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Metformin improves the glucose homeostasis of Wuchang bream fed high-carbohydrate diets: a dynamic study

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Abstract

After a 12-week feeding trial, the glucose tolerance test was performed in *Megalobrama amblycephala* to evaluate the effects of metformin on the metabolic responses of glycolipids. Plasma insulin peaked at 2 h, then decreased to the basal value at 8–12 h post-injection. Plasma triglyceride levels and liver glycogen contents of the control group was decreased significantly during the first 2 and 1 h, respectively. Then, they returned to basal values at 12 h. During the whole sampling period, the high-carbohydrate groups had significantly higher levels of plasma metabolites and liver glycogen than those of the control group, and metformin supplementation enhanced these changes (except insulin levels). Glucose administration lowered the transcriptions of *ampk α1*, *ampk α2*, *pepck*, *g6pase*, *fbpase*, *cpt IA* and *aco*, the phosphorylation of Ampk α and the activities of the gluconeogenic enzymes during the first 2–4 h, while the opposite was true of *glut 2*, *gs*, *gk*, *pk*, *accα* and *fas*. High-carbohydrate diets significantly increased the transcriptions of *ampk α1*, *ampk α2*, *glut 2*, *gs*, *gk*, *pk*, *accα* and *fas*, the phosphorylation of Ampk α and the activities of the glycolytic enzymes during the whole sampling period, while the opposite was true for the remaining indicators. Furthermore, metformin significantly upregulated the aforementioned indicators (except *accα* and *fas*) and the transcriptions of *cpt IA* and *aco*. Overall, metformin benefits the glucose homeostasis of *Megalobrama amblycephala* fed high-carbohydrate diets through the activation of Ampk and the stimulation of glycolysis, glycogenesis and fatty acid oxidation, while depressing gluconeogenesis and lipogenesis.

Introduction

Carbohydrates are the cheapest energy and carbon source for vertebrates including fish, due to their relatively high abundance and low price (1). In aquaculture, the incorporation of carbohydrates in diets is regarded as a promising approach to reduce feed cost, and to spare protein from being used as an energy source (1, 2). However, unlike the case of mammals, the ability of fish to use dietary carbohydrates as an energy-yielding substrate is quite limited. In general, most teleost fish are considered to be glucose-intolerant with a persistent hyperglycemia often observed after a glucose load or the ingestion of carbohydrate-rich diets (1, 2, 3). At present, several valid hypotheses have been proposed to explain the poor utilization of dietary carbohydrates by fish, including a relatively low number of insulin receptors, a poor capacity for hepatic lipogenesis from glucose,

a higher potency of amino acids as insulin secretagogues than glucose and an imbalance between hepatic glucose uptake and production (1, 2, 4, 5, 6). However, the physiological basis for such apparent glucose intolerance in fish is still poorly understood. Hence, investigating the underlying mechanisms is of great significance, as it may promote the carbohydrates utilization by fish.

Recently, glucose tolerance tests (GTTs) have been widely used to investigate the metabolic utilization of carbohydrates by fish, since they are easy to perform and could provide a reliable indication of glucose utilization (1, 2, 7, 8, 9, 10). After a GTT, the duration and degree of hyperglycemia and the metabolic responses of the peripheral tissues are highly variable among different fish species, as might be closely related to the feeding habits of fish, the adaption to different carbohydrate levels and sources, the routes of glucose administration, fish sizes and so on (3, 11, 12, 13). Generally, both omnivorous and herbivorous fish exhibit a significantly shorter period of hyperglycemia than carnivorous species (3). In fact, a more efficient regulation of plasma glucose has been observed in both omnivorous and herbivorous fish, as may be attributed to an accelerated glycolysis and lipogenesis, but a decelerated gluconeogenesis (2). However, to date, the energy metabolism of fish after a GTT has barely been investigated. It is now widely acknowledged that, the energy metabolism of fish is monitored by the intracellular energy sensors (1, 10). Indeed, several studies have suggested that the low utilization of carbohydrates by fish might be due to the poor postprandial supervision of certain energy sensors that are directly involved in glucose metabolism (1, 10, 14). Among them, AMP-activated protein kinase (AMPK) has attracted considerable attention, due to its central role in governing glucose homeostasis. The activation of AMPK can lead to a series of physiological responses of the glycolipid metabolism by affecting intracellular signal transduction pathways, such as the enhancement of glycogenesis by increasing the phosphorylation of glycogen synthase kinase-3β (Gsk3β) (15), the inhibition of fatty acid synthesis by reducing the activity of acetyl-CoA carboxylase and the depression of the expressions of lipogenic genes (16, 17). Until now, extensive studies have confirmed that the activity of AMPK can be modulated by the phosphorylation of a threonine at position 172 (Thr172) in the α-subunit (18, 19). As an activator of AMPK, metformin (an antidiabetic drug) has attracted considerable attention, since it is effective in improving insulin sensitivity, decreasing plasma triglyceride and

low-density lipoprotein concentrations, depressing hepatic gluconeogenesis and inhibiting glycogenolysis, while having no effect on insulin secretion (20, 21). In general, all of these metabolic processes are mediated by AMPK. In teleost fish, several studies have demonstrated that oral administration or infusion of metformin can improve the glucose homeostasis of common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) (22, 23, 24, 25). In addition, our previous study has indicated that metformin supplementation can remarkably enhance the energy-sensing, insulin-signaling pathway and glycolipid metabolism of Wuchang bream (*Megalobrama amblycephala*) after a long-term adaption to a carbohydrate-enriched diet (26). However, literature concerning the physiological functions of metformin on the glucose homeostasis of teleost fish is still quite limited. Moreover, it remains unclear whether metformin can improve the AMPK-signaling pathway in fish, and consequently promote its glucose tolerance, although the dynamic values of plasma glucose have been reported in our previous study (26).

