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Dietary Ascorbic Acid Requirements of Fingerlings of Genetically Improved Rohu (*Labeo rohita* Ham)

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Key words: *Labeo rohita*, ascorbic acid

Abstract

Indian major carp, including *Labeo rohita*, are incapable of biosynthesizing ascorbic acid due to the absence of the enzyme L-gulono-γ-lactone oxidase. To assess their ascorbic acid requirements, improved rohu fingerlings (2.33±0.18 g) were fed one of six semi-purified formulated diets containing 0, 20, 40, 60, 80, or 100 mg ascorbyl-2-polyphosphate (APP) per kg feed for 60 days in water of 28-30°C. Fish fed diets deficient in ascorbic acid had a significantly lower weight gain, poor feed conversion (FCR) and protein efficiency (PER) ratios, lower survival, and behavioral abnormalities such as lethargic movements and poor feed intake. The best FCR and PER were recorded in the 60 mg APP diet. Ascorbic acid in the kidney dropped from 36.62 to 5.09 mg/kg by the end of the experiment. Weight gain analysis by regression indicated that the dietary ascorbic acid requirement for maximum growth and survival of rohu fingerlings can be achieved with 53.5 mg APP incorporated into 1 kg diet.

Introduction

Rohu (*Labeo rohita* Ham.) is the most important cultured species in the freshwater ecosystem of India (Ayyappan and Jena, 1998). Following the classic approach of selective breeding for improved growth, an improved variety of rohu called Jayanti was developed from rohu stocks of the Ganga, Gomati, Yamuna, Sutlej, and Brahmaputra Rivers and the Central Institute of Freshwater Aquaculture (CIFA) farm stock. The fifth generation of improved rohu exhibits 17% growth enhancement over the original farm stock (Reddy, 2003; Das Mahapatra et al., 2007). To achieve its genetic potential for growth, it must be fed a reasonably-priced nutritionally-adequate diet.

Ascorbic acid is a multifunctional indispensable vitamin involved in vital physiologi-

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cal functions. Multiple signs of this deficiency have been reported in fish (Mahajan and Agrawal, 1980; Teskeredzic et al., 1989; Adham et al., 2000; Chen et al., 2003; Xie and Niu, 2006). Rohu cannot synthesize ascorbic acid *de novo* due to its lack of the enzyme L-gulono-γ-lactone oxidase (Sahoo and Mukhopadhyay, 2005a,b). Therefore, this species depends on an exogenous supply for normal growth, health, and reproduction (Mukhopadhyay et al., 1998).

As ascorbic acid in crystalline form is unstable and thermolabile, bioavailable forms such as ascorbyl-2-polyphosphate (APP), ascorbyl sulfate (AS), ascorbyl palmitate (AP), and ascorbyl monophosphate (AMP) are now used in feed formulation. Phosphate derivatives of ascorbic acid provide good feed stability, superior bioactivity, and confirmed resistance to oxidation (Grant et al., 1989; Sandnes, 1991; Waagbo et al., 1991; Cho and Cowey, 1993).

The purpose of this investigation was to determine the optimum dietary requirement of vitamin C in diets for genetically improved rohu fingerlings using APP as the source of vitamin C.

Materials and Methods

Fish and rearing system. Three hundred fingerlings (avg 2.33±0.18 g) of improved rohu (Jayanti) were collected from the nursery ponds of the Fish Genetics and Biotechnology Division of CIFA. The fish were acclimated to laboratory conditions in a 700-l tank for one week during which they were fed a diet free of ascorbic acid to deplete ascorbate pools in tissues and body stores as much as possible.

Experimental diets. A basal diet containing no ascorbic acid was prepared as the control (Table 1). Five experimental diets containing various levels of ascorbic acid were prepared by adding 20, 40, 60, 80, or 100 mg ascorbyl 2-polyphosphate (Rovimix Stay C 25%, Hoffmann LaRoche Basel) to one kg feed. The actual amounts of ascorbic acid in the feeds were insignificantly lower than planned. Finely powdered ingredients were thoroughly mixed. Gelatin was added separately after being moistened in a measured volume of lukewarm water. The ingredients were kneaded thoroughly with necessary additions of water and blended to produce dough. The dough was spread on a flat surface, cut into small cubes, and dried in atmospheric temperature in a glass chamber. The prepared diets were kept in air-tight plastic containers and stored in a refrigerator until use. To prevent loss of ascorbic acid during storage, no more than a week's ration was prepared at a time.

