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Ammonia stress-induced physiological and histological changes in *Heteropneustes fossilis* (Bloch, 1794)

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Abstract

Ammonia is a major environmental pollutant in freshwater aquatic systems, significantly impacting the survival and growth of aquatic organisms. In the present study, we investigated the changes in blood physiological parameters and gill structure in *Heteropneustes fossilis* under hyper-ammonia stress (25 mM NH_4Cl) for experimental exposure durations of 1, 3, 6 and 9 h, as well as after a 24 h recovery period in normal water following 9 h of exposure (9h+N). Results indicated a progressive increase in blood ammonia levels up to 6 h, with fluctuations thereafter, however, even after recovery period, it was significantly more than that of control ($p<0.05$). This was accompanied by a corresponding rise in urea concentration, reaching up to a four-fold increase at 6 h exposure, followed by a decrease at 9 h and to that of control level at 9h+N. Blood glucose exhibited a continuous increase with prolonged exposure till 9 h exposure and then significantly decreased during recovery period. Serum glutamic-oxalacetic transaminase (SGOT) showed significant increase at 6 h, while serum glutamic-pyruvic transaminase (SGPT) at both 3 and 6 h. There was a fluctuating pattern of serum lactate dehydrogenase (LDH). No significant changes were observed for haemoglobin, haematocrit, serum protein and serum creatinine. Histological examination of gill tissues revealed extensive damage, particularly after 9 h of ammonia exposure. At this time point, 78% of the gills exhibited severe structural alterations, including the loss of secondary lamellae tips and 51% showed oedematous changes.

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Introduction

In aquaculture, ammonia is one of the most important water quality parameters that affect and influence fish behaviour, health and productivity. In pond water, ammonia occurs in two forms *i.e.,* ionised ammonia ($NH4^+$) which is relatively non-toxic and the toxic un-ionised form of ammonia (NH_3) (Hargreaves and Tucker, 2004). The toxicity is controlled by the values of pH, temperature and salinity in the aquatic environment (Emerson *et al.,* 1975). The major source of ammonia in water is excretion by fish, primarily through diffusion across the gill epithelium during feed metabolism, as well as through urine and faeces. In addition, fertilisers, decomposition of uneaten feed, plant material and other organic matter also contribute significantly to ammonia accumulation in the aquatic environment (Hargreaves and Tucker, 2004).

Aquatic plants and algae in the pond ecosystem act as the ammonia sinks, as they need nitrogen as an important component of photosynthesis; seasonal as well as day to day variations largely depend on pH changes, caused by photosynthesis (Collos and Harrison, 2014). However, during late summers, consistent production of ammonia with limited uptake by algae may cause an increase in ammonia levels. In winter months, nitrification, the most important process in the nitrogen cycle, slows down due to reduced bacterial processes at cold temperature. Under these circumstances, ammonia concentrations (un-ionised) accumulate that may have sub-lethal effects and fish are subjected to stress for a few hours each day (Milne *et al.,* 2000). Fishes have evolved a number of alternate strategies against ammonia toxicity, such as conversion of ammonia into urea or glutamine (Chew *et al.,* 2003), up-regulation of ammonia excretion against

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gradient, reduction of amino acid catabolism to decrease the production of ammonia (Randall *et al.,* 2004) and detoxification of ammonia to the less toxic form, as urea (ureogenesis) (Zhang *et al.,* 2019).

Heteropneustes fossilis (Bloch), commonly known as stinging catfish (local name Singhi), is a commercially important and popular indigenous catfish in Asian aquaculture, particularly in countries like India, Thailand, Bangladesh, Pakistan, Nepal, Sri Lanka, Myanmar, Indonesia and Cambodia (Burgess 1989; Akand *et al.,*1991; Hossain *et al.,* 2015; Kumar *et al.,* 2019). This fish is mostly preferred for its tender flesh, delicious taste and low-fat content, whereas it is also appreciated in traditional medicine. Interestingly, *H. fossilis* is rich in iron content (226 mg per 100 g) compared to many other freshwater fishes (Chakraborty and Nur, 2012) and due to its high nutritive value, has been recommended as a food for anaemic patients (Kohli and Goswami 1989; Alok *et al.,* 1993). All these characteristics are responsible for a high market demand and economic value (US$ 6–8 per kg) of this catfish species. *H. fossilis* is considered as an extremely promising candidate species for diversification of freshwater aquaculture in India (Kumar *et al.,* 2018; Kutty 2001), due to its adaptability to survive in adverse ecological conditions such as oxygen-depleted waters, tolerance to crowding stress (Tharakan and Joy, 1996) and high ammonia levels (Saha *et al.,* 2001). They are often exposed to high concentrations of total ammonia (ammonia exists as molecular NH_3 and cationic NH_4^+) especially when they are trapped in puddles of water or while burrowing inside the mud peat during summer, where continual excretion of endogenous ammonia into a small volume of external media can lead to high ammonia concentrations. Previously, it was reported as ureogenic, having a functional ornithine-urea cycle (OUC) that could be upregulated during high ammonia levels. However, Chew *et al.* (2019) confirmed that *H. fossilis* is ammonotelic and does not switch to ureotelism when exposed to environmental ammonia.

