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Haemato-Biochemical and Hepatic Changes in *Labeo rohita* Fingerlings Exposed to Multiple Stressors of Crowding and Feed Deprivation

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ABSTRACT

In the present study, we observed the effect of crowding and short term (48 h) feed deprivation in Labeo rohita fingerlings. A total of 150 fingerlings (9 ± 0.5g) were distributed in three treatment groups in triplicates. Group with normal stocking density (10 no/ 75L) and normal feeding (satiation) was referred as control (C) whereas a high stocking density (20 no/ 75L) and normal feeding was referred as T1 group. A high stocking density and absolute starvation group was considered as T2 group. Blood samples were collected at regular intervals from 3h, 9h, 24 h and 48 h, and tissue samples were collected at 48 h. Higher blood glucose level was recorded at 9 and 48 h in T2 and T1 group, respectively. Nitroblue tetrazolium assay value also followed similar trend like blood glucose after 9 h. Haemoglobin level was significantly higher in T1 (6.02 ± 0.30) and T2 (6.27±0.18) group after 48h compared to control (5.27 ± 0.13) .The RBC, glutathione-s-transferase (GST) and glutamate oxaloacetate transaminase (GOT) activity in the liver showed no significant difference among the groups. Activities of hepatic superoxide dismutase (SOD), catalase, glutamate pyruvate transaminase (GPT) and glucose-6- phosphate dehydrogenase (G6PDH) followed a similar trend with the highest activity of all these enzymes wererecorded in the liver of T1 group at 48h. But, T2 group showed highest (P<0.05) lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) activities in liver while there was no significant difference (P>0.05) in liver glutathione peroxidase activity between T1 and T2 group. A significantly higher (p<0.01) hepatic glutathione and malondialdehyde concentration were observed in T1 group.

***Key words:** Acute Stress, Stocking Density, Liver Enzymes, Serum Parameters, Feed Deprivation*

INTRODUCTION

Fish either from wild or cultured condition undergo short and long term stress[2] caused due to starvation, crowding, and or environmental stressors etc, which often result in several biochemical and physiological alteration in order to cope up the stress. But such metabolic modifications can affect the growth and reproductive capacity of the animal.

Increase in stocking density improves the total production and efficiency of the farm but, it can also cause stress and diseases in aquatic organism. Studies on crowding stress have mainly focussed on the chronic effect[46] on growth, survival, food intake and hormonal variation in fish[25]. However, high stocking density may cause stress at acute level, due to high oxygen demand and competition for food.

Rohu (*Labeo rohita*) is a widely cultured Indian major carp and to ensure the seed availability and getting the benefit of compensatory growth, farmers rear them at higher stocking density with restricted ration and according to Tejpal *et al*[39]., crowding is considered as a chronic stressor in rearing system of mrigal. But, crowding results in competition for food, space and oxygen. Crowding as well as starvation can cause increased energy demand and metabolic shifting in fish[5,44], thereby results in the mobilization of reserved energy in order to compensate the metabolic demand generated due to stress. This can lead to down regulation of enzymes of some of the metabolic pathways like lipogenesis and up-regulation of others like gluconeogenesis and pentose phosphate shunt[23,26]. Up-regulation of oxidative metabolic enzymes will result in continuous synthesis of reactive oxygen species triggering oxidative stress in the system. Eventhough, there is an antioxidant defence system in fish, but an increased production of reactive oxygen species (ROS) can reduce the efficiency of the cells to deactivate the reactive species. The free radical scavenging is done by enzymatic and non-enzymatic antioxidants present in fish. The enzymatic antioxidants like superoxide dismutase (SOD), glutathione peroxidases (GPx) and catalase are highly active during oxidative stress in many vertebrates[42] while there are other substances like vitamin A, E, C, glutathione, selenium, zinc etc to support the free radical scavenging process in cells.

The mechanism of stress response in fish has been studied using several biomarkers[4]. Cortisol levels are considered to be a major key response to stress in animals but a holistic study of antioxidant defence along with haematological parameters can give a clear picture of the stress response in fish, which can also help us to derive other biomarkers for multiple stresses. So, the present study is mainly aimed to understand the effect of multiple stressors on various haematological, biochemical and antioxidant enzyme parameters of *Labeo rohita.*

MATERIALS AND METHODS
Experimental animals
A total of 150 rohu (*Labeo rohita*) fingerlings of size 8-10g were procured from Prem fisheries, Ankaleswar, Gujarat, India, and left undisturbed overnight. Fish were reared with control diet (35% protein) for 14 days for acclimatization in the laboratory condition before commencing the experiment. The fish were distributed in three groups in triplicates as control (C) with normal stocking density (10 fish/ 75 l) and satiation feeding, high stocking density (20 fish /75 l) and satiation feeding (T1, crowding group) and high density and absolute starvation (T2, crowding-with-feed deprivation group). The experiment started at 9.00 am and continued for the next 48h. Samples were collected at 3h, 9h, 24h and 48h. There were additional fish kept in separate tanks with similar condition to avoid the sampling repetition. Round the clock aeration was provided in all the experimental tubs. Control and T1 groups were fed to satiation level twice daily with a purified diet containing 35% crude protein.

Water quality parameters
The experiment was conducted in 150 L capacity rectangular tubs (80 x 57 x 42 cm), and the water quality was monitored throughout the experimental period. The ammonia level was within the range of 0.09-0.05 mg/L while the hardness was within a range of 234-242 mg/L.

