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Research Article

Impact of low water pH on health and disease susceptibility of *Labeo rohita* fingerlings in changing climate conditions

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Abstract

The present study evaluated the impact of low water pH on the health and disease susceptibility of *Labeo rohita* fingerlings (mean length 11.7±0.5 cm; mean weight 14±0.5 g) under changing climate conditions. *L. rohita* fingerlings were exposed to water with pH levels of T1 (4.0-4.5), T2 (5.2-5.7), and control (6.4-6.9) for 120 h. Haematological, biochemical, and immunological parameters were measured at 0, 6, 12, 24, 48, 72, and 120 h intervals, along with mortality rates following a 10-day exposure to *Aeromonas hydrophila* infection. Results showed a significant ($p<0.05$) decrease in haemoglobin (Hb), packed cell volume (PCV), and total protein levels in fish exposed to low pH, while white blood cell count, glucose, SGOT, SGPT, alkaline phosphatase, lysozyme, and phagocytic activity were significantly ($p<0.05$) elevated. The challenge study with *A. hydrophila* infection revealed significantly higher mortality rates ($p<0.05$) in fish kept at low water pH (T1 and T2) compared to the control. The most pronounced alterations in blood parameters and increased mortality rates occurred at pH 4.0-4.5. These findings suggest that low water pH induces physiological stress, leading to altered blood parameters and increased susceptibility to bacterial infections in *L. rohita* fingerlings, highlighting potential risks under changing climate conditions.

Keywords: Biochemical response, Haematology, Immunological response, Mortality, Stress

Highlights

- Hemoglobin, PCV, and protein levels drop significantly at lower pH levels.
- Elevated WBC count, glucose, SGOT, SGPT, and immune responses were observed.
- Low water pH increases *L. rohita* fingerlings' susceptibility to infections.
- Mortality is highest at pH 4.0-4.5, indicating severe stress and infection risks.

INTRODUCTION

Water quality is a critical determinant of fish productivity and plays a central role in the overall health, normal metabolism, and growth of aquatic organisms. Among the various water quality parameters, pH, which measures the concentration of hydrogen ions (H^+), is particularly significant due to its influence on numerous chemical and biological processes essential for maintaining the physiological well-being of aquatic animals (Viadero Jr, 2019). In recent years, the issue of water quality has been further exacerbated by climate change, which has led to an increase in the acidity of rainfall. This phenomenon poses a significant challenge for aquaculture, as it lowers the pH of natural water bodies, creating adverse conditions that directly impact fish productivity (Maulu *et al*., 2021).

Fish have specific pH tolerances, with the optimal range for productivity being 6.5 to 8.5, while levels between 5 and 9 are considered safe for survival. pH levels below 6.5 cause stress and levels below 5.0 can lead to high mortality in fry and juveniles. A pH below 4.0 is typically lethal (Parra and Baldisserotto, 2019). Low pH disrupts ion regulation and acid-base balance in fish, particularly affecting the gills, which are vital for respiration and ion exchange. These disruptions can impair growth, reproduction, and overall performance, making fish more vulnerable to disease (Swain *et al*., 2020). Zahangir *et al*. (2015) reported the impact of low pH on zebrafish (*Danio rerio*), while Ghanbari *et al*. (2012) studied its effects on *Cyprinus carpio*. These studies highlight the broad susceptibility of fish to pH-induced stress and decreased performance.

Environmental stressors, such as altered water pH, significantly increase fish susceptibility to various pathogens (Walters and Plumb, 1980; Wise *et al*., 1993; Tort *et al*., 1998; Sherif and Soad, 2013). *Aeromonas hydrophila*, in particular, is linked to severe disease outbreaks in stressed, pond-cultured fish (Semwal *et al*., 2023). With climate change exacerbating environmental fluctuations, understanding the impact of low pH on fish health is critical for sustainable aquaculture. Haematological, biochemical, and immunological

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parameters are valuable tools for assessing the physiological effects of environmental stressors. These parameters provide insights into how fish respond to changes in their environment, such as fluctuations in water chemistry and temperature, which are becoming more common due to climate change (Shahjahan *et al*., 2022).

Despite the growing recognition of these challenges, the interaction between climate change-induced factors, such as low water pH, and the susceptibility of fish to *A. hydrophila* infections has not been extensively studied. This gap in knowledge highlights the need for research focused on the effects of varying low water pH levels on the haematological, biochemical, and immunological parameters of *L. rohita* fingerlings. Such studies are essential for understanding the mortality risk of *A. hydrophila* infection and for developing strategies to mitigate these risks, thereby ensuring the sustainable development of aquaculture in the face of climate change.

MATERIALS AND METHODS

Collection and maintenance of experimental animals: A total of 315 *L. rohita* fingerlings (mean length 11.7±0.5 cm; mean weight 14±0.5 g) were collected from the College Pond (College of Fisheries, Lembucherra, Tripura West, India). The fish were acclimatized for 15 days in 500 L FRP tanks before the experiment. During acclimatization, the fish were provided with adequate aeration and fed extruded floating pellets containing 30% crude protein, procured from the Department of Aquaculture, College of Fisheries, Lembucherra. Feeding was done at 3% of body weight twice daily, and 30% of the water was exchanged daily. The pH of the water was maintained between 6.4 and 6.9 by adding 4(N) HCl or 4(N) NaOH.