The present investigation aimed to study the physiological and histological changes on exposure to high external ammonia concentration (25 mM NH4Cl, of which 6.9 mg l^{-1} is unionised ammonia), for shorter periods followed by 24 h in normal (control) water after 9 h of ammonia exposure, in *H. fossilis*.

Materials and methods

Experimental animal

H. fossilis specimens were obtained from commercial catches in Lucknow, Uttar Pradesh, India and acclimatised in the laboratory, at 22±2ºC in FRP tanks for one month. During this period, fishes were fed with powdered commercial feed mixed with chicken (1:1) twice daily and 50% water was changed every day to remove extra feed and faecal waste. Feeding was suspended 48 h prior to start of the experiments.

Experimental setup for ammonia exposures

For exposure experiments, fishes of 40-50 g were placed in 20 l of 25 mM NH4Cl solution prepared in bacteria-free water, that provide free swimming environment. The exposure experiments were for periods of 1, 3, 6, 9 h and followed by 24 h in normal (control) water (9h+N). Another set of fish was kept in 20 l of bacteria-free water that served as controls. During the experimental period, water parameters recorded in the rearing tanks were, temperature - 22±1°C, pH - 7.0±1, dissolved oxygen - 4.5 to 5 mg l^{-1} and total ammonia - 15 mg l^{-1}.

After the exposure to NH_4Cl solution, three fish from each treatment were anaesthetised with 4 ml of 2-Phenoxyethanol in 1.5 l of water for 4-5 min (Varkey and Sajeevan, 2014) and immediately tissue samples were collected. Blood was collected from the caudal vein with a heparinised syringe and immediately centrifuged at 5000 rpm for 10 min for collection of serum. Gills were dissected out from three fish of each treatment and control fish group. Immediately after removal, gills were fixed in 10% neutral buffered formatin for further histological analyses.

Blood parameters

Haemoglobin (Hb) and Hematocrit (Hct) were estimated from whole blood, while glucose and protein from serum. Haemoglobin was determined by Cyan methaemoglobin method (Dacie and Lewis, 1991) using Agappe Mispa Nano Analyser, India. Hematocrit value was calculated following centrifugation of micro hematocrit capillary tube filled with blood, at 10,000 rpm for 5 min (Van Assendelft and England, 1982).

Serum glucose was analysed by Glucose oxidase (GOD) and Peroxidase (POD) calourimetric method (Basak, 2007), based on end point method using Autozyme kit, Agappe Mispa Nano (fully auto-biochemistry Analyser), India. The glucose present reacts with O_2 and H_2O to form D-gluconate and hydrogen peroxide. The hydrogen peroxide reacts with P-hydroxybenzoic and 4-aminoaqntipirine and forms quinonimine dye and water. The amount of NADPH formed through the combined action of hexokinase and glucose -6-P dehydrogenase measured at 340 nm, stoichiometric with the amount of D-glucose in sample volume.

Serum protein was measured by Biuret method (Riegler, 1914) using Autozyme kit, Microlab 300-Semi Auto-Analyser, ELITech Group, France. The -CO-NH-bond of the polypeptide reacts with copper sulphate and gives a purple colour, which is observed at 540 nm showing the presence of protein in the serum.

Biochemical assays

Blood ammonia was estimated based on Kinetic-UV method (Limeres *et al.,* 2017) using EcolineTM Ammonia kit, Microlab 300- Semi Auto-Analyser, ELITech, France. In this method the ammonia present in the serum is converted to urea by the action of urease, the ammonia also reacts with NADH for the production of glutamate and NAD. The TC Matrix system monitors the change in absorbance at 340 nm. The change in absorbance is directly proportional to the concentration of urea in the sample and is used by TC Matrix system to calculate and express the ammonia concentration.

Blood urea level was estimated using GLDH technique (Sampson *et al.*, 1980), using Microlab 300-Semi Autoanalyser, ELITech Group, France. Two types of reagents were used *viz.*, R1 Buffer (TRIS Ph7.8, α-ketoglutarate, urease) and R2 Enzyme (GLDH and NADH). The urea present in the serum gets converted to ammonium which then reacts with α-ketoglutarate catalysed by GLDH with simultaneous

oxidation of NADH. The decrease in the concentration of NADH is proportional to urea concentration in the sample.