Sample preparation for analytical procedures
Blood and serum samples were collected at regular intervals of 3, 9, 24 and 48h without killing the fish so that the stocking density could maintain uniformly, while the tissue samples (liver and gill) were collected at the end of the experiment (48h). Six fish from each treatment were collected for blood and serum collection. EDTA coated vials were

used for blood collection from caudal vein without anesthetizing the fish, and with less handling time[31]. After termination of the experiment, tissue samples were collected from the respective groups after anesthetized in clove oil (50μl/L). Utmost care was taken to avoid handling stress to the fish. Serum was separated by centrifuging at 3000 rpm for 10 min at 4^0C. Tissue homogenates (5%) were prepared in 0.25M sucrose with the help of a mechanical tissue homogeniser (Miccra D-9, ART Prozess & Labortechnik, Germany), centrifuged at 5000 rpm for 10 min at 4° C (REMI CPR-24, India), and supernatants were collected and used for analysis.

Determination of blood and serumparameters

Total erythrocyte count (RBC) was done using the method of Hendrick[15] and the hemoglobin content of blood was analyzed using cyanmethemoglobin method[43] at Suburban diagnostic laboratory, Mumbai, India.

Blood glucose and Nitro blue tetrazolium (NBT) assay

Blood glucose was estimated[41] using Liquixx Glucose kit (Erba® Diagnostic Mannheim, Solan, HP, India).

Respiratory burst activity (NBT assay) was done by the method of Stasiak and Baumann[38]. Wells of a flat bottom micro titre plate were placed with 50μl of blood and incubated at 37^0C for 1 h to facilitate the adhesion of cells. In order to remove the non-adhesive cells, the wells were washed thrice with phosphate buffer saline. After washing, 100 μl of 0.2% NBT were added and incubated for 1 h. Methanol (100%) was used to fix the cells (2-3 min) and washed thrice with 70% methanol. The plates were air-dried and 120 μl of potassium hydroxide (KOH, 2N) and 140 μl of dimethyl sulphoxide (DMSO) were added to each well and mixed well. The OD of the turquoise blue coloured solution was then read in microplate (BioTek Power Wave 340, India) reader at 620 nm.

Serum protein, albumin, globulin and A/G ratio

Serum total protein was estimated according to biuret method[32] using Liquixx Total Protein kit (Erba® Diagnostic Mannheim, Transasia Bio-medicals Ltd, Solan, HP, India). Albumin was estimated by spectrophotometric method by measuring bromocresol green binding[8] using kit (Erba® Diagnostic Mannheim, Transasia Bio-medicals Ltd, Solan, HP, India). Globulin was calculated by subtracting albumin values from total serum protein, and A/G ratio calculated

Determination of TBARS and glutathione (GSH)

Thiobarbituric acid reacting substances were estimated as Malondialdehyde (MDA) content in the liver as described by Sharma and Krishnamurthy[35]. The OD was measured at 535nm and TBARS was expressed in nmoles of MDA/mg protein.

Reduced glutathione was estimated in liver tissue according to the method of Moron *et al*[27]. To a 500μl tissue homogenate, 125 μl of 25% TCA was added and kept in ice for 5 min. To the cooled mixture, 500 μl of 5% TCA was added and subjected to centrifugation (3000g, 5 min). From the supernatant collected 150 μl was mixed with 350 μl of 0.2M phosphate buffer (pH 8.0) and 0.6mM DTNB prepared in buffer (1ml). The yellow colour thus formed was measured against TCA containing blank at 412 nm. The reduced glutathione was measured using standard graph and expressed as n mol/g protein.

Enzyme assays

Liver and gill tissues were used to study the following enzyme activities conducted at 25 ^{o}C

Enzymatic antioxidants

The superoxide dismutase (SOD): SOD activity was assayed according to the method described by Misra and Fridovich[24] based on the oxidation of epinephrine–adrenochrome transition by the enzyme.Tissue homogenate (50μl) was taken in the cuvette and 1.5 ml 0.1M carbonate – bicarbonate buffer containing 57 mg/ dl EDTA (pH 10.2) and 0.5 ml epinephrine (3mM) was added and mixed well. Change in optical density was measuredin a Shimadzu – UV spectrophotometer at 480 nm for 3 min. SOD expressed as unit activity which gives amount of protein utilized for inhibition of epinephrine (50%).

Catalase: Catalase was assayed according to the method described by Takahara *et al.*, and the decrease in absorbance was measured at 240 nm at 15 sec intervals for 3 min. Blank was runwith 1.0 ml distilled water. Enzyme activity was expressed as nano moles H_2O_2 decomposed / min / mg protein.

Glutathione peroxidise (GPx) and Glutathione S transferases: GPxactivity was determined according to the method of Hafeman *et al*[13]., while GST activity was determined by the method of Habig *et al*[12].

Metabolic enzyme assay

Alanine aminotransferases (ALT) and Aspartate amino transferases (AST): The AST and ALT activity was assayed in different tissue homogenates as described by Wooten[47]. The reaction mixture consist of 0.2M D, L-aspartic acid and 2mM α-ketoglutarate in 0.05M phosphate buffer (pH 7.4). The reaction was started by adding enzyme and terminated after an incubation period of 60 min at 37^0C by adding 1mM 2, 4 - dinitrophenyl hydrazine (DNPH). In control tubes, the tissue homogenate was added after this step and incubate for 20 min at room temperature. Further, 0.4M NaOH solution (5ml) was added, mixed well and reading was taken at 540nm. The ALT was assayed using same protocol but the substrate used was L- alanine instead of aspartic acid.

Lactate dehydrogenase (LDH) and Malate dehydrogenase (MDH): The method described by Wroblieuski and Ladue[48] (1955) was used to assay LDH activity in tissues, while the MDH activity was assayed following the method of Ochoa. For both the reaction, a reaction mixture of 2.9 ml prepared out of 0.1M phosphate buffer (pH 7.5), 0.1ml of NADH solution (dissolve 2 mg NADH in 1ml buffer), 0.1ml of tissue homogenate and 0.1ml of sodium pyruvate. The substrate sodium pyruvate (0.1ml) is used by LDH and oxaloacetate by MDH. The OD was recorded at 340nm at 30 sec intervals for 3 min. The results were expressed as units/ mg protein/ min, where 1 unit was equal to Δ0.01OD/ min.

