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Responses of digestive and metabolic enzymes to different feeding strategies with sub-optimal protein level in *Labeo rohita* fingerlings

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ABSTRACT

A 60-days feeding trial was conducted to study the effect of different feeding strategies with normal and low protein diet on digestive and metabolic activity in *Labeo rohita* fingerlings. One hundred thirty five fingerlings were distributed in triplicate groups of each treatment. Three experimental isocaloric (401.32 to 410.28 kcal/100g) diets of 30%, 25% and 20% crude protein designated as diet A, diet B and diet C respectively were prepared, using locally available feed ingredients. Three different feeding schedules of normal protein diet continuously (diet A-30%) throughout the experiment, alternate feeding of 1-day diet A followed by 1-day diet B (1A/1B) and alternate feeding of 1-day diet A followed by 1-day diet C (1A/1C) throughout the experiment were tested by feeding the fishes at 5% body weight daily. Results showed that digestive enzymes activity such as protease and amylase was higher in the group fed 1A/1B. Glucose 6 phosphatase (G6Pase) were also analysed. The metabolic enzymes such as lactate dehydrogenase (LDH), malate dehydrogenase (MDH) are reported to be significantly difference ($p<0.05$) in among the different feeding schedules. The proteolytic activity such as alanine amino transaminase (ALT), aspartate amino transaminase (AST), alkaline phosphatise (ALP) were reported to be higher in the group fed with 1A/1B feeding schedule.

KEYWORDS

Alternate feeding strategies;
Digestive enzymes;
Metabolic enzymes;
Labeo rohita.

INTRODUCTION

One of the main objectives of fish nutrition research is to minimize the amount of protein in diets and cover energy requirements using carbohydrates or lipids. Excess dietary protein with respect to growth demands leads to increased amino acid degradation and the loss of nitrogen to ambient waters, which in turn could result in water eutrophication[1, 2, 3]. The protein used in fish diets is mainly from fish meal, therefore any reduction in its use could also lead to decreased pressure on overexploited marine fisheries;

as in many cases, a significant part of catches is converted to fish meal[4].

Fish have the enzymes and metabolic pathways required to regulate the metabolism of carbohydrates ingested through diet[5-15]. However, the intermediary metabolism of carnivorous fish, like rainbow trout, shows only moderate adaptation to high levels of dietary carbohydrate[5, 14, 16]. Integrated studies combining enzyme regulation, nutrient digestibility and growth performance in relation to dietary carbohydrates are required for cultured species like gilthead sea bream.

Digestive enzyme pattern can reflect the feeding habit of fish (whether it is herbivore, detritivore, omnivore or carnivore) and also reflects the digestive capacity of fish[17]. The type, source and amount of nutrients can alter the enzyme profile/concentration of digestive tract. This adaptive characteristic of the enzymes can be successfully used to take advantage of the nutrient content of diets[18]. Digestive enzyme activities in fish vary among species, which can be influenced by the age as well as by the quantity and composition of diet[19]. Several comparative studies of the digestive enzymes in different fish species have been reported[20-27].

Labeo rohita (commonly called rohu) is the most important Indian Major Carp (IMC), which is cultivated commercially across the country. Traditionally and commercially rohu is being fed with farm-made rice bran and oil cake-based diet containing 30–35% protein. To our knowledge no studies were conducted to investigate the digestive enzymes and metabolic profile in this fish species as a means to optimize the nutrient requirements. Studies on the digestive enzymes in fish might elucidate some aspects of their nutritive physiology and thus could support developing nutritional strategies for fish feeding and diet formulation. Metabolic profile may reflect the availability of nutrients which in turn is associated with digestion and absorption[18, 28], although in warm water fish, correlation between growth and metabolism is controversial[29].

In this context, the present study was undertaken to study the effect of mixed feeding schedule of normal and low protein diet on digestive and metabolic activity in *Labeo rohita* fingerlings.

EXPERIMENTAL

Experimental animal

L. rohita juveniles were procured from Khopoli fish farm, Maharashtra, India. The fishes were transported in a circular container (500 l) with sufficient aeration to Central Institute of Fisheries Education, Mumbai, India and were acclimatized to the experimental rearing conditions for 15 days.

Experimental design and feeding

Three experimental isocaloric (401.32 to 410.28 kcal/100g) diets of 30%, 25% and 20% crude protein designated as diet A, diet B and diet C, respectively were prepared, using locally available feed ingredients as shown in TABLE 1. One hundred thirty five fingerlings were distributed in triplicate groups of each treatment following a completely randomized design (CRD). Feeding trial was conducted for 60 days.

