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Physicochemical property optimization and nutrient redistribution in the muscle of sub-adult grass carp (*Ctenopharyngodon idella*) by conjugated linoleic acid

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ABSTRACT

We studied the effects of conjugated linoleic acid (CLA) on the amount of nutrients, flavour substances, and healthcare fatty acids, the physicochemical properties, and the potential molecular mechanisms in the muscles of sub-adult grass carp (*Ctenopharyngodon idella*). Fish were fed graded levels of CLA (0.0, 3.1, 6.4, 9.6, 12.7, and 15.9 g/kg diets) for 60 days. Protein, glutamic acid, alanine, inosine monophosphate (IMP), eicosapentaenoic acid (EPA; 20:5n-3), docosahexaenoic acid (DHA; 22:6n-3), and total CLA contents ($p < 0.05$) increased in CLA 3.1 ~ 12.7, 6.4 ~ 9.6, 6.4 ~ 9.6, 6.4 ~ 15.9, 3.1 ~ 9.6, 3.1 ~ 9.6, and 3.1 ~ 15.9 g/kg diet, respectively ($p < 0.05$). In addition, optimal CLA significantly increased pH_{24}, shear force, collagen content, and myofibre density in the muscle ($P < 0.05$); however, it decreased myofibre diameter ($p < 0.05$). We concluded that 6–9 g/kg CLA in the diet could improve the flesh quality of sub-adult grass carp.

Introduction

Fish and fish products have attracted the attention of consumers and the aquaculture industry because they are nutritionally rich and beneficial to human health. Recently, high-quality fish products, which not only provide nutrition but also have health and other benefits, have been favoured by consumers. Conjugated linoleic acid (CLA) is a group of isomers of linoleic acid (C18:2n6) with conjugated double bonds, which has the potential to decrease body fat and cholesterol, increase eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) in muscle, enhance the lean meat rate, and improve muscle texture in pigs (Joo, Lee, Ha & Park, 2002; Rossi, Pastorelli, Musella, & Corino, 2005). There are great differences in the muscle between mammals and fish. Myofibre hyperplasia terminates immediately after birth in mammals, while proliferation and enlargement of myofibre occurs throughout life in fish (Yu et al., 2017). However, the studies on the effects of dietary CLA on flesh quality are limited and not systematic.

Flesh quality includes nutrition, flavour, and health quality (Yang et al., 2019). Amino acids such as aspartic acid (Asp), glycine (Gly), glutamate (Glu), and inosine monophosphate (IMP) contribute to the flavour of meat (Fang et al., 2021). Some polyunsaturated fatty acids (such as CLA, EPA, and DHA) have health benefits such as anti-cancer and immune enhancement in humans (Koba & Yanagita, 2014; Liu & Ma, 2014). Grass carp (*Ctenopharyngodon idella*), one of the four major fish species in China, is highly favoured by consumers because of its rich nutrition and delicious meat. Grass carp can be classified into juvenile grass carp, on-growing grass carp, and sub-adult grass carp, according to their growth stage; among them, sub-adult grass carp are usually marketed. The effects of CLA on muscle nutrition and health in grass carp have been studied only in the juvenile stage. However, the effects of CLA

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on muscle lipids and fatty acids in fish at different stages vary. In large yellow croaker (*Larmichthys crocea*), dietary CLA had no effects on EPA and DHA contents, but increased the lipid content in muscle in the juvenile stage (7.56 g) (Zuo, Ai, Mai, & Xu, 2013). Dietary CLA could increase EPA and DHA content, but had no effects on lipid content in the muscle of the youth stage (146.25 g) (Zhao, Wu, Tang, Pan, & Zhang, 2008). Studies on the effects of CLA on muscle protein and fatty acids in sub-adult grass carp are lacking and require further exploration. Surprisingly, there are no reports on the effects of CLA on amino acids and IMP in fish. CLA could increase the serum insulin levels in mice (Zhang et al., 2016). In *Fundulus heteroclitus*, insulin could promote the absorption of amino acids into muscle (Lu, Wang, Song, Chibbar, & Meng, 2008). Nevertheless, whether CLA affects the nutrition and health of sub-adult grass carp is unknown and requires further study.

In addition to the above qualities, the physicochemical quality of flesh is also an important indicator for evaluating flesh quality, among which the potential of hydrogen (pH) and hardness are key factors. After slaughter, the respiration and oxygen supply in fish muscle cells are interrupted; this enhances anaerobic glycolysis; therefore, the pH decreases due to the accumulation of a large amount of lactic acid (Indergård, Tolstorebrov, Larsen, & Eikevik, 2014). The effect of CLA on anaerobic glucose metabolism in grass carp remains unclear and requires further study. In addition, cathepsins B and L can reduce the muscle hardness of rainbow trout (*Oncorhynchus mykiss*) (Godiksen, Morzel, Hyldig, & Jessen, 2009). In addition to cathepsins, muscle hardness is also closely related to collagen synthesis [regulated by transforming growth factor β1 (TGF-β1)/ drosophila mothers against decapentaplegic protein (Smads) and target of rapamycin (TOR) signalling] and myofibre growth [regulated by mitogen-activated protein kinase (MAPK) and myostatin (MSTN) signalling] (Yang et al., 2019). There are no reports on the effects of CLA on fish cathepsins, collagen synthesis, muscle fibre growth, and related signalling pathways. n3 and n6 fatty acids can reduce the activity of cathepsin B and L in the muscle of Atlantic salmon (Bahuaud et al., 2009). Dietary CLA can increase the level of adiponectin in the muscle of grass carp (Zou et al., 2018), which is the upstream signalling molecule of TGF-β1/Smad signalling in rats (Gao, Liu, Han, Wu, & Li, 2017). Therefore, CLA might affect cathepsins' activity, collagen synthesis as well as muscle fibre growth, thereby affecting muscle hardness, which requires further study.

Therefore, it is important to determine an appropriate amount of CLA. Studies on the optimal amount of CLA in grass carp have focused only on the juvenile stage (Dong et al., 2014; Zou et al., 2018). However, CLA plays different roles in different growth stages of aquatic animals. In the large yellow croaker (*Pseudosciaena crocea* R.), 0.42 % CLA could promote juvenile growth (Zuo, Ai, Mai, & Xu, 2013); however, it is not needed in the youth stage (Zhao, Wu, & Tang, 2009). To the best of our knowledge, the optimal amount of CLA to be added to sub-adult grass carp was not determined and needs to be investigated.

Therefore, this study is the first to explore the effects of CLA on flavour quality indicators, collagen synthesis, and muscle growth in fish muscle; this could provide a theoretical basis for CLA supplementation for improving flesh quality. The optimal supplemental amount in sub-adult grass carp was determined based on the growth performance and flesh quality indices, which provide guidance for the production of high-quality grass carp.

Materials and methods

Animal

All procedures in this study were approved by the University of Sichuan Agricultural Animal Care Advisory Committee. Grass carp were obtained from a local fishery (Sichuan, China) and domesticated to adapt to the culture conditions for 30 d before experimentation.

Experimental design and feeding management

The formulations and nutrient compositions of the experimental diets are listed in Table S1. In brief, fish meal, cottonseed meal, rapeseed meal, and soybean meal were chosen to formulate the main protein sources, and the main lipid sources were provided by fish oil and soybean oil, which satisfied the omega-3 polyunsaturated fatty acid (n3-PUFA) and omega-6 polyunsaturated fatty acid (n6-PUFA) needs of the grass carp. CLA (containing 94.2 % CLA mixture) was purchased from Qingdao Aohai Biotech Co., ltd. (Qingdao, China), and the contents of c9, t11-CLA and t10, c12-CLA were 45.4 % and 44.5 %, respectively. Graded levels (0.0 %, 0.30 %, 0.60 %, 0.90 %, 1.20 %, and 1.50 %) of CLA oils were supplemented to the basal diet to present six isonitrogens, isolipids, and isoenergy diets, with the expense of coconut oil (Qingdao Haizhiyuan Life Technology Co., ltd) according to Zou et al. (2018). The diets were then mixed, granulated, and stored at −20 °C. CLA content in the treatment groups was determined to be 0.0 %, 0.32 %, 0.64 %, 0.96 %, 1.27 %, and 1.59 %, respectively. Each diet was randomly distributed into one treatment with three net cages (L1.4 m × W1.4 m × H1.4 m), with 25 grass carps in each cage. Each net cage was equipped with a 50 cm radius disc which was made of 1 mm gauze at the bottom to collect the residual feed. The fish were fed their respective experimental diets to apparent satiation four times a day (8:00, 11:00, 15:00, and 19:00) for 60 days, and residual feed was collected to calculate feed intake. The dissolved oxygen was above 6 mg/L, the water temperature was 27.7 ± 1.8 °C, and the pH was 7.6 ± 0.2. A feeding trial was conducted under natural light conditions.

Sample collection

Grass carp were weighed at the beginning and end of the experiment to calculate the percentage weight gain (PWG) and feed coefficient rate (FCR). All fish were subjected to fasting for 12 h before sampling. Subsequently, six healthy grass carp from each treatment were anaesthetised with *para*-aminobenzoic acid to avoid catching stress. Blood was collected quickly from the caudal vein using a syringe. Blood samples were centrifuged to separate the serum. Fish were sacrificed by a sharp blow to the head according to our previous laboratory study (Jiang et al., 2016). Muscle samples of grass carp were intercepted from the left side and immediately stored for biochemical parameters and molecular analysis. Simultaneously, muscle samples from the other side of the same fish were rinsed with normal saline and soaked in 4 % paraformaldehyde fixative. All sampling processes were performed on ice.

