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Feeding restriction alleviates high carbohydrate diet-induced oxidative stress and inflammation of *Megalobrama amblycephala* by activating the AMPK-SIRT1 pathway

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ABSTRACT

This study investigated the effects of restricted feeding on the growth performance, oxidative stress and inflammation of *Megalobrama amblycephala* fed high-carbohydrate (HC) diets. Fish (46.94 ± 0.04 g) were randomly assigned to four groups containing the satiation of a control diet (30% carbohydrate) and three satiate levels (100% (HC1), 80% (HC2) and 60% (HC3)) of the HC diets (43% carbohydrate) for 8 weeks. Results showed that HC1 diet remarkably decreased final weight (FW), weight gain rate (WGR), specific growth rate (SGR), feed conversion ratio (FCR), hepatic activities of total anti-oxidation capacity (T-AOC), superoxide dismutase (SOD) and catalase (CAT), the AMP/ATP ratio, the p-AMPKα/t-AMPKα ratio, sirtuin-1 (SIRT1) protein expression and hepatic transcriptions of AMPKα2, SIRT1, nuclear factor erythroid 2-related factor 2 (Nrf2), catalase (CAT), manganese superoxide dismutase (Mn-SOD), glutathione peroxidase 1 (GPx1) and interleukin10 (IL 10) compared to the control group, whereas the opposite was true for protein efficiency ratio (PER), nitrogen retention efficiency (NRE), energy retention efficiency (ERE), plasma glucose levels, alanine transaminase (AST) and aspartate aminotransferase (ALT) activities, hepatic contents of malondialdehyde (MDA), tumour necrosis factor α (TNF α) and interleukin 1β (IL 1β), ATP and AMP contents and hepatic transcriptions of kelch-like ECH associating protein 1 (Keap1), IkB kinase α (IKK α), nuclear factor kappa B (NF-κB), TNF α, IL 1β, interleukin 6 (IL 6) and transforming growth factor β (TGF β). As for the HC groups, fish fed the HC2 diet obtained relatively high values of SGR, PER, NRE, ERE, hepatic activities of T-AOC, SOD and CAT, the AMP/ATP ratio, the p-AMPKα/t-AMPKα ratio, SIRT1 protein expression and hepatic transcriptions of AMPKα2, Nrf2, CAT, copper/zinc superoxide dismutase (Cu/Zn-SOD), Mn-SOD, GPx1, glutathione S-transferase (GST) and interleukin10 (IL 10), while the opposite was true for hepatic content of IL 6 and transcription of IKK α. Overall, an 80% satiation improved the growth performance and alleviated the oxidative stress and inflammation of blunt snout bream fed HC diets via the activation of the AMPK-SIRT1 pathway and the up-regulation of the activities and transcriptions of Nrf2-modulated antioxidant enzymes coupled with the depression of the levels and transcriptions of the NF-κB-mediated pro-inflammatory cytokines.

1. Introduction

Carbohydrates (CHO) are regarded as the cheapest energy source and organic carbon for vertebrates including fish [1,2]. In aquaculture, the incorporation of this nutrient can not only improve the pelleting quality of feed, but also effectively reduce the use of some expensive nutrients such as protein and lipid, for energy needs [3,4]. However, unlike the case of mammals, most fish species are considered to be glucose-intolerant with a limited capability of carbohydrates utilization [2,5]. To date, some possible reasons involving the glucose intolerance have been proposed, such as the imbalance between hepatic glucose uptake and production, a relatively low hepatic lipogenesis capacity from glucose, a higher sensitivity of insulin to amino acids rather than glucose, etc [6]. But, the physiological basis for it in fish still remains unclear. In mammals, high-carbohydrate intakes could induce an excessive increase in the oxidative stress and inflammation of tissues, as

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might consequently result in their low insulin sensitivity [7,8]. This has been regarded as a key cause of the type II diabetes [9]. However, such correlations in fish have still been poorly understood. Recently, several studies have suggested that the inadequate regulation of glucose homeostasis in fish may be partly attributed to the impaired immunity induced by hyperglycemia [3,10–12]. However, the underlying mechanisms still remain obscure, as warrants further studies.

At present, accumulating evidence indicates that hyperglycemia, inflammation and glucose intolerance are inter-related and are reciprocal causation [7]. In fact, the long-term postprandial hyperglycemia caused by high-carbohydrate diets could exacerbate the oxidative stress of tissues, as is reflected by the increased generations of superoxide radical O_2^{-1} [13]. Excessive O_2^{-1} could activate some major pro-inflammatory transcription factors, which are redox sensitive, such as nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1), leading to the increased transcriptions of the pro-inflammatory genes (like tumour necrosis factor-α (TNF α), interleukin 1β (IL 1β) and interleukin 8 (IL 8)) and thus inflammation [14–17]. In return, the increased productions of the pro-inflammatory cytokines (such as TNF α, IL 1β and IL 6) can reduce the insulin sensitivity of tissues by up-regulating the expressions of several proteins that could suppress and impair the specific pathways of insulin signaling, thus resulting in glucose intolerance [18–21]. Therefore, glucose intolerance, commonly known as a manifestation of hyperglycemia, is pro-inflammatory [7]. Recently, several studies have suggested that the pronounced glucose intolerance in fish may be attributed to the poor postprandial supervision of certain energy sensors, which are closely involved in glucose metabolism and inflammatory responses [22]. Among them, AMP-activated protein kinase (AMPK) has attracted considerable attention due to its potent effects on the regulations of intracellular energy balance as well as protecting cells from damage [23]. Generally, AMPK can be activated by phosphorylation when cellular stresses increase the AMP/ATP ratio, such as hypoxia, exercise, energy restriction, etc [24]. Once activated, AMPK can activate silent information regulator 1 (SIRT1) by altering the NAD^+ (nicotinamide adenine dinucleotide) to nicotinamide adenine dinucleotide reduced form (NADH) ratio [25]. Then, the activated AMPK/SIRT1 network can regulate a series of physiological consequences concerning glucose metabolism, oxidative responses and inflammation, such as 1) the inhibition of hepatic glucose production and the stimulation of glucose uptake in skeletal muscle [26,27]; 2) the activation of the Nrf2 (NF-E2-related factor 2) antioxidant pathway [28]; 3) the inhibition of NF-κB inflammatory pathway [29]. However, these results have generally been obtained in mammals. Such information in aquatic species is still quite limited. Recently, several studies have demonstrated that a long-term intake of high-lipid diet could induce chronic inflammation, apoptosis and DNA damage in fish, resulting in hepatic metabolic disorders [30,31]. These results clearly indicate a close correlation between inflammation and the disturbance of nutrients metabolism in fish [32]. However, the potential mechanisms are still barely unknown. In addition, in mammals, an appropriate restriction of energy intake could decrease the circulating levels of inflammatory cytokines and inflammatory signaling activities in a wide variety of tissues, thus benefiting glucose homeostasis [33]. However, the exact mechanisms underlying these metabolic processes are still barely understood until now. Meanwhile, whether restricted feeding could alleviate the oxidative stress and inflammation of fish fed high-carbohydrate diets thus improving its glucose metabolism and carbohydrate utilization is still unknown, as warrants further studies.

