Biochemical and haematological responses to thermal stress in *Labeo rohita* (Hamilton, 1822) fingerlings

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ABSTRACT

An experiment was conducted to understand the effect of thermal shock on stress and recovery in *Labeo rohita* (Hamilton, 1822) fingerlings. *L. rohita* fingerlings were exposed to a temperature of 38°C for 2 h and permitted to recuperate afterwards for 48 h. Primary and secondary stress parameters were analysed after 2, 4, 8, 12, 24 and 48 h of recovery. The activity of glucose-6-phosphate dehydrogenase and acetylcholine esterase reduced significantly (p<0.05). Erythrocytes, haemoglobin, respiratory burst activity and lysozyme levels increased significantly (p<0.05) by 7.31, 23.91, 109.09 and 70.13% after thermal shock, whereas leucocytes, serum protein, albumin and globulin showed a reverse trend. During recovery, biochemical parameters normalised in 48 h. However, immunological variables such as serum total protein, albumin and globulin, and histopathological changes in gill and kidney tissues did not return to normal condition.

Keywords: Enzymes, Haematology, Histopathology, *Labeo rohita*, Stress recovery, Thermal stress

Introduction

Rohu *Labeo rohita* (Hamilton, 1822) is an economically important aquaculture species in India. The water temperature in freshwater bodies in India goes up to 34-37°C during summer months, which is beyond the optimum temperature for growth of this species (Das et al., 2005). During farming, fish are often exposed to several kinds of stressors, among which temperature is a major one, owing to the poikilothermic nature of fish. Therefore, any change in water temperature directly affects physiology of the fish (Jonassen et al., 1999). Stress responses activate the neuro-endocrine system that alters the body metabolism (Chatterjee et al., 2006). Most commonly studied stress response in fish and other vertebrates are primary responses which are measured as the level of cortisol and catecholamine (Barton and Iwama, 1991). Commonly measured parameters for secondary stress responses are blood glucose and lactate levels (Chatterjee et al., 2006). There is ample literature available on thermal stress responses in fishes; however, there is very limited information on the time required for normalisation of the stress responses and on the histopathological changes due to thermal stress. Therefore, the present study was carried out to understand the level of stress responses against heat shock and duration for recouping to normal state in *L. rohita*.

Materials and methods

Experimental animal

Fingerlings *L. rohita* (mean weight of 6.5±0.05 g) were transported from a private fish hatchery to the laboratory in an open container (500 l) under oxygenation. The stock was acclimatised to laboratory conditions for a month and fed with a commercial diet.

Experimental setup

Ideal water temperature for Indian major carps (*Catla catla*, *L. rohita* and *Cirrhinus mrigala*) is 28°C (Akhtar et al., 2012). However, carps can grow well between the temperature range of 18.3 and 37.8°C (Jhingran, 1975). Therefore, in this study *L. rohita* fingerlings were given heat shock at 38°C. Six fishes maintained at 28°C were used as control. Forty-two fishes were randomly selected and exposed to thermal shock at 38°C for 2 h in a 70 l glass aquarium, using a digital water heater to bring the water temperature to the desired experimental temperature of 38°C. Immediately after 2 h of thermal shock (0 h of recovery), six fishes were sampled. The remaining fishes were transferred back to the acclimatisation tank (28°C) and sampled (n=6) at an interval of 2, 4, 8, 12, 24 and 48 h, of recovery.

Six fish from each samplings were anesthetised with clove oil (50 µl l⁻¹) and blood was collected by
caudal vein puncture and pooled. Thereafter fishes were decapitated. Tissue homogenates of liver, brain, gill and muscle were prepared separately in sucrose solution (0.25 M) and centrifuged at 5000 g for 10 min at 4°C. The supernatants were collected and stored at -20°C for further assays. Blood samples were allowed to clot for 2 h, centrifuged at 3000 g for 5 min at 4°C and then kept at -80°C until use. Pooled blood sample before clotting, was used for blood glucose estimation.

