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Changes in Blood, Serum Antioxidant Status, and Lipid Profile of *Labeo rohita* on Short-Term Multiple Stress Exposure

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Abstract

The present study focused on the effect of crowding and crowding-with-feed restriction in *Labeo rohita* fingerlings of an average weight of 8.5 ± 0.5 g. The experiment was conducted with 180 fingerlings distributed randomly in four distinct groups with each group in triplicate viz, Control (C) (stocking density at 10 fish/75 L with satiation feeding), T1 (stocking density at 10 fish/ 75 L with restricted feeding at 1% of body weight), T2 (stocking density at 20 fish / 75 L with satiation feeding) and T3 (stocking density at 20 fish / 75 L with restricted feeding at 1% body weight) for 48 h. Serum sample were collected at regular intervals of 3, 9, 24 and 48h. The serum total antioxidant status of the control group was significantly higher ($p<0.05$) than the stressed groups from 9 h onwards while the catalase and glutathione peroxidase showed an increasing trend after 24 h. There was no significant effect of stress on serum triglyceride profile except for the triglyceride levels at 3 h of stress exposure. The serum antioxidants started varying only after 9 h of stress exposure. Blood glucose levels varied only after 9 h of stress exposure and haemoglobin content were higher in feed restricted and crowding-with feed-restricted groups compared to control. Blood and serum parameters are often used as an indicator of stress in fish, and the variation in serum triglyceride profile and the activity of enzymatic antioxidants indicated that crowding as well as crowding-with-feed restriction are acute stressors in aquaculture farms.

Keywords:
Carp culture, Restriction feeding, Natural stressors, Biochemical analysis

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Introduction

The total carp production of the country is around 5.95 million tonnes and rohu contributes more than 30% of the total carp production in India (FAO, 2009). Among the Indian major carps, the most preferred species is *Labeo rohita* and is produced through monoculture and polyculture practices (Nandeesha *et al*. 1993) in the country. Being herbivorous column feeder, rohu can easily digest plant-based ingredients and can thrive well in conventional feed ingredients. Andhra Pradesh is the hub of carp culture and farmers have adopted several innovative technologies to increase the seed availability and improved growth in carps, especially rohu. In order to get the benefit of the compensatory growth effect and to reduce the cost of production, the farmers adopted a practice called "stunting" in rohu culture. Stunting is associated with stocking of fish at high density and under diet restriction. But high density and feed restriction are considered as stressors in aquatic system (Vijayan and Leatherland, 1988; Yin *et al*., 1995) and in stunting, maintenance of fish under crowding and inadequate diet even continue up to a year. These stressors can change the immunological, physiological and antioxidant status of the animal (Ellis 1981). Crowding can cause oxygen deficiency and competition among fish for food, that can further result in hypoxic condition at cellular level with high reactive oxygen species (ROS) production. Uncontrolled ROS synthesis in body can cause oxidative stress and enhance the oxidative damage of cell. During this period, the immunity and antioxidant system of the fish gets weakened. This can predispose fish to disease and mortality.

There is plethora of biochemical responses in fish to mitigate the stress effect by adjusting the metabolic pathways to meet the increasing energy demand (Mommsen *et al*., 1999). The primary responses to stress results in an increased serum level of cortisol, and the secondary responses switch on and off various biochemical reactions in the system. The study of cortisol, metabolites and enzyme activities in serum can help to understand the stress responses during acute stress. Literature has posited that, unlike in chronic stress studies, the level of cortisol is analysed to determine the level of acute stress effects (Barton, 2002) but other metabolites like glucose, triglycerides, etc can also indicate the effect of short-term stress exposure (Rossi *et al*., 2017). Though enzymatic antioxidants are the second line of defence in stress mitigation, their activity can get initiated even at short term exposure to stressors, especially when exposed to multiple stressors (Akhtar *et al*., 2012). Lipid is a major energy source in fish and is a major component of cell membranes. During short term starvation, fish mobilize lipid class of triglycerides which provide energy readily than the structural lipids (Henderson and Tocher, 1987), while on prolonged starvation the phospholipid (structural lipid) also found to be decreased. So, the triglyceride concentration is a better indicator of nutrient mobilization during short term starvation to satisfy the energy demand (Yengkokpam *et al*., 2013). Similarly, the antioxidant enzymes called superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) are playing a major role in mitigating the stress effect in fish (Martinez-Alvarez *et al*., 2005; Shamna *et al*., 2018; Nazeemashahul *et al*., 2021b). Though there are many studies on the effect of stress in fish, including those done in our lab, the effect of multiple stressors like high stocking density and starvation on the antioxidant system and lipid metabolism is scanty. Hence, the present study tries to observe the effect of acute level multiple stress exposure on the blood, serum antioxidant and lipid profile of rohu.

