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Effects of dietary vitamin C levels on tissue ascorbic acid concentration, hematology, non-specific immune response and gonad histology in broodstock Japanese eel, *Anguilla japonica*

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ABSTRACT

A feeding trial was conducted to evaluate the effects of dietary vitamin C (L-ascorbic acid, AA) levels on tissue AA concentration, hematology, non-specific immune response and gonad histology in male broodstock Japanese eel. A basal commercial diet was used as a control, and four other diets were prepared by supplementing 200, 400, 800 or 1600 mg AA kg^{-1} diet in the form of L-ascorbyl-2-monophosphate (AMP). The analyzed AA concentrations of the diets were 32, 206, 423, 840 and 1686 mg kg^{-1} diet, respectively. Triplicate groups of fish (initial body weight, 360 g) were fed one of the test diets at a ratio of 2% body weight for 16 weeks. At the end of the feeding trial, AA concentration in liver, kidney, muscle and testes was increased significantly in a dose dependent manner by increment of dietary AA level ($P < 0.05$). Significantly higher hematocrit value was found in fish fed 1686 mg AA kg^{-1} diet compared to 32 and 423 mg AA kg^{-1} diets. White blood cells count of fish fed 1686 mg AA kg^{-1} diet was significantly higher than those of fish fed 32–206 mg AA kg^{-1} diet. Plasma glucose concentration was increased significantly with increasing dietary AA levels up to 840 mg AA kg^{-1} diet; also significant enhancements in aspartate aminotransferase and alanine aminotransferase values were obtained at 1686 mg AA kg^{-1} diet. Significantly higher superoxide dismutase activity was recorded in fish fed 840–1686 mg AA kg^{-1} diet compared to 32–206 mg AA kg^{-1} diet. The results of gonad histology showed an enhanced number of spermatogonia by dietary AA increment. The optimal dietary AA requirement level was estimated at approximately 410.8 and 911.8 mg AA kg^{-1} diet by broken-line regression analysis based on liver and testes AA concentration, respectively.

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1. Introduction

Vitamins are organic compounds that can be synthesized from other essential nutrients to spare a portion of the dietary requirements; however, they are required in trace amounts from an exogenous source for normal growth, reproduction and health (NRC, 2011). Vitamin C (L-ascorbic acid, AA) is an essential micronutrient for normal growth and physiological function of most aquatic animals (Lim and Lovell, 1978; Ren et al., 2007) due to the absence of L-gulonolactone oxidase that is responsible for AA biosynthesis (Ai et al., 2006; Fracalossi et al., 2001). AA is a strong antioxidant that is capable of scavenging reactive oxygen species (Bae et al., 2012). It participates in synthesis of steroid hormones and collagen (Cavalli et al., 2003; Hunter et al., 1979; Lightner et al., 1979), tolerance to toxicants and environmental stressors (Agrawal et al., 1978; Ishibashi et al., 1992; Merchie et al., 1995). It has also been proposed to be potentially beneficial in increasing immune response (Lin and Shiau, 2005) and reducing oxidative damage to tissues (Dabrowski, 2001; Khassaf et al., 2003). One frequently overlooked problem is that AA uptake is highly dose dependent. Inadequate supply of dietary AA usually results in a number of deficiency symptoms such as impaired collagen formation, internal hemorrhaging, retarded growth, suppressed immunity, anorexia, erratic swimming behavior (Ai et al., 2006; Al-Amoudi et al., 1992; Gouillou-Coustans et al., 1998; Zehra and Khan, 2012) and increased mortality in fish (Dabrowski, 1992; Zehra and Khan, 2012). However, subjects saturated with AA through their daily diet will efficiently excrete any surplus and are therefore highly unlikely to benefit from further AA supplementation (Lykkesfeldt and Poulsen, 2010). This and several other issues should be taken into account while drawing conclusions from randomized controlled trials with the purpose of studying the effects of AA.

