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http://www.aquaculturehub.org

ISSN 0792 - 156X

PUBLISHER:

Israeli Journal of Aquaculture - BAMIGDEH -
Kibbutz Ein Hamifratz, Mobile Post 25210,
ISRAEL
Phone: + 972 52 3965809
http://siamb.org.il

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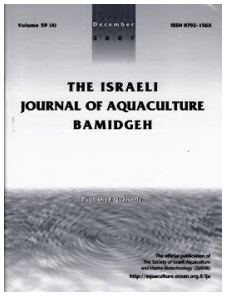

Growth Performance, Nutrient Utilization, Metabolic and Digestive Enzymes Studies in Mrigal (*Cirrhinus mrigala*) Juveniles Fed Graded Levels of Carbohydrate

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Key words: mrigal fish (*Cirrhinus mrigala*), mrigal digestive enzymes, mrigal nutrition, digestive enzymes sequencing, digestive enzymes expression

Abstract

A 45-day experiment was carried out to evaluate the growth performance, digestive enzyme activity, and gene expression in mrigal fish *Cirrhinus mrigala*, fed graded levels of carbohydrate. Three isonitrogenous and isolipidic diets containing either 30, 40 or 50% carbohydrate were formulated using purified ingredients. One hundred and eight *Cirrhinus mrigala* fingerlings (av. wt. 5.83-6.08g) were equally divided into three groups in 9 plastic tanks of 150L capacity each. Each of the three experimental diets was fed to triplicate groups of fish held under natural light cycle of 12h daylight and 12h darkness. % Weight Gain (WGR), specific growth rate (SGR), feed conversion ratio (FCR) and hepato-somatic index (HSI) were evaluated and found to vary significantly ($P<0.05$) in relation to the level of carbohydrate in the experimental diets. No significant difference ($P>0.05$) was found in % weight gain, SGR, and FCR, between the 30% and 50% carbohydrate fed groups but HSI increased significantly across the inclusion levels. Amylase enzyme activity was highest in 40% fed group relative to the 30% and 50% groups. Chymotrypsin and trypsin activities were not significantly different between the 40% and 50% carbohydrate fed groups. Various metabolic enzyme activities in the muscle [aspartate aminotransferase (AST), alkaline amino transaminase (ALT) and malate dehydrogenase (DH)] varied significantly ($P<0.05$) in response to increased levels of carbohydrate. Gene expression among the digestive enzymes reflected both transitional and translation effect; amylase and trypsin exhibited higher expression with the increasing level of carbohydrate of the experimental diets but results were reversed for chymotrypsin expression. In our study, gene expression was initially significantly higher but then decreased. No consistent pattern was found among the different digestive enzymes.

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Introduction

Carbohydrates, which serve as an energy source in feeds, constitute one of the three major components of fish diets. They are the most economical and inexpensive sources of energy in fish diets and are widely included in feed to reduce cost and contribute to protein and lipid sparing (Ren et al 2011). The utilization of dietary carbohydrate has also been found to vary with the molecular complexity or chemical structure of the carbohydrate, source, and inclusion level (Kumar et al. 2006). In general, warm water omnivorous or herbivorous fish species such as common carp, channel catfish, and eels, have been found to be more tolerant to high dietary carbohydrate levels. Excess inclusion of carbohydrate in the fish diet may cause metabolic stress and hence reduce growth rate (Yengkokpam et al. 2007).

Therefore, it is necessary to be aware of the maximum inclusion level of carbohydrate for any species to optimize production costs. From the traditional growth rate and feed efficiency based nutrient requirements of fish, there is a paradigm shift from growth maximization concept to digestive and metabolic optimization studies (Lundstedt et al. 2004). Studies on fish digestive enzymes, serum metabolites, and hematological parameters, could thus emphasize physiological mechanisms, and better use of nutrients. This may invariably resolve nutritional problems, such as the optimal level of inclusion of carbohydrate and the nutrient utilization capacity of fish.

Results from digestive and metabolic enzyme studies sometimes vary, probably due to the methodology adopted, difference in substrates, temperatures, instrument calibration, unit of activity, species, and ontogenetic stages examined, as well as the quality and composition of the diet (Chan et al. 2004). Thus, enzyme studies alone may not give conclusive information regarding nutrient requirements and utilization in fish.

The application of cellular and molecular biology techniques in fish nutritional studies can determine whether changes in digestive enzymes are reflected at the level of transcription or translation and identification of the master gene involved in the regulation of gastrointestinal development (Zambonino-Infante and Cahu, 2001).

Expression studies of the amylase gene include *Haliotis discus discus*, in which MRNA expression of the α-amylase gene appeared to be negatively affected by starvation, and positively affected by food availability (Nikapitiya et al. 2009). Regulation of digestive gene expression has been used to study the relationship between growth and nutrient digestive processes in *Pelteobagrus fulvidraco* larvae (Wang et al. 2006). Gene expression studies could therefore consolidate the results from both growth and enzyme analyses in fish nutritional studies. In the present experiment, the gene expression study was proposed to study the effect of feeding graded levels of carbohydrate.

Decisions regarding new feed formulation for the maximization of carbohydrate utilization for mrigal should probably rely on results of the chemical analyses and gene expression studies of digestive and metabolic enzymes activities. The present experiment was designed to test this hypothesis.

Materials and methods

Experimental fish and feeding

One hundred and eight fingerlings of *Cirrhinus mrigala* (av. wt. 5.83–6.08g) were equally distributed into three treatment groups with three replications of each treatment, in 9 plastic tanks of 150 L (60X60X42 cm) capacity.