TABLE 1 : Composition of the experimental diets (% Dry matter basis)

Ingredients	Diet - A	Diet - B	Diet -C
Fish meal	21	18	13
Soybean meal	16	10.6	7
MOC	11.6	10	6
GNOC	16	11	6
Wheat bran	8	15	20.6
Corn flour	11	14	20
Rice Bran	10	15	21
Fish Oil	4	4	4
Premix	2	2	2
Vit C	0.2	0.2	0.2
BHT	0.2	0.2	0.2

Composition of vitamin mineral mix (PREEMIX PLUS) (quantity/2.5kg)

Vitamin A, 55,00,000 IU; Vitamin D_3, 11,00,000 IU; Vitamin B_2, 2,000 mg; Vitamin E, 750 mg; Vitamin K, 1,000 mg; Vitamin B_6, 1,000 mg; Vitamin B_{12}, 6 mcg; Calcium Pantothenate, 2,500 mg; Nicotinamide, 10 g; Choline Chloride, 150 g; Mn, 27,000 mg; I, 1,000 mg; Fe, 7,500 mg; Zn, 5,000 mg; Cu, 2,000 mg; Co, 450 L-lysine, 10 g; DL- Methionine, 10 g; Selenium, 50 ppm; Satwari, 2500 mg.

Diet - A (30% CP); Diet - B (25 % CP); Diet - C (20% CP)

Tissue homogenate preparation

The muscle, liver and intestine of the fishes were removed carefully and were weighed. It was homogenized with chilled sucrose solution (0.25 M) in a glass tube using Teflon coated mechanical tissue homogenizer. The tube was continuously kept in ice to avoid heating. The homogenate was centrifuged at 5000 rpm for 10 min at 40°C in a cooling centrifuge. The supernatant was stored at 40°C until use. A 5% homogenate was prepared for muscle, liver and intestine. The intestinal content was removed before homogenization.

Enzyme assays

Protease

Protease activity was determined by casein digestion method as described by Drapeau[30]. The enzyme reaction mixture consisted of 1% casein in 0.05M Tris PO4 buffer (pH – 7.8) and incubated for 5 min at 37 °C. Ten min latter reaction was stopped by adding 10% TCA. Then the sample was filtered after 10 min of the reaction. Adding tissue homogenate just before stop the reaction and with no incubation was made the reagent blank. One unit of enzyme activity was defined as the amount of enzyme needed to release acid soluble fragments equivalent to 0.001A280 per min at 37°C and pH 7.8.

Amylase

The reducing sugars produced due to the action of glucoamylase and amylase on carbohydrates was estimated using Dinitro-salicylic-acid (DNS) method by Rick and Stegbauer[31]. The reaction mixture consisted of 1% (w/ v) starch solution, phosphate buffer (pH-6.9) and the tissue homogenate. The reaction mixtures were incubated at 37°C for 30 min. DNS was added after incubation and 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. Amylase activity was expressed as mole of maltose released from starch per min at that temperature.

Lactate Dehydrohenase (LDH)

The LDH activity in the liver tissue was assayed by the method of Wroblewski and Ladue[32]. Total 3 ml of the reaction mixture comprised of 2.7 ml of 0.1 M phosphate buffer (pH 7.5), 0.1 ml of NADH solution (2 mg NADH dissolved in 1ml of phosphate buffer solution), 0.1ml of tissue homogenate and 0.1 ml sodium pyruvate. The reaction was started after addition of substrate sodium pyruvate. The OD was recorded at 340nm at 30 seconds interval for 3 min. The enzyme activity was expressed as units/mg protein/min at 25°C where 1 unit was equal to D0.01 OD/min.

Glucose-6-Phosphatase (G-6-Pase)

The G-6-Pase activity in the liver tissue was assayed by the method of Marjoric[17]. The assay mixture consisted of 0.3ml of malate buffer (pH 6.5), 0.1 ml of 0.1M glucose 6-phosphate solutions and 0.1 ml of tissue homogenate and was incubated for 15 min at 370C. The reaction was terminated by addition of 1 ml of 10% TCA solution. One milli litre of the aliquot of the supernatant was used for phosphate (Pi) estimation by method of Fiske and Subbarow.