Setting up the experiment: The experiment was conducted in rectangular glass aquariums (22 x12 x 12), following a Completely Randomized Design (CRD). A total of 315 well-acclimatized *L. rohita* fingerlings were evenly distributed into 63 aquariums, with five fish per aquarium, each containing 45 L of water. The fish were exposed to three pH treatments: T1 (pH 4.0-4.5), T2 (pH 5.2-5.7), and a control (pH 6.4-6.9), with three replicates for each treatment at six exposure times 0, 6, 12, 24, 48, 72, and 120 h (Li and Chen, 2008). The pH of the experimental water was adjusted using 4(N) HCl or 4(N) NaOH, and the fish were not fed during the experiment. Aeration was provided to ensure proper oxygenation, and 30% of the water was exchanged every 12 h to maintain water quality. The pH of the experimental water was maintained according to the treatment conditions throughout the exposure time.

Collection of blood and serum: Sampling for different haemato-biochemical and immunological parameters were done at 0, 6, 12, 2 , 48, 72 and 120 h of exposure time. Blood was drawn from the caudal vein using a 1.0 mL syringe and 24-gauge needle after the fish were anesthetized with clove oil (at the rate of 0.2 mL per liter of water). Blood samples were transferred into EDTA-coated vials. Serum samples were collected without EDTA after allowing the blood to clot for 2 h, then stored at -20°C.

Determination of haematological parameters: Haematological parameters were assessed following Schaperclaus (1991). RBC and WBC counts were done using a haemocytometer. Packed Cell Volume (PCV) was determined using a haematocrit reader after centrifugation for 5 minutes at 5000xg, and haemoglobin (Hb) levels were estimated using a Sahli Haemometer.

Determination of biochemical parameters: Biochemical parameters, including blood glucose, total protein, serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), and alkaline phosphatase (ALP), were measured using a standard diagnostic kit (as per the manufacturer's instructions; Crest Biosystems, India).

Determination of immunological parameters: Immunological parameters were assessed following Anderson and Siwicki (1995) with slight modifications. Lysozyme activity was measured by adding 0.9 mL of *Micrococcus lysodeikticus* (Sigma, St Louis, MO, USA) (0.75 mg mL^{-1} in phosphate-buffered saline, pH 6.2) to 0.1 mL of serum, mixing thoroughly, and measuring the absorbance in a spectrophotometer at 450 nm at 1-minute intervals for 10 minutes. Change of absorbance was calculated.

Respiratory burst activity was determined using nitroblue tetrazolium (NBT) assay, where 0.1 mL of blood was mixed with an equal amount of 0.2% NBT solution. Incubated for 30 minutes at room temperature, and then 0.05 mL of the NBT-blood suspension was added to 1.0 mL of N, N-dimethyl formamide (DMF). The tube was then centrifuged at 3000 rpm for 5 minutes, and the absorbance of the supernatant was measured at 540 nm.

Total immunoglobulin level was estimated by adding 0.1 mL of 12% polyethylene glycol to 0.1 mL of serum, incubating the mixture for 2 h, and centrifuging at 7000 rpm for 10 minutes to collect the supernatant. The immunoglobulin concentration was determined by measuring total protein in the supernatant using a total protein analysis kit (Crest Biosystems, India), with the protein absorbed by polyethylene glycol subtracted from the total plasma protein. Total immunoglobulin (g dL^{-1}) = Total protein in individual samples - Total protein taken out by absorption to polyethylene glycol.

Phagocytosis activity was evaluated by adding 0.1 mL of blood to 0.1 mL of *Pseudomonas aeruginosa* ($1x10^7$cells) suspended in phosphate-buffered saline

(pH 7.2), incubating for 20 minutes at room temperature, and preparing a smear on a glass slide. The smear was air-dried, fixed in 95% ethanol for 5 minutes, stained with 10% Giemsa (pH 6.8) for 10 minutes, and examined under a light microscope. Approximately 100 phagocytes were counted, and the percentage of active phagocytes engulfing antigens was determined. (Phagocytic activity (%) = number of phagocytizing cells/total number of phagocytes counted X 100).

Challenge study: A pure culture of *A. hydrophila* COF_AHE58 (GenBank accession number: OQ244503) was obtained from the Department of Aquatic Health and Environment, College of Fisheries, Tripura. The bacteria were grown on nutrient broth for 24 h at 37°C. Following the lethal dose 50 (LD_{50}) protocol by Das *et al*. (2013), a bacterial suspension was prepared with an optical density of 0.5 at 456 nm, corresponding to 1x10w cells/mL. For the challenge study, *L. rohita* fingerlings (mean length 11.7±1.5 cm; mean weight 14±2.5 g) were injected intraperitoneally with 0.2 mL of the bacterial suspension and randomly distributed across aquariums (10 fish per aquarium) under three pH conditions: T1 (pH 4.0-4.5), T2 (pH 5.2-5.7), and control (pH 6.4-6.9). A Completely Randomized Design (CRD) with three replicates per treatment was employed. Mortality was recorded daily over a 10-day period, and *A. hydrophila* was re-isolated from the liver of moribund or freshly dead fish to confirm infection. Mortality percentage was calculated according to Khan *et al*. (2018).

$$\text{Mortality (\%)} = \frac{\text{Total number of fish died}}{\text{Total number of fish infected}} \text{ X } 100$$

Analysis of water quality parameters: Water pH was measured using a water analyzer (Multi 340i/SET). Other parameters like temperature, dissolved oxygen, ammonia, and alkalinity were measured every sampling hour according to APHA (2005).

