



Changes in weight gain, digestive and metabolic enzyme activities in *Labeo rohita* fingerlings in response to multiple stress exposure and dietary nutraceutical

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Abstract

A feeding trial was conducted for 4 months to study the effect of a nutraceutical conglomerate at different levels (0, 0.1, and 0.5%) on stress-exposed (high stocking density, H and feed restriction, R) *Labeo rohita* fingerlings. Six isonitrogenous diets with a crude protein of 35% were prepared for the different treatments, viz. THR0, THR1, THR5, TNS0, TNS1 and TNS5. Stress had significantly reduced the weight gain and feeding of 0.1% nutraceutical improved it in both stress-exposed and non-exposed groups from 3 months onwards. Two- and three-month stress-exposed groups exhibited lower ($p < 0.05$) protease activity, while amylase activity was significantly higher in 2-month stress-exposed groups. Higher lipase activity was found in stress-exposed groups irrespective of the duration of stress. Higher serum glutathione peroxidase (GPx), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and hepatic and muscle lactate dehydrogenase (LDH) activities were observed in the THR0 groups; however, THR1 showed a lower activity of these enzymes. Increased malate dehydrogenase (MDH) and glucose-6-phosphate dehydrogenase (G6PDH) activity was recorded in the 1st and 2nd months stress-exposed groups. However, the THR1 group exhibited a reduced MDH activity and a reverse trend for G6PDH compared to THR0. Hence, it can be concluded that the duration of multiple stress exposure adversely affected the digestive and metabolic enzymes activities and feeding of 0.1% nutraceutical conglomerates could restore the activities of digestive and metabolic enzymes in rohu fingerlings.

Keywords Crowding · Glutathione peroxidase · Lipase · Protease · Amylase · Lactate dehydrogenase

Introduction

Labeo rohita is the most preferred Indian major carps in the Asian subcontinent which is produced either in monoculture or polyculture production system (Nandeesh et al. 1993). Rearing of stunted fingerling is a common practice, where farmers produce table size fish of around a kilogram within 4-month period of compensatory feeding. In such practices, the fish are mostly exposed to multiple stressors like high stocking density (crowding stress) and restricted feeding to

limit the growth. However, high stocking density and feed restriction are considered stressors in aquatic system (Klinger et al. 1983; Blazer and Wolke 1984). Though common stressors like crowding and diet restriction or starvation can be experienced in wild as well as in culture conditions (Madrid et al. 2001; TorfiMozanzadeh et al. 2017), but the stress should be within the tolerance level of the fish. Beyond the tolerance level, it reaches a point of no return and that can cause detrimental effect on the fish (Wedemeyer 1997). In a stressed condition, there is release of stress hormones within the animal, which alter the physio-metabolic responses of fish (Mommsen et al. 1999). This stress response increases the energy demand for meeting the maintenance requirement which can further lead to growth retardation. Stress condition can alter the normal physiology, immunity, and antioxidant status of the fish (Ellis 1981). Moreover, the increased demand of energy to ameliorate the stress is achieved mostly by rearranging and adapting the metabolic pathways of fish (Mommsen et al. 1999). This is possible only when the fish

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has a capacity to counter oxidative stress. But most of these mitigation responses are species-specific and the real mechanism is still unclear (Wang et al. 2000; McCue 2010).

The digestive and metabolic enzyme activities in fish may be altered during the exposure to various stressors like crowding (Tejpal et al. 2009), nitrite exposure (Ciji et al. 2012), feed restriction (Yengkokpam et al. 2013), and exposure to antinutrients (Shamna et al. 2015). Activities of these enzymes can be used as indicators of stress in fish and can be a potential cue for regulation of growth and metabolism during multiple stress exposure. Insufficient availability of nutrients in digestive tract reduces the activity of digestive enzymes and redirects the body nutrients for the maintenance of life. Such kind of metabolic shift alter the intermediary metabolism (Morales et al. 2004) and result in variation of enzymes associated with different metabolic pathways like alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Sanchez-Muros et al. 1998; Tejpal et al. 2009), lactate dehydrogenase (LDH), pyruvate kinase, and glucose-6-phosphate dehydrogenase (G6PDH), catalase, superoxide dismutase, and malate dehydrogenase (MDH) (Shimeno et al. 1997; Metón et al. 2003; Yengkokpam et al. 2013; Shamna et al. 2018). However, the individual effect of stressors may be different from the multiple stressors as it may cause a different pattern of up- and downregulation of these enzymes (Matthaei and Lange 2016; Shamna et al. 2017).

Fish has a well-developed antioxidative defense system with two major components, viz., the enzymatic antioxidants and non-enzymatic antioxidants. The non-enzymatic antioxidants ameliorate the reactive oxygen species generated during stress either by its own independent action or by supporting the enzymatic antioxidants. Some non-enzymatic antioxidants like glutathione and vitamin E donate electrons and deactivate reactive oxygen species generated during stress (Martinez-Alvarez et al. 2005; Ciji et al. 2012), while some others like zinc, selenium, and copper support the faster action of enzymes or other physiological processes (Finkelstein 2000; Choi et al. 2005; Nazeemashahul et al. 2020). Hence, feeding of such biological antioxidants like vitamin E, methionine, glutathione, and zinc can support the antioxidant system in animals and reduce the higher energy demands for maintenance (Choi et al. 2005; Li et al. 2010; Ciji et al. 2012). There are few studies reported on nutritional strategy to mitigate stress in fish by feeding nutraceuticals which has the capacity to improve feed intake, digestion, and metabolism (Akhtar and Ciji 2021; Ciji and Akhtar 2021). In our previous study, we could observed an increase in the expression of IGF-1 (insulin like growth factor-1) and growth, when stress-exposed rohu were fed with nutraceutical conglomerates, containing methionine (360 mg), selenium (50 mg), zinc (1000 mg), and vitamin E (21 g) compared to their non-nutraceutical-fed counterpart (Nazeemashahul et al. 2020). However, the

action of this mixture in fish to improve the growth may be related to its action on various digestive and metabolic enzymes. According to Ciji and Akhtar (2021), as nutritional requirement varies under stressful conditions, dietary interventions are found to be a sustainable strategy among all stress mitigation strategies to develop stress resistance in fish. Hence, the present study on rohu, *Labeo rohita*, aimed at revealing the effects of multiple stressors (feed restriction and overstocking) on various digestive and metabolic enzymes and mitigating the stress level by feeding nutraceutical conglomerates (vitamin E, selenium, zinc, and methionine) on the enzyme activities during stress exposure at different periods of time.

Materials and methods

Experimental animal and diets

Labeo rohita (Hamilton, 1822) fingerlings were procured from a private farm in Mumbai, India. Acclimatization was done under aerated conditions for 7 days in the laboratory of ICAR-CIFE, Mumbai, India. During this time period, the fish were fed with a diet of 35% crude protein. Purified ingredients like gelatin and casein (protein sources), starch and dextrin (carbohydrate sources), vegetable oil and fish oil (lipid sources), carboxymethyl cellulose (binder), vitamin and mineral mixture, and BHT (synthetic antioxidant) procured from Hi-Media were used to prepare three experimental diets, viz. control (C), diet A, and diet B with supplementation of nutraceutical conglomerate at 0, 0.1, and 0.5% level, respectively (Table 1). The nutraceutical mixture contained methionine (360 mg), selenium (50 mg), zinc (1000 mg), and vitamin E (21 g) per kilogram. Hence, when given in diet at 0.1 and 0.5% levels, these nutrients were above the requirement levels of the fish (Table 1).