The Wuchang bream is an economically important freshwater fish in China with a worldwide distribution (27). As an herbivore, it could efficiently utilize dietary carbohydrates for energy purposes. Therefore, the diets formulated for this species generally contain large amounts of carbohydrates in practical aquaculture. However, an excessive intake of dietary carbohydrates usually results in a severe metabolic burden of this species coupled with a compromised glucose homeostasis, despite its relatively high dietary carbohydrates tolerance (the optimal level is approximately 30%) (13, 28). To better understand the molecular events involved in the glucose homeostasis of this fish, the aim of the present study is to elucidate the long-term effects of metformin on the metabolic responses of *Megalobrama amblycephala* subjected to a GTT after the adaption to different dietary carbohydrate levels.

Materials and methods

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 permission of the Animal Research Committee of Nanjing Agricultural University (Nanjing, China) (permit number: SYXK (Su) 2011-0036).

Animals and GTT

Fish were obtained from our previous 12-week feeding trial (26). During this feeding trial, fish were fed one of the four isonitrogenous and isolipidic diets, including a control diet (C, 30% carbohydrate), a control diet supplemented with 0.25% metformin (CM), a high-carbohydrate diet (HC, 43% carbohydrate) and a high-carbohydrate diet supplemented with 0.25% metformin (HCM). Dietary metformin level was adopted according to the previous studies (24, 29). The diet was intended to supply the fish with 250 mg metformin per day per kg body weight (based on the initial weight) at the beginning of the feeding trial. The formulation and approximate composition of the experimental diets are shown in Table 1. After the initial sampling, the remaining fish in each group were starved for 24 h, and then a GTT was performed as described by Li *et al*. (13, 30) and Xu *et al*. (31). Briefly, 20 fish per treatment were anesthetized with 100 mg/L MS-222 (tricaine methanesulfonate; Sigma) after the body mass was estimated. Then, an intraperitoneal injection of glucose (1.67 g/kg body weight) was administered. This dosage corresponds to 100 g glucose per 60 kg body weight in a human (7), which is also used for other fish (3, 7, 32). A saline solution (0.9%) containing 100 mg

glucose per mL was used for that purpose. After injection, fish were immediately transferred to five tanks (100 L each) at a density of four fish per tank. After the glucose administration, the blood and liver were sampled at 1, 2, 4, 8 and 12 h post-injection. Before the GTT, blood and liver samples were collected from fish starved for 24 h. These samples were designated as 0 h. The blood samples were rapidly centrifuged (3000 g, 10 min, 4°C) to obtain plasma. All samples were immediately frozen in liquid nitrogen, and were kept at −80°C until use.

Analysis of plasma and liver metabolites and enzymes

Plasma insulin level was analyzed by the radioimmunoassay method (33). Insulin concentration was expressed in ng/mL. This method was previously validated in common carp (*Cyprinus carpio* L.) by Hertz *et al*. (22). It is noteworthy that *M. amblycephala* belongs to the carp family. Plasma triglycerides (TG) were quantified by the protocols detailed previously (34). Liver glycogen levels were determined following the method detailed by Keppler *et al*. (35). Hepatic glucokinase (GK) and pyruvate kinase (PK) activities were estimated according to Panserat

Table 1 Formulation and proximate composition of the experimental diets.

	C	CM	HC	HCM
Formulation (%)				
Fish meal	8.00	8.00	8.00	8.00
Soybean meal	26.00	26.00	26.00	26.00
Rapeseed meal	17.00	17.00	17.00	17.00
Cottonseed meal	17.00	17.00	17.00	17.00
Fish oil	2.00	2.00	2.00	2.00
Soybean oil	2.00	2.00	2.00	2.00
Corn starch	12.00	12.00	25.00	25.00
Metformin	0	0.25	0	0.25
Microcrystalline cellulose	13.00	13.00	0.00	0.00
Calcium biphosphate	1.80	1.80	1.80	1.80
Premix[a]	1.20	1.20	1.20	1.20
Proximate composition (% dry matter basis)				
Moisture	6.96	6.90	6.85	6.92
Crude lipid	5.93	5.98	5.71	5.78
Ash	8.46	8.51	8.28	8.12
Crude protein	29.82	29.91	30.12	30.31
Crude fiber	16.97	16.82	6.18	6.29
Nitrogen-free extract[b]	31.86	31.88	42.86	42.58
Energy (MJ/kg)	19.09	19.12	19.14	19.18

[a]Premix supplied the following minerals and/or vitamins (per kg): $CuSO_4 \cdot 5H_2O$, 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).
C, diet with 30% carbohydrate; CM, diet with 30% carbohydrate and 0.25% metformin; HC, diet with 43% carbohydrate; HCM, diet with 43% carbohydrate and 0.25% metformin.

et al. (36) and Foster & Moon (37), respectively. The phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-biphosphatase (FBPase) and glucose-6-phosphatase (G6Pase) activities were assayed using the methods described by Gong *et al.* (38).

Real-time quantitative RT-PCR (qPCR) and Western blot analysis

Hepatic transcriptions of the genes involved in intermediary metabolism were determined via q-PCR (26, 28). They included *ampk α1*, *ampk α2*, *glut 2* (glucose transporter 2), *gs* (glycogen synthase), *gk* (glucokinase), *pk* (pyruvate kinase), *pepck* (phosphoenolpyruvate carboxykinase), *g6pase* (glucose-6-phosphatase), *fbpase* (fructose-1,6-biphosphatase), *cpt IA* (carnitine palmitoyl transferase IA), *aco* (acyl-CoA oxidase), *acc α* (acetyl-CoA carboxylase α) and *fas* (fatty acid synthetase). Primers were designed using the known sequences of *M. amblycephala* found in the nucleotide databases (GenBank and INRA-Sigenae), and the partial cDNA sequences of the target genes were obtained by transcriptome analysis (39) (Table 2). Hepatic mRNA levels of these genes were quantified using *ef1α* as a reference gene (40), which had stable expression levels in this study. Relative abundance of the transcripts was calculated following the $2^{-\Delta\Delta CT}$ method (40).

Protein extraction and Western blot analysis (20 μg of liver protein) were performed as previously described (26, 31, 41) using anti-Ampk α (#2532; Cell Signaling Technology), anti-phospho-Ampk α (#2535; Cell Signaling Technology) and anti-β-actin (BM3873; Boster, Wuhan, China) antibodies. These antibodies have been shown to successfully cross-react with *Megalobrama amblycephala* proteins (26, 31, 41).

