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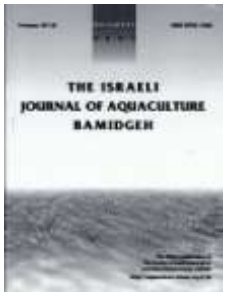

Dietary Iron Requirement of Goldfish (*Carassius auratus*) Fry

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Abstract

To estimate the dietary iron requirement of common goldfish, *Carassius auratus* (Linnaeus, 1758), eight isoproteinous diets containing graded levels of dietary iron ($FeSO_4.7H_2O$) ranging 34.27-537.14 mg/kg were fed to triplicate groups of *C. auratus* fry in glass tanks. The rearing medium was groundwater passed through a reverse osmosis water treatment unit. Fish were fed 3% of their body weight per day. After 75 days, the fish were randomly sampled and anesthetized, blood was withdrawn from the caudal vein, and red blood cell count, hemoglobin content, and hematocrit value were evaluated. Tissue samples were randomly collected for muscle, liver, and whole-body iron analysis. The iron concentrations in the diets and fish tissues were measured by atomic absorption spectrophotometer. Broken-line regression analysis and analysis of variance (ANOVA) with multiple mean comparisons of the data indicate that the dietary iron requirement for optimal hematological values and tissue iron levels of *C. auratus* fry should be at least 139.06 mg/kg dry diet.

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Introduction

Iron is an essential micronutrient involved in oxygen transport and cellular respiration through its oxidation-reduction activity and electron transfer (NRC, 1993). It occurs in the animal body as a component of hemoglobin, myoglobin, cytochromes, and many other enzyme systems (Davis and Gatlin, 1996). Iron deficiencies in several species of fish have been documented. Iron deficiency causes hypochromic microcytic anemia in brook trout, *Salvelinus fontinalis* (Kawatsu, 1972) and common carp, *Cyprinus carpio* (Sakamoto and Yone, 1978a). Iron deficiency suppressed growth and feed efficiency and reduced hematocrit, hemoglobin, and erythrocyte count in catfish (Gatlin and Wilson, 1986). In addition to deficiencies causing physiological problems, excessive iron levels can also be toxic. Dietary iron toxicity signs developed in rainbow trout fed more than 1,380 mg Fe/kg diet (Desjardins et al., 1987). The major effects of iron toxicity include reduced growth, increased mortality, diarrhea, and histopathological damage to liver cells (NRC, 1993).

Fish have the ability to absorb soluble iron from water across the gill membrane (Roeder and Roeder, 1966). Therefore, because both dietary intake and waterborne iron uptake must be taken into consideration, dietary iron requirement studies in fish are more complicated than in terrestrial animals. Nevertheless, the diet is considered the major source of iron for fish because of the low concentrations of soluble iron in natural waters (NRC, 1993). An adequate dietary supply of iron reduces the dependency on gill absorption of iron in guppies (Segner and Storch, 1985). Many practical diets contain considerable levels of endogenous iron, but little is known about its form and availability (Lall, 1989). In cereals, it may be in a complex form with phytin (Watanabe et al., 1997). Because the absorption of endogenous iron in most feedstuffs is low and the presence of other compounds may inhibit iron absorption, practical fish diets may require iron supplementation to ensure diet adequacy. However, supplementation of iron to the diet may affect diet stability by increasing lipid oxidation and reducing ascorbic acid stability (Hilton, 1989). Hence, supplementation of iron from a readily available source for fish and restriction of ingredients with high levels of iron in a complex form are recommended for practical diet formulations.

The dietary iron requirements for optimum growth and prevention of deficiency vary depending on species and iron source. The guppy, *Poecilia reticulata*, requires 80 mg Fe/kg diet (Shim and Ong, 1992), tilapia requires 85 mg Fe/kg diet (Shiau and Su, 2003), and juvenile gibel carp, *Carassius auratus gibelio*, requires at least 202 mg Fe/kg diet (Pan et al., 2009). Dietary iron supplementation affects the bioavailability of trace elements such as zinc, copper, and manganese. Goldfish is one of the most important domesticated and traded freshwater ornamental fish species in India. It is successfully reared in glass aquaria, ponds, outdoor gardens, cement cisterns, and FRP tanks. Because a low-cost and well-balanced nutrient-rich diet is essential for the good health of all ornamental fishes, the present study was designed to estimate the optimum dietary level of iron required by goldfish fry.

