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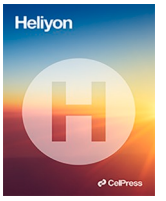

Research article

Combined effects of ammonia-N exposure and salinity changes on hematological and serum biochemical factors and thyroid hormones in Nile tilapia (*Oreochromis niloticus*)

Javad Motamedi-Tehrani [a,*], Rahim Peyghan [a], Ali Shahriari [b], Mohammad Razijalali [a], Eisa Ebrahimi [c]

[a] *Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran*
[b] *Department of Basic Sciences, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran*
[c] *Department of Natural Resources, Isfahan University of Technology, Isfahan, Iran*

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ABSTRACT

The aim of this research was to evaluate the interaction effects of ammonia-N levels and salinity on hematological and serum biochemical parameters in Nile tilapia (*Oreochromis niloticus*). The fish were randomly divided into 12 treatments including the levels of salinity (0, 4, 8 and 12 ppt) and 0, 50% of LC50-96 h of ammonia-N and 30% of LC50-96 h of ammonia-N in a factorial design (4 salinity levels x 3 ammonia levels). Hemoglobin value in all treatments, except for salinity treatments, namely 2, 3, 4, showed a significant decrease than the control (0 ppt and no poisoning). Also, red blood cells in treatment ammonia-N levels were significantly less than the control. Serum protein concentration, in treatments 9 (50% of LC50-96 h of ammonia-N) and 5 and also with increasing salinity (treatments 2, 3 and 4) had a significant decrease compared to the control. There is a significant increase in serum glucose, cortisol, ammonia and urea levels in 50% and 30% of LC50-96 h of ammonia-N treatments compared to the control, meanwhile these parameters were significantly increased with increasing salinity. Serum thyroid stimulating hormone (TSH), T3 and T4 levels in acute and sub-acute ammonia-N treatments were significantly lower than the control. Moreover, with increasing salinity in 50% and 30% of LC50-96 h of ammonia-N treatments, TSH showed a decreasing pattern. According to the results, fluctuations in blood biochemical factors, increase of stress and decrease of thyroid hormones show that the salinity, ammonia, and their interaction caused adverse effects on fish health during the 96 h of testing.

1. Introduction

Nitrogen pollution in aquatic animals is largely due to ammonia, which is produced during the final stage of protein catabolism [1]. It can also pollute aquatic environments in various ways such as agricultural effluents, industrial pollution and biological wastes from decomposition [2,3]. In aquatic environments, ammonia has two chemical forms, unionized ammonia (NH_3) and ionized ammonium (NH_4^+) [4]. Environmental parameters such as temperature, pH, oxygen and salinity can affect the toxicity of ammonia in aquatic

* Corresponding author.
E-mail address: jmotamedi124@gmail.com (J. Motamedi-Tehrani).

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animals [5,6]. NH_3, unlike NH_4^+, can pass through the branchial epithelium [7]. The increased concentration of the ammonia in water pond will have adverse effects like fish growth reduction, tissue damage, immune suppression, a disorder in Kidney and liver function, and high mortality [5,6]. It is well accepted that ammonia is one of the main contaminants in aquaculture [7,8], therefore dwindling the ammonia concentration is one of the most important issues in aquaculture.

Salinity is one of the factors affecting the osmotic pressure of the aquatic environment and changing the osmotic pressure of the environment leads to a change in aquatic metabolism [9]. Salinity refers to the value of soluble salts in water and is expressed as parts per thousand (ppt) or Practical Salinity Unit (PSU) or g/L. Most of the salinity of water resources can be attributed to factors such as evaporation from the water surface; dissolution of evaporite outcrops in the reservoir; the inflow of saline surface water; discharge of saline groundwater from adjacent aquifers through subaquatic springs or seepage areas [10–13]. Temperature and rainfall are two main parameters in seasonal salinity changes that can change the physiology of aquatic organisms [14,15]. On the other hands, salinity is one of the factors that can reduce ammonia toxicity. Indeed, the concentration of the non-ionized ammonia was decreased along with increasing water salinity level.