Statistical analyses

SPSS (SPSS) software (version 22.0) was used for data analysis. Two-way ANOVA was used to analyze plasma parameters, tissue glycogen contents, related gene and protein expression levels, and hepatic enzyme activities. Tukey's multiple test was used to compare the means at a significance level of $P<0.05$. All data are presented as the mean ± s.e.m. of four replicates.

Results

Plasma insulin and TG levels and liver glycogen contents after the GTT

After the GTT, plasma insulin and TG levels and liver glycogen content were significantly ($P<0.01$) affected by sampling time, dietary treatments and their interaction (Fig. 1). Glucose loading resulted in a significant ($P<0.001$) increase of plasma insulin levels with the maximum value attained at 2 h. Thereafter, the levels decreased significantly ($P<0.001$) to the basal value at 12 h. TG levels of the HC group increased significantly ($P<0.001$) with time up to 8 h. Thereafter, it decreased significantly ($P<0.001$) to the basal value at 12 h. However, a significant ($P<0.001$) decrease in the TG levels of the C group was observed

Table 2 Primers used in the experiment.

Target gene	Forward (5'-3)	Reverse (5'-3)	Accession numbers or references
ampk α1	AGTTGGACGAGAAGGAG	AGGGCATACAAAATCAC	ARF07712.1
ampk α2	ACAGCCCTAAGGCACGATG	TGGGTCGGGTAGTGTTGAG	KX061841
glut 2	ACGCACCCGATGTGAAAGT	TTGGACAGCAGCATTGATT	KC513421.1
gs	CCTCCAGTAACAACTCACAACA	CAGATAGATTGGTGGTTACGC	39
pepck	TGGCCCGTGTGGAGAGTAAAA	ATGTGTTCTGCCAGCCAG	39
fbpase	TACCCAGATGTCACAGAAT	CACTCATACAACAGCCTCA	KJ743995.1
g6pase	TGAGACCCGGTTTTATGGAG	CATGCAGACCACCAGCTCTA	39
gk	AAAATGCTGCCCACTTAT	AATGCCCTTATCCAAATC	KJ141202.1
pk	GCCGAGAAAGTCTTCATCGCACAG	CGTCCAGAACCGCATTAGCCAC	39
fas	AGCGAGTACGGTGATGGT	GGATGATGCCTGAGATGG	KF918747.1
acc α	TCTGCCCTCTATCTGTCT	ATGCCAATCTCATTTCCT	39
cpt IA	TACTTCCAAAGCGGTGAG	AGAGGTATTGTCCGAGCC	39
aco	GCTCAACCCTGGCATACT	CTGGCTCAGCTTTACACG	39
ef 1α	CTTCTCAGGCTGACTGTGC	CCGCTAGCATTACCCTCC	X77689.1

acc α, acetyl-CoA carboxylase α; *aco*, acyl-CoA oxidase; *ampk α1*, AMP-activated protein kinase α1; *ampk α2*, AMP-activated protein kinase α2; *cpt IA*, carnitine palmitoyl transferase IA; *ef 1α*, elongation factor 1, α; *fas*, fatty acid synthetase; *fbpase*, fructose-1,6-biphosphatase; *g6pase*, glucose-6-phosphatase; *gk*, glucokinase; *glut 2*, glucose transporter 2; *gs*, glycogen synthase; *pepck*, phosphoenolpyruvate carboxykinase; *pk*, pyruvate kinase.
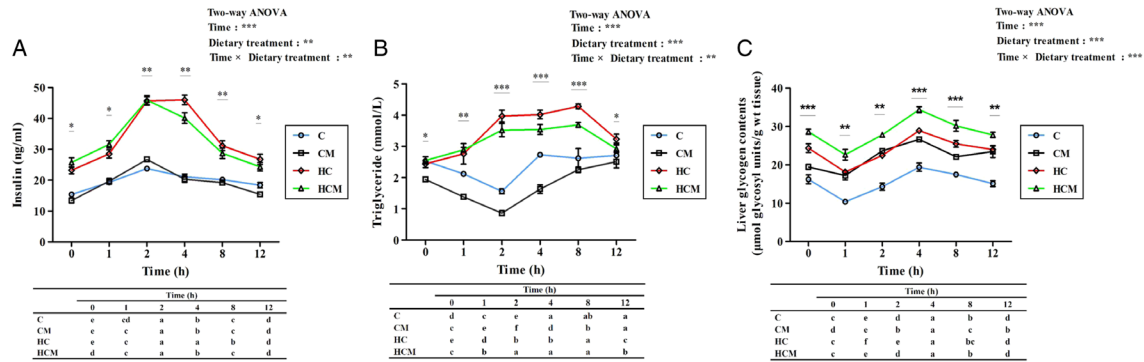
Figure 1

Plasma insulin (A) and triglyceride (B) levels and liver glycogen (C) contents of *Megalobrama amblycephala* subjected to a glucose load after the adaption to different experimental diets. Each data represents the mean ± s.e.m. of four replicates. Significant differences ($P < 0.05$) among sampling times within each treatment are indicated by the lowercase letters. *Indicates a significant difference ($P < 0.05$) among these four treatments at each sampling point. ns $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

during the first 2h. Thereafter, it gradually increased and returned to the basal value with further increasing times. Liver glycogen contents decreased significantly ($P < 0.05$) during the first 1h, then increased significantly ($P < 0.001$) to the maximum value by 4h. With regard to dietary treatments, plasma insulin and TG levels and liver glycogen contents of the HC group were significantly ($P < 0.05$) higher than those of the C treatment (except for the liver glycogen contents of the HC group at 2h). Additionally, insulin and TG levels and liver glycogen contents were significantly affected ($P < 0.01$) by the interaction between sampling time and dietary treatment. The insulin levels of the HCM group were significantly ($P < 0.05$) lower than that of the HC treatment during the period of 4–8h. The TG levels of the CM and HCM treatments were both significantly ($P < 0.05$) lower than those of the C and HC groups during the entire sampling

period (except for those of the HCM group at 0 and 1h), respectively, while the opposite was true for liver glycogen contents.

