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Aquaculture Science and Management (2024) 1:3
https://doi.org/10.1186/s44365-024-00005-z

Aquaculture Science and Management

RESEARCH

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Histo-architectural and histochemical changes in the liver and intestine of *Labeo rohita*: integrative effect of water temperature and dietary protein level

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Abstract

This study aimed to study the histo-architectural and histochemical changes in the liver and intestine of *Labeo rohita* fingerlings due to the integrative effect of short-term exposure to higher temperature and dietary protein levels. Six hundred *Labeo rohita* fingerlings (average weight 6.78 ± 0.05 g) were distributed into 24 tanks, with 25 fingerlings in each tank. The tanks were divided into eight experimental groups, with three replicates for each tank. Half of the experimental groups were maintained at an ambient water temperature of 26^{0}C, while the other half were kept at 32^{0}C for one week and then shifted back to 26^{0}C for four weeks. The fingerlings were fed with either of four diets, each containing different protein levels of 20%, 30%, 40% or 45% protein. At the end of the experiment, the hepatosomatic index (HSI) and visceral index (VI) of the juveniles were increased with the increase in dietary protein level, however significantly lower HSI was recorded at 32^{0}C compared to 26^{0}C. Juveniles exposed to elevated temperature (32^{0}C) for one week registered significantly higher ($p < 0.05$) liver glycogen content compared to 26^{0}C. Amylase, protease, and alkaline phosphatase activity in the intestine of juveniles were significantly ($p < 0.05$) higher at elevated temperature (32^{0}C) and decreased after 4 weeks of decrease in temperature from elevated temperature (32^{0}C) to ambient temperature (26^{0}C). The 45% CP fed group at 26^{0}C showed mildly desquamated mucosa in the liver whereas mucosa was found absent at 32^{0}C. Marked loss of mucosa was found in the intestine of *L. rohita* fed with 45% dietary protein after five weeks either at 26^{0}C or 32^{0}C. Overall it concludes that the dietary protein level and water temperature interactions are more effective in maintaining the histo-architectural structure of the liver and intestine, which responds to increased digestive enzyme activity.

Keywords Dietary protein level, *Labeo rohita*, Water temperature, Glycogen, Digestive enzyme, Histology of liver and intestine

Introduction

The digestive system associated with the digestion and absorption processes, is one of the prime regulators of growth performance and feed utilization [5, 16]. Digestion is an important physio-metabolic activity in animals because it provides energy and nutrients required for survival, growth, and maintenance. Among ectotherms, typically in fish, the sustainable energy budget is governed by the digestive capacity to assimilate

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nutrients from food. Thereby, the growth, metabolic, and physiological performance of the fish are closely linked to the health and efficiency of their digestive system. The digestion process is catalyzed in the gastrointestinal tract by a cascade of enzymatic activities which are typically localized at the lumen and brush border membrane. Undoubtedly, the functionality of the digestive enzyme has a direct impact on the capacity of fish to digest and absorb food and also influences the overall efficacy with which gained energy is mobilized [17, 59]. Accordingly, the kinetics of the digestive enzymes reflect the functional capacity and the physiognomies of the digestive system in the fish. Besides enzymatic functionality, the structural and morphological characteristics of the gastrointestinal tract and the associated organs are also coupled with the absorptive capacity of the digestive system. The liver plays a pivotal role in processing nutrients from food and supporting their absorption during digestion. In essence, the macro- and microstructure profile of the gastrointestinal tract and liver offers an excellent indication of the nutritional status and digestive capacity of fish [9, 21, 23]. Therefore, for assessing the functionality of digestion and the physiological impact of environmental cues on the overall digestive process, the comprehensive examination of these organs (liver and intestine) at the histological level along with the dynamics of enzymatic activity is crucial [50, 55].

The quantity and composition of nutrients are important factors, which can also influence the digestive enzyme activities in fish [14]. It is interesting to note that through digestive enzyme profiing, it is possible to predict a fish species ability to prioritize nutrient use [31]. In addition, it has been recommended that to promote the greatest possible utilization of other nutrients, a suitable level of a preferred nutrient must be included in the diet [56]. There's ample evidence indicating that when there's a shortage of non-protein energy, the body tends to prioritize using protein as the primary energy source for growth [46].

The digestive process in fish involves the participation of numerous enzymes. As *Labeo rohita* is a stomachless fish, digestion starts from the intestine and proteases are synthesized by the pancreas, and then cause selective hydrolysis. Certain enzymes in the intestine aid in both breaking down and transporting nutrients. For instance, alkaline phosphatase helps to absorb and transport lipids and carbohydrates, while glutamyl transferase transports amino acids and oligopeptides through the intestinal lining [20]. Therefore, the ability of fish to utilize proteins might be constrained by either the rate at which digestive enzymes produce nutrients or the capacity of nutrient transport systems.

There is a close relationship between teleost fish and their environment. The body temperature of fish aligns closely with the temperature of the water they inhabit, thereby impacting their overall physiology. As an important ecological factor, temperature also has a significant influence on the vital physiological processes in fish [35]. In general, fish temperatures are optimal for growth and feeding [57]. Growth frequency increases as the water temperature increases to the optimum temperature and then decreases as the temperature increases [29]. Blier et al. [5] found that digestive feed can determine the maximum growth rate under optimal conditions. This is because digestive enzymes have an impact on digestibility, thereby placing physiological limits on both the growth rate and feed conversion ratio [47].

The effect of water temperature on the protein requirement of fish has been well documented for fish [12, 42, 52]. Kumar et al. [35] found that when *L. rohita* fingerlings were exposed to a higher temperature of 32^0C, their metabolic activities were activated and the increase in metabolic rate was observed to last for three weeks. In this timeframe, it was documented that a dietary protein level of 40% could sustain the growth rate. Tahir et al. [54] also reported that *L. rohita* requires more protein at higher temperatures for optimum growth. In contrast, Blier et al. [5] reported that the maximum growth rate can be determined by the ability of digestion to absorb nutrients. Therefore, it is assumed that digestibility can be stimulated by short-term exposure to higher temperatures that lasts longer and contributes to increased growth and protein retention of *L. rohita* fingerlings.

Therefore, the main aim of the experiment was to determine if exposing *L. rohita* fingerlings to high temperature in combination with optimal dietary protein level, which has been shown to increase growth and protein retention capacity in previous studies [35], could also result in an improvement in digestive capacity and morphological changes in the gastrointestinal tract of *L. rohita* or not.

Material and methods

Ethical statements

After approval from concerned committee of ICAR-Central Institute of Fisheries Education (ICAR-CIFE), India, the present study was conducted with recommended guidelines.

Diets

Four isocaloric diets were formulated with different levels of crude protein (20%, 30%, 40% & 45%) using a mixture of ingredients that were combined with gelatin to form dough. The dough was then conditioned and steamed before adding vitamin & mineral mixture and vitamin C.