Data analysis: Data were analyzed using SPSS-15.0 (SPSS Inc., Chicago, IL, USA). Results are expressed as mean ± standard deviation. One-way Analysis of Variance (ANOVA) and the TUKEY test were used to compare the mean values at 0.05 levels of significance.

RESULTS

Haematological parameters: The haematological results show a declining trend in total haemoglobin for both T1 and T2 from 24 to 120 h, with a significant reduction ($p<0.05$) in T1 at 120 h compared to the control (Fig. 1). Packed cell volume (PCV) decreased in T1 and T2 from 24 to 120 h, significantly lower ($p<0.05$) than the control at 48, 72, and 120 h (Fig. 2). Total red blood cells (RBCs)

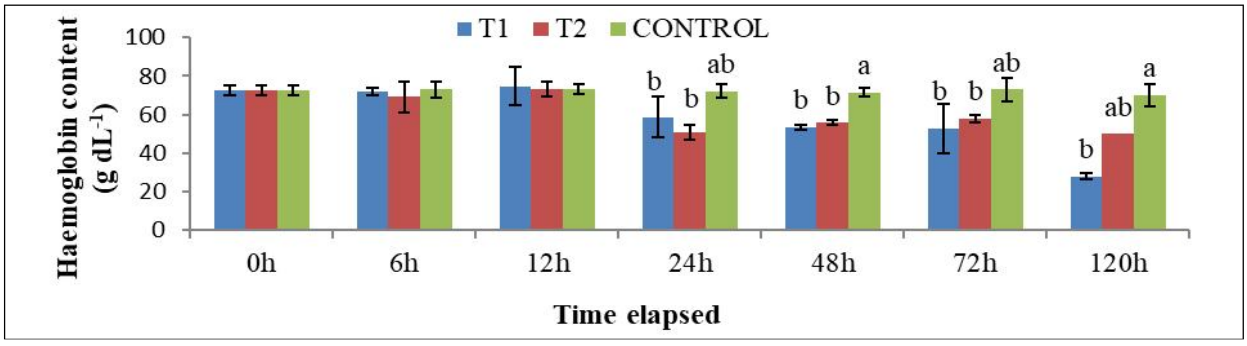

Fig. 1. Hemoglobin content (g dL^{-1}) of different experimental groups on various sampling hours with different levels of acid stress. Means without a common superscript letter differ significantly (P<0.05).

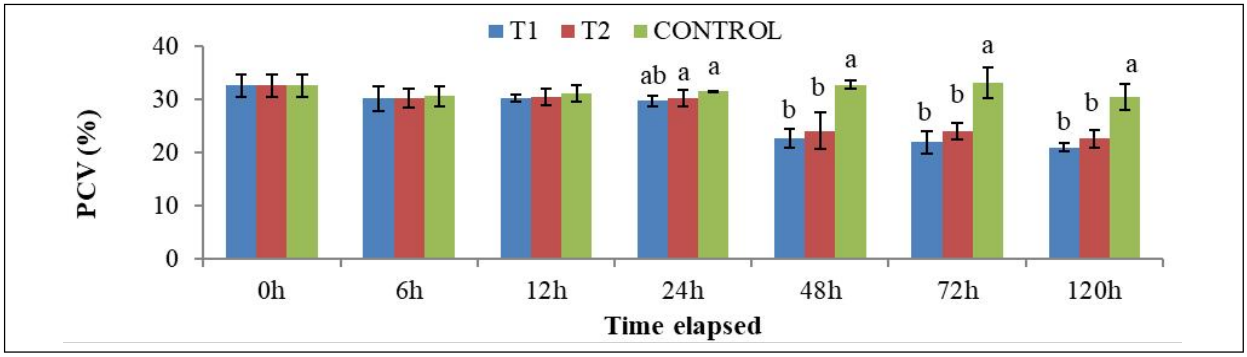

Fig. 2. PCV (%) of different experimental groups on various sampling hours with different levels of acid stress. Means without a common superscript letter differ significantly (P<0.05).

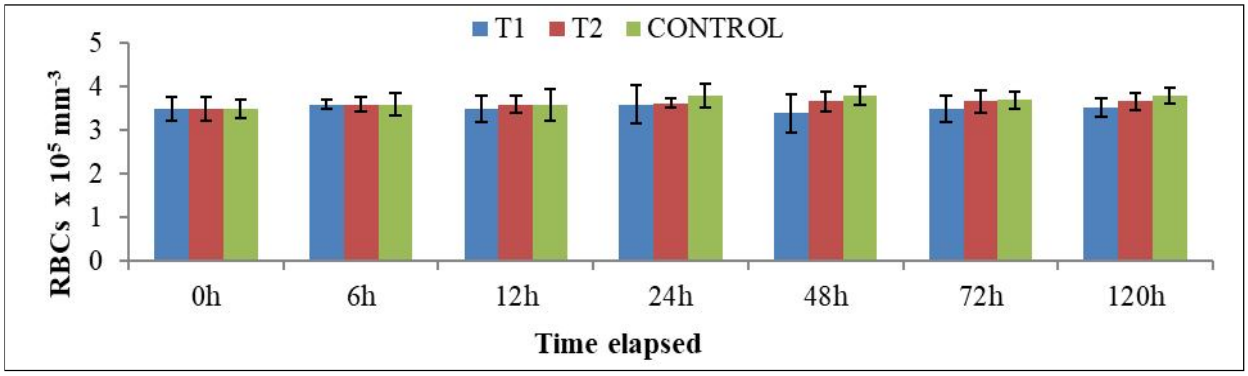

Fig. 3. RBC count (x 10^5 mm^{-3}) of different experimental groups on various sampling hours with different levels of acid stress. Means without a common superscript letter differ significantly (P<0.05).

