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Effect of Dietary Iron Levels on Tissues, Intestinal Digestive Enzyme Activity, and Muscle Nutrient Compositions of Juvenile Bighead Carp (*Aristichthys nobilis*)

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Keywords: *Aristichthys nobilis;* trypsin; lipase; amylase; muscle.

Abstract

A 60-day feeding trial was conducted to estimate the effects of dietary iron (Fe) levels on iron concentration in tissues, intestinal digestive enzyme activities, and muscle nutrient composition of bighead carp (*Aristichthys nobilis*). Six experimental diets were formulated to contain different Fe levels (0, 43.1, 84.2, 123.3, 162.2 and 203.1 mg/kg of dry diets) using ferrous sulfate ($FeSO_4$) as the source. When Fe dietary content increased to 43.1 mg/kg, trypsin activity in the intestine significantly increased and thereafter decreased. Lipase and amylase activity in the intestine significantly increased with increasing dietary Fe levels up to 123.3 mg/kg diet and thereafter decreased. With lipase and amylase activity in the intestine as the main indicators, Fe content of 123.3 mg/kg was the most suitable dietary Fe level for *A. nobilis.* Crude protein content in the muscle of the 84.2 mg/kg Fe group was the highest in all groups. Results indicated that the appropriate levels of dietary Fe alter muscle nutrient composition of *A. nobilis.* Fe content in the muscle and vertebrae significantly increased with increasing dietary Fe levels up to 203.1 mg/kg diet. Fe contents in different tissues were as follows: Vertebra >intestine >muscle.

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Introduction

Fe is an essential mineral in living organisms and is necessary for several biological processes, including oxygen transport, cellular respiration, and lipid oxidation (Lee et al., 1981). Previous studies have demonstrated that different organs and tissues of fish had different concentrations of microelements (Xiao, 2009).

Fe plays an active part in oxidation and reduction reactions and electron transport associated with cellular respiration. It is found in complex bonds to proteins such as haem, in enzymes such as microsomal cytochromes catalase, and in non-haem compounds such as transferrin, ferritin and flavin iron enzymes (Watanabe et al., 1997). Digestive enzymes, such as trypsin, lipase, and amylase, are synthesized in the exocrine pancreas and secreted into the intestinal lumen, and therefore play a vital role in the food utilization and growth of fish (Infante and Cahu, 2001). Digestive enzymes play a key role in digesting nutrients, and their activities directly reflect digestive capacity, and affect fish growth rate (Blier et al., 1997; Ling et al., 2010). A study on yellowtail showed that trypsin and amylase are synthesized and increase after feed ingestion (Murashita et al. 2007). Digestive enzymes are responsible for nutrient digestion and availability so that evaluation of such enzyme activities provides reliable information about precocious fish nutritional status and performance evaluation. Proteins and lipids are the major constituents in the muscle of fish, these should be evaluated for their nutritional value.

To our knowledge, no attempt has ever been made to study the effect of dietary Fe levels on the digestive enzyme activities of bighead carp (*Aristichthys nobilis*). Bighead carp is one of the most important commercial aquaculture fish in China (Hong et al., 2012). Therefore, the purpose of the present study was to investigate the effects of dietary Fe levels on Fe concentrations in the muscle, vertebra, intestine, and intestinal digestive enzyme activities as well as muscle nutrient compositions of *A. nobilis.* This study provides basic data to further explore the physiological functions of Fe at the cellular and molecular levels.

Materials and Methods

Bighead carp were obtained from a hatchery in Hanchuan, Hubei Province, China and acclimated to experimental conditions for 3 weeks prior to onset of the feeding trial. During the trial period, the fish were fed to satiation twice daily (09:00 and 16:00) with a standard diet (0 mg/kg of dietary Fe). As presented in Table 1, the experimental basic diets included 46.77% crude protein, and 7.81% crude lipid were formulated to contain different Fe levels (0, 43.1, 84.2, 123.3, 162.2, 203.1 mg/kg of dry diets) by using the same source ($FeSO_4$). The feed with a particle size of 0.63 mm was air-dried at a low temperature, and then stored at 4°C for further use.

Table 1. Formulations and chemical compositions of experimental diets (g/100 g of dry matter).

