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Elevation in Rearing Temperature within Optimum Range Mitigate Immunosuppressive and Metabolic Stress Effect of High or Low Dietary Protein Level in *Labeo Rohita* Fingerlings

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Abstract

In the present study, *Labeo rohita* fingerlings were maintained either at ambient water temperature (26ºC) for five weeks or exposed to 32ºC for one week then later maintained at 26ºC for four weeks. Fingerlings reared under different temperature regimes were fed with any of the four experimental diets containing 20, 30, 40 or 45% protein. Serum cortisol level was higher at 26ºC compared to 32ºC, and decrease with the increase in dietary protein level up to 40%. Fingerlings fed 30% and 40% protein recorded similar WBC_S count and respiratory burst activity which was lower and higher respectively compared to 20% and 45% protein fed fingerlings. Correspondingly, lower WBC_S count and higher NBT were recorded following exposure to higher temperature (32ºC) for one week compared to 26ºC exposure group. Significantly lower survival was recorded in groups fed with lowest (20%) and highest (45%) dietary protein level whereas fingerlings exposed to 32ºC for one week exhibited higher survival (%) compare to 26ºC. Present results indicate that both lower and higher level of dietary protein may cause metabolic stress to fingerlings, as might consequently lead to the depressed immunity and exposure of elevated temperature (32ºC) for one week mitigates this immunosuppressive effect.

Introduction

Protein is a major constituent of fish tissues and organs, which also act as the precursors of other nitrogenous compounds like enzymes, hormones, neurotransmitters, cofactors, etc. beside as an important energy source for fish. Therefore, a consistent dietary intake of protein is required by the fish to build new proteins. Amino acids (AA) are building blocks for protein which regulate key metabolic pathways that are crucial to maintenance, growth, reproduction, and immune responses (Li et al., 2009). Thus, inadequate protein levels in the diet results in reduction of growth as well as immune response also. Although, the dietary protein level and their relationship to immunological function in mammals have been widely studied, only a few attempts have been made to study the interactions of protein levels with water temperature in fish immunity (Thomas & Woo, 1990; Kiron et al., 1993).

Growth rate and growth efficiency of rohu (*Labeo rohita*), a popular Indian major carp, have been shown to be temperature dependent (Kumar et al., 2013) which is also known to have strong influences on the immunity of fish (Schreck, 1996). Ambient temperature is a critical factor in the development of host immunity (Watts et al., 2001) and fluctuation in the temperature can have an effect on the immune response (Lillehaug, 1993). Regardless of the fish species examined, elevated

water temperature that remains within the physiological range of the species has been shown to alter immune function (Bly and Clem, 1992).

There is an intense co-ordination that exists between teleost fish and their environment. In general, fish possess a body temperature that is essentially regulated by the temperature of the surrounding water (Fry, 1967); as such their entire physiology, including immune function, is influenced by the water temperature. Therefore, an increase in the water temperature may enhance the metabolic rate and hence increase the growth and immunity of fish. But continuous exposure to the elevated temperature may not be economically and commercially feasible due to the high operational cost involved. Hence, metabolic activity may be triggered by exposure to elevated temperature for few days, which may continue to persist for certain duration and tend to enhance the growth and the immunity responses. During such circumstances of exposure to elevated temperature, feeding the fish with higher level of protein may complement the growth and immunity.

In our previous study (Kumar et al., 2013), we revealed that metabolic activities in *L. rohita* fingerlings are triggered by exposing the fish to an elevated temperature (32°C) for one week and the metabolic rate prolonged for 3 weeks during which the 40% dietary protein level was found to support better growth rate than the either lower or higher dietary protein levels. However, the effect of change in water temperature and protein level in the diet on metabolic stress and immunological response is still lacking. Therefore, the present investigation, paralleling our previous study (Kumar et al., 2013), was performed to provide a comprehensive investigation on the impact of short term exposure to elevated temperature under different dietary protein regime on metabolic stress and immune response of *L. rohita* fingerlings.

Material and Methods

Diets

Four isocaloric diets were formulated with crude protein (CP) level of 20, 30, 40 or 45%. All ingredients except gelatin, vitamin and mineral mixture, and vitamin C were mixed in a big plastic bowl. Gelatin crystals were mixed in lukewarm water so as to form a gel. The ingredients were then mixed with gelatin form dough with the addition of necessary quantity of water. The dough was then kept for 1 h for proper conditioning followed by steaming for 20 min in a pressure cooker. The vitamin mineral mixture and vitamin C were mixed after cooling. Pellets were prepared by hand pelletizer having 2 mm diameter size. Finally, the pellets were air dried for some time and kept in oven at 50°C till complete drying. After drying, the pellets were packed in airtight polythene bags and labeled properly. Ingredient compositions of the experimental diets are presented in Table 1.

Experimental Animals

Fingerlings of *Labeo rohita* were brought from Prem Fisheries Consultancy, Gujarat, India to the Fish Nutrition and Biochemistry laboratory, CIFE (Mumbai,

Table 1. Feed formulation of the different experimental diets.

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

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

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

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

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

[d] Vitamin-mineral mix (Emix™ plus) (quantity/2.5kg): Vitamin A-55,00,000 IU; Vitamin D_3-11,00,000 IU; Vitamin B_2-2,000 mg; Vitamin E-750 mg; Vitamin K-1,000 mg; Vitamin B_6-1,000 mg; Vitamin B_{12}- 6 mg; Calcium panthothenate-2,500 mg; Niacinamide-10 gm; Choline chloride-150 gm; Mn-27,000 mg; Iodine-1,000 mg; Fe-7,500 mg; Cu-2,000; Zn-5,000 mg; Co- 450 mg; Ca- 500 g; P-300 g; Se-50 ppm; L-Lysine-10 g; DL-methionine-10g.

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

CP: Crude protein

* Digestible energy (DE) (Kcal/100 g) = CP (%) x 4 + EE (%) x 9 + TC (%) x 4 (Halver, 1976)

India). The stock was acclimatized under aerated conditions at ambient temperature (26ºC) for a period of 15 days and was fed with a practical diet containing 30% crude protein.

Experimental Design

Six hundred *Labeo rohita* fingerlings (average weight 6.78±0.05 g) were randomly distributed into eight treatment groups with three replicates each, following a completely randomized design in 24 tanks (150 L). Half of the experimental groups were maintained at ambient temperature (26ºC), whereas other half were exposed to 32ºC by using thermostatic water heater (General Trading Corporation, Mumbai, India). After one week, all experimental groups were maintained at ambient temperature (26ºC). The water temperature of the treatments exposed to 32ºC was decreased to 26ºC within 24 hrs. Continuous aeration was provided to all the tanks from a compressed air pump and water was exchanged every other day. The experiment was continued for five weeks. During this period the groups were fed twice a day (08:00 and 18:00 h) to near satiation with either 20%, 30%, 40% or 45% CP. Samplings were done at first, third and fifth weeks for different parameters.

Sampling and Analysis of Samples

During each sampling, six fingerlings from each tank i.e eighteen fingerlings from each treatment group were randomly anaesthetized with clove oil (50 µl L^{-1}) and blood was collected from the caudal vasculature of all fingerlings using a medical syringe. Out of six, blood of three fingerlings was collected by medical syringe rinsed with 2.7% ethylene di-amine tetra acetic (EDTA) solution and transferred immediately to an eppendorf tube containing a pinch of EDTA powder (as an anticoagulant). This blood sample was used for the determination of all hematological parameters and respiratory burst activity. For serum, blood of rest three fingerlings were withdrawn by medical syringe without having rinsed with anticoagulant and allowed to stay in a tilted position for 1 h to collect the serum, which were used subsequently for the estimation of serum proteins, cortisol, triglyceride, cholesterol, urea, creatinine and enzyme activities (creatinine kinase, lactate dehydrogenase and alkaline phosphatase). Blood or serum samples collected from fish of each tank were pooled in one eppendrof tube. Proximate composition of the feed was analysed by standard methods (AOAC, 2000).

Hematological Parameters

Total erythrocytes (RBC) and leukocytes (WBC) were counted in a haemocytometer using erythrocyte and leucocyte diluting fluids (Qualigens, India), respectively. The following formula was used to calculate the number of erythrocytes and leukocytes per ml of the blood sample: Number of cells ml^{-1} = (Number of cells counted × dilution) / (Area counted × depth of fluid) (Kumar et al., 2005). The haemoglobin (Hb) percentage was determined by estimating cyanmethemoglobin using Drabkin's fluid (Qualigens, India).

The mean cell volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated as follows:

$$MCV = (Hematocrit\ (Hct)/RBC) \times 10,\ MCH = (Hb / RBC) \times 10,\ MCHC = (Hb \times Hct) \times 100$$

Hematocrit (Hct) and differential count were analysed by using Abacus Hematology Analyzer (Fully Automated 3 Part Differential Counter, Austria).

Respiratory Burst Activity

Respiratory burst activity of phagocytes was quantified by using the reduction of nitroblue etrazolium (NBT) to formazon as a measure of superoxide anion (O^{2-}) production (Secombe, 1990; Stasiack and Baumann, 1996).

