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Responses of channel catfish (*Ictaluris punctatus*) fed iron-deficient and replete diets to *Edwardsiella ictaluri* challenge

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Abstract

Juvenile channel catfish were fed egg-white-based diets with or without 20 mg supplemental iron/kg from iron methionine for 13 weeks. Subsequently, fish from each treatment were divided into two groups and fed either the iron-deficient or replete diets for another 4 weeks. Growth response, hematological values, serum iron level, nonspecific immune response and mortality after *Edwardsiella ictaluri* challenge were determined. At the end of week 13, fish fed the iron-deficient diet had significantly lower weight gain, feed efficiency ratio, hematocrit, total cell count, and serum iron and transferrin saturation than those fed the replete diet. These values remained consistently low or decreased for the groups that continued to receive, or were switched to the iron-deficient diet, but were improved for those fed the replete diet. The differences, however, were not always significant. Chemotactic response of peritoneal macrophages to *E. ictaluri* exoantigen was suppressed for fish fed the iron-deficient diet, but the abnormality was reversed by feeding the iron-replete diet. However, the challenge study showed that dietary iron did not protect channel catfish against mortality from *E. ictaluri*, but the onset of mortality was earlier for fish fed the iron-deficient diet, probably due to the combined effect of iron deficiency and infection.

Keywords: Dietary iron; Growth; Hematology; Immune response; Disease resistance

1. Introduction

Edwardsiella ictaluri is the causative bacterium of enteric septicemia of catfish (ESC) (Hawke, 1979; Hawke et al., 1981). The high morbidity and mortality associated

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with ESC and its rapid onset in the spring and fall make it a major threat to catfish farming (MacMillian, 1985). Losses of cultured fish from this disease parallel the intensification and expansion of the industry. It is estimated that ESC costs the catfish industry US $20–30 million annually (Plumb and Vinitnantharat, 1993). Attempts to prevent this disease by immunization have not been successful. The control of the disease by use of antibiotics, medicated-feed or treatment of water have been met with some success. Some of the drawbacks associated with these methods of treatment are high costs, development of antibiotic-resistance bacteria, and environmental contamination.

It has long been recognized that nutrition is a key factor in host defense against pathogens. Deficiency or excess of nutrients can alter immune response and susceptibility of animals to infection (Chandra and Newberne, 1977). Iron is one of the most important micronutrients in terms of its effect on immune system functions and host defense against infections (Beisel, 1982; Bhaskaram, 1988). The total dietary iron requirement for optimum growth and hematological values of juvenile channel catfish has been determined to be about 30 mg/kg diet (Gatlin and Wilson, 1986; Lim et al., 1996). Iron sulfate and iron methionine were equally effective in preventing anemia in channel catfish (Lim et al., 1996). However, the effect of dietary iron on immune function and resistance of channel catfish to bacterial pathogens is poorly understood. In a recent 8-week feeding study in this laboratory, no conclusive evidence could be established on the influence of dietary levels of iron from iron sulfate or iron methionine on immune response and resistance of channel catfish to *E. ictaluri*, although it was observed that some immune parameters and the resistance of fish against *E. ictaluri* appeared to be affected by iron deficiency (Sealey et al., 1997). Thus, a longer term feeding study was conducted to determine the effect of iron deficiency and adequacy on the immune response of channel catfish and their resistance to *E. ictaluri* challenge. Growth response and hematological parameters were also evaluated.

2. Materials and methods

2.1. Experimental diets

The egg-white-based diet used in this study was modified from Gatlin and Wilson (1986) and is given in Table 1. The basal diet was supplemented with 0 (deficient-diet) or 20 mg (replete-diet) of iron/kg from iron methionine (Zinpro, Chaska, MI) in place of cellulose. The diets were formulated to contain approximately 34% crude protein and 3.1 kcal of digestible energy/g based on feedstuff values reported in NRC (1993). Diets were prepared as 3-mm diameter, semimoist (approximately 25% moisture) pellets as described by Lim et al. (1996). Pellets were broken into small pieces and stored at −18°C until needed. Iron content of the basal diet without iron supplementation was determined to be 9.2 mg iron/kg by an inductively-coupled argon plasma (ICAP) spectrometer according to the method of Campbell and Plank (1992).