Serum creatinine was analysed based on the picrate kinetic reaction in alkaline medium (Lustgarten and Wenk, 1972) with ERBA CHEM 7 Biochemistry Analyser, Hyderabad, India. Creatinine in alkaline solution reacts with picric acid to form coloured complex. The amount of the complex formed is directly proportional to creatinine concentration. The reaction is monitored using picrate selective electrodes and increase in the electrode potential during a fixed period of time is measured and calculated directly to creatinine concentration. During the reaction, alkaline picrate reacts with creatinine to produce a red coloured complex, the rate of red coloured complex formation is directly proportional to the creatinine concentration.

Enzyme assays

Determination of serum glutamic-pyruvic transaminase (SGPT) and glutamic-oxalacetic transaminase (SGOT) were done from blood serum by UV-Kinetic method, using Mispa Nano Auto Biochemistry Analyser, India, following manufacturer's instructions. SGOT catalyses the transfer of an amino group from L-aspartate to α-ketoglutarate. Malate forms from oxaloacetate by the activity of MDH along with oxidation of NADH. Its oxidation is measured by monitoring the decrease in absorbance at 340 nm.

Lactate dehydrogenase (LDH) activity was determined in serum by IFCC Method using LDH (P-L) Kit (ERBA CHEM 7 Biochemistry Analyser, Hyderabad, India), following the manufacture's protocol. LDH catalyses the reduction of pyruvate with NADH, to form NAD. The rate of oxidation of NADH to NAD is calculated as a decrease in absorbance, which is proportional to the LDH activity in the sample.

Histopathological studies of gills

For histopathological studies, control and ammonium chloride (25 mM) exposed gill tissues for different durations of treatment periods was fixed in 10% neutral-buffered formaldehyde. Preserved samples were dehydrated in a graded ascending series of ethanol, cleared in chloroform, infiltrated with wax and then embedded in paraffin block using TEC 2800-M Embedding Dispensing Console and TEC 2800-C Cryo Console, TEC2800 Embedding Centre, Amos Scientific Ltd, Australia. Serial sagittal sections (3-4 µm thick) were cut from each paraffin block using a Leica RM 2135 rotary microtome, mounted on glass slides and air dried. After that, tissue sections were de-paraffined with xylene, rehydrated in graded series of alcohol and stained with haematoxylin and eosin (H&E) for general histomorphological observations. All stained tissue sections were permanently mounted on slide with Entellan (Merck, Darmstadt, Germany) and observed under an Olympus (BX 53, Japan) light microscope.

Results

Blood parameters

Haemoglobin and haematocrit concentration in *H. fossilis* exposed to 25 mM ammonium chloride for different duration did not show significant changes (p>0.05) (Figs. 1 and 2). The concentration of haemoglobin for control was observed as 11.7±0.6 g% and that for 9 h was 13.5±1.0 g% which shows no significant changes in the Hb value. Similarly, no significant changes were observed in the value of haematocrit when compared to the control. There was gradual increase in the concentration of serum glucose from control (88.3±4.04 mg%) to 9 h (166.5±3.8 mg%) and then decline in its concentration for 9h+N (Fig. 3). The blood glucose level was found to be significantly high for specimens treated for 9 h and the lowest level of blood glucose was observed for 9h+N (68.3±3.05). Serum protein level showed no significant variation (p>0.05) when compared between control and fish exposed with 25 mM ammonium chloride for 3 h, 6 h, 9 h and 9h+N (Fig. 4). However, serum protein level was observed lowest in 9h+N (4.2±0.3 mg%) and the highest value was observed in 6 h group (4.8±0.2 mg%).

Biochemical assays

Blood urea and blood urea nitrogen (BUN) showed significant difference (p<0.05) when compared between control, 3, 6 and 9 h but, no significant difference (p>0.05) between control and 9h+N (Figs. 5 and 6). Blood urea concentration showed gradual increase with the increase in duration of exposure with 25 mM ammonium chloride till 6 h exposure period from 5.7±1.1 mg% in control to significantly high value 19.3±2.5 at 6 h. However, its concentration decreased to 7±2 in 9h+N mg%. BUN showed a similar trend with significant increase from control to 6 h and then decreased to control level in respiratory burst. Comparison of blood ammonia level of control and experimental groups showed significant difference (p<0.05) (Fig. 7).