Ingredients	*Dietary Fe level(mg/kg)*					
	0	*43.1*	*84.2*	*123.3*	*162.2*	*203.1*
Casein	45.00	45.00	45.00	45.00	45.00	45.00
Soybean meal	24.60	24.60	24.60	24.60	24.60	24.60
Flour	20.00	20.00	20.00	20.00	20.00	20.00
$FeSO_4$	0.000	0.011	0.022	0.033	0.044	0.055
Soybean oil	6.00	6.00	6.00	6.00	6.00	6.00
Mineral premix[1]	1.00	1.00	1.00	1.00	1.00	1.00
Vitamin premix[2]	0.50	0.50	0.50	0.50	0.50	0.50
Choline chloride	0.30	0.30	0.30	0.30	0.30	0.30
$Ca(H_2PO_4)_2$	2.50	2.50	2.50	2.50	2.50	2.50
Cellulose	0.100	0.089	0.078	0.067	0.056	0.045
Chemical composition(g/100g in dry matter)						
Crude lipid (%)	7.85	7.66	7.77	7.81	7.86	7.89
Crude protein (%)	46.77	46.96	46.78	46.72	46.84	46.86
Crude ash (%)	6.65	6.55	6.59	6.51	6.54	6.57
Water(%)	10.29	10.27	10.31	10.32	10.30	10.31

[1]Per kilogram of mineral premix containing (g/kg mixture): $MgSO_4 \cdot 7\ H_2O$, 100 g; NaCl, 20 g; $AlCl_3 \cdot 6\ H_2O$, 0.6 g ; KI, 0.6 g ; KCl, 40 g; $CuSO_4 \cdot 5\ H_2O$, 2 g ; $MnSO_4 \cdot H_2O$, 4 g; $CoCl_2 \cdot 6\ H_2O$, 2 g; $ZnSO_4 \cdot 7\ H_2O$, 20 g; and cellulose powder, 810.8 g.

[2]Per kilogram of vitamin premix containing: vitamin A, 700000 IU; vitamin D_3, 350000 IU; vitamin K_3, 3.5 g; vitamin E, 16 g; vitamin B_1, 3.5 g; vitamin B_2, 7 g; vitamin B_6, 7 g; vitamin B_{12}, 0.007 g; vitamin C, 35 g; biotin, 0.03 g; folic acid, 1.6 g; niacin, 35 g; Ca-D-pantothenate, 16 g; inositol, 35 g; corn starch, 840.12 g.

The trial was conducted in a circulating system consisting of 18 cylindrical plastic tanks (diameter, 80 cm; height, 60 cm; water volume, 300 L). At the beginning of the trial, the fish were fasted for 24 h. Then, a total of 540 fish of similar size (initial body weight, 6.18 ± 0.11 g) were randomly selected, weighed, and stocked in 18 tanks, and were fed six different diets. There were six groups, each group with three replicates. Each tank contained 30 fish. During the experiment, aeration was provided to each tank to maintain a dissolved oxygen level of 7–8 mg/L. The water temperature was maintained at about 26°C and recorded daily. The pH was maintained at about 7–7.5, and the ammonia-N content was monitored once a week. Fish were hand-fed to apparent satiation twice daily. The amount of food supplied was recorded daily.

All fish in each tank were sampled after 1 day of food deprivation on day 60. Ten random fish from each tank were weighed and the sampled fish were dissected immediately. The vertebra, midgut, and dorsal white muscle (from posterior edge of operculum to end of dorsal-fin base above the lateral line) were collected and frozen in liquid nitrogen and stored at −80°C for subsequent analysis.

Tissue samples were dried in an oven at 105°C until they reached constant weight. Dried tissue samples were turned into ash by incineration for 7 h at 550°C in a muffle furnace. Then the ash was dissolved in 5 mL of hydrogen chloride solution and the resulting solution was combined with 20 mL of ultrapure water. The solutions obtained were stored at -20°C until analysis (Xu, 2017).

Fe content was determined by flame atomic absorption spectrophotometry (TAS-990; Beijing Purkinje General Instrument Co., Ltd., Beijing, China) (Xu, 2017).

Proximate composition of diets and fish carcass were analyzed using standard methods of the Association of Official Analytical Chemists. Intestine samples were each homogenized in 10 volumes (*w/v*) of ice-cold physiological saline solution and centrifuged at 6000×g for 20 min at 4°C. The supernatant was collected for enzyme activity analysis.

The protein contents of all tissues were determined by the Bradford method (Bradford 1976). Estimation of crude lipid was performed using Soxhlet extract system. The trypsin, lipase, and amylase activities were determined according to a previous study (Jiang et al. 2016).

Statistical analysis of the resulting data was performed using SPSS Statistics for Windows, version 17.0 (SPSS, Inc., Chicago, IL, USA). Variables among treatments were compared using one-way analysis. Significant differences ($p < 0.05$) were reanalyzed using Duncan's shortest significant ranges test.

Results

After feeding for 60 days with diets containing different Fe levels, the trypsin, lipase, and amylase activities in the intestine of *A. nobilis* were as follows: trypsin activity in the intestine significantly increased with increasing dietary Fe levels up to 43.1 mg/kg diet and thereafter decreased; trypsin activity in the intestine of the control group was significantly lower than that of the 43.1, 84.2 and 123.3mg/kg Fe groups ($p < 0.05$). The trypsin activity in the intestine of the 43.1 mg/kg Fe group was significantly higher ($p < 0.05$) than that of the 123.3, 162.2 and 203.1 mg/kg Fe groups. (see Table 2.)