Materials and Methods

Diets. Purified ingredients supplemented with ferrous sulfate heptahydrate ($FeSO_4.7H_2O$) as the source of dietary iron were used to formulate eight experimental diets (Table 1). Supplemental levels of iron were 0 (basal diet), 20, 40, 60, 80, 100, 250, and 500 mg/kg diet. Cellulose was replaced by $FeSO_4.7H_2O$ to achieve the desired iron levels. Carboxymethyl cellulose was added as a binding agent that, together with starch soluble, stabilizes feed and thus reduces leaching of the water-soluble FeSO4.7H2O. Gelatin crystals were mixed in warm distilled water in a plastic container and the dry ingredients were added. Oil was added to the mixture and dough was prepared with the necessary amount of distilled water. The dough was cooked in a pressure cooker for 30 min and cooled, then the vitamin and mineral mixtures were added. $FeSO_4.7H_2O$ was dissolved separately in water and mixed with the dough. Pellets were prepared with a hand pelletizer, air dried for 30 min, and oven dried at 52ºC for 12 h. The dried pellets were packed in zip lock polythene bags and stored in an air sealed container until use.

Table 1. Ingredients and proximate composition of diets containing different iron contents for goldfish fry.

Ingredient	*Diet (mg supplemented iron/kg diet)*							
	0	*20*	*40*	*60*	*80*	*100*	*250*	*500*
Casein (vitamin-free)	32.0	32.0	32.0	32.0	32.0	32.0	32.0	32.0
Starch soluble	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0
Dextrin white	15.7	15.7	15.7	15.7	15.7	15.7	15.7	15.7
Gelatin	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00
Sunflower oil	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Iron-free mineral mix[1]	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Cod liver oil	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Cellulose	1.50	1.49	1.48	1.47	1.46	1.45	1.375	1.25
Carboxymethyl cellulose	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Vitamin mix[2]	0.48	0.48	0.48	0.48	0.48	0.48	0.48	0.48
DL-methionine	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
L-tryptophan	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
BHT	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
$FeSO_4.7H_2O$	0.00	0.01	0.02	0.03	0.04	0.05	0.125	0.25
Proximate composition (% dry matter basis)								
Crude protein	35.23	35.00	35.47	34.77	34.53	35.23	34.77	35.47
Ether extract	6.93	7.29	6.80	7.22	7.27	7.22	7.00	7.07
Ash	5.44	5.39	5.53	5.37	5.49	5.52	5.62	5.65
Iron (mg/kg)	34.27 ± 1.09^a	59.75 ± 1.19^b	80.72 ± 1.03^c	95.54 ± 1.69^d	119.04 ± 1.36^e	139.06 ± 1.79^f	285.84 ± 1.35^g	537.14 ± 1.78^h

Values with different superscripts vary significantly ($p<0.05$).
[1] per kg diet: $CaCO_3$ 5.8 g; $CaHPO_4.2H_2O$ 12 g; KH_2PO_4 12.9; $MgSO_4.7H_2O$ 5.5 g; NaCl 3 g; $ZnSO_4.7H_2O$ 400 mg; $MnSO_4.H_2O$ 352 mg; $CuSO_4.5H_2O$ 30 mg; KI 6 mg; $NaMoO.2H_2O$ 8 mg; $CoCl.6H_2O$ 2 mg; Na_2SeO 2 mg
[2] per kg diet: thiamine hydrochloride 20 mg; riboflavin 30 mg; calcium D pantothenate 50 mg; niacin 160 mg; vitamin B_6 20 mg; vitamin B_{12} 0.05 mg; vitamin C 450 mg; folic acid 6 mg; inositol 1000 mg; biotin 2.5 mg; choline chloride 3000 mg; vitamin A 6000 IU; vitamin D 800 IU; vitamin E 75 IU; vitamin K 10 mg.

Proximate compositions of the experimental diets were analyzed by standard methods (AOAC, 1995).

