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The influence of ammonia-N and salinity levels on oxidative stress markers, hepatic enzymes, and acid phosphatase activity in Nile tilapia (*Oreochromis niloticus*)

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The point of our study was to examine the interaction of ammonia-N poisoning and salinity on serum enzymes and oxidative stress factors of blood and liver in Nile tilapia (*Oreochromis niloticus*). The 50% lethal concentration (LC_{50}) in 96 h was 0.86 mg/L of ammonia-N. A random allocation was used to divide the fish into 12 treatments. These treatments encompassed various combinations of acute ammonia-N levels (0 and 50% of LC_{50}-96 h), sub-acute ammonia-N levels (30% of LC_{50}-96 h), and salinity levels (0, 4, 8, and 12 ppt). The experimental design employed a factorial arrangement of 3 × 4. The findings revealed that the amounts of aspartate transferase (AST) and alanine transaminase (ALT) in treatments 3 and 4 increased significantly compared to the treatment 2 (4 ppt) and control. Salinity levels did not affect serum glutathione levels (GSH), nevertheless the reduction of serum GSH and levels of total antioxidant capacity (TAC) and superoxide dismutase (SOD) and catalase activities (CAT) in ammonia poisoning treatments, 5 and 9, compared to the control, states ammonia can stimulate oxidative stress in fish. Similar to the serum measurements, increasing salinity in acute ammonia poisoning treatments (5, 6, 7 and 8) caused an increasing effect on the liver TAC value, which was presumably due to the improving effect of salinity in reducing ambient ammonia. The findings indicate that while elevated salinity levels can be beneficial in mitigating the effects of ammonia toxicity in water, the combined presence of salinity, ammonia, and their interaction had detrimental impacts on the physiological well-being of fish over a 96-hour testing period.

Keywords Ammonia, Liver enzymes, Oxidative stress, Salinity, Toxicity, Tilapia

The final product of the protein catabolism pathway is ammonia, which significantly contributes to nitrogen pollution in aquatic organisms[1]. Aquatic environments can be impacted by a variety of sources, including agricultural effluents, industrial pollution, and biological waste from decomposition[2,3]. In aquatic environments, ammonia exists in two distinct chemical forms: un-ionized ammonia (NH3) and ionized ammonium (NH4+). The toxicity of ammonia to aquatic organisms can be influenced by a range of environmental parameters, including temperature, pH, oxygen concentration, and salinity[4–6]. The level of the ammonia increased in pond water will have destructive efficacy on aquatic animals such as reduction of growth, degeneration and necrosis, suppression of the immune system, disorders of kidney and liver function and may lead to high mortality[4,5]. It is widely acknowledged that ammonia is a significant contaminant in the field of aquaculture[7,8]. Thus, reducing the concentration of ammonia is a critical concern in the field of aquaculture.

Salinity is a key factor that influences the osmotic pressure in aquatic environments. Altering the osmotic pressure of the environment can have a significant impact on the metabolic processes of aquatic animals[9]. The main contributors to the salinity levels in water resources are evaporation from the water surface, soil dissolution within reservoirs, inflow of saline surface water, and discharge of salty groundwater from surrounding sources and areas[10–13]. The variations in temperature and rainfall play a significant role in the seasonal changes

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observed in salinity levels. These fluctuations have the potential to impact the physiological functions of aquatic organisms[14,15]. Additionally, salinity plays a significant role in mitigating ammonia toxicity. An increase in water salinity levels corresponds to a reduction in the concentration of non-ionized ammonia.

Oxidative stress is a physiological state that arises when reactive oxygen species (ROS) chemically interact with lipids, proteins, or nucleic acids[16–18]. Reducing or disrupting ROS formation is one of the most important precautions of living organisms[16,19]. Aquatics against oxidative stress have two defenses, including non-enzymatic ROS scavengers like glutathione, flavonoids, β-carotene and vitamins (C, A and E), and also an enzymatic system that consists of antioxidant enzymes[16,17,20–22]. Researches have proven that a large number of environmental factors like temperature[23], air exposure[24,25], exposure to ammonia[26], salinity[27–29], pH or alkalinity, even photoperiod[30,31], other seasonal parameters[28,29,31] and pollutants[32,33] could affect the oxidative condition in aquatic animals.

Iran is situated in a semi-arid region characterized by annual precipitation levels that are less than one-third of the global average.Additionally, the rainfall in this region is not evenly distributed temporally or spatially[34]. By 2025, Iran is projected to be one of the 27 countries at risk of facing water shortages[35]. Improper utilization of groundwater has negative impacts on the integrity and availability of subterranean aquifers[34]. The provided description suggests that there may have been an increase in the salinity levels of both surface and groundwater resources[36,37]. Consequently, it may be necessary to focus on the development of fish species that are adapted to higher salinity conditions.In the environment, it is possible that ammonia-N poisoning occurs at different salinity levels. It is noteworthy to highlight that there is a significant absence of data regarding the antioxidant defense responses of Oreochromis niloticus to simultaneous variations in salinity and ammonia-N levels. Consequently, our study was conducted with the aim of assessing the impact of different salinity levels on the occurrence of ammonia poisoning in Nile tilapia, *Oreochromis niloticus*. This particular species is known for its remarkable ability to adapt and thrive in brackish water environments, and there is even a possibility that it may exhibit superior growth compared to freshwater conditions[38,39]. Nile tilapia, a highly favored species, serves as a crucial substitute for freshwater species in arid and water-scarce regions such as Iran[40,41]. This study aims to enhance our understanding of alterations in the components of the antioxidant system within the liver and serum in response to salinity, ammonia-N, and their combined impact in an aquaculture environment.

Materials and methods

Fish husbandry

This study was carried out in accordance with the guidelines established by The National Ethical Framework for Animal Research in Iran, receiving approval under the number EE/95.11.3.51112/scu.ac.ir.Our investigation follows the protocols outlined in the ARRIVE guidelines. The Vice-Chancellor of Graduate Studies of Shahid Chamran University of Iran has granted approval for the design and methodology employed in this research.

Juvenile Nile tilapia (*Oreochromis niloticus*) was acquired from a fish breeding center located in Qom city, Iran, and then transported to the Partako breeding center in Tiranchi village, Isfahan, Iran, in cellophane bags filled with oxygen. For the experiments, a random selection of fish weighing 57.32 ± 16.7 g and exhibiting a healthy appearance was made. To ensure their adjustment to the laboratory settings, the fish were placed in thoroughly disinfected aquariums (100 L) for a period of 7 days. In this study, the fish were nourished by specialized commercial food provided by Kimiyagaran Co. located in Shahrekord, Iran. The food composition consisted of 40% protein, 30% carbohydrates, 7% crude fat, 6% crude fiber, 7% ash, and 10% moisture. The fish were fed this diet twice a day, in the morning and evening. Throughout the adaptation period, no instances of mortality were recorded. Additionally, buffer water was employed for the purpose of this research.

Adjust acidity

To maintain a consistent level of acidity in the water, a precise quantity of hydrochloric acid (0.4 N, Merck, Germany) and sodium hydroxide (0.4 N, Merck, Germany) were introduced into the tank water to regulate its acidity. Following the addition of acid and base, the pH level of the tank water was determined[42]. The acidity of the water was monitored consistently over the course of a week. Following its adjustment, the water's acidity was stabilized and subsequently utilized in the experiment.

Physical and chemical factors of water

The Köldahl system and titration were employed to assess the total ammonium nitrogen, TAN[43]. NH3 was calculated based on the temperature and pH values, in addition to the standard Table[44]. The EC meter (Conductivitymeter 4310, Jenway, UK) was utilized to measure electrical conductivity (EC), while the pH meter (744 pH meter, Metrohm, UK) was employed to determine acidity levels. Aeration was continuously provided throughout the experiment.

Measuring 50% lethal concentration (LC50) of ammonia-N

The LC50 determination test utilizing a static method involved the replacement of water every 24 h and the preparation of desired concentrations. The study focused on two concentrations of ammonium chloride (Merck, Germany) that resulted in 100% mortality without any losses. Subsequently, six concentrations of ammonium chloride were exponentially calculated within the range of the initial two doses. Subsequently, following the adjustment phase, the fish were segregated into six distinct groups, each comprising ten fish. The fish were monitored continuously for a period of 96 h, with mortality rates being noted every 24 h[45]. Throughout the duration of the experiment, the temperature and pH levels were maintained at a constant value. Following the observation of mortality, the LC_{50} values were determined at 24, 48, 72, and 96 h using probit software.