Glucose-6-phosphate Dehydrogenase (G6PDH): The G6PDH activity was assayed in liver by the method of De Moss[7] (1953). The total reaction volume was 3ml and comprised of 1.5 ml of 0.1M Tris buffer (pH 7.8), 0.2ml of 2.7mM NADP, 0.1ml of tissue homogenate, 1.05ml of distilled water and 0.1ml of 0.02M glucose-6-phosphate (G6P). The OD was recorded at a wavelength of 340nm for 3 min against distilled water and the activity was expressed as unit's/ mg protein/ min.

Statistical analysis

Data were statistically analysed using one-way analysis of variance (ANOVA - SPSS version 16) and significance differences between the means were determined by Duncan's multiple range tests. Comparisons were made at 5% probability level and data expressed as mean ± standard error.

RESULTS

Haematological parameters

Blood glucose level varied significantly ($P<0.05$) among the groups ($p<0.01$) only after 3 h. Highest level ($P<0.05$) of blood glucose was observed in T2 group at 9 and 24 h butT1 group exhibited highest value at 48h (Figure 1). Respiratory burst activity varied significantly among the groups in all the four sampling period. In 3h sampling, both T1 and T2 groups showed higher value than the control group while at 9 and 24 h T2 group showed a significantly higher value ($P<0.05$). During 48h sampling significantly higher ($p<0.01$) NBT was observed in T1 group, similar to glucose level (Figure1). RBC value showed no significant difference among the various groups. However, significantly higher haemoglobin level was observed in T2 group compared to other groups (Table 2).

Serum parameters

Serum total protein varied significantly among the groups in the 3h and 9h of sampling ($p<0.05$) but 24 and 48h sampling showed no significant difference among the groups. Albumin level in the serum was significantly higher in T1 group in 3h sampling. All other sampling time showed no significant variation among the groups. Serum globulin level was found to be significantly ($p<0.05$) lower in T1 group during 9h sampling, whereas 3, 24, 48h samples exhibited non-significant variation ($p>0.05$). No significant variation was

observed in the A/G ratio among the various groups.

Enzyme parameters

GOT activity in liver showed no significant difference among the groups while GPT activity in the liver was significantly higher (P<0.05) in T1 group compared to others. LDH and MDH activity in liver showed significantly higher activity (p<0.01) in T2 group. LDH activity in gill did not vary significantly (P>0.05). SOD, catalase and GPx activity in liver showed a significant difference among the groups. SOD and catalase activity in liver of T1 was significantly higher (p<0.01) than T2 and control group, while Gpx activity in liver of T1 was significantly higher than the control but similar to T2. Catalase activity in gill showed a significantly higher activity (p<0.01) in T2 group while GST activity in liver did not vary significantly (p>0.05).

TBARS and Glutathione

MDA content and glutathione followed a similar trend with a significantly higher (p<0.01) value in T1 group (Table1) compared to other groups. T2 groups also showed higher value compared to the control.

DISCUSSION

The level of stress in fish can be assessed by changes in various biochemical and physiological parameters and their return to normal conditions[18]. During crowding stress, haematological response is reported to be a stress biomarker[3]. In the present study, higher haemoglobin contents were recorded in both the crowding and crowding-with-Feed deprivation groups compared to the control group. The increase in heamoglobin without an increase in RBC reflects the increased hemopoeisis due to high oxygen demand. Nikinma and Rees[29] reported that hypoxia induces erythropoiesis, and Akhtar *et al*[1]., also reported that crowding stress can cause hypoxia in fish. Hence, the increase in heamoglobin observed in this study is a strategy by the fish to cope up hypoxia caused due to the crowding.

Blood glucose has been considered as an indicator of stress[22,37]. As a primary response the chromaffin tissues secrete corticosteroids and catecholamines, which stimulate the carbohydrate metabolism, thereby elevate blood glucose level during stress[45]. In the present study, blood glucose showed no significant differences at 3h, while there was a significant variation among the groups after 9, 24 and 48 h. The higher blood glucose recorded in the treatment groups compared to the control is indicative of the fish ability to produce glucose from othersource through gluconeogenesis. In line with our observation Vijayan *et al*[44]., reported that following short-term exposure of gilthead seabream and tilapia to crowding stress, blood glucose level increased significantly. Similarly, in carps, hypoxia given at 10 ^{0}C resulted in increased blood glucose after 10h. It is well known that fish phagocytes are able to generate superoxide anions ($O2^-$) and its reactive derivatives during a period of intense oxygen intake[33]. This is an immunological defence of the animal against external pathogens. In the present study, respiratory burst activity significantly varied among the groups throughout the experiment. At all hours of sampling both the stressed groups exhibited significantly higher NBT values than the control, except in 24 h. It was also noticed that the NBT and blood glucose values followed similar trend at 48h. In congruent with our findings in crowding-with-Feed deprivation at 48h, long-term application of multiple stressors caused a reduction in respiratory burst activity in *Labeo rohita*[1], and application of repetitive stress reduced the activity in gilthead seabream[25]. This indicates a gradual adaptation in multiple stressed groupsat the earliest than the single stressed group. Serum proteins show the health and well-being of fish and are associated with protein malnutrition[10]. In the present study, total protein, and albumin showed a significantly lower value in crowding-with- feed deprivation group after 3h compared to crowding group alone. Crowding-with- feed deprivation group also showed a significant variation (P<0.05) in serum total protein and globulin content after 9h. After 24 and 48h none of these parameters varied significantly (P>0.05). This can be due to reduced synthesis of protein and non-

specific degradation so as to compensate for high energy demand during stress.