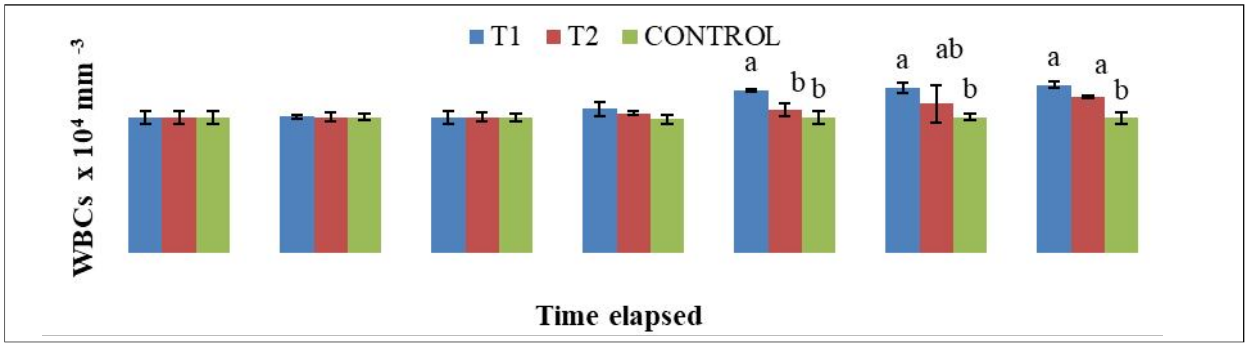

Fig. 4. WBC count (x 10^4 mm^{-3}) of different experimental groups on various sampling hours with different levels of acid stress. Means without a common superscript letter differ significantly (P<0.05).

count slightly decreased in T1 and T2, though not significantly (Fig. 3). Total white blood cells (WBCs) count increased significantly (p<0.05) in T1 at 48 and 72 h, and in both T1 and T2 at 120 h compared to the control (Fig. 4).

Biochemical parameters: Glucose levels in both T1 and T2 showed a consistent increase, with a significant rise (p<0.05) at 72 and 120 h compared to the control (Fig. 5). Total protein levels decreased over time, with a significant reduction (p<0.05) in T1 from 6 to 120 h (Fig. 6). SGOT and SGPT levels also increased significantly (p<0.05) in both groups compared to control, with a higher elevation in T1, especially at 72 and 120 h (Fig. 7 & 8). ALP activity significantly increased (p<0.05) in T1 and T2 at 48, 72, and 120 h, though no difference was noted between treatments (Fig. 9).

Immunological parameters: Lysozyme activity was significantly elevated (p<0.05) in both treatment groups at all times except 0 h compared to control, with T1

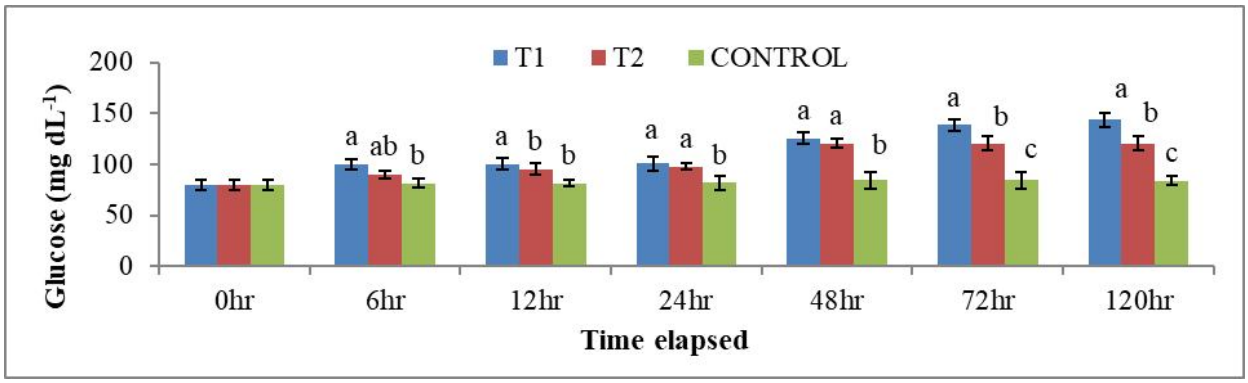

Fig. 5. Glucose level (mg dL^{-1}) of different experimental groups on various sampling hours with different levels of acid stress. Means without a common superscript letter differ significantly (P<0.05).