Iran is located in a semi-arid region with annual rainfall lower than one third of the world average. As well as, the temporal and spatial distribution of rainfall is not integrated [16]. It is predicted that Iran will be one of the 27 countries to face increasing water shortage until 2025 [17]. Uncontrolled use of groundwater has adversely effects on the quality and quantity of aquifers [16]. According to the given description, it is possible that surface and groundwater resources become salty, and Iran will be a good candidate for fostering fish adapted to salinity.

It is possible that ammonia poisoning can occur at various salinity levels, as mentioned in the previous paragraphs [16,18,19]. Therefore, the need to investigate the interaction between ammonia and salinity on the physiology of freshwater fish is clear, On the other hand, so far there has been little discussion about this interference effect, Salinity and ammonia. Consequently, we designed the present study to evaluate the interference effect of salinity levels on ammonia-N levels in Nile tilapia, *Oreochromis niloticus*, which has a good adaptation and growth in brackish water, and even illustrates better growth than in freshwater [20,21]. Nile tilapia is one of the most popular species and an essential alternative [22–24] for cultured freshwater species in dry and water-deficient areas like Iran. This study will assist in a better understanding of changes in physiological factors such as hematological and serum biochemical indicators in response to salinity, ammonia-N and their interference effect in an aquaculture setting.

2. Materials and methods

2.1. Fish husbandry

This research was performed in accordance with the standards and animal care committee of Shahid Chamran University of Ahvaz from October 2019 to December 2020. Nile tilapia juveniles (*Oreochromis niloticus*) were obtained from a fish breeding center in Qom city, then fish were transported in oxygenated cellophane bags to the Partako breeding center, Tiranchi village, Isfahan. The fish, 57.32 $\pm$ 16.7 gr and healthy in appearance, were randomly selected for experiments. In order to adapt to the conditions of the laboratory, the fish were kept in clean and completely disinfected aquariums (100 L) for one week. The fish were fed with commercial food, 40% protein-30% carbohydrates-7%Crude fat-6%Crude fiber-7% ash- 10% moisture, (Kimiagaran, Iran) in the morning and the evening. During the time of adaptation, no mortality was observed.

2.2. Adjust acidity

In order to adjust acidity of water with constant acidity, a specific amount of hydrochloric acid (0.4 N, Merck, Germany) and sodium hydroxide (0.4 N, Merck, Germany) was added to the tank water. Having added acid and base, the acidity of the tank water was measured. Water acidity was continuously measured for one week. Having been fixed, the acidity of the water, this water was used throughout the experiment.

2.3. Physical and chemical factors of water

Total ammonium nitrogen (TAN) was evaluated using the Köldahl system and titration [25]. Owing to the value of temperature and pH along with the standard table, NH_3 was calculated [26]. Electrical conductivity (EC) and acidity were measured using an EC meter (Conductivitymeter 4310, Jenway, UK) and a pH meter (744 pH meter, Metrohm, UK), respectively.

2.4. Determination of 50% lethal concentration (LC_{50}) of ammonia in tilapia fish

LC_{50} determination test based on static method, changing water once every 24 h and replacing the desired concentrations again, was investigated. In this method, two concentrations of ammonium chloride (Merck, Germany) with 100% mortality and without losses were prepared. Afterward, six concentrations of ammonium chloride were exponentially determined between the range of the two previous doses. Then, after the adaptation period, the fish were divided into 6 groups (10 fish per group). The fish were checked for 96 h and the mortality rate was recorded every 24 h [27]. The temperature and pH were kept constant during the experiment period. After recording fatality, LC_{50} was calculated at 24, 48, 72 and 96 h with probit software.

2.5. Experimental design

Twelve treatments including 3 levels of ammonia-N (0, 30% and 50% of LC50-96 h), 4 levels of salinity (0, 4, 8, and 12 ppt) and interactive treatments of ammonia toxicity and salinity were designed (Table 1). 50% and 30% of LC_{50}-96 h was considered as acute and sub-acute poisoning, respectively [28]. Each treatment consisted of five repetitions. The water was changed daily and completely during 96 h. Total ammonium-N, pH and EC were daily measured. Aeration was saturately carried out throughout the experiment.