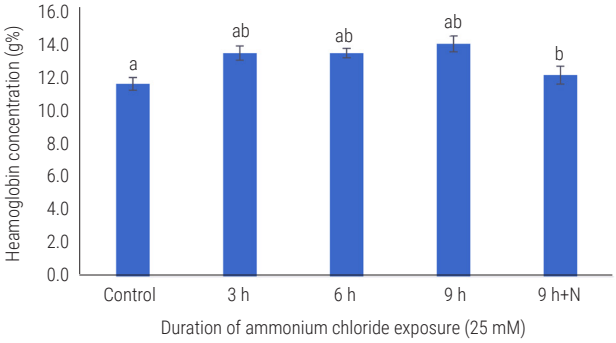

Fig. 1. Haemoglobin concentrations in blood of *H. fossilis* after exposure with 25 mM ammonium chloride for different durations (p<0.05)

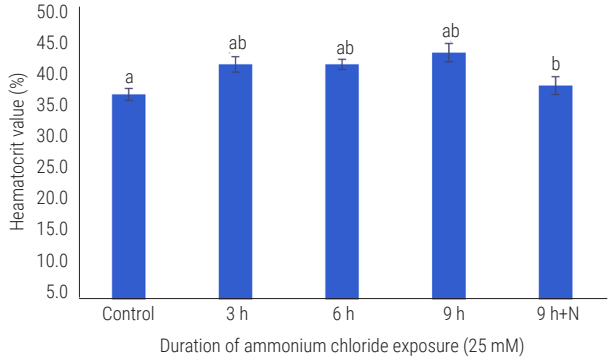

Fig. 2. Haematocrit values of *H. fossilis* after exposure with 25 mM ammonium chloride for different durations (p<0.05)

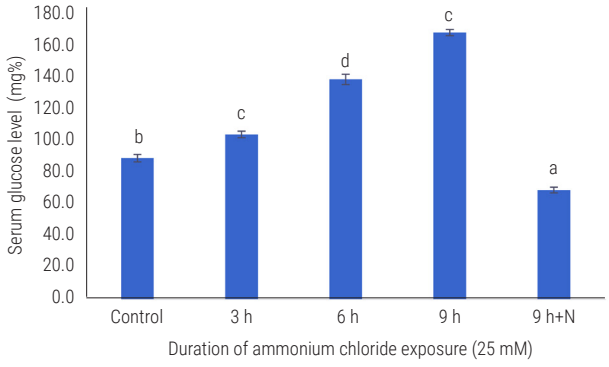

Fig. 3. Blood sugar levels of *H. fossilis* after exposure with 25 mM ammonium chloride for different durations (p<0.05)

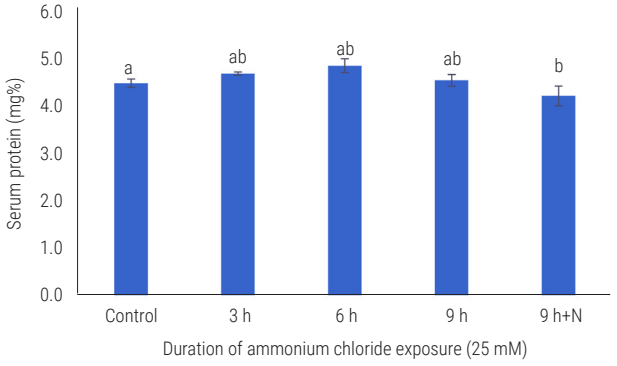

Fig. 4. Serum protein levels in *H. fossilis* after exposure with 25 mM ammonium chloride for different durations (p<0.05)

Blood ammonia concentration showed 3.5-fold increase at 3 h (2281.3±49.9 mg dl^{-1}) exposure with ammonium chloride when compared to control (653.3±34.7 mg dl^{-1}) and then decreased at 6 h exposure period. However significantly highest level was observed in 9 h of exposure (3236.7±53.5 mg dl^{-1}). All 3, 6 and 9 h showed in significant difference between each other as well as with control. Serum creatinine level showed no significant change (p>0.05) when compared between control and fish in all treatments (Fig. 8). However, the concentration of serum creatinine was highest at 9 h (0.53±0.06 mg%) and 3 h (0.52±0.02 mg%) of exposure period.