SOD, catalase and glutathione peroxidise are the major enzymes playing important role in antioxidant defence in vertebrates[37]. During the generation of free radicals in the system, SOD catalyses dismutation of superoxide into hydrogen peroxide while catalase and Gpx help to decompose the hydrogen peroxide generated by the action of SOD. It has been reported that the SOD concentration increases with the intensity of stress but the activity of catalase and Gpx can vary depending upon the type of stress[11,14,34]. In the present study, SOD and catalase activities in the liver after 48h showed a higher value in group with crowding alone compared to other groups, while there was no significant difference in Gpx activity between crowding and crowding-with-Feed deprivation group. Similarly, increase in activity of SOD, Gpx and catalase were observed in liver of *Dentex dentex*[26] and *Mesopotaichthys sharpeyi*[28] on short-term starvation. Han *et al*[14]., also reported that on exposure to acidic stress the enzyme activity as well as expression of SOD remained high from 24 to 48 h. Moreover, Caipeng *et al*[4]., reported that Cu/Zn SOD, a variant of SOD, showed a higher expression even after 72 h in crowding population of Atlantic cod and can be considered as a biomarker. An energetic restriction like hypoxia on aerobic organisms keeps the antioxidant activity high. This can be the reason for increased antioxidant activity in rohu fingerlings in both T1 and T2 groups (as crowding generate hypoxia) compared to the control, while lower catalase activity in liver of T2 compared to T1 group may be due to feed deprivation as reported by Hidalgo *et al*[16]., and Pascaul *et al*[30]., who reported that nutrient deficiency can cause reduced activity of antioxidant enzymes. But the catalase activity in the gill of crowding-with-Feed deprivation group was significantly higher than the crowding alone group, which is a reverse trend of the activity in the liver. The difference in the activity of catalase and GPx in different tissues indicates that the production of H_2O_2 is higher in the former than the latter as catalyse hasa lower affinity towards H_2O_2 than Gpx[20]. Hence, gill is more sensitive to acute oxidative stress generated by crowding-with- feed deprivation than crowding alone in rohu fingerlings.

The LDH enzyme is highly active when there is high energy demand in the cell and catalyzes the conversion of lactate to glucose through gluconeogenesis. This condition also indicates the oxygen deficiency in the system. In the present study, LDH activity was significantly higher in crowding-with-feed deprivation groups but not in crowding group alone. Malate dehydrogenase is an enzyme which helps in the conversion of malate to oxaloacetate. A significantly higher activity of MDH and lower activity of LDH in liver of the crowding group shows that the increase in energy requirement in this group is satisfied through oxaloacetate based gluconeogenesis. The significantly higher GPT activity in crowding group supports this observation as amino acids metabolism is controlled by GOT and GPT enzymes. A reverse trend was observed in crowding-with-feed deprivation group, which reflects that the lactate based gluconeogenesis was more active for this group after 48h and GPT activity in this group was similar to the control group. In line with our study, Das *et al*[6]., observed higher LDH and MDH activities in rohu exposed to thermal stress and Tejpal *et al*[39]., on crowding stress. Similarly, higher GPT activity was observed in rohu during thermal stress[6] and tilapia in confinement[44].

G6PDH mediates the conversion of glucose-6-phosphate to 6-phospho-gluconolactone in pentose phosphate shunt with the liberation of NADPH. NADPH is required for the synthesis of fatty acids and regeneration of reduced glutathione[40].The higher value in crowding alone group may be due to regeneration of the glutathione used for stress mitigation as the glutathione (GSH) level in the same group showed significantly higher values. There are several reports indicating that moderate oxidative stress can increase the level of GSH as an adaptation by means of enhanced synthesis[9,49]. Additionally, Pascaul *et al*[30]., reported that on absolute starvation the GSH level in gilthead seabream was significantly lower compared to 2% fed group, which

supports the lower level of GSH observed in crowding-with- feed deprivation groups in the present study.

Oxidative damage can be measured by MDA, an indicator of lipid peroxidation[19]. Fenton reaction produces MDA which can be measured in terms of TBARS[18]. The MDA level in liver differed significantly among the groups, and both the stressed groups showed significantly higher MDA content compared to the control. This indicates higher oxidative stress in T1 and T2 groups compared to the control at48 h. But T1 group showed a