Three iso-nitogenious and iso-lipidic experimental diets were formulated with graded levels of carbohydrate at 30, 40 and 50%, respectively (Table 1). Water was exchanged manually twice a week and continuous aeration was supplied from an air pump to maintain an adequate level of dissolved oxygen. The average water temperature recorded ranged between 28-30^{o}C. Fish were fed to satiation for 45 days. Total feed was divided into two and given at 10:00 and 18:00h.

Table 1 Feeding formulation

Ingredients %	*Diet 1*	*Diet 2*	*Diet 3*
Casein	34.5	34.5	31
Gelatin	6	6	8
Dextrin	20	19.5	29.5
Starch	10	20.5	20.5
Cellulose	17.5	7.5	0
Oil	8	8	8
V-M mix	2.7	2.7	2.7
Vit. C	0.1	0.1	0.1
CMC	1	1	0
Glycine	0.2	0.2	0.2
TOTAL	100	100	100
Calculated CP%	35%	35%	35%
Calculated lipid %	8%	8%	8%
Calculated CHO (NFE)	30	40	50

V-Mmix=Vitamin-mineral mix (Emix™ plus) (quantity/ 2.5 kg): Vitamin A-55,00,000 IU; Vitamin D3—11,00,000 IU; Vitamin B2—2000 mg; Vitamin E—750 mg; Vitamin K—1000 mg; Vitamin B6—1000 mg; Vitamin B12—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; mg; Carrier-q.s; Lactobacillus— 120 million units and yeast culture—3000 crore units. CMC Carboxymethylcellulose; CP%= Crude Protein Percentage; CHO= Carbohydrate, Casein fat free: 75% CP (HImedia), Gelatin: 96% CP (HImedia).

Growth study. Fish from each tank were bulk weighed at 15-day intervals and feeding rate was adjusted accordingly. Growth and feed efficiency parameters were calculated using the following formulae:

Weight Gain (%) =(Final weight – Initial Weight)/Initial weight x100

SGR (%/day) = ln final body weight - ln initial body weight
Time (in days)

FCR =dry weight of feed fed/ Fish live body mass gain (g)

Protein efficiency ratio (PER) =Weight gain (g) / crude protein intake (g)

Hepato-somatic index= Liver weight/ body weight x 100

Sampling. The fish were fasted for 24 h before sampling. Two fish from each replicate (six fish per treatment) were sampled and anesthetized with clove oil at 50µl/l water. Blood was withdrawn from the caudal vein using a 1ml medical syringe and immediately transferred to an Eppendorf tube containing 2.7% dry ethylene diamine tetraacetic acid (EDTA) disodium salt to prevent clotting. Blood serum was similarly transferred without the EDTA anticoagulant. Both serum and blood were later centrifuged at 5^0C for 10 min at 5000 ×g. Intestine, liver and muscle samples were collected and frozen immediately at -20^0C for enzyme assays and metabolites.

Enzyme assays. Whole intestine was homogenized in cold 0.25 M sucrose in a teflon coated motor driven homogenizer to prepare a 5% homogenate. The homogenate was centrifuged at 5000 ×g for 15 min in a cooling centrifuge (5^0C), the supernatant was collected and frozen in sample vials and stored at −20^0C until assayed for the different digestive enzymes (amylase, trypsin, chymotrypsin and lipase). Amylase (E.C. 3.2.1.1) activity was assayed with 2% (w/v) starch solution as substrate. The 2% starch solution was prepared in phosphate buffer (pH 7) and the reaction mixture was incubated at 37 ^{0}C for 30 min. Then dinitrosalicylic acid (DNS) was added to stop the reaction and the reaction mixture was kept in a boiling water bath for 5 min. After cooling, the reaction mixture was diluted with distilled water and absorbance recorded at 540 nm. Activity was determined from the maltose standard curve and expressed as mole of maltose released from starch/ min/mg protein at 37^0C.

Tissue protein. The amount of soluble protein in the gut extract was determined by the Lowry method (Lowry et al. 1951) using bovine serum albumin as a standard protein. 0.1 ml of gut extract sample or the standard was added to 0.1 ml of 2 N NaOH. The mixture was then hydrolyzed at 100°C for 10 min in boiling water-bath and cooled to room temperature; 1 ml of freshly mixed complex-forming reagent was added. After 10 min, 0.1 ml Folin reagent was added and mixed using a vortex mixer. The absorbance was read at 550 nm after 30 min.

Lipase (E.C. 3.1.1.3) activity was determined based on Cherry and Crandell (1932). The quantity of fatty acid released in unit time was measured by the quantity of NaOH required to maintain constant pH. The reaction mixture consisted of distilled water, tissue homogenate, phosphate buffer solution (pH 7), and olive oil emulsion. The mixture was shaken well and incubated at 4^0C for 24 h. Then 95% alcohol and 2 drops of phenolphthalein indicator were added and titrated against 0.05 N NaOH until it was a permanent pink color. A control was taken using an enzyme source that was inactivated prior to addition of buffer and olive oil emulsion. The milliequivalent of alkali consumed was taken as a measure of the activity of the enzyme.