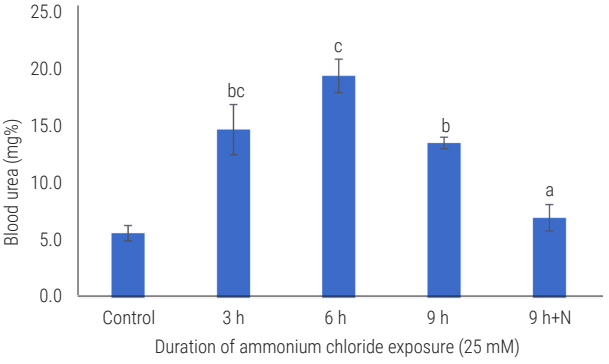

Fig. 5. Blood urea concentrations in serum of *H. fossilis* after exposure with 25 mM ammonium chloride for different durations (p<0.05)

Enzyme assays

Serum lactate dehydrogenase (LDH) of *H. fossilis* after exposure with 25 mM ammonium showed no significant changes (p>0.05) when compared between control, 3 h, 6 h, 9 h and 9h+N (Fig. 9). The highest concentration of serum LDH was observed in 9h+N (4513±1237 ul^{-1}) and the lowest concentration was observed for experimental group, 3 h (2682±522 ul^{-1}). Both SGOT and SGPT (Fig. 10a and b) showed no significant changes (p>0.05) but the concentration showed a slight variation at different exposure periods, concentration of SGOT increased from control to 6 h showing highest value of 572±309 ul^{-1} and then decreased till 9h+N (353±148.09 ul^{-1}). While the concentration of SGPT increased for 3 h (128.6±46.6 ul^{-1}) and 6h which then decreased till 9 h (69±17 ul^{-1}), then showed an increase for 9h+N.

Gill histopathology

Histopathological observations of the gill tissues are summarised in Table 1. Congested blood capillaries were observed in the treated fish, highest (60%) recorded in 3 h groups and it declined by 38.3% in 9h+N group (Fig. 11). There was no congested blood capillaries observed in control group of animals. Hyperplasia in the gill tissues was observed maximum (80%) at 3 h of exposure group and then gradually declined as the period of exposure increased and finally at 24 h+N, it was observed lowest (36%). Similarly, the apical regions of primary lamellae were observed without secondary lamellae, in

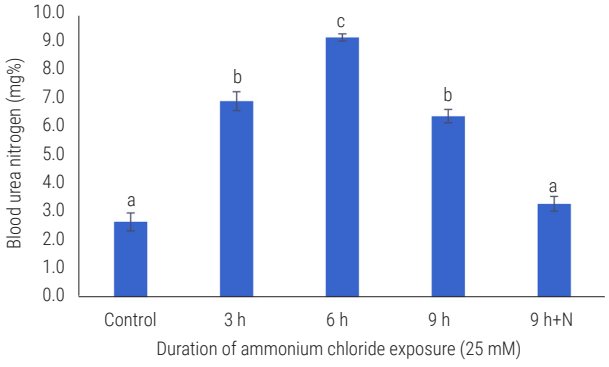

Fig. 6. Blood urea nitrogen in serum of *H. fossilis* after exposure with 25 mM ammonium chloride for different durations (p<0.05)

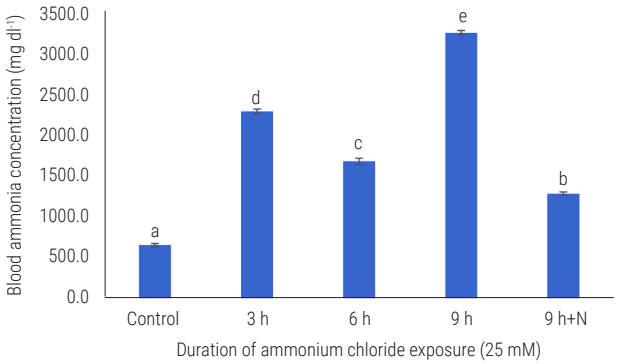

Fig. 7. Ammonia concentrations in blood serum of *H. fossilis* after exposure with 25 mM ammonium chloride for different durations (p<0.05)

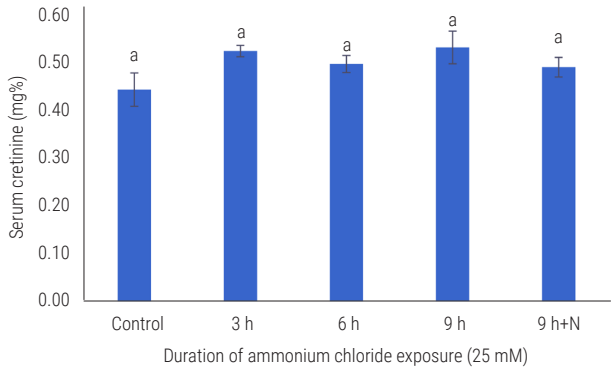

Fig. 8. Serum creatinine in blood serum of *H. fossilis* after exposure with 25 mM ammonium chloride for different durations ($p<0.05$)