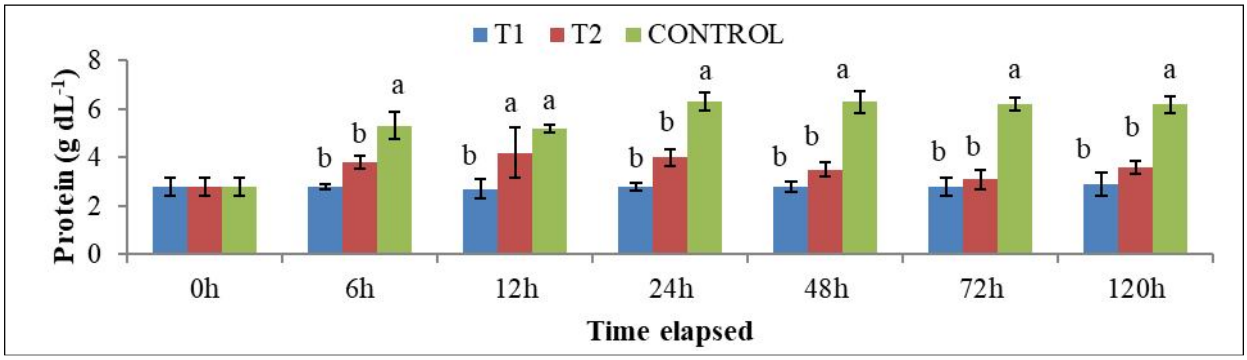

Fig. 6. Total protein level (g dL^{-1}) of different experimental groups on various sampling hours with different levels of acid stress. Means without a common superscript letter differ significantly (P<0.05).

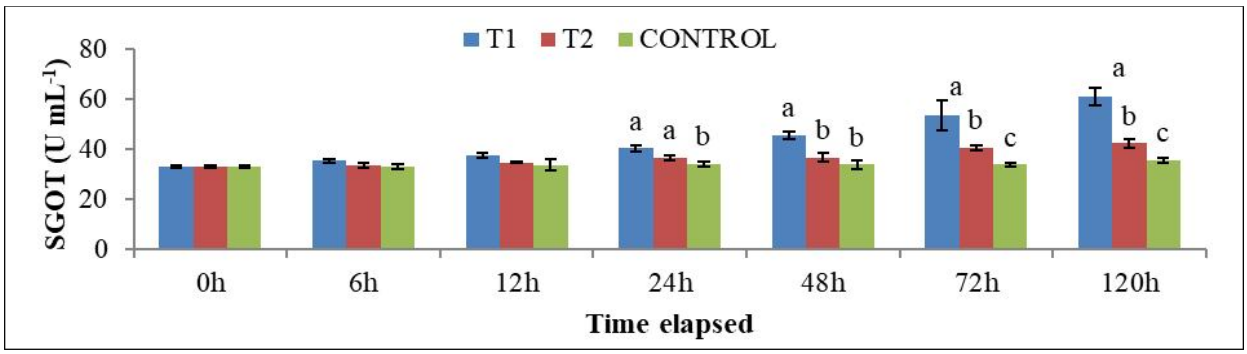

Fig. 7. SGOT level (U mL^{-1}) of different experimental groups on various sampling hours with different levels of acid stress. Means without a common superscript letter differ significantly (P<0.05).

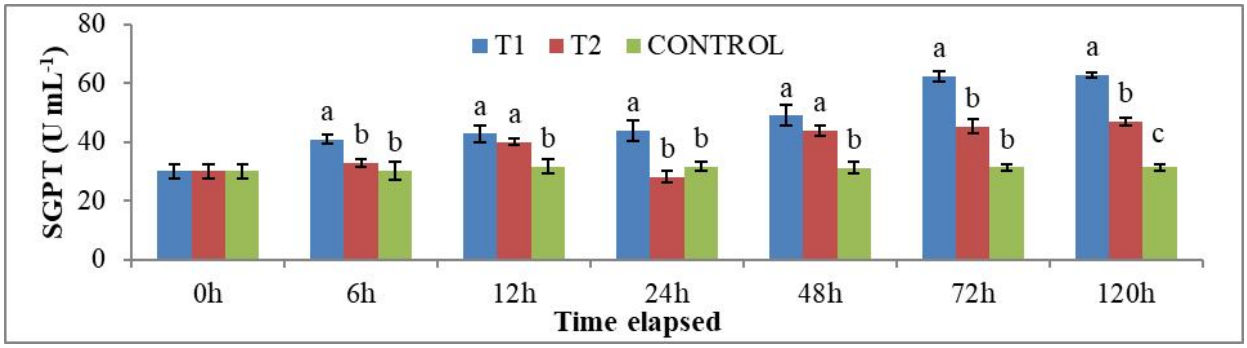

Fig. 8. SGPT level (U mL^{-1}) of different experimental groups on various sampling hours with different levels of acid stress. Means without a common superscript letter differ significantly (P<0.05).