Aspartate amino transferase (AST) and Alanine amino transfearse (ALT)

The AST activity was assayed in different tissue homogenates as described by Wooten[34]. The substrate comprised of 0.2M D, L- aspartic acid and 2mM α-ketoglutarate in 0.05M phosphate buffer (pH 7.4). In the experimental and control tubes, 0.5ml of substrate was added. The reaction was started by adding 0.1ml of tissue homogenate. The assay mixture was incubated at 37°C for 60 min. The reaction was terminated by adding 0.5ml of 1mM 2,4 dinitrophenyl hydrazine (DNPH). In the control tubes the enzyme source was added after DNPH solution. The tubes were held at room temperature for 20 min with occasional shaking. Then 5ml of 0.4N NaOH solution was added, the contents were thoroughly mixed. After 10 min, the OD was recorded at 540nm against the blank. The procedure adopted for ALT activity was same as that for AST activity except the substrate comprised of 0.2 M D,L-alanine instead of aspartic acid.

Alkaline phosphatase (ALP) (E.C. 3.1.3.1)

ALP activity was determined by Garen and Levinthal[35]. The assay mixture consisted of bicarbonate buffer (0.2M, pH 9.5), 0.1 M MgCl2, tissue homogenate and freshly prepared 0.1Mpara-nitrophenyl phosphate (p-NPP) as substrate. The reaction mixture was incubated in water bath at 37 °C for 15 min and then stopped with 0.1 N NaOH. Optical density was recorded at 410 nm. ALP activity was expressed as nanomoles p-nitrophenol released/ min/mg protein at 37 °C. Acid phosphatase (ACP) (E.C. 3.1.3.2) activity was estimated using the same method as ALP, except that acetate buffer (0.2M, pH 5) was used in place of bicarbonate buffer. Tissue protein content was determined following the standard method by Lowry[36] and expressed as mg protein/g wet tissue.

Statistical analysis

Mean values of all parameters were subjected to one-way ANOVA to study the treatment effect and Duncan's multiple range tests were used to determine the significant differences between any two means, if they were significant. Comparisons were made at 5% level probability level. All the data were analysed using statistical package SPSS (Version 16).

RESULTS AND DISCUSSION

Protein requirement studies on fish are the most important aspects of aquaculture. Requirement studies in fish are generally carried out using purified research diets to enable consistency, though these diets find limited or no use in commercial level feeding practices. For this reason, practical diets were formulated in the present study. *L. rohita* fingerlings showed best growth in alternate day feeding strategy of normal and low protein diet in group T 1 fed (1A/1B). Growth of fish is an outcome of numerous factors, the reason why growth alone cannot be selected as a criterion to formulate the best artificial feed for a species. Moreover, dietary protein and growth rate are not always correlated[37], which also depends on environmental factors. Therefore, digestive capacity and metabolite concentrations were used in optimizing the dietary nutrient balance for *L. rohita* fingerlings in the present paper.

Body weight

The body weight of the experimental groups was recorded at 15 days interval. There was significant difference ($P<0.05$) in the body weight gain among different treatment groups at the end of the experimental period. The significantly improved growth was observed in the group T1 fed (1A/1B) diet and the lowest was in the group T2 fed (1A/1C) diet. The initial body weight among the treatment group varied from 41.56±0.241g to 42.9±0.321g and the final body weight varied from 78.03±0.95 g to 86.23±0.41g. This result is supported with the findings of Ali[38] demonstrated that the mixed feeding schedule of a Low Protein (LP) alternated with a High Protein (HP) - (1LP/1HP) resulted in the best growth, feed utilization and production compared with feeding sutchi catfish and silver carp with a High Protein (HP) continuously. Conducting experiments on mixed feeding schedules, similar types of growth response have also been reported in common carp, in which low- and high-protein-based diets were fed alternately resulted in higher growth and this was considered as a possible way of reducing feed cost[39, 40].

Enzyme assays

Protease

In the intestine, the protease activity was significantly higher ($P<0.05$) in the T1 fed (1A/1B) group followed by control (30% CP) and T2 fed (1A/1C) groups. The significantly lowest activity was found in T2 fed (1A/1C) group. Similarly same results were observed for liver.

Protease activity in intestine showed significant difference in among the different treatment groups as the dietary inclusion were of the carbohydrate source so the non chalancy of protease activity was obvious. In contrast to this, trypsin was found to be unresponsive to dietary CP in early-weaned sea bass[41]. Trypsin and chymotrypsin activities in pintado were unresponsive to diet composition in pintado by Lundstedt *et al*[28]. Lopez-lopez[42] also could not find any correlation between protease activity and dietary CP and between protease activity and growth in *Homarus americanus*.