Assay of dietary iron concentration. Nitric acid, 65% w/v, and hydrochloric acid, 30% w/v (Suprapur Reagents, Merck) were used with high-purity water produced with a Milli-Q system (Millipore, MA, USA). Calibration was obtained with external standards. Standard solutions were prepared by diluting a 1000 mg/l ICP multi-element standard solution IV (Merck, Darmstadt, FRG) with deionized water. Glassware was cleaned by soaking with the contact overnight in a 10% (w/v) H_2SO_4 solution and then rinsing with deionized water. A feed sample (0.5) was digested with 8 ml HNO_3 and 1 ml HCl in a microwave reaction system (Multiwave 3000, Perkin Elmer) at 190°C and a pressure of 25 bar @ 0.4 bar/s for 20 min. Dietary iron concentrations were measured with an atomic absorption spectrophotometer (AAnalyst 800, Perkin Elmer). Parameters were set according to the manufacturer's instructions. Hollow-cathode lamps were operated at a current of 30 mA to obtain a clear lean blue flame (oxidizing condition). The wave length was set at 248.3 nm, and the slit width at 0.2 nm, for characteristic concentration checks at 6.0 mg/l.

Fish and management. Carassius auratus fry were obtained from the hatchery unit of the Institute, acclimatized in an aerated 500-l FRP tank, and fed 3% of their body weight twice a day for seven days before commencement of the experiment. Fish averaging 2.961±0.026 g were randomly distributed into 45 × 22 × 30 cm adequately aerated glass tanks at 10 fish/tank with triplicates of each treatment. Tanks were filled with groundwater passed through a reverse osmosis water treatment unit (RO unit) that reduced the total dissolved solids in the groundwater. Fish were fed their respective diets at 3% body weight twice a day for 75 days. Fish were weighed fortnightly to adjust the feeding rate. Tanks were manually cleaned once a week to prevent algae growth and 50% of the water was siphoned each day to remove fecal matter.

Water quality. The dissolved iron in two water sources (Bombay Municipal Corporation water and well water) were analyzed and, because the groundwater contained less iron

than the Bombay municipal water (0.115±0.003 mg/l vs 0.136±0.003 mg/l), the groundwater was passed through an RO unit and used to rear the *C. auratus* fry. The RO unit comprises a series of a sand filter, a coarse filter (20 µm), a reverse osmosis membrane filter, an activated charcoal filter, a sterilizer, and a fine filter (5 µm). After treatment in the RO unit, the water contained 0.034±0.001 mg/l dissolved iron, which was higher than the iron concentration of water used by Shiau and Su (2003) in their estimation of the dietary iron requirement for juvenile tilapia. The iron content of the water was determined using atomic absorption spectrometry. Water (250 ml) was evaporated to reduce the volume to no more than approximately 25 ml in a conical flask that was chromic acid washed and dried. The content was then digested by adding 2 ml HNO_3 and 1 ml HCl (Suprapur, Merck), made up to a volume of 50 ml in a volumetric flask, and analyzed according to the manufacturer's instructions.

Water quality was recorded throughout the experiment (APHA-AWWA-WEF, 1998) and within normal ranges: temperature 26-29°C, pH 6.5-7.0, dissolved oxygen 6.1-7.4 mg/l, nitrate 0.02-0.04 mg/l, nitrite 0.001-0.003 mg/l, and phosphate 0.08-0.11 mg/l. At the beginning of the experiment, the water temperature was 26ºC, the ideal temperature for goldfish. The dissolved oxygen content of the water was periodically adjusted by regulating the air flow to a sponge filter using a plastic controller.

Collection and assay of blood. At the end of the feeding trial, three fish per tank were randomly sampled and anesthetized with clove oil (50 µl/l water). Blood was withdrawn from the caudal vein with a 1-ml disposable medical syringe previously rinsed with anticoagulant (2.7% EDTA solution) and transferred immediately to Eppendorf tubes containing a thin layer of EDTA powder and well-shaken to prevent hemolysis.