Analysis of blood biochemical indexes

Serum was used to determine the lipid composition of blood using the Nanjing Jiancheng Kit [total triglyceride (TG), total cholesterol (*T*-CHO), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C)] and Shanghai Sinovax Kit (fatty acid synthase, FAS), according to the manufacturer's instructions. The details are as follows: the colour intensity of the quinone compounds is proportional to the content of TG and *T*-CHO; the absorbance of the standard and sample was measured at 510 nm, and the concentrations of TG and *T*-CHO in the sample were calculated. The serum levels of LDL-C and HDL-C were determined using the direct method by measuring absorbance at 546 nm. FAS content was determined using the double antibody sandwich method, with absorbance measured at 450 nm.

Determination the muscle colour of grass carp

The muscle colour of grass carp was measured using Sanenshi NR145, according to the method of Liu, Li, Xia, Regenstein and Zhou (2013). After removing the viscera, the carcase of grass carp was equally divided into three parts to measure the muscle colour. The measured

values were expressed as L* (+represents white, - represents dark), a* (+represents red, while - represents green), and b* (+represents yellow, - represents dark blue). Whiteness = 100-[(100-L*) ^{2} + a*^{2} + b*^{2}]$^{0.5}$.

Histological observation

The muscle samples were stored in 4 % paraformaldehyde solution and embedded in paraffin. The paraffin-embedded samples were cut into 4 μm slices for haematoxylin-eosin (HE) staining. A Nikon light microscope (TS100) was used to observe the stained slides. According to Yu et al. (2017), 300 myofibres were randomly selected from each sample, and the cross-sectional area of the myofibres was measured using Image-Pro Plus 6.0. The cross-section of the myofibres was assumed to be a circle; therefore, the diameter was calculated using the formula d = [(s/π) $^{1/2}$] * 2 (where "s" is the myofibre area and "d" is the myofibre diameter). Myofibre density was calculated as the number of fibres per mm^2 of the muscle cross-sectional area.

Biochemical analysis

The muscle pH, shear force, and cooking loss were determined according to the method described by Yang et al. (2019). Muscle moisture, crude protein, and lipid contents were determined according to the method of AOAC (2000) (Cunniff, Horwitz, & Latimer, 2000). The muscle collagen content was determined using the method described by Fang et al. (2021). Cathepsin B/L content was determined using an appropriate kit (Qisong Bioengineering Institute, Beijing, China). The activities of lactate dehydrogenase (LDH), pyruvate kinase (PK), phosphofructokinase (PFK), and hexokinase (HK) were determined using an assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), similar to that in Yang et al. (2021). The muscle glycogen and lactic acid contents were determined using the anthrone colorimetric method and nitroblue tetrazolium (NBT) reduction method, according to Yang et al. (2021) and Zeng et al. (2018). The free amino acid content in the muscle was measured using an automatic amino acid analyser, and the fatty acid constituents were determined using the gas chromatographic method, as described by Fang et al. (2021). Muscle protein content was measured using Coomassie Bright Blue, which was used to calculate enzyme activity.

Real-time polymerase chain reaction (PCR) analysis

The target gene expression was determined using real-time fluorescence quantitative PCR. Total ribonucleic acid (RNA) in the muscle of grass carp was extracted using the RNAiso Plus kit (Takara Bio, Japan). Agarose gel electrophoresis was used to verify the integrity of total RNA, and spectrophotometric values at 260 and 280 nm was used to determine the purity of total RNA. RNA samples with high integrity and purity were selected and reverse-transcribed into single-stranded cDNA using the PrimeScript^{TM} RT reagent kit (Takara Bio, Japan). The specific primers were designed based on the grass carp sequence information obtained through cloning in our laboratory and published in the National Centre for Biotechnology Information (NCBI) (Table S2). Subsequently, the specificity and purity of the PCR products were determined by analysing the melting curves after amplification. β-actin was chosen as the reference gene, based on the preliminary experiments for internal reference gene evaluation. The amplification efficiency was ~ 100 %. Expression levels were calculated using the $2^{-\Delta\Delta CT}$ method, as described by Kenneth and Thomas (2002).

Western blotting

We analysed the effects of different levels of dietary CLA on the synthesis of type I collagen-related signal molecular proteins in the muscle of grass carp through western blotting, as described earlier with a few modifications (Jiang et al., 2016). The muscle samples of grass carp (six samples per treatment) stored in an ultra-low temperature refrigerator were placed into 2 mL tubes preloaded with lysate [8 μL phenylmethylsulfonyl fluoride (PMSF) + 400 μL Radio Immunoprecipitation Assay (RIPA)]. Protein homogenates were prepared using a tissue grinder and were incubated for 15 min; the homogenate was centrifuged at 13,000 r/min (4 °C, 15 min) to obtain the supernatant. The protein concentration in the supernatant was measured using a bicinchoninic acid (BCA) protein assay kit (Biyuntian, Shanghai). We adjusted the protein concentration of the supernatant in each tube to the same level as that of the appropriate lysate. Subsequently, equal amounts of protein samples were loaded from the respective treatments per lane and electrophoresed on a sodium dodecyl sulphate (SDS)-glycine polyacrylamide gel. The protein samples were then transferred to a polyvinylidene difluoride (PVDF) membrane for western blot analysis. The PVDF membrane was sealed for 1.5 h at room temperature and then incubated with primary antibodies (anti-total-TOR, p-TOR Ser2448, total-Smad2, p-Smad2 Ser467, Smad4 and β-actin) overnight at 4 °C. β-actin used as the internal reference protein for cytoplasmic and total proteins, as described previously (Jiang et al., 2016). The PVDF membranes were washed thrice with Tris-buffered saline with Tween (TBST), followed by incubation with horseradish peroxidase (HRP)-labelled secondary antibodies (A0208, 1:8000 diluted, Biyuntian, Shanghai) for 1.5 h. The bands were visualised and quantified using an enhanced chemiluminescence (ECL) luminescent agent (Affinity) and Image Lab software (version 3.0).

Data statistics and analysis

All statistical analyses were performed using Statistical Product and Service Solutions (SPSS) 27.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to evaluate the results. When there were significant differences between the experimental groups ($p < 0.05$), the groups were further compared using Duncan's multiple range test. All data are presented as mean ± standard deviation (SD). Correlation and regression analyses were performed on biologically correlated and statistically significant indicators. Based on the indicators of growth performance and muscle quality, the optimal supplemental level of dietary CLA for sub-adult grass carp was determined using a matching mathematical model. Growth performance indicators were calculated as follows:

PWG = [final body weight (g/fish) - initial body weight (g/fish)]/ initial body weight (g/fish) × 100

Food coefficient rate = weight gain (g/fish)/ feed intake (g/fish) × 100

Results

Effects of CLA on growth performance and blood biochemical index of sub-adult grass carp

The effects of CLA on the growth performance of sub-adult grass carp are shown in Table 1. The survival rate in each experiment was 100 %. Under the experimental conditions, no significant differences were observed in the FCR of grass carp ($p > 0.05$). The grass carp that consumed the diet supplemented with 6.4 g CLA /kg diet had significantly higher feed intake (FI) and PWG than that in the control ($p < 0.05$). Dietary CLA significantly reduced the levels of LDL-C, *T*-CHO, and TG in the serum of sub-adult grass carp ($p < 0.05$); they were the highest in fish fed diet with 6.4 g CLA /kg diet. However, serum HDL-C and FAS contents gradually increased and then plateaued (at 6.4 and 12.7 g/kg diet, respectively) with the increase in CLA ($p < 0.05$). Subsequently, quadratic regression based on PWG was performed to determine the optimal supplemental level of CLA for sub-adult grass carp; it was 6.4 g/kg diet.

Table 1
Effect of dietary CLA on growth performance, blood biochemical indices, and muscle colour of sub-adult grass carp.

	Dietary CLA levels (g/kg diet)					
	0.0	3.1	6.4	9.6	12.7	15.9
Growth performance						
IBW	664.00 ± 3.67^{a}	663.20 ± 3.20^{a}	664.53 ± 1.67^{a}	661.60 ± 2.12^{a}	664.00 ± 2.12^{a}	662.67 ± 2.81^{a}
FBW	1341.80 ± 12.52^{ab}	1402.13 ± 3.28^{bc}	1454.40 ± 76.11^{c}	1352.27 ± 32.42^{ab}	1358.93 ± 77.00^{abc}	1287.73 ± 44.90^{a}
FI	1145.74 ± 8.81^{b}	1225.99 ± 0.87^{d}	1308.08 ± 1.07^{f}	1231.25 ± 0.77^{e}	1158.33 ± 0.83^{c}	1099.32 ± 0.84^{a}
PWG	102.09 ± 3.00^{ab}	111.41 ± 6.35^{bc}	118.86 ± 11.38^{c}	104.39 ± 4.77^{abc}	104.65 ± 11.26^{abc}	94.34 ± 7.29^{a}
FCR	1.70 ± 0.03^{a}	1.66 ± 0.10^{a}	1.67 ± 0.16^{a}	1.79 ± 0.08^{a}	1.68 ± 0.17^{a}	1.77 ± 0.13^{a}
Blood biochemical indices						
LDL-C	5.77 ± 0.48^{e}	4.65 ± 0.29^{b}	2.64 ± 0.38^{a}	3.32 ± 0.13^{b}	3.90 ± 0.46^{c}	4.24 ± 0.41 ^{cd}
HDL-C	2.33 ± 0.25^{a}	3.04 ± 0.4^{b}	3.69 ± 0.57^{c}	3.82 ± 0.42^{c}	3.69 ± 0.57^{c}	3.53 ± 0.35^{bc}
T-CHO	6.49 ± 0.34^{c}	5.15 ± 0.26^{ab}	5.04 ± 0.39^{a}	5.62 ± 0.38^{b}	6.42 ± 0.47^{c}	6.53 ± 0.55^{c}
TG	4.33 ± 0.33^{d}	3.27 ± 0.13^{b}	2.25 ± 0.15^{a}	4.00 ± 0.24^{c}	4.33 ± 0.33^{d}	4.18 ± 0.29 ^{cd}
FAS	132.88 ± 11.55^{a}	127.31 ± 17.15^{a}	130.38 ± 19.95^{a}	137.03 ± 17.16^{a}	161.61 ± 25.56^{b}	161.94 ± 24.27^{b}
Regression						
$y_{PWG} = -0.2399x^2 + 3.0751x + 103.52$				X = 6.41	$R^2 = 0.7858$	$P = 0.099$

Values are means ± SD for three replicate groups. And different superscripts in the same row are significantly different (P < 0.05), the following table shows the same. IBW, Initial body weight (g/fish); FBW, Finish body weight (g/fish); FI, Feed intake (%); PWG, Percentage weight gain (%); FCR, Food coefficient rate (%); LDL-C, low density lipoprotein cholesterol (mmol/L); HDL-C, high-density lipoprotein cholesterol (mmol/L); *T*-CHO, total cholesterol (mmol/L); TG, triglyceride (mmol/L); FAS, fatty acid synthetase (nmol/L).