2.6. Blood sampling

At the end of the 96 h of the test period, blood sampling was done with insulin syringes. The fish were anesthetized by 2-phenoxyethanol and were dried with a towel. Blood sampling was done with heparin and non-heparin syringes via a caudal vein. Blood samples were spun down at 3000×*g* for 10 min in non-heparinized tubes to extract serum. The supernatant was removed, separated into aliquots, and stored at −70 °C (Ultra Low Temperature Freezer, MDF-U71VC, Sanyo, Japan) until analyses were performed. Blood samples in heparinized tubes were used to measure hematological factors.

2.7. Hematological factors

Hematocrit (Hct), total hemoglobin (Hb), and red blood cell (RBC) count were done in total blood. Hct was determined in blood samples centrifuged at 12,000×*g* for 5 min in capillary tubes [29]. In order to measure Hb concentration, kit of hemoglobin measurement was used (Zistshimi, Iran). Total Hb was determined via the use of the spectrophotometric device (JENWAY 6400 spectophotomet, UK) at 540 nm [30]. In order to separate the nuclei of lysed red blood cells, one centrifugation step, 3000 rpm for 5 min, was performed. RBC pipette was used to count the number of RBC. RBC were counted under a light microscope with a Neubauer chamber [31].

2.8. Measurement of serum factors

2.8.1. Total protein

According to the standard Bioreh method, the total protein of blood serum was measured using a Pars Azmoon laboratory kit (Protein kit, Biorex, Pars Azmoon, Iran) and the auto-analyzer device (Auto Analyser Biotechnica, BT 1500, Italy).

2.8.2. Determination of cholesterol

Based on colorimetric method, cholesterol measurement was done using the auto-analyzer device (Auto Analyser Biotechnica, BT 1500, Italy) and the Pars Azmoon laboratory kit (Cholesterol kit, Pars Azmoon, Iran).

2.8.3. Glucose assay

Owing to the production of the quinonemine which has a direct relationship with glucose levels, glucose level was measured using the auto-analyzer device (Auto Analyser Biotechnica, BT 1500, Italy) and the Pars Azmoon laboratory kit (Glucose kit, Pars Azmoon, Iran).

2.8.4. Cortisol hormone measurement

Cortisol hormone level was assayed using the Monobind test kit (Monobind Inc, USA) and ELISA method, based on the enzyme immunoassay including the primary reaction between antigen and antibody using a marker.

2.8.5. Serum ammonia and urea

The measurement of serum ammonia was performed by the Milad Pathobiology Laboratory, Isfahan, Iran. Based on the Urease-

Table 1
Experimental treatments during 96 h.

Treatment	
$\mathbf{T_1}$	Adjusted water (control)
$\mathbf{T_2}$	4 ppt of salinity
$\mathbf{T_3}$	8 ppt of salinity
$\mathbf{T_4}$	12 ppt of salinity
$\mathbf{T_5}$	50% of LC_{50}-96 h
$\mathbf{T_6}$	50% of LC_{50}-96 h- 4 ppt of salinity
$\mathbf{T_7}$	50% of LC_{50}-96 h- 8 ppt of salinity
$\mathbf{T_8}$	50% of LC_{50}-96 h- 12 ppt of salinity
$\mathbf{T_9}$	30% of LC_{50}-96 h
$\mathbf{T_{10}}$	30% of LC_{50}-96 h- 4 ppt of salinity
$\mathbf{T_{11}}$	30% of LC_{50}-96 h- 8 ppt of salinity
$\mathbf{T_{12}}$	30% of LC_{50}-96 h)- 12 ppt of salinity

GLDH enzyme method, urea measurement was done using UREA UV diagnostic kit of Pars Azmoon (Urea kit, Pars Azmoon, Iran) and photometric method.

2.8.6. Thyroid hormones

According to the competitive ELISA method and monoclonal antibodies, the measurement of thyroid stimulating hormone (TSH), T_4, and T_3 hormones was designed using a laboratory kit (Thyroid hormones kit, Pishtazteb, Iran).

2.9. Statistical analysis

This research was carried out in a factorial design (4 salinity levels x 3 ammonia levels). Results are presented as means ± standard deviation (SD). All data were subjected to the Kolmogorov-Smirnov test to check their normality, followed by Levene's test for homogeneity of variance. All data were analyzed using two-way analysis of variance (ANOVA), save for physical and chemical factors of water assessed using one-way ANOVA. In order to evaluate differences between means, the data were analyzed by Tukey's post hoc test. Only differences where $P < 0.05$ were regarded as statistically significant. Statistical analysis was performed by SPSS software version 24.

