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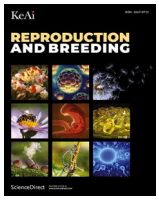

Comparative study of carbohydrate levels on growth, oxidative stress and glucolipid metabolism of hybrid fish between *Megalobrama amblycephala* (♀) × *Culter alburnus* (♂) and *Culter alburnus*

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ABSTRACT

The present study used hybrid fish (*Megalobrama amblycephala* ♀ × *Culter alburnus* ♂, abbreviated as BT) and topmouth culter (*Culter alburnus*, abbreviated as TC) as research subjects to assess and compare the effects of carbohydrate on the growth, oxidative stress and glucolipid metabolism of the two fish. The TC (initial weight, 13.10 ± 0.10 g) and BT (initial weight, 12.85 ± 0.13 g) were fed isonitrogenous (43% crude protein) and isolipidic (6.5% crude lipid) diets with carbohydrate levels of 10% (LC diet) and 30% (HC diet) for 11 weeks, respectively. The results showed that the HC diet resulted in a significant decrease in specific growth rate (SGR) of both fish. However, the SGR in BT was significantly higher than in TC, regardless of diet. Furthermore, HC diet significantly increased serum alanine transaminase (GPT) and hepatic malondialdehyde (MDA) content, decreased hepatic catalase (CAT) activity and resulted in increased hepatocyte volume, nucleus deficiency and nucleus deviation from the center of cytoplasm in both fish. However, BT had significantly higher glutathione peroxidase (GPx) activity and lower serum GPT and aspartate transaminase (GOT) content compared to TC. The HC diet induced significant increases in hepatosomatic index (HSI), liver lipid droplet area, liver glycogen, serum glucose (GLU) and triglyceride (TG) content in both fish. However, HSI and lipid droplet area were significantly higher in BT than in TC, while serum GLU and TG content were significantly lower in BT than in TC. Both fish can adapt to the HC diet by up-regulating the expression of glycolysis-related gene (glucokinase (*gk*)) and lipogenesis-related genes (acetyl-CoA carboxylase a (*acca*), fatty acid synthase (*fas*)). However, the expression levels of glucose transporter 2 (*glut2*), glycolysis-related gene (phosphofructokinase liver b (*pfklb*)) and lipogenesis-related genes (sterol regulatory element-binding protein 1 (*srebp1*), ATP citrate lyase a (*aclya*)) were increased only in BT fed HC diet. In summary, the BT liver has stronger glucose transport, glycolysis, and lipid tolerance compared to TC, thus exhibiting better glucose homeostasis and growth performance. It indicates that hybridization may help to produce new strain of fish with high carbohydrate utilization capacity.

1. Introduction

Carbohydrate is one of the most abundant organic compounds in nature, which is commonly used as dietary energy source for aquatic feed due to its relatively low price and extensive source [1]. The application of carbohydrate in aquatic feed can enhance various physical properties of pellets [2], reduce the catabolism of protein and lipid for energy purposes, increase protein and lipid retention, and provide metabolic intermediates for biological syntheses [3–5]. Nevertheless, unlike most mammals that rely on carbohydrate as their primary energy source, the low glucose metabolism rate of fish limits their capacity to utilize dietary carbohydrate. Most fish typically exhibit persistent hyperglycemia, after ingestion of high carbohydrate feed or direct glucose administration, similar to the symptoms of type 2 diabetes mellitus in humans [3,6]. Prolonged hyperglycemia has a negative impact on the growth performance of fish, as well as induce metabolic disturbances and even various pathological conditions such as, lipid and glycogen accumulation [7,8], histopathology [7,9], oxidative stress [10,11] and suppress innate immunity [11]. Therefore, understanding the mechanisms of carbohydrate utilization in fish and exploring effective strategies to improve carbohydrate utilization are popular research topics and major challenges in the field of aquatic animal nutrition.

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The ability of fish to utilize carbohydrate and the mechanisms of glucolipid metabolism differ significantly, which can be attributed to a variety of factors, including biological, dietary, and environmental factors [1]. Various strategies have been proposed to improve carbohydrate utilization in fish, including genetic selection [12,13], dietary additives supplementation (eg. probiotics, prebiotics, herbal extracts, exogenous enzymes, etc.) [14–16], dietary macronutrient balancing [9, 17] and modification of the rearing water environment [18]. Genetic selection has attracted considerable interest as a promising strategy to improve carbohydrate utilization capacity in fish. Fish populations exhibit high genetic variability and phenotypic plasticity, which makes it possible to selectively breed genotypes with high carbohydrate utilization capacity. For instance, previous studies have revealed that glucose utilization differed significantly between two rainbow trout lines that were divergently selected on the basis of their muscle lipid content [13,19]. In recent studies, the fat line of rainbow trout was found to maintain glucose homeostasis more efficiently than the lean line, and this different was closely related to enhanced levels of hepatic glycolysis, glycogen storage and lipogenesis [13,20–22]. Similarly, studies on different strains of gibel carp found that glucose uptake, glycolysis and lipid utilization ability in the F strain were stronger than in the A strain [23]. Moreover, the F strain can inhibit endogenous glucose synthesis through regulation of gluconeogenesis [12]. It is evident that genotype might be an essential factor influencing glucose homeostasis, and the introduction of favorable genes through breeding techniques may be a novel way to overcome the metabolic barriers of carbohydrate utilization. Unfortunately, there are few studies on the carbohydrate utilization capacity of hybrid fish, and the differences in glucolipid metabolism between hybrid fish and their parents are not clear. Therefore, more studies are still necessary to further clarify the complete characteristics and metabolic mechanism of glucolipid metabolism in different fish, especially for those newly formed hybrid fish, which could provide a reference value for subsequent studies to improve carbohydrate utilization in fish.

Blunt snout bream (*Megalobrama amblycephala*, BSB) and topmouth culter (*Culter alburnus*, TC) are two freshwater fish species belonging to the Cyprinidae. Both fish are commercially important aquaculture species in China with different characteristics and advantages. Blunt snout bream exhibits a fast growth rate, a strong resistance to stress, and a broad feeding spectrum, while topmouth culter possesses a high nutritional value of muscle tissue [24,25]. To integrate the excellent characters of blunt snout bream and topmouth culter, a hybrid fish (blunt snout bream ♀ × topmouth culter ♂, abbreviated as BT) was obtained by intergeneric distant hybridization in our laboratory. As a new type of aquatic germplasm resource, the hybrid fish has the advantages of fast growth rate and high flesh quality, which has important economic value and development potential in aquaculture industry [25]. The current research on the hybrid fish is mainly focused on genetic characteristics (including morphology, ploidy, karyotype, etc.) and genetic mechanisms [26,27]. For instance, Previous studies have shown that the hybrid fish, containing one set of chromosomes from each parent, is fertile and exhibits different degrees of phenotypic variation [27]. Based on a comparative analysis of the gut microbiota communities and gastrointestinal tract, it was found that the feeding habits of this hybrid fish were biased towards its mother parent, blunt snout bream [28]. In addition, the total and essential amino acid levels in the muscle of this hybrid fish were significantly higher than those of its parents [29]. Previous studies on nutrition found that the optimal dietary protein content of this hybrid fish was 33.67% (unpublished), which was lower than that of topmouth culter at 43.75% [30]. Based on these findings, we speculate that the difference in utilization of dietary carbohydrate between the hybrid fish and topmouth culter lead to differences in their optimal dietary protein requirement. Nevertheless, there is a lack of knowledge regarding the ability of the hybrid fish to utilize carbohydrate and the regulation mechanism of glucolipid metabolism after the intake of carbohydrate. Therefore, two diets with different carbohydrate levels (100, 300 g/kg) were designed in the study to compare the effects of dietary carbohydrate on the growth performance, oxidative stress, glucolipid metabolism between the hybrid fish and topmouth culter. The results of the present study not only help us to better understand the relationship between genetic and nutritional characteristics, but also provide some valuable information for subsequent hybrid breeding.

