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Dietary protein enhances non-specific immunity, anti-oxidative capability and resistance to *Aeromonas hydrophila* in *Labeo rohita* fingerlings pre-exposed to short feed deprivation stress

Sona Yengkokpam [a, *, 1], Dipesh Debnath [a, 1], N.P. Sahu [a], A.K. Pal [a], K.K. Jain [a], Kartik Baruah [b]

[a] Division of Fish Nutrition, Biochemistry and Physiology, ICAR-Central Institute of Fisheries Education, Fisheries University Road, Versova, Mumbai 400 061, India

[b] Laboratory of Aquaculture & Artemia Reference Centre, Faculty of Bioscience Engineering, Department of Animal Production, Ghent University, 9000 Ghent, Belgium

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ABSTRACT

Present experiment was conducted to study the effect of dietary protein levels on growth, immunity and anti-oxidative status of *Labeo rohita* fingerlings during feed deprivation followed by refeeding. Fish (5.44 ± 0.10 g) were deprived of feed for 3 weeks and then re-fed to satiation for 5 weeks with one of the diets containing 25 (25P), 30 (30P), 35 (35P) or 40 (40P) percent crude protein (CP) level. In addition to these groups, a control group (C) was also maintained by feeding to satiation level twice daily with a diet containing 30% CP throughout the experimental period. At the end of 8-weeks' trial, fish were challenged with *Aeromonas hydrophila* and survival was recorded for the next 7 days. Complete recovery of growth in terms of weight gain percentage was achieved in the fish fed 35 and 40% protein during refeeding. The body indices (condition factor and hepatosomatic index), haematological parameters and serum protein contents at the end of the experimental trial were not significantly different ($P > 0.05$) among different groups suggesting that the overall health of the fish was not compromised. However, respiratory burst activity and serum lysozyme activity were indicative of a better immune function in the higher protein fed groups (35P and 40P) than the lower protein groups (25P and 30P). Following challenge with *Aeromonas hydrophila*, survival rate, blood monocyte%, respiratory burst activity, serum lysozyme activity, serum protein and globulin were significantly higher ($P < 0.05$) in the 35P and 40P groups compared to the other groups. Further, fish fed lower dietary protein were not able to restore the activities of anti-oxidative enzymes (superoxide dismutase and catalase) in the liver. Conclusively, an improved disease resistance capability and immune status was observed in the fish fed a higher dietary protein (35–40%), even out-performing the daily-fed fish.

1. Introduction

Aquaculture management strategies such as short-term feed deprivation and refeeding are generally adopted by farmers to induce compensatory growth during refeeding regime. Such practices reduce feeding cost as well as save time required for feed dispensing without affecting the growth of the fish. There is a growing interest in this area with various studies of compensatory growth on fish reporting complete compensation [1–4], partial compensation [4,5], no compensation [6] or overcompensation [7]. Majority of the past studies focused on growth and body composition [1,3,4,8], metabolic responses [9–11], digestive capacity [11–14] and a few on anti-oxidative defenses [15–17] and immunological responses [18–20]. Starvation studies in fish have been less controversial [13] as many fish species face natural starvation periods in part of their life cycle due to winter, spawning or pre-spawning stage, and fish has the capacity to withstand long periods of feed deprivation [21].

Fish immune systems are affected by nutritional state [22,23]. The non-specific functions are often reduced in undernourished

* Corresponding author.
E-mail address: sonayen@gmail.com (S. Yengkokpam).
[1] Present address: ICAR-Central Inland Fisheries Research Institute, Regional Centre, HOUSEFED Complex, Dispur, Guwahati 781006, India.

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fish, while antibody production is less affected [24]. There is a direct relationship between resistance to some diseases and good nutritional status, since stress caused due to lack of nutrients intensifies infections caused by opportunistic etiologic agents [25]. For instance, in channel catfish, *Ictalurus punctatus*, it was observed that the fish that were not fed (2 and 4 weeks) were more susceptible to bacterial (*Flavobacterium columnare*) infection than the daily- and alternate-day fed groups [26]. On the other hand, there were also reports that nutritional restriction or starvation sometimes augments innate immunity, as observed in mammal [27,28]. However, little is known about the immune status of a fish subjected to feed deprivation and refeeding, except for a study in red porgy, *Pagrus pagrus*, where refeeding for 7 days after a 14-day fast did not significantly affect the haematological, biochemical and immunological parameters [20]. In addition, the influence of diet composition on such adaptations has been scarcely studied.

Starvation is known to cause oxidative stress and increase the activities of anti-oxidative enzymes, such as superoxide dismutase (SOD), catalase and glutathione peroxidase as reported in fish [15,16]. It is well established that superoxide anions (O_2^-) are released as a result of oxidative stress. The SOD catalyzes superoxide anions to molecular oxygen and hydrogen peroxide, while catalase reduces the hydrogen peroxide, thus providing antioxidant protection [29]. It is also a fact that starvation-induced stress leads to heat shock protein (HSP) 70 production in fish [30,31].

Labeo rohita is one of the most widely cultured freshwater carps in the Indian subcontinent. In our previous study, *L. rohita* fingerlings that were deprived of feed for 1 or 2 weeks and refed for 5 weeks could achieve complete compensatory growth, while feed deprivation of 3 weeks followed by refeeding for 5 weeks only supported a partial compensatory growth [4]. A dietary protein content of 30%, which was optimum protein requirement for *L. rohita* fingerlings [32], was used in this study during refeeding. However, feeding higher dietary protein may further improve growth of the fish as nutrient composition of a feed can play important roles in compensatory growth and also in restoring the immune status of the fish. With this background, the present study was conducted to study the effect of dietary protein on the growth, immune status and anti-oxidant status of *L. rohita* fingerlings which were starved for 3 weeks and refed with diets containing varying protein levels for 5 weeks.

