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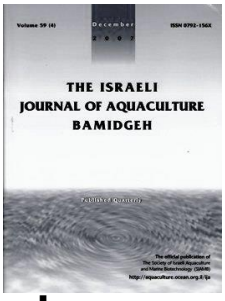

Influence of High-Carbohydrate Diet on Growth, Muscle Composition, and Lipid Metabolism of *Megalobrama amblycephala*

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Keywords: *Megalobrama amblycephala*; high carbohydrate; growth; lipid metabolism; liver structure

Abstract

The aim of this research was to study the influence of a high-carbohydrate diet on the growth and lipid metabolism of *Megalobrama amblycephala.* Wheat starch (carbohydrate source) and soybean oil (lipid source) were used to prepare a semipurified diet (control) and a high-carbohydrate diet (experimental). A series of measurements were conducted, which included growth performance, blood biochemical indices, muscle components, fatty acid composition, liver lipid deposition, and tissue structure. Results indicated that high levels of carbohydrate significantly reduce weight gain, specific growth rate, protein efficiency, and muscle protein content and increase lipids in fish. Composition of the muscle fatty acids was also altered and the activity of the hepatopancreatic lipid-metabolism enzymes decreased. A large quantity of lipid droplets, and vacuole degeneration, were observed in fish liver sections of the experimental group, indirectly suggesting a negative effect of a high-carbohydrate diet on the liver of *M. amblycephala*.

Ethics statement. This study was approved by the Animal Care and Use Committee of the Centre for Applied Aquatic Genomics at Chinese Academy of Fishery Sciences.

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Introduction

Fish meal and fish oil are considered essential raw materials for aquatic cultured organisms. In terms of economy, and ecology of the environment, it is very important to find an alternative to marine aquatic resources commonly used as raw materials for aquatic feeds.

Previous studies have demonstrated the effects of vegetable feed on fish growth, feed conversion efficiency, metabolic response, immunity, and the nutritional quality of fish (Kissil and Lupatsch, 2004; Leaver et al., 2008; Panserat et al., 2009; Geay et al., 2011; Morais et al., 2012). Given the high carbohydrate content in many vegetable materials, replacing fish meal with vegetable materials increases dietary carbohydrate levels. A carbohydrate is naturally low in many fish diets, it cannot be used by many fish, therefore it can cause metabolic disorders and glucose intolerance. The dietary carbohydrate level also plays an important role in regulating fat metabolism in fish (Enes et al., 2011; Kamalam et al., 2012, 2013). Many hormones, including growth hormones and thyroid hormones, influence carbohydrate and lipid metabolism, and dietary carbohydrates strongly influence those enzymes and hormones. Excessive carbohydrate can be metabolically converted into lipid and other substances and stored in the liver. Dietary carbohydrate intake reportedly affects the blood lipid level, causing formation and deposition of liver lipids, promoting the metabolism and transformation of fatty acids (Enes et al., 2011). Carbohydrate levels also greatly influence hepatic enzymes and expression of associated genes. In normal fish, higher carbohydrate levels mean higher lipid content, lipid metabolic enzyme activity, and enzyme gene expression. Interestingly, dietary lipid levels also affect the carbohydrate metabolism of fish (Menoyo et al., 2006).

Thus, carbohydrate metabolism correlates closely with lipid metabolism, and lipid utilization is clearly higher than carbohydrate utilization in fish. Therefore, the carbohydrate/lipid ratio is critical to fish growth and development (Li et al., 2012). If the dietary carbohydrate level is high, excessive carbohydrate will be converted to fat, causing lipid deposition in the muscles and organs, and may affect growth, taste, and health of the fish (Gao et al., 2009). In this study, we used soybean oil (lipid source) and wheat starch (carbohydrate source) to test the influence of a high-carbohydrate diet on growth, lipid deposition, and metabolism in the organs of *Megalobrama amblycephala*.

Materials and Methods

Experimental diets.

Based on the nutritional requirements for *M. amblycephala* (Ren et al. 2015), wheat starch (glucose source) and soybean oil (fat source) were used to prepare a semipurified diet (Normal Wheat Starch, NWS, 25.85% digestible carbohydrate, 32.73% crude protein, and 8.54% crude fat) for the control group, and a high-carbohydrate high-energy diet (High Wheat Starch, HWS, 45.25% digestible carbohydrate, 33.32% crude protein, 8.58% crude fat) for the experimental group (Table 1). All the ingredients were mixed and converted into a 2 mm sinking-pellet diet.