3. Results

3.1. LC_{50}-96 h value of ammonia

The results obtained from measurement of LC_{50} ammonia-N were shown in Table 2. The LC_{50} ammonia-N of 24, 48, 72 and 6 h was 1.42, 1.365, 1.187 and 0.86 mg/L, respectively. Ammonia-N concentrations in our research were calculated based on the LC50- 96 h.

3.2. Water quality factors

Based on Table 3, there were no significant differences in temperature and pH factors. In treatments 5, 6 and 7, NH_3 concentration showed a significant difference compared to other treatments ($P < 0.05$). NH_3 concentration in 50% of LC_{50}-96 h ammonia-N −12 ppt of salinity, namely treatment 8, was less than the treatment 5 ($P < 0.05$). NH_3 measured in treatments 1, 2, 3 and 4 was the lowest value among experimental treatments.

3.3. Hematological factors

The ammonia variable showed significant effects on hematological factors (Table 4). The effect of salinity variable and the interaction between salinity and ammonia poisoning on the hemoglobin factor were not significant. The salinity and the interaction effect in RBC factor did not show any significant effect (Table 4).

Because there were many treatments and variables to some extent, in order to understand clearly the effects of variables, we compared treatments 2, 3 and 4 with the control treatment, treatments 5 and 9 with the control treatment, treatments 6, 7 and 8 with the treatment 5, and treatments 10, 11 and 12 with the treatment 9.

Table 5 states, in Hb, all treatments, except for treatment 2, 3 and 4, showed a significant decrease compared to the control treatment ($P < 0.05$). Salinity treatments, namely 2, 3, 4, and acute ammonia poisoning treatment, namely 5, had a significant increase compared to the control treatment, in hematocrit factor ($P < 0.05$). RBC factor in salinity treatments, 2, 3 and 4, and treatment of 30% of LC_{50}-96 h of ammonia-N, namely treatment 9, has no significant difference compared to the control treatment, yet RBCs in treatment of 30% of LC_{50}-96 h of ammonia-N, namely treatment 5, were less compared to the control treatment ($P < 0.05$).

3.4. Serum biochemical indices

The effect of ammonia-N level, salinity levels and their interaction on total protein, cholesterol, glucose, and cortisol factors was performed using two-way variance test (Table 6). Salinity and ammonia variables and their interaction effects on all mentioned factors were significant ($P < 0.05$).

The protein concentration, in treatments 9 and 5 and also with increasing salinity (treatments 2, 3 and 4) had a significant decrease compared to the control treatment [Fig. 1 (a-d)]. Based on the results of Fig. 1, there were significant changes in the total protein concentration with increasing salinity in 50% of LC_{50}-96 h of ammonia-N treatments (namely 6, 7 and 8) compared to treatment 5,

Table 2
LC_{50} values of ammonia in different times in Nile tilapia.

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 3
Physical and chemical factors of water during 96 h (mean ± standard deviation, n = 4).

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

The presence of different letters in each column indicates a significant difference ($P < 0.05$).

Table 4
The p value obtained from two-way analysis of variance showing the effects of ammonia-N poisoning, salinity, and their interactions on hematological factors in Nile tilapia.

	Hemoglobin	Hematocrit	RBC
Ammonia	0.00	0.00	0.00
Salinity	0.31	0.03	0.128
Ammonia*salinity	0.095	0.00	0.218

$P < 0.05$ indicates a significant difference in the desired variable.

Table 5
The effects of different levels of ammonia-N and salinity on hematological indices of Nile tilapia after 96 h (mean ± standard deviation, n = 5).

