

Growth and Biochemical Studies of Mrigal Fish (*Cirrhinus mrigala*) Fed Graded Levels of Oil

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Abstract: A 45-day experiment was carried out to evaluate the growth performance, digestive enzymes activity and biochemical response of *Cirrhinus mrigala* fed graded levels of oil. Three isonitrogenous purified diets (6%, 8% and 10% of oil) were formulated. A total of 108 juveniles of *Cirrhinus mrigala* (5.95±0.25) g were equally distributed in triplicate groups in nine plastic tanks of 100 L capacity and fed under natural light cycle. Growth performance of the fish significantly reflected the effect of inclusion levels of oil tested but the diet with 8% lipid had the best significant growth performance [% weight gain, Feed Conversion Ratio (FCR) and Specific Growth Rate (SGR)], across different experimental groups. Levels of secretion of digestive enzymes in the fish fed different experimental diets resulted in significant values of ($p<0.05$) amylase and lipase, while Aspartate transaminase (AST), Alanine transaminase (ALT), Lactate dehydrogenase (LDH) and Malate dehydrogenase (MDH) values in the muscle and liver decreased significantly ($p<0.05$) as the levels of oil in the diet increased. However, serum biochemical parameters did not record any significant difference ($p>0.05$) with the exception of the total lipid across diets. The study confirmed the inclusion effect of graded levels of oil in juvenile Mrigal diet, but the inclusion should not be more than 8% to avert growth depression.

Key words: Mrigal, oil level, growth, enzyme activity

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Introduction

Mrigal (*Cirrhinus mrigala*) is one of the three economically important India major carp, constituting between 25%-31% of the cultivated carp population in India. Production cost of carp is constituted by 50% feed cost and for the targeted doubled production target, the understanding of cost effective feed production through adequate utilization of dietary lipid will be of great importance.

Dietary lipids are important sources of energy, Essential Fatty Acids (EFA), sterols and phospholipids for the fish. Dietary lipids are necessary for proper

functioning of physiological processes, maintenance of biological structures and act as carriers of nutrients such as the fat-soluble vitamins (A, D, E and K), carotenoids and hormones (Ghanawi *et al.*, 2010). The addition of lipids to a diet also contributes to the effective utilization of dietary protein, through the protein sparing effect in fish. Lipid composition in fish affects fillet texture and flavor and keen interest in fish lipids has increased, due to commercial values of hepatic and muscular lipids and its increased relevance in human nutrition.

Fish ability to utilize dietary lipids is limited, beyond a certain level; growth may be retarded owing to reduced feed consumption (Ellis and Reigh, 1991).

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The needs for nutrient requirement studies, justified by growth rate and feed efficiency measurements, are very essential for effective, economic and viable culture of fish but beyond this traditional method in fish nutrition, a paradigm shift from growth maximization concept to digestive and metabolic enzyme activity optimization is of unquestionable importance in fish feeding procedures (Lundstedt *et al.*, 2004).

Growth rate in fish is dependent on digestive capacity, oxygen availability and metabolic capacity require supporting tissue protein synthesis (Blier *et al.*, 1997). Therefore, the measurement of digestive, metabolic enzymes and metabolites can facilitate in determining the optimal dietary nutrient composition which can be effectively utilized.

Lipase is a category of digestive enzymes which hydrolyse the ester bonds between fatty acids and glycerols, and carnivorous and marine fishes are said to have higher activity of this enzyme than omnivorous and herbivorous fish (Tengjaroenkul *et al.*, 2000).

Fish are plastic in production of digestive enzymes and according to Chan *et al.* (2004), variations do exist in digestive enzymes resulting from different studies, due to methodology used, differences in assay, substrates, assay temperatures, instruments used for analyses, units of measurement of activities, species and ontogenetic stages examined, as well as quantity and composition of diet.

The objective of the present study was to find out the optimum lipid levels for Mrigal fish and to evaluate its effects on growth performance, biochemical and haematological parameters as well digestive enzyme activities in fish fed graded levels of oil.