Hepatic *ampk α* transcriptions and phosphorylation status after the GTT

After the glucose loading, hepatic transcriptions of *ampk α1* and *ampk α2* were significantly ($P < 0.01$) affected by sampling time, dietary treatments and their interaction (Fig. 2). The transcripts of *ampk α1* and *ampk α2* both decreased sharply after 2h post-injection. Then, it increased significantly ($P < 0.05$) to the maximum value at 8h, and gradually decreased to the basal value at 12h. In terms of dietary treatment, the transcriptions of *ampk α1* and *ampk α2* of the HCM group were significantly ($P < 0.05$) higher than those of the other groups. In addition,

Figure 2
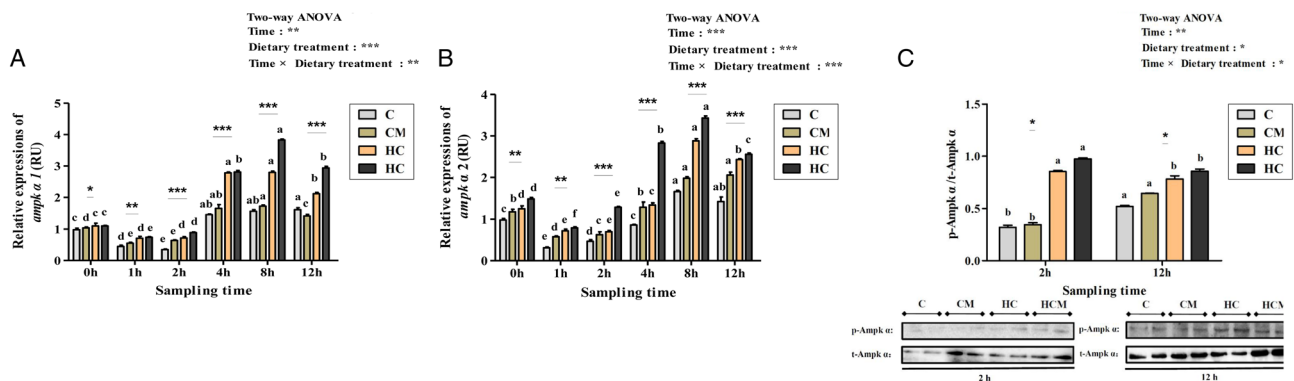
Hepatic transcriptions of *ampk α1* (A) and *ampk α2* (B) and protein expressions of Ampk α at 2 and 12 h (C) of *Megalobrama amblycephala* subjected to a glucose load after the adaption to different experimental diets. Each data represents the mean ± s.e.m. of four replicates. Significant differences ($P < 0.05$) among sampling times within each treatment are indicated by the lowercase letters. *Indicates a significant difference ($P < 0.05$) among these four treatments at each sampling point. ns $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

the transcriptions of *ampk α1* and *ampk α2* were significantly ($P<0.01$) affected by the interaction between sampling time and dietary treatment with significant ($P<0.05$) differences observed among all groups during the entire sampling period.

In addition, after the GTT, the hepatic p-Ampk α/t-Ampk α ratio were also significantly ($P<0.05$) affected by sampling time, dietary treatments and the interaction thereof. With increasing sampling time from 2 to 12 h, the hepatic p-Ampk α/t-Ampk α ratio of the C group was significantly ($P<0.01$) increased, but the opposite trend was observed in the HC group. In terms of dietary treatment, the hepatic p-Ampk α/t-Ampk α ratio in the fish fed with the HC diets were significantly ($P<0.05$) higher than those of the other groups. Furthermore, a significant interaction ($P<0.05$) between dietary carbohydrate level and metformin supplementation was observed with

regard to the p-Ampk α/t-Ampk α ratio. The p-Ampk α/t-Ampk α ratio of the HCM group at 2 h were significantly ($P<0.05$) higher than those of the other groups.

Hepatic transcripts and activities of the enzymes involved in glucose metabolism after the GTT