Table 1 Feed formulation of the different experimental diets

Ingredients	Experimental diets			
	20% CP	30% CP	40% CP	45% CP
Fish meal[a]	5	15	30	30
Soybean meal[a]	21	36	40	40
Wheat flour[a]	18	15	10	5
Wheat bran[a]	44	22	5	5
Casein + Gelatin (4:1)[b]	2	2	5	10
Soybean oil	4	4	4	4
Cod liver oil	2	2	2	2
CMC[c]	2	2	2	2
Emix (Vit-Min mix)[d]	1.99	1.99	1.99	1.99
Vitamin C[e]	0.01	0.01	0.01	0.01
Proximate composition of diet (% DM basis)				
Dry Matter	92.87	93.44	93.52	93.68
Crude protein (CP)	20.08	30.35	40.25	44.85
Ether extract (EE)	9.57	9.69	10.10	10.05
Total Carbohydrate (TC)	60.46	49.42	38.60	33.95
Total ash	9.89	10.54	11.05	11.15
Digestible energy (kcal/100 g)[f]	408.29	406.29	406.30	405.65

[a] Procured from Central poultry farm, Mumbai, India

[b] Casein fat free: 75% CP (HImedia ltd, India)

[b] Gelatin: 96% CP (HImedia ltd, India)

[c] Carboxymethylcellulose (Sd Fine Chemicals Ltd., India)

[d] Vitamin-mineral mix (Emix™ plus) (quantity/2.5 kg): Vitamin A-55,00,000 IU; Vitamin D_3—11,00,000 IU; Vitamin B_2—2,000 mg; Vitamin E-750 mg; Vitamin K-1,000 mg; Vitamin B_6—1,000 mg; Vitamin B_{12}- 6 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; Zn-5,000 mg; Co- 450 mg; Ca- 500 g; P-300 g; Se-50 ppm; L-Lysine-10 g; DL-methionine-10 g

[e] Stay C (Hoffman La Roche, Nutley, N.J., USA) 15% ascorbic acid activity

[f] DE (Kcal/100 g) = CP (%) × 4 + EE (%) × 9 + TC (%) × 4

Hand pelletizer was used to prepare pellets, which were air-dried and then dried completely in the oven at 50^0 C. Finally, the pellets were packed in airtight bags and labeled appropriately. Table 1 shows the compositions of the experimental diets.

Experimental animals

Fingerlings of *L. rohita* were acquired from Prem Fisheries Consultancy, Gujarat, India, and were transported to the Fish Nutrition and Biochemistry Laboratory in CIFE, Mumbai, in a round container equipped with sufficient aeration. The subsequent day, a 3% NaCl treatment was administered to the fingerlings to alleviate handling stress. Following this, the fingerlings were acclimatized for 15 days in aerated conditions at an ambient temperature of 26^0C. A practical diet (30% crude protein) was fed to the fingerlings during the acclimatization period.

Experimental design

Six hundred fingerlings of *Labeo rohita*, averaging a mean weight of 6.78 ± 0.05 g, were distributed among 8 treatment groups. Every group consisted of 3 replicates, and the arrangements was carried out in a completely randomized design. The fingerlings were distributed among 24 tanks, each with a capacity of 150 L. Experimental design is mentioned in Fig. 1. Half of the experimental groups were maintained at the ambient temperature of 26^0C. The water temperature for the groups exposed to 32^0C was gradually decreased to 26^0C within 24 h. The water temperature of the groups exposed to 32^0C was decreased to 26^0C within 24 h. All tanks were continuously aerated with a compressed air pump and water was changed alternately. The trial extended over a span of five weeks, during which the groups were provided diets with varying crude protein levels of 20%, 30%, 40% or 45%. Fish in both sets were fed twice daily, at 08:00 and 18:00 h, until they reached satiation. Sampling was carried out in the 1st, 3rd, and 5th weeks to assess different parameters.

Sampling and analysis of samples

The proximate analysis followed AOAC's [2] standard methods. Moisture content was assessed by drying the

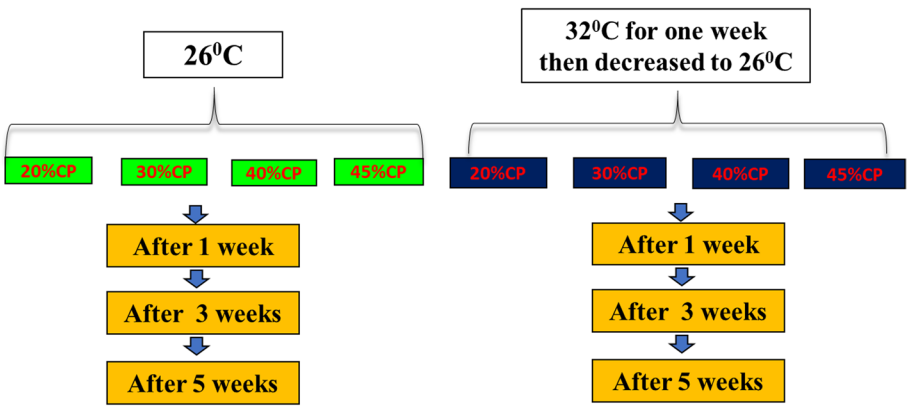

Fig. 1 Experimental design

sample at 105^0C until a consistent weight was achieved. Nitrogen content was determined through a Kjeltec system (2200 Kjeltec Auto distillation Foss Tecator, Sweden), and crude protein (CP) was computed by multiplying the nitrogen percentage by 6.25. Ether extract (EE) was gauged using the Soxtec system (1045 Soxtec Extraction Unit, Foss Tecator, Höganäs, Sweden), with diethyl ether (boiling point, 40–60^0C) as the solvent. Ash content was derived by incinerating the sample in a muffle frnace at 600^0C for 6 h. Total carbohydrate (TC) was calculated by deducting the percentage of CP, EE, and ash from 100. The digestible enegy (DE) of the experimental diets was determined utilizing the methodology outlined by Halver [24].

Sample preparation

During each sampling, liver and intestine samples were gathered from three fish in each tank per treatment group. The fingerlings were anesthetized using clove oil at a concentration of 50 µl L^{-1} and euthanized by a quick blow to the head. Liver and intestine samples were collected, immediately frozen in liquid nitrogen and stored at -70^0 C until further use for assessing digestive enzymes like amylase, protease, and lipase. Another three fingerlings were anaesthetized with clove oil and dissected to collect the liver and muscle samples for glycogen estimation. Muscle samples were taken from the caudal peduncle region after scraping off the scales.

Liver & muscle glycogen

The glycogen content in liver and muscle was analysed colorimetrically by the method described by Hassid and Abraham [26] and Kumar et al. [33]. The tissue samples were placed in pre-weighed centrifuge tubes containing 3 ml of 30% KOH. The weight of the tissue was recorded before placing the tubes in a boiling water bath for 20 min. After cooling, 5 ml of 95% ethanol was added to the tubes to precipitate the glycogen. The precipitate obtained was dissolved in 1 ml of distilled water and then precipitated again with 95% ethanol and centrifuged. The glycogen precipitate was then dissolved in distilled water to estimate the quantity of glycogen. To estimate the glycogen content, 0.1 ml of the glycogen solution was taken and added to 5 ml of anthrone reagent, which was then mixed by swirling the tube. The tubes were covered with glass marbles and heated for 10 min in boiling water, followed by cooling. The absorbance of the solution was then recorded at 590 nm, and the reading was compared with a standard glycogen solution to estimate the amount of glycogen in the sample.