Design of experiments

Twelve different experimental conditions were set up, which included different concentrations of ammonia-N (0, 30%, and 50% of LC_{50}-96 h) and various levels of salinity (0, 4, 8, and 12 ppt). Furthermore, interactive treatments that combined both ammonia toxicity and salinity were incorporated into the experimental setup (Table 1). The levels of 50% and 30% of LC_{50}-96 h were classified as acute and sub-acute poisoning, respectively[46]. Each experimental group was replicated five times. The water was replaced in its entirety on a daily basis over a period of 96 h. Daily assessments were conducted to measure total ammonium levels, pH, and EC.

Blood sampling

At the conclusion of the 96-hour testing period, blood samples were collected using insulin syringes. The fish were rendered unconscious using 2-phenoxyethanol and subsequently dried with a towel. Blood sampling was performed through the caudal vein using non-heparin syringes. Blood samples were centrifuged at a speed of 3000 × g for a duration of 10 min using tubes that did not contain heparin in order to obtain serum[47]. The resulting liquid above the sediment was carefully collected, divided into smaller portions, and stored at a temperature of -70 °C in an Ultra Low Temperature Freezer (MDF-U71VC, Sanyo, Japan) until further analysis could be conducted.

Lactate measurement

Lactate levels were assessed through the UV test enzyme method employing lactate dehydrogenase, the Pars Azmoun test kit (Pars Azmoon, Iran), and an autoanalyzer (Auto Analyser Biotechnica, BT 1500, Italy).

Tissue sampling

The fish were euthanized by delivering a swift and forceful blow to the head, after which they were weighed. In order to collect a liver sample, a targeted portion of the right liver lobe was meticulously removed on ice, ensuring that the tissue was not subjected to excessive pressure during the procedure.Subsequently, the excised liver sample was rinsed in ice-cold isotonic NaCl saline solution. To maintain its integrity, the liver sample was promptly placed on an aluminum sheet and stored on dry ice until the sampling process was concluded. Finally, the liver tissue samples were frozen at a temperature of -70 °C for preservation.

Homogenization of liver tissue

The liver tissue, with a ratio (1:5 w/v), was homogenized in potassium phosphate buffer 100 mM, KCl 100 mM and EDTA 1 mM at pH 7.4 using homogenizer (Miccra, Germany) at 22,000 rpm for 20 s. The centrifugation process was carried out on the homogenized sample using a Hettich centrifuge from Germany. The centrifugation was performed at a speed of 2000 × g for a duration of 30 min at a temperature of 4 °C. The resulting supernatant was then utilized for the measurement of oxidative stress parameters[48].

Serum enzymes and biochemical parameters

The activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and acid phosphatase (ACP) enzymes was measured using an autoanalyzer (BT-1500, Italy) and laboratory kits of Pars Azmoun company (Pars Azmoun, Iran). The determination of serum lactate value was conducted by employing a commercial kit (Biorex Diagnostics, Antrim, UK) in accordance with the established protocol provided by the company.

Serum and liver oxidative stress factors

The amount of liver tissue protein was measured according to Bradford method[49]. The total antioxidant capacity (TAC) was determined according to the procedure of Benzie and Strain[50]. Reduced glutathione (GSH) was measured based on protocol of Ellman[51]. Based on the method of Koroluk, et al.[52], catalase (CAT) enzyme activity was measured. In order to measure the level of malondialdehyde (MDA), the procedure described by

Treatment	
T_1	Adjusted water in terms of acidity (control)
T_2	4 ppt of salinity
T_3	8 ppt of salinity
T_4	12 ppt of salinity
T_5	50% of LC_{50}-96 h (As acute ammonia poisoning)- 0 ppt of salinity
T_6	50% of LC50-96 h (As acute ammonia poisoning)- 4 ppt of salinity
T_7	50% of LC50-96 h (As acute ammonia poisoning)- 8 ppt of salinity
T_8	50% of LC50-96 h (As acute ammonia poisoning)- 12 ppt of salinity
T_9	30% of LC50-96 h (As sub-acute ammonia poisoning)- 0 ppt of salinity
T_{10}	30% of LC50-96 h (As sub-acute ammonia poisoning)- 4 ppt of salinity
T_{11}	30% of LC50-96 h (As sub-acute ammonia poisoning)- 8 ppt of salinity
T_{12}	30% of LC50-96 h (As sub-acute ammonia poisoning)- 12 ppt of salinity

Table 1. Experimental groups over a span of 96 h.

Duration (h)	LC_{50} (mg/L)	95% confidence interval
24	1.42	(0.611–1.77)
48	1.365	(1.131–1.625)
72	1.187	(0.982–1.397)
96	0.86	(0.708–1.09)

Table 2. LC_{50} values of ammonia at various time intervals in Nile tilapia.

Treatment	Temperature (°C)	pH	NH_3 (mg/L)	EC (ms)
1	27.8 ± 0.26	7.02 ± 0.02	0.047 ± 0.036^{a}	0.52 ± 0.02^{a}
2	27 ± 0.2	7.04 ± 0.06	0.04 ± 0.02^{a}	7.5 ± 0.24^{ab}
3	28 ± 0.15	7.07 ± 0.03	0.02 ± 0.001^{a}	$14.79 \pm 0.58d^{ef}$
4	27.8 ± 0.26	7.01 ± 0.02	0.09 ± 0.03^{a}	19.2 ± 0.18^{f}
5	27.76 ± 0.2	7 ± 0.01	1.62 ± 0.83^{e}	3.6 ± 0.01^{abc}
6	28.13 ± 0.32	7.02 ± 0.04	1.62 ± 0.51^{de}	9.6 ± 3.3^{cde}
7	28.03 ± 0.05	7.04 ± 0.4	1.11 ± 0.12^{de}	15.41 ± 7.6^{ef}
8	28 ± 0.9	6.98 ± 0.02	0.73 ± 0.037^{cd}	17.12 ± 8^{f}
9	27.86 ± 0.15	7.04 ± 0.05	0.45 ± 0.14^{bc}	1.44 ± 0.01^{ab}
10	27.92 ± 0.13	7 ± 0.07	0.59 ± 0.05^{ab}	8.44 ± 0.09^{cd}
11	28 ± 0.1	7.03 ± 0.03	0.54 ± 0.11^{bc}	15.96 ± 1.08^{ef}
12	27.9 ± 0.17	7.07 ± 0.035	0.71 ± 0.11^{ab}	19.87 ± 0.24^{f}
P value	0.442	0.183	0.00	0.00

Table 3. Water's physical and chemical characteristics over a period of 96 h (mean ± standard deviation, $n = 4$). The existence of distinct letters in every column indicates a notable disparity. ($P<0.05$).

Placer, et al.[53] was used with some changes. In this study, the activity of superoxide dismutase enzyme was measured using the method of Kono, 1978.

Statistical analysis

The present study employed a factorial design (3 × 4) to conduct the research. The findings were reported in terms of means ± standard deviation (SD). To ensure normality, the data underwent the Kolmogorov-Smirnov test, while the homogeneity of variance was assessed through Levene's test. The statistical analysis involved a two-way analysis of variance (ANOVA), except for the examination of physical and chemical factors of water, which were evaluated using a one-way ANOVA. Tukey's post hoc test was employed to assess the disparities between means. Statistical significance was determined for differences where $P < 0.05$. The statistical analysis was conducted using SPSS software version 24.

Results

LC50 values of ammonia

Table 2 presents the findings derived from the assessment of LC50 ammonia-N. The LC50 ammonia-N values for time intervals of 24, 48, 72, and 96 h were recorded as 1.42, 1.365, 1.187, and 0.86 mg/l, respectively. In our study, the concentrations of ammonia-N were determined by considering the LC50 value at the 96-hour mark.

The assessment of water quality

Based on the data presented in Table 3, there were no significant differences observed in the temperature and pH factors. However, treatments 5, 6, and 7 displayed a significant variance in NH3 concentration when compared to the other treatments ($P < 0.05$). It is significant to note that the NH3 concentration in treatment 8, characterized by acute ammonia toxicity at a salinity level of 12 ppt, was observed to be lower than that in treatment 5 ($P < 0.05$). The experimental conditions demonstrated the lowest NH3 levels in treatments 1, 2, 3, and 4.

Serum enzymes and biochemical parameters

Table 4 declares the effects of ammonia poisoning levels, salinity levels and salinity and ammonia interaction on the activity of ALT, AST, ALP, LDH, ACP and lactate in tilapia blood serum.

