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ORIGINAL ARTICLE

Effects of ammonia on the cellular, physiological, biochemical and genetic traits of Indian major carp (*Labeo rohita*) fry in artisanal Bangladeshi aquaculture

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Abstract

The major carp Rohu (*Labeo rohita*) is a prime freshwater aquaculture species across the Indian subcontinent that faces various production-related issues associated with water quality parameters. The present study examined the effects of three different doses of NH_3 ($T1$ = 1 mg/L, $T2$ = 2 mg/L and $T3$ = 3 mg/L) on cellular (gill ultrastructure), physiological (growth and oxygen consumption rate), biochemical (blood cell counts, blood cortisol and glucose levels) and genetic (expression of five genes involved in growth, immunity and metabolism) traits of Rohu. The experimental ammonia dose significantly affected the tested biological parameters ($p < 0.05$), causing moderate-to-severe gill tissue damage. In general, compared with those in the control group, 16%–25% slower growth, 12%–30% lower survival and 15%–56% higher O_2 consumption were observed for the treatment groups. Blood glucose and cortisol levels increased with increasing ammonia levels, but blood cell counts decreased. The five selected candidate genes showed a differential expression pattern in response to the ammonia dose, with higher expression in the control group and lower expression in the treatment groups. The results indicate that different concentrations of ammonia impose stress on different orders of magnitude in the experimental fishes. Therefore, it can be inferred that the presence of ammonia in aquatic/farming environments can adversely affect production performance; the severity of damage during production depends on the concentration of ammonia. Therefore, maintaining no or minimum ammonia levels in farming environments is urgently needed for sustainable aquaculture production of Rohu.

KEYWORDS
aquaculture, chemical stressor, gene expression, Indian major carp, stress hormone

1 | INTRODUCTION

Aquaculture has become one of the fastest growing food production systems worldwide over the last few decades, attempting to fill the gap in supplying fish for human consumption that wild catch fisheries cannot meet (Food and Agriculture Organization [FAO], 2020; Hunter et al., 2017). Aquaculture alone has contributed 17% of the total animal protein supply for human consumption in recent years, and the demand for aquaculture products is increasing gradually (Boyd et al., 2022; FAO, 2020). To meet the growing demand for fish, various aquaculture techniques, including the use of different types of feed ingredients, chemicals, fertilizers and probiotics, are employed; these techniques generally result in increased nitrogen production (particularly ammonia [NH_3]) (He et al., 2000; Rahi & Shah, 2012a). The presence of NH_3 (above the tolerance limit) in aquaculture ponds can severely affect production systems, causing growth retardation, reducing feed intake (FI), damaging different tissues (adversely affecting the nervous system) and increasing vulnerability to diseases (Constable et al., 2003; Muhammad et al., 2023; Randall & Tsui, 2002). All of these factors ultimately cause economic loss to farmers through production failure.

Ammonia is known to adversely affect the cellular, physiological, biochemical and genetic traits (entire biological system) of aquatic organisms. At the cellular level, NH_3 can rupture different cells and tissues (e.g. gill, kidney, liver), create barriers in the gill lamellae that restrict O_2 intake and affect the central nervous system (Moshtaghi et al., 2018; Muhammad et al., 2023; Rahi et al., 2020). As a result, fish may suffocate, and ruptured/damaged tissues can become easy targets for different microbes, subsequently increasing susceptibility to disease. From a physiological perspective, lower O_2 consumption reduces metabolic activity and FI, resulting in lethargic conditions with growth retardation (Rahi, Mather, et al., 2021). Damage at the biochemical level includes hormonal imbalance, blood cell lysis and/or deformity and changes in the amount of amino acids (Rahi, Mahmud, et al., 2021). The genetic aspects include changes in gene expression and DNA damage (Rahi & Shah, 2012a; Rahi, Azad, et al., 2021). All of these factors increase susceptibility to diseases, reduce growth and cause fish to become weak and moribund. Therefore, the presence of NH_3 in aquaculture farms is adversely linked to growth (Lemarie et al., 2004), survival (Tarazona et al., 1987) and overall production performance.

There are several ways through which fish farmers may attempt to reduce ammonia. These include reducing feeding rates and/or using low-protein diets, water exchange and adequate aeration (Hunter et al., 2017; Patoczka & Wilson, 1984). Feed may remain an issue due to nutritional constraints on the species being cultured, but the latter two methods for reducing ammonia should be easily applied (Constable et al., 2003). However, in many farms of developing countries, uncontaminated water and/or facilities for sufficient aeration are unavailable (Afroz et al., 2021; Rahi & Shah, 2012b). Therefore, the negative effects of higher levels of ammonia in developing countries pose challenges for many aquaculture species.

The Indian major carp Rohu (*Labeo rohita*) is an important freshwater aquaculture species and a major species in inland open water systems across the entire Indian Subcontinent, including Bangladesh (Rahi et al., 2013; Shah et al., 2011; Sabbir et al., 2017). This single species contributes ≈8% of the total freshwater fish production in Bangladesh (Ali et al., 2015; Department of Fisheries [DOF], 2021b). In the last fiscal year, almost 300,000 MT of Rohu were produced in Bangladesh (DOF, 2021b; Mridul et al., 2024). Although Rohu is the major freshwater aquaculture species in Bangladesh, the production of this fish is severely hampered due to relatively slow growth and disease-related issues. The major challenges associated with the production system of Rohu include inappropriately designed farms (i.e. without aeration or limited access to good-quality water), cheap and low-quality local feed ingredients (lower consumption rates due to rapid braking down, resulting in a higher decomposition rate) and nitrogen loads, which inevitably result in poor production performance (Ali et al., 2008; Islam et al., 2015; Mridul et al., 2024). Ammonia is commonly found in Rohu farms, which regularly experience poor production performance. Therefore, there is an urgent need to investigate the effects of different doses of ammonia (NH_3) on different biological parameters in Rohu. This approach can help detect the intensity of stress and damage caused by different NH_3 concentrations on Rohu, which will provide a foundation of knowledge for minimizing adverse effects.

Although Rohu is the major freshwater aquaculture species in Bangladesh, no studies have investigated the effects of ammonia on different biological parameters of this species. Several important biological markers widely used to investigate stress levels in fish include cellular (blood cell deformity, gill ultrastructure), physiological (growth, survival rate, developmental duration, oxygen consumption and FI), biochemical (blood glucose and hormone levels) and genetic (mRNA expression levels of selected genes) aspects (Akram et al., 2023; Islam et al., 2014; Mridul et al., 2024; Rahi, 2017; Rahi et al., 2022). Some important stress-responsive genetic markers include changes in the expression patterns of growth regulatory (e.g. insulin-like growth factor I [IGF-I], growth hormone [GH]), metabolic (e.g. glycerol-3-phosphate [G-3-P]) and immune (e.g. hepcidin, interleukin-1) genes, among others (Chowdhury et al., 2023; Rahi et al., 2023). Testing the effects of different doses of ammonia on these biological markers in Rohu will help to measure the level of imposed stress and the mechanisms (how ammonia affects the production of Rohu) involved in the loss or failure of aquaculture production. Several specific attributes, including the easy availability of seeds, good domestication status and acceptability of artificial feed, have made this species an ideal candidate for testing the effects of different doses of ammonia. Therefore, this study was conducted to investigate the effects of three different doses of ammonia (in the form of NH_4Cl) on the selected cellular, physio-biochemical and genetic changes in Rohu.

2 | MATERIALS AND METHODS

2.1 | Experimental Rohu collection

Animal ethics approval was obtained from the appropriate authority (the Animal Ethics Committee) of Khulna University (Ref. No.: KUAEC-2021/09/21). Rohu fry (30-day old with ≈0.5 g mean body weight) were collected from a government hatchery (Fish Seed Multiplication Farm)

located beside the Khulna University campus. A total of 300 fry from the same cohort were collected from the hatchery and brought to the Wet Laboratory of Fisheries and Marine Resource Technology (FMRT) Discipline, Khulna University. Rohu fry were transported with supplied oxygen during transportation.