Materials and Methods

Experimental animals and design:

Fingerlings of rohu (8-10g) were obtained from Prem fisheries, Ankaleswar, India and reared with a control diet (35% protein) for 14 days to acclimatize the fish to lab condition. For conducting the experiment, the fish were randomly distributed in different treatments namely Control (C) (stocking density at 10 fish/75 L with satiation feeding), T1 (stocking density at 10 fish/ 75 L with restricted feeding at 1% of body weight), T2 (stocking density at 20 fish / 75 L with satiation feeding) and T3 (stocking density at 20 fish / 75 L with restricted feeding at 1% body weight) for 48 h. The experiment was conducted for 48 h in 150 L capacity rectangular tubs (80 x 57 x 42 cm. Samples were collected at definite intervals (3 h, 9 h, 24 h and 48 h). Round the clock aeration was arranged in all the tubs.

Serum collection

Serum collection was done from six fish per tank after anesthetizing it with clove oil (50 µl/l). Blood samples were collected using a non-heparinised syringe and transferred to 2 ml Eppendorf tubes. The tubes were kept in a slanting position for 4h and allowed to clot. The tubes were then centrifuged at 4000 rpm for 20 min and the supernatant (serum) was collected. The serum was stored at -20 until further use.

Total antioxidant (TAO) activity

Total antioxidant activity was estimated following the method described by Yen and Duh (1994). To the 50 µl serum, 1 ml methanol and 50 µl normal saline were added and vortexed well. Further, the solution was centrifuged at 3000 rpm for 30 min followed by collection of the supernatant. 1.5 ml methanol and 0.5 ml 2,2-diphenyl-1-picrylhydrazyl (DPPH) were added to the supernatant. Reading was taken at 517 nm against blank.

Superoxide dismutase (SOD) activity

SOD activity was measured from serum using Cayman's SOD assay kit (Cayman chemicals, Ann Arbor, USA). The activity of the enzyme was calculated using the following formula

SOD (Uml^{-1}) = ((Sample OD-Y intercept x 0.23 x sample dilution))/(slope x 0.01 ml)

Catalase activity

Serum catalase was assayed using catalase assay kit (Cayman chemicals, Ann Arbor, USA) which utilizes the peroxidation function of catalase to determine the activity. To a cocktail of 100 µl of assay buffer, 30 µl of methanol and 20 µl of sample, 20 µl of hydrogen peroxide was quickly added and kept in a shaker for 20 min. By adding 30 µl of potassium hydroxide the reaction was stopped and added a chromogen again. The mixture was kept for incubation (10 min) in a shaker at room temperature. To this, 10 µl of potassium periodate was added and incubated for 5 min. Reading was taken at 540 nm and values were calculated from the following formula and expressed in nmol/min/ml (1unit = amount of enzyme that cause the formation of 1nm of formaldehyde/min at 25°C)

CAT activity (Uml^{-1})=((µM of sample/20))x sample dilution

Glutathione peroxidase (GPx) activity

Cayman's GPx assay Kit (Cayman chemicals, Ann Arbor, USA) was used for studying the serum GPx activity. The reaction rate was calculated using the following formula and one unit was equivalent to the enzyme that can oxidize 1.0 nmol of NADPH to NAD+/ min at 25 °C.

