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Dietary L-Tryptophan potentiates non-specific immunity in *Labeo rohita* fingerlings reared under elevated temperature

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ABSTRACT

At present environmental scenario global climate change is a reality and its affect all living organism including fish. The aquatic ecosystem is the most affected system as it is the biggest sink for global warming and elevated temperature and obviously affects all the aquatic life forms. With this hypothesis an experiment was conducted to evaluate the effect of elevated temperature on *Labeo rohita* fingerlings and potential of dietary L-tryptophan (TRP) in mitigating the effects elevated temperature and enhancing the non-specific immunity. Seven hundred and twenty fishes were randomly distributed in three different thermal groups each with three replicates. The thermal groups were ambient temperature (26 °C), 34 and 38 °C. Then each thermal groups were fed with four different formulated diets containing 0. 0.36%, 0.72% and 1.44% TRP. The effect of dietary TRP supplementation was studied on stress responses, such as cortisol, blood glucose, histopathological changes in liver and kidney and immuno-hematological changes such as red blood cell count (RBC), haemoglobin (Hb), white blood cell count (WBC), lysozyme, nitroblue tetrazolium (NBT), total serum protein, albumin, globulin and albumin-globulin ratio. Subsequently the treated fish were subjected to challenge test with *Aeromonas hydrophila*. In the present study, primary stress markers were noticeably ($p < 0.01$) elevated with temperature stress and levels were reduced with nutritional supplementation of TRP. Similarly, immuno-hematological parameters were altered with the exposure of temeparture stress and got improved with dietary TRP supplementation. Results of the present study suggest that dietary supplementation of 1.44% tryptophan has definitive role in the mitigation of temperature stress and gives protection against bacterial infection to *L. rohita*.

1. Introduction

Labeo rohita is one of the most economically important aquaculture species worldwide including India, and its farming usually passes throughseasonal variations including change in water temperature. During summer months water temperature reaches up to a range of 34–37 °C that is beyond the optimal temperature for this species (Das et al., 2005), which disturbs the ecophysiology of fish. During culture period, fish usually come across to many type of stressors. Among different stressors, temperature is one of the prime concerns with respect to fish as it is an ectothermic animal. Alteration in water temperature has a direct effect on the key physiological process and behavioural activities of fish (Jonassen et al., 1999). Exposure of cells or whole organisms to heat shock results in a reversible increase in the synthesis of some acute phase proteins, known as heat shock proteins (HSPs) (Iwama et al., 1998), which play an important role in maintaining homeostasis. Scientists have long been trying to find stress mitigation strategies for different stressors, using a number of chemical compounds, such as vitamin C, vitamin E, beta-glucan, pyridoxine, microbial levan and lecithin. Many studies have shown that immunomodulators can trigger immune system of fish under stressful conditions, and therefore, ameliorate the deleterious effects mediated

Abbreviations: 5-HT, 5-hydroxytryptamine; A, albumin; ANOVA, analysis of variance; BSA, bovine serum albumin; CF, Crude fat; CP, crude protein; CRD, complete randomized design; DE, digestible energy; ELISA, enzyme-linked immunosorbent assay; G, globulin; Hb, haemoglobin; HSPs, heat shock proteins; NBF, Neutral buffer formalin; NBT, nitroblue tetrazolium; NBT, nitroblue tetrazolium; PBS, phosphate-buffered saline; PVDF, polyvinylidene fluoride; RBC, red blood cell count; RIA, radioimmunoassay; RPS, Relative percentage survival; STP, Serum total protein; TBS, Tris Buffer Saline; TRP, L-tryptophan; WBC, white blood cell count

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Table 1
Feed and proximate composition of the experimental diets (% dry matter basis) fed to *L. rohita* fingerlings.

Ingredient	T1 (0% TRP)	T2(0.36% TRP)	T3 (0.72%TRP)	T4 (1.44%TRP)
Soybean meal	40	40	40	40
Fish meal	20.50	20.50	20.50	20.50
Corn flour	15	15	15	15
Wheat flour	14	13.64	13.28	12.44
Fish oil	8	8	8	8
Carboxy Methyl Cellulose	1	1	1	1
L-tryptophan	0	0.36	0.72	1.44
Vitamin-Mineral mix[a]	0.5	0.5	0.5	0.5
Vitamin B complex[b]	0.05	0.05	0.05	0.05
Vitamin C	0.95	0.95	0.95	0.95
Total	100	100	100	100
Proximate composition of diets				
Organic matter	96.44 ± 0.31	96.53 ± 0.07	96.83 ± 0.02	96.70 ± 0.01
Crude protein	34.33 ± 0.23	34.73 ± 0.16	35.06 ± 0.06	35.81 ± 0.18
Total carbohydrate	54.56 ± 0.02	54.07 ± 0.17	54.19 ± 0.21	53.08 ± 0.10
Ether extract	7.55 ± 0.10	7.73 ± 0.08	7.59 ± 0.29	7.81 ± 0.07
Ash	3.57 ± 0.32	3.48 ± 0.08	3.17 ± 0.02	3.30 ± 0.01
Digestible energy[c]	423.49 ± 1.76	424.75 ± 0.70	425.27 ± 1.53	425.85 ± 0.31

Vitamin C: (Hoffman La Roche, Nuetly. NJ.USA), 15% ascorbic acid activity.
L-tryptophan: (Himedia).
[a] Composition of vitamin-mineral mix (Agrimin) (quantity kg^{-1}): Vitamin A-6,25,000 IU; Vitamin D_3–62,500 IU; Vitamin E- 250 mg; Nicotinamide-1 g; Cu-312 mg; Co- 45 mg; Mg- 6 g; Fe- 1.5 g; Zn- 2.13 g; I- 156 mg; Se- 10 mg; Mn- 1.2 g; Ca- 247.34 g; P- 114.68 g; S-12.2 g; Na- 5.8 mg; K- 48.05 mg.
[b] Composition of vitamin B complex (quantity g^{-1}): Thiamine mononitrate-20 mg; Riboflavin-20 mg; Pyridoxine hydrochloride-6 mg; Vitamin B_{12}–30 mcg; Niaciamide-200 mg; Ca pantothenate-100 mg; Folic acid-3 mg; Biotin-200 mcg.
[c] Digestible energy (Kcal/100 g) = (% CP x 4) + (%EE x 9) + (% TC x 4).