Table 2. The activities of trypsin, lipase, and amylase, in intestine of *A. nobilis*

Dietary Fe level(mg/kg)	*Parameters*		
	Trypsin(U/mg)	*Lipase(U/g)*	*Amylase(U/g)*
0	2916.53±135.86^{a}	33.36±3.14^{a}	95.07±16.87^{a}
43.1	4251.30±444.71^{d}	38.16±7.20^{ab}	140.67±28.40^{ab}
84.2	3859.48±65.64^{cd}	46.59±5.74^{bc}	160.77±39.50^{bc}
123.3	3647.77±303.97^{bc}	63.68±7.72^{d}	198.60±32.80^{c}
162.2	3345.08±265.47^{ab}	58.12±6.48^{cd}	156.27±13.70^{bc}
203.1	3253.06±218.46^{ab}	54.81±6.61^{cd}	102.77±13.41^{a}

Each parameter is presented as the mean ± standard error. Superscript letters indicate significant differences ($p < 0.05$). The lack of a superscript letter indicates no significant differences among diets ($p > 0.05$).

Lipase activity in the intestine significantly increased with increasing dietary Fe levels up to 123.3 mg/kg diet and thereafter decreased. Lipase activity in the intestine of the 0 and 43.1 mg/kg Fe groups was significantly lower than that of the 123.3, 162.2 and 203.1 mg/kg Fe groups ($p < 0.05$). Lipase activity in the intestine of the 123.3 mg/kg Fe group was significantly higher than those of the 43.1 and 123.3 mg/kg Fe groups ($p < 0.05$). Fe level of 123.3 mg/kg was the most suitable dietary Fe level with the lipase activity in the intestine as the main indicator for *A. nobilis.*

Amylase activity in the intestine significantly increased with increasing dietary Fe levels up to 123.3 mg/kg diet and thereafter decreased. The amylase activity in the intestine of the 0 and 43.1 mg/kg Fe groups was significantly lower than that of 162.2 and 203.1 mg/kg Fe groups ($p < 0.05$). The amylase activity in the intestine of the 123.3 mg/kg Fe group was significantly higher than that of the 43.1 and 203.1 mg/kg Fe groups ($p < 0.05$)

Crude protein content and crude lipid content in the muscle of *A. nobilis* are presented in Table 3. The crude protein content in muscle of the 43.1 and 84.2 mg/kg Fe groups was significantly higher than that of the 203.1 mg/kg Fe group ($p < 0.05$). The crude protein content in the muscles of the 84.2 mg/kg Fe group was the highest in all groups. The crude lipid content of muscle was not significantly affected by different dietary Fe levels ($p > 0.05$).

Table 3. Crude protein and crude lipid (% of dry weight) in muscle of *A. nobilis*

Dietary Fe level(mg/kg)	*Parameters*	
	protein	*lipid*
0	72.89±2.89^{ab}	6.15±0.64^{a}
43.1	73.57±2.47^{b}	5.79±0.98^{a}
84.2	75.37±6.34^{b}	7.01±2.07^{a}
123.3	72.15±6.78^{ab}	7.11±1.30^{a}
162.2	68.87±1.01^{ab}	6.56±2.18^{a}
203.1	65.06±2.27^{a}	7.26±2.18^{a}

Each parameter is presented as the mean ± standard error. Superscript letters indicate significant differences ($p < 0.05$). The lack of a superscript letter indicates no significant differences among diets ($p > 0.05$).

Fe content in the muscle, vertebra, and intestine of *A. nobilis* are presented in Table 4. The basic trend was that the Fe content in the muscle increased with increasing dietary Fe levels up to 203.1 mg/kg diet. The Fe content in the muscle of the 123.3 and 162.2 mg/kg Fe groups was significantly higher than that in the 0, 43.1 and 84.2 mg/kg Fe groups ($p < 0.05$). Fe content in the muscle of the 203.1 mg/kg Fe group was significantly higher than in the other five groups ($p < 0.05$).