2.2 | Experimental tank preparation and acclimation

In total, 12 glass tanks (30 L each) were cleaned and prepared for maintaining Rohu fry. The experimental tanks were filled with water collected from the nursery pond of the hatchery (up to 25 L) and maintained with continuous aeration. In each tank, 25 Rohu fry were randomly allocated and maintained under continuous aeration for 10 days to acclimate to the tank environment. Fingerlings were fed high-quality commercial nursery feed (CP Co. Ltd.; containing 33% crude protein) at a rate of 10% of the total biomass. Bottom debris (uneaten food particles and faecal material) was siphoned out daily to maintain optimum water quality in the experimental tanks and to estimate FI and the feed conversion ratio (FCR) (growth-related parameters).

2.3 | Ammonia challenge test

Following initial acclimation for 10 days, Rohu fry were challenged with three different doses of ammonia, using ammonium chloride (NH_4Cl). The control group consisted of no ammonia, whereas the treatments included three different doses of ammonia: *T*1 = 1 mg/L NH_4Cl, *T*2 = 2 mg/L NH_4Cl and *T*3 = 3 mg/L NH_4Cl. A stock solution (250 mg/L) was prepared by mixing 500 mg of NH_4Cl with 2 L of tap water. An appropriate amount of this solution was added to the experimental tanks to achieve the target concentrations for *T*1–*T*3. The concentrations of ammonia in the experimental tanks were increased at a rate of 1 mg/L per day. The concentration of ammonia was increased by slowly pouring 100 mL of stock solution into the experimental tanks (100 mL of stock solution containing 25 mg of NH_4Cl, which provides an ammonia concentration of 1 mg/L because each of the tanks holds 25 L of water). It took 12 h to add the mL of stock solution into the replicate treatment tanks (25 mL of stock solution was added at 3 h intervals). To achieve the three treatments simultaneously, stock solution was added to the replicate tanks of *T*2 and *T*3 1 and 2 days before *T*1. Rohu farms that experience higher rates of mortality and production failure are found to have ammonia levels ranging between 1 and 2.8 mg/L (DoF, 2021a). This report provides us with a baseline for selecting three doses of NH_4Cl for the current study.

2.4 | Growth and survival

To estimate growth performance, the body weights of the experimental Rohu cockroaches were measured at 15-day intervals using 30 individuals from each treatment (10 fish were randomly collected from each of the replicate tanks). Survival rates were estimated by deducting the number of individuals from the beginning to the end of the experiment. Further growth-related parameters were measured according to the following equations (Rahman et al., 2022; Zeynali et al., 2020):

$$\mathrm{DWG}\,(\%) = \{(\mathrm{BWf} - \mathrm{BWi}) / (\mathrm{BWi} \times t)\} \times 100$$

$$\mathrm{SGR}\,(\%) = \{(\ln \mathrm{BWf} - \ln \mathrm{BWi}) / t\} \times 100$$

$$\mathrm{FCR} = \text{feed intake}\,(g) / \text{weight gain}\,(g)$$

$$\mathrm{FI}\left(\text{g fish 1 days}^{-1}\right) = (\text{dry diet given} - \text{dry remaining diet recovered}) / \text{number of fish}$$

where DWG is daily weight gain, BW_f is final body weight, BW_i is initial body weight, *t* is total experimental time (60 days), SGR is specific growth rate, FCR is feed conversion ratio and FI is feed intake.

2.5 | Oxygen consumption rates

Experimental Rohu plants were sampled at nine different time intervals to measure differential changes in O_2 consumption rates. The sampling times for measuring specific changes in ammonia levels and O_2 consumption were as follows: day 1 (immediately after achieving the target ammonia levels); day 2; day 3; day 4; day 5; day 10; day 20; day 30; and day 60. O_2 consumption rates were estimated in a 250 mL flow respirometric chamber (Q-Box Aqua Respiratory System, Qubit) according to Rahi et al. (2017, 2022) using the following equation (Rosas et al., 2001):

$$O_2\text{consumption} = [O_2\text{en} - O_2\text{ex}] \times \mathrm{FR}$$

where the water flow rate is 1.5 L h^{-1} in the respirometric chamber, O_2 en is the amount of O_2 at the entry of the respirometric chamber and O_2 ex is the amount of O_2 during exit.

2.6 | Blood cell counts

Three replicate fish were collected from each experimental condition, after which the total blood cell count was determined. From each fish, 50 μL of blood was collected via heparinized microinjection, after which the blood was immediately transferred to Eppendorf tubes containing an equal volume (50 μL) of 20 mM EDTA. Following this step, 100 μL of 10% neutral buffered formalin was added to the blood samples, which were then maintained at ambient temperature for 30 min to fix the samples. The samples were then serially diluted 2, 4, 8, 16 and 32 times using ice-cold phosphate-buffered saline (20 mM, pH 7.2)

(Witeska et al., 2022). Finally, the total number of normal blood cells was counted using a hemocytometer (Boeco) and checked under a microscope (SOLARIS-TLED) at 100× magnification.

2.7 | Determination of blood glucose and cortisol levels

In total, 200 μL of blood was collected from each fish (three replicate fish from each experimental group) for estimating blood glucose and stress hormone (cortisol) levels. Collected blood samples were immediately transferred to 1.5 mL tubes containing 200 μL of heparin (Zentiva) to avoid blood clotting (Pravda & Svobodová, 2003). The anticoagulated blood of 100 μL was centrifuged at 800 × *g* (4°C) for 10 min to isolate blood plasma for the determination of glucose levels. Glucose assays were performed using a commercial kit (Glu L 1000, PLIVA-Lachema). The remaining 300 μL blood samples were used to obtain adequate quantities of plasma for assaying cortisol levels. Blood samples were centrifuged at 16,000 × *g* (at 4°C) for 2 min. Finally, 80 μL plasma samples were used for determining cortisol levels according to Islam et al. (2011), Moshtaghi et al. (2017) and Akram et al. (2023) by using a monoclonal antibody enzyme-linked immunosorbent assay kit (Enzo Life Sciences).

2.8 | Gene expression profiling

All individuals were required to investigate the expression patterns of different genes due to their small size. Sampling was performed in triplicate across nine different time series, as mentioned earlier for O_2 consumption rate analysis. Total RNA was extracted from the experimental fish samples using the TRIzol/chloroform extraction method and a commercial RNA extraction kit (SERVA). Total RNA integrity (quality and quantity) for each sample was evaluated using 2% agarose gel electrophoresis and a NanoDrop 2000 Spectrophotometer (Nabi). The high-quality RNA samples were preserved at −80°C for subsequent analysis. Complementary DNA (cDNA) was synthesized from the total RNA samples (using 1 μg of RNA for each sample) by using the SensiFAST cDNA synthesis kit (Bioline) according to the manufacturer's protocol. cDNA samples were then preserved at −20°C until further analysis.

Five different genes with different functional roles were used for gene expression analysis; these included genes related to growth and metabolism (GH, IGF-I and G-3-P) and immunity (hepcidin and interleukin-1). We chose elongation factor 1 alpha as the reference gene because its suitability has been well documented in numerous aquatic species (Chowdhury et al., 2023; Moshtaghi et al., 2018; Sahu et al., 2015; Zeynali et al., 2020). The candidate genes were selected due to their inferred functional roles in several earlier studies (Aziz et al., 2017; Moshtaghi et al., 2016; Rogl et al., 2018; Sahu et al., 2015). Sequences of the target genes were collected from Sahu et al. (2015) to design gene-specific primers (Table 1) using Primer3 software (Untergasser et al., 2012). Reactions were performed in 20 μL mixtures containing 10 μL of 2× SensiFAST SYBR No-ROX Mix (Bioline), 3 μL of ultrapure water, 5 μL of template cDNA and 1 μL of each forward or reverse primer. Reactions were then performed in triplicate for each sample using a real-time PCR system (Bio-Rad). At the end of each reaction, standard melt curve analysis was performed to confirm the amplification of a single-qPCR product. Finally, gene expression (relative gene expression) data were analysed using the ΔΔ*Ct* method (Aziz et al., 2018; Pfaffi, 2001; Rahi et al., 2019) according to the following equation:

$$\text{relativegeneexpression (R)} = 2^{-[\Delta Ct\ \text{targetgene} - \Delta Ct\ \text{reference gene}]}$$

2.9 | Gill ultrastructure through scanning electron microscopy (SEM)

Fish samples from each condition were dissected to obtain fresh gill tissue, which was immediately preserved in 2.5% glutaraldehyde solution. The preserved gill samples were dehydrated gradually through a series of ethanol (different concentrations of ethanol: 30%–100%) washing steps (Figure S1). Samples were subjected to another round of serial dehydration steps using various concentrations of hexamethyldisilane (HDMS) (25%–100%) according to Figure S2. The gill samples were then air-dried at room temperature for overnight. Next, the samples were gold coated for 180 s (~40 mA) by using an Edwards Sputter Coater for examination with an FEI Quanta 200 ESEM using the conventional mode (high vacuum) and a Thornley–Everhart secondary electron detector.