by stress (Akhtar et al., 2012; Kumar et al., 2014, 2017). Tryptophan (TRP), an essential amino acid, is a precursor for serotonin (5-hydroxytryptamine, 5-HT), an indoleamine neurotransmitter which have been implicated in several behavioural patterns including fear, stress, aggression, appetite regulation, social dominance and sex behaviour in human and various animals including fishes (Winberg et al., 2001; Lepage et al., 2002; Hseu et al., 2003; Grimmett and Sillence, 2005; Herrero et al., 2006; Fernandez and Strathe, 2009; LaranjaJr et al., 2010). Serotonin (5-HT) is very important in maintaining regulation of the hypothalamic pituitary interrenal (HPI) axis in fish (Hoglund et al., 2000). The ratio of 5-hydroxyindoleacetic acid to 5-HT brain concentrations has been very much related with plasma level of cortisol (Hoglund et al., 2000). Brain serotonergic pathways are very important in respect to coping with stress through initiating and terminating the adrenocortical stress (Markus et al., 2000). Several studies have found that dietary TRP can augment growth, modulate aggressive behaviour or reduce cannibalism and stress-induced anorexia and cortisol rise in different fish species (Tejpal et al., 2009; Hoseini and Hosseini, 2010; Costas et al., 2012; Akhtar et al., 2012; Kumar et al., 2014).

The tryptophan requirement of *L. rohita* for optimum growth and feed efficiency was worked out to be in the range of 0.36–0.38% of the diet (Abidi and Khan, 2010). In this backdrop, the present work has been carried out to delineate the immunomodulatory role of dietary TRP in mitigating thermal stress in *L. rohita* fingerlings.

2. Materials and methods

2.1. Experimental fish and Experimental design

L. rohita fingerlings (weight: 4.5 ± 0.05 g) were transported from the Government Fish Farm, Khopoli, Maharashtra and transported in an open container (500 L) with adequate oxygenation to the wet laboratory of Central Institute of Fisheries Education, Mumbai. During acclimation of seven days, fish were fed with practical diets. After acclimatization, fish were distributed in to 36 uniform size experimental tanks (80 × 57 × 42 cm) of 150 L capacity and reared for 45 days. Four iso-caloric (423.49–425.85 kcal $100\,g^{-1}$) and iso-nitrogenous (34.33–35.81% crude protein) purified diets were prepared with different levels (0, 0.36%, 0.72% and 1.44%) of L-tryptophan (Himedia Laboratories, Mumbai, India). Seven hundred and twenty fishes were randomly distributed into three major groups viz. ambient temperature (26 °C), 34 °C and 38 °C following a completely randomized design (CRD). Acclimation of fishes (20 individuals/tub) to experimental temperature of 34 and 38 °C over average ambient temperatures were carried out at 1 °C per day. After that each groups were fed with experimental diet for forty five days. Hence, total twelve experimental groups viz. T_1 (Amb. X 0%TRP), T_2 (Amb. X 0.36%TRP), T_3 (Amb. X 0.72%TRP), T_4 (Amb. X 1.44%TRP), T_5 (34 °C x 0%TRP), T_6 (34 °C x 0.36%TRP), T_7 (34 °C x 0.72%TRP), T_8 (34 °C x 1.44%TRP), T_9 (38 °C x 0%TRP), T_{10} (38 °C x 0.36%TRP), T_{11} (38 °C x 0.72%TRP), and T_{12} (38 °C x 1.44%TRP) were arranged in triplicate. The physicochemical parameters of water, such as dissolved oxygen: 6.6–7.6 mg L^{-1}; pH: 7.6–8.1; ammonia nitrogen: 0.15–0.28 mg L^{-1}; nitrite nitrogen: 0.001–0.005 mg L^{-1}; nitrate nitrogen: 0.02–0.05 mg L^{-1} were determined and they were within optimum range throughout the experimental period. Feed was given at 3% of body weight twice daily at 10:00 and 18:00 h under a normal light regime (light/dark 12/12). Uneaten feed and faecal matter were siphoned out daily and 40% water was exchanged with bore well water, maintained at the same temperature. Round the clock aeration was provided during the entire experimental period.

2.2. Proximate analysis of feed

The proximate composition of the experimental diets was determined following the standard methods of AOAC (1995), and is presented in Table 1. Dry weight of feed was calculated after drying at 105 °C for 12 h. Crude fat (CF) of the feed was estimated using Soxtec system (Model HT2, 1045 extraction unit Foss Tecator, Sweden) using organic solvent, diethyl ether. Nitrogen in the feed and carcass was estimated by a micro Kjeldahl method (Foss Tecator 2200 Kjeltec) and crude protein (CP) was calculated as N x 6.25. Total carbohydrate was calculated as: total carbohydrate (%) = 100-(CP% + CF% + ash %). The digestible energy (DE) of the feed and carcass was calculated following Halver (1976).

2.3. Growth study

The growth performance of *L. rohita* fingerlings was evaluated in term of Weight gain% using the following formula:

Weight gain% = (final weight-initial weight)/initial weight x 100.

2.4. Sample preparation

At the end of the 45 days of experiment, fish from each treatment were anesthetized with clove oil at 100 µl l^{-1}, and blood was collected without anticoagulant and allowed to clot for 2 h, centrifuged (3000 g for 5 min at 4 °C) and then kept at − 80 °C until use. Blood was also collected in a vial previously rinsed with 2.7% EDTA solution as an anticoagulant for estimation of blood glucose and other hematological parameters.