**Enzyme analyses**

Fructose-1, 6-diphosphatase (D-FDP-1-Phosphohydrolase; E.C. 3.1.3.11; FDPase) was assayed according to Freeland and Harper (1959). Adenosine triphosphatase (adenosine triphosphate phosphohydrolase, E.C. 3.6.1.3; ATPase) activity was measured as described by Post and Sen (1967). Phosphate released was estimated at OD of 660 nm (Kaplan, 1953). Glucose-6-phosphate dehydrogenase (α-D-glucose-6 phosphate: NADP oxidoreductase; E.C.1.1.1.49; G6PDH) activity was measured by the method of DeMoss (1953). Alkaline phosphatase (Orthophosphoric monoester phosphohydrolase, E.C.3.1.3.1; ALP) activity was measured according to the procedure detailed by Garen (1960). Acetylcholine esterase (acetyl hydroxylase, E.C.3.1.1.7; AChE) was estimated by the method of Hestrin (1949). Total protein in the sample was determined as per Lowry et al. (1951) for calculating the activity of enzymes. The absorbance of all the samples was measured in a UV-Vis spectrophotometer (E-Merck, Germany).

**Glucose and cortisol**

Estimation of blood glucose was done as per Nelson-Somogii (1945). Cortisol in serum was assessed by radioimmunoassay (RIA) as per the methods of Winberg and Lepage (1998).

**Haematological parameters**

**Blood haemoglobin (Hb)**

Blood Hb level was estimated by cyanmethemoglobin method using Drabkins fluid (Qualigens Diagnostics, Division of Glaxo Smithkline Pharmaceutical Ltd., India). Readings were taken at 540 nm in a spectrophotometer and haemoglobin content in the blood was calculated as:

\[
\text{Haemoglobin (g%) = [OD (T)/OD(S)] X [251/100] X 60}
\]

where, OD (T) = Absorbance of test; OD (S) = Absorbance of standard

**Red blood cells and white blood cell counts**

Total erythrocyte and leucocyte counts were made using leucocyte and erythrocyte diluting fluid (Qualigens, India) in haemocytometer. Twenty microlitre blood was diluted with 3980 μl of diluting fluid in a test tube and mixed well to suspend the cells uniformly. Erythrocytes and leucocyte counts per ml of the blood sample were calculated as:

\[
\text{Number of cells ml}^{-1} = \frac{\text{Number of cells counted x dilution}}{\text{Area counted/Depth of fluid}}
\]

**Serum total protein, albumin (A), globulin (G) and A/G ratio**

Total protein and albumin kit (Qualigens Diagnostics) was used to estimate plasma total protein and albumin following Biuret and bromocresol green (BCG) dye binding method (Reinhold, 1953; Doumas et al., 1971). The value of globulin was calculated by subtracting albumin values from total protein and A/G ratio was calculated by dividing albumin values with globulin values.

**Respiratory burst activity and serum lysozyme activity**

The respiratory burst activity was measured following the method of Secombes (1990). Fifty microlitre each of the blood sample was added to the wells of a U-bottom microtiter plates and incubated at 37°C for 1 h and washed thrice with phosphate buffered saline (PBS). After washing, 50 μl of 0.2% nitroblue tetrazolium (NBT) was added and incubated for 1 h. Adhering cells were fixed with 100% methanol, plates were air dried, and 60 μl of 2 N KOH and 70 μl dimethyl sulphoxide (DMSO) were added. Optical density was measured at 540 nm in an enzyme-linked immunosorbent assay (ELISA) reader.

For analysing serum lysozyme activity, serum sample was diluted with phosphate buffer (pH 7.4) to a final protein concentration of 0.33 mg ml⁻¹. Three millilitre of *Micrococcus luteus* was taken in a cuvet, to which 50 μl of diluted serum sample was added. Optical density was measured in a spectrophotometer at 450 nm exactly 60 s after addition of serum sample and the absorbance was compared with standard lysozyme of known activity.