significantly higher MDA level similar to catalase and Gpx activity, thus, stressing that the higher level of H_2O_2 produced in the system oxidized the cellular lipids. After the exposure to multiple stressors, elevated MDA level was observed in European seabass with higher H_2O_2 in liver[36]. Similarly, Liu *et al*[21]., observed that the MDA levels in liver after 24 h was higher in rainbow trout exposed to crowding stress. Further, Hidalgo *et al*[17]., reported that seabream subjected to starvation stress also showed a higher hepatic MDA.
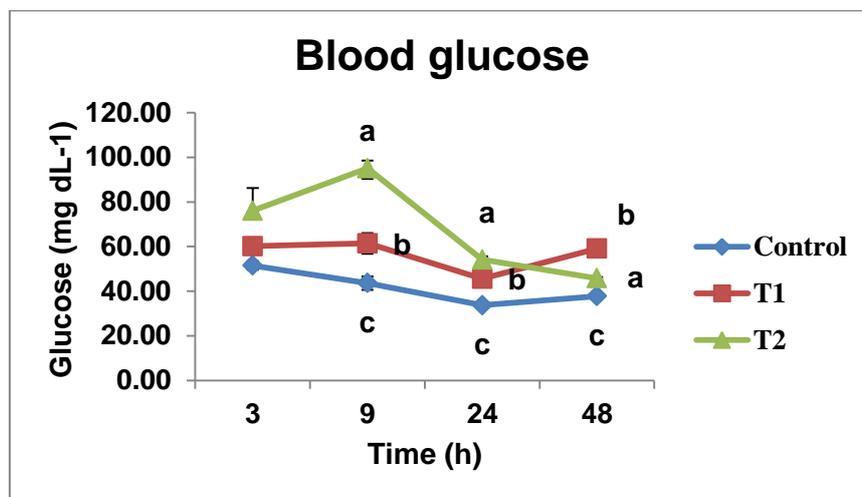
(a)
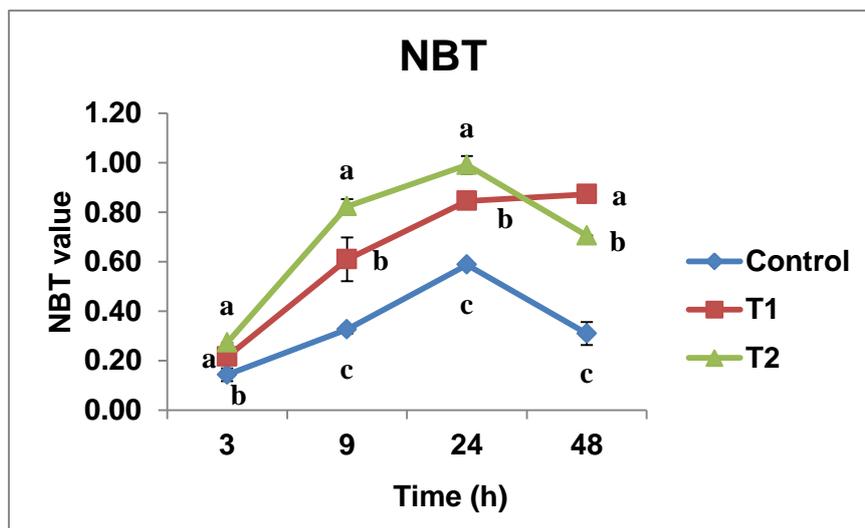
(b)

Fig. 1: Effect of crowding and crowding-with- feed deprivation on blood glucose (a) and respiratory burst activity (b) in *Labeo rohita* at different time intervel. Data expressed in terms of mean ± SD (n=6). Level of significance was measured at p <0.05.
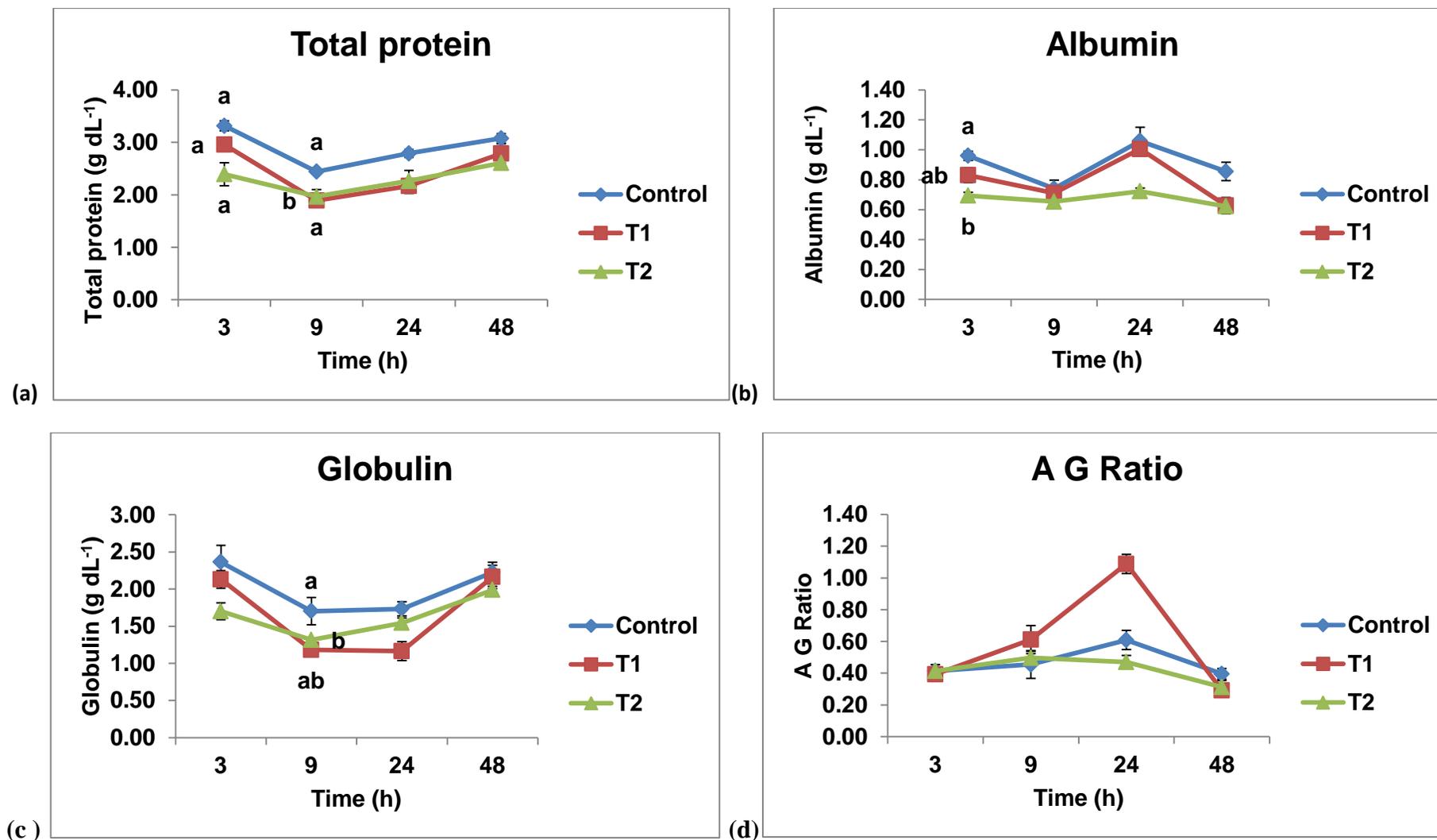
Fig. 2: Effect of crowding and crowding-with-starvation on serum parameters in *Labeo rohita* at different time intervals. Data expressed in terms of mean ± SD (n=6).;Level of significance was measured at p <*0.05*