Serum Proteins

Serum protein was estimated by the Biuret and BCG dye binding method (Reinhold, 1953) using a kit (total protein and albumin kit, Qualigens Diagnostics, Glaxo Smithkline). Albumin was estimated by the bromocresol green binding method (Doumas et al., 1971). Globulin was calculated by subtracting albumin values from total serum protein. A/G ratio was calculated by dividing albumin values by globulin values.

Serum Cortisol

Cortisol in fish serum was estimated by using a validated radioimmunoassay (EIAKIT DSL -10-2000) kit method. The kit was purchased from Diagnostic Systems Laboratories, Mumbai. Serum cortisol was expressed as ng ml^{-1}.

Serum Creatinine

Serum creatinine was estimated by the method described by Yatzidis (1974). To each of sample tubes, 0.10-ml aliquots of serum sample and 0.75 ml of the alkaline picrate reagents pH 9.5, respectively, were added. The solution was kept for 45 min at 37°C and measured at 500 nm against a blank prepared with 0.10 ml of water. A standard curve was prepared in the same way, with use of the aqueous creatinine standard solution.

Serum Triglycerides, Cholesterol and Urea

Serum triglycerides were estimated using triglyceryl reagent GPO kit (Qualigens Fine Chemicals, Mumbai, India) and serum cholesterol was estimated by enzymatic method using cholesterol kit (Qualigens Fine Chemicals, Mumbai, India) and both were expressed as g%. Serum urea was estimated by Berthelot method using kit provided by Qualigens Fine Chemicals, Mumbai, India and the quantity of urea was expressed as g%.

Serum Enzyme Assay

Creatine kinase (ATP creatine N-phosphotransferase; EC 2.7.3.2) activity in serum was determined using CK-NAC kit (NAC activated method, kinetic, Erba Mannheim, Transasia Bio-medicals, Daman, India). The activity was expressed as IU/L.

Serum lactate Dehydrogenase (L- Lactate: NAD+ oxidoreductase; E.C.1.1.1.27) was estimated using the kit (LD F245 CH) provided by Chema Diagnostica following the "Sample as Starter" Procedure. The activity was expressed as U/L.

Serum alkaline phosphatase (Orthophosphoric monoester phosphohydrolase, E.C. 3.1.3.1) activity was estimated using the kit provided by Qualigens Fine Chemicals, Mumbai, India following the p-nitrophenyl phosphate (PNPP) Procedure. The activity was expressed as IU/L.

Challenge Study

After 5 weeks of feeding trial, eighteen fish from each experimental group were challenged with virulent *Aeromonas hydrophila* obtained from Fish Pathology and Microbiology Division, Central Institute of Fisheries Education (CIFE), Mumbai. First the pathogenic isolates of *A. hydrophila* were grown on nutrient broth for 24 hr at 30°C in Bio-oxygen demand (BOD) incubator. The *A. hydrophila* cells were harvested by centrifuging the culture broth at 10,000 rpm for 10 min at 4°C. The cells were then washed thrice in sterile phosphate buffered saline (PBS) (pH 7.2) and finally maintained in PBS at a concentration of 1×10^8 CFU (colony forming unit) cells ml^{-1}. The fish in each experimental group were intra-peritonealy injected 0.1 ml of bacterial suspension. Mortality was observed for seven days post-challenge and survival percentage was calculated. After seven days of post-challenge, blood was withdrawn from the survived fish of all treatment groups to analyse the hematological parameters, respiratory burst activity and serum parameters. Before challenge, the dose of *A. hydrophila* necessary for challenge of fish was determined in pre challenge experiment. In the pre challenge experiment, group of 20 fish were given 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 ml of 1×10^8 CFU cells ml^{-1} by intraperitoneal injection. Bacterial doses resulting in 50-60% accumulated mortality (0.1 ml) were chosen for challenge.

Statistical Analyses

The main effect was analysed by using two-way analysis of variance (ANOVA) with protein level (20%, 30%, 40% and 45%) and temperature (26ºC and 32ºC) as two fixed factors. Where significant interactions were found between main effects, a one-way ANOVA was used to compare simple effects. When results were significant, comparison between means were made using the Duncan's multiple range test (DMRT). Means were considered significant at $P<0.05$. Statistical evaluation of the data was carried out using the software SPSS version 14.0 (SPSS, Richmond, CA, USA).

Results

Total Leucocyte Count (WBCSS)

Both temperature and dietary protein level had significant effect on the WBCSS counts of fingerlings (Table 2). Lowest WBCSS count was recorded in the treatment groups fed with 30% and 40% dietary protein compared to 20% and 45%, irrespective of the temperature exposure during pre challenge period (Figure 1A). Regardless of the dietary protein level, increase in temperature significantly decrease ($P<0.05$) the WBCSS count (Figure 2A) and the decreased count continued for next four weeks after lowering the temperature from 32ºC to 26ºC. There was a significant ($P<0.05$) interaction between temperature and dietary protein level. Post challenge proliferation of WBCSS count was visible in all treatment groups.

Differential Count

The 30% and 40% CP fed groups showed similar lymphocyte count which was significantly lower ($P<0.05$) than 20% and 45% CP fed groups in both the temperature. Challenge with *Aeromonas hydrophila* significantly reduced ($P<0.05$) the lymphocyte count in 30% and 40% CP fed groups than the other groups irrespective of the temperature exposure. The 20% and 45% dietary protein level fed groups showed similar monocyte count which was significantly lower ($P<0.05$) than 30% and 40% CP fed groups in both the temperature exposure groups. After challenge with *Aeromonas hydrophila*, monocyte count significantly augmented ($P<0.05$) in 30% and 40% CP fed groups. The granulocyte content also exhibited the same trends as for the lymphocyte and monocytes.

Total Erythrocyte Count (RBC), Hemoglobin Content (Hb) and Hematocrit Value (Hct)

The RBC count, Hb content and Hct value of different experimental groups was not affected either due to dietary protein or temperature exposure

Table 2. WBCSS count, differential count and respiratory burst activity (NBT) in *L. rohita* fingerlings exposed to short term elevated temperature and fed diet differing in protein level.