Treatment	Hemoglobin (g/dL)	Hematocrit (%)	**RBC ($*10^6$ cell/mm^3)**
1	14.09 ± 0.69^{bc}	24.2 ± 2.48^{b}	1.878 ± 0.074^{bcd}
2	14.19 ± 0.43^{bc}	27 ± 1.73^{def}	1.93 ± 0.014^{cde}
3	14.75 ± 0.41^{bc}	28.6 ± 0.88^{f}	1.95 ± 0.011^{cde}
4	13.44 ± 0.56^{b}	27.6 ± 0.89^{ef}	1.92 ± 0.042^{cde}
5	10.04 ± 0.24^{a}	26.2 ± 0.83^{cde}	1.68 ± 0.046^{a}
6	10.56 ± 0.69^{a}	25.4 ± 0.54^{bcd}	1.77 ± 0.088^{ab}
7	10.39 ± 0.8^{a}	26.2 ± 1.09^{cde}	1.83 ± 0.015^{abc}
8	9.81 ± 0.76^{a}	24 ± 1.58^{b}	1.66 ± 0.47^{a}
9	10.19 ± 0.24^{a}	24.33 ± 1.03^{bc}	1.97 ± 0.047^{cdef}
10	9.79 ± 0.57^{a}	24.24 ± 0.95^{bc}	2.06 ± 0.085^{ef}
11	10.48 ± 1.23^{a}	22 ± 2.16^{a}	2 ± 0.081^{de}
12	10.87 ± 2.01^{a}	21.75 ± 1.06^{a}	2.1 ± 0.018^{f}

The presence of different letters in each column indicates a significant difference ($P < 0.05$).

Table 6
The p value obtained from two-way analysis of variance showing the effects of ammonia-N level, salinity, and their interactions on serum biochemical indices in Nile tilapia.

	Total protein	Cholesterol	Glucose	Cortisol
Ammonia	0.00	0.00	0.00	0.00
Salinity	0.00	0.00	0.00	0.00
Ammonia*salinity	0.00	0.031	0.005	0.00

$P < 0.05$ indicates a significant difference in the desired variable.

however, the protein concentration showed no significant difference in 30% of LC_{50}-96 h of ammonia-N treatments (namely 10, 11 and 12) compared to treatment 9.

The amount of serum cholesterol was decreased with increasing salinity up to 8 ppt, treatment 3, and increased in the 12 ppt, namely treatment 4, ($P < 0.05$). The cholesterol level in treatment 5 was significantly increased compared to the control treatment, nevertheless treatment 9 showed no significant difference compared to the control treatment [Fig. 1 (a-d)]

The results of Fig. 1 declare there is a significant increase in glucose and cortisol levels in 50% and 30% of LC_{50}-96 h of ammonia-N, namely treatments 5 and 9 respectively, compared to the control treatment, meanwhile glucose and cortisol levels were significantly

