Mixed feeding schedule of low and high protein in the diet of *Labeo rohita* (Hamilton) fingerlings: effect on growth performance, haemato-immunological and stress responses

B. XAVIER[1,2], K.K. JAIN[1], A.K. PAL[1], N.P. SAHU[1], G. MAHESWARUDU[2], D. GAL[3] & S. KUMAR[1,3]

[1] *Department of Fish Nutrition and Biochemistry, Central Institute of Fisheries Education, Mumbai, India;* [2] *Regional Centre of Central Marine Fisheries Research Institute, Visakhapatnam, India;* [3] *National Agricultural Research and Innovation Centre, Research Institute for Fisheries and Aquaculture (NAKI – HAKI), Szarvas, Hungary*

Abstract

Four experimental diets D_1, D_2, D_3 and D_4 having 100, 300, 350 and 400 g protein kg^{-1} diet, respectively, were prepared. Fingerlings of *Labeo rohita* (5.45 ± 0.03 g) were fed with different diets following a mixed feeding schedule with the feeding cycle of 28 days that consisted of either 28 days feeding of diet D_1 or D_2 or 21 days feeding with D_1 diet and 7 days with either D_3 or D_4 diet. Different treatments were T_1 (D_1 for 28 days), T_2 (D_2 for 28 days), T_3 (D_1 for 21 days and D_2 for 7 days), T_4 (D_1 for 21 days and D_3 for 7 days) or T_5 (D_1 for 21 days and D_4 for 7 days). Fingerlings were sampled after three feeding cycles (84 days). Higher ($P < 0.05$) growth performance and protein utilization efficiency were found in the T_3 group, which was similar to the T_2 group. The blood count, NBT, lysozyme activity, serum parameters and stress indicator parameters also complement for the better immunity in the T_2 and T_3 groups. Therefore, it could be concluded that fingerlings feeding alternately a low-protein diet (D_1) for 21 days followed by a normal-protein diet (D_2) for 7 days in a cycle of 28 days for 84 days maintained better growth and health status.

KEY WORDS: cortisol, growth performance, *Labeo rohita*, lysozyme, mixed feeding schedule, nitroblue tetrazolium

Introduction

The increasing cost of the fish feed ingredients has drawn the attention of researchers to reduce the cost of the most expensive item, the protein source (Wee & Wang 1987), through proper formulation along with adopting economic feeding strategies. However, regular feeding of such fish protein feed to carp raises the question whether this much protein is a actual requirement on daily basis. Indian major carp need 300–400 g protein kg^{-1} diet (Shetty & Nandeesha 1988). De Silva (1985) reported that feeding of fish everyday with the same level of protein is not economical. Use of mixed feeding schedules in reducing feed costs and improving nutrient utilization has been demonstrated by various authors in different fish species such as Indian major carp, *Catla catla* (Nandeesha *et al*. 1993, 1994); common carp, *Cyprinus carpio* (Srikanth *et al*. 1989; Nandeesha *et al*. 1995, 2002); nile tilapia, *Oreochromis niloticus* (Patel & Yakupitiyage 2003); sutchi catfish, *Pangasius hypophthalmus*; and silver carp, *Hypophthalmichthys molitrix* (Ali *et al*. 2005).

Labeo rohita, an Indian major carp, is one of the most preferred species in the Indian subcontinent, which contributes more than 60% of the total carp production in India (Mohanta *et al*. 2008). *Labeo rohita* is primarily herbivorous to omnivorous species and prefers to feed on plant materials (Talwar & Jhingran 1991). Over the last decade, the culture of this fish in India has intensified, and so there is an increasing demand for the development of a cost-effective aqua feed that could maintain the growth and health status of this species.

The health of fish has often been reported in terms of the relationship between the increase in weight and length.

However, there is a need to understand the physiological concept of fish health in relation to blood parameters and the quality of dietary protein fed. Any changes in the constituent component of blood sample when compared to the normal values could be used to interpret the metabolic state of the animal and the state of health (Babatunde *et al.* 1982). Therefore, this study was aimed to assess the effect of mixed feeding schedule on growth, haemato-immunological and stress responses in *Labeo rohita* fingerlings and justifies the effect of such schedule on the fish health.

Materials and methods

Diet

Four diets were formulated that contained four levels of crude protein (CP): 100 g kg^{-1} diet (D1), 300 g kg^{-1} diet (D2), 350 g kg^{-1} diet (D3) or 400 g kg^{-1} diet (D4). Ingredient compositions of the different experimental diets are presented in Table 1. All the ingredients except vitamin–mineral mixture and vitamin C were mixed in a big plastic bowl to get a homogeneous ingredient mixture. Dough was formed with the addition of a required amount of water. Then, oil was added to the dough and mixed thoroughly

for uniform distribution. The dough was then allowed for 1 h at room temperature for proper conditioning followed by steaming for 10 min in a pressure cooker. The vitamin–mineral premix (Emix™ plus, India) along with vitamin C was added after cooling. Finally, the dough was mixed and pressed through a hand pelletizer to get uniform-sized pellets. After that, the pellets were air-dried for 4 h and then kept in an oven at 50 °C until complete drying. After drying, the pellets were packed in an airtight polythene bag, labelled properly and stored at 4 °C until use.

Experimental animals

Fingerlings of *Labeo rohita* were brought from fish seed farm (Kosamba, Gujarat, India). Fish were transported in a circular container (500 L) with sufficient aeration to the wet laboratory of Fish Nutrition and Biochemistry Lab, Central Institute of Fisheries Education, Mumbai, India. They were carefully transferred to a circular tank (1000 L) and were left undisturbed the whole night. To ameliorate the handling stress, the fingerlings were given a mild salt treatment (30 g kg^{-1}) on the next day. About 50% of water was exchanged every day. The stock was acclimatized under aerated conditions for a period of 30 days.