Hepatosomatic index (HSI) and visceral index (VI)

The hepatosomatic index (HSI) and visceral index (VI) was determined using the following formulae:

$$\text{HSI (\%)} = \left(\text{wet weight of liver/whole wet body weight}\right) \times 100$$

$$\text{VI (\%)} = \left(\text{wet weight of gastrointestinal tract/whole body weight}\right) \times 100$$

Digestive enzymes

Amylase (E.C. 3.2.1.1) activity was assayed with 2% (w/v) starch solution as substrate [49]. The starch solution was prepared in phosphate buffer (pH 7). The reaction mixture was incubated at 37^0C for 30 min. Then dinitrosalicylic acid (DNS) was added to stop the reaction and kept in boiling water bath for 5 min. After cooling, reaction mixture is diluted with distilled water and absorbance recorded at 540 nm. Activity was determined from the maltose standard curve and expressed as micromole of maltose released from starch/min/g protein at 37^0C.

The determination of protease activity followed the procedure outlined by Drapeanu [15]. The reaction mixture comprised 1% casein in 0.05 M Tris PO_4 buffer (pH 7.8) and was incubated at 37^0 C for 5 min. Subsequently, tissue homogenate was introduced. After ten minutes, the reaction was halted by adding 10% TCA, followed by sample filtration. A reagent blank was prepared by adding tissue homogenate just before stopping the reaction, without incubation. Enzyme activity was quantified as one unit, corresponding to the enzyme quantity required to release acid-soluble fragments equal to $0.001A_{280}$ per minute at 37^0 C and pH 7.8.

Lipase (E.C. 3.1.1.3) activity was assessed following the method described by Cherry and Crandell [13]. The quantity of fatty acids released within a specific duration was gauged by the amount of NaOH required to sustain a constant pH. The reaction mixture comprised distilled water, tissue homogenate, phosphate buffer solution (pH 7), and an olive oil emulsion. After thorough agitation, the mixture was incubated at 4^0C for 24 h. following this incubation, 95% alcohol and two drops of phenolphthalein indicator were introduced, and titration was conducted using 0.05 N NaOH until a permanent pink color appeared. A control test was conducted using an enzyme source that had been inactivated before the addition of buffer and olive oil emulsion. The milliequivalent of alkali consumed were regarded as a measure of lipase activity.

The determination of Alkaline Phosphatase (ALP) (E.C. 3.1.3.1) activity followed the protocol outlined by Garen and Levinthal [18] the assay mixture included bicarbonate buffer (0.2 M, pH 9.5), 0.1 M $MgCl_2$, tissue homogenate and freshly prepared 0.1 M para-nitrophenyl phosphate (p-NPP) as substrate. The mixture underwent

incubation in a water bath at 37^{0}C for 15 min, followed by termination with 0.1N NaOH. Optical density was measured at 410 nm. ALP activity was expressed as nanomole of p-nitrophenol released per minute per milligram of protein at 37^{0}C. for Acid Phosphatase (ACP) (E.C. 3.1.3.2) activity, the same method as for ALP was employed, but with acetate buffer (0.2 M, pH 5) instead of bicarbonate buffer.

Histo-architectural studies

Liver and intestinal sections from the experimental subjects were procured following the first and fifth weeks of the experiment. These sections were promptly preserved in neutral buffered formalin, embedded in paraffin wax, cut into 5 µm slices, and stained using hematoxylin and eosin (H & E), following the methodology outlined by Roberts [50]. The prepared slides underwent examination and photography under a light microscope.

Statistical analysis

Two-way analysis of variance (ANOVA) was used to analyse the main effect with protein levels (20%, 30%, 40%, and 45%) and temperature (26^{0}C and 32^{0}C). Duncan's multiple range test was used to compare means when the results were significant. At $P<0.05$, the means were considered to be significant. Statistical evaluation of the data was conducted using SPSS version 14.0 (SPSS, Richmond, CA, USA).

Results

Hepato-somatic Index (HSI) and Visceral Index (VI)

The HSI of *L. rohita* fingerlings was affected significantly *($P<0.05$)* by both the dietary protein evel and the water temperature, while only the dietary protein level had a notable effect on visceral index (Table 2). The HSI was significantly lower *($P<0.05$)* at 32^{0}C compared to 26^{0}C, and the reduced HSI at 32^{0}C increased significantly *($P<0.05$)* after two weeks of decreasing the temperature from 32^0 to 26^{0}C. Despite he temperature change, an increase in dietary protein level decreased the HSI and VI significantly *($P<0.05$)*. The highest and lowest HSI and VI were observed in groups fed with 20% and 45% CP, respectively.

Glycogen content in the liver and muscle

The glycogen content in the liver of *L. rohita* fingerling was found to be significantly *($P<0.05$)* influenced by both temperature and different dietary protein levels (Table 2). However, neither water temperature nor dietary protein level had an impact on muscle glycogen (Table 2). The liver glycogen content was found to be significantly lower at higher temperature (32^{0}C) in comparison to 26^{0}C, and the reduced glycogen content was found to increase after four weeks of decreasing the temperature from 32^0 to 26^{0}C. Regardless of temperature, a decreasing trend of liver glycogen content was found with an increase in dietary protein level, and the highest content was found in fish fed a 20% CP diet. An interaction effect ($p<0.05$) between temperature and dietary protein level was evident for glycogen concentration in the liver of *L. rohita*.

Digestive enzyme activity

The study found that both elevated temperature and dietary protein levels significantly affected the amylase activity in the intestine of *L. rohita* fingerlings (Table 3). Interestingly, the liver of fingerlings was found to be unaffected. Specifically, exposure to elevated temperature (32^{0}C) resulted in significant ($p<0.05$) increase in amylase activity in the intestine of fingerlings. However, this increased activity was significantly reduced ($p<0.05$) after 4 weeks of the decrease in temperature from 32^0 to 26^{0}C. Furthermore, regardless of temperature, amylase activity in the intestine of fingerlings increased gradually with the increase in dietary CP level up to 40%, which was similar to the 45% CP fed group. Lastly, the study revealed a significant ($p<0.05$) interaction effect between temperature and starch type on amylase activity in the intestine of fingerlings.

The protease activity in the liver of *L. rohita* fingerlings remained unchanged ($p>0.05$) of whether the temperature was elevated to 30^{0}C or they were fed different dietary protein levels (Table 3). However, exposure to elevated temperature (32^{0}C) resulted in a significant increase ($p<0.05$) the protease activity in the fingerling's intestine. This increase was found to be significantly decreased ($p<0.05$) after 4 weeks of lowering the temperature from 32^0 to 26^{0}C. Additionally, an increase in dietary protein level led to a significant increase in protease activity in the fingerling's intestine, up to 40% CP fed. This increase was similar to the protease activity observed in the 45% CP fed groups.

