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Influence of Feeding Regimes on the Digestive Enzyme Profile and Ultrastructure of Digestive Tract of First Feeding *Catla catla* (Hamilton) Larvae

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Abstract

The present investigation aimed to evaluate the effect of feeding regimes composed of various combinations of live food (LF), artificial food (AF) and mixed food (MF: LF and AF 50% each) on the digestive physiology of first feeding *Catla catla* (catla) larvae. Larvae (0.92 ± 0.21 mg) were cultured for 30 days under six different feeding regimes of LF, AF, MF, two groups were shifted from LF to AF on day-13 (LF-AF-13) and day-18 (LF-AF-18) and one group from LF to MF on day-12 (LF-MF-13). Significantly ($p<0.05$) higher survival and average weight of catla were found in MF, LF, and LF-MF-13, respectively compared to others. Chitinase and chitinobiase activities were significantly ($p<0.05$) higher in LF compared to others. This group was followed by LF-MF-13. Amylase, carboxypeptidase A and B activities were significantly ($p<0.05$) higher in LF-AF-18, MF and LF-AF-13, respectively compared to others. Total protease, trypsin, chymotrypsin and lipase activities were significantly ($p<0.05$) higher in LF-MF-13 compared to the other feeding regimes. SDS-PAGE and substrate SDS-PAGE showed the presence of 14 (14.34-97.57 kDa) and 8 (15.26-72.88 kDa) bands, respectively in the digestive tissue extract. The ultrastructure of the digestive tract viz. the height of microvilli, number and size of mitochondria, lipid droplet and goblet cell were influenced by the feeding regime of larvae. LF-MF-13 group was conspicuous by the abundance of mitochondria, nucleus and goblet cell and the absence of lipid droplet. Considering the digestive enzyme profile, survival and growth of larvae, it seems LF-MF-13 feeding regime is suitable for catla larvae and co-feeding should be started after 12 days of hatching.

Keywords: Catla; Feeding regimes; Digestive enzymes; Ultrastructure

Introduction

The feeding of larvae at early developmental stage with artificial diet is one of the major constrains for larviculture. The poor performance is usually found when the inert diets are fed to larvae from the onset of exogenous feeding [1]. This may be due to composition, palatability or physical characteristics of dry feed. Difference in growth and survival rates between fish fed live and compound diets are related to the nutritional value of the feed [2] and/or food digestion, nutrient absorption and metabolism [3]. The lower growth in fish larvae fed with inert diets is related to poor attractiveness and low acceptance of diet; also inadequate digestion and assimilation compared to live food [4]. Though total replacement of live food with formulated diet is not possible for the larvae of many species, partial replacement of live food with formulated diet may be possible [5]. Co-feeding (combined feeding of live food and artificial diet) from the start of exogenous feeding is an alternative strategy. The co-feeding of live food and formulated diet of Senegalese sole *Solea senegalensis* from first-feeding shows a toll in terms of growth and lipid digestibility but do not seem to compromise lipid metabolic utilization [6]. Nhu et al. [7] have suggested that the nutritional requirements of cobia *Rachycentron canadum* are age-dependent and prolongation of live food co-feeding during weaning may be necessary. Co-feeding may reduce stress in early larval stage of longsnout catfish *Leiocassis longirostris* [8]. In common carp *Cyprinus carpio*, mixed feeding of zooplankton and dry food resulted into better growth compared to the only dry food or zooplankton [9].

The understanding of temporal appearance of key enzymes in the gut of cultivable species is essential for the formulation of age-specific feed that contributes to rapid and efficient growth [10]. Biochemical studies of the digestive enzyme classes and their changes during the fish ontogenesis can give important information on stomach functionality. This information is very useful for assessing the optimal moment for weaning and also for developing suitable feeding strategies for fish larvae [11]. At first feeding enzyme activity is relatively low compared to the adult fish and each enzyme develops independently during ontogenesis [12]. The quality of food also influenced the digestive enzyme activities and thereby, growth of fish.

The mucosa of the intestine plays important role in digestion, re-absorption and metabolic processes in animals. In general gastrointestinal morphology is related to different feeding habit including the nature of the food and frequency of food intake, as well as taxonomy, body size and shape [13-15]. Several ultrastructural investigations of the digestive tract of stomachless fish have demonstrated regional differences in the microscopic appearances of absorptive cells [16-17].

Freshwater aquaculture production in India is dominated by carps. Indian major carp *Catla catla* (catla) is a major contributor in this total production. Catla is a stomachless, surface feeder, zooplaktivore species. High larval mortality and poor growth have been recorded in this species in absence of live food [18]. Problems associated with feeding of dry diets results into poor survival, growth and lower enzyme production in Indian major carp larvae [19].