After the glucose administration, the transcripts and activities of glucose metabolism-related enzymes were significantly ($P<0.05$) affected by sampling time, dietary treatments and their interaction (Figs 3 and 4). The glucose loading resulted in a significant ($P<0.01$) increase of *glut 2*, *gs*, *gk* and *pk* expressions with the highest values being obtained at 1 (*glut 2*), 4 (*gs*) and 2 h (*gk* and *pk*), respectively. Thereafter, the values decreased significantly ($P<0.05$) to the basal level at 12 h. However, the transcriptional levels of *pepck*, *g6pase* and *fbpase* decreased significantly
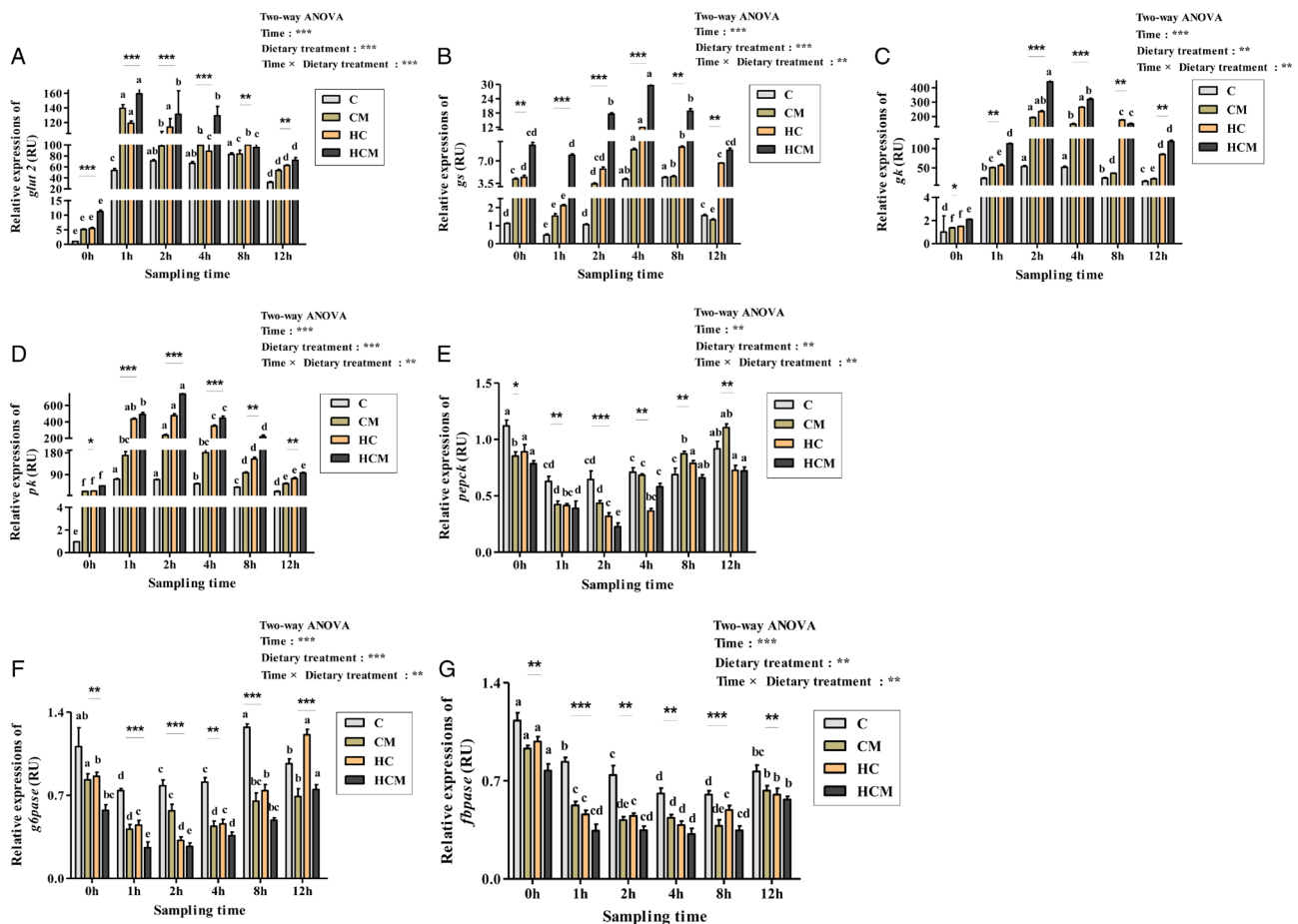

Figure 3

Relative transcriptions of glucose metabolism-related genes (*glut 2* (A), *gs* (B), *gk* (C), *pk* (D), *pepck* (E), *g6pase* (F) and *fbpase* (G)) in the liver of *Megalobrama amblycephala* subjected to a glucose load after the adaption to different experimental diets. Each data represents the mean ± s.e.m. of four replicates. Significant differences ($P < 0.05$) among sampling times within each treatment are indicated by the lowercase letters. *Indicates a significant difference ($P < 0.05$) among these four treatments at each sampling point. ns $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
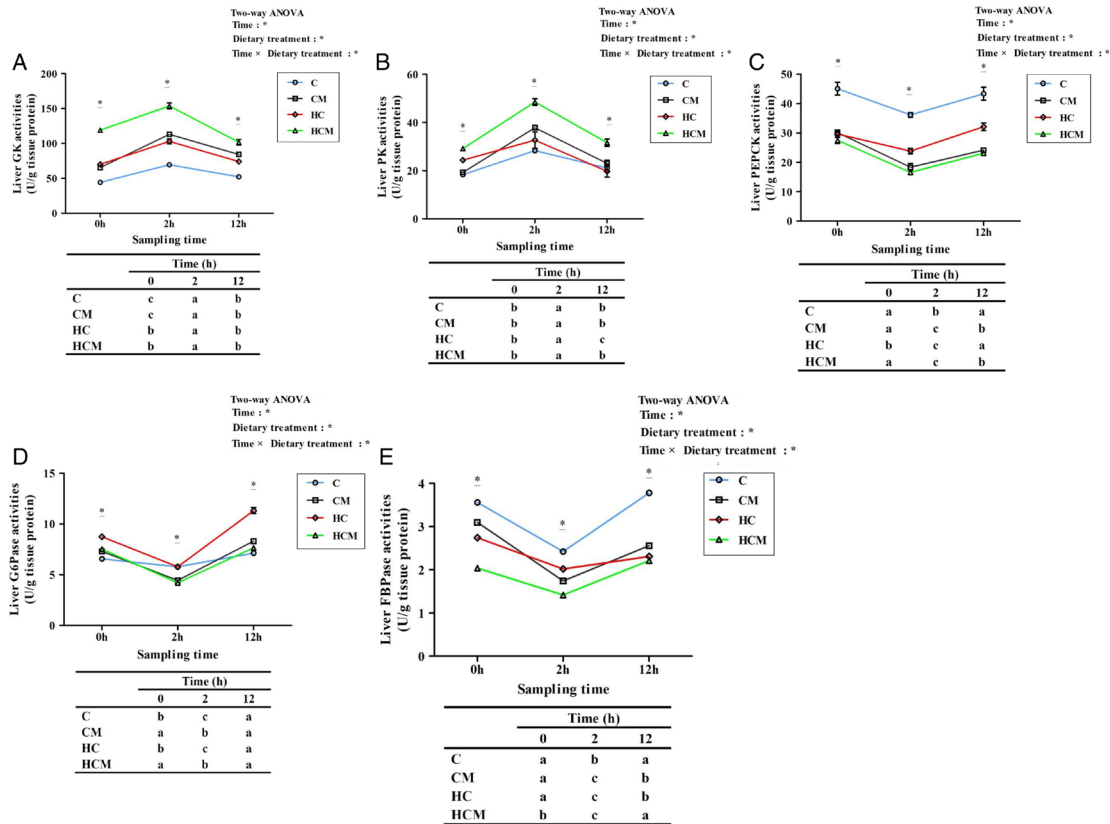
Figure 4