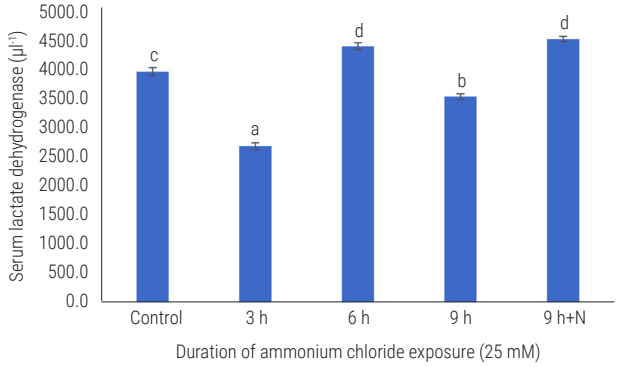

Fig. 9. Serum LDH of *H. fossilis* after exposure with 25 mM ammonium chloride for different durations ($p<0.05$)

specimens exposed to 25 mM ammonium chloride. The highest level (100%) of apical lamellar disintegration was observed in fishes exposed for 9h+N with ammonium chloride and lowest (0%) observed in control group of animals. Along with disintegrated secondary lamellae, deformed lamellae were also noticed which included shortened, curled and fused secondary lamellae. Most disintegrated secondary lamellae were observed at 3 h of exposure, whereas it was lowest in control group. Most deformed secondary lamellae (23%) were observed in fishes exposed for 9h+N and most epithelial lifting (55%) were observed at 9 h exposure group. Similarly, oedema was highest (51%) in 9 h exposure group.

Discussion

The present study aimed to evaluate the blood, biochemical and enzyme parameters of *H. fossilis* exposed to 25 mM ammonium chloride (NH_4Cl) for various durations (3, 6, 9 and 9h+N). Significant changes were observed in several parameters, indicating that exposure to ammonium chloride induces physiological and biochemical alterations in the fish. The findings from this study provide valuable insights into the toxic effects of ammonium chloride exposure on *H. fossilis*. Haematological parameters, such as haemoglobin (Hb) and haematocrit levels, did not exhibit significant changes ($p>0.05$) across all exposure durations. This suggests that while ammonium chloride exposure affected other physiological functions, it did not significantly impact the oxygen-carrying capacity or blood volume in *H. fossilis*. The lack of significant changes in Hb and hematocrit values implies that *H. fossilis* may have a certain degree of tolerance to ammonium chloride, at least over the exposure durations examined. Similar results have been reported in other fish species exposed to ammonia, where no significant changes in haemoglobin levels were observed during short-term

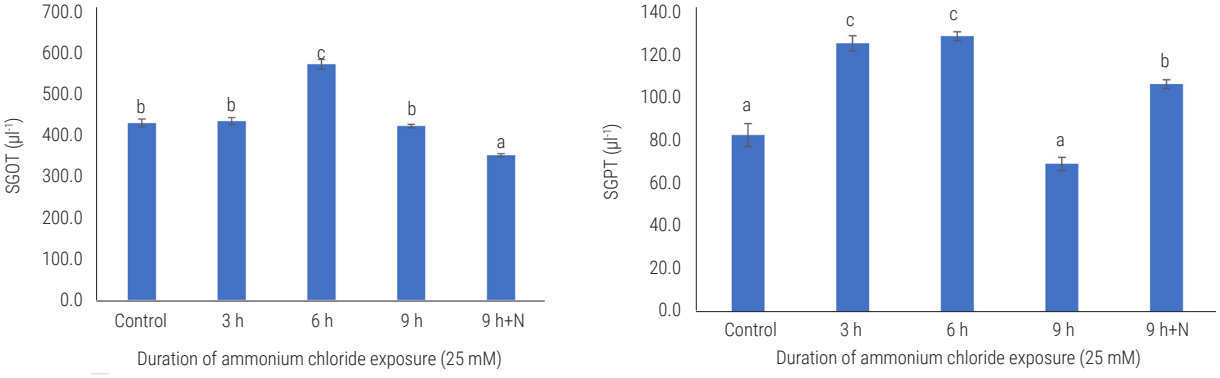

Fig. 10. Significant differences ($p<0.05$) in the value of SGPT and SGOT in blood serum of *H. fossilis* after exposure with 25 mM ammonium chloride for different durations ($p<0.05$)

Table 1. Histopathological observation of gills tissues of *H. fossilis* treated with ammonium chloride (25 mM) for different durations and in comparison, to control groups

Treatments	Congested blood capillaries	Hyper plasia	Tips without secondary lamellae	Disintegrated secondary lamellae	Deformed secondary lamellae	Epithelial lifting	Oedema
Control	0.0%	8.3%	0.0%	0.0%	4.2%	2.0%	1.9%
3h	60.0%	80.0%	57.5%	18.2%	21.4%	1.6%	0.0%
6h	13.3%	38.3%	70.0%	9.4%	24.06%	8.4%	0.9%
9h	30.0%	43.3%	78.3%	6.5%	11.44%	55.8%	51.0%
9h+ N	38.3%	36.0%	100.0%	1.7%	23.07%	11.4%	4.5%