Table 1 Ingredient composition of the diets and proximate composition of the diets

Ingredients (g kg^{-1})	D1 (100 g protein kg^{-1} diet)	D2 (300 g protein kg^{-1} diet)	D3 (350 g protein kg^{-1} diet)	D4 (400 g protein kg^{-1} diet)
Fish meal	–	150	200	250
Soybean	20	360	410	480
Rice bran	400	120	90	50
Wheat bran	360	150	90	50
Wheat flour	150	150	140	100
Cod liver oil: sunflower oil (1 : 2)	50	50	50	50
CMC	5	5	5	5
Vitamin–mineral mix[1]	14	14	14	14
Vitamin C	1	1	1	1
Proximate composition of the diets (g kg^{-1} DM)				
Organic matter	908	908	906	905
Protein	128	309	360	412
Lipid	57	53	53	56
Ash	151	98	95	92
Crude fibre	163	115	105	102
Nitrogen free extract	505	426	388	338

CMC, carboxy methyl cellulose.
[1] Vitamin–mineral mix = composition of vitamin–mineral mix (EMIX PLUS) (quantity/2.5 kg): Vitamin A 55 00 000 IU; Vitamin D$_3$ 1 100 000 IU; Vitamin B$_2$ 2000 mg; Vitamin E 750 mg; Vitamin K 1000 mg; Vitamin B$_6$ 1000 mg; Vitamin B$_{12}$ 6 μg; Calcium pantothenate 2500 mg; Nicotinamide 10 g; Choline chloride 150 g; Mn 27 000 mg; I 1000 mg; Fe 7500 mg; Zn 5000 mg; Cu 2000 mg; Co 450 mg; Ca 500 g; P 300 g; L-lysine 10 g; DL-methionine 10 g; Selenium 50 ppm.

Experimental design

Two hundred and twenty-five *Labeo rohita* fingerlings (average weight 5.45 ± 0.03 g) were equally distributed in the five treatments groups with three replicates each, following a completely randomized design in 15 tanks (150 L). Continuous aeration was provided to all tanks from a compressed air pump, and water was exchanged on alternate days. The experiment was continued for 84 days. During this period, the fingerlings were fed with different diets following a mixed feeding schedule with the feeding cycle of 28 days that consisted of either 28 days feeding of diet D_1 (100 g protein kg^{-1} diet) or D_2 (300 g protein kg^{-1} diet) or 21 days feeding with D_1 (100 g protein kg^{-1} diet) diet and 7 days with either D_2 (300 g protein kg^{-1} diet), D_3 (350 g protein kg^{-1} diet) or D_4 (400 g protein kg^{-1} diet) diet. Different treatments were T_1 (D_1 fed for 28 days), T_2 (D_2 fed for 28 days), T_3 (D_1 for 21 days and D_2 for 7 days), T_4 (D_1 for 21 days and D_3 for 7 days) or T_5 (D_1 for 21 days and D_4 for 7 days) (Table 2). Fingerlings were fed twice a day (08:00 and 18:00 h) to apparent satiation.

Growth study

Fingerlings in each tank were bulk-weighed at each cycle to assess the weight gain. Growth performance of fish such as percentage weight gain, specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER) and apparent net protein utilization (ANPU) was calculated based on the following standard formulae:

$$\text{Weight gain}\,(\%) = [(\text{Final weight} - \text{Initial weight})/ \text{Initial weight}] \times 100$$

$$\text{SGR}\,(\%) = [\ln \text{Final weight} - \ln \text{Initial weight}/\text{Number of days}] \times 100$$

Table 2 Details of experimental diet fed groups with normal and mixed feeding schedule

Experimental groups	Details
T_1	D1 diet (100 g protein kg^{-1} diet) for 28 days
T_2	D2 diet (300 g protein kg^{-1} diet) for 28 days
T_3	D_1 (100 g protein kg^{-1} diet) for 21 days and D_2 (300 g protein kg^{-1} diet) for 7 days
T_4	D_1 (100 g protein kg^{-1} diet) for 21 days and D_3 (350 g protein kg^{-1} diet) for 7 days
T_5	D_1 (100 g protein kg^{-1} diet) for 21 days and D_4 (400 g protein kg^{-1} diet) for 7 days

$$\text{FCR} = \text{Total dry feed intake}\,(g)/\text{wet weight gain}\,(g)$$

$$\text{PER} = \text{Net weight gain}\,(\text{wet weight})/\text{Protein fed}$$

$$\text{ANPU} = [(\text{Final carcass protein} - \text{Initial carcass})/\text{Protein fed}] \times 100$$

Proximate analysis of experimental diets and whole body

Proximate composition of all the diets and whole body tissue was analysed by the standard methods of AOAC (1995). Moisture was determined by drying the sample at 105 °C to a constant weight. Nitrogen content of the sample was analysed using Kjeltec system (2200 Kjeltec Auto distillation; Foss Tecator, Hoganas, Sweden), and crude protein (CP) was calculated by multiplying the nitrogen percentage by 6.25. Ether extract (EE) was determined using Soxtec system model SD2 (1045 Soxtec Extraction Unit; Tecator) using diethyl ether (boiling point, 40–60 °C) as a solvent, and ash content was estimated by incinerating the sample in a muffle furnace at 600 °C for 6 h. The digestible energy (DE) value of experimental diets and tissue was calculated as described by Halver (1976).

Sample preparation

At the completion of the experiment, the liver and intestine were taken from three fish from each tank for each treatment. Fingerlings were anaesthetized with CIFECALM (50 µL L^{-1}) (Verma *et al.* 2007), an herbal anaesthetic formulation containing natural alcoholic extracts of *Eugenia caryophyllata* and *Mentha arvensis* (developed by Central Institute of Fisheries Education, Mumbai, India), and dissected to collect the liver and intestine for digestive enzyme (amylase and protease) estimation. Samples of liver and intestine were homogenized in chilled sucrose solution (0.25 M) in a mechanical tissue homogenizer to prepare 5% homogenate and were then centrifuged (5000 *g* at 4 °C for 20 min). Supernatant was collected and frozen (−20 °C) for enzyme assays. Whole intestine was used for amylase and protease assays. Before homogenization, the intestinal contents were removed.