Table 1: enzymatic and non-enzymatic antioxidant activities of liver and gill in *Labeo rohita* under acute crowding and crowding-with- feed deprivation stress

Treatments	SOD (L)	CAT (L)	CAT (G)	GPx (L)	GST (L)	G6PDH (L)	GSH (L)	MDA (L)
C	$51.67^b \pm$ 0.33	$10.45^c \pm$ 0.70	$10.37^b \pm$ 0.70	$2.44^b\pm$ 0.009	$8.12\pm$ 0.35	$0.05^b\pm$ 0.001	$7.28^c \pm$ 0.36	$0.33^c\pm$ 0.008
T1	$57.60^a \pm$ 1.65	$55.69^a \pm$ 0.31	$10.93^b \pm$ 0.15	$2.51^a\pm$ 0.008	$6.71\pm$ 0.89	$0.80^a\pm$ 0.17	$100.39^a \pm$ 5.3	$0.44^a\pm$ 0.004
T2	$52.76^b \pm$ 0.36	$16.45^b \pm$ 2.05	$25.60^a \pm$ 0.43	$2.48^{ab}\pm$ 0.007	$6.41\pm$ 0.73	$0.09^b\pm$ 0.02	$15.94^b \pm$ 0.51	$0.40^b\pm$ 0.002
P value	0.012	0.000	0.000	0.005	0.253		0.000	0.000

Data expressed as Mean ± SD, n=6. Mean values in the same column with different superscript differ significantly (p<*0.05*). SOD- superoxide dismutase, specific activity was expressed as 50% inhibition of epinephrine auto-oxidation min^{-1} mg $protein^{-1}$, Catalase activity was expressed as nano moles of H_2O_2 decomposed min^{-1}mg $protein^{-1}$,GST and GPx- nmoles $mgprotein^{-1}$ min^{-1} , G6PDH- Unit mg $protein^{-1}min^{-1}$,GSH and MDA-micromoles gm $tissue^{-1}$

Table 2: Metabolic enzyme activities of*Labeo rohita* under acute crowding and crowding-with- feed deprivation stress

Treatments	GOT (L)	GPT (L)	LDH (L)	LDH (G)	MDH (L)	RBC(10^6 cells /mm^3)	Heamoglobin (g/dl)
C	0.10 ± 0.007	$16.05^b \pm$ 1.03	$0.71^b \pm$ 0.09	1.16 ± 0.15	$4.30^b\pm$ 0.21	1.07 ± 0.07	$5.27^b\pm$ 0.13
T1	0.10 ± 0.02	$29.29^a \pm$ 0.04	$1.15^b \pm$ 0.18	1.22 ± 0.24	$2.57^b\pm$ 0.16	1.10 ± 0.05	$6.03^a \pm$ 0.30
T2	0.04 ± 0.01	$16.68^b \pm$ 3.80	$3.29^a \pm$ 0.82	1.78 ± 0.30	$6.44^a\pm$ 0.91	1.09 ± 0.06	$6.27^a \pm$ 0.18
P value	0.118	0.010	0.021	0.220	0.007	0.908	0.012

Data expressed as Mean ± SD, n=6. Mean values in the same column with different superscript differ significantly (p<*0.05*). Enzyme activities are expressed in the following units : GOT-specific activities expressed as nano moles of sodium pyruvate formed min^{-1} mg $protein^{-1}$ at 37°C; GPT specific activities expressed as nano moles of oxaloacetate released min^{-1} mg $protein^{-1}$ at 37°C; LDH and MDH: was expressed as specific activity expressed as Units min^{-1} mg $protein^{-1}$ at 37°C

CONCLUSION

In summary, the results obtained in the present study reflect that the stress caused due to crowding and crowding-with-feed deprivation had an adverse effect on the physio-metabolic condition of the fish. Crowding stress take more time to acclimatize with the situation than crowding-with-feed deprivation stress in rohu when fish subjected to short term stress of 48h. But further long term study is required to understand the exact mechanism of action of multiple stressors like crowding and feed deprivation in fish as these are common stressors in an aquaculture system.

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References

1. Akhtar, M.S., Pal, A.K., Sahu, N.P., Ciji, A. and Meena, D.K., Effects of dietary pyridoxine on growth and physiological responses   of *Labeorohita* fingerlings reared at higher water temperature. *Isr J Aquac Bamid*., **64:** 777 (2012).

2. Antonopoulou, E., Kentepozidou, E., Feidantsis, K., Roufidou, C., Despoti, S. and Chatzifotis, S., Starvation and re-feeding affect Hsp expression, MAPK activation andantioxidant enzymes activity of European Sea Bass (*Dicentrarchuslabrax*). Comp. BiochemPhysiol, Part A **165:** 79–88 (2013).

3. Barcellos, L.J.G., Kreutz, L.C., Quevedo, R.M., Fioreze, I., Soso, A.B., Cericato, L., Fagundes, M., Conrad, J., Baldissera, R., Bruschiand, A. and Ritter, F., Nursery rearing of jundiá, *Rhamdiaquelen* (Quoy and Gaimard) in cages: cage type, stocking density and stress response to confinement. *Aquaculture*, **232:** 383 – 394 (2004).

