Iranian Journal of Fisheries Sciences 21(6) 1343-1366 2022
DOI: 10.22092/ijfs.2022.128167

Research Article

Alterations in Hematological indices, histopathology and p450 gene expression in stellate sturgeon (*Acipenser stellatus* Pallas, 1811) fingerlings exposed to different salinities levels and ammonia

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Received: December 2021 Accepted: August 2022

Abstract

This study was conducted in two stages. At the first stage, the lethal concentrations (LC50 96h) of ammonia in different salinities including, 0ppt (7.42 mgL^{-1}), 4ppt (8.24 mgL^{-1}), 8ppt (9.60 mgL^{-1}) and 12 ppt (10.22 mgL^{-1}) to *A. stellatus* fingerlings were determined. At the second stage, 240 fish (15.23±2.17 g, mean weight and 17±1.96 cm, total length) were exposed to half (50%) median lethal concentration (LC50 96h) under the same salinities for four days (8 treatments in triplicates). Maximum Red blood cells, white blood cell, hematocrit, hemoglobin, mean corpuscular volume ean corpuscular hemoglobin, mean corpuscular hemoglobin concentration , an differentiation of leucocytes indices were observed in combined treatments (salinity with ammonia) with salinity and ammonia ($p\leq0.05$). Cortisol, glucose, and lactate decreased, meanwhile immunoglobulin M, lysozyme and total immunoglobulin blood serum levels significantly increased with increasing salinity and ammonia in combination treatments ($p\leq0.05$). The activity of superoxide dismutase and glutathione peroxidase blood serum increased in combined salinity and ammonia treatments but catalase decreased significantly ($p\leq0.05$). Alanine aminotransferase and aspartate aminotransferase enzyme levels increased in combined salinity and ammonia treatments ($p\leq0.05$). But a significant decrease in the levels of alkaline phosphatase and lactate dehydrogenase blood serum enzymes was observed ($p\leq0.05$). The highest gill and liver damage was observed in salinity of 12 ppt and also combined treatment of salinity of 12 ppt with ammonia. The highest hepatic P450 gene expression was observed in the last treatment ($p\leq0.05$). As a result, increasing salinity increasing tolerance, reduced stress and increasing immunity in stellate sturgeon fingerlings at higher concentrations of ammonia. But salinity could not reduce the toxic effects of ammonia on other indicators.

Keywords: *Acipenser stellatus*, Ammonia, Salinity, Hematological indices, Histopathological, Gene expression p450

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[DOR: 20.1001.1.15622916.2022.21.6.1.7]

Introduction

The starry sturgeon (*A. stellatus*) is one of the most valuable commercial fish of the Caspian Sea, which migrates to the rivers of the southern basin this lake, including Sefirod River to spawn. The artificial breeding of this species is pivotal to restore the populations and aquaculture purposes. Ammonia is one of the most important common toxic compounds in aquaculture systems. Fish excrete ammonia into the culture water as a result of amino acid catabolism. Ammonia is also produced by the decomposition of organics such as non-consumed feed and faeces in the aquaculture systems . A high level of ambient ammonia has a series of toxicological effects in fish: it changes their energy metabolism, decreases growth , disrupts ion balance , increases sensitivity to disease, and even causes mortality (Kir *et al*., 2019) ammonia can concentrate aquaculture systems especially in high stocking density production ones and can reduce water-quality (Frias-Espericueta *et al.,* 1999). High ammonia concentration in aquaculture systems may increase to the sub-lethal levels that reduce fish growth and compromise fish welfare or reach to lethal doses. The toxic effect of ammonia on aquatic animals is ascribed to the unionized form of total ammonia nitrogen (TAN) (NH_3-N). The ionized form (NH_4 +-N) is considered non-toxic because it cannot pass through cell membranes. The NH_3-N penetrates cell membrane and leads to cell malfunction and mortality in fish (Jeney *et al*., 1992; Kir and Sunar, 2018). The toxicity of NH_3 depends on water pH, temperature, and salinity. Salinity is one of the most important environmental variables that can affect the toxicity of NH_3 (Kir and Sunar, 2018). There are many reports that the effects of ammonia in saltwater and freshwater can affect toxicity (Barbieri, 2012; Kir and Sunar, 2018; Kir *et al*., 2019), hematological and biochemical indices (Bonnie and Liu, 2004; Das *et al*., 2004; Shin *et al*., 2016; Zeitoun *et al*., 2016), histopathological indices (Benli *et al*., 2008; Rodrigues *et al*., 2014; Senthamilselvan *et al*., 2014; Mazandarani *et al*., 2015; Termeh Yousefi *et al*., 2018), and gene expression (Gunal *et al*., 2008; Sinha *et al*., 2012; Hangzo *et al*., 2017; Li *et al*., 2020; Puntila-Dodd *et al*, 2021; Zarantoniello *et al*., 2021) of fish species. Therefore, any change in the above-mentioned indices can be a benchmark for the presence of contaminants in an aquatic environment (Oliveira Ribeiro *et al*., 2002). This study was aimed to determine the effects of ammonia toxicity at different salinities and investigate the combined effects of salinity and ammonia on physiological responses, liver enzymes, P450 gene expression, and the gill and liver tissues of the stellate sturgeon fingerlings.

Materials and methods

This study was conducted at the International Sturgeon Research Institute in Chaboksar, Guilan, Iran. Prior to the experimental procedures, the stellate sturgeon fingerlings were acclimated to the experimental salinities

[DOR: 20.1001.1.15622916.2022.21.6.1.7]

0 ppt (well water), 4 ppt (A combination of Caspian Sea water and well water), 8 ppt (A combination of Caspian Sea water and well water) and 12 (Caspian Sea water) ppt for four weeks. The experiment was conducted in two steps:

Step 1: Determining median lethal concentration (LC50 96h) of ammonia at different salinities:

In this experiment, 480 stellate sturgeon (12.8±1.19cm total length and 14.56±1.84g mean weight) were used as the sample. The acute toxicity assay was conducted to determine the median lethal concentration (LC50 96h) of ammonia under semi-static renewal test conditions, as recommended by the Organization for Economic Cooperation and Development. Ammonium chloride (NH_4Cl) (purity: ≥98%, Merck, Darmstadt, Germany) was used to prepare different concentrations of ammonia. Groups of 10 stellate sturgeon (*A. stellatus*) fingerlings were randomly exposed to ammonia (3, 20, and 50 mgL^{-1}) (Banihashemi *et al*., 2014) at different salinities (0, 4, 8, and 12 ppt) (Bahmani and Yousefi Jourdehi, 2011) in separate 40 L plastic tanks (Table 1) for 96 h. During this 4-day trial, the water quality indices were set as follows: temperature, 21±0 °C; dissolved oxygen, 7.42±0.18 mgL^{-1}, and pH 8.04±0.21. Th e fish were not fed during this period to avoid the adverse effects of waste and feed. The stellate sturgeon fingerlings were not fed during this period. The mortality rate was calculated at 24, 48, 72, and 96 h after exposure to ammonia. Based on the Probit analysis test (Finney, 1971), the LC_{50} of ammonia at salinities 0, 4, 8, and 12 ppt was obtained. To minimize ammonia loss, 75% of the water was renewed daily then the ammonium chloride was added to the stock solution.

Table 1: LC_{50} of ammonia to stellate sturgeon fingerlings at different salinities.