Red blood cells (RBC) were counted in a hemocytometer using RBC diluting fluid (Qualigens, India). Blood (20 µl) was mixed with 3,980 µl diluting fluid in a glass test tube and the mixture was well-shaken to suspend the cells uniformly in the solution. A small drop of the mixture was charged to Neubauer's counting chamber of the hemocytometer and counted under a light microscope. The number of RBC/mm^3 was calculated as (no. RBC × dilution)/(area in mm^2 × depth of fluid in mm). The hemoglobin (Hb) content of the blood was assayed by estimating cyanmethemoglobin using Drabkins Fluid (Qualigens, India). Drabkins working solution (5 ml) was added to 20 µ blood in a clean dry test tube. Absorbance was measured with a spectrophotometer (Merck, Nicolet, evolution 100) at a wavelength of 540 nm. The final concentration was calculated by comparing with the standard cyanmethemoglobin (Qualigens, India). Hematocrit (Hct) was measured by drawing non-dotted blood by capillary action into microhematocrit tubes. One end of the tubes was sealed with a synthetic sealant. The sealed tube was centrifuged in a microhematocrit centrifuge for 5 min at 10,500 rpm. The packed cell volume measured by the microhematocrit reader was expressed as a percentage.

Assay of iron concentrations in body tissues. Four fish from each tank were anesthetized and immediately dissected, muscle tissues were collected from two fish, and liver tissues were pooled to estimate iron concentrations. For estimation of whole-body iron concentrations, the whole fish was cut into small pieces and homogenized using a mortar and pestle. Samples (0.5 g) were digested by 5 ml Suprapure HNO_3 and 1 ml Suprapure HCl in a microwave reaction system. The digested samples were analyzed by the atomic absorption spectrometry according to the manufacturer's instructions.

Statistical analysis. Data were subjected to one-way analysis of variance (ANOVA) using SPSS version 16.0 for Windows. Data are expressed as means±SE. Duncan's multiple range test was used to determine the significance of differences between means. Comparisons were made at the 5% probability level (p<0.05). The dietary iron requirement was determined using R.J. Oosterbaan's Segmented Regression Model, SegReg 1.7.0.0.

Results

Hematological parameters. There were significant differences in mean RBC counts, Hb content, and hematocrit value, but no significant differences between groups fed the diet witth 100-500 mg supplementary iron (Table 2). Therefore, based on ANOVA and

multiple mean comparison tests, *C. auratus* fry require 139.06 mg Fe/kg diet for optimal RBC, Hb content, and Hct.

Muscle, liver, and whole-body iron concentrations. There were significant differences in mean muscle iron concentrations up to 100 mg supplementary iron but no differences beyond this level. Similarly, there were no significant differences in mean hepatic iron concentrations in fish fed 80-500 mg supplementary iron or in mean whole-body iron concentration in fish fed 250 or 500 mg supplementary iron. Based on ANOVA and the multiple mean comparison test, *C. auratus* fry require 139.06 mg Fe/kg diet for maintenance of muscle iron concentration, 119.04 mg Fe/kg diet for maintenance of hepatic iron concentration, and somewhere between 139.06 and 285.84 mg Fe/kg diet for maintenance of whole-body iron concentration.

Table 2. Hematological values and tissue iron levels of goldfish fry (*Carassius auratus*) fed diets with different amounts of iron supplementation for 75 days (means±SE; n = 6 for muscle iron; n = 3 for liver and whole body).

	Diet (mg supplemented iron/kg diet)							
	0	*20*	*40*	*60*	*80*	*100*	*250*	*500*
Dietary iron (mg/kg)	34.27±1.09	59.75±1.19	80.72±1.03	95.54±1.69	119.04±1.36	139.06±1.79	285.84±1.35	537.14±1.78
Hematological values								
RBC (10^6/mm)	1.59±0.01^{a}	2.07±0.06^{b}	2.78±0.04^{c}	2.84±0.03^{cd}	2.78±0.03^{c}	2.93±0.04^{d}	2.87±0.03^{cd}	2.91±0.03^{d}
Hb (g/dl)	4.71±0.02^{a}	5.97±0.04^{b}	7.88±0.06^{c}	8.15±0.03^{d}	8.10±0.03^{cd}	8.50±0.12^{e}	8.70±0.17^{e}	8.70±0.06^{e}
Hct (%)	15.23±0.23^{a}	19.53±0.78^{b}	25.81±0.06^{c}	27.28±1.32^{de}	26.76±0.56^{cd}	27.71±0.21^{e}	28.40±0.23^{e}	29.03±0.90^{e}
Tissue iron concentration (µg/g)								
Muscle	9.20±0.28^{a}	11.26±0.71^{a}	15.66±0.81^{bcd}	14.48±0.70^{bc}	14.20±0.49^{b}	16.44±0.39^{bcd}	16.97±0.99^{cd}	17.31±0.68^{d}
Liver	56.54±2.66^{a}	71.53±4.65^{b}	92.62±1.43^{c}	91.11±2.59^{c}	102.26±4.80^{cd}	100.67±2.04^{cd}	103.50±7.33^{cd}	107.17±5.87^{d}
Whole body	15.55±1.58^{a}	26.17±1.99^{ab}	36.66±4.10^{bc}	43.06±2.61^{c}	40.62±5.20^{c}	46.85±5.73^{c}	59.05±3.35^{d}	66.03±2.62^{d}

