Revista Brasileira de Zootecnia
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ISSN 1806-9290
www.sbz.org.br

R. Bras. Zootec., 43(12):627-635, 2014

Fasted and postprandial response of serum physiological response, hepatic antioxidant abilities and HSP70 expression in Wuchang bream (*Megalobrama amblycephala*) fed different dietary carbohydrate levels

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ABSTRACT - The effect of dietary carbohydrate (CHO) level on serum physiological response, hepatic antioxidant abilities and heat shock protein 70 (HSP70) expression of Wuchang bream (*Megalobrama amblycephala*) was studied. Two isonitrogenous (28.56% crude protein) and isolipidic (5.28% crude lipid) diets were formulated to contain 30% or 53% wheat starch. Diets were fed for 90 days to fish in triplicate tanks (28 fish per tank). At the end of feeding trial, significantly higher serum triglyceride level, insulin level, cortisol level, and malondialdehyde (MDA) content were observed in fish fed the 53% CHO diet, while significantly lower serum total protein content, alkaline phosphatase activity, superoxide dismutase activity and total antioxidative capacity were found in fish fed the 53% CHO diet compared with those fed the 30% diet. The relative level of hepatic heat shock protein 70 mRNA was significantly higher in the 53% CHO group than that in the 30% CHO at 6, 12 and 48 h after feeding. Ingestion of 53% dietary CHO impacts the nonspecific immune ability and causes metabolic stress in *Megalobrama amblycephala*.

Key Words: biochemical parameters, gene expression, herbivorous fish, immunity, starch

Introduction

Fish are known to have a limited ability for digesting and metabolizing carbohydrates, and excessive intake of this nutrient may result in nutritional problems (Jauncey, 1982; Roberts and Bullock, 1989; Roberts, 1989; Lall, 1991). The carbohydrate utilization by fish varies among fish species and carbohydrate sources (Wilson, 1994; Krogdahl et al., 2005). In general, herbivorous fish are more capable of utilizing dietary carbohydrate than carnivorous and omnivorous fish (Hemre et al., 2002). Fish with excess glucose are assumed to be under constant metabolic stress (Pieper and Pfeffer, 1980; Fletcher, 1981), which may lead to suppressed immune functions (Erfanullah, 1995; Fu and Xie, 2005). Thus, high dietary carbohydrate intake may affect the antioxidation ability and increase the incidence of diseases in fish.

Wuchang bream (*Megalobrama amblycephala*), a freshwater herbivorous species, was originally found in Newshan Lake and Yuli Lake (Ke, 1975). Its main distribution is in the mid reach of the Yangtze River, China (Zhu, 1995). Due to the merits of this species including tender flesh, fast growth, feeding on natural foods, high disease resistance, economic profitability, and cultural values (Ke, 1986; Zhou et al., 2008), Wuchang bream is widely cultured in China with the output of 625,789 t in 2009 – an increase of 31.50% in the past decade. Shi et al. (1988) reported that Wuchang bream requires 27.04-30.39% protein for optimal growth when the water temperature is about 20 ºC, and the optimum protein requirement ranges from 25.58% to 41.40% when water temperature varies from 25 °C to 30 ºC. Zou et al. (1987) reported that Wuchang bream required a diet containing 21.05-30.83%, using methods of linear and polynomial regression analysis. According to the previous reports (Yang et al., 1989; Liu et al., 1992; Zhou et al., 1997; Li et al., 2010), the carbohydrate requirement was reported to be between 25% and 30%. However, little research has been done on the health implications of high dietary carbohydrate on Wuchang bream.

Therefore, the objectives of the present study were to investigate the effect of dietary carbohydrate (CHO) on serum physiological response, hepatic antioxidative enzyme activities and HSP70 expression of *M. amblycephala* to gain a better understanding of the health status of *M. amblycephala* fed high CHO diets.

Received March 11, 2014 and accepted August 29, 2014.
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http://dx.doi.org/10.1590/S1516-35982014001200001

Material and Methods

Wuchang bream juveniles were obtained from a fish farm at the Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences, in China and reared in six 300-L cylindrical fiberglass tanks. Fish were allocated to each tank and acclimated for 15 days before the feeding trial. Thereafter, the fish in the control group and the high-CHO group in triplicate (three tanks, 28 fish per tank, 34.73±0.67 g initial body weight) were fed 30 and 53% CHO, respectively (Table 1).

Two isonitrogenous (28.6% crude protein) and isolipidic (5.3% crude lipid) diets were formulated to contain 30 and 53% CHO, respectively. The dietary crude protein and lipid contents have been evidenced for optimal growth of Wuchang bream (Shi et al., 1988; Yang et al., 1989; Liu et al., 1992). Dry ingredients were well ground and mixed thoroughly in a mixer, and then water was added. The 2-mm-diameter pellets were wet-extruded. The diets were dried in a forced air oven at 40 °C to a moisture content of 10% and stored at −20 °C until use.

Wuchang bream juveniles were acclimated in cylindrical fiberglass tanks for 15 days and then fed the trial diet in a quantity equivalent to about 2.0-4.0% BW three times a day at 06.00-06.30, 11.00-11.30, and 16.00-16.30 h, provided with a continuous flow of sand-filtered freshwater (2 L/min) with continuous aeration. Tanks were thoroughly cleaned biweekly. Water quality parameters were monitored daily. Feeding trials lasted 90 days. During the test period, the water quality on average was as follows: water temperature 26.0±1.5 °C, dissolved oxygen (DO) >5 mg L^{-1}, NH_3<0.05 mg L^{-1}, H_2S<0.1 mg L^{-1}, and pH 7.8-8.0. The feeding amount was increased every other week.

Table 1 - Basal diet and nutritional levels of Wuchang bream (*Megalobrama amblycephala*)

Ingredients	Composition	
	30.42% CHO	52.92% CHO
Fish meal[1]	42	42
α-starch[2]	30	52.5
Microcrystalline cellulose	22.5	0
Fish oil	1.5	1.5
Premix[3]	1	1
Carboxyl-methyl cellulose	2	2
Calcium dihydrogen phosphate	1	1
Total	100	100
Dry matter (%)	92.32	90.52
Crude protein (%)	28.56	28.56
Ether extract (%)	5.28	5.28
Digestible carbohydrate (%)[4]	30.42	52.92
Ash	8.90	9.31
Gross energy (kJ/g)[5]	14.06	17.92

[1] Fish meal: Tecnologica de Alimentos S.A., Perú.
[2] α-starch: purchased from Jin Lingta Co., Ltd. China.
[3] Premix supplied the following minerals (g/kg of diet) and vitamins (IU or mg/kg of diet): $CuSO_4$•$5H_2O$ - 2.0 g; $FeSO_4$•$7H_2O$ - 25 g; $ZnSO_4$•$7H_2O$ - 22 g; $MnSO_4$•$4H_2O$ - 7 g; Na_2SeO_3 - 0.04 g; KI - 0.026 g; $CoCl_2$•$6H_2O$ - 0.1 g; vitamin A - 900,000 IU; vitamin D - 200,000 IU; vitamin E - 4,500 mg; vitamin K_3 - 220 mg; vitamin B_1 - 320 mg; vitamin B_2 - 1,090 mg; vitamin B_5 - 2,000 mg; vitamin B_6 - 500 mg; vitamin B_{12} - 1.6 mg; vitamin C - 5,000 mg; pantothenate - 1,000 mg; folic acid - 165 mg; choline - 60,000 mg.
[4] Carbohydrate contents of feed were analyzed by the 3,5-dinitro salicylic acid method (Yu et al., 1997).
[5] Gross energy (kJ/g) was calculated using energy equivalents 23.64 kJ/g, 39.54 kJ/g, and 17.15 kJ/g for protein, lipid and digestible carbohydrate, respectively.