Table 1 Composition and proximate analysis of the experimental diets

Ingredient (%)	*Diets*	
	NWS	*HWS*
Casein[1]	20	20
Gelatin	5	5
Fish meal[2]	16	16
Wheat starch[3]	25	45
Soya bean oil[4]	7	7
Vitamin premix[5]	1	1
Mineral premix[5]	1	1
Carboxymethylcellulose	24	4
Monocalcium phosphate[5]	1	1
Approximate composition (% dry matter)		
Crude protein	32.73	33.32
Crude lipid	8.54	8.58
Digestible carbohydrate	25.85	45.25
Energy (kJ/g)	15.01	18.55
Calcium	1.11	1.06
Total phosphorus	1.34	1.32

[1] Provided byFeiya Science & Technology Development Co., Ltd (Shanghai, China), protein content 88.7%.
[2] Provided by Tongwei Co., Ltd (Wuxi, China), protein content 61.2%.
[3] Provided by Jinglingta Co., Ltd (Wuxi, China).
[4] Fatty acid content: 0.096% C14:0, 11.479% C16:0, 0.105% C16:1, 0.095% C17:0, 4.264% C18:0, 20.132% C18:1, 55.706% C18:2, 7.247% C18:3n3, 0.319% C20:0, 0.246% C20:1, 0.313% C22:0
[5] Provided by Wuxi Hanove Animal Health Products Co., Ltd (Wuxi, China). The vitamin premix and mineral premix were according to Ren et al. (2013).

Experimental fish.

M. amblycephala were provided by the Nanquan breeding base of the Freshwater Fisheries Research Center, the Chinese Academy of Fishery Sciences. The fish were temporarily raised in a circulating-water temperature-controlled aquaculture system and were fed a commercial freshwater-fish sinking-pellet diet (31.13% crude protein, 5.05% crude fat, 1.0 mm particle size; Tongwei Group Co., Ltd, Wuxi, China). The fish were raised in eight circular fish tanks (Φ 1300 mm × 700 mm) before the experiment. After acclimatization for 15 days, 240 healthy fish of uniform size were randomly classified into two groups, with four parallel sets per group, with each parallel set consisting of 30 fish (initial weight 27.54 ± 0.16 g).

Feeding and management. In the 8-week feeding experiment, the fish were fed daily at 0800, 1100, 1500, and 1800 hours, and their feeding activities were recorded 30 min after feeding. The residual feed was removed, and feed consumption was calculated. The amount consumed was adjusted each week according to feeding activity. The goal was to make the fish eat, leaving no residual feed. The aquaculture system and filtering equipment were cleaned daily, and oxygen was supplied day and night during the experiment. Water conditions were: average water temperature 26.0 ± 1.5°C, dissolved oxygen ≥ 6 mg/L, ammonia nitrogen ≤ 0.2 mg/L, nitrite ≤ 0.02 mg/L, pH 6.8-7.0. After 8 weeks of the feeding experiment, the fish were weighed, and blood and tissue samples were collected. The use of experimental fish was conducted under scientific research protocols of Chinese Academy of Fishery Sciences (CAFS) and Ministry of Agriculture, PR China.

Sample collection.

The fish were fasted for 24 h after the feeding experiment. Three fish were randomly selected from each tank and anesthetized with 100 mg/L MS-222. The fish blood samples were collected from the caudal vein, with sodium citrate as the anticoagulant. The blood sample was centrifuged at 4000 rpm for 10 min at 4°C. Plasma was isolated and stored at -20°C before the serum biochemical indices were determined. Meanwhile, liver muscle tissue of the sampled fishes were stripped immediately. The liver tissues were added to 4°C buffer (10 ×) ice bath homogenate to generate a 10% homogenate. The homogenate was centrifuged at 4000 rpm for 10 min, and the supernatant was stored at -20°C before analysis of the hepatic lipid-metabolism enzymes. The descaled muscles of fish backs were collected to determine the muscle nutritional components and fatty acid composition. Two liver-tissue blocks from other fish were collected quickly and fixed in paraformaldehyde solution for oil-red-O staining and hematoxylin–eosin (HE) staining, respectively.