GPx activity (Uml^{-1}) = (rate of change in absorbance x 0.19ml x sample dilution)/ (0.00373 µmol M^{-1} x 0.02 ml)

The myeloperoxidase activity

Total myeloperoxidase content was estimated from the serum sample following Quade and Roth (1997) method with slight modification. About 15 µl of serum was diluted with 135 µl of Hank's Balanced Salt Solution (HBSS) without Ca^{2+} or Mg^{2+} in 96 well plates. 25 µl of 20 mM 3,3',5,5'-Tetramethylbenzidine (TMB) (Himedia, India) and 25 µl of 5 mM H_2O_2 (Qualigens, India) were added. The colour change reaction was stopped after 2 min by adding 50 µl of 4M sulphuric acid (H_2SO_4). Plate was centrifuged at 400 x g for10 min, and 150 µl of the supernatants, from each well, were filled in a fresh 96 well plates. The OD was recorded using a microplate reader at 450 nm wavelength (BioTek Power Wave 340, India).

Total lipids estimation

The total lipid in serum was estimated using Labkit total lipid assay kit (Merck, India) which follows sulfo-phospho-vanillin (SPV) method. Acid digest was prepared by boiling the mixture of sulphuric acid (2.5 ml) and standard (100 µl) or serum (100µl) for 10 min. This acid digest was cooled for a while and to a 50 µl of acid digest added 1ml of Labkit reagent. After vigorous shaking, it incubated for 15 min. Reading was taken at 520nm and calculation was done using the formula

$$\text{Total lipid (mg/dl)} = \frac{\text{Absorbance of Test (T)} \times 7.5}{\text{Absorbance of standard (S)}}$$

Phospholipids estimation

The phospholipids were assayed on the principle of enzymatic colourimetric method using Labkit assay kit (Merck india). To the sample tube containing 10µl of serum,1 ml of reagent was added, mixed thoroughly and incubated at 37 °C for 10 min. Standard tube was also kept similar way with 10µl of standard taken from a stock of 300 mg/dl. Reading was taken at 505 nm against blank and calculation done using the formula. The absorbance was measured at 505 nm and the calculations were done as follows:

Phospholipids (mg/dl) = Absorbance of test (T) x 3/Absorbance of standard (S)

Triacylglycerides (TAGs) estimation

Trinder (1969) method was followed for estimating TAG as mentioned by the manufacturer of Erba triglyceride kit (ERBA diagnostics Manheim, Germany). To a sample of 5 µl, 500 µl of reagent was added and kept in incubation for 10 min at 37 °C. A blank with distilled water and standard (std) was also kept similar way and absorbance was read at 546nm against reagent blank. Calculation was done using the following formula

Triglycerides (mg/dl) = (Abs of Test/Abs of std) x Concentration of std (mg/dl)

Statistical Analysis

Statistical analysis of data was done using one-way analysis of variance (ANOVA) in SPSS 19.0 version. Duncan's multiple range test (DMRT) was used for comparison of means among different experimental groups and significance was observed at 5% probability level ($P<0.05$). Data presented in the text, figures and tables were expressed as mean ± standard error (SE).

Results and discussion

Physico-chemical parameters of water

The water quality parameters viz, temperature (°C), pH, dissolved oxygen (mg/L), total hardness (mg/L) and ammonia (mg/ L) ranged from 25 - 29, 7.5 - 8.1, 6.3 – 7.5, 236 - 242 and 0.05 – 0.09, respectively.

Serum antioxidant profile

Total antioxidant status (TAS)

In the present study, the serum total antioxidant status was significantly different ($P <0.01$) among the groups at all duration of stress exposure except at 3h exposure (Fig 1). The total antioxidant status was significantly higher ($P <0.05$) in control group compared to the feed restriction (T1), crowding (T2), and crowding with feed restriction group (T3) at 9, 24 and 48 h of stress exposure.

Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)

The serum SOD, CAT and GPx values are presented in Fig 1. The SOD activity of fish was significantly different from 9 h onwards of stress exposure whereas the CAT and GPx activities of fish were significantly different among the groups at 24 and 48 h of stress exposure. From 9h onwards of stress exposure, the fish of T3 group (crowding plus feed restriction) showed a significantly higher activity ($P <0.05$) of SOD, which was not different from the fish of T1 group (feed restriction alone) at 24 and 48 h of stress exposure. The fish of other groups showed an increasing trend in SOD activity with the time of stress exposure. Also, GPx and CAT activities in fish of T1, T2 and T3 groups were significantly higher than the control group at 24 h of stress exposure (Fig. 1) while these values in the fish of T1 group were not significantly different from the fish of control at 24hrs of stress exposure.

Total lipid in the serum was significantly different in 24 and 48 h stress exposed fish. The higher values of serum total lipid were observed in fish of T3 group at 24 and 48hrs of stress exposure. The fish in T1 group was not significantly different from T3 at 24 and 48hrs, whereas T2 showed a significantly lower value at 24 h ($P <0.05$) (Fig 2).

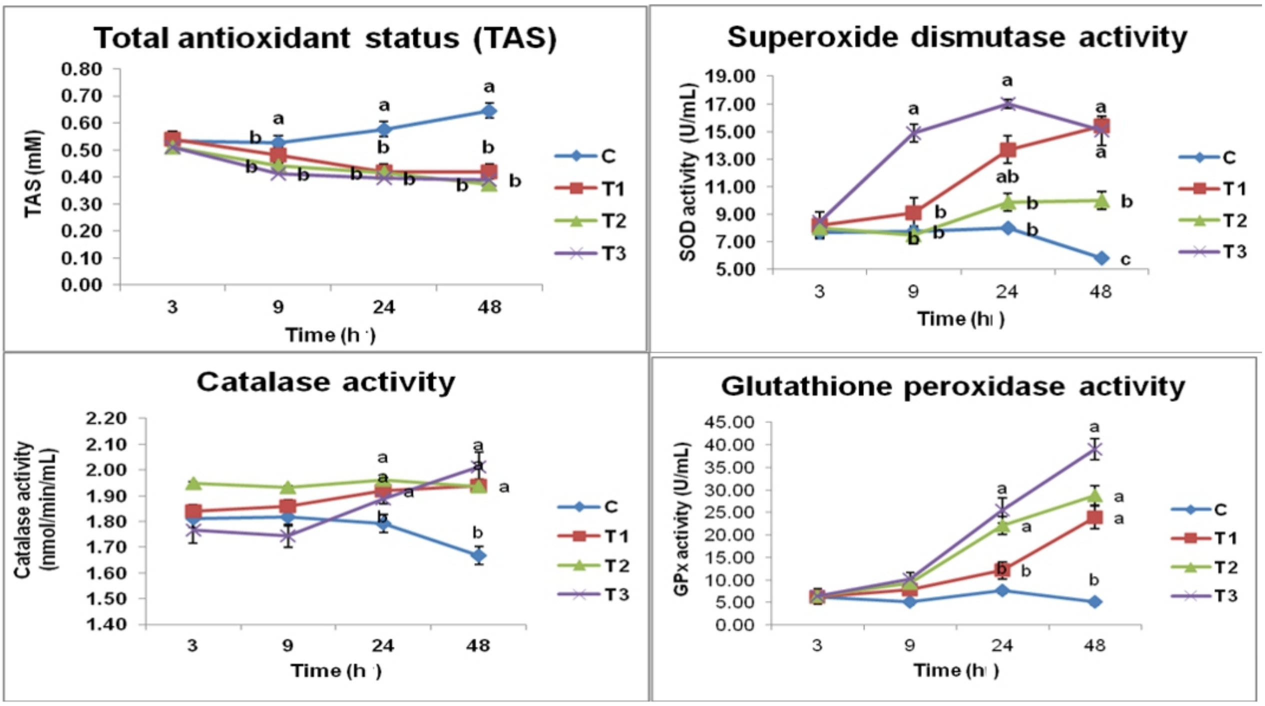

Fig. 1 Effect of crowding and crowding-with-feed restriction on total antioxidant status, SOD, catalase and glutathione peroxidase activity in *Labeo rohita* at different time intervals *Total lipid*