Due to the presence of numerous treatments and variables, it was necessary to conduct a comparative analysis to gain a comprehensive understanding of the effects of these variables.

In this investigation, we conducted a comparative analysis of treatments 2, 3, and 4 against the control treatment. Furthermore, treatments 5 and 9 were evaluated in relation to the control treatment, whereas treatments 6, 7, and 8 were assessed in comparison to treatment 5.

	ALT	AST	ALP	LDH	ACP	Lactate
Ammonia	0.00	0.00	0.00	0.00	0.00	0.00
Salinity	0.00	0.00	0.00	0.00	0.00	0.00
Ammonia*salinity	0.032	0.003	0.00	0.00	0.00	0.017

Table 4. The obtained p value from the two-way ANOVA analysis which demonstrated the impact of ammonia-N poisoning, salinity, and their interactions on the levels of ALT, AST, ALP, LDH, ACP and lactate in the Nile tilapia. A p-value less than 0.05 indicates a statistically significant difference in the variable of interest. ALT (Alanine aminotransferase), AST (Aspartate Aminotransferase), ALP (Alkaline Phosphatase), LDH (Lactate dehydrogenase), ACP (Acid Phosphatase).

In conclusion, treatments 10, 11, and 12 were evaluated in relation to treatment 9. This systematic methodology enabled the identification of the unique effects of each treatment, facilitating the formulation of significant conclusions.

The results of measuring the activity of serum enzymes indicated, with increasing salinity level and ammonia content of water, ALT value increased significantly compared to the control treatment, Fig. 1, ($P<0.05$). The results of measuring AST activity, Fig. 1, were similar to ALT, and enzyme activity increased with increasing salinity in acute ammonia poisoning (i.e. treatments 6, 7 and 8) and sub-acute ammonia poisoning treatments, i.e. treatments 10, 11 and 12.

The results of ALP, LDH and ACP activities (Fig. 1) were almost similar, as such ALP, LDH and ACP activities in treatment 3 and 4 were significantly higher than treatment 1 and 2. The activity of these enzymes in the treatments of acute and sub-acute ammonia poisoning was significantly increased compared to the control treatment. The increase of the activity of these enzymes in the interference treatments of salinity and poisoning was evident (Fig. 1).

The serum lactate levels exhibited a significant increase in response to the elevated salinity levels observed in treatments 2, 3, and 4, as depicted in Fig. 1. Furthermore, the findings indicated a substantial elevation in lactate levels during acute ammonia poisoning (treatment 5) when compared to the control treatment.

Serum oxidative stress factors

Relying on the data of Table 5, the impact of ammonia poisoning, salinity, and their interplay exhibited noteworthy influences on all five factors, with the exception of reduced glutathione (GSH).

The concentration of GSH shows no significant difference among salinity treatments (2, 3 and 4) compared to the control treatment (Fig. 2), yet the concentration of that in acute and sub-acute ammonia poisoning treatments (5 and 9, respectively) was lower than the control ($P<0.05$). Among salinity treatments (2, 3 and 4), catalase activity expressed significant fluctuations ($P<0.05$). The decrease to some degree was recorded for catalase activity in acute and sub-acute ammonia treatments (5 and 9, respectively) compared to the control (Fig. 2).

In conjunction with elevated salinity levels during acute and sub-acute ammonia poisoning interventions, a corresponding rise in catalase activity was observed. The SOD activity stated a significant decrease in the treatments of acute and sub-acute ammonia poisoning (5 and 9, respectively) compared to the control treatment (Fig. 2). According to the results of Fig. 2, with increasing salinity in ammonia poisoning treatments, a significant increase in SOD activity was recorded ($P<0.05$). The value of MDA in salinity treatments (namely 2, 3 and 4), acute ammonia treatment (namely 5) and sub-acute ammonia treatment (namely 9) was lower than the control (Fig. 2).

The serum total antioxidant capacity (TAC) showed fluctuations among salinity treatments, namely 2, 3 and 4 (Fig. 2). Figure 2 declares the values of TAC in acute and sub-acute ammonia poisoning treatments (namely 5 and 9, respectively) is lower than the control. Significant increases in serum TAC were observed with increasing salinity in acute (treatment 6, 7 and 8) and sub-acute ammonia poisoning treatments (namely 10, 11 and 12).

Liver oxidative stress factors

The impact of ammonia, salinity, and their interaction on the oxidative stress factors in liver tissue was assessed using a two-way ANOVA. The results, as presented in Table 6, indicated that CAT, SOD, GSH, MDA, and TAC were significantly influenced by these variables ($P<0.05$).

The results of measuring the liver GSH showed that the changes of this factor among the treatments were less compared to other factors (Fig. 3). The obtained results declared the liver CAT activity in treatments 3, 7, 8 and 11 had no significant difference compared to the control. The liver SOD measurement mentioned there were significant differences among all treatments except for treatment 2 and 12 ($P<0.05$). There were significant differences in the liver MDA content among the test treatments (Fig. 3). The results of comparing the average of the liver TAC, Fig. 3, expressed there were outstanding differences between all treatments but treatment 5 with treatment 6 and treatment 9 with 10.

Discussion

The present study aimed to examine the impact of acute ammonia toxicity on Nile tilapia fish. The study produced significant results concerning the 50% lethal concentration (LC50) of ammonia in tilapia fish across various time periods. Specifically, the LC_{50} values for ammonia were determined as 1.42 mg/L after 24 h, 1.356 mg/L after 48 h, 1.187 mg/L after 72 h, and 0.86 mg/L after 96 h.