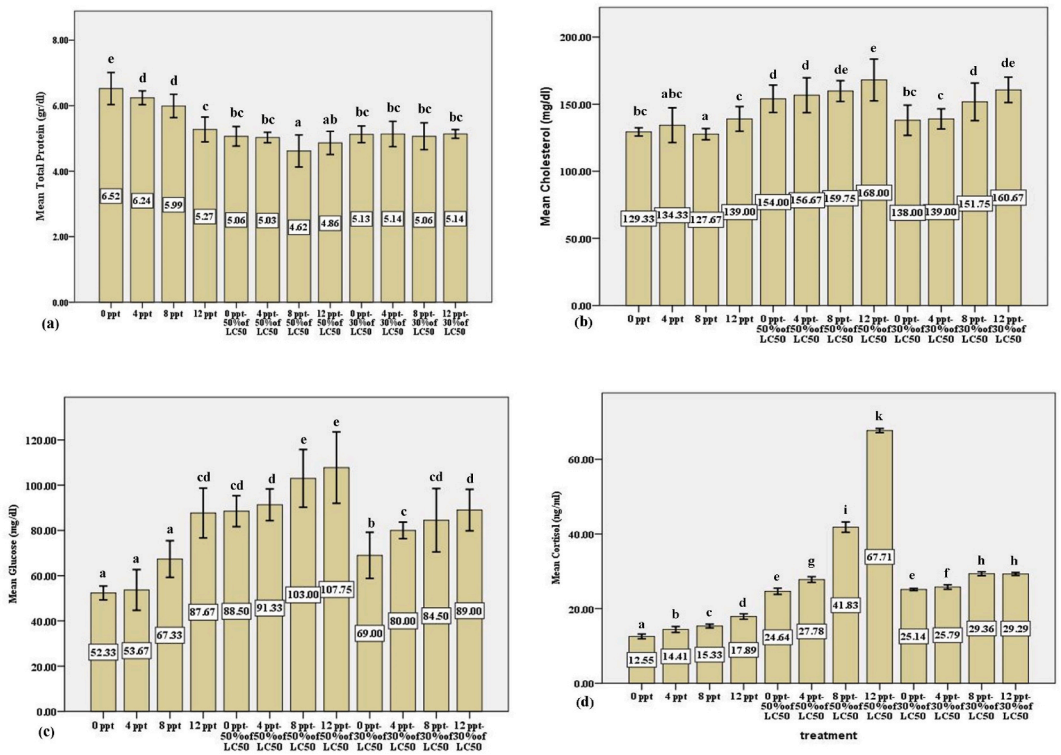

Fig. 1. The effects of different levels of ammonia-N and salinity on serum factors of Nile tilapia after 96 h (mean ± SD, n = 5), (**a**): Total protein, (**b**): Cholesterol, (**c**): Glucose, (**d**): Cortisol. The presence of different letters indicates a significant difference ($P < 0.05$).

increased with increasing salinity among 50% of LC_{50}-96 h of ammonia-N treatments (6, 7 and 8) compared to treatment 5 and 30% of LC_{50}-96 h of ammonia-N treatments (10, 11 and 12) compared to treatment 9.

3.5. Serum ammonia and urea

The results of two-way ANOVA of ammonia-N and urea are presented in Table 7. The variables of salinity, ammonia and their interaction had significant effects on ammonia and urea of the serum ($P < 0.05$).

Blood ammonia concentration in treatments 5 and 9 was significantly increased compared to the control treatment [Fig. 2 (a-b)]. Also, almost increasing salinity in all treatments caused an increase effect on the serum ammonia level.

Based on the results of Fig. 2, increasing salinity (treatments 2, 3 and 4) had an increasing effect on serum urea ($P < 0.05$). The concentration of urea in the treatment of 50% of LC_{50}-96 h of ammonia-N (treatment 5) and 30% of LC_{50}-96 h of ammonia-N (treatment 9) had a significant increase compared to the control treatment [Fig. 2 (a-b)].

3.6. Serum thyroid hormones

The variables of salinity, ammonia-N and their interaction had a significant effect on serum thyroid hormones, T_3, T_4 and TSH, except for the salinity variable on the TSH (Table 8).

The results of Fig. 3(a–c) expressed salinity treatments (namely 2, 3 and 4) had a decreasing effect on T_3 and T_4 concentration compared to the control treatment ($P < 0.05$). Also, the levels of T_3 and T_4 in the treatments of 50% and 30% of LC_{50}-96 h of ammonia-N, namely 5 and 9 respectively, were lower than the control treatment ($P < 0.05$).

Table 7
The p value obtained from two-way analysis of variance showing the effects of ammonia-N level, salinity, and their interactions on serum ammonia and urea in Nile tilapia.

	Ammonia	Urea
Ammonia	0.00	0.00
Salinity	0.00	0.00
Ammonia*salinity	0.00	0.00

$P < 0.05$ indicates a significant difference in the desired variable.