Analytical methods.

Glucose (GLU), cholesterol (CHOL), triglyceride (TG), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) detection kits were purchased from Junshi Biological Technology (Shanghai, China) Co., Ltd, and results were analyzed with a Beckman C×4 automatic biochemical analyzer (United States).

Compositional analyses of the experimental feeds and whole fish bodies were performed according to the Association of Official Analytical Chemists (AOAC) method (2003). Fatty acid composition of the fish muscle was measured using the method of Hong et al. (2015), and the area normalization method was used to determine the relative fatty acid content.

Lipases, including lipoprotein lipase (LPL), hepatic lipase (HL), and nonesterified fatty acids (NEFA), were measured using detection kits. The kits were brought from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

HE staining of paraffin sections of liver tissue was as in Fischer et al. (2008). Optical microscopy (Nikon H500S, Japan) was used for image collection and analysis.

Lipid deposition in the liver was analyzed in oil-red-O-stained frozen sections (Wang et al., 2014).

Statistical data and analysis.

SPSS (ver. 20.0) was used for single-factor analysis of variance (one-way ANOVA). The results are expressed as means ± SE. Differences between groups were analyzed with an independent-samples *t* test.

Results

Influence of high-carbohydrate diet on growth and muscle composition of M. amblycephala. The dietary carbohydrate levels significantly influenced the growth performance of *M. amblycephala*. Weight gain rate (WGR) and specific growth rate (SGR) were highly significantly less in the HWS group than in the NWS group ($P < 0.01$), while feed conversion rate (FCR) was highly significantly greater in the HWS group than in the NWS group ($P < 0.01$), and the protein efficiency was significantly lower ($P < 0.05$) (Table 2).

Table 2. Growth performance and feed utilization of Megalobrama amblycephala fed with NWS and HWS diets

	NWS	*HWS*
Initial bodyweight (g)	27.52±0.07	27.55±0.08
Final bodyweight (g)	64.89±1.47	46.29±0.25**
WGR (%)[1]	135.80±5.06	68.02±4.11**
SGR(%)[2]	1.43±0.14	0.86±0.09**
FCR[3]	1.68±0.05	2.65±0.06**
PER(%)[4]	126.18±2.02	117.39±1.20*

Note: Values are means ± S.E., n = 4. Differences between groups were analyzed with an independent-samples t test. *$P < 0.05$, significant difference; **$P < 0.01$, extremely significant difference.
[1] Specific growth rate (SGR, %) = 100 × ([Ln final bodyweight − Ln initial bodyweight]/test days).
[2] Weight gain rate (WGR, %) = 100 × (average final bodyweight − average initial bodyweight)/average initial bodyweight.
[3] Feed conversion rate (FCR) = feed intake/weight gain.
[4] Protein efficiency ratio (PER, %) = 100 × (wet weight gain)/(crude protein intake).

Crude protein content of the muscle was significantly lower in the HWS fish than in the NWS fish ($P < 0.05$), whereas the crude fat content was significantly higher ($P < 0.05$). However, the moisture and ash content were the same in both groups (Table 3).

Table 3. Muscle composition of Megalobrama amblycephala fed the NWS or HWS diet

	NWS	*HWS*
Moisture (%)	77.74±2.32	77.37±1.12
Protein (%)	76.62±0.48	74.75±0.14*
Lipid (%)	15.87±0.43	17.93±0.52*
Ash (%)	7.70±0.17	7.57±0.43

Note: Values are means ± S.E., n = 12. Differences between groups were analyzed with an independent-samples *t* test. *$P < 0.05$, significant difference.

No significant difference was observed in total amounts of saturated fatty acids (SFA) in the muscle of the two groups. However, myristic acid and hexadecanoic acid contents were significantly lower in the HWS fish than in the NWS fish ($P < 0.05$), whereas stearic acid content was significantly higher ($P < 0.05$). No significant difference was observed in the total amounts of unsaturated fatty acids (UFA) in the muscle of the two groups, whereas the total amount of monounsaturated fatty acids (MUFA) was higher in the HWS fish than in the NWS fish ($P < 0.05$), and total polyunsaturated fatty acids (PUFA) was significantly lower ($P < 0.05$). In terms of MUFA classification, muscle oleic acid increased significantly in the HWS fish ($P < 0.01$). On the contrary, docosapentaenoic acid (DPA) was significantly reduced ($P < 0.05$), and eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and EPA+DHA were highly significantly reduced ($P < 0.01$), all in terms of PUFA classification (Table 4).

