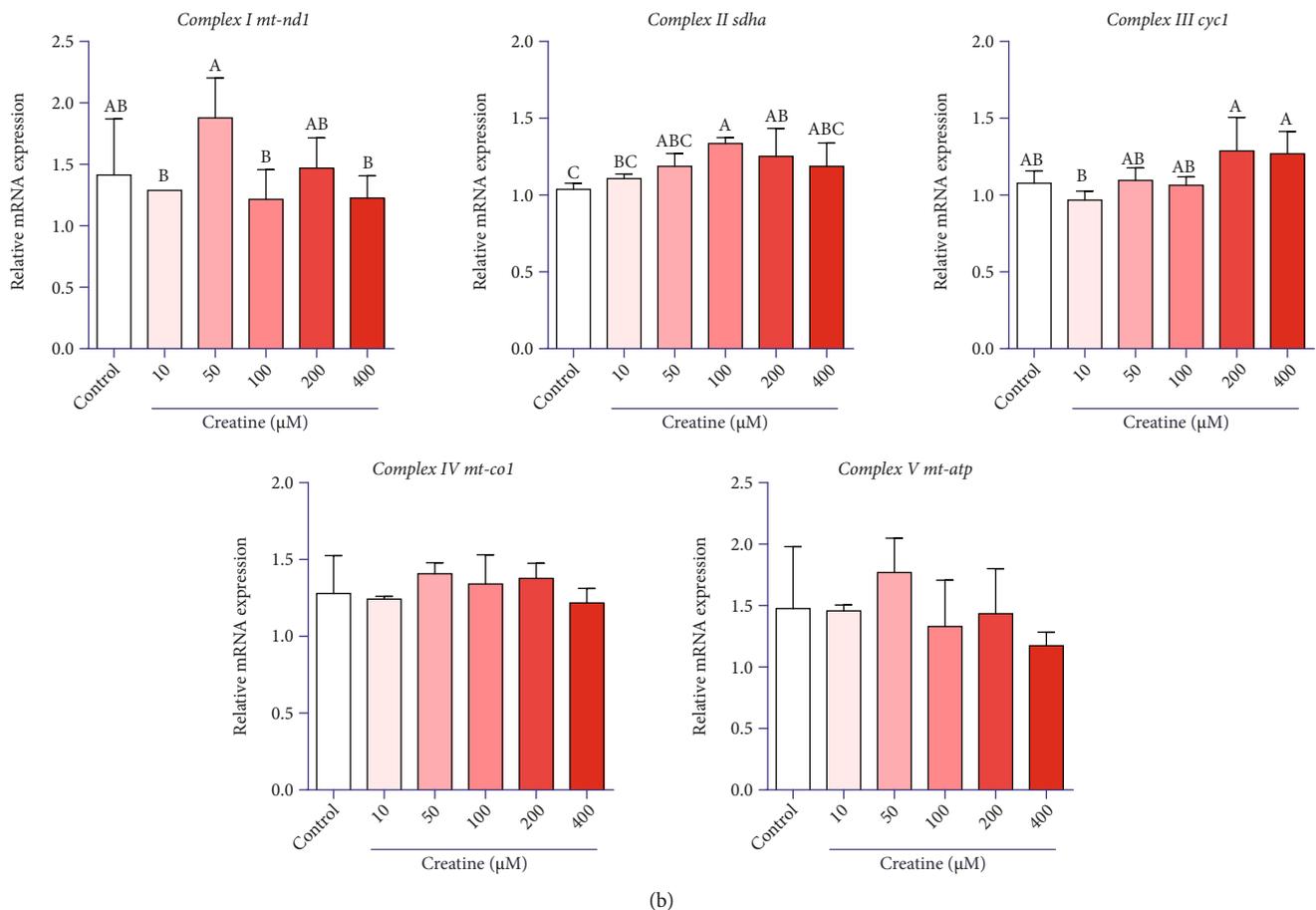


FIGURE 4: Mitochondrial β -oxidation-related (a) and mitochondrial respiratory chain complex-related (b) gene expression in grass carp hepatocytes following treatment with different concentrations of creatine for 24 h. Data shown as means \pm standard deviation (shown as error bars; $n = 3$). The average in each panel with absent common letter is statistically significant ($p < 0.05$; ANOVA). *Cpt-1*: carnitine palmitoyltransferase 1; *acadvl*: acyl-CoA dehydrogenase very long chain; *acadm*: acyl-CoA dehydrogenase medium chain; *acads*: acyl-CoA dehydrogenase short chain; *hadh*: hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex; *mt-nd1*: NADH dehydrogenase 1, mitochondrial; *sdha*: succinate dehydrogenase complex, subunit A; *cyc1*: cytochrome c-1; *mt-co1*: cytochrome c oxidase I, mitochondrial; *mt-atp*: ATP synthase, mitochondrial.

protein-sparing function. However, we did not find differences in the whole-body protein content among treatments. Importantly, the numerical decrease in the FCR may be a clue that dietary creatine increases protein deposition. The influence of dietary creatine on protein metabolism, such as protein synthesis and degradation, still requires further investigation.

Several indices related to fish health are shown in the *in vivo* study. The HFD increased the serum SOD, ALT, and AST activities and MDA levels, whereas these indices were all suppressed by dietary creatine. Lipid peroxidation produces MDA. Therefore, MDA levels are a direct reflection of lipid peroxidation [39]. SOD is pivotal crucial antioxidant that catalyzes O_2^- to H_2O_2 [40]. A study in red tilapia showed that dietary creatine reduced MDA level and enhanced SOD activity [14], which is similar to our study and suggests that dietary creatine can reduce oxidative stress in fish. It is believed that the tissue lipid content is responsible for lowering oxidative stress [41]. Generally, serum ALT and AST activities are strong predictors of hepatic or other tissue (like spleen, kidney, and red blood cells) health [42]. Our study demonstrated that dietary creatine can reduce cell

damage and should be beneficial to fish health. It was reported that serum activities of ALT and AST are increased when lipids are excessively deposited in the liver of fish [31, 43]. Thus, the reduced lipid accumulation of hepatocytes by dietary creatine should be responsible for the decreased ALT and AST activities in the serum. In mammals, creatine utilization is limited in specific circumstances as it may affect the gut health [44]. Hence, more indices related to health are therefore required to assess creatine usage in fish diets.

5. Conclusions

In conclusion, our study showed that dietary creatine can reduce lipid accumulation in GC hepatocytes. These alterations can be partly explained by the enhanced levels of molecules linked to lipolysis, β -oxidation, and oxidative phosphorylation. In this study, we used semipurified diets. It remains to be confirmed if creatine addition in commercial diets would also affect lipid accumulation. The optimal supplementation level, as well as its impact on the protein-sparing function and fish health should be evaluated further.

Data Availability

The study data are available upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Jing-jing Tian and Yu-ping Li contributed equally to this work.

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