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

Chao Xu¹, Xiang-Fei Li¹, Hong-Yan Tian², Hua-Juan Shi¹, Ding-Dong Zhang¹, Kenneth Prudence Abasubong¹, Wen-Bin Liu¹

¹, Key Laboratory of Aquatic Nutrition and Feed Science of Jiangsu Province, College of Animal Science and Technology, Nanjing Agricultural University, No.1 Weigang Road, Nanjing 210095, People’s Republic of China.
², Department of Ocean Technology, College of Marine and Biology Engineering, Yancheng Institute of Technology, Yancheng 224051, Province Jiangsu, China.

Corresponding author: Key Laboratory of Aquatic Nutrition and Feed Science of Jiangsu Province, College of Animal Science and Technology, Nanjing Agricultural University, No. 1 Weigang Road, Nanjing 210095, Jiangsu Province, People’s Republic of China. Tel (Fax): 86-025-84395382. E-mail: wbliu@njau.edu.cn (Wen-Bin Liu).

A short title: Metformin improves glucose homeostasis in fish

Word count: 7792 words
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 triglycerides levels and liver glycogen contents of the control group 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 up-regulated 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.

**Keywords:** Intraperitoneal glucose load, Metformin, Carbohydrate, Glycolipid metabolism, *Megalobrama amblycephala*
1. 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 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 adaptation to different carbohydrate levels and sources, the routes of glucose administration, fish sizes, etc. (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 (ACC), 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 anti-diabetic 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 adaptation 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 world-wide 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 adaptation to different dietary carbohydrate levels.

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

2.2. Animals and glucose tolerance test (GTT)

Fish were obtained from our previous 12-week feeding trial (26). During this feeding trial, fish were fed one of 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 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, twenty fish per treatment were anesthetized with 100 mg/L MS-222 (tricaine methanesulfonate, Sigma, USA) 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 4 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.

**Table 1 here**

2.3. 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 *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).

**Fig. 1 here**

2.4. 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 a1*, *ampk a2*, *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 1A* (carnitine palmitoyl transferase 1A), *aco* (acyl-CoA oxidase), *acc a* (acetyl-CoA carboxylase a) 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, USA), anti-phospho-Ampk α (#2535, Cell Signaling Technology, USA) and anti-β-actin (BM3873, Boster, China) antibodies. These antibodies have been shown to successfully cross-react with *Megalobrama amblycephala* proteins (26, 31, 41).

**Table 2 here**

2.5. Statistical analyses

SPSS (SPSS Inc., Chicago, USA) 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.

3. Results

3.1. 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 during the first 2 h. 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 1 h, then increased significantly ($P < 0.001$) to the maximum value by 4 h. 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 2 h). 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 4-8 h. 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 1 h), respectively, while the opposite was true for liver glycogen contents.

**Fig. 1 here**

3.2. Hepatic *ampk a* transcriptions and phosphorylation status after the GTT

After the glucose loading, hepatic transcriptions of *ampk a1* and *ampk a2* were significantly ($P < 0.01$) affected by sampling time, dietary treatments and their interaction (Fig. 2). The transcripts of *ampk a1* and *ampk a2* both decreased sharply after 2 h post injection. Then, it increased significantly ($P < 0.05$) to the maximum value at 8 h, and gradually decreased to the basal value at 12 h. In terms of dietary treatment, the transcriptions of *ampk a1* and *ampk a2* of the HCM group were significantly ($P < 0.05$) higher than those of the other groups. In addition, the transcriptions of *ampk a1* and *ampk a2* 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 high-carbohydrate 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.

**Fig. 2 here**

### 3.3. 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 $\text{glut} \ 2$, $\text{gs}$, $\text{gk}$ and $\text{pk}$ expressions with the highest values being obtained at 1 ($\text{glut} \ 2$), 4 ($\text{gs}$) and 2 h ($\text{gk}$ and $\text{pk}$), respectively. Thereafter, the values decreased significantly ($P < 0.05$) to the basal level at 12 h. However, the transcriptional levels of $\text{pepck}$, $\text{g6pase}$ and $\text{fhpase}$ decreased significantly ($P < 0.05$) 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 $\text{glut} \ 2$, $\text{gs}$, $\text{gk}$ and $\text{pk}$ 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.

**Fig. 3 and 4 here**

3.4. 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.