Temp.	26ºC				One week at 32ºC then at 26ºC				Two Way ANOVA Variation Source		
Treatment	20%CP	30%CP	40%CP	45%CP	20%CP	30%CP	40%CP	45%CP	Temp.	Protein	Inter.
					WBCSS (10^3 cells mm^{-3})						
1st week	$162.5^{a}\pm4.5$	$145.8^{b}\pm1.7$	$144.8^{b}\pm2.8$	$165.5^{a}\pm3.5$	$146.7^{b}\pm2.6$	$117.5^{c}\pm0.9$	$117.8^{c}\pm2.6$	$145.6^{b}\pm7.7$	P<0.05	P<0.05	P<0.05
3rd Week	$161.0^{a}\pm4.0$	$146.0^{b}\pm2.0$	$145.0^{b}\pm2.0$	$159.5^{a}\pm4.5$	$145.0^{b}\pm5.0$	$115.0^{c}\pm2.0$	$112.5^{c}\pm2.5$	$144.5^{b}\pm4.5$	P<0.05	P<0.05	P<0.05
5th week	$158.0^{Ya}\pm3.5$	$143.0^{Yb}\pm3.0$	$145.0^{Yb}\pm2.0$	$167.0^{Ya}\pm2.0$	$148.0^{Yb}\pm4.0$	$119.5^{Yc}\pm2.5$	$116.0^{Yc}\pm4.0$	$148.0^{Yb}\pm2.0$	P<0.05	P<0.05	P<0.05
Post challenge	$172.5^{Xa}\pm2.5$	$162.5^{Xb}\pm2.5$	$164.5^{Xb}\pm2.5$	$175.0^{Xa}\pm3.0$	$157.5^{Xbc}\pm2.5$	$134.0^{Xc}\pm4.0$	$130.5^{Xd}\pm1.5$	$159.5^{Xbc}\pm2.5$	P<0.05	P<0.05	P<0.05
					Lymphocyte (%)						
1st week	$88.1^{a}\pm1.1$	$77.8^{b}\pm1.7$	$77.0^{bc}\pm2.0$	$88.3^{a}\pm2.1$	$82.0^{ab}\pm2.0$	$70.8^{c}\pm1.3$	$71.0^{c}\pm2.0$	$82.5^{ab}\pm2.5$	P<0.05	P<0.05	P<0.05
3rd Week	$86.5^{a}\pm1.5$	$78.5^{bc}\pm1.5$	$75.5^{cd}\pm2.5$	$86.5^{a}\pm1.5$	$84.0^{ab}\pm1.0$	$72.0^{d}\pm1.0$	$72.5^{cd}\pm2.5$	$84.0^{ab}\pm2.0$	P<0.05	P<0.05	P<0.05
5th week	$86.5^{ab}\pm2.5$	$79.0^{Xbc}\pm1.0$	$76.0^{Xc}\pm3.0$	$88.0^{a}\pm2.0$	$86.0^{ab}\pm2.0$	$74.0^{Xc}\pm1.0$	$75.0^{Xc}\pm2.5$	$86.5^{ab}\pm3.5$	NS	P<0.05	P<0.05
Post challenge	$83.0^{a}\pm2.0$	$72.0^{Yb}\pm2.0$	$70.0^{Yb}\pm3.0$	$84.0^{a}\pm1.0$	$82.0^{ab}\pm1.0$	$66.0^{Yc}\pm1.0$	$68.0^{Ybc}\pm2.0$	$82.5^{a}\pm2.5$	NS	P<0.05	P<0.05
					Monocyte (%)						
1st week	$7.6^{c}\pm0.7$	$14.1^{b}\pm1.2$	$13.5^{b}\pm1.1$	$7.8^{c}\pm0.9$	$9.1^{c}\pm0.3$	$16.1^{ab}\pm0.6$	$16.5^{a}\pm0.6$	$9.2^{c}\pm0.8$	P<0.05	P<0.05	P<0.05
3rd Week	$7.5^{b}\pm0.5$	$14.5^{a}\pm1.5$	$13.8^{a}\pm0.8$	$7.8^{b}\pm0.8$	$9.2^{b}\pm0.8$	$16.5^{a}\pm0.5$	$16.0^{a}\pm0.5$	$8.8^{b}\pm0.8$	P<0.05	P<0.05	P<0.05
5th week	$7.4^{b}\pm0.8$	$13.0^{Ya}\pm1.0$	$14.0^{Ya}\pm1.0$	$7.5^{b}\pm0.7$	$8.5^{b}\pm0.7$	$15.5^{Ya}\pm0.7$	$14.9^{Ya}\pm0.9$	$7.8^{b}\pm0.8$	P<0.05	P<0.05	P<0.05
Post challenge	$7.9^{b}\pm0.8$	$16.0^{Xa}\pm1.0$	$17.0^{Xa}\pm1.0$	$9.5^{b}\pm0.5$	$9.9^{b}\pm0.4$	$18.5^{Xa}\pm0.5$	$18.0^{Xa}\pm1.5$	$8.8^{b}\pm0.8$	P<0.05	P<0.05	P<0.05
					Granulocyte (%)						
1st week	$4.3^{c}\pm0.4$	$8.2^{bc}\pm0.6$	$9.6^{b}\pm0.9$	$3.9^{c}\pm0.1$	$8.9^{ab}\pm0.6$	$13.1^{a}\pm0.8$	$12.6^{ab}\pm1.1$	$8.3^{bc}\pm0.7$	P<0.05	P<0.05	P<0.05
3rd Week	$6.0^{c}\pm0.3$	$7.0^{bc}\pm0.5$	$10.8^{ab}\pm0.7$	$5.8^{c}\pm0.4$	$6.8^{bc}\pm0.2$	$11.5^{a}\pm0.5$	$11.5^{a}\pm1.0$	$7.3^{abc}\pm0.2$	NS	P<0.05	P<0.05
5th week	$6.2^{bc}\pm0.6$	$8.0^{Yab}\pm0.2$	$10.0^{a}\pm1.0$	$4.5^{c}\pm0.5$	$5.5^{c}\pm0.5$	$10.5^{Ya}\pm1.1$	$10.1^{Ya}\pm0.9$	$5.8^{c}\pm0.3$	NS	P<0.05	P<0.05
Post challenge	$9.1^{bcd}\pm0.2$	$12.0^{Xabc}\pm1.0$	$13.0^{ab}\pm1.2$	$6.5^{d}\pm0.5$	$8.2^{cd}\pm0.7$	$15.5^{Xa}\pm1.5$	$14.0^{Xa}\pm0.5$	$8.7^{bcd}\pm0.7$	P<0.05	P<0.05	P<0.05
					NBT (OD at 540 nm)						
1st week	$0.24^{c}\pm0.01$	$0.32^{b}\pm0.02$	$0.35^{b}\pm0.01$	$0.22^{c}\pm0.01$	$0.26^{c}\pm0.01$	$0.42^{a}\pm0.01$	$0.43^{a}\pm0.02$	$0.26^{c}\pm0.01$	P<0.05	P<0.05	P<0.05
3rd Week	$0.23^{c}\pm0.01$	$0.33^{b}\pm0.02$	$0.34^{b}\pm0.01$	$0.21^{c}\pm0.01$	$0.22^{c}\pm0.01$	$0.42^{a}\pm0.02$	$0.43^{a}\pm0.02$	$0.22^{c}\pm0.01$	P<0.05	P<0.05	P<0.05
5th week	$0.21^{Xc}\pm0.01$	$0.34^{b}\pm0.02$	$0.35^{b}\pm0.01$	$0.20^{Xc}\pm0.01$	$0.22^{Xc}\pm0.01$	$0.39^{a}\pm0.01$	$0.38^{a}\pm0.01$	$0.21^{Xc}\pm0.01$	P<0.05	P<0.05	P<0.05
Post challenge	$0.15^{Yc}\pm0.01$	$0.32^{b}\pm0.01$	$0.33^{b}\pm0.01$	$0.13^{Yd}\pm0.01$	$0.17^{Yc}\pm0.01$	$0.38^{a}\pm0.01$	$0.37^{a}\pm0.01$	$0.15^{Ycd}\pm0.01$	P<0.05	P<0.05	P<0.05

CP: Crude protein; NS: Not significantly different

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

Mean values in a column (5th week and post challenge) under each parameter bearing different superscript (X, Y) differ significantly (P<0.05).

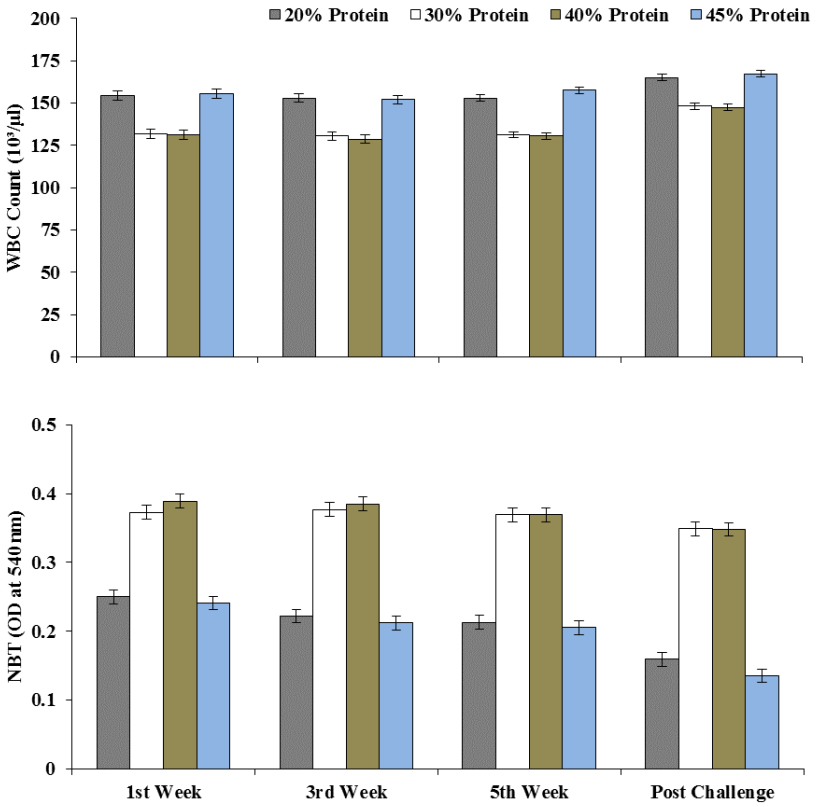

Figure 1. Figure 1: Effect of dietary protein level on WBCSS count [A] and respiratory burst acrivity (NBT) [B] in *Labeo rohita*. Significant differences indicated with (*) were determined using Duncan's multiple range test (DMRT) (P<0.05).