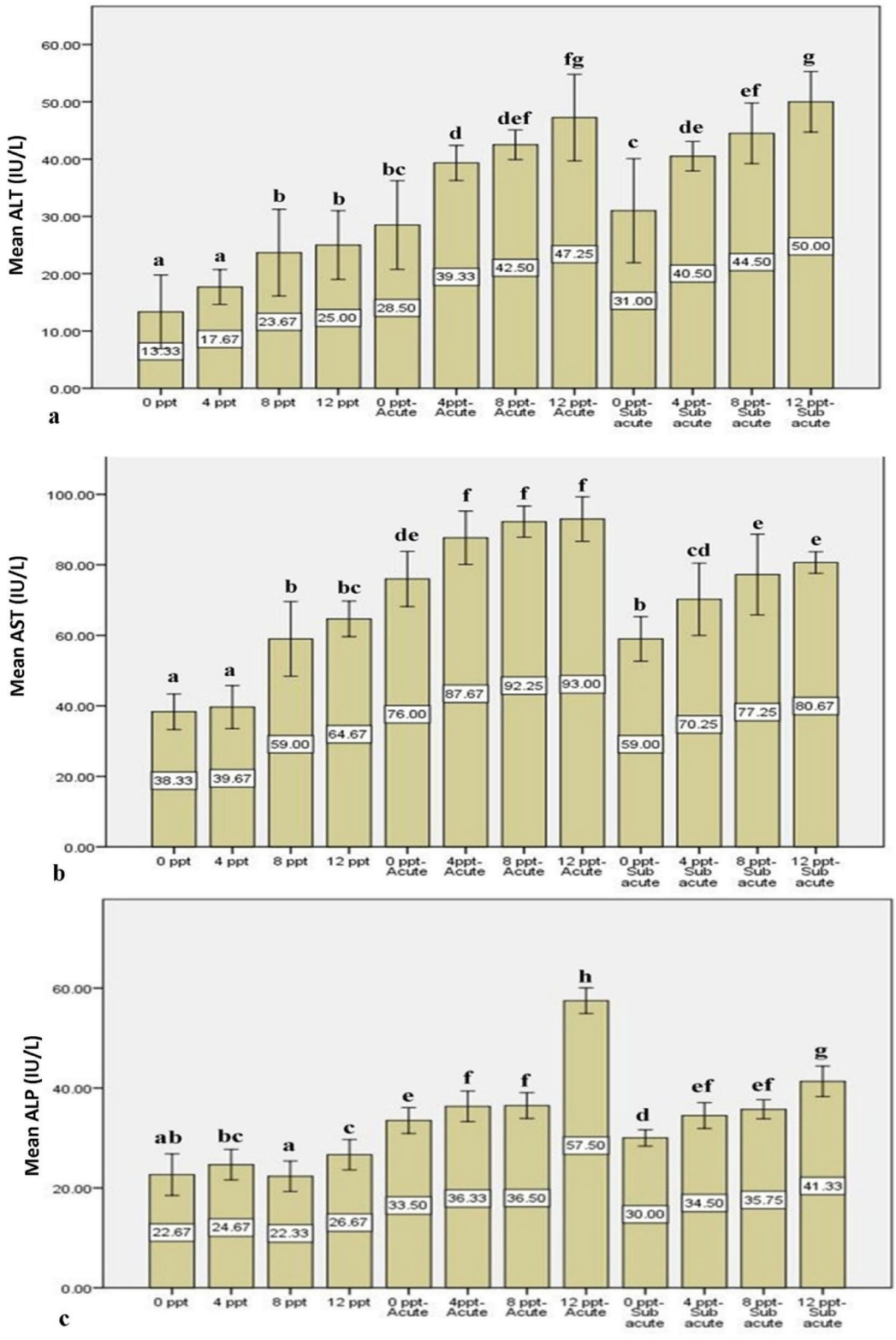

Fig. 1. The impact of varying degrees of ammonia-N poisoning, salinity, and their interplay on the levels of ALT (**a**), AST (**b**), ALP (**c**), LDH (**d**), ACP (**e**), and lactate (**f**) in Nile tilapia after a duration of 96 h (mean ± SD, $n = 5$). The existence of distinct letters indicates a significant difference ($P < 0.05$). ALT (Alanine Aminotransferase), AST (Aspartate Aminotransferase), ALP (Alkaline Phosphatase), LDH (Lactate dehydrogenase), ACP (Acid Phosphatase), IU (International Unit), dL (deciliter), mg (mili gram).

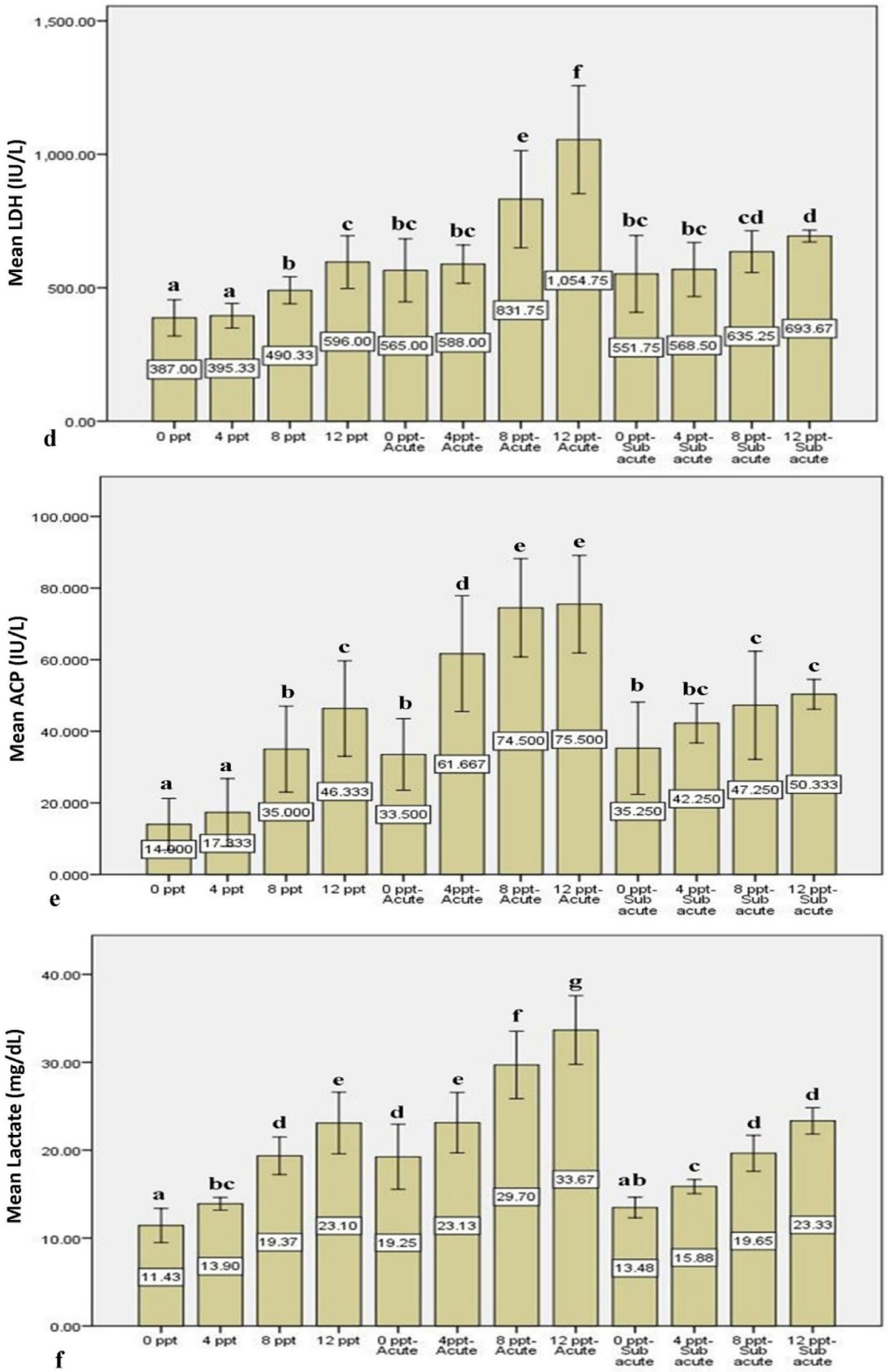

Figure 1. (continued)

The findings illuminate the possible detrimental effects of ammonia toxicity on Nile tilapia and offer significant guidance for future investigations in this field. Typically, the toxicity of chemicals in aquatic organisms is influenced by factors such as the age, size, and health conditions of the species. Various physical and chemical properties, including quality, dissolved oxygen, temperature, pH, water turbidity, type and quantity of aquatic plants, as well as the concentration and composition of chemicals, can fundamentally impact the lethal

	GSH	CAT	SOD	MDA	TAC
Ammonia	0.001	0.00	0.008	0.00	0.00
Salinity	0.197	0.002	0.00	0.00	0.00
Ammonia*salinity	0.166	0.00	0.00	0.00	0.00

Table 5. The p value obtained from two-way ANOVA showing the effects of ammonia-N poisoning, salinity and their interactions on oxidative stress factors of serum in Nile tilapia. A p-value less than 0.05 indicates a statistically significant difference in the variable of interest. GSH (Glutathione), CAT (Catalase), SOD (Superoxide dismutase), MDA (Malondialdehyde) and TAC (Total antioxidant capacity).