**Histopathological studies**

Tissue samples were fixed in neutral buffered formalin (NBF), dehydrated in ascending grades of ethanol (70-100%), cleared in xylene, embedded in paraffin wax, and sections of 5 μ thickness were cut using a microtome. The sections were stained with haematoxylin and cosin (H&E) (Roberts, 1989) and observed under light microscope.

**Statistical analysis**

Differences among treatments for different stress responses were tested by one-way ANOVA and comparison.
of mean values was done using Duncan’s multiple range tests at 5% level of significance (p<0.05). All statistical analyses were performed with SPSS 17.0 for Windows.

**Results and discussion**

Data on biochemical parameters analysed are presented in Table 1. Cortisol and blood glucose are the indicators of primary and secondary stress responses respectively (Kumar et al., 2014). A significant surge (p<0.05) in glucose and cortisol by 631.81 and 610%, respectively was noticed after heat shock (Kumar et al., 2014) (Fig. 1a,b). However, both the parameters returned to normal values after 48 h. Significant increase (p<0.05) in the activity of FDPase, ATPase, and ALP and decrease in the activity of G6PDH and AChE were noticed immediately after thermal shock. The activity of FDPase, a gluconeogenic enzyme, increased by 1212.62 and 2048.52% in liver and gill tissues respectively, which would have activated the gluconeogenic pathway to deal with the high energy needed during thermal stress. Further substrate for gluconeogenesis would have been delivered by transaminase activity (Kumar et al., 2014). In the current study, increase in ALP activity by 375.32 and 221.19% in liver and muscle tissues respectively would have caused breakdown of high-energy phosphate bonds that tolerate stress. ATPase hydrolyses the adenosine triphosphate (ATP) to sustain ionic gradient through the plasma membrane (Das, 2002). In the present investigation,

**Table 1.** Glucose-6-Phosphate Dehydrogenase (G6PDH in liver and gill), fructose-1, 6-Diphosphatase (FDPase in liver and gill), alkaline phosphatase (ALP in liver and muscle), adenosine triphosphatase (ATPase in liver and gill) and acetylcholine esterase (AChE in brain) activity in *L. rohita* fingerlings exposed to thermal shock of 38°C for 2 h

| Recovery period (h) | Biochemical parameters |
|---------------------|------------------------|
|                     | Liver | Gill | Liver | Gill | Liver | Muscle | Liver | Gill | Brain |
| Control             | 6.74±0.74 | 5.07±1.75 | 10.78±0.33 | 8.85±0.65 | 25.91±0.01 |
| 0                   | 6.74±0.74 | 5.07±1.75 | 10.78±0.33 | 8.85±0.65 | 25.91±0.01 |
| 2                   | 6.74±0.74 | 5.07±1.75 | 10.78±0.33 | 8.85±0.65 | 25.91±0.01 |
| 4                   | 6.74±0.74 | 5.07±1.75 | 10.78±0.33 | 8.85±0.65 | 25.91±0.01 |
| 8                   | 6.74±0.74 | 5.07±1.75 | 10.78±0.33 | 8.85±0.65 | 25.91±0.01 |
| 12                  | 6.74±0.74 | 5.07±1.75 | 10.78±0.33 | 8.85±0.65 | 25.91±0.01 |
| 24                  | 6.74±0.74 | 5.07±1.75 | 10.78±0.33 | 8.85±0.65 | 25.91±0.01 |
| 48                  | 6.74±0.74 | 5.07±1.75 | 10.78±0.33 | 8.85±0.65 | 25.91±0.01 |

Different superscripts in the same column indicate significant difference (p<0.05) amongst different recovery periods (Duncan’s multiple range test, α = 0.05). Values are expressed as mean ± SE (n = 6). Unit: ∆ 0.01 optical density mg protein⁻¹ min⁻¹ (G6PDH), µg phosphorous released mg protein⁻¹ min⁻¹ at 37°C (FDPase); µM acetylcholine hydrolysed mg protein⁻¹ min⁻¹ at 37°C (ALP); Nanomoles P-nitrophenol released mg protein⁻¹ min⁻¹ at 37°C (AChE); Nanomoles P-nitrophenol released mg protein⁻¹ min⁻¹ at 37°C (ATPase)