Effects of CLA on colour, nutrient components, and physicochemical property in muscle of sub-adult grass carp

The influence of dietary CLA on muscle colour of sub-adult grass carp is shown in Table 2. The brightness (L*) of the muscle was significantly increased in response to 3.1 g/kg CLA supplementation ($p < 0.05$). CLA supplementation significantly increased redness (a*) of the muscle compared to that in the non-supplementation group ($p < 0.05$). Furthermore, the yellowness (b*) of muscle was significantly higher than that in the control group when the diet was supplemented with12.7–15.9 g/kg CLA ($p < 0.05$). However, CLA had no significant effect on muscle whiteness ($p > 0.05$).

The moisture content in the muscle gradually increased with increasing CLA concentration ($p < 0.05$). When CLA supplementation

Table 2
Effect of dietary CLA on nutrient components and physicochemical property in the muscle of sub-adult grass carp.

	Dietary CLA levels (g/kg diet)					
	0.0	3.1	6.4	9.6	12.7	15.9
Muscle color						
L*	42.59 ± 0.48^{ab}	43.35 ± 0.24^{c}	42.93 ± 0.40^{bc}	42.68 ± 0.57^{ab}	42.67 ± 0.69^{ab}	42.25 ± 0.47^{a}
a*	−2.84 ± 0.25^{a}	−2.48 ± 0.16^{b}	−2.28 ± 0.46^{b}	−2.27 ± 0.22^{b}	−2.40 ± 0.38^{b}	−2.42 ± 0.12^{b}
b*	−2.13 ± 0.25^{a}	−2.14 ± 0.37^{a}	−2.05 ± 0.37^{ab}	−1.95 ± 0.20^{ab}	−1.44 ± 0.16^{c}	−1.74 ± 0.17^{bc}
Whiteness	42.47 ± 0.47^{ab}	43.07 ± 0.23^{b}	43.04 ± 0.39^{b}	42.58 ± 0.56^{ab}	42.60 ± 0.69^{ab}	42.17 ± 0.47^{a}
Muscle nutrition components						
Moisture	69.15 ± 1.63^{a}	68.34 ± 1.95^{a}	71.07 ± 2.75^{ab}	70.74 ± 3.42^{ab}	71.46 ± 3.92^{ab}	73.87 ± 2.63^{b}
Lipid	3.50 ± 0.28^{b}	3.09 ± 0.28^{a}	3.04 ± 0.27^{a}	3.25 ± 0.27^{ab}	3.34 ± 0.14^{ab}	3.35 ± 0.28^{ab}
Protein	16.36 ± 0.47^{ab}	18.07 ± 1.17^{bc}	18.96 ± 1.79^{c}	18.43 ± 1.89^{c}	17.89 ± 1.73^{bc}	16.04 ± 1.12^{a}
Cathepsin B	90.64 ± 4.94^{c}	74.58 ± 4.20^{a}	80.88 ± 4.18^{b}	91.76 ± 3.80^{c}	92.28 ± 5.72^{c}	89.37 ± 4.06^{c}
Cathepsin L	6.03 ± 0.58^{a}	5.76 ± 0.35^{a}	5.57 ± 0.35^{a}	5.95 ± 0.02^{a}	6.02 ± 0.20^{a}	5.98 ± 0.32^{a}
FAS	1235 ± 130.01^{a}	1088 ± 81.34^{a}	1180 ± 148.51^{a}	1260 ± 111.31^{a}	1512 ± 155.07^{b}	1633 ± 184.02^{b}
LPL	1.01 ± 0.16^{a}	1.26 ± 0.21^{b}	1.29 ± 0.13^{b}	1.19 ± 0.22^{ab}	1.11 ± 0.22^{ab}	1.04 ± 0.19^{ab}
HSL	1.02 ± 0.21^{a}	3.98 ± 1.34^{c}	4.59 ± 1.00^{c}	2.90 ± 0.76^{b}	2.44 ± 0.51^{b}	0.77 ± 0.26^{a}
Muscle physicochemical property						
pH_{24h}	6.71 ± 0.02^{a}	6.73 ± 0.05^{ab}	6.79 ± 0.04^{b}	6.71 ± 0.04^{a}	6.74 ± 0.06^{ab}	6.75 ± 0.08^{ab}
Cooking loss	12.49 ± 1.52^{a}	12.90 ± 1.40^{a}	13.74 ± 2.40^{ab}	14.10 ± 1.86^{ab}	15.60 ± 1.00^{b}	15.15 ± 1.36^{b}
Shear force	1.11 ± 0.16^{a}	1.75 ± 0.35^{c}	1.63 ± 0.36^{bc}	1.51 ± 0.33^{bc}	1.49 ± 0.31^{bc}	1.33 ± 0.30^{ab}
ATP	24.62 ± 1.49^{b}	23.82 ± 2.20^{b}	23.41 ± 2.57^{b}	23.86 ± 2.71^{b}	23.48 ± 2.05^{b}	14.03 ± 1.62^{a}
CK	1.40 ± 0.07^{a}	1.41 ± 0.03^{a}	1.36 ± 0.08^{a}	1.34 ± 0.03^{a}	1.32 ± 0.02^{a}	1.23 ± 0.12^{b}
Muscle glycogen	1.93 ± 0.10^{b}	2.00 ± 0.07^{b}	4.00 ± 0.14^{e}	3.71 ± 0.10^{d}	2.83 ± 0.13^{c}	1.63 ± 0.20^{a}
LDH	23.85 ± 2.21^{c}	16.86 ± 0.65^{b}	13.11 ± 0.93^{a}	14.32 ± 0.83^{a}	16.71 ± 1.10^{b}	27.73 ± 0.85^{d}
Lactate	1.75 ± 0.21^{b}	1.47 ± 0.18^{a}	1.39 ± 0.07^{a}	1.44 ± 0.04^{a}	1.67 ± 0.11^{b}	1.63 ± 0.07^{b}
PK	66.67 ± 6.84 ^{cd}	62.05 ± 7.25^{bc}	43.97 ± 4.59^{a}	55.41 ± 5.58^{b}	68.00 ± 6.03 ^{cd}	73.55 ± 5.10^{d}
HK	5.91 ± 0.37^{d}	4.50 ± 0.61^{ab}	4.23 ± 0.48^{a}	4.35 ± 0.62^{a}	5.13 ± 0.56^{bc}	5.46 ± 0.59 ^{cd}
PFK	1.28 ± 0.12^{b}	0.92 ± 0.09^{a}	0.83 ± 0.11^{a}	1.26 ± 0.17^{b}	1.29 ± 0.15^{b}	1.36 ± 0.14^{b}
Collagen	1.47 ± 0.06^{b}	1.57 ± 0.13^{b}	2.25 ± 0.23^{d}	2.25 ± 0.20^{d}	1.85 ± 0.10^{c}	1.24 ± 0.14^{a}
Regression						
$Y_{\text{crude protein in muscle}} = -0.0437x^2 + 0.6582x + 16.404$				X = 7.53	$R^2 = 0.9847$	$P < 0.01$
$Y_{\text{collagen}} = -0.0144x^2 + 0.2229x + 1.3235$				X = 7.74	$R^2 = 0.8378$	$P = 0.065$
$Y_{\text{myofibre density}} = -0.7908x^2 + 11.558x + 96.985$				X = 7.31	$R^2 = 0.8926$	$P < 0.05$

Values are means ± SD for three replicate groups. And different superscripts in the same row are significantly different ($P < 0.05$), the following table shows the same. L* (brightness), "+"means white, "-"means dark; a* (redness), "+"means red, "-"means green; b* (yellowness), "+"means yellow, "-"means blue. Moisture (%); Protein (%); Lipid (%); Cathepsin B (ng/g tissue); Cathepsin L (ng/g tissue); FAS (nmol/g tissue). LPL (lipoprotein lipase) and HSL (hormone-sensitive lipase) were mRNA levels; Cooking loss (%); Shear force (Kgf); ATP (adenosine-triphosphate, μmol/g protein); Muscle glycogen (mg/g tissue); LDH (lactate dehydrogenase, U/mg protein); Lactate (mmol/g protein); PK (pyruvate kinase, U/g protein); HK (hexokinase, nmol/min/mg protein); PFK (phosphofructokinase, U/mg protein); Collagen (mg/g tissue weight).

was 6.4 g/kg diet, the crude protein in muscle was the highest, while crude lipid in muscle was the lowest for the experimental fish. Compared with that in the non-supplemented group, the CLA group fed with diet supplemented with 3.1 g CLA/kg diet had a lower cathepsin B content ($p < 0.05$). Similarly, the cathepsin L content slightly increased with increasing CLA levels, although there was no significant effect within the range of 0–15.9 g CLA/kg diet ($p > 0.05$). The content of FAS was not significantly different when the CLA supplementation level was 0–9.6 g/kg, but it increased significantly when the CLA supplementation level increased continuously. We further determined the mRNA levels of lipoprotein lipase (LPL) and hormone-sensitive lipase (HSL); the mRNA levels of LPL and HSL at 3.1–6.4 g/kg CLA supplementation were significantly higher than those in the other groups ($p < 0.05$).

