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Effect of short-term hyper-ammonia exposure in Indian catfish, *Clarias magur* (Hamilton, 1822) on haematological parameters, tissue structure and gene expression profiles

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Abstract

In the present study, pattern of changes in blood physiological parameters, structural changes in gills, liver and kidnney as well as gene expression patterns for ornithine-urea cycle (OUC) were studied, under hyper-ammonia stress (25 mM NH_4Cl) for 1, 3, 6 and 9 h and 24 h in normal water condition after 9 h exposure (9h+N), in *Clarias magur* (Hamilton, 1822). Analysis showed increase in blood ammonia upto 3 h, with decreasing trend (upto 2 folds reduction) subsequently. It was accompanied by simultaneous increase in urea levels upto 6 folds at 9 h exposure. However, there were no significant changes in blood parameters, except for serum protein, serum glutamic pyruvic transaminase (SGPT) and lactate dehydrogenase (LDH) levels after 1 h exposure. Histological analysis of gills revealed highest (88%) percentage of damaged gills with congested blood capillaries, treated for 9 h, but declined by 65% at 9h+N. Histological alterations in liver and kidney were prominent only at 9 h exposure, with nuclear hypertrophy and necrosis of hepatic cells in liver tissues and distension as well as degeneration of Bowman's capsule in kidneys. Significant up-regulation of *cpsIII* and *otc* genes at 9 h and 9h+N, points to activation of OUC in liver at 6 h exposure and unique physiological characteristics of turning towards ureotelism from ammoniotelism, after short term exposure to high ammonia level.

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Introduction

In aquaculture, ammonia is one of the most critical water quality parameters that affects the fish productivity. Of the two forms, un-ionised ammonia is toxic than the ionised one, whose 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 the excretion of ammonia into water by fish through the gill epithelium, during the feed metabolism and also in the form of urine and faeces. In addition, decomposition of the plant material in the aquatic environment also contributes to it (Hargreaves and Tucker, 2004). As a result, an ammonia spike may occur and larger volume of water is required to reduce its effect.

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 process 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 strategies against ammonia toxicity, such as conversion of ammonia into glutamine, up-regulation of ammonia excretion against gradient, inhibition of breakdown of amino acids to reduce the production of ammonia and detoxification of ammonia to the less toxic form, as urea (ureogenesis) (Zhang *et al*., 2019).

Clarias magur (Hamilton, 1822), an air-breathing catfish found in the Indian subcontinent, is a potential aquaculture species, because of its high value and customers' preference (Khan *et al.,* 2000), in addition to comparative hardiness, good growth and efficient feed conversion (Argungu *et al.,* 2013). *C. magur* is a facultative air-breather (Mohindra *et al.,* 2016), usually inhabiting stagnant, slow-flowing swampy water bodies or wetlands, which are often covered with macrovegetation, such as water hyacinth. These waters are also characterised by low dissolved oxygen, and high bicarbonate and ammonia levels (Saha and Ratha, 1998). Extreme tolerance to a high concentration of ambient ammonia has been reported for this species, with a reported capacity to survive an exposure of 25 mM NH_4Cl for long durations in experimental conditions, by stimulation of ureogenesis (Saha *et al.,* 2002; Saha *et al.,* 2003) *via* ornithine-urea cycle (OUC), as an adaption strategy and the genes responsible for it have been reported to be induced after long term exposure, between seven to fourteen days of the exposure (Banerjee *et al.,* 2020; Varkey and Sajeevan, 2014). However, in the real situations of the pond conditions during aquaculture, the fish experiences high ammonia exposures in short durations, followed by normal water conditions. However, there is no information on the response of *C. magur* on physiological, biochemical and histological changes in various organs as well as for gene expression profiles, for acute (short term) exposures as in culture conditions. So, to study the effects of these exposures, the experiment was planned for short duration. Consequently, authors have hypothesised that chronic exposure to ammonia-N for short periods could cause oxidative stress damage, physiological response and histological changes in different organs of fish.

The present investigation aimed to study the physiological and histological changes on exposure to high external ammonia concentration (25 mM NH_4Cl, 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 *C. magur*. Gene expression profiles of four genes belonging to ornithine-urea cycle (OUC) were also studied, to verify if OUC has been initiated after short term ammonia exposures.

Materials and methods

Experimental animal

C. magur 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 NH_4Cl 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, liver and kidney were dissected out of three fish from each treatment and control fish group. Immediately after removal, gills were fixed in 10% neutral buffered formaldehyde 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 determined 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 estimated by Glucose oxidase (GOD) and Peroxidase (POD) calorimetric 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 determined by Biuret reaction (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 measured 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. With the increase in the amount of the urea formed there will be decrease in the absorbance. 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 determined using GLDH technique (Sampson *et al.*, 1980), using Microlab 300-Semi Autoanalyzer, ELITech Group, France. Two types of reagents were used viz., R1 Buffer (TRIS Ph7.8, α-ketoglutarate, urease) and R2 Enzyme (GLDH & 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 determined 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 measured as a decrease in absorbance, which is proportional to the LDH activity in the sample.

Histology

For histopathological studies, control and ammonium chloride (25 mM) exposed gills, liver and kidney for different durations were fixed in 10% neutral-buffered formaldehyde for further histological analyses. Preserved samples were dehydrated in a graded series of methanol, ethanol, chloroform, 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 graded series of xylene 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.

RNA isolation and cDNA preparation

Kidney tissue samples were collected at 0 (control), 3 and 9 h of exposure to ammonia and 9 h+N exposure, flash frozen in liquid nitrogen and stored at -80^{0}C for further analysis. Total RNA was extracted using RaFlexTM Total RNA isolation kit (Genei, Bangalore, India) and the RNA quality and quantity were assessed with NanoDrop™ Onec (Thermo Fisher Scientific, USA). For quantitative real time PCR analysis, first strand cDNA was synthesised using RevertAid H minus First strand cDNA Synthesis Kit (Thermo Scientific, USA) following standard protocols and stored at -20^{0}C, for further use.

Quantitative Real Time PCR (qRT-PCR) analysis

For the qRT-PCR analysis, gene expression patterns of four genes from arginine biosynthesis pathway and urea cycle (Kanehisa and Goto, 2000; Kanehisa *et al.*, 2017; Kanehisa *et al.*, 2019) (Fig. 1): *cps III* (Carbamoyl-phosphate synthase), *otc* (Ornithine carbamoyl transferase), *nags* (N-acetylglutamate synthases) and *gls* (Glutamine synthetase) were analysed. The primers used (Table 1) were according to Banerjee *et. al.* (2020) and for gls, primers were