Materials and Methods

Experimental fish and feeding

A total of 108 fingerlings of *Cirrhinus mrigala* (5.95 ± 0.25) g were equally distributed in nine plastic tanks of 100 L ($60 \times 60 \times 42$ cm³) capacity. Three experimental diets were formulated to have equal amount of protein and carbohydrate levels, but varied levels of lipid at

6%, 8% and 10%, respectively. The diets were made using purified ingredients (Himedia Laboratory, India) as shown in Table 1. Fat free casein and gelatin were used as the protein sources; cod liver oil and sunflower oil (ratio 1 : 2) were used as lipid sources, dextrin, starch soluble and cellulose as carbohydrate sources and carboxymethylcellulose as binder. Ingredients were mixed properly to form dough with the addition of adequate quantity of water. The dough was steam cooked for about 5 min in a pressure cooker and after cooling, oil, butylated hydroxytoluene and vitamin-mineral mixture were added appropriately. After incorporation of these ingredients, the dough was mixed properly and pelletized using twin-screw extruder. Pelletized feed was spread out on a paper, air dried overnight and stored in a refrigerator at 4°C until needed.

Table 1 Feed formulation

Ingredient	Diet		
	1	2	3
Inclusion (%)	6% oil	8% oil	10% oil
Casein	34.5	34.5	34.5
Gelatin	6	6	6
Dextrin	20	20	20
Starch soluble	10	10	10
Cellulose	19.48	17.50	15.48
Oil	6	8	10
V-M mix	2.7	2.7	2.7
Vit. C	0.1	0.1	0.1
CMC	1	1	1
BHT	0.02	0.02	0.02
Glycine	0.2	0.2	0.2
Total	100	100	100

V-M mix=Vitamin-mineral mix (EmixTM plus) (quantity/2.5 kg): Vitamin A-5 500 000 IU; Vitamin D₃-1 100 000 IU; Vitamin B₂-2 000 mg; Vitamin E-750 mg; Vitamin K-1 000 mg; Vitamin B₆-1 000 mg; Vitamin B₁₂-6 mg; Calcium panthothenate-2 500 mg; Niacinamide-10 gm; Choline chloride-150 gm; Mn-27 000 mg; Iodine-1 000 mg; Fe-7 500 mg; Cu-2 000 mg; Zn-5 000 mg; Co-450 mg; Ca-500 g; P-300 g; Se-50 mmol·L⁻¹; L-Lysine-10 g; DL-methionine-10 g. CMC-Carboxymethylcellulose.

Each of the three experimental diets was fed to a triplicate group of fish under natural light cycle of 12 h day light and 12 h darkness. Initially feed was given at 5% of the biomass and gradually adjusted based on

the observation of daily feed consumption. The total feed was divided into two installments and fed for 45 days at 10: 00 a.m. and 6: 00 p.m. Water exchange was done manually twice a week and continuous aeration was supplied from an air pump to maintain the dissolved oxygen at $7.37\text{--}7.85\text{ mg}\cdot\text{L}^{-1}$. The average water temperature recorded was between $28\text{--}30^{\circ}\text{C}$.

Growth study

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

$$\text{Weight gain (\%)} = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \times 100$$

$$\text{SGR (\%/Day)} = \frac{\ln \text{Final body weight} - \ln \text{Initial body weight}}{\text{Time (days)}}$$

$$\text{FCR} = \frac{\text{Dry weight of feed fed}}{\text{Fish weight gain}}$$

$$\text{Protein Efficiency Ratio (PER)} = \frac{\text{Weight gain (g)}}{\text{Crude protein intake (g)}}$$

$$\text{Hepato-somatic index (\%)} = \frac{\text{Liver weight}}{\text{Body weight}} \times 100$$

Sampling procedure

The fish were fasted for 24 h prior to sampling; six fish per treatment were sampled and anaesthetized with clove oil at $50\text{ }\mu\text{L}\cdot\text{L}^{-1}$ water to reduce stress. Blood was drawn from the caudal vein using a 1 mL medical syringe and immediately transferred to an Eppendorf tube containing 2.7% dry Ethylene Diamine-Tetraacetic Acid (EDTA) disodium salt to prevent clotting. Serum on the other hand was collected in a similar way but without the anticoagulant and later separated by centrifuging at 4°C for 10 min at $5\,000\times g$. Intestine, liver and muscle samples were collected and frozen immediately at -20°C for enzyme assays.

Digestive enzyme assays

The whole intestine was homogenized in cold

$0.25\text{ mol}\cdot\text{L}^{-1}$ sucrose in a teflon coated motor driven homogenizer to prepare a 5% homogenate. The homogenate was centrifuged at $5\,000\times g$ for 15 min in a cooling centrifuge; supernatant was collected, frozen in sample vials and stored at -20°C until use. Amylase (Rick & Stegbauer, 1974), trypsin and chymotrypsin (Kunitz, 1947) and lipase (Cherry and Crandell, 1932) enzyme activities were assayed following the standard procedure.