There was no significant difference in cooking loss when grass carp were supplemented with CLA from 0 to 9.6 g/kg diet ($p > 0.05$), but cooking loss increased with increasing CLA ($p < 0.05$). The shear force reached a maximum when grass carp were supplemented with CLA at 3.1 g/kg diet ($p < 0.05$) and then gradually decreased. Muscle pH, muscle glycogen, and collagen content peaked at 6.4 g CLA /kg diet ($p < 0.05$) and slowly decreased thereafter. Conversely, the contents of lactate and HK, as well as the activities of LDH, PK, and PFK, reached a minimum at 6.4 g CLA /kg diet ($p < 0.05$) and gradually increased thereafter. Subsequently, the quadratic regression based on crude protein content, collagen content, and myofibre density in muscle were performed to determine the optimal dietary CLA level for sub-adult grass carp, which was 7.53, 7.74, and 7.31 g/kg diet, respectively.

Effects of CLA on fatty acid composition and Δ-6 desaturase (Δ-6D) mRNA level in muscle of sub-adult grass carp

Dietary CLA changed the fatty acid composition of the muscles of the sub-adult grass carp (Table 3). The content of C12, C14, C16:1, C17:1, C18:1n9c, C20:1n9, and C22 fatty acids decreased with CLA supplementation ($p < 0.05$). In contrast, with an increase in CLA, the content of C18:1n9t was sustainably enhanced ($p < 0.05$). Compared with the non-supplemented group, the group fed with diet supplemented with 3.1 g CLA/kg diet had markedly increased contents of C15 and C22:6n3 ($p < 0.05$). The C16, C18:3n3 (ALA), C20:2, and C20:5n3 contents were the highest when sub-adult grass carp were fed diet supplemented with 6.4 g CLA/kg diet ($p < 0.05$). In addition, when the supplemental level of CLA was 9.6 g/kg diet, the content of C17 in the muscle of sub-adult grass carp was significantly higher than that in the other groups ($p < 0.05$). C18 content peaked at 12.7 g CLA/kg diet ($p < 0.05$). Additionally, we summarised the saturated fatty acid (SFA), unsaturated fatty acid (UFA), monounsaturated fatty acid (MUFA), PUFA, n3-PUFA, n6-PUFA, and total CLA contents in the muscle of sub-adult grass carp, as well as the ratio of n3-PUFA to n6-PUFA. The highest SFA content was observed when the sub-adult grass carp was supplemented with 6.4 g CLA/kg diet ($p < 0.05$). Conversely, the lowest content of UFA was obtained with the diet supplemented with 6.4 g CLA/kg diet ($p < 0.05$). MUFA decreased continuously with CLA supplementation ($p < 0.05$), whereas PUFA and *T*-CLA were sustainably enhanced with increasing CLA ($p < 0.05$). Dietary CLA supplementation did not markedly affect n6-PUFA. However, the ratio of n3-PUFA to n6-PUFA increased to a maximum when sub-adult grass carp were fed with a diet supplemented with 3.1 g CLA/kg diet ($p < 0.05$). We tested the mRNA level of *Δ-6D*; the addition of CLA increased the mRNA level of *Δ-6D*. The mRNA level of *Δ-6D* at 6.4–12.7 g/kg CLA supplementation was significantly higher than that in the other groups ($p < 0.05$).

Effects of CLA on free amino acids and inosine monophosphate content in the muscle of sub-adult grass carp

The effects of CLA on muscle-free amino acids and IMP content in sub-adult grass carp are summarised in Table 4. Compared with that in the control group, the CLA group showed no significant difference in the

Table 3
Effects of CLA on the fatty acid composition (% of total fatty acid methyl esters) and Δ-6 desaturase mRNA levels in the muscle of sub-adult grass carp.

	Dietary CLA levels (g/kg diet)					
Fatty acids	0.0	3.1	6.4	9.6	12.7	15.9
C12:0	3.57 ± 0.06^{f}	2.63 ± 0.09^{e}	2.11 ± 0.05^{d}	1.44 ± 0.01^{c}	0.83 ± 0.03^{b}	0.09 ± 0.00^{a}
C14:0	3.93 ± 0.03^{f}	3.48 ± 0.05^{e}	3.35 ± 0.04^{d}	3.15 ± 0.01^{c}	2.72 ± 0.06^{b}	2.21 ± 0.02^{a}
C15:0	0.21 ± 0.004^{b}	0.23 ± 0.003^{e}	0.21 ± 0.002^{c}	0.22 ± 0.001^{d}	0.21 ± 0.004^{b}	0.20 ± 0.003^{a}
C16:0	21.67 ± 0.20^{a}	22.21 ± 0.47^{b}	23.15 ± 0.11^{c}	22.41 ± 0.28^{b}	22.18 ± 0.11^{b}	22.49 ± 0.26^{b}
C16:1	7.26 ± 0.02^{c}	5.50 ± 0.06^{b}	5.58 ± 0.02^{b}	5.08 ± 0.02^{a}	5.01 ± 0.18^{a}	4.99 ± 0.02^{a}
C17:0	0.37 ± 0.02^{a}	0.42 ± 0.01^{b}	0.42 ± 0.01^{b}	0.47 ± 0.01^{c}	0.46 ± 0.01^{c}	0.46 ± 0.01^{c}
C17:1	0.21 ± 0.00^{b}	0.21 ± 0.00^{b}	0.21 ± 0.00^{c}	0.21 ± 0.003^{b}	0.20 ± 0.003^{a}	0.20 ± 0.002^{a}
C18:0	4.45 ± 0.04^{a}	6.54 ± 0.09^{b}	6.54 ± 0.04^{b}	7.58 ± 0.02^{c}	8.22 ± 0.07^{d}	7.73 ± 0.03^{e}
C18:1n9t	0.28 ± 0.02^{a}	0.35 ± 0.01^{b}	0.40 ± 0.03^{c}	0.49 ± 0.002^{d}	0.48 ± 0.02^{d}	0.52 ± 0.02^{e}
C18:1n9c	35.54 ± 0.06^{c}	32.71 ± 0.48^{b}	32.42 ± 0.19^{ab}	32.54 ± 0.03^{ab}	32.42 ± 0.21^{ab}	32.32 ± 0.07^{a}
C18:2n6c	11.50 ± 0.20^{a}	11.45 ± 0.30^{a}	11.51 ± 0.12^{a}	11.67 ± 0.28^{a}	11.73 ± 0.07^{a}	11.49 ± 0.31^{a}
C20:0	0.22 ± 0.03^{a}	0.24 ± 0.01^{a}	0.20 ± 0.03^{a}	0.23 ± 0.04^{a}	0.21 ± 0.04^{a}	0.22 ± 0.03^{a}
C18:3n6	0.21 ± 0.01^{a}	0.20 ± 0.04^{a}	0.20 ± 0.01^{a}	0.20 ± 0.03^{a}	0.20 ± 0.03^{a}	0.21 ± 0.02^{a}
C20:1n9	0.17 ± 0.0004^{c}	0.16 ± 0.001^{b}	0.16 ± 0.002^{b}	0.16 ± 0.0003^{b}	0.16 ± 0.002^{a}	0.16 ± 0.001^{a}
C18:3n3 (ALA)	1.38 ± 0.003^{a}	1.42 ± 0.01^{c}	1.43 ± 0.01^{c}	1.40 ± 0.002^{b}	1.40 ± 0.03^{b}	1.39 ± 0.002^{ab}
c9,t11-CLA	0.10 ± 0.004^{a}	0.79 ± 0.02^{b}	1.36 ± 0.01^{c}	2.10 ± 0.001^{d}	2.65 ± 0.01^{e}	3.24 ± 0.005^{f}
t10,c12-CLA	0.03 ± 0.01^{a}	0.56 ± 0.07^{b}	1.07 ± 0.03^{c}	1.74 ± 0.03^{d}	2.25 ± 0.03^{e}	2.79 ± 0.003^{f}
C20:2	0.58 ± 0.006^{c}	0.61 ± 0.009^{d}	0.67 ± 0.004^{e}	0.50 ± 0.005^{b}	0.49 ± 0.005^{a}	0.49 ± 0.004^{a}
C22:0	0.42 ± 0.001^{d}	0.40 ± 0.011^{c}	0.35 ± 0.004^{a}	0.34 ± 0.003^{a}	0.36 ± 0.011^{b}	0.41 ± 0.001^{c}
C20:3n6	0.62 ± 0.002^{a}	0.61 ± 0.009^{a}	0.60 ± 0.030^{a}	0.61 ± 0.010^{a}	0.61 ± 0.017^{a}	0.60 ± 0.020^{a}
C23:0	1.64 ± 0.05^{a}	1.58 ± 0.22^{a}	1.49 ± 0.02^{a}	1.49 ± 0.11^{a}	1.64 ± 0.21^{a}	1.61 ± 0.13^{a}
C20:5n3 (EPA)	1.19 ± 0.01^{a}	1.52 ± 0.01^{c}	1.56 ± 0.06^{d}	1.43 ± 0.01^{b}	1.21 ± 0.03^{a}	1.22 ± 0.03^{a}
C22:6n3 (DHA)	3.75 ± 0.02^{b}	5.59 ± 0.04^{e}	4.30 ± 0.03^{d}	3.94 ± 0.04^{c}	3.77 ± 0.05^{b}	3.55 ± 0.03^{a}
SFA	36.74 ± 0.24^{b}	37.89 ± 0.50^{d}	38.00 ± 0.10^{d}	37.44 ± 0.32^{c}	36.89 ± 0.23^{b}	35.45 ± 0.38^{a}
MUFA	43.69 ± 0.06^{d}	39.12 ± 0.48^{c}	38.99 ± 0.19^{c}	38.68 ± 0.03^{b}	38.48 ± 0.16^{ab}	38.40 ± 0.06^{a}
PUFA	19.55 ± 0.20^{a}	22.97 ± 0.33^{b}	22.99 ± 0.17^{b}	23.86 ± 0.30^{c}	24.62 ± 0.12^{d}	25.29 ± 0.34^{e}
UFA	63.24 ± 0.24^{c}	62.09 ± 0.49^{a}	61.98 ± 0.11^{a}	62.54 ± 0.32^{b}	63.10 ± 0.24^{c}	63.68 ± 0.34^{d}
T-CLA	0.12 ± 0.01^{a}	1.35 ± 0.09^{b}	2.43 ± 0.03^{c}	3.84 ± 0.03^{d}	4.90 ± 0.03^{e}	6.03 ± 0.01^{f}
n-3PUFA	6.42 ± 0.02^{b}	8.64 ± 0.05^{f}	7.39 ± 0.04^{e}	6.87 ± 0.04^{d}	6.48 ± 0.07^{c}	6.26 ± 0.06^{a}
n-6PUFA	12.40 ± 0.19^{ab}	12.33 ± 0.29^{a}	12.44 ± 0.15^{ab}	12.58 ± 0.30^{ab}	12.66 ± 0.09^{b}	12.39 ± 0.33^{ab}
n-3/n-6	0.52 ± 0.01^{a}	0.70 ± 0.02^{d}	0.59 ± 0.01^{c}	0.55 ± 0.01^{b}	0.51 ± 0.01^{a}	0.51 ± 0.01^{a}
Δ-6D mRNA levels	1.05 ± 0.30^{a}	1.12 ± 0.18^{a}	1.72 ± 0.45^{bc}	2.00 ± 0.48^{c}	1.84 ± 0.37^{bc}	1.45 ± 0.23^{ab}
Regression						
$Y_{EPA} = -0.005x^2 + 0.07x + 1.2564$				X = 7.00	R^2 = 0.6839	P = 0.178