Digestive enzymes

Amylase activity of liver and intestine was measured by estimating the reducing sugars produced due to the action

of glucoamylase and α-amylase on carbohydrates (Rick & Stegbauer 1974). The reaction mixture consisted of 1% (w/v) starch solution, phosphate buffer and the tissue homogenate. The reaction mixture was incubated at 37 °C for 30 min. Dinitro salicylic acid (DNS) was added to stop the reaction and was kept in boiling water bath for 5 min. After cooling, the reaction mixture was diluted with distilled water and absorbance was measured at 540 nm. Maltose was used as the standard, and amylase activity was expressed as μmol of maltose released from starch per min per 100 mg protein at 37 °C.

Total proteolytic activity of liver and intestine was measured using the casein hydrolysis method (Walton 1986). Enzyme reaction mixture consisted of 1% (w/v) casein solution, phosphate buffer (pH 8.0) and the tissue homogenate, which was incubated for 1 h at 37 °C. The reaction was stopped by adding 6% trichloroacetic acid (TCA). After holding for 1 h at 4 °C, samples were centrifuged (Remi, India Ltd., India) at 10 000 *g* for 10 min and the absorbance of the supernatant was recorded at 280 nm. The reagent blank was made by adding the supernatant just before stopping the reaction with TCA without incubation. Tyrosine was used as the standard, and one unit of enzyme activity is defined as the amount of enzyme needed to catalyse the formation of 1 μg of tyrosine per min.

Blood collection

At the end of the feeding trial, three fishes from each replicate, with a total of nine fishes per treatment, were anaesthetized with clove oil (50 μL L^{-1}). Blood samples were drawn from the caudal peduncle using a syringe, which was previously rinsed with 2.7% EDTA solution as an anticoagulant. The blood samples were used immediately for analysis of haemoglobin, total erythrocyte count (RBC) leucocyte count (WBC) and NBT assay. Blood was collected from another nine fishes from each treatment without anticoagulant and was allowed to clot for 2 h. The collection of straw-coloured serum was performed with micropipette and stored at −20 °C for the serum parameter estimation.

Haematological parameters

The haemoglobin percentage was determined by estimating cyanmethemoglobin using Drabkin's fluid (Qualigens, India). Five millilitres of Drabkin's working solution was taken in a clean and dry test tube, and 20 μL of blood was added into it. The absorbance was measured using a spectrophotometer (MERCK, Nicolet, evolution 100) at a wavelength of 540 nm. The final concentration was calculated by comparing with standard cyanmethamoglobin (Qualigens).

The total erythrocyte (RBC) and leucocyte (WBC) counting was performed using the respective diluting fluids (Qualigens). Twenty microlitres of blood was mixed with 3980 μL of diluting fluid in a clean glass vial. The mixture was shaken well to suspend the cells uniformly in the solution. The cells were counted using a haemocytometer. The number of RBC and WBC per mL of the blood sample was calculated using the formula:

$$\text{No of cells} \,(mL^{-1}) = \text{No of cells counted} \times \\ \text{Dilution/Area counted} \times \\ \text{depth of fluid}$$

Respiratory burst activity

The respiratory burst activity of the phagocytes was measured by nitroblue tetrazolium (NBT) assay following the method of Secombes (1990) subsequently modified by Stasiack & Bauman (1996). Fifty microlitres of blood was placed into the wells of 'U' bottom microtitre plates and incubated at 37 °C for 1 h to facilitate the adhesion of cells. Then, the supernatant was removed and the loaded wells were washed three times with PBS. After washing, 50 μL of 0.2% NBT was added and plate was incubated for further 1 h. The cells were then fixed with 100% methanol for 2–3 min and again washed thrice with 30% methanol. The plates were then air-dried. Sixty microlitres of 2 N potassium hydroxide and 70 of microlitres dimethyl sulphoxide were added to each well to dissolve the formazon blue precipitate formed. The OD of the turquoise blue-coloured solution was then read in ELISA reader at 540 nm.

Serum lysozyme activity

Serum lysozyme activity was measured using an ion exchange chromatography kit (Bangalore Genei, India). Serum samples were diluted with phosphate buffer (pH 7.4) to a final concentration of 0.33 mg mL^{-1} protein. In a suitable cuvette, 3 mL of *Micrococcus luteus* (Bangalore Genei) suspension in phosphate buffer (A450 = 0.5–0.7) was taken, to which 50 μL of diluted serum sample was added. The content of the cuvette was mixed well for 15 s, and a reading was taken in a spectrophotometer at 450 nm exactly 60 s after the addition of the serum sample. This absorbance was

compared with the standard lysozyme of known activity following the same procedure as above. The lysozyme activity was expressed as U min^{-1} mg^{-1} protein of serum.

Serum total protein, albumin and globulin

Serum protein was estimated by the Biuret and BCG dye binding method using a kit (total protein and albumin kit; Qualigens Diagnostics, Glaxo Smithkline). Albumin was estimated by the bromocresol green binding method. The absorbance of the standard and test was measured against a blank in a spectrophotometer at 630 nm.

$$Serum\ total\ protein\,(g\,\%) = (Absorbance\ of\ sample/$$
$$Absorbance\ of\ Standard) \times 6$$

$$Albumin\,(g\%) = (Absorbance\ of\ sample/$$
$$Absorbance\ of\ standard) \times 4$$

Serum globulin was determined by subtracting the albumin values from the total serum protein. The albumin:globulin ratio was determined.