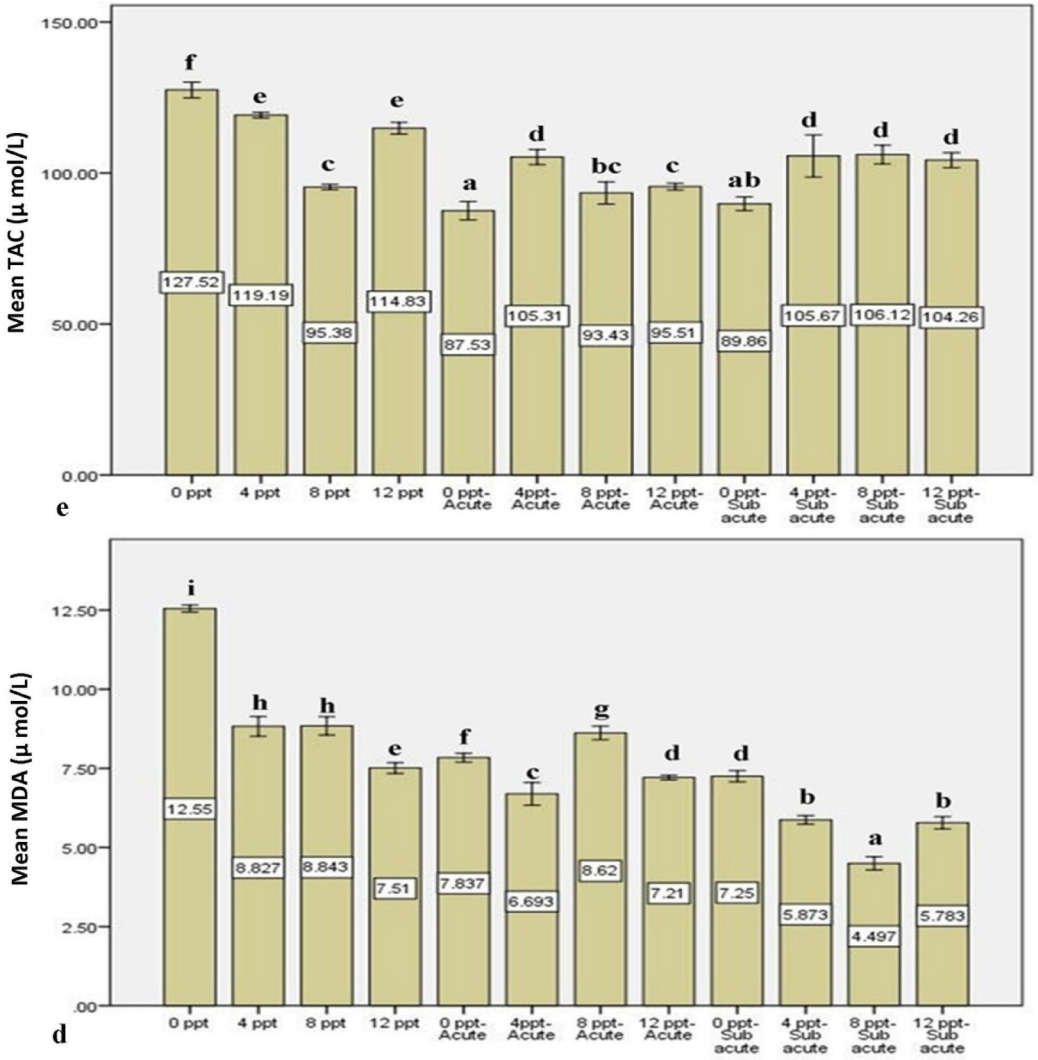

Fig. 2. The impact of varying degrees of ammonia-N toxicity, salinity, and their combined influence on the levels of serum SOD (**a**), CAT (**b**), GSH (**c**), MDA (**d**) and TAC (**e**) in Nile tilapia following a 96-hour exposure period (mean ± SD, $n = 5$). Statistical significance was indicated by the presence of distinct letters ($P < 0.05$). GSH (Glutathione), CAT (Catalase), SOD (Superoxide dismutase), MDA (Malondialdehyde) and TAC (Total antioxidant capacity). µmol (micromolar), L (litr), U (One unit of catalase activity is defined as the amount of enzyme required to decompose 1 micromole of H2O2 per minute at pH 7.0 and 25°C at a substrate concentration of 65 mM H2O2.)

concentration and toxicity tests[54]. The most crucial environmental variables influencing ammonia-N poisoning are pH, temperature, dissolved oxygen levels, and salinity[4,5,55–57]. To enhance the identification of the impact of salinity on ammonia levels, the study maintained two primary variables, namely pH and temperature, at nearly constant levels (refer to Table 3). NH3 levels were found to be the lowest in treatments 1, 2, 3, and 4 compared to the other experimental treatments. Typically, in the case of chronic ammonia poisoning, there is

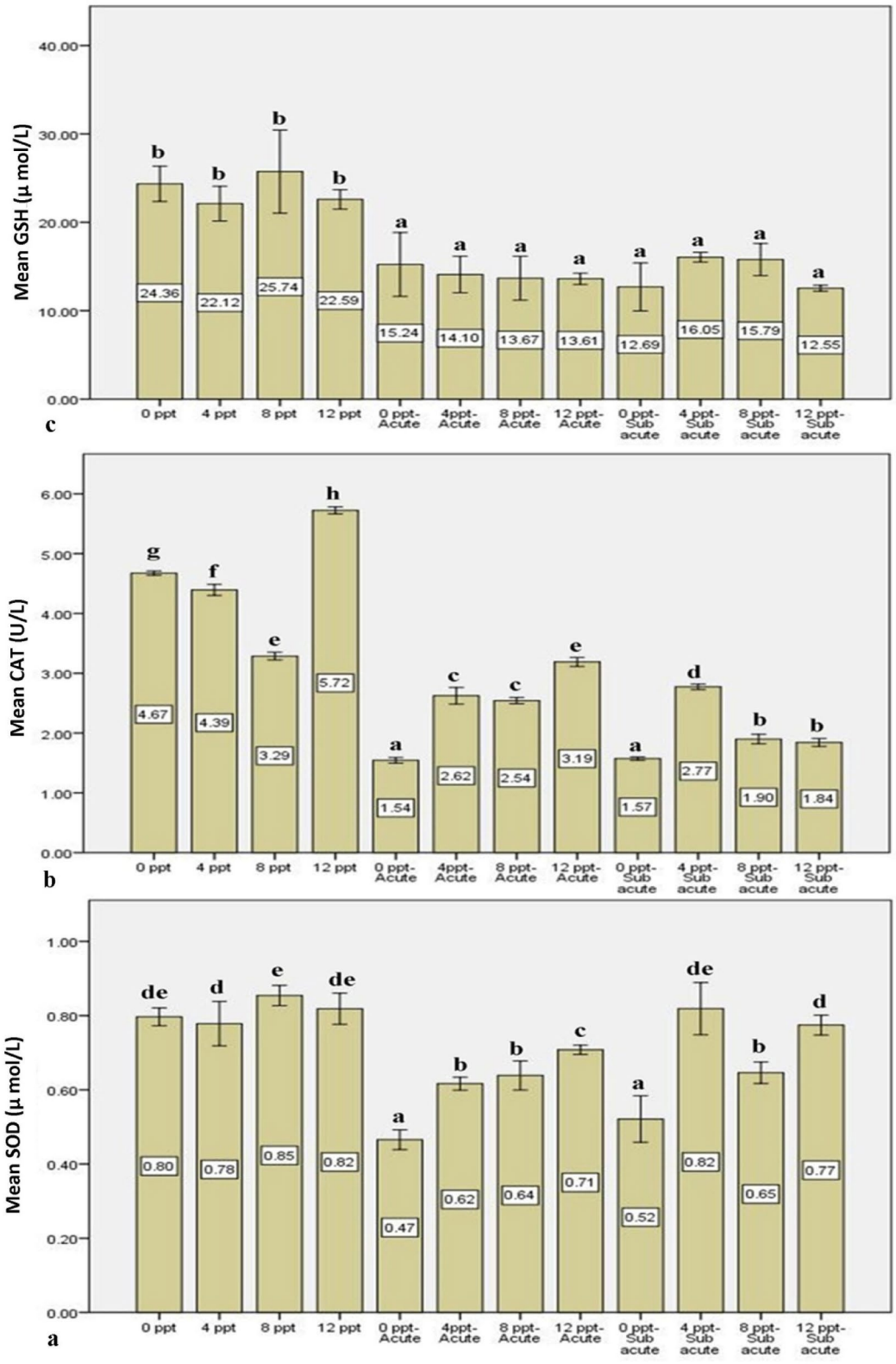

Figure 2. (continued)

a decrease in both the rate of growth and the percentage of survival among fish. Fish exposed to elevated levels of ammonia experience swift alterations in plasma composition[58] and accumulation of ammonia within their bodies (Rasmussen and Korsgaard, 1998).

	GSH	CAT	SOD	MDA	TAC
Ammonia	0.00	0.00	0.00	0.00	0.00
Salinity	0.004	0.00	0.00	0.00	0.00
Ammonia*salinity	0.00	0.00	0.00	0.00	0.00

Table 6. The obtained p value from the two-way ANOVA analysis showing the impact of ammonia-N poisoning, salinity, and their interactions on the oxidative stress factors of the liver in Nile tilapia. A *p-value* less than 0.05 indicates a statistically significant difference in the variable of interest. GSH (Glutathione), CAT (Catalase), SOD (Superoxide dismutase), MDA (Malondialdehyde) and TAC (total antioxidant capacity).