The enzymatic activities of glycolysis (GK (A) and PK (B)) and gluconeogenesis (PEPCK (C), G6Pase (D) and FBPase (E)) in the liver of *Megalobrama amblycephala* subjected to a glucose load after the adaption to different experimental diets. Each data represents the mean ± S.E.M. of four replicates. Significant differences ($P < 0.05$) among sampling times within each treatment are indicated by the lowercase letters. *Indicates a significant difference ($P < 0.05$) among these four treatments at each sampling point. ns $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

($P < 0.05$) to the minimum value at 2 h post-injection. Then they gradually increased to the basal value with further increasing times. In addition, after the GTT, hepatic GK and PK activities were both significantly higher ($P < 0.05$) at 2 h than those of 1 and 12 h, whereas the opposite was true for PEPCK, G6Pase and FBPase activities. In terms of dietary treatments, the transcriptional levels of *glut 2*, *gs*, *gk* and *pk* as well as the activities of GK and PK of the HCM group were significantly ($P < 0.05$) higher than those of the other groups, whereas the opposite was true for that of the gluconeogenic enzymes. In addition, the transcriptional levels and activities of these enzymes were all significantly ($P < 0.05$) affected by the interaction between sampling time and dietary treatment.

Hepatic transcripts of enzymes involved in lipid metabolism after the GTT

After the glucose administration, the relative transcriptions of the lipid metabolism-related enzymes were significantly ($P < 0.01$) affected by sampling time, dietary treatments

and their interaction ([Fig. 5](#)). Glucose loading resulted in a significant ($P < 0.001$) decrease in the transcripts of both *cpt IA* and *aco* with a minimum value being obtained at 2 h. Thereafter, the values increased significantly ($P < 0.05$) to the basal at 12 h. However, the transcriptional levels of both *acc α* and *fas* increased significantly ($P < 0.05$) to the maximum value at 4 h post-injection. Then, the levels gradually decreased to the basal value with further increasing times. In terms of dietary treatments, the transcriptional levels of both *cpt IA* and *aco* of the CM group were significantly ($P < 0.05$) higher than those of the other groups. However, the highest transcripts of *acc α* and *fas* were both recorded in the HC group. In addition, they were all significantly ($P < 0.01$) affected by the interaction between sampling time and dietary treatment.

Discussion

Like the case in mammals, plasma glucose levels of fish are remarkably influenced by pancreatic hormones ([42](#)).
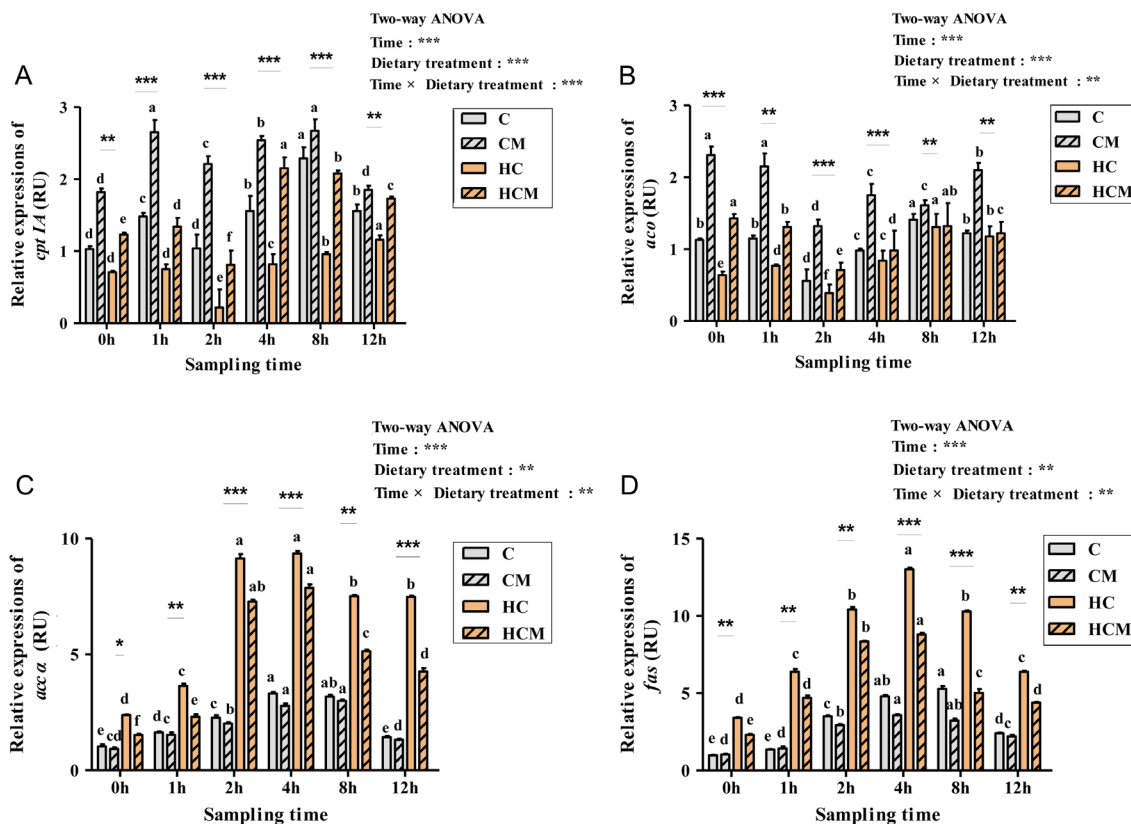
Figure 5

Relative transcriptions of lipid metabolism-related genes (*cpt IA* (A), *aco* (B), *acca* (C) and *fas* (D)) in the liver of *Megalobrama amblycephala* subjected to a glucose load after the adaption to different experimental diets. Each data represents the mean ± s.e.m. of four replicates. Significant differences ($P < 0.05$) among sampling times within each treatment are indicated by the lowercase letters. *Indicates a significant difference ($P < 0.05$) among these four treatments at each sampling point. ns $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