Experimental design

The experiment was conducted with one thousand and eighty *Labeo rohita* (Hamilton 1822) fingerlings (average weight 8.5 ± 0.5 g) in 6 different experimental groups (random distribution). The experimental fish were subjected to different stressors like crowding stress (high stocking density at 20 no. of fish per 75 L water) and feed restriction (restricted feeding at 1% of body weight) and normal stocking density (10 no. of fish per 75 L) and satiation feeding (SF). Three levels of nutraceuticals were also given (0, 0.1, and 0.5%). Hence, the groups were THR0 (crowding stress, feed restriction, and 0% nutraceutical), THR1 (crowding stress, feed restriction, and 0.1% nutraceutical), THR5 (crowding stress, feed restriction, and 0.5% nutraceutical), TNS0 (normal stocking density, satiation feeding, and 0% nutraceutical),

Table 1 Ingredient composition of the experimental diets (%)

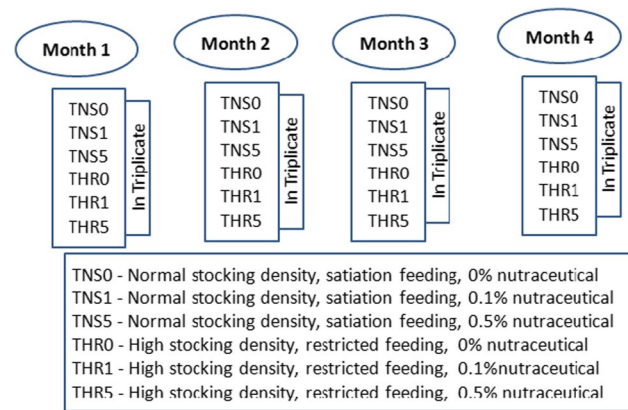
Ingredient	Control (C)	Diet A	Diet B
Casein (vitamin free) ¹	30	30	30
Gelatin ¹	9	9	9
Dextrin ¹	10	10	10
Starch soluble ¹	30	30	30
Cellulose powder ¹	10.89	10.79	10.39
Vegetable oil ²	3	3	3
Cod liver oil ²	3	3	3
Carboxymethyl cellulose ¹	2	2	2
Vitamin ³	2	2	2
Butylated hydroxyl toluene ¹	0.01	0.01	0.01
Choline chloride ¹	0.1	0.1	0.1
Nutraceutical ⁴	0	0.1	0.5
Proximate composition of feed (%)			
Moisture	8.49	7.15	7.16
Crude protein	35.45	35.78	35.98
Ether extract	6.12	6.13	6.11
Total ash	5.20	4.81	4.74
Crude fiber	10.63	10.20	10.68
Nitrogen-free extract	42.59	43.03	42.37

¹Himedia Laboratories, India²Procured from local market³composition (quantity 2.5 kg⁻¹): vitamin A, 55,00,000 IU; vitamin D3, 11,00,000 IU; vitamin B2, 2000 mg; vitamin E, 750 mg; vitamin K, 1000 mg; vitamin B6, 1000 mg; vitamin B12, 6 mcg; calcium pantothenate, 2500 mg; nicotinamide, 10 g; Mn, 27,000 mg; I, 1000 mg; Fe, 7500 mg; Zn, 5000 mg; Cu, 2000 mg; Co, 450 mg L-lysine, 10 g; DL-methionine, 10 g; selenium, 125 mg; vitamin C, 2500 mg⁴Includes four compounds (selenium 50 mg/kg, zinc 1000 mg/kg, vitamin E 21 g/kg, and methionine, 36 mg/kg) 0.1% nutraceutical contains 25% extra methionine, 16.66% extra vitamin E, 2.5% extra zinc, and 5% extra selenium than the requirement level while 0.5% contains 46% extra methionine, 80% extra vitamin E, 12.5% extra zinc, and 25% extra selenium than the requirement level

TNS1 (normal stocking density, satiation feeding, and 0.1% nutraceutical), and TNS5 (normal stocking density, satiation feeding, and 0.5% nutraceutical). The groups were in triplicate with four similar experimental set for the duration of 1, 2, 3, or 4 months, respectively (Fig. 1). The feeding trial was conducted in plastic rectangular tubs (57×36×47 cm, 75 L capacity) having perforated cover lids and continuous aeration.

Sample collection and processing

Weight of all the fish was measured at regular intervals following standard protocols, and the weight gain was calculated by subtracting the monthly weights from initial weights. Six ($n=6$) fish were sacrificed from each tank and the liver, muscle, and intestine samples were collected for enzyme assay. The tissues were collected in

**Fig. 1** Experimental design showing various treatment groups

chilled sucrose solution (0.25 M) and homogenization was done using Micra D-9 (ART Prozess & Labortechnik, Germany) tissue homogenizer and then subjected to centrifugation at 5000 rpm for 15 min at 4 °C (REMI CPR-24, India), and supernatants were collected and stored at –20 °C until further use for digestive and metabolic enzyme assay. Blood sample was collected from caudal vein after anesthetizing the fish with clove oil (50 µL/L). The separation of the serum was done by centrifuging the blood clots at 3000 rpm for 10 min at 4 °C. The serum samples were used for the analysis of serum glutathione peroxidase (GPx). The entire procedure followed the ethical standard as approved by the ICAR-Central Institute of Fisheries Education, Mumbai, India.

Digestive enzyme assays

Protease

Protease enzyme activity was estimated by standard method described by Drapeau (1976) which follows the principle of casein digestion. The reaction mixture was prepared out of 1% casein in 0.05 M triphosphate buffer (pH 7.8) and the mixture was kept at 37 °C for 5 min, to which homogenate prepared from intestine tissue was added. The experimental reaction was terminated with the help of 10% trichloroacetic acid (TCA) after 10 min and the whole contents were filtered. A reagent blank was prepared for the assay by making a small modification in the treatment procedure. For blank, the intestine homogenate was added just before the termination of reaction using TCA without incubation. The enzyme activity is expressed in terms of quantity of enzyme needed for the release of equivalent acid-soluble fragments to $\Delta 0.001 A_{280}$ per min at 37 °C.

Lipase

The activity of lipase enzyme was studied following the standard method (Cherry and Crandall 1932). Three milliliters of distilled water and 1 mL of intestine tissue homogenate were added to clean, dried, and properly labeled test tubes, and the control tubes were allowed to heat in boiling water for 5 min. After cooling the tubes, 0.5 mL of phosphate buffer and 2 mL of olive oil emulsion were added to all the tubes. Furthermore, the tubes were shaken thoroughly and, after that, incubated at 37 °C for 24 h. Titration was done with N/20 NaOH solution after adding 2 drops of phenolphthalein. The activity was calculated based on the following formula:

$$\text{Lipase activity (U mg protein}^{-1}\text{)} = \frac{\text{Volume of NaOH}}{\text{g of sample} \times \text{mg protein g tissue}^{-1}}$$

Amylase

Amylase activity was determined using the dinitrosalicylic acid (DNS) method described by Rick and Stegbauer (1974). This method follows the principle of estimation of reducing sugars produced from carbohydrate by the action of glucoamylase and alpha-amylase. The reaction mixture was prepared using 1% (w/v) starch solution, intestine tissue homogenate, and phosphate buffer (pH 6.9) and subjected to 37 °C incubation for 30 min. After the addition of DNS, the mixture was kept in boiling water bath (5 min). The mixture was diluted with distilled water once it is cooled, and absorbance was recorded against 540 nm in a spectrophotometer (Shimadzu, Japan).

Metabolic enzymes

Aspartate aminotransferase and alanine aminotransferase

The AST and ALT activities were studied using the method described by Wooten (1964). In the experimental and control tubes, 0.5 mL of substrate (0.2 M D,L-aspartic acid and 2 mM α -ketoglutarate in 0.05 M phosphate buffer (pH 7.4)) was taken. The initiation of reaction was done by the addition of 0.1 mL of tissue homogenate and the mixture was subjected to incubation at 37 °C for 60 min. The termination of reaction was done with the help of 0.5 mL of 1 mM 2,4-dinitrophenyl hydrazine (DNPH). Control group was maintained by adding the homogenate after the addition of DNPH. Occasional shaking was done for all tubes for 20 min at room temperature (RT). Then, the tubes were shaken well after the addition of 0.4 mL NaOH (5 mL) solution. The OD was noted at 540 nm against the blank after 10 min. The assay followed for ALT activity was similar for AST

activity, but instead of aspartic acid, 0.2 M D,L-alanine was used as the substrate.