2. Materials and methods

2.1. Experiment designs and diets

The nutrient formulation and approximately chemical composition of experimental diets are shown in Table 1. Two isonitrogenous (43% crude protein) and isolipidic (6.5% crude lipid) diets were formulated comprising 100 g/kg (low carbohydrate diet, LC diet) and 300 g/kg (high carbohydrate diet, HC diet) corn starch, respectively. Fishmeal and poultry by-product meal were used as the main protein sources. Fish oil and soybean oil were added in equal amounts as dietary lipids. The dietary digestible carbohydrate was provided by corn starch. All ingredients were adequately ground and passed through a 40-mesh sieve. The ingredients were mixed thoroughly step by step in accordance with the recipe, stirred with 15% water, and processed into pellets of 1.5 mm diameter using a laboratory feed machine. The drying of all diets was carried out in a ventilated oven at 60 °C and storage at 4 °C until use.

2.2. Experimental fish and feeding trial

The feeding trial was conducted in a pond in Changde, Hunan Province, China. Topmouth culter (TC) for the experiment was purchased from Hechi Agricultural Development Co., Ltd., Changsha, China. The hybrid fish of blunt snout bream ♀ × topmouth culter ♂ (BT) were obtained from The Engineering Research Center of Polyploid Fish Reproduction and Breeding of the State Education Ministry, Hunan

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

Ingredients (% dry matter)	Diets	
	LC[c]	HC[d]
Fish meal	30	30
Poultry by-product meal	30	30
Fish oil	0.75	0.75
Soybean oil	0.75	0.75
Corn starch	10	30
$Ca(H_2PO4)_2$	2	2
Vitamin premix[a]	0.2	0.2
Mineral premix[b]	0.2	0.2
Choline chloride	0.2	0.2
Antioxidants (Ethoxyquin)	0.05	0.05
Mold inhibitor (Propionate)	0.1	0.1
Cellulose	25.75	5.75
Total	100	100
Proximate analysis (%)		
Dry matter (%)	90.77	92.02
Crude protein (%)	42.62	43.51
Crude lipid (%)	6.64	6.25
Starch	9.20	27.29

[a] Vitamin premix (mg or IU/kg): vitamin A 120000 IU; vitamin B1, 200 mg; vitamin B2, 280 mg; vitamin B8, 240 mg; vitamin B12, 0.6 mg; VC phosphatase, 6850 mg; vitamin D3, 40,000 IU; vitamin E, 480 mg; vitamin K3, 200 mg; calcium pantothenate, 720 mg; nicotinic acid, 1000 mg; folic acid, 60 mg; biotin, 1.2 mg; creatine, 3200 mg.

[b] Mineral premix (g/kg): magnesium, 4000 mg; iron, 4800 mg; zinc, 2000 mg; manganese, 800 mg; copper, 160 mg; cobalt, 12 mg; iodine, 40 mg; selenium, 4 mg.

[c] LC: low carbohydrate diet.

[d] HC: high carbohydrate diet.

Normal University, Changsha, China. Both TC and BT were cultured in a floating cage for 1 week in order to acclimatize, respectively. All fish were fasted for approximately 24 h prior to the experiment. Then, TC (13.10 ± 0.10 g) and BT (12.85 ± 0.13 g) were respectively divided into 6 floating cages (1.5 × 1.5 × 1.5 m), each containing 30 fish, with three replicates per diet. Fish were fed twice times per day at 6:00 and 18:00 to apparent satiation during the 11 weeks feeding trial. During the experiment, the water quality was maintained as follows: water temperature was 28 ± 5 °C, pH was 7.2 ± 0.3, dissolved oxygen was >6.0 mg/L.

2.3. Sample collection

At the end of the experiment, fish that had endured 24 h of food deprivation were anesthetized with diluted MS-222 solution. Then, the total number of fish from each cage was counted, and all fish were batched-weighed for subsequent calculation of growth indices. Three fish were randomly collected from each replicate cage, weighed, measured for body length and dissected to obtain the viscera and hepatopancreas in order to calculate morphological indices. Three additional fish were randomly selected from per cage, and blood samples were obtained via the caudal vein and centrifuged at 3000 *g*, 4 °C for 10 min. Then, the upper serum was instantly frozen and maintained at −80 °C for subsequent biochemical index determination. For further analysis, the liver and dorsal muscle from these fish were immediately dissected, frozen and stored at −80 °C until use. Besides, another two fish from per cage were dissected to obtain liver, and the liver were immersed in 4% paraformaldehyde in order to histomorphological analysis.

2.4. Growth performance and fish morphological indices analysis

Weight gain rate (WGR), specific growth rate (SGR), condition factor (CF), hepatosomatic index (HSI), and viscerosomatic index (VSI) were calculated according to the following equations:

$$\mathrm{WGR}\ (\%) = (W_{af} - W_{ai})/W_{ai}$$

$$\mathrm{SGR}\ (\%/\mathrm{d}) = [\mathrm{Ln}\ (W_{af}) - \mathrm{Ln}\ (W_{ai})]/\mathrm{d} \times 100$$

$$\mathrm{CF}\ (\mathrm{g/cm^3}) = W_f/L_b^3 \times 100$$

$$\mathrm{HSI}\ (\%) = W_l/W_f \times 100$$

$$\mathrm{VSI}\ (\%) = W_v/W_f \times 100$$

Where W_{af} represents average final body weight, W_{ai} represents average initial body weight, d represents feeding days, W_f represents final body weight of a fish, L_b represents body length of a fish, W_l represents liver wet weight of a fish, and W_v represents viscera wet weight of a fish.

2.5. Biochemical analysis

Proximate composition of diets and muscle tissue were analyzed in conformity to standard methods of Association of Official Analytical Chemists (AOAC) (1995). Moisture was determined by drying to constant weight in an oven at 105 °C. Crude protein was determined by the Kjeldahl method in FOSS Kjeltec 2300 (Foss Analytical Instruments Co., Ltd., Hillerød, Denmark) after an acid digestion. Crude lipid was determined using ether extraction in FOSS Soxtec 2050 (Foss Analytical Instruments Co., Ltd., Hillerød, Denmark). The starch content of diets was calculated by the commercial starch content kit (A148-1-1, Jiancheng Bioengineering Institute, Nanjing, China). Serum glucose (GLU), total cholesterol (T-CHO), triglyceride (TG), alanine transaminase (GPT), aspartate transaminase (GOT) and liver glycogen were measured using commercial kits from Nanjing Jiancheng Bioengineering Institute, following the requirements of the manual.

2.6. Histological analysis

The liver samples that had been fixed in 4% polyformaldehyde solution were sequentially dehydrated with gradient ethanol. Then, the samples were trimmed to an appropriate size, rendered transparent by treatment with xylene and embedded in paraffin. The paraffin blocks were cut into sections of 6 μm thickness using a microtome, and stained with hematoxylin and eosin (HE). The sections were observed and photographed under an optical microscope. To observe the lipid droplet content of liver, the same liver samples were embedded with OCT compound. The samples were frozen sectioned on a cryosicicle. The sections were then soaked in 60% isopropyl alcohol for 20–30s, and stained with Oil Red O staining solution. The relative area of lipid droplets in liver sections was measured using ImageJ software.

2.7. Liver enzyme activities

The liver samples were analyzed for total antioxidant capacity (T-AOC), catalase (CAT), glutathione peroxidase (GPx), reduced glutathione (GSH), total superoxide dismutase (T-SOD), malondialdehyde (MDA), hexokinase (HK), pyruvate kinase (PK) using commercial kits (Jiancheng Bioengineering Institute, Nanjing, China). The activity of phosphofructokinase (PFK), glucose-6-phosphatase (G6P) in liver were determined by commercial ELISA kit (Jiancheng Bioengineering Institute, Nanjing, China).