Table 1
Composition of basal diet

Ingredient	Diet (dry matter basis, g/kg)
Egg white	399.0
Corn starch	475.8
Cellulose	5.0
Cod liver oil	32.5
Corn oil	32.5
Iron-free mineral mix[a]	40.0
Vitamin mix[b]	15.0
Ethoxyquin	0.2

[a]Contains (as g/kg of premix): calcium carbonate, 300.0; potassium phosphate, dibasic, anhydrous, 319.0; sodium phosphate, monobasic, 200.34; magnesium sulfate heptahydrate, 132.0; zinc sulfate heptahydrate, 3.00; sodium chloride, 43.50; cobalt chloride, 1.00; manganous sulfate monohydrate, 0.80; cuprous chloride, 0.20; potassium iodide, 0.15; sodium selenite, 0.011.

[b]Contains (as g/kg of premix): retinyl acetate, 1.20; cholecalciferol, 0.17; menadione, 3.33; DL-alpha tocopheryl acetate, 4.00; L-ascorbyl-2-polyphosphate (15% vitamin C activity), 600; inositol, 10.00; choline chloride, 150.0; niacin, 9.00; riboflavin, 2.00; pyridoxine hydrochloride, 2.00; thiamin hydrochloride, 2.00; D-calcium pantothenate, 6.00; biotin, 0.31; folic acid, 0.18; cyanocobalamin, 0.0027; celufil, 295.62.

2.2. Experimental fish and feeding

Channel catfish (*Ictaluris punctatus*) fingerlings from a single Auburn × Kansas spawn, which had been maintained at the USDA, Fish Diseases and Parasites Research Laboratory and fed a commercial diet to an average weight of 4.7 g, were randomly stocked into 18, 110-l aquaria at a density of 100 fish per aquarium. Aquaria were supplied with flow-through dechlorinated tap water at a rate of 0.6–1.2 l/min. Water flow rates were checked and adjusted daily to insure proper water exchange rate. Water temperature was maintained by a centralized heater at 26 ± 2°C. Water was continuously aerated and photoperiod was maintained at 12:12 h light:dark schedule. The water contained less than 0.5 mg iron/l.

Fish in nine aquaria were randomly assigned to each of the two experimental diets and fed their respective diets twice daily (between 0730–0800 and 1430–1500) to satiation for a period of 13 weeks. However, only fish from three randomly chosen aquaria of each treatment were regularly sampled. At the end of week 13, these groups of fish were used for hematological and immunological assays, and challenge study. The remaining fish in the other six aquaria of the same treatment were pooled and then randomly divided into six groups of 50 fish of approximately equal weight and stocked in another set of 110-l aquaria. Fish in three random aquaria of the same treatment stayed on the same diet, whereas those in the other three aquaria were switched to either the iron-deficient or replete diet for an additional 4-week period. During each feeding, feed was offered by hand three to four times until satiation was reached. The quantity of feed consumed was recorded daily by calculating the differences in weights of feeds before the first and after the last feeding. All aquaria were cleaned weekly by scrubbing and siphoning accumulated wastes. On cleaning days fish were fed only in the afternoon. Fish in three aquaria of each treatment were counted and weighed at biweekly

intervals except from weeks 10 to 13 when fish were sampled only once. No feeding was done on sampling days.

2.3. Blood analysis

Blood samples were obtained from fish at weeks 13 and 17. Ten fish from each of the triplicate aquarium were randomly chosen and anesthetized with tricane methanesulfonate (MS-222, Argent Chemical, Redmond, WA) at 125 mg/l. Blood samples were collected from the caudal vein of five fish per aquarium with a 27-gauge needle and tuberculin syringe. Serum samples for determination of iron and total iron binding capacity were collected after centrifugation of whole blood from five fish at $1000 \times g$. Sera from each of the five fish from the same aquarium were pooled to obtain one composite sample. Total iron was determined by the colorimetric assay using the Stanbio Total Iron Kit (Stanbio Laboratory, San Antonio, TX). In this procedure, iron is released from its combination with transferrin in acid medium, reduced to its ferrous form by hydroxylamine, and reacted with ferrozine to form a violet colored complex that is measured at 560 nm. Transferrin saturation was determined as described by Sherman and Moran (1984).

The remaining five fish from each replicate tank were bled for determination of hematocrit and total cell counts with heparinized needles and syringes (20 units/ml). Hematocrit was determined by the microhematocrit method described by Brown (1988). Total cell counts were determined by diluting whole blood and enumeration in a hemacytometer.

