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Effect of High Dietary Carbohydrate on Growth, Serum Physiological Response, and Hepatic Heat Shock Protein 70 Expression of Wuchang Bream (*Megalobrama amblycephala*) at Two Temperatures

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Abstract

The effects of high carbohydrate diet on growth, serum physiological response, and hepatic heat shock protein 70 expression in Wuchang bream were determined at 25 °C and 30 °C. The fish were reared at 25 °C had significantly higher serum C3, respiratory burst activity and hepatic superoxide dismutase activity, lower hepatic malonaldehyde than those were reared at 30 °C (P<0.05). At each temperature, the fish fed the control diet (31% CHO) had significantly higher weight gain, specific growth rate, respiratory burst activity, lysozyme, alkaline phosphatase, hepatic total antioxidative capacity, lower feed conversion ratio, glutamic-oxaloacetic transaminase, cortisol, hepatic malonaldehyde and the relative level of hepatic HSP70 mRNA than those fed the high-carbohydrate diet (47% CHO) (P<0.05). Significant interactions between temperature and diet were found for glutamic-oxaloacetic transaminase, C3, lysozyme, alkaline phosphatase and respiratory burst activity and hepatic superoxide dismutase activity (P<0.05). The present study suggested that higher dietary carbohydrate impact growth performance, the nonspecific immune ability and hepatic antioxidant abilities of Wuchang bream at higher temperature.

Keywords: *Megalobrama amblycephala*; Dietary carbohydrate; Temperature; Growth; Immunity; HSP70.

Introduction

The carbohydrate utilization of fish varies between fish species and carbohydrate sources (Wilson, 1994; Krogdahl *et al*., 2005). Fish are known to have a limited ability for digestion and metabolism of carbohydrate and hence, excessive intake of this nutrient may result in nutritional problems (Jauncey, 1982; Robert and Bullock, 1989; Roberts, 1989; Lall, 1991). Excess carbohydrates reduce the growth rate and are often accompanied by poor feed utilization (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 and Jafri, 1995; Fu and Xie, 2005). And excess dietary CHO excess dietary CHO caused change of heat shock protein 70 (HSP70) content in gilthead sea bream *Sparus aurata* (Enes *et al*., 2006).

Temperature is one of the key factors influencing the physiological characteristics of ectotherms (Jobling, 1994; Gillooly *et al*., 2001; Hochachka and Somero, 2002; Clarke, 2004), and it also modulates utilization of nutrients by fish (Keembiyehetty and Wilson, 1998). Ambient temperature is a critical factor in the development of both specific and non-specific host immunity (Watts *et al*., 2001) and any changes in temperature can affect the immune response (Lillehaug *et al*., 1993). In general, regardless of the fish species examined, elevated water temperature that still remains within the physiological range of the species has been shown to alter immune function (Bly and Clem, 1992). No effect of temperature on carbohydrate utilization was, however, found in rainbow trout (Capilla *et al*., 2003).

Wuchang bream (*Megalobrama amblycephala*) is a Chinese freshwater herbivorous species (Ke, 1975) with high potential for aquaculture. Its optimum temperature is about 25 °C (Ke, 1986). Due to the merits of this species including tender flesh, fast growth, economic profitability and cultural values (Zhou *et al*., 2008; Li *et al*., 2010), Wuchang bream is widely cultured in China with the output of 625,789 ton in 2009 — an increase of 31.50% in the past decade. This species has been introduced to North America (northern Canada to southern Mexico), Africa, Europe and other Asian countries (Ke, 1986).

A recent study showed that capacity for carbohydrate utilization was reported to be between 25% and 30% (Yang *et al*., 1989). However, the

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effect of high dietary CHO level on the health and immunological responses of Wuchang bream are not known. Thus, the objectives of this study are to examine whether the effects of high dietary CHO on the growth, serum metabolites, immune parameters, antioxidation enzymes, and expression of hepatic heat shock protein 70 (HSP70) in Wuchang bream at different water temperatures and to try to identify the molecular mechanisms for these effects.

Materials and Methods

Fish and Diets

Juvenile Wuchang bream, *M. amblycephala* (15.73±0.03g), were obtained by fish farm of Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences in China. Fish were acclimated at either 25±0.5 °C or 30±0.5 °C in cylindrical fibreglass tanks in a re-circulating system for 15days before the feeding experiment. During the period, fish were fed to apparent satiation with a control diet every day. The water oxygen content was approximately 6.0 mg/L during the experimental period. A 12L: 12D photoperiod was used.