Lipase activity in the liver and intestine did not vary either due to exposure to elevated temperature (32 ^{0}C) or different dietary protein levels (Table 3).

Alkaline phosphatase (ALP) activity both in the liver and intestine of the fingerlings was significantly different ($p<0.05$) due to exposure to elevated temperature as well as different dietary protein levels (Table 4). Exposure to a higher temperature (32 ^{0}C) significantly increased ($p<0.05$) the ALP activity in the intestine whereas decreased ($p<0.05$) in the liver. Significantly increased activity in the liver and a decrease in the intestine were found after 2 weeks and 4 weeks of decrease in temperature from 32 ^{0}C to 26 ^{0}C, respectively. Regardless of

Table 2 Hepatosomatic index (HSI), visceral index (VI) and glycogen content in liver and muscle of *L. rohita* juvenile exposed to short term higher temperature and fed diet differing in protein level

Temp.	26^0C				One week at 32^0C then at 26^0C				Two Way ANOVA		
									Variation Source		
Treatment	20%CP	30%CP	40%CP	45%CP	20%CP	30%CP	40%CP	45%CP	Temp.	Protein	Inter.
HSI											
1st week	$1.11^{a}\pm0.1$	$0.91^{bc}\pm0.1$	$0.96^{b}\pm0.1$	$0.78^{cd}\pm0.1$	$0.92^{Bbc}\pm0.1$	$0.68^{Bdc}\pm0.1$	$0.69^{Bdc}\pm0.1$	$0.60^{Bc}\pm0.1$	$P<0.05$	$P<0.05$	NS
3rd Week	$1.09^{a}\pm0.1$	$0.96^{bc}\pm0.1$	$0.97^{bc}\pm0.1$	$0.88^{c}\pm0.1$	$1.05^{Aab}\pm0.1$	$0.89^{Ac}\pm0.1$	$0.90^{Ac}\pm0.1$	$0.78^{Ad}\pm0.1$	$P<0.05$	$P<0.05$	NS
5th week	$1.10^{a}\pm0.1$	$0.90^{bc}\pm0.1$	$0.93^{b}\pm0.1$	$0.85^{cd}\pm0.1$	$1.12^{Aa}\pm0.1$	$0.91^{Ab}\pm0.1$	$0.90^{Abc}\pm0.1$	$0.83^{Ad}\pm0.1$	NS	$P<0.05$	NS
VI											
1st week	$4.48^{a}\pm0.1$	$3.65^{b}\pm0.2$	$3.26^{cd}\pm0.1$	$3.07^{d}\pm0.1$	$4.39^{a}\pm0.1$	$3.46^{bc}\pm0.08$	$3.19^{d}\pm0.09$	$3.12^{d}\pm0.07$	NS	$P<0.05$	NS
3rd Week	$4.20^{a}\pm0.1$	$3.62^{b}\pm0.1$	$3.24^{d}\pm0.1$	$3.08^{e}\pm0.1$	$4.30^{a}\pm0.1$	$3.44^{c}\pm0.15$	$3.23^{d}\pm0.07$	$3.06^{e}\pm0.14$	NS	$P<0.05$	NS
5th week	$4.29^{a}\pm0.1$	$3.42^{bc}\pm0.1$	$3.21^{cd}\pm0.1$	$3.11^{d}\pm0.2$	$4.34^{a}\pm0.1$	$3.50^{b}\pm0.10$	$3.18^{cd}\pm0.14$	$3.09^{d}\pm0.12$	NS	$P<0.05$	NS
Glycogen (Liver) (mg/gm wet tissue)											
1st week	$46.39^{a}\pm2.85$	$34.65^{b}\pm1.10$	$30.54^{bc}\pm2.11$	$26.46^{cd}\pm1.59$	$33.25^{Bb}\pm1.39$	$27.22^{Bcd}\pm1.45$	$23.16^{Bde}\pm1.40$	$18.83^{Be}\pm0.94$	$P<0.05$	$P<0.05$	$P<0.05$
3rd Week	$46.72^{a}\pm1.40$	$33.60^{bc}\pm2.06$	$30.11^{cd}\pm1.95$	$28.40^{cd}\pm1.56$	$38.45^{Bb}\pm1.89$	$29.68^{ABbc}\pm1.10$	$26.81^{ABcd}\pm0.94$	$24.73^{ABd}\pm0.97$	$P<0.05$	$P<0.05$	$P<0.05$
5th week	$46.48^{a}\pm1.72$	$33.93^{bc}\pm2.39$	$30.89^{bc}\pm2.41$	$26.54^{c}\pm2.31$	$46.17^{Aa}\pm1.56$	$35.26^{Aa}\pm2.85$	$30.49^{Abc}\pm1.98$	$27.68^{Ac}\pm1.91$	NS	$P<0.05$	NS
Glycogen (Muscle) (mg/gm wet tissue)											
1st week	6.55±0.45	5.20±0.20	6.06±0.34	5.88±0.21	5.97±0.41	5.89±0.37	7.14±0.55	5.99±0.45	NS	NS	NS
3rd Week	5.88±0.37	5.57±0.22	6.05±0.45	4.42±0.15	6.07±0.25	5.71±0.17	5.02±0.32	5.29±0.42	NS	NS	NS
5th week	5.50±0.31	5.92±0.37	5.75±0.44	5.07±0.26	5.99±0.19	5.55±0.25	5.45±0.31	5.70±0.27	NS	NS	NS

Mean values in the same row with different superscript (a, b, c, d) differ significantly ($P < 0.05$)

Mean values in a column (1st, 3rd and 5th week) under each parameter bearing different superscript (A, B, C) differ significantly ($P < 0.05$)

CP Crude protein, *NS* Not significantly different ($P > 0.05$)

Table 3 Digestive enzyme profile in the liver and intestine of *L. rohita* juvenile exposed to short term higher temperature and fed diet differing in protein level