Stress parameters

Blood glucose Blood glucose was estimated by the method of Nelson & Somogyi (1945). Blood was deproteinized with zinc sulphate and barium hydroxide and filtered, and the supernatant was used for the estimation of glucose. The supernatant was taken in a test tube, and alkaline copper sulphate was added and then placed in a boiling water bath for 20 min. The test tubes were then cooled to room temperature, arsenomolybdate reagent was added, and absorbance was recorded at 540 nm against a blank.

Serum cortisol Cortisol in fish serum was estimated using a validated radioimmunoassay (EIAKIT DSL -10–2000) kit method. The kit was purchased from Diagnostic Systems Laboratories, Mumbai. The serum cortisol was expressed as $ng\ mL^{-1}$. The procedure follows the basic principle of enzyme immunoassay where there is competition between an unlabelled antigen and an enzyme-labelled antigen for a fixed number of antibody-binding sites. The amount of enzyme-labelled antigen bound to the antibody is inversely proportional to the concentration of the unlabelled analyte present. Unbound materials are removed by decanting and washing the wells.

Statistical analysis

Statistical analysis of the data was carried out using the software SPSS version 14.0. ANOVA was used to compare the treatment effects. Comparison among the means was made using Duncan's multiple range tests (Duncan 1955).

Results

Proximate composition of the diets and whole body

The proximate composition of the different experimental diets ($g\ kg^{-1}$ DM) and whole body tissue ($g\ kg^{-1}$ DM) is presented in Tables 1 and 3, respectively. Among the different feeding schedules, the T_1 group registered significantly the lowest ($P < 0.05$) tissue protein and the T_2 group registered the highest ($P < 0.05$), which was similar to the T_3 group. Increase in dietary protein level during mixed feeding schedule from 300 g protein kg^{-1} diet (T_3) to 350 g protein kg^{-1} diet (T_4) significantly ($P < 0.05$) decreased the tissue protein content and similarly between 350 g protein kg^{-1} diet (T_4) and 400 g protein kg^{-1} diet (T_5). The lowest ($P < 0.05$) moisture content and the highest ($P < 0.05$) lipid content in the tissue were found in the T_2 group.

Growth and nutrient utilization

Significantly highest ($P < 0.05$) weight gain (%) and SGR were recorded in the T_3 group, which was similar ($P > 0.05$) to the T_2 group and lowest ($P < 0.05$) in the T_1 group (Table 4). Weight gain (%) and specific growth rate (SGR) increased significantly ($P < 0.05$) with the increase in dietary protein level from 100 g protein kg^{-1} diet (T_1) to 300 g protein kg^{-1} diet (T_2), whereas among the cyclic feeding groups, they decreased significantly ($P < 0.05$) with the increase in dietary protein level from 300 g protein kg^{-1} diet (T_3) to 350 g protein kg^{-1} diet (T_4) and 400 g protein kg^{-1} diet (T_5) (Table 3). The growth rate was almost similar in all groups up to 56 days, and it was observed that as the level of protein increased in the cyclic feeding group, the growth rate was gradually decreased (Fig. 1). Significantly decreased ($P < 0.05$) FCR and increased ($P < 0.05$) PER and ANPU were found with increase in the dietary protein level from 100 g protein kg^{-1} diet (T_1) to 300 g protein kg^{-1} diet (T_2) (Table 4). The increased ($P < 0.05$) FCR and decreased ($P < 0.05$) PER and ANPU were observed in the cyclic

Table 3 Proximate composition (g kg^{-1} wet weight) of body tissue of *Labeo rohita* fingerlings maintained on mixed feeding schedule for 84 days (Mean ± SE)

Tissue composition	Treatments				
	T_1	T_2	T_3	T_4	T_5
Moisture	762^d ± 13	739^a ± 20	747^b ± 13	755^c ± 12	755^c ± 1
Protein	127^a ± 8	165^d ± 13	159^c ± 4	145^b ± 10	149^b ± 1
Lipid	24^a ± 1	33^c ± 0	26^b ± 0	26^b ± 0	26^b ± 0
Ash	38^b ± 8	31^a ± 1	30^a ± 0	30^a ± 0	31^a ± 0

Mean values bearing different superscripts in a row are significantly ($P < 0.05$) different.

Table 4 The growth performance of *Labeo rohita* fingerlings maintained on mixed feeding schedule for 84 days (Mean ± SE)
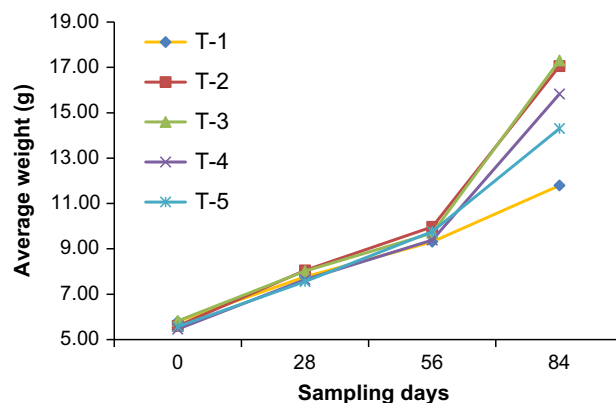
Parameters	Treatments				
	T_1	T_2	T_3	T_4	T_5
Initial body weight (gm)	5.65 ± 0.01	5.65 ± 0.04	5.65 ± 0.03	5.46 ± 0.03	5.57 ± 0.04
Final body weight (gm)	11.79^a ± 0.06	17.06^d ± 0.01	17.31^d ± 0.07	15.83^c ± 0.03	14.31^b ± 0.05
Weight gain (%)	109.56^a ± 1.42	206.02^d ± 2.30	209.11^d ± 2.54	189.85^c ± 2.62	156.69^b ± 1.47
SGR	2.45^a ± 0.01	2.82^d ± 0.01	2.83^d ± 0.01	2.74^c ± 0.01	2.64^b ± 0.01
FCR	3.77^d ± 0.05	2.09^a ± 0.02	2.00^a ± 0.04	2.23^b ± 0.01	2.69^c ± 0.04
PER	1.62^a ± 0.02	1.87^b ± 0.03	2.64^d ± 0.08	2.23^c ± 0.01	1.71^a ± 0.02
ANPU	15.12^a ± 0.24	34.67^d ± 0.77	34.52^d ± 0.77	30.20^c ± 0.09	21.62^b ± 0.47

Mean values bearing different superscripts in a row are significantly ($P < 0.05$) different.

feeding group with the increase in dietary protein level from 300 g protein kg^{-1} diet (T_3) to 350 g protein kg^{-1} diet (T_4) and 400 g protein kg^{-1} diet (T_5). Among all groups, the lowest FCR and highest ANPU were observed in the T_3 group, which was similar to the T_2 group, whereas the highest FCR and lowest ANPU were recorded in the T_1 group.