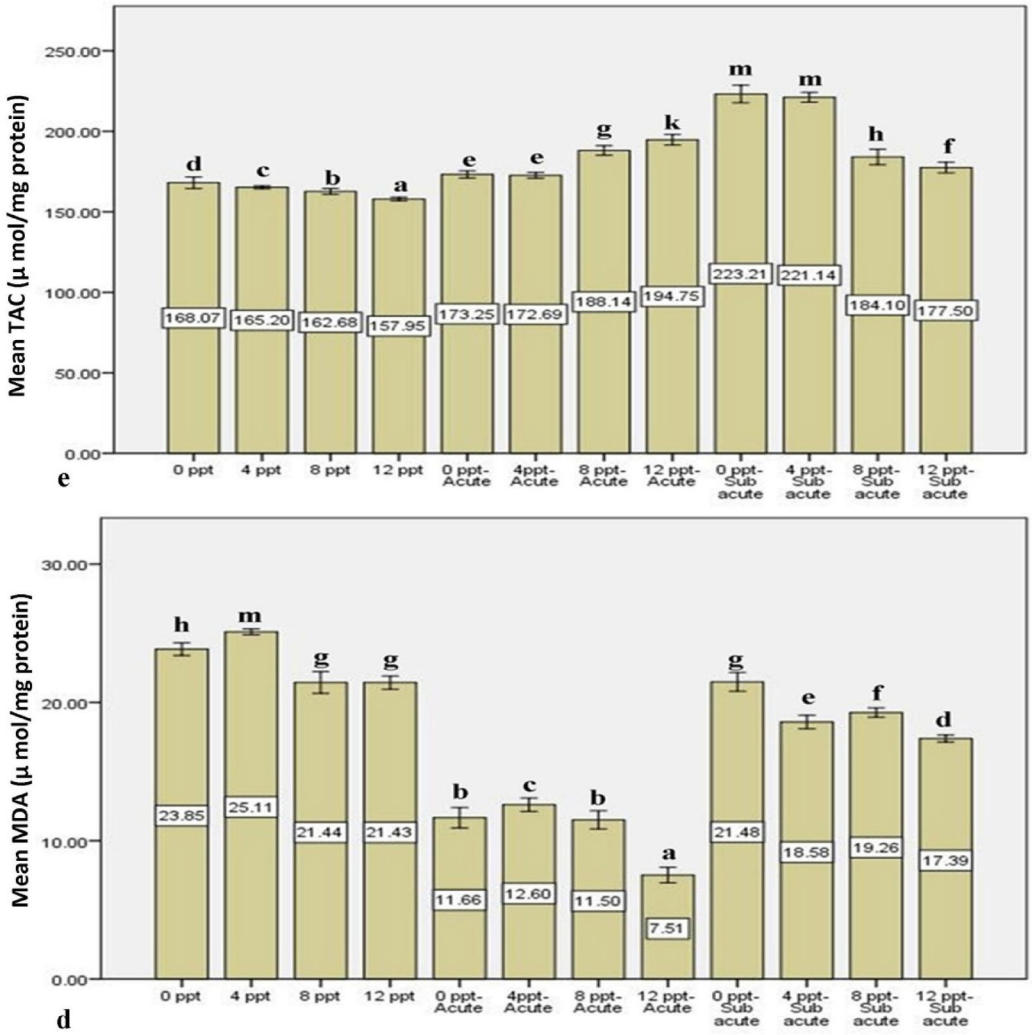

Fig. 3. The impact of varying degrees of ammonia-N toxicity, salinity, and their combined influence on the levels of liver GSH, CAT, SOD, MDA, and TAC in Nile tilapia following a 96-hour exposure period (mean ± SD, $n = 5$). The presence of different letters indicates a dramatically different ($P < 0.05$). GSH (Glutathione), CAT (Catalase), SOD (Superoxide dismutase), MDA (Malondialdehyde) and TAC (Total antioxidant capacity), µmol (micromolar), L (litr), U (One unit of catalase activity is defined as the amount of enzyme required to decompose 1 micromole of H2O2 per minute at pH 7.0 and 25°C at a substrate concentration of 65 mM H2O2.)

Serum enzymes and biochemical factors

Assessing stress induced by various stressors is commonly done through the evaluation of serum levels of glucose, lactate, and cortisol, which is widely regarded as a reliable approach[59]. The typical response to the elevation of catecholamines and corticosteroids is generally an augmentation in the synthesis of serum glucose[60]. Lactic acid is the predominant end product of anaerobic glycolysis[61]. Typically, the secretion of lactate is the subsequent reaction observed in response to stress[60]. Treatment 2, 3, and 4, which involved an elevation in salinity levels,

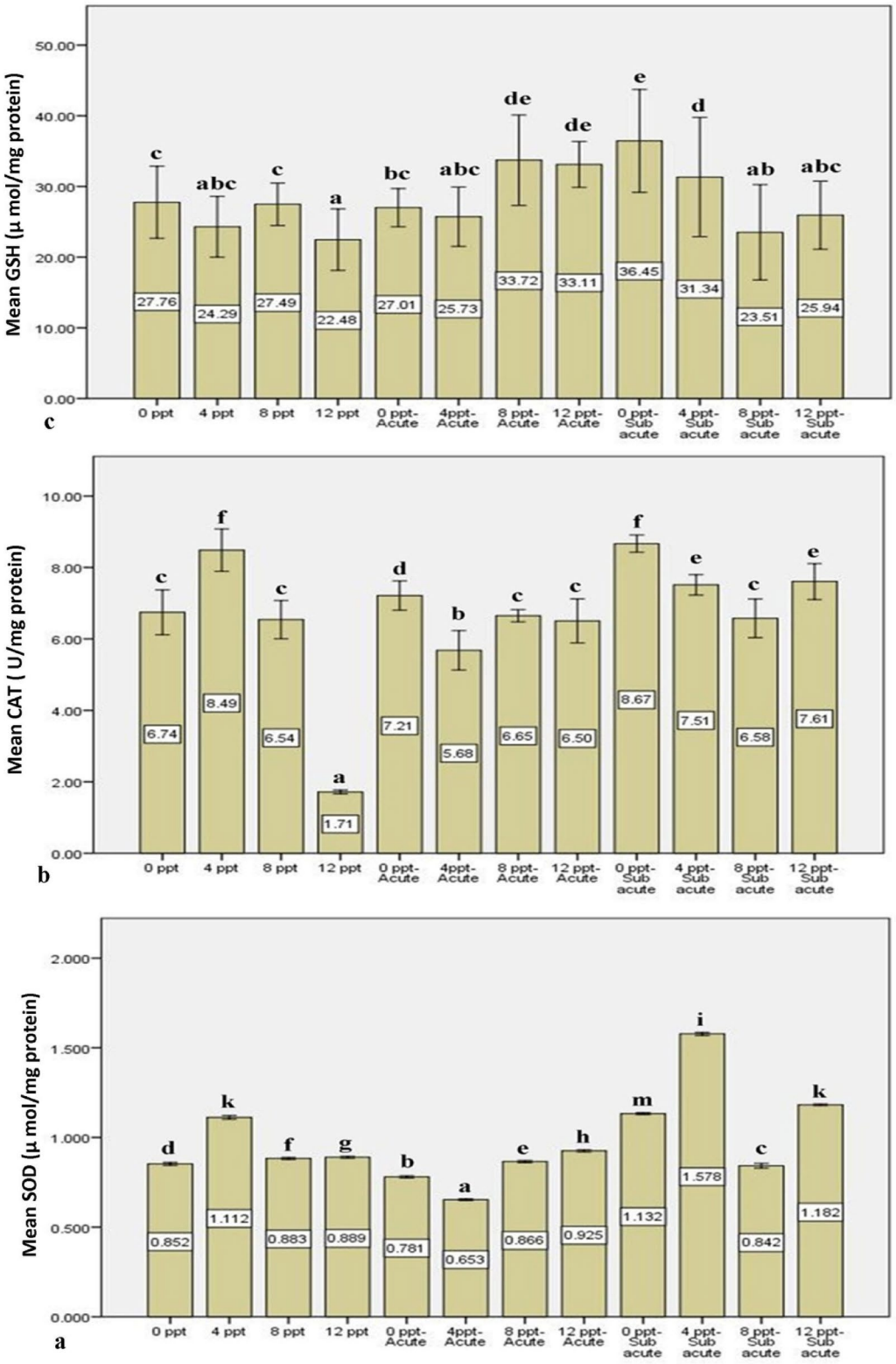

Figure 3. (continued)

resulted in a notable rise in lactate levels when compared to the control group. Tsui, et al.[62] investigated the impact of an abrupt salinity change on stress-related factors in *Epinephelus malabaricus* fish. Furthermore, it was observed that the concentration of lactate in the experimental treatments exhibited a relatively stable pattern for a duration of up to 60 min. The observed inconsistency in the results of the current study may be attributed to the specific species of fish examined.