**Fig. 5 here**

4. Discussion

Like the case in mammals, plasma glucose levels of fish are remarkably influenced by pancreatic hormones (42). In this study, plasma insulin levels drastically increased from 0 to 2 h after the glucose administration. The time to reach the insulin peak in blunt snout 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 1 h 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 2-12 h 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 2-fold 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 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). Unlike, 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 adaptation to high-carbohydrate 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 of 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 adaptation 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, as in turn might stimulate glycogen synthesis in peripheral tissues (5, 52). Another explanation may be that a long-term feeding of a high-carbohydrate 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 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 up-regulated from 2 to 12 h post glucose load. This might be due to a decline in insulin level during this period, as mitigated 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 high carbohydrate 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 \textit{ampk} \( \alpha_1 \) and \( \alpha_2 \) transcriptions and p-Ampk \( \alpha/t\)-Ampk \( \alpha \) ratios. This may be explained by the fact that metformin can up-regulate 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 \textit{glut 2}, \textit{gs}, \textit{gk} and \textit{pk} as well as GK and PK activities during the first 2 h, 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 excessive exogenous glucose could be uptake by hepatocytes, as 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 blunt snout 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 12 h, hepatic transcriptions of \textit{glut 2}, \textit{gk} and \textit{pk} gradually decreased to the basal level, whereas the opposite was true for \textit{pepck} and \textit{g6pase}. In addition, although the transcriptions of \textit{gs} and \textit{fbpase} were constant at 0-4 h, they gradually returned to the basal values at 12 h. 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 2-8 h were higher than that at 12 h. This may be partly attributed to the activation of Ampk by decreasing insulin levels during this period, as stimulates the transcriptions of the genes 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 high-carbohydrate 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) stimulate glycolysis by up-regulating the expressions of glycolytic enzymes (60); (2) enhance glycogenesis by phosphorylating Gsk3β (15); and (3) inhibiting hepatic glucose production by down-regulating the transcriptions of pepck, g6pase and fbpase (61, 62).

In this study, glucose administration induced a down-regulation of cpt IA and aco expression (from 0-2 h) but the up-regulation of acc α and fas expression (from 0-4 h). 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 up-regulated at 1 h 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 12 h. 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 \textit{acc a} and \textit{fas}. This indicated that high carbohydrate intake further promoted fatty acid biosynthesis in fish, but decreased fatty acid \(\beta\)-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 \textit{cpt IA} and \textit{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 \(\beta\)-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 \textit{Megalobrama amblycephala} fed high-carbohydrate diets after the GTT through the activation of Ampk \(\alpha\) and the stimulation of glucose transportation, glycolysis, glycogenesis and fatty acid oxidation, while inhibiting lipogenesis and gluconeogenesis.

**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.

**Author Contributions**

X-FL, CX, and W-BL conceived and designed the experiments. CX and D-DZ analyzed the data. CX, H-YT, H-JS, and LZ performed the experiments and contributed reagents, materials, and analysis tools. CX and X-FL wrote the paper. All authors read and approved the final version of the manuscript.

**Funding**

This research was funded by the National Technology System of Conventional Freshwater Fish Industries of China (CARS-45-14), the Postgraduate Research and Practice Innovation Program of Jiangsu Province (KYCX18_0697) and the China
Scholarship Council (Contract 201806850082).

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Figure legends

Fig. 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 lower-case letters. * Indicates a significant difference ($P < 0.05$) among these four treatments at each sampling point. ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 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 lower-case letters. * Indicates a significant difference ($P < 0.05$) among these four treatments at each sampling point. ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 3. Relative transcriptions of glucose metabolism-related genes 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 lower-case letters. * Indicates a significant difference ($P < 0.05$) among these four treatments at each sampling point. ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 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 lower-case letters. * Indicates a significant difference ($P < 0.05$) among these four treatments at each sampling point. ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 5. Relative transcriptions of lipid metabolism-related enzymes 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 lower-case letters. * Indicates a significant difference ($P < 0.05$) among these four treatments at each sampling point. ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. 
Table 1. Formulation and proximate composition of the experimental diets.

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<th>HC</th>
<th>HCM</th>
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<td></td>
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<td><strong>Proximate composition (% dry matter basis)</strong></td>
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<td>Ash</td>
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<td>Crude protein</td>
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<tr>
<td>Crude fiber</td>
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<td>16.82</td>
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<td>31.88</td>
<td>42.86</td>
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<tr>
<td>Energy (MJ/kg)</td>
<td>19.09</td>
<td>19.12</td>
<td>19.14</td>
<td>19.18</td>
</tr>
</tbody>
</table>

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, the same below.

1 Premix supplied the following minerals and/or vitamins (per kg): CuSO₄·5H₂O, 2.0 g; FeSO₄·7H₂O, 25 g; ZnSO₄·7H₂O, 22 g; MnSO₄·4H₂O, 7 g; Na₂SeO₃, 0.04 g; KI, 0.026 g; CoCl₂·6H₂O, 0.1 g; Vitamin A, 900,000 IU; Vitamin D, 200,000 IU; Vitamin E, 4500 mg; Vitamin K₃, 220 mg; Vitamin B₁, 320 mg; Vitamin B₂, 1090 mg; Vitamin B₅, 2000 mg; Vitamin B₆, 500 mg; Vitamin B₁₂, 1.6 mg; Vitamin C, 5000 mg; Pantothenate, 1000 mg; Folic acid, 165 mg; Choline, 60,000 mg.