Blunt snout bream (*Megalobrama amblycephala*) is an herbivorous freshwater fish, which is native and economically important in China. Due to its herbivorous feeding habit, the practical feeds formulated for this species usually contain large amounts of carbohydrates to meet its energy requirement as well as to reduce the feed cost. However, excessive intakes of carbohydrates inevitably lead to the liver metabolic dysfunction and immune depression of this fish [34,35]. Whether this is induced by the enhanced oxidative stress and inflammatory responses due to high-carbohydrate feeding is still unknown. In addition, whether an appropriate feeding regime could improve the glucose metabolism and carbohydrate utilization of this species fed high-carbohydrate diets by alleviating the oxidative stress and inflammation is also uncharted. Bearing these in mind, this study was conducted to investigate the potential effects of feeding restriction on the growth performance, oxidative stress and inflammation of blunt snout bream fed a carbohydrate enriched diet. The findings obtained here might benefit our understandings of the glucose metabolism in fish, as might also facilitate the development of effective and practical approaches to improve its carbohydrate utilization.

2. Materials and methods

2.1. Ethics statement

The present study was performed in compliance with the rules and regulations established by the Animal Care and Use Committee of Nanjing Agricultural University (Nanjing, China) with the permissions obtained (permit number: SYXK (Su) 2011–0036).

2.2. Feeding trial

Two experimental diets were formulated to contain two dietary carbohydrate (namely nitrogen-free extract) levels: 30% and 43%. According to our previous studies, the optimal dietary carbohydrate level for juvenile blunt snout bream is 29–32% [36,37]. Therefore, a diet containing 30% carbohydrate was adopted as the control, while that of 43% was designated as the high-carbohydrate (HC) diet. Formulation and proximate composition of the experimental diets were presented in Table 1. Dietary protein and lipid levels have been evidenced to meet the optimal growth of *Megalobrama amblycephala* at the juvenile stage [38,39]. Soybean oil was used as lipid source. Fish meal, soybean meal, rapeseed meal and cottonseed meal served as protein sources. Corn starch was used as the main carbohydrate source. Microcrystalline cellulose was used to compensate for the carbohydrate levels required.

Table 1
Formulation and proximate composition of the experimental diets.

	Control diet	High-carbohydrate diet
Formulation (%)		
Fish meal	5.00	5.00
Soybean meal	30.00	30.00
Rapeseed meal	18.40	18.40
Cottonseed meal	15.00	15.00
Soybean oil	3.60	3.60
Corn starch	12.00	25.00
Microcrystalline cellulose	13.00	0.00
Calcium biphosphate	1.80	1.80
Premix[a]	1.20	1.20
Proximate composition (% air-dry basis)		
Dry matter	8.79	8.96
Crude lipid	4.97	5.25
Ash	7.00	6.96
Crude protein	31.71	31.94
Crude fiber	15.76	3.00
Nitrogen-free extract[b]	31.77	43.89
Energy (MJ/kg)	18.89	18.82

[a] Premix supplied the following minerals and/or vitamins (per kg of premix): $CuSO_4 \cdot 5H_2O$, 2.0 g; $FeSO_4 \cdot 7H_2O$, 25 g; $ZnSO_4 \cdot 7H_2O$, 22 g; $MnSO_4 \cdot 4H_2O$, 7 g; Na_2SeO_3, 0.04 g; KI, 0.026 g; $CoCl_2 \cdot 6H_2O$, 0.1 g; Vitamin A, 900,000 IU; Vitamin D, 200,000 IU; Vitamin E, 4500 mg; Vitamin K_3, 220 mg; Vitamin B_1, 320 mg; Vitamin B_2, 1090 mg; Vitamin B_5, 2000 mg; Vitamin B_6, 500 mg; Vitamin B_{12}, 1.6 mg; Vitamin C, 5000 mg; Pantothenate, 1000 mg; Folic acid, 165 mg; Choline, 60,000 mg.

[b] Calculated by difference (100 - moisture - crude protein - crude lipid - ash - crude fiber).

Blunt snout bream were obtained from the fish hatchery of Yangzhou (Jiangsu, China). Prior to the feeding trial, fish were acclimated to the experimental conditions for 2 weeks by feeding a commercial diet (feed No. 191, Tongwei feed group Co., Ltd., Wuxi, China) containing 32% protein and 31% nitrogen-free extract. After the acclimation, a 2-week preliminary experiment was conducted to evaluate the 100% satiate levels of fish fed the control and the HC diets, respectively. Accordingly, we set two other satiate levels (namely 80% and 60%) of fish fed the HC diet. Fish of similar sizes (average weight: 46.94 ± 0.04 g) were randomly distributed into 16 tanks (300 L volume) at a rate of 15 fish per tank. Then fish were randomly assigned to four groups containing a full satiation of the control diet (the control group) and three satiate levels (100%, 80% and 60%) of the HC diet. These four treatments were named as control, HC1, HC2 and HC3, respectively. Each group was tested in four replicates. Fish were hand-fed three times daily (07:30, 11:30 and 16:30) for 8 weeks. During the feeding trial, fish were batch-weighed by tanks once a week. Then, feed amounts were adjusted accordingly. Fish were reared under the following conditions: water temperature ranged from 29 ± 3 °C; dissolved oxygen was maintained at 5.0–6.0 mg/L; pH fluctuated between 7.1 and 7.3; and total ammonia nitrogen was kept less than 0.04 mg/L.

2.3. Sampling procedures

After the 8-week feeding trial, fish were starved for 24 h to evacuate the alimentary tract contents prior to sampling. All fish in each tank were counted and weighed. Then, 4 fish were randomly collected from each tank, and were anesthetized in diluted MS-222 (100 mg/L) (tricaine methanesulfonate, Sigma, USA). Thereafter, blood samples were obtained from caudal vein using heparinized syringes, and were centrifuged at 3000 g at 4 °C for 10 min. The supernatant was then stored at −80 °C for subsequent analysis. Meanwhile, individual liver samples were dissected, and then kept at −80 °C for subsequent analysis.

2.4. Analytic procedures

2.4.1. Proximate composition analysis

Proximate compositions of the diets were analyzed following the AOAC protocols [40]. Moisture was determined by oven drying at 105 °C until constant weight. Crude protein (nitrogen × 6.25) was measured by the micro-Kjeldahl method using an Auto Kjeldahl System (FOSS KT260, Switzerland). Crude lipid was measured by solvent extraction using a Soxtec System (Soxtec System HT6, Tecator, Höganäs, Sweden). Ash was analyzed by combustion at 550 °C for 6 h. Gross energy was determined using a Bomb Calorimeter (PARR 1281, Parr Instrument Company, Moline, IL, USA). Crude fiber was analyzed by fritted glass crucible method using an automatic analyzer (ANKOM A2000i, Macedon, New York, NY, USA).

2.4.2. Analysis of plasma and liver metabolites

Plasma glucose was measured by the glucose oxidase method as described by Asadi et al. [41]. Plasma aspartate aminotransferase (AST) and alanine transaminase (ALT) activities were determined according to Habte-Tsion et al. [42]. Hepatic protein content was determined following the methods by Bradford [43]. Hepatic activities of total superoxide dismutase (T-SOD), total antioxidant capacity (T-AOC) and catalase (CAT) were all determined following the procedures detailed by Zhou et al. [34] and Aebi [44], respectively. Hepatic malondialdehyde (MDA) content was estimated using the thiobarbituric acid test [45]. Hepatic contents of tumour necrosis factor α (TNF α), interleukin 1β (IL 1β) and interleukin 6 (IL 6) were measured following the methods by Yildirim and Danabas [46]. In addition, hepatic contents of adenosine triphosphate (ATP) and adenosine monophosphate (AMP) were both assessed as described by Lund et al. [47].