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

Plasma protein, albumen and metabolite concentrations

Plasma protein and albumen were estimated using the total proteins and albumin kit (Qualigens Fine Chemicals, Mumbai, India). Globulin was calculated by subtracting the albumin value from the total plasma protein value, and albumen/globulin (A/G) ratio was calculated. The total lipids and cholesterol were measured using Labkit range clinical kit (Spain) following manufacturer's guideline.

Assays of metabolic enzymes

Lactate Dehydrogenase (LDH)

LDH activity was assayed by the method of Wroblewski and Ladue (1955). A total of 3 mL of the reaction mixture comprised of 2.7 mL of $0.1\text{ mol}\cdot\text{L}^{-1}$ phosphate buffer (pH 7.5), 0.1 mL of NADH solution (2 mg NADH dissolved in 1 mL of phosphate buffer solution), 0.1 mL of tissue homogenate and 0.1 mL of sodium pyruvate. The reaction was started after addition of substrate sodium pyruvate. The Optical Density (OD) was recorded at 340 nm at 30 s inter-

vals. The enzyme activity was expressed as unit's mg protein⁻¹ min⁻¹ at 25°C, where 1 unit was equal to $\Delta 0.01 \text{ OD min}^{-1}$.

Malate dehydrogenase (MDH)

MDH activity was estimated following Ochoa (1955). The reaction mixture comprised of 0.1 mol·L⁻¹ phosphate buffer (pH 7.5), NADH solution (2 mg/1 mL phosphate buffer), tissue homogenate and freshly prepared oxaloacetate solution (2 mg in 2 mL ice cold phosphate buffer). The reaction was started by adding oxaloacetate solution as substrate and change in OD was recorded at 340 nm at 30 s intervals for 3 min. The enzyme activity was expressed as units per mg protein per min at room temperature where 1 unit was equal to $\Delta \text{OD/min}$.

Aspartate amino transferase (AST) and alkaline amino transferase (ALT)

AST activity was assayed in different tissue homogenates as described by Wooten (1964). The substrate comprised of 0.2 mol·L⁻¹ D, L-aspartic acid and 2 mmol·L⁻¹ α -ketoglutarate in 0.05 mol·L⁻¹ phosphate buffer (pH 7.4). In the experimental control tubes, 0.5 mL of substrate was added. The reaction was started by adding 0.1 mL of tissue homogenate. The assay mixture was incubated at 37°C for 60 min. The reaction was terminated by adding 0.5 mL of 1 mmol·L⁻¹ 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. After which, 5 mL of 0.4 mL NaOH solution was added, the contents were thoroughly mixed and after 10 min, and OD was recorded at 540 nm against the blank. ALT activity was determined using the 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 per min per mg protein at 37°C.

Haematological parameter analyses

Complete blood analysis was done with automated Sysmex XS-1000iTM Auto Hematology Analyzer SYS-XS-1000i from Sysmex Corporation, Kope, Japan.

Results

Growth and nutrient utilization parameters

Growth performance among the groups fed different experimental diets reflected the effect of varied inclusion levels of oil. The fish fed 8% lipid had the best performance in terms of growth [% weight gain and Specific Growth Rate (SGR)] and nutrient utilization [Feed Conversion Ratio (FCR) and PER]. However, further increases in the oil inclusion (10%) levels showed no significant effects ($p > 0.05$) on all the performance parameters studied compared to that of the group fed 8% (Table 2).

Digestive enzyme activities

The dietary lipid levels had no significant ($p > 0.05$) impact on trypsin and chymotrypsin enzyme activities, wherein amylase and lipase enzymes responded significantly ($p < 0.05$), with the highest value recorded in 10% group, compared to 6% fed group (Table 3).

Serum biochemical parameters

The total protein, albumin, globulin and cholesterol levels among various groups did not differ significantly ($p > 0.05$). Serum total lipid contents showed that the fish fed 10% lipid recorded the lowest value compared to other fed groups ($p < 0.05$) (Table 4).

Table 2 Growth and nutrient utilization parameters

Level	% Weight gain	SGR	PER	FCR	HSI
6%	39.77±1.90a	0.74±0.03a	1.44±0.07a	1.99±0.18b	1.48±0.13a
8%	52.50±3.70b	0.93±0.06ab	1.85±0.06b	1.54±0.09a	2.34±0.40ab
10%	46.83±1.23ab	0.85±0.02b	1.66±0.02b	1.71±0.04a	2.84±0.33b

Different letters in the same column differ significantly ($p < 0.05$) (mean±S.E.M.) ($n=3$).