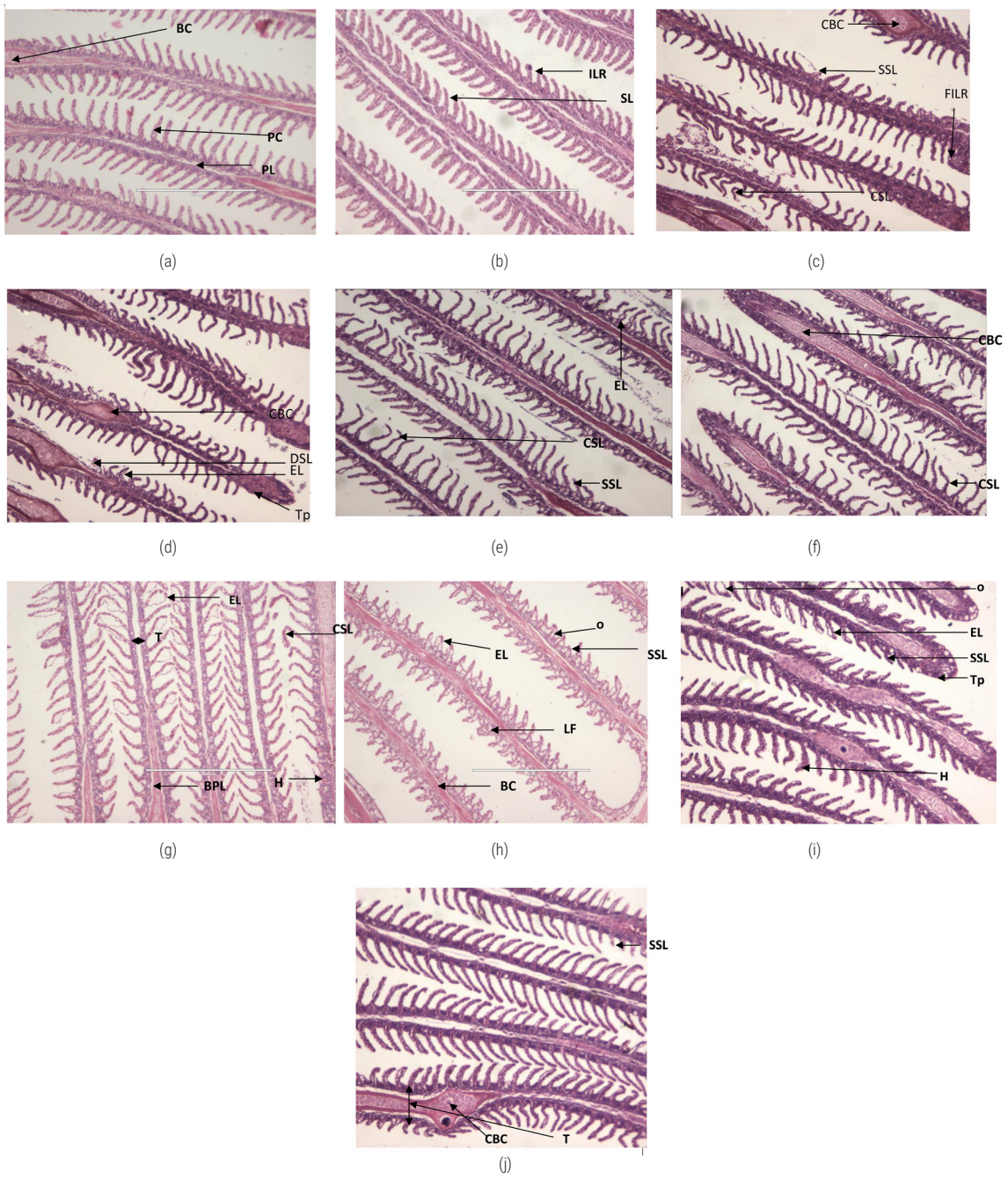

Fig. 11. Effects of ammonium chloride (25 mM) on gills exposed for different time durations. (a and b) Control; (c and d) 3 h; (e and f) 6 h; (g and h) 9 h and (i and j) 9h+N. BC-Blood capillaries, CBC-Congested blood capillaries, SL-Secondary lamellae; PL-Primary lamellae, PC-Pillar cells, ILR-Inter lamellar region; DSL-Disintegrated secondary lamellae, FILR-Filled inter lamellar region, LF-Lamellar fusion, EL-Epithelial lifting, O-oedema, T-Thickness, Tp-Tips without the secondary lamellae, BPL-Bulging tip of primary lamellae, CSL-Curling of secondary lamellae, SSL- Shortening of secondary lamellae, H- Hyperplasia.