Using fishmeal as protein source, fish oil as lipid source and cassava starch as carbohydrate source, two isonitrogenous (34% crude protein) and isolipidic (8.1% crude lipid) semi-purified diets were formulated to contain 31% and 47% carbohydrate, and these were referred to as a control and a high carbohydrate level respectively (Table 1). Dry ingredients were mixed thoroughly in a mixer, then water was added and mixed. Two-mm-diameter pellets were wet-extruded. The pellets were dried in a forced air oven at 40 °C to a moisture content of 10% and then stored at –20 °C until use.

Rearing Management

At the start of the experiment, the fish were fasted for 24 h and then weighed. Nine fish from each temperature were sampled. The fish from each temperature were then divided into two groups, each group comprising three tanks of 20 fish, and fish fed to satiation with the control and high-carbohydrate diets three times (06:30, 11:30 and 16:30 hours respectively) daily for 8 weeks. Water was exchanged once a week, and one-third of the volume in the tank was exchanged each time. Water was oxygenated at all times using an aerator. Feces and debris were removed daily using a siphon. Water temperature was measured every day, and water quality was measured every week. During this period, feed consumption was recorded daily. The number and weight of the dead fish was also recorded. During the test period, the water quality on average was as follows: dissolved oxygen (DO) > 6mgL^{-1}, NH_3 < 0.05 mg L^{-1}, H_2S < 0.1 mg L^{-1}, and pH 6.8–7.3. After the completion of the test period (70 days), serum and liver samples were collected. At the end of the feeding trial, the fish were fasted for 24 h, and then counted and weighed. The serum and liver samples were collected.

Table 1 Formulation and nutrient compositions of experimental diets

Ingredient	Dietary carbohydrate levels (%)	
	Control diet	High-carbohydrate diet
Fish meal[a]	47	47
Cassava starch[b]	30	45
Microcrystalline cellulose[c]	15	0
Carboxyl-methyl cellulose[d]	2	2
Fish oil[e]	4	4
Vitamin mixture[f]	0.5	0.5
Mineral mixture[g]	1	1
Zeolite power[h]	0.5	0.5
Proximate composition (% dry matter)		
Dry matter (%)	94.79	94.79
Crude protein (% DM)	33.66	33.71
Gross energy (kJ/g DM)[i]	16.56	19.25
Fat (% DM)	8.12	8.15
Digestible carbohydrate (% DM)	31.42	47.01

[a] Fish meal (CP 67.70 %, EE 7.90 %, carbohydrate 0.45 %, ash 16.70 %, Norway fish meal, Shanghai Imports and Exports Co. Ltd., China)
[b] Cassava starch (Wuxi Yongfeng starch Engineering Co., Ltd., China)
[c] Microcrystalline cellulose (Zhejiang Joinway Pharmaceutical Co., Ltd.)
[d] Carboxyl-methyl cellulose (Shanghai Jiande Industrial Co., Ltd.)
[e] Fish oil (Wuxi Xunda Ocean Biological Co. Ltd., China)
[f] Vitamin (IU or per kg premix): vitamin A 900000 IU, vitamin D 250000 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 116 mg, vitamin C 5000 mg, pantothenate 1000 mg, folic acid 165 mg, folic acid 165 mg, choline 60000 mg
[g] Mineral (per kg premix): $CuSO_4 \cdot 5H_2O$ 2.5 g, $FeSO_4 \cdot 7H_2O$ 28 g, $ZnSO_4 \cdot 7H_2O$ 22 g, $MnSO_4 \cdot 4H_2O$ 9 g, Na_2SeO_3 0.045 g, KI 0.026 g, $CoCl_2 \cdot 6H_2O$ 0.1 g
[h] Zeolite powder (Nanjing Huamu Animal Research Institute, China)
[i] Gross energy (GE) kJ g^{-1}: protein 23.64 kJ/g, fat 39.54 kJ/g, carbohydrate 17.15 kJ/g, and the others are measured in the nutrition levels

Serum and Liver Sample Collection

At the end of the growth trial, the fish were starved for 24 h and weighed. Nine individuals from each group (3 tanks, 3 fish per tank) were anesthetized with MS-222 and sampled. Blood was sampled from the caudal vein, centrifuged at 3000 g for 10min (4 °C), and kept frozen until analysis. The liver was excised, frozen in liquid nitrogen and then stored at −80 °C until assay.