At the end of the trial, the fish were starved for 48 h and then fed once. Nine fish from each group (three tanks, three fish per tank) were anesthetized with MS-222 and sampled at 0, 6, 12, 24, and 48 h after the meal. Gut contents were systematically checked to make sure that the fish had actually consumed the test diets. Blood was sampled from the caudal vein, centrifuged for 10 min (4 °C, 3,000 × g), and kept frozen until analysis. The liver was excised, frozen in liquid nitrogen, and stored at −80 °C until analysis.

Serum total protein and albumin contents were estimated by the Biuret and BCG dye binding method and the bromocresol green binding method (Reinhold, 1953), respectively. Serum total protein content was measured using the biuret method (kit purchased from Shanghai Fudan Zhangjiang Biopharmaceutical Co., Ltd., China) in a Beckman Cx-4 type auto biochemical analyzer (Beckman Coulter, USA). Bovine serum albumin was used as standard (BSA: 66 kDa; Nanjing Jiancheng Biological Engineering Research Institute of China). Serum globulin was determined by subtracting serum albumin from total protein. Activity of serum aspartate aminotransferase (GOT) was estimated according to the methods described by the study of Krajnović-Ozretić and Ozretić (1978). Serum GOT activity was determined using a colorimetric kit (Shanghai Fudan Zhangjiang Bio Medical Co., Ltd., China) in a Beckman Cx-4 type Auto Bio-chemical Analyzer (Beckman Coulter, USA).

Serum glucose (GLU), cholesterol (CHOL) and triglyceride (TGL) were measured by the glucose oxidase, enzymatic (cholesterol oxidase) and colorimetric and enzymatic (glycerol phosphate oxidase) and colorimetric (PAP) methods, respectively, using kits purchased from Junshi Biotechnology (Shanghai) Co., Ltd., China.

Serum insulin was measured by radioimmunoassay (RIA) using a test kit (Beijing Beifang Biotech Research Institute, China) and following the method described by Clark and Hales (Clark and Hales, 1994; Li et al., 2012a). Serum cortisol was measured by RIA using a test kit (Beijing Beifang Biotech Research Institute, China) and following the method described by Pickering and Pottinger (Liu et al., 2012a). Serum alkaline phosphatase (AKP) activity was measured according to the procedure described

by Dabrowski (1990) using kits from Junshi Biotechnology (Shanghai) Co., Ltd., China.

Hepatic superoxide dismutase (SOD) activity, malondialdehyde (MDA) content and total antioxidation capacity (T-AOC) were determined by following the methods described in the previous studies (Marklund and Marklund, 1974; Drape et al., 1993; Rice-Evans and Miller, 1994). Test kits for these assays were purchased from Nanjing Jiancheng Biological Engineering Research Institute in China.

The *M. amblycephala* cDNA sequences in GenBank were used to design the primers for HSP70 (accession No EU884290.2) and β-actin (accession No AY170122.2) (Ming et al., 2010). The primers were: (1) 5'- CTTTATCAGGGAGGGATGCCAGC-3' and 5'- CCC TGCAGCATTGAGTTCATAAGGT-3' for HSP70 cDNA and; (2) 5'- TCGTCCACCGCAAATGCTTCTA-3' and 5' –CCGTCACCTTCACCGTTCCAGT-3' for β-actin cDNA. All primers were synthesized by Shanghai Generay Biotech co., LTD. China. The PCR products were 100-150 bp long.

Total RNA was extracted from the liver tissue using RNAiso Plus (Dalian Takara Co. Ltd., China). RNA samples were treated with RQ1 RNase-Free DNase (Dalian Takara Co. Limited, China) to avoid genomic DNA amplification. cDNA was generated from 500 ng DNase-treated RNA using ExScript™ RT-PCR Kit (Dalian Takara Co. Ltd., China). The reverse transcription PCR reaction solution consisted of 500 ng RNA, 2 μL 5× buffer, 0.5 μL dNTP mixture (10 mM each), 0.25 μL RNase inhibitor (40 U/μL), 0.5 μL dT-AP primer (50 mM), 0.25 mL ExScript™ RTase (200 U/μL), and DEPC H_2O, up to a final volume of 10 μL. The reaction conditions were as follows: 37 ºC for 15 min, 85 ºC for 5 s, and 4 ºC thereafter.