Table 1. Composition of basal diet (46.25% crude protein, 5.0% lipid) to which 20, 40, 60, 80, or 100 mg ascorbyl 2-polyphosphate per kg feed was added.

Ingredient	*%*
Casein[1]	40
Dextrin	25
Gelatin	20
Vitamin/mineral mixture[2]	10
Soya lecithin	2
Vegetable oil	3

[1]Casein (protein content 85.32 %) without vitamin C (Himedia, Mumbai 400086, India)
[2]Vitamin mineral premix (no vitamin C): 500,000 IU vitamin A; 100,000 IU vitamin D_3; 0.2 g vitamin B_2; 75 units vitamin E; 0.1 g vitamin K; 0.25 g calcium pantothenate; 1.0 g nicotinamide; 0.6 g vitamin B_{12}; 15 g choline chloride; 75 g calcium; 2.75 g manganese; 0.1 g iodine; 0.75 g iron; 1.5 g zinc

Feeding, sampling, analyses. Fingerlings were stocked in eighteen 25-l glass aquaria (30 x 30 x 30 cm) at a rate of ten fish per aquarium and three replicates per treatment. A constant water level of 22 l was maintained. Feed was provided twice daily (10:00 and 17:00) at a rate of 2% of the body weight per day for 60 days. Fecal matter was carefully siphoned from the water every day. Water was exchanged at a daily rate of 75% to maintain water quality. Replenishing water passed through a bolting silk cloth (no. 29) to prevent entry of phyto and zooplankton. Water temperature, recorded daily, was 28-30°C. Dissolved

oxygen, pH, etc., were measured weekly following standard methods (APHA, 1989).

Every week, fish were removed from the aquaria, anesthetized in MS-222 (100 mg/l), measured, and weighed. After sampling, fish were dipped into a dilute $KMnO_4$ solution (0.2%) to prevent bacterial and fungal infestation. The voluntary feed intake (VFI), growth (percent body weight gain), and feed conversion ratio (FCR) were calculated and the feed quantity was adjusted accordingly.

At the end of the feed trial, the fingerlings were removed, anesthetized in MS-222 (100 mg/l), measured, and weighed. Kidney samples were removed from each fingerling and preserved at -80°C for subsequent analysis of ascorbic acid. Since the kidney (head and trunk) is the most important storage organ for ascorbic acid in fish (Gabaudan and Verlhac, 2001), this tissue was chosen to analyze ascorbic acid content and determine the vitamin C requirement of the species. The ascorbic acid content of the kidney of fish fed the control diet was monitored weekly during the 60-day trial to determine the changes that would occur in fish fed a diet free of ascorbic acid.

Tissue ascorbic acid assay. Kidney tissues from the ten fish in each aquarium were pooled and homogenized in ice-cold 250 mM $HClO_4$ containing 5% trichloroacetic acid (TCA). The homogenates were centrifuged at 10,000 x *g* for 10 min, then transferred to fresh tubes. Total ascorbic acid was estimated spectrophotometrically coupling with 2,4-dinitrophenylhydrazine (DNPH) as described by Dabrowski and Hinterleitner (1989).

Statistical analysis. Growth performance and survival were analyzed by one way ANOVA using the SPSS/PC program. When ANOVA identified differences in mean weight gain, FCR, PER, or SGR, multiple comparisons were made with the Duncan New Multiple Range Test (Puri and Mullen, 1980). Statistical significance was determined by setting the aggregate type I error at 5% ($p<0.05$) for each comparison set. Quadratic regression was taken for all cases (weight gain, FCR, and PER) because there was little difference between r^2 values of quadratic and cubic regression.

Results

In the control and 20 mg treatment, behavioral abnormalities including lethargic movements and poor feed intake were observed 38 days into the feeding trial. Morphological changes were seen in about 30% of the fish in these groups, including caudal fin erosion and discoloration (dark black) of the body. These abnormalities were not apparent in other groups.