Serum Total Protein, Globulin, and Glutamic-Oxaloacetic Transaminase (GOT) Measurement

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 (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).

Respiratory Burst Activity, Serum Complement C3 and C4

The respiratory burst activity of the leucocytes was carried out following the method described in the other study (Li *et al*., 2011). The leucocytes were then incubated at room temperature for 20 min with 2',7'-dichlorofluorescin diacetate (DCFH-DA) (Vuorte *et al*., 1996). After diffusing through the cell membrane, DCFH-DA is deacetylated by cytosolic enzymes to dichlorofluorescin (DCFH). DCFH is polar and thus trapped within the cells. Zymosan A (Sigma) was used directly to stimulate the cells producing active oxygen. The active oxygen oxidises the nonfluorescent DCFH to highly fluorescent 2',7'-dichlorofluorescein (DCF). After 40 minutes, the fluorescence value of the cells was detected by flow cytometry (Goedken and Guise, 2004). The respiratory burst activity of the cells was measured by reduction of mean fluorescence value.

The serum complement C3 level and the activity of the serum complement C4 level were determined following the method described in another study (Sun *et al*., 2010). Results of C3 and C4 are presented as C3 mg/ml and C4 mg/ml, respectively (Thomas, 1998; He *et al*., 2009). Test kits for these assays were purchased from Zhejiang Elikan Biological Technology Co., LTD in China.

Serum Cortisol, Lysozyme, and Alkaline Phosphatase (AKP) Measurement

Serum cortisol was measured by RIA using a test kit (Beijing Beifang Biotech Research Institute, China) and following the method described in the other studies (Pickering and Pottinger, 1983; Xie *et al*., 2008). Serum lysozyme activity was measured using a kit (Nanjing Jiancheng Biological Engineering Research Institute of China) in a Beckman Cx-4 type Auto Biochemical Analyzer (Beckman Coulter, USA), following the method described in the other study (Muona and Soivio, 1992). Serum AKP activity was determined by the colorimetric method of Pinoni *et al*. (2004) (test kit from Shanghai Fudan Zhangjiang Bio Medical Co., Ltd., China) in a Beckman Cx-4 type Auto Biochemical Analyzer.

Hepatic SOD, MDA and T-AOC Measurement

Hepatic samples were homogenized in ice-cold phosphate buffer (1:10 dilution) (phosphate buffer: 0.064 M, pH 6.4). The homogenate was then centrifuged for 20 min (4 °C, 3000 × g) and aliquots of the supernatant were used to quantify hepatic SOD, MDA and T-AOC. Hepatic SOD activity and MDA content were measured using a xanthine oxides (Marklund and Marklund, 1974) and barbituric acid reaction chronometry (Drape *et al*., 1993), respectively. T-AOC was measured by the method described in the other study (Benzie and Strain, 1996) using commercial kits (Jiancheng Institute of Biotechnology, Nanjing, China). We measured the hepatopancreas protein content using the Folin method (Lowry *et al*., 1951), with bovine serum albumin as the standard.

Real-Time PCR Measurement of Hepatic HSP70

We used M. *amblycephala* cDNA sequences in GenBank 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'- CCCTGCAGCATTGAGTTCATAAGGT-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.

We extracted total RNA 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. We generated cDNA 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^{TM} RTase (200 U/μL), and DEPC H_2O, up to a final volume of 10 μL. The reaction conditions were as follows: 37 °C for 15 min, 85 °C for 5 sec, and 4 °C thereafter.

We used real-time PCR to determine mRNA levels with an SYBR Green I fluorescence 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 SYBR $^{\circ}$,R premix Ex Taq^{TM} (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), 9.5 μL dH_2O. The reaction conditions were as follows: 95 °C for 2 min, followed by 44 cycles consisting of 95 °C for 10 s, 59 °C for 20 s, and 72 °C for 20 s. The florescent flux was then recorded and the reaction continued at 72 °C for 3 min. We measured the dissolution rate between 65 and 92 °C. Each increase of 0.2 °C was maintained for 1 s and the fluorescent flux was recorded. We calculated the relative quantification of the target gene transcript (HSP70) 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. We measured the PCR efficiency 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.