2.4.3. Analysis of western blot and real-time-PCR

Protein extraction and western blot analysis were performed according to the method described in our previous studies [48–50]. The specific primary antibodies are anti-Ampk α (#2532, Cell Signaling Technology, United States, 1:2000 dilution), anti-phospho-Ampk α (#2535, Cell Signaling Technology, United States, 1:2000 dilution), anti-SIRT1(13161-1-AP, Proteintech, United States, 1:1000 dilution) and anti-β-actin (BM3873; Boster, China, 1:5000 dilution) antibodies. The secondary antibody is anti-rabbit antibody (#7074, Cell Signaling Technology, United States, 1:2000 dilution). These antibodies have been shown to successfully cross-react with blunt snout bream proteins [48–51].

Total RNA extraction and cDNA synthesis were performed using the liver samples according to our previous studies [35,48–50]. Then, the transcriptions were investigated for several target genes, including AMPKα1, AMPKα2, SIRT1, Nrf2, Keap1 (kelch-like ECH associating protein 1), CAT, Cu/Zn-SOD (copper/zinc superoxide dismutase), Mn-SOD (manganese superoxide dismutase), GPx1 (glutathione peroxidase 1), GST (glutathione S-transferase), IKK α (IkB kinase α), NF-κB, TNF α, IL 1β, IL 6, IL 8, IL 10 and TGF β (transforming growth factor-β). Specific primers for AMPKα1, AMPKα2, Cu/Zn-SOD, Mn-SOD, GPx1, GST, NF-κB, TNF α, IL 1β and IL 6 were designed using the Primer 5 software according to the known sequences of this fish found in the nucleotide databases and the partial cDNA sequences of these target genes obtained by transcriptome analysis [52], respectively. The primers for SIRT1, Nrf2, Keap1, CAT, IKK α, IL 8, IL 10 and TGF β were designed using the published sequences of blunt snout bream (Table 2). Hepatic transcriptions of these target genes were quantified by a reference gene (namely the elongation factor 1 alpha, EF1α) using the $2^{-\triangle\triangle CT}$ method.

2.4.4. Calculations and statistical analysis

The growth parameters adopted in this study were calculated as follows:

Weight gain rate (WGR) = (Final body weight − initial body weight) × 100 / initial body weight.

Specific growth rate (SGR) = $(\mathrm{LnW_t} - \mathrm{LnW_0}) \times 100 / T$

where W_0 and W_t are the initial and final body weights, and T is the culture period in days.

Feed conversion ratio (FCR) = Feed intake / total wet weight gain.

Relative feed intake (RFI) = Feed intake (g) × 100 / [(initial fish weight (g) + final fish weight (g) + dead fish weight (g)) × days reared / 2].

Protein efficiency ratio (PER) = Wet weight gain / total protein fed.

Nitrogen and energy retention (NRE and ERE) (%) = $[(W_t \times C_t) - (W_0 \times C_0)] \times 100 / (C_{diet} \times$ feed intake)

where W_0 and W_t are the initial and final body weights, C_0 and C_t are the initial and final nitrogen/energy contents in body respectively, and C_{diet} is the nitrogen/energy content in the diets.

2.4.5. Statistical analyses

The data were analyzed by one-way ANOVA test using the SPSS program version 22.0 (SPSS Inc., Michigan Avenue, Chicago, IL, USA), taking into account the normality of the data distribution and the homogeneity of variances. If significant ($P < 0.05$) differences were found, Tukey's HSD multiple range test was conducted to rank the means. All data were presented as mean ± S.E.M (standard error of the mean).

Table 2
Nucleotide sequences of primers used to quantify gene expressions by real-time PCR.

Target gene	Forward (5′-3)	Reverse (5′-3)	Accession numbers or references
AMPKα1	AGTTGGACGAGAAGGAG	AGGGCATACAAAATCAC	ARF07712.1
AMPKα2	ACAGCCCTAAGGCACGATG	TGGGTCGGGTAGTGTTGAG	KX061841
SIRT1	TCGGTTCATTCAGCAGCACA	ATGATGATCTGCCACAGCGT	[51]
Nrf2	GGGGAAGTCCTTGAACGGAG	AACCAGCGGGAATATCTCGG	[53]
Keap1	AATATCCGCCGGCTGTGTAG	TGAGTCCGAGGTGTTTCGTG	[53]
CAT	CAGTGCTCCTGATACCCAGC	TTCTGACACAGACGCTCTCG	[53]
Cu/Zn-SOD	AGTTGCCATGTGCACTTTTCT	AGGTGCTAGTCGAGTGTTAGG	KF479046.1
Mn-SOD	AGCTGCACCACAGCAAGCAC	TCCTCCACCATTCGGTGACA	KF195932.1
GPx1	GAACGCCCACCCTCTGTTTG	CGATGTCATTCCGGTTCACG	KF378713.1
GST	AACCTCTGTGGGGAAACTGAAGAAG	TAGAGTTCCTGGCAGATTCTCATCG	[52]
IKK α	GGCTACGCCAAAGACCTG	CGGACCTCGCCATTCATA	[54]
NF-κB	GAAGAAGGATGTGGGAGATG	TGTTGTCGTAGATGGGCTGAG	[52]
TNF α	TGGAGAGTGAACCAGGACCA	AGAGACCTGGCTGTAGACGA	KU976426.1
IL 1β	ACGATAAGACCAGCACGACC	CTGTTTCCGTCTCTCAGCGT	[52]
IL 6	CAGCAGAATGGGGGAGTTATC	CTCGCAGAGTCTTGACATCCTT	KJ755058.1
IL 8	CAGAGAGTCGACGCATTGGT	ATTCACGGTGCTTTGTTGGC	[53]
IL 10	GTGTTTTCGGGTGCAAGTGG	ATGAACGAGATCCTGCGCTT	[53]
TGF β	ACTGGACAAACAGAGAGGCG	CAGGGGAGTTGCCGTTAGAG	[53]
EF 1α	CTTCTCAGGCTGACTGTGC	CCGCTAGCATTACCCTCC	X77689.1

AMPKα1, AMP-activated protein kinase α1; AMPKα2, AMP-activated protein kinase α2; SIRT1, sirtuin 1; Nrf2, nuclear factor erythroid 2-related factor 2; Keap1, kelch-like ECH associating protein 1; CAT, catalase; Cu/Zn-SOD, copper/zinc superoxide dismutase; Mn-SOD, manganese superoxide dismutase; GPx1, glutathione peroxidase 1; GST, glutathione S-transferase; IKK α, IkB kinase α; NF-κB, nuclear factor kappa B; TNF α, tumour necrosis factor α; IL 1β, interleukin 1β; IL 6, interleukin 6; IL 8, interleukin 8; IL 10, interleukin10; TGF β, transforming growth factor-β; EF 1α, elongation factor 1, α.

3. Results

3.1. Growth performance and feed utilization

As can be seen from Table 3, the final weights (FW), weight gain rate (WGR), specific growth rate (SGR) and feed conversion ratio (FCR) of the HC1 group were all significantly ($P < 0.05$) lower than those of the control group. But no statistical difference was observed in the relative feed intake (RFI), protein efficiency ratio (PER), nitrogen retention efficiency (NRE) and energy retention efficiency (ERE) ($P > 0.05$). As for the HC groups, the FW, WGR and SGR of the HC2 treatment were all significantly higher than those of the other two groups ($P < 0.05$). The FCR and RFI of the HC2 group were both significantly lower ($P < 0.05$) than those of the HC1 group, but showed no statistical difference with those of the HC3 group, while the opposite was true for both PER and NRE.