Number	Treatments	Number	Treatments
1	Salinity 0 (ppt) + non-ammonia (control)	9	Salinity 0 (ppt) + 20 (mgL^{-1}) ammonia
2	Salinity 4 (ppt) + non-ammonia (control)	10	Salinity 4 (ppt) + 20 (mgL^{-1}) ammonia
3	Salinity 8 (ppt) + non-ammonia (control)	11	Salinity 8 (ppt) + 20 (mgL^{-1}) ammonia
4	Salinity 12 (ppt) + non-ammonia (control)	12	Salinity 12 (ppt) + 20 (mgL^{-1}) ammonia
5	Salinity 0 (ppt) + 3 (mgL^{-1}) ammonia	13	Salinity 0 (ppt) + 50 (mgL^{-1}) ammonia
6	Salinity 4 (ppt) + 3 (mgL^{-1}) ammonia	14	Salinity 4 (ppt) + 50 (mgL^{-1}) ammonia
7	Salinity 8 (ppt) + 3 (mgL^{-1}) ammonia	15	Salinity 8 (ppt) + 50 (mgL^{-1}) ammonia
8	Salinity 12 (ppt) + 3 (mgL^{-1}) ammonia	16	Salinity 12 (ppt) + 50 (mgL^{-1}) ammonia

Step 2: Testing effects of half (50%) median lethal concentration (LC50 96h) of ammonia at different salinities

In this step, 240 stellate sturgeon (*A. stellatus*) fingerlings (15.23±2.17g and 17.0±1.95 cm) were randomly assigned to 24 Plastic tanks of 40 L (10 fish per tank) to establish eight treatments with triplicates. To minimize ammonia loss, 75% of the water was renewed daily by adding the ammonium chloride stock solution (Table 2).

[DOR: 20.1001.1.15622916.2022.21.6.1.7]

Table 2: Half (50%) of ammonia LC50 96h at different salinities

Number	Control (non-ammonia)	Number	Exposed (half (50%) LC50 96h of ammonia)
1	Salinity 0 (ppt)	5	Salinity 0 (ppt) + 3.71 mgL^{-1}
2	Salinity 4 (ppt)	6	Salinity 4 (ppt) + 4.12 mgL^{-1}
3	Salinity 8 (ppt)	7	Salinity 8 (ppt) + 4.80 mgL^{-1}
4	Salinity 12 (ppt)	8	Salinity 12 (ppt) + 5.11 mgL^{-1}

During this 4-day trial, the water quality indices were set as follows: temperature, 16.5±0.51 °C; dissolved oxygen, 7.0±0.11 mgL^{-1}, and pH 8±0.12. After 24 h of starving, the stellate sturgeon (*A. stellatus*) fingerlings were anesthetized with a mixture of 0.5g of clove powder in one liter of water (Rahbar *et al*., 2019). In each treatment, 7-8 fish were caught and after anesthetization, blood sampling was done through the caudal vein. The blood samples collected from the fish in each treatment were pooled, half in a heparinized vial and the rest in a non-heparinized tube and immediately transferred to the laboratory. Plasma was extracted from blood samples by centrifugation (3000 rpm, 4°C, 10 min) and stored at -80°C for further biochemical analysis (Adel *et al*., 2015). Also, one of the stellate sturgeon (*A. stellatus*) fingerlings from each replicate were randomly selected for histopathological, and gene expression examinations.

Water physicochemical characteristics

Water physicochemical characteristics, including pH, oxygen, and temperature, were measured using a HACH multi-parameter device (made in Germany), and salinity was measured using a refractometer (Atago S. mill – E ophthalmometer model, made in Japan).

Hematological indices

Red blood cells (RBC), white blood cell (WBC), hematocrit (Hct), hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and differentiation of leucocytes were evaluated based on standard methods (Kazemi *et al.,* 2010).

Biochemical analysis of plasma

Lysozyme activity level was measured by turbidity method using Micrococcus lysodeikticussuspension and Moramydaz enzyme. The turbidity was measured at a wavelength of 450nm. IgM levels in plasma were measured using Fish Immunoglobulin M (IgM) ELISA kit and Elisa Reader (Rs232, BIOTEK, USA). Total IgM concentration , Immunoglobulin M (IgM), and lysozyme were measured using the methods described by Siwicki and Anderson (1993), and Ellis (1990), respectively. Cortisol and glucose levels were determined using enzyme immunoassays (Kit Monobind, Lake Forest, CA; Tintos *et al*., 2006) and colorimetric methods (Kit Pars Azmon, Iran; Bayunova *et al*., 2002). Lactate was measured with a commercial kit (Lactate LS, Ziestchim Diagnostics, Tehran, Iran). The levels of alanine aminotransferase (ALT), aspartate

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aminotransferase (AST), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) were determined using commercial kits (Pars Azmoon Company, Tehran, Iran) and a biochemical auto-analyzer (prestige 24i, Boeki, Japan) (Strik *et al*., 2007). The activities of superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT) were measured using methods described by Yousefi (2018).

Histopathological studies

The stellate sturgeon (*A. stellatus*) fingerlings gills and liver were dissected and fixed in Bouin's solution (48 h) for histological evaluation (Haschek *et al*., 2010). Tissue samples were dehydrated in a series of alcoholic grades before being embedded in paraffin and sectioned into pieces with a thickness of 5 µm. After staining the samples with hematoxylin and eosin method, they were analyzed using a light microscope equipped with a camera (BEL photonic BIO2T, Monza, Italy). In addition, the histopathological changes in tissues were graded using a histological grading system as follows: no tissue damage (-), less than 25% (+), less than 75% (++), and more than 75% (+++).

Samples collection and gene analysis

At the end of experimental trial, fish were anesthetized using clove powder and killed before sampling. The liver tissues were then collected using sterile scissors and a scalpel blade and was transferred to separate tubes and immediately placed in a nitrogen tank. At the end of sampling, the tubes containing the sample were stored in the −80°C until RNA was extracted. BIOZOL RNA extraction kit (Bioflux-Bioer, China) was used for total RNA was extraction. Agarose gel electrophoresis and spectrophotometric analysis were used to determine RNA quality and quantity. The complementary DNA (cDNA) was synthesized using Super Script III reverse transcriptase (Invitrogen) with an oligo dT18 primer. Real-time PCR was performed with an iCycler (BioRad, USA) using SYBR Green qPCR master mix (Fermentase, France) as follows: 3 min at 95°C and then 45 cycles of 20 s at 95°C, 20 s at 60°C and 20 s at 72°C. The reaction was carried out with triplicates for each sample. Table 3 represents the primer used in this study. Betaactin was used as a reference gene to normalize the expression of the target genes. After verification of primers efficiencies, gene expression was determined using 2^{-DDCT} method (Livak and Schmittgen, 2001).

Statistical analysis

LC_{50} for 24, 48, 72 and 96 h were estimated following probit analysis (Finney, 1971). The percentage mortality was calculated and transformed into probit value using the probit table. The Pfaffl formula was used to calculate relative gene expression (Pfaffl *et al*., 2002).

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Table 3: Name, sequence, melting temperature (Tm), and product length of primers used in this study to quantify P450 1A transcript of the stellate sturgeon (*A. stellatus*) fingerlings through Real-time PCR.

Primer name	Primer sequence	Tm	Product length	Primer performance
Ap p450q-PCRF	GTCATCTGTGCCATGTGCTT	56	237	99%
Ap p450q-PCRR	TCTTGTCGAAGGAGCGGTAG	56		
β- actin q-PCRF	TTGCCATCCAGGCTGTGCT	56	215	
β- actin q-PCRR	TCTCGGCTGTGGTGAA	56		

All data statistical analysis and plotting were conducted using SPSS 22.0 (SPSS-22.0, IBM software Inc, Chicago, IL) and EXCEL 2013 . The experiment was designed for analysis by using repeated measures analysis of variance (two-way ANOVA) to determine the effects of ammonia and salinity exposure on treatments. The two-way ANOVA first assessed interaction between ammonia and salinity on responses. As interaction occurred, one-way ANOVA followed by Tukey's a significance level of $p<0.05$ was used to determine treatment responses. All data were reported as mean±SD.