2.5. Blood glucose, Serum cortisol and L-tryptophan

Blood glucose was estimated at 540 nm by the method of Nelson and Somogyi (1945). Serum cortisol was estimated using a validated radioimmunoassay (RIA) modified by Olsen et al. (1992) as described by Winberg and Lepage (1998) and expressed as ng ml^{-1}. Plasma tryptophan was quantified using high- performance liquid chromatography (HPLC) with the oxidizing potential set at 600 mV (Lepage et al., 2002).

2.6. Heat shock protein

Heat shock protein 70 (HSP 70) expression in muscle tissue of all the experimental groups was studied. HSP 70 expression was analysed by SDS-PAGE and western blotting (Towbin et al., 1979). Muscle tissues were homogenised in tris buffer (pH 7.5) with protease inhibitor (0.1 mM phenyl methane sulfonyl fluoride, PMSF). Homogenate was centrifuged (3000 g at 4 °C for 10 min) and the supernatant collected and stored at − 20 °C for HSP 70 analysis. Total protein in the sample was analysed as per method of Lowry et al. (1951). Sample buffer was immediately added to each sample and heated to 95 °C for 2 min. Subsamples of protein (50 µg) were separated by SDS-PAGE with 12% separating and 5% stacking polyacrylamide gels using an electrode buffer (Laemmli, 1970). Heat shocked, Hela cell lysate (Cat No. LYC 101 F); Stressgen,Canada) (20 µg) was loaded to one lane to serve as an internal standard for blotting efficiency. Proteins were separated at 1,5 mA per well for 3 h and then electroblotted on to a total PVDF (polyvinylidene fluoride) transfer membrane (E578-10 × 10 cm SQ, USA) at 200 Ma for 3 h. After blotting, gels were stained with coomassie blue to ensure complete transfer. Membranes were blocked with 3% bovine serum albumin (BSA) and Tris Buffer Saline (TBS 7.4). Tween 20 (0.05%) in TBS was used as washing solution. Primary monoclonal antibodied HSP 70 (1:2000) dilution, Bioreagents-SPA-810; Stressgen) was used as probe. Horseradish peroxidase-conjugated goat antimouse IgG (1:2000 dilution, Bioreagents –SAB- 100; Stressgen) was used to detect HSP 70 probes. Bound antibodies were visualized by Gel Documentation system (Syngene, UK).

2.7. Haemato-immunological parameters

2.7.1. Blood haemoglobin

Blood haemoglobin (Hb) level was analysed following the cyanmethaemoglobin method using Drabkins Fluid (Qualigens Diagnostics, Division of Glaxo Smithkline Pharmaceutical Limited, India). Five millilitre of Drabkin's working solution was taken in a clean and dry test tube and 20 µl of blood was added to it. The absorbance was measured using a spectrophotometer at 540 nm. The final concentration was calculated by comparing with standard cyanmethaemoglobin (Qualigens). The haemoglobin concentration was then calculated by using the following formula:

Haemoglobin Content (*g*%) = [OD (T)/OD(S)]X[251/100]X60.

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

2.7.2. Total erythrocytes and leucocytes

Total erythrocytes (RBC, red blood cells) and leucocytes (WBC, white blood cells) were counted in a haemocytometer using erythrocyte and leucocyte diluting fluid (Qualigens, India), respectively. Blood sample (20 µl) was mixed with 3980 µl of diluting fluid in a clean and dry test tube. The mixture was shaken well to suspend the cells uniformly in the solution for counting. Following formula was used to calculate the number of erythrocytes and leucocytes per ml of the blood sample:

Number of cells ml^{-1} = (Number of cells counted x dilution) / (area counted/depth of fluid)

2.7.3. Serum total protein (STP), albumin (A), globulin (G) and A/G ratio

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

2.7.4. Respiratory burst activity

The respiratory burst activity of the neutrophils was measured by nitroblue tetrazolium (NBT) assay following the method of Secombes (1990) subsequently modified by Stasiack and Bauman (1996). Blood (50 µl) was placed into the wells of a 'U' bottom microtitre plates and incubated at 37 °C for 1 h to allow adhesion of cells. The supernatant was discarded and the wells were washed three times with phosphate-buffered saline (PBS). After washing, 50 µl of 0.2% NBT was added and incubated for 1 h. The cells were then fixed with 100% methanol for 2 min and washed three times with 30% methanol. The plates were air dried and 60 µl of 2 N potassium hydroxide and 70 µl dimethyl sulphoxide were added to each well. The OD was recorded in an enzyme-linked immunosorbent assay (ELISA) reader at 540 nm.

2.7.5. Serum lysozyme activity

Serum lysozyme activity was measured using an ion exchange chromatography kit (Banglore Geni, India). Serum samples were diluted with phosphate buffer (pH 7.4) to a final concentration of 0.33 mg ml^{-1} protein. In a suitable cuvette, 3 ml of *Micrococcus luteus* (Bangalore Genie, India) suspension in phosphate buffer (A_{450} = 0.5–0.7) was taken, to which 50 µl of diluted serum sample was added. The contents of the cuvette were mixed well for 15 s and reading was taken in a spectrophotometer at 450 nm exactly 60 s after addition of serum sample. This absorbance was compared with standard lysozyme of known activity following the same procedure as above. The lysozyme activity was expressed as U min^{-1} mg^{-1} protein of serum.

2.8. Histopathological studies

Neutral buffer formalin (NBF) fixed tissue samples such as liver and kidney were dehydrated by means of increasing ethanol concentrations (70–100%), followed by dipping in acetone and cleansing in benzene. The tissues were embedded in paraffin and serial sections were cut in to 5 µ thickness using microtome. The sections were stained with haematoxylin and eosin (H and E), as described by Roberts (1989). The tissue slides were cleaned in xylene, mounted in DPX, and then observed under light microscope.