4. Caipang, C.M.A., Potential molecular biomarkers of crowding stress in Atlantic cod, *Gadusmorhua*and their importance in health management. *ABAH Bioflux*, **4(2):** 79-83 (2012).

5. Cara, J.B., Aluru, N., Moyano, F.J. and Vijayan, M.M., Food deprivation induces HSP 70 and HSP 90 protein expression in larval and gilthead sea bream and rainbow trout. *Comp Biochem Physiol B*. **142:** 426-431 (2005).

6. Das, Pal, A.K., Chakraborty, S.K., Manush, S.M., Chatterjee, N. and Apte, S.K., Metabolic elasticity and induction of heat shock protein 70 in *Labeorohita* acclimated to three temperatures. *Asian-Aust. J. Anim. Sci*., **19:** 1033–1039 (2006).

7. De Moss, R.D., Gunsalus, I.C. and Bard, R.C., A glucose 6-phosphate dehydrogenase in *Leuconostocmesenteroides*. *J. Bacteriol*., **66:** 10-16 (1953).

8. Doumas, B.T., Watson, W. and Biggs, H.G., Albumin standards and measurement of serum albumin with bromocresol green. *Clin Chim Acta*., **31:** 87-96 (1971).

9. Doyotte, A., Cossu, C., Jacquin, M.C., Babut, M. and Vasseur, P., Antioxidant enzymes,glutathione and lipid peroxidation relevant biomarkers of experimental or field exposure in gillsand the digestive gland of the fresh water bivalve *Uniotumidis Aquat Toxicol*., **39:** 93-110 (1997).

10. Eggum, O.L., The protein quality of cassava leaves. *Br. J. Nutr*., **24:** 761-769 (1970).

11. Fawole, F.J., Sahu, N.P., Pal, A.K. and Kumar, A., Haemato-immunological response of *Labeorohita* (Hamilton) fingerlings fed leaf extracts and challenged by *Aeromonashydrophila. Aquacult Res*., **47(12):** 3788–3799 (2015).

12. Habig, W.H., Pabst, M.J. and Jakoby, W.B., Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *J Biol Chem*., **249:** 7130-7139 (1974).

13. Hafeman, D.G., Sunde, R.A., Hoekstra, W.G., Effect of dietary selenium on erythrocyte and liver glutathione peroxidase in the rat. *J Nutr*., **104:** 580-587 (1974).

14. Han, C.Y., Zeng, Q.M. and Sun, Z.T., Gene expression of antioxidant enzymes in hybrid tilapia, *Oreochromisniloticus* X *Oreochromisaureus* under acute pH stress. J. Worlaquacul. Soc. 47(2) doi:10.1111/jwas.12260 (2016).

15. Hendrick, L.J., Erythrocytes counts and haemoglobin determinations for two species of sucker, genus Catostomus from Colorado. *Copeia*, **4:** 265–266 (1952).

16. Hidalgo, M.C., Expo, sito, A., Palma, J.M. and de la Higuera, M., Oxidative stress generated by dietary Zn-deficiency: studies in rainbow trout

(*Oncorhynchusmykiss*). *Int J Biochem Cell Biol*., **34:** 183 – 193 (2002).

17. Hidalgo, V., Villada, C., Almela, M., Espín, L., Gómez-Amor, J. and Salvador, A. Enhancing effects of acute psychosocial stress on priming of non-declarative memory in healthy young adults. *Stress*, **15:** 329–338 (2012).

18. Hoseini, S.M., Hosseini, S.A. and Nodeh, A.J., Serum biochemical characteristics of Beluga, *Husohuso*(L.), in response to blood sampling after clove powder solution exposure. *Fish Physiol Biochem*,, **37(3):** 567-572 (2011).

19. Huang, B., Ban, X.Q., He, J.S., Tong, J., Tian, J. and Wang, Y.W., Hepato-protective and antioxidant activity of ethanolic extracts of edible lotus (*Nelumbonucifera*Gaertn.) leaves. *Food Chem*., **120(3):** 873–878 (2010).

20. Izawa, Y., Inoue, and Kimura, A., Importance of catalase in the adaptive response to hydrogen peroxide: analysis of acatalasaemic *Saccharomycescerevisiae. Biochem. J*., **320:** 61–67 (1996).

21. Liu, F., Shi, H.Z., Guo, Q.S., Yu, Y.B., Wang, A.M., Lv, F. and Shen, W., Effects of astaxanthin and emodin on the growth, stress resistance and disease resistance of yellow catfish (*Pelteobagrusfulvidraco*). *Fish Shellfish Immunol*., **51:** 125-35. doi: 10.1016/j.fsi.2016.02.020. Epub 2016 Feb 17 (2016).

22. Manush, S.M., Pal, A. K., Chatterjee, N., Das, T. and Mukherjee, S.C., Thermal tolerance and oxygen consumptionof*Macrobrachiumrosenbergii* acclimated to threetemperatures. *J. Therm. Biol*., **29:** 15-19 (2004).

23. Meton, I., Fernandez, F. and Baanante, I.V., Short- and long-term effects of refeeding on key enzyme activities in glycolysis-gluconeogenesis in the liver of gilthead seabream (*Sparusaurata*). *Aquaculture*, **225:** 99 – 107 (2003).

24. Misra, H.P. and Fridovich, I., The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J Biol Chem*., **247:** 3170–3175 (1972).

25. Montero, D., Izquierdo, M.S., Tort, L., Robaina, L. and Vergara, J.M., High stocking density produces crowding stress altering some physiological and biochemical parameters in gilthead seabream, *Sparusaurata*, juveniles *Fish Physiol Biochem*., **20:** 53–60 (1999).

26. Morales, A.E., AmaliaPerez-Jimeneza, Hidalgo, M.C., Abellan, E. and Cardenete, G., Oxidative stress and antioxidant defenses after prolonged starvation in *Dentexdentex* liver. *CompBiochemPhysiol Part C*., **139:** 53 – 161 (2004).