Dietary AA requirement has been determined for several cultured juvenile fish species including parrot fish, *Oplegnathus fasciatus* (Wang et al., 2003); rohu, *Labeo rohita* (Misra et al., 2007); tilapia, *Oreochromis karongae* (Nsonga et al., 2009); Jian carp, *Cyprinus carpio* (Liu et al., 2011); Japanese eel, *Anguilla japonica* (Bae et al., 2012) and Mrigal, *Cirrhinus mrigala* (Zehra and Khan, 2012). The estimated AA requirements vary among species (NRC, 2011). The variations in AA requirements

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have been attributed to fish species, fish size, and the differences in diet formulation and culture conditions (Ai et al., 2006).

The Japanese eel, *A. japonica*, is a freshwater fish and very important cultured species in East Asia due to its high market value, desirable taste and recent supply shortage (Ren et al., 2007). Five major producers of this species are China, Japan, Korea, Malaysia and Taiwan. The eel aquaculture industry in these countries has been growing e.g. in Korea its production increased from 2739 tons in 2000 to 6766 tons in 2009 (Son et al., 2011). Nevertheless, the eel farming industry relies exclusively on wild-caught juveniles (Butts et al., 2014). Broodstock nutrition, particularly in the case of Japanese eel, is one of the most poorly understood and researched areas of finfish nutrition (Izquierdo et al., 2001). To a large extent, this has been due to the necessity of suitable indoor or outdoor culture facilities for maintaining large groups of broodstock fish and the consequent higher cost of running and conducting extended broodstock feeding trials. However, as in human and livestock nutrition, it is clear that the dietary nutrient requirements of broodstock will be different from those of rapidly growing juvenile animals (Leboulanger, 1977). Moreover, it is also clear that many of the deficiencies and problems encountered during the early rearing phases of newly hatched finfish larvae are directly related to the feeding regime (including nutrient level and duration) of the broodstock. To our knowledge, there is no available study on regarding to dietary AA supplementation in male broodstock Japanese eel. Therefore, the present study was undertaken to evaluate the effects of varying dietary AA levels on growth, tissue AA concentration, hematology, non-specific immune response and histological changes of testes in male broodstock Japanese eel.

2. Materials and methods

2.1. Experimental diets

A basal commercial diet (Woosung feed Co., South Korea) (Table 1) without AA supplementation was used as a control diet, and four other diets were prepared by supplementing 200, 400, 800 or 1600 mg AA kg^{-1} to the basal diet. In this study L-ascorbyl-2-monophosphate (AMP) (Sigma-Aldrich, Germany) was used as AA sources due to its high heat resistance in comparison to unprotected AA and its excellent availability to fish (Dabrowski et al., 1994). The actual AMP levels of the experimental diets were determined by High-Pressure Liquid Chromatography (HPLC; Dionex, Softron, USA). The analyzed AA concentrations of the diets were 32, 206, 423, 840 and 1686 mg kg^{-1} diet, respectively. The targeting levels of AA were mixed with the powdered commercial diet at the expense of cellulose, and then stored at −20 °C until diet preparation. Tap water was added to the feeds (1.5% of diet weight) and a ball-shaped dough was made before feeding.

