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Effect of Vitamin C on Growth, Survival and Resistance to *Lernaea* Infection in Mrigal (*Cirrhinus mrigala*) Fingerlings

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Abstract.- Vitamin C was administered to three groups, each of twenty *Cirrhinus mrigala* fingerlings at a concentration of 60, 500 and 3000 mg/kg of diet for 53 days in separate 1000 L fibreglass tank to evaluate its efficacy on growth, survival and resistance to disease. A control group was not given vitamin C. There was no mortality throughout the rearing period. The fish nourished on feed containing 60 $mg.kg^{-1}$ vitamin C gained the highest weight, while control group grew the lowest. Fish in the rest of the two treatments grew equally well, though their weight increase was significantly lower than that of 60 mg vitamin C group. When these fish were exposed to thermal shock, there was gradual decrease in mortality with increasing vitamin C concentration. Likewise fish getting higher vitamin C concentration showed better resistance to *Lernaea* infection. Intensity of invasion gradually declined in fish fed on diets with elevated levels of vitamin C. The present studies suggest that vitamin C concentration up to 60 $mg.kg^{-1}$ of diet is the most appropriate concentration. Any quantity beyond this will not be economical at least for growth purposes, though higher concentration can enhance fish immunity.

Key words: Vitamin C, mrigal fingerlings.

INTRODUCTION

Vitamin C is essential for normal physiological functions in animals including fish (Wilson and Poe, 1973; Lim and Lovell, 1978). It is a biological reducing agent and is involved in many intra and extracellular processes, including assembly of collagen by hydroxylation of tryptophan, tyrosine and proline for use in cartilage synthesis. Collagen which is a principal constituent of skin, scales, mucous cartilaginous and conjunctive tissue formation, is heavily dependent on body storage of this vitamin and its supply from outside sources (Mc-Dowell, 1989). It is involved in carnitine and adrenal steroids synthesis and detoxifies pesticides and other toxicants using cytochrome P_{450} system (WHO, 1970; De Silva and Anderson, 1995). Vitamin C also plays a critical role in its repair and wound healing (Halver, 2002). It is highly labile to cooking and lengthy or improper storage. Hence its 10 fold excess addition to formulated feeds is mandatory (De Silva and Anderson, 1995). Like other teleost fishes *Cirrhinus mrigala* lacks the capability of biosynthesis of vitamin C due to absence of an essential enzyme gulono-lactone oxidase (Fracalossi *et al*., 2001). Thus ascorbic acid must be provided in the diet in stable form. Deficiency results in impaired collagen formation, spinal deformation, hemorrhage and growth retardation (Halver *et al*., 1969; Al-Amoudi *et al*., 1992; Gouillou Coustans *et al*., 1998).

Quantitative vitamin C requirements have been determined for several fish species. Values normally ranged from 20-50 mg ascorbic acid kg^{-1} of fish biomass (NRC, 1993). These variations in requirement have been attributed to species difference, stage of fish, type of diet, its processing methodologies and experimental environment (Halver, 2002). Not much work has been done on this aspect of this particular fish.

Cirrhinus mrigala is an important member of prevalent polyculture system of Indian and Chinese major carps. It is bottom feeder and thrives on detritus accummulated at pond bottom. Its growth rate is comparatively less than *Labeo rohita* but there is however, not much difference in flesh quality. It is less hardy than *Labeo* and hence is more amenable to common external and internal infections. Minor changes in feed or water quality

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can easily predispose this fish to *Lernaea* attack.

Keeping in mind these problems, studies were planned and primarily focused to improve its growth when cultured and resistance to disease or other routine stresses when exposed to them in daily life.

MATERIALS AND METHODS

Animals and experimental design

Cirrhinus mirgala fingerlings of average body weight 3.34±0.40 to 4.28±0.02 g, obtained from the Central Fish Hatchery, Lahore, were acclimated for two weeks. During this period fish were fed control diet. Proper water quality was maintained to avoid any unnecessary prior stress to the fish. Experiment was designed as one way analysis of variance (ANOVA).