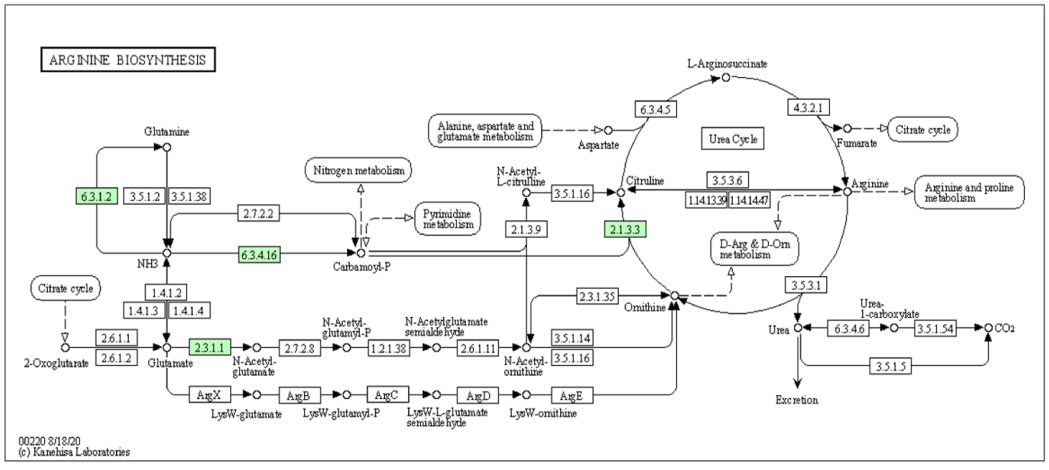

Fig. 1. Depiction of the *ornithine–urea cycle* (OUC). The genes studied in this cycle have been marked in green : EC 6.3.1.2: *gls* (Glutamine synthetase); EC 6.3.4.16: *cpsIII* (Carbamoyl-phosphate synthase); EC 2.3.1.1: *nags* (N-acetylglutamate synthases); EC 2.1.3.3: *otc* (Ornithine carbamoyl transferase) (Reprinted with permission from Kyoto Encyclopedia of Genes and Genomes)

Table 1. Primers for qRT-PCR used the present study

S.No.	Gene*	Primer	Primer sequence 5' to 3'	Reference
1	*nags*	Forward	AGCAGTGGTGCTGCCTTTAT	Banerjee *et al.* (2020)
		Reverse	ATTGAGGTGAGTGGAGCAGC	
2	*otc*	Forward	TTGTCTCCCACGAAAGTCCG	
		Reverse	ACACACAAGCAACCCCATGA	
3	*cpsIII*	Forward	CCACAAGCACCGTGAAATGG	
		Reverse	TGATGCTGTTCTCCACCCAC	
4	gls	Forward	GCCCTGTAGTGAGCTTCGAC	Designed for present study
		Reverse	ACACCATCCTCGGAACAGAC	
5	*B2M* (housekeeping gene)	Forward	AGGCTGGGTGATAGGATACAAAT	Mohindra *et al.* (2014)
		Reverse	ACCACAGATAAGTGATACTACACATCTTGC	

**cpsIII* (Carbamoyl-phosphate synthase); *otc* (Ornithine carbamoyltransferase); *nags* (N-acetylglutamate synthases); *gls* (Glutamine synthetase); *B2M*: Beta 2 microglobulin

designed for the present analysis from NCBI accession JX457351. Beta 2 microglobulin (B2M) gene was used as the house keeping gene (Mohindra *et al.,* 2014).

The standard curves of each primer were generated using 10-fold serial dilutions of PCR products. PCR amplification reaction mixture (25 µl) contained 2.5 µl 10X buffer, 5 mM dNTPs (2.5 µl), 25 mM $MgCl^2$ (0.5 µl), 1.5 units Taq polymerase (Genei, India), 5 pM of each primer and 100 ng template cDNA. The PCR cycling was set as initial denaturation for 5 min at 95ºC, followed by 40 cycles of denaturation for 30 s at 95ºC, annealing for 15 s at 55ºC and elongation 15 s at 72ºC and final elongation for 10 min at 72ºC. The PCR products were visualised by gel electrophoresis and quantified using NanoDrop™ One (DeNovix Inc. DE, USA). Targeted amplicons were diluted to 1 ng μl^{-1} and 10-fold serial dilutions were prepared. For the time points for each treatment and control, qRT-PCR was conducted in 3 individuals and the reaction mixture (25 µL) contained 1 µl cDNA (1 ng μl^{-1}) and 12.5 µl SYBR Green master mix (Thermo Fishers Scientific, Waltham, Massachusetts, USA) and 0.3 µM (0.75 µl) of each primer. Initial denaturation step was at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 60°C for 30 s and extension at 72°C for 30 s and for melting curve analysis, 20 s at 95ºC and 20 s at 65ºC, cooling 30 s at 40ºC in a LightCycler 480 (Roche Applied Science) system. Primer efficiencies (E) for each gene were calculated from tenfold dilution series, that were used to make standard curves for genes analysed, according to the formula E = 10-1/slope. The specificities of the PCR amplifications were confirmed by melting curve analysis. Absolute quantification was used to derive the concentration values in LightCycler (TM) 480 (Roche Holding AG, Basel, Switzerland) software. Fold changes of expression of OUC genes in treated fish compared to untreated controls were calculated using Relative Expression Software Tool (REST, Pfaffl *et al.,* 2002) and was used for group-wise comparison and statistical analysis of expression results in qPCR.

Statistical analysis

The generated data were subjected to statistical analysis to test for level of significance between ammonia treated with control values from fish exposed to normal water conditions. As the observations on eleven parameters were for three specimens in each of six groups, data matrix 18x11 was generated. The samples of fish specimens were different for each group, which may cause non-equality of variances of six groups of fish specimens. In this situation, one of the assumption of "equality of variances of groups under comparison" for application of Classical ANOVA may not be fulfilled, therefore, to deal with this, Welch's analysis of variance (ANOVA) was applied and for group comparisons, Games-Howell *post hoc* test was used. Games-Howell *post hoc* test, like Welch's ANOVA, does not require the groups to have equal variances (Jan and Shieh, 2014).

The multivariate analysis, principal components analysis (PCA) and canonical discriminant function analysis (CDFA) of data matrix 18x11 were performed. PCA was applied to identify the role of parameters through significant principal components (PC1, 2), the variations among specimens were analysed and graphically displayed over scores of PC1, as PCA reduces the dimensionality of variables (Veasey *et al.,* 2001), in decreasing redundancy among the variables (Samaee *et al.,* 2006) and by removing a number of independent variables for group of specimens differentiation (Samaee *et al.,* 2009). In PCA, according to Dunteman (Dunteman, 1989), 70-90% (cumulative) of total variation is required to be applied to preserve principal components. In PCA, Kaiser's, 1960 criterion of retaining eigenvalues greater than 1 for determining the number of components was applied (Jolliffe, 2002). CDFA was applied for identification of important discriminant functions and computation of the scores for discriminant functions (Function1 vs. Function2). Under this, the variations in parameters of specimens over groups were analysed and graphically displayed by scores over Function1 vs Function2. The variations among specimens over group were analysed and graphically displayed over scores of function1. Further analysis by application of multivariate method: partial least square discriminant analysis (PLS-DA) on data matrix used for projection of functions (1,2) and also factors (PLS-DA factor1 and PLS-DA factor2), which are used for score based graphical presentation of eleven parameters in projection and also graphically demonstrated the linking of factors (factor1, 2) to functions (Function1, 2) through eleven parameters. In all these cases, 5% (p=0.05) level of significance was selected to indicate statistically significant differences. All statistical tests were performed with SPSS version 16 (SPSS Inc. Released, 2007), SAS version 9.3 (SAS Institute Inc., 2012).