Amylase

The specific activities of amylase in the intestine of the fishes of different experimental groups are shown in the TABLE 3. The amylase activity of intestine was significantly different ($P<0.05$). In the intestine, the amylase activity was found to be higher in the T2 fed (1A/1C) group followed by T1 fed (1A/1B) and control fed group.

Low amylase activity in carnivorous fish (with stomach) and high activity in omnivorous fish (without stomach) is the general assumption[43, 44]. In the present study, amylase activity did not change significantly, which is in agreement with Hoyle[45] and Lopez-Lopez[42], who did not find any correlation between amylase activity and

TABLE 2 : Feeding strategies during the experimental period

C	Diet A (30%) throughout the experiment.
T1	Diet A (30%) followed by Diet B (25%) in alternate days.
T2	Diet A (30%) followed by Diet C (20%) in alternate days.

C – Control, T1 - Treatment 1, T2 –Treatment 2
Diet A – 30% protein, Diet B – 25% protein, Diet C – 20% protein

TABLE 3 : Enzyme activity in different treatment groups fed different diet at end of the experiment.

Treatments	Amylase (Int)	Protease (Int)	ALP (Int)	G-6-Pase (Liver)
C	$0.0566^a \pm 0.023$	$0.186^b \pm 0.0562$	$143.26^b \pm 12.21$	$5.40^a \pm 0.45$
T1	$0.0899^b \pm 0.0015$	$0.264^c \pm 0.0183$	$150.17^a \pm 15.87$	$33.15^b \pm 3.44$
T2	$0.0911^b \pm 0.032$	$0.155^a \pm 0.00255$	$104.82^b \pm 6.24$	$22.38^b \pm 1.76$

Mean values in the same column with different superscript differ significantly (P<0.05).
Int: - Intestine, Unit: U/mg protein.
Amylase as micromole of maltose released/min/mg protein
Protease as micromole of tyrosine released/min/mg protein
ALP as nanomoles p-nitrophenol released/min/mg protein at 37 °C;
G-6-Pase expressed as nano moles phosphorous released /mg protein/min at 37°C
Data expressed as Mean ± SE n=6

carbohydrate/starch content of the diet in *Homarus americanus* and *Cherax quadricarinatus*, respectively. However, Cahu and Zambonino Infante[41] reported that diet induced amylase activity in sea bass, *Dicentrarchus labrax* larvae. Amylase activity was found to increase in rainbow trout (*Oncorhynchus mykiss*) fed diets containing increasing rates of dietary protein[46, 47]. Amylase and protease activities increase with increase in dietary carbohydrate or protein[48]. As a result, amylase/protease (A/P) ratio can be used to study the influence of diet composition on these enzymatic activities. When the optimal dietary level of carbohydrate and protein is surpassed, enzyme activities responsible for their breakdown begin to decrease[49].

Lactate Dehydrogenase (LDH) and Malic Enzyme (ME)

The Lactate Dehydrogenase and Malic enzyme activity in the liver and muscle of *L. rohita* fingerlings of the different experimental groups are presented in the TABLE 4. The Lactate Dehydrogenase activity in the liver was found to be significantly higher than in the muscle. In the liver, significantly highest activity was found in the T1 fed (1A/1B) group and lowest activity was recorded in T2 fed (1A/1C) group.

TABLE 4 : Lactate dehydrogenase (LDH) and Malate dehydrogenase (MDH) activity in Muscle and Liver of *L rohita* fingerlings fed with different experimental diet.

Treatments	LDH		MDH	
	MUSCLE	LIVER	MUSCLE	LIVER
C	$1.75^a \pm 0.48$	$1.03^b \pm 0.025$	$0.78^a \pm 0.023$	$0.55^b \pm 0.01$
T1	$1.87^b \pm 0.071$	$1.09^a \pm 0.23$	$0.94\ ^ab \pm 0.25$	$0.61^a \pm 0.041$
T2	$1.58^b \pm 0.03$	$0.79^b \pm 0.34$	$0.71^a \pm 0.073$	$045^b \pm 0.15$

Values in the same column with different superscript differ significantly (P<0.05)
LDH: specific activity expressed as Units/ min/ mg protein at 37°C
MDH: specific activity expressed as Units/ min/ mg protein at 37°C
Data expressed as mean ±SE, n=6.

In the liver, significantly (P<0.05) highest malate dehydrogenase activity was found in the group T1 fed (1A/1B) and the relatively low activity has been found in T2 fed (1A/1C) group. In the muscle, the highest activity was found same as in liver and the lowest activity was recorded in T2 fed (1A/1C) group.