Lactate dehydrogenase and malate dehydrogenase

The LDH activity was assayed using the method of Wroblewski and Ladue (1955), while the MDH activity was estimated using the method of Ochoa (1955). The enzyme activity was reported as units/milligram of protein/minute at 25 °C, where 1 unit was equivalent to $\Delta 0.01$ OD/min.

Glucose-6-phosphate dehydrogenase

The G6PDH activity measured using the method of De Moss (1953) and the optical density was measured at 340 nm at 15-s interval against distilled water (blank) for 3 min and the expression was done as units/milligram of protein/minute.

Serum glutathione peroxidase

GPx assay was performed by using Cayman's GPx assay Kit (Cayman chemicals, Ann Arbor, USA). The reaction rate was calculated using the formulae below. One unit was equivalent to the enzyme that can oxidize 1.0 nmol of NADPH to NAD⁺/min at 25 °C.

$$\text{GPx activity (U/mL)} = \frac{\text{Rate of change in absorbance} \times 0.19 \text{ mL} \times \text{sample dilution}}{0.00373 \text{ } \mu\text{mol/M} \times 0.02 \text{ mL}} \times 100$$

Statistical analysis

All the data were subjected to two-way and one-way ANOVA in SPSS version 22 software after checking the normality and homogeneity of the variance. The results were expressed as mean $\pm$ SE in the case of one-way ANOVA and as mean and SEM in the case of two-way ANOVA. DMRT was used for post hoc analysis. The significance level was considered at 5% probability ($p < 0.05$).

Results

Weight gain

The weight gain was significantly lower ($p < 0.05$) in stress-exposed groups than the non-stress-exposed counterpart in all the 4 months of the experimental period (Table 2). TNS1 showed an increase in weight gain from 3 months onwards. Similarly, feeding of 0.1% nutraceutical significantly ($p < 0.05$) increased the weight gain in THR1 groups at the end of the 2nd, 3rd, and 4th months. An interaction effect ($p < 0.05$) was observed between nutraceutical and stress throughout the experimental period.

Table 2 Effects of stress (high stocking density and feed restriction), level of nutraceutical, and interaction between stress and nutraceutical on weight gain of rohu, *Labeo rohita*, fingerlings

Two-way ANOVA	Weight gain (g)			
	1st month	2nd month	3rd month	4th month
Treatments ¹				
TNS0	4.36 ^a ± 0.08	6.54 ^a ± 0.05	11.47 ^b ± 0.27	13.32 ^b ± 0.05
TNS1	4.41 ^a ± 0.03	6.49 ^a ± 0.04	12.55 ^a ± 0.13	14.96 ^a ± 0.16
TNS5	4.28 ^a ± 0.11	6.46 ^a ± 0.11	10.31 ^b ± 0.12	12.79 ^b ± 0.14
THR0	0.27 ^c ± 0.003	0.58 ^c ± 0.008	1.14 ^d ± 0.006	1.28 ^d ± 0.005
THR1	0.66 ^b ± 0.04	1.06 ^b ± 0.07	1.56 ^c ± 0.008	2.40 ^c ± 0.02
THR5	0.48 ^b ± 0.004	0.81 ^c ± 0.006	1.21 ^d ± 0.01	1.90 ^d ± 0.01
Effect of stress				
Without stress	4.35 ^a	6.50 ^a	10.60 ^a	12.89 ^a
With stress	0.43 ^b	0.78 ^b	1.09 ^b	1.59 ^b
SEM	0.04	0.05	0.08	0.06
p value	0.001	0.008	0.004	0.001
Effect of nutraceutical level				
0.0	2.27 ^b	3.56 ^b	6.31 ^b	7.30 ^b
0.1	2.44 ^a	3.77 ^a	7.06 ^a	8.68 ^a
0.5	2.47 ^a	3.76 ^a	5.75 ^c	7.33 ^b
SEM	0.04	0.06	0.09	0.08
p value	0.004	0.016	0.002	0.006
Effect of stress nutraceutical (two-way ANOVA)*				
p value	0.003	0.019	0.000	0.014

Data expressed as mean and SEM, $n=3$ (two-way ANOVA); mean values in the same column with different superscripts differ significantly. Significance of mean value was observed at 5% probability level ($P < 0.05$)

Digestive enzymes

Protease activity

Stress and nutraceutical level had a significant interaction effect on protease activity among the groups throughout the 4 months of the experiment, but the main effect of stress was observed only at the 2nd and 4th months of the experiment (Table 3). Stress exposure to fish significantly reduced the protease activity in the 2nd and 4th months. The main effect of dietary nutraceutical on protease activity was observed in the 2nd, 3rd, and 4th months of the experiment (Table 2) and the fish of 0.1% nutraceutical-fed group showed a significantly higher ($p < 0.05$) protease activity within these periods. One-way ANOVA analysis indicated that protease activity was significantly low in THR0 groups at the 2nd, 3rd, and 4th months of stress exposure. THR1 and THR5 showed a significantly higher protease activity in the 2nd and 3rd months; however, only THR1 showed a similar value like non-stress-exposed groups in the 4th month period.

Lipase activity

Two-way ANOVA analysis indicated that stress exposure had a significant effect on the lipase activity in all 4 months of the experiment while the effect of nutraceutical, and the interaction of stress and nutraceutical were found only in the 1st month of the experiment (Table 4). Stress caused a reduction in lipase activity in rohu in the 1st, 3rd, and 4th months of experiment, whereas at the 2nd month, the activity increased. The one-way ANOVA analysis indicated that the activity of lipase in stress-exposed experimental groups (THR0, THR1, and THR5) was considerably reduced in the 1st, 3rd, and 4th months. THR0 showed a lower ($p < 0.05$) activity in the 4th month.

Amylase activity

The effect of stress and interaction effect of both stress and nutraceutical on amylase activity in rohu varied significantly during all the 4 months of the experiment, while the individual effect of nutraceutical on amylase activity was observed in the 2nd, 3rd, and 4th months of the experiment (Table 5). During all 4 months, stress showed a significant reduction ($p < 0.05$) in the amylase activity. Feeding of dietary nutraceutical at 0.1% level improved the activity of amylase in the

Table 3 Effect of duration of stress (high stocking density and feed restriction) and levels of nutraceuticals on protease activity in rohu, *Labeo rohita*, fingerlings

One-way ANOVA	Protease (Units/mg protein) ²			
	1st month	2nd month	3rd month	4th month
Treatments ¹				
TNS0	5.47 ^b ± 0.17	13.22 ^a ± 0.01	12.39 ^b ± 0.29	7.94 ^a ± 0.07
TNS1	6.58 ^b ± 0.07	14.56 ^a ± 0.22	12.46 ^b ± 0.09	8.13 ^a ± 0.01
TNS5	7.21 ^{ab} ± 1.43	15.58 ^a ± 0.97	12.92 ^b ± 0.75	7.01 ^a ± 0.08
THR0	10.19 ^a ± 1.48	8.68 ^c ± 0.18	7.81 ^c ± 0.05	6.09 ^b ± 0.01
THR1	4.05 ^b ± 0.90	12.68 ^b ± 0.29	15.19 ^a ± 0.02	7.82 ^a ± 0.007
THR5	7.23 ^{ab} ± 1.04	10.29 ^b ± 0.36	14.68 ^a ± 0.32	6.60 ^b ± 0.39
P value	0.021	0.016	0.005	<0.001
Effect of stress (two- way ANOVA)				
Without stress	6.42	14.45 ^a	12.59	11.54 ^a
With stress	7.16	10.55 ^b	12.56	6.84 ^b
SEM	0.59	0.26	0.21	0.10
p value	0.386	<0.001	0.065	<0.001
Effect of nutraceutical level				
0.0	7.83		10.95 ^c	10.14 ^c
0.1	5.32		13.62 ^a	14.06 ^a
0.5	7.22		12.94 ^b	13.53 ^b
SEM	0.72		0.32	0.25
p value	0.101		0.001	<0.001
Effect of stress nutraceutical*				
p value	0.024		0.01	0.016
				<0.001