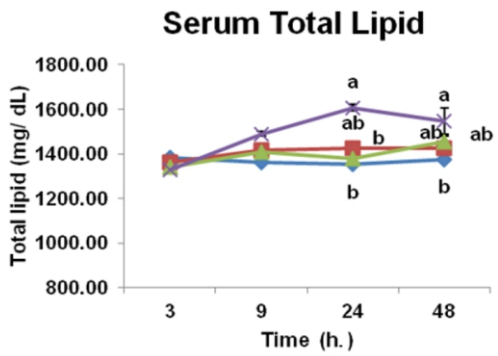

Fig.2: Effect of crowding and crowding-with-feed restriction on total lipid content of *Labeo rohita* fingerlings at different time intervals.

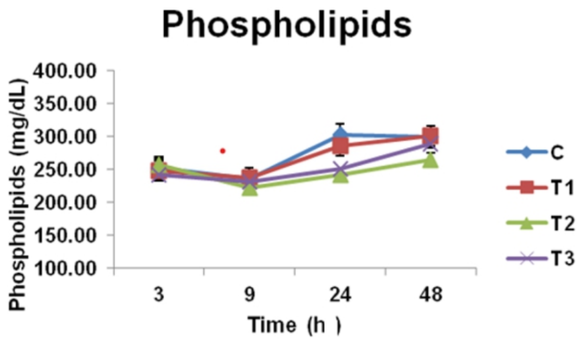

Fig.3: Effect of crowding and crowding-with-feed restriction on serum phospholipid levels of *Labeo rohita* fingerlings at different time intervals.

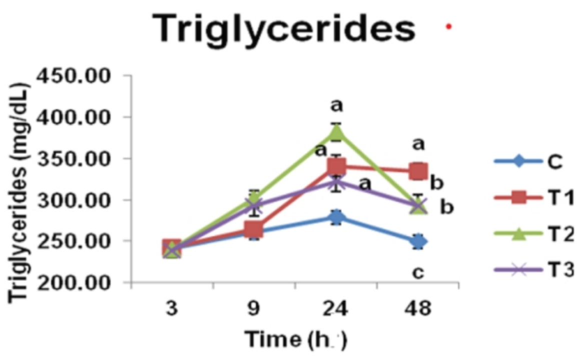

Fig.4: Effect of crowding and crowding-with-feed restriction on serum triglyceride levels of *Labeo rohita* fingerlings at different time intervals.

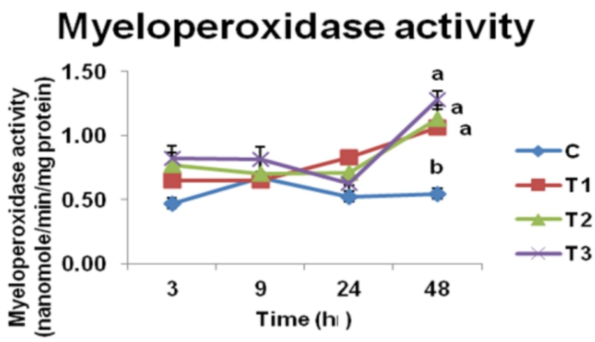

Fig. 5 Effect of crowding and crowding-with-feed restriction on myeloperoxidase activity in *Labeo rohita* at different time intervals.

Phospholipids

Phospholipids contents among the different groups did not show any significant difference (P >0.05) irrespective of the time of the stress exposure (Fig 3).

Triglycerides

The triglyceride levels were significantly higher (P >0.05) in fish of the T1, T2 and T3 groups at 24 and 48 h of stress exposure compared to control (Fig. 4).

Serum myeloperoxidase activity

The myeloperoxidase activity in serum was not significantly different among the groups at 3, 9 and 24 h of stress exposure, whereas a significantly higher (P <0.01) value was observed in fish of T1, T2 and T3 groups compared to control at 48h (Fig.5).