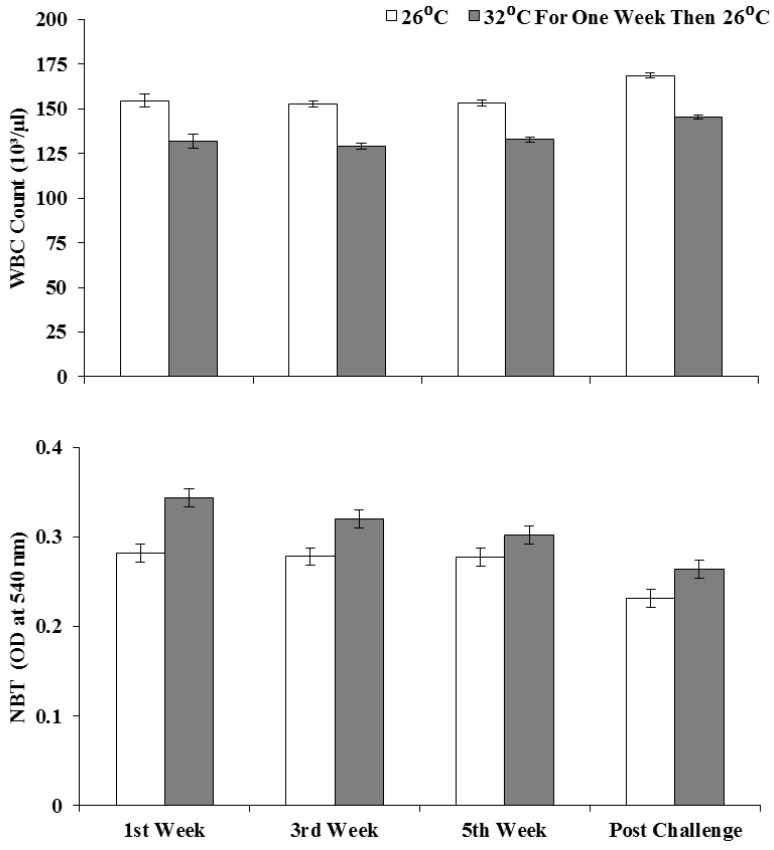

Figure 2. Effect of temperature on WBCSS count [A] and respiratory burst acrivity (NBT) [B] in *Labeo rohita*. Significant differences indicated with (*) were determined using Duncan's multiple range test (DMRT) (P<0.05).

(Table 3). Challenge with *A. hydrophila* significantly reduced (P<0.05) the RBC count, Hb content and Hct value of different experimental groups at both the temperature.

MCV, MCH and MCHC

The Mean cell volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) are presented in Table 4. After challenge with *A. hydrophila,* the MCV value significantly decreased whereas no effect on MCH and MAHC value was observed.

Respiratory Burst Activity (NBT)

The respiratory burst activities of various experimental groups are presented in Table 3. Treatments fed with 30% and 40% dietary protein level registered similar NBT value which was significantly higher (P<0.05) than the 20% and 45% dietary fed protein either at 26ºC or 32ºC of exposure (Figure 1B). Significantly decrease in the NBT value was found in the treatment fed with 20% and 45% CP fed groups after challenge with *Aeromonas hydrophila* than their pre challenge counterpart (Table 3). Fingerlings exposed to elevated temperature (32ºC) for one week registered significantly higher (P<0.05) NBT value compare to fingerlings exposed to 26ºC (Figure 2B).

Serum Total Protein, Albumin (A), Globulin (G) and A/G Ratio

Irrespective of temperature, different level of dietary protein had significant effect (P<0.05) on serum total protein, albumin and globulin in *L. rohita* fingerlings (Table 4). Fingerlings fed with 30% dietary protein irrespective of temperature exposure registered highest (P<0.05) serum total protein, albumin and globulin, whereas lowest (P<0.05) content in 45% dietary protein fed groups. Similar A/G ratio was found in the fingerlings fed with 30%, 40% and 45% dietary protein which was significantly higher (P<0.05) than 20% dietary protein fed fingerlings (Table 5). Fingerlings challenged with *A. hydrophila* registered significantly decreased (P<0.05) serum total protein and globulin content whereas albumin content was unaffected. Significant reduction in the A/G ratio of 20%, 40% and 45% CP fed group was found due to challenge with *A. hydrophila*.

Serum Cortisol

The protein levels in the diet as well as the exposure of fish to the elevated water temperature had significant effect *(*P<0.05) on the serum cortisol level of *L. rohita* fingerlings. However, the temperature was effective only for the first week. The cortisol level was significantly lower (P<0.05) at elevated temperature exposure of 32ºC compare to 26ºC and the reduced cortisol level increased after two week of decrease in temperature from 32ºC to 26ºC. Irrespective of temperature, the increase in dietary protein level from 20% to 40% significantly decreased (P<0.05) the serum cortisol level, whereas significantly higher cortisol level was observed in 45% CP fed group as compare to the 40% CP fed group. During post-challenge period the protein level alone had significant (P<0.05) effect on serum cortisol level among different treatments (Table 6).

Serum Creatinine

Serum creatinine level in *L. rohita* fingerlings was neither affected due to temperature nor protein level (Table 2). However, creatinine content of post challenge fingerlings exhibited significantly lower than in their pre challenge period.

Serum Enzyme

The serum creatine kinase (CK), lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) activity in *L. rohita* fingerlings were also not affected neither due to exposure of elevated temperature nor dietary protein level (Table 2). Significantly higher (P<0.05) post challenge CK, LDH and ALP activity in the serum was observed (Table 2).

Serum Triglycerides, Cholesterol and Urea

Exposure of elevated temperature significantly affected the serum triglycerides and cholesterol level of fingerlings whereas no effect was observed on serum urea level (Table 5). The serum triglycerides and cholesterol content were significantly higher (P<0.05) in the fingerlings exposed to elevated temperature (32ºC) and the increased triglycerides and cholesterol content was significantly reduced (P<0.05) after 4 week of the decrease of temperature from 32ºC to 26ºC (Table 6). Irrespective of temperature, different levels of dietary protein had significant effect on serum triglycerides, cholesterol and urea content in *L. rohita* fingerlings (Table 6).

Survival (%)

After injection with *Aeromonas hydrophilla*, the first mortality was recorded after 24 h. Mortality was recorded up to 7 days after injection. Fingerlings fed with 30% and 40% dietary protein registered highest (P<0.05) survival (%) compared to fingerlings fed with 20% and 45% dietary protein (Figure 3). Irrespective of dietary protein level, fingerlings exposed to 32ºC for one week registered significantly higher survival (%) (Figure 4).

Table 3. RBC count, hemoglobin, hematocrit (hct), mean cell volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) in *L. rohita* fingerlings exposed to short term elevated temperature and fed diet differing in protein level.

Temp.	26ºC				One week at 32ºC then at 26ºC				Two Way ANOVA Variation Source		
Treatment	20%CP	30%CP	40%CP	45%CP	20%CP	30%CP	40%CP	45%CP	Temp.	Protein	Inter.
					RBC (10^6 cells mm^{-3})						
1st week	1.85±0.1	2.16±0.1	2.13±0.1	2.05±0.1	2.01±0.1	2.01±0.1	1.90±0.1	2.19±0.1	NS	NS	NS
3rd Week	1.99±0.1	2.11±0.1	2.12±0.1	2.01±0.2	1.96±0.0	2.02±0.1	2.03±0.2	1.99±0.1	NS	NS	NS
5th week	1.97^{X}±0.1	2.07^{X}±0.1	2.07^{X}±0.1	1.99^{X}±0.1	1.93^{X}±0.1	2.09^{X}±0.1	2.06^{X}±0.1	1.95^{X}±0.1	NS	NS	NS
Post challenge	1.76^{Y}±0.1	1.79^{Y}±0.2	1.68^{Y}±0.1	1.74^{Y}±0.1	1.69^{Y}±0.1	1.74^{Y}±0.1	1.72^{Y}±0.1	1.70^{Y}±0.1	NS	NS	NS
					Hemoglobin (gm%)						
1st week	9.93±0.1	10.90±1.0	10.65±0.6	10.55±1.1	9.70±0.4	10.80±0.9	10.50±1.2	9.95±0.5	NS	NS	NS
3rd Week	10.05±0.3	10.90±0.5	10.20±0.6	9.95±0.9	10.50±0.6	10.45±1.2	10.05±0.9	10.50±0.8	NS	NS	NS
5th week	10.85^{X}±0.3	10.50^{X}±.7	10.00^{X}±0.4	10.35^{X}±1.1	10.40^{X}±0.8	10.25^{X}±0.9	10.35^{X}±1.2	10.70^{X}±0.8	NS	NS	NS
Post challenge	8.65^{Y}±0.6	8.30^{Y}±0.7	7.80^{Y}±0.4	8.20^{Y}±0.6	8.30^{Y}±0.5	8.30^{Y}±0.7	8.40^{Y}±0.6	8.50^{Y}±0.5	NS	NS	NS
					Hct (%)						
1st week	23.25±2.1	24.80±1.6	24.35±0.6	23.90±2.0	22.85±1.05	25.65±0.9	24.35±1.2	23.60±0.6	NS	NS	NS
3rd Week	24.15±2.3	25.25±1.8	24.45±1.8	23.25±1.2	23.30±2.3	25.90±0.7	24.15±1.6	22.75±1.6	NS	NS	NS
5th week	22.80^{X}±1.2	24.25^{X}±2.1	24.15^{X}±0.9	23.00^{X}±2.0	24.40^{X}±2.1	25.90^{X}±1.1	24.55^{X}±1.5	23.25^{X}±1.5	NS	NS	NS
Post challenge	15.80^{Y}±1.3	17.60^{Y}±1.4	17.90^{Y}±0.9	15.20^{Y}±1.2	16.50^{Y}±1.5	16.20^{Y}±3.2	16.70^{Y}±1.7	14.50^{Y}±0.9	NS	NS	NS
					MCV (fL)						
1st week	125.5±3.6	114.9±6.9	114.7±9.9	116.4±6.9	113.9±4.4	128.1±10.6	121.8±6.4	108.1±4.9	NS	NS	NS
3rd Week	121.6±10.7	120.3±12.5	116.8±10.7	116.9±9.6	118.7±9.8	128.6±8.6	119.5±5.3	114.3±6.9	NS	NS	NS
5th week	116.0^{X}±9.1	117.7^{X}±10.4	117.2^{X}±7.8	115.4^{X}±7.5	127.5^{X}±11.1	124.6^{X}±10.3	120.1^{X} ±11.6	119.5^{X}±9.1	NS	NS	NS
Post challenge	89.9^{Y}±4.1	103.1^{Y}±9.9	106.8^{Y}±7.9	87.8^{Y}±7.7	98.7^{Y}±14.5	93.4^{Y}±20.8	97.9^{Y}±7.1	85.4^{Y}±3.9	NS	NS	NS
					MCH (pg)						
1st week	53.8±2.5	50.8±6.6	50.3±4.6	51.3±4.8	48.4±5.5	54.1±7.1	52.5±6.7	45.5±3.5	NS	NS	NS
3rd Week	50.5±2.9	51.9±3.9	48.3±3.1	49.5±2.7	53.7±4.2	51.7±4.5	50.6±6.6	53.2±4.1	NS	NS	NS
5th week	55.4±4.2	50.9±4.5	48.5±3.5	51.8±2.9	54.3±6.2	49.4±5.9	50.1±2.9	54.9±3.4	NS	NS	NS
Post challenge	49.5±4.9	48.6±4.9	46.7±3.6	47.5±4.7	49.8±2.7	47.9±3.8	48.4±4.8	49.7±2.9	NS	NS	NS
					MCHC (gm dl^{-1})						
1st week	42.9±3.3	44.4±4.8	43.8±3.2	44.0±2.5	42.5±3.1	42.0±2.1	43.5±4.8	42.2±2.9	NS	NS	NS
3rd Week	41.9±3.0	43.3±2.2	42.1±4.6	43.1±4.2	45.8±4.2	40.2±3.7	42.0±4.2	46.6±3.6	NS	NS	NS
5th week	47.8±3.6	43.4±2.7	41.4±4.1	44.9±2.5	42.7±4.3	39.5±2.9	42.6±3.6	45.9±4.1	NS	NS	NS
Post challenge	55.4±4.7	47.1±2.2	43.7±2.5	54.0±4.9	50.2±2.5	51.8±2.1	51.5±4.2	58.4±7.6	NS	NS	NS