Lactate dehydrogenase (LDH) is an important enzyme in glycolytic pathway. LDH converts pyruvate to lactate in the presence of coenzyme NADH that is converted to NAD^+. In presence of enough oxygen pyruvate enters the Kreb's cycle but under anaerobic condition, LDH helps in ATP production by converting pyruvate to lactate. Thus LDH assay serves as a useful stress indicator. LDH activity increases with stress viz. temperature stress[50], starvation stress and confinement stress[51]. LDH activity increases with increase energy demand. In the present study LDH activity in liver increased significantly in treatment groups as compared to control group.

Glucose-6-Phosphatease (G6Pase)

Significantly highest G-6-Pase value of liver was recorded in the group T2 fed (1A/1B) followed by T2 fed (1A/1C) group and control (30% CP) fed group. The lowest activity was recorded in control group which was significantly different from all other groups.

G6Pase is an enzyme, which catalyzes the conversion of glucose-6phosphate to glucose. The brain tis-

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sues essentially require glucose. Gluconeogenesis is the process by which glucose is produced from non-carbohydrate sources like proteins, fatty acids and glycerol etc. G6Pase activity in liver of *Labeo rohita* fingerlings in experimental groups were significantly reduced as compared to the control group.

Alkaline phosphatase activity (ALP)

The Alkaline phosphatase activity in the intestine of *L. rohita* fingerlings of the different experimental groups are shown in the TABLE 3. The ALP activity of intestine vary significantly (P<0.05) among the different experimental groups. Significantly highest ALP value of liver was recorded in the group T1 fed (1A/1B) followed by T2 fed (1A/1C) group and control (30% CP) fed group. The lowest activity was recorded in 1A/1C group which was significantly different from all other groups.

Alkaline phosphatase (ALP) activity was reported to be an indicator of the intensity of nutrient absorption in enterocytes of fish[52, 53]. Blier et al.[54] also did not find any significant difference in ALP activity in growth hormone transgenic coho salmon that was supposed to have higher growth rate compared to non-transgenic coho salmon. But, the growth rate of Atlantic cod was found to be positively correlated with ALP activity[55]. The herbivorous fishes are reported to have lesser ALP activities than carnivorous fishes[52]. The herbivorous fishes are reported to have lesser ALP activities than carnivorous fishes[52].

Aspartate amino transaminase (AST) activity and Alanine amino transaminase (ALT) Activity

The AST and ALT activity of *Labeo rohita* fingerlings fed with different experimental diet is shown in TABLE 5. The activity of enzyme in liver and muscle differ significantly (p<0.05). In the muscle, the highest activity was observed in T1 fed (1A/1B) group which was significantly different from all other groups and the lowest activity was recorded in T2 fed (1A/1C) group. In the liver, the highest activity was found in the group T1 fed (1A/1B)) and the lowest activity has been recorded in T2 fed (1A/1C) group.

This suggests that muscle tissue is very efficient in utilizing amino acid for metabolic purposes. Both liver and muscle showed an increased activity of AST and ALT in diet of 1A/1B. This may be due to the utilization of the glucose derived TCA intermediates for protein synthesis which has given the highest growth in T1 (1A/1B) group. In liver and muscle tissue both, AST predominates over ALT where the formation of amino acids via energy cycle is more through glutamate-aspartate pathway. This represents the aerobic tendency of the tissues and glucose utilization for energy production. High protein intake enhances gluconeogenesis and amino acid catabolism[29, 56-59]. Alanine aminotransferase activity in liver and muscle showed opposite trend to that of AST in both the tissues. It seems there is complementary activity of these two amino acid metabolizing enzymes in a particular tissue (liver or muscle).

TABLE 5 : Aspartate amino transaminase (AST) or GPT and alanine amino transaminase (ALT) or GOT activity in liver and muscle of different experimental group

Treatments	AST		ALT	
	Muscle	Liver	Muscle	Liver
C	49.48[a] ±1.75	10.41[a] ±1.38	42.32[a] ±3.14	35.2±0.96
T1	50.28[a] ±2.3	14.2[b] ±1.12	45.13[a] ±1.04	36.28[a]±0.45
T2	45.79[a]±1.47	9.9±1.01	41.27[a] ±1.10	32.01±2.81

Mean values bearing different superscripts under each column vary significantly (P<0.05)
ALT: specific activities expressed as nano moles of sodium pyruvate formed/mg protein/minute at 37⁰C.
AST specific activities expressed as nano moles of oxaloacetate released/min/mg protein at 37⁰C
Data expressed as mean ±SE, n = 6.

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