2.10 | Statistical analyses

Different types of data obtained in this study were tested for normality and homogeneity of variance using the Kolmogorov–Smirnov test and Levene's test, respectively, in SPSS (version 23) software. Different types of data were also evaluated for one- and two-way analyses of variance (ANOVAs) using a 5% level of significance ($p < 0.05$). Treatments and sampling times were considered independent variables for a two-way ANOVA, whereas comparisons were made only between treatments for one-way ANOVA. The dependent variables were different *physiological* (*growth and O_2 consumption*), *biochemical* (*number of blood cells, glucose and cortisol levels*) and *genetic* (*gene expression*) parameters. Tukey's HSD test was performed to compare the means of different parameters. The different types of results are presented in the tables and graphs as the mean ± standard error. The R package (version 3.5.1) was used for correlation plotting among the different parameters: (i) *growth and O_2 consumption*, (ii) *growth, glucose and cortisol levels*, (iii) *growth and expression of growth-related genes* and (iv) *blood cell counts and expression of immunity genes*.

TABLE 1 Gene-specific forward (F) and reverse (R) primer sequences with specific details of product length (base pair) and annealing temperature (Mridul et al., 2024; Sahu et al., 2015).

Candidate gene	Primer sequence (5′–3′)	Product length (bp)	Annealing temperature
Insulin-like growth factor I (IGF-I)	**F**: CCCGGGGTCAAAATGCAGCT	120	62
	R: GGGGTAACTCAGGCCACGGA		
Growth hormone (GH)	**F**: AGAAGCTCTTGCAGCCCTCG	118	58
	R: CCACAGAACGTGTTGCGGGA		
Glycerol-3-phosphate (G-3-P)	**F**: CGTCCTGTTCACTGCACCCAG	112	60
	R: ATGCCACAGCAGACGTCGCT		
Hepcidin	**F**: TGCAGGTCTCTGTGGTGGTG	130	61
	R: ACAGCTGGATGCTGGGCAGT		
Interleukin-I	**F**:AGCAGGAAGGATTTGAGGCACT	110	56
	R: GGGCGCACTTTGCTTCCTCT		
Elongation factor 1 alpha (EF 1α)	**F**:TTCGAGCAGGAGATGGGCACTG	114	60
	R: GCATCCTGTCAGCAATGCCA		

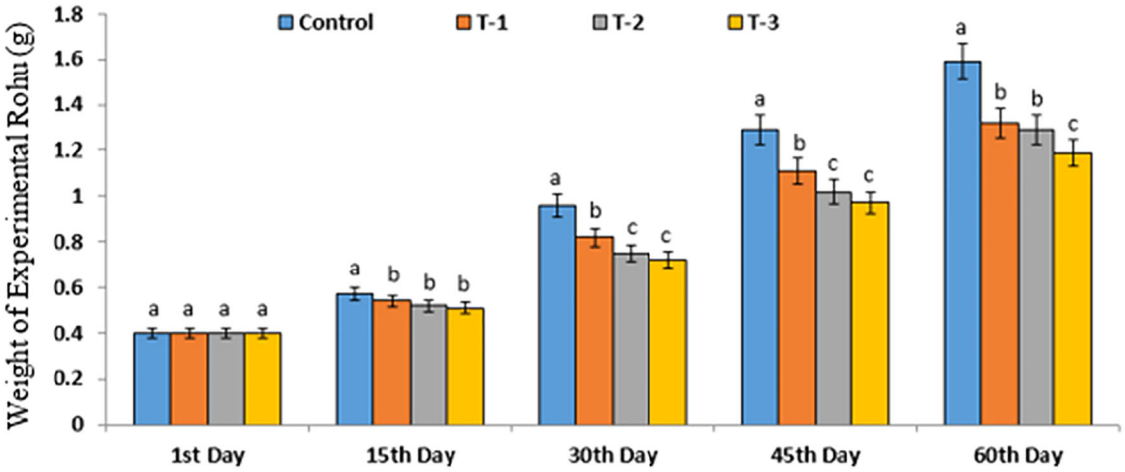

FIGURE 1 Changes in body weight (±S.E.) of experimental Rohu at 15-day interval ($N = 30$ fish samples per sampling time). Different letters above the bars indicate significant difference at 5% level of significance.

3 | RESULTS

3.1 | Ammonia-induced changes in growth and survival

Experimental ammonia treatments significantly altered the growth and survival performance ($F_{(3,120)} = 83.7$, $p = 0.01$) of Rohu (Figure 1 and Table 2). Significantly, greater growth ($p < 0.05$) was observed for the control treatment than for the other treatments throughout the experiment. Different doses of ammonia also had significant effects on various growth-related parameters (DWG, SGR, FI and FCR) and survival in the Rohu population (Table 2). No significant differences were observed between the treatments (*T*1–*T*3) up to the 15th day, after which significant differences ($p < 0.05$) were evident between the treatment groups (Figure 1). *T*1 showed significantly greater growth than did *T*2 and *T*3 up to the 45th day, after which *T*1 showed significantly greater growth than did *T*3. No significant differences were observed between *T*2 and *T*3 from the beginning to the 45th day, but *T*2 showed significantly greater growth than *T*3 by the end of this study.

3.2 | Changes at the cellular level (gill ultrastructure and blood cell deformity)

Ammonia treatments were found to adversely affect the gill ultrastructural view (SEM imaging) of the experimental Rohu (*L. rohita*). Clear structures of gill lamellae and filaments were observed for the control group, whereas the treatment groups showed slight-to-severe damage (Figure 2). Slight damage to gill tissue was observed at the lower ammonia dose (*T*1), but severe damage was observed at *T*2 and *T*3.

TABLE 2 Effects of ammonia treatments on different growth parameters of Rohu (*Labeo rohita*).

Growth parameters	Control	*T* – 1	*T* – 2	*T* – 3
Initial weight (BW_i) (g)	0.4 ± 0.06[a]	0.4 ± 0.05[a]	0.4 ± 0.07[a]	0.4 ± 0.06[a]
Final weight (BW_f) (g)	1.59 ± 0.5[a]	1.32 ± 0.4[b]	1.29 ± 0.4[c]	1.19 ± 0.3[c]
Daily weight gain (DWG) (%)	4.96 ± 0.6[a]	3.83 ± 0.8[b]	3.71 ± 0.8[b]	3.29 ± 0.6[c]
Specific growth rate (SGR) (%)	2.30 ± 0.2[a]	1.99 ± 0.3[b]	1.95 ± 0.4[b]	1.82 ± 0.3[c]
Feed intake (g g^{-1} day^{-1})	0.152 ± 0.008[a]	0.157 ± 0.009[b]	0.159 ± 0.009[b]	0.161 ± 0.008[b]
Feed conversion ratio (FCR)	3.06 ± 0.3[a]	4.10 ± 0.4[b]	4.28 ± 0.4[b]	4.89 ± 0.5[c]
Survival rate (%)	96 ± 2.3[a]	77 ± 5.2[b]	64 ± 6.1[c]	51 ± 4.2[d]

Note: Different superscripts indicate significant difference at 5% level of significance.