3.2. Plasma and liver metabolites

As can be seen from Table 4, the plasma glucose levels and ALT activities as well as the hepatic MDA, TNF α, IL 1β and IL 6 contents of the HC1 group were all higher than those of the control group, but no statistical difference was observed in hepatic IL 6 content ($P > 0.05$). However, hepatic activities of T-AOC, SOD and CAT of fish fed the HC1 diet were all significantly ($P < 0.05$) lower than those of the C group. As for the HC groups, plasma glucose levels and AST and ALT activities as well as hepatic MDA, TNF α, IL 1β and IL 6 contents were all decreased when the satiate levels decreased from 100% to 60%, but no statistical difference was observed in hepatic IL 6 content ($P > 0.05$). In addition, hepatic activities T-AOC, SOD and CAT of the HC2 group were all significantly higher ($P < 0.05$) than those of group HC1 and HC3 except for the CAT activities, which showed no statistical ($P > 0.05$) difference between group HC2 and HC3.

3.3. Liver ATP and AMP contents

As can be seen from Fig. 1, liver ATP and AMP contents of the HC1 group were all significantly ($P < 0.05$) higher than those of the control, whereas the opposite was true for the ATP/AMP ratio. As for the HC groups, liver ATP and AMP contents both decreased significantly ($P < 0.05$) as the satiate levels decreased from 100% to 60%, while the opposite was true for the ATP/AMP ratio.

Table 3
Growth performance and feed utilization of blunt snout bream subjected to different treatments.

	Control	HC1	HC2	HC3
Initial weight (g)	46.97 ± 0.12	46.9 ± 0.14	46.88 ± 0.06	46.82 ± 0.07
Final weight (g)	134.86 ± 3.24^{a}	123.16 ± 6.68^{b}	134.25 ± 3.62^{a}	120.02 ± 2.29^{b}
WGR (%)	180.70 ± 3.55^{a}	160.79 ± 3.62^{b}	179.70 ± 5.89^{a}	166.92 ± 8.49^{b}
SGR	1.84 ± 0.01^{a}	1.68 ± 0.01^{b}	1.88 ± 0.01^{a}	1.70 ± 0.02^{b}
FCR	2.15 ± 0.09^{a}	1.84 ± 0.03^{b}	1.47 ± 0.11^{c}	1.39 ± 0.07^{c}
RFI	3.35 ± 0.03^{a}	3.11 ± 0.07^{a}	2.43 ± 0.03^{b}	2.39 ± 0.01^{b}
PER	1.40 ± 0.06^{c}	1.66 ± 0.04b^{c}	2.40 ± 0.21^{a}	1.98 ± 0.03^{ab}
NRE	27.77 ± 1.30^{b}	30.96 ± 0.46^{b}	42.38 ± 1.11^{a}	39.18 ± 2.41^{a}
ERE	28.93 ± 1.04^{b}	35.70 ± 1.97^{ab}	48.68 ± 1.94^{a}	39.83 ± 1.93^{ab}

Control, the satiated level at 100% of the control diet, HC1, the satiated level at 100% of the high-carbohydrate diet, HC2, the satiated level at 80% of the high-carbohydrate diet, HC3, the satiated level at 60% of the high-carbohydrate diet (the same below).
WGR, weight gain rate; SGR, specific growth rate; FCR, feed conversion ratio; RFI, relative feed intake; PER, protein efficiency ratio; NRE, nitrogen retention efficiency; ERE, energy retention efficiency.
Values are mean ± S.E.M of four replications. Means in the same line with different superscripts are significantly different ($P < 0.05$).

Table 4
Plasma and liver metabolites of blunt snout bream subjected to different treatments.

	Control	HC1	HC2	HC3
Plasma metabolites				
Glucose (mmol/L)	2.33 ± 0.12^{c}	3.66 ± 0.12^{a}	2.94 ± 0.09^{b}	2.06 ± 0.06^{d}
AST (U/L)	21.01 ± 1.61^{ab}	24.95 ± 1.92^{a}	21.82 ± 0.83^{ab}	18.65 ± 1.07^{b}
ALT (U/L)	34.41 ± 0.12^{b}	46.18 ± 0.37^{a}	33.67 ± 0.37^{b}	31.01 ± 0.12^{b}
Liver metabolites				
T-AOC (U/mg protein)	0.31 ± 0.01^{b}	0.11 ± 0.01^{c}	0.45 ± 0.02^{a}	0.30 ± 0.01^{b}
SOD (U/mg protein)	26.11 ± 0.21^{b}	13.48 ± 0.14^{c}	30.23 ± 0.30^{a}	26.25 ± 0.27^{b}
CAT (U/mg protein)	17.21 ± 0.14^{b}	9.34 ± 0.21^{c}	23.1 ± 0.18^{a}	21.86 ± 0.13^{a}
MDA (nmol/mg protein)	5.28 ± 0.10^{b}	8.59 ± 0.38^{a}	6.03 ± 0.15^{b}	5.46 ± 0.23^{b}
TNF α (pg/mg protein)	142.30 ± 10.41^{c}	319.57 ± 12.10^{a}	160.39 ± 8.41^{b}	146.19 ± 5.83^{c}
IL 1β (pg/mg protein)	48.18 ± 4.20^{bc}	71.11 ± 3.72^{a}	51.49 ± 1.37^{b}	42.64 ± 0.31^{c}
IL 6 (pg/mg protein)	12.40 ± 0.31	14.25 ± 0.72	11.39 ± 0.58	12.74 ± 0.56

AST, alanine transaminase; ALT, aspartate aminotransferase; T-AOC, total anti-oxidation capacity; SOD, superoxide dismutase; CAT, catalase; MDA, malondialdehyde; TNF α, tumour necrosis factor α; IL 1β, interleukin 1β; IL 6, interleukin 6.
Values are mean ± S.E.M of four replications. Means in the same line with different superscripts are significantly different ($P < 0.05$).

3.4. Hepatic p-AMPKα/t-AMPKα ratio, protein expressions of SIRT1 and transcriptions of AMPKα and SIRT1

As can be seen from Fig. 2, the transcription of AMPKα1 showed no statistical difference ($P > 0.05$) among all the treatments. The hepatic p-AMPKα/t-AMPKα ratio, protein expressions of SIRT1 and the transcription of AMPKα2 of the HC1 group were all significantly ($P < 0.05$) lower than those of the control group. As for the HC groups, their values of the HC2 group were all significantly ($P < 0.05$) higher than those of the other groups except for the p-AMPKα/t-AMPKα ratio and the SIRT1 transcription, both of which showed no statistical difference ($P > 0.05$) between HC2 and HC3.