Trypsin (E.C.3.4.2.1.4) and chymotrypsin (E.C.3.4.2.1.1) activity was evaluated by the casein digestion method of Kunitz (1947). The reaction mixture for trypsin consisted of 1% casein as substrate, phosphate buffer (pH 7.5), and tissue homogenate which was incubated at 37ºC for 20 min. The reaction was stopped by addition of 5% TCA and kept for one hour at 2ºC. Samples were later filtered and optical density (OD) of the supernatant was measured at 280nm. A reagent blank was prepared by adding tissue homogenate without incubation, just before stopping the reaction. Chymotrypsin reaction mixture consisted of 1% casein solution, borate buffer (pH 8.0), tissue homogenate and CaCl2. The remaining procedure was the same as for the trypsin. The activity of trypsin, and chymotrypsin, was determined from tyrosine standard curve and expressed as micromole of trypsin released/ minute/ g protein at 37ºC.

Plasma protein and albumin were estimated using total protein and albumin kit (Biuret and BCG dye binding method, Qualigens Fine Chemicals, Mumbai, India). Globulin was calculated by subtracting the albumin values from the total plasma protein.
Globulin (gdL) =total protein (g/dL) − albumin (g/dL).
Albumin/globulin (A/G) ratio was calculated by dividing albumin values by globulin values. Total lipids and cholesterol were measured with Labkit range clinical kit (Spain) strictly following manufacturer guidelines.

Aspartate amino transfarase (AST) and alkaline amino transaminase (ALT). Aspartate amino transfarase (AST) or glutamate oxaloacetate transaminase, was measured by the method of Wooten (1964). The mixture consisted of substrate (alpha-ketoglutarate and DL-aspartic acid) and the homogenate (enzyme source), which was incubated for one hour at 37 ºC. The reaction was stopped by the addition of 2,4 dinitrophenyl hydrazine (DNPH). After waiting for 20 min at room temperature, 0.4 N NaOH was added and the color read at 540nm. A control and standard (sodium pyruvate) were run along with the sample. The enzyme activity was expressed as nanomoles oxaloacetate formed/ min/mg protein at 37 ºC.

Alkaline amino transaminase (ALT) or glutamate pyruvate transaminase (E.C.2.6.1.2), activity was determined using same method as AST except that the substrate had DL-alanine instead of DL-aspartic acid. The enzyme activity was expressed as nanomoles oxaloacetate formed/ min/mg protein at 37ºC (Wooten 1964). Complete blood count was conducted using an automated Sysmex XS-1000i™ Auto Hematology Analyzer SYS-XS-1000i from Sysmex Corporation based in Kobe, Japan.

Molecular analysis. For quantitative real-time PCR (qPCR) assays, 0.1 g tissue of intestine samples from three fish from each replicate group were cut to ≤0.5 cm and then submerged in 1 mL RNA (Ambion, Life Technologies, Carlsbad, CA, USA). Samples were stored at 4°C overnight to allow the solution to thoroughly penetrate the tissue and samples were stored at -80°C until further processing.

RNA extraction and cDNA synthesis. Total RNA was purified from intenstines of juvenile Mrigal fish. Samples were homogenized in Trizol reagent (Life Technologies) according to the manufacturer's protocol using an IKA Ultra Turrax T25 Homogenizer (24,000 rpm). RNA purity and concentrations were measured using the Nano Drop ND-1000 spectrophotometer (NanoDrop Technologies, Fisher Scientific, Hampton, NH, USA), and OD260/280 nm ratio for all samples ranged between 1.80 and 2.10. Integrity of the RNA samples was evaluated by running an aliquot of the RNA sample on a 1% agarose

gel. Prior to reverse transcription, total RNA from all samples were subjected to DNase treatment using a TURBO DNA-free kit (Ambion Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol.

First strand cDNA synthesis was performed using 1.0 µg total RNAs from all samples using Superscript III and Oligo (dT)20 primers (Invitrogen, Life Technologies). Negative controls were performed in parallel by omitting RNA or enzyme. The obtained cDNA was diluted at 1:10 and stored at -20°C.

Primer design, cloning and sequencing. Degenerate primers for cloning of partial sequences of *Danio rerio*, *Oreochromis niloticus*, *Thunnus thynnus*, *Cyprinus carpio* were designed based on highly conserved regions from sequences registered in GenBank. Partial sequences were amplified by PCR in the presence of each of the designed primer sets (Table 2) under the following conditions; 4 min at 95 °C; 30 cycles of 30 s at 95°C, 30 s at 52°C, 45 s at 72°C; 7 min at 72 °C. (Annealing temperature varies with primers: Amylase; 52°C, lipase, chymotrypsin and trypsin at 48°C respectively). PCR products were separated on a 1.0% agarose gel, using a 100 bp ladder as a marker. Bands of expected sizes were excised from the gel, purified using a QIAquick Gel Extraction Kit (Qiagen, Venlo, Netherlands) and cloned on Escherichia coli. Transformed cells were selected by spreading on selective agar (LB agar with Ampicilin/IPTG/X-Gal).

Plasmid DNA was isolated from positive recombinants using a PureLink Quick Plasmid Miniprep kit and sequenced by Eurofins MWG Operon (Ebersberg, Germany). All sequences were confirmed by BLASTX analysis (http://www.ncbi.nlm.nih.gov/BLAST) and submitted to GenBank (Table 3). Specific primers for quantitative PCR were designed using Primer3 (http://frodo.wi.mit.edu/primer3/) on the basis of partial sequences obtained.