Temp	26^0C				One week at 32^0C then at 26^0C				Two Way ANOVA		
									Variation Source		
Treatment	20%CP	30%CP	40%CP	45%CP	20%CP	30%CP	40%CP	45%CP	Temp	Protein	Inter.
Amylase (Liver) (micromole maltose released/min/gm protein)											
1st week	29.61 ± 0.48	30.21 ± 0.94	30.63 ± 0.42	29.40 ± 0.51	29.89 ± 0.31	29.86 ± 0.73	30.68 ± 0.47	30.09 ± 0.45	NS	NS	NS
3rd Week	27.47 ± 0.57	30.02 ± 0.18	28.65 ± 0.40	26.80 ± 0.44	30.61 ± 0.39	27.85 ± 0.63	30.01 ± 0.34	28.68 ± 0.51	NS	NS	NS
5th week	28.84 ± 0.36	29.93 ± 0.99	29.86 ± 0.38	29.75 ± 0.59	30.72 ± 0.37	30.07 ± 0.23	29.63 ± 0.38	29.89 ± 0.22	NS	NS	NS
Amylase (Intestine) (micromole maltose released/min/gm protein)											
1st week	12.79^{e} ± 0.97	18.82^{d} ± 0.87	23.66^{bc} ± 0.98	21.25^{cd} ± 0.77	18.49^{Ad} ± 1.46	24.48^{Abc} ± 1.32	29.80^{Aa} ± 0.84	26.59^{Aab} ± 1.36	$P < 0.05$	$P < 0.05$	NS
3rd Week	12.87^{e} ± 0.52	17.65^{d} ± 0.83	23.27^{bc} ± 0.41	22.21^{bc} ± 0.65	15.66^{ABd} ± 0.64	21.02^{ABc} ± 1.01	26.33^{ABa} ± 1.17	24.09^{ABab} ± 0.93	$P < 0.05$	$P < 0.05$	NS
5th week	12.01^{c} ± 1.01	19.09^{b} ± 0.39	22.43^{a} ± 0.56	21.23^{ab} ± 0.72	12.78^{Bc} ± 0.72	19.29^{Bb} ± 0.80	23.04^{Ba} ± 0.91	22.33^{Ba} ± 0.82	NS	$P < 0.05$	NS
Protease (Liver) (micromole tyrosine released/min/gm protein)											
1st week	0.57 ± 0.04	0.64 ± 0.02	0.55 ± 0.04	0.67 ± 0.02	0.61 ± 0.01	0.61 ± 0.02	0.60 ± 0.03	0.52 ± 0.01	NS	NS	NS
3rd Week	0.51 ± 0.02	0.54 ± 0.01	0.48 ± 0.01	0.59 ± 0.04	0.49 ± 0.01	0.53 ± 0.03	0.47 ± 0.04	0.47 ± 0.03	NS	NS	NS
5th week	0.46 ± 0.02	0.44 ± 0.01	0.48 ± 0.01	0.51 ± 0.03	0.50 ± 0.04	0.51 ± 0.02	0.49 ± 0.02	0.47 ± 0.01	NS	NS	NS
Protease (Intestine) (micromole tyrosine released/min/gm protein)											
1st week	4.37^{e} ± 0.33	9.63^{d} ± 0.19	13.31^{bc} ± 0.61	10.36^{cd} ± 0.87	5.20^{e} ± 0.50	12.12^{bcd} ± 0.41	17.37^{Aa} ± 0.51	15.14^{Aab} ± 0.23	$P < 0.05$	$P < 0.05$	NS
3rd Week	5.03^{d} ± 0.25	8.91^{c} ± 0.53	12.82^{ab} ± 0.88	10.26^{bc} ± 0.55	4.91^{d} ± 0.22	11.13^{bc} ± 0.31	14.58^{ABa} ± 0.69	12.07^{Bab} ± 0.36	NS	$P < 0.05$	NS
5th week	5.13^{b} ± 0.34	10.30^{a} ± 0.51	11.86^{a} ± 0.42	10.95^{a} ± 0.74	4.37^{b} ± 0.33	10.22^{a} ± 0.84	11.65^{Ba} ± 0.63	11.13^{Ca} ± 0.31	NS	$P < 0.05$	NS
Lipase (Liver) (Units/mg protein)											
1st week	0.29 ± 0.01	0.27 ± 0.02	0.33 ± 0.01	0.33 ± 0.03	0.22 ± 0.02	0.23 ± 0.01	0.31 ± 0.03	0.21 ± 0.01	NS	NS	NS
3rd Week	0.28 ± 0.02	0.29 ± 0.01	0.25 ± 0.01	0.32 ± 0.03	0.31 ± 0.02	0.27 ± 0.02	0.28 ± 0.01	0.25 ± 0.01	NS	NS	NS
5th week	0.33 ± 0.03	0.30 ± 0.02	0.19 ± 0.01	0.24 ± 0.01	0.32 ± 0.03	0.23 ± 0.02	0.24 ± 0.01	0.21 ± 0.02	NS	NS	NS
Lipase (Intestine) (Units/mg protein)											
1st week	0.64 ± 0.04	0.67 ± 0.05	0.74 ± 0.05	0.63 ± 0.03	0.52 ± 0.04	0.57 ± 0.03	0.51 ± 0.02	0.65 ± 0.05	NS	NS	NS
3rd Week	0.43 ± 0.02	0.44 ± 0.03	0.50 ± 0.04	0.52 ± 0.04	0.45 ± 0.03	0.51 ± 0.04	0.42 ± 0.03	0.49 ± 0.04	NS	NS	NS
5th week	0.54 ± 0.04	0.43 ± 0.02	0.52 ± 0.02	0.48 ± 0.01	0.56 ± 0.04	0.47 ± 0.03	0.43 ± 0.02	0.54 ± 0.02	NS	NS	NS

Mean values in the same row with different superscript (a, b, c, d) differ significantly ($P < 0.05$)

Mean values in a column (1st, 3rd and 5th week) under each parameter bearing different superscript (A, B, C) differ significantly ($P < 0.05$)

CP Crude protein, *NS* Not significantly different ($P > 0.05$)

Table 4 Aalkaline phosphatase and acid phosphatase activity in the liver and intestine of *L. rohita* juvenile exposed to short term higher temperature and fed diet differing in protein level