The increase of lactate levels, after 96 h of exposure, confirms that secondary responses to salinity stress are activated in tilapia fish. The findings of the current study indicated that the levels of lactate in the treatment of acute ammonia poisoning, specifically treatment 5, exhibited a significant increase in comparison to the control group. Shi, et al.[63] explored the potential protective properties of *Sargassum horneri* against ammonia stress in black sea bream, *Acanthopagrus schleglii*. The study revealed a significant elevation in the lactate levels of the fish, attributed to the stress induced by ammonia, which is in agreement with the results of the present investigation. The results of our research were confirmed in *Megalobrama amblycephala*, *Labeo rohita*, and *Anoplopoma fimbria* when they were subjected to elevated concentrations of ammonia, as reported by Zhang, et al.[64], Acharya, et al.[65], and Kim, et al.[66], respectively. When fish are subjected to acute stressors, they activate anaerobic metabolism to satisfy urgent energy needs, which results in an unusual increase in lactate levels[67]. According to the findings of this study, the rise in salinity levels in treatments 6, 7, and 8 during acute ammonia poisoning, as well as treatments 10, 11, and 12 during sub-acute poisoning, resulted in an upward trend in lactate levels. This increase in salinity was observed in comparison to treatment 5 for acute poisoning and treatment 9 for sub-acute poisoning. The concurrent rise in ammonia and salinity clearly demonstrates a synergistic impact on the lactate level, serving as a reliable stress indicator.

The role of enzymes is very crucial in the normal metabolism of cells and their activity level is considered as a sensitive biochemical index[68]. Liver enzymes, alanine transaminase (ALT) and aspartate aminotransferase (AST), are used as a standard laboratory index to investigate liver disorders in animals. The present results stated the amounts of AST and ALT in 8 and 12 ppt, treatments 3 and 4, increased significantly compared to the treatment 2 and control. In normal conditions, the amount of these enzymes inside the cell is more than outside the cell. As a result, the increase in their values indicates the occurrence of cell damage in contrast to the types of pollutants introduced into the body[69,70]. The presence of AST and ALT enzymes has been detected in various tissues including the liver[71], heart[72], kidney, pancreas, spleen, red blood cell and gill tissues[73]. In the final stages of protein breakdown in order to produce adenosine triphosphate, these enzymes play an important role[72]. The increase in the levels of these enzymes indicates the consumption of amino acids in the processes of oxidation or glycogenesis[74]. According to our results, the salinity of 8 and 12 g/L causes damage to liver tissue during 96 h and probably the salinity of 4 g/L has quickly adapted. In terms of physiology, increase of the activity of ALT and AST in order to neutralize ammonia toxicity is one of the organism's behaviors[75]. The liver is a vital organ involved with pollutants, and liver damage and dysfunction affect the amount of these enzymes[76]. Therefore, the increase of the enzyme activities, LDH, ALT, AST and ALP, could be due to liver tissue damage. Based on researches, environmental parameters (season, salinity, temperature and density), sampling time, physiological parameters (fish species, age, gender and nutritional condition), how to prepare samples, accuracy and sensitivity of measurement procedures may affect the activity of these enzymes[77].

The results obtained from measuring ACP were almost similar to LDH. Increasing the levels of salinity (treatments 2, 3 and 4) and ammonia poisoning alone caused a considerable increase in ACP activity than the control treatment. Das, et al.[78], in the study of acute ammonia poisoning in *Cirrhinus mrigala*, Li, et al.[79], in investigating the effects of ammonia poisoning in yellow catfish, *Pelteobagrus fulvidraco*, and Das, et al.[80], in investigating the effect of nitrite poisoning in Indian carp species, *Catla catla*, *Labeo rohita* and *C. mrigala*, observed the activity of ACP increased in the serum.

Oxidative stress occurs when oxidants or oxidizing agents exceed the amount of antioxidant substances[81–83]. Accumulation of toxic substances causes the production of free radicals, especially ROS[84]. To counteract the toxic impact of reactive oxygen species (ROS), living organisms employ both enzymatic and non-enzymatic antioxidants to counterbalance or eliminate the presence of free radicals[84,85]. Enzymatic antioxidants (SOD, CAT) and non-enzymatic antioxidants like GSH play an essential role in removing free radicals[86].

Glutathione is a site for the enzymes glutathione peroxidase and S-glutathione transferase, playing a role in neutralizing toxins[87,88] Our results showed the level of GSH in tilapia serum had significant differences only in ammonia poisoning levels. According to the present results, salinity levels had no effect on serum GSH levels, nevertheless the reduction of GSH in poisoning treatments, 5 and 9, compared to the control treatment, indicates that ammonia levels can stimulate oxidative stress in fish[89–91]. GSH functions as a substrate in enzymatic reactions, facilitating the conversion of H2O2 into water and the reduction of lipid hydroperoxides. Consequently, alterations in the measured GSH levels serve as a crucial indicator of the antioxidant system's status[91].

The results of this study highlighted considerable fluctuations in liver GSH levels, attributable to three key factors: salinity, ammonia, and the interaction between these two variables. In treatment 4, the GSH level of the liver exhibited a notable reduction when compared to the control treatment. Several research investigations have demonstrated a clear correlation between salinity levels and the onset of oxidative stress in fish and various other aquatic organisms[57,92,93]. Treatment of sub-acute ammonia poisoning (T_9) compared to the treatment of acute ammonia poisoning and the control treatment (T_5 and T_1) showed a significant increase in GSH. Research has indicated that elevated levels of toxic ammonia have the potential to induce oxidative stress by either generating reactive oxygen species (ROS) or oxidizing lipids[89–91]. There was an increasing trend in liver GSH among different salinity levels in treatments of acute ammonia poisoning, T_5 to T_8, nonetheless values of liver GSH showed a decreasing trend among different salinity levels in treatments of sub-acute ammonia poisoning, T_9 to T_{12}. The decline in liver GSH levels could be attributed to the heightened utilization of GSH for the purpose of neutralizing or eliminating ROS. Based on the findings, it seems that increasing salinity in high concentrations of ammonia has a protective effect but in sub-acute level of ammonia, increasing salinity has no significant protective effect.

Superoxide dismutase serves as the primary enzymatic barrier against reactive oxygen species (ROS), with heightened SOD activity signifying an escalation in ROS generation and the subsequent onset of oxidative stress[94]. SOD is considered as an early warning of the environmental pollution and oxidative stress, and the

antioxidant capabilities of a biological system are estimated by measuring activity of SOD[95]. According to the findings derived from the analysis, there was no significant disparity was observed in the fish serum SOD activity levels among the salinity levels T2, T3, and T4, when compared to the control group. The findings indicated the formation and activity of ROS in the liver, unlike the serum, have been able to affect the antioxidant system of the tissue. Research has shown that environmental stresses that involve the osmotic regulation system can cause the formation of free radicals[57,96]. The decrease in levels of antioxidant enzymes could potentially be attributed to the engagement or elimination of the superoxide anion radical[97]. Conversely, a reduction in SOD activity may suggest a rise in lipid oxidation[98].

The control group exhibited higher levels of serum SOD activity compared to the ammonia poisoning treatment groups (T5 and T9), indicating a decline in SOD activity in these treatment groups. The data of the present research show the fact that with the increase in the amount of ammonia, the activity of SOD in the serum and liver decreased. Low level of ammonia stimulates more organs to produce more ROS, and as a result, high activity of SOD is seen in order to deal with more ROS[99]. The decrease in SOD activity, which could be due to the inability of SOD to overcome ROS, was observed with high doses of ammonia, and excessive ROS can inactivate SOD[100]. The reduction of SOD activity can be used as an indicator of the ability to remove free radicals in stressful situations, meanwhile it shows that the antioxidant defense system is weakened by ROS[95].

Sun, et al.[90], in investigating the effect of ammonia and microcysteine on the antioxidant system of bighead carp (*Hypophthalmythys nobilis*), stated with the increase of ammonia, SOD activity decreased significantly. Hong, et al.[101] investigated the immune response of Chinese crab (*Eriocheir sinensis*) in the face of increasing the amount of ammonia and observed that SOD activity decreased along with the increase of ammonia dose.

The results of the previously mentioned study are in strong agreement with the data collected in the present research. In the present study, with increasing salinity in acute (T_6, T_7 and T_8) and sub-acute ammonia poisoning (T_{10}, T_{11} and T_{12}), the serum SOD activity level exhibited a notable rise in comparison to the acute and sub-acute treatments (T5 and T9, respectively), and probably increased salinity had the effect of improving the activity of this enzyme. The liver SOD activity exhibited a significant decline followed by a substantial rise as the salinity levels increased during the occurrence of acute ammonia poisoning. Increasing salinity in the treatments of sub-acute ammonia poisoning (9, 10, 11 and 12) had a significant effect without a specific pattern on the level of liver SOD activity. It was said that in lower doses of ammonia, the production of ROS in the organs increases, and the decrease and increase of SOD activity in treatment 9 to 12 can be related to the consumption of SOD in order to deal with ROS (the reason for the decrease in SOD activity) and the replacement of SOD (The cause behind the rise in SOD activity) in dealing with oxidative stress.