Table 2. Primers design for digestive enzymes

Reference genes used for quantitative real time PCR.						
Code						
S/n	Primer	sequence (all sequence in 5′ to 3′ direction)				
	Name	(5′–3′)				
1	Amylase forward	GGCAGTGGAAACATTGAGAACTACC				
2	Amylase reverse	CCATGTCCTCTCGGTTATCATG				
3	Trypsin forward	TGGGTTGTGTCTGCTGCTCACT				
4	Trypsin reverse	TAACCCCAGGACACAATACCCTG GCAGGGAGATGGGCTGTACA				
5	Chymotrypsin forward	CACCATCAACAAYGACATCCTGCT TCTCCTGTGTGCCTCCTCGA				
6	Chymotrypsin reverse	ACCRCCAGAGTCRCCCATGCA TTGGTCAGCAGAGGCAGAGC				

Table 3. Primer pair sequences, annealing temperature and amplicon size of target genes and reference genes used for quantitative real time PCR.

S/n	*Primer Name*	*Nucleotide sequence (5′–3′)*	*Base pass*	*Annealing Temp C*	*Genbank acc. no*
1	Amylase	CTGTCGTCTGGTTGGTCTT	119BP	60 oC	KJ586127
	Amylase Forward	CTGTCGTCTGGTTGGTCTT CTG	119BP	60°C	KJ586127
2	Amylase Reverse	AGGCGTCCACTCTGAATCC A			
3	Trypsin Forward	CAGTTCATCGATTCTGCAA AGG	115BP	60°C	KJ716487
4	Trypsin Reverse	GCAGGGAGATGGGCTGTA CA			
5	Chymotrypsin Forward	TCTCCTGTGTGCCTCCTCG A	115BP	60°C	
6	Chymotrypsin Reverse	TTGGTCAGCAGAGGCAGA GC.			

Quantitative real time PCR (qPCR). The expression of target genes was analyzed using the LightCycler 480 (Roche Diagnostics, Basel, Switzerland). Each 10 µL amplification contained 3µL PCR-grade water, 5 µL Brilliant III ultra-fast SYBR Green

QPCR Master mix (Agilent Technologies), 1 µL cDNA template of each forward and reverse primer. Each sample was assayed in duplicate, including a no template control. The three step qPCR program included an enzyme activation step at 95°C (3 min) and 40 cycles of 95°C (5 s), annealing temperature of each primer set (10 s) and 60°C (10 s). For determination of the quantification cycle (Cq) the second derivative maximum method measuring a maximum increase rate of newly synthesized DNA per cycle was used on the basis of the LightCycler 480 software release 1.5.0 (Roche Diagnostics). To confirm amplification specificity, the PCR products from each primer pair were subjected to melting curve analysis and visual inspection by agarose gel electrophoresis. Two sets of house-keeping gene β-actin (actb), elongation factor-1α (ef-1α) were evaluated for use as reference genes by ranking relative gene expression according to their overall CV and their interspecific variance as described elsewhere (Kortner et al. 2011). The geometric average expression of β-actin (actb) was used as a normalization factor. Mean normalized expression of the target genes was calculated from raw Cq values by relative quantification (Müller et al. 2002).

Statistical analysis. Data were analyzed by one-way analysis of variance (ANOVA) and the significant difference among the treatments was determined by Duncan's Multiple Range Test using SPSS (Version 12.0). Results are reported as mean ± SEM (n=6) and each fish was considered as an experimental unit. The level of significance was 0.05.

Results

Growth performance. Best growth performance (percentage weight gain, FCR, PER and SGR) was observed in the 40% carbohydrate fed group. Further increase in the level of carbohydrate significantly reduced the growth performance. However, there was no significant difference between the growth performance of the 30% and 50% carbohydrate fed groups (Table 4). There was no mortality recorded in fish fed any of the experimental diets.

Table 4. Growth of *Cirrhinus mrigala* fed graded level of carbohydrate.

Parameter	*Diet 1*	*Diet 2*	*Diet 3*
	30% CHO	*40% CHO*	*50% CHO*
Weight gain (%)	46.79±2.96^{a}	72.51±6.97^{b}	54.17±1.65^{a}
FCR	1.72±0.08^{b}	1.21±0.07^{a}	1.54±0.04^{b}
PER	1.67±0.07^{a}	2.60±0.17^{b}	1.85±0.04^{a}
SGR (%)	0.85±0.04^{a}	1.21±0.09^{b}	0.96±0.02^{a}
Hepatosomatic index	1.38±0.12^{a}	2.23±0.93^{b}	2.41±0.80^{b}

Different superscripts in the same row signify statistical differences (P<0.05) (mean±S.E.M.) (n=6).

Digestive and metabolic enzymes Maximum amylase activity was recorded in the 40% carbohydrate fed group and activity reduced significantly (P<0.05) in the 50% carbohydrate fed group. However, 50% carbohydrate fed group registered significantly higher activity than its 30% fed counterpart. The same trend was also observed in trypsin and chymotrypsin but no significant difference was noticed between these enzymes at the 40% and 50% inclusion level of carbohydrate. Lipase enzyme showed no significant difference (p>0.05) at any level of carbohydrate inclusion (Table 5).

AST and ALT values in the liver were not significantly different (P>0.05) at any level of carbohydrate inclusion in the experimental diets. Muscle ALT and AST on the other hand, increased significantly in response to the carbohydrate levelS up to 40%. No significant difference was found in the muscle LDH. Significant difference in both liver and muscle MDH was observed between the experimental groups (Table 5). The MDH activity in muscle increased with the inclusion level of carbohydrate but in the liver maximum activity was found only at 50% carbohydrate fed group.