Results

Blood and physiological parameters

Overall statistical analysis

Welch's ANOVA for the eleven blood physiological parameters of *C. magur* exposed with 25 mM ammonium chloride for different time

intervals and control samples indicated that out of these parameters studied, all parameters were found to be significantly different ($p \leq 0.05$), except for haemoglobin (Hb) and haematocrit (Hct). These results were also supported by the Games-Howell *post hoc* multiple comparison test ($p<0.05$) for pairs of parameters also

Discrimination among specimen groups

Based on Welch's ANOVA and Games-Howell *post hoc* test for equality of means of 11 parameters over Welch Statistic (Asymptotically F distributed), nine parameters were observed as significant ($p<0.05$), with two parameters (haematocrit, haemoglobin) being non-significant. The Welch's (ANOVA) for eleven parameters over group-wise analysis indicated that seven parameters have significant ($p<0.05$) variations among fish groups while four parameters (Hb, HCT, SGPT and blood sugar) are non-significant. The Games-Howell *post hoc* multiple comparison tests for pair-group comparisons indicated that 74 out of 330 pairs (22%) showed significant variations ($p \leq 0.05$) of parameters and highest number of group-comparisons were observed to be significant for parameters related to ammonia exposed group.

PCA analysis indicated significant (eigenvalue greater than one) principal components (PC1, 2), which constitute 33.53 and 24.96% variations, respectively and both (PC1, 2) constitutes 58.49% of total variation of 18x11 data matrix. The graphical distribution of 18 specimens over scores from principal component (PC1) indicated that scores were in the range from (-) 2.5 to 7.5, clearly demonstrating the presence of parameter-linked variations among specimens.

CDFA analysis showed that functions (1 and 2) jointly control 92.18% of total variations (function 1, 2 as 83.37, 8.82%) in data matrix of 18x11 and functions (1, 2) have values for six specimen-groups as (-4.76, -1.94), (5.76, 2.44), (-7.49, 3.44), (-1.45, -1.33), (11.60, -0.59), (-3.66, -2.20). These values of functions (function 1 vs. 2) clearly indicated that centroids of group 2, 3, 4 and 5 have differences over parameters, whereas group 1 (control) and group 6 (9h+N) have merged centroids, which indicated that specimens in these two groups (control and 9h+N) are similar over different parameters (Fig. 2). The score based discrimination of 18 specimens through discriminant function (Function1) indicated that scores were in range from (-)13 to 17.0 and graphical analysis clearly demonstrated the presence of parameters linked variations among specimens, also specimens may be discriminated among six groups.

Further, analysis on distribution of specimens over scores on factors (Factor1, Factor 2) from partial least squares discriminant analysis (PLS-DA) indicated the association of PLS-DA factors (Factor1, Factor2) with the discriminant functions (Function1, Function2) in explaining the percent variation among eleven parameters (X-Effects) (Fig. 3a). However, individually Factor1 explains variations among eleven parameters and more than 90% variation is explained for parameters; BUN (blood urea nitrogen) and blood urea followed by other parameters. The parameters that have important role in principal components (PC1, 2), discriminant functions (Function1, 2) and partial least square discriminant analysis factors (Factor 1, 2) are BUN, blood urea, serum protein, SGPT, SGOT, BAL (blood ammonia level) and LDH as these seven parameters have important contribution (values more than 0.85) within parameter variations (Fig. 3b).

Blood parameters

When control samples were compared with treated samples, Hb and Hct concentrations, serum glucose, protein and creatine, in *C. magur* exposed with 25 mM ammonium chloride for different durations showed non-significant differences except for serum protein at 1 h exposure (Fig. 4-8). It was found to be significantly ($p \leq 0.05$) reduced at 1 h (3.53±0.50) exposure as compared to that of control (4.8±0.2) (Fig. 6).

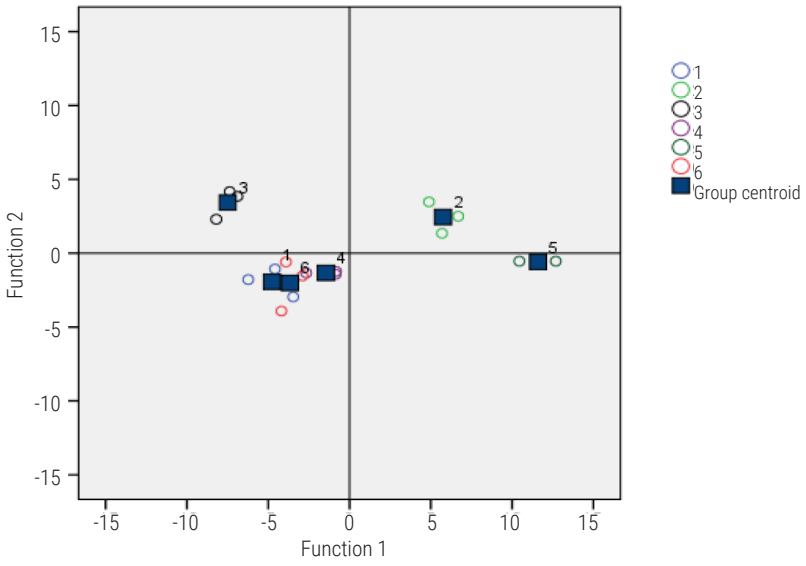

Fig. 2. The distribution of group of specimens (1-6) over Function1 vs. Function 2. (Group 1: control, 2: 1h, 3: 3h, 4: 6h, 5: 9h, 6: 9 h+N)

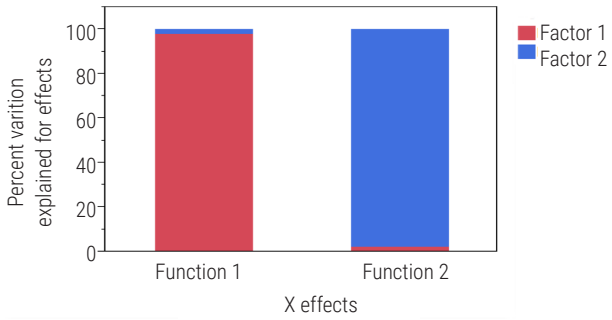

Fig. 3a. The factors (Factor 1, Factor 2) associated with discriminant functions (Function1, 2) in explaining the variations among eleven parameters (X Effects); Factor1 individually explains more than 90% variation among parameters (X Effects)

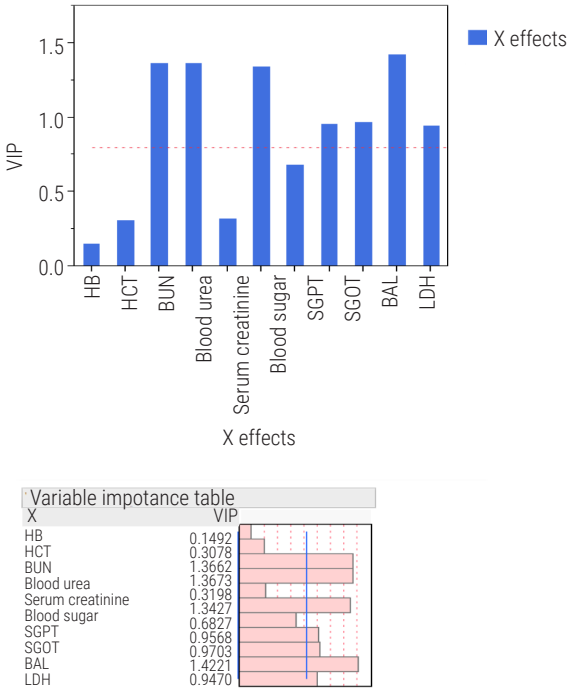

Fig. 3b. The distribution of eleven parameters (X) in variable importance plot and table. The seven parameters; BUN (blood urea nitrogen), blood urea, serum protein, SGPT, SGOT, BAL (blood ammonia level) and LDH identified as variables of importance have higher contribution (value more than 0.85) in discrimination of 18 specimens over six groups