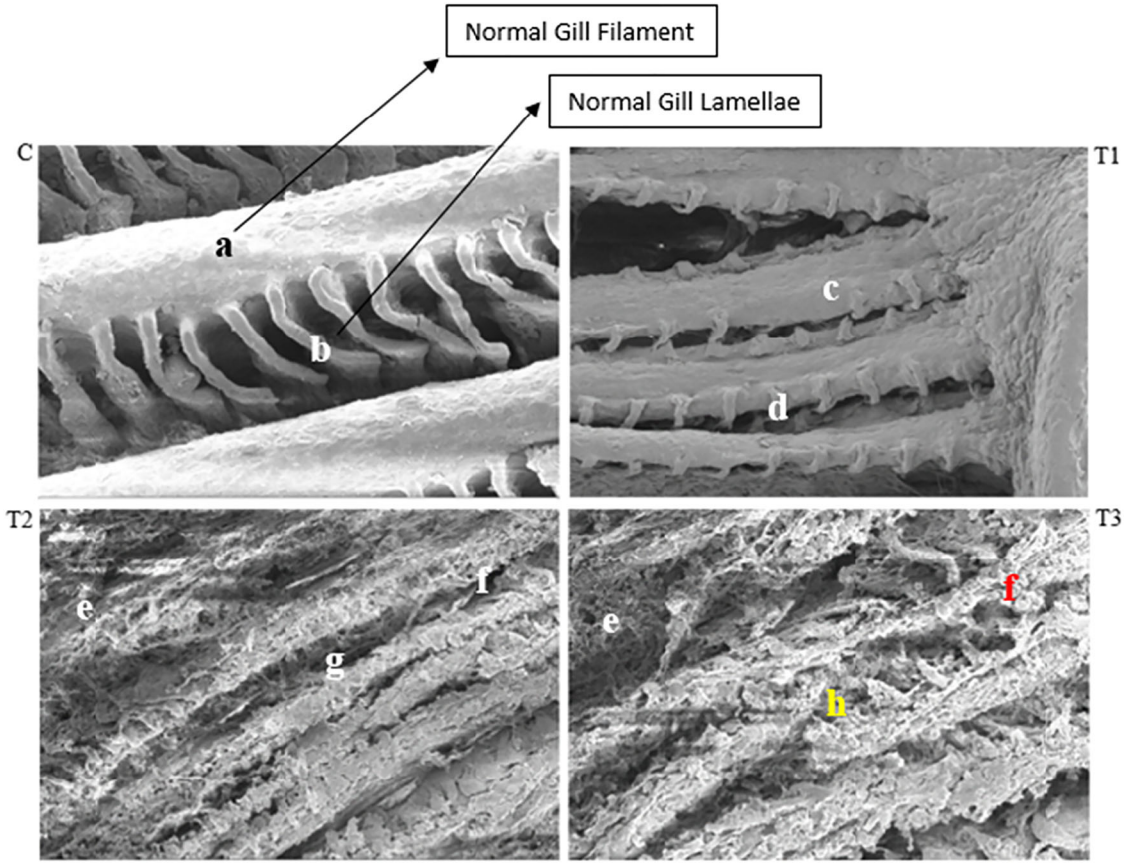

FIGURE 2 Gill ultrastructure of Rohu (*Labeo rohita*) through scanning electron microscopy (SEM): C = control, *T*1 = 1 mg/L NH_3, *T*2 = 2 mg/L NH_3 and *T*3 = 3 mg/L NH_3. Labels with different letters and colour in the figure indicate the degree of change/damage in the gill tissue, including (a) normal structure of gill filament in control condition, (b) normal structure of gill lamellae in control condition, (c and d) slightly damaged gill filament and lamellae in *T*1 (1 mg/L NH_3), (e) heavy load of mucus/slime cells in *T*2 (2 mg/L NH_3) and *T*3 (3 mg/L NH_3), (f) damaged gill filament in *T*2 and *T*3, (g) ruptured gill lamellae in *T*2 and (h) severely ruptured gill lamellae in *T*3. Images were taken at 1000× magnification covering 20 μm area.

Heavy loads of mucus/slime cells were observed at higher ammonia doses (*T*2 and *T*3). Both the gill lamellae and gill filaments were ruptured in *T*2 and *T*3. Like those of the gill ultrastructure, the blood cells of the experimental Rohu were also adversely affected by the ammonia treatments. The type of blood cell deformity and the number of deformed blood cells (Figure 3) were found to vary significantly ($p < 0.05$) among the treatments. No deformed cells were observed in the control group, whereas variable numbers of deformed cells or deformity types were observed at *T*1–*T*3. The number of deformed blood cells was significantly different ($p < 0.05$) among the treatment groups; a greater number of deformed cells was observed with increasing ammonia concentrations (i.e. *T*3 > *T*2 > *T*1).

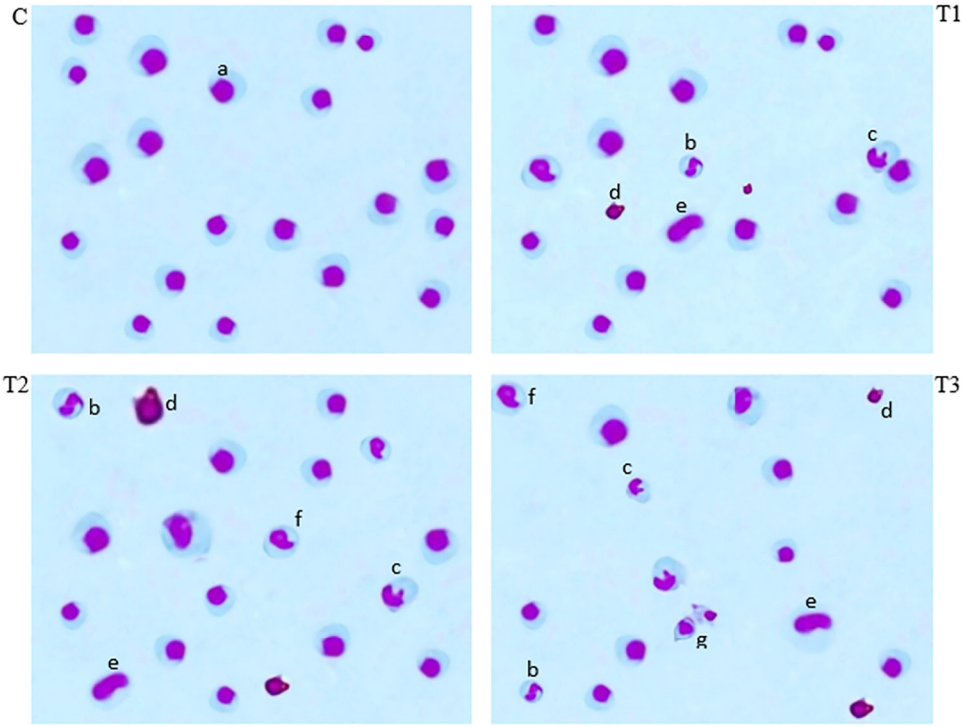

FIGURE 3 Types of blood cell deformities of experimental Rohu (*Labeo rohita*) at different doses of ammonia (image was taken at 100× magnification covering 5 μm area): C = control, *T*1 = 1 mg/L NH_3, *T*2 = mg/L NH_3 and *T*3 = mg/L NH_3. Here, the labels with different letters indicate (a) normal blood cells of control group (no deformity of blood cells). Different types of blood cell deformities in treatment groups include (b) change in shape/size of nucleus, (c) breakdown of nucleus, (d) complete lysis/breakdown of cell wall, (e) kidney shaped nucleus, (f) micro nuclei formation and (g) double nucleus formation.

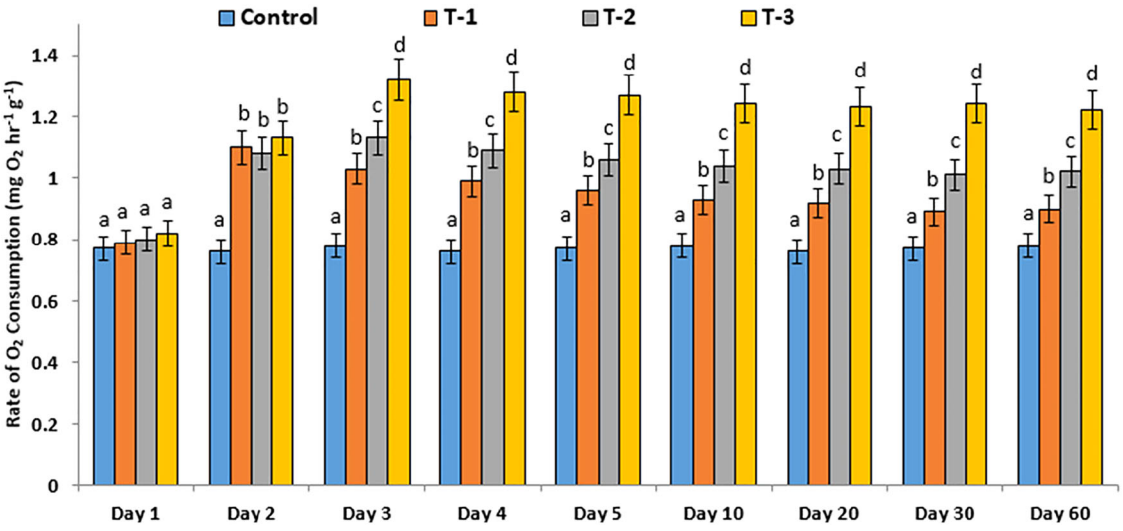

FIGURE 4 Ammonia induced changes (Mean ± S.E.) in the rate of O_2 consumption in experimental Rohu (*Labeo rohita*) across the sampling times. Different letters above the bar indicate significant difference at 5% level of significance.