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Digestive enzymes like amylase, protease, trypsin, chymotrypsin and lipase are found in first feeding catla, but in less quantity and enzyme activities gradually increase during ontogenic development [20-21]. In the present investigation, an attempt has been made to study the effect of six different types of food on the digestive enzyme profile and ultrastructure of the digestive tract of first feeding *Catla catla* (Hamilton). The knowledge of the ultrastructure of the intestine is essential to understand the physiological conditions of the fish. This study may help to understand the basic mechanism of digestion of ingested food at cellular level in catla larvae.

Materials and Methods

Culture of fish

Four-day-old catla *Catla catla* larvae (0.92 ± 0.21 mg) were procured from Chatterjee Brothers' Fish Farm, Mogra, West Bengal, India. Larvae were cultured in 15 L aquarium having connection with recirculating system [22]. The stocking density was 125 larvae aquarium^{-1}. Larvae were cultured under six different feeding regimes: (1) live food for 30 days (from day-4 to 34, LF), (2) artificial food for 30 days (AF), (3) mixed food (50% live food and 50% artificial food for 30 days, MF), (4) live food for initial 12 days (from day-4 to 12), then shifted to artificial food from 13-day onward (LF-AF-13), (5) live food for initial 17 days (from day-4 to 17) and then shifted to artificial food from day-18 (LF-AF-18) and (6) live food for initial 12 days (from day-4 to12) and then shifted to mixed food from day-13 onward (LF-MF-13). Three replicates were used for each feeding scheme. The live food composed of *Brachionus calyciflorous* (38 to 82%), *Ceriodaphnia cornuta* (5-10%), *Mesocyclops* sp. (8-31%), nauplii (24-38%), *Cypris* sp. (4-8%) and the rest was phytoplankton. Artificial food containing 45% protein (Table 1) was prepared in the laboratory. Food was given at the rate of 5% of initial body weight of larva. Survival rate and weight of individual larva were recorded after 30 days of culture. Larvae were cleaned with distilled water and stored immediately at -20°C for further study.

Enzyme assays

Larvae were dissected on a glass plate maintained at 0°C. Digestive system from individual fish was collected and pooled (12-40 larvae) to make 100 mg of tissue. Then pooled sample was homogenized with 1 ml of chilled distilled water. The homogenate was centrifuged at 10000 x g for 15 min at 4°C and the supernatant was used for analysis. Three replicates were used for each feeding scheme. Total soluble protein was measured [23].

Amylase activity was estimated by measuring the increase in reducing power of buffered starch (SRL, Mumbai, India) solution with 3, 5-dinitro salicylic acid (SRL, Mumbai, India) according to the method of Bernfeld [24]. Specific amylase activity was expressed as mg of maltose liberated mg protein^{-1} h^{-1} at 37°C. Chitinase and chitinobiase activities were assayed following the method of Gutowska et al. [25]. Enzyme activity was expressed as mg NAG g^{-1} wet weight h^{-1}. Total protease activity was measured using 1% azocasein (Sigma, St Louis, MO, USA) as substrate in 50 mM Tris-HCl, pH 7.5 [26]. The enzyme activity was expressed as mUnits mg protein^{-1} min^{-1}. Trypsin and chymotrypsin activities were measured using N-α-Benzoyl D, L-arginine-*para*-nitroanilide (BAPNA) and succinyl-Ala-Ala-Pro-Phe-*para*-nitroanilide (SAPNA) (Sigma, St Louis, MO, USA) as substrate, respectively. Change of absorbance was recorded under kinetic mode [27]. The activity was expressed in mU mg protein^{-1} min^{-1}. Carboxypeptidase A [28] and B [29] were measured using hippuryl-*L*-phenylalanin and hippuryl-*L*-arginine as substrate, respectively. The activity was expressed as the change in absorbance g^{-1} tissue min^{-1}. Lipase activity was measured following the method of Winkler and Stuckman [30]. The enzyme activity was expressed in mUnits mg protein^{-1}.

Ingredients	Amount (g Kg^{-1} dry weight)
Fish meal	583.3
Wheat flour	402.7
Cod liver oil	10.0
Vitamin and mineral premixes	4.0
Proximate analysis (% dry matter basis)	
Crude protein	45.625
Crude fat	7.1
Ash	7.0
Energy value (cal g^{-1})	3.7

Table 1: Composition of artificial diet fed to *Catla catla* larvae.

SDS-PAGE and substrate SDS-PAGE

Separation of proteins in the enzyme extract was done by 12% SDS-PAGE according to Laemmli [31]. Enzyme extract (30 µg protein sample^{-1}) was loaded into each well and electrophoresis was performed on a vertical dual mini gel electrophoresis device (Hoefer SE-260, Amersham Pharmacia) at 4°C. Gel was stained for 2 h using Coomassie brilliant blue (SRL, Mumbai, India) and then distained. The protease composition was studied after separation of proteins by substrate SDS-PAGE [32]. Sample containing 5 mU of activity was loaded into each well. After electrophoresis, the gel was immersed in 3% casein solution containing 50 mM Tris-HCl (pH 7.5) for 30 min at 5°C in order to allow the substrate to diffuse into the gel at low enzyme activity. The temperature was raised to 25°C and incubated for 60 min. The gel was then washed, stained and destained. Clear bands with blue background were identified as protease activity bands.