CP: Crude protein; NS: Not significantly different

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

Mean values in a column (5th week and post challenge) under each parameter bearing different superscript (X, Y) differ significantly (P<0.05).

Table 4. Serum total protein, albumin (A), globulin (G) and A/G ratio in *L. rohita* fingerlings exposed to short term elevated temperature and fed diet differing in protein level.

Temp.	26ºC				One week at 32ºC then at 26ºC				Two Way ANOVA Variation Source		
Treatment	20%CP	30%CP	40%CP	45%CP	20%CP	30%CP	40%CP	45%CP	Temp.	Protein	Inter.
					Total Protein (gm %)						
1st week	3.49^{b}±0.2	4.39^{a}±0.2	2.70^{cd}±0.1	2.35^{d}±0.2	3.64^{b}±0.1	4.64^{a}±0.1	3.00^{c}±0.1	2.70^{cd}±0.1	P<0.05	P<0.05	NS
3rd Week	3.39^{bc}±0.1	4.47^{a}±0.2	2.79^{de}±0.1	2.37^{e}±0.2	3.63^{b}±0.1	4.69^{a}±0.3	2.98^{cd}±0.1	2.74^{de}±0.1	P<0.05	P<0.05	NS
5th week	3.56^{Xb}±0.3	4.51^{Xa}±0.2	2.72^{Xcd}±0.1	2.41^{Xd}±0.1	3.55^{Xb}±0.1	4.58^{Xa}±0.1	2.91^{Xc}±0.2	2.73^{Xcd}±0.2	NS	P<0.05	NS
Post challenge	3.04^{Yb}±0.1	3.94^{Ya}±0.1	2.25^{Ycd}±0.1	1.92^{Yd}±0.1	2.88^{Yb}±0.2	3.93^{Ya}±0.1	2.34^{Yc}±0.1	2.16^{Ycd}±0.1	NS	P<0.05	NS
					Albumin (gm %))						
1st week	1.24^{b}±0.1	1.78^{a}±0.1	1.07^{bc}±0.1	0.90^{c}±0.1	1.29^{b}±0.1	1.88^{a}±0.1	1.22^{b}±0.1	1.22^{bc}±0.1	P<0.05	P<0.05	NS
3rd Week	1.20^{b}±0.1	1.76^{a}±0.1	1.16^{b}±0.1	0.97^{b}±0.1	1.33^{b}±0.1	1.82^{a}±0.1	1.20^{b}±0.1	1.08^{b}±0.1	NS	P<0.05	NS
5th week	1.37^{b}±0.1	1.84^{a}±0.1	1.09^{cd}±0.1	1.00^{d}±0.1	1.25^{bc}±0.1	1.79^{a}±0.1	1.14^{cd}±0.1	1.07^{cd}±0.1	NS	P<0.05	NS
Post challenge	1.30^{b}±0.1	1.60^{a}±0.1	1.05^{cd}±0.1	0.93^{d}±0.1	1.19^{bc}±0.1	1.70^{a}±0.1	1.08^{cd}±0.1	0.91^{d}±0.1	NS	P<0.05	NS
					Globulin (gm %))						
1st week	2.25^{c}±0.1	2.61^{ab}±0.1	1.62^{de}±0.1	1.45^{e}±0.1	2.35^{bc}±0.1	2.76^{a}±0.1	1.78^{d}±0.1	1.55^{de}±0.1	P<0.05	P<0.05	NS
3rd Week	2.19^{b}±0.1	2.71^{a}±0.1	1.63^{c}±0.1	1.40^{d}±0.1	2.30^{b}±0.1	2.87^{a}±0.1	1.78^{c}±0.1	1.66^{c}±0.1	P<0.05	P<0.05	NS
5th week	2.18^{Xb}±0.1	2.67^{Xa}±0.1	1.62^{Xcd}±0.1	1.41^{Xd}±0.1	2.31^{Xb}±0.1	2.79^{Xa}±0.1	1.77^{Xc}±0.1	1.65^{Xc}±0.1	P<0.05	P<0.05	NS
Post challenge	1.73^{Yb}±0.1	2.34^{Ya}±0.1	1.21^{Yc}±0.1	0.99^{Yc}±0.1	1.68^{Yb}±0.1	2.22^{Ya}±0.1	1.26^{Yc}±0.1	1.25^{Yc}±0.1	NS	P<0.05	NS
					AG Ratio (gm %))						
1st week	0.54^{c}±0.1	0.67^{bc}±0.1	0.65^{ab}±0.1	0.62^{ab}±0.1	0.55^{c}±0.1	0.68^{bc}±0.1	0.68^{ab}±0.1	0.73^{a}±0.1	NS	P<0.05	NS
3rd Week	0.55^{c}±0.1	0.65^{bc}±0.1	0.70^{a}±0.1	0.68^{a}±0.1	0.58^{c}±0.1	0.63^{bc}±0.1	0.68^{ab}±0.1	0.64^{ab}±0.1	NS	P<0.05	NS
5th week	0.62^{Ybcd}±0.1	0.68^{bcd}±0.1	0.67^{Yab}±0.1	0.71^{Ya}±0.1	0.54^{Xd}±0.1	0.64^{cd}±0.1	0.65^{Ybc}±0.1	0.65^{Xbc}±0.1	NS	P<0.05	NS
Post challenge	0.75^{Xcd}±0.1	0.69^{d}±0.1	0.86^{Xb}±0.1	0.93^{Xa}±0.1	0.71^{Yd}±0.1	0.76^{d}±0.1	0.86^{Xbc}±0.1	0.73^{Ybcd}±0.1	NS	P<0.05	NS

CP: Crude protein; NS: Not significantly diferent

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

Mean values in a column (5th week and post challenge) under each parameter bearing different superscript (X, Y) differ significantly (P<0.05).