Data expressed as mean ± SE, *n* = 3 (one-way ANOVA) and mean and SEM, *n* = 3 (two-way ANOVA); mean values in the same column with different superscripts differ significantly. Significance of mean value was observed at 5% probability level (*P* < 0.05)

*TNS0, normal stocking density, satiation feeding, 0% nutraceutical; TNS1, normal stocking density, satiation feeding, 0.1% nutraceutical; TNS5, normal stocking density, satiation feeding, 0.5% nutraceutical; THR0, high stocking density, restricted feeding, 0% nutraceutical; THR1, high stocking density, restricted feeding, 0.1% nutraceutical; THR5, high stocking density, restricted feeding, 0.5% nutraceutical

²Protease activity is expressed as units/milligram of protein

3rd and 4th months of the experiment compared to the fish of all other groups. One-way ANOVA showed that among the fish of stress-exposed and nutraceutical-fed groups, the fish of THR1 groups showed increased amylase activity in the 2nd, 3rd, and 4th months, whereas the fish of THR5 showed a similar activity with that of THR1 in the 4th month of the experiment.

Metabolic enzyme activities in the tissue

Aspartate aminotransferase and alanine aminotransferase

AST activity in the liver significantly increased in first 3 months and decreased in the 4th month of the experiment in stress-exposed groups (Table 6). Similarly, liver AST activity increased in the nutraceutical-fed groups in the 2nd and 4th months, although the fish of 0.1% nutraceutical-fed group showed lower value

of AST activity in the 1st month of the experiment. In the 3rd month, there was no significant effect on liver AST activity in fish due to feeding of nutraceutical. A significantly higher (*p* < 0.05) activity was observed in liver AST enzyme of THR0 group during the 1st and 2nd months, whereas both THR0 and THR1 groups registered lower AST activity in the 4th month of the experiment (Table 5).

Liver ALT activity of stress-exposed groups was significantly increased in first 3 months of the experiment (Table 5). Hepatic ALT activity was significantly reduced in nutraceutical-fed groups and feeding of 0.1% nutraceutical exhibited the lowest activity of liver ALT in fish at the 1st, 2nd, and 3rd months of the experiment. After one-way ANOVA analysis, it was observed that THR0 group exhibited a significantly higher ALT activity in the liver of fish in the 1st, 2nd, 3rd, and 4th months.

Table 4 Effect of duration of stress (high stocking density and feed restriction) and levels of nutraceutical on lipase activity in rohu, *Labeo rohita*, fingerlings

One-way ANOVA	Lipase (Units/mg protein)			
	1st month	2nd month	3rd month	4th month
Treatments ¹				
TNS0	30.85 ^a ± 4.62	35.17 ^b ± 4.90	36.21 ^a ± 8.63	36.44 ^a ± 3.69
TNS1	20.46 ^a ± 0.44	34.47 ^b ± 8.32	34.66 ^a ± 4.40	41.00 ^a ± 4.40
TNS5	20.33 ^a ± 0.46	31.31 ^b ± 3.80	32.16 ^a ± 8.29	53.49 ^a ± 8.29
THR0	15.37 ^b ± 0.40	41.26 ^a ± 5.20	13.17 ^b ± 4.28	9.73 ^c ± 3.31
THR1	15.43 ^b ± 0.25	40.74 ^a ± 13.1	13.23 ^b ± 10.17	13.23 ^b ± 5.16
THR5	17.15 ^b ± 1.27	34.29 ^b ± 9.51	15.50 ^b ± 7.53	11.50 ^b ± 7.46
p value	0.012	0.043	0.005	<0.001
Effect of stress (two-way ANOVA)				
Without stress	23.21 ^a	33.65 ^b	33.96 ^a	34.82 ^a
With stress	15.98 ^b	38.76 ^a	10.63 ^b	11.48 ^b
SEM	1.14	6.83	1.06	3.29
p value	<0.001	0.006	0.022	0.001
Effect of nutraceutical level				
0.0	23.11 ^a	38.21	24.69	23.09
0.1	17.95 ^b	37.60	23.94	27.12
0.5	17.74 ^b	57.80	23.83	32.50
SEM	1.40	8.37	2.35	2.03
p value	0.000	0.368	0.889	0.230
Effect of stress nutraceutical*				
p value	<0.001	0.676	0.855	0.509

Data expressed as mean ± SE, $n=3$ (one-way ANOVA), and mean and SEM, $n=3$ (two-way ANOVA); mean values in the same column with different superscripts differ significantly. Significance of mean value was observed at 5% probability level ($P < 0.05$)

*TNS0, normal stocking density, satiation feeding, 0% nutraceutical; TNS1, normal stocking density, satiation feeding, 0.1% nutraceutical; TNS5, normal stocking density, satiation feeding, 0.5% nutraceutical; THR0, high stocking density, restricted feeding, 0% nutraceutical; THR1, high stocking density, restricted feeding, 0.1% nutraceutical; THR5, high stocking density, restricted feeding, 0.5% nutraceutical

Lactate dehydrogenase and malate dehydrogenase

The main effect of stress and nutraceutical and interaction of stress and nutraceuticals on both hepatic and muscle LDH activity were significantly different in all the 4 months of the experiment (Table 7). Stress caused an increase in the activity of both hepatic and muscle LDH activity during all the 4 months. A significantly lower liver LDH activity was observed in 0.1% nutraceutical fed group in the 1st, 2nd, and 3rd months. However, feeding of nutraceutical (0.1%) showed a significantly lower muscle LDH only in the 2nd and 3rd months. Fish fed 0% nutraceutical and stress-exposed (THR0) showed significantly higher ($p < 0.05$) hepatic and muscular LDH activity during the experimental period (Table 7).

Stress caused a significant increase ($p < 0.05$) of liver MDH in rohu at the 1st, 2nd, and 3rd months of the experiment. Feeding of 0.1% nutraceutical caused a decrease in liver MDH activity in the 1st and 4th months while feeding

of 0.5% nutraceutical decreased only in the 1st month of the experiment. The interaction effect was observed during all the 4 months of the experiment (Table 8). From one-way ANOVA analysis, it was noticed that the THR0 showed an increase in hepatic MDH activity in the 1st and 2nd months, while a significantly lower ($p < 0.05$) activity was observed in the 3rd and 4th months of the experiment. The MDH activity was found to be higher in TNS5 groups compared to all non-stress-exposed groups in the 2nd, 3rd, and 4th months.

Stress showed a significant effect on muscle MDH activity in fish during each of all the 4 months of the experiment with a higher ($p < 0.05$) value in the 1st and 4th months and lower value in the 2nd and 3rd months of the experiment (Table 8). The level of dietary nutraceutical showed a significant effect on muscle MDH activity in fish at the 1st, 2nd, and 3rd months. The interaction between stress and nutraceutical showed a significant ($p < 0.05$) effect on muscle MDH activity in fish in the first

Table 5 Effect of duration of stress (high stocking density and feed restriction) and levels of nutraceuticals on amylase in rohu, *Labeo rohita*, fingerling