Protease inhibition assay

Protease inhibition assay was carried out to estimate the protease class by treating the enzyme with different specific inhibitors [33] using Sigma chemicals (St Louis, MO, USA). Soybean trypsin inhibitor (SBTI, 250 µM in distilled water) and phenyl methyl suphonyl fluoride (PMSF, 100 mM in 2-propanol) were used as serine protease inhibitors. N-α-*p*-tosyl-L-lysine chloromethylketone (TLCK, 1 mM in HCl) and N-tosyl-L phenylalanine chloromethylketone (TPCK, 5 mM in methanol) were used as specific inhibitor for trypsin and chymotrypsin, respectively. All these inhibitors were pre-incubated with enzyme extract in the ratio of 1:1 for 1 h at room temperature.

Transmission electron microscopy

After 30 days of culture, larvae were anesthetized with MS 222 and digestive tract from individual larva was dissected out. The intact digestive tract was examined under Carl Zeiss Microscope (Axio Vision 0.1, Germany). Each digestive tract was divided into three segments -proximal, middle and distal for transmission electron microscopic study. Each segment was fixed separately in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 3 to 4 h, then post fixed in 1% OsO_4 for 1 h. Tissue was dehydrated in graded acetone series and embedded in araldite. Ultrathin sections (60-90 nm) were stained with uranyl acetate and lead citrate and observed under Transmission Electron Microscope (Morgagni 268D, Fei Electron Optics).

Statistical analysis

Various cell organelles were examined and quantified. All data were compiled as means ± SE. Data were analyzed using one-way analysis of variance (ANOVA) and Duncan's multiple range test, DMR [34]. Statistical significance was accepted at $p<0.05$ level.

Results

Survival and average weight of fish

Significantly ($p<0.05$) higher survival of catla was recorded in MF and LF compared to the other feeding regimes (Figure 1a). The survival rate was 1 to 34% higher in MF compared to others. There was no significant ($p>0.05$) difference in the survival rate of fish cultured in LF-AF-13, LF-AF-18 and LF-MF-13. The average weight of catla cultured in LF-MF-13 was significantly ($p<0.05$) higher compared to others (Figure 1b). This group was followed by LF and MF.

Enzyme profile

Significantly ($p<0.05$) higher amylase activity was found in feeding regime LF-AF-18 (15-61% higher) compared to others. Minimum amylase activity was recorded in larvae fed with only live food (LF). Whereas, chitinase and chitinobiase activities were significantly ($p<0.05$) higher in the live food fed larvae compared to the other feeding regimes. This group was followed by LF-MF-13. These enzyme activities were 4-21% higher in LF-MF-13 compared to MF. Chitinase and chitinobiase activities were 1.5-3 folds lower in AF compared to LF-AF-13 and LF-AF-18. Total protease activity was significantly ($p<0.05$) higher in LF-MF-13 compared to others. This group was followed by MF. Trypsin and chymotrypsin activities were also significantly ($p<0.05$) higher in LF-MF-13 compared to the other feeding regimes. Significantly ($p<0.05$) higher carboxypeptidase A and B activities were found in the in MF (1.3-9 folds higher) and LF-AF-13 (1.5-6.7 folds higher) compared to others. Significantly ($p<0.05$) higher lipase activity was recorded in LF-MF-13 (1.2-2.0 folds higher) compared to others. This group was followed by MF, LF-AF-18, LF-AF-13, AF and LF (Table 2).

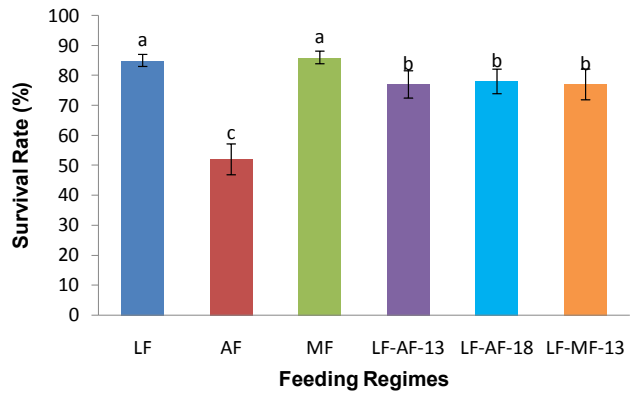

Figure 1a: Survival rate of *Catlacatla* larvae cultured in six different fee dingregimes (n=3). Bars with different superscripts are significantly ($p<0.05$) different.