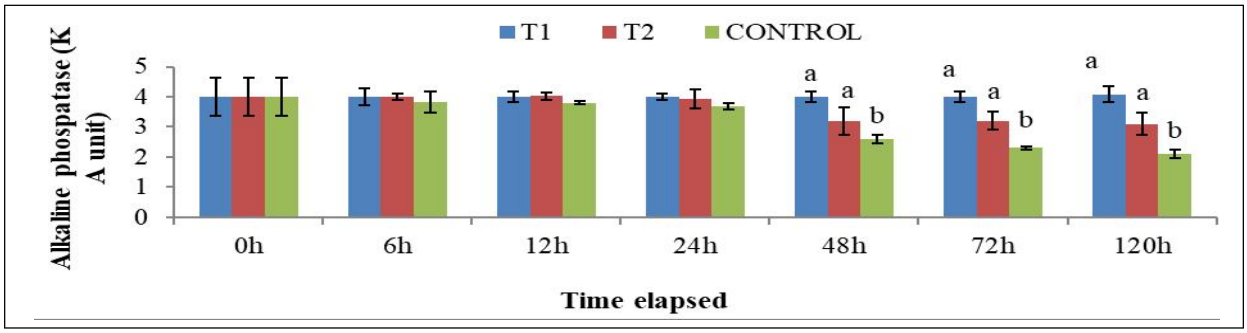

Fig. 9. Alkaline phosphatase activity (K A unit) of different experimental groups on various sampling hours with different levels of acid stress. Means without a common superscript letter differ significantly (P<0.05).

showing a significant increase at 48, 72, and 120 h (Fig.10). NBT levels were significantly higher ($p<0.05$) in T1 at all time points except 0 h compared to the control (Fig. 11). Total immunoglobulin levels did not differ significantly ($p<0.05$) among the control, T1, and T2 (Fig. 12). Phagocytic activity in T1 and T2 was significantly higher ($p<0.05$) at 24, 48, 72, and 120 h than in the control, though no difference was observed between T1 and T2 (Fig. 13).

Challenge study: A 10 days' challenge study revealed that mortality rates in T1 and T2 were significantly higher ($p<0.05$) compared to the control group. Among the experimental groups, T1 exhibited higher mortality rates, which was significantly greater ($p<0.05$) than the experimental group, T2 (Fig. 14).

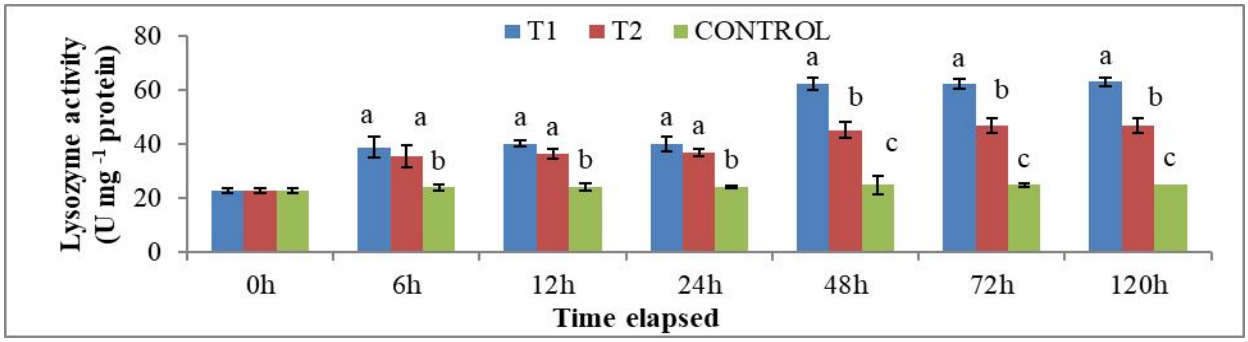

Fig. 10. Lysozyme activity (U mg^{-1} protein) of different experimental groups on various sampling hours with different levels of acid stress. Means without a common superscript letter differ significantly ($P<0.05$).

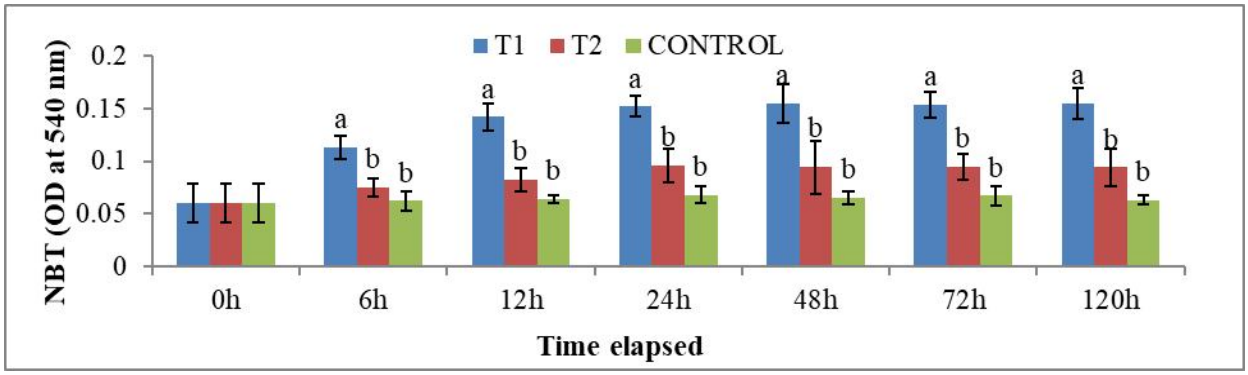

Fig. 11. NBT level (OD at 540 nm) of different experimental groups on various sampling hours with different levels of acid stress. Means without a common superscript letter differ significantly ($P<0.05$).