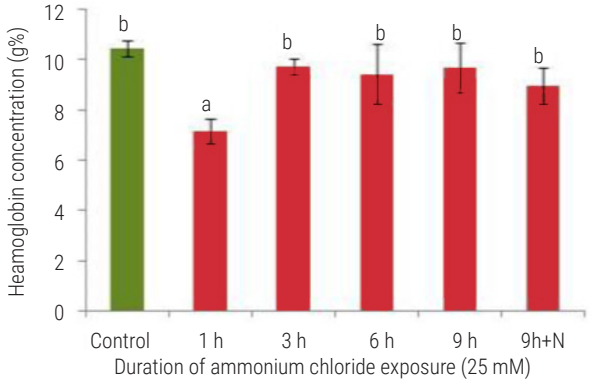

Fig. 4. Haemoglobin in the blood of *C. magur* treated with 25 mM ammonium chloride for different exposure period. Different letters indicate statistical differences among groups (ANOVA, $p<0.05$)

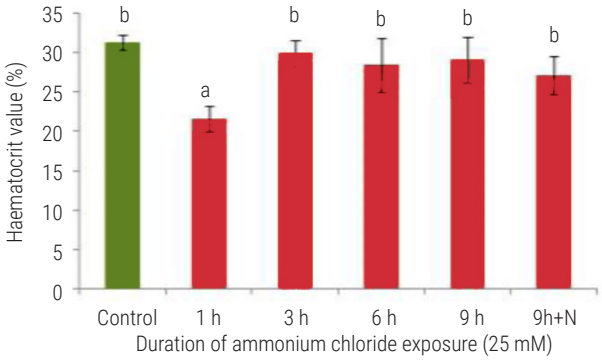

Fig. 5. Haematocrit in the blood of *C. magur* treated with 25 mM ammonium chloride for different exposure periods. Different letters indicate statistical differences among groups (ANOVA, $p<0.05$)

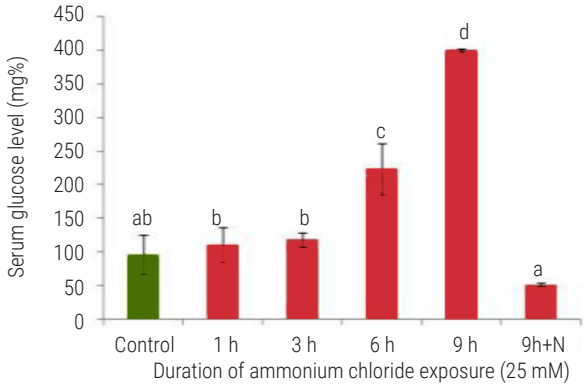

Fig. 6. Glucose in the serum of *C. magur* treated with 25 mM ammonium chloride for different exposure periods. Different letters indicate statistical differences among groups (ANOVA, p<0.05)

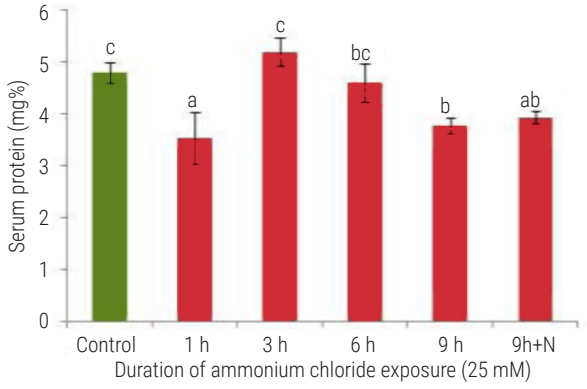

Fig. 7. Serum protein in *C. magur* treated with 25 mM NH_4Cl for different exposure periods. Different letters indicate statistical differences among groups (ANOVA, p<0.05)

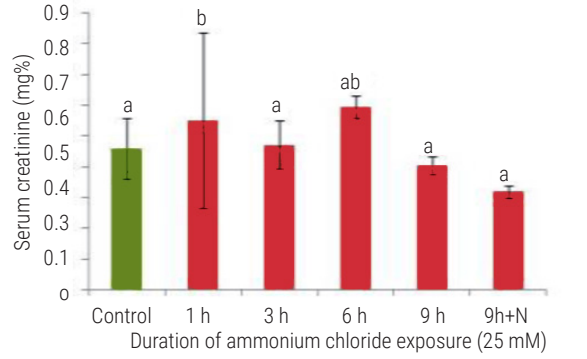

Fig. 8. Serum creatinine in *C. magur* exposed to 25 mM NH_4Cl at different exposure time interval and control. Different letters indicate the existence of statistical differences among groups (ANOVA, p<0.05)

Biochemical assays

Blood ammonia

Blood ammonia concentration in *C. magur* showed a gradual increasing trend from 1 to 3 h exposure with 25 mM ammonium chloride, as compared to that of control group (Fig. 9) and highest ammonia accumulation in blood was found at 3 h exposure, (910.6±135.43). Interestingly, 6 h (855.3±33.23) and 9 h (67.0±4.0) exposures showed significant (p<0.05) decreasing trend in blood ammonia concentration. At 9 h, it was even lower than that of control. Moreover, returning to normal conditions after 9 h exposure the blood ammonia level was found to be similar (585±74.5) to that of control (p>0.05).

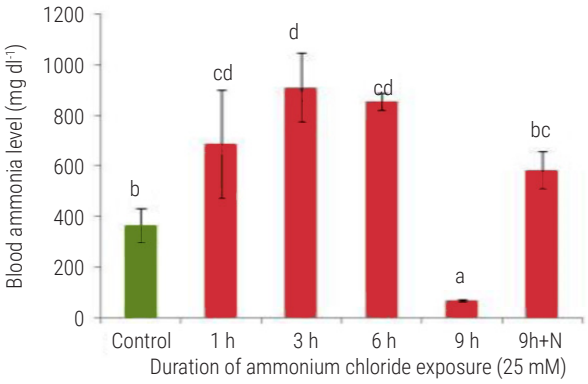

Fig. 9. The level of blood ammonia in *C. magur* exposed to high external ammonia (25 mM NH_4Cl) at different exposure periods. Different letters indicate statistical differences among groups (ANOVA, p<0.05)

Blood urea and blood urea nitrogen

Blood urea and BUN concentration of *C. magur* exposed to 25 mM ammonium chloride for different duration are depicted in Fig. 10 a and b. The level of urea and BUN were found to be significantly ($p \leq 0.05$) higher at 1 and 9 h of exposures. However, at 1 h, the concentration was found to be slightly higher than that of control, while found highest at 9 h of exposure. Interestingly, beyond 9 h exposure after returning to normal conditions, the urea and BUN levels were found to be non-significant ($p \geq 0.05$) similar to that for control and 3 and 6 h of exposures.