Histopathological symptoms in gill and liver were determined qualitatively with 4 different categories: no tissue damage (-), less than 25% (+), less than 75% (++), and more than 75% (+++).

Results

LC_{50} of ammonia ($NH_4^+CL^-$)

The LC_{50} at various salinities was determined after 24, 48, 72, and 96 h (Table 4). The LC_{50} values at each salinity decreased over time (from 24 to 96 h) depending on the exposure time. The results indicated that the median lethal concentration increased from 7.42 mgL^{-1} at salinity of 0 ppt to 10.22 mgL^{-1} at salinity of 12 ppt after 96 h.

Table 4: Median lethal concentrations of ammonia to the sturgeon (*A. stellatus*) fingerlings.

		Concentration (mgL^{-1}) with 95% confidence limits				half (50%) of median lethal concentration (LC50 96h) of ammonia at different salinities (mgL^{-1})
		Time				
		24 h	48 h	72 h	96 h	
Salinity (ppt)	0	19.13	18.31	17.09	7.42	3.71
	4	33.66	30.85	10.23	8.24	4.12
	8	25.00	25.05	25.05	9.60	4.80
	12	95.83	95.83	30.85	10.22	5.11

Hematological indices

According to the two-way analysis of variance, it was found that salinity and ammonia simultaneously have an effect on WBC, RBC, Hct, Hb, MCH, differentiation of leucocytes ,Cortisol, glucose, lactate, immunoglobulin M, lysozyme, total immunoglobulin, superoxide dismutase ,glutathione peroxidase, catalase, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase ,

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and lactate dehydrogenase (p<0.05). In other words, there is an interaction effect between the two factors of salinity and ammonia on the hematological indices. therefore , the treatments were merged with each other and using one-way analysis of variance and Tukey's test it was checked . he results showed that WBC, RBC, Hct, Hb, MCV, MCH, neutrophils, and monocytes significantly increased with the increase in ammonia concentration at different salinities (p<0.05). The MCH levels significantly increased in treatment 6 (salinity: 4 ppt + ammonia: 4.12 mgL^{-1}, 50% of LC50 96h) (p<0.05). Eosinophils were also observed in stellate sturgeon (A. stellatus) fingerlings of both the test group (exposed to ammonia) and the control group (not exposed to ammonia) at a salinity of 8 ppt (p<0.05). There was no significant difference between treatments in MCHC and Lymphocytes (p>0.05) (Tables 5 and 6).

Table 5: Effects salinity and ammonia on hematological indices of the stellate sturgeon (*A. stellatus*) fingerlings.

Treatments	RBC (mm^{-3})	Hb ($g\ dL^{-1}$)	Hct (%)	MCH (pg)	MCHC ($g\ dL^{-1}$)	MCV (fl)	WBC (mm^{-3})
1	582333.3±3055.1 ^{cd}	4.30±0.20^{a}	25 ± 1 ^{ab}	75.33 ± 2.08 ^{ab}	19.33 ± 2.08^{a}	427.67 ± 7.51 ^{ab}	4233.33 ± 208.2 ^{ab}
2	569333.3±14011.9 ^{bcd}	4.47±0.25^{ab}	25.67 ± 2.08 ^{ab}	77.63 ± 2.31^{ab}	18.03 ± 1.48 ^{a}	427 ± 6.56 ^{ab}	4566.67 ±152.7 ^{bc}
3	597000±14106.7^{d}	4.4± 0.10 ^{ab}	25 ± 1^{ab}	73.80 ± 2.60^{a}	17.37 ± 1.07 ^{a}	419.67 ± 5.03 ^{a}	5100 ± 264.6 ^{cd}
4	515666.7±9712.5 ^{abc}	4.03 ±0.35 ^{a}	21± 1.73 a	78.50 ± 3.10 ^{ab}	18.27 ± 0.94 ^{a}	424 ± 3.61 ^{ab}	4800 ± 300 ^{bcd}
5	57100±793.2^{bcd}	4.4 ± 0.25 ^{ab}	25 ± 2.65 ^{ab}	79 ±3.61 ^{ab}	18.43 ± 0.90^{a}	434 ±7.21^{ab}	3566.7 ± 251.7^{a}
6	50933.3±8144.5^{ab}	4.4 ± 0.25 ^{ab}	22.33 ± 2.52 ^{a}	83.57 ± 3.06^{b}	19.23 ± 0.76^{a}	431.67 ± 4.62^{ab}	5133.3 ± 208.2 cd
7	497000±10816.6^{a}	4.43 ± 0.31^{ab}	21.67 ± 2.08 ^{a}	83.47 ± 3.40 ^{b}	19.47 ± 1.60 ^{a}	425.67 ± 4.16^{ab}	6200 ± 360.6 ^{e}
8	599333.3±65271.2 e	5.03 ±0.31 ^{c}	29.33 ± 2.08 ^{b}	77.33 ± 3.51^{ab}	17.40 ± 1.35^{a}	439 ± 4.58 ^{b}	54667 ± 450.9^{de}

Data are presented as mean±SD. Uncommon Latin letters indicate a significant difference between treatments (p<0.05).

Table 6: Effects salinity and ammonia on differentiated leukocytes count of the stellate sturgeon (*A. stellatus*) fingerlings.

Treatments	Neutrophil (%)	Lymphocyte (%)	Monocyte (%)	Eosinophil (%)
1	13.00 ± 1.00 ^{ab}	83.00 ± 3.61^{a}	4.00± 0.58 ^{ab}	0.00 ± 0.00 ^{a}
2	14.33 ± 1.53^{b}	79.00± 2.08 ^{a}	5.00 ± 1.00 ^{ab}	0.00 ± 0.00 ^{a}
3	14.67 ± 1.53^{b}	78.67 ± 3.51 ^{a}	5.67 ± 0.58 ^{ab}	1.00 ± 0.00 ^{b}
4	12.00 ± 1.00 ^{ab}	80.67 ± 1.53^{a}	4.67 ± 0.58 ^{ab}	0.00 ± 0.00 ^{a}
5	10.67 ± 0.58 ^{a}	83.67 ± 3.51 ^{a}	4.00 ± 1.00 ^{a}	0.00 ± 0.00 ^{a}
6	14.67 ± 1.53^{b}	79.00± 3.51 ^{a}	6.33 ± 0.58 ^{ab}	0.00 ± 0.00 ^{a}
7	14.67 ± 1.15^{b}	78.00 ± 3.00 ^{a}	6.67 ± 1.16 ^{b}	1.00 ± 0.00 ^{b}
8	14.00 ± 1.00 ^{ab}	81.00 ± 3.51 ^{a}	5.00 ± 1.00 ^{ab}	0.00 ± 0.00 ^{a}

Uncommon Latin letters indicate a significant difference between treatments (p<0.05).

Cortisol, glucose and lactate showed a significant decrease in combination treatments with increasing salinity and ammonia (p<0.05), except for salinities 8 and 12 ppt (Table 7). IgM, total immunoglobulin, and lysozyme significantly increased with the increase in ammonia concentration.

[DOR: 20.1001.1.15622916.2022.21.6.1.7]

Table 7: Effects salinity and ammonia on blood serum stress indices of the stellate sturgeon (*A. stellatus*) fingerlings.