2.9. Challenge study

After 45 days of feeding trial, 12 fish from each group were challenged with virulent *Aeromonas hydrophila* obtained from Aquatic

Animal Health Division, ICAR-Central Institute of Fisheries Education, Mumbai. The fish in each experimental group were intraperitoneally injected with 0.2 ml of bacterial suspension (1.8×10^8 CFU). Mortality was recorded for 10 days. Tissues were taken from the dead fish for bacteriological culture to confirm *A. hydrophila* as the cause of death. The relative percentage survival (RPS) was calculated as follows:

RPS = mortality (%) control–mortality (%) treatment /mortality (%) control x 100

2.10. Statistical analysis

Each experimental assay was performed in triplicate and values are expressed as mean ± SE. Two way analysis of variance (ANOVA) was used to determine difference between different temperature and graded L-tryptophan for each parameter, and interaction between temperature and L-tryptophan level on different parameters by Duncan's multiple range test to determine the significant difference at the 5% ($p < 0.05$) level. All the statistical analyses were performed with Statistical Package for the Social Sciences (SPSS Chicago, IL) Version 20.0 for Windows.

3. Results

3.1. Proximate composition of diet and growth performance

There was no significant difference in nutritional value of different experimental diets (Table 1). However, different experimental diets showed a concomitant increase in TRP level. Weight gain% in different experimental groups i.e. temperature and TRP interaction is shown in Fig. 1A. The interaction study showed that the weight gain% of different experimental groups varied significantly ($p < 0.05$) with incorportration of dietary TRP. The highest weight gain% was found in group exposed to 34 °C and fed with 1.44% of TRP, and lowest in group exposed to 38 °C and fed with 0% of TRP.

3.2. Blood glucose, cortisol and L- tryptophan

Effects of different temperatures and graded levels of dietary TRP and their interaction on blood glucose, cortisol and TRP are shown in Table 2 and Fig. 1B and 2 D. Both serum cortisol and blood glucose increased with the temperature ($p < 0.05$) and subsided with dietary TRP supplementation level ($p < 0.05$). The interaction of temperature and TRP in different experimental groups revealed that the glucose and cortisol level varied significantly ($p < 0.05$). The highest and the lowest values of cortisol and glucose were observed in groups exposed to 38 °C fed without TRP and ambient temperature group fed with 1.44% of TRP.

3.3. Hematological parameters

Data on RBC, WBC, Hb, NBT and lysozyme in fish under different temperatures and graded levels of dietary TRP and their interactions are shown in Tables 2, 3 and Figs. 1(C, D) and 2(B, C). Significant increases ($p < 0.05$) in values of RBC, Hb and NBT, and decrease in values of WBC and lysozyme were noticed with increase in temperature. TRP supplementation increased WBC, lysozyme and reduced respiratory burst activity, however it did not alter RBC count. The highest and lowest RBC, Hb and NBT value were recorded in group exposed to 38 °C, fed without TRP and ambient temperature group, fed with 1.44% TRP, respectively. Effect of different temperatures and graded levels of dietary TRP and their interaction on serum total protein, albumin, globulin and albumin-globulin ratio are presented in Table 3 and Fig. 2(A) and (B). Total serum protein, albumin, globulin and albumin-globulin ratio significantly decreaseed with increase in temperature whereas, dietary supplementation of TRP enhanced total protein and globulin values, and reduced albumin-globulin ratio.