Table 4. Muscle fatty acid profiles of Megalobrama amblycephala fed the NWS or HWS diet (%)

	NWS	*HWS*
Lauric acid (C12:0)	0.28±0.08	0.18±0.07
Myristic acid (C14:0)	2.51±0.11	1.35±0.20*
Pentadecanoic acid (C15:0)	0.23±0.03	0.12±0.05
hexadecanoic acid (C16:0)	19.88±0.74	16.84±0.67*
Daturic acid (C17:0)	0.25±0.03	0.16±0.03
Stearic acid (C18:0)	4.80±0.22	5.90±0.37*
Arachidic acid (C20:0)	0.14±0.02	0.16±0.03
Saturated fatty acids (SFA)	28.08±1.81	24.69±1.67
Palmitoleic acid (C16:1)	5.21±0.55	4.59±0.32
Oleic acid (C18:1)	31.32±1.78	42.52±2.03**
Eicosaenoic acid (C20:1)	0.79±0.25	1.03±0.22
Docosenoic acid (C22:1)	0.23±0.07	0.20±0.06
Linoleic acid (C18:2)	20.34±1.06	19.00±0.98
Eicosandienoic acid (C20:2)	0.53±0.11	0.62±0.21
Linolenic acid (C18:3)	2.42±0.43	2.01±0.38
Eicosatrienoic acid (C20:3)	0.66±0.19	0.93±0.21
Docosatrienoic acid (C22:3)	0.17±0.03	0.17±0.02
Arachidonic acid (C20:4)	1.04±0.10	1.12±0.09
Docosatetraenoic acid (C22:4)	0.22±0.05	0.22±0.04
Docosapentaenoic acid (C22:5)	0.87±0.07	0.33±0.01*
Eicosapentaenoic acid (EPA) C20:5	2.16±0.17	0.57±0.08**
Docosahexaenoic acid (DHA) C22:6	5.97±0.56	2.01±0.11**
EPA+DHA	8.13±0.89	2.58±0.23**
Monounsaturated fatty acids (MUFA)	37.55±1.88	48.34±2.41*
Polyunsaturated fatty acids (PUFA)	34.38±1.76	26.97±1.64*
Unsaturated fatty acids (UFA)	71.92±2.12	75.31±2.37

Note: Values are means ± SE, n = 12. Differences between groups were analyzed with an independent-samples *t* test. *$P < 0.05$, significant difference;
**$P < 0.01$, highly significant difference.

Influence of high-carbohydrate diet on blood and hepatic lipid metabolism in M. amblycephala.

The data suggest that the different levels of carbohydrates had no significant effect on serum HDL levels. However, serum GLU level increased significantly in the HWS fish ($P < 0.05$), and CHOL, TG and LDL were highly significantly ($P < 0.01$) elevated (Table 5).

Table 5. Blood biochemical parameters of Megalobrama amblycephala fed the NWS or HWS diet

	NWS	*HWS*
GLU (mmol/L)	14.76±1.11	21.30±2.37*
CHOL (mmol/L)	6.23±0.13	8.54±0.30**
TG (mmol/L)	1.64±0.14	2.36±0.09**
LDL (mmol/L)	1.27±0.08	2.49±0.16**
HDL (mmol/L)	3.67±0.59	4.63±0.23

Note: GLU, Glucose; CHOL, cholesterol; TG, triglyceride; LDL, low-density lipoprotein; HDL, high-density lipoprotein. Values are means ± SE, n = 12. Differences between groups were analyzed with an independent-samples *t* test. *$P < 0.05$, significant difference; **$P < 0.01$, highly significant difference.

Liver lipase activity was influenced by the dietary carbohydrate levels. Our results suggest that lipase activity, including LPL, HL, and NEFA, were highly significantly ($P < 0.01$) less in the HWS fish than in the NWS fish (Table 6).