All data are presented as means ± SD (n = 6). SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; UFA, unsaturated fatty acid; *T*-CLA, total conjugated linoleic acid; n-3PUFA, n-3 polyunsaturated fatty acids; n-6 PUFA, n-6 polyunsaturated fatty acid; Δ-6D, Δ-6 desaturase.

sub-adult grass carp muscle Asp, Gly, valine (Val), and leucine (Leu) contents ($p > 0.05$). The content of muscle Glu, serine (Ser), alanine (Ala), lysine (Lys), threonine (Thr), and arginine (Arg) reached a maximum with 6.4 g CLA /kg supplementation ($p < 0.05$). The isoleucine (Ile) content gradually increased with dietary CLA supplementation ($p < 0.05$). Phenylalanine (Phe) content peaked at 12.7 g CLA /kg diet and then plateaued ($p < 0.05$). The highest histidine (His), tyrosine (Tyr), and proline (Pro) contents were observed when sub-adult grass carp were fed diet supplemented with 9.6 g CLA/kg diet ($p < 0.05$). IMP content was sustainably enhanced at a CLA concentration of 6 g/kg diet ($p < 0.05$) and then slowly decreased.

Effects of CLA on type I collagen synthesis and muscle growth of sub-adult grass carp

The influence of CLA on the morphological structure of the muscle tissue of sub-adult grass carp is shown in Fig. 1A; the diameter and density of myofibres are presented in the form of a line chart in Fig. 1B. CLA supplementation increased myofibre density in sub-adult grass carp but decreased myofibre diameter. When the CLA supplementation level was 6.4 g/kg diet, myofibre density reached the highest value ($p < 0.05$), while myofibre diameter had the lowest value ($p < 0.05$). For myofibre density, the optimal supplemental level of CLA was 7.31 g/kg diet.

The mRNA levels of the genes involved in muscle growth and type I collagen synthesis are shown in Fig. 1C. *CyclinB*, *CyclinE,* and *MyoG* mRNA levels in the 3.1–9.6 g CLA/kg diet group increased prominently compared to that in the non-supplemental groups ($p < 0.05$). In addition, *CyclinD*, *myogenic differentiation antigen* (*MyoD)*, *myogenic regulatory factor 4* (*MRF4),* and *myogenic factor 5 (Myf5)* mRNA levels in the 3.1–6.4 g CLA/kg diet group increased prominently compared to that in the non-supplemented group ($p < 0.05$). The mRNA expression of *p38 mitogen-activated protein kinase (p38MAPK)* gradually increased with increasing CLA levels, and the group fed diet supplemented with 6.4 g CLA/kg diet had the highest value ($p = 0.13$). In addition, compared with that in the other groups, the group fed with diet supplemented with 6.4 g CLA/kg diet had the highest mRNA expression of *MSTN1* and *MSTN2* ($p < 0.05$). The mRNA level of *TGF-β1* was markedly higher when sub-adult grass carp were fed with a diet supplemented with 3.1 g CLA/kg diet ($p < 0.05$). The *TOR*, *collagen type I alpha 1 (Col1α1)*, *Col1α2*, *Smad2,* and *Smad4* mRNA levels increased until they reached a maximum in the group supplemented with 6.4 g CLA/kg diet ($p < 0.05$) and then gradually decreased thereafter.

CLA supplementation had a positive impact on TOR (target of rapamycin), Smad2 protein levels, and phosphorylation in sub-adult grass carp muscle (Fig. 1D & E). Total Samd2, total TOR, and phosphorylation TOR (p-TOR) protein levels were at the maximum with the supplementation of 6.4 g CLA/kg diet for grass carp ($p < 0.05$), and the protein levels of phosphorylated Smad2 (p-Smad2) and Samd4 protein were markedly upregulated until they reached a maximum at 9.6 g CLA/kg diet ($p < 0.05$); they slightly decreased thereafter.

Discussion

CLA improved the growth performance of sub-adult grass carp

Appropriate CLA supplementation (6.4 g/kg diet) increased the growth performance of sub-adult grass carp in terms of feed intake and percentage weight gain. The enhancement of growth performance was similar to that in the large yellow croaker (Zuo, Ai, Mai, & Xu, 2013). In addition, we observed that the deposition of CLA in the muscle gradually increased with increasing dietary CLA content. Thus, CLA can be directly deposited in the muscles. Therefore, we explored the effects of dietary CLA on the muscle quality of grass carp.

CLA improved the colour in muscle of fish

The colour of fish flesh is an important factor in determining the freshness and market value of fish flesh, which is mainly determined by haemoglobin and myoglobin (Grunwald, Richards, & Chemistry, 2006). In general, the increase in brightness (L*) and redness (a*) of the flesh indicates that the fresher it is, the more popular it is with consumers. Dietary CLA increased the brightness (L*) and redness (a*) of muscle, suggesting that CLA could improve muscle colour in fish. CLA-mediated increase in the brightness (L*) and redness (a*) in fish muscle might be partly related to the increase in type I fibres. The haemoglobin and myoglobin in type I fibres are relatively high, which makes flesh fresh, ruddy, and shiny (Newcom, Stalder, Baas, Goodwin, & Wiegand, 2004). Dietary CLA promotes mRNA expression of myosin heavy chain I (MyHC I) in sows; this myosin heavy chain subtype corresponds to type I fibres (Qi, Chen, Peng, Jiang, Xiao, & Huang, 2015). Therefore, CLA could increase muscle brightness and redness, partly by promoting the mRNA expression of MyHC I, which requires further investigation.

Table 4
Effects of CLA on the contents of free amino acids and inosine monophosphate (IMP) (mg/100 g tissue) in the muscle of sub-adult grass carp.