2.8. Gene expression analysis

The total RNA was extracted from liver tissue of each treatment by using RNA isolater Total RNA Extraction Reagent (Vazyme, China) for genes expression analysis. The quality of total RNA was determined by spectrophotometry, and the OD260/280 ratio ranging 1.8 to 2.2 indicated that the total RNA was of high purity, which could be used for real-time polymerase chain reaction (qRT-PCR). Afterward, the total RNA was reversely transcribed into cDNA by using HiScript III RT SuperMix for qRT-PCR (Vazyme, China) according to the manufacturer's instructions. The qRT-PCR analysis was performed with the reaction system including 5 μL 2 × ChamQ Universal SYBR qPCR Master Mix (Vazyme, China), 3 μL sterile water, 1 μL cDNA, 0.5 μL forward primer (10 μmol/mL) and 0.5 μL reverse primer (10 μmol/mL) in QuantStudio 5 (Thermo Fisher Scientific Inc., Waltham, MA, USA). A melt-curve was then performed in order to ensure the amplification of a single product. The primers used in this experiment were designed using Primer 5.0 for qRT-PCR, and their sequences are listed in Table 2. The housekeeping gene, β-actin, was chosen as an internal reference due to the stability of β-actin. The relative expression of target genes was analyzed in three fish (n = 3) and calculated using $2^{-\Delta\Delta CT}$ method.

2.9. Statistical analyses

All of the statistical analyses were performed using the statistical software SPSS for Windows (version 26.0; SPSS Inc., Chicago, IL, USA). All values were expressed as means ± standard error of means (SEM). The independent samples *t*-test was used to compare significant differences between TC-LC and TC-HC; BT-LC and BT-HC; TC-LC and BT-LC; and TC-HC and BT-HC. To test whether there was an interaction between dietary carbohydrate content and fish strain in each parameter, the significant difference was made between all groups using the one-way ANOVA, and then the two-way ANOVA was used to measure the individual or combined effects of dietary carbohydrate content and fish strain. In Supplemental Table 1-7, the statistical results of two-way ANOVA were presented. The significant differences were considered to exist when the p-value was less than 0.05.

Table 2
Primers designed in the qRT-PCR.

Target Gene	Primer (5′ to 3′)	GenBank No.
β-actin	F: CCCATCTACGAGGGTTATG R: TCGAAGTCAAGAGCCACA	XM_048192430.1
glut2	F: ACATCAAACAGGGCAAAG R: CACCGAGGAACGGAGTAG	KC513421.2
gk	F: CAACGCTCATCATACACTT R: TCTCCGCATTCTCCTCAT	KJ141202.1
pfklb	F: TTGTGGGTGCCAAAGTCT R: TGCCGCCAAGTTGAATGA	XM_048172371.1
pk	F: CCGTATCAGGCGTTACTCTG R: CTGGTGTTGCGTGCTGTG	MT747911.1
g6p	F: TGATGGATACAGTGCATGGGTT R: TGGGAAAGAAGATGAAGAAGG	DQ323499.1
fbp	F: ACCCAGATGTCACAGAAT R: CACTCATACAACAGCCTCA	KJ743995.1
pepck	F: CCTATTGATGCCATTGTATT R: GTCGCCGAAGTTGTAGCC	MK986677.1
srebp1	F: CTGCCCATCAATCGCATCG R: CCCAGCCACCAGGTCTTT	MH633449.1
aclya	F: CCGAGCGGTTAGTCGTTA R: GCTACAAAGGGCTCAATCA	XM_048209568.1
acca	F: ATGGTTTCCTTCCGCTCCT R: TTGGCTCATCTTGAATGCTCTT	MT747913.1
fas	F: CATAAACAGCCCATCCTT R: TCACCATTCCCACCACTC	KF918747.1
cpt1	F: TGTGCCCGTAATGCCTAC R: AACCACCTGTCATAACACTTCC	KJ141198.1
aco3	F: TCCCAGTGGAGCAGAAGA R: ATCCCTTTGTTAAGGAAGTA	XM_048185264.1

3. Results

3.1. Growth performance and fish morphological indices

The intake of HC diet resulted in a significant decrease in the weight gain rate (WGR) and specific growth rate (SGR) of BT and TC, while the hepatosomatic index (HSI) and viscerosomatic index (VSI) increased significantly (Fig. 1B and C, E, F). As compared to TC, the BT had significantly higher final body weight (FBW), WGR, SGR, HSI, and condition factor (CF) in both LC diet and HC diet (Fig. 1A–E), while the significantly higher VSI was only observed in the LC diet group (Fig. 1F). We observed an interaction between strain and diet with respect to HSI (Supplemental Table 1).

3.2. Approximate muscle composition

The HC diet significantly increased the content of muscle crude lipid in TC and BT, but did not affect the content of muscle crude protein (Fig. 2B and C). In addition, the BT had higher muscle crude protein content, while lower muscle crude lipid than the TC under the same diet conditions (Fig. 2B and C). Moisture, crude protein and crude lipid of muscle did not show significant interactions between strain and diet (Supplemental Table 2).

3.3. Serum and liver biochemical indicators

The HC diet significantly enhanced serum glucose, triglyceride and liver glycogen content, and increased serum GPT activities in both TC and BT (Fig. 3A and B, D, F). After feeding the HC diet, the significantly increased serum GOT activities were only observed in TC (Fig. 3E). Compared to TC, the BT all had significantly lower serum glucose and triglyceride, and had significantly lower serum GPT and GOT activities irrespective of diet (Fig. 2A and B, D, E). No statistical difference was observed in total cholesterol in serum, either between TC and BT, or between LC diet and HC diet (Fig. 3C). The significant interaction between strain and diet was observed in serum GPT and GOT activities (Supplemental Table 3).

3.4. Hepatic histomorphology

As shown in the H&E staining results (Fig. 4A), the hepatocytes in the LC diet group had clearer cell boundaries and more nuclei irrespective of strain. In contrast, hepatocytes in the HC diet exhibited obvious enlargement, nucleus deficiency and nucleus deviation from the center of cytoplasm. Under the same feed conditions, there was no significant difference in the size and cell integrity of the hepatocytes between TC and BT. The statistical results of Oil Red O staining showed that the HC diet significantly increased the area of liver lipid droplets in both strains (Fig. 4C). Furthermore, the area of liver lipid droplets was significantly greater in BT fed the HC diet compared with TC (Fig. 4C).

3.5. Liver antioxidant indicators and enzymes involved in carbohydrate metabolism

With the increasing of carbohydrate concentration, the CAT activity significantly decreased and MDA content significantly increased in the liver (Fig. 5C, F), irrespective of strain. The hepatic T-AOC decreased in

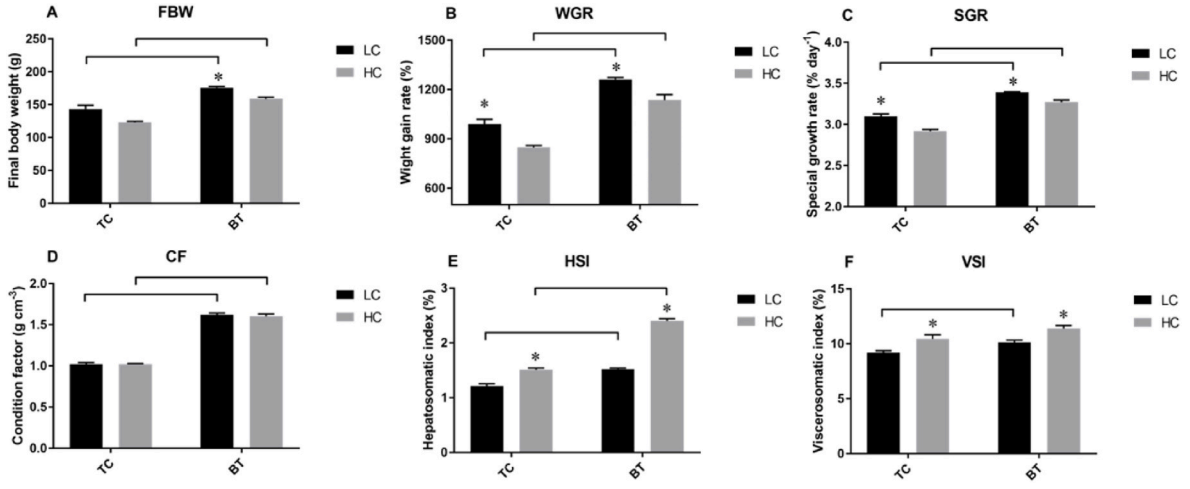

Fig. 1. Effects of dietary carbohydrate levels on growth performance and fish morphological indices in TC and BT. (A) Final body weight (g); (B) Wight gain rate (%); (C) Special growth rate (% day^{-1}); (D) Condition factor (g cm^{-3}); (E) Hepatosomatic index (%); (F) Viscerosomatic index (%); Data are presented as mean ± SEM (n = 3 in A-F). The asterisk (*) indicates significant differences between LC and HC diet within the same strain of fish, while a line above the bars shows significant differences between TC and BT within the same dietary carbohydrate content ($p < 0.05$).