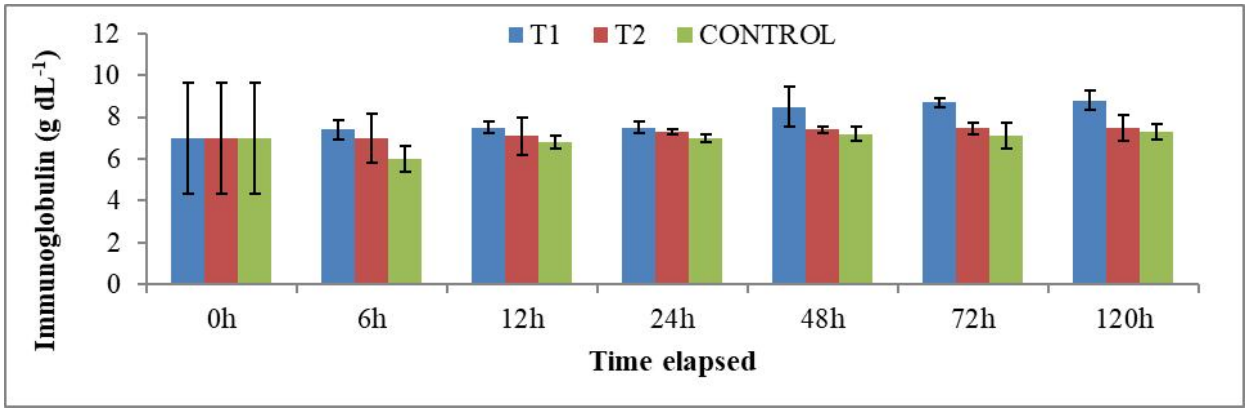

Fig. 12. Total immunoglobulin level (g dL^{-1}) of different experimental groups on various sampling hours with different levels of acid stress. Means without a common superscript letter differ significantly ($P<0.05$).

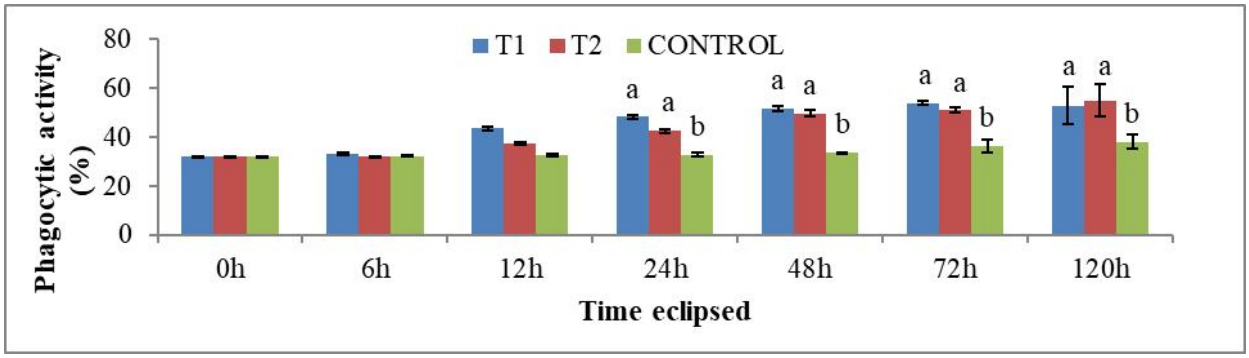

Fig. 13. Phagocytic activity (%) of different experimental groups on various sampling hours with different levels of acid stress. Means without a common superscript letter differ significantly (P<0.05).

Physicochemical parameters of water: Throughout the experiment, all water parameters- temperature, dissolved oxygen, alkalinity, and ammonia-N were within acceptable limits (Table 1).

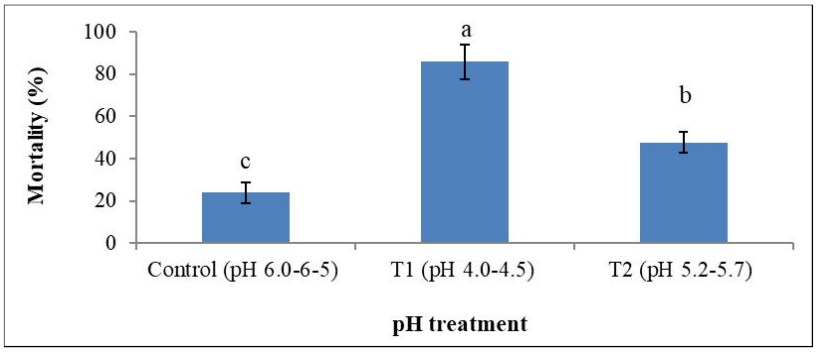

Fig. 14. Mortality percentage of *L. rohita* fingerlings infected with *A. hydrophila* exposed to selected water pH. Means without common superscript letter differ significantly (P<0.05).