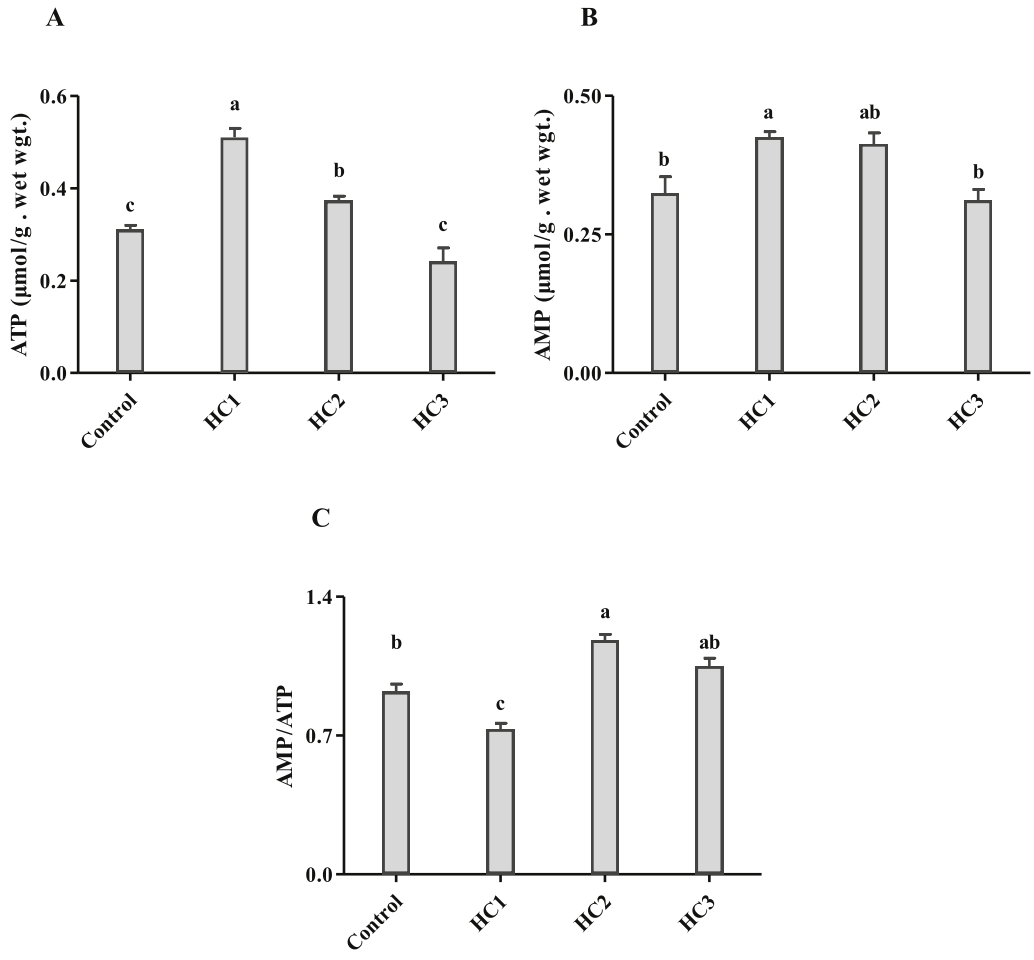

Fig. 1. ATP (A) and AMP (B) contents and ATP/AMP ratio (C) in the liver of blunt snout bream subjected to different treatments. ATP, adenosine triphosphate; AMP, adenosine monophosphate. Values are mean ± S.E.M of four replications. Bars assigned with different superscripts are significantly different ($P < 0.05$). Control, the satiated level at 100% of the control diet, HC1, the satiated level at 100% of the high-carbohydrate diet, HC2, the satiated level at 80% of the high-carbohydrate diet, HC3, the satiated level at 60% of the high-carbohydrate diet (the same below).

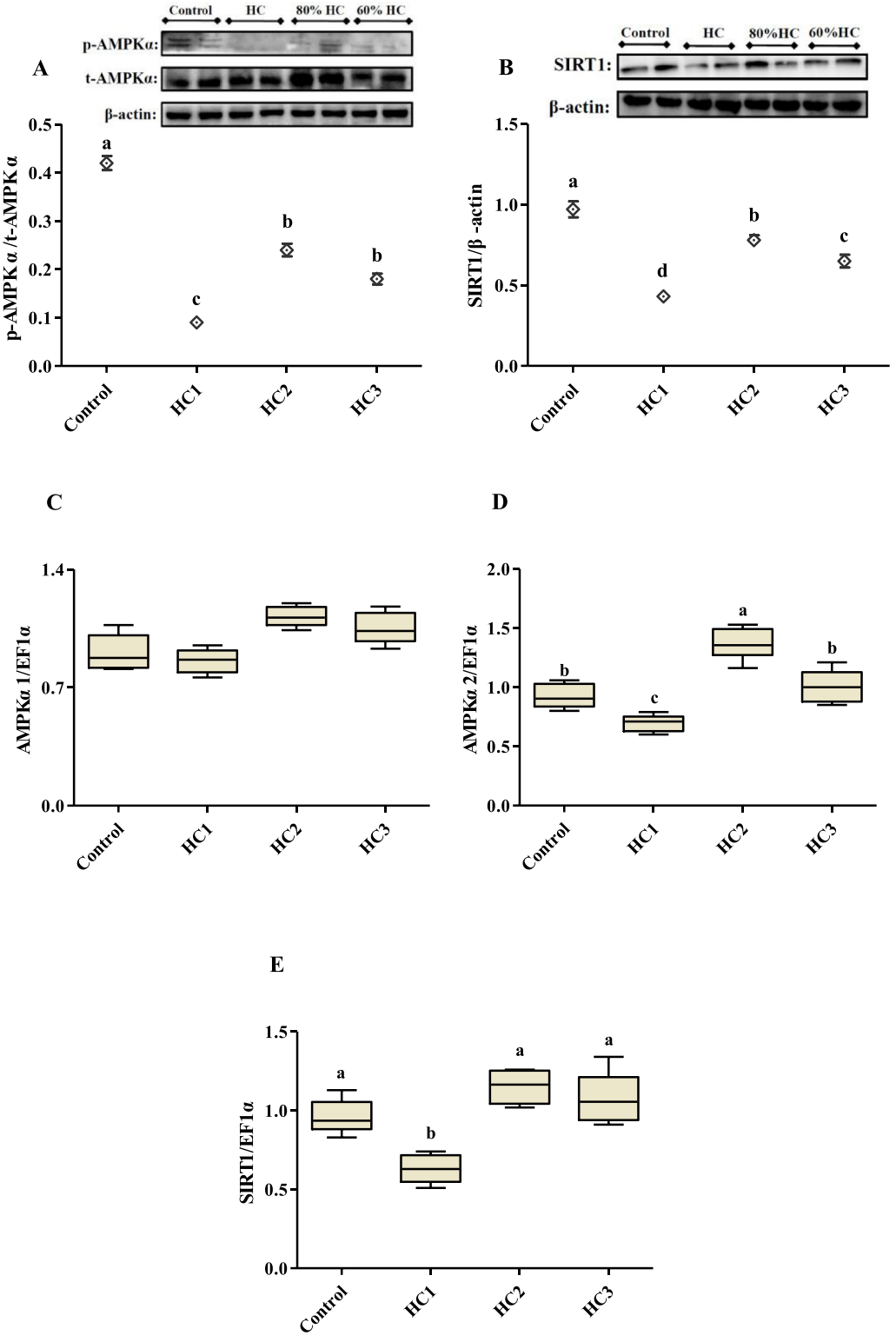

Fig. 2. Hepatic p-AMPKα/t-AMPKα ratio (A), protein expressions of SIRT1 (B) and the transcriptions of AMPKα1 (C), AMPKα2 (D) and SIRT1 (E) of blunt snout bream subjected to different treatments. Gels were loaded with 20 mg total protein per lane. Protein and phosphorylation levels were normalized to liver β-actin level. Values are mean ± S.E.M of four replications. Bars assigned different superscripts are significantly different ($P < 0.05$).