In this study, plasma insulin levels drastically increased from 0 to 2h after the glucose administration. The time to reach the insulin peak in Wuchang bream was similar to that observed in several omnivorous species, such as Nile tilapia (*Oreochromis niloticus*) (6, 43), white sea bream (*Diplodus sargus*) (12) and common carp (7). However, it was shorter than that of carnivorous fish receiving the same glucose dose (7, 32). This result further supports the finding that differences in the time required to attain insulin peak can be related to the differences of feeding habit among different species (12). Moreover, the increase of insulin levels post-injection is usually considered a positive signal, since it can efficiently relieve the physiological state of hyperglycemia by accelerating glucose uptake in peripheral tissues (44). In addition, time to reach insulin peak is slower than that of plasma glucose, whose highest value was obtained at 1h after the GTT (26). This result has been partially attributed to the fact that high exogenous glucose can stimulate somatostatin secretion in pancreatic D cells, which in

turn can inhibit insulin secretion during the initial period after glucose injection (8, 45). A subsequent decrease of plasma insulin was observed at 2–12h post-injection, which may be attributed to the gradual decrease of plasma glucose levels during this time. Indeed, the declining glucose level can weaken the stimulation of pancreatic cells, thereby decreasing insulin secretion (8). In terms of dietary carbohydrate levels, the plasma insulin levels of fish fed HC diets were approximately twofold higher than those of the control. This may be due to the long-term metabolic adaption of fish to high dietary carbohydrate levels. Indeed, the intake of carbohydrate-enriched diets by fish usually leads to elevated plasma glucose levels with subsequent enhancement of insulin synthesis and release (1). Furthermore, such high insulin level in the HC group is also considered a positive signal for glucose homeostasis, which was in line with our previous study (26) that fish fed HC diets had a relatively low plasma glucose level after glucose administration. Meanwhile, no statistical difference was observed in plasma insulin levels

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with regard to metformin supplementation. This may be explained by the fact that metformin effectively lowers the glycemic level without affecting insulin secretion (46). For TG, the decreased levels observed in the C group at 2 h post-injection were in line with the results obtained previously in rainbow trout and European sea bass (*Dicentrarchus labrax*) (8, 11, 47, 48). This may be ascribed to the differences in the activities of the metabolic enzymes observed among different fish species. In fact, glucose loading can lead to high hepatic triacylglycerol lipase activity and low glucose-6-phosphate dehydrogenase activity in fish, as may enhance lipolysis thus inhibiting TG synthesis (8). Furthermore, this catabolic pattern might also be related to the elevated plasma somatostatin concentrations induced by the GTT, which could improve hepatic lipolysis in fish (8, 42). Unlikely, the glucose injection led to a prompt increase of plasma TG in the HC group during the first 2 h. This suggested an enhanced lipid synthesis of fish after the adaption to HC diets. According to previous studies, excess glucose could be converted into lipids in the peripheral tissues of fish, thus helping to maintain glucose homeostasis (5, 10). In addition, metformin supplementation resulted in a low TG levels during the sampling period, indicating that metformin reduced tissue lipid deposition. Indeed, a long-term intake of metformin can activate Ampk, thereby lowering plasma TG levels through the inhibition of lipogenesis and the enhancement of mitochondrial fatty acid β-oxidation (49).

In this study glucose administration induced a remarkable decrease in liver glycogen content during the first 1 h, as is similar to the results of *gs* transcription. This result may be attributed to the fact that the pancreatic D cells of fish generally have higher glucose sensitivities than the B cells (50). Indeed, excessive glucose initially stimulates the D cells to release glucagon and somatostatin, as might accelerate the catabolism of liver glycogen (8). Subsequently, glycogen content increased remarkably with time up to 4 h, suggesting an enhanced glucose metabolism in fish. This result was in line with the decline of plasma glucose during this period of time (26). In fact, increased insulin levels induced by glucose administration can effectively enhance glucose uptake and glycogen synthesis in peripheral tissues, as is beneficial in improving the glucose homeostasis of fish (51). Furthermore, in terms of dietary carbohydrate levels, the liver glycogen content of the HC treatment was higher than that of the C groups, as may be attributed to the enhanced glycogen synthesis in fish after the adaption to HC diets. Indeed, previous studies have demonstrated

that dietary carbohydrate intake can increase insulin secretion in fish, thereby decreasing the level of cyclic adenosine monophosphate and the activities of total glycogen phosphorylase (GPase) and GPase-a, which could stimulate glycogen synthesis in peripheral tissues (5, 52). Another explanation may be that a long-term feeding of a HC diet would force fish to adapt to this energy state. Indeed, under high-energy stress, Ampk may be activated to maintain glucose homeostasis in fish by coordinating glucose metabolism, such as the enhancement of glycogen synthesis (15). Furthermore, liver glycogen contents were significantly affected by the interaction between sampling time and dietary treatment. Metformin supplementation led to higher glycogen contents, which may be partly ascribed to the possible activation of Ampk. According to a previous study, the activation of Ampk by metformin can upregulate the phosphorylation of Gsk-3β, thus increasing the glycogen stores in liver (15).

AMPK plays an important role in improving glucose homeostasis, since it can regulate the intermediary metabolism of fish (1). In this study, hepatic *ampk α1* and *α2* transcripts responded promptly to the glucose loading with the minimum values being attained at 2 and 1 h post-injection, respectively. Furthermore, hepatic p-Ampk α/t-Ampk α ratio of the C groups at 2 h was lower than that at 12 h. The plausible explanation for this might be an increase in insulin and insulin-like growth factor levels after glucose administration, as it directly upregulates Ampk α phosphorylation at Ser485/491 to inhibit both the enzymatic activity and gene expression of *ampk α* (8, 53). Then, hepatic *ampk α1* and *α2* transcriptions were both significantly upregulated from 2 to 12 h post glucose load. This might be due to a decline in insulin level during this period, as mitigated by the inhibitory effects on AMPK α activity (54, 55). Moreover, the results were in line with the decreased plasma glucose levels during the same period (26) in this fish, because the activation of AMPK can improve the glucose homeostasis of fish in response to a glucose load by stimulating glucose uptake and inhibiting glucose production in tissues (16, 17). In terms of dietary carbohydrate levels, hepatic *ampk α1* and *α2* transcriptions and p-Ampk α/t-Ampk α ratio of the HC group were significantly higher than those of the control during the entire sampling period, as might suggest a high-energy stress. Under HC stress, Ampk α may be activated to participate in a series of physiological responses of glucose metabolism, thus enhancing the glucose homeostasis of fish (14, 16, 56). In addition, metformin supplementation led to higher *ampk α1* and *α2* transcriptions and p-Ampk α/t-Ampk α ratios. This may be

explained by the fact that metformin can upregulate the AMP/ATP ratio by inhibiting the activity of mitochondrial complex 1, thus enhancing the AMPK activity required to maintain glucose homeostasis (57, 58).