Table 3 Digestive enzyme activities

Level	Amylase	Lipase	Trypsin	Chymotrypsin
6%	6.50±0.00034a	0.249±0.015a	7.78±0.040	7.40±0.014
8%	9.61±0.0015ab	0.329±0.031ab	9.37±0.022	8.88±0.0001
10%	14.83±0.0009b	0.440±0.076b	9.94±0.013	8.35±0.006

Activities are expressed as follows: protease as micromol of tyrosine released per min per g protein; amylase as micromol of maltose released per min per g protein; lipase as units per mg protein; different letters in the same column differ significantly ($p<0.05$) (mean±S.E.M.) ($n=3$).

Table 4 Serum biochemical parameters

Level	Total protein (g·dL ⁻¹)	Albumin (g·dL ⁻¹)	Globulin (g·dL ⁻¹)	A/G ratio	Cholesterol (mg·dL ⁻¹)	Total lipid (mg·dL ⁻¹)
6%	3.81±0.22	2.34±0.09	1.47±0.17	1.64±0.17	284.28±22.12	1699.03±66.5b
8%	4.65±0.38	2.46±0.11	2.19±0.36	1.19±0.21	289.33±28.81	1851.72±99.6b
10%	3.95±0.37	2.48±0.04	1.47±0.002	1.69±0.03	260.82±9.79	1005.76±41.2a

Different letters in the same column differ significantly ($p<0.05$) (mean±S.E.M.) ($n=3$).

made in LDH and MDH enzyme activities (Table 5).

Metabolic enzymes activities

The activities of AST and ALT enzymes among various dietary groups in both the muscle and liver showed a decreasing trend with the lowest value recorded in 10% fed group. Similar observation was

Haematological parameters and lysozyme activity

Feeding varied levels of lipid to *C. mrigala* had no significant effects on the haematological parameters and lysozyme activity (Table 6).

Table 5 Metabolic enzyme activities

Level	ALT (M)	AST (M)	ALT (L)	AST (L)	LDH (M)	MDH (M)
6%	236.91±23.81b	77.87±2.49b	210.20±16.63b	129.56±15.90b	19.31±0.25b	657.31±40.91b
8%	202.11±13.85b	68.22±4.14b	125.59±2.53a	75.99±8.39a	11.16±0.19a	351.74±38.21a
10%	101.09±4.60a	29.33±1.76a	94.38±2.05a	70.55±8.78a	11.08±0.25a	244.43±17.39a

Different letters in the same column differ significantly ($p<0.05$) (mean±S.E.M.) ($n=3$). M, Muscle; L, Liver. ALT, Alanine transaminase; AST, Aspartate transaminase; LDH, Lactate dehydrogenase; MDH, Malate dehydrogenase.

Table 6 Haematological parameters

Level	WBC	RBC	HG	HCT	MCV	MCH	MCHC	Platelet	Lysozyme
6%	247.20±8.43	1.91±0.12	7.70±0.35	27.70±1.07	145.03±6.48	40.23±0.88	27.90±1.82	28.33±4.80	97.50±0.25
8%	224.43±11.91	1.82±0.14	7.63±0.64	25.77±1.07	143.90±15.04	41.90±0.27	29.87±3.47	18.00±3.76	98.26±0.34
10%	237.60±4.87	1.93±0.34	7.60±1.13	25.67±3.32	135.30±7.92	39.70±1.11	29.43±1.10	19.00±3.00	98.13±0.22

Different letters in the same column differ significantly ($p<0.05$) (mean±S.E.M.) ($n=3$).

Discussion

Growth of fish is one of the most desirable qualities

sought by aquaculturist and this depends on species, culture conditions, feeds and feeding regimes. Growth in fish could partly be said to be a function of digestive capacity, oxygen availability or metabolic capacity

required to support tissue protein synthesis (Blier *et al.*, 1997; Fawole *et al.*, 2016).

Generally, the increase of dietary lipid levels improves growth, feed and protein utilization efficiency. Possible explanation for this could be due to the protein sparing action of lipids. According to Li *et al.* (2010) nitrogen retention increased significantly as protein levels decreased and lipid levels increased. However, some authors have observed no protein sparing effect on lipid (Thoman *et al.*, 1999). As observed in this study, optimum limit for lipid in mrigal diets was 8% beyond which dietary fat could cause growth depression. Similar observation has been reported in other findings (Du *et al.*, 2005; López-López *et al.*, 2005). Most studies attributed the reduced growth of fish fed on high lipid diets to the excessive energy that could cause reduced feed intake and invariably the uptake of some essential nutrients like protein and vitamins, coupled with limited ability to digest and absorb high amounts of lipid (Wang *et al.*, 2005).