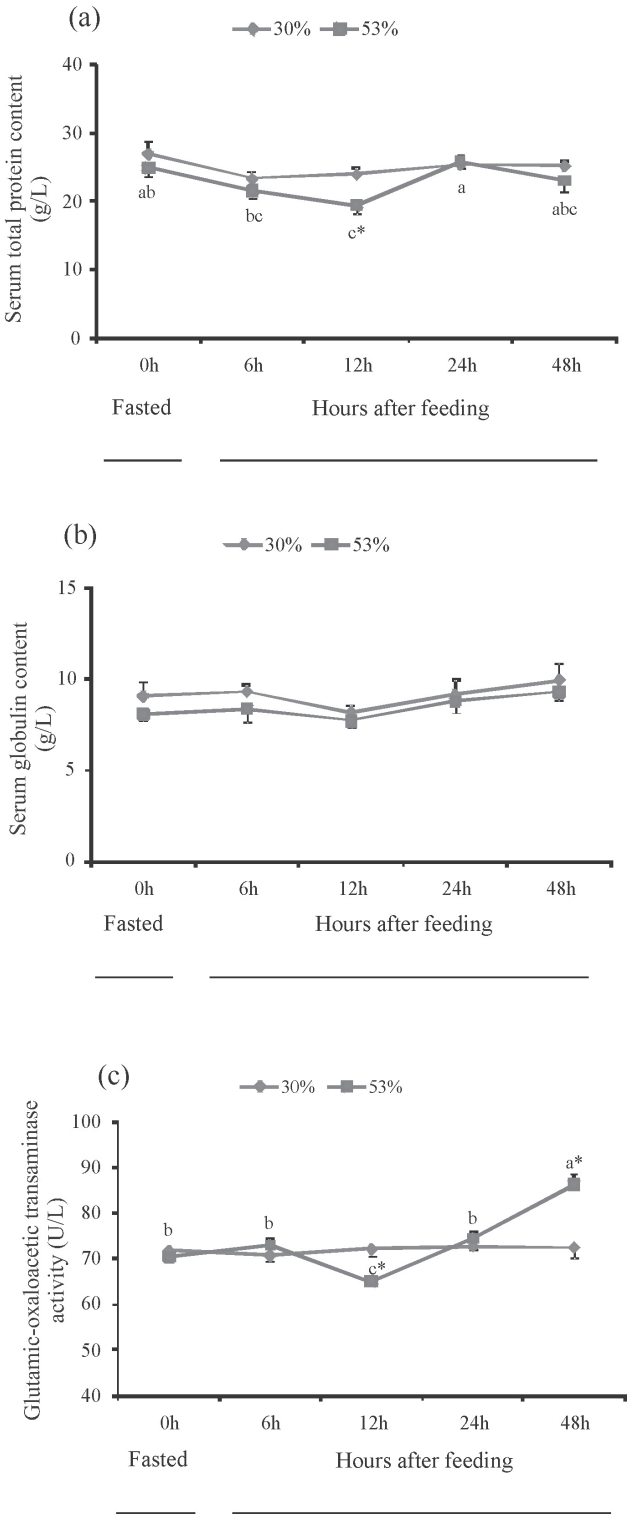
Real-time PCR was used to determine mRNA levels with an SYBR Greenlfluorescence kit (Ming et al., 2010). Real-time PCR was performed in a Mini Opticon Real-Time Detector (Bio-Rad, USA). The fluorescent quantitative PCR reaction solution consisted of 12.5 μL SYBRR premix Ex Taq™ (2×), 0.5 μL PCR Forward Primer (10 μM), 0.5 μL PCR Reverse Primer (10 μM), 2.0 μL RT reaction mix (cDNA solution), and 9.5 μL dH_2O. The reaction conditions were as follows: 95 ºC for 2 min, followed by 44 cycles consisting of 95 ºC for 10 s, 59 ºC for 20 s, and 72 ºC for 20 s. The florescent flux was then recorded and the reaction continued at 72 ºC for 3 min. Dissolution rate was measured between 65 and 92 ºC. Each increase of 0.2 ºC was maintained for 1 s and the fluorescent flux was recorded. The relative quantification of the target gene transcript (HSP70) was calculated with a chosen reference gene transcript (β-actin) using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). This mathematical algorithm, which does not require a calibration curve, computes an expression ratio based on real-time PCR efficiency and the crossing point deviation of the sample versus a control. The PCR efficiency was measured by constructing a standard curve using a serial dilution of cDNA; $\Delta\Delta C_T = (C_{T,\ Target} - C_{T,\ \beta\text{-actin}})$ time x − $(C_{T,\ Target} - C_{T,\ \beta\text{-actin}})$time 0.

The SPSS (version 16.0) software was used to perform Duncan's multiple range tests and Independent-Samples t-tests to determine differences among treatments. Different little letters above histogram bars denote statistically significant differences ($P<0.05$) in different CHO groups of each sampling point in Duncan's multiple range tests. Significant differences ($P<0.05$) between values obtained from normal and high CHO groups are indicated by asterisks above histogram bars in Independent-Samples t-tests. All the results were expressed as means ± standard error ($\overline{X}$ ±SE).

Results and Discussion

There was no significant change in serum total protein content in the control group (Figure 1A). Serum total protein content at 12 h after feeding was significantly lower than that at 0 h in the 53% CHO group ($P<0.05$) (Figure 1A). Serum total protein content was significantly lower in the 53% CHO group than that in the control group at 12 h ($P<0.05$) (Figure 1A). There was no significant change in serum globulin content in both groups (Figure 1B). No significant change in serum GOT activity was observed in the control group (Figure 1C). Serum GOT activity at 12 h after feeding was significantly lower than that at 0 h in the 53% CHO group, but serum GOT activity at 48 h after feeding was significantly higher than that at 0 h in the 53% CHO group ($P<0.05$) (Figure 1C). Serum GOT activity was significantly lower in the 53% CHO group than that in the control group at 12 h, but higher at 48 h ($P<0.05$) (Figure 1C).

Proteins are the most important compounds in the serum. The serum protein content is used as an immune parameter that can indicate whether or not a fish is healthy (De Smet and Blust, 2001). Excess dietary CHO can impact serum metabolites such as serum total protein and globulin (Kumar et al., 2005). In the present study, the serum total protein content was significantly lower in the 53% CHO group than that in the control group at 12 h. It indicated that the excess dietary CHO have an immuno-suppressive effect on Wuchang bream. The results were in agreement with the previous study in fish in which the serum total protein content was negatively correlated with dietary carbohydrate (Hemre et al., 1995). Serum aspartate aminotransferase,



Data are expressed as the mean of triplication of each group ± standard error (n = 9).
Significant differences (P<0.05) between values obtained in control group and high CHO group are marked by asterisk by t-tests.
Different letters indicate significant differences (P<0.05) in different sampling points by Duncan's multiple range tests.

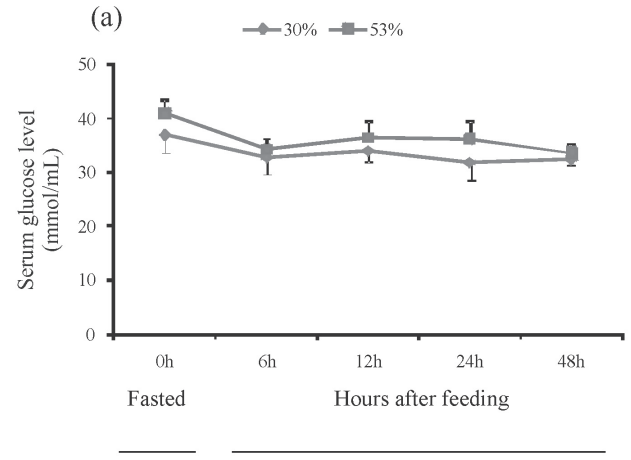
Figure 1 - Fasted and postprandial patterns of serum total protein (a), globulin (b) and glutamic-oxaloacetic transaminase (c) in *M. amblycephala* fed the different-carbohydrate-level diets for 10 weeks.

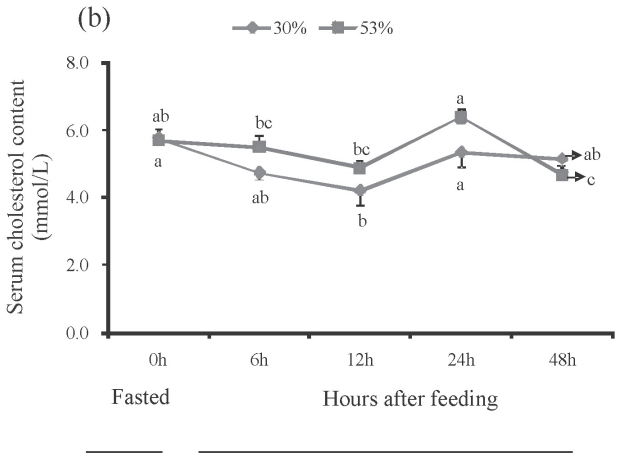
ubiquitous aminotransferase located in the mitochondria of fish, is an important index for the diagnosis of liver function and damage (Cho et al., 1994; Liu et al., 2010). In this study, the serum GOT activity was significantly higher in the 53% CHO group than that in the control group at 48 h, which indicated that, to some extent, the excess dietary carbohydrate caused damage to the liver function of *M. amblycephala*. Similar findings were obtained in top mouth culter (*Erythroculter ilishaeformis* Bleeker) (Liu et al., 2012b). However, in this study, the serum GOT activity was significantly lower in the 53% CHO group than that in the control group at 12 h. Miao et al. (2011) also found that *Carassius auratus gibelio* fed higher levels (50%) of CHO had lower GOT activity.