Treatments	Cortisol (ng mL^{-1})	Glucose (mg dL^{-1})	Lactate (mg dL^{-1})	Cortisol (ng mL^{-1})
1	28.00 ± 2.00 ^{cd}	55.00 ± 3.61 ^{bc}	23.73 ± 1.56 ^{bcd}	28.00 ± 2.00 ^{cd}
2	30.00 ± 2.65 ^{cd}	52.00 ± 2.65 ^{abc}	20.17 ± 1.72^{ab}	30.00 ± 2.65 ^{cd}
3	31.67 ± 1.53 ^{de}	50.67 ± 4.04 ^{abc}	27.97 ± 1.11^{e}	31.67 ± 1.53 ^{de}
4	36.33 ± 2.08 ^{e}	59.67 ± 5.03 ^{c}	27.23 ± 1.12^{de}	36.33 ± 2.08 ^{e}
5	24.33 ± 2.52^{bc}	54.33 ± 2.08 ^{abc}	25.50 ± 1.50 ^{cde}	24.33 ± 2.52^{bc}
6	36.67 ± 2.52 ^{e}	52.33 ± 2.52 ^{abc}	22.97 ± 0.96^{abc}	36.67 ± 2.52 ^{e}
7	21.33 ± 2.08 ^{ab}	45.67 ± 1.15 ^{a}	19.60 ± 1.30^{a}	21.33 ± 2.08 ^{ab}
8	18.00 ± 2.00 ^{a}	46.33 ± 3.06 ^{ab}	20.97 ± 1.30 ^{ab}	18.00 ± 2.00 ^{a}

Moreover, the highest IgM and total immunoglobulin were observed in treatment 8 (salinity 12 ppt+5.11 mgL^{-1}) and the highest lysozyme was related to treatment 7 (salinity 8 ppt+4.80 mgL^{-1}) (p<0.05) (Table 8). SOD, Catalase, and GPX significantly increased with the increase ammonia and salinity(from treatment 5 to 8).. The highest levels of SOD and GPX were observed in treatment 8 (salinity 12 ppt+5.11 mgL^{-1}) and the highest catalase level was related to treatment 6 (salinity 4 ppt+4.12 mgL^{-1}) (p<0.05) (Table 9). The results demonstrated that ALT and AST of serum significantly increased with the increase ammonia and salinity(from treatment 5 to 8) , whereas LDH and ALP significantly decreased (p<0.05) (Table 10).

Table 8: Effects salinity and ammonia on blood serum immunological indices of the stellate sturgeon (*A. stellatus*) fingerlings.

Treatments	IgM (mg dL^{-1})	Lysozyme Activity (U/mL/min^{-1})	Total Immunoglobulin (mg mL^{-1})
1	47.67 ± 2.52 ^{ab}	29.00 ± 2.00^{ab}	15.53 ± 0.83^{b}
2	45.00 ± 3.61 ^{ab}	32.33 ± 2.08 ^{bcd}	12.07 ± 0.78 ^{a}
3	43.33 ± 3.06 ^{ab}	29.33 ± 0.58 ^{ab}	12.33 ± 0.76^{a}
4	41.67 ± 3.21 ^{a}	24.67 ± 1.53 ^{a}	10.67 ± 0.42^{a}
5	43.67 ± 1.15 ^{ab}	29.67 ± 2.52 ^{bc}	11.23 ± 0.71^{a}
6	41.00 ± 1.00 ^{a}	30.67 ± 0.58 ^{bc}	12.37 ± 0.35^{a}
7	50.67 ± 3.51^{bc}	37.00 ± 1.73 ^{d}	15.60 ± 0.52^{b}
8	55.33 ± 2.08^{c}	34.33 ± 1.53 ^{cd}	16.63 ± 1.22 ^{b}

Uncommon Latin letters indicate a significant difference between treatments (p<0.05).

Table 9: Effects salinity and ammonia on blood serum antioxidant activities of the stellate sturgeon (*A. stellatus*) fingerlings.

Treatments	Catalase (u/mL)	SOD (u/ml)	GPX (u/mL)
1	42.67 ± 2.52 ^{bcd}	54.67 ± 1.53 ^{abc}	121.00 ± 5.57 ^{abc}
2	42.67 ± 3.21 ^{bcd}	57.67 ± 2.52^{abcd}	126.33 ± 7.02 ^{bcd}
3	36.33 ± 2.08 ^{ab}	59.33 ± 3.21 ^{bcd}	120.33 ± 3.78 ^{abc}
4	41.67 ± 2.08 ^{bcd}	61.67± 3.52 ^{cde}	124.33 ± 4.73 ^{abcd}
5	44.00 ± 1.00 ^{cd}	51.67 ± 2.08 ^{ab}	113.67 ± 4.16 ^{ab}
6	48.33 ± 3.06^{d}	50.33 ± 2.31 ^{a}	112.67 ± 3.06 ^{a}
7	34.67 ± 1.53^{a}	63.33 ± 4.51 ^{de}	129.33 ± 3.06 ^{cd}
8	38.33 ± 3.06^{abc}	67.33 ± 1.53 ^{e}	134.33 ± 3.06 ^{d}

Uncommon Latin letters indicate a significant difference between treatments (p<0.05).

[DOR: 20.1001.1.15622916.2022.21.6.1.7]

Table 10: Effects salinity and ammonia on blood serum liver enzymes of the stellate sturgeon (*A. stellatus*) fingerlings.

Treatments	(ALT) (U/L)	(AST) (u/L)	ALP (u/L)	LDH (u/L)
1	16.33 ± 2.08 ^{ab}	121.33 ± 4.73 ^{a}	201.67 ± 6.51^{bc}	917.00 ± 15.39^{a}
2	18.33 ± 1.53 ^{bc}	173.67 ± 6.11 ^{d}	187.67 ± 6.81^{b}	884.00 ± 11.53^{a}
3	13.33 ± 1.53 ^{a}	159.67 ± 4.16^{c}	242.67 ± 7.09 ^{d}	1021.00 ± 15.87 ^{b}
4	14.67 ± 0.58 ^{a}	137.33 ± 6.66 ^{b}	217.33 ± 3.06 ^{c}	997.33 ± 12.06 ^{b}
5	16.10± 1.00 ^{ab}	148.33 ± 4.04 ^{bc}	254.00 ± 7.21^{d}	1356.33 ± 14.84 ^{c}
6	19.33 ± 0.58 ^{bc}	185.33 ± 5.03 ^{de}	142.00 ± 6.56 ^{a}	1012.00 ±10.53^{b}
7	21.33 ± 1.53 ^{c}	192.33 ± 3.79^{e}	137.00 ± 4.00 ^{a}	891.67 ± 17.24^{a}
8	26.67 ± 0.58 ^{d}	218.67 ± 3.51 ^{f}	195.67 ± 5.51 ^{b}	891.33 ± 9.29 ^{a}

Uncommon Latin letters indicate a significant difference between treatments (p<0.05).

Histology of gill and liver

Table 1 shows changes in the gill tissue of stellate sturgeon (*A. stellatus*) fingerlings in different treatments. The most important changes were curling in secondary lamellae, destruction of the pilar apparatus and congestion in the lamellae, edema in lamellae, necrosis of secondary lamellae, necrosis of primary lamellae, fusion of some secondary lamellae, hyperplasis of epithelial cells, hemorrhage, loss of gill cartilage, disruption of cartilaginous core, aneurysm in the lamellae, destruction of epithelium layer in the secondary lamella, clubbing of the primary and secondary lamellae (Table 1 and Fig. 1). The highest gill lesions were observed at the salinity of 12 ppt in both the test group (exposed to ammonia) and the control group (not exposed to ammonia). As shown in Table 11 and Figure 2, the sinusoid contains red blood cells and hepatocytes suffer from atrophy. Other common lesions observed in the liver tissue were karyolysis, pyknotic nucleus, karyorrhexis, necrosis, melanomacrophage center, sinusoid, cloudy swelling, hemorrhage, blood congestion in sinusoid, and vacuolar degeneration. The highest prevalence of liver lesions was related to fingerlings of stellate sturgeon (*A. stellatus*) exposed to different concentrations of ammonia. The severity of lesions was dependent on ammonia concentration and salinity. Degradation was observed along with the increasing concentration of ammonia in treatment 8 (salinity: 12 ppt+ammonia: 5.11 mgL^{-1}) (50% of LC50 96h).