Experimental procedure

Experimental system consisted of 8 fiberglass tanks, two for each treatment. After acclimation, 20 fish were randomly transferred in each half filled fiberglass tank of 1000 L water capacity. These tanks were then randomly assigned to four treatment groups. Each treatment has two replicates. Uniform aeration was provided to each tank when required. Fish fasted for 24 hours. Five fish were randomly collected from each tank anesthetized with MS 222 and weighed. Each diet was randomly assigned to a group of duplicate tanks. Fish was fed manually to satiation in each group at 8.00, 12.00 and 18.00 hours. Every third day, 1/3rd of total water volume was exchanged with tap water. Left over feed and fish feces deposited at the bottom was siphoned out to maintain proper water quality and tank hygiene.

Feeding trial for growth studies lasted for 4 weeks, while remaining 25 days were devoted to infection studies. At the termination of experiment, fish were starved for 24 hours. Water was lowered to quarter of the total volume. All the fish were harvested. Number of fish was counted to calculate survival rate and then weighed to estimate growth increments.

Preparation of diets

The practical diets were formulated from locally available feed ingredients (fish meal, 25%; corn gluten meal, 9.95%; sesame oil cake, 22%; wheat flour, 30%; molasses, 2%; bone meal, 1%; blood meal, 5%; mustard oil cake, 2%; vitamin premix, 2%; Dl-methionine 0.05% and gelain, 1%). Individual feed ingredients were not analyzed. Formulation was based on literature values. Formulated feed, however, was analyzed for crude protein, total lipids, ash contents, moisture and nitrogen free extract. Formulated diet with 32.73±3.5% crude protein, 7.89±1.6% total lipids, 31.40±4.7% nitrogen free extract, 4.6±0.4% ash and 9.52±2.3% moisture was well pulverized in a coffee grinder and then passed through 250 µm mesh and divided into 4 groups. Group 1 without vitamin C supplementation, was kept as control. Group 2 was supplemented with 60 mg L-ascorboyl-2-polyphosphate equivalent kg^{-1} of diet, group 3, 500 mg and group 4 received 3000 mg vitamin C kg^{-1} of diet. Vitamin C contents of feed were analyzed to confirm the presence of desired levels of vitamin C (2±0.3 in group 1, 55±5.2 in group 2, 475±20.3 in group 3, and 2800±75.7 in group 4). Boiled gelatin was incorporated into each dietary group @ 1% as binder. Diets were then soaked in water, well mixed and then dried in oven at 60^{o}C. Oven dried diets were crumbled and finely ground to match mouth gape of fish for easy ingestion of diet particles.

Exposure to thermal shock

Five fish were randomly removed from each tank and confined in 5 L 38^{o}C temperature water for 5 minutes. Dead fish were collected, counted and recorded to assess the intensity of thermal shock to individual treatment group.

Exposure to Lernaea

After weighing, remaining 15 fish in each tank, were challenged with *Lernaea* to measure level of resistance to infection in respective group. Water was raised to previous level. Fish was fed on control diet and similar water quality parameters were maintained as observed in growth trial. One infected fish bearing 5 *Lernaea* specimens was introduced into each tank. Experiment was run for another 25 days. On the termination of experiment each fish was individually observed for the number of *Lernaea* present. Total number of *Lernaea* specimens present at each fish, were counted and recorded to estimate the intensity of infection.

Chemical analysis

Proximate composition of diets was determined using standard methods (AOAC, 1995). A duplicate sample from each diet was dried overnight in convection oven at 105°C to constant weight to determine moisture (AOAC, 1995). Protein was determined by measuring nitrogen (N x 6.25) using Microkjeldahl method. Total lipids were extracted in petroleum ether for quantitative estimation using Soxhlet Lipid Extraction Method (AOAC, 1995). Ash contents were determined after incinerating the feed samples in a muffle furnace at 550°C. The concentrations of ascorbic acid in diets were determined by Reverse Phase HPLC (HP 1100, USA) with an ODS column (4.6 x 25 mm). An aqueous solution of 0.5 M KH_2 PO_4 (pH 2.8 with phosphoric acid) with flow rate of 0.6 ml min^{-1}, was used as mobile phase. The effluent was detected by a UV-detector at wave length of 254 nm.