Calculations and Statistics

Weight gain (WG) (%) = (Final body weight - initial body weight)×100//initial body weight.

Specific growth rate (SGR) (%) = (LnW_t - LnW_0) ×100/*T*, where W_0 and W_t are the initial and final body weights, and *T* is the culture period in days.

Feed conversion ratio (FCR) (%) = total diet fed (kg)/total wet weight gain (kg).

Results are presented as mean ± S.E. Data were analysed by two-way ANOVA using the SPSS 16.0 (SPSS, IL USA). $P < 0.05$ indicated that there was a significant difference.

Results

The Effect of High Dietary Carbohydrate Diet on Growth of Wuchang bream (*M. amblycephala*) at Different Temperatures

At each temperature, the final weight, WG and SGR of the fish fed the control diet were significantly higher than those fed the high-carbohydrate diet, and the FCR of the fish fed the control diet was lower than that fed the high-carbohydrate diet (P<0.05) (Table 2).

The Effect of High Dietary Carbohydrate Diet on Serum Total Protein, Globulin and Glutamic-Oxaloacetic Transaminase (GOT) of Wuchang Bream (*M. amblycephala*) at Different Temperatures

At each temperature, the GOT of the fish fed the control diet was lower than that fed the high-carbohydrate diet (P<0.05). The total protein, globulin and GOT were not affected by temperature (P>0.05). The total protein and globulin were not significantly different between diets at each temperature. Significant interaction between temperature and diet was found for GOT (P<0.05) (Table 3).

Table 2 Effect of high dietary carbohydrate diet on weight gain, specific growth rate and feed conversion efficiency of Wuchang bream (*M. amblycephala*) at different temperatures

Diets(Temperature/CHO) (%)	Initial weight (g)	Final weight (g)	WG(%)	SGR	FCR
25/31	15.80±0.06	53.40±0.04a	237.97±7.75	2.17±0.03	1.60±0.04
25/47	15.77±0.07	49.31±0.86bc	212.76±4.83	2.04±0.03	2.31±0.20
30/31	15.67±0.03	49.51±0.62b	216.07±4.61	2.05±0.03	2.23±0.14
30/47	15.67±0.09	47.04±0.92c	200.26±7.34	1.96±0.04	2.93±0.31
Temperatures (°C)					
25	15.79±0.07	51.36±0.45	225.36±6.75	2.11±0.04	1.96±0.18
30	15.67±0.06	48.28±0.77	208.16±5.25	2.01±0.03	2.58±0.22
CHO levels (%)					
31	15.74±0.05	51.46±0.33	227.02±6.11	2.11±0.03	1.92±0.15
47	15.72±0.08	48.18±0.89*	206.51±4.82*	2.00±0.03*	2.62±0.22*
Two- way ANOVA					
Temperature	NS	NS	NS	NS	NS
CHO	NS	*	*	*	*
Interaction	NS	NS	NS	NS	NS

Values are means ± SE of 3 replications. Means in the same column with different superscripts are significantly different (P<0.05).
ns not significant. * P<0.05, ** P<0.01, *** P<0.001

Table 3. Effect of high dietary carbohydrate diet on serum total protein, globulin and glutamic-oxaloacetic transaminase(GOT) of Wuchang bream(*M. amblycephala*) at different temperatures

Diets(Temperature /CHO) (%)	Total protein (g/L)	Globulin (g/L)	GOT (U/L)
25/31	33.92±0.43	12.11±0.17	229.34±7.22[c]
25/47	31.53±1.88	12.88±0.72	322.78±5.08[a]
30/31	31.63±2.66	13.17±0.86	266.33±2.08[b]
30/47	25.69±0.72	11.69±0.62	319.11±7.78[a]
Temperatures (°C)			
25	32.73±1.01	12.50±0.37	276.06±21.26
30	28.66±1.81	12.43±0.58	292.72±12.34
CHO levels (%)			
31	32.78±1.31	12.64±0.46	247.83±8.93
47	28.61±1.59	12.28±0.50	320.94±4.24[*]
Two- way ANOVA			
Temperature	NS	NS	NS
CHO	NS	NS	***
Interaction	NS	NS	**