	Dietary CLA levels (g/kg diet)					
	0.0	3.1	6.4	9.6	12.7	15.9
Aspartate	0.71 ± 0.21^{a}	0.73 ± 0.28^{a}	0.62 ± 0.37^{a}	0.69 ± 0.13^{a}	0.81 ± 0.28^{a}	0.55 ± 0.20^{a}
Glutamate	8.48 ± 0.63^{ab}	8.41 ± 0.23^{ab}	9.80 ± 1.63^{c}	9.24 ± 1.51^{bc}	8.16 ± 0.29^{ab}	7.81 ± 0.42^{a}
Serine	6.85 ± 0.86^{a}	6.90 ± 0.78^{a}	9.01 ± 2.34^{b}	9.26 ± 1.25^{b}	7.29 ± 1.07^{a}	6.24 ± 0.74^{a}
Glycine	136.89 ± 7.69^{a}	135.79 ± 4.94^{a}	140.04 ± 8.57^{a}	143.10 ± 5.23^{a}	142.50 ± 3.86^{a}	141.88 ± 5.27^{a}
Alanine	25.00 ± 0.32^{b}	25.64 ± 1.38^{bc}	31.00 ± 1.89^{d}	27.72 ± 3.64^{c}	24.54 ± 0.72^{b}	21.98 ± 1.68^{a}
Methionine	1.72 ± 0.19^{a}	1.75 ± 0.29^{a}	1.89 ± 0.29^{a}	1.90 ± 0.24^{a}	1.88 ± 0.26^{a}	1.70 ± 0.18^{a}
Lysine	27.81 ± 0.63^{a}	27.96 ± 2.44^{a}	40.96 ± 10.87^{c}	34.47 ± 1.38^{b}	30.63 ± 2.79^{ab}	29.46 ± 1.31^{ab}
Valine	5.82 ± 0.30^{a}	5.81 ± 0.23^{a}	6.46 ± 0.76^{a}	6.45 ± 0.47^{a}	6.29 ± 0.79^{a}	5.92 ± 0.39^{a}
Isoleucine	3.04 ± 0.28^{a}	3.10 ± 0.17^{ab}	3.45 ± 0.35^{c}	3.43 ± 0.32^{bc}	3.64 ± 0.23^{c}	3.78 ± 0.32^{c}
Phenylalanine	3.79 ± 0.18^{a}	3.78 ± 0.14^{a}	4.16 ± 0.38^{ab}	4.12 ± 0.60^{ab}	4.37 ± 0.35^{b}	4.36 ± 0.32^{b}
Leucine	6.81 ± 0.21^{ab}	6.38 ± 0.22^{a}	6.96 ± 1.24^{ab}	7.29 ± 0.67^{b}	7.27 ± 0.55^{b}	7.41 ± 0.50^{b}
Threonine	18.91 ± 0.68^{a}	18.31 ± 0.56^{a}	29.04 ± 2.01^{d}	26.16 ± 3.31^{c}	23.60 ± 0.87^{b}	22.06 ± 1.31^{b}
Arginine	32.29 ± 17.33^{ab}	35.30 ± 5.95^{ab}	49.92 ± 13.96^{c}	45.42 ± 8.84^{bc}	33.30 ± 9.80^{ab}	29.89 ± 4.24^{a}
Histidine	552.78 ± 17.64^{ab}	558.61 ± 25.10^{ab}	588.14 ± 59.43^{bc}	602.37 ± 31.08^{c}	563.22 ± 18.30^{abc}	532.71 ± 26.42^{a}
Tyrosine	3.96 ± 0.68^{a}	4.12 ± 0.41^{a}	5.42 ± 1.52^{b}	5.60 ± 0.83^{b}	5.29 ± 0.32^{b}	4.76 ± 0.52^{ab}
Proline	41.21 ± 7.45^{a}	39.28 ± 3.05^{a}	50.76 ± 12.87^{b}	51.67 ± 6.02^{b}	39.02 ± 6.61^{a}	35.50 ± 2.82^{a}
IMP	91.42 ± 8.33^{a}	91.28 ± 6.15^{a}	134.68 ± 10.91^{c}	126.30 ± 12.2^{bc}	123.34 ± 10.93^{bc}	115.10 ± 10.84^{b}

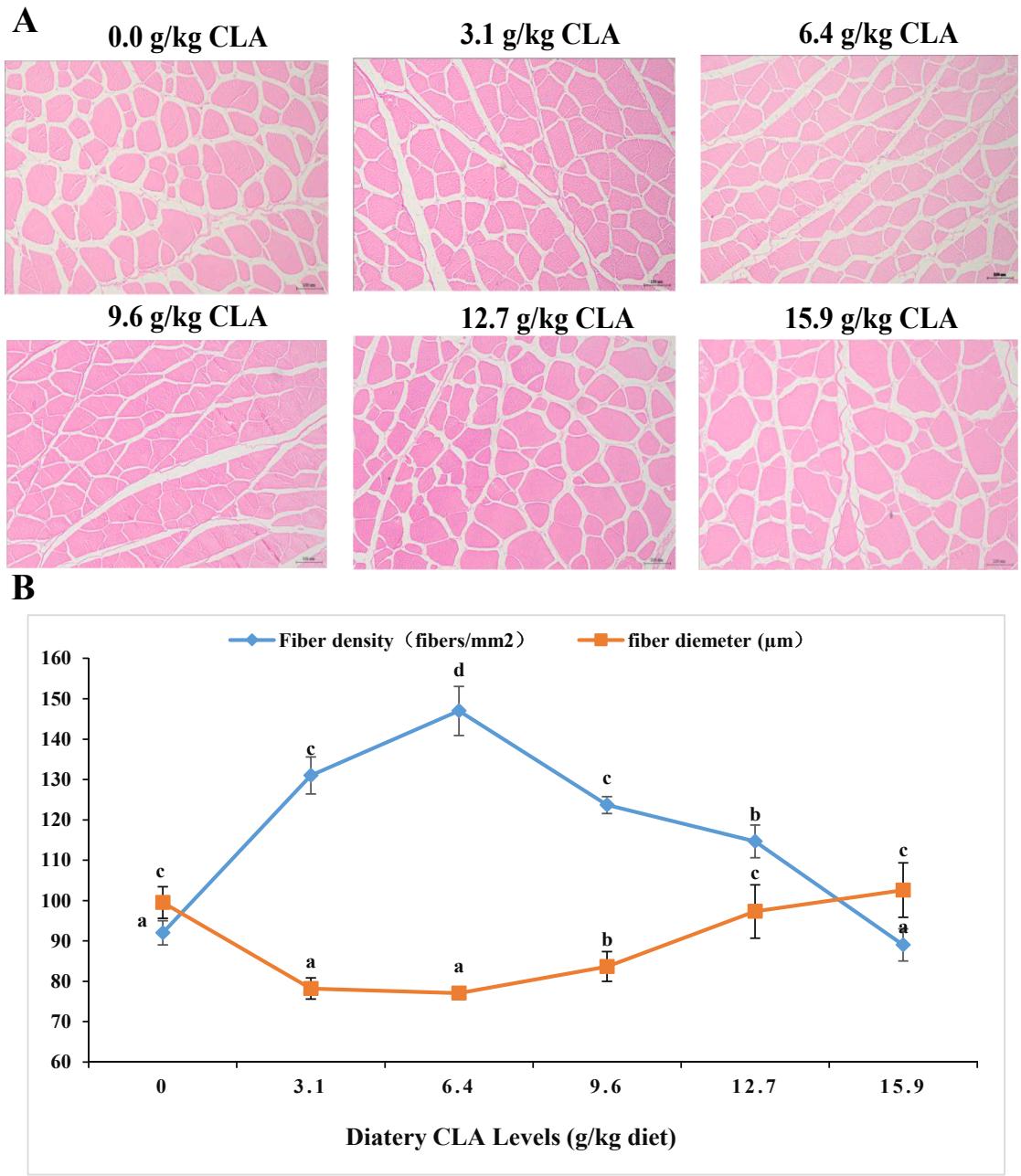

Fig. 1. Effects of conjugated linoleic acid on mRNA and protein levels of factors related to muscle type I collagen synthesis and myofibre growth in sub-adult grass carp. (A) Muscle transverse section microstructure (x100); (B) muscle fiber density (fibers/mm^2) and diameter (μm); (C) the mRNA levels of molecular related to type I collagen synthesis and myofibre growth; (D) the protein levels of T-Smad2, p-Smad2 and Smad4 in muscle; (E) the protein levels of p-TOR and *T*-TOR in muscl.

CLA promoted nutrition redistribution in muscle of fish

Muscle is the main edible part of aquatic products and its nutritional composition includes moisture, lipids, and proteins. The proper moisture content in the muscle makes the flesh tender and juicy; however, when the moisture is too high, the flesh becomes soft. CLA had no significant effect on grass carp muscle moisture content when supplemented at low levels; however, the highest level of CLA (15.9 g/kg) increased the moisture content, which is consistent with the results from Atlantic salmon (Berge, Ruyter, & ASgaRd, 2004).

CLA decreases body fat and increases lean tissue mass in mammals (Dong et al., 2014). CLA supplementation at appropriate levels (3.1–6.4 g/kg diets) reduced the lipid content in sub-adult grass carp muscle. This could be attributed to two reasons. First, CLA might be related to the reduced amount of lipids transported from the liver/blood to muscle. In grass carp, high amount of lipids derived from enhanced serum TG and *T*-CHO contents are more likely to induce deposition in the muscle rather than uptake by adipose tissue (Yuan et al., 2016). LDL-C and HDL-C are two forms that transport endogenous cholesterol from the liver/blood to other tissues and remove excess cholesterol, respectively (Marques et al., 2018). Appropriate CLA supplementation (6.4 or 6.4–9.6 g/kg diets) decreased the contents of TG, *T*-CHO, and LDL-C, and increased the HDL-C content in the blood of sub-adult grass carp, supporting our hypothesis. Second, the decrease in lipid content may be associated with the promotion of fatty acid catabolism (not anabolism) by CLA in fish muscle. Fat content is affected by anabolism and