3.3 | Changes in the rate of O_2 consumption

The O_2 consumption rates of the experimental Rohu plants were significantly affected ($F_{(3, 120)} = 98.2$, $p = 0.00$) by the treatments. Initially, no significant differences were observed among the treatments (Figure 4). Then, significantly greater O_2 consumption rates ($p < 0.05$) were observed for the treatments than for the control from day 2 to the end of the experiment. No significant differences were initially observed

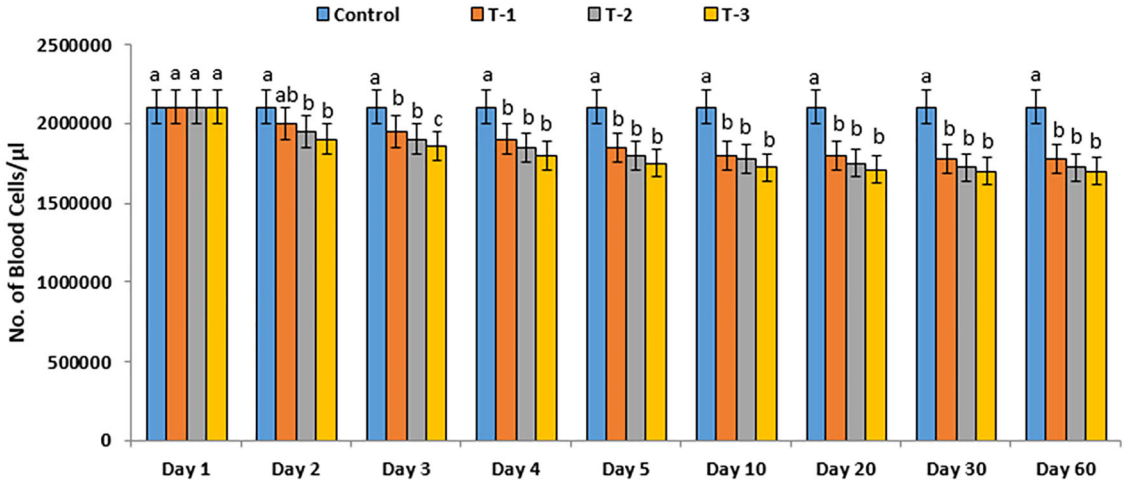

FIGURE 5 Changes in the number of blood cell counts for experimental Rohu (*Labeo rohita*) across different sampling times.

between the treatments (*T*1–*T*3) (up to day 2), after which significant differences were observed among the treatments. Ammonia-treated Rohu plants exhibited increasing trends in O_2 consumption up to day 3, followed by a declining trend up to the fourth day, after which O_2 consumption remained stable for the remaining time.

3.4 | Blood cell counts

Significant differences were observed for the total number of blood cells ($F_{(3, 120)} = 55.9$, $p = 0.02$) among the experimental Rohu pigs (Figure 5). Blood cell counts were significantly greater in the Rohu group than in the treatment group ($p < 0.05$) from the second day to the end of the experiment. No significant differences were observed for blood cell counts between the treatments (*T*1–*T*3) from beginning to end. The total number of blood cells generally decreased through the fifth day, followed by a stable trend until the end of the study (Figure 5).

3.5 | Changes in blood cortisol levels

Different doses of ammonia significantly altered the blood cortisol levels in the Rohu study ($F_{(3, 120)} = 76.4$, $p = 0.00$), whereas a dose-specific response (higher levels of cortisol with increased ammonia doses) was also observed (Figure 6). Significantly, lower (and stable) cortisol levels were found in the control group for the entire experimental period. Ammonia treatments (*T*1–*T*3) produced significantly greater levels of cortisol ($p < 0.05$) than did the control throughout the course of the experiment. The general trend in cortisol levels was found to be an increasing trend up to the 5th day (the peak level of cortisol for the three treatments), followed by a declining trend up to the 10th day and a stable trend for the remainder (Figure 6). No significant differences were detected between the treatments (*T*1–*T*3) up to the 2nd day, after which *T*3 had significantly greater cortisol levels ($p < 0.05$) than did *T*1 and *T*2 throughout the course of the experiment, with a few exceptions (3rd, 10th and 30th days: No significant differences were evident between *T*2 and *T*3 during these sampling times). No significant differences were observed between *T*1 and *T*2 up to the 3rd day, after which the differences between these two treatments were irregular (significant differences were found only at the 4th, 5th, 20th and 60th days).

3.6 | Changes in glucose levels

Changes in blood glucose levels were significantly affected by the ammonia treatments ($F_{(3, 120)} = 68.1$, $p = 0.03$). *T*1–*T*3 showed almost double the glucose concentration compared to that of the control (Figure 7). The generalized pattern in ammonia dose-specific glucose levels was found to be an initial increasing trend up to the 5th day (the peak levels for blood glucose), followed by a declining trend up to the 30th day and, finally, a stable pattern for the remainder. All three ammonia treatments resulted in significantly greater glucose levels than those in the control from the beginning to the end of the treatment. No significant differences were found between the treatments (*T*1–*T*3) on the 1st day, but significant differences were observed among the treatments; in particular, *T*3 had significantly higher glucose levels than did *T*1 and *T*2 from the 2nd day to the end (Figure 7), with some exceptions (there was no difference between *T*2 and *T*3 on the 2nd and 30th days). Significant differences were observed between *T*1 and *T*2 from the fourth day to the end of the study.

3.7 | Relative expression levels of target candidate genes

Different doses of ammonia significantly affected the relative expression levels of growth regulatory genes (IGF-I and GH) in the Rohu population (Figures 8 and 9). In comparison to those in the control group, the expression levels of the two growth regulatory genes in the control group were ≈twofold higher expression levels ($F_{(3, 120)} = 96.5$ & 97.3, $p = 0.00$). No significant differences were initially observed

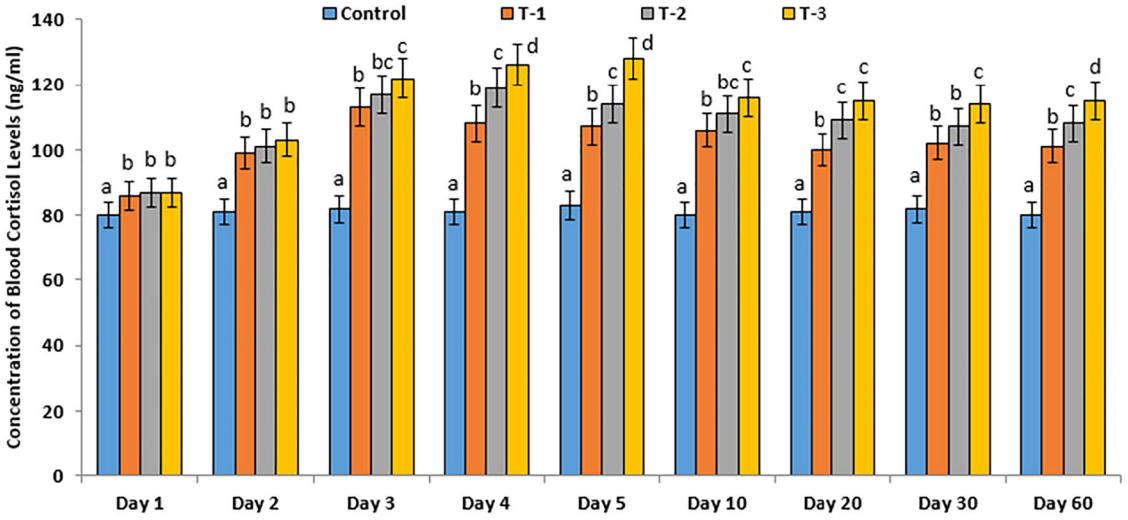

FIGURE 6 Experimental ammonia dose specific changes in blood cortisol levels of Rohu (*Labeo rohita*) across the sampling times.