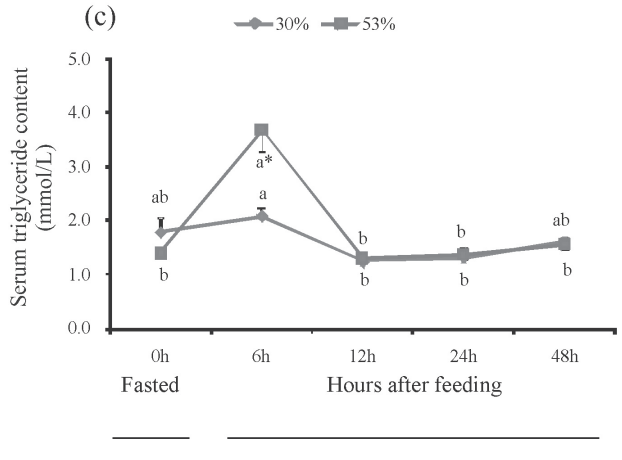
There was no significant change in serum glucose concentration in both groups (Figure 2A). Serum cholesterol content at 12 h after feeding was significantly lower than that at 0 h in the control group (P<0.05) (Figure 2B). Serum cholesterol content at 48 h after feeding was significantly lower than that at 0 h in the 53% CHO group (P<0.05) (Figure 2B). Serum triglyceride content at 6 h after feeding was significantly higher than those at 0 h in the 53% CHO group and then serum triglyceride content in the 53% CHO group returned to the level at 0 h (P<0.05) (Figure 2C). There was no significant change in serum triglyceride content in the control group (Figure 2C). Serum triglyceride content was significantly higher in the 53% CHO group than those in the control group at 6 h (P<0.05) (Figure 2C).

The serum triglyceride contents in fish fed the 52.94% CHO diet were significantly higher than those fed the control diet at 6 h, which confirmed that the high dietary CHO had a detrimental effect on Wuchang bream (Thorpe and Ince, 1974). Similar findings were obtained in top mouth culter (*Erythroculter ilishaeformis* Bleeker) (Liu et al., 2008) and European whitefish (*Coregonus lavaretus*) (Vielma et al., 2003). However, some other studies reported no significant differences in gilthead sea bream (*Sparus aurata*) (Enes et al., 2008), largemouth bass (*Micropterus salmoides*) (Amoah et al., 2008) and grass carp (*Ctenopharyngodon idellus*) (Gao et al., 2010).

Serum insulin at 6 and 12 h after feeding was significantly higher than that at 0 h in the 53% CHO group and then serum insulin in the 53% CHO group at 24 and 48 h after feeding returned to the level at 0 h. Serum insulin at 6, 12, 24 and 48 h after feeding was significantly higher than that at 0 h in the control group (Figure 3A). Serum insulin was significantly higher in the 53% CHO group than that in the control group at 0 h, 24 h and 48 h (P<0.05) (Figure 3A). Serum cortisol levels at 6, 12, 24 and 48 h after feeding were significantly lower than those at 0 h in

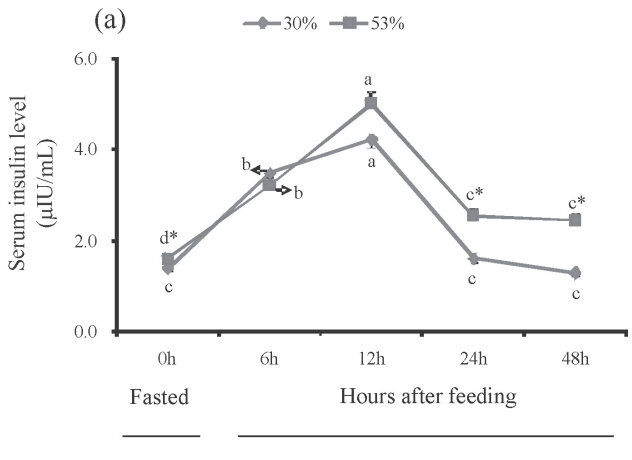


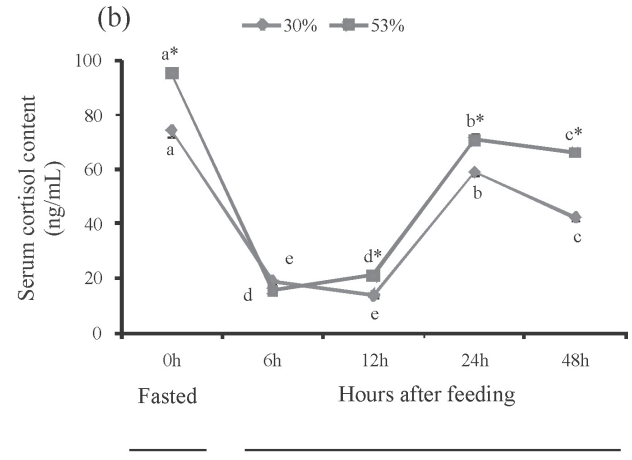


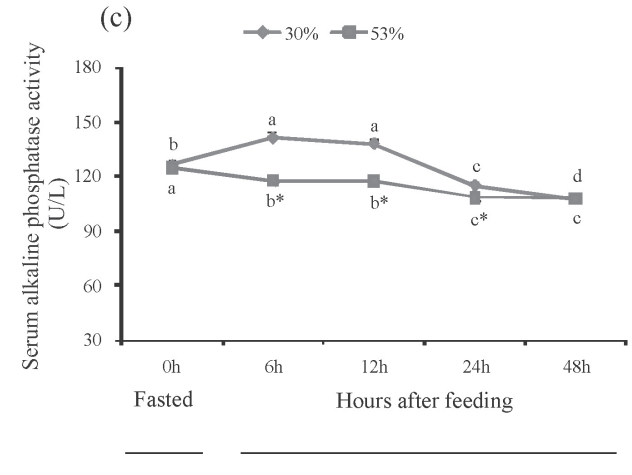


Data are expressed as the mean of triplication of each group ± standard error (n = 9).
Significant differences (P<0.05) between values obtained in control group and high CHO group are marked by asterisk by t-tests.
Different letters indicate significant differences (P<0.05) in different sampling points by Duncan's multiple range tests.