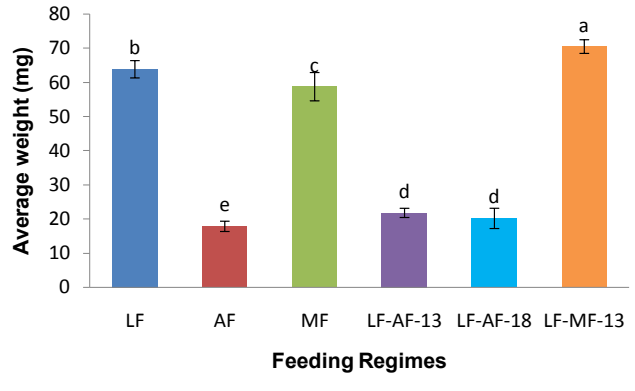

Figure 1b: Average weight of *Catlacatla* larvaecultured in six different feedingregimes (n=3). Bars with different superscripts are significantly ($p<0.05$) different.

SDS-PAGE and substrate SDS-PAGE

SDS-PAGE of digestive tissue protein of 34-days-old catla showed total 14 bands (14.34 - 97.57 kDa) in all these six feeding regimes. The intensity of bands was higher in LF, MF and LF-MF-13 compared to others. Total eight bands with molecular mass of 15.26, 18.36, 27.34, 31.17, 39.6, 44.76, 64.57 and 72.88 KDa were found in substrate SDS-PAGE. Five bands (15.26, 31.17, 39.6, 44.76 and 64.57 kDa) had higher intensity compared to others regardless of feeding regimes (Figure 1a, 1b, supplementary materials).

Inhibition assay

Inhibition assay of enzyme extract of catla with SBTI showed clear inhibition of six bands, which were found in substrate SDS-PAGE. The molecular masses of two uninhibited bands were 15.26 and 64.57 kDa. PMSF inhibited 3 bands (15.26, 18.36 and 27.34 kDa) regardless of feeding regimes. The following activity bands were visible: 31.17, 39.6, 44.76, 64.5 and 72.88 kDa in the sample treated with PMSF. TLCK-treated sample showed inhibition of three bands (18.36, 27.34 and 31.17 kDa). Enzyme extract treated with TPCK showed inhibition of two bands of 18.36 and 27.34 kDa (Figure 2a-2d, supplementary materials).

Morphology of digestive system

The body of the larva was very transparent up to day-12 due to less pigmentation and all the internal structures were visible from outside the body during microscopic study. The morphological feature of the digestive tract of stomachless catla was very simple without marked coiling at the age of 34 days. The proximal segment of the intestine was wider and formed a bulb-like structure. The bile duct opened at the beginning of the bulb region. The distal region was narrower compared to the proximal region (Figure 3, supplementary materials).

Ultrastructure of the digestive tract

Proximal segment: The absorptive cell of proximal segment of the digestive tract had many uniformly distributed microvilli regardless of feeding regimes. The height of microvilli varied among the feeding regimes (Table 3). Significantly ($p<0.05$) larger sized microvilli were recorded in the proximal part of the larvae fed with AF (1.6 ± 0.03 µm) compared to other feeding regimes. Large number of mitochondria was found in the apical cytoplasm of larvae cultured in LF-MF-13 and this was followed by LF (Table 4). Larger sized mitochondria were present in LF-MF-13 and AF (Figures 2a and 2b). The other organelles like nucleus, goblet cells and rough endoplasmic reticulum were observed in the central region of the proximal part. Lipid droplet of variable size was scattered in the cytoplasm. Highest number of lipid droplet was observed in the larvae fed with artificial food (AF) followed by LF and LF-AF-13 (Figure 3). Lipid droplet was absent in MF and LF-MF-13.