Enzyme assays

SGPT and SGOT

SGPT levels remained unchanged at 3, 6 and 9 h of exposure as compared to control, except for 1 h and 9h+N, where the levels were significantly low ($p \leq 0.05$), when compared to control (Fig. 11a). The concentrations of SGPT were in the range of 70.3±9.3 to 118±1.52 μ l^{-1}. Similarly, the concentration of SGOT also showed no significant changes between control and for all the exposure periods (Fig. 11b) and the values ranged from 222±26.5 to 447±291 μ l^{-1}.

Serum LDH

Serum LDH of *C. magur* after exposure with 25 mM ammonium showed non-significant variation (p>0.05) among the different treatment groups and control (Fig. 12), except at 9 h, where it showed was slight but significant increase. The levels of LDH varied from 364.7±67.6 μ l^{-1} (control) to 5315±287.5 μ l^{-1} (9 h exposure).

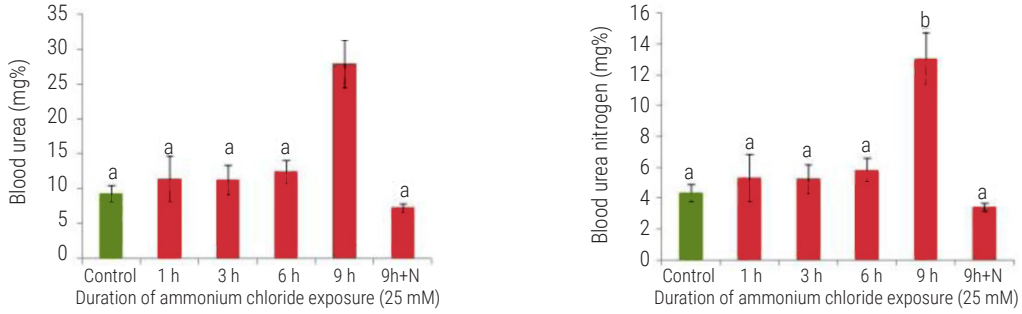

Fig. 10. The level of (a) blood urea and (b) blood urea nitrogen (BUN) in *C. magur* treated with high external ammonia (25 mM NH_4Cl) at different exposure time periods. Different letters indicate statistical differences among groups (ANOVA, p<0.05)

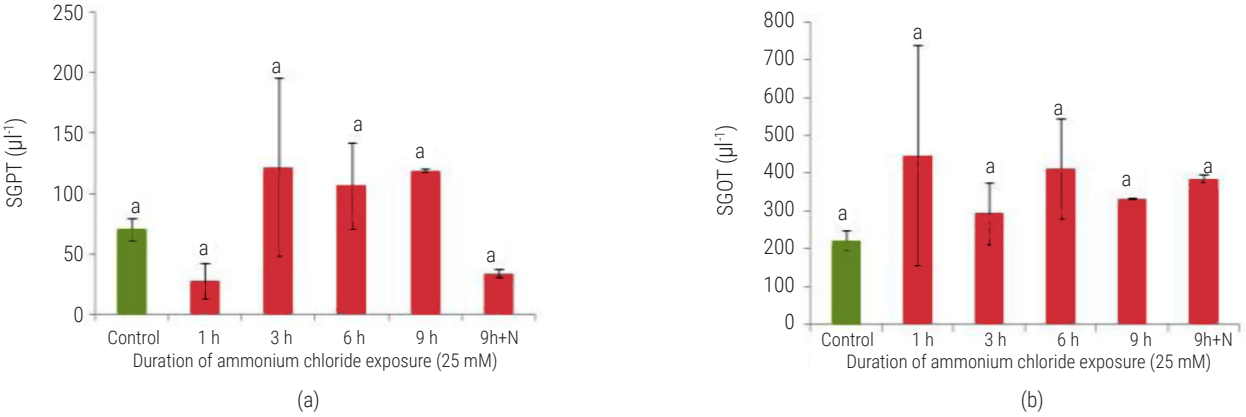

Fig. 11. The levels of (a) SGPT and (b) SGOT in *C. magur* treated with high external ammonia (25 mM NH_4Cl) at different exposure time intervals. Different letters indicate statistical differences among groups (ANOVA, p<0.05)

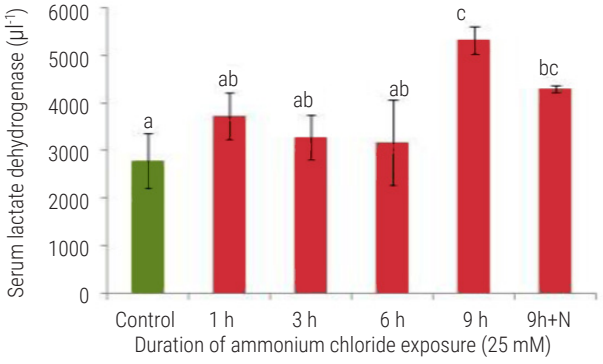

Fig. 12. Lactate dehydrogenase (LDH) concentration in the serum of *C. magur* treated with 25 mM ammonium chloride at different exposure periods. Different letters indicate statistical differences among groups (ANOVA, p<0.05)

Gene expression profiles

Quantitative Real Time PCR (qRT-PCR) analysis

Primer efficiencies (E) of target genes and the specificities of the PCR amplifications are given in Table 2. A significant increase in the expression of mRNAs for different OUC genes was observed during exposure to ammonia (25 mM NH4Cl) for 9 h and 9h+N as compared to the experimental control (Fig. 13). The mRNA level for the *cps III* gene increased significantly at 9 h (13.95 fold) and 9h+N (74.12). The mRNA level for the *otc* gene also showed increasing trend by 3.35 and 5.51 fold changes for 9 h and 9h+N, respectively. The expression levels for *nags* showed significant increase of 9.27 fold only at 9h+N, while *gls* showed no fold changes in the expression of mRNA levels in the tissues on exposure to ammonia, during the present study.

Histopathological studies of gills, liver and kidney

Histopathological observations of the gill tissues are summarised in Table 3. Congested blood capillaries were observed in the treated fish, highest in 9 h (88%) and it declined by 65% at 9h+N (23%) (Fig. 14). There were no congested blood capillaries observed in control group of animals. Similarly, the apical regions of primary lamellae were observed without secondary lamellae, in specimens exposed to 25 mM ammonium chloride. The highest level of apical lamellar disintegration was observed in fishes exposed for 9 h with ammonium chloride and lowest observed in control group of animals. Along with deteriorated secondary lamellae, deformed lamellae were also noticed which included shortened, curled and

Table 2. qRT-PCR Primer efficiencies (E) of target genes of ornithine urea cycle and the specificities of the PCR amplifications in *C. magur*

Parameters	*B2M*	*otc*	*cpsIII*	*nags*	*gls*
Slope	-3.66	-3.57	-3.65	-3.16	-3.54
SE (slope)	±0.42409	±0.35769	±1.03379	±0.82258	±1.85502
Efficiency	1.87	1.91	1.88	2.07	1.92
SE (E)	±0.13636	±0.12326	±0.33528	±0.39478	±0.65247

**cpsIII* (Carbamoyl-phosphate synthase); *otc* (Ornithine carbamoyltransferase); *nags* (N-acetylglutamate synthases); *gls* (Glutamine synthetase); *B2M* (Beta 2 microglobulin).