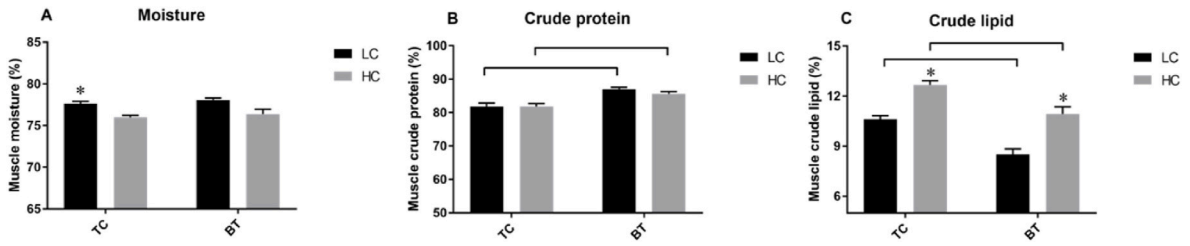

Fig. 2. Effects of dietary carbohydrate levels on approximate composition in TC and BT. (A) Moisture (%); (B) Crude protein (%); (C) Crude lipid (%); Data are presented as mean ± SEM (n = 3). The asterisk (*) indicates significant differences between LC and HC diet within the same strain of fish, while a line above the bars shows significant differences between TC and BT within the same dietary carbohydrate content ($p < 0.05$).

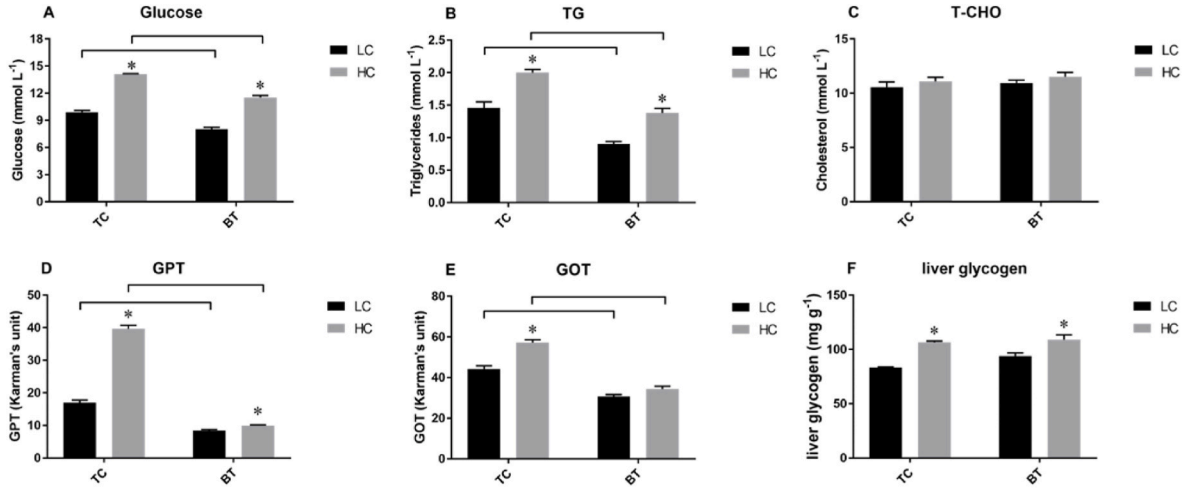

Fig. 3. Effects of dietary carbohydrate levels on serum and liver biochemical indicators in TC and BT. (A) Serum glucose; (B) Serum triglyceride; (C) Serum total cholesterol; (D) Serum alanine transaminase; (E) Serum aspartate transaminase; (F) liver glycogen; Data are presented as mean ± SEM (n = 3). The asterisk (*) indicates significant differences between LC and HC diet within the same strain of fish, while a line above the bars shows significant differences between TC and BT within the same dietary carbohydrate content ($p < 0.05$).

the HC diet compared with the LC diet group (Fig. 5A), but no significant difference was observed in TC. The HC diet significantly reduced hepatic T-SOD activity of BT (Fig. 5B), but reduced hepatic GSH content of TC (Fig. 5E). Compared to TC, the BT showed the significantly higher GPx activity in liver (Fig. 5D), regardless of diet. Furthermore, the liver CAT activity of BT was significantly higher (Fig. 5C), while MDA was significantly lower than that of TC at the LC diet (Fig. 5F). The interaction of strain and diet only appeared in hepatic GSH content (Supplemental Table 4).

High dietary carbohydrate significantly enhanced the activity levels of liver HK in both TC and BT (Fig. 6A). In addition, high dietary carbohydrate significantly increased the activity of PFK in the liver of BT and G6P in the liver of TC (Fig. 6B, D). Under the same carbohydrate level, the PFK activity in the liver of BT was significantly higher (Fig. 6B), while the liver PK activity was significantly lower than that of TC (Fig. 6C). Additionally, the BT showed significantly higher activity of liver HK compared with TC at the LC diet (Fig. 6A). The interaction between strain and diet had no effects in activity of HK, PFK, PK, G6P (Supplemental Table 5).

3.6. Expression levels of genes related to glucolipid metabolism

With respect to glucose metabolism in the liver, the transcription levels of the genes for glucose transporter 2 and key enzymes of glycolysis were analyzed. The mRNA expression levels of *gk* in the liver of TC and BT were significantly up-regulated with increasing carbohydrate content (Fig. 7B). In addition, the HC diet significantly increased the transcription levels of PK in TC liver and *glut2* and *pfklb* in BT liver (Fig. 7A, C, D). The mRNA expression levels of *pk* in the liver of BT were significantly lower than that of TC, irrespective of diet (Fig. 7D). In contrast, the mRNA expression levels of *glut2*, *gk* and *pfklb* in the liver of BT were significantly higher than those of TC under the HC diet (Fig. 7A, B, C). At the same time, the mRNA expression levels of three key genes encoding gluconeogenic enzymes were also determined. The elevated mRNA expression of *pepck* was found in the liver of TC fed the HC diet, whereas *fbp* was lower expressed in the liver of BT (Fig. 7F and G). The BT showed significantly higher mRNA expression levels of *fbp* in liver compared with TC (Fig. 7F), regardless of diet. There was significant interaction between strain and diet in the expression of *glut2*, *gk*, *pfklb*, *pk* and *pepck* mRNA in liver (Supplemental Table 6).

Subsequently, we further analyzed lipid metabolism in the liver by measuring the mRNA expression levels of specific enzymes involved in hepatic lipogenesis. The mRNA expression levels of *acca* and *fas* were significantly up-regulated in the liver of TC and BT with the increase of dietary carbohydrate content (Fig. 8C and D). And the increased expression levels of *srebp1* and *aclya* was only found in the liver of BT fed the HC diet (Fig. 8A and B). In comparison with TC, the expression levels of *acca* and *fas* were more abundant in the liver of BT fed the LC diet (Fig. 8C and D). Regarding fatty acid oxidation in the liver, we found that the expression levels of *cpt1* in TC significantly increased with increasing dietary carbohydrate levels (Fig. 8E). In contrast, the expression levels of *cpt1* in BT significantly decreased (Fig. 8E). Compared with TC, the BT fed with the HC diet showed lower expression of *cpt1* (Fig. 8E). There was significant interaction between strain and diet in the expression of *acca* and *cpt1* mRNA in liver (Supplemental Table 7).