One-way ANOVA	Amylase (nanomoles of maltose released/min/mg protein)			
	1st month	2nd month	3rd month	4th month
Treatments ¹				
TNS0	0.86 ^a ± 0.17	0.34 ^{ab} ± 0.001	0.33 ^b ± 0.004	0.16 ^a ± 0.011
TNS1	0.38 ^b ± 0.12	0.39 ^a ± 0.008	0.41 ^a ± 0.007	0.17 ^a ± 0.009
TNS5	0.33 ^b ± 0.05	0.33 ^a ± 0.02	0.15 ^{bc} ± 0.023	0.06 ^b ± 0.003
THR0	0.20 ^b ± 0.03	0.41 ^a ± 0.004	0.11 ^c ± 0.014	0.05 ^b ± 0.020
THR1	0.39 ^b ± 0.08	0.60 ^a ± 0.006	0.41 ^a ± 0.004	0.11 ^a ± 0.016
THR5	0.37 ^b ± 0.05	0.16 ^b ± 0.012	0.06 ^c ± 0.002	0.17 ^a ± 0.034
P-value	0.006	0.014	0.021	0.008
Effect of stress (two-way ANOVA)				
Without stress		0.53 ^a	0.35 ^a	0.30 ^a
With stress		0.32 ^b	0.25 ^b	0.19 ^b
SEM		0.05	0.008	0.007
p value		0.018	<0.001	0.001
Effect of nutraceutical level				
0.0	0.53	0.37 ^a	0.22 ^b	0.11 ^b
0.1	0.39	0.22 ^b	0.41 ^a	0.14 ^a
0.5	0.35	0.24 ^b	0.11 ^c	0.11 ^b
SEM	0.46	0.07	0.01	0.008
p value	0.298	0.001	<0.001	0.038
Effect of stress nutraceutical*				
p value	0.016	<0.001	0.001	0.001

Data expressed as mean ± SE, *n* = 3 (one-way ANOVA), and mean and SEM, *n* = 3 (two-way ANOVA); mean values in the same column with different superscripts differ significantly. Significance of mean value was observed at 5% probability level (*P* < 0.05)

*TNS0, normal stocking density, satiation feeding, 0% nutraceutical; TNS1, normal stocking density, satiation feeding, 0.1% nutraceutical; TNS5, normal stocking density, satiation feeding, 0.5% nutraceutical; THR0, high stocking density, restricted feeding, 0% nutraceutical; THR1, high stocking density, restricted feeding, 0.1% nutraceutical; THR5, high stocking density, restricted feeding, 0.5% nutraceutical

3 months of experiment (Table 8). THR0 showed a significantly higher value in the 2nd month of the experiment.

Glutathione peroxidase activity

Stress exposure increased the activity of GPx enzyme in serum throughout the experimental period (Table 9). However, dietary nutraceutical had significant (*p* < 0.05) effect only in the 3rd and 4th months. Feeding of 0.5% nutraceutical enhanced the GPx activity while 0.1% nutraceutical decreased the GPx activity in the 3rd and 4th months. The interaction effect on serum GPx activity significantly varied only in the 3rd and 4th months. The one-way ANOVA showed significantly higher serum GPx activity during all the 4 months in THR0 group, whereas THR5 and THR0 showed similar value (*p* > 0.05) in the 2nd, 3rd, and 4th months (Fig. 2). However, THR1 showed a significantly lower value in all the 4 months compared to other stress-exposed groups. The

TNS5 also showed a significantly (*p* < 0.05) higher value in the 3rd and 4th months compared to TNS0 and TNS1 in the non-stress groups.

Glucose-6-phosphate dehydrogenase

The G6PDH activity in liver was significantly reduced by stress exposure throughout the experiment (Table 9). Feeding of 0.1% nutraceutical increased the enzyme activity in the 3rd and 4th months (*p* < 0.05). Interaction effect on the hepatic G6PDH in rohu was significantly different throughout the 4 months of the experiment. One-way ANOVA revealed that hepatic G6PDH activity was significantly (*p* < 0.05) higher in the TNS0 and TNS1 groups throughout the experimental period (Fig. 3). However, the THR0 groups showed a significantly higher activity in the 1st and 2nd months,

Table 6 Effect of stress (high stocking density and feed restriction) and nutraceutical and the interaction of both on the activity of AST and ALT in liver of rohu, *Labeo rohita*, fingerlings

One-way ANOVA	AST ² (nanomoles of sodium pyruvate released/min/mg protein) (L)				ALT ² (nanomoles of oxaloacetate released/min/mg protein) (L)			
	1st month	2nd month	3rd month	4th month	1st month	2nd month	3rd month	4th month
Treatments ¹								
TNS0	12.48 ^c ± 0.39	8.18 ^c ± 0.63	10.83 ^b ± 0.17	8.82 ^b ± 0.32	4.49 ^c ± 0.97	2.62 ^c ± 0.12	2.75 ^b ± 0.83	4.66 ^b ± 0.16
TNS1	12.90 ^c ± 0.46	13.60 ^{bc} ± 0.33	9.98 ^b ± 0.14	16.12 ^a ± 0.96	4.88 ^c ± 0.97	1.80 ^c ± 0.10	1.89 ^c ± 0.29	5.05 ^b ± 0.16
TNS5	17.03 ^{bc} ± 0.44	11.19 ^c ± 0.04	11.53 ^{ab} ± 0.63	13.10 ^a ± 1.25	5.16 ^{bc} ± 0.43	2.44 ^c ± 0.27	3.92 ^b ± 0.90	11.98 ^a ± 0.13
THR0	26.39 ^a ± 0.83	18.93 ^a ± 0.24	12.58 ^a ± 1.42	5.57 ^c ± 0.39	13.76 ^a ± 0.08	6.67 ^a ± 0.87	11.04 ^a ± 0.62	10.25 ^a ± 0.08
THR1	16.32 ^{bc} ± 0.74	16.76 ^b ± 0.25	12.59 ^a ± 0.48	6.17 ^c ± 0.18	9.98 ^{bc} ± 1.05	1.55 ^c ± 0.83	2.63 ^b ± 1.73	6.85 ^b ± 0.14
THR5	19.57 ^b ± 0.48	15.09 ^b ± 0.03	12.40 ^a ± 1.10	7.45 ^b ± 0.47	12.14 ^b ± 1.94	4.64 ^b ± 0.12	2.49 ^b ± 1.70	3.99 ^b ± 0.04
P value	0.011	0.004	0.015	0.021	0.002	<0.001	0.032	0.017
Effect of stress (two-way ANOVA)								
Without stress	14.14 ^b	11.66 ^b	10.78 ^b	12.68 ^a	4.84 ^b	2.29 ^b	2.85 ^b	7.90
With stress	20.76 ^a	16.93 ^a	12.52 ^a	6.39 ^b	9.51 ^a	3.95 ^a	5.39 ^a	7.03
SEM	0.62	0.30	0.66	0.07	0.34	0.19	0.47	0.41
p value	0.003	0.005	0.006	<0.001	0.002	<0.001	0.002	0.159
Effect of nutraceutical level								
0.0	19.44 ^a	13.55 ^b	11.70	7.18 ^b	9.13 ^a	4.15 ^a	6.89 ^a	7.45
0.1	14.61 ^b	15.18 ^a	11.28	11.14 ^a	7.43 ^c	1.68 ^c	2.26 ^c	6.95
0.5	18.30 ^a	14.14 ^a	11.97	10.25 ^a	8.65 ^b	3.54 ^b	3.21 ^b	7.99
SEM	0.76	0.36	0.81	0.09	0.41	0.23	0.57	0.50
p value	0.002	0.023	0.07	0.001	<0.001	0.002	0.004	0.368
Effect of stress nutraceutical*								
p value	<0.001	0.002	0.057	<0.001	0.492	0.001	0.004	<0.001

Data expressed as mean ± SE, *n* = 3 (one-way ANOVA), and mean and SEM, *n* = 3 (two-way ANOVA); mean values in the same column with different superscripts differ significantly. Significance of mean value was observed at 5% probability level (*P* < 0.05)

L, liver

*TNS0, normal stocking density, satiation feeding, 0% nutraceutical; TNS1, normal stocking density, satiation feeding, 0.1% nutraceutical; TNS5, normal stocking density, satiation feeding, 0.5% nutraceutical; THR0, high stocking density, restricted feeding, 0% nutraceutical; THR1, high stocking density, restricted feeding, 0.1% nutraceutical; THR5, high stocking density, restricted feeding, 0.5% nutraceutical

but decreased significantly (*p* < 0.05) as the feeding progresses to the 3rd and 4th months.