2.4. Collection of peritoneal exudate cells

Collection and isolation of peritoneal exudate cells followed the procedure of Klesius and Sealey (1996). At the end of weeks 13 and 17, five fish from each of the three replicate tanks were randomly chosen, injected intraperitoneally (IP) with 0.25 ml of squalene (Sigma Chemical, St. Louis, MO) and transferred into 57-l aquaria where they continued to be fed the various experimental diets. Five to seven days later, fish were anesthetized with MS-222 and injected (IP) with 15 ml sterile, cold phosphate buffered solution (PBS). Then, PBS was removed along with the squalene-elicited exudate cells using a 20-gauge needle attached to a 3-way valve into a 50-ml centrifuge tube. The peritoneal fluids of five fish from the same tank were combined and centrifuged at $300 \times g$ for 10 min. The supernatant was discarded, and the cells suspended in calcium- and magnesium-free Hank's Balance Salt Solution (HBSS) without phenol red (Gibco, Grand Island, NY) for chemotaxis assay. Cell counts and viability were established following enumeration with a hemocytometer in 5% Trypan blue counting solution.

2.5. Chemotaxis assay

Chemotaxis was determined by a modification of the lower-surface method of Boyden (1962) as described by Klesius and Sealey (1996). Blind well chemotactic chambers (Corning CoStar, Cambridge, MA) and 5-μm pore diameter polycarbonate

membrane filters (Nucleopore, Pleasonton, CA) were used to determine the number of macrophages reaching the lower surface of the filter. In the bottom of the chamber, 0.2 ml of either *E. ictaluri* exoantigen (0.53 mg/ml) (Klesius, 1993) or RPMI-1640 (Gibco) containing 1% horse serum (control) was added. Peritoneal macrophages were added to the upper compartment of the chamber (separated from the bottom chamber by a filter) at a concentration of approximately 400,000 exudate cells/chamber. The chambers were incubated on a rotating platform (100 rpm) for 100 min at 25°C. The filters were removed, inverted, placed on a slide, attached with clear fingernail polish and stained with Leukostat. The number of macrophages on the surface of the filter were counted in five fields of duplicate filters at 100 ×.

2.6. *Edwardsiella ictaluri* challenge

E. ictaluri (AL-75-94) from a virulent outbreak of ESC was grown in brain–heart infusion (BHI) broth for 24 h and used for bacterial challenge (Klesius, 1992). At the end of week 13, 20 fish from each of three replicate tanks per diet were randomly selected, placed in perforated 5-gallon plastic buckets and immersed for 1 h in static, aerated aquaria containing $1-2 \times 10^7$ cells/ml of *E. ictaluri*. Each group of fish was then transferred to their new, randomly assigned 57-l aquarium provided with flowing water (0.5–0.6 l/min) maintained at 25 ± 2°C. Water flow and feeding were discontinued for the first 24 h after challenge. Mortality was monitored and recorded twice daily before feeding for 14 days (or less if all the fish died). Due to poor condition of fish fed the iron-deficient diet throughout, challenge study with *E. ictaluri* was not performed at the end of week 17.

2.7. *Statistical analysis*

Data were analyzed by one-way analysis of variance, and Duncan's multiple-range test was used to determine differences among treatment means (SAS Institute, 1993, Carey, NC). Differences were considered significant at the 0.05 probability level.

3. Results

The average body weight of channel catfish fed the iron-deficient and replete diets are presented in Fig. 1. Mean weight gains, survival and feed efficiency ratios at the end of week 13 and from weeks 14–17 are given in Table 2. Fish fed the iron-deficient diet had consistently lower weight gain than fish fed the iron-replete diet throughout the 13-week period. By the end of week 6, the weight gain of this group was significantly lower than those fed the iron-replete diet. At the end of week 17, fish that continued to receive the iron-deficient diet (0 to 0) lost weight, whereas those switched to the iron-replete diet (0 to 20) had positive weight gain, but it was significantly lower than that of fish fed the iron-replete diet for the first 13 weeks and continued on this diet (20 to 20) or switched to the iron-deficient diet (20 to 0). Feed efficiency ratio, at the end of week 13, was significantly lower for fish fed the iron-deficient diet than those fed the