Table 6. Lipid-metabolism enzyme activities in Megalobrama amblycephala fed the NWS or HWS diet (%)

	NWS	*HWS*
LPL (U/mg prot)	3404.17±177.64	2308.33±132.67**
HL (U/mg prot)	4264.92±239.09	2709.59±177.32**
NEFA (μmol/mg prot)	536.98±24.09	419.82±21.08**

Note: LPL, lipoprotein lipase; HPL, hepatic lipase; NEFA, nonesterified fatty acids. Values are means ± SE, n = 12. Differences between groups were analyzed with an independent-samples *t* test. **$P < 0.01$, highly significant difference.

Influence of high-carbohydrate diet on liver lipid deposition and liver structure in M. amblycephala.

Frozen liver sections were stained with oil red O (lipid red and nuclei light blue). The stained frozen liver sections indicated that lipid content was higher in the HWS fish than in the NWS fish, and the red cells in the hepatic portal were surrounded by large amounts of lipids. According to the positive area quantified in the frozen sections, lipid deposition in the liver was highly significantly greater in the HWS fish ($P < 0.01$) (Figure 1; Table 7).

Table 7. Liver fat deposition in *Megalobrama amblycephala* fed the NWS or HWS diet (%)

	NWS	*HWS*
Liver fat area	67.53±15.28	81.02±4.30**

Note: Values are means ± S.E., n = 36. Differences between groups were analyzed with an independent-samples *t* test. **$P < 0.01$, extremely significant difference.

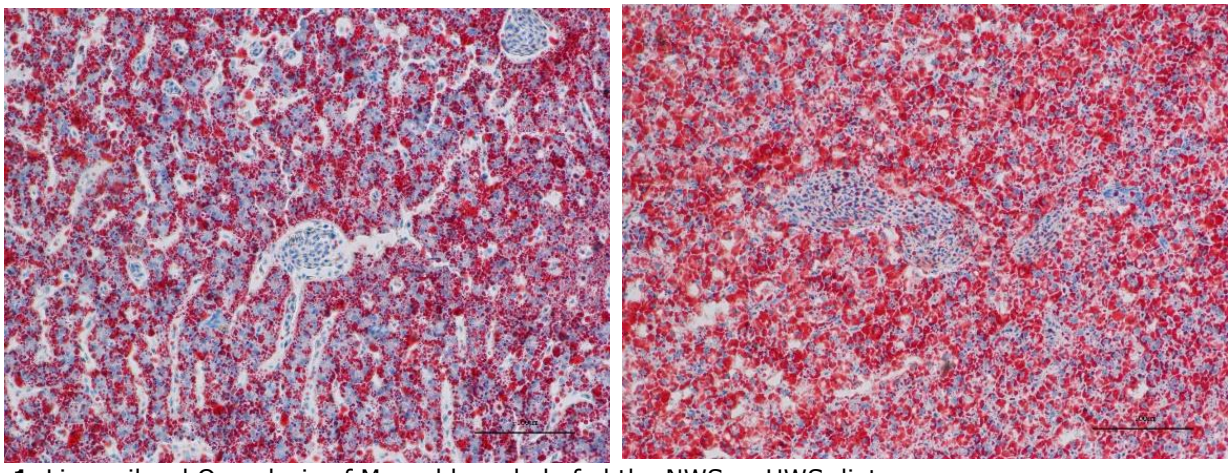
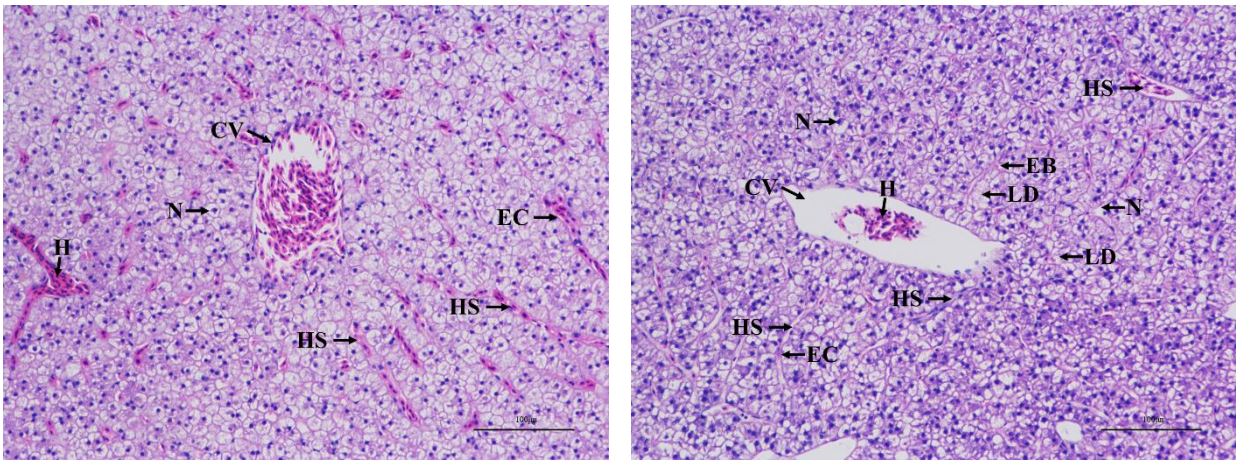