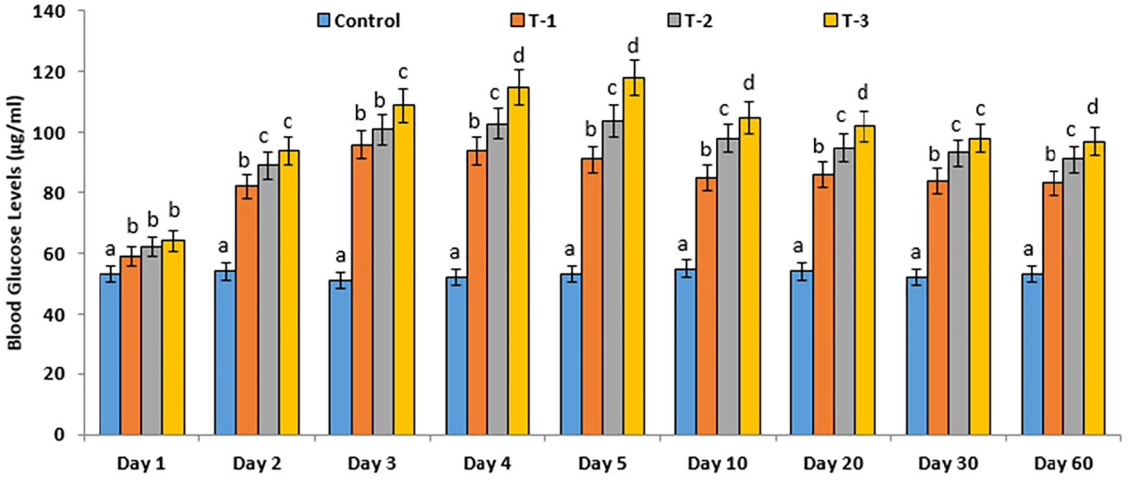

FIGURE 7 Changes in blood glucose levels of experimental Rohu (*Labeo rohita*) across the sampling times.

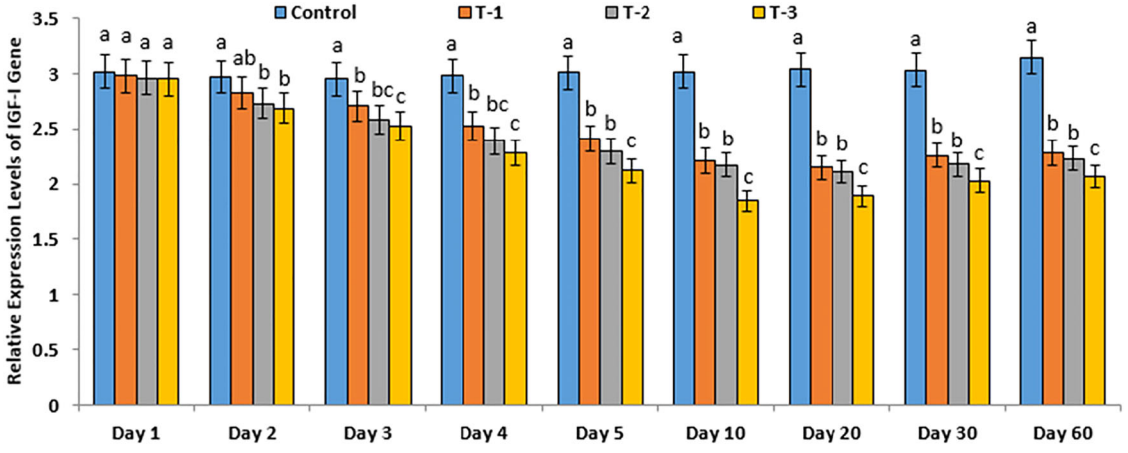

FIGURE 8 Changes in relative expression levels of insulin-like growth factor I (IGF-I) gene for experimental Rohu (*Labeo rohita*) across the sampling times.

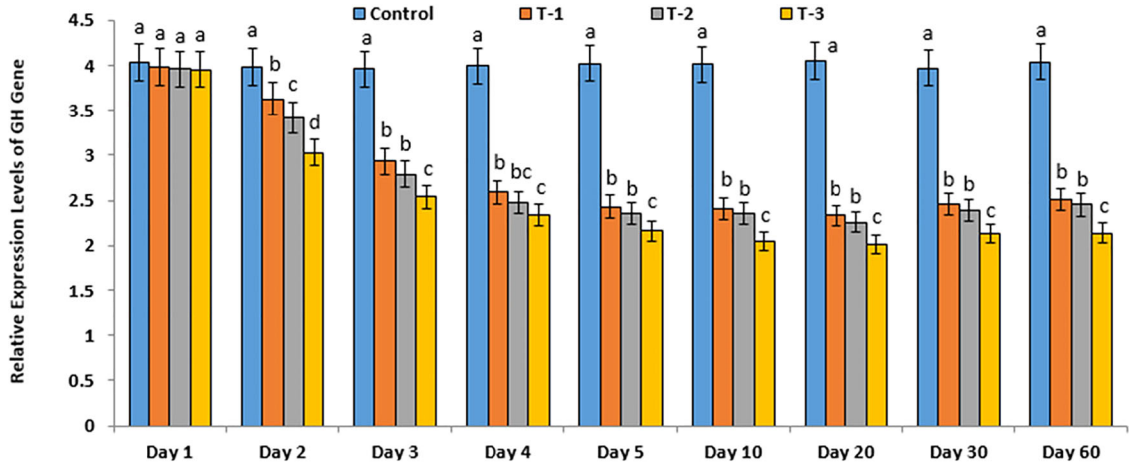

FIGURE 9 Changes in relative expression levels of growth hormone (GH) gene for experimental Rohu (*Labeo rohita*) across the sampling times.

between the control and ammonia treatments for the two growth genes (first day), after which the control group showed significantly greater expression levels for the entire experimental period. No significant differences were detected for IGF-I expression among the ammonia treatments up to the second day, after which *T*1 and *T*2 showed significantly greater expression levels than *T*3 during the remaining time frame (Figure 8). No significant differences were observed between *T*1 and *T*2 for IGF-I throughout the experiment. For the GH gene, no significant differences were found between the control and treatment groups at the beginning (first day), after which the expression of the GH gene significantly increased ($p < 0.05$) in the control group compared with that in the ammonia treatment group throughout the experiment (Figure 9). *T*1 and *T*2 presented significantly greater expression levels than *T*3 from the third day to the end, whereas significant differences were observed between all three treatments on the second day.

Similarly, the expression of the metabolic gene G-3-P exhibited the opposite pattern from that of the growth genes, in which all three ammonia-treated Rohu plants (*T*1–*T*3) exhibited significantly greater ($F(3, 120) = 82.6, p = 0.00$) expression levels than did the control plants (Figure 10). At the start of this experiment, no significant differences were found among the treatments, after which the ammonia treatments resulted in significantly greater expression ($p < 0.05$) than did the control for the entire experimental period. *T*3 showed significantly greater expression levels than *T*1 and *T*2 from the second day to the end, whereas no significant differences were evident between *T*1 and *T*2 from the third day to the end.

Like for the growth genes, ammonia treatments considerably influenced the expression levels (F (3, 120) = 92.8 & 89.4, p = 0.00) of immune response genes (hepcidin and interleukin-1) of Rohu (Figures 11 and 12). The control group exhibited significantly greater ($p < 0.05$) expression levels (almost double) of these two genes than did the three ammonia-treated groups from the second day to the end of the treatment. The expression of hepcidin was found to be highly variable among the three treatment groups from the second day to the end of this study (Figure 11). No significant difference was detected between treatments up to the second day. *T*1 showed significantly greater expression than *T*3 from the 3rd day to the 10th day, followed by no significant difference from the 20th to the 30th days and finally a significant difference on the 60th day. Similarly, the expression of interleukin-1 was similar to that of hepcidin among the treatment groups (Figure 12).