Survival was highest (95%) in the 40 mg group, followed by the 60 and 80 mg groups (90%), 100 mg (85%) group, and 20 mg group (80%); the lowest survival (65%) was in the control (Fig 1). The best growth, FCR, and PER were obtained in the 60 mg treatment (Table 2). The ascorbic acid content of the kidney in the control group dropped consistently with time (Fig. 2). At the end of the trial, the ascorbic acid content in the kidney was lowest in the control and rose with the increasing level of ascorbyl 2-polyphosphate (Fig. 3). The ascorbic acid content in the kidney positively correlated with growth to the 60 mg level and then decreased. Using regression analysis for weight gain, FCR, and PER, the dietary requirement for the genetically improved rohu fingerlings was 53.5 mg ascorbyl 2-polyphosphate per kg feed.

Discussion

Results indicate the need for dietary ascorbic acid for survival and normal growth of genetically improved rohu fingerlings. The requirement for optimal growth was 53.5 mg/kg diet, as provided in the 60 mg treatment. In common carp larvae, the dietary requirement was 45 mg ascorbic acid/kg feed, based on growth performance, while the required level of ascorbic acid stored in tissues was higher, i.e., 350 mg ascorbic acid/kg diet (Gouillou-Coustans et al., 1998). It is expected that tissue levels reach a maximum or saturation level beyond which they do not increase with further increases in dietary concentration; the excess intake is excreted or metabolized (Cho and Cowey, 1993; Gouillou-Coustans and Kaushik, 2001). In newly hatched *Cirrhina mrigala*, an Indian major carp, fed purified diets with graded levels of crystalline ascorbic acid,

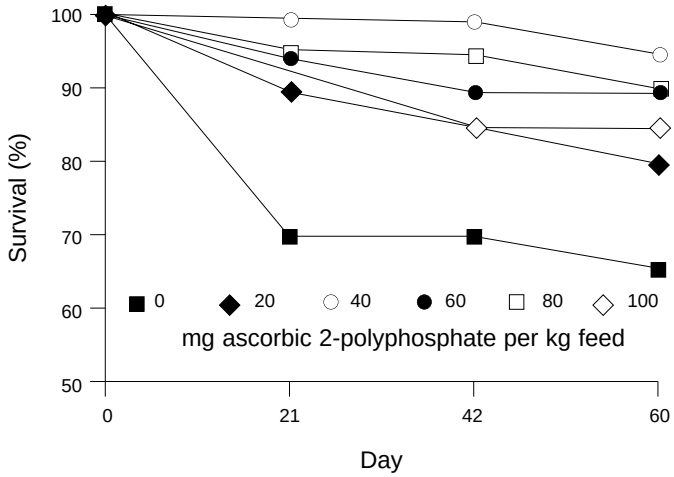

Fig. 1. Survival (%) of rohu fingerlings fed different amounts of dietary ascorbyl 2-polyphosphate (from 0-100 mg per kg feed).

Table 2. Weight gain (%), specific growth rate (SGR), feed conversion ratio (FCR), and protein efficiency ratio (PER) in rohu fingerlings fed diets with different amounts of ascorbyl-2-polyphosphate (vitamin C)

	Vitamin C (mg/kg diet)					
	0	*20*	*40*	*60*	*80*	*100*
Wt gain (%)	54.75±0.55^{a}	65.3±7.4^{ab}	68.4±6.3^{ab}	77.5±5.95^{b}	60.17±6.17^{ab}	56.9±1.0^{a}
SGR	0.78±0.01^{a}	0.84±0.08^{ab}	0.87±0.12^{ab}	0.95±0.1^{b}	0.86±0.07^{ab}	0.75±0.01^{a}
FCR	2.77±0.08^{a}	2.38 ±0.15^{ab}	2.31 ±0.24^{ab}	2.13±0.13^{b}	2.59±0.11^{ab}	2.76±0.04^{a}
PER	0.55±0.01^{a}	0.64±0.04^{ab}	0.66±0.07^{ab}	0.71±0.04^{b}	0.58±0.02^{ab}	0.55±0.01^{a}

Values in a row with different superscripts differ significantly at $p<0.05$.