Figure 1. Liver oil red O analysis of M. amblycephala fed the NWS or HWS diet
a: Liver oil red O stain in NWS group (×200) b: Liver oil red O stain in HWS group (×200)
Note: Red means lipid droplets.

Liver paraffin sections were stained with HE (nuclei blue and cytoplasm red). Under a light microscope (200×), the liver cell structure in the NWS group (Figure 2A) remained intact and the cells were arranged closely, when lightly HE stained. The centrally located nuclei in the liver cells were starboard and full. Cells of the central vein wall were intact, uniformly arranged, and surrounded by many radially arranged liver sinusoids and liver cells. The endothelial cells of the liver sinusoids were intact, and abundant internal blood cells were closely apposed. Liver sections from the HWS group showed many lipid droplet vacuoles within the cells and the liver cells were closely compressed, causing pyknosis and dark staining. Many nuclei were compressed to the other sides of the cells. The section in Figure 2B shows that the liver cell cytoplasm was more eosinophilic, the nuclei were pyknotic or had been lost, and acidophilic corpuscles had formed and fallen into the hepatic sinusoid or Disse's space. Lipid droplets were apparent, compressing Disse's space, and causing the hepatic sinusoidal space to decrease. Vacuolar degeneration was present. Red blood cell count in the central veins and hepatic sinusoid decreased dramatically (Figure 2).



a：Hepatic HE staining in NWS group (×200)　　b：Hepatic HE staining in HWS group (×200)

Figure 2. HE analysis of the M. amblycephala liver after consumption of the NWS or HWS diet
Note: N, hepatic cell nucleus; CV, central vein; HS, hepatic sinusoid; H, hemocyte; EC, endothelial cell; LD, lipid droplet; EB, eosinophilic body.

Discussion

Influence of high-carbohydrate diet on growth and muscle composition of M. amblycephala. The carbohydrate content of many fish diets is low, and carbohydrate cannot be effectively used by many fish, particularly carnivorous fish. To optimize the use of fish meal and fish oil, scientists have investigated addition of carbohydrates to aquatic animal feed. Previous studies have suggested that α-starch can be utilized by *Elopichthys bambusa*, but nutritional physiological stress was induced when starch exceeded 20%, thus inhibiting fish growth (Zhou et al., 2011). Weight gain rate and the specific growth rate of *Larimichthys crocea* decreased with increasing dietary glucose levels (Ma et al., 2016). In this study, a diet containing 45% carbohydrate significantly reduced the WGR, SGR, and PER, as well increasing FCR of *M. amblycephala.* However, some researchers have argued that overuse of carbohydrates does not influence fish growth or feed utilization (Lee & Lee, 2004).