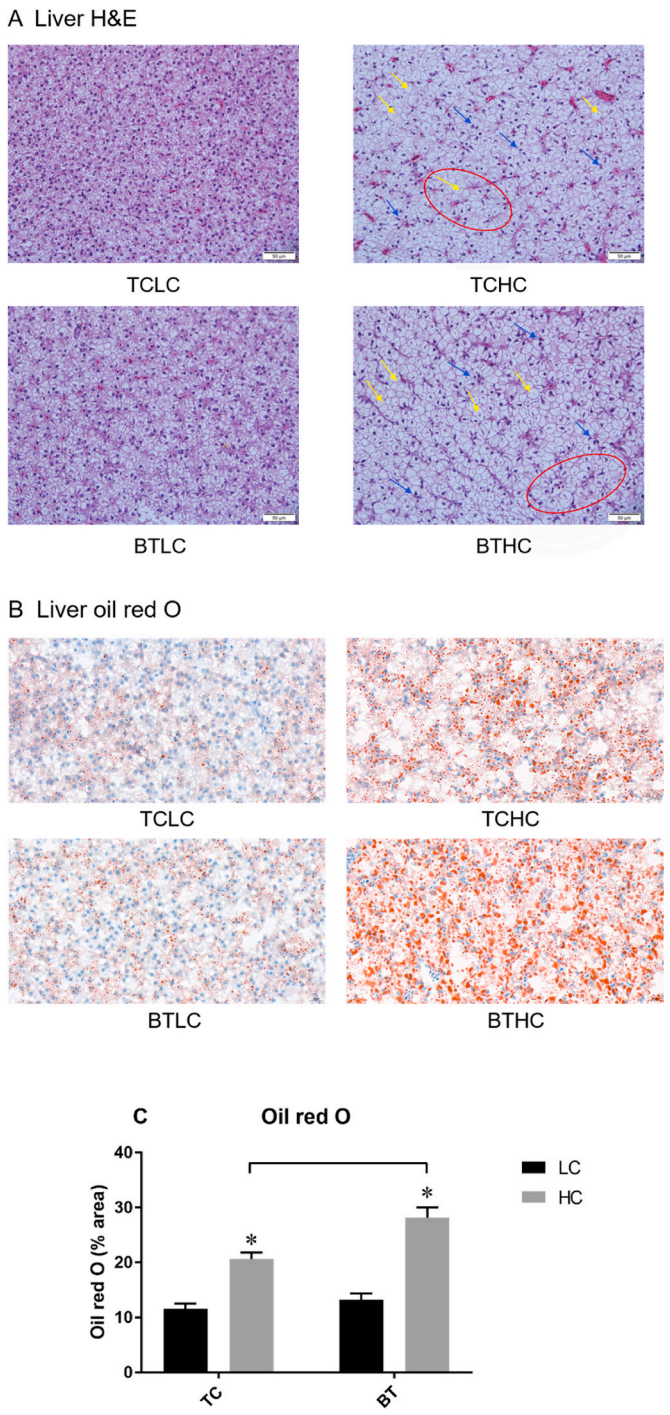

Fig. 4. Effects of dietary carbohydrate levels on histological characteristics of liver in TC and BT. (A) Liver histological sections of H&E staining. Red circles: enlarged cell volume; Yellow arrows: absence of nuclei; Blue arrows: deviated nuclei. Scale bars, 100 μm (20 ×) (B) liver histological sections of oil red O staining, Scale bars, 100 μm (20 ×) (C) oil red O staining area; Data are presented as mean ± SEM (n = 3). The asterisk (*) indicates significant differences between LC and HC diet within the same strain of fish, while a line above the bars shows significant differences between TC and BT within the same dietary carbohydrate content ($p < 0.05$).

4. Discussion

4.1. Effects of dietary carbohydrate levels on growth performance and fish morphological indices in TC and BT

Previous studies have shown that dietary carbohydrate content no more than 20% can improve the growth performance of topmouth culter, a typical carnivorous fish [31]. However, excessive carbohydrate intake can lead to persistent hyperglycemia in fish, which is considered as a physiological stress response that hinder the growth rate and cause metabolic disorders [32]. In the present study, the results showed that the ingestion of 30% carbohydrate diet caused a decrease in growth performance of TC and BT. The phenomenon that excessive carbohydrate in diets inhibit the growth performance has been reported in cobia [33], gibel carp [34], blunt snout bream [8], european sea bass and gilthead sea bream [3]. In the study, FBW, WGR and SGR of BT were higher than those of TC, regardless of diet. It indicated that the carbohydrate utilization capacity and demand of BT were greater than that of TC. In other words, hybridization makes BT have better growth potential. Hybrid progeny with excellent growth performance has been confirmed in several studies, such as *Megalobrama amblycephala* ♀ × *Culter mongolicus* ♂ [35], *Megalobrama terminalis* ♀ × *Culter alburnus* ♂ [36], *Epinephelus fuscogutatus* ♀ × *E. lanceolatus* ♂ [37].

On the other hand, the most direct change in internal organs of fish after consuming high carbohydrate diets is an increase in relative weight. The HSI is considered as a sensitive indicator to measure digestible energy of fish feed. In the present study, the HSI and VSI of both fish increased with increasing dietary carbohydrate levels, which is in accordance with the results obtained in largemouth bass [7], gibel carp [23] and nile tilapia [38]. It indicated that excessive digestible energy is converted into glycogen or lipid and stored in the liver, resulting in liver enlargement. In addition, the HSI and VSI of BT were always higher than TC in the study. There were similar results in early studies on the hybrid fish, which always had significantly higher HSI than topmouth culter, regardless of dietary protein levels [39]. The two-way ANOVA test indicated that the higher HSI of BT is due to the interaction between strain and carbohydrate levels in the diet. From the perspective of metabolism, BT inherently had higher HSI, possibly indicating a stronger nutritional metabolic function [40]. Thus, the liver of BT has a greater carbohydrate utilization capacity and tolerance.

4.2. Effects of dietary carbohydrate levels on approximate muscle composition in TC and BT

As the main edible portion of fish, muscle provides numerous nutrients required by humans [41]. Excessive carbohydrate diets will not only cause growth and metabolism disorders, but also have an impact on muscle nutritional value [42]. Generally speaking, the higher protein and essential amino acids content in muscle is associated with a relatively higher nutritional value [43]. In the present study, it is worth noting that different carbohydrate levels had no significant effect on muscle crude protein content, which is also consistent with the findings in nile tilapia [42]. One view is that the protein content of each fish species seems to be predetermined when dietary essential nutrients are sufficient to satisfy fish growth and metabolism [41]. Nevertheless, the BT had higher muscle crude protein content than TC, regardless of the diet, which is similar to the results found in earlier studies of the hybrid fish [24,29]. It suggested that one of the hybridization advantages of BT is the relatively higher nutritional value of muscle. It was observed in previous studies on nile tilapia and grass carp that crude lipid content of whole fish or muscle increased significantly with the increase of carbohydrate level [38,44]. As expected, an increase of muscle crude lipid content was observed with the increase of carbohydrate level in diet in the present study, which also provided evidence that high carbohydrate diets can induce lipogenesis in fish.

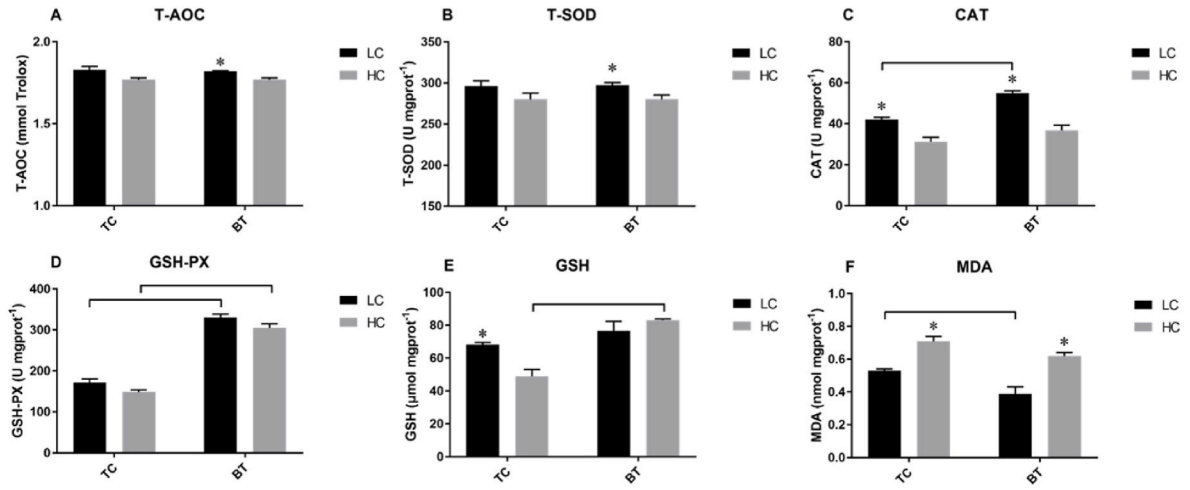

Fig. 5. Effects of dietary carbohydrate levels on antioxidant capacity of liver in TC and BT. (A) Total antioxidant capacity; (B) Total superoxide dismutase activity; (C) Catalase activity; (D) Glutathione peroxidase activity; (E) Reduced glutathione content; (F) Malondialdehyde content; Data are presented as mean ± SEM (n = 3). The asterisk (*) indicates significant differences between LC and HC diet within the same strain of fish, while a line above the bars shows significant differences between TC and BT within the same dietary carbohydrate content ($p < 0.05$).