Discussion

Understanding the stress and immune response in fish is of prime importance for successful fish husbandry practice to enhance the production. The present study elucidates the stress and immune response of *L. rohita* fingerlings due to short term exposure of elevated temperature and different dietary protein level. Being reliable indicator of stress response, serum cortisol has been widely measured in fish stress studies (Martínez-Porchas et al., 2009). Changes in the concentration of these hormones might provide a means by which the severity and duration of fish stress response can be quantitatively measured (O'Connor et al., 2011). In the present study, serum cortisol level of *L. rohita* fingerlings decreased with the increase in temperature and increased later, after two weeks of decrease in temperature from 32°C to 26°C. This indicates that fish were under stress at low temperature (26ºC) as cortisol is generally released when an organism undergoes stressful condition (Barton, 2000). It was also reported that a temperature range of 31°C to 33°C is the optimum temperature for better growth in *L. rohita* fry (Das et al., 2005). Irrespective of temperature, the cortisol level was similar in fingerlings fed with 30% & 40% CP diet which was significantly lower than fingerlings fed with 20% & 45% CP diet. This suggests that *L. rohita* fingerlings were in stress condition when fed with either low (20%) or high (45%) CP diet than the optimum level (30% or 40%). This may be supported by our earlier findings (Kumar et al., 2013), illustrating an increment in weight gain (%) and specific growth rate of *L. rohita* fingerlings with the increase in dietary protein level up to 40% but declined when fed with 45% dietary protein level. It is very likely that, the reduced growth rate of fingerlings at higher dietary protein level (45%) is due to the metabolic stress. Challenge with *A. hydrophila* significantly increased (P<0.05) the cortisol level in the serum of *L. rohita* fingerlings and increase in dietary protein level significantly decrease the serum cortisol level. This can be explained by the findings of Kumar et al., (2011), who reported that high protein diet enriches

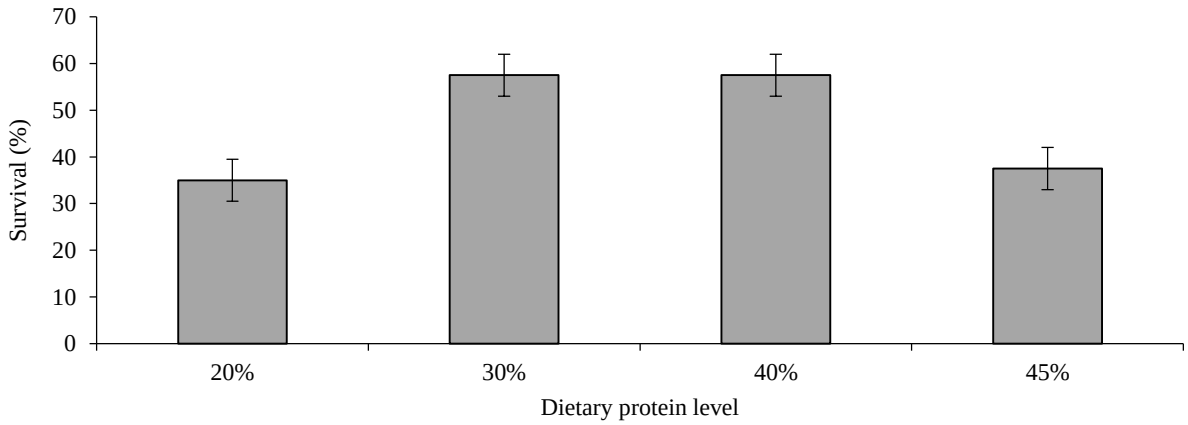

Figure 3. Effect of dietray protein level on survival (%) after challenge with *Aeromonas hydrophila*. Significant differences indicated with (*) were determined using Duncan's multiple range test (DMRT) (P<0.05).

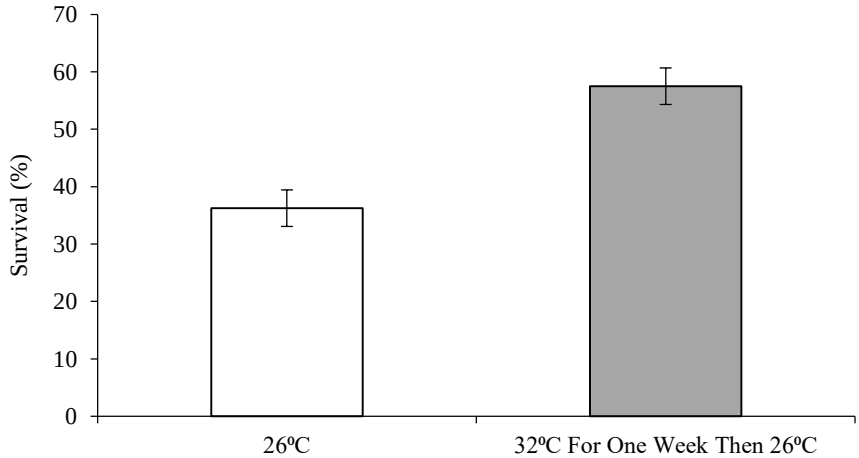

Figure 4. Effect of temperature on survival (%) after challenge with *Aeromonas hydrophila*. Significant differences indicated with (*) were determined using Duncan's multiple range test (DMRT) (P<0.05).

Table 5. Serum triglycerides, cholesterol and urea level in *L. rohita* fingerlings exposed to short term elevated temperature and fed diet differing in protein level.

Temp.	26ºC				One week at 32ºC then at 26ºC				Two Way ANOVA Variation Source		
Treatment	20%CP	30%CP	40%CP	45%CP	20%CP	30%CP	40%CP	45%CP	Temp.	Protein	Inter.
					Triglycerides (mg dl^{-1})						
1st week	18.18^{d}±1.5	30.99^{c}±1.9	32.22^{c}±1.4	30.06^{c}±2.1	44.34^{Ab}±3.5	61.58^{Aa}±4.1	59.49^{Aa}±4.6	61.70^{Aa}±5.3	P<0.05	P<0.05	NS
3rd Week	18.34^{c}±1.4	29.64^{b}±2.1	29.46^{b}±3.3	28.97^{b}±1.6	39.65^{Ab}±3.1	55.67^{Aa}±3.3	55.47^{Aa}±2.7	55.30^{Aa}±3.5	P<0.05	P<0.05	NS
5th week	17.35^{Xb}±0.8	28.57^{Xa}±1.7	28.19^{Xa}±2.1	30.13^{Xa}±1.7	18.78^{BXb}±1.3	35.09^{BXa}±2.6	31.89^{BXa}±2.9	30.19^{BXa}±2.6	NS	P<0.05	NS
Post challenge	10.98^{Yc}±0.6	19.19^{Yb}±1.2	23.23^{Yab}±0.8	19.90^{Yab}±1.5	12.06^{Yc}±1.7	24.68^{Ya}±1.1	24.06^{Ya}±1.8	21.25^{Yab}±1.4	NS	P<0.05	NS
					Cholesterol (mg dl^{-1})						
1st week	26.71^{d}±2.1	32.57^{c}±1.6	36.50^{bc}±1.9	35.27^{bc}±1.2	35.79^{Abc}±1.8	42.78^{Aa}±2.0	41.21^{Aab}±1.7	39.69^{Aab}±2.2	P<0.05	P<0.05	NS
3rd Week	25.18^{d}±2.3	33.18^{bc}±2.6	37.27^{ab}±2.3	34.03^{bc}±1.1	31.38^{ABcd}±1.7	41.02^{Aa}±1.9	39.69^{ABab}±1.2	38.29^{ABab}±2.0	P<0.05	P<0.05	NS
5th week	26.97^{c}±1.5	33.19^{ab}±1.8	36.68^{a}±1.4	34.84^{a}±1.6	28.05^{Bbc}±2.1	33.74^{Bab}±1.8	36.96^{Ba}±1.9	34.13^{Ba}±2.1	NS	P<0.05	NS
Post challenge	27.78^{b}±1.6	32.20^{ab}±1.7	37.37^{a}±2.1	35.40^{a}±3.1	28.47^{b}±1.5	32.62^{ab}±2.1	35.56^{a}±1.3	35.55^{a}±1.8	NS	P<0.05	NS
					Urea (mg dl^{-1})						
1st week	7.84^{d}±0.6	10.45^{cd}±1.1	14.34^{c}±1.2	21.45^{b}±1.6	7.70^{d}±0.7	10.12^{cd}±0.9	12.80^{cd}±1.1	27.35^{a}±1.0	NS	P<0.05	NS
3rd Week	7.24^{d}±0.8	10.06^{bcd}±0.7	14.54^{b}±1.1	23.46^{a}±2.1	8.37^{cd}±0.5	10.12^{bcd}±0.9	12.80^{bc}±1.1	24.67^{a}±1.4	NS	P<0.05	NS
5th week	8.04^{c}±0.5	9.65^{c}±0.3	19.96^{Yb}±0.8	25.60^{Ya}±2.1	9.45^{c}±0.8	10.79^{c}±1.1	13.20^{Ybc}±1.3	26.27^{Ya}±1.7	NS	P<0.05	NS
Post challenge	12.06^{c}±0.5	13.01^{c}±0.9	27.01^{Xb}±1.2	34.99^{Xa}±0.4	12.13^{c}±0.2	13.47^{c}±1.27	27.95^{Xb}±0.46	37.00^{Xa}±0.9	NS	P<0.05	NS

CP: Crude protein; NS: Not significantly different

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

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

Mean values in a column (5th week and post challenge) under each parameter bearing different superscript (X, Y) differ significantly (P<0.05).

the amino acid pool in the cells (non-essential amino acids) and act to produce substrate for energy, which aids in combating the stress.