Digestive enzymes activity

Amylase and protease activities in liver and intestine of *L. rohita* fingerlings were significantly ($P < 0.05$) affected due to different dietary protein levels as well as to cyclic feeding schedule (Table 5). The liver and intestinal amylase activities significantly ($P < 0.05$) increased with the increase in dietary protein level from 100 g protein kg^{-1} diet (T_1) to 300 g protein kg^{-1} diet (T_2), whereas decreased ($P < 0.05$) liver amylase activity was found with the increase in dietary protein level from 300 g protein kg^{-1} diet (T_3) to 350 g protein kg^{-1} diet (T_4) and 400 g protein kg^{-1} diet (T_5) during different cyclic feeding schedules. The highest amylase activity in the liver as well as in the intestine was recorded in the T_3 group. Similarly, protease activity in both the liver and intestine was also recorded the highest in the T_3 group.

Figure 1 The mean growth increment of *Labeo rohita* fingerlings maintained at mixed feeding schedules over 84 days.

Haematological parameters

Different feeding schedules had significant effect on total leucocyte (WBC) count and differential cell count of *L. rohita* fingerlings (Table 6). Total leucocyte count and differential cell (monocyte, lymphocyte and granulocyte) count were increased significantly ($P < 0.05$) with the increase in dietary protein level from 100 g protein kg^{-1} diet (T_1) to 300 g protein kg^{-1} diet (T_2), whereas among the cyclic feeding groups, they decreased significantly

Table 5 Effect of mixed feeding schedule on digestive enzymes activity (μmol min^{-1} 100 mg^{-1} protein) of *Labeo rohita* fingerlings (Mean $\pm$ SE)

Treatments	Amylase		Protease	
	Liver	Intestine	Liver	Intestine
T_1	54.82^a $\pm$ 0.74	83.59^b $\pm$ 0.12	16.35^a $\pm$ 0.20	26.75^a $\pm$ 1.04
T_2	72.75^b $\pm$ 1.75	100.03^c $\pm$ 0.17	24.84^d $\pm$ 0.14	35.62^c $\pm$ 0.34
T_3	78.18^b $\pm$ 5.49	103.40^d $\pm$ 0.59	25.86^e $\pm$ 0.19	39.88^d $\pm$ 0.80
T_4	47.41^a $\pm$ 1.92	77.67^a $\pm$ 0.47	18.74^b $\pm$ 0.25	28.47^a $\pm$ 0.49
T_5	46.24^a $\pm$ 1.29	85.74^b $\pm$ 1.60	22.24^c $\pm$ 0.62	31.03^b $\pm$ 0.56

Mean values bearing different superscripts in a row are significantly ($P < 0.05$) different.

Table 6 Haematological parameters of *Labeo rohita* fingerlings maintained on mixed feeding schedule for 84 days (Mean $\pm$ SE)

Treatments	WBC (10^3 cells mm^{-3})	Lymphocyte (%)	Monocyte (%)	Granulocyte (%)	RBC (10^6 cells mm^{-3})	Haemoglobin (g%)	Haematocrit (%)
T_1	107.50^a $\pm$ 0.30	95.70^a $\pm$ 3.50	4.60^b $\pm$ 0.22	2.52^a $\pm$ 0.24	1.77^a $\pm$ 0.02	10.65^a $\pm$ 0.09	17.45^a $\pm$ 0.25
T_2	142.50^c $\pm$ 0.50	128.00^c $\pm$ 1.00	8.22^c $\pm$ 0.18	4.50^c $\pm$ 0.30	2.57^b $\pm$ 0.12	12.80^c $\pm$ 0.20	31.65^c $\pm$ 0.85
T_3	162.50^d $\pm$ 0.50	148.70^d $\pm$ 0.70	11.42^d $\pm$ 0.32	3.70^b $\pm$ 0.14	2.79^b $\pm$ 0.07	13.25^d $\pm$ 0.15	32.60^c $\pm$ 1.10
T_4	110.50^b $\pm$ 0.50	102.50^b $\pm$ 0.50	4.69^b $\pm$ 0.19	3.71^b $\pm$ 0.17	1.88^a $\pm$ 0.01	11.65^b $\pm$ 0.15	23.25^b $\pm$ 0.45
T_5	107.50^a $\pm$ 0.50	98.55^{ab} $\pm$ 0.45	3.90^a $\pm$ 0.25	2.62^a $\pm$ 0.13	1.53^a $\pm$ 0.12	10.50^a $\pm$ 0.01	24.15^b $\pm$ 0.65

Mean values bearing different superscripts in a column are significantly ($P < 0.05$) different.

($P < 0.05$) with the increase in dietary protein level from 300 g protein kg^{-1} diet (T_3) to 350 g protein kg^{-1} diet (T_4) and 400 g protein kg^{-1} diet (T_5). Significant increase ($P < 0.05$) in RBC count, haemoglobin content and haematocrit value was recorded with the increase in dietary protein level from 100 g protein kg^{-1} diet (T_1) to 300 g protein kg^{-1} diet (T_2), whereas among the cyclic feeding groups, they decreased significantly ($P < 0.05$) with the increase in dietary protein level from 300 g protein kg^{-1} diet (T_3) to 350 g protein kg^{-1} diet (T_4) and 400 g protein kg^{-1} diet (T_5).