Digestive Enzyme activity	Feeding regimes					
	LF	AF	MF	LF-AF-13	LF-AF-18	LF-MF-13
Specific amylase activity (mg maltose mg protein^{-1} h^{-1})	0.09 ± 0.01^{d}	0.15 ± 0.02^{c}	0.13 ± 0.01^{c}	0.16 ± 0.02^{c}	0.23 ± 0.01^{a}	0.19 ± 0.01^{b}
Chitinase (mg NAG g^{-1} wet weight h^{-1})	44.31 ± 0.21^{a}	5.91 ± 0.85^{e}	19.56 ± 3.02^{b}	8.73 ± 0.2^{d}	17.73 ± 0.84^{c}	20.4 ± 2.33^{b}
Chitinobiase (mg NAG g^{-1} wet weight h^{-1})	44.73 ± 0.36^{a}	5.13 ± 0.46^{d}	17.64 ± 0.15^{c}	5.22 ± 0.20^{d}	17.68 ± 2.61^{c}	21.33 ± 0.49^{b}
Protease (mUnits mg protein^{-1} min^{-1})	569.72 ± 52.0^{d}	682.5 ± 57.4^{c}	1219.32 ± 20^{b}	432.66 ± 28.8^{e}	661.64 ± 28.8^{c}	1261 ± 52.0^{a}
Trypsin (mUnits mg protein^{-1} min^{-1})	38.62 ± 4.14^{d}	49.31 ± 6.64^{c}	60.77 ± 7.86^{b}	38.0 ± 2.49^{d}	57.15 ± 2.60^{c}	74.86 ± 5.78^{a}
Chymotrypsin (mUnits mg protein^{-1} min^{-1})	519.21 ± 10^{b}	172.14 ± 5^{e}	485.81 ± 10^{c}	363.64 ± 20^{d}	471.30 ± 15^{c}	552.43 ± 9^{a}
Carboxypeptidase A (Δ Abs g^{-1} tissue min^{-1})	2.66 ± 0.4^{f}	12.46 ± 1.42^{c}	24.32 ± 1.41^{a}	18.37 ± 2.18^{b}	9.70 ± 0.93^{d}	6.68 ± 0.48^{e}
Carboxypeptidase B (Δ Abs g^{-1} tissue min^{-1})	1.16 ± 0.26^{d}	1.4 ± 0.11^{d}	5.08 ± 0.98^{b}	7.74 ± 0.12^{a}	5.14 ± 1.37^{b}	3.24 ± 0.57^{c}
Specific lipase activity (mUnits mg protein^{-1})	109.34 ± 6.73^{e}	131.64 ± 8.1^{d}	179.53 ± 10.04^{b}	150.27 ± 8^{c}	167.31 ± 7.1^{b}	227.93 ± 17^{a}

Table 2: Digestive enzyme profiles of *Catla catla* cultured in six different feeding regimes. Mean (n= 3) followed by different letters in the same row are significantly (p<0.05) different.

Segment	Feeding regimes					
	LF	AF	MF	LF-AF-13	LF-AF-18	LF-MF-13
Proximal	1.2 ± 0.02^{b}	1.6 ± 0.03^{a}	1.07 ± 0.04^{b}	1.15 ± 0.04^{b}	1.12 ± .04^{b}	1.12 ± 0.02^{b}
Middle	1.02 ± 0.04^{b}	1.32 ± 0.05^{a}	0.99 ± 0.03^{b}	0.91 ± 0.08^{b}	0.94 ± 0.02^{b}	0.95 ± 0.03^{b}
Distal	0.64 ± 0.02^{b}	1.16 ± 0.05^{a}	0.94 ± 0.07^{b}	0.59 ± 0.02^{b}	0.58 ± 0.04^{b}	0.72 ± 0.04^{b}

Table 3: Height of microvilli (µm) found in three different segments of digestive tract of *Catla catla* cultured in six different feeding regimes. Mean (n=3) followed by different letters in the same row are significantly (p<0.05) different.

Feeding regime	Segment	Mitochondria	Lipid droplet	Goblet cell	Vacuole	Nucleus
LF	Proximal	***	**	-	-	+++++
	Middle	**	**	+	*	+
	Distal	**	-	-	**	++
AF	Proximal	**	****	++	*	++++
	Middle	****	*	+++	**	++
	Distal	****	-	-	*	+++
MF	Proximal	*	-	+	*	+++
	Middle	*	-	++	*	+++++
	Distal	*	**	++	*	+++
LF-AF-13	Proximal	**	**	-	*	+
	Middle	*	*	+	-	++
	Distal	*	*	+	*	++++
LF-AF-18	Proximal	**	*	-	*	+++
	Middle	*	*	++	*	++
	Distal	*	*	++	**	+++++
LF-MF-13	Proximal	****	-	+	**	+++++
	Middle	*	-	+++	*	+
	Distal	*	*	++++	*	++++

Note: =0, +=1, ++=2, +++=3, ++++=4, +++++=5 and
- = 0, *=number<10, ** =number>10, ***=number>20, ****=number>30, *****=number>40.

Table 4: Abundance of goblet cell and various cell organelles in three different segments of digestive tract of *Catla catla* cultured in six different feeding regimes.

Significantly (p<0.05) higher number of goblet cell was observed in AF compared to the MF and LF-MF-13. But the size of goblet cell was larger in two latter feeding regimes compared to the former one. Goblet cell was absent in larvae cultured in LF, LF-AF-13 and LF-AF-18. The vacuole was absent in the proximal segment of the larvae cultured in LF. Significantly (p<0.05) higher number of vacuole was found in LF-MF-13 (Figure 4). The nucleus was found in the basal region of the cell in all feeding regimes. Significantly (p<0.05) higher number of nucleus was found in the larvae cultured in LF and LF-MF-13 compared to others (Figure 5).