Table 4. Fe concentrations (mg/kg of weight) in the muscle, vertebra and intestines of *A. nobilis*

Dietary Fe level(mg/kg)	*Parameters*		
	muscle	*Vertebra*	*intestine*
0	34.72±2.99^{a}	73.79±4.65^{a}	71.98±1.74^{a}
43.1	35.56±1.71^{a}	81.88±7.36^{ab}	80.30±5.02^{bc}
84.2	36.12±2.34^{a}	87.89±7.63^{bc}	82.77±6.58^{c}
123.3	42.30±3.91^{b}	92.67±7.17^{bc}	79.80±4.63^{abc}
162.2	43.88±3.47^{b}	96.95±3.12^{c}	75.86±1.02^{abc}
203.1	51.86±3.53^{c}	98.32±7.62^{c}	74.16±3.34^{ab}

Each parameter is presented as the mean ± standard error. Superscript letters indicate significant differences ($p < 0.05$). The lack of a superscript letter indicates no significant differences among diets ($p > 0.05$).

Fe content in the vertebra of the 84.2, 123.3, 162.2 and 203.1 mg/kg Fe groups was significantly higher than the control group ($p < 0.05$). Fe content in the vertebra of the 162.2 and 203.1 mg/kg Fe groups was significantly higher than that of the 43.1 mg/kg Fe group ($p < 0.05$). Fe content in the vertebra significantly increased with increasing dietary Fe levels up to 203.1 mg/kg diet.

The Fe content in the intestine of the 43.1 and 84.2 mg/kg Fe groups was significantly higher than that of the control group ($p < 0.05$). The Fe content in the intestine of the 203.1 mg/kg Fe group was significantly lower than that of the 84.2 mg/kg Fe group ($p < 0.05$). Fe content in the intestine significantly increased with increasing dietary Fe levels up to 84.2 mg/kg diet and thereafter declined.

Discussion

In a previous study, it has been considered that metals might have negative effects on digestive enzymes (Clearwater et al., 2002). The present results showed that trypsin activity in the intestine significantly increased with increasing dietary Fe levels up to 43.1 mg/kg diet and thereafter decreased. This may have resulted from different studies *in vitro and vivo.* The present results showed that the lipase and amylase activities in the intestine significantly increased with increasing dietary Fe levels up to 123.3 mg/kg diet

and thereafter decreased. In the previous study when Fe was supplemented to the basal diet, the lipase and amylase activities in the intestine of *Oreochromis niloticus* (L.) ×*Oreochromis aureus* increased (Li et al., 2007). The effects of Fe on digestive enzyme activities in vitro were inconsistent with or even opposite to those seen in vivo. These differences are presumably attributed to the following: The secretion of digestive enzymes may have changed with the supplementation of Fe to fish diets, and long periods of access to high levels of Fe could also have improved nutritional status of the whole body and altered the physicochemical characteristics of the intestinal membrane, which in turn can influence digestive enzymes activity (Li et al., 2007).

The muscle nutrient composition of comestible fish such as *Epinephelus septemfasciatus* (Cheng et al., 2009) and *Plectropomus leopardus* in China have already been reported (You et al., 2014), however, there is little information about the nutrient composition of *A. nobilis*. The present results showed that the crude protein and crude lipid content in the muscles of *A. nobilis*. The crude protein content of muscle in the dietary Fe level of the 43.1 and 84.2 mg/kg groups were significantly higher than that of the 203.1 mg/kg Fe group ($p < 0.05$) suggesting that appropriate levels of dietary Fe increase the crude protein content in the muscles and inappropriate Fe levels decrease the level of muscle's crude protein. The crude protein content in the muscle in the 84.2 mg/kg Fe group was the highest in all groups. The crude lipid content of muscle was not significantly affected by different dietary Fe levels ($p > 0.05$). A previous study showed that the crude protein content was the highest and crude lipid content was lowest in the muscles of *Cenopharyngodon idellus* fed with 300 mg/kg Fe diet (Fu et al., 2007). This result may be related to the type of fish, the composition and palatability of the feed, the size of the experimental fish, the number of feedings, the experimental conditions, certain species-specific effects of Fe on growth, and difference in digestion and absorption of Fe among species (Feng et al., 2012).

The present study showed the Fe contents in the muscle and vertebra significantly increased with increasing dietary Fe levels. The Fe content in the intestine increased significantly with increasing dietary Fe levels up to 84.2 mg/kg diet and thereafter declined. Other previous results demonstrated that Fe absorption capacity and tissue levels in *Sparus aurata* (Rigos et al., 2010) and *Salmo salar* (Andersen et al., 1996) are not proportional to the dietary administrated levels. These results support the hypothesis that there is a regulation mechanism for iron metabolism in the fish that is not thoroughly known yet (Andersen et al., 1996). The present study showed that the sequence of Fe in different tissues of all groups were as follows: Vertebra >intestine >muscle.

Fe content of 123.3 mg/kg was the most suitable dietary Fe level with for the activity of lipase in the intestine as the of *A. nobilis.* These results indicate that appropriate levels of dietary alter the muscle nutrient compositions of *A. nobilis* and therefore provide valuable information for formulating *A. nobilis* feed in the future.

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