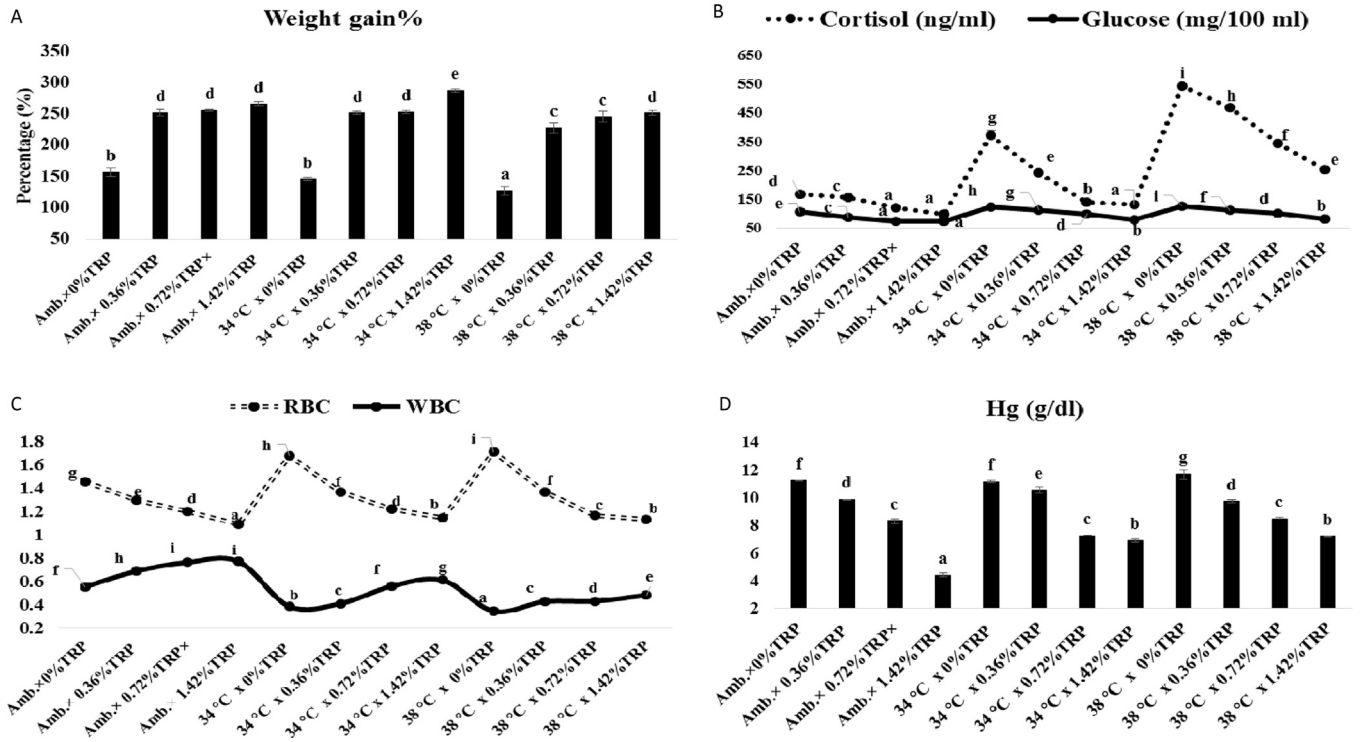

Fig. 1. (A–D): Interaction between different temperature and different level of dietary L-tryptophan on: (A) weight gain (%); (B) cortisol (ng ml^{-1}) and glucose (mg 100 ml^{-1}); (C) RBC (x 10^6 cells ml^{-1}) and WBC (x 10^6 cells ml^{-1}); (D) haemoglobin (g dl^{-1}) of *L. rohita* fingerlings. Mean value on the bar with different alphabets differs significantly ($p < 0.05$). Values are expressed as mean ± SE (n = 3).

Table 2
Effect of different temperature and graded dietary L-tryptophan (TRP) on serum cortisol, blood glucose, tryptophan, red blood cells (RBC), white blood cells (WBC) and haemoglobin (Hb) of *L. rohita* fingerlings.

Treatments	Cortisol	Glucose	TRP	RBC	WBC	Hg
Temperature (°C)						
Ambient	$135.51^{c} \pm 8.28$	$84.15^{b} \pm 3.88$	$0.58^{a} \pm 0.14$	$1.26^{b} \pm 0.04$	$0.70^{a} \pm 0.52$	$8.45^{b} \pm 0.50$
34	$220.38^{b} \pm 29.64$	$103.43^{a} \pm 5.20$	$0.11^{b} \pm 0.01$	$1.35^{a} \pm 0.06$	$0.66^{b} \pm 0.58$	$9.05^{a} \pm 0.86$
38	$400.92^{a} \pm 33.68$	$102.83^{a} \pm 4.66$	$0.10^{c} \pm 0.02$	$1.35^{a} \pm 0.07$	$0.66^{b} \pm 0.30$	$9.13^{a} \pm 0.44$
TRP (%)						
0	$360.45^{a} \pm 54.27$	$117.09^{a} \pm 3.21$	$0.05^{d} \pm 0.01$	1.32 ± 0.04	$0.43^{d} \pm 0.64$	11.34 ± 0.13
0.36	$288.16^{b} \pm 46.47$	$102.59^{b} \pm 4.16$	$0.10^{c} \pm 0.01$	1.35 ± 0.01	$0.51^{c} \pm 0.90$	10.02 ± 0.14
0.72	$200.22^{c} \pm 35.59$	$90.64^{c} \pm 4.26$	$0.43^{b} \pm 0.14$	1.28 ± 0.01	$0.59^{b} \pm 0.97$	12.00 ± 0.20
1.44	$160.24^{d} \pm 23.43$	$76.89^{d} \pm 1.18$	$0.47^{a} \pm 0.16$	1.32 ± 0.01	$0.62^{a} \pm 0.83$	11.15 ± 0.44

Mean values of all the assays were subjected to one way analysis of variance. Different superscripts in the same column indicate significant difference ($p < 0.05$) amongst different treatments. Values are expressed as mean ± SE (n = 3). Unit: Cortisol (Nano gm ml^{-1} of plasma), blood glucose (mg 100 ml^{-1}.), TRP (mg ml^{-1} serum), RBC (x 10^{6} cells mm^{-3}), WBC (x 10^{5} cells mm^{-3}), haemoglobin content (g dl^{-1}).

Table 3
Effect of different temperature and graded dietary L-tryptophan (TRP) levels on nitroblue tetrazolium (NBT), lysozyme, serum total protein (TP), albumin (A), globulin (G) and albumin/globulin ration (A/G) of *L. rohita* fingerlings.

Treatments	NBT	Lysozyme	TP	A	G	A/G
Temperature (°C)						
Ambient	$0.15^{c} \pm 0.01$	$652.74^{a} \pm 35.94$	$3.45^{a} \pm 0.10$	$1.06^{a} \pm 0.02$	$2.93^{a} \pm 0.12$	$0.37^{a} \pm 0.02$
34	$0.16^{b} \pm 0.01$	$431.29^{b} \pm 1.79$	$3.21^{b} \pm 0.10$	$0.84^{c} \pm 0.06$	$2.91^{a} \pm 0.15$	$0.31^{b} \pm 0.04$
38	$0.18^{a} \pm 0.01$	$419.22^{c} \pm 9.15$	$2.91^{c} \pm 0.10$	$0.93^{b} \pm 0.03$	$2.72^{c} \pm 0.15$	$0.36^{c} \pm 0.03$
TRP (%)						
0	$0.21^{a} \pm 0.01$	$338.18^{d} \pm 9.26$	$2.72^{d} \pm 0.08$	$1.06^{a} \pm 0.01$	$2.19^{d} \pm 0.07$	$0.49^{a} \pm 0.01$
0.36	$0.19^{b} \pm 0.01$	$440.95^{c} \pm 5.40$	$3.16^{c} \pm 0.09$	$1.01^{b} \pm 0.02$	$2.74^{c} \pm 0.09$	$0.38^{b} \pm 0.01$
0.72	$0.14^{c} \pm 0.01$	$550.53^{b} \pm 5.14$	$3.33^{b} \pm 0.08$	$0.92^{c} \pm 0.05$	$3.12^{b} \pm 0.05$	$0.30^{c} \pm 0.02$
1.44	$0.13^{d} \pm 0.01$	$674.68^{a} \pm 1.94$	$3.54^{a} \pm 0.09$	$0.77^{d} \pm 0.06$	$3.37^{a} \pm 0.04$	$0.23^{d} \pm 0.02$

Mean values of all the assays were subjected to one way analysis of variance. Different superscripts in the same column indicate significant difference ($p < 0.05$) amongst different treatments. Values are expressed as mean ± SE (n = 3). Unit: NBT (absorbance at 540 nm), lysozyme activity (unit min^{-1} mg^{-1} serum protein), serum total protein (g dl^{-1}), albumin (g dl^{-1}), globulin (gdl^{-1}).