Calculations and statistical analysis

One Way Analysis of Variance followed by Duncan's Multiple Range test was used to evaluate the statistical significance of differences among the treatments. Differences between the treatment means were considered significant at $p < 0.05$ level. Specific growth rate of fish and survival was calculated by the following mathematical expressions (Ricker, 1979).

$$\text{Specific growth rate} = (\log_e Y_2 - \log_e Y_1) \div (t_2 - t_1)$$

$$\text{Survival} = N_t \times 100 \div N_o$$

where Y_1 is initial weight of fish; Y_2 is final weight of fish; t_1 is initial time; t_2 is final time; N_o is initial number of fish in each replicate; and N_t is final number of fish in each replicate.

RESULTS

Growth and survival

After one month of experimental period, fish fed on diet with or without ascorbic acid supplementation did not show any physical deficiency signs as scoliosis, lordosis or caudal fin erosion. No mortality was observed and all the fishes in each treatment survived equally well. Differences, however, were prominent in growth. Fish supplemented with different concentrations of vitamin C, grew significantly better than control. Concentrations higher than 60 $mg.kg^{-1}$ of diet gradually depressed growth. Similar trend was observed in other growth parameters also and values proportionated to growth increments (Table I).

Table I.- Initial and final weights of the fingerlings in different dietary treatments. Values have been presented as Mean±SE.

Weights	Control	Diet with vitamin C		
		60 mg	500 mg	300 mg
Initial wt. (g)	4.28±0.02[a]	3.41±0.01[a]	3.34±0.40[a]	3.90±0.33[a]
Final wt. (g)	4.41±0.22[a]	4.29±0.22[b]	4.08±0.35[c]	4.39±0.23[c]
Wt. gain (g)	0.13	0.88	0.74	0.49
Specific growth rate	0	0.001	0.0007	0.0003

[a]Values in the columns having the same superscripts are not significantly different from each other at P<0.05.

Performance of fish to thermal shock

When fish were transferred to water kept at 38°C and confined there for 5 minutes, mortality rate was in proportion to vitamin C concentrations. Highest mortality (70%) in control group and lowest (30%) in fish fed on 3000 $mg.kg^{-1}$ vitamin C supplemented diet (Table II). There was decreasing trend in mortality when we moved from lower to high concentrations.

Table II.- Response of *Cirrhinus mrigala* fingerlings reared on artificial feed with different concentrations of vitamin C when exposed to temperature shock (38°C) and *Lernaea* infection.

Parameters	Control	Diet with vitamin C		
		60 mg	500 mg	300 mg
No. of fish exposed to thermal shock	10	10	10	10
No. of fish died	7	5	3	3
% mortality	70±6.0[a]	50±5.0[b]	30±6.0[c]	30±6.0[c]
No. of fish exposed to *Lernaea* infection	30	30	30	30
Average number of *Lernaea* observed at each fish	4±0.9[a]	3±0.8[b]	3±0.8[b]	2±0.07[c]

[a]Values sharing the same superscripts are not significantly different from each other at $p<0.05$.

Performance of fish to Lernaea *infection*

When fish were exposed to *Lernaea* infection, intensity of infection followed the same trend as in growth in the beginning but at higher concentrations unlike growth better resistance was observed in fish fed on feed containing higher concentrations of vitamin C. Number of *Lernaea* specimens were highest on control group while the lowest on the fish fed on diet containing the highest vitamin C concentration (Table II).