Discussion

Enhancing aquaculture production from the existing level has become a very big challenge as demand for fish consumption is continuously increasing across the world (FAO 2016). Vertical expansion of aquaculture is considered the best possible strategy in this regard, and hence, stocking of fish at a higher rate is inevitable. Crowding stress and feed restriction are found to be common stressors in aquaculture (Wedemeyer 1998), and therefore, it is essential to study the combined effect of these stressors on various digestive and metabolic enzymes, which play

a major role in nutrient utilization and growth of fish, and find out an effective strategy to mitigate the stress through the feeding of nutraceutical conglomerates.

Weight gain and digestive enzymes

In this study, the reduced growth in stress-exposed groups might be due to crowding and feed restriction. Crowding might be resulting in competition for food and which was aggravated due to diet restriction. Das et al. (2015) reported that exposure to feed restriction or high stocking density or a combination of both the stressors can result in growth retardation in fish. Feeding of nutraceutical at 0.1% level improved the weight gain both in stress-exposed and non-stress-exposed groups from the 3rd month onwards. This might be due to the stress mitigation and growth-promoting

Table 7 Effect of stress (high stocking density and feed restriction) and nutraceuticals and the interaction between both of them on the enzymes of lactate dehydrogenase (LDH) in rohu, *Labeo rohita*, fingerlings

One-way ANOVA	Lactate dehydrogenase, LDH ² (L)				Lactate dehydrogenase, LDH ² (M)			
	1st month	2nd month	3rd month	4th month	1st month	2nd month	3rd month	4th month
Treatments ¹								
TNS0	2.10 ^b ± 0.02	1.95 ^c ± 0.20	2.08 ^c ± 0.10	1.44 ^d ± 0.10	5.07 ^b ± 0.13	3.38 ^c ± 0.08	3.12 ^c ± 0.13	5.11 ^c ± 0.42
TNS1	1.02 ^c ± 0.02	1.22 ^c ± 0.07	0.74 ^d ± 0.07	3.78 ^b ± 0.24	9.84 ^b ± 0.67	3.72 ^c ± 0.36	3.49 ^c ± 0.21	6.63 ^c ± 1.24
TNS5	1.73 ^b ± 0.25	4.32 ^b ± 0.73	2.50 ^c ± 0.26	3.08 ^{bc} ± 0.09	6.48 ^b ± 0.36	3.92 ^c ± 0.07	6.26 ^{bc} ± 0.36	9.85 ^b ± 1.32
THR0	3.17 ^a ± 0.38	7.10 ^a ± 0.85	6.61 ^a ± 0.22	4.93 ^a ± 0.45	13.15 ^a ± 0.15	26.93 ^a ± 0.33	24.89 ^a ± 0.62	10.20 ^{ab} ± 1.44
THR1	2.21 ^b ± 0.15	4.90 ^b ± 0.67	2.89 ^c ± 0.06	3.64 ^b ± 0.35	17.29 ^a ± 0.43	11.35 ^b ± 0.31	16.68 ^b ± 2.76	23.85 ^a ± 2.23
THR5	1.84 ^b ± 0.13	3.94 ^b ± 0.03	5.12 ^b ± 0.75	2.69 ^c ± 0.22	14.30 ^a ± 0.26	38.46 ^a ± 0.28	23.07 ^a ± 0.26	19.49 ^a ± 2.43
<i>p</i> value	< 0.001	0.010	0.001	0.032	0.032	< 0.001	0.001	0.048
Effect of stress (two-way ANOVA)								
Without stress								
With stress	1.61 ^b	2.49 ^b	1.77 ^b	2.76 ^b	7.13 ^b	10.34 ^b	4.29 ^b	7.19 ^b
SEM	2.40 ^a	5.31 ^a	4.88 ^a	3.75 ^a	14.91 ^a	32.25 ^a	15.44 ^a	17.85 ^a
<i>p</i> value	0.118	0.313	0.197	0.158	0.513	1.284	0.319	0.538
Effect of nutraceutical level	< 0.001	0.002	0.004	< 0.001	0.002	0.007	0.008	< 0.001
0.0	2.63 ^a	4.53 ^a	4.35 ^a	3.19 ^{ab}	9.11 ^b	15.15 ^b	13.99 ^a	7.66 ^b
0.1	1.61 ^b	3.06 ^b	1.82 ^b	3.71 ^a	13.57 ^a	7.54 ^c	10.08 ^b	15.24 ^a
0.5	1.78 ^b	4.13 ^a	3.81 ^a	2.89 ^b	10.39 ^{ab}	21.19 ^a	5.51 ^c	16.68 ^a
SEM	0.145	0.383	0.241	0.194	0.628	1.573	0.391	0.659
<i>p</i> value	0.001	0.049	< 0.001	0.032	0.001	< 0.001	0.007	0.01
Effect of stress nutraceutical*								
<i>p</i> value	0.042	0.001	0.01	< 0.001	0.940	0.002	0.005	< 0.001

Data expressed as mean ± SE, *n* = 3 (one-way ANOVA), and mean and SEM, *n* = 3 (two-way ANOVA); mean values in the same column with different superscripts differ significantly. Significance of mean value was observed at 5% probability level (*P* < 0.05)

L, liver; M, muscle

*TNS0, normal stocking density, satiation feeding, 0% nutraceutical; TNS1, normal stocking density, satiation feeding, 0.1% nutraceutical; TNS5, normal stocking density, satiation feeding, 0.5% nutraceutical; THR0, high stocking density, restricted feeding, 0% nutraceutical; THR1, high stocking density, restricted feeding, 0.1% nutraceutical; THR5, high stocking density, restricted feeding, 0.5% nutraceutical

²units/milligram of protein/minute

Table 8 Effect of stress (high stocking density and feed restriction) and level of nutraceuticals and the interaction between both of them on the enzymes of malate dehydrogenase (MDH) in rohu, *Labeo rohita*, fingerlings

One-way ANOVA	Malate dehydrogenase, MDH ² (L)				Malate dehydrogenase, MDH ² (M)			
	1st month	2nd month	3rd month	4th month	1st month	2nd month	3rd month	4th month
Treatments ¹								
TNS0	2.81 ^b ± 0.45	2.33 ^d ± 0.48	5.50 ^c ± 0.50	5.57 ^d ± 0.07	3.03 ^b ± 0.50	3.58 ^b ± 0.20	5.33 ^b ± 0.37	4.08 ± 0.43
TNS1	1.62 ^{bc} ± 0.13	2.31 ^d ± 0.24	3.33 ^c ± 0.34	6.72 ^d ± 0.83	3.66 ^b ± 0.67	2.20 ^b ± 0.80	7.71 ^{ab} ± 0.45	9.85 ± 0.88
TNS5	0.98 ^c ± 0.16	3.70 ^c ± 0.35	11.00 ^b ± 0.90	17.66 ^a ± 1.45	3.27 ^b ± 0.24	1.17 ^c ± 0.24	15.70 ^a ± 0.77	15.25 ± 1.24
THR0	6.57 ^a ± 0.68	6.24 ^a ± 0.35	3.38 ^c ± 0.24	10.72 ^c ± 0.77	8.07 ^a ± 1.43	10.64 ^a ± 3.03	3.51 ^b ± 0.37	14.51 ± 0.33
THR1	1.44 ^c ± 0.18	4.75 ^b ± 0.07	15.71 ^a ± 1.10	9.34 ^c ± 0.12	6.58 ^{ab} ± 0.87	2.54 ^b ± 0.21	10.88 ^a ± 0.57	15.59 ± 1.25
THR5	1.95 ^{bc} ± 0.46	4.67 ^b ± 0.21	12.27 ^b ± 0.08	13.74 ^b ± 0.55	10.55 ^a ± 1.08	2.73 ^b ± 1.83	4.75 ^b ± 0.67	16.06 ± 1.01
P value	0.005	<0.001	0.012	<0.001	0.008	0.015	<0.001	0.086
Effect of stress (two-way ANOVA)								
Without stress	1.80 ^b	2.78 ^b	6.61 ^b	9.98	3.32 ^b	6.96 ^a	9.58 ^a	9.72 ^b
With stress	3.32 ^a	5.22 ^a	10.46 ^a	11.27	12.6 ^a	4.63 ^b	6.38 ^b	15.38 ^a
SEM	0.23	0.18	0.37	0.455	0.22	0.15	0.91	1.45
p value	0.005	0.001	<0.001	0.069	0.001	0.010	0.005	<0.001
Effect of nutraceutical level								
0.0	4.69 ^a	4.28	4.44 ^b	8.15 ^b	5.55 ^b	7.11 ^a	4.42 ^c	9.83
0.1	1.53 ^b	3.53	9.52 ^a	8.03 ^b	5.12 ^c	1.00 ^b	9.30 ^b	12.72
0.5	1.46 ^b	4.19	11.64 ^a	15.70 ^a	6.91 ^a	1.45 ^b	10.23 ^a	15.65
SEM	0.28	0.22	0.45	0.56	0.27	0.19	1.11	1.77
p value	<0.001	0.066	0.008	0.004	0.003	<0.001	0.002	0.065
Effect of stress nutraceutical*								
p value	0.001	0.002	<0.001	<0.001	0.002	0.024	0.004	0.080