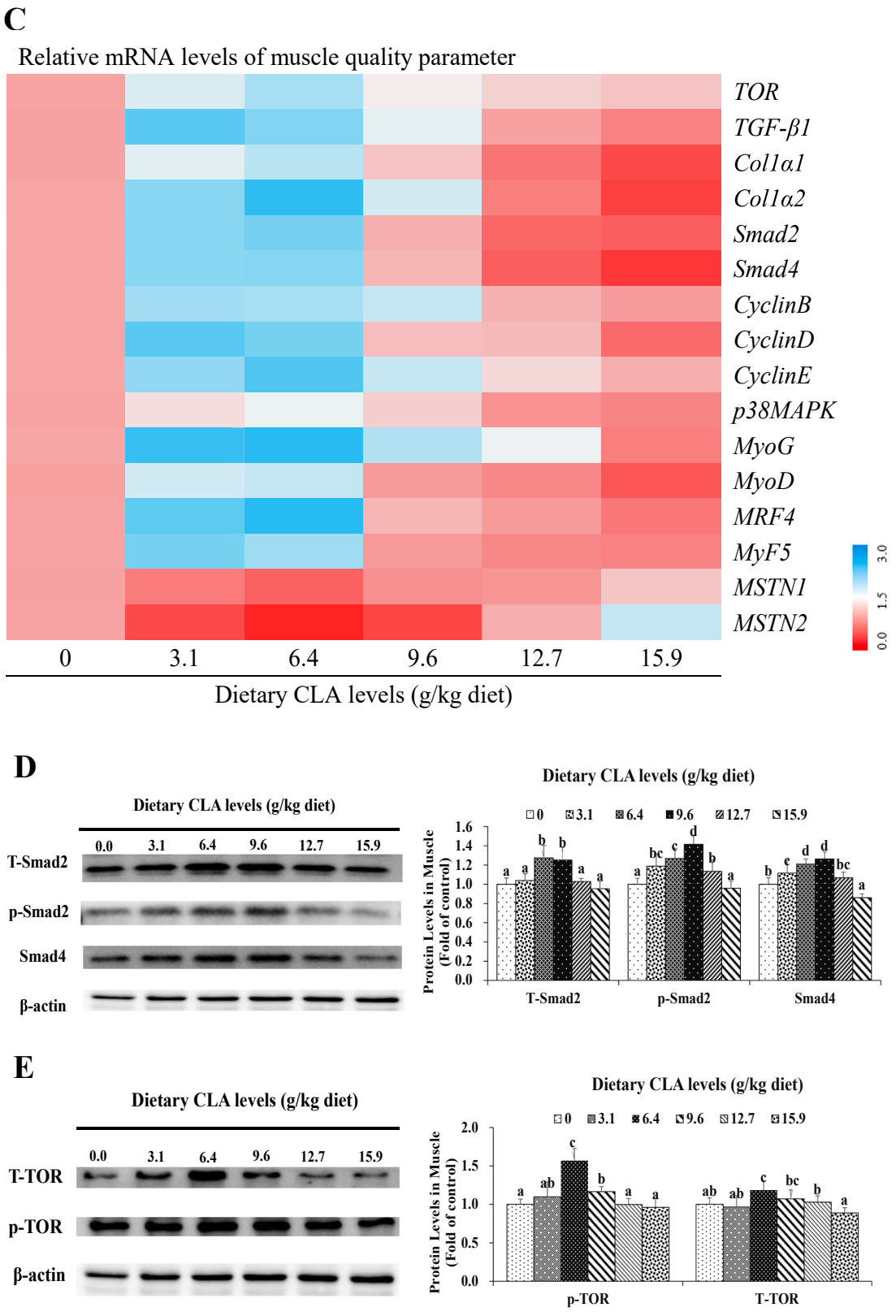

Fig. 1. (*continued*).

catabolism. FAS is the rate-limiting enzyme in fatty acid synthesis, whereas lipoprotein lipase (LPL) and hormone-sensitive lipase (HSL) are the key enzymes for lipolysis (Dong et al., 2014). Low levels of CLA (0–9.6 g/kg) have no significant impact on the FAS content in serum and muscle of sub-adult grass carp, while optimal levels of CLA increased the mRNA levels of LPL and HSL in sub-adult grass carp muscle; this in line with the results for young grass carp (Dong et al., 2014). Therefore, optimal levels of CLA could reduce the lipid content of grass carp muscle

by modulating the levels of LPL and HSL rather than regulating FAS.

Fish and fish products with high protein contents have higher nutritional value and are more popular among consumers. This study, for the first time, showed that CLA increased the protein content of sub-adult grass carp muscle. The CLA-induced increase in protein content may be attributed to two reasons. The CLA-elevated protein level might be partially attributed to decreased cathepsin B level (not that of L). Following slaughter, cathepsins can degrade myofibril protein in fish muscle and break them down into amino acids, small peptides, and amine compounds, thereby reducing muscle protein content (Ahmed, Donkor, Street, & Vasiljevic, 2013). CLA supplementation significantly reduced the content of cathepsin B in grass carp muscle but had no significant effect on cathepsin L. The CLA-induced elevation in protein levels could be attributed to the activation of TOR signalling. Protein synthesis in the muscle cells of rainbow trout is mainly regulated by the TOR signalling pathway, which is activated by the p-TOR level (Wang, Wu, Liu, Zhang, & Zhang, 2018). Addition of CLA increased the levels of *TOR* mRNA and p-TOR protein in the muscle of sub-adult grass carp. Therefore, the optimal level of CLA-mediated increase in protein content might be partly related to the decrease in cathepsin B (not L) and activation of the TOR pathway in the muscle of fish.

CLA improved flavour and health of flesh

Flavour is an important indicator for evaluating flesh quality and usually determines consumer acceptance of fish and fish products. Free amino acids and nucleotides are key flavouring substances in aquatic products. Glu, Asp, and IMP impart the umami taste of meat, while Gly and Ala provide the sweetness of meat (Yang et al., 2021). Dietary supplementation of 6.4–9.6 or 6.4–15.9 g/kg of CLA increased the contents of flavour-imparting amino acids (Ala and Glu) and IMP, suggesting that appropriate amount of CLA could enhance umami and sweetness in the muscle of sub-adult grass carp.

Flesh, as an important source of food fatty acids for humans including essential fatty acids not synthesised in humans, has important health effects on human (Yang et al., 2019). Among them, n3-PUFAs, including EPA (C20:5n3) and DHA (C22:6n3), have been extensively studied; they are recognised as fatty acids with health benefits (Liu & Ma, 2014). CLA has anticancer and antiatherosclerosis properties (Koba & Yanagita, 2014). To the best of our knowledge, this is the first study to show that CLA increased the deposition of EPA and DHA in sub-adult grass carp muscle, which might be related to the increase in Δ6-D. In fish, Δ6-D is a rate-limiting enzyme involved in the biosynthesis of highly unsaturated fatty acids (including EPA and DHA) (Yang et al., 2019). CLA enhanced the mRNA levels of Δ6-D in sub-adult grass carp, supporting our hypothesis.

CLA improved physicochemical property of flesh and the potential mechanisms

Following slaughter, respiration and oxygen supply in fish muscle cells are interrupted; this causes a decrease in the physicochemical properties of the flesh, such as muscle water holding capacity (WHC) and pH (Indergård, Tolstorebrov, Larsen, & Eikevik, 2014). In earlier studies on fish, cooking loss is usually used to reflect WHC; they are negatively correlated (Jiang et al., 2016). Low-dose CLA (0–9.6 g/kg) had no significant effect on cooking loss in grass carp muscle. Similar results were observed in pigs (Joo, Lee, Ha, & Park, 2002). In addition, this is the first study to show that supplementation with appropriate amount of CLA increases the pH of sub-adult grass carp muscle. The CLA-induced increase in pH might be partly related to the decrease in lactic acid. Our earlier study showed that the pH of grass carp meat after slaughter was negatively correlated with the content of lactic acid (Yang et al., 2021). In this study, supplementation with optimal amount of CLA reduced lactic acid content in the muscle of sub-adult grass carp, supporting our hypothesis. The CLA-induced decrease in lactic acid content could be mainly attributed to the anaerobic glycolysis of muscle glycogen. Under hypoxia conditions, degradation of muscle glycogen by the combined action of PFK, HK, PK, and LDH leads to the production of a large amount of lactic acid (Indergård, Tolstorebrov, Larsen, & Eikevik, 2014). Interestingly, appropriate amount of CLA could increase the content of muscle glycogen while significantly reducing the activities of PK, HK, PFK, and LDH in sub-adult grass carp muscle. Therefore, CLA could increase muscle pH partly by inhibiting aerobic glycolysis of muscle glycogen to reduce lactic acid content.

In addition to pH and WHC, hardness is commonly used to evaluate muscle quality. Hardness is assessed using the shear force of meat (Zhong, Zhang, Li, Huang, & Wang, 2011). Optimal CLA supplementation could enhance the shear force in the muscles of sub-adult grass carp. CLA causes an increase in the muscle shear force for three reasons.

First, the CLA-enhanced shear force may be partly attributed to the reduction in cathepsins. Cathepsins are mainly involved in the degradation of myofibrillar protein, which leads to the destruction of myofibrillar structure, thereby decreasing muscle hardness in rainbow trout (Godiksen, Morzel, Hyldig, & Jessen, 2009). CLA could decrease the content of cathepsin B but had no effect on cathepsin L. Therefore, CLA-induced enhancement of shear force might be partly related to the reduction in cathepsin B content (not L).

Second, the CLA-mediated enhancement in the shear force may be partly attributed to the increase in collagen content. Collagen is the main component of fish connective tissue. Collagen in grass carp muscle is mainly type I collagen, encoded by *Col1α1* and *Col1α2,* (Yang et al., 2021). CLA increased the content of collagen as well as the mRNA levels of *Col1α1* and *Col1α2* in sub-adult grass carp muscle. CLA-induced enhancement of collagen content and *Col1α1* and *Col1α2* might be linked to the activation of TGF-β/Smad signalling. The TGF-β/Smad signalling pathway is the main pathway that regulates the expression of type I collagen (Yang et al., 2021). CLA at 3.1–6.4 g/kg increased the mRNA levels of *TGF-β1*, as well as the mRNA and protein levels (phosphorylation) of Smad2 and Smad4 in grass carp muscle. In addition, the TOR pathway positively regulates the production of type I collagen (Shegogue & Trojanowska, 2004). Usually, phosphorylation of TOR can be used to monitor the activation of TOR signalling (Yang et al., 2019). CLA increased the mRNA and protein levels of TOR and p-TOR in sub-adult grass carp muscle. Consequently, CLA may regulate collagen synthesis in muscles partly by activating TGF-β/Smad and TOR signalling.