2.2. Experimental fish and feeding trial

One-year-old broodstock of Japanese eel were obtained from an eel Research Center (Yeonggwang, South Korea). Prior to the start of the feeding trial, the health status of the fish was checked and they were starved for 24 h. All the fish were fed the basal commercial diet for four weeks to become acclimatized to the experimental conditions and facilities. Three hundred fish averaging at 360 ± 10 g (mean ± SD) were weighed and randomly distributed into 15 indoor fiberglass tanks (20 fish/tank) with 500-l volume receiving a constant flow (4 l min^{-1}) of filtered freshwater. Males and females were stocked together; there were about 15 males and 5 females at the beginning of the experiment. Each tank was then randomly assigned to one of three replicates of 5 dietary treatments. During the experiment, supplemental aeration was provided in each tank to maintain enough dissolved oxygen, and also water heated by electric heaters in a concrete reservoir. Water temperature and pH during the experiment were maintained at 28.3 ± 0.05 °C and 7.83 ± 0.02, respectively. Fish were fed twice daily (05:00 and 17:00 h) for 16 weeks at a fixed rate of 2% body weight per day. Dead fish were removed immediately and weighed, and the amounts of feed for the tanks were adjusted to the proper percentage of the remaining fish weight in the tanks. Uneaten feed was siphoned out after 1 h of feeding and tank inside was scrubbed once per week to minimize algal and fungal growth.

Table 1
Proximate analyses (dry basis) of the basal commercial diet.[1]

Calculated composition	Amount
Moisture (%)	6.64 ± 0.06
Crude protein (%)	48.8 ± 0.05
Crude lipid (%)	5.21 ± 0.06
Crude ash (%)	11.5 ± 0.01
Crude fiber (%)	2.39 ± 1.03
Carbohydrate (%)	25.4 ± 1.78
Gross energy (kcal g^{-1})	3.43 ± 0.05

[1] Values are mean of duplicate samples and presented as mean ± SD.

2.3. Sample collection and analysis

At the end of the feeding trial, fish were starved for 24 h, and the total number and weight of fish in each tank were determined for calculation of final weight (FW), specific growth rate (SGR), feed efficiency (FE) and survival. Three fish per tank were randomly selected, individually weighed, then dissected to obtain liver and testes organs for determination of hepatosomatic index (HSI) and gonadosomatic index (GSI); thereafter, the same testes were used for histology observations. Three additional fish were randomly sampled from each tank, and then liver, kidney, muscle, brain and testes were removed from each fish and pooled for determining AA concentration. Another three fish per tank were randomly captured, anesthetized with ethylene glycol phenyl ether (200 mg l^{-1} for 5–10 min), and blood samples were collected from the caudal vein with heparinized syringes for determination of white blood cell (WBC) and red blood cell (RBC) count, hematocrit (PCV), hemoglobin (Hb) and respiratory burst activity (NBT). After the abovementioned measurements with whole blood, plasma was separated by centrifugation at 5000 ×*g* for 10 min and stored at −70 °C for determination of blood biochemical parameters including plasma total protein, alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose, cortisol and cholesterol. Another set of blood samples of the same fish were taken without heparin and allowed to clot at room temperature for 30 min. Then, the serum was separated by centrifugation at 5000 ×*g* for 10 min and stored at −70 °C for the analysis of non-specific immune responses including lysozyme, myeloperoxidase (MPO) and superoxide dismutase (SOD) activities; and thiobarbituric acid-reactive substances (TBARS) activity as lipid peroxidation indicator.

The testes tissue fixed in the 10% neutral buffered formalin were dehydrated in a graded ethanol series and embedded in paraffin. Tissue blocks were sectioned (4 μm thick) and stained with hematoxylin and eosin (H&E). Tissue sections were examined under an AX70 Olympus (Japan) microscope for common and/or significant lesions.

Analysis of moisture, crude protein, lipid, ash and fiber in the feed was performed using standard methods (AOAC, 1995), and carbohydrate content was measured by Bomb Calorimeter (PARR 1351, Co., Illinois, USA). The energy value was determined on the basis of physiological fuel value, i.e., 3.99 kcal g^{-1} proteins or carbohydrates and 9.01 kcal g^{-1} lipids (Lee and Putnam, 1973). Samples of diets, fish and liver were dried to constant weights at 105 °C to determine their moisture contents. Ash was determined by incineration at 550 °C, crude lipid was determined by soxhlet extraction using the Soxtec system 1046 (Tecator AB, Hoganas, Sweden), and crude protein content was determined by the Kjeldahl method (N × 6.25) after acid digestion.

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