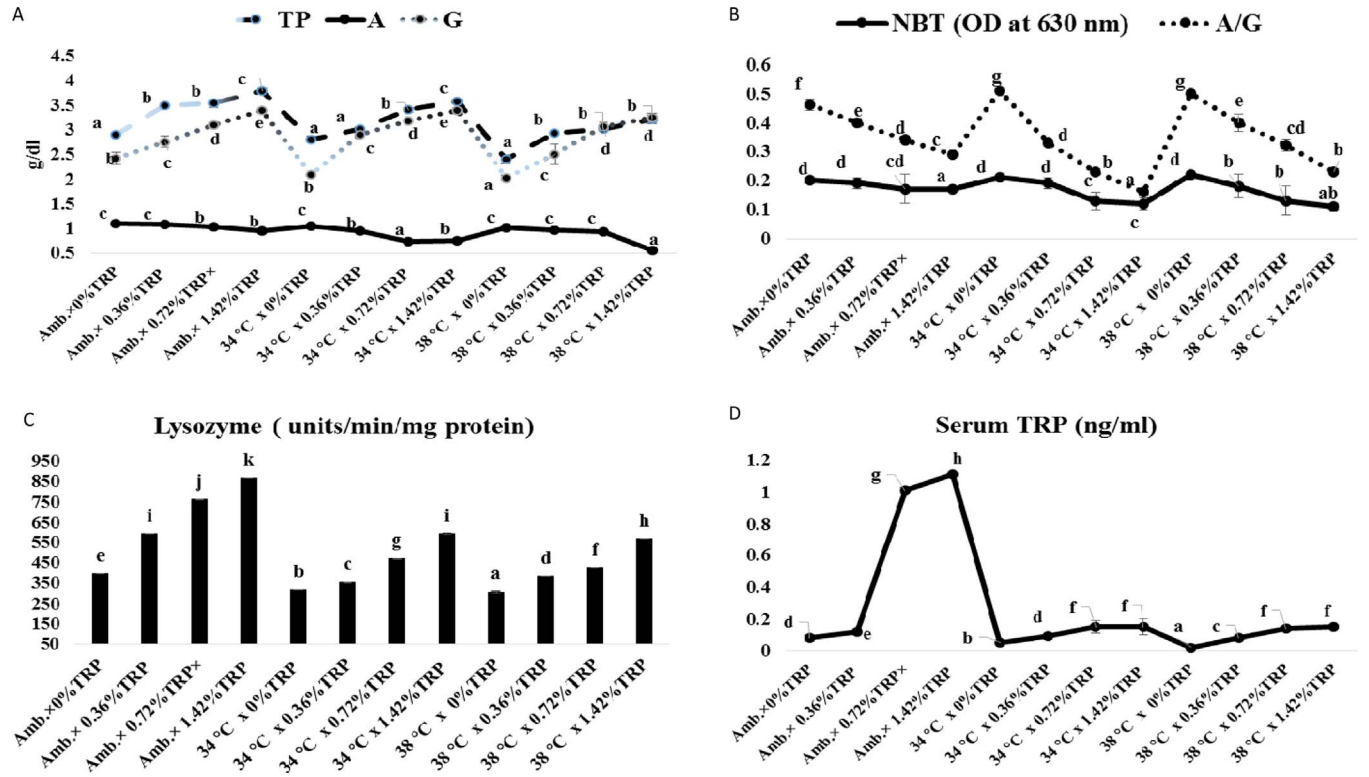

Fig. 2. (A–D): Interaction between different temperature and different level of dietary L-tryptophan on: (A) serum total protein (TP, g dl^{-1}), albumin (A, g dl^{-1}) and globulin (G, g dl^{-1}); (B) NBT (absorbance at 540 nm) and A:G ratio; (C) lysozyme (unit min^{-1} mg^{-1} serum protein) and (D) serum TRP (ng ml^{-1}) of *L. rohita* fingerlings. Mean value on the bar or line with different alphabets differs significantly ($p < 0.05$). Values are expressed as mean ± SE (n = 3).

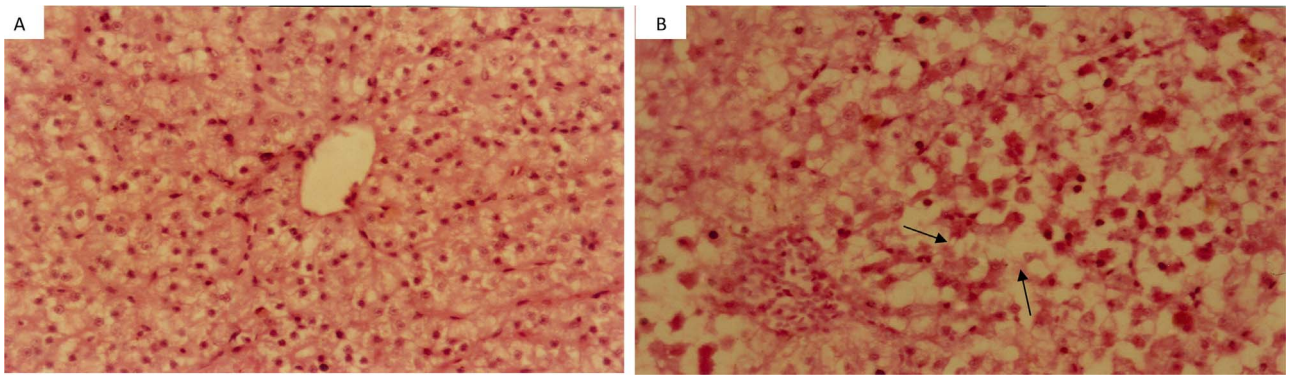

Fig. 3. (A–B): A-Liver tissues of *L. rohita* fingerlings showed normal histoarchitecture (H & E 160X), B: vacuolation of disc and patches (arrow) in liver parenchyma with obliterated architecture of liver tissues (H & E 80X).