Middle segment: The height of microvilli was recorded in the middle segment of the digestive tract of catla. Largest microvilli was found (Table 3) in larvae cultured in AF (1.32 ± 0.05 µm). Large number of mitochondria was found in AF compared to others. Significantly (p<0.05) higher number of lipid droplets was found in LF compared to AF, LF-AF-13 and LF-AF-18. Lipid droplet was absent in MF and

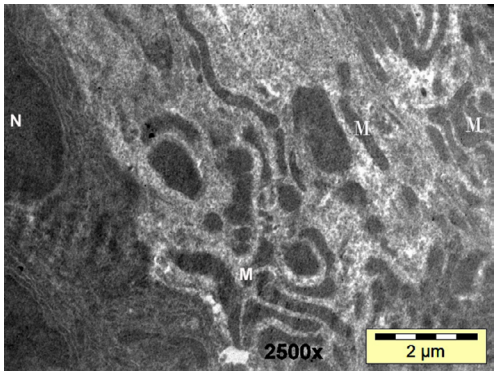

Figure 2a: Ultrastructure showing mitochondria at the proximal segment of digestive tract of *Catlacatla* cultured in feeding regime LF-MF-13. M=mitochondria, N=nucleus.

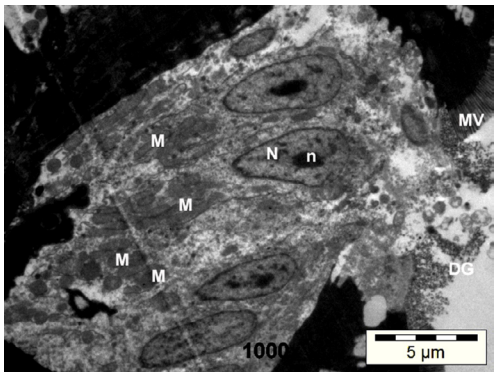

Figure 2b: Ultrastructure showing mitochondria at the proximal segment of digestive tract of *Catlacatla* cultured in feeding regime AF. M=mitochondria, MV=microvilli, N=nucleus.

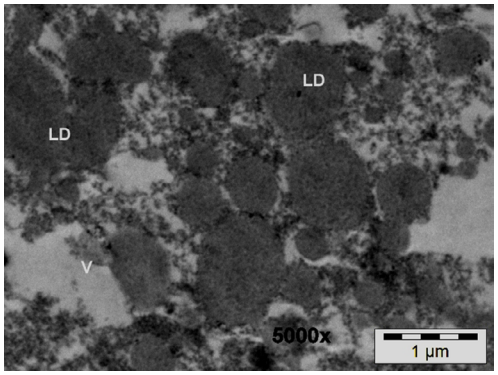

Figure 3: Ultrastructure showing lipid droplet at the proximal segment of digestive tract of *Catlacatla* cultured in feeding regime AF. LD=lipid droplet, V=vacuole.

LF-MF-13. Goblet cell was observed in all the feeding regimes, but the size was larger in the larvae cultured in MF and LF-MF-13 compared to others (Figures 6a and 6b). Maximum number of goblet cell was found in AF and LF-MF-13. Higher number of vacuole was found in catla cultured in AF compared to others (Table 4). Significantly ($p<0.05$) higher number of nucleus was observed in MF compared to others.

Distal segment: The height of microvilli in the distal segment was maximum (Table 3) in the larvae cultured in AF (1.16 ± 0.05 µm) compared to others. Significantly ($p<0.05$) higher number of mitochondria was recorded in AF compared to others (Figure 7). Lipid droplets were absent in LF and AF (Table 4). Significantly ($p<0.05$) higher number of lipid droplet was observed in MF compared to others (Figure 4, supplementary). Significantly ($p<0.05$) higher number of goblet cell was found in LF-MF-13 compared to others. The goblet cell was larger in MF compared to others, this was oval in shape. Goblet cell was absent in LF and AF. Significantly ($p<0.05$) higher number of vacuole was observed in LF and LF-AF-18 compared to others. In AF,

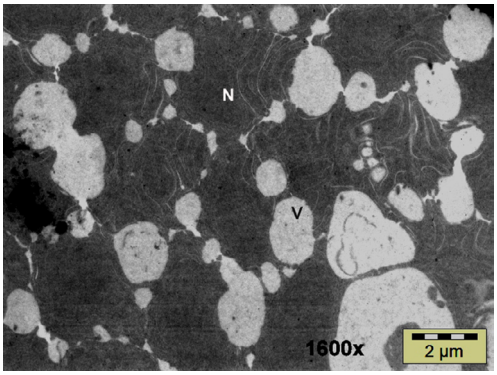

Figure 4: Ultrastructure showing vacuole at the proximal segment of digestive tract of *Catlacatla* cultured in feeding regime LF-MF-13. N=nucleus, V=vacuole.