Parameter	Diet 1	*Diet 2*	*Diet 3*
	30% CHO	40% CHO	50% CHO
Amylase	7.52±1.29[a]	11.53±1.44[c]	10.15±2.32[b]
Lipase	0.320±0.03	0.463±0.04	0.342±0.09
trypsin	15.68±2.82[a]	22.51±2.96[b]	24.32±3.14[b]
Chymotrypsin	16.98±2.58[a]	23.72±3.68[b]	21.72±2.57[b]
ALT Muscle	11.17±1.88[a]	23.95±1.97[b]	20.78±1.89[b]
ALT Liver	21.99±1.79	17.62±1.47	15.53±1.44
AST Muscle	39.97±2.96[a]	52.94±3.78[b]	44.45±8.19[ab]
AST Liver	21.58±4.72	43.96±7.14	19.66±4.56`
LDH Muscle MDH	14.70±1.53	15.94±1.43	18.11±1.68
LDH Liver	2.34±0.16[b]	1.27±0.24[a]	3.42±0.13[c]
MDH Muscle	179.34±14.30 [a]	361.65±15.84 [b]	521.39±26.08 [c]
MDH Liver	101.76±10.84 [a]	89.29±4.09 [a]	143.54±7.01[b]

Table 5. Digestive and metabolic enzyme activities of *cirrhinus mrigala* fed graded level of carbohydrate activity is expressed as follows: protease as micromol of tyrosine released/min/g protein; amylase as micromol of maltose released/min/g protein; lipase as units/mg protein; Different superscripts in the same row signify statistical differences (P<0.05) (mean±S.E.M.) (n=6).

Hematological parameters. Total serum protein, albumin, globulin and cholesterol levels did not vary significantly with the inclusion level of the carbohydrate in the experimental diets. Similarly, other blood parameters like total RBC, WBC, Hg, HCT values did not vary significantly among the groups. However, total serum lipid content was significantly higher in the 40% carbohydrate fed group (Table 6).

Table 6. Serum and Heamatological parameters of *cirrhinus mrigala* fed graded level of carbohydrate.

Parameter	*Diet 1*	*Diet 2*	*Diet 3*
	30% CHO	*40% CHO*	*50% CHO*
Total protein(g/dL)	3.85±0.61	3.69±0.20	3.83±0.06
Albumin (g/dL)	2.36±0.16	2.43±0.07	2.33±0.122
Globulin (g/dL)	1.49±0.46	1.26±0.15	1.50±0.19
Albumin/globulin	1.80±0.36	1.97±0.21	1.63±0.28
Cholesterol (mg/dL)	295.20±66.40	341.27±58.73	208.81±6.12
Total lipid (mg/dL)	1210.83±19.93 [b]	2050.83±109.55 [c]	749.35±76.65[a]
WBC (K/uL)	237.70±15.85	237.67±5.36	237.23±14.01
RBC (M/uL)	2.07±0.35	1.87±0.22	1.96±0.34
HGM (g/dl)	8.10±1.66	7.93±0.86	7.93±0.86
HCT (%)	31.57±5.64	27.33±2.39	28.30±4.65
MCV (Fl)	152.50±6.45	147.40±4.39	145.43±6.19
MCH (pg)	38.57±1.95	42.57±0.33	41.13±1.98
MCHC (g/dl)	25.43±1.99	28.93±0.64	28.43±2.35
PLATLET (m/uL)	19.33±5.36	16.33±4.91	17.50±4.50
LYSOZOME (%)	97.55±0.35	98.10±0.26	98.00±0.40

Different superscripts in the same row signify statistical differences (P<0.05) (mean±S.E.M.) (n=6).

Gene expression of digestive enzymes. Amylase enzyme expression varied with the level of carbohydrate in the experimental groups. Fish group fed with 50% carbohydrate had significantly higher expression (p<0.05) than their 30% and 40% counterparts. Amylase expression was also found to increase across the study period in the 50%CHO fed group, while the 30% fed group showed no significant difference over the different study period. (Figure 1)

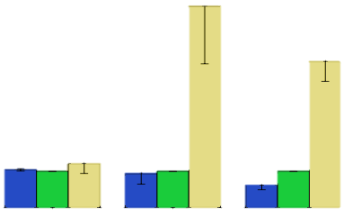

Figure 1. Shows the relative expression of amylase gene in the experimental groups across the different periods.

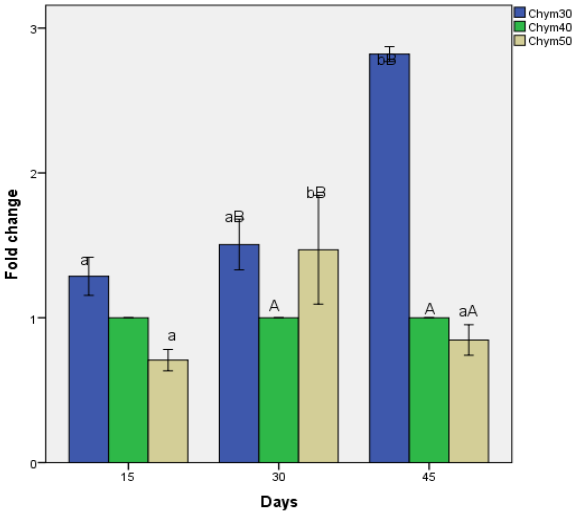

Fig. 2. The relative expression of chymotrypsin gene in the experimental groups across the different periods.