3.4. Histological changes in liver and kidney

Liver and kidney tissues of *L. rohita* fingerlings of all the experimental groups, except group exposed to 34 °C and 38 °C, and fed with 0% of TRP appeared normal, with normal cellular architecture and staining character (Fig. 3A and 4A). Liver tissue of fish at higher temperature (34 and 38 °C) without TRP supplementation in diet showed mark vacuolation of disc and patches in liver parenchyma with obliterated architecture of liver tissues (Fig. 3B). Similarly, Kidney tissue at higher temperature without TRP supplementation in diet showed marked hyperplasia (Fig. 4B).

3.5. Relative percentage survival

After challenge with *A. hydrophila,* the first mortality was recorded after 18 h. The relative percentage survival is presented in (Fig. 5). The highest survival (90%) survival was recorded in ambient temperature group, fed with 0.72% and 1.44% TRP followed by group exposed to 38 °C, fed without TRP. The lowest survival (20%) was recorded in group exposed to 38 °C, fed with 0% of TRP.

3.6. HSP 70

It was very interesting to note that none of the experimental groups showed expressions of HSP 70 in muscle tissue as shown in Fig. 6.

4. Discussion

In this study, primary, secondary and tertiary stress responses in terms of cortisol; blood glucose, immune-hematological, histoapthological changes; survival, and growth performaces were assessed in *L. rohita* reared under elevated temperatures such as 34 and 38 °C.

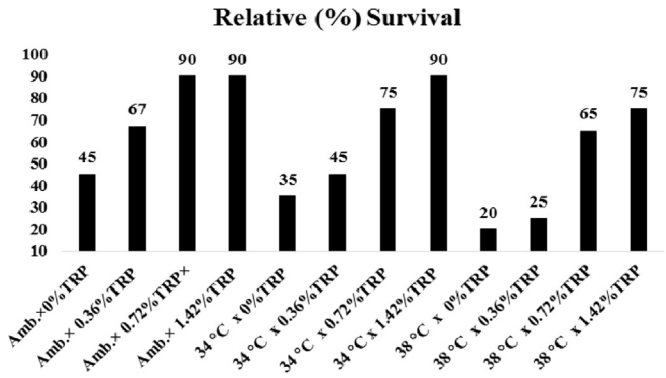

Fig. 5. Interaction between different temperature and different level of dietary L-tryptophan on relative percentage survival (RPS) of *L. rohita* fingerlings. Mean value on the bar with different alphabets differs significantly ($p < 0.05$). Values are expressed as mean ± SE (n = 3). HSP70 + T1 T2 T3 T4 T5 T6 HSP70 + T7 T8 T9 T10 T11 T12.

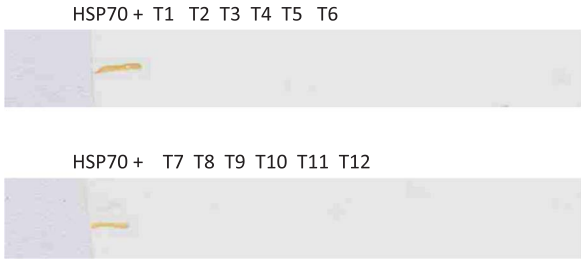

Fig. 6. Western blot analysis showing HSP70, Lane 1- PC- Positive control (recombinant Chinook salmon HSP70) and other lanes represent all treatments (T1, T2, T3, T4, T5,T6,T7,T8,T9,T10,T11 and T12 respectively.

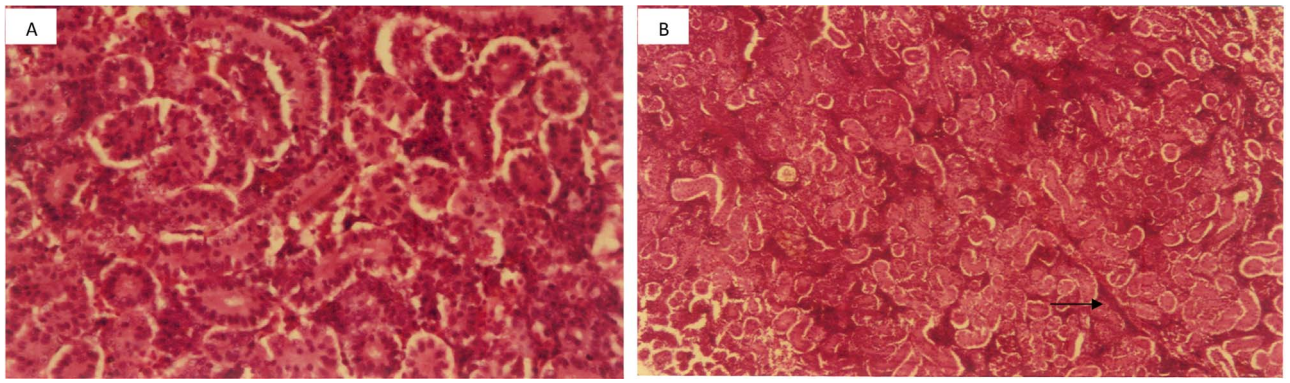

Fig. 4. (A–B): A-Kidney tissues of *L. rohita* fingerlings showed normal appearance (H & E 160X), B: Kidney tissues of *L. rohita* fingerlings showed many fold increased cell number (arrow) (H&E 80X).