exposure (9 h), while longer exposure (96 h) produced more pronounced effects (Kim *et al.,* 2019; Molayemraftar *et al.,* 2022). In cases of prolonged exposure, reduced haemoglobin levels in fish exposed to nitrite have been linked to increased methemoglobin formation, which occurs due to nitrite toxicity and its impact on haemoglobin (Jensen, 2003; Jensen, 2009). In the present study, no significant changes in Hb or haematocrit were observed, likely due to the shorter exposure duration (9 h). Blood urea and blood urea nitrogen (BUN) levels showed a significant increase after 3 and 6 h of exposure, followed by a decrease at 9 h. The initial increase in urea and BUN levels may reflect impaired renal function or the accumulation of nitrogenous waste products due to metabolic stress induced by ammonium chloride. The subsequent decrease at 9 h could indicate a compensatory mechanism or a reduction in the fish's ability to excrete urea after prolonged exposure. This pattern of an initial increase followed by a gradual reduction

has been observed in fish exposed to nitrogenous pollutants, where disruptions in osmoregulation and nitrogen metabolism are common (Guo *et al.,* 2023). Ammonia accumulation in blood plasma was notably high, with a significant 4.5-fold increase at 3 h, followed by a peak at 9 h of exposure. Elevated ammonia levels indicate a significant impairment in the detoxification mechanisms of *H. fossilis* (Choudhury *et al.,* 2012). Ammonia toxicity can disrupt cellular functions, particularly in the gills, where ammonia excretion typically occurs (Ip and Chew, 2010). Prolonged exposure to ammonium chloride may reduce the fish's ability to effectively excrete ammonia, contributing to the observed physiological stress, as evidenced in previous research on ammonia toxicity in fish (Xu *et al.,* 2021). Serum creatinine and serum protein levels did not show significant changes across the exposure durations. Although creatinine is often used as an indicator of kidney function, the lack of significant variation here suggests that renal function may not have been severely impacted, at least within the 9 h exposure window. However, previous studies have shown that serum protein and creatinine concentrations increase with prolonged exposure to pollutants (Guo *et al.,* 2023; Dawood *et al.,* 2023). Lactate dehydrogenase (LDH) and transaminases (SGOT and SGPT) serve as markers for tissue damage and stress responses. While LDH activity showed a significant increase ($p<0.05$) at 9 h, suggesting some degree of tissue damage or cellular injury, SGOT and SGPT did not exhibit significant changes. This may indicate that while ammonium chloride exposure triggered metabolic shifts, it did not lead to widespread liver or muscle damage, or that such damage occurred later in the exposure timeline (Wang *et al.,* 2021). Histopathological examination of gill tissues revealed a progressive accumulation of damage with increased exposure duration. The most significant changes occurred at 9 h and during the 9h+N, with prominent gill abnormalities such as edema, epithelial lifting, hyperplasia and the loss of secondary lamellae. These alterations suggest that prolonged exposure to ammonium chloride disrupts gill architecture, which could impair both respiratory and osmoregulatory functions. The observed gill damage aligns with findings from previous studies on ammonia toxicity, where ammonia accumulation in the blood causes damage to gill tissues, disrupts respiration and gas exchange, induces metabolic disorders, suppresses immune function, hinders growth and development and may lead to abnormal behaviour (Sun *et al.,* 2024; Kim *et al.,* 2015; Kim *et al.,* 2017; Kim *et al.,* 2020). These structural changes in the gills likely contribute to the physiological stress observed and reduced ammonia excretion efficiency (Sun *et al.,* 2024).

The findings from this study demonstrate that exposure to ammonium chloride induces significant physiological, biochemical and histopathological alterations in *H. fossilis*. Although haematological parameters such as haemoglobin and haematocrit levels remained largely unaffected, key indicators of metabolic and renal stress, including blood urea nitrogen, ammonia accumulation, and enzyme activity (LDH), exhibited marked changes, suggesting metabolic disruptions and potential impairment of detoxification mechanisms. Histopathological analysis revealed progressive gill damage, including edema, epithelial lifting, and hyperplasia, which likely impaired respiratory and osmoregulatory functions. These results underscore the toxic effects of ammonium chloride on fish, particularly in the context of ammonia toxicity, and highlight the need for further research into the long-term consequences of such exposure. The study contributes valuable insights into the physiological and biochemical responses of *H. fossilis* to ammonium chloride, providing a foundation for future studies on the ecological and environmental implications of ammonium chloride contamination in aquatic systems.

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