Gene expression

The results of P450 gene expression in the face of different levels of salinity and ammonia stress showed that different levels of salinity and ammonia separately, as well as the interaction effect of both salinity and ammonia stress, showed a significant difference in P450 gene expression (p≤0.05). In examining the interaction effect, the highest level of P450 gene expression was observed in the treatment with higher salinity and ammonia, and in the treatments with salinity equal to the increase in ammonia and in the treatments with ammonia equal to the increase in salinity, a significant difference was observed in the treatments (p≤0.05).

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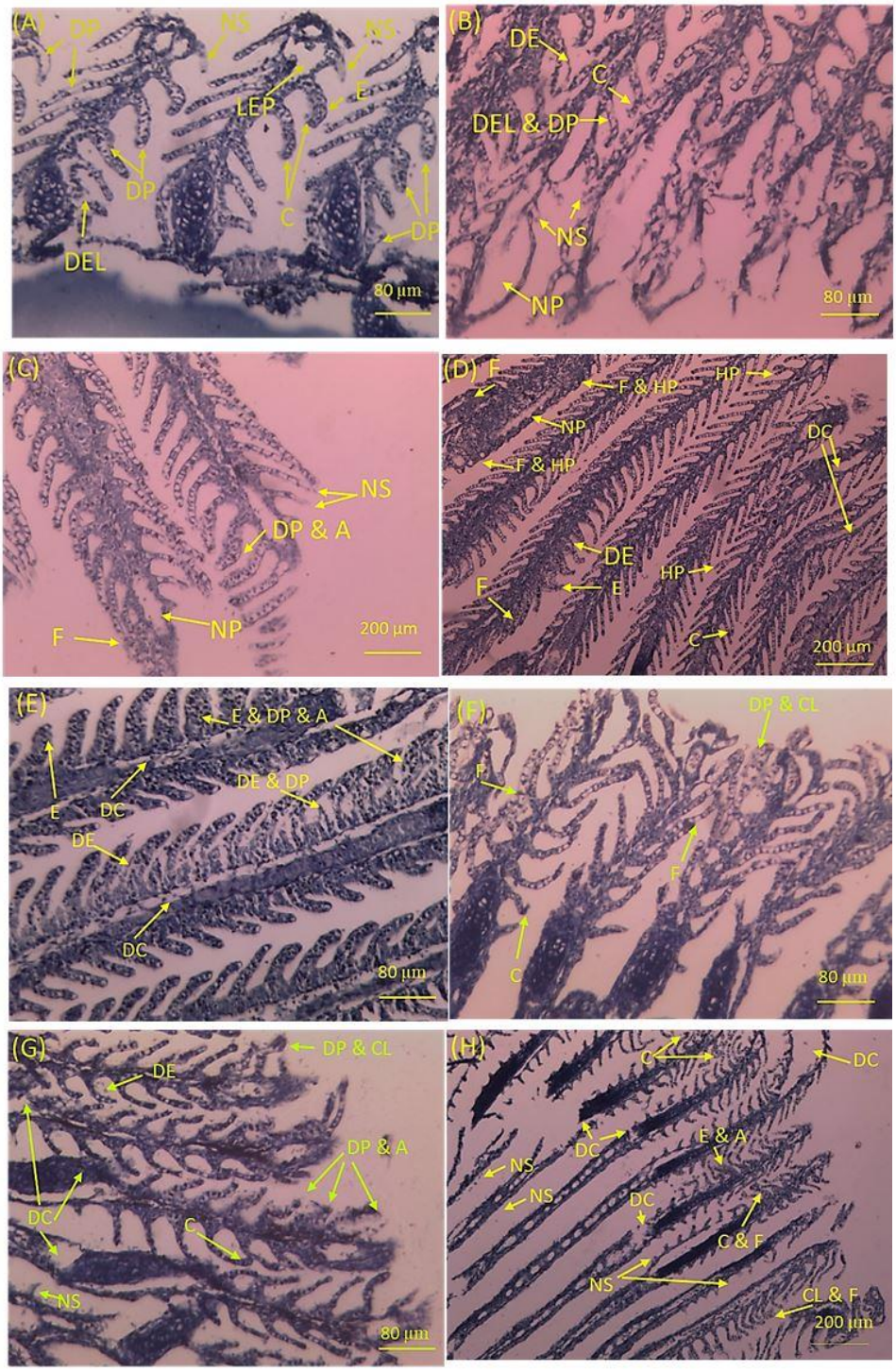

Figure 1: Histopathological changes of the gill tissue of starry sturgeons at different salinities and ammonia concentrations after 4 days (H&E staining). LEP: Loss of epithelium in the primary lamellae; DE: Detachment of epithelium layer in the secondary lamellae; C: Curling of secondary lamellae; DP: Destruction of the pilar apparatus and congestion in the lamellae; E: Edema in lamellae; NS: Necrosis of Secondary lamellae; NP: Necrosis of primary lamellae; F: Fusion of some secondary lamellae; HP: Hyperplasia of epithelial cells; H: Hemorrhage; Lg: Loss of gill cartilage; DC: Disruption of cartilaginous core; A: Aneurysm in the lamellae; DEL: Destruction of epithelium layer in the secondary lamellae; CL: Clubbing of the primary and secondary lamellae. (A) Treatment 1, (B) Treatment 2, (C) Treatment 3, (D) Treatment 4, (E) Treatment 5, (F) Treatment 6, (G) Treatment 7, and (H) Treatment 8.