Figure 2 - Fasted and postprandial patterns of serum glucose (a), cholesterol (b) and triglyceride (c) in *M. amblycephala* fed the different-carbohydrate-level diets for 10 weeks.







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Different letters indicate significant differences (P<0.05) in different sampling points by Duncan's multiple range tests.

Figure 3 - Fasted and postprandial patterns of serum insulin (a), cortisol (b) and alkaline phosphatase (c) in *M. amblycephala* fed the different-carbohydrate-level diets for 10 weeks.

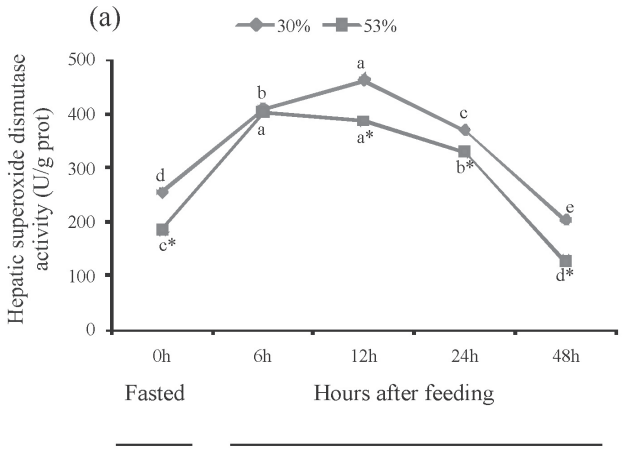
both groups (P<0.05) (Figure 3B). Serum cortisol levels were significantly higher in the 53% CHO group than those in the control group at 0 h, 12 h, 24 h and 48 h (P<0.05) (Figure 3B). Serum alkaline phosphatase at 6 and 12 h after feeding was significantly higher than that at 0 h in the control group, but significantly lower at 24 and 48 h after the meal than those at 0 h in the control group (P<0.05) (Figure 3C). Serum alkaline phosphatase at 6, 12, 24 and 48 h after feeding was significantly lower than that at 0 h in the 53% CHO group (Figure 3C). Serum alkaline phosphatase was significantly lower in the 53% CHO group than that in the control group at 6, 12 and 24 h (P<0.05) (Figure 3C).

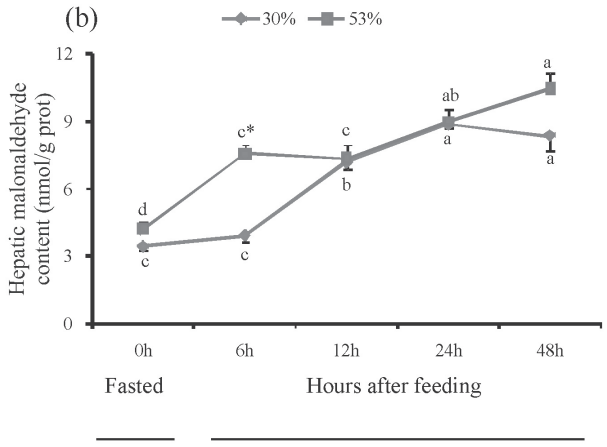
Insulin is very effective in stimulating glucose and amino acid uptake in fish (Castillo et al., 2004). In this study, the serum insulin was significantly higher in the 53% CHO group than that in the control group at 0 h, 24 h and 48 h. However, the results obtained here were quite preliminary. Further studies concerning effects of dietary CHO in this species are needed. Stress results in adrenergic and hypothalamus–pituitary–interrenal (HPI) axis responses, and the HPI response culminates in an increase in the plasma cortisol level. Increase in the blood cortisol level is an indication that a fish is under stress (Hsieh et al., 2003). The plasma cortisol levels increased significantly as the dietary CHO:L ratios decreased from 5.64 to 1.62 in *M. amblycephala* (Li et al., 2012b). The dietary intake of high CHO was shown to improve the serum cortisol level (Waagbø et al., 1994), whereas a high-CHO diet did not significantly affect the serum cortisol level in rainbow trout (*Oncorhynchus mykiss*) (Hilton et al., 1987) or cod (*Gadus morhua*) (Hemre et al., 1991). In the present study, the serum cortisol levels at 6, 12, 24 and 48 h after feeding were significantly lower than those at 0 h in both groups. Serum cortisol levels were significantly higher in the 53% CHO group than those in the control group at 0 h, 12 h, 24 h and 48 h. These results indicate that a diet containing higher CHO would lead to a stress response in *M. amblycephala*. Alkaline phosphatase (AKP) is an important enzyme that regulates a number of essential functions in all living organisms. In *Megalobrama amblycephala*, supplementation with 0.1% anthraquinone extract significantly increased serum AKP activity at 24 h after infection (Liu et al., 2012a). The AKP activity increased dramatically in *Labeo rohita* that were fed *Achyranthes aspera* (Rao et al., 2006). The AKP activity in the high-glucose group (40% CHO) was significantly lower than that in the low-glucose group (20% CHO)(Cai, 2004). The AKP activity decreased in allogynogenetic crucian carp (*Carassius auratus gibelio*) fed high-CHO diet (Miao et al., 2011). In our study, serum AKP activities at 6, 12, 24 and 48 h after feeding were significantly lower than those at 0 h in the 53% CHO group. Serum AKP activities were significantly lower in the 53% CHO group than those in the control group at 6, 12 and 24 h (P<0.05). In the present study, the results suggested that supplementation with higher dietary CHO decreased the levels of serum AKP.

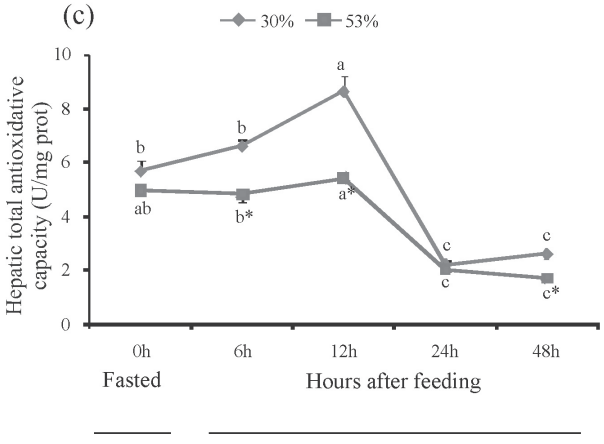
Hepatic superoxide dismutase activity at 6, 12 and 24 h after feeding was significantly higher than at 0 h in both groups, but significantly lower at 48 h after the meal than at 0 h in both groups (P<0.05) (Figure 4A). Hepatic SOD activity was significantly lower in the 53% CHO group than in the control group at 0 h, 12 h, 24 h and 48 h (P<0.05) (Figure 4A). Hepatic MDA contents at 12, 24 and 48 h after feeding were significantly higher than those at 0 h in the control group, and significantly higher at 6, 12, 24 and 48 h after the meal than those at 0 h in the 53% CHO group (P<0.05) (Figure 4B). Hepatic MDA content was significantly higher in the 53% CHO group than in the control group at 6 h (P<0.05) (Figure 4B). Hepatic T-AOC at 12 h after feeding was significantly higher than at 0 h in the control group, but significantly lower at 24 and 48 h after feeding than those at 0 h in both groups (P<0.05) (Figure 4C). Hepatic T-AOC was significantly lower in the 53% CHO group than in the control group at 6 h, 12 h and 48 h (P<0.05) (Figure 4C).