**Fig. 1.** (a) Blood glucose level and (b) Serum cortisol level of *L. rohita* fingerlings exposed to thermal shock of 38°C for 2 h and its recovery. Bars with different alphabets indicate significant difference during recovery period (p<0.05). Values are expressed as mean ± SE (n = 6)
ATPase activity increased by 144.30 and 451.86% in liver and gill tissues respectively, which signifies that there was no depletion of ATP after the thermal stress of 38°C for 2 h. Similarly, increased activity of ATPase in response to stress to meet the energy requirement is reported in teleosts by Anderson (2016). G6PDH converts glucose-6-phosphate to 6-phosphogluconolactone in pentose phosphate pathway using NADP⁺ as a coenzyme and produces NADPH. This enzyme activity is expected to increase during high temperature (Verma et al., 2007). However, in the present study, G6PDH activity decreased by 25.64 and 16.66 % in liver and gill tissues respectively after thermal shock, which might be due to the inhibitory action of cortisol on G6PDH (Tripathi and Verma, 2003). Reduction in the activity of AChE by 69.08% in brain tissue is similar to the observation made by Verma et al. (2007) in carp.

Data on total leucocyte count, erythrocyte count, haemoglobin, respiratory burst activity, plasma total protein, albumin, globulin and lysozyme activity are given in Table 2. Total erythrocyte count and haemoglobin level increased by 48.36 and 23.91% after thermal shock and returned to normal levels after 24 h of acclimation. The surge in erythrocyte and haemoglobin might be due to excess release of RBC from spleen into the general circulation to cope up with thermal stress by increasing the oxygen-carrying capacity. A similar observation was made by Randall and Perry (1992) due to acute stress in fishes. However, reduction in RBC and haemoglobin has been reported in L. rohita by Verma et al. The total plasma protein, A, G and its ratio decreased significantly (p<0.05) by 15.67, 17.68, 13.30 and 5.08% respectively after thermal shock, which is in agreement with the observations of Akhtar et al. (2012) in L. rohita exposed to thermal and salinity stress. Such reductions might be due to the mobilisation of these compounds to fulfill energy requirement to cope with stress condition (Akhtar et al., 2012).

Histopathological changes are widely used as biomarkers in the evaluation of the health status of fish (Camargo and Martinez, 2007). Histopathology of gills describes the general health condition of fish and quality of water (Peters et al., 1984). Thermal shock of 2 h did not affect liver tissue architecture in most of the fishes examined during the present study (Fig. 2), however in some fishes, the liver showed congested blood vessels (Fig. 3). After 48 h, liver tissues appeared normal in architecture. Kidney tissue, after thermal shock showed congested blood vessels, vasodilatation and thickening of...

### Table 2. Levels of white blood cells (WBC), red blood cells (RBC), haemoglobin (HG), respiratory burst activity in NBT assay, total serum protein, albumin (A), globulin (G), A/G ratio and lysozyme activity of L. rohita fingerlings exposed to thermal shock of 38°C for 2h