Serum total protein, albumin (A), globulin (G) and albumin/globulin (A/G) ratio

A significant difference ($P > 0.05$) in the serum total protein, albumin, globulin and A/G ratio was found among the various treatment groups at the end of the experiment (Table 7). Increase in dietary protein level from 100 g protein kg^{-1} diet (T_1) to 300 g protein kg^{-1} diet (T_2) significantly ($P < 0.05$) increased the total protein, albumin (A), globulin (G) and A/G ratio, whereas among the cyclic feeding groups, they decreased significantly ($P < 0.05$) with the increase in dietary protein level from 350 g protein kg^{-1} diet (T_4) to 400 g protein kg^{-1} diet (T_5) and similar between 300 g protein kg^{-1} diet (T_3) and 350 g protein kg^{-1} diet (T_4).

Respiratory burst activity

The respiratory burst activity (NBT reduction) of neutrophils of *L. rohita* fingerlings of the experimental groups is shown in Table 7. The NBT assay showed significant difference ($P > 0.05$) in respiratory burst activity among the

Table 7 Effect of mixed feeding schedule on serum biochemical parameters and immunological parameters (NBT and Lysozyme) of *Labeo rohita* fingerlings (Mean $\pm$ SE)

Treatments	Total protein (mg dL^{-1})	Albumin (A) (mg dL^{-1})	Globulin (G) (mg dL^{-1})	A/G	NBT (OD at 540 nm)	Lysozyme (U min^{-1} mg^{-1} protein of serum)
T_1	2.49^a $\pm$ 0.13	0.83^b $\pm$ 0.05	1.66^a $\pm$ 0.04	0.50^a $\pm$ 0.06	0.15^a $\pm$ 0.004	65.71^a $\pm$ 0.14
T_2	3.88^{cd} $\pm$ 0.19	1.47^{cd} $\pm$ 0.06	2.41^{cd} $\pm$ 0.12	0.61^c $\pm$ 0.03	0.21^b $\pm$ 0.005	262.17^c $\pm$ 0.13
T_3	3.45^c $\pm$ 0.14	1.30^c $\pm$ 0.15	2.15^c $\pm$ 0.05	0.60^c $\pm$ 0.05	0.21^b $\pm$ 0.004	131.42^b $\pm$ 0.27
T_4	3.53^c $\pm$ 0.19	1.35^c $\pm$ 0.13	2.18^c $\pm$ 0.16	0.62^c $\pm$ 0.06	0.20^b $\pm$ 0.005	131.30^b $\pm$ 0.15
T_5	3.07^b $\pm$ 0.24	1.08^b $\pm$ 0.16	1.99^b $\pm$ 0.18	0.54^b $\pm$ 0.05	0.15^a $\pm$ 0.002	65.71^a $\pm$ 0.14

Mean values bearing different superscripts in a column are significantly ($P < 0.05$) different.

treatments. The highest ($P < 0.05$) activity was found in the T_3 and T_4 groups, which was similar ($P > 0.05$) to the T_2 group, and the lowest ($P < 0.05$) activity was observed in the T_1 and T_5 groups.

Serum lysozyme activity

Lysozyme activity was significantly ($P < 0.05$) influenced by different feeding schedules (Table 7). Significantly increased ($P < 0.05$) lysozyme activity was found with the increase in dietary protein level from 100 g protein kg^{-1} diet (T_1) to 300 g protein kg^{-1} diet (T_2). Among the cyclic feeding groups, similar lysozyme activity was found in the group fed with 300 g protein kg^{-1} diet (T_3) and 350 g protein kg^{-1} diet (T_4), whereas it decreased significantly ($P < 0.05$) with the increase in dietary protein level from 350 g protein kg^{-1} diet (T_4) to 400 g protein kg^{-1} diet (T_5).

Stress parameters

Blood glucose   There was significantly decrease ($P < 0.05$) in blood glucose level with the increase in dietary protein level from 100 g protein kg^{-1} diet (T_1) to 300 g protein kg^{-1} diet (T_2) (Table 8). Among the cyclic feeding groups, blood glucose level increased significantly ($P < 0.05$) with the increase in dietary protein level from 300 g protein kg^{-1} diet (T_3) to 350 g protein kg^{-1} diet (T_4) and 400 g protein kg^{-1} diet (T_5) (Table 8). Similar blood glucose level was found among treatments T_2 and T_3.

Serum cortisol   The serum cortisol level was significantly ($P < 0.05$) influenced by different feeding schedules (Table 8). Significantly highest ($P < 0.05$) cortisol level was found in the T_5 group and lowest ($P < 0.05$) in the T_3 group, which was similar ($P > 0.05$) to the T_2 group (Table 8). Increase in dietary protein among cyclic feeding group significantly ($P < 0.05$) increased the serum cortisol level.

Table 8 Effect of mixed feeding schedule on stress parameters of *Labeo rohita* fingerlings (Mean $\pm$ SE)

Treatments	Glucose (mg dL^{-1})	Cortisol (ng mL^{-1})
T_1	48.55[b] $\pm$ 1.60	161.02[b] $\pm$ 1.94
T_2	37.56[a] $\pm$ 0.62	139.07[a] $\pm$ 1.74
T_3	37.43[a] $\pm$ 0.69	143.17[a] $\pm$ 1.54
T_4	56.26[bc] $\pm$ 1.19	151.07[ab] $\pm$ 4.38
T_5	65.72[c] $\pm$ 0.92	188.57[c] $\pm$ 2.81

Mean values bearing different superscripts in a column are significantly ($P < 0.05$) different.