The reduced growth and PER of the fish fed the high lipid diet in the present study indicated that excessive dietary lipid levels could impair growth performance. Similar results have also been reported for common dentex (*Dentex dentex*) and turbot (*Psetta maxima*) (Espinós *et al.*, 2003; Wang *et al.*, 2005).

The differences in Hepatosomatic index (HSI) and serum total lipids were due to the graded dietary lipid levels in the experimental diets and this was equally observed for the following fish: grouper (*Epinephelus coioides*) (Luo *et al.*, 2005), haddock (*Melanogrammus aeglefinus*) (Tibbetts *et al.*, 2005), rockfish (*Sebastes schlegeli*) (Lee *et al.*, 2002) and cobia (Wang *et al.*, 2005). On the contrary, HSI and VSI were found not significantly different among varied lipid levels in the following species: shortfin eel (*Anguilla australis*) (De Silva *et al.*, 2001), white seabream (Sá *et al.*, 2006) and brown meagre (Chatzifotis *et al.*, 2006). Trypsin and chymotrypsin levels in mrigal fish were not significantly affected by the graded levels of lipid, which agreed with previous results from Correa *et al.* (2007). The possible explanation for this in the present study

could be due to similar levels of dietary protein across different diets tested.

Amylase and lipase digestive enzymes showed a significant response to dietary lipids tested. Lipase is the digestive enzyme responsible for the breakdown of bonds between fatty acids and glycerol and since fish are plastic in production of digestive enzymes in response to diet (Caviedes-vidal *et al.*, 2000), the increases in levels of these lipid digestive enzymes are thus expected. According to Cho (1987), up to 20%-80% energy loss could be recorded in fish when the synthesis of digestive enzymes failed to respond to dietary nutrient level.

Cholesterol, the total proteins, albumin, globulin and albumin/globulin ratio did not vary significantly among various groups. The experimental diets were similar in proteins and carbohydrate levels, which could probably be the reason for the trend observed. Decreased serum total protein may be associated with onset of disease due to decreased capacity for synthesis (Petrescu-Mag *et al.*, 2007), reduced absorption or protein loss (Yang and Chen, 2003). Serum proteins were made up of molecular chaperones and directly involved in fish health homeostasis, and could explain why no mortality was recorded in fish fed all the experimental diets. Significant decrease in cholesterol levels could indicate a possible disease situation, an increase in physiological discomfort and likelihood dysfunction in lipidic metabolism (Patriche *et al.*, 2011). The total lipid in serum varied significantly with dietary lipid inclusion level up to 8% beyond which the value fell due to reduced feed intake and probably metabolic stress as observed.

ALT and AST are responsible for the conversion of amino acids towards the synthesis of non-essential amino acid. The levels of ALT and AST decreased significantly among different dietary lipid levels. This trend was also supported by the finding of Dalal *et al.* (2001), who reported that the level of GPT decreased with increased carbohydrate level. On the contrary, Fernandez *et al.* (2007) in *S. aurata* found that most enzymes (transaminases) that initiate amino

acid catabolism in the liver was unaffected by the changes in dietary treatment. Lactate dehydrogenase catalyses the inter-conversion of pyruvate and lactate with concomitant inter-conversion of NADH to NAD. When concentration of pyruvate is high, the enzyme exhibits feedback inhibition and rate of conversion of pyruvate to lactate is decreased. This is the scenario obtained in this study as a result of increased feed energy from increased inclusion of oil. Malate dehydrogenase also decreased like LDH with increased oil levels in the experimental diets, MDH being a TCA cycle enzyme that catalyses the conversion of malate to oxaloacetate. MDH could increase when there is need for energy demand and according to Das *et al.* (2006) increased MDH was found in fish acclimatized to high temperature. Increased oil contents of experimental diets at equal inclusion levels of protein and carbohydrate resulted in higher energy feed and the digestive system was thus loaded with high energy and a negative feedback thus generated by MDH enzyme. Haematological values in this experiment did not respond significantly to the levels of lipids tested and this was supported by findings of Shimeno *et al.* (1996) who recorded no significant variation in the haematological parameters of juvenile yellowtail fed varying levels of carbohydrate and lipid.

Conclusions

Both growth and biochemical parameters gave more comprehensive information about the nutritional, physiological status and lipid requirement needs of *Cirrhinus mrigala* and thus concluded that though increasing lipid favoured better growth, nutritional and physiological performance of *Cirrhinus mrigala*, but definitely not beyond 8% inclusion level in order to avoid poor growth and reduction in nutrient utilization.

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