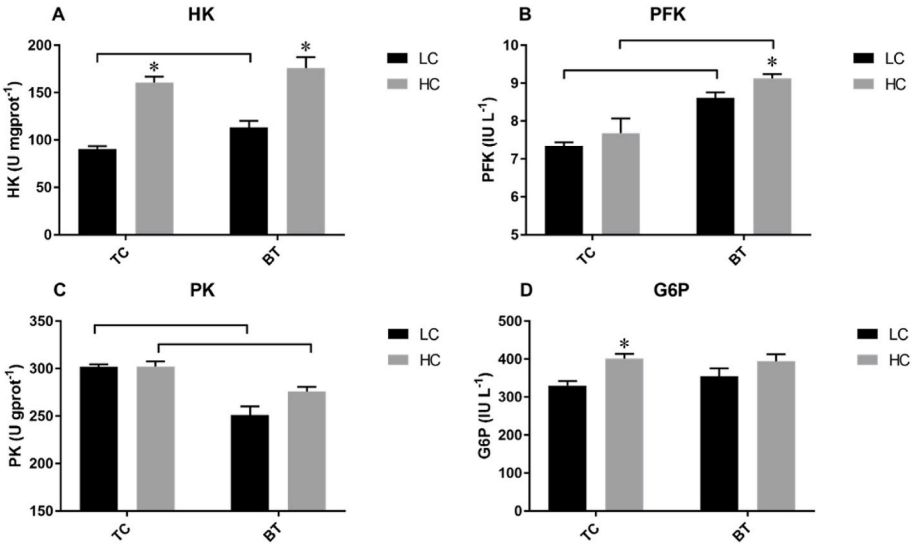

Fig. 6. Effects of dietary carbohydrate levels on the activity levels or concentrations of enzymes involved in carbohydrate metabolism of liver in TC and BT. (A) Hexokinase activity; (B) Phosphofructokinase activity; (C) Pyruvate kinase activity; (D) Glucose-6-phosphatase activity; Data are presented as mean ± SEM (n = 3). The asterisk (*) indicates significant differences between LC and HC diet within the same strain of fish, while a line above the bars shows significant differences between TC and BT within the same dietary carbohydrate content ($p < 0.05$).

4.3. Effects of dietary carbohydrate levels on oxidative stress of liver in TC and BT

In the present study, the HC diet significantly increased the activities of GPT and GOT of TC serum, which have been widely recognized as markers for diagnosing liver function and injury [45]. Meanwhile, the BT fed with HC diet only observed a significant increase in serum GPT activity. Furthermore, the GPT and GOT activities in serum of BT were always lower than those of TC in the study, suggesting that the HC diet led to liver damage in both TC and BT, but the liver of BT was healthier and less damaged compared to TC. High carbohydrate-induced oxidative stress and damage have been demonstrated in many fish, such as yellow catfish [9], largemouth bass [46] and black carp [11]. To further investigate the effect of carbohydrate on TC and BT liver health, the antioxidant capacity of fish liver was analyzed and the histological characteristics of the liver were observed.

Under normal physiological conditions, the fish generates ROS (reactive oxygen species) during the process of nutrient metabolism. In order to mitigate cytotoxicity, the body responds to the ROS through an enzymatic antioxidant system and a non-enzymatic antioxidant system [47,48]. T-AOC is a comprehensive indicator of the capacity of the body's antioxidant system and is directly influenced by health status [49]. The CAT, SOD and GPx are three representative antioxidant enzymes that are widely exist in the body and can scavenge harmful oxidants [50,51]. The GSH is an important non-enzymatic antioxidant that can effectively eliminate ROS directly and indirectly through enzymatic reaction [52,53]. As the main product of lipid peroxidation, MDA has strong biological toxicity and can indirectly reflect the extent of oxidative damage [54]. The results of this experiment showed that as the level of dietary carbohydrate increased, liver CAT activity decreased significantly, T-AOC showed a downward trend and MDA content increased significantly, regardless of fish strain. It meant that 30% carbohydrate diet reduced the antioxidant capacity of the fish, resulting in an imbalance of ROS production and elimination in the fish and inducing oxidative stress. This result is in general agreement with the findings of studies on largemouth bass (*Micropterus salmoides*) and black carp (*Mylopharyngodon piceus*) in which high carbohydrate levels (20%, 37.9%) led to a decrease in CAT activity and an increase in MDA content in the liver [11,46]. However, the influence of HC diet on the T-SOD activity and GSH content of the two fish was significantly different,