the optimum requirement was 650-700 mg ascorbic acid/kg feed based on weight gain, mortality, and behavioral and morphological criteria (Mahajan and Agrawal, 1980). This level appears to be on the high side, possibly due to the use of crystalline ascorbic acid which is an unstable form of vitamin C. Using enriched zooplankton with ascorbyl palmitate, the ascorbic acid requirement of rohu larvae was 1409 mg/kg (Mitra and Mukhopadhyay, 2003). This also appears to be high, possibly due to the increasing requirements of larvae as they grow (Mahajan and Agrawal, 1980). Recently Mishra et al. (2007) reported better immunity, growth, and survival in rohu fingerlings after feeding diets containing 500 mg/kg ascorbic acid for eight weeks. Their results agree with Sahoo and Mukherjee (2003) that high dietary vitamin C enhanced the non-specific immunity of fish, including an enhanced phagocyte ratio, increased serum lysozyme activity, and protection against *Aeromonas hydrophila* infection.

In channel catfish, there is no significant

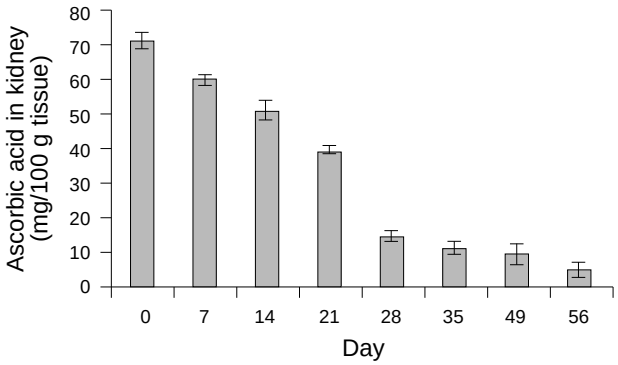

Fig. 2. Ascorbic acid contents in kidneys of control fish at weekly intervals throughout the experiment.

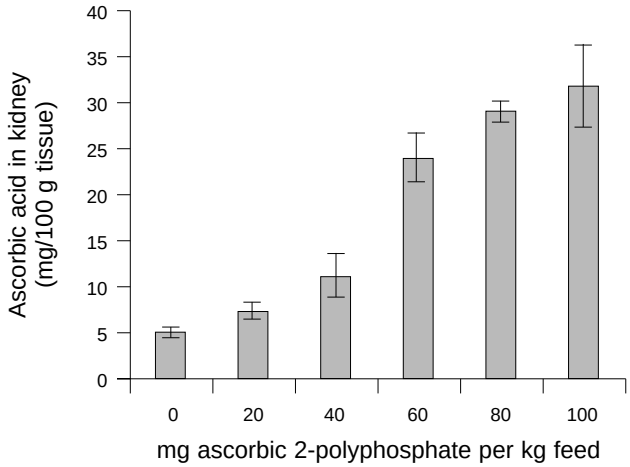

Fig. 3. Ascorbic acid contents in kidneys of experimental fish at the end of the experiment.

difference in dietary ascorbic acid requirements when crystalline and polyphosphate forms of ascorbic acid are used (Andrews and Murai, 1975; Murai et al., 1978). In tilapia (*Oreochromis niloticus*), however, the polyphosphate form of ascorbic acid is more effective than the crystalline form as Soliman et al. (1994) found the requirement to be 1250 mg crystalline ascorbic acid/kg for normal growth and feed efficiency while Abdelghany (1996) found that fingerlings require only 50 mg ascorbic acid/kg body weight when fed the polyphosphate form. The loss of activity in crystalline ascorbic acid during manufacture and storage of feed exceeds that in other forms of ascorbic acid, such as coated (Marchetti et al., 1999), sulfate (Shiau and Hsu, 1993), monophosphate (Shiau and Hsu, 1993), and polyphosphate (Volker and Fenster, 1994). However, ascorbyl polyphosphate proved to be a highly stable form of vitamin C, resistant to leaching and oxidation

during feed processing and storage (Moreau et al., 1998). Ascorbyl polyphosphate in fish feeds is stable during feed processing and highly bioavailable. The polyphosphate protects the vitamin C from oxidation and is easily acted upon by phosphatases in the digestive tract to make the ascorbic acid available.

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