Mean values in a row with different superscripts differ significantly (p<0.05).

Dietary iron requirement. The dose-response relationships between dietary iron concentrations and hematological values and tissue iron levels were examined using Segmented Regression Model and the optimum breakpoint values (BP_x) are considered the dietary iron requirements of *C. auratus* fry (Table 3). To ensure enough iron is available to maintain optimum values of these parameters, the dietary iron level should be at least 134.8 mg/kg diet. While the dose-response relationship indicates that an optimum RBC is maintained when dietary iron is at least 119.8 mg Fe/kg (Fig. 1), when examined using ANOVA and multiple mean comparison tests, the estimated dietary iron requirement of common goldfish fry for optimum hematological values and tissue iron concentration should be at least 139.06 mg/kg diet.

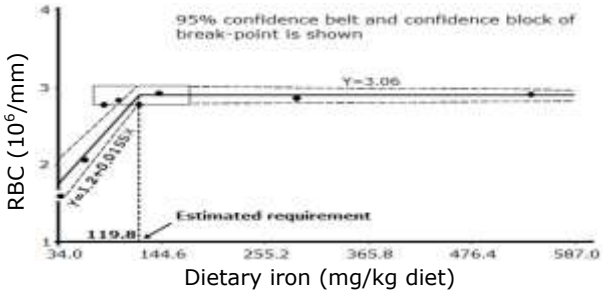

Fig. 1. Dose-response relationship between dietary iron and RBC count of *Carassius auratus* fry fed diets with different iron contents.

Table 3. Optimum breakpoint (BP_X) values of dietary iron (X) for optimum hematological values and tissue iron level of goldfish *Carassius auratus* fry.

Parameter	*Regression equation (Y =)*		BP_X
	When X<BP_X	*When X>BP_X*	
RBC count	1.20+0.0155X	3.06	119.8
Hb content	2.61+0.0603X	8.91	104.7
Hct value	11.1+0.155X	29.6	119.8
Muscle iron	7.57+0.0693X	16.9	134.8
Hepatic iron	37.1+0.604X	103.0	109.7
Whole-body iron	-1.20+0.434X	57.3	134.8

Discussion

A major obstacle in estimating the dietary iron requirement of fish is obtaining an iron-free basal diet because it is difficult to obtain iron-free ingredients (Lall, 1989). The iron sources in our basal diet were dextrin white, starch soluble, and casein.

The supplemental iron in the experimental diets was $FeSO_4.7H_2O$, one of the cheapest and most available

sources of iron. Ferrous iron (Fe^{+2}) is absorbed more efficiently than ferric iron (Fe^{+3}) at a neutral pH (Guillaume et al., 2001) and the effectiveness of ferric citrate as an iron source is half that of ferrous sulfate in meeting the iron requirement of juvenile tilapia (Shiau and Su, 2003). Hence, it was assumed that $FeSO_4.7H_2O$ would be readily available to the *C. auratus* fry. However, ferrous iron is a more potent catalyst of lipid peroxidation than ferric iron (Chvapil et al., 1974; Lee et al., 1981). Lipid peroxidation increases with iron supplementation which in turn adversely affects feed stability (Desjardins et al., 1987). Therefore, 0.02% BHT was added to the experimental diets to prevent lipid peroxidation.