3.5. Hepatic transcriptions of the genes involved in the antioxidant defense and inflammation

As can be seen from Figs. 3 and 4, the transcription of IL 8 showed no statistical difference ($P > 0.05$) among all the treatments. The hepatic expressions of Nrf2, CAT, Mn-SOD, GPx1 and IL 10 of the HC1 group were all significantly ($P < 0.05$) lower than those of the control, whereas the opposite was found in the transcriptions of Keap1, IKK α, NF-κB, TNF α, IL 1β, IL 6 and TGF β. As for the HC groups, the hepatic expressions of Nrf2, CAT, Cu/Zn-SOD, Mn-SOD, GPx1, GST and IL 10 of

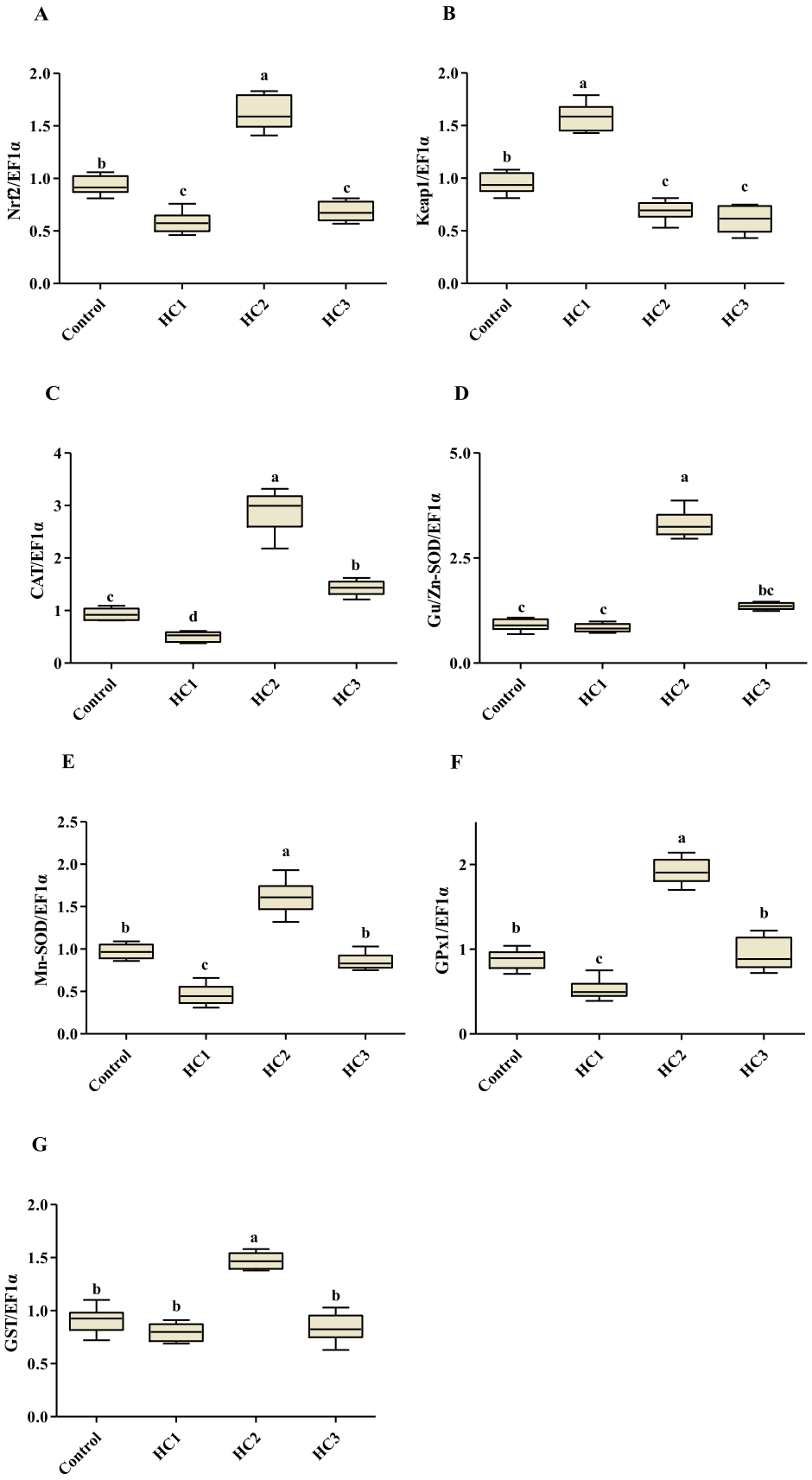

Fig. 3. The transcriptional levels of antioxidant-related genes in the liver of blunt snout bream subjected to different treatments. Values are mean ± S.E.M of four replications. Bars assigned with different superscripts are significantly different ($P < 0.05$).