Table 3. Histopathological observation of gills tissues of *C. magur* treated with ammonium chloride (25 mM) for different durations and in comparison to control groups

Treatment	Congested blood capillaries (%)	Tips without secondary lamellae (%)	Deformed secondary lamellae (%)	Hyperplasia of primary epithelial cells (low) (%)	Hyperplasia of primary epithelial cells (high) (%)
Control	0	16	23	3	0
3 h	14	71	31	30	69
6 h	65	63	42	22	69
9 h	88	88	27	9.27	90
9 h+N	23	58	24	8.30	91

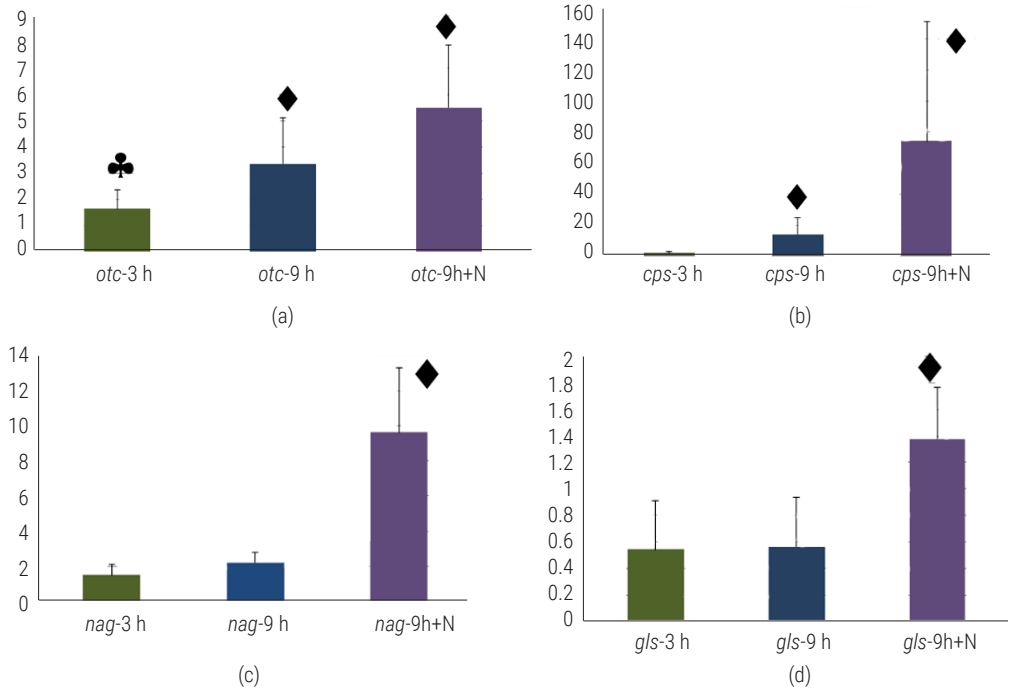

Fig.13. Expression profiles of genes of OUC pathway in *C. magur* after 3 and 9 h and 9h+N after ammonia exposure, as compared to 0 h. (a) Ornithine carbamoyltransferase, *otc;* (b) Carbamoyl-phosphate synthase, cps*III;* (c) N-acetylglutamate synthases, *nag* and (d) Glutamine synthetase, *gls* □p≤0.05¨p≤0.01

fused secondary lamellae. Most deformed secondary lamellae (42%) were observed in fishes exposed for 6 h and most fused secondary lamellae were also observed in the same group of animals. It was observed that the percentage deformity in control and after 9h+N was almost similar. The thickness of secondary lamellae gradually started increasing from 6 h exposure which was observed at both 9 h and 9h+N. Furthermore, hyperplasia was observed after 3 and 6 h of exposure in 69% of primary epithelial cells and maximum was observed after 9 h (90%) and 9h+N (91%).

The histopathological changes in the liver and kidney of *C. magur* exposed to 25 mM ammonium chloride were examined after 3, 6, 9 and 9h+N (Fig. 15). Changes in liver were not prominent when control samples were compared to that of treatments. Nuclear hypertrophies in a few cells were observed, which signified the increase in the overall size of the hepatocytes. The level of nuclear hypertrophy showed a slight increase during 9 h of exposure than during 3 h and 6 h. The histological changes occurred in liver as a result of exposure with ammonium chloride were more notable at 9 h of exposure, in which dilation of sinusoids were observed along with necrosis of a few hepatic cells, diffused vacuolation and nuclear hypertrophy. Liver of 9 h+N also did not show any significant changes, when compared with that of control. Similarly, kidney of control group clearly showed proximal tubules, glomerulus and bowman's space. There were not much changes in at 3 h exposure time, except for the diffusion of blood cells in some areas. The