27. Moron, M.S., De Pierre, J.W., and Vik, B.M., Levels of glutathione, glutathione reductase and Glutathione-S-transferase activities in rat and lung liver, *Biochem Biophys Acta*., **582:** 3170-3185 (1979).

28. Najafi, A., Salati, A.P., Yavari, V. and Asadi, F., Effects of short-term starvation and re-feeding on antioxidant defense status in*Mesopotamichthyssharpeyi* (Günther, 1874) fingerlings. *Inter J. Aqua Biol*., **2(5):** 246-252 (2014).

29. Nikinmaa, M. and Rees, B.B., Oxygen-dependent gene expression in fishes. *Am J Physiol: Regulatory, Integrative and Comparative Physiology*, **288:** 1079- 1090 (2005).

30. Pascual, P., Pedrajas, J.R., Toribio, F., J. Lopez-Barea, J. and Peinado, Effect of food deprivation on oxidative stress biomarkers in fish (*Sparusaurata*) ChemBiolInt 145: 191-199 (2003).

31. Pickering, A.D., Pottinger, T.G. and Christie, P., Recovery of the brown trout, *Salmotrutta*L., from acute handling stress: a time-course study. *J Fish Biol*., **20:** 229–244 (1982).

32. Reinhold, J.G., Manual for determination of serum total protein, albumin and globulin fractions by Biuret method. In: Reiner M (ed). Standard method of clinical chemistry. New York: Academic Press pp. 88. (1953).

33. Secombes, C.J., The nonspecific immune system: cellular defence In G. Iwama, T.

Nakanishi (Eds.), The Fish Immune System, Academic Press, San Diego, California, pp. 63–103 (1996).

34. Shamna, N., Sardar, P., Sahu, N.P., Pal, A.K., Jain, K.K. and Phulia, V., Nutritional evaluation of fermented Jatropha protein concentrate in *Labeorohita* fingerlings *Aquacult Nutr*., **21:** 33–42 (2015).

35. Sharma, S.K. and Krishnamurthy, C.R., Production of lipid peroxidation of brain. *J. Neurochem.* **15:** 147–149 (1968).

36. Sinha, A.K., Elgawad, H.A., Zinta, G., Dasan, A.F., Rasoloniriana, R., Asard, H., Blust, R. and De Boeck, G., Nutritional Status as the Key Modulator of Antioxidant Responses Induced by High Environmental Ammonia and Salinity Stress in European Sea Bass (*Dicentrarchuslabrax*) plos one | DOI:10.1371/journal.pone.0135091 (2015).

37. Slaninova, A., Smutna, M., Modra, H. and Svobodova, S., A review: Oxidative stress in fish induced by pesticides. *Neuroendocrinology Letters*, **30(1):** (2009).

38. Stasiak, A.S. and Baumann, C.P., Neutrophil activity as a potential bioindicator for contaminant analysis. Fish shellfish Immunol 6:537-539 Takahara S, Hamilton HB, Neel JV, Kobara TY, Ogura Y, Nishimura ET, (1960).Hypocatalasemia, a new genetic carrier state. *J Clin Invest*., **39:** 610-619 (1996).

39. Tejpal, C.S., Pal, A.K., Sahu, N.P., Jha, A.K., Muthappa, N.A., Sagar, V. and Rajan, M.G., Dietary l-tryptophan mitigates crowding stress and augments the growth in *Cirrhinusmrigala* fingerlings. *Aquaculture*, **293:** 272–277 (2009).

40. Trenzado, C.E., Morales, A.E., Palma, J.M. and de la Higuera, M., Blood antioxidant defenses and hematological adjustments in crowded/uncrowded rainbow trout (*Oncorhynchusmykiss*) fed on diets with different levels of antioxidant vitamins and HUFA. *Comp Biochem Physiol. C*, **149:** 440–447 (2009).

41. Trinder, P., Determination of glucose in blood using glucose oxidase with an alternative oxygen receptor, *Ann Clin Biochem*., **6:** 24-27 (1969).

42. Valavanidis, A., Vlahogianni, T., Dassenakis, M. and Scoullos, M., Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotoxicol Environ Saf*., **64:** 178–189 (2006).

43. Van Kampen, E.J. and Zijlstra, W.G., Recommendations for haemoglobinometry in Human blood. *Bri. J. Haematol*., **13:** 71(1961).

44. Vijayan, M.M., Pereira, C., Grau, E.G. and Iwama, G.K., Metabolic responses associated with confinement stress in tilapia: the role of cortisol. *Comp Biochem Physiol C*., **116:** 89–95 (1997).

45. Wedemeyer, G.A., Effects of rearing conditions on the health and physiological quality of fish in intensive culture. In Fish Stress and Health in Aquaculture Pp. 35–71. Edited by G.K. Iwama, A.D. Pickering, J.P. Sumpter, and C.B. Schreck. Cambridge University Press, Cambridge (1997).

46. Wendelaar Bonga, S.E., The stress response in fish. *Physiological Reviews*, **77:** 591-625 (1997).

47. Wooten, I.D.P., Micro-analysis.*In*: Medical Biochemistry 4th edn(ed. Churchill, J. and Churchill, A.). London, pp. 101-107 (1964).

48. Wrobleiuski, F. and Ladue, J.S., LDH activity in blood. *Proc. SOC. Exp. Biol. Med.,* **90:** 210-213 (1955).

49. Zhang, J.F., Shen, H., Wang, X.R., Wu, J.C. and Xue, Y.Q., Effects of chronic exposure of 2,4-dichlorophenol on the antioxidant system in liver of freshwater fish *Carassiusauratus. Chemosphere*, 5516-7174 (2004).