Discussion

Dietary protein is always considered to be of primary importance in fish feeding (Jauncey & Ross 1982); thus, sufficient supply of dietary protein is needed for rapid growth (Lovell 1989). Feeding daily at a constant rate is the most prevalent practice in fish farming. De Silva (1985) proved the existence of daily variations in dry matter and protein digestibility and opined that feeding fish everyday with the same level of protein is not economical. Based on this theory, this experiment was conducted to test the applicability of cyclic feeding schedule in *Labeo rohita* fingerlings with low-protein diet followed by normal- or high-protein diets.

Whole body tissue composition of *L. rohita* fingerlings at the end of the experiment varies considerably among the treatments. In the present experiment, it is evident that crude protein content of fish was significantly influenced by dietary protein level, showing conversion and deposition of protein with the increase in dietary protein level from 100 g protein kg^{-1} diet to 300 g protein kg^{-1} diet. Similar results were observed in other fish species (Shyong *et al.* 1998; Gunasekera *et al.* 2000). The decrease in tissue crude protein content with the increase in dietary protein level from 300 g protein kg^{-1} diet to 350 g protein kg^{-1} diet during mixed feeding schedule may be due to the utilization of protein for energy at higher dietary protein level (Hidalgo & Alliot 1988; Kumar *et al.* 2009). Debnath *et al.* (2007) reported that 300 g protein kg^{-1} diet in practical diet is the optimal dietary protein level for *L. rohita* fingerlings. The increase in moisture content was reflected in the decrease in ether extract in fish, confirming the inverse relationship between moisture and lipids. The lowest fat accumulation was recorded in fish receiving the lowest protein level (T_1), which was similar to the finding in common carp (Srikanth *et al.* 1989; Nandeesha *et al.* 2002).

In the present study, results revealed that the weight gain (%), SGR, FCR, PER and ANPU were influenced by the different feeding schedules. Maximum growth performance was exhibited by the T_3 group fed with 100 g protein kg^{-1} diet for 21 days and then 300 g protein kg^{-1} diet for 7 days, which was comparable to the T_2 group fed with 300 g protein kg^{-1} diet daily. Feeding of high-protein levels in T_4 and T_5 did not exhibit enhancement in the fish growth rate. This result may be due to the fact that each fish has a certain protein limit after which excess protein level could not be utilized efficiently (Wilson 1989; Ahmad *et al.* 2004; Debnath *et al.* 2007). The highest PER and

ANPU values were recorded in the T_3 group. These results may be because the major part of weight gain is related to the deposition of proteins, and the protein accretion is a balance between protein anabolism and catabolism. Jauncey (1982) reported that protein efficiency ratio and protein retention are known to be high at low level of protein inputs. Hossain *et al.* (2006) demonstrated that FCR and PER values were lower when fed high-protein diet and other mixed feeding schedules as compared to continuous feeding with low-protein diets. Similar trends were reported for Indian major carps and common carp (Srikanth *et al.* 1989 Nandeesha *et al.* 1993, 1995). This suggests that nutrient utilization were similar in both the T_2 and T_3 groups as evident from the ANPU value. However, PER value was more in the T_3 group. This is due to the less protein fed to the T_3 group than the T_2 group. Both PER and ANPU gradually decreased as the protein content increased in feeding schedules T_4 and T_5. This indicates that protein utilization was less at higher inclusion level. The efficiency with which fish converts dietary protein into tissue growth decreases as the protein content of feed increases (Kumar *et al.* 2013). Mohapatra *et al.* (2004) and Kumar *et al.* (2006) also found decrease in PER at increasing dietary protein level. Similar findings have been reported for other fish species (Jobling & Wandsvik 1983; Daniels & Robinson 1986; Brown *et al.* 1992; Arzel *et al.* 1995; Yang *et al.* 2002). This is probably because more dietary protein is used as energy when high-protein diets are fed to fish (Jobling & Wandsvik 1983; Hidalgo & Alliot 1988; Kim *et al.* 1991; Kumar *et al.* 2009).

The activity of digestive enzymes is known to be affected by dietary nutrient composition (Kawai & Ikeda 1972). The gut enzyme profile is the indicator of nutrient digestibility and utilization. In the present study, the activity of both protease and amylase in the liver and intestines of the fingerlings fed the experimental diets was higher in the T_3 group than the rest of the groups. The results clearly indicate a correlation between formulated diet intake and digestive enzyme activity, resulting in diet-related growth differences. Similar diet-related differences in growth and digestive enzyme activity have been reported with rainbow trout (Kawai & Ikeda 1973). In the present study, the digestive enzyme (amylase and protease) activities in both the liver and intestine of *Labeo rohita* fingerling were found to be higher in the T_3 treatment. Amylase and protease activities increase with the increase in dietary carbohydrate or protein, respectively (Le Moullac & Van Wormhoudt 1994). When the optimal dietary level of carbohydrate and protein is surpassed, enzyme activities responsible for their

breakdown begin to decrease (Ceccaldi 1997). Similar results were observed in the present experiment.

Diet management can lead to deformation and functional changes in the blood cells (Duncan *et al.* 1993; Klinger *et al.* 1996). There is a relationship between the blood characteristics (haematocrit, size and number of red blood cells, white blood cells and haemoglobin concentration) and physiological condition of the fish (Rios *et al.* 2002 and Rios *et al.* 2005). In the present study, the RBC count, haemoglobin content and haematocrit value were increased with the increase in dietary protein level from 100 g protein kg^{-1} diet to 300 g protein kg^{-1} diet, whereas among the cyclic feeding group, they decreased with the increase in dietary protein level from 300 g protein kg^{-1} diet to 400 g protein kg^{-1} diet. It has been reported that red blood cell (RBC) count can determine, at least in part, the efficiency of oxygen transport to the tissue (Holland & Forster 1966; Nikinmaa & Salama 1998) and any change in their number and volume may influence the metabolic performance (Hlavova 1993; Rios *et al.* 2002 and Rios *et al.* 2004). Therefore, this result complements the better growth and metabolic efficiency in the T_3 group. The leucocyte (WBC) counts can reveal changes in the immune function (Wedemeyer & McLeay 1981). It is an indicator of the health status of fish because of its role in non-specific or innate immunity (Roberts 1978). A reduction in WBC count was observed in the group fed with lowest protein (100 g protein kg^{-1}) diet (T_1) as well as with highest protein (400 g protein kg^{-1}) diet (T_5), which suggests that the immunity of fish is adversely affected when fed with either very less protein or excess proteins. Further, provision of higher than required protein level to fish may have caused metabolic stress to the fish, which finally decreases the immunity of fish (Roberts 1978).