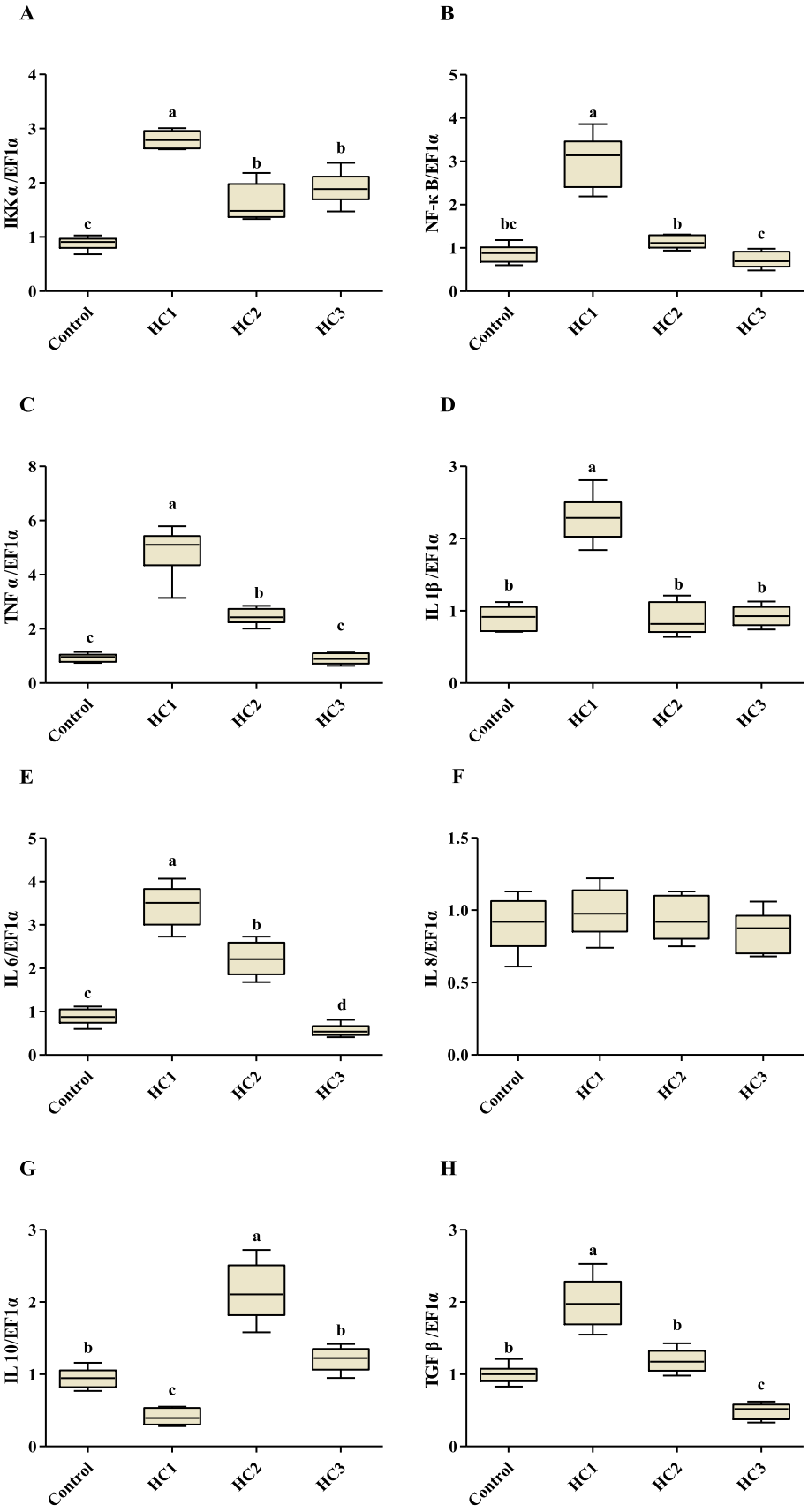

Fig. 4. The transcriptional levels of inflammation-related genes in the liver of blunt snout bream subjected to different treatments. Values are mean ± S.E.M of four replications. Bars assigned with different superscripts are significantly different ($P < 0.05$).

the HC2 group were all significantly higher ($P < 0.05$) than those of the other groups. However, the Keap1, IKK α, NF-κB, TNF α, IL 1β, IL 6 and TGF β of the HC1 group were all significantly higher ($P < 0.05$) than those of the other groups.

4. Discussion

In the present study, the final weight, WGR, SGR, FCR and RFI of the HC1 group were all lower than those of the control group, whereas the opposite was true for PER, NRE and ERE. These findings indicated the growth retardation but an enhanced nutrients-sparing effect in this fish fed carbohydrate-enriched diets. In fact, high-carbohydrate intakes usually cause a series of physiological stresses concerning glucose homeostasis and immune responses, as might consequently lead to the retarded growth of fish [1,3,55]. The decreased FCR may be partly due to the fact that high dietary carbohydrate levels can reduce feed palatability and accelerate fish satiety, thus resulting in a decrease in feed consumption [55]. Indeed, the reduced RFI observed in this study further supports this. Similar results have also been observed in other fish species [56,57]. In addition, the increased PER, NRE and ERE in the HC1 group indicated a nutrients-sparing effect of carbohydrates [1,2], as may be explained by the fact that high-carbohydrate intakes could depress the gluconeogenic pathway of fish, thereby improving the protein and lipid retentions [58]. As for the HC groups, final weight, WGR, SGR, PER, NRE and ERE were all significantly improved as the satiated levels decreased from 100 to 80%, while the opposite was true for both FCR and RFI. In addition, the SGR, PER, NRE and ERE of the HC2 group were all relatively higher than those of the control, while the opposite was true for both FCR and RFI. This suggested that an 80% satiation could significantly increase the growth performance and nutrients-sparing effect of this fish fed the HC diet. According to previous studies, feeding restriction can decrease the leptin secretion and activate the growth hormone pathway of fish fed high-energy diets [59,60], thereby promoting the growth performance. Moreover, in mammals, the activated AMPK/SIRT1 network by feeding restriction can effectively enhance tissue insulin sensitivity via inhibiting the glucose-induced oxidative and inflammatory stresses and activating the insulin signaling pathway [28,29,61], thereby improving the carbohydrates utilization and then the growth performance. However, such information is still absent in aquatic species. So, we conducted a follow-up study to investigate the corresponding underlying molecular mechanisms in fish.

According to previous studies, excess dietary carbohydrates could not only reduce the growth rate and feed utilization of fish, but also cause severe physiological stresses [1,34]. In this study, fish fed the HC1 diet exhibited relatively high values of plasma glucose levels, AST and ALT activities and liver MDA, TNF α, IL 1β and IL 6 contents compared with the control, whereas the opposite was true for hepatic activities of T-AOC, SOD and CAT. These findings clearly indicated that carbohydrate enriched diets induced a hyperglycemic state and inflammation in blunt snout bream coupled with a low antioxidant capacity. This was supported by the fact that the protective effects against oxidative damage could be directly reflected by the activities of several antioxidant enzymes such as SOD, CAT and T-AOC in fish [62]. Moreover, the pro-inflammatory cytokines including TNF α, IL 1β and IL 6 are the sensitive mediator of host response inflammation [46]. These results can be explained by the fact that the prolonged postprandial hyperglycemia induced by excess carbohydrates intake could inevitably increase the generation of superoxide radical O_2^{-1} by activating nicotinamide adenine dinucleotide phosphateoxidase and inducing mitochondrial membrane hyperpolarization [63]. This might result in intracellular chronic oxidative and inflammatory stresses, thereby damaging the metabolic functions of the liver. This can be further supported by the fact that AST, ALT and MDA are all the effective and sensitive indicators of tissue oxidative damage [64]. In fact, high glucose-induced glucolipotoxicity can lead to the oxidative damage of tissues marked as the boost in some specific indicators, such as AST and ALT activities and MDA content [65,66]. In addition, previous researches have confirmed that excessive lipid and glycogen accumulations in liver is considered as a negative signal, since it can cause the metabolic dysfunctions of tissues characterized by inducing mitochondrial damage, the overproduction of reactive oxygen species (ROS) and inflammation [30,31,34,63]. As for the HC groups, feeding restriction significantly increased hepatic activities of T-AOC, SOD and CAT, whereas the opposite was true for the values of plasma glucose, AST and ALT levels and liver MDA, TNF α, IL 1β and IL 6 contents. These findings indicated that feeding restriction improved the hepatic antioxidant capacity, inflammation and glucose metabolism of blunt snout bream fed carbohydrate-enriched diets. According to previous studies, restricted feeding can decrease the energy state of an organism, as might be reflected by the decreased plasma glucose levels [67,68]. Meanwhile, the decreased energy levels in tissues can activate the AMPK/SIRT1 axis, thereby alleviating the high glucose-induced oxidative stress and inflammation by affecting intracellular signal transduction pathways such as the Nrf2 pathway and NF-κB inflammatory pathway [28,29,69]. Additionally, the relatively high hepatic activities of antioxidant enzymes and low plasma glucose levels, plasma AST and ALT activities as well as liver pro-inflammatory cytokines contents were all observed in the HC2 group. These results indicated that an 80% satiation could effectively enhance the antioxidant capacity and inhibit inflammation of blunt snout bream fed a high-carbohydrate diet.

Generally, high energy intakes usually elevate the energy status of cells which could be indicative of the AMP/ATP ratio [70]. In this study, hepatic ATP and AMP contents of the HC1 group were all significantly higher than those of the control, whereas the opposite was true for AMP/ATP ratio. The reasonable explanation for this may be that high-carbohydrate diets could enhance glycolysis and glucose oxidation in liver, thereby increasing ATP production [71]. Subsequently, the excessive ATP was hydrolyzed, thus resulting in the increased AMP content. As for the HC groups, feeding restriction significantly increased hepatic AMP/ATP ratio with the maximum value observed in the HC2 group. Meanwhile, the hepatic AMP/ATP ratio of the HC2 group was also significantly higher than that of the control. This suggested that feeding restriction modified the intracellular energy state of blunt snout bream. This may be due to the fact that under high-energy status, restricted feeding could up-regulate the expressions of ATP metabolism-related genes (such as nucleoside diphosphate kinase-Z2), thereby leading to low liver ATP content [72]. Meanwhile, the up-regulated AMP/ATP ratio is considered as a positive signal in maintaining glucose homeostasis, since it could activate some metabolic sensors such as AMPK, thereby coordinating the glycolipid metabolism of fish [23,24].