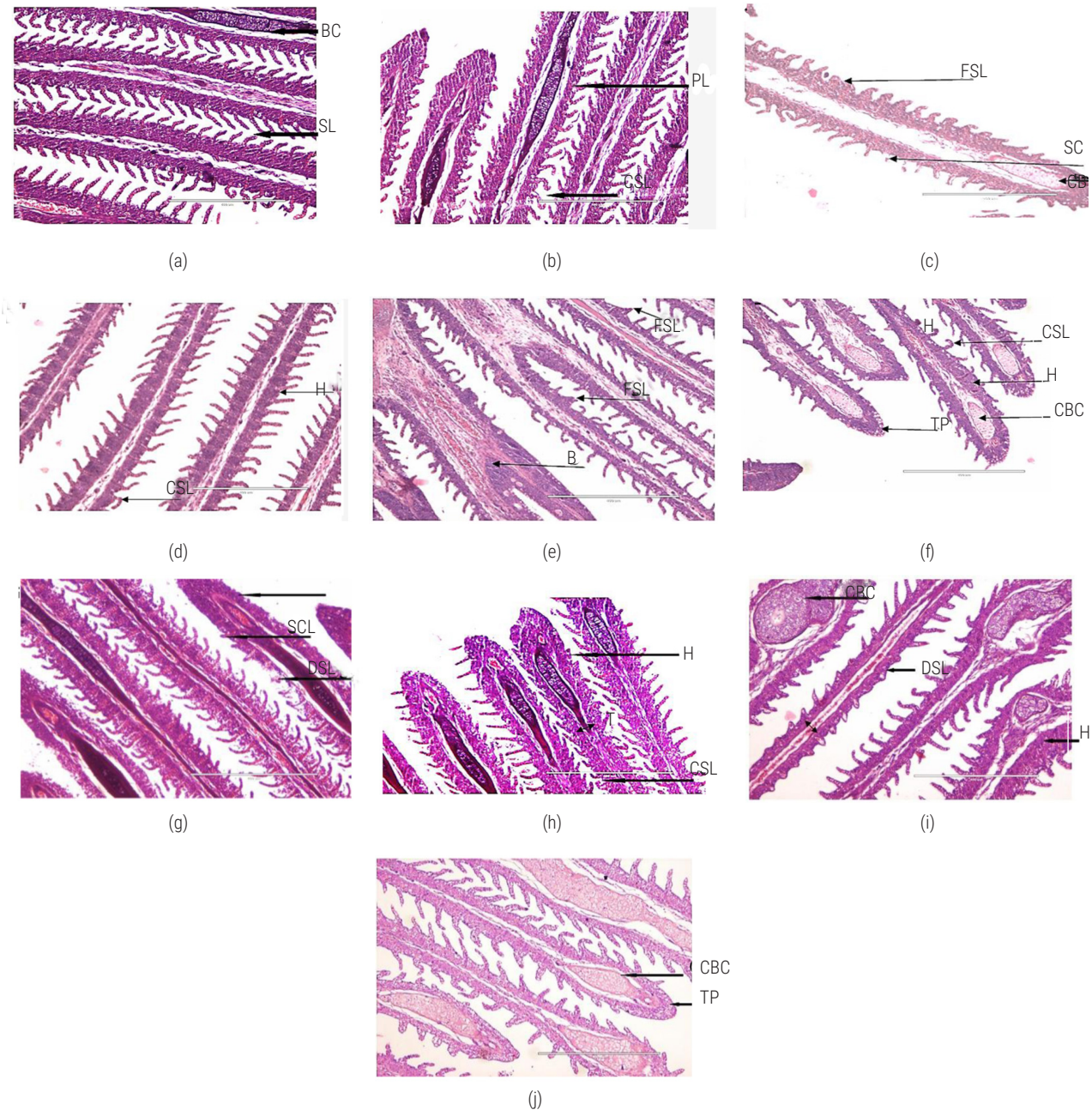

Fig. 14. Histopathological changes in the gills of *C. magur* exposed to 25 mM ammonium chloride for different duration. Control (a and b), 3 h (c and d), 6 h (e and f), 9 h (g and h) and 9 h+N (i and j).

BC: Blood capillaries, SL: Secondary lamellae; PL: Primary lamellae, CSL: Curled secondary lamellae; FSL: Fused secondary lamellae, SC: Shortening of secondary lamellae, CBC: Congested blood capillaries' H: Hyperplasia of primary epithelial cells; B: Branching of the primary lamellae; CBC: Congested blood capillaries, Tp: Tips without secondary lamellae; SCL: Shortened secondary lamellae, DSL: Disintegrated secondary lamellae; T: Decreased thickness of primary lamellae; DSL: Deformed secondary lamellae; TP: Tips of primary lamellae are with more secondary lamellae, when compared to the normal.

6 h treatment group showed karyolysis of nuclear material to some extent. The changes in the kidney were more prominent in 9 h exposure group, showing distension of Bowman's capsule and degeneration of Bowman's capsule along with karyolysis of nucleic material. It has been observed that liver and kidney tissues of *C. magur* showed prominent changes when exposed to 25 mM of ammonium chloride for at least 9 h.

Discussion

The physiological status of fishes is directly reflected by changes in biochemical and physiological level of fish blood on exposure to toxic substances in the aquatic environment (Kim and Kang, 2015; Shin *et al.,* 2016). Ammonia is one of the important environmental

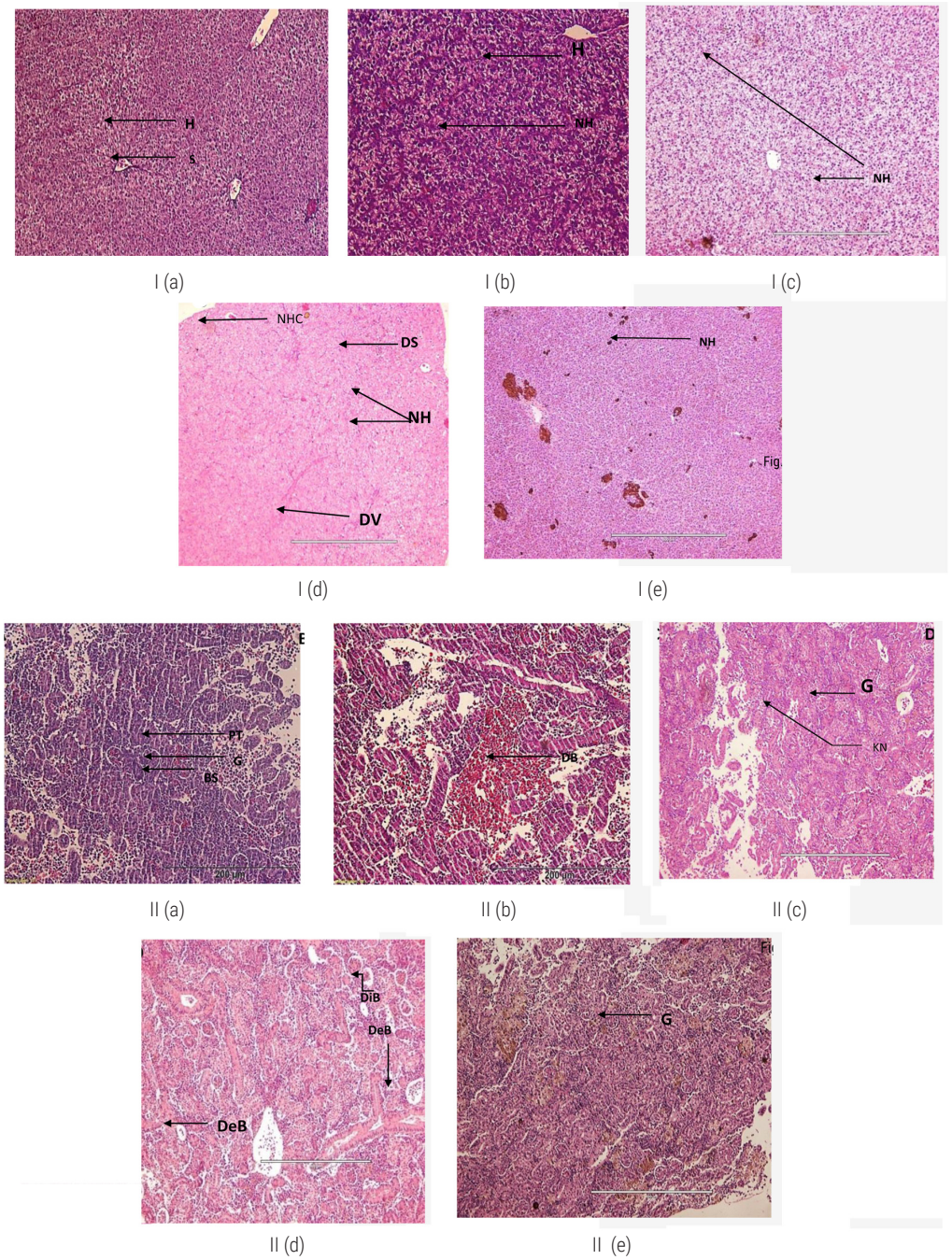

Fig.15. Histopathological changes of *C. magur* exposed to 25 mM ammonium chloride for different durations. (I). Liver : (a) control, (b) 3 h, (c) 6 h, (d) 9 h, (e) 9 h+N, H-Hepatocytes, S-Sinusoids, NH-Nuclear hypertrophy, DS-Dilatation of sinusoids, NHC-Necrosis of hepatic cells, DV-Diffused vacuolation. (II). Kidney: (a) Control, (b) 3 h, (c) 6 h, (d) 9 h+N, PT: Proximal tubules; G: Glomerulus; BS: Bowman space; DB: Diffused blood, KN: Karyolitic of nucleic material; DiB: Distension of Bowman's capsule; DeB: Degeneration of Bowman's capsule