Total erythrocyte count (TEC) and haemoglobin (Hb) content

At the end of 48 hrs of stress exposure, the TEC value showed no significant difference among the groups. However, a significantly higher (P <0.05) Hb content was observed in T2 and T3 groups compared to the control and T1 groups (Table 1).

Table 1: The total erythrocyte count and haemoglobin content in *Labeo rohita* fingerlings of different treatments

Treatments[1]	Parameters	
	TEC[2] ($X10^6$ cells/ mm^3)	Haemoglobin (g/ dL)
C	1.07 ± 0.07	5.27^b ± 0.13
T1	1.06 ± 0.05	5.87^b ± 0.22
T2	1.10 ± 0.05	6.03^a ± 0.30
T3	1.09 ± 0.06	6.27^a ± 0.18
p-value	***0.908***	***0.012***

Data expressed as Mean ± SE; n=6; Means bear different superscripts differ significantly (*P* <0.05)

[1]C, Control without stress; T1, Crowding; T2, Feed restriction; T3, Crowding-with-feed restriction; [2]TEC, Total erythrocytic count

Blood Glucose

Blood glucose level varied significantly (P <0.05) among the groups (*P* <0.01) only after 9 h. of exposure, where T2 and T3 groups showed significantly higher values compared to the control at 9, 24 and 48h of exposure which was not significantly different from T1 group (Fig 6).

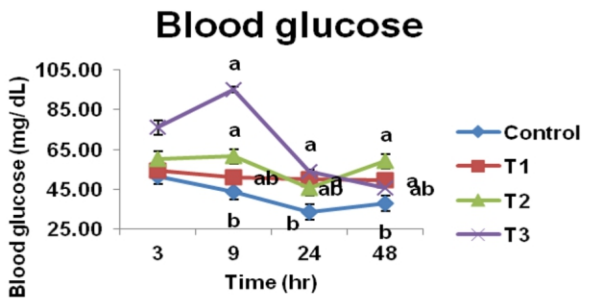

Fig 6: Effect of crowding and crowding-with-feed restriction on blood glucose levels in *Labeo rohita* fingerlings at different intervals

Discussion

Serum enzymatic antioxidants

Starvation and crowding can cause stress and generation of harmful ROS in the system (Robinson *et al*. 1997; Tejpal *et al*. 2009; Nazeemashahul *et al*., 2021a). This ROS can further convert to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD), however this metabolite is also toxic to the cells. Nevertheless, H_2O_2 so produced can be detoxified by the catalase (CAT) or glutathione peroxidase (GPx). In the present experiment, the activity of SOD was increased in fish of T3 (crowding along with feed restriction) at 9h, T2 (feed restriction alone) at 24h and T1 (crowding alone) groups, respectively, at 9, 24 and 48h compared to the fish of the control. This observation denoted that the response of SOD in fish subjected to the crowding with feed restriction (multiple stressor) was initiated at 9hr, whereas in fish subjected to the stress in the form of feed restriction or crowding alone, the response of this enzyme was initiated at 24 and 48h, respectively. That means SOD activity was significantly higher in multiple stress exposed groups at 9h, while crowding alone group showed a similar higher activity from 24hr. Though feed restriction caused stress, the SOD activities were not as high as in other treatment groups. Further, the activities of CAT and GPx in fish exposed to stress in terms of crowding and crowding with feed restriction were increased from 24h onwards, and this response might be for the conversion of toxic H_2O_2 generated due to the action of SOD. Catalase activities were higher in all treatment groups at 24 and 48h, whereas, along with the multiple stress-exposed group, the feed restricted groups showed a higher GPx activity at 24h. It is reported that non-enzymatic antioxidants are the first line of free radical scavengers, and the enzymatic antioxidants act along with or after the exhaustion of certain non-enzymatic antioxidants (Kossman, 1988; Nazeemashahul *et al*., 2021a). This can be the reason for the non-significant activity of SOD, catalase and GPx up to 9h and the higher enzymatic antioxidant activity in T3 might be due to the faster exhaustion of non-enzymatic antioxidants in this group compared to single stress-exposed groups and the control. There are reports saying that there will be parallel induction of SOD and CAT during heavy metal toxicity stress (Dimitrova *et al*. 1994). In corroboration with our results, Caipang *et al*. (2008) also reported that enzymatic antioxidants responded with a higher expression after 2h of overcrowding in Atlantic cod, and a significantly higher CAT activity was observed in Nile tilapia due to stress mediated through short-term exposure to copper and zinc (Metwally,1998).