It was found that the dietary carbohydrate level had no significant effect on crude protein, moisture, or crude ash content of African electric catfish, whereas lipid content increased dramatically (Ali & Jauncey, 2004). When corn starch was used as the carbohydrate source, the abdominal and whole-fish lipid content of *Tilapia mossambica* increased as the dietary corn starch content increased (Wang et al., 2005). These findings were in accordance with our study. After consumption of the HWS diet for 8 weeks, muscle lipid content of the fish increased, whereas protein was decreased, suggesting that *M. amblycephala* has a limited capacity to convert carbohydrate to protein, whereas excess digestible carbohydrate accumulated in the muscle where excess energy is stored in the form of fat. However, another study (Sangmin & Kyoungduck, 2009) reported that muscle lipid content decreased after fish were fed excessive amounts of carbohydrate. The discrepancies in these results may be attributable to differences in the different type of fish studied, the growth phase analyzed, and the carbohydrate source. It has been reported that growth performance of fish is largely influenced by the carbohydrate source, and that WGR and SGR increase as the molecular weight of carbohydrate increases (Dong et al., 2016).

Carbohydrate intake also accelerated fatty acid metabolism and transformation (Kamalam et al., 2013). PUFA and MUFA are the two intermediate fatty acids, which can be synthesized by fatty acid elongase and desaturase. A study of the composition of turbot muscle fatty acids suggested that a high-carbohydrate, low-protein, diet caused UFA desaturation and lipid accumulation (Li & Ma, 2010), consistent with the results of previous studies of lipid-metabolism enzymes. Similarly, our study suggests that a high-carbohydrate diet significantly increases the MUFA content in fish muscle.

Influence of a high-carbohydrate diet on blood and liver lipid metabolism of M. amblycephala.

Most fish are glucose intolerant, and a diet with excessive carbohydrate significantly increased the blood glucose content of *M. amblycephala* in this study, as in other studies. However, carbohydrate plays an important role in regulating the lipid metabolism of fish (Kamalam et al., 2012). For example, dietary carbohydrate intake affects blood lipid levels, causing the formation and deposition of liver lipids, and accelerating metabolism and transformation of fatty acids (Enes et al., 2011; Kamalam et al., 2013). Some researchers have also reported that the phosphopentose pathway can be used for lipid synthesis, providing an important route for glucose digestion in the fish body (Hemre et al., 2002). As dietary glucose content increases, fish liver enzyme activity increases and the phosphopentose digestion pathway is strengthened. Glucose 6-phosphate (G6P) is catalyzed to produce reduced $NADP^+$, and synthesis of fatty acid is accelerated (Brauge et al., 1994), thus affecting lipid metabolism. Lipids can be decomposed into a mixture of glycerides and free fatty acids by lipase action, allowing them to be absorbed by fish. As the rate-limiting enzyme of TG catabolism, LPL catalyzes the TG carried by very-low-density lipoproteins and chylomicron. HL participates in HDL transportation and decomposition, regulating the role of TG in energy generation and accumulation in adipose tissues (Mead et al., 2002). However, our study has shown that excessive carbohydrate significantly and highly significantly increased the CHOL, TG, and LDL contents in the blood of *M. amblycephala*. The liver LPL and HL activity and the NEFA content also decreased significantly.

Influence of high-carbohydrate diet on hepatic lipid deposition and tissue composition of M. amblycephala.

A high-carbohydrate diet can cause the formation and deposition of liver lipids (Kamalam et al., 2013). High carbohydrate levels can induce more liver glycogen to be synthesized. The synthesized glycogen is deposited in the fish liver and abdominal cavity in the form of lipids, which can damage the physiological functions of the liver (Vielma et al., 2003), causing enlargement and vacuolation of the liver cells (Lee & Lee, 2004). The intake of excessive carbohydrates can induce nutritional stress in terms of the ichthyological histology, such as significant rupture of the liver cell membrane and nuclear migration (Cheng et al., 2007), together with cytoplasm loss and vacuolar degeneration (Jiang et al., 2013). In this study, we obtained similar results with oil-red-O-stained, and HE-stained, liver sections which were consistent with the results for muscle composition and activity of liver lipid-metabolism enzymes.

Conclusions

Dietary carbohydrate level has a significant influence on the growth, muscle composition, and lipid metabolic mechanisms of *M. amblycephala*. Excessive carbohydrate exerts a series of negative effects on fish health, including nutritional metabolic disorder, reduced growth performance, changes in the lipid content and fatty acid composition of the muscle, decreased activity of hepatopancreatic lipid-metabolism enzymes, vacuolar degeneration, and liver lipid droplets.

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