limiting factors for fishes, with notable effect on growth, ionic balance, immunity, histopathology, energy metabolism and hormone regulation (Foss *et al.,* 2003). Several species of fish have adapted to unique environmental circumstances that preclude this route of ammonia detoxification; by expressing high levels of the ornithine-urea cycle (OUC) enzymes (Anderson, 2001) and in *C. batrachus* (Saha and Ratha, 2007), OUC has been reported to be induced after seven to fourteen days of high external ammonia. In the present study, up-regulation of two of the five main genes coding for enzymes, *cpsIII* and *otc* significantly at 9 h and 9h+N, and decrease in level of ammonia and increase in urea at six hours of exposure, indicated activation of ornithine urea cycle in liver around six hours of exposure. The enzymes, *cps III* (form in fishes) is involved in arginine-biosynthesis reactions, while *otc* is one of the first few in the urea cycle (Saha and Ratha, 2007). This indicated the presence of unique physiological characteristics of turning towards

ureotelism from ammoniotelism which recovered with improvement in environmental conditions, after short term exposure.

The evaluation of eleven blood physiological parameters of *C. magur*, where samples were exposed for five different time intervals and compared with control created design of experiment layout (D-E-L) such that observations on eleven blood physiological parameters over eighteen fish samples would be dependent, non-normal, non-equality of variances and each sample may have different distribution pattern. Prior to the analysis of variance, the test of homogeneity of variance (*e.g.,* Bartlett's test) was used to assess the homogeneity of within-group variances. Therefore, analysis of variance for this data matrix (rows as specimens, column as parameters) would not be possible through Classical ANOVA and multiple group comparisons by a method which assumes equality of variances. Therefore, Welch's analysis of variance (ANOVA) and Games-Howell *post hoc* test for multiple group comparisons was applied for analysis. The Games-Howell *post hoc* test, like Welch's analysis of variance (Jan and Shieh, 2014) does not require the groups to have equal variances. The application of Welch's ANOVA with Games-Howell *post hoc* test (in place of Classical ANOVA along with any *post hoc* test which assumes equality of variances) ensures selection of appropriate technique for analysis, higher accuracy of results and unbiased pair-group comparisons (Ruxton and Beauchamp, 2008).

The haematological parameters *i.e.*, haemoglobin and haematocrit values, are used to assess the physiological and health status of fish or used as stress indicators (Nussey *et al.,* 1995). Discriminant functions (function 1 vs. 2) scores over groups in the present study clearly indicated that specimens in control and 9h+N were almost similar in terms of blood parameters. This may point out to the recovery of the fish (discussed below), even after nine hours of ammonia exposure, if the water conditions are restored. Zhang *et al*. (2019) suggested the decrease in RBC and hemoglobin levels in fish may be due to be inhibition of haemoglobin synthesis. However, in the present study, no significant change in serum glucose and protein levels was noticed, which indicate that the short term exposure to the level of ammonia concentration tested may not be adequate to cause significant stress to the fish, or may be linked with the activation of ornithine urea cycle in liver at 6 h of exposure, for conversion of ammonia to non-toxic urea-N for secretion (Grosell *et al.,* 2010) and can be correlated to rapid increase of blood urea and reduction of blood ammonia. However, sudden reduction of serum protein in our findings may be stress-induced deleterious effect on protein synthesis causing its depletion in the serum (Kumar *et al.,* 2017), which recovered in later after exposure period, due to induction of IOU cycle.

Serum creatinine, SGOT and SGPT are sensitive indicators of kidney and liver damage, respectively (Agrahari *et al.,* 2007). However, except for one-hour exposure, the levels of SGOT and SGPT indicated that liver functions were not affected by the short-term ammonia exposures. Elevated SGPT level after one-hour exposure, may indicate temporary impaired liver function.

Glucose is one of the most important sources of energy and essential substrate for cell metabolism in fishes and used as a complement of stress tests (Manush *et al.*, 2005). Stress may increase the level of adrenaline and nor-adrenaline and activate the secretion of catecholamine. Further, catecholamine converts liver glycogen to blood glucose to fulfil the energy demands (Shin *et al*., 2016; Kumar *et al.,* 2017). However, in the present study, non-significant increase in the level of blood sugar found at 9 h exposures, points to the stress caused by toxicity of ammonia and once the urea cycle starts, the toxicity level gradually decreases, which leads to the reduction of glucose in serum. Begum (1987) reported decrease in sugar level along with increase in levels of phosphorylase and aldolase activities, due to increase in oxidation of glucose through glycolytic pathway, to provide energy for fish under ammonia stress. Increases in blood glucose levels on exposure to external ammonia have been reported in wide range of species *i.e.,* catfish, carps and cichlids (Acharya *et al.,* 2005; Shin *et al.,* 2016). In addition, sudden induction of oxidative stress by high ammonia exposure (Zhao *et al*., 2020) caused corresponding increase in LDH concentrations at 1 h exposure, which would result in increased lactate accumulation and lactate dehydrogenase activity (Tripathi *et al.,* 2013).

Exposure to high environmental ammonia can also cause histological changes in different organs *viz.,* gills, liver and kidney. In fish, the gill tissue is considered as the major and primary site of toxic impact from any toxicants (Zhao *et al.,* 2020). In our study, the damages due to high ammonia exposure varied with the tissues. More severe damaging effects were observed in gill structure, as compared to kidney and liver. This is most obvious as the gills are in direct contact with the high toxic environment and are main receptors for the toxic substances. Similar results were also reported by Zhao *et al.* (2020) in largemouth bass. It was interesting to note that when high ammonia was withdrawn, the process of recovery of the structural damages could be observed in gills. The slow recovery in gills in a carp fish after copper exposure was also observed by Velcheva *et al.* (2013). However, Velmurugan *et. al.* (2009) reported the period for the gills to recover is dependant upon the concentration of pesticide the fish is exposed to. As the gills are in continuous contact with the external environment and have important functions to perform, the extent and mechanisms leading to recovery of gill structures after the repeated exposures to high ammonia content would be valuable in a culture system.

The current study provided valuable information, for understanding the initial physiological responses of air-breathing catfish, *C. magur* and histological changes in response to acute ammonia toxicity. This study was able to elucidate the underlying adaptation mechanisms during stressful environment of short term high ammonia exposure. Study of blood physiological parameters indicated induction of oxidative stress and corresponding changes in blood parameters. The findings also indicated, turning towards ureotelism from ammonotelismin, which helps this fish to overcome the ammonia toxicity. This involved activation of OUC cycle to convert ammonia to less toxic urea-N for secretion around six hours of exposures. The findings of this research points towards the importance of maintaining a healthy aquatic environment, which is crucial for growing healthy *C. magur*, for achieving satisfactory production and improving culture efficiency.

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