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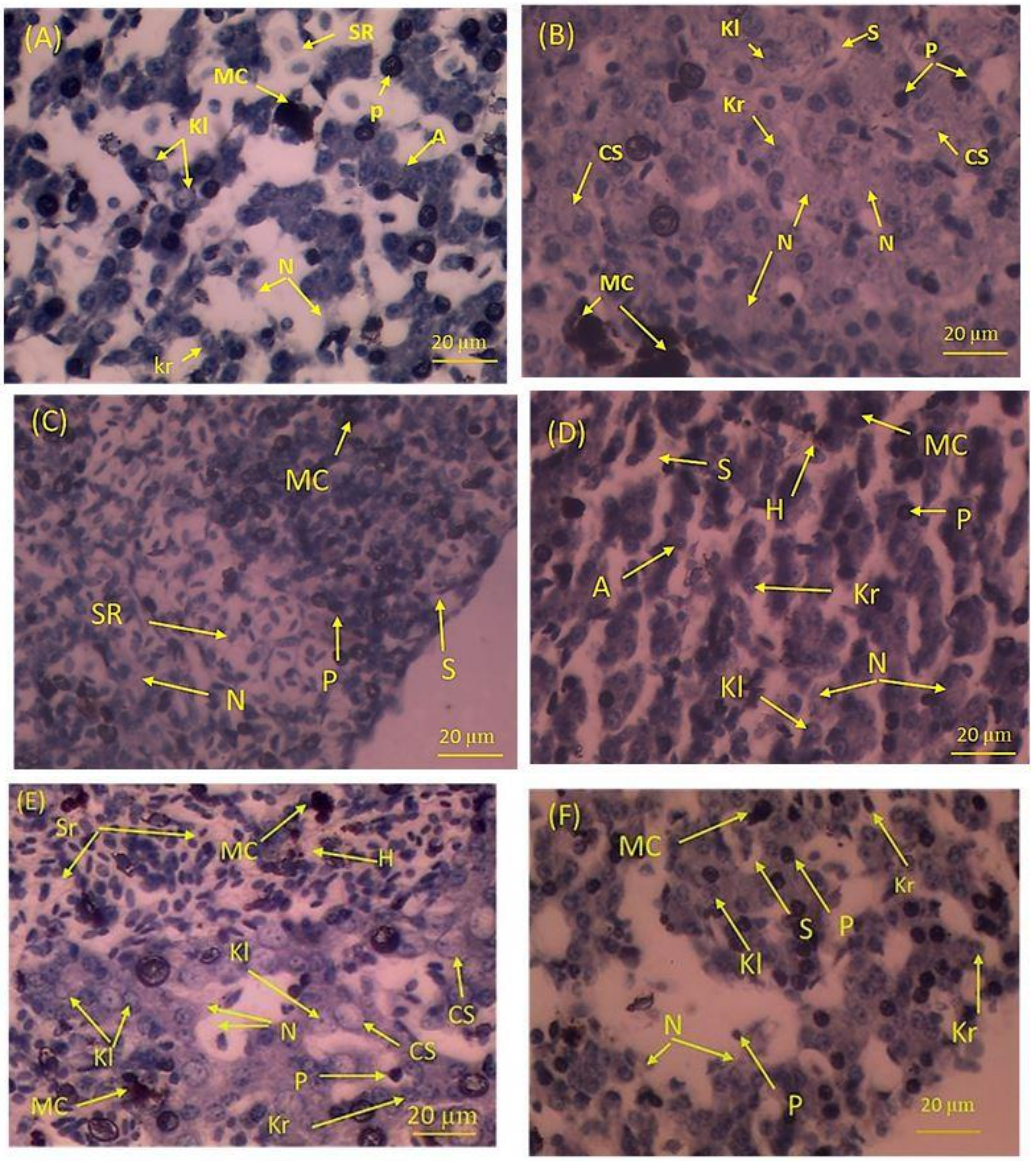
Table 11: Histopathological changes in the gill tissue of sturgeon (*A. stellatus*) fingerlings exposed to different levels salinity and ammonia.

Treatments	DE	NS	HP	H	DP	NP	F	E	LEP	C	Lg	DC	A	DEL	CL
1	-	+	-	-	++	-	-	+	+	+	-	-	-	+	-
2	+	+++	-	-	+++	+++	-	-	-	+	-	-	-	+++	-
3	-	++	-	-	++	+	+	-	-	-	-	-	-	-	-
4	+	+	++	-	++	+	++	+	-	++	-	++	+	-	-
5	++	-	-	-	+++	-	-	+++	-	-	-	++	+	-	-
6	-	++	-	-	++	-	+++	-	-	+	-	-	-	-	+
7	+	++	-	+	++	-	-	-	-	+	-	++	++	-	+
8	-	+++	-	-	-	+++	++	+	-	++	-	+++	+	-	+

-: No tissue damage; +: less than 25%; ++: less than 75%; +++: more than 75%. 2). C (Control): No ammonia; S: Salinity; A: Ammonia



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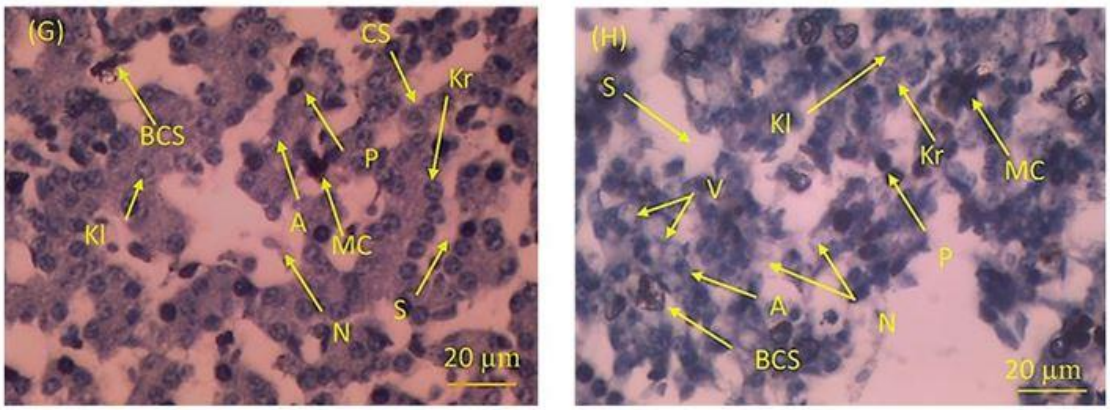

Figure 2: Histopathological changes of the liver tissue of starry sturgeon at different salinities and ammonia concentrations after 4 days (H&E staining). SR: The sinusoid contains red blood cells; A: Hepatocytes suffer from atrophy; K: Karyolysis; P: Piknosis; Kr: Karyorrhexis; N: Necrosis; MC: Melanomacrophage center; S: Sinusoid; CS: Cloudy Swelling; H: Hemorrhage; BCS: Blood congestion in sinusoid; V: Vacuolar degeneration. A) Treatment 1, (B) Treatment 2, (C) Treatment 3, (D) Treatment 4, (E) Treatment 5, (F) Treatment 6, (G) Treatment 7, and (H) Treatment 8.

In the comparison between the mutual effect of salinity and ammonia and in different treatments showed a significant difference only at the level of 8 (grams per liter) of salinity and half (50%) of the LC50 of ammonia ($p \leq 0.05$) (Fig. 3 and Table 12).

The t-test results on the salinity-ammonia interaction in different treatments revealed a significant difference only at a salinity of 8 ppt and LC50 96h of ammonia ($p \leq 0.05$) (Table 13).

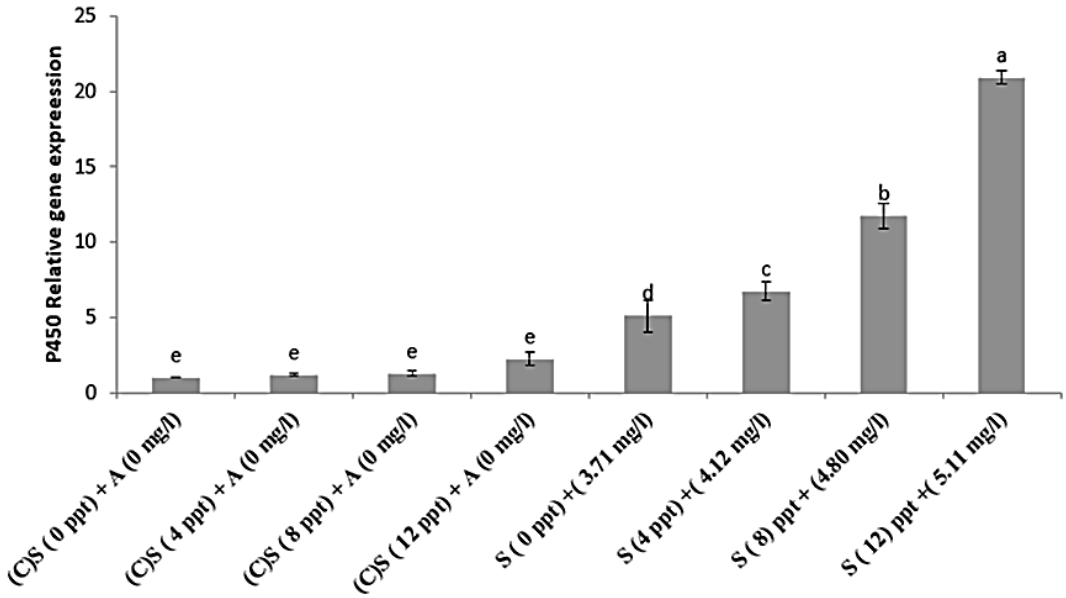

Figure 3: The P450 gene expression in the liver tissue of sturgeon (*A. stellatus*) fingerlings exposed to different levels of ammonia and salinity. The same letters were assigned where the differences were not statistically significant ($p<0.05$). C (Control): No ammonia; S: Salinity; A: Ammonia.