Lastly, the CLA-enhanced shear force might be partly attributed to the growth of the myofibres. Myofibre growth in fish is a dynamic process that can be regulated by nutritional strategies (Qi, Chen, Peng, Jiang, Xiao, & Huang, 2015). Dietary CLA (3.1–9.6 g/kg diets) could increase the density of sub-adult grass carp myofibres and reduce their diameter. Therefore, CLA promoted the proliferation and differentiation of myocytes. CLA-induced enhancement of myofibre proliferation may be linked to increased Cyclin mRNA levels. During the cell cycle, Cyclin B, Cyclin D, and Cyclin E mainly promote the transition of cells from the G1 to M phase (Hochegger, Takeda, & Hunt, 2008). CLA significantly increased the levels of *Cyclin B*, *Cyclin D,* and *Cyclin E* mRNAs in the muscle. In addition, CLA-enhanced myofibre differentiation may be attributed to an increase in the mRNA levels of myogenic regulatory factors. Study on mice have clarified that myogenic regulatory factors (such as MyoD, Myf5, MRF4, and myogenin) can transform non-muscle cells into myogenic cells (Lee, Tachibana, Morinaga, Fujimura, & Yamada, 2009). Supplementation with the appropriate amount of CLA increased mRNA levels of the myogenic factors, *MyoD*, *Myf5*, *MRF4,* and *myogenin* in sub-adult grass carp muscle. The CLA-induced increase in myogenic regulatory factor mRNA levels might be attributed to the activation of MAPK signalling. MAPK signalling pathway regulates the expression of myogenic regulatory factors (MyoD, Myf5, MRF4, and myogenin) in C2C12 muscle cells (Lee, Tachibana, Morinaga, Fujimura, & Yamada, 2009). Addition of CLA increased the level of *p38MAPK* mRNA, revealing that CLA-regulated expression of myogenic factors

might be partly related to p38MAPK signalling. MSTN is a member of the TGF-β family that transmits signals to the nucleus by binding to receptors, thereby downregulating the expression of regulatory genes related to muscle differentiation, such as MyoD and Myf5 (Pan, Wang, Song, Chen, Li, & Zhao, 2007). CLA treatment reduced the levels of *MSTN1* and *MSTN2* mRNAs. Therefore, CLA increased the expression of myogenic factors, which might be partly related to the promotion of p38MAPK and the inhibition of MSTN signalling.

The adverse effects of high level of CLA on grass carp

An appropriate level of CLA could help improve the growth performance and meat quality of aquatic animals; however, a high level of CLA exerted the opposite effects. Excessive intake of CLA (15.9 g/kg diet) significantly reduced the feed intake of sub-adult grass carp. CLA-mediated decrease in feed intake might be related to an increase in leptin levels. Leptin inhibits feed intake through signals from the brain (Zhang, Proenca, Maffei, Barone, Leopold, & Friedman, 1994). Zou et al. (2018) reported that high levels of CLA (25–30 g/kg diets) increased leptin content in the muscle of juvenile grass carp. Therefore, the feed intake inhibition in response to high level of CLA might be related to an increase in leptin content; this requires further studies. In addition, high levels of CLA (12.7–15.9 g/kg diets) increased the yellowness (b*) and cooking loss in sub-adult grass carp muscle. CLA-induced muscle cooking loss and yellowness (b*) may be linked to the oxidation of lipids on the muscle cell membrane. Lipid peroxidation can destroy the cell membrane structure, causing intracellular fluid to flow out and increase cooking loss (Joo, Lee, Ha, & Park, 2002). Meanwhile, free radicals produced by fat oxidation attack the electrolysis of iron ions in myoglobin, which destroys the enzyme methaemoglobin reductase, resulting in dark or greyish-brown flesh (Decker, Chan, Livisay, Butterfield, & Faustman, 2010). In *M. nipponenseed*, high levels of CLA (compared with the control) increased the MDA content, which is a marker of lipid peroxidation (Ding et al., 2017). In addition, this is the first study to show that high levels of CLA (12.7–15.9 g/kg diets) significantly reduced collagen content. This could be attributed to the reduction in the mRNA and protein levels of Smad4. Smad4 positively regulates Smad2 phosphorylation and transmits signals to the nucleus to regulate the expression of collagen genes (such as *Col1α1* and *Col1α2*) (Fang et al., 2021). High levels of CLA decreased the mRNA levels of *Col1α1* and *Col1α2*, suggesting that high levels of CLA could suppress collagen synthesis, which may be partly due to the inhibition of samd4 signalling.

The optimal supplemental level of CLA

PWG, muscle EPA content, collagen content, and myofibre density were used to determine the appropriate supplemental level of CLA in sub-adult grass carp (663.3–1454.4 g), which were 6.41, 7.39, 7.74, and 7.31 g/kg diet, respectively. The optimal dietary CLA supplementation level for sub-adult grass carp determined by meat quality indices was slightly higher than that determined using the growth performance index (PWG). Therefore, we speculated that improving fish quality may require more CLA supplementation than that required for improving growth performance.

Conclusion

Supplementation with the appropriate amount of CLA could improve the growth performance and meat quality of sub-adult grass carp. There were some interesting and innovative results regarding the improvement in meat quality (Fig. 2). (1) Appropriate CLA supplementation could improve the flesh colour of grass carp, redistribute protein and fat in muscle, and improve muscle flavour quality (Glu, Ala, and IMP content), health quality (EPA, DHA, and CLA content), and physicochemical quality (pH and shear force). (2) The increase in EPA and DHA levels induced by CLA may be attributed to Δ-6 desaturase. (3) CLA can mitigate the post-mortem pH decline by inhibiting anaerobic metabolism. (4) CLA-increased shear force in muscle partly due to enhancement of cathepsin B (not L) content as well as promotion of collagen synthesis and myofibre growth; CLA-induced increase in collagen content is attributed to the upregulation of TGF-β/Smads and TOR signallings; CLA-induced increase in myofibre growth is not only attributed to myofibre proliferation partly related to upregulation of Cyclin, but also to myofibre differentiation partly related to the upregulation of p38MAPK signalling and inhibition of MSTN signalling, thereby improving myofibre density. Based on PWG, muscle EPA content, collagen content, and myofibre density, the appropriate levels of

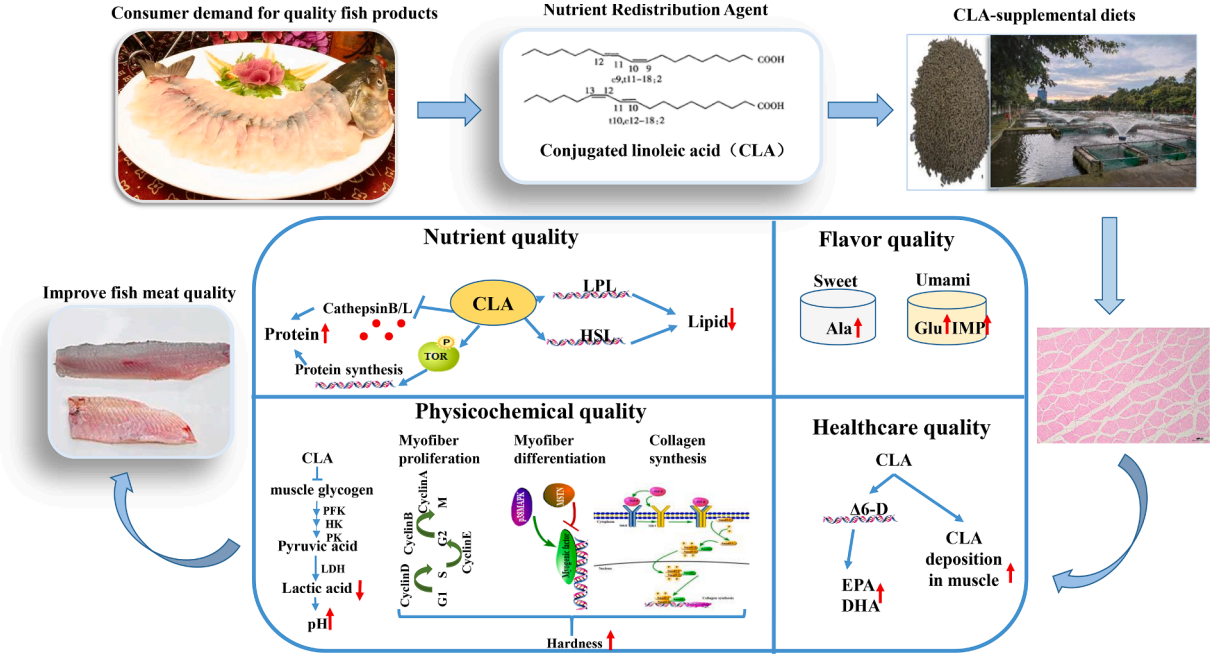

Fig. 2. The potential pathway of CLA supplementation on the meat quality.

CLA for sub-adult grass carp was determined to be 6.41, 7.39, 7.74, and 7.31 g/kg diets, respectively. Therefore, CLA as a nutrient redistributive agent could meet consumer and aquaculture demand for high-quality fish products by improving flesh quality.

CRediT authorship contribution statement

Xiao-Qing Liu: Formal analysis, Investigation, Writing – original draft. **Lin Feng:** Conceptualization, Methodology, Project administration. **Pei Wu:** Conceptualization, Methodology. **Yang Liu:** Project administration. **Hong-Mei Ren:** Methodology, Project administration. **Xiao-Wan Jin:** Methodology, Project administration. **Sheng-Yao Kuang:** Resources. **Shu-Wei Li:** Resources. **Ling Tang:** Resources. **Lu Zhang:** Project administration. **Hai-Feng Mi:** Project administration. **Xiao-Qiu Zhou:** Conceptualization, Methodology, Supervision, Funding acquisition, Resources. **Wei-Dan Jiang:** Data curation, Validation, Methodology, Supervision, Project administration, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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