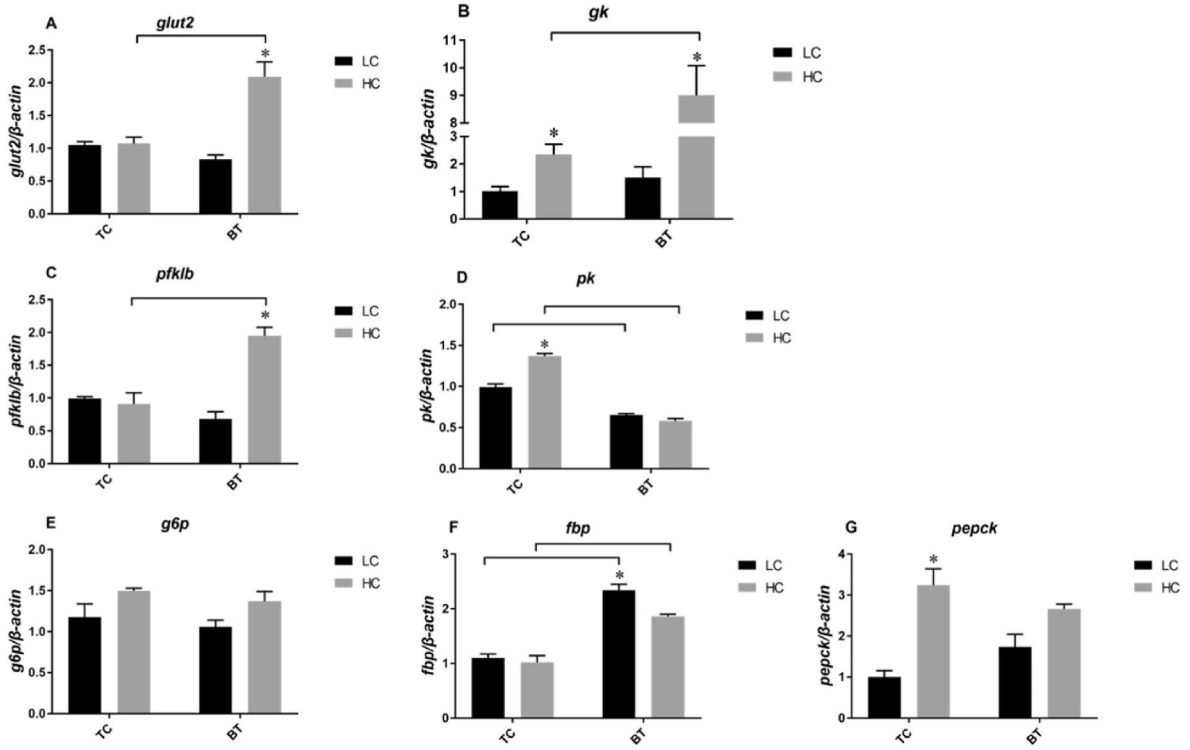

Fig. 7. Effects of dietary carbohydrate levels on the gene expression levels of glucose transporter, glycolysis enzymes and gluconeogenesis enzymes in the liver of TC and BT. (A) Glucose transporter type 2 (*glut2*) mRNA expression; (B) glucokinase (*gk*) mRNA expression; (C) phosphofructokinase liver b (*pfklb*) mRNA expression; (D) pyruvate kinase (*pk*) mRNA expression; (E) glucose-6-phosphatase (*g6p*) mRNA expression; (F) fructose 1,6-bisphosphatase (*fbp*) mRNA expression; (G) phosphoenolpyruvate carboxylase kinase (*pepck*) mRNA expression; Data are presented as mean ± SEM (n = 3). The asterisk (*) indicates significant differences between LC and HC diet within the same strain of fish, while a line above the bars shows significant differences between TC and BT within the same dietary carbohydrate content ($p < 0.05$).

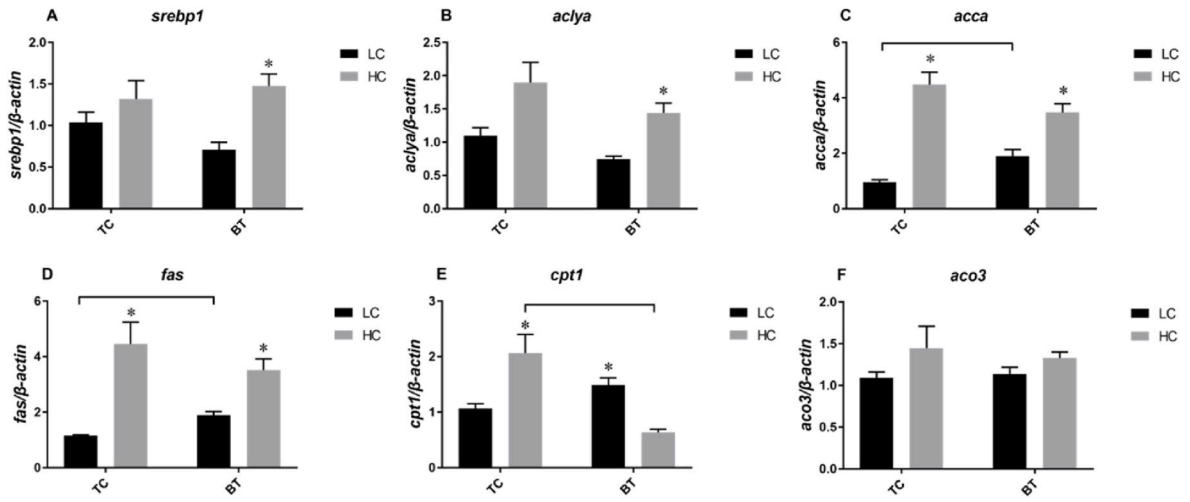

Fig. 8. Effect of dietary carbohydrate levels on the expression levels of genes related to lipogenesis and fatty acid β-oxidation in the liver of TC and BT. (A) Sterol regulatory element binding protein 1 (*srebp1*) mRNA expression; (B) ATP citrate lyase a (*aclya*) mRNA expression; (C) acetyl-CoA carboxylase a (*acca*) mRNA expression; (D) fatty acid synthase (*fas*) mRNA expression; (E) Carnitine palmitoyl transferase 1 (*cpt1*) mRNA expression; (F) acyl-CoA oxidase 3 (*aco3*) mRNA expression; Data are presented as mean ± SEM (n = 3). The asterisk (*) indicates significant differences between LC and HC diet within the same strain of fish, while a line above the bars shows significant differences between TC and BT within the same dietary carbohydrate content ($p < 0.05$).

which may imply that the two fish may have different pathways to resist oxidative stress, and the reasons for this result remains to be further studied. In the LC diet, the liver CAT activity of BT was significantly higher, while liver MDA was significantly lower than those of TC. It demonstrated that the CAT in liver of BT may respond more positively to oxidative damage.

To observe the morphology of hepatocytes, paraffin sections of fixed liver tissue were made and stained with hematoxylin and eosin (HE) and Oil red O solution, respectively. In the present study, significant enlargement of cell volume, absence of nucleus and deviation of nucleus from the center of cytoplasm were observed in the liver of fish fed HC diet, which may be attributed to the accumulation of lipid droplets and

glycogen in the liver caused by the nutritional stress of high carbohydrate. The same phenomenon was observed in the liver of yellow catfish and largemouth bass fed with high carbohydrate diets [7,9]. From the above results, it suggested that excessive carbohydrate diet caused liver damage and interfered with normal liver function. However, oil red O staining results showed that BT liver accumulated significantly higher lipid droplets than TC in the HC diet, while GPT and GOT activities in BT serum were significantly lower than TC. GPT and GOT have been widely considered as markers for diagnosing liver function and injury [45]. It suggested that in the HC diet, the BT liver was in better health despite depositing more lipids.

4.4. Effects of dietary carbohydrate levels on glucolipid metabolism of liver in TC and BT

Biochemical indicators of blood, such as triglyceride, cholesterol and blood glucose, are important components for evaluating the metabolic and physiological status of the animal organism [9]. Differences in the nutritional status of fish inevitably cause differences in blood biochemical parameters. The results of this experiment showed that fish fed the HC diet exhibited higher serum glucose and triglyceride. Generally, the breakdown of carbohydrate raises blood glucose, and fish in a persistent hyperglycemic state promote the transfer of excess glucose from the blood to the liver, where de novo lipogenesis takes place. Some of the lipids in the liver are transferred to other tissues for storage through the blood circulation, which may correspondingly increase the content of serum triglyceride and total cholesterol [1,55]. In the present study, the BT always contained lower levels of serum glucose than TC, which indicated that the glucose clearance of BT is stronger than that of TC. These may be attributed to the fact that BT contains half of the genes of the herbivorous fish blunt snout bream, and therefore it has better glucose homeostasis ability. Furthermore, serum triglyceride levels were also always lower in BT than in TC, which may be because more lipids endogenously synthesized in the liver of BT were stored in the liver, which also corroborates with the higher HSI in BT.

Liver is a key organ of fish with an important role in the utilization and regulation of glucose [56,57]. Glucose in the liver is either produced by glycolysis to pyruvate, which is then oxidized to provide energy, or accumulated in the form of glycogen or lipids [1,32]. It was shown in the study that HC diet increased both liver lipid droplet area and liver glycogen content in both fish, which was consistent with the findings on nile tilapia [38] and largemouth bass [7]. It suggested that after consuming excess dietary carbohydrates, a portion of carbohydrates were converted into glycogen and lipids that were deposited in the liver, rather than providing energy. In addition, it was observed in the study that the lipid droplet area of BT liver was significantly higher than that of TC under HC diet, while there was no difference in liver glycogen content between the two fish. This result suggested that the higher HSI of BT than TC may be due to the difference in liver lipogenesis capacity of the two fish.

To further understand the differences in carbohydrate utilization between the two fish, we investigated the regulatory effect of dietary carbohydrate on glucolipid metabolism at the mRNA expression level. As in mammals, different members of the glucose transporters (GLUTs) have been reported in fish, and GLUT2 is an important isoform of the glucose transporters family, whose important function is to participate in the bidirectional transport of glucose in hepatocytes and plasma to maintain the same glucose concentration inside and outside the cell [58,59]. In the present study, the mRNA expression levels of *glut2* in BT liver were significantly up-regulated after feeding the HC diet, while there was no significant alteration in the mRNA expression levels of *glut2* in TC liver. Additionally, the mRNA expression levels of *glut2* in BT liver were significantly higher than that of TC under the HC diet. It indicated that hyperglycemia stimulated more glucose uptake in the liver of BT. Consistent with the results observed in Chinese longsnout catfish, gibel carp and grass carp, the mRNA expression levels of *glut2* were enhanced in the liver of fish fed high carbohydrate diet or intraperitoneally injected with glucose, stimulating the liver to uptake more glucose [23,60]. However, the hepatocytes of TC failed to respond, and the delivery of glucose into hepatocytes is one of the rate-limiting steps in glucose utilization, which might be one of the reasons for the higher blood glucose in TC than that in BT. The above results also indicated that the expression of *glut2* was affected by factors both genotype differences and dietary carbohydrate levels.