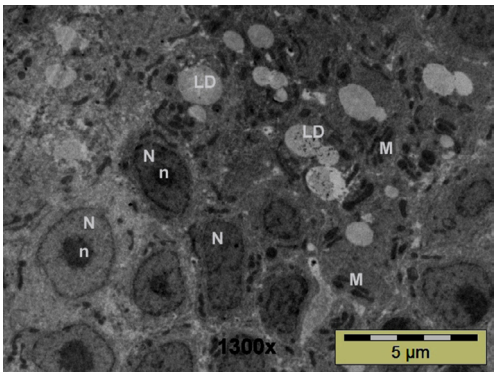

Figure 5: Ultrastructure showing nucleus at the proximal segment of digestive tract of *Catlacatla* cultured in feeding regime LF. LD=lipid droplet, M=mitochondria, N=nucleus, n=nucleolus, V=vacuole.

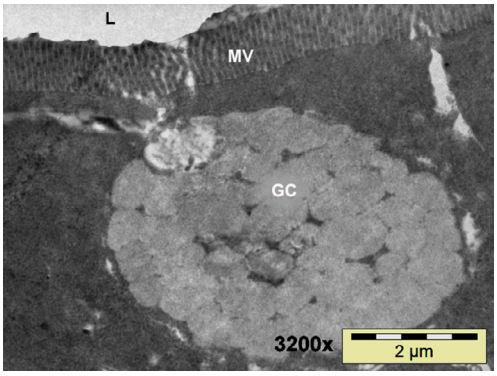

Figure 6a: Ultrastructure showing the goblet cell at the middle segment of digestive tract of *Caltacatla* cultured in feeding regime MF. GC=gobletcell, L=lumen, MV=microvilli.

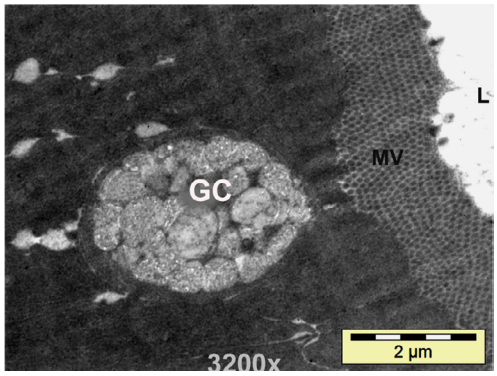

Figure 6b: Ultrastructure showing the goblet cell at the middle segment of digestive tract of *Caltacatla* cultured in feeding regime LF-MF-13. GC=gobletcell, L=lumen, MV=microvilli.

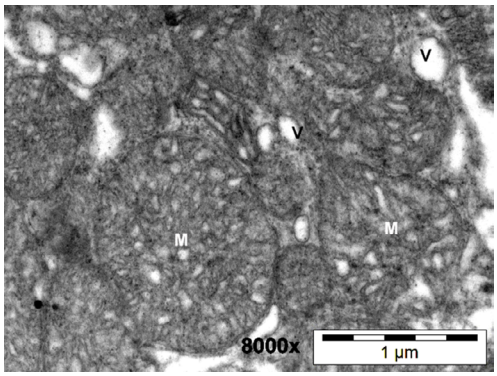

Figure 7: Ultrastructure showing mitochondria at the distal segment of digestive tract of *Catlacatla* cultured in the feeding regime AF. M=mitochondria, V=vacuole.

nuclei were very conspicuous. Pinocytotic channels (PC) were found in LF and LF-MF-13 (Figures 5a and 5b, supplementary).

Discussion

The positive effect of feeding of carp larvae with live food was found in the present study. Feeding of catla larvae with live food or mixed food resulted into higher survival and average weight compared to the larvae fed with artificial food at first feeding or shifted to artificial food after 13 and 18 days. The shifting of larvae from live food to mixed food on day-13, resulted into 10.42% higher average weight of larvae compared to the fish fed with only live food. Whereas, this shifting resulted into 11% reduced survival of larvae in the former feeding scheme compared to the latter one. This indicates that feeding of catla larvae with live food is crucial for initial 12 days. Tandler and Kolkovski [35] achieved 80% survival with no growth problem using the co-feeding technique for rearing 10-days-old gilthead seabream larvae.

The lower growth and survival of larvae fed with artificial diet may also be related to the poor digestibility of artificial diet, because of presence of 80 to 90% of dry matter in artificial food compared to 10% in zooplankton [4]. Moreover, the ingestion of microparticles might be enhanced by visual or chemical stimulation of the larvae in the presence of live prey organisms in the co-feeding [36]. The effect of food as substrate on the digestive enzyme activities is very clear in the present study. Presence of chitinous exoskeleton in the live food influenced the secretion of chitinase and chitinobiase in the digestive tract of larvae. High chitinase activity was associated with the feeding habit of the fish [37]. The simple nature of the composition of zooplankton does not require the presence of other digestive enzymes in higher quantity compared to the larvae fed with mixed and artificial diets. This resulted into low enzyme activities in larvae fed with live food. Several studies showed the influence of live food during early developmental stages of carps [19,38,39]. It has been suggested that the live food assist in the digestion process by contributing their digestive enzymes either by autolysis or as zymogens that activate the endogenous enzymes of larvae. Kumar et al. [40] showed the presence of various digestive enzymes in *Daphnia carinata*. The gut neuropeptides and nutritional growth factors of live food also enhance digestion [4].