Data expressed as mean ± SE, $n=3$ (one-way ANOVA), and mean and SEM, $n=3$ (two-way ANOVA); mean values in the same column with different superscripts differ significantly. Significance of mean value was observed at 5% probability level ($P<0.05$)

L, liver; M, muscle

*TNS0, normal stocking density, satiation feeding, 0% nutraceutical; TNS1, normal stocking density, satiation feeding, 0.1% nutraceutical; TNS5, normal stocking density, satiation feeding, 0.5% nutraceutical; THR0, high stocking density, restricted feeding, 0% nutraceutical; THR1, high stocking density, restricted feeding, 0.1% nutraceutical; THR5, high stocking density, restricted feeding, 0.5% nutraceutical

²units/milligram of protein/minute

ability of zinc, selenium, methionine, and vitamin E. Similar effects of these nutraceuticals are observed individually in various species (Tocher et al., 2002; Frassinetti et al. 2006; Shan et al., 2017). Our earlier study also showed a higher weight gain percentage and protein efficiency ratio in 0.1% nutraceutical-fed groups (Nazeemashahul et al. 2020).

Variation in activity of digestive enzymes to duration of feed restriction may be due to the conservation and mobilization of nutrients according to the requirement during the stress (Harms et al. 1991; Johnston et al. 2004). In this study, the effect of stress on amylase and lipase activity was observed in all the 4 months; however, the protease activity was affected only in the 2nd and 4th months. There are reports indicating that starvation reduces the activity of digestive enzymes in Atlantic salmon (Krogdahl and Bakke-McKellep 2005), which could be due to a low level of required substrate since the concentration of substrate

determines the activity of enzymes. During diet restriction, the availability of substrate is limited, thereby causing a reduction in digestive enzyme activity. Similar to our results, Belanger et al. (2002) also reported lower digestive enzyme activity in starved Atlantic cod. The trypsin and lipase activity in sturgeon and rainbow trout were found to be significantly lower after starvation (Furné et al. 2008).

The significantly lower protease activity observed in the restricted feeding groups (THR) compared to their satiation feeding counterpart during the 2nd and 4th months might be due to the low level of substrate available to restricted fed group. This result agrees with the observation of Abolfathi et al. (2012), who reported a decreased protease activity in *Rutilus rutilus* subjected to restricted feeding. The significantly higher protease activity observed in THR0 in the 1st month might be due to compensatory increase of enzyme activity to supply more nutrients during early feed restriction period.

Table 9 Effect of stress (high stocking density and feed restriction), level of nutraceutical, and the interaction between both serum GPx and G6PDH enzyme activities in rohu, *Labeo rohita*, fingerlings

Two-way ANOVA	Serum GPx ¹				Glucose-6-phosphate dehydrogenase (L) (G6PDH) ²			
Treatments	1st month	2nd month	3rd month	4th month	1st month	2nd month	3rd month	4th month
Effect of stress								
Without stress	6.14 ^b	6.84 ^b	8.87 ^b	9.29 ^b	2.38 ^a	2.49 ^a	2.43 ^a	2.20 ^a
With stress	16.70 ^a	19.84 ^a	16.11 ^a	15.48 ^a	1.87 ^b	2.00 ^b	1.37 ^b	1.30 ^b
SEM	0.75	0.92	1.68	0.42	0.097	0.054	0.005	0.047
<i>p</i> value	0.000	0.001	0.002	0.002	<0.001	0.002	<0.001	0.003
Effect of nutraceutical level								
0.0	13.24	14.50	12.59 ^b	12.61 ^b	2.15	2.38	1.68 ^b	1.68 ^b
0.1	10.77	12.01	10.26 ^c	10.45 ^c	2.20	2.26	2.05 ^a	1.99 ^a
0.5	10.26	13.51	14.63 ^a	14.10 ^a	2.04	2.11	1.97 ^{ab}	1.59 ^b
SEM	0.92	1.13	2.05	0.51	0.119	0.067	0.006	0.058
<i>p</i> value	0.062	0.761	0.018	0.014	0.301	0.470	0.004	0.003
Effect stress nutraceutical*								
<i>p</i> value	0.225	0.912	0.031	0.021	<0.001	0.004	0.004	0.001

Data expressed as mean and SEM, $n=3$ (two-way ANOVA); mean values in the same column with different superscripts differ significantly. Significance of mean value was observed at 5% probability level ($P<0.05$)

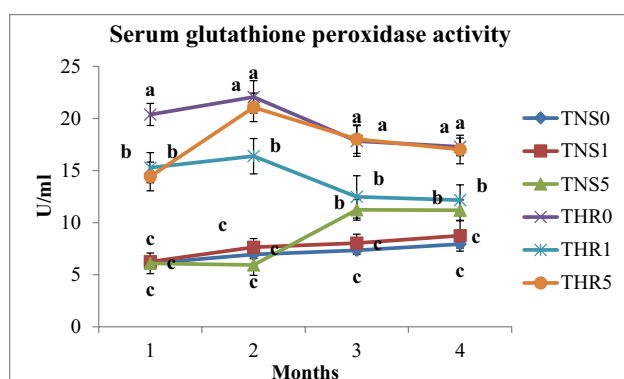


Fig. 2 Serum GPx activity in *Labeo rohita* fingerlings of different treatments during different experimental period with or without stress exposure; TNS0, normal stocking density, satiation feeding, 0% nutraceutical; TNS1, normal stocking density, satiation feeding, 0.1% nutraceutical; TNS5, normal stocking density, satiation feeding, 0.5% nutraceutical; THR0, high stocking density, restricted feeding, 0% nutraceutical; THR1, high stocking density, restricted feeding, 0.1% nutraceutical; and THR5, high stocking density, restricted feeding, 0.5% nutraceutical

Stress caused a significant reduction in the amylase activity in all the 1st, 3rd, and 4th months, and this may be due to change in carbohydrate utilization during stress as suggested by Eroldogan et al. (2008). The stress-exposed groups showed 2.7 times lower amylase activity than the control group in the 3rd month. The increase in amylase activity during the 2nd month in the THR0 group could be due to increased effort of the fish to utilize the glycogen

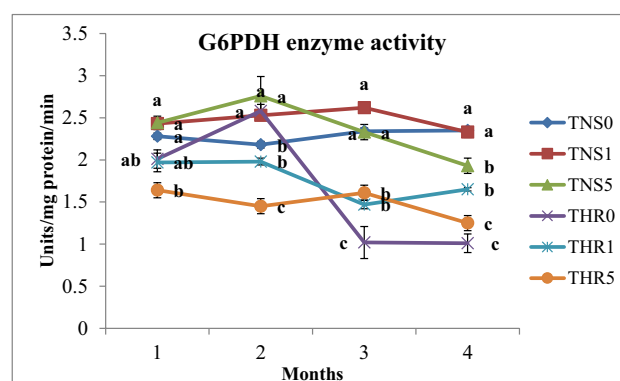


Fig. 3 Hepatic glucose-6-phosphate dehydrogenase (G6PDH) enzyme activity in *Labeo rohita* fingerlings of different treatments during different experimental period with or without stress exposure; TNS0, normal stocking density, satiation feeding, 0% nutraceutical; TNS1, normal stocking density, satiation feeding, 0.1% nutraceutical; TNS5, normal stocking density, satiation feeding, 0.5% nutraceutical; THR0, high stocking density, restricted feeding, 0% nutraceutical; THR1, high stocking density, restricted feeding, 0.1% nutraceutical; and THR5, high stocking density, restricted feeding, 0.5% nutraceutical

mobilized from tissues. This assertion is in congruent with the submission of Sangiao-Alvarellos et al. (2005) who observed that the capacity to export glucose derived from stored liver glycogen considerably increased in gilthead sea bream when subjected to 2-week food deprivation.