Glycolysis is a common stage that organisms must pass through to utilize glucose, and the transcriptional expression and activity of key glycolysis enzymes directly affect the rate of glycolysis [56]. Glucokinase (GK) is the rate-limiting enzyme in the glucose metabolic pathway and is involved in the first step of hepatic glycolysis [61,62]. In the present study, the HC diet induced a significant increase in the activity of HK and the mRNA expression levels of *gk* in the liver. The results of this study were consistent with previous studies, such as rainbow trout [63], nile tilapia [64] and grass carp [60]. At the same time, it was observed that the mRNA expression levels of *gk* in BT liver was significantly higher than that of TC, in the HC diet. It indicated that the *gk* of both fish responded efficiently to high dietary carbohydrate at the molecular level, while the *gk* of BT was more strongly feedback regulated by carbohydrate. The activity and mRNA expression levels of PFK are considered to be influenced by dietary carbohydrate levels, however, it does not always respond positively to dietary carbohydrate levels as GK does [64,65]. In the present study, the HC diet promoted PFK activity and *pfklb* mRNA expression levels in BT. In addition, both hepatic PFK activity and *pfklb* mRNA expression levels were higher in BT fed the HC diet than in TC. It suggested that hepatic glycolysis of BT is up-regulated by dietary carbohydrate and more pyruvate is subsequently available for energy metabolism. The above findings may explain the poor glycemic control observed in TC. Pyruvate kinase (PK) is responsible for catalyzing the final step of the glycolytic pathway, and there are no consistent findings on the regulation of hepatic PK by dietary carbohydrate in fish. Several authors found that in some carnivorous fish, the regulatory effect of high carbohydrate on *pk* was not observed [60,66]. On the contrary, high carbohydrate significantly increased PK activity and *pk* mRNA expression levels in grass carp [67] and nile tilapia [38]. In the present study, the *pk* mRNA expression levels of TC increased with carbohydrate levels, but did not change in BT, and *pk* mRNA expression levels and enzyme activities were always higher in TC than in BT. It suggested that the lack of coherent regulation of glycolysis rate-limiting enzymes may be an important factor affecting the rate of liver glycolysis in BT. Since this study is the first to explore the differences in glucolipid metabolism between these two fish, more subsequent studies are still necessary to more fully explain the results.

Gluconeogenesis is the main pathway for converting non-glycosidic substrates into glucose or glycogen, and PEPCK, FBP and G6P are key enzymes regulating hepatic gluconeogenesis [55]. Studies on some fish have shown that excess glucose inhibits the activity and mRNA expression of gluconeogenic enzymes to reduce the release of endogenous glucose [38]. However, in carnivorous fish, such as rainbow trout [68,69], European sea bass and gilthead sea bream [3], the gene expression and activity of key enzymes involved in the gluconeogenic pathway (FBP, G6P and PEPCK) remained at high levels despite the HC diet intake. In the present study, mRNA expression levels of *fbp* in BT liver were down-regulated by HC diet, while the mRNA expression of *g6p*, *pepck* and the enzyme activity of G6P were not significantly affected. In contrast, the mRNA expression levels of *pepck* and the enzyme activity of G6P in TC liver increased significantly with increasing dietary carbohydrate levels. It could be due to the fact that the distant hybridization resulted in BT being more biased toward herbivorous fish in terms of feeding habits, and thus BT had some inhibitory effect on gluconeogenesis. Earlier studies on gut microbial community and gastrointestinal tract also found BT to be more biased towards herbivorous fish [28]. In contrast, TC, as a carnivorous fish, lacked the inhibitory effect on gluconeogenesis after ingesting the HC diet. Similar

results were found in the study by Song et al. [63], where some genes (*pck1, fbp1b*) encoding key enzymes of gluconeogenesis were down-regulated and others genes (*g6pca, g6pcb1a, g6pcb2a, fbp1a*) were up-regulated in the liver of rainbow trout fed high carbohydrate diet. Unexpectedly, the mRNA expression levels of *fbp* in BT liver was significantly higher than that in TC. The activity or expression levels of *fbp* was reported to be less regulated by dietary carbohydrate, while more significantly affected by protein levels [70,71]. The protein content of the diets in the study was designed according to the optimal protein for TC, which may be high enough for BT, so that the mRNA expression levels of *fbp* was at high levels.

As we all know, the metabolic relationship between glucose and lipids is mutually regulated. Generally speaking, dietary carbohydrate affect the synthesis and catabolism of lipid, and excess glucose can be converted to fatty acids and triglyceride by lipogenesis [6]. The SREBP1 is a transcription factor regulated by membrane binding, which is involved in the regulation of key lipogenic enzymes such as ACLY and FAS [1]. The results of this study showed that *srebp1* mRNA expression levels were significantly up-regulated in the liver of BT after the HC diet intake, which in turn induced up-regulation of the mRNA expression levels of key enzymes in fatty acid synthesis (*acca*, *aclya* and *fas*). The above findings were consistent with the results of study in grass carp, which indicated that carbohydrate-induced lipogenesis was enhanced [60]. However, the HC diet only induced up-regulation of *acca* and *fas* in TC liver. Similar results were obtained in the study of rainbow trout, in which mRNA expression levels of *srebp1* were not regulated by dietary carbohydrate, but the HC diet increased the enzyme activity of FAS and mRNA expression levels of *acly* [21]. These results may imply that carbohydrate-induced lipogenesis has different pathways in different fishes.

Fatty acids play an important role in the liver in supplying energy, and their predominant oxidation pathway is β-oxidation. Current studies on the effect of dietary carbohydrate on fatty acid β-oxidation have yielded conflicting results. Even in studies on the same fish (rainbow trout), either an induction or an inhibition of fatty acid oxidation by carbohydrate was found [20,72]. CPT1 is the key rate-limiting enzyme controlling the entry of fatty acids into mitochondria [20], and ACO3 is the key enzyme catalyzing the oxidation reaction in peroxisomal β-oxidation [73]. The mRNA expression of *cpt1* in BT liver was suppressed by the HC diet in the present study, whereas *aco3* were not affected by dietary carbohydrate levels. In contrast, the mRNA expression levels of *cpt1* in the liver of TC were significantly increased with the increase of dietary carbohydrate levels. Thus, the mRNA expression levels of *cpt1* were significantly higher in TC than in BT, in HC diet. The up-regulation of genes related to fatty acid β-oxidation in TC liver by carbohydrate may be due to the accumulation of lipids in TC liver leading to severe oxidative stress and liver damage, while β-oxidation of fatty acid contributed to avoid the accumulation of lipid droplets and lipotoxicity [74]. However, the up-regulation of genes related to fatty acid β-oxidation was not observed in BT, which may be because the liver of BT is more tolerant and can deposit more lipid. It is also consistent with the larger liver lipid droplet area and higher HSI observed in BT.

5. Conclusion

The study is the first to compare the differences between BT and TC in the utilization of dietary carbohydrate. The results indicated that BT had higher carbohydrate utilization capacity and requirement than TC, as evidenced by better glucose homeostasis and growth performance under high carbohydrate diet. High carbohydrate diet resulted in liver glycogen and lipid accumulation in both fish, but BT exhibited lower oxidative stress and liver damage. Both fish could adapt to high carbohydrate diet to some extent by enhancing glycolysis and inducing lipogenesis. However, glucose transport and glycolysis in the BT liver were more strongly regulated by the high carbohydrate diet.

Ethical statement

This experimental protocol was recognized by the Animal Ethics Experimental Committee of Hunan Normal University (Changsha, China), and all experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals.

Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.repbre.2023.07.002.

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