Values are means ± SE of 3 replications.
ns not significant. * $P<0.05$, ** $P<0.01$, *** $P<0.001$

The Effect of High Dietary Carbohydrate Diet on Serum Complement 3, Complement 4 and Respiratory Burst Activity of Leukocyte of Wuchang Bream (*M. amblycephala*) at Different Temperatures

C3 and respiratory burst activity were significantly higher in fish at 25 °C compared with those reared at 30 °C ($P<0.05$). At each temperature, respiratory burst activity of the fish fed the control diet was higher than that fed the high-carbohydrate diet ($P<0.05$). The C4 and respiratory burst activity were not significantly different between diets at each temperature ($P>0.05$). The C4 was not affected by temperature ($P>0.05$). Significant interactions between temperature and diet were found for C3 and respiratory burst activity ($P<0.05$) (Table 4).

The Effect of High Dietary Carbohydrate Diet on Serum Cortisol, Lysozyme and Alkaline Phosphatase (AKP) of Wuchang Bream (*M. amblycephala*) at Different Temperatures

At each temperature, the cortisol was significantly lower and the lysozyme and AKP were significantly higher in fish fed the control diet compared with those fed the high-carbohydrate diet ($P<0.05$). The cortisol, lysozyme and AKP were not significantly different between two temperatures ($P>0.05$). Significant interactions between temperature and diet were found for lysozyme and AKP ($P<0.05$) (Table 5).

The Effect of High Dietary Carbohydrate Diet on Hepatic SOD, MDA, T-AOC and the Relative Level of Hepatic HSP70 mRNA of Wuchang Bream (*M. amblycephala*) at Different Temperatures

Hepatic SOD were significantly higher and the hepatic MDA was significantly lower in fish at 25 °C compared with those reared at 30 °C ($P<0.05$). At each temperature, the hepatic T-AOC of the fish fed the control diet were significantly higher than that fed the high-carbohydrate diet, and the hepatic MDA of the fish fed the control diet was lower than that fed the high-carbohydrate diet ($P<0.05$). The hepatic SOD was not significantly different between diets at each temperature ($P>0.05$). The hepatic T-AOC was not affected by temperature ($P>0.05$). Significant interaction between temperature and diet was found for hepatic SOD ($P<0.05$) At each temperature, The relative level of hepatic HSP70 mRNA of the fish fed the control diet were significantly lower than that fed the high-carbohydrate diet ($P<0.05$). The expression levels of hepatic HSP70 were not affected by temperature ($P>0.05$) (Table 6).

Discussion

The previous studies suggest that incorporation of appropriate levels of dietary carbohydrates in fish feeds can improve their growth performance, physical quality and to provide an inexpensive non-nitrogenous energy source (Alliot *et al*., 1979; Hemre *et al*., 1995; Hung *et al*., 1989; Hemre and Hansen, 1998; Peragón *et al*., 1999; Vielma *et al*., 2003). However, excess levels of carbohydrates reduce the growth rate and are often accompanied by poor feed utilization (Hemre *et al*., 2002; Enes *et al*., 2006; Vielma *et al*., 2003). The previous study shows that depressed growth and feed utilization in common carp fed diets high in carbohydrate content (Furuichi and Yone, 1980). In this study, we found that the weight gain, specific growth rate were affected by high dietary carbohydrate. Similar results were observed in other species (Gao *et al*., 2010; Tan *et al*., 2009; Miao *et al*., 2011). Temperature influencs the physiological characteristics of ectotherms (Jobling, 1994; Gillooly *et al*., 2001; Hochachka and Somero, 2002; Clarke, 2004), and it also modulates utilization of nutrients by

Table 4. Effect of high dietary carbohydrate diet on serum complement 3, complement 4 and respiratory burst activity of leukocyte of Wuchang bream(*M. amblycephala*) at different temperatures

Diets(Temperature /CHO) (%)	Complement 3 (g/L)	Complement 4 (g/L)	Respiratory burst activity
25/31	0.32±0.01[a]	0.04±0.01	13.95±0.18[c]
25/47	0.22±0.01[bc]	0.03±0.00	15.00±0.80[c]
30/31	0.23±0.01[b]	0.04±0.01	23.11±0.55[a]
30/47	0.20±0.01[c]	0.03±0.01	17.57±0.79[b]
Temperatures (°C)			
25	0.27±0.02	0.03±0.01	14.48±0.44
30	0.22±0.01	0.04±0.02	20.34±1.31[*]
CHO levels (%)			
31	0.27±0.02	0.04±0.01	18.53±2.06
47	0.21±0.01[*]	0.03±0.01	16.29±0.77
Two- way ANOVA			
Temperature	NS	NS	**
CHO	*	NS	NS
Interaction	**	NS	**