2. Materials and methods

2.1. Fish and experimental design

Labeo rohita fingerlings, procured from Palghad fish farm, Mumbai, Maharashtra, were acclimated to the experimental conditions for one month. During the acclimation period, fish were fed a diet containing 30% crude protein (CP) twice daily. Three hundred fingerlings (av. wt. 5.44 ± 0.10 g) were distributed in five treatment groups with quadruplicates following a completely randomized design. Four experimental diets were formulated containing 25, 30, 35 or 40% CP (Table 1). Fish in the control group (C) were fed to satiation level twice daily with the diet containing 30% CP throughout the experimental period. Fish in the other four treatment groups were deprived of feed for 3 weeks and then re-fed to satiation for 5 weeks with a diet containing 25 (25P), 30 (30P), 35 (35P) or 40 (40P) % CP. Measured amount of feed were given to the fish and after 1 h, uneaten feed were siphoned, dried and weighed. Total feed was divided into two halves and given at 08:00 and 18:00 h. Water exchange was done manually at every alternate day and continuous aeration was done. The water quality parameters, i.e. temperature, pH, dissolved oxygen (DO), carbon dioxide (CO_2), ammonia-nitrogen and nitrite-nitrogen were recorded every week following the standard methods [33] and were found to be within the acceptable range for carp culture. DO and pH ranged from 7.3 to 7.9 ppm and 7.4 to 8.5, respectively. The ammonia and nitrite levels varied between 0.11–0.27 ppm and 0.03–0.15 ppm, respectively and the water temperature ranged from 26.2 to 28.0 °C.

Table 1
Ingredients and proximate composition of the experimental diets.

Ingredients	Experimental diets			
	25% CP	30% CP	35% CP	40% CP
Fish meal	15	15	15	15
Soybean meal	24	32	44	56
Wheat flour	15	15	15	13
Rice polish	35	27	15	5
Soybean oil	4	4	4	4
Cod liver oil	2	2	2	2
CMC[a]	2	2	2	2
Emix (Vit-Min mix)[b]	2.9	2.9	2.9	2.9
Vitamin C	0.1	0.1	0.1	0.1
Proximate composition (% dry matter basis) (mean ± SE; n = 3)				
Crude protein (%)	25.1	30.54	34.55	40.3
Ether extract (%)	6.12	6.2	6.32	6.58
Nitrogen free extract (%)	42.86	37.99	34.63	29.15
Crude fibre (%)	6.01	5.71	4.01	3.58
Total ash (%)	12.6	12.3	12.8	13.2
Moisture	7.31	7.26	7.69	7.19
Digestible energy (kcal/100 g)	326.92	329.92	333.60	337.02

[a] Carboxymethylcellulose.
[b] Vitamin-mineral mix (Emix™ plus) (quantity/2.5 kg): Vitamin A-55,00,000 IU; Vitamin D_3-11,00,000 IU; Vitamin B_2-2000 mg; Vitamin E-750 mg; Vitamin K-1000 mg; Vitamin B_6-1000 mg; Vitamin B_{12}-6 mg; Calcium panthothenate-2500 mg; Niacinamide-10 gm; Choline chloride-150 gm; Mn-27,000 mg; Iodine-1000 mg; Fe-7500 mg; Cu-2000; Zn-5000 mg; Co-450 mg; Ca-500 g; P-300 g; Se-50 ppm; L-Lysine-10 g; DL-methionine-10 g; Carrier-q.s; Lactobacillus-120 million units and Yeast culture-3000 crore units.

2.2. Sampling

At the end of the feeding trial, 2 fish from each replicate (8 fish per treatment) were anaesthetized with clove oil at 50 μl/l. Blood was withdrawn from the caudal vein using a medical syringe previously rinsed with 2.7% ethylene diamine tetra-acetic acid (EDTA) disodium salt (as an anticoagulant), immediately transferred to a microcentrifuge tube containing a thin layer of EDTA powder and shaken well to prevent blood haemolysis. The blood was used for the determination of haemoglobin content, total erythrocyte and leukocyte counts, and for respiratory burst activity. For serum, another 2 fish from each replicate were anaesthetized. Blood was collected without anticoagulant, allowed to clot for 2 h followed by collection of straw coloured serum and stored at −20 °C until use. For the assay of anti-oxidative enzymes, liver tissues of the fish were dissected, weighed and homogenized in chilled sucrose solution (0.25 M) in a glass tube using a tissue homogenizer (MICCRA D-9, Digitronic, Germany) so as to prepare a 5% homogenate. The tube was continuously kept in ice bath while homogenizing. The homogenate was centrifuged at 10,000×*g* for 20 min at 4 °C in a cooling centrifuge, supernatant collected and stored at −20 °C for further analysis.

2.3. Growth and body indices

The various growth parameters of the fish were calculated as follows: weight gain % = (final weight − initial weight)/initial weight × 100; Feed conversion ratio (FCR) = total feed consumed (g)/weight gain; Protein efficiency ratio (PER) = weight gain (g)/protein fed (g); Apparent net protein utilization (ANPU) = increase