Table 1. Water quality parameters (mean ± SD) during the experimental period

Parameter	Control	T1	T2
Temperature (°C)	27.25±0.75	27.25±0.75	27.25±0.75
pH	6.65±0.25	5.45±0.25	4.25±0.25
DO (mg L^{-1})	4.99±0.61	5.40 ± 0.20	5.15±0.35
Alkalinity (mg L^{-1})	104±4	100±3	106.5±3.5
Ammonia-N (mg L^{-1})	0.02±0.01	0.015±0.005	0.02±0.01

DISCUSSION

Haemato-biochemical and immunological parameters are crucial for assessing fish health, particularly in response to environmental stressors such as those associated with climate change (Shahjahan *et al*., 2022). In this study, significant reductions in haemoglobin (Hb) and pack cell volume (PCV) levels were observed in the treatment group, similar to findings in *Catla catla*, *Labeo rohita*, and *Cirrhinus mrigala* exposed to low pH levels of 5.5 (Das *et al*., 2006). The reductions in Hb and PCV observed in *L. rohita* fingerlings exposed to low water pH, especially over extended periods, indicate a decline in the blood's oxygen-carrying capacity under stress. This decline is likely due to red blood cell distortion and lysis, potentially caused by the depletion of hematopoietic activity in the kidney, or haemodilution resulting from low pH (Kim *et al*., 2021). Climate change-induced water acidification can further disrupt ion regulation and acid-base balance at the gills, leading to imbalances in internal pH, electrolytes, and osmotic pressures, which may trigger catecholamine release and disrupt red blood cell homeostasis (Ghanbari *et al*., 2012). Additionally, a significant increase in total WBCs count was observed, consistent with findings in zebrafish (Zahangir *et al*., 2015) and *L. rohita* (Kandari and Rauthan, 2013) exposed to similarly low pH levels. The rise in WBCs count likely reflects a stress response and immune system activation to combat environmental stressors, including those linked to climate change (Das *et al*., 2006).

Blood glucose levels are well-established indicators of stress in fish and serve as reliable secondary stress response markers (Dara *et al*., 2023). In this study, *L. rohita* fingerlings exposed to acidic water showed significantly higher glucose levels compared to controls, consistent with findings in other fish species under low pH stress (Das *et al*., 2006; Ghanbari *et al*., 2012; Zahangir *et al*., 2015). This increase in glucose, driven by the hypothalamo-pituitary axis and sympathetic nervous system activation, leads to catecholamine release, which promotes glycogen breakdown to meet the body's energy demands during stress (Malini *et al*., 2018). The study also found a proportional rise in glucose with increasing acidity, suggesting greater liver glycogen depletion as

fish mobilize energy to maintain homeostasis. The reduction in total protein levels observed under acid stress in this study appears to result from low pH-induced hemolysis, leading to plasma dilution and decreased protein content (Das *et al*., 2004), along with protein catabolism to meet the increased energy demands during stress (Ghanbari *et al*., 2012). Significantly increased levels of liver enzymes SGPT, SGOT, and ALP, proportional to acidity in the present study, indicate liver damage and stress (Haque *et al*., 2017). These findings suggest that climate change-induced water acidification may exacerbate stress and liver damage.

The immune system is vital for defending against pathogens and maintaining homeostasis in fish (Mokhtar *et al*., 2023). Environmental stressors, often exacerbated by climate change, can negatively impact fish immune responses, with factors like temperature fluctuations, salinity changes, heavy metals, and pollutants altering immune function (Makrinos and Bowden, 2016; Shahjahan *et al*., 2022). In this study, *L. rohita* fingerlings exposed to low water pH showed significant increases in lysozyme, respiratory burst (NBT), and phagocytic activity, consistent with earlier findings in stressed fish (Pulsford *et al*., 1994; Jia *et al*., 2023). These heightened immune responses, likely driven by stress-induced cortisol and catecholamine production (Pulsford *et al*., 1994), suggest a short-term stimulatory effect on the innate immune system, as seen in acute stress (Guo and Dixon, 2021). However, no significant changes in immunoglobulin levels were observed, indicating that short-term acid stress did not affect antibody production. Overall, the results suggest that while low pH, a potential consequence of climate change, enhances non-specific immune responses, it does not impact specific humoral immunity within the short exposure period.

A challenge study with *A. hydrophila* at varying water pH levels revealed significantly higher mortality rates at lower pH, indicating increased fish susceptibility to bacterial infections under acidic conditions. This heightened vulnerability is likely due to stress induced by low pH, which weakens immune defenses. Similar studies have shown that environmental stressors, increasingly common due to climate change, can greatly increase disease susceptibility in fish. Walters and Plumb (1980) found that elevated ammonia (NH*f*) and carbon dioxide (CO,) levels significantly raised mortality in bacterial-infected channel catfish. Likewise, Wise *et al*. (1993) reported reduced survival in stressed channel catfish infected with *Edwardsiella ictaluri*. Sherif and Soad (2013) and Tort *et al*. (1998) also observed higher mortality in *Oreochromis niloticus* and gilthead sea bream under similar conditions. In this study, prolonged exposure to acidic water was linked to suppressed immune responses (Guo and Dixon, 2021), resulting in higher mortality in *L. rohita* infected with *A. hydrophila*.

These findings highlight the significant impact of climate change-driven acidification on fish health, as lower water pH disrupts haematological, biochemical, and immunological parameters and increases disease susceptibility.

Conflict of interest: All authors declare that they have no conflicts of interest related to this research.

Author's contribution: KRS: Formal analysis, investigation, visualization, data curation and writing-original draft; HS: Conceptualization, methodology, supervision, writing-review and editing; RKS: Resources, supervision and validation; CL: Writing-review and editing.

Data availability statement: The data supporting the findings of this study are available upon request from the corresponding author.

Ethical approval: This study was reviewed and approved by the Institutional Animal Ethics Committee (IAEC), CAU-CF/48/IAEC/2018/03-21, dated 1st October 2021, of the College of Fisheries, Central Agricultural University, Imphal. All the live fish were handled humanely, and efforts were made to minimize the suffering.

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