Values are means ± SE of 3 replications.
ns not significant. * $P<0.05$, ** $P<0.01$, *** $P<0.001$

Table 5. Effect of high dietary carbohydrate diet on serum cortisol, lysozyme and alkaline phosphatase (AKP) of Wuchang bream (*M. amblycephala*) at different temperatures

Diets(Temperature /CHO) (%)	Lysozyme (U/mL)	Cortisol (ng/mL)	AKP (μmol/L)
25/31	10.37±0.67[a]	318.71±3.07	66.44±0.95[b]
25/47	6.11±0.40[b]	380.61±6.30	63.11±0.97[b]
30/31	6.81±0.25[b]	326.53±2.93	72.89±1.28[a]
30/47	5.92±0.26[b]	389.31±1.17	64.00±1.35[b]
Temperatures (°C)			
25	8.24±1.01	349.66±14.19	64.77±0.96
30	6.36±0.26	357.92±14.11	68.44±2.15
CHO levels (%)			
31	8.59±0.86	322.62±2.58	69.67±1.61
47	6.02±0.22[*]	384.96±3.46[*]	63.56±0.77[*]
Two- way ANOVA			
Temperature	NS	NS	NS
CHO	*	***	**
Interaction	**	NS	*

Values are means ± SE of 3 replications.
ns not significant. * $P<0.05$, ** $P<0.01$, *** $P<0.001$

Table 6. Effect of high dietary carbohydrate diet on hepatic SOD, MDA,T-AOC and the relative level of hepatic HSP70 mRNA of Wuchang bream (*M. amblycephala*) at different temperatures

Diets(Temperature /CHO) (%)	SOD (U/mL)	MDA (nmol/mL)	T-AOC (U/mL)	HSP70
25/31	162.95±0.73[a]	13.07±0.93	7.33±0.51	1.08±0.02
25/47	146.08±0.71[b]	15.57±0.43	4.48±0.34	1.76±0.05
30/31	121.11±382[c]	15.27±0.41	7.04±0.61	1.28±0.02
30/47	116.43±1.10[c]	17.56±0.35	3.95±0.44	1.93±0.05
Temperatures (°C)				
25	154.52±3.80	14.32±0.72	5.91±0.69	1.42±0.15
30	118.77±2.06[*]	16.41±0.56[*]	5.49±0.77	1.61±0.15
CHO levels (%)				
31	142.03±9.52	14.17±0.67	7.19±0.36	1.18±0.05
47	131.26±6.66	16.56±0.51[*]	4.22±0.28[*]	1.85±0.05[*]
Two- way ANOVA				
Temperature	***	*	NS	NS
CHO	NS	*	***	***
Interaction	*	NS	NS	NS

Values are means ± SE of 3 replications.
ns not significant. * $P<0.05$, ** $P<0.01$, *** $P<0.001$

fish (Keembiyehetty and Wilson, 1998). However no effect of temperature on the growth performance was found in this study.

GOT is ubiquitous aminotransferase that is found in the mitochondria of fish. The enzyme is important index for the diagnosis of hepatopancreas function and damage (Cho *et al*., 1994). The excess carbohydrates may cause liver damage (Hemre *et al*., 2002; Shoemaker *et al*., 2001; Holland and Lambris, 2002). In this study, we also found that GOT activity was affected by high dietary carbohydrate.

Phagocytes are cellular components of natural immunity. Phagocyte activation in mammals is usually associated with an abrupt rise in oxygen consumption, leading to the production of reactive oxygen species (ROS) which have potent microbicidal activities and which play an important role in the host defence against microorganisms. This metabolic event is called the respiratory burst (Castro *et al*., 2004). High dietary carbohydrate decrease the respiratory burst activity of phagocytes (Kumar *et al*., 2005). In our study, a similar phenomenon was observed at each temperature. In addition, we found that temperature negatively modulated respiratory burst activity and C3 level. Thus, both dietary composition and temperature seemed to affect the immunity of Wuchang bream.