The increased activity of protease observed in the 2nd, 3rd, and 4th months in 0.1% nutraceutical-fed group was probably due to increased utilization of proteins present in the diet as the nutraceutical reduced the stress effects in this

group. 0.1% nutraceutical groups had 2.5% extra zinc than the requirement, and this might be the reason for improved amylase activity in the 3rd and 4th months compared to all other groups. In bacteria, the stabilization of lipase and amylase is facilitated by zinc (Alexei et al. 2002; Choi et al. 2005). Furthermore, Tan et al. (2011) reported that supplementation of zinc enhanced lipase and amylase activity in Jian carp. These findings opined that zinc plays an important role in the intestinal enzyme activity in fish; but, the mechanism of action needs more investigation. There are no literatures available on the effect of other compounds on amylase activity in fish.

Alanine amino transferase and aspartate amino transferase

Aspartate aminotransferase (AST) and alanine amino transferase (ALT) are the mostly studied transaminases whose activities increase during an elevated protein catabolism and gluconeogenesis (Das 2002). Such elevated levels were observed in different animals exposed to higher levels of pesticides (Gupta and Paul 1978; Kumar and Muni 1997), pollutants like nitrite (Ciji et al. 2012) etc. In addition, Tok et al. (2016) had also reported that when there is an availability of dietary amino acids, synthesis of new protein from these amino acids is also indicated by transaminases. In the present study, the transaminase activities were significantly higher in the THR0 group indicating that the protein catabolism was higher in these groups to produce the citric acid cycle intermediate for ATP production to fulfill the energy requirement of the fish exposed to stress. Excessive protein intake, starvation, or stress cause increased catabolism of amino acids for energy production through the energy generation-pathway (Kerret al. 2014). Similar observations were also made in different fish species subjected to food deprivation or confinement/crowding stress (Sanchez-Muros et al. 1998; Vijayan et al. 1997; Tejpal et al. 2009).

Lactate dehydrogenase and malate dehydrogenase activity

As a terminal enzyme of glycolysis, LDH is responsible for the reverse conversion of pyruvate to lactate which is present in almost all tissues. During stress, the LDH activity gets upregulated (Semenza et al. 1996). The presence of oxygen ensures the entry of pyruvate to Krebs's cycle, but its absence leads to its conversion to lactate (Murray et al. 2000). Lactate is a substrate for gluconeogenesis in the liver during anaerobic metabolism to supply the energy (Wright et al. 1989), which is mediated by cortisol and catecholamine (Vijayan et al. 1997). It is demonstrated that LDH activity gets elevated during crowding stress (Tejpal et al., 2009) and diet restriction stress (Vijayaraghavan and Rao 1986).

The current study observed higher LDH activity in the non-nutraceutical-fed stress-exposed group (THR0). This suggests that during feed restriction, the activity of LDH enzyme increases to convert lactate to glucose. The higher level of LDH observed in the muscle could also be due to the same reason. This has been reported as a survival strategy in anoxia-adapted fishes (goldfish) and hypoxia-resistant fish (common carp) as reported by Childress and Seibel (1998). Similarly, increased LDH activity was reported in yellow perch subjected to food deprivation (Foster and Moon 1991).

The enzyme MDH is involved in Krebs's cycle, which is important for energy production. Significantly higher MDH activity in the THR0 and THR5 groups suggested that the stress level in these groups were very high and the cycles were highly active to provide the extra energy required for stress mitigation. The lower LDH activity in 0.1 and 0.5% nutraceutical-fed groups with lower levels of other parameters related to stress suggests the effective amelioration of stress responses in these groups by the nutraceutical. Kumar et al. (2012) reported the ability of methyl donors to ameliorate the endosulfan-induced stress in rohu fingerlings by lowering the LDH and MDH activities.

Serum glutathione peroxidase and hepatic glucose-6-phosphate dehydrogenase activities

The pentose phosphate shunt is facilitated by G6PDH which liberates NADPH. NADPH plays a vital role in the regeneration of glutathione (reduced) and synthesis of fatty acids (Trenzado et al. 2009). Activity of G6PDH was significantly higher in control group in all the 4 months indicating an active pentose phosphate pathway (PPP), which supports fatty acid synthesis, nucleotide synthesis etc. The higher values of GPx activity in serum and higher activity of G6PDH in the liver in stress-exposed and non-nutraceutical-fed groups (THR0) in the first 2 months might be due to active PPP, and the GPx activity indicates that the NADPH synthesized in PPP were channeled for GSH regeneration from oxidized glutathione (GSSG), which is essential for the action of GPx enzyme. Reports are saying that moderate oxidative stress can result in an adaptive increase in the level of GSH and, hence, show an enhanced synthesis (Doyotte et al. 1997; Zhang et al. 2004). Pascual et al. (2003) reported a significantly lower GSH level in gilthead seabream exposed to absolute starvation compared to 2% diet-fed group, which supports the lower level of G6PDH activity in the 3rd and 4th months of stress-exposed group fed 0% nutraceutical in the present study. The enzymes involved in pentose phosphate pathway were reported to decrease in starved *Salvelinus saureus* (Meton et al. 1999), coho salmon (Lin et al. 1977), and *Salvelinus fontinalis* (Yamauchi et al. 1975). Several studies have also reported a decrease in G6PDH activity

during prolonged feed restriction (Hung et al. 1997; Shimeno et al. 1997).

The decreased G6PDH activities in nutraceutical-supplemented groups (both stress-exposed and normal stocking) indicate that the compounds (selenium, vitamin E, zinc, and methionine) are not driving the pentose phosphate shunt. De novo methionine biosynthesis uses 3 molecules of NADPH per molecule of methionine, and the pentose phosphate pathway is a key to supply of NADPH for this (Campbell et al. 2016). In the present study, external supplementation of methionine might have reduced this requirement and, hence, reduced the G6PDH activity. GSH is synthesized from cysteine, glycine, and glutamate, but cysteine is the major limiting amino acid for its synthesis (Wu et al. 2004). Moreover, the same trend observed in GPx and G6PDH activity in nutraceutical-fed stress-exposed groups, especially 0.1% level, in all the 4 months supports the effectiveness of the supplied molecules in mitigating oxidative stress in these groups. Therefore, the increased level of GSH against the excess ROS produced during stress in the 2nd and 3rd months 0.1% nutraceutical-fed group might have been mediated through enhanced level of cysteine synthesized from methionine supplemented at 25% extra level in the diet. However, supplementation of 46% extra methionine in diet did not give any extra benefit in relation to stress mitigation.

Hence, it can be concluded that the duration of multiple stress exposure adversely affected the weight gain; digestive and metabolic enzymes; and feeding of a mixture of zinc, selenium, vitamin E, and methionine at 0.1% level could enhance the activities of these enzymes in rohu fingerlings along with an improved weight gain.

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Data availability The data that support the findings of this study are available on request from the corresponding author.

Declarations

Ethics approval and animal rights The study was approved by the institute ethics committee, and the trial/sampling procedure was carried out following ethical standards. The manuscript does not contain clinical studies or patient data.

Conflict of interest The authors declare no competing interests.

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