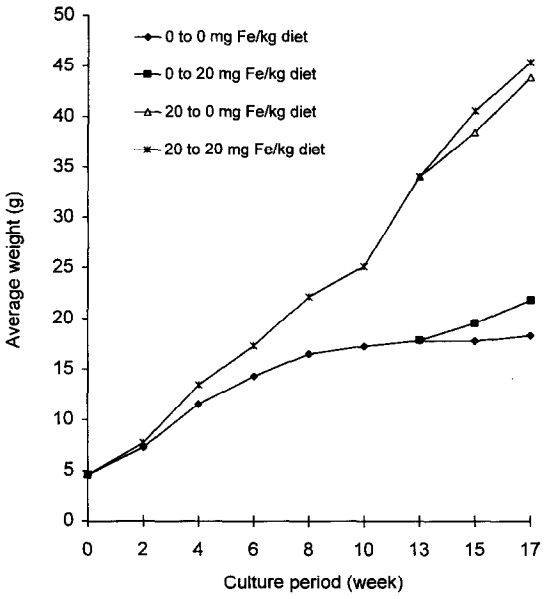

Fig. 1. Average weight of channel catfish fed the iron-deficient and/or replete diets for 17 weeks.

iron-replete diet. These values for weeks 14–17 did not differ, except for the 0 to 0 treatment that had a negative value. Survival at week 13 was not significantly affected by dietary iron supplementation. The survival significantly decreased when fish fed the iron-deficient diet were continued on that diet for another 4 weeks. No significant differences were observed among the survival of fish in 0 to 20, 20 to 0 and 20 to 20 treatments.

Fish fed the iron-deficient diet had significantly lower hematocrit and total blood cell count at the end of weeks 13 and 17 (Table 3) than fish fed the iron-repleted diet. These values significantly increased for the group switched to the iron-replete diet (0 to 20).

Table 2
Average weight gain, feed efficiency ratio and survival of channel catfish fed iron-deficient and replete diets[1]

Period (week)	Dietary iron supplement (mg/kg)	Weight gain (g)	Feed efficiency ratio	Survival (%)
0–13	0	13.3 ± 0.6^{b}	0.62 ± 0.01^{b}	88.7 ± 1.2
	20	29.4 ± 3.7^{a}	0.74 ± 0.02^{a}	94.7 ± 2.4
14–17	0–0	-1.5 ± 0.5^{c}	–	65.3 ± 12.1^{b}
	0–20	4.0 ± 1.2^{b}	0.43 ± 0.16	80.0 ± 11.4^{ab}
	20–0	9.8 ± 1.1^{a}	0.67 ± 0.03	98.0 ± 0.0^{a}
	20–20	11.3 ± 0.7^{a}	0.70 ± 0.02	99.3 ± 0.7^{a}

[1]Column means ($\pm$SE) within the same period having the same superscript are not significantly different ($P > 0.05$).

Table 3
Mean hematocrit and whole blood cell count of channel catfish fed iron-deficient and replete diets[1]

Period (week)	Dietary iron supplement (mg/kg)	Hematocrit (%)	Total blood cell count ($\times 10^6$/mm^3)
13	0	16.0 ± 0.8^b	1.91 ± 0.08^b
	20	30.8 ± 1.2^a	3.56 ± 0.21^a
17	0–0	15.4 ± 2.1^c	1.79 ± 0.08^b
	0–20	24.8 ± 1.6^b	2.53 ± 0.22^a
	20–0	24.2 ± 1.3^b	2.56 ± 0.19^a
	20–20	33.9 ± 0.5^a	3.03 ± 0.21^a

[1]Values represent means of $n = 15$ determinations/treatment. Column means ($\pm$SE) within the same period having the same superscript are not significantly different ($P > 0.05$).

For fish that were fed the iron-replete diet throughout (20 to 20), hematocrit and total blood cell count remained consistently high but decreased for fish that were switched to the iron-deficient diet (20 to 0).

Serum iron and transferrin saturation at weeks 13 and 17 for fish fed the iron-deficient diet were significantly lower than those of fish fed the iron-replete diet (Table 4). No significant differences were found for total iron binding capacity (TIBC) at week 13, but this value significantly increased at week 17 for the 0 to 0 treatment (Table 4). There were no significant differences among fish in treatments 0 to 20, 20 to 0 and 20 to 20 for serum iron content, TIBC and transferrin saturation at week 17.