Trypsin gene expression also responded significantly to the level of CHO in the diets following the same pattern with the amylase gene, the highest trypsin expression obtained on day 15 after a drop on 30 days and again a significant rise in 45 days. This same pattern was repeated in both the 30 and 50% inclusion of CHO.

Chymotrypsin expression increased over the time period, but unlike the trypsin expression, 30% carbohydrate fed group exhibited higher expression than the 50%CHO fed group. (Figs. 2 and 3)

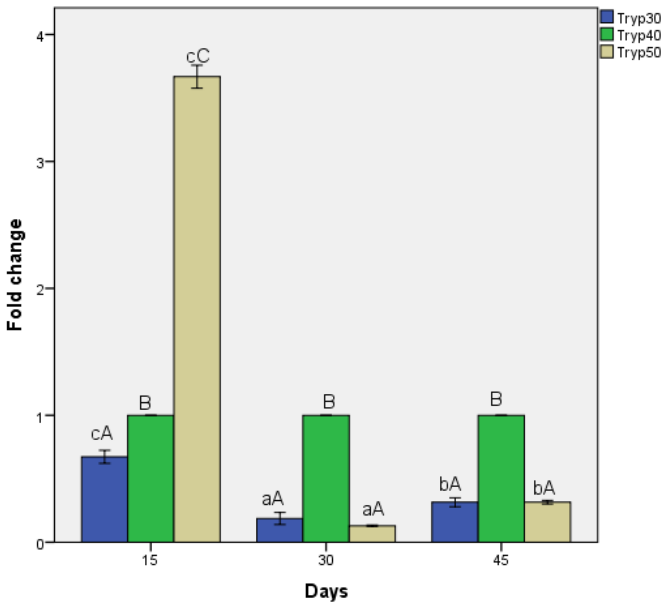

Fig. 3. The relative expression of trypsin gene in the experimental groups across the different periods.

Discussion

Excess carbohydrate has been implicated to cause metabolic stress (Zhou et al 2013) in fish. This is because increased cortisol secretion and nutrient absorption is impaired. This may be due to feeding to satiation which leads to reduced fish growth (Hemre et al 2001). Lower growth performance and SGR of fish fed 50% level of carbohydrate in this study agrees with findings of Tan et al (2009), Gao et al (2010) and Zhou et al (2013), who fed excess carbohydrate levels to different species of fish

Growth is also limited when fish are fed insufficient carbohydrate; this ultimately results in metabolism of protein to produce energy. This process is both uneconomical and un-ecofriendly. At a young age, the feeding pattern of rohu, catla and mrigal fish are similar, 40% carbohydrate and 35% protein was optimal for rohu fish (Saha and Ray 2001), while, 40% carbohydrate and 30% protein, was optimum for maximum protein sparing in mrigal fish (Singh et al. 2006).

In this study, weight gain and specific growth rate were affected by feeding high dietary carbohydrate. Similar results were also observed in other species (Zhou et al. 2013). Hepatosomatic index increased significantly in experimental diets; this could be due to either excess glycogen or stored lipid. Significant increase in HSI in rohu fed varied CHO levels were observed (Debnath et al. 2007).

The α-amylase, responsible for hydrolysis of α-1,4 glycoside bond in glycogen and starch as well as related polysaccharides and oligosaccharides (Lazo et al. 2011) increased significantly with the inclusion level of CHO (Kumar et al. 2006).

The reported optimum carbohydrate level for rohu fish was 35% with inclusion of 43.48% protein (Debnath et al. 2007), but our present study suggests that 40% CHO is sufficient for optimum growth in mrigal fish. Beyond this, growth was reduced.

Lipase enzyme activity was not significant among fish fed varying levels of carbohydrate. This is probably due to similar levels of lipid inclusion among the different diets, (Debnath et al. 2007). Both chymotrypsin and trypsin digestive enzymes did not respond significantly to carbohydrate inclusion levels beyond 40%. There was an increase in protease enzyme in *Catla catla* fed varying gelatinized starch (Yengkokpam et al. 2007). On the other hand, trypsin and chymotrypsin level in fish did not vary significantly even up to 50% inclusion level of corn starch in the diet of *Colossoma macropomum* (Corrêa et al. 2007). This variation appears to be species specific as reported by many authors (Zhou et al. 2013).

Total lipid in serum varied significantly with the carbohydrate inclusion level, though the diets are iso-lipids, however, increased supply of carbohydrate, induced lipogenesis (Enes et al. 2006). This may probably explain the increased total lipid as obtained in this study.

ALT and AST are responsible for the conversion of amino acids towards the synthesis of non-essential amino acids. In the carbohydrate fed groups the levels of ALT and AST in the muscle increased up to 40%, after which it significantly decreased. No significant changes of those enzymes were found in the liver, which is in agreement with the report that most enzymes (transaminases) that initiate amino acid catabolism in the liver were unaffected by the changes in dietary treatment (Fernández et al. 2007).

The adaptive digestive capacities of fish under different feeding regimes and their growth, is of major interest in aquaculture. Digestive enzyme activity and mechanisms controlling them may, be important in maximizing absorption efficiency and food utilization. Indeed, digestive enzyme activity has been reported to affect absorption efficiency for their corresponding substrates, and this could probably be one of the principal factor explaining in situ growth differences in fish (Huvet et al. 2012).