The nonspecific defense mechanisms of fish include neutrophil activation, production of peroxidase and oxidative free radicals, and initiation of other inflammatory factors (Ainsworth et al., 1991). The stress response might also impact factors such as total antioxidation capacity and levels of SOD, MDA and various peroxidases (Itou et al., 1996; Liu et al., 2010). In this study, the hepatic SOD activities were significantly lower in the 53% CHO group than those in the control group at 0 h, 12 h, 24 h and 48 h. Hepatic MDA content was significantly higher in the 53% CHO group than in the control group at 6 h. Hepatic T-AOC was significantly lower in the 53% CHO group than in the control group at 6 h, 12 h and 48 h. These results indicated that the antioxidant ability of Wuchang bream fed high-CHO diets decreased. Similar results were obtained in previous studies on Wuchang bream (*Megalobrama amblycephala*) (Zhou et al., 2013), top-mouth culter (*Erythroculter ilishaeformis* Bleeker) (Liu et al., 2012b) and black carp (*Mylopharyngodon piceus*) (Cai et al., 2009).

The relative levels of hepatic HSP70 mRNA at 6 h and 12 h after the meal were significantly higher than those at 0 h in both groups (P<0.05) (Figure 5). However, levels in both groups at 24 and 48 h were significantly lower than those at 0 h (P<0.05) (Figure 5). Furthermore, the relative level of hepatic HSP70 mRNA was significantly higher in the 53% CHO group than in the control group at 6, 12 and 48 h after feeding (P<0.05) (Figure 5).







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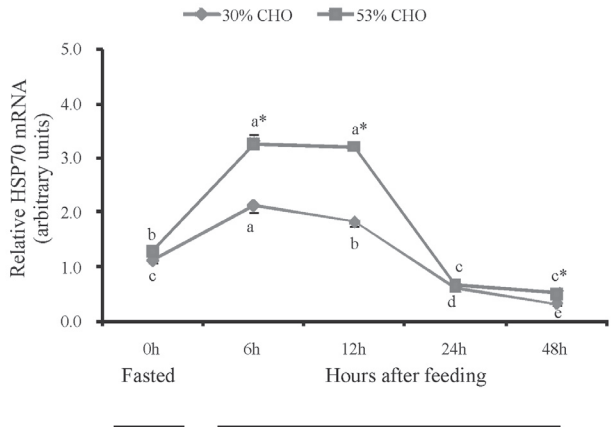
Significant differences (P<0.05) between values obtained in control group and high CHO group are marked by asterisk by t-tests.
Different letters indicate significant differences (P<0.05) in different sampling points by Duncan's multiple range tests.

Figure 4 - Fasted and postprandial patterns of heaptic superoxide dismutase (a) malondialdehyde (b), and total antioxidative capacity (c) in *M. amblycephala* fed the different-carbohydrate-level diets for 10 weeks.



Data are expressed as the mean of triplication of each group ± standard error (n = 9).
Significant differences (P<0.05) between values obtained in control group and high CHO group are marked by asterisk by t-tests.
Different letters indicate significant differences (P<0.05) in different sampling points by Duncan's multiple range tests.

Figure 5 - Fasted and postprandial patterns of the relative level of hepatic HSP70 mRNA in *M. amblycephala* fed the different-carbohydrate-level diets for 10 weeks.

Heat shock proteins (HSP) are one of the most conserved and important protein families, present in all organisms including fish (Basu et al., 2002). HSP70 is mainly involved in stress protection, improving cell survival and raising tolerance to environmental stressors or harm (Basu et al., 2002). Thus, HSP70 has been most widely used as a bioindicator of stress. HSP70 is induced by heat and chemical shocks in fish, like in mammals (Gornati et al., 2004). Gornati et al. (2004) observed that HSP70 was also shown to be inducible by increasing stocking density in sea bass. Enes et al. (2006) reported that HSP70 gene expression was affected by water temperature and dietary carbohydrate. In this study, the relative level of hepatic HSP70 mRNA was significantly higher in the 53% CHO group than in the control group at 6, 12 and 48 h after feeding. The results of this study indicated that high dietary CHO may lead to metabolic stress in *M. amblycephala*.

Conclusions

High dietary carbohydrate intake impacts the non-specific immunity of *M. amblycephala* to some extent and also causes metabolic stress. The underlying mechanisms of carbohydrate metabolism are far from being understood in *M. amblycephala*, and the effect of dietary carbohydrates on the health status in *M. amblycephala* requires further study.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (31472289); Open Fund of Key Laboratory of Freshwater Fisheries and Germplasm Resources Utilization of Ministry of Agriculture (KF201311); the National Non-profit Institute Research Grant of Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences (2013JBFR03); the National Technology System for Conventional Freshwater Fish Industries, the Modern Agriculture Industrial Technology System (CARS-46); the Special Fund for Agro-Scientific Research in the Public Interest (201003020); Special Scientific Research Funds for Central Non-profit Institutes, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences (2014YD01).

References

Ainsworth, A. J.; Dexiang, C. and Waterstrat, P. R. 1991. Changes in peripheral blood leukocyte percentages and function of neutrophils in stressed channel catfish. Journal of Aquatic Animal Health 3:41-47.

Amoah, A.; Coyle, S. D.; Webster, C. D.; Durborrow, R. B. M.; Bright, L. A. and Tidwell, J. H. 2008. Effects of graded levels of carbohydrate on growth and survival of largemouth bass, *Micropterus salmoides*. Journal of the World Aquaculture Society 39:397-405.

Basu, N.; Todgham, A. E.; Ackerman, P. A.; Bibeau, M. R.; Nakano, K.; Schulte, P. M. and Iwama, G. K. 2002. Heat shock protein genes and their functional significance in fish. Gene 295:173-183.

Cai, C. F. 2004. Study on the utilization of dietary carbohydrate by *Mylopharyngodon piceus* Richardson and *Carassius auratus* and their mechanism of metabolism. Doctoral dissertation. East China Normal University, Shanghai.

Cai, C. F.; Chen, L. Q.; Ye, Y. T.; Song, X. H.; Yang, C. G. and Chen, G. F. 2009. Effects of kind and level of dietary carbohydrate on growth performance and physiological indices of juvenile black carp (*Mylopharyngodln piceus Richardson*). Chinese Journal of Animal Nutrition 21:212-218 (in chinese).

Castillo, J.; Codina, M.; Martínez, M. L.; Navarro, I. and Gutiérrez, J. 2004. Metabolic and mitogenic effects of IGF-I and insulin on muscle cells of rainbow trout. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 286:935-941.