Peritoneal elicited macrophages from fish fed the iron-deficient diet had decreased chemotactic ratios at weeks 13 and 17, but the difference was not significant at week 13 (Table 5). When the fish were switched from the iron-deficient to the iron-replete diet (0 to 20), macrophage chemotaxis ratio significantly increased to a level comparable to that of fish fed the iron-replete diet throughout (20 to 20). On the contrary, this value significantly decreased for fish switched from the iron-replete to the iron-deficient diet

Table 4
Mean serum iron, total iron binding capacity (TIBC) and transferrin saturation of channel catfish fed iron-deficient and replete diets[1]

Period (week)	Dietary iron supplement	Serum iron (μg/d l)	TIBC (μg/d l)	Transferrin saturation (%)
13	0	16.9 ± 2.3^b	175.6 ± 4.5	9.6 ± 1.2^b
	20	44.7 ± 3.9^a	158.4 ± 18.9	29.2 ± 5.6^a
17	0–0	4.9 ± 3.4^b	251.5 ± 11.3^a	1.9 ± 1.3^b
	0–20	26.6 ± 2.1^a	170.6 ± 13.6^b	15.8 ± 2.5^a
	20–0	21.5 ± 3.1^a	157.8 ± 18.4^b	14.0 ± 3.6^a
	20–20	30.6 ± 3.1^a	146.2 ± 6.5^b	20.9 ± 2.4^a

[1]Values represent means of $n = 6$ determinations/treatment for week 13 and $n = 9$ for week 17. Column means($\pm$SE) within the same period having the same superscript are not significantly different ($P > 0.05$).

Table 5
Mean macrophage chemotaxis ratio of channel catfish fed iron-deficient and replete diets

Period (week)	Dietary iron supplement (mg/kg)	Macrophage chemotaxis ratio[1]
13	0	0.63 ± 0.09
	20	0.82 ± 0.05
17	0–0	0.67 ± 0.03^{c}
	0–20	0.82 ± 0.02^{ab}
	20–0	0.77 ± 0.02^{b}
	20–20	0.84 ± 0.01^{a}

[1]Ratio represents the number of migrating cells with *E. ictaluri* exoantigen divided by the number of migrating cells in the control (without *E. ictaluri* exoantigen) plus the number of migrating cells with *E. ictaluri* exoantigen. Values represent means of $n = 6$ determinations/treatment. Column means ($\pm$SE) within the same period having the same superscript are not significantly different ($P > 0.05$).

(20 to 0). However, no significant differences were observed among chemotactic ratios of fish in treatments 0 to 20 and 20 to 0.

The average daily cumulative mortality of fish following *E. ictaluri* challenge is presented in Fig. 2. Fish fed the iron-deficient diet began to die on day 1 post-challenge. Mortality started to occur 5 days post-challenge in fish fed the iron-replete diet. However, there were no significant differences in the cumulative mortality on days 5, 6 and 7 post-challenge among the treatments. All fish in both treatments died 7 days after challenge.

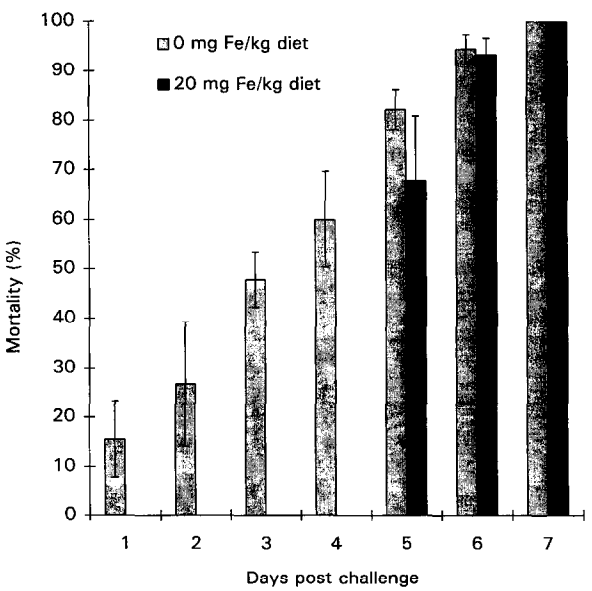

Fig. 2. Average daily cumulative mortality ($\pm$SE) of channel catfish fed the iron-deficient and replete diets after immersion challenge with *E. ictaluri* at the end of week 13.