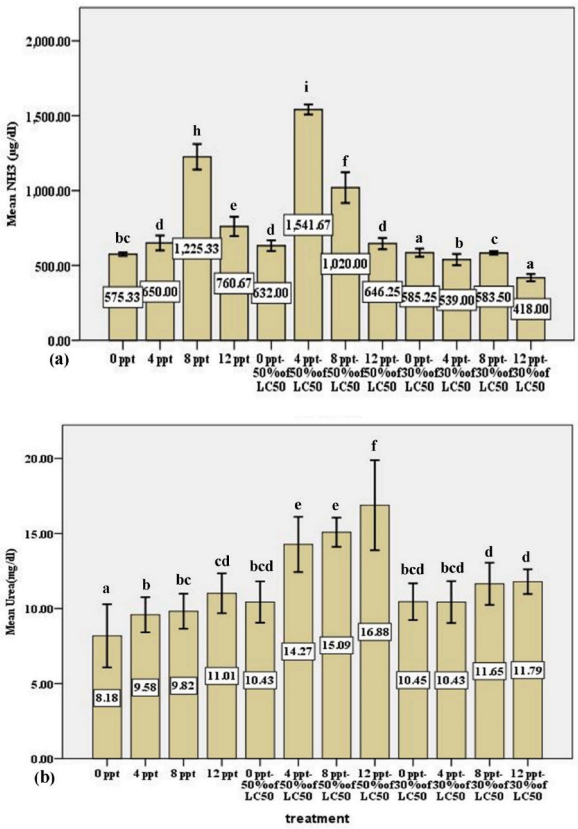

Fig. 2. The effects of different levels of ammonia-N and salinity on nitrogenous excreta of serum of Nile tilapia after 96 h (mean ± SD, n = 5), (**a**): NH_3, (**b**):Urea. The presence of different letters indicates a significant difference ($P < 0.05$).

Table 8
The p value obtained from two-way analysis of variance showing the effects of ammonia-N levels, salinity, and their interactions on serum thyroid hormones in Nile tilapia.

	T_3	T_4	TSH
Ammonia	0.00	0.00	0.00
Salinity	0.00	0.00	0.535
Ammonia*salinity	0.00	0.00	0.014

$P < 0.05$ indicates a significant difference in the desired variable.

The value of TSH in 50% and 30% of LC_{50}-96 h of ammonia-N (treatments 5 and 9 respectively) was significantly lower than the control treatment [Fig. 3 (a-c)]. Moreover, with increasing salinity in 50% of LC_{50}-96 h of ammonia-N (treatments 6, 7 and 8) and 30% and 30% of LC_{50}-96 h of ammonia-N (treatments 10, 11 and 12), TSH showed a decreasing pattern ($P < 0.05$).

4. Discussion

The increase of ammonia in water is one of the major problems in raising and keeping aquatic animals [32]. Ammonia is precious toxic and deadly not only for aquatic animals, but also for humans. Several studies have evaluated the harmful effect of ammonia on human health [33–35]. In this research, the effects of high level of ammonia-N on Nile tilapia fish were investigated, and the results of the investigation in a tilapia fish expressed the 50% lethal concentration (LC_{50}) in 24, 48, 72 and 96 h is 1.42, 1.356, 1.187 and 0.86 mg/L of ammonia-N, respectively. In general, the age, size and health conditions of the species have an effect on the toxicity of chemicals in aquatic organisms. Physical and chemical characteristics such as quality, dissolved oxygen, temperature, pH, water turbidity, type and amount of aquatic plants, concentration and composition of chemicals can basically affect the lethal concentration and toxicity tests [36]. The most important environmental factors affect ammonia-N level, such as pH, temperature, dissolved oxygen and salinity, [5,6,37–39]. In order to better detect the effect of salinity on ammonia levels, two main variables, pH and temperature, affecting ammonia were kept almost constant (Table 3). Generally, the rate of growth and the percentage of survival of fish in chronic ammonia poisoning decreases, and the sensitivity of fish to infectious agents increases. Exposure of fish to high levels of ammonia