Cho, Y. J.; Kim, Y. Y.; Lee, N. G. and Choi, Y. J. 1994. Basic studies on developing equipment for waterless transportation of live fish. Bulletin of the Korean Fishery Society 27:501-508.

Clark, P. and Hales, C. 1994. How to measure plasma insulin. Diabetes/Metabolism Reviews 10:79-90.

Dabrowski, K. 1990. Assay of ascorbic phosphates and in-vitro availability assay of ascorbic mono- and polyphosphates. Journal of the Science of Food and Agriculture 52:409-420.

Drape, H. H.; Squires, E. J.; Mahmoodi, H.; Wu, J.; Agarwal, S. and Hadley, M. 1993. A comparative evaluation of thiobarbituric acid methods for the determination of malondialdehyde in biological materials. Free Radical Biology Medicine 15:353-363.

De Smet, H. and Blust, R. 2001. Stress responses and changes in protein metabolism in carp *Cyprinus carpio* during cadmium exposure. Ecotoxicology and Environmental Safety 3:255-262.

Enes, P.; Panserat, S.; Kaushik, S. and Oliva-Teles, A. 2006. Rapid metabolic adaptation in European sea bass (*Dicentrarchus labrax*) juveniles fed different carbohydrate sources after heat shock stress. Comparative Biochemistry and Physiology A-Molecular & Integrative Physiology 145:73-81.

Enes, P.; Panserat, S.; Kaushik, S. and Oliva-Teles, A. 2008. Growth performance and metabolic utilization of diets with native and waxy maize starch by gilthead sea bream (*Sparus aurata*) juveniles. Aquaculture 274:101-108.

Erfanullah, J. A. K. 1995. Protein-sparing effect of dietary carbohydrate in diets for fingerling *Labeo rohita*. Aquaculture 136:331-339.

Fletcher, T. C. 1981. Dietary effects on stress and health. p.147-169. In: Fish stress and health in aquaculture. Iwama, G. K.; Pickering, A. D.; Sumpter, J. P. and Schreck, C.B., eds. Academic Press, London/ New York.

Fu, S. J. and Xie, X. J. 2005. Effect of dietary carbohydrate levels on growth performance in *Silurus meridionalis* Chen. Acta Hydrobiologica Sinica 29:393-398.

Gao, W.; Liu, Y. J.; Tian, L. X.; Mai, K. S.; Liang, G. Y.; Yang, H. J.; Huai, M. Y. and Luo, W. J. 2010. Effect of dietary carbohydrate-to-lipid ratios on growth performance, body composition, nutrient utilization and hepatic enzymes activities of herbivorous grass carp (*Ctenopharyngodon idella*). Aquaculture Nutrition 16:327-333.

Gornati, R.; Papis, E.; Rimoldi, S.; Terova, G.; Saroglia, M. and Bernardini G. 2004. Rearing density influences the expression of stress-related genes in sea bass (*Dicentrarchus labrax* L.). Gene 341:111-118.

Hemre, G. I.; Lambertsen, G. and Lie, Ø. 1991. The effects of dietary carbohydrate on the stress response in cod (*Gadus morhua*). Aquaculture 95:319-328.

Hemre, G. I.; Mommsen, T. P. and Krogdahl, Å. 2002. Carbohydrates in fish nutrition: effects on growth, glucose metabolism and hepatic enzymes. Aquaculture Nutrition 8:175-194.

Hemre, G. I.; Sandnes, K.; Lie, Ø.; Torrissen, O. and Waagbø, R. 1995. Blood chemistry and organ nutrient composition in Atlantic salmon (*Salmo salar*) fed graded amounts of wheat starch. Aquaculture Nutrition 1:37-42.

Hilton, J. W.; Plisetskaya, E. M. and Leatherland, J. F. 1987. Does oral 3,5,30 -triiodo-L-thyronine affect dietary glucose utilization and plasma insulin levels in rainbow trout (*Oncorhynchus mykiss*)? Fish Physiology and Biochemistry 4:113-120.

Hsieh, S. L.; Chen, Y. N. and Kuo, C. M. 2003. Physiological responses, desaturase activity and fatty acid composition in milkfish (*Chanos chanos*) under cold acclimation. Aquaculture 220:903-918.

Itou, T.; Lida, T. and Kawatsu, H. 1996. Kinetics of oxygen metabolism during respiratory burst in Japanese eel neutrophils. Developmental Comparative Immunology 20:323-330.

Jauncey, K. 1982. Carp (*Cyprinus carpio* L.) nutrition - a review. p.215-263. In: Recent advances in aquaculture. Muir, J. F. and Roberts, R. J., eds. Croom Helm Ltd., London.

Ke, H. 1975. An excellent freshwater food fish, *Megalobrama amblycephala*, and its propagating and culturing. Acta Hydrobiologica Sinica 5:293-312.

Ke, H. 1986. Cultivation of blunt snout bream (*Megalobrama amblycephala*) in China. Fisheries Science and Technology Information 5:1-5.

Krajnović-Ozretić, M. and Ozretić, B. 1978. Estimation of the enzymes LDH, GOT and GPT in plasma of grey mullet Mugil auratus and their significance in liver intoxication. Diseases of Aquatic Organisms 3:187-193.

Krogdahl, Å.; Hemre, G. I. and Mommsen, T. P. 2005. Carbohydrates in fish nutrition: digestion and absorption in postlarval stages. Aquaculture Nutrition 11:103-122.

Kumar, S.; Sahu, N. P.; Pal, A. K.; Choudhury, D.; Yengkokpam, S. and Mukherjee, S. C. 2005. Effect of dietary carbohydrate on haematology, respiratory burst activity and histological changes in *L. rohita* juveniles. Fish and Shellfish Immunology 19:331-344.

Lall, S. P. 1991. Salmonid nutrition and feed production. p.107-123. In: Proceedings of the Special Session on Salmonid Aquaculture. Cook, R. H. and Pennel, W., eds. World Aquaculture Society, Los Angeles, CA.

Li, X. F.; Liu, W. B.; Jiang, Y. Y.; Zhu, H. and Ge, X. P. 2010. Effects of dietary protein and lipid levels in practical diets on growth performance and body composition of blunt snout bream (*Megalobrama amblycephala*) fingerlings. Aquaculture 303:65-70.

Li, X. F.; Jiang, Y. Y.; Liu, W. B. and Ge, X. P. 2012a. Protein-sparing effect of dietary lipid in practical diets for blunt snout bream (*Megalobrama amblycephala*) fingerlings: effects on digestive and metabolic responses. Fish Physiology and Biochemistry 38:529-541.

Li, X. F.; Liu, W. B.; Lu, K. L.; Xu, W. N. and Wang, Y. 2012b. Dietary carbohydrate/lipid ratios affect stress, oxidative status and non-specific immune responses of fingerling blunt snout bream, *Megalobrama amblycephala*. Fish and Shellfish Immunology 33:316-323.