AMPK and SIRT1 are evolutionary conserved partners, and are considered as the vital targets for improving metabolic disorders [73]. Studies have shown that AMPK activation can enhance SIRT1 activity by increasing cellular NAD^+ levels, resulting in the modulation of the activities of SIRT1 downstream targets involved in the energy metabolism and immune response processes [28,69,74]. However, such information in fish is still barely available. In this study, fish fed the HC1 diet exhibited relatively low values of the p-AMPKα/t-AMPKα ratio, SIRT1 protein expression and the transcriptions of AMPKα2 and SIRT1 compared with the control. This result indicated the long-term intake of carbohydrate enriched diets impaired the energy sensing of blunt snout bream, as might further result in the impaired glucose homeostasis. This was supported by the fact that AMPK/SIRT1 is an energy sensing network that regulates and controls a series of physiological processes such as energy expenditure, metabolic homeostasis and immune response [74]. According to previous studies, high-carbohydrate diets usually increase intracellular ATP contents, thus inhibiting the activity of AMPK [75,76], as might be reflected by the down-reregulated p-AMPKα/t-AMPKα ratio and AMPKα1 and AMPKα2 expressions. Subsequently, the inactive AMPK attenuates the stimulation of SIRT1

characterized by decreasing both the transcriptions and protein contents of SIRT1. As for the HC groups, feeding restriction significantly increased hepatic p-AMPKα/t-AMPKα ratio, SIRT1 protein expression and the transcriptions of AMPKα1, AMPKα2 and SIRT1 with the maximum values all observed in the HC2 group. In addition, hepatic transcriptions of AMPKα1, AMPKα2 and SIRT1 of the HC2 group were also higher than those of the control, although both the p-AMPKα/t-AMPKα ratio and SIRT1 protein expression exhibited an opposite trend. This indicated that an appropriate feeding restriction could improve the energy sensing of fish, thus improving the glucose metabolism [72].

Accumulating evidence suggests that the activated AMPK/SIRT1 network can enhance the activity of the oxidative stress regulator-Nrf2, thereby regulating the antioxidant defense of organisms [77,78]. However, such information in aquatic animals is extremely scarce. In this study, the hepatic transcriptions of Nrf2, CAT, Mn-SOD and GPx1 of the HC1 group were all significantly lower than those of the control, whereas the opposite was true for Keap1. These results were in accordance with that of the antioxidant enzymes activities, suggesting that a long-term satiety of the HC diet resulted in a decreased hepatic antioxidant capacity of blunt snout bream. According to a previous study, high-energy diets could induce the changes of the Nrf2 antioxidant response element signaling pathway [79]. Indeed, the decreased Nrf2 activity could down-regulate the transcriptional levels of Nrf2-modulated antioxidant enzymes including CAT, GPx1, Cu/Zn-SOD and Mn-SOD [53,80], thereby decreasing the antioxidant capacity. Meanwhile, the high Keap1 expressions found in the HC1 group were also justifiable, since Keap1 can down-regulate the activity of Nrf2 by mediating the ubiquitination [81]. Subsequently, the transcriptional levels of Nrf2, CAT, Cu/Zn-SOD, Mn-SOD, GPx1 and GST were all significantly improved as the satiated levels decreased from 100 to 80%, while the opposite was true for Keap1. Meanwhile, they were also significantly higher than those of the control. This suggested that an 80% satiation could significantly enhance the antioxidant capacity of this fish fed a HC diet. This was supported by the fact that the activation of SIRT1 can significantly up-regulate the transcriptional activities of Nrf2 and the Nrf2-mediated genes by decreasing the Keap1-mediated ubiquitination, thus promoting the cellular defense against oxidative stress [77,82,83].

In addition, the activated AMPK/SIRT1 network can also suppress the high glucose-induced chronic inflammatory responses via the inhibition of the NF-κB pathway, thereby improving the glucose intolerance [29]. In the present study, the hepatic transcriptions of IKK α, NF-κB, TNF α, IL 1β, IL 6, IL 8 and TGF β of the HC1 group were all higher than those of the control, whereas the opposite was true for the transcription of IL 10. These results were in line with that of the pro-inflammatory cytokines contents in liver. This indicated that a long-term satiety of high-carbohydrate diets might cause the inflammation in the liver of blunt snout bream. This was supported by the fact that the phosphorylation of IKK induced by high-glucose can accordingly trigger NF-κB signaling [84], thus resulting in the increased transcriptions of NF-κB-mediated pro-inflammatory cytokines including TNF α, IL 1β, IL 6 and IL 8 and then inflammation [29,85]. Subsequently, the accumulation of certain pro-inflammatory factors (such as TNF α and IL 6) in turn suppresses the activity of AMPK by up-regulating the expressions of protein phosphatase 2C, which further promotes the development of inflammation [86,87]. Meanwhile, excessive pro-inflammatory cytokines could inevitably increase the vulnerability of pancreatic beta cells in response to stress by producing large amounts of nitric oxide (NO), thereby decreasing the glucose-stimulated insulin secretion [88], as may result in the glucose intolerance of fish. Furthermore, the increased transcription of TGF β in the HC1 group was considered to be a positive signal, since it can inhibit the IL 1-induced NO production, thereby suppressing the inflammation in tissues [89]. However, although TGF β is effective in suppressing inflammation, the over-expression of it induced by high-glucose intakes usually leads to an excessive accumulation of extracellar matrix proteins in tissues, as eventually causes the impaired metabolic function [90], thus further aggravating the inflammation. It should be stated here that this information was mainly derived from mammals. The underlying mechanisms in fish still need further in-depth studies. As for the HC groups, feeding restriction significantly increased hepatic transcription of IL 10 with the maximum value observed in the HC2 group. In addition, the transcriptions of IKK α, NF-κB, TGF β and pro-inflammatory cytokines in the HC2 group were all lower than those of the HC1 group. However, the transcriptions of NF-κB, IL 1β, IL 8 and TGF β all showed no statistical difference between control and HC2 group. Those results indicated an 80% satiation could alleviate the inflammation in the liver of fish fed high-carbohydrate diets. The most plausible explanation may be that the activated AMPK/SIRT1 by feeding restriction can deacetylate the p65 protein (an activator of NF-κB signaling) at lysine 310, thus inhibiting the NF-κB-mediated pro-inflammatory pathways [29,91]. Moreover, the activated AMPK/SIRT1 can also up-regulate IL 10 expression and inhibit TGF β over-expression, thus decreasing the inflammatory stress of tissues [92]. Furthermore, Nrf2 activation induced by the AMPK/SIRT1 network can up-regulate the activity of the downstream target protein HO-1 (heme oxygenase-1), resulting in the decreased transcriptional activity of NF-κB and the transcriptions of NF-κB-dependent pro-inflammatory genes [93], which further inhibits the development of inflammation in fish liver.

In conclusion, the results obtained in this study suggested that an 80% satiation could improve the growth performance and alleviate the oxidative stress and inflammation of blunt snout bream fed HC diets, through the activation of the AMPK-SIRT1 pathway, the up-regulation of the activities related to the Nrf2-modulated antioxidant enzymes coupled with the depression of the transcriptions and activities of the NF-κB-mediated pro-inflammatory cytokines.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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