DISCUSSION

The studies were conducted in fiberglass tanks and totally on formulated artificial feeds. Any type of nutritional contribution from natural food was quite minimal if not negligible. Over one month of observation did not produce any deformity like scoliosis or lordosis, caudal fin erosion or mortality. They might have appeared if fish were exposed to low concentrations for longer duration as it have occurred in Japanese sea bass (Ai *et al*., 2004). All the fish survived equally well. Possibly deficiency level where death could ensue, was not reached in this limited period.

Growth increments, however, were very much obvious. Fish fed on 60 $mg.kg^{-1}$ vitamin C containing diet yielded the highest weight while control group (without vitamin C supplementation) the lowest. The other two groups ranked in the middle of these two extremes, comparatively higher in 500 $mg.kg^{-1}$ and lower in 3000 $mg.kg^{-1}$ group. Ai *et al*. (2004) reported similar growth trends in Japanese sea bass, Kumari and Sahoo (2005) in Asian catfish, Ai *et al*. (2006) in large yellow croaker (*Pseudosciaena crocea*) and Misra *et al*. (2007) in *Labeo rohita* when fed on diets with different concentrations of vitamin C. In our studies growth rates reached plateau at or above 60 $mg.kg^{-1}$ vitamin level which could not be differentiated due to wide ranged concentrations. This may be the requirement level of *Cirrhinus* at this age group and adequate enough to maintain normal growth and physiological functions, which agree favorably well with previous studies (Al-Amoudi *et al*., 1992; Gouillou-Coustans *et al*., 1998; Shiau and Hsu, 1999 and Wang *et al*., 2003). Growth rate not only levelled off at higher doses but showed inverse relationship with increased vitamin C concentration. Hence declining trend was observed at 3000 mg vitamin C kg^{-1}. Although similar observations have been recorded by Kumari and Sahoo (2005) on Asian catfish but reasons for this return to initial levels is not clear at this moment and demands further comprehensive investigations.

When fish was exposed to thermal shock, survival rate of fish paralleled to the relative rise in vitamin C concentrations in the diet. Maximum individuals died in control group but lowest in 3000 vitamin C concentration group. Although it had an inhibitory effect on growth but it certainly improved the resistance of fish against routine stressor at hatcheries which can result into mass mortality. Other stressors like high stocking density (Monterro *et al*., 1999) and 2 hours confinement (Thompson, 1993) have been reported to have little or no effect after feeding fish on vitamin C containing diets.

Lernaea is an external parasite. *Catla,* mrigal and grass carp are more prone to its infestation than *Labeo rohita* and common carp (personal observation). It does not spare any developmental stage of these fish species and is a real nuisance at hatcheries. It does not cause immediate death but leaves a long lasting growth inhibitory effect on fish. It was a meager initiative and an attempt to control or at least retard its infestation in fish body. Although our deliberations were not conclusive but they were definitely suggestive. Gradual decrease in intensity was obvious from control group towards higher concentrations. The elevated vitamin C concentrations may have caused some changes in collagen formation, unfavorable to massive *Lernaea* attack which inhibited its access and made penetration of its horn difficult in fish skin. Feeding high levels of ascorbic acid has been reported to enhance protection against bacterial infections, *viz*., *Edwardsiella tarda* (Durve and Lovell, 1982), *E. ictaluri* (Li and Lovell, 1985), *Vibrio angillarum* (Navarre and Halver, 1989), *Aeromonas salmonicida* (Waagbo *et al*., 1993), *Aeromonas hydrophila* (Sobhana *et al*., 2002) and against parasitic infection (*Ichthyophthirius multifilis*) (Wahli *et al*., 1986; Shariff *et al*., 1986).

Most of the previous studies have been focused on the effect of vitamin C supplementation on the non-specific immune response of fish where it worked as modulator to immune system and created resistance against disease in Atlantic salmon (Hardie *et al*., 1991), Japanese seabass (Ai *et al*., 2004) and Asian catfish (Kumari and Sahoo, 2005). In the present study we are reporting the effect of vitamin C on the infestation rate of an external parasite.

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