Temp	26^0C					One week at 32^0C then at 26^0C			Two Way ANOVA		
									Variation Source		
Treatment	20%CP	30%CP	40%CP	45%CP	20%CP	30%CP	40%CP	45%CP	Temp	Protein	Inter.
Alkaline phosphatase (Liver) (nano moles p-nitrophenol released/min/mg protein at 37^0C)											
1st week	$15.31^{a} \pm 1.34$	$11.65^{b} \pm 0.67$	$8.26^{c} \pm 0.77$	$9.93^{bc} \pm 0.47$	$9.94^{Bbc} \pm 0.47$	$8.26^{Bc} \pm 0.28$	$7.74^{c} \pm 0.43$	$12.07^{b} \pm 0.81$	$P < 0.05$	$P < 0.05$	$P < 0.05$
3rd Week	$15.79^{a} \pm 1.05$	$12.08^{b} \pm 0.73$	$7.30^{d} \pm 0.26$	$10.35^{bc} \pm 0.35$	$12.75^{Ab} \pm 0.35$	$9.93^{ABbcd} \pm 0.46$	$8.56^{cd} \pm 0.47$	$10.94^{bc} \pm 0.54$	NS	$P < 0.05$	NS
5th week	$14.65^{a} \pm 0.67$	$11.87^{b} \pm 0.24$	$8.22^{c} \pm 0.53$	$9.70^{bc} \pm 0.68$	$14.89^{Aa} \pm 0.62$	$11.93^{Ab} \pm 0.68$	$9.26^{c} \pm 0.49$	$10.14^{bc} \pm 0.47$	NS	$P < 0.05$	NS
Alkaline phosphatase (Intestine) (nano moles p-nitrophenol released/min/mg protein at 37^0C)											
1st week	$7.99^{f} \pm 0.29$	$13.78^{e} \pm 1.14$	$17.69^{d} \pm 0.96$	$16.39^{de} \pm 1.29$	$26.57^{Ac} \pm 0.55$	$30.86^{Ab} \pm 0.80$	$37.90^{Aa} \pm 0.92$	$31.13^{Ab} \pm 0.81$	$P < 0.05$	$P < 0.05$	$P < 0.05$
3rd Week	$7.31^{d} \pm 0.61$	$13.93^{c} \pm 0.16$	$18.31^{b} \pm 0.70$	$15.95^{bc} \pm 0.81$	$14.01^{Bc} \pm 0.91$	$18.19^{Bb} \pm 0.53$	$22.11^{Ba} \pm 1.01$	$17.50^{Bb} \pm 0.67$	$P < 0.05$	$P < 0.05$	$P < 0.05$
5th week	$7.38^{d} \pm 0.74$	$13.78^{c} \pm 1.14$	$18.16^{ab} \pm 0.68$	$15.92^{bc} \pm 0.61$	$8.71^{Cd} \pm 0.69$	$14.13^{Cc} \pm 0.66$	$18.52^{Ba} \pm 0.58$	$15.71^{Bbc} \pm 0.69$	NS	$P < 0.05$	NS
Acid phosphatase (Liver) (nano moles p-nitrophenol released/min/mg protein at 37^0C)											
1st week	3.51 ± 0.17	3.69 ± 0.29	4.13 ± 0.39	4.34 ± 0.11	3.70 ± 0.16	4.37 ± 0.24	4.33 ± 0.29	4.56 ± 0.15	NS	NS	NS
3rd Week	3.94 ± 0.24	4.42 ± 0.33	4.28 ± 0.32	4.04 ± 0.24	3.95 ± 0.19	4.79 ± 0.33	4.17 ± 0.32	4.39 ± 0.41	NS	NS	NS
5th week	4.02 ± 0.37	3.81 ± 0.25	4.37 ± 0.18	3.72 ± 0.31	4.30 ± 0.25	4.03 ± 0.12	4.87 ± 0.28	4.28 ± 0.35	NS	NS	NS
Acid phosphatase (Intestine) (nano moles p-nitrophenol released/min/mg protein at 37^0C)											
1st week	$3.64^{e} \pm 0.21$	$6.15^{cd} \pm 0.40$	$7.71^{bc} \pm 0.57$	$9.67^{ab} \pm 0.35$	$4.01^{de} \pm 0.38$	$6.45^{c} \pm 0.41$	$8.51^{abc} \pm 0.71$	$10.21^{a} \pm 0.82$	NS	$P < 0.05$	NS
3rd Week	$3.89^{f} \pm 0.11$	$5.71^{de} \pm 0.48$	$8.21^{bc} \pm 0.34$	$10.77^{a} \pm 0.51$	$4.73^{ef} \pm 0.50$	$6.71^{cd} \pm 0.45$	$8.75^{b} \pm 0.46$	$9.69^{ab} \pm 0.54$	NS	$P < 0.05$	NS
5th week	$4.02^{ef} \pm 0.27$	$5.85^{de} \pm 0.47$	$8.85^{bc} \pm 0.50$	$10.92^{a} \pm 0.50$	$3.87^{f} \pm 0.20$	$7.35^{cd} \pm 0.45$	$9.89^{ab} \pm 0.53$	$10.70^{ab} \pm 0.32$	NS	$P < 0.05$	NS

Mean values in the same row with different superscript (a, b, c, d) differ significantly ($P < 0.05$)

Mean values in a column (1st, 3rd and 5th week) under each parameter bearing different superscript (A, B, C) differ significantly ($P < 0.05$)

CP Crude protein, *NS* Not significantly different ($P > 0.05$)

temperature, ALP activity in both the liver and intestine increased gradually with an increase in dietary CP level up to 40% significantly, which was similar to the 45% CP fed group.

Acid phosphatase (ACP) activity in the liver was found unaffected due to different dietary protein levels as well as exposure to higher temperatures (32 ^{0}C). However, in the intestine, ACP was significantly ($p < 0.05$) affected only due to different dietary protein levels (Table 4). An increasing trend of ACP activity in the intestine of fingerlings was found with the increase in dietary CP level.

Histo-architectural changes

The liver structure of fish fed 20% CP diet either at 26^{0}C or 32^{0}C showed no visible histological alteration with normal cellular architecture and hepatocyte staining after one week (Fig. 2a). Similarly, the liver section of fish fed 30% CP diet at 26^{0}C showed no visible changes however, at 32^{0}C showed slightly increase in the size of the hepatocyte i.e. mild swollen of the liver cell (Fig. 2b). It was evident that liver cells were highly swollen after one week of feeding of 40% and 45% dietary CP level at 26^{0}C (Fig. 2c). Whereas fish fed 40% dietary protein at 32^{0}C showed slightly higher swollen liver cells compared to the 30% dietary protein fed at the same temperature after one week (Fig. 2d). Sclerosis of the artery i.e. thickening of the wall of blood vessels and mass degeneration of hepatocytes with picnotic nuclei are evident in the liver of the fish fed with 45% dietary protein at 32^{0}C for one week (Fig. 2e). At the end of the experiment (i.e. after five weeks) no visible change was seen in the treatment fed with 20% dietary protein at 26^{0}C whereas fish fed with 30% or 40% CP at 26^{0}C appeared normal with slightly swollen hepatocytes. Similarly, hepatocytes of the treatment fed with 20%, 30%, and 40% dietary protein at 32^{0}C for one week after that decreased to 26^{0}C also appeared normal with swollen cells. The liver cells of the fingerlings were fed 45% dietary protein either at 26^{0}C or 32^{0}C for one week then at 26^{0}C showed swollen liver with sclerotic arteries.

Based on the microscopy of intestinal sections of the treatments after one week of feeding with 20%, 30%, and 40% dietary protein either at 26^{0}C or 32^{0}C, no consistent histo-architectural changes were observed and there were no marked differences among these groups (Fig. 3a). The 45% CP fed group at 26^{0}C showed mildly desquamated mucosa (Fig. 3b) whereas defused mucosa was found at 32^{0}C (Fig. 3c). Treatment fed with 20% dietary protein for five weeks at 26^{0}C showed that intestinal mucosa became edematous with less intensity of stain (Fig. 3d) whereas denaturation of mucosa with lots of edema was found in the treatment fed with 20% dietary protein at 32^{0}C for one week and then decreased to 26^{0}C (Fig. 3e). No specific change has been found in the treatment fed with 30% and 40% dietary protein after five weeks either at 26^{0}C or 32^{0}C for one week after that at 26^{0}C. Marked loss of mucosa around the lamina propria was found in the intestine of rohu fed with 45% dietary protein after five weeks either at 26^{0}C or 32^{0}C for one week after that at 26^{0}C (Fig. 3f).