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Table 12: Histopathological changes in the liver tissue of sturgeon (*A. stellatus*) fingerlings exposed to different levels salinity and ammonia.

Treatments	A	SR	Kl	MC	N	P	S	H	BCS	CS	V	Kr
1	++	+	++	+	++	+	++	-	-	-	-	++
2	-	-	+	+	++	+	++	-	-	+	-	++
3	-	++	-	++	++	+	+	-	-	-	-	-
4	++	-	++	+	+++	++	++	-	-	-	-	++
5	-	+++	++	++	+++	+	+	++	++	++	-	++
6	-	-	++	+	+++	++	+++	-	-	-	-	++
7	+	-	+	+	+++	+	++	-	+	+	-	+++
8	+	-	+	+++	+++	+	++	-	-	-	++	+

-: No tissue damage; +: less than 25%; ++: less than 75%; +++: more than 75%. 2).

Table 13: A comparison of the effect of interaction between salinity and LC50 96h of ammonia.

Salinity (ppt)	Ammonia 0 (mgL^{-1})	Half the concentration of semi-lethal ammonia (LC50 96h (mgL^{-1}))
0	1±0.1	4.16±0.1
4	1.18±0.1	6.02±0.1
8	1.27±0.19	6.2±0.19
12	2.24±0.47	20.58±0.47

Discussion

During 96 hours, no deaths were observed for the control group. The results obtained from this research showed that the amount of LC50 96h of ammonia on sturgeon (*A. stellatus*) fingerlings decreased with decreasing salinity and also increasing the duration of exposure to the test. A lower concentration of ammonia is necessary to destroy 50% of sturgeon fingerlings and the value of LC50 in the first hour and 12 ppt salinity was always higher than its value in 0 ppt salinity and the final hours of the experiment. In fact, it can be said that with the increase in salinity, the sturgeon fingerlings tolerance to ammonia toxicity increased, as a result, the LC50 value at 12 ppt salinity was higher than that at 0 ppt salinity . Similar results have been reported in cobia (*Rachycentron canadum*) juvenile (Barbieri, 2012), sea bream(*Sparus aurata)* juvenile (Kir and Sunar, 2018), and European sea bass *Dicentrarchus labrax* juvenile (Kir *et al*., 2019). The increase in ammonia toxicity with a decrease in salinity can be justified due to the high absorption of ammonia in low salinity. The beneficial effects of elevated salinity with respect to reducing the toxic effects of NH3-N exposure are thought to be due to increased sodium levels, which have been shown to facilitate ammonia excretion via $NH4^+$ and Na^+ exchange at the gill membrane and to prevent influx of NH3-N by decreasing membrane permeability (Weirich and Riche *et al*., 2006). So fish farmers and organizations in charge of stock restoration should pay attention to the difference in ammonia toxicity in different salinities during pool management and when sturgeon fingerlings are released.

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Complete blood count (CBC) indices showed the most changes in combined treatments with increased salinity and ammonia. It seems that salinity did not reduce the toxic effects of ammonia on these indicators. Usually, salinity has a profound effect on ionization equilibrium of (total) ammonia into toxic gaseous (NH_3) and non-toxic ionized (NH_4^+) form. A reduction of 10 units in salinity (at 20°C) would results in approximately 9% increase in NH_3 fraction, and, therefore, higher ammonia toxicity would be expected at lower salinity (Sinha *et al*., 2015). This finding is consistent with the results of some previous studies that found an increase in WBC in red tilapia after 3 days of exposure to 3 mg of NH_4Cl (Bonnie and Liu, 2004). Zeitoun *et al*. (2016) found that ammonia exposure increased WBCin the Nile tilapia (*Oreochromis niloticus*) adult. Furthermore, Das *et al*. (2004) discovered a similar trend, concluding that an increase in WBC as a result of stress is involved in the regulation of fish immunological function. An increase in total leukocytes is caused by an increase in lymphopoiesis and or enhanced lymphocyte release from lymphoid tissues (Zeitoun *et al.,* 2016). The increase in RBC can be attributed to the release of red blood cells from the spleen following fish stress (Vijayan and Leatherland, 1989; Pulsford *et al.,* 1994).

Cortisol, glucose, and lactate levels in salinity treatments decreased with increasing ammonia concentration. Lactate depletion in this study indicates a lack of anaerobic metabolism, which is indirectly attributed to haemoglobin levels. Inadequate oxygen delivery to the tissue can result in anaerobic metabolism and lactate buildup. The absence of lactic acid secretion indicates the absence of anaerobic metabolism. High levels of ammonia stress the body and cause harmful physiological responses such as osmoregulatory disturbances, kidney and branchial epithelial damage, and growth retardation (Meade, 1985; Soderberg, 1994). Inefficient immune response and increased plasma glucose are the results of catecholamine mobilizing energy resources to support responses (Cheng *et al*., 2004; Pinto *et al*., 2007) and decreased survival (Jobling, 1994). Shokr (2015) reported a significant increase in glucose and cortisol levels in Nile tilapia (*Oreochromis niloticus*) adult exposed to ammonia.

Furthermore, Shin *et al*. (2016) found an increase in glucose, cortisol, and lactate in Sebastes schlegelii, the Korean rockfish, after exposure to different levels of ammonia and salinity. However, it is not fully understood why salinity may mitigate the effects of nitrite. We suggested that as $NH4^+$ is thought to compete for the same transport site as chloride ions in the $NH4^+/Na^+$ exchanger (located in the apical side of the gill cells), then increased salinity prevents the direct effect of ammonia on of cortisol, glucose and lactate within the blood.The findings showed the reduction of toxic effects of ammonia with increasing salinity. The innate immunity of fish exposed to high

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levels of ammonia was found to be enhanced in this study. Shokr (2019) discovered that ammonia stress reduced IgM, lysozyme, and total immunoglobulin activity in the Nile tilapia (*Oreochromis niloticus)* adult. The ammonia exposure significantly reduced the lysozyme activity of yellowhead catfish (*Pelteobagrus fulvidraco)* juvenile (Zhang *et al*., 2018), as well as the total immunoglobulin of this species (Li *et al*., 2020).

In this study, SOD and GPX activity increased significantly during 4 days of exposure to high levels of ammonia and salinity. Whereas, CAT activity showed a significant decrease. In control treatments (no ammonia), decrease in CAT and increase in SOD and GPX were not significant. The increase in these enzymes could be attributed to the destructive effect of ammonia on these enzymes (Rodrigues *et al*., 2015) or superoxide radical accumulation (Safari, 2015). Zhang *et al*. (2019) reported that the SOD, CAT, and GPX activity gradually decreased in stellate fingerlings of the blunt snout bream(*Megalobrama amblycephala)* juvenile, over 9 weeks of exposure to high levels of ammonia. Consistent with this study, Jia *et al*. (2017) showed that the SOD and CAT activity in the liver significantly increased after exposure to high levels of ammonia (20 and/or 40 mgL^{-1}, 1 TANN) in turbot (*Scophthalmus maximus*) juvenile, depending on the duration of exposure.