The significant increase in weight gain with a corresponding increase in expression of amylase gene is in line with the findings of Huvet et al. (2012), but beyond 40% CHO level, a significant decrease in weight gain was observed at 50% CHO. This corresponds with decreased amylase enzyme activity as reported in this study. The differences observed between enzyme activity and expression results suggest a post transcriptional (Gawlicka and Horn, 2006) or translational (Wang et al. 2006) regulation, respectively. Similar result was recorded for trypsin expression on Jian carp fish fed different isoleucine inclusion levels (Zhou et al. 2013).

Trypsin is important as the key enzyme for feed utilization and growth through its role in the protein digestion processes (Rungruangsak-Torrissen and Male 2000). Growth mechanisms are primarily affected by trypsin expression and thus influence protein and amino acid utilization and growth.

The group fed 40% CHO exhibited the highest trypsin gene expression on day 30 and 45 despite the same protein inclusion level in all the experimental diets. This trend could be explained thus; when the protein /energy ratio is adequate, the utilization of protein in the diet will be devoted for growth and rate of conversion to protein to generate energy will be reduced, thus maximum growth could be achieved as obtained in this study. Variations in the expression of trypsin have been related to feed conversion efficiency and/or growth rate by affecting the rates of protein digestion and resulting in differences in amino acid absorption and transport (Sunde et al. 2004).

30% CHO level produced the lowest growth rate and low CHO inclusion, produced the highest level of chymotrypsin specific activity. This is in line with the findings of Rungruangsak-Torrissen et al. (2006). High chymotrypsin specific activity is usually observed under conditions of starvation or food deprivation. This is in contrast to the trypsin specific activity.

A new feed formulation approach providing approximate nutrient needs of juvenile fish, would enable us to draw conclusions from the combined studies of the above methodologies since both amylase and trypsin gene expression responded to the inclusion level of carbohydrate. Trypsin is the most sensitive protease for growth, while chymotrypsin, plays a major role when growth is limited or depressed.

Acknowledgements

The authors would like to thank Dr. W.S. Lakra, Director, Central Institute of Fisheries Education, Mumbai for providing infrastructural facilities for carrying out the work. The first author is grateful to TWAS-DBT for providing the fellowship and to the University of Lagos Nigeria for granting study leave.

References

Chan A.S., Horn M.H., Dickson K.A. and A. Gawlicka, 2004. Digestive enzyme activities in carnivoures and herbivoures: Comparision among four closely related Prickleback fishes (*Teleostei stichaeidae*) from California rocky intertidal habitat. *J. Fish Biol*, 65:848-858.

Cherry I.S. and L.A. Crandell Jr., 1932. The specificity of pancreatic lipase: its appearance in blood after pancreatic injury. *Am. J. Physiol,* 100: 266–273.

Corrêa C.F., De Aguiar L.H. and L.M. Lundstedt, 2007. Responses of digestive enzymes of tambaqui (*Colossoma macropomum*) to dietary cornstarch changes and metabolic inferences. *Comp. Biochem. Physiol.,* 147: 857-862.

Debnath D., Pal A.K., Sahu N.P., Yengkokpam S., Baruah K., Choudhury D. and G. Venkateshwarlu, 2007. Digestive enzymes and metabolic profile of *Labeo rohita* fingerlings fed diets with different crude protein levels. *Comp. Biochem. Physiol,* B 146: 107–114.

Enes P., Panserat S. Kaushik S. and A. Oliva-Teles, 2006. Rapid metabolic adaptation in European sea bass (*Dicentrarchus labrax*) juveniles fed different carbohydrate sources after heat shock stress. *Comp. Biochem. Physiol,* Part A. *Mol Integr. Physiol.* 145:73-81.

Fernández F., Anna G. M., Marlon C., Manuel V., Isidoro M., Anna C. and V.B. Isabel, 2007. Effects of diets with distinct Protein-to-carbohydrate ratios on nutrient digestibility, growth performance, body composition and liver intermediary enzyme activities in gilthead sea bream (*Sparus aurata*, L.) fingerlings. *J. Exp. Mar. Bio. Eco,* 343: 1–10.

Gao W., Liu Y.J., Tian L.X., Mai K.S., Liang G.Y., Yang H.J., Huai M.Y. and W.J. Luo, 2010. Effect of dietary carbohydrate-to-lipid ratios on growth performance, body composition, nutrient utilization and hepatic enzymes activities of herbivorous grass carp (*Ctenopharyngodon idella*). *Aquac Nutr.* 16:327–333.

Gawlicka A.K. and M.H. Horn, 2006. Trypsin gene expression by quantitative *in situ* hybridization in carnivorous and herbivorous prickle back fishes (Teleostei: stichaeidae): ontogenic, dietary and phylogenetic effects. *Physiol. Biochem. Zool.* 79:120-132.