Liu, B.; Xie, J.; Su, Y. T.; Yu, J. H.; Tang, Y. K. and Ge, X. P. 2008. Effect of high carbohydrate levels of dietary on growth, GK activities and GK mRNA levels in top mouth culter (*Erythroculter ilishaeformis* Bleeker). Acta Hydrobiologica Sinica 32:47-53.

Liu, B.; Xie, J.; Ge, X. P.; Xu, P.; Wang, A. M.; He, Y. J.; Zhou, Q. L.; Pan, L. K. and Chen, R. L. 2010. Effects of anthraquinone extract from *Rheum officinale* Bail on the growth performance and physiological responses of *Macrobrachium rosenbergii* under high temperature stress. Fish and Shellfish Immunology 29:49-57.

Liu, B.; Ge, X. P.; Xie, J.; Xu, P.; He, Y. J.; Cui, Y. T.; Ming, J. H.; Zhou, Q. L. and Pan, L. K. 2012a. Effects of anthraquinone extract from *Rheum officinale* Bail on the physiological responses and HSP70 gene expression of *Megalobrama amblycephala* under Aeromonas hydrophila infection. Fish and Shellfish Immunology 32:1-7.

Liu, B.; Xie, J.; Ge, X. P.; Miao, L. H. and Wang, G. Y. 2012b. Effect of high dietary carbohydrate on growth, serum physiological response, and hepatic heat shock cognate protein 70 expression of the top-mouth culter *Erythroculter ilishaeformis* Bleeker. Fisheries Science 78:613-623.

Liu, M. Z.; Shi, W. L.; Zhu, C. W.; Lu, M. Y.; Wang, G. L. and Wang, F. Q. 1992. The effect of dietary lipid level on the growth of blunt snout bream (*Megalobrama amblycephala*) fingerling. Journal of Fisheries of China 4:330-336.

Livak, K. J. and Schmittgen, T. D. 2001. Analysis of relative gene expression data using Real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. Methods 25:402-408.

Marklund, S. and Marklund, G. 1974. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. European Journal of Biochemistry 47:469-474.

Miao, L. H.; Liu, B.; Ge, X. P.; Xie, J.; Zhou, C. P.; Pan, L. K.; Chen, R. L. and Zhou, Q. L. 2011. Effcet of high carbohydrate levels in the dietary on growth performance, immunity and transmission electron microscopy (TEM) on hepatic cell of allogynogenetic crucian carp (*Carassius auratus gibelio*). Journal of Fisheries of China 35:221-230.

Ming, J. H.; Xie, J.; Xu P.; Liu, W. B.; Ge, X. P.; Liu, B.; He, Y. J.; Cheng, Y. F.; Zhou, Q. L. and Pan, L. K. 2010. Molecular cloning and expression of two HSP70 genes in the Wuchang bream (*Megalobrama amblycephala* Yih). Fish & Shellfish Immunology 28:407-418.

Pieper, A. and Pfeffer, E. 1980. Studies on the effect of increasing proportions of sucrose or gelatinized maize starch in diets for rainbow trout (*Salmo gairdneri*, R.) on the utilization of dietary energy and protein. Aquaculture 20:333-342.

Rao, Y. V.; Das, B. K.; Jyotyrmayee, P. and Chakrabarti, R. 2006. Effect of *Achyranthes aspera* on the immunity and survival of *Labeo rohita* infected with *Aeromonas hydrophila*. Fish and Shellfish Immunology 3:263-273.

Reinhold, J. G. 1953. Manual determination of serum total protein, albumin and globulin fractions by Biuret method. In: Standard method of clinical chemistry. Reiner, M., ed. Academic Press, New York. p.88.

Rice-Evans, C. and Miller, N. J. 1994. Total antioxidant status in plasma and body fluids. Methods in Enzymology 234:279-293.

Roberts, R. J. 1989. Nutritional pathology of teleosts. p.337-362. In: Fish pathology. Roberts, R.J., ed. Bailliere Tindall, London.

Roberts, R. J.; Bullock, A. M. 1989. Nutritional pathology. p.423-473. In: Fish nutrition. Halver, J. E., ed. Academic Press Inc., London.

Shi, W. L.; Shan, J.; Liu, M. Z.; Yan, H.; Huang, F. Q.; Zhou, X. W. and Shen, L. 1988. A study of the optimum demand of protein by blunt snout bream (*Megalobrama amblycephala*). FAO library. Report No: FAO-FI-RAS/86/047; FAO-FI-NACA/WP/88/68. Accession No: 289611.

Thorpe, A. and Ince, B. W. 1974. The effects of pancreatic hormones, catecholamines, and glucose loading on blood metabolites in the northern pike (*Esox Lucius* L.). General and Comparative Endocrinology 23:29-44.

Vielma, J.; Koskela, J.; Ruohonen, K.; Jokinen, I. and Kettunen, J. 2003. Optimal diet composition for European whitefish (*Coregonus lavaretus*): carbohydrate stress and immune parameter responses. Aquaculture 225:3-16.

Waagbø, R.; Glette, J.; Sandnes, K. and Hemre, G. I. 1994. Influence of dietary carbohydrate on blood chemistry, immunity and disease resistance in Atlantic salmon, *Salmo salar* L. Journal of Fish Diseases 17:245-258.

Wilson, R. P. 1994. Utilization of dietary carbohydrate by fish. Aquaculture 124:67-80.

Yang, G. H.; Dai, Q. X.; Gu, L. D. 1989. Nutrition, feed formulation and high-yield aquaculture techniques of blunt snout bream (*Megalobrama amblycephala*). Feed Industry 1:7-10.

Yu, S. K.; Olsen, C. E. and Marcussen, J. 1997. Methods for the assay of 1, 5-anhydro-D-fructose and α-1,4-glucanlyase. Carbohydrate Research 305:73-82.

Zhou, C. P.; Liu, B.; Ge, X. P.; Xie, J. and Xu, P. 2013. Effect of dietary carbohydrate on the growth performance, immune response, hepatic antioxidant abilities and heat shock protein 70 expression of Wuchang bream, *Megalobrama amblycephala*. Journal of Applied Ichthyology 29:1348-1356.

Zhou, W. Y.; Yu, C. Y.; Liu, J. Z. and Yang, G. H. 1997. Effects of quality and quantity of fat in diet on growth of blunt snout bream (*Megalobrama amblycephala*). Fisheries Science and Technology Information 1:3-9.

Zhou, Z.; Ren, Z.; Zeng, H. and Yao, B. 2008. Apparent digestibility of various feedstuffs for blunt nose black bream *Megalobrama amblycephala* Yih. Aquaculture Nutrition 14:153-165.

Zhu, S. Q. 1995. Synopsis of freshwater fishes of China. Jiangsu Science and Technology Publishing House, Nanjing, Jiangsu, China.

Zou, Z.; Yuan, F. and Chen, S. 1987. The optimal protein requirement of blunt nose black bream *Megalobrama amblycephala* Yih. Freshwater Fisheries 3:21-24.