| Recovery period (h) | WBC | RBC | HG | NBT | Total protein | Albumin (A) | Globulin (G) | A/G ratio | Lysozyme |
|---------------------|-----|-----|----|-----|--------------|-------------|--------------|-----------|----------|
| Control             | 0.451 ± 0.05 | 1.22 ± 0.01 | 8.07 ± 0.09 | 0.11 ± 0.00 | 5.74 ± 0.08 | 3.11 ± 0.01 | 2.63 ± 0.08 | 1.18 ± 0.01 | 221.23 ± 3.38 |
| 0                   | 0.353 ± 0.01 | 1.81 ± 0.03 | 10.00 ± 0.00 | 0.23 ± 0.01 | 4.84 ± 0.03 | 2.56 ± 0.06 | 2.28 ± 0.04 | 1.12 ± 0.04 | 740.74 ± 2.11 |
| 2                   | 0.21 ± 0.01 | 1.66 ± 0.01 | 9.57 ± 0.00 | 0.22 ± 0.00 | 4.72 ± 0.01 | 2.36 ± 0.02 | 2.06 ± 0.03 | 1.15 ± 0.03 | 689.48 ± 5.61 |
| 4                   | 0.216 ± 0.06 | 1.56 ± 0.01 | 9.14 ± 0.03 | 0.19 ± 0.00 | 4.13 ± 0.01 | 2.21 ± 0.01 | 1.92 ± 0.01 | 1.15 ± 0.01 | 604.21 ± 4.01 |
| 8                   | 0.171 ± 0.01 | 1.52 ± 0.00 | 9.77 ± 0.05 | 0.18 ± 0.00 | 4.10 ± 0.00 | 2.17 ± 0.00 | 1.92 ± 0.00 | 1.13 ± 0.01 | 533.34 ± 2.12 |
| 12                  | 0.170 ± 0.02 | 1.44 ± 0.01 | 9.39 ± 0.03 | 0.13 ± 0.01 | 3.92 ± 0.03 | 2.09 ± 0.01 | 1.83 ± 0.03 | 1.14 ± 0.02 | 485.94 ± 3.19 |
| 24                  | 0.396 ± 0.03 | 1.28 ± 0.01 | 8.47 ± 0.03 | 0.12 ± 0.01 | 3.69 ± 0.02 | 1.91 ± 0.04 | 1.56 ± 0.05 | 1.22 ± 0.07 | 264.79 ± 8.90 |
| 48                  | 0.449 ± 0.03 | 1.19 ± 0.01 | 8.49 ± 0.03 | 0.11 ± 0.00 | 3.47 ± 0.02 | 1.24 ± 0.01 | 2.41 ± 0.03 | 0.51 ± 0.02 | 224.39 ± 6.41 |

Different superscripts in the same column indicate significant difference (p<0.05) among different recovery periods (Duncan’s multiple range test, α = 0.05). Values are expressed as mean ± SE (n = 6). Unit: RBC (x 10³ cells mm⁻³), WBC (x 10³ cells mm⁻³), haemoglobin content (g dl⁻¹), NBT (absorbance at 540 nm), serum total protein (g dl⁻¹), albumin (g dl⁻¹), globulin (g dl⁻¹), lysozyme activity (unit min⁻¹ mg serum protein⁻¹)
Fig. 2. Liver tissue section of *L. rohita* showing normal architecture and staining characteristics of the hepatocytes (H&E; X160)

Fig. 3. Liver tissue section of *L. rohita* exposed to thermal shock of 38°C for 2 h showing congested blood vessels (arrow) (H&E; X160)

Fig. 4. Kidney tissue of *L. rohita* fingerlings exposed to thermal shock of 38°C for 2 h showing thickening of arterial wall (large arrow), endothelial cells sloughing (arrow head) and vasodilatation (small arrow) (H&E; X160)

Fig. 5. Gill tissue of *L. rohita* fingerlings exposed to thermal shock of 38°C for 2 h showing lamellar tissue destruction (arrow) (H&E; x40)

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arterial wall and sloughing of endothelial cells (Fig. 4). Gill tissue of fishes exposed to heat shock showed lamellar tissue destruction and mild hyperplasia at the base of gill lamellae (Fig. 5). Similar observation was made by Solid et al. (2005) in *Carassius carassius*. Degeneration of lamellar tissue could be attributed to direct heat shock, which is in par with the observation of Temmink et al. (1983) in rainbow trout.

In conclusion, acute thermal stress noticeably affects the physiology and stress responses of *L. rohita* fingerlings. During recovery, biochemical parameters recouped to normal levels in 48 h post-thermal shock. However, immunological variables such as serum total protein, albumin and globulin and histopathological changes in gill and kidney tissues did not return to normal conditions in 2 h. These findings have ecological significance from the perspective of environmental changes and global warming.

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