Other elements may inhibit iron absorption. Excess dietary calcium may reduce the absorption of the trace elements zinc, iron, and manganese (Lall, 1979). Therefore, an iron-free mineral mixture was prepared as per the requirements of freshwater aquarium fish. Juvenile goldfish require 29% protein, in comparison to 53% for larvae (Lochmann and Phillips, 1994). Goldfish lack a stomach and have only an intestinal tract. Thus they cannot digest excessive protein. However, carbohydrate digestibility is high (70%) in goldfish (Pannevis, 1993). Therefore, we formulated diets to contain 35% crude protein as casein and gelatin, and a high percentage of carbohydrate as starch soluble and dextrin.

The dose-response relationships between dietary iron and hematological parameters and body composition were examined using segmented regression analysis (the one-slope method) and ANOVA with a multiple mean comparison test. Both statistical methods predicted almost similar requirements. The dietary iron requirement of *C. auratus* fry was estimated as the iron level that produced the maximum responses, using basic hematological parameters and tissue iron levels as indicators of the iron status in *C. auratus*. These parameters were significantly affected by the level of dietary iron supplementation. There were no adverse effects on hematological values when dietary iron was increased to 537.14 mg/kg diet. In comparison, a diet containing 1250 mg Fe/kg showed no toxicity signs in rainbow trout (Desjardins et al., 1987).

The goldfish required 139.06 mg Fe/kg diet for optimal RBC count, Hb content, Hct, and maintenance of muscle iron, and 119.04 mg Fe/kg diet for maintenance of hepatic iron. However, according to the ANOVA and multiple mean comparison tests, they require somewhere between 139.06 and 285.84 mg Fe/kg diet for maintenance of whole-body iron. This seemingly high dietary iron requirement was due to the absence of treatment groups between the 139.06 and 285.84 mg/kg diet levels. Based on broken-line regression analysis of the hematological values and body iron concentrations, the iron requirement lies between 104.7 and 134.8 mg Fe/kg diet. Using this method, a relatively low concentration of dietary iron satisfied the hepatic iron storage and blood hemoglobin content, maybe because the liver and blood are the primary iron absorption sites in the body, possibly have better absorption routes, and thus respond more readily to changes in dietary iron intake (Bjørnevik and Maage, 1993). We consider the minimum dietary iron requirement of *C. auratus* fry to be 139.06 mg/kg diet because the broken-line regression method and ANOVA frequently underestimate the requirement (Shearer, 2000) and because it is within the iron requirement ranges reported for fish (30-170 mg/kg dry diet; Watanabe et al., 1997).

The requirement of goldfish fry for dietary iron is higher than the requirements of guppy (80 mg/kg diet) and tilapia (85 mg/kg diet) when $FeSO_4$ is used as the source of iron. However, tilapia requires much more dietary iron (150-160 mg Fe/kg diet) when fed diets supplemented with ferric citrate (Shiau and Su, 2003). Using ferric chloride as the iron source for common carp, a dietary iron concentration of 199 mg Fe/kg diet is required to prevent iron deficiency symptoms (Sakamoto and Yone, 1978a). The lower requirement in the present study may be because ferrous iron is absorbed more efficiently than ferric iron. Our requirement is comparable to the minimum dietary iron concentration (150 mg/kg diet) needed to prevent iron deficiency symptoms in red sea bream (Sakamoto and Yone, 1978b).

The dietary iron requirement for juvenile gibel carp, which belongs to the same family (Cyprinidae) and genus (*Carassius*) as our goldfish, is 202 mg/kg diet. The different

dietary iron requirements result not only because of differences in species, but also may be due to differences in body iron demand, soluble iron present in the rearing water, feeding efficiency, or source of iron (Shearer, 1995). To ensure enough available iron for *C. auratus* fry, we recommend dietary iron of at least 139.06 mg Fe/kg dry diet when $FeSO_4$ is used as the iron source.

Feeds of animal origin such as fishmeal and meat meal are rich sources of iron, containing about 400-800 mg/kg (Watanabe et al., 1997) and are efficiently absorbed by fish. Therefore, practical goldfish diets that contain proteins of animal origin should not require iron supplementation. However, feeds of plant origin contain much less iron. Oil seeds contain 100-200 mg Fe/kg, while cereals contain 30-60 mg Fe/kg (Watanabe et al., 1997). Some sources contain iron in a complex form that is less available to fish. Like carps, goldfish do not have an acidic stomach and cannot digest iron of low solubility. Therefore, goldfish diets that contain plant sources require iron supplementation of at least 100 mg/kg diet with an iron source of high solubility.

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