4 | DISCUSSION

The significantly lower growth and survivability ($p < 0.05$) of the ammonia-treated Rohu plants (*L. rohita*) (Table 2 and Figure 1) clearly indicated adverse effects on the experimental fishes. O_2 consumption increased with increasing experimental ammonia dose (Figure 4). The stable O_2 consumption rates and significantly greater growth of the control group throughout the experiment indicated that there was no imposed stress. A significant positive interaction ($R^2 = 0.72$; $p < 0.05$) between growth and O_2 consumption (Figure S3) indicated the important role of O_2 consumption in the growth of Rohu. A higher rate of O_2 consumption generally indicates greater metabolic performance, which in turn enhances growth (Loughland & Seebacher, 2020; Rahi, Mather, et al., 2021; Rahi, Mahmud, et al., 2021). Fish also tend to consume more O_2 under stressful conditions to meet the increasing energy demand, which helps to counterbalance the adverse effects of stressors (Rahi et al., 2020; Rajendiran et al., 2016). Thus, the decreased growth of Rohu coupled with increased O_2 consumption in the treatment groups (*T*1–*T*3) likely occurred due to an ammonia-induced stress response.

Under stressful conditions (particularly when organisms face tissue damaging stressors), fishes tend to secrete specific cell masses (i.e. mucus/slime cells, chloride cells and free fatty acids) to the damaged tissue region (Yada & Tort, 2016). Depending on the intensity of the stressor, fishes fail to eliminate cell damage (Schreck et al., 2001; Somero, 2020). An increase in the amount of mucus/slime cells in the gill tissue of ammonia-treated Rohu plants suggested a stress response strategy (an attempt to eliminate ammonia from the gill

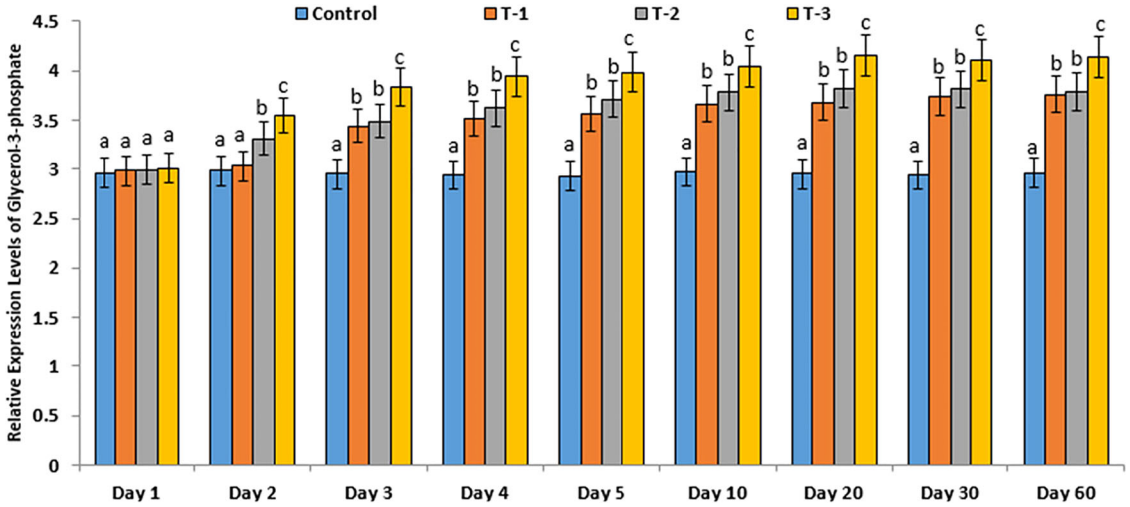

FIGURE 10 Experimental ammonia dose-specific changes in relative expression levels of glycerol-3-phosphate (G-3-P) gene for Rohu (*Labeo rohita*) across the sampling times.

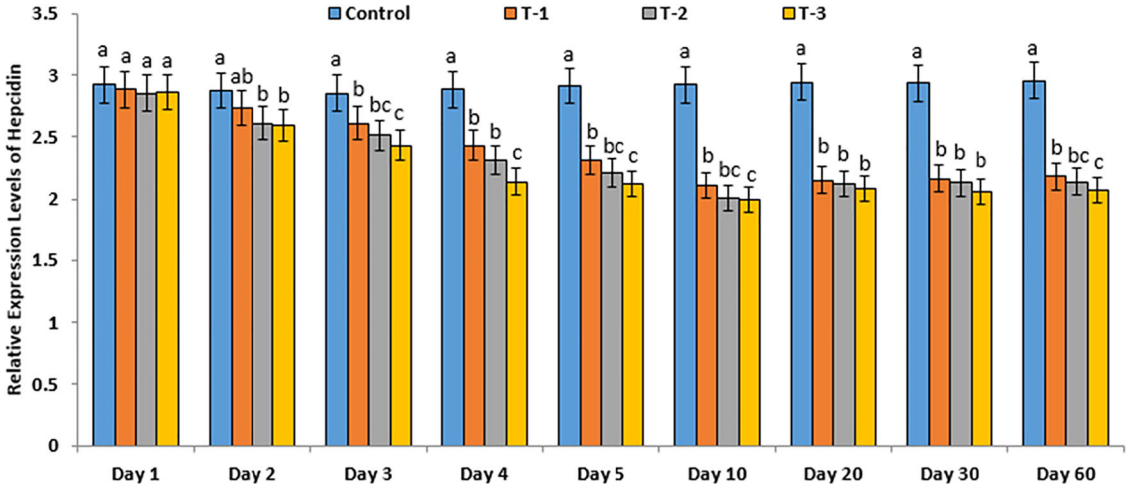

FIGURE 11 Changes in relative expression levels of hepcidin for experimental Rohu (*Labeo rohita*) across the sampling times.

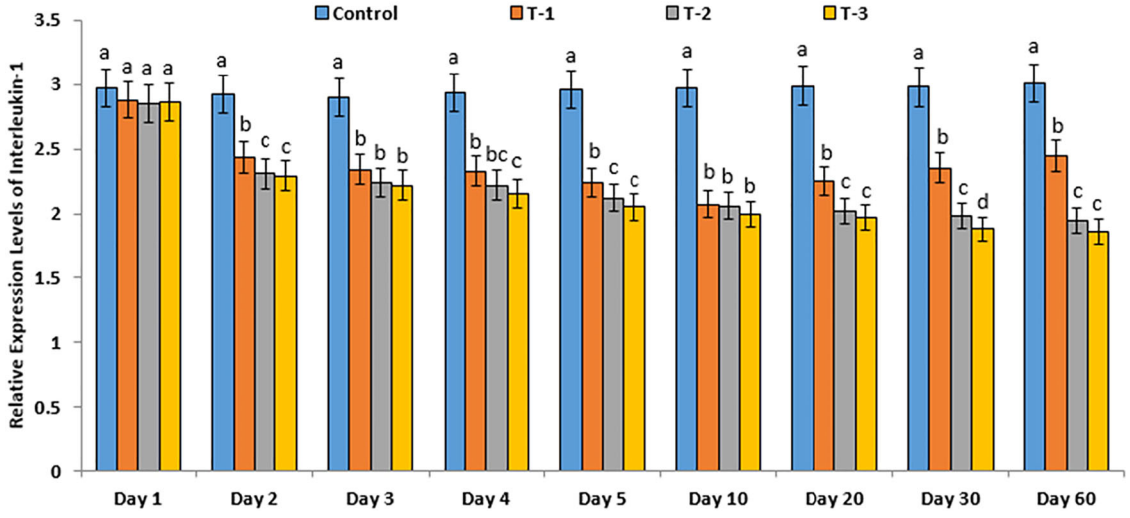

FIGURE 12 Changes in relative expression levels of interleukin-1 for experimental Rohu (*Labeo rohita*) across the sampling times.

region) (Figure 2). The slight damage to gill tissue in *T*1 (1 mg/L NH_4Cl) indicated that Rohu was able to eliminate the catastrophic effect of ammonia to some extent but was unable to protect gill tissue in *T*2 and *T*3. Therefore, the gill tissue of Rohu at *T*2 and *T*3 is an easy target for different microbes and is extremely vulnerable to diseases. Moreover, blood cell lysis occurs (different types of cellular deformity and a reduced number of blood cells) in fishes that are exposed to different types of stressors that, in turn, reduce immunity (Aliko et al., 2018). Therefore, counting blood cells and blood cell deformities are reliable indicators of the immune status of fish, where a lower number of blood cells and elevated deformity types indicate poor immunological status (Rahi et al., 2022). Six types of blood cell deformities have been recognized in fish and the presence of more deformity types indicates increased stress (Esmaeili, 2021; Rahi et al., 2018; Seibel et al., 2021; Witeska et al., 2022). The absence of deformed blood cells in the control group indicated no stress, whereas the presence of all six deformity types indicated the highest levels of imposed stress at *T*3 (Figure 5). A lower blood cell count and more deformity types in the ammonia-treated Rohu individuals (Figure 3) imply reduced immunity. Experimental ammonia levels were also found to damage gill lamellae and filaments (Figure 2). In addition, the damaged tissue (i.e. gill) of Rohu has become an easy target for microbes to create infections, whereas reducing immunity increases susceptibility to disease (the inability to resist microbes at the infection site due to poor immunity).