Among the serum proteins, albumin and globulin are the major proteins that play a significant role in the immune response. The serum protein level can be used to estimate the protein requirements and protein catabolism. Any changes in serum total protein are associated with infectious disease, kidney disease, nutritional imbalance, hemoconcentration and impaired water balance (Wedemeyer & McLeay 1981). Increasing trend of serum total protein, globulin, albumin and A/G ratio with the increase in dietary protein level from 100 g protein kg^{-1} diet (T_1) to 300 g protein kg^{-1} diet (T_2) and decreasing trend with the increase in dietary protein level from 350 g protein kg^{-1} diet (T_4) to 400 g protein kg^{-1} diet (T_5) in the cyclic feeding schedule suggests an immunosuppressive action of <300 g protein kg^{-1} diet and higher than 350 g

protein kg^{-1} diet dietary protein in the diet of *L. rohita* fingerlings. Positive balance is seen during the growth and gain of lean body mass, and negative balance indicates inadequate protein intake or excessive catabolism.

Respiratory burst activity of neutrophils gives a measure of oxygen-dependent defence mechanism in vertebrate phagocytic cells. There is generation of reactive oxygen species (ROS) intermediates in phagocytes with powerful microbicidal activity (Itou *et al.* 1997). Hence, increased respiratory burst activity can be correlated with increased pathogen killing activity of phagocytes (Sharp & Secombes 1993) and better immunity (Jha *et al.* 2007). In the present study, the respiratory burst activity of phagocytes was measured by reduction of nitroblue tetrazolium (NBT) by intracellular superoxide radicals produced by leucocyte. The group fed with 100 g protein kg^{-1} diet (T$_1$) registered significantly less NBT than the group fed with 300 g protein kg^{-1} diet (T$_2$), whereas among the cyclic feeding groups, NBT value was similar between 300 g protein kg^{-1} diet (T$_3$) and 350 g protein kg^{-1} diet (T$_4$) dietary protein fed group, which significantly decreased in the 400 g protein kg^{-1} diet (T$_5$) dietary protein fed group. This observation suggested that T$_3$ and T$_4$ groups had a same immunity as the T$_2$ group.

Lysozyme appears in the blood as secretion from the lysozymes of neutrophils and macrophages (Goldstein *et al.* 1975; Murray & Fletcher 1976). Lindsay (1986) has confirmed the role of lysozyme in the disease defence mechanism in fish. It was observed that lysozyme plays important role in the innate immunity by lysis of bacterial cell wall and thus stimulates the phagocytosis of bacteria (Ellis 1990). In the present study, low levels (100 g protein kg^{-1} diet, T$_1$) of protein in the diet reduced the lysozyme activity compared with optimal levels (300 g protein kg^{-1} diet, T$_2$) of protein. This results corresponds to the results of Kiron *et al.* (1995). The lysozyme activity was found maximum in the T$_3$ and T$_4$ groups, which suggest better immunity in these groups compared to other groups.

Glucose has been extensively used as an indicator of stress and is one of the energy substrates used by fish to cope with stressful events. When fish are stressed, increased cortisol levels are thought to play an important role in the production of glucose (Mommsen *et al.* 1999). In the present study, blood glucose level and serum cortisol level increased with the decrease in dietary protein level from 300 g protein kg^{-1} diet (T$_2$) to 100 g protein kg^{-1} diet (T$_1$). The same trend was also observed by Lundstedt *et al.* (2004) in *Pseudoplatystoma corruscans*. The main reason behind this could be increase in total carbohydrate inclu-

sion with the increase in diet CP to maintain isocaloric nature of the diets, and higher glucose concentration and cortisol level in the low-protein fed group are a result of stress due to high dietary carbohydrate (Kumar *et al.* 2011). In different mixed feeding schedule groups, increase in dietary protein level increased the blood glucose and serum cortisol level, suggesting that high level of dietary protein also induces stress to fish. During stress, insulin resistance impairs glucose transport into tissues; therefore, protein is broken down, and amino acids are released into the bloodstream and transported to the liver where they can be used for the production of glucose through gluconeogenesis. Chronic exposure to cortisol suppresses the immune system and decreases growth rates (Hazon & Balment 1998).

It could be concluded from the present study that fish feeding alternately a low-protein diet (100 g protein kg^{-1} diet) for 21 days followed by a normal-protein diet (300 g protein kg^{-1} diet) for 7 days in a cycle of 28 days maintained the growth rate similar to the normal protein fed group (300 g protein kg^{-1} diet) and also maintained the better health status on the basis of haematological as well as of serum biochemical parameters. The stress indicator parameters such as glucose and cortisol also complement for the better immunity in the same group. This suggests that daily feeding a normal-protein diet is not necessary, because the requirement of protein of a species is an average figure of an extended period. Hence, adopting a specific feeding strategy by feeding variable protein for short duration probably satisfies the requirement. The survival was similar in all the groups, and the proximate composition was not affected much due to adopting the above strategy, indicating feeding a low-protein diet followed by a normal-protein diet is an ideal strategy for reducing the production cost. However, long-term studies need to be evaluated as no reports available in these aspects.

Acknowledgements

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