Serum lipid profile

Lipid is a major energy source in fish (MacFarlance *et al*. 1990), and during stress, lipids are mobilized to supply energy. This is the reason triglycerides and cholesterol levels in serum are considered indicators of stress (Lupatsch *et al*. 2010). The present study exhibited that the serum total lipid was increased in crowding along with the feed restriction (T3) group and also in the feed restriction (T1) group, which was increased after 24hr, while triglyceride levels were increased in feed restriction, crowding, and crowding with feed restriction groups from 24hr. The increased level of total lipid and triglycerides in stress-exposed groups were due to the mobilization of stored lipids to fulfill the energy demand of the fish (Lupatsch *et al*. 2010). It is well known that during starvation, lipid is utilized to fulfill the energy demand, and triglycerides are preferentially catabolized during this phase without altering the levels of other body lipid components (Love, 1980). Unlike our study, Mathew *et al*. (2013) reported that the triglycerides were considerably decreased in the serum of GIFT tilapia due to crowding stress mediated through high stocking density and a zero-water exchange system. In the present study, the unaltered serum phospholipid levels throughout the experimental period, indicate that phospholipids were not preferred by the fish for catabolism during short term crowding and crowding with feed restriction stress.

Haematological parameters and blood glucose

Changes in various biochemical and physiological parameters can be an excellent indicator of level of stress in fish (Hoseini *et al*, 2011). During crowding stress, haematological changes are considered as stress biomarkers (Barcellos *et al*., 2004; Trenzado *et al*., 2006). The higher haemoglobin content was recorded in this study in both the crowding and crowding along with feed restriction groups compared to other groups. However, the fish with feed restriction alone and the control groups showed similar haemoglobin contents. The increase in haemoglobin content without an increase in RBC numbers in crowding and crowding along with feed restriction groups is due to the increased haemopoiesis as a result of high oxygen demand during stress-induced hypoxic conditions. Akhtar *et al*. (2012) also reported that hypoxia is observed in crowding stress. Nikinma and Rees (2005) reported that hypoxia condition can promote erythropoiesis. Hence, in the present study, the increased haemoglobin content in fish exposed to crowding stress suggests it as a mechanism to cope with stress induced hypoxia.

In the present study, blood glucose level showed no significant differences at 3 h, while there was a significant variation among the groups after 9, 24 and 48 h. The higher blood glucose level in the stress exposed groups compared to the control is indicative of the ability of fish to produce glucose through gluconeogenesis and glycogenolysis during stress. In line with our observation, Ortuño *et al*. (2001) and Vijayan *et al*. (1997) had also reported that short-term exposure of crowding stress in gilthead seabream and tilapia caused significant elevation of blood glucose level. Similarly, in carps, exposure to hypoxia at 10 °C resulted in increased blood glucose after 10 h (Mazeaud *et al*., 1977). The higher blood glucose level in T3 group at 9 h is going in parallel with the increased enzymatic antioxidant status, reduced total protein in blood and increased NBT activity indicate that there was an immediate stress response at 9. To the best of our knowledge there is no literature available to support such variation on short term exposure of crowding and feed restriction in fish.

Conclusion

Based on the observations of the current study, it can be concluded that crowding alone or crowding-with-feed restriction had a strong influence on the antioxidant enzyme system and the serum antioxidants and blood glucose started varying only after 9h of stress exposure. Furthermore, it was noted that crowding and/or crowding-with-feed restriction increased the triglyceride level, thus they can be considered as acute stressors in farms and should be avoided.

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