Almost all creatinine is excreted by the kidney, so increased concentration may reflect kidney dysfunction due to structural damage (Pakhira, et. al., 2000). Data from serum creatinine was found unaffected due to the exposure of elevated temperature as well as dietary protein level and suggest that there were no adverse effect due to increase in temperature from 26ºC to 32ºC as well as dietary protein level from 20% to 45% on renal function of *L. rohita*.

Serum enzyme activities are excellent tools in monitoring health status of aquatic animals providing information on the metabolic disorders and chronic stress induced by various external and ecological stressors, including feeding regime (Zhou et al., 2009). The enzyme CK, which is concentrated in muscle and heart tissue, is an indicator of damage to one or both of these tissue, and plays a key role in the energy homeostasis of cells. Higher CK level can be caused by many stressful events (Shahsavani et al., 2010) but no significant differences in CK activities due to exposure of elevated temperature as well as different dietary protein level was observed. Besides CK, LDH and ALP are also useful indices of tissue damage because they may be released quite readily even during minor change in the morphology and function of cells (Wagner and Congleton, 2004). Raised activity of LDH and ALP is generally associated with liver dysfunction due to stress (Gora et. al., 2018). Their activities were also found unaffected due to exposure to elevated temperature for one week as well as different dietary protein level. Challenge with *A. hydrophila* significantly increased ($P<0.05$) the CK, LDH and ALP activity in the serum. Post-challenge activity of LDH and ALP were significantly decreased with the increase in dietary protein level from 20% to 30%, which was similar to 40%, whereas significantly higher activity was observed in 45% fed group compare to 40% CP fed group. This suggests an immunosuppressive action of low (20%) and high (45%) dietary protein in the diet of *L rohita*.

The numbers of white blood cells is an indicator of the health status of the fish because of its role in nonspecific or innate immunity (Kumar et. al., 2015). The observed trend of elevated WBC counts in the lowest (20%) or highest (45%) dietary protein as well as at lower temperature (26ºC) could be due to the metabolic stress, confirmed by the higher cortisol level in these groups. It was also stated that stressful conditions (due to infection, dietary imbalance, etc) raise the WBC count (Kumar et. al., 2015). At the same time, higher lymphocyte and lower monocyte and granulocyte count was evident in the fish fed with either 20% or 45% CP irrespective of temperature as well as in the lower temperature (26ºC) exposed group irrespective of dietary protein level. Likewise, it was reported that increase in the proportion of monocytes enhances the cellular immunity (Irianto and Austin, 2002). Thus, the findings of the hematological responses in our experiment demonstrate a low immunity and depressed metabolic performance in the fish fed with 20% or 45% dietary protein as well as exposed to lower temperature (26ºC). In the present study, post challenge increase the leucocyte count irrespective of dietary protein level as well as water temperature, thereby affirm a possible increased inflammatory response mediated by leucocytes against bacterial infection (Kumar et. al., 2015).

Respiratory burst activity of neutrophills provides a measure of oxygen dependent defense mechanism in vertebrate phagocytic cells. During this process, reactive oxygen species (ROS) intermediates are generated in phagocytes possessing powerful microbicidal activity (Hardie et al., 1996; Itou et al., 1997). In the present study, the respiratory burst activity of phagocytes was measured by reduction of nitroblue tetrazolium (NBT) by intracellular superoxide radicals produced by leucocytes. It is generally accepted that fish phagocytes are able to generate superoxide anion (O_2^-) and its reactive derivatives (i.e., hydrogen peroxide and hydroxyl radicals) during a period of intense oxygen consumption referred as the respiratory burst (Secombes and Fletcher, 1992; Secombes, 1996). As such, increased respiratory burst activity can be associated with increased pathogen killing activity of phagocytes (Sharp and Secombes, 1993) and hence a better immunity. The results of the present experiment shows reduced NBT activity in fish fed 20% and 45% CP diet indicating compromised immune status. The significant decrease in respiratory burst activity in the 20% and 45% CP fed groups during post challenge was presumably due to exhaustion of the respiratory burst activity of the phagocytes following infection of the fish after *A. hydrophila* challenge. The respiratory burst activity of 30% and 40% CP fed group was not affected due to challenge with *A. hydrophila.* This indicates that 30% and 40% CP fed groups enhanced the immunity to overcome the stress caused by *A. hydrophila.* The result shows that after challenge the respiratory burst activity of the fingerlings exposed to elevated temperature (32ºC) was higher compared to fingerlings exposed to 26ºC. This indicates an increased production of superoxide anion in fingerlings exposed to elevated temperature (32ºC). Likewise, it was observed that NBT value were higher in the fingerlings exposed to elevated temperature (32ºC) compare to ambient temperature (27^0C) (Alexander et al., 2011). Reduction in respiratory burst activity during outbreak of winter syndrome was also observed in farmed gilthead sea bream, *Sparus aurata* (Contessie et al., 2006).

Diet management can lead to deformation and functional changes in the blood cells (Duncan et al., 1993; Wise et al., 1993; Klinger et al., 1996). The red blood cells (RBC) count can determine, at least in part, the efficiency of oxygen transport from the surface to tissue (Nikinmaa and Salama, 1998) and any change in their number and volume may influence metabolic

Table 6. Stress response in *L. rohita* fingerlings exposed to short term elevated temperature and fed diet differing in protein level.

Temp.	26ºC				One week at 32ºC then at 26ºC				Two Way ANOVA Variation Source		
Treatment	20%CP	30%CP	40%CP	45%CP	20%CP	30%CP	40%CP	45%CP	Temp.	Protein	Inter.
Cortisol (n mole gm^{-1})											
1st week	$176.86^{a}\pm7.1$	$140.88^{c}\pm8.5$	$127.07^{d}\pm6.8$	$147.07^{bc}\pm7.9$	$148.42^{Ab}\pm10.3$	$121.85^{Ad}\pm5.1$	$120.27^{d}\pm5.2$	$146.54^{bc}\pm5.7$	P<0.05	P<0.05	NS
3rd Week	$170.5^{a}\pm11.5$	$145.04^{b}\pm11.9$	$130.40^{c}\pm4.9$	$150.89^{b}\pm9.4$	$171.79^{Ba}\pm11.2$	$148.30^{Bb}\pm8.7$	$126.84^{c}\pm7.8$	$145.50^{b}\pm10.5$	NS	P<0.05	NS
5th week	$172.50^{Ya}\pm11.5$	$146.5^{Yb}\pm11.4$	$131.00^{Yc}\pm6.0$	$145.56^{Yb}\pm6.5$	$176.83^{BYa}\pm7.8$	$148.94^{BYb}\pm11.9$	$127.50^{Yc}\pm2.5$	$142.41^{b}\pm9.4$	NS	P<0.05	NS
Post challenge	$253.00^{Xa}\pm12.3$	$227.50^{Xab}\pm4.5$	$194.50^{Xc}\pm6.5$	$214.00^{Xbc}\pm6.0$	$246.00^{Xa}\pm9.0$	$229.50^{Xab}\pm8.5$	$191.00^{Xc}\pm7.0$	$209.00^{bc}\pm6.2$	NS	P<0.05	NS
Creatinine (mg dl^{-1})											
1st week	1.18 ± 0.03	1.17 ± 0.01	1.18 ± 0.02	1.26 ± 0.03	1.27 ± 0.04	1.21 ± 0.02	1.25 ± 0.04	1.25 ± 0.01	NS	NS	NS
3rd Week	1.23 ± 0.04	1.21 ± 0.03	1.24 ± 0.05	1.27 ± 0.03	1.23 ± 0.04	1.24 ± 0.04	1.21 ± 0.04	1.23 ± 0.01	NS	NS	NS
5th week	$1.24^{X}\pm0.03$	$1.25^{X}\pm0.04$	$1.23^{X}\pm0.02$	$1.19^{X}\pm0.01$	$1.23^{X}\pm0.03$	$1.21^{X}\pm0.02$	$1.23^{X}\pm0.04$	$1.25^{X}\pm0.04$	NS	NS	NS
Post challenge	$1.11^{Y}\pm0.01$	$1.12^{Y}\pm0.01$	$1.10^{Y}\pm0.01$	$1.10^{Y}\pm0.01$	$1.09^{Y}\pm0.01$	$1.09^{Y}\pm0.01$	$1.10^{Y}\pm0.02$	$1.11^{Y}\pm0.01$	NS	NS	NS
Creatinine Kinase (IU L^{-1})											
1st week	560.08 ± 45.6	541.29 ± 38.5	567.55 ± 35.2	566.55 ± 24.8	558.38 ± 22.8	527.72 ± 36.9	592.81 ± 12.9	588.90 ± 41.6	NS	NS	NS
3rd Week	557.55 ± 35.7	543.12 ± 39.2	563.09 ± 37.8	523.29 ± 28.4	550.70 ± 37.7	565.03 ± 36.1	570.15 ± 37.9	506.46 ± 45.2	NS	NS	NS
5th week	$520.84^{Y}\pm26.3$	$502.15^{Y}\pm36.5$	$529.01^{Y}\pm33.8$	$563.69^{Y}\pm38.1$	$547.10^{Y}\pm44.4$	$526.20^{Y}\pm33.6$	$555.15^{Y}\pm47.1$	$575.15^{Y}\pm32.9$	NS	NS	NS
Post challenge	$911.38^{X}\pm35.1$	$898.44^{X}\pm42.8$	$931.79^{X}\pm50.1$	$952.69^{X}\pm53.1$	$895.67^{X}\pm52.6$	$966.55^{X}\pm40.7$	$968.65^{X}\pm42.1$	$934.96^{X}\pm55.0$	NS	NS	NS
Lactate Dehydrogenase (IU L^{-1})											
1st week	35.18 ± 3.1	34.62 ± 3.5	31.10 ± 2.1	35.00 ± 3.0	36.66 ± 3.5	35.04 ± 2.0	32.62 ± 2.5	31.50 ± 2.5	NS	NS	NS
3rd Week	39.40 ± 2.8	36.46 ± 2.9	31.50 ± 3.5	31.00 ± 2.5	39.50 ± 2.5	33.50 ± 2.5	32.50 ± 2.5	34.50 ± 3.5	NS	NS	NS
5th week	$36.50^{Y}\pm2.5$	$33.00^{Y}\pm3.0$	$32.50^{Y}\pm3.5$	$33.00^{Y}\pm2.0$	$36.50^{Y}\pm4.5$	$36.50^{Y}\pm3.5$	$34.50^{Y}\pm3.5$	$31.50^{Y}\pm1.5$	NS	NS	NS
Post challenge	$90.00^{Xa}\pm6.0$	$69.00^{Xbc}\pm4.0$	$58.50^{Xc}\pm4.5$	$79.50^{Xab}\pm3.5$	$87.50^{Xb}\pm7.5$	$65.50^{Xbc}\pm4.5$	$60.00^{Xc}\pm3.0$	$81.00^{Xab}\pm3.0$	NS	P<0.05	NS
Alkaline Phosphatase (IU L^{-1})											
1st week	68.18 ± 4.1	70.19 ± 5.5	73.62 ± 4.7	67.41 ± 4.4	64.78 ± 5.4	71.43 ± 5.4	78.03 ± 4.5	70.48 ± 3.6	NS	NS	NS
3rd Week	61.72 ± 5.6	64.32 ± 5.4	71.55 ± 5.9	66.56 ± 5.1	70.56 ± 4.1	68.84 ± 5.3	75.80 ± 3.7	70.07 ± 4.5	NS	NS	NS
5th week	$64.73^{Y}\pm3.9$	$68.38^{Y}\pm6.5$	$69.55^{Y}\pm2.5$	$70.95^{Y}\pm5.5$	$69.69^{Y}\pm5.5$	$71.87^{Y}\pm4.1$	$77.34^{Y}\pm3.1$	$71.27^{Y}\pm4.6$	NS	NS	NS
Post challenge	$142.27^{Xa}\pm8.2$	$120.78^{Xbc}\pm7.8$	$126.44^{Xcc}\pm5.2$	$136.94^{Xab}\pm4.2$	$145.07^{Xa}\pm9.2$	$122.91^{Xbc}\pm5.5$	$119.94^{Xcc}\pm4.7$	$140.28^{Xab}\pm6.8$	NS	P<0.05	NS