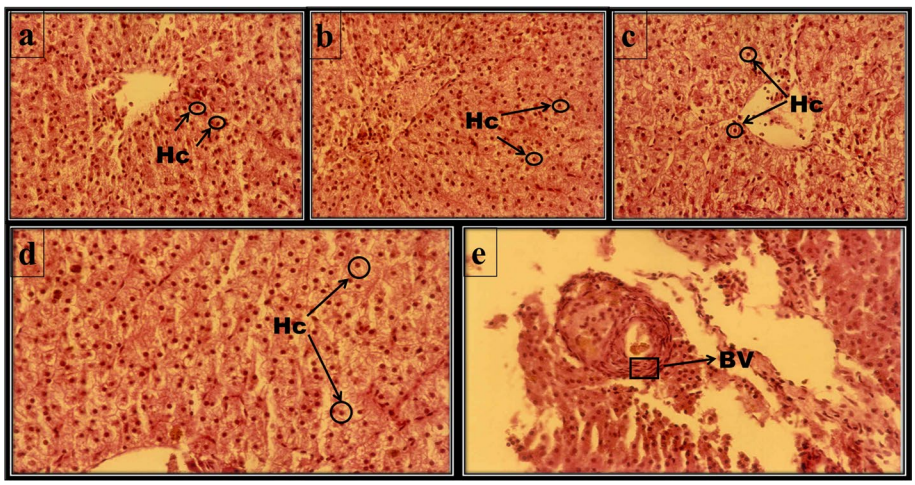

Fig. 2 **a**-**e** Histological pictures of the liver of *Labeo rohita* fingerlings fed different diets. **a** Liver of *Labeo rohita* fingerlings fed with 20% protein for seven days either on 26^{0}C or 32^{0}C showing no visible changes (H & E, 160X). **b** Liver of *Labeo rohita* fingerlings fed with 30% protein for seven days on 32^{0}C showing mild swollen cells i.e. increase in the size of hepatocyte (H & E, 160X). **c** Liver of *Labeo rohita* fingerlings fed with 40% or 45% protein for seven days on 26^{0}C, cells are highly swollen (H & E, 160X). **d** Liver of *Labeo rohita* fingerlings fed with 40% protein for seven days on 32^{0}C showing comparatively higher swollen cells than fed with 30% protein at same temperature (H & E, 160X). **e** Liver of *Labeo rohita* fingerlings fed with 45% protein for seven days on 32.^{0}C showing sclerosis of the artery i.e. thickening of the wall of blood vessels (H & E, 160X)

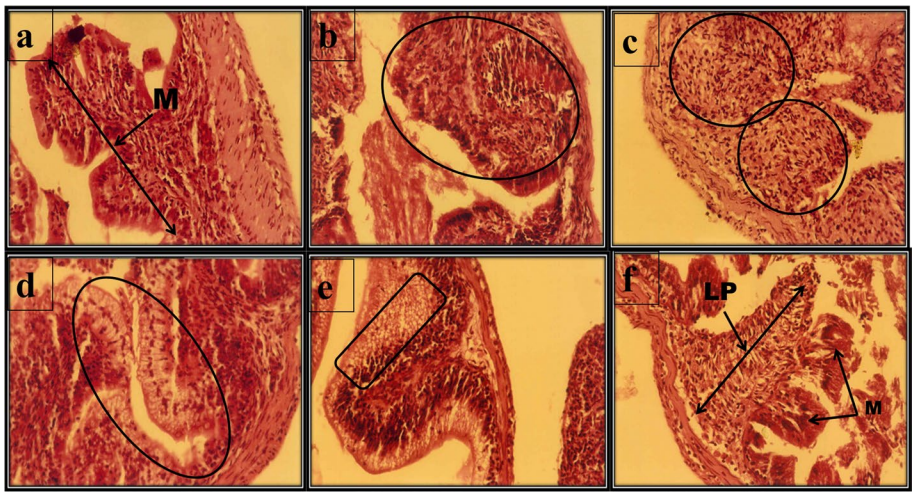

Fig. 3 **a**-**f** Histological pictures of the intestine of *Labeo rohita* fingerlings fed different diets. **a** Intestine of *L. rohita* fingerlings fed with 20% protein for seven days on 26^{0}C showing no visible changes (H & E, 320X), **b** Intestine of *L. rohita* fingerlings fed with 45% protein for seven days on 26^{0}C showing mildly desquamated mucosa (M) in the circle (H & E, 160X), **c** Intestine of *L. rohita* fingerlings fed with 45% protein for seven days on 32^{0}C showing defused mucosa in both circle (H & E, 320X), **d** Intestine of *L. rohita* fingerlings fed with 20% protein for five weeks on 26^{0}C showing that mucosa become edimatous with less intensity of stain in circle. (H & E, 160X), **e** Intestine of *L. rohita* fingerlings fed with 20% protein for seven days on 32^{0}C showing denaturation of mucosa with lots of edema after five week in rounded rectangle (H & E, 160X), **f** Intestine of *L. rohita* fingerlings fed with 45% protein for seven days on 32.^{0}C showing marked loss of mucosa of surrounding the lamina propria (Lp) after five week. (H & E, 160X)

Discussion

The changes in tissue metabolite concentrations with the variation in feed composition, indicate biochemical modifications of animal metabolism. A reduction in liver glycogen levels accompanied the increase in dietary protein levels. The primary cause of this could be the steady reduction in total carbohydrate inclusion as the quantity of dietary protein gradually rises to preserve the isocaloric character of the test diets. Additionally, the fact that glycogen levels dropped as dietary protein levels rose suggests that amino acids satisfied the body's need for energy at higher protein levels.

The hepatosomatic index explains the relationship between somatic growth and liver weight. In the present study, lower HSI has been found in the treatment exposed to higher temperatures (32^{0}C). Moreira et al. [44] found that HSI was higher at the lowest temperature. Increased HSI may be either due to high deposition of glycogen or lipid. The hepato-somatic index was inversely related to the dietary protein levels and positively related to the dietary carbohydrate levels. Several other researchers [1, 7, 44, 58] have reported such relationships, suggesting that an increase in hepato-somatic index with increasing dietary digestible carbohydrate was due to the deposition of glycogen in the liver.

The visceral index explains the relationship between the weight of the intestine and somatic weight. In the present study, no effect of temperature was found on visceral index, irrespective of dietary protein level. Irrespective of temperature, an increase in dietary protein level significantly decreased the visceral index in *L.* rohita. Moreira et al. [44] also observed a decrease in the visceral index in European sea bass with the increase in temperature.