Fish in aquaculture are consistently affected by various stressors such as nutritional factors, high temperature, stocking density, transport and storage (Barton 2002, Liu *et al*., 2012). If fish are in long-standing stress during culture process, the axis of hypothalamus - pituitary-interrenal axis of fish will be continuously stimulated so as to culminate in an increase in plasma cortisol (Barton 2002; Fevolden et al., 2003). The change of plasma cortisol has been widely as an indication that a fish is under stress (Iwama *et al*., 1997; Hsieh *et al*., 2003). High dietary CHO improved the serum cortisol level in Atlantic salmon (Waagbø *et al*., 1994) and top-mouth culter (*Erythroculter ilishaeformis* Bleeker) (Liu *et al*., 2012). In our study, a similar phenomenon was observed at each temperature.

Lysozyme has bactericidal activity and can be an opsonin that activates the complement system and phagocytes to prevent infection and disease (Alexander and Ingram, 1992). High-CHO diet may reduce the lysozyme level of *E. ilishaeformis* (Liu *et al*., 2012). In the present study, the lysozyme was significantly higher in fish fed the control diet than that fed the high-CHO diet at each temperature. Thus, the high-CHO diet may reduce the immune ability of the fish to some degree.

AKP is an important enzyme that regulates a number of essential functions in all living organisms (Rao *et al*., 2006). Stress made blood cortisol increase apparently and in turn caused some immune index change e.g. serum AKP activity (Fevolden et al., 1999). The previous study shows that the AKP activity in the normal carbohydrate group (35%CHO) was higher than that in 50%CHO group, but no significant difference (Miao *et al*., 2011). In our study, AKP activity was significantly higher in fish fed the control diet compared with those fed the high-carbohydrate diet. Therefore the above results of the present study indicated that high dietary CHO could somewhat reduce the serum ALP activities of M. amblycephala to some degree.

The stress response might also increase free radical contents, leading to increased lipid peroxidation and lipid peroxidation injury (Zhou and Liang, 2003; Zhou *et al*., 2003; Chen *et al*., 2006; He *et al*., 2007). In Atlantic salmon, dietary intake of high CHO can impact on the immunity and fish mortality after *Aeromonas Salmonicida* infection (Waagbø *et al*., 1994). The hepatic lipid peroxide will decompose further to produce a large amount of aldehydes, alcohols and hydrocarbon of which MDA is a substance with strong toxicity. SOD plays a significant role in resisting oxide damage (Gu *et al*., 1995). The stress response might also impact factors such as total antioxidation capacity (Lewis *et al*., 1995). In this study, at each temperature, the hepatic MDA of the fish fed the control diet was lower than that fed the high-carbohydrate diet, and the hepatic T-AOC of the fish fed the control diet were significantly higher than that fed the high-carbohydrate diet suggesting that excessive CHO lowered the hepatic antioxidant abilities of Wuchang bream. Temperature plays an important role in cold-blooded animals and temperatures below or above the thermal limit can affect physiological functions, including adaptive and innate immunity, increase susceptibility to infection and even cause death (Watts et al., 2001; Ndong et al., 2007). In our study, we found that hepatic SOD were significantly lower and the hepatic MDA was significantly higher in fish at higher temperature.

Heat shock proteins (HSPs) are one of the most conserved and important protein families and have been studied extensively (Basu *et al*., 2002). HSP70 is known to assist the folding of nascent polypeptide chains, act as a molecular chaperone, and mediate the repair and degradation of altered or denatured proteins (Basu *et al*., 2002). Thus, HSP70 has been most widely used as a biomarker of stress. In fish, like in mammals, HSP70 is induced by heat and chemical shocks (Gornati *et al*., 2004). In sea bass, HSP70 was also shown to be inducible by rearing density (Gornati *et al*., 2004). HSP70 gene expression was similar in both cold water and warm water acclimated seabass fed different carbohydrate source, and weak downregulation was observed after heat shock only in fish fed the glucose diet (Enes *et al*., 2006). In this study, the relative level of hepatic HSP70 mRNA of the fish fed the control diet were significantly lower than that fed the high-carbohydrate diet. This finding indicated that high dietary CHO may lead to metabolic stress in Wuchang bream.

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