Hemre G.I., Mommsen T.P. and A. Krogdahl, 2001. Carbohydrates in fish nutrition: effects on growth, glucose metabolism and hepatic enzymes. *Aquac. Nutr,* 7: 1–20.
Huvet A., Fleury E., Corporeau C., Quillien V., Daniel J.Y., Riviere G., Boudry P. and C. Fabioux, 2012. In vivo RNA interference of a gonad-specific transforming growth factor-β in the Pacific oyster Crassostrea gigas. *Mar. Biotech.* 14:402-410.
Kortner T.M., Overrein I., Øie G., Kjørsvik E., Bardal T., Wold P.A. and A. Arukwe, 2011. Molecular ontogenesis of digestive capability and associated endocrine control in Atlantic cod (Gadus morhua) larvae. *Comp. Biochem. Physiol. - Part A: Mol. & Integr. Physio*. 160: 190-199.
Kumar S., Sahu N.P., Pal, A.K., Choudhury D. and S.C. Mukherjee, 2006. Studies on the digestibility and digestive enzymes activities of *Labeo rohita* Hamiilton juveniles: effect of microbial amylase supplementation in non-gelatinised or gelatinized corn-based diet at two protein levels. *Fish Physiol. and Biochem* 32: 209-220
Kunitz M., 1947. Crystalline soybean trypsin inhibitor; 2. General properties. *J. Gen. Physiol,* 30: 291-310.
Lazo J.P., Darias M.J. and E. Gisbert, 2011. Chapter 1 *Ontogeny of the digestive tract*. In: Holt, G.J. (Ed.), Larval Fish Nutrition. John Wiley & Sons, Inc., pp. 5–46.
Lopez-Lopez S., Nolasco H., Villarreal-Colmenares H., and R. Civera-Cerecedo, 2005. Digestive enzyme response to supplemental ingredients in practical diets for juvenile freshwater crayfish *Cherax quadricarinatus*. *Aquac. Nutr,* 11: 79–85.
Lowry D.H., Rosenbrough N.J. and A.L. Far, 1951. Protein measurement with folin phenol reagent. *J. Biol. Chem.,* 193: 265-275.
Lundstedt L.M., Fernando J., Melo B. and G. Moraes, 2004. Digestive enzymes and metabolic profile of Pseudoplatystoma corruscans (Teleostei: Siluriformes) in response to diet composition. *Comp. Biochem. and Physiol.* part B 137: 331-339.
Müller R., Owen C.A., Xue Z.T., Welander M. and B.M. Stummann, 2002. Characterization of two CTR-like protein kinases in *Rosa hybrida* and their expression during flower senescence and in response to ethylene. *J. Exper. Bot.,* 53:1223-1225.
Nikapitiya C., De Zoysa M., Whang I., Kim C., Lee Y., Kim S. and J. Lee, 2009. Molecular cloning, characterization and expression analysis of peroxiredoxin 6 from disk abalone *Haliotis discus discus* and the antioxidant activity of its recombinant protein. *Fish Shellfish Immunol.*, 27: 239–249.
Ren, M.C., Ai, Q.H., Mai, K.S., Ma, H.M., Wang, X.J., 2011. Effect of dietary carbohydrate level on growth performance, body composition, apparent digestibility coefficient and digestive enzymeactivities of juvenile cobia, *Rachycentron canadum* L. Aquaculture Research 42, 1467-1475.
Rungruangsak-Torrissen, K. and R. Male, 2000. Trypsin isozymes: development, digestion and structure. In: *Seafood Enzymes, Utilization and Influence on Postharvest Seafood Quality*. pp. 215–269. Edited by N.F. Haard and B.K. Simpson. Marcel Dekker, Inc., New York.
Rungruangsak-Torrissen K., Moss R., Andresen L.H., Berg A. and R. Waag, 2006. Different expressions of trypsin and chymotrypsin in relation to growth in Atlantic salmon (Salmo salar L.). *Fish Physiol. Biochem.* 32: 7-23.
Saha A.K. and A.K. Ray, 2001. Optimum dietary carbohydrate requirement of rohu, Labeo rohita (Hamilton), fingerlings. *ActaIchthyologicaet Piscatoria,* 31: 81-96.
Singh R. K., Balange A.K. and M. M. Ghughuskar, 2006. Protein sparing effect of carbohydrates in the diet of *Cirrhinus mrigala* (Hamilton, 1822) fry. *Aquaculture,* 258:680-684.
Sunde J., Eiane S.A., Rustad A., Jensen H.B., Opstvedt J., Nyga˚rd E., Venturini G. and K. Rungruangsak-Torrissen, 2004. Effect of fish feed processing conditions on digestive protease activities, free amino acid pools, feed conversion efficiency and growth in Atlantic salmon (*Salmo salar* L.) *Aquac. Nutr.*, 10: 261–277.
Tan QS, Wang F, Xie SQ, Zhu XM, Lei W. and JZ Shen, 2009**.** Effect of high dietary starch levels on the growth performance, blood chemistry and body composition of gibelio crucian carp (*Carassius auratus* var. gibelio) Aquaculture Res.2009;40:1011–1018.

Wang C., Xie S., Zhu X., Lei W., Yang Y. and J. Liu, 2006. Effects of age and dietary protein level on digestive enzyme activity and gene expression of *Pelteobagrus fulvidraco* larvae. *Aquaculture.* 254: 554-562.
Wooten, D.P., 1964. Microanalysis In: *Medical biochemistry* 4th edn. J. and A. Churchill London; pp: 101-107.
Yengkokpam S., Sahu N.P., Pal A.K., Mukherjee S.C. and D. Debnath, 2007. Gelatinised carbohydrates in the diet of *Catla catla* fingerlings: Effect of levels and sources on nutrient utilization, body composition and tissue enzyme activities. *Asian-Aust. J. Anim. Sci.* 20, 9 1: 89-99.
Zambonino Infante J. L., and C.L. Cahu, 2001. Ontogeny of the gastrointestinal tract of marine fish larvae. *Comp. Biochem. and Physiol.,* 130(4): 477-487.
Zhou C., Liu B., Ge X., Xie J. and P. Xu, 2013. Effect of dietary carbohydrate on the growth performance, immune response, hepatic antioxidant abilities and heat shock protein 70 expression of Wuchang bream, *Megalobrama amblycephala*. *J. Appl. Ichthyol.,* 29:1348–1356.