In LF-MF-13, fish were fed with live food for initial 12 days and then shifted to mixed food (i.e. live and artificial diet). The presence of mixed food in the digestive tract influenced the secretion of protease, trypsin, chymotrypsin and lipase. The movement of live food inside the digestive tract resulted in better digestion of ingested artificial food. Feeding of seabass *Dicentrarchus labrax* larvae with dry microdiet and *Artiemia* nauplii, influenced the rates of assimilation and growth of fish [41]. Several recent studies showed the influence of food on the digestive enzyme activities of larvae. In spotted rose snapper (*Lutjanus guttatus*), maximum levels of trypsinogen expression (at 25 dph) and trypsin activity (at 35 dph) were observed, when larvae were fed with *Artemia* nauplii and artificial diet, respectively [42]. In large yellow croaker (*Larimichthys crocea*) larvae specific activities of alanine and aspartate aminotransferases were influenced by dietary amino acid patterns [43]. Taurine supplementation in diet resulted into increased specific amylase and trypsin activities in early larval stages of cobia *Rachycentron canadum* [44]. Feeding of catla larvae with live food for initial 12 days and then shifting to mixed food resulted into higher digestive enzyme activities in larvae. These heightened enzymatic activities may lead enhanced nutrient availability and enhanced survival and growth.

In SDS-PAGE and substrate SDS-PAGE, higher intensity of bands was found in LF, MF and LF-MF-13 compared to others. This might be related to better protein synthesis in these three feeding schemes. Inhibition assay of enzyme extract with SBTI indicated the presence of two non-serine protease bands. Rathore et al. [21] also found the presence of non-serine proteases in the digestive tissue extract of catla larvae. The enzyme extract treated with TLCK showed clear inhibition of three trypsin-like bands. The presence of more than one isoform of trypsin in many bony fishes had been reported [12,45].

In the present investigation, significant differences were found in the ultrastructure of proximal, middle and distal segments of catla regardless of feeding regime. The height of microvilli gradually decreased from proximal to middle and distal segments. Kumar et al. [46] reported a progressive change in the number and size of microvilli in catla larvae during ontogenic development. In rainbow trout *Oncorhynchus mykiss*, villi showed progressively shorter length towards the posterior intestine [47]. There might be link between feeding habits of the fish and the structure of the enterocyte brush border. The height of microvilli was maximum in catla larvae feed with artificial food. Microvilli increased the digestive and transportation surface of the enterocytes in comparison with the smooth membrane by 30 to 60 times [48] and these were the structural basis of the processes of membrane digestion [49]. The elevation of the mucosa into villi favoured the absorptive role of it [50]. In the present study, the type of food also influenced the ultrastructure of the digestive tract. Feeding of larvae with artificial food showed the presence of higher number of

lipid droplets in the digestive tract, whereas lipid droplets were either totally absent or very less in number in mixed food fed larvae. This showed the immature digestion capacity in AF feeding regime. Fish larvae which are devoid of gastric glands are known to have immature digestive mechanisms characterized by temporary lipid storage in the anterio-median intestine cells [51,52]. The number of goblet cells was possibly related to the fish diet [53]. The mucus secreted by the goblet cells might help in the lubrication of undigested materials for onward progression. Cinar and Senol [13] suggested that goblet cells were most densely distributed in posterior intestine. In LF-MF-13, highest number of goblet cells was found at the posterior segment. The presence of vacuoles in the digestive tract of catla till day-34 might be due to the absence of stomach in this species.

The timing of co-feeding and the nutritional composition, palatability and digestibility of the artificial diets determine the success of weaning [54,55]. Early co-feeding is beneficial, since it reduces the use of live food, which are cumbersome to produce and difficult to manipulate nutritionally [56]. However, the starting time for co-feeding is species-specific according to the maturity of the digestive system [57]. In pikeperch *Sander lucioperca* [58] and Chinese longsnout catfish *Leiocassis longirostris* [8] larvae, co-feeding at 19 and 6 days post hatching, respectively were found to be beneficial. Pradhan et al. [59] suggested that butter catfish (*Ompok bimaculatus*) larvae might be weaned between 15 and 21 dph, as larvae achieved the complete maturation of their digestive capacities. The present study showed that in catla larvae co-feeding may be useful at 12 days post hatching. These data were evidence of the marked influence of feeding regimes on the digestive enzyme profile and ultrastructure of digestive tract of first feeding catla larvae.

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