4. Discussion

Channel catfish fed the iron-deficient diet had decreased appetite and feeding activity, suppressed growth, reduced hematocrit, total blood cell count, serum iron and transferrin saturation as previously reported by Gatlin and Wilson (1986) and Lim et al. (1996). A significant decrease in feed efficiency reported by Gatlin and Wilson (1986) in catfish fed iron-deficient diets was also observed in this study. Lim et al. (1996), however, reported that feed conversion was not affected by dietary iron level. Studies with red sea bream (Sakamoto and Yone, 1976), yellowtail (Ikeda et al., 1973), common carp (Sakamoto and Yone, 1978) and Atlantic salmon (Andersen et al., 1996) failed to detect the adverse effects of iron deficiency on growth and feed efficiency. Gatlin and Wilson (1986) and Lim et al. (1996) indicated that dietary iron had no effect on the mortality of channel catfish and the total iron binding capacity of the serum. An increased total iron binding capacity is one of the characteristics of iron-deficiency anemia in domestic animals (Kaneko, 1980). In the present study, these values did not differ at week 13. However, when fish were continued on the iron-deficient diet for another 4 weeks, a significant reduction in survival and increase in the level of total iron binding capacity were observed. Thus, based on the results of this study and those of Gatlin and Wilson (1986) and Lim et al. (1996), a feeding duration of more than 13 weeks was necessary to significantly affect survival and total serum iron binding capacity of channel catfish.

The chemotactic response of macrophages to *E. ictaluri* exoantigen as shown by the chemotactic ratios was consistently lower for fish fed the iron-deficient diet. The chemotactic ratio also decreased when the group fed the iron-replete diet were switched to the iron-deficient diet. This indicates that iron deficiency suppressed macrophage migration in the presence of exoantigen. Sealey et al. (1997) also observed a significant decrease in macrophage migration for fish fed iron-deficient diets. The suppression of macrophage chemotaxis, however, was reversed when the iron-deficient fish were fed the iron-replete diet for 4 weeks. Chandra and Saraya (1975) and MacDougall et al. (1975) showed that phagocytosis, as determined by the nitroblue tetrazolium (NBT) assay, was impaired in neutrophils of iron-deficient children, but the abnormality quickly returned to normal after treatment with iron. Berger (1996) reported that either a deficiency or an excess of iron could compromise the immune system.

It is generally believed that anemic animals are more susceptible to infection than those with adequate iron. Sealey et al. (1997) observed increased mortality of iron-deficient channel catfish due to ESC. However, with Atlantic salmon, it has been observed that fish fed a low-iron diet had some protection against *Vibrio anguillarum* (S.P. Lall, Institute of Marine Biosciences, National Research Council, Halifax, Canada, personal communication). Ravndal et al. (1994) observed a significant association between high concentrations of serum iron and mortality of Atlantic salmon infected with *V. anguillarum*. This is consistent with previous studies in infants that showed that mild iron deficiency provided protection against infection, whereas excessive iron enhanced infectious illness (Sherman, 1992). The present study, however, showed that dietary iron did not protect against mortality of channel catfish from *E. ictaluri*. However, the onset of mortality was delayed in fish fed the iron-replete diet. Fish fed the iron-deficient diet were observed to develop signs of hypoxemia after handling, which resulted in slough-

ing of mucus, skin discoloration and stiffening of the body. Thus, the earlier onset of mortality observed in this group of fish could be due to the synergistic effect of iron deficiency and *E. ictaluri* infection. The differences between the results of this study and those of Sealey et al. (1997) could not be explained. Even in humans in which numerous studies have been conducted, reports on the relationship between iron deficiency and infection is still a dilemma (Dallman, 1987; Bhaskaram, 1988). Bhaskaram (1988) suggested that iron deficiency has an adverse effect on the immunocompetence of the host and potentially increases susceptibility to bacterial infection. However, Sherman (1992) indicated that a delicate balance exists between the need for iron for host defense mechanisms and the need for iron to sustain microbial growth.

The results of this study show that fish fed an iron-deficient diet developed characteristic signs of hypochromic microcytic anemia as has been previously reported. Severe iron deficiency reduced the macrophage chemotactic response to *E. ictaluri* exoantigen. This abnormality was remedied by feeding an iron-replete diet for 4 weeks. Dietary iron, however, did not protect juvenile catfish against *E. ictaluri* infection, but the onset of mortality was delayed for fish fed the iron-replete diet. Further studies are needed to provide better understanding of the relationship between iron deficiency, immunocompetence and resistance to ESC.

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