2 Calculated by difference (100 - moisture - crude protein - crude lipid - ash - crude fiber).
### Table 2. Primers used in the experiment.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Forward (5′-3′)</th>
<th>Reverse (5′-3′)</th>
<th>Accession numbers or references</th>
</tr>
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<tr>
<td><strong>ampk α1</strong></td>
<td>AGTTGGACGAGAAGGAG</td>
<td>AGGGCATACAAATTACAC</td>
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<tr>
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<td>ACAGCCCTAAGGCAGATG</td>
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<td><strong>glut 2</strong></td>
<td>ACGCACCAGATGGAAAATG</td>
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<td><strong>gs</strong></td>
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<td>TGGCCCGTGTGAGAGTAAA</td>
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<tr>
<td><strong>fbpase</strong></td>
<td>TACCCAGATGTCAACAGAAT</td>
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<td><strong>g6pase</strong></td>
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<td><strong>gk</strong></td>
<td>AAAATGCTGCCACCTTAT</td>
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<td><strong>pk</strong></td>
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<td><strong>fas</strong></td>
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<td><strong>aco</strong></td>
<td>GCTCAACCCTGCACTGACT</td>
<td>CGGCTAGCATTACCTCC</td>
<td>39</td>
</tr>
</tbody>
</table>

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

A

Two-way ANOVA
Time: ***
Dietary treatment: **
Time × Dietary treatment: **

![Graph showing Insulin levels over time for different groups]

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>C</th>
<th>CM</th>
<th>HC</th>
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<td>b</td>
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<tr>
<td>8</td>
<td>d</td>
<td>c</td>
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<tr>
<td>12</td>
<td>d</td>
<td>c</td>
<td>a</td>
<td>b</td>
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</table>

B

Two-way ANOVA
Time: ***
Dietary treatment: ***
Time × Dietary treatment: **

![Graph showing Triglyceride levels over time for different groups]

<table>
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<td>12</td>
<td>c</td>
<td>b</td>
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Copyright © 2019 the authors
Two-way ANOVA
Time : ***
Dietary treatment : ***
Time × Dietary treatment : ***

Liver glycogen contents (μmol glycogen units/g wet tissue)

<table>
<thead>
<tr>
<th>Time (h)</th>
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<th>HC</th>
<th>HCM</th>
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<td>d</td>
<td>d</td>
</tr>
</tbody>
</table>

Copyright © 2019 the authors
Fig. 2.

A

Two-way ANOVA
Time : **
Dietary treatment : ***
Time × Dietary treatment : **

B

Two-way ANOVA
Time : ***
Dietary treatment : ***
Time × Dietary treatment : ***
Two-way ANOVA
Time: **
Dietary treatment: *
Time × Dietary treatment: *
Fig. 3.

Two-way ANOVA
Time : ***
Dietary treatment : ***
Time × Dietary treatment : ***

Sampling time
Relative expressions of glut 2 (RU)

A

Sampling time
Relative expressions of gs (RU)

B
Two-way ANOVA
Time : ***
Dietary treatment : **
Time × Dietary treatment : **

Sampling time
Relative expressions of gk (RU)

C

Sampling time
Relative expressions of pk (RU)

D

Copyright © 2019 the authors
Two-way ANOVA
Time : **
Dietary treatment : **
Time × Dietary treatment : **

### E

**Relative expressions of pepck (RU)**

**Sampling time**

### F

**Relative expressions of g6pase (RU)**

**Sampling time**
Two-way ANOVA

Time: ***

Dietary treatment: **

Time × Dietary treatment: **

Sampling time

Relative expressions of fbpase (RU)
Fig. 4

A

Two-way ANOVA
Time : *
Dietary treatment : *
Time × Dietary treatment : *

Liver GK activities (U/g tissue protein)

Sampling time

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</table>

B

Two-way ANOVA
Time : *
Dietary treatment : *
Time × Dietary treatment : *

Liver PK activities (U/g tissue protein)

Sampling time

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Two-way ANOVA
Time : *
Dietary treatment : *
Time × Dietary treatment : *

Liver FBPase activities
(Ug tissue protein)

Sampling time

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</table>
Fig. 5.

Two-way ANOVA
Time : ***
Dietary treatment : ***
Time × Dietary treatment : ***

A

Sampling time

Relative expressions of cpt IA (RU)

B

Sampling time

Relative expressions of aco (RU)
Two-way ANOVA

Time : ***
Dietary treatment : **
Time × Dietary treatment  : **

C

Sampling time

Relative expressions of acc α (RU)

D

Sampling time

Relative expressions of fas (RU)