AST and ALP activity in stellate sturgeon fingerlings increased after 4 days of exposure to high levels of ammonia and salinity in combination treatments. Whereas, ALT and LDH activity decreased in the same period. In control treatments (no ammonia), decrease in ALT and increase in AST, ALP and LDH were not significant. Taheri Mirghaed *et al*. (2019) reported that the serum activity of LDH, ALP, AST, and ALT increased in Common carp (*Cyprinus carpio*) adult after exposure to ammonia, which can indicate tissue damage and immunosuppression. Kumar *et al*. (2018) found that AST, ALT, LDH, and AChE levels were higher and the ALP level was lower in a group of Indian major carp the rohu, *Labeo rohita*, exposed to ammonia, compared to the control group. The increase in plasma AST and ALP levels could be regarded as sensitive enzymatic biomarkers for assessing hepatic damage. The reduced hepatic ALT and LDH levels may be caused by toxicant-induced liver damage and necrosis (Bernet *et al*, 2001; Okechukwu and Auta 2007). The differences observed in the enzyme activity can be attributed to differences in fish species, toxicant type, dose and duration of exposure, or other unknown factors. As a result, it appears that changes in the activity of the AST, ALT, LDH, and ALP enzymes may be important in the stress response to pollutants.

Pollutants primarily target the gills, which also serve as indicators of pollutant exposure in fish. In this study, exposure to salinity alone and salinity plus ammonia caused different damages on the gill tissue of stellate sturgeon. The

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highest level of damage was observed in exposure to a salinity of 12 ppt alone and a salinity of 12 ppt plus LC50 96h of ammonia. The most common gill lesions observed in this study were necrosis of primary lamellae, disruption of cartilaginous core, destruction of the pilar apparatus and congestion in the lamella, necrosis of primary lamellae, and fusion of some secondary lamellae. Several authors have reported similar changes in the gills of various fish species exposed to ammonia (Mallatt, 1985; Benli *et al*., 2008). Salin and Williot (1991) discovered modified epithelium of the secondary lamellae and a slightly turgescent base of the filament in 270-g Siberian sturgeon (*Acipenser baerii*) larvae and juveniles, exposed to more than 60 mgL^{-1} of ammonia. According to Benli *et al*. (2008), the most significant histopathological effects of median lethal concentration of ammonia on the gill were chloride hyperplasia, telangiectasis on lamella, and epithelial hyperemia. Similar results have been reported by Senthamilselvan *et al*. (2014) in the common carp (*Cyprinus carpio*) adult, Mazandarani *et al*. (2015) in Fingerlings of Caspian Roach (*Rutilus rutilus caspicus*), Farhangi (2010) in Persian sturgeon (*Acipenser persicus*) and beluga (*Huso huso*) adult, Banihashemi *et al*. (2016) in Persian sturgeon (*Acipenser persicus*) and beluga (*Huso huso*) fingerling, Termeh Yousefi *et al*. (2018) in Banded Cichlid (*Heros severus)* Fingerlings, and Gunal *et al*. (2008) in Nile tilapia (*Oreochromis niloticus)* adult. The most common complications observed in this study were melanomacrophage center, necrosis, Sinusoid, and karyorrhexis. The liver tissue samples of the stellate sturgeon were exposed to high levels of salinity and ammonia. Because the liver is the primary organ for many key metabolic pathways, toxic effects of chemicals usually manifest first in the liver. Ammonia can be carried to the liver as a nutrient by the hepatic portal vein and enter liver metabolic pathways (Kucuk, 1999). Benli *et al*. (2008) showed cloudy swelling and hydropic degeneration in the liver of the Nile tilapia (*Oreochromis niloticus*) adult, exposed to 5–10 mgL^{-1} of TAN (0.35–0.71 mgL^{-1} of NH_3-N) for 42 days. Rodrigues *et al*. (2014) observed dilatation, hepatic sinusoids, and fatty deposition in hepatocytes and Mallory bodies in the maroon clownfish (*Premnas biaculeatus*) juvenile, exposed to 20 and 30 mgL^{-1} of NH_3-N for 4 days.

The high ammonia accumulation in stellate sturgeon exposed to ammonia was accompanied by a significant increase in p450 mRNA transcript levels. Based on these findings, it is clear that ammonia stress causes the expression of p450 genes in the stellate sturgeon, most likely as a result of increased ammonia accumulation in the tissue liver. The highest expression of the P450 gene was observed in the treatment with higher salinity and ammonia (p<0.05). According to Hangzo *et al*. (2017), high ammonia concentrations in ammonia-exposed in mud eel (*Monopterus cuchia*) adult were accompanied by a significant increase in

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the levels of hsp70 and hsp90a mRNA transcripts and increased abundance of Hsp70 and Hsp90a proteins in the liver tissue of the Pacific mud eel. After 14 days of exposure, the abundance of hsp90a mRNA transcript increased by 3.02 times in the liver (p<0.001). Thus, ammonia stress of exposure to HEA causes the induction of hsp70 and hsp90a genes in the mud eel, most likely as a result of increased ammonia accumulation in different tissues and or other ammonia-induced insults. Zhang *et al*. (2015) found that the expression of both proteins (Hsp70 and Hsp90a) in the liver increased in the first 12 hours of exposure to ammonia but then decreased over time. Similar results have been reported by Sung *et al*. (2012) in the Common carp (*Cyprinus carpio*) fingerlings, Sinha *et al*. (2012) in the Common carp (*Cyprinus carpio*) juveniles, Luckenbach *et al*. (2003) in the tiger trout (*Salmo trutta fario*) early life stages, and Gunal *et al*. (2008) in the Nile tilapia (*Oreochromis niloticus*) adult. The high expression of P450 in the liver is attributed to the metabolic role of this organ (Da Cuna *et al*., 2011). The results showed that changes in environmental conditions firstly cause biological responses at the molecular level; as the environmental changes increase, biological responses extend to biochemical, cellular, histologic, organ, organism, population, and ecosystem levels. The study findings suggested that changes in this enzyme can be considered a biomarker of contamination with ammonia as well as exposure to salinity stress.

At the end of this part of the experiment (96 h of exposure), no death occurred at different treatment. The results showed that with increasing salinity, ammonia concentration also increased. Thus, it showed that the tolerance of fish against increasing ammonia concentrations increased. With increasing salinity, fish susceptibility to ammonia toxicity decreased. Ammonia at high concentrations had destructive effects on gill tissue and liver. The gene expression p450 of the liver showed a rising trend in mixed treatments with high salinity and ammonia concentration indicating that exposure to ammonia along with salinity induced tension trigger internal defense system of fishes. It may be stated in conclusion that although ammonia toxicity in the stellate sturgeon (*A. Stellatus*) fingerlings was alleviated with increased salinity, but there were tissue damages (gills, kidneys, liver and intestines), blood parameters, and increased p450 gene expression (in the liver) occurred with increased salinity in combination treatments with ammonia. As a result, the use of seawater and brackish water in ammonia concentrations declared in this study is suitable for culture of stellate sturgeon (*A. stellatus*) fingerlings, and also, considering the concentration of ammonia obtained in different salinities at this study, it makes it possible that Iran Fisheries Organization (IFO) can use the results of this study to select the best release point (sea and river) for young *A. stellatus*.

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Acknowledgements

The authors would like to thank the staff of the International Sturgeon Research Institute, Chaboksar Sturgeon Research Station, and Inland Waters Aquaculture Research Center (Bandar Anzali), for their kind help during the experiment.

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