Previous studies have demonstrated that the activation of Ampk can enhance glucose uptake and oxidation in peripheral tissues, especially in the liver and muscle (1). In the present study, glucose administration resulted in a prompt increase of hepatic transcriptions of *glut 2*, *gs*, *gk* and *pk* as well as GK and PK activities during the first 2h, whereas the opposite was true for that of gluconeogenic enzymes. This suggested an enhancement of the anabolic metabolism involving glucose transport, glycolysis and glycogenesis after the glucose load. In these metabolic processes, the increasing plasma glucose levels during this time may play an important role, since the uptake of exogenous glucose by hepatocytes could be excessive, as it requires the assistance of GLUT 2 (5). Subsequently, high glucose concentrations in hepatocytes could stimulate glycolysis and glycogenesis while depressing gluconeogenesis by coordinating the transcriptional levels and activities of related enzymes (1, 5, 26). In addition, the increased insulin levels during this period may also serve as a positive signal for the regulation of these processes, since insulin is the primary pancreatic hormone that lowers blood glucose (42, 47). The results obtained here were different from those observed in most carnivorous fish, which generally exhibit an enhancement of gluconeogenesis after a glucose load (7, 59). This indicated that Wuchang bream, as an herbivorous fish, might be highly capable of maintaining glucose homeostasis via the inhibition of gluconeogenesis compared to carnivorous species (30). During 2 to 12h, hepatic transcriptions of *glut 2*, *gk* and *pk* gradually decreased to the basal level, whereas the opposite was true for *pepck* and *g6pase*. In addition, although the transcriptions of *gs* and *fbpase* were constant at 0–4h, they gradually returned to the basal values at 12h. These changes may be a metabolic adjustment of fish to restore normoglycemia after starvation (10). Indeed, a decline of blood glucose concentration can weaken the stimulation of the anabolic and catabolic processes related to glucose metabolism (6, 26). However, the transcriptional levels of *glut 2*, *gs*, *gk* and *pk* at 2–8h were higher than that at 12h. This may be partly attributed to the activation of Ampk by decreasing insulin levels during this period, as it stimulates the transcriptions of the genes that participate in glucose uptake in peripheral tissues (16, 17). With regard to dietary carbohydrate levels, hepatic transcriptions of *glut 2*, *gs*, *gk* and *pk* as well as the activities of GK and PK in the

HC group were all significantly higher than those of the control, whereas the opposite was true for gluconeogenic enzymes. This indicated that the tolerance to a glucose load was improved in fish after a long-term adaption to a HC diet. In addition, fish treated with metformin exhibited relatively higher transcriptions of *glut 2*, *gs*, *gk* and *pk* as well as the activities of glycolytic enzymes, whereas the opposite was true for the gluconeogenic enzymes. This result might be attributed to the activation of Ampk by the long-term administration of metformin, as might (1) stimulating glycolysis by upregulating the expressions of glycolytic enzymes (60); (2) enhancing glycogenesis by phosphorylating Gsk3β (15); and (3) inhibiting hepatic glucose production by downregulating the transcriptions of *pepck*, *g6pase* and *fbpase* (61, 62).

In this study, glucose administration induced a downregulation of *cpt IA* and *aco* expression (from 0 to 2h) but the upregulation of *acc α* and *fas* expression (from 0 to 4h). This indicated an inhibition of fatty acid β-oxidation coupled with an enhancement of lipogenesis after the GTT. A plausible explanation is that glucose administration usually induces an increase in insulin level, which consequently accelerates the intracellular conversion of excessive glucose into fatty acids (2, 63). Furthermore, it should be noted that the expression of *cpt IA* was significantly upregulated at 1h post-injection. This may be due to an initial increase of somatostatin induced by glucose loading, thus enhancing lipolysis and inhibiting TG synthesis (8). Then, the transcriptions of *cpt IA* and *aco* gradually increased with increasing sampling time, whereas the opposite was true for the lipogenic genes. This result was in line with plasma TG levels that declined from 8 to 12h. In terms of dietary carbohydrate levels, *cpt IA* and *aco* transcriptions of the HC group was both lower than that of the control, whereas the opposite was true for both *acc α* and *fas*. This indicated that HC intake further promoted fatty acid biosynthesis in fish, but decreased fatty acid β-oxidation after the glucose loading. This result is supported by the fact that carbohydrate-rich diets can activate the target of rapamycin (TOR) signaling pathway, thereby increasing the transcriptions of lipogenic genes in fish liver (64). Furthermore, fish receiving the HCM diet exhibited remarkably higher transcriptions of *cpt IA* and *aco* compared with that of the HC group, whereas the opposite was true for lipogenic genes. This indicated that metformin can inhibit hepatic lipid deposition of fish after glucose loading, which may be due to the enhancement of AMPK activity by metformin treatment. Indeed, metformin could increase malonyl-CoA decarboxylase activity, leading to low malonyl-CoA levels, as might

consequently promote mitochondrial β-oxidation while simultaneously suppressing fatty acid synthesis (65).

In conclusion, our results indicated that a long-term administration of metformin could improve the glucose homeostasis of *Megalobrama amblycephala* fed HC diets after the GTT through the activation of Ampk α and the stimulation of glucose transportation, glycolysis, glycogenesis and fatty acid oxidation, while inhibiting lipogenesis and gluconeogenesis.

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