The activity of digestive enzymes in fish may be influenced by the type of feeding [28, 39], temperature of acclimatization [57], biochemical composition of the food [33]. Fish's digestive capacity and dietary habits (herbivore, detritivore, omnivore, or carnivore) may be inferred from their digestive enzyme patterns [8]. The digestive system's enzyme profile might change depending on the kind of source and quantity of nutrients. It is possible to successfully utilize the enzymes' adaptive nature to benefit from the nutritional content of diets [43]. One of the most important carbohydrases, amylase hydrolyzes the glycosidic bonds between the sugar residues. Starch is particularly broken down into glucose molecules by amylase. The common belief is that omnivore fish (fish without a stomach) have high amylase activity whereas carnivorous fish (fish with a stomach) have low amylase activity [27]. Amylase activity in the intestine increased significantly with the increase in temperature as well as the increase in dietary protein level up to 40% and similar between 40 and 45% CP fed group. The amylase activity in fish can be influenced by the feeding habit [19], type of carbohydrate [33], and temperature [37]. Increased amylase activity was reported in striped

catfish (*Pangasius hypophthalmus*) fed with diets containing increasing dietary protein levels [4]. Sun et al. [53] reported that increasing the dietary starch level in *Micropterus salmoides* reduced intestinal amylase activity. In the later study, the amylase reduction was considered to be because of inhibition of the enzyme after adsorption to the starch molecule. Irrespective of dietary protein level, the increase in amylase activity due to the temperature rise decreased after 4 weeks of the decrease in temperature. The present study also indicates that high dietary carbohydrate content induces or suppresses amylase activity.

Proteases are digestive enzymes that hydrolyze the peptide bonds between the adjacent amino acids in proteins. Protease activity in the intestine was higher than the hepatic protease activity, which is supported by the results of Hidalgo et al. [27] and Kumar et al. [33]. In the present study liver protease activity was not affected either due to temperature or dietary protein level whereas significantly higher protease activity in the intestine was found at higher temperatures and increased with the increase in dietary protein level. In contrast, early-weaned sea bass was shown to have trypsin that was insensitive to dietary CP [10]. Similarly, Lundstedt et al. [41] observed very minute changes in trypsin and chymotrypsin activities in pintado, *P. corruscans* as the dietary protein level changed, suggesting that both these enzymes are unresponsive to diet composition in pintado. Lopez-lopez et al. [40] also did not find any correlation between protease activity and dietary CP and between protease activity and growth in *H. americanus*.

Lipases hydrolyze the ester bonds between the fatty acids and glycerol in fats. Albeit our experimental feeding design did not consider the study of the inductive character of lipases, because lipid levels were constant in the diet composition. Similar observation was reported by Lundstedt et al. [41] pintado. Moreover, the occurrence of lipase activity is more important in carnivorous fish as they feed on food rich in fat [11]. Exposure to higher temperatures also did not affect the lipase activity.

According to Harpaz and Uni [25] and Gawlicka et al. [20], alkaline phosphatase activity in fish enterocytes serves as a gauge of how well nutrients are absorbed. In the present study, ALP activity in both the liver and intestine was found to be significantly affected due to temperature as well as dietary protein level. The growth rate was found to be positively correlated with ALP activity in Atlantic cod [38], which normally feeds on invertebrates and fish in the natural environment. Contrarily, Blier et al. [6] found no appreciable differences in ALP activity in growth hormone transgenic coho fish, which were predicted to develop at a faster pace than non-transgenic coho salmon. Herbivorous fish are reported to have lesser ALP activities than carnivorous fish [25]. The ACP activity in the liver and intestine was found not affected by the change in the temperature whereas an increase in the dietary protein level significantly increased the ACP activity in the intestine.

Normal functioning of the vital organ viz., liver, and intestine are essential for the wellbeing of all living animals. It is obvious that severe or irreversible anatomical and histopathological changes in these vital organs would seriously affect their normal processes and eventually lead to the death of the fish. The relation between physiological and behavioral changes in fishes can be observed by histo-architectural examination. The histological changes in the liver and intestine due to different feeding strategies are one of the important tools to determine the suitability of the ingredient for feed formulation. The most often used stain in histology and histopathology is hematoxylin and eosin (H&E). Nuclei are colored blue by hematoxylin, while the cytoplasm is pink by eosin. Swollen of the liver cell was evident in the fish exposed to 32^0C compared to 26^0C in the 20%, 30%, and 40% CP fed groups. The probable reason for swollen liver cells is more utilization of nutrients at higher temperatures. Sclerosis of the artery i.e. thickening of the wall of blood vessels was found in the liver of the fish fed with 45% CP at 32^0C for one week as well as after 4 weeks of the decrease in temperature from 32^0 to 26^0C. The high dietary protein level may create an overabundance of energy for consumption by intestinal cells during growth, which further leads to an increase in the weight of the intestine [22]. The higher inclusion level of soybean meal in 45% CP groups might have adversely affected the liver histology, an effect of heat-stable anti-nutritional factors present in the soybean meal. On the contrary, no consistent histopathological changes were observed in intestinal sections in 20%, 30% and 40% CP fed groups. Whereas desquamated mucosa or defused mucosa was found in the 45% CP fed group. Dietary soybean meal was found to induce morphological changes in the distal intestine [3, 51]. These are associated with a profound infiltration of a mixed population of inflammatory cells [3] and reduced relative weight of the distal intestine [45]. Soy non-starch carbohydrates and heat-stable anti-nutritional factors have been claimed as major factors responsible for impaired growth performance because they reduce nutrient digestibility and digestive-absorptive capabilities in fish [30, 32, 48].

Conclusion

The present study demonstrated that the activities of digestive enzymes, specifically amylase and protease, were more pronounced at 32^0C compared to 26^0C.

Moreover, this heightened activity persisted for the subsequent two weeks following a temperature decrease from 32^0 to 26^0C. Further, there was an increased activity of amylase and protease in the intestine was observed in correlation with dietary protein levels, reaching a peak at 40%. The histological changes of swollen liver cells at higher temperatures suggest increased nutrient utilization at higher temperatures, however, desquamated mucosa of the intestine in 45% dietary protein fed group acclaimed reduction in the digestive-absorptive capabilities. Our data corroborate previous findings that exposure to higher temperature (32^0C) for one week increases the growth rate, feed efficiency, and protein retention through efficient utilization of dietary carbohydrates [34] and protein [35], through higher (45%) level of dietary protein resulted in metabolic stress [36] and impaired growth performance. Overall, it concludes that the dietary protein level and water temperature interactions are more effective in maintaining the histo-architectural structure of the liver and intestine, which responds to the increased digestive capacity of the fish and results in higher nutrient utilization.

Acknowledgements
The first author is thankful to the Director, Central Institute of Fisheries Education for providing financial assistance in the form of an Institutional fellowship for Ph.D during the research period.

Authors' contributions
Shivendra Kumar: Investigation, Methodology, Writing – original draft, review & editing N. P. Sahu: Conceptualization, Supervision, Validation, Writing - review& editing.

Funding
The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Data availability
The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interest
The authors declare no competing interests.

Received: 26 June 2024 Accepted: 29 November 2024
Published online: 12 December 2024

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