TRP supplementation with appropriate inclusion to fish feed enhanced growth performance in term of weight gain (%), which might be due to improved protein utilization triggered by high TRP, an essential amino acid (EAA) (Abidi and Khan, 2010). Whereas, low weight gain (%) was noticed in high temperature and low level of TRP inclusion, which might be due to stress rendered by higher temperature and impaired protein synthesis due to deficiency of TRP. In contrast, higher inclusion of TRP causes amino acid toxicity and TRP catabolism, which results in poor growth (Abidi and Khan, 2010; Pianesso et al., 2015). Alam et al. (2002) also suggested that excessive amino acid levels in diet may become toxic and adversely affected growth performance of Japanese flounder due to disproportionate ingestion, absorption and utilization of other amino acid. The reduction in weight gain (%) in fish reared under high temperature might be due to thermal stress and unbalanced body homeostasis (Das et al., 2005; Kumar et al., 2017). Supplementation of dietary TRP at 1.44% of diet significantly improved weight gain% at higher temperature of 34 °C. This may be due to stress mitigating effect of TRP as reported by Akhtar et al. (2012) and Kumar et al. (2014).

Blood glucose level is an ideal parameter to study the secondary stress response (Wedemeyer and Mcleay, 1981). In the current study, blood glucose level increased with increase in temperature, which is in accordance with the findings of Costas et al. (2012). Reduction in blood glucose level in dietary TRP supplemented groups, could be due to inhibitory action of TRP on release of cortisol, which is responsible for gluconeogenesis during stress. Gradual decrease in blood glucose level due to dietary supplementation of TRP during thermal stress confers the thermal stress mitigating effect of TRP. Similar observations were made by Akhtar et al. (2012) in *L. rohita* in which dietary supplementation of TRP reduces blood glucose level in stressed fish. Cortisol is an extensively used indicator of primary stress response in fish, and it generally increases after stress (Hoseini and Hosseini, 2010; Costas et al., 2012). In the present study, maximum serum cortisol was found in higher temperature groups fed without dietary TRP, indicating thermal stress. Gradual decrease in cortisol level due to dietary supplementation of TRP in all the higher temperature groups (34 and 38 °C) confirms the thermal stress mitigating effect of TRP. Similarly, crowding and osmotic stress mitigation effects of TRP in *Cyprinus carpio* and *L. rohita* were reported by Hoseini and Hosseini (2010) and Akhtar et al. (2012), respectively. We have also observed a significant reduction of cortisol level in unstressed fish (ambient temperature group), which is similar to the observations of Martins et al. (2013).

Gradual reduction in plasma TRP level in higher temperature groups indicates the utilization of plasma TRP for synthesis of serotonin (5-hydroxytryptamine), which might be essential to combat the stress (Costas et al., 2012). Thermal stress significantly reduces the erythrocyte count and Hb content in *L. rohita* fingerlings, which might be due to change in the haemoglobin-oxygen affinity caused by alternation in blood pH to diminish the need of oxygen (Moyle and Cech, 1982), or lysis of erythrocyte cells (Ciji et al., 2012; Akhtar et al., 2012). Dietary supplementation of TRP does not restore the erythrocytes and Hb levels. Further, it is suggested that TRP is not capable to stimulating erythropoiesis in *L. rohita* fingerlings. However, there is inadequate literature available on this aspects in fish, and further studies are required to validate these results. Leucocyte is an indicator of health status and play an important role in immunity of fish. In the present study, total leucocyte count significantly decreased due to higher temperature stress, which is similar to the finding of Akhtar et al., 2012., contradictory to the observations made by Alexander (2011) in *L. rohita* fingerlings due to thermal stress. Interactions of different temperature and graded levels of tryptophan showed an improvement in leucocyte counts by dietary supplementation of 1.44% tryptophan. Respiratory burst activity measures the phagocytic activity of macrophages, which is measured by reduction of NBT by intracellular superoxide radicals produced by leucocytes. In this study, respiratory burst activity increased due to thermal stress which is contradictory to the reports of Akhtar et al. (2012) in *L. rohita*. Lysozyme plays an important role in innate immunity by lysis of the bacterial cell wall and thus stimulating phagocytosis of bacteria (Ellis, 1990). Lysozyme activity increases concomitantly with the number of leucocytes (Fletcher and White, 1973). In the current study, lysozyme activity significantly decreased in higher temperature groups, however dietary supplementation of 1.44% tryptophan resumed the lysozyme activity. Among the serum proteins, albumin and globulin are the major proteins that play a significant role in the immune response (Kumar et al., 2007). In the present study, total protein, albumin, and globulin levels decreased due to thermal stress. Similar observation was made by Akhtar et al. (2012). Dietary supplementation of 1.44% tryptophan resulted in restoration of serum total protein and globulin, suggesting an immuno-stimulating effect of tryptophan. Histopathological changes in liver and kidney tissue of higher temperature groups without tryptophan supplementation in diet showed a clear vcuolation of disc and patches in parenchyma, and many-fold increase in cell membrane, compared to other groups fed with dietary supplementation of tryptophan. This indicates the tissue repairing effect of tryptophan in liver and kidney, which was damaged due to thermal stress. However, there are no published literatures to support this observation. After challenge test with *Aeromonas hydrophila*, the RPS was found maximum (90%) in 1.44% tryptophan supplemented diet fed fish at ambient temperature and 34 °C groups, whereas it was minimum (20%) in 38 °C group without dietary supplementation of tryptophan. It was very interesting to observed that expression of HSP 70 in any of the experimental groups wasnot noticed, which might be due to thermal adaptation to the experimental temperature in 45 days of experiment. However, there is inadequate literature available on this aspects in fish, and further studies are required to validate this result.

From the study, it is concluded that dietary supplementation of 1.44% dietary L-tryptophan mitigates thermal stress and gives protection against bacterial infection to *L. rohita* fingerlings.

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