Blood cortisol (a vertebrate stress hormone) and glucose levels are important biochemical markers for identifying the intensity of imposed stress on fishes (Rahi et al., 2022; Sabbir et al., 2010). Fish usually release higher amounts of cortisol and glucose under stressful conditions by using protein reserves for gluconeogenesis in the liver to minimize stress levels (Aziz et al., 2018; Rahi, Mahmud, et al., 2021). The significantly higher blood cortisol and glucose levels ($p < 0.05$) in the Rohu ammonia treatment group throughout the course of the experiment (Figures 6 and 7) indicated the negative effects of ammonia. The superior growth of Rohu coupled with significantly lower (but stable) cortisol and glucose levels throughout the experiment in the control condition indicates no stress. A significantly ($p < 0.05$) greater interaction ($R^2 = 0.81$ for cortisol and $R^2 = 0.79$ for glucose) between growth and glucose and between growth and cortisol levels (Figure S4) suggested that increasing ammonia levels adversely affects the growth of Rohu.

Fish growth is known to be directly influenced by stocking density (Rahi et al., 2017, 2022); the slower growth in *T*1–*T*3 (the treatment groups had a lower density due to higher mortality) than in the control (higher density due to no mortality) clearly indicates that ammonia induced slower growth. The higher levels of blood cortisol and glucose in the treatment groups up to the 60th day (Figures 8 and 9) also indicated that the ammonia-treated Rohu plants were under persistent stress. The survival performance of aquatic species depends on the duration and intensity/magnitude of stress, where moderate-to-higher levels of stress for a longer period of time can cause massive (and even total) mortality (Luo et al., 2020; Moshtaghi et al., 2018; Sabbir et al., 2017). This likely explains the significant difference in mortality rates among the four experimental conditions (Table 2) in this study.

IGF-I and GH are known to play important functional roles in growth regulation in different fish species, whereas G-3-P is associated with metabolic activities (Li et al., 2016; Tian et al., 2020; Triantaphyllopoulos et al., 2020). Therefore, any environmental stressor can reduce the expression of growth genes that ultimately cause slower growth performance (Casu et al., 2017; Sinha et al., 2012; Zarantoniello et al., 2021). Experimental ammonia treatment significantly reduced the expression levels of the IGF-I and GH genes (Figures 8 and 9), indicating that ammonia has adverse effects on these two growth regulatory genes. Significant ($p < 0.05$) and negative interactions ($R^2 = -0.82$ for IGF-I and $R^2 = -0.71$ for GH) between growth and the expression of IGF-I and GH (Figure S5) further validated the roles of these two genes as important growth regulators in Rohu; reduced expression of these two genes was associated with slower growth in Rohu in this study. Increasing expression of the metabolic gene G-3-P with increasing experimental ammonia dose (Figure 10 and Figure S5) indicated elevated metabolic activity under stressful conditions, most likely to counterbalance the imposed stress. Under stressful conditions, fish tend to exhibit increased expression levels of G-3-P but reduced expression of IGF-I and GH (Albertyn et al., 1994). The relatively higher and stable expression levels of IGF-I and GH and the lower and more stable expression of G-3-P (coupled with greater growth) in the control group throughout the trial suggest that there was no imposition under these conditions.

Hepcidin and interleukin-I are two important immune response genes for different fish species that exhibit relatively lower expression levels under stressful conditions (Srole & Ganz, 2021). These two genes play important roles in fighting pathogens and eliminating infections and other disease-causing agents (Hsieh et al., 2010; Lee & Beutler, 2009; Sun et al., 2020). Therefore, any environmental stressor (i.e. ammonia) can decrease the expression levels of interleukin-1 and hepcidin. The expression of these two genes was significantly reduced in Rohu plants exposed to ammonia during the whole study period ($p < 0.05$), demonstrating the detrimental effects of ammonia (Figure S6) on the regular activities of these two genes. Total blood cell counts exhibited significant ($p < 0.05$) and positive interactions ($R^2 = 0.95$ for hepcidin and $R^2 = 0.97$ for interleukin-I) with the expression levels of hepcidin and interleukin-I (Figure S6), suggesting that the immune system of Rohu is strongly associated with the expression of these two genes.

Previous investigations have shown that ammonia hampers the overall biological activity of aquatic organisms in numerous ways, hampering O_2 transport and growth, damaging different tissues and nervous systems, reducing immunity and consequently increasing susceptibility to disease (Cabillon & Lazado, 2019). Therefore, ammonia is considered a major limiting factor for the production performance of farmed aquatic species by negatively influencing major biological traits (Tian et al., 2020). All the biological markers tested in this study exhibited significant changes in response to the experimental ammonia concentration, indicating the critical role of this abiotic stressor in the aquaculture of Rohu and, broadly, other fish species. Several earlier studies have indicated that an ammonia concentration less than 0.25 mg/L does not adversely affect fish growth

or production (Barbieri, 2010; Romano & Zeng, 2013; Zhang et al., 2015). Therefore, appropriate facilities must be developed (for fish farms, water exchange, aeration, feeding tray use, bottom debris removal and high-quality feed) to maintain minimum or no ammonia levels.

In the present study, the effects of ammonia treatments on Rohu (*L. rohita*) were examined for cellular (gill ultrastructure), physiological (growth and O_2 consumption), biochemical (blood cell counts, cortisol and glucose levels) and genetic (gene expression) responses. Experimental ammonia treatments significantly altered growth, O_2 consumption, survival, cortisol and glucose levels and gene expression patterns. There were significant correlations between growth and gene expression (IGF-I, GH and G-3-P) and between blood cell counts and gene expression (hepcidin and interleukin-1), indicating important roles for these genes in the growth, metabolism and immunity of Rohu. The findings of this study clearly indicate that ammonia has an adverse effect on the overall biological traits of Rohu and, broadly, other fish species. Even a lower amount of ammonia can impose adequate stress and increase vulnerability to different disease-causing agents. Therefore, farming environments must maintain optimum water quality (with no or minimum ammonia) to improve aquaculture productivity.

AUTHOR CONTRIBUTIONS

Md Shariar Zeehad: Conceptualization; data curation; formal analysis; investigation; methodology; software; validation; visualization; writing—original draft. **Md Monirul Mridul**: Conceptualization; data curation; formal analysis; investigation; methodology; software; validation; visualization. **Dipankar Chakrobortty**: Data curation; resources; visualization. **Sarower Mahfuj**: Software; visualization; writing—review and editing. **Dania Aziz**: Conceptualization; methodology; software; writing—review and editing. **David Hurwood**: Conceptualization; methodology; supervision; writing—review and editing. **Md Lifat Rahi**: Conceptualization; funding acquisition; investigation; methodology; project administration; resources; supervision; writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

This is to confirm that we (seven authors: Md. Shariar Kabir Zeehad, Md. Monirul Islam Mridul, Dipankar Chakrobortty, Sarower Mahfuj, Dania Aziz, David A. Hurwood and Md. Lifat Rahi) conducted a study titled "**Effects of Ammonia on the Cellular, Physiological, Biochemical and Genetic Traits of Freshwater Carp, Rohu (*Labeo rohita*) in Artisanal Bangladeshi Aquaculture**". This is our own research work, and we do not have any conflicts of interest. The authors confirm that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

All of the data generated for this study are presented in the form of different Tables and Figures. Therefore, we do not have any data to share.

ETHICS STATEMENT

Animal ethics approval was obtained from the Animal Ethics Committee of Khulna University (Ref. No.: KUAEC-2021/09/21).

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PEER REVIEW

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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