Catalase, an enzyme present in the majority of bodily organs, facilitates the conversion of free radicals of H2O2 into water and oxygen[102]. The study demonstrates that fluctuations in salinity levels had a considerable effect on the functioning of the CAT enzyme.The activity of serum and liver CAT, along with increasing salinity, showed a fluctuating pattern. Physiologically, salinity changes in aquatic organisms affect stress hormones, electrolyte disturbance, homeostasis change and osmotic regulation mechanism[96,103]. Catalase prevents damage to macromolecules by converting H_2O_2 to water[104]. Increasing the enzyme activities, SOD and CAT, reduces the oxidation of lipids[105]. The general trend of decreasing CAT activity of serum and liver and on the other hand, decreasing the amount of MDA in T_2, T_3 and T_4 treatments enunciates that presumably the decrease of CAT activity was due to the reduction of the fight against free radicals (ROS) and oxidation. Caxico Vieira, et al.[93], in investigating the effect of salinity stress on the expression of antioxidant enzyme genes in Nile tilapia, observed that increasing the salinity up to 21 g/L after 24 h caused a significant decrease in the expression of CAT enzyme.

A significant decrease in CAT enzyme activity was noted in the treatments of acute and sub-acute ammonia poisoning (T5 and T9, respectively) when compared to the control group. This decline can be attributed to the elevated utilization of serum CAT in eliminating free radicals. The hepatic catalase activity in the treatments of acute and sub-acute ammonia poisoning exhibited a considerable increase compared to the control group. Moreover, the catalase activity level in sub-acute poisoning (T9) was markedly higher than that in acute poisoning (T5). It has been reported that the stimulation of organs in the production of ROS is higher in low ammonia concentrations[106], hence the mentioned report to great extent confirms the results of the present research. Considering the toxicity of ammonia, the reason for the increase of the activity of CAT in the liver is probably the increase of the amount of H_2O_2 resulting from the activity of SOD. The results of several researches show that ammonia-N poisoning increases the CAT activity of liver[106–108]. Probably, the metabolism of ammonia, which can ultimately lead to an increase in level of CAT, causes the production of free oxyradicals. Conversely, elevated CAT activity levels may suggest a rise in hydrogen peroxide levels as part of the detoxification mechanism within the cell.

The findings of our study indicated that elevated salinity levels in acute ammonia poisoning (treatments 6, 7 and 8 compared to 5) as well as sub-acute poisoning (treatments 10, 11 and 12 compared to 9) had an increasing effect on serum CAT activity. Presumably, this result is due to less toxicity of ammonia in higher salinity. The CAT function of the liver exhibited a significant reduction in activity in response to elevated salinity levels during acute ammonia-N poisoning (treatments 6, 7, and 8 in contrast to treatment 5) and sub-acute ammonia-N poisoning (treatments 10, 11, and 12 in contrast to treatment 9). Probably, the combined impact of salinity and ammonia caused the reduction of ammonia poisoning and subsequently the reduction of CAT activity in liver.

The research findings indicated that with rising salinity levels, there was a significant reduction in the concentration of MDA, which serves as an indicator of the oxidative degradation of omega-3 and omega-6 fatty acids in blood serum[109]. According to these results, with the increase of salinity in the water, the oxidation of serum fats declined. As salinity levels rise to 12 ppt in treatment 4, the amount of liver MDA decreased compared to the control, which states increasing salinity caused reduced lipid oxidation in the liver. The concentration of MDA found in serum and liver samples following treatments for acute and sub-acute ammonia-N poisoning (T5 and T9, respectively) was significantly reduced compared to the control group. It seems that the antioxidant

system of tilapia fish has a good performance in the face of ammonia levels from the oxidation of lipids against the generated ROS. Ding, et al.[110], in the study of the impact of salinity stress on the antioxidant response of a type of turtle (*Trachemys scripta elegans*), exhibited that there was a significant increase in liver MDA at 15 g/L salinity from 6 to 48 h and after that, the changes were not significant until 30 days. Sinha, et al.[108] stated that the amount of MDA in the liver of rainbow trout (*Oncorhynchus mykiss*) exposed to ammonia increased significantly compared to the control group at the end of 180 h. Sun, et al.[111] conducted a study on acute nitrite poisoning (0.1, 15 and 30 mg/L) for 48 h at *Megalobrama amblycephala* fish and observed a significant increase in MDA values. The differences in results between the previous studies and the present investigation could be attributed to variations in the species examined, the length of exposure, and the salinity conditions.

The combined impact of ammonia-N and salinity levels showed that the amount of MDA fluctuated with increasing salinity in acute ammonia (treatments 5, 6, 7 and 8) and sub-acute ammonia poisoning (treatment 9, 10, 11 and 12), and MDA changes probably in serum and liver with increasing salinity in ammonia poisoning treatments did not follow a specific pattern.

In the realm of antioxidant defense, the comprehensive measure of all enzymatic and non-enzymatic elements is referred to as total antioxidant capacity (TAC)[112]. Therefore, its measurement can help researchers with useful information regarding the changes in the system of total antioxidant defense and the reaction of the organism's body.

The findings indicated that as salinity levels increased in treatments 2, 3, and 4, there was a significant decrease in the serum TAC level compared to the control treatment. Probably, the increase in salinity alone caused an oxidative challenge for fish. The control treatment exhibited a significant decrease in the level of serum TAC when compared to both acute ammonia-N poisoning (treatment 5) and sub-acute ammonia-N poisoning (treatment 9). The reason for the reduction in TAC can probably be related to the production of ROS, which has involved the antioxidant system of the body. In general, it can be argued that with the increase of effective factors in creating oxidative conditions by ROS, the TAC also decreases[113].

Increasing the salinity in acute ammonia poisoning (treatments 6, 7 and 8) increased the level of serum TAC compared to acute ammonia poisoning (treatment 5), and also, the amount of serum TAC in the treatments of sub-acute ammonia poisoning and salinity (treatments 10, 11 and 12) was higher than the treatment of sub-acute ammonia poisoning (treatment 9). The reason for the increase of the serum TAC can be related to the decrease of ammonia due to the increase of salinity. As a result, the level of ROS produced is less and the antioxidant system is less affected by oxidant agents.

The results of TAC measurements in the liver of tilapia showed that in treatments 2, 3, and 4, as salinity levels rise, TAC decreased significantly. As ammonia levels rise in the environment, the level of TAC also shows a significant increase (control treatment < acute poisoning < sub-acute poisoning). The cause of this increase is probably related to the increase in the amount of ROS in the liver, which has caused more stimulation of the body in the production of antioxidant agents. According to the results, it is possible that the amount of ROS in the liver is higher in the treatment of sub-acute ammonia poisoning than in the treatment of acute poisoning and in order to neutralize the stimulatory factors of oxidative stress, the production of the antioxidant factors in the liver has increased. Similar to the results of serum measurements, increasing salinity in the treatments of acute ammonia poisoning (treatments 5, 6, 7 and 8) caused an increasing effect on the TAC value of the liver, which was presumably due to the improving effect of salinity in reducing ambient ammonia. Nonetheless, increasing salinity in the treatments of sub-acute ammonia poisoning (treatments 9, 10, 11 and 12) caused a significant decrease in the level of TAC of the liver. The cause of this decrease can likely be related to the higher amount of ROS in sub-acute treatments and the increase of salinity not only had no ameliorating effect, but the increase in salinity itself was a contributing factor in increasing ROS in the liver and subsequently reducing TAC.

Conclusions

The results of our study suggest that while salinity can decrease the concentration of ammonia in water, it also leads to stress in test subjects over a 96-hour period. Also, serum lactate level in acute ammonia poisoning was significantly increased. The reduction of serum GSH and TAC levels and SOD and CAT activities in ammonia poisoning treatments indicates that ammonia can stimulate oxidative stress in fish. Increasing salinity in acute ammonia poisoning treatments (T_6,T_7 and T_8) caused an increasing effect on the liver TAC. During the course of the experiment, it can be observed that the combination of salinity, ammonia, and their interplay had detrimental impacts on the physiological aspects of the fish. To obtain more comprehensive data, it is recommended to assess immune factors and pathology in addition to extending the duration of the experiment.

Data availability

Data will be made available from the corresponding author on credible request.

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Author contributions

Motamedi-Tehrani .J wrote the main manuscript text and prepared figures . All authors reviewed the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Ethics approval

This research was carried out according to the standards of the animal protection and ethics committee of Shahid Chamran University, Ahvaz, Iran.

Additional information

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