CP: Crude protein; NS: Not significantly different

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

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

Mean values in a column (5th week and post challenge) under each parameter bearing different superscript (X, Y) differ significantly (P<0.05).

performance (Hlavova, 1993; Rios et al., 2002; Rios et al., 2004). There was no variation in RBC count, hemoglobin content, Hct, MCV, MCH and MCHC among the treatment groups either in pre or post-challenge period. Hence, either increased dietary protein or exposure of 32ºC for one week did not affect the blood parameters. A significant decrease in RBC count in all groups during the post challenge period may be the effect of *A. hydrophila* infection, which can be correlated with the observation in Nile tilapia that showed decreased erythrocyte count after bacterial inoculation (Ranzani-Paiva et al., 2004). Similarly, reduction in the RBC count and hemoglobin content when challenged with *A. hydrophila*, corroborates with the findings of decreased hemoglobin content in *L. rohita* infected with *Aeromonas salmonicida* (Misra et al., 2006; Kumar et al., 2007).

Proteins are the most important compounds in the serum, and serum proteins are called the circulating mobile proteins. The serum proteins are divided into two major groups, albumin and globulin. The liver synthesizes albumin and it creates an osmotic force that maintains fluid volume within the vascular space. Globulin like gamma globulin is absolutely essential for maintaining a healthy immune system. Relative and total amounts of serum protein fractions are affected by infections, inflammation, nutritional, and physiological status and are therefore important health indicators in higher animals (Grasman et al., 2000). The changes in serum total protein are associated with infectious disease, kidney disease, nutritional imbalance, hemoconcentration and impaired water balance (Kumar et. al., 2015). There was significantly higher serum globulin level found in 30% CP fed group compared to other levels, both during pre and post-challenge period. This suggests an immunosuppressive action of less and higher than 30% dietary protein in the diet of *L. rohita* fingerlings. Total serum protein and globulin content was significantly reduced after challenge with *A. hydrophila.* This reduction may be due to vascular leaking of serum protein (Green, 1978; Ellis et al., 1981) along with impaired synthesis and nonspecific proteolysis of serum protein (Ellis et al., 1981).

The concentration of serum triglycerides and cholesterol were positively associated with dietary protein level. This may be due to reduced dietary lipid intakes as fingerlings could not properly utilise all the ration of low protein diet (20%). Also, the exposure of elevated temperature (32ºC) for one week registered higher ($P<0.05$) concentration of serum triglycerides and cholesterol compare to lower temperature (26ºC) and the increased concentration was significantly reduced after 4 week of the decrease of temperature from 32ºC to 26ºC. Furthermore, urea cycle induction can be observed in fasting (Walsh et al. 1990), dehydration (Polez et al., 2003), confinement stress (Hopkins et al., 1995), water alkalinity (Polez et al., 2004) and external ammonia concentration (Saha & Ratha, 1994). Also, the increase of protein content in the diets can enhance the activity of urea cycle enzymes in fish (Chiu et al., 1986). Similarly, enhanced serum urea content was found in fingerlings fed high protein diet.

Bacterial challenge tests have often been employed as a final indicator of fish health status after the termination of feeding trial (Misra et al., 2006; Waagbø et al., 1994). The nutritional status and rearing condition of fish determines its susceptibility to pathogens (Ndong et al., 2007; Kiron et al., 1995). As per the result of mortality under *A. hydrophila* infection, it can be suggested that low temperature (26°C) and high (45%) and low (20%) level of dietary protein would increase susceptibility against *A. hydrophila*. The hypothesis that low water temperatures can be immunosuppressive has been supported earlier study (Bly & Clem, 1992). Further, it was reported that spring viraemia of carp (*Cyprinus carpio*) occur at lower environmental temperatures (Baudouy et al., 1980). Thus, the pathological situation in fish depends on temperature dependent immune system regulation. In addition, the immunosuppressive effect of low and high dietary protein level has been confirmed in rainbow trout (Kiron et al., 1995), suggesting that a low protein diet invariably led to greater mortalities but higher level also seemed to depress disease resistance. Pathological invasion at higher levels of dietary protein was also observed in chinook salmon (Hardy et al., 1979). The increased susceptibility of fish fed with low (20%) and high (45%) level of dietary protein to infection may be due to the reduced immuno-modulatory effect of high carbohydrate and protein as reflected in the considerably lower leucocyte (WBCSS) count and NBT value in the post-challenge period. The lower mortality in optimum level of dietary protein (30% and 40%) fed fingerlings suggesting the acquisition of nonspecific immunity due to optimum dietary protein level.

Conclusions

In conclusion, present study revealed that both lower (20%) and higher (45%) level of dietary protein compare to optimum level (30%-40%) induced stress in *L. rohita* fingerlings, which was associated with the immune-depression and decline in the resistivity against the *A. hydrophila* infection. Further, exposure to elevated temperature (32ºC) for one week increased the immuno-status of the fingerlings as reflected by higher WBCSS count and respiratory burst activity during pre- and post-challenge period. The dietary protein level and water temperature interactions are effective in modulating the leucocyte count, differential count, respiratory burst activity and survival after challenge with *A. hydrophila*. In general, it is concluded that immune responses are enhanced by a short term (one week) exposure to elevated temperature (32ºC) in *L. rohita* fingerlings which prolongs for 4 week during which optimum level of dietary protein inclusion was found to increase the survival against *A. hydrophila* infection. In brief, we can recommend that for

improving the metabolic and immunological fitness of tropical fish such as *L. rohita*, it is advisable to expose the fish to elevated water temperature (32°C) for a short period and rear them with diet containing optimal range of protein (30%-40%). Nevertheless, the feasibility of this operation has yet to be evaluated in economic terms.

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Not applicable

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Author Contribution

1st Author (SK): Investigation, Methodology, Writing – original draft, review & editing

2nd Author (NPS): Conceptualization, Supervision, Validation, Writing - review & editing

3rd Author (SKG): Investigation, Resources

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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