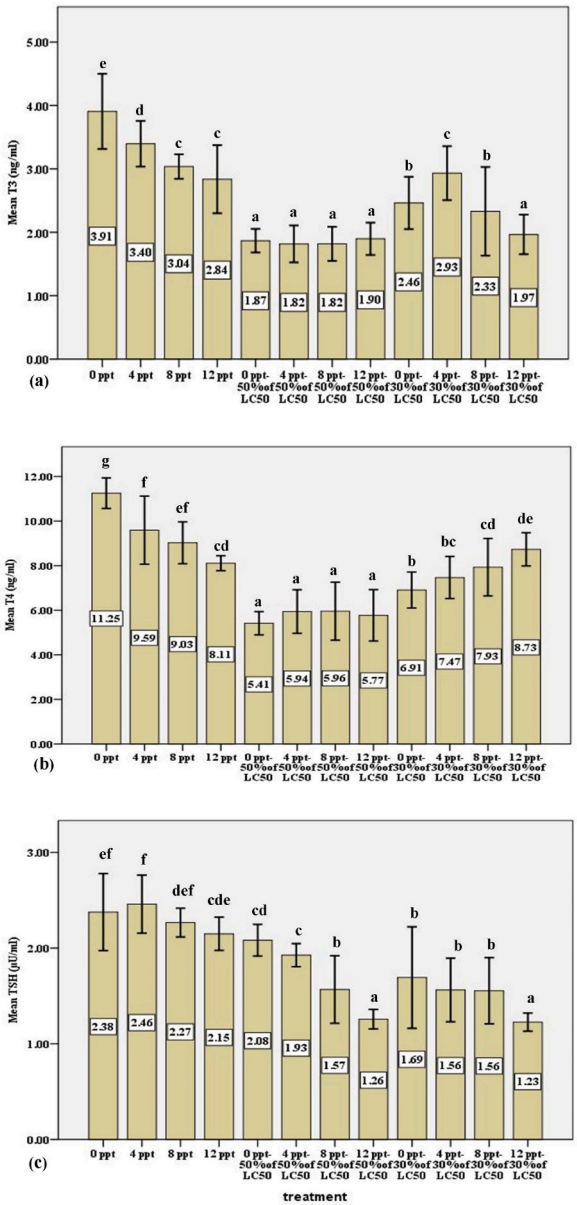

Fig. 3. The effects of different levels of ammonia-N and salinity on thyroid hormones of serum of Nile tilapia after 96 h (mean ± SD, n = 5), (**a**): T_3, (**b**): T_4, (**c**): TSH. The presence of different letters indicates a significant difference ($P < 0.05$).

causes rapid changes in plasma composition [40] and ammonia accumulation in fish body [41].

Hematological factors in fish are very useful as physiological signs of stress response [42]. The reason for the lack of significant change in hemoglobin with the increase in salinity can be attributed to the independence of osmotic changes with the oxygen demand of the studied species [43]. Imsland et al. [44], in investigating the effect of reducing salinity on growth factors and hematological indices of Atlantic halibut (*Hippogloss hippoglossus*), pronounced hemoglobin and hematocrit did not change significantly due to salinity reduction. Luz et al. [45] reported, in a research on golden crucian carp exposed to salinity for a long time, that hematocrit and hemoglobin levels were not affected by the increase in salinity, and stated the reason for this result was the high adaptability of the species, the gradual increase and the longer test period. The changes of environmental factors on blood indices can be caused by the difference in the optimal range of salinity of each fish and the power of adaptability, and the reason for the increase in hematocrit, in stress conditions, can be due to the absorption of water in red blood cells, swelling of red blood cells, reduction of plasma volume and the release of more erythrocytes from hematopoietic tissues [46].

In the present study, treatments of 50%-30%of LC_{50}-96 h of ammonia-N (namely treatments 5 and 9) caused the decrease of hemoglobin. Similarly, Thangam et al. [47], in a study on the effect of ammonia toxicity (1.05 mg/L of ammonia) on the amount of

hemoglobin in common carp (5–6 gr), reported that the amount of hemoglobin decreased by 15% compared to the control group. Moreover, *Brycon Cephalus* fish exposed to environmental nitrite-N showed a decrease in hemoglobin, as shown by Avilez et al. [48]. The increase in oxygen consumption can be a cause of the hemoglobin decrease [49]. The value of hemoglobin can fluctuate depending on various factors, such as geographical location, physical and chemical conditions, diet, and microbial condition [50].

Based on the results of our study, changes in salinity alone had no significant effect on the number of red blood cells. The results showed that, with increasing water salinity, RBCs of tilapia, euryhaline fish, did not expel their water. Probably, the lack of change in plasma volume prevented the alteration in blood factors such as red blood cells [46]. RBCs in treatment 5 (50% of LC_{50}-96 h of ammonia-N) were significantly lower than the control treatment in our study. Thangam et al. [47], in a research titled "the effect of ammonia poisoning on the blood factors of common carp (*Cyprinus carpio*), reported that RBCs decreased significantly due to subacute ammonia poisoning, and mentioned the reason for the reduction of RBCs can be due to anemia caused by the reduction of erythropoietin production in hematopoietic organs. Ren et al. [51]who investigated the effect of acute 96-h ammonia poisoning on *Carassius auratus* fish stated RBCs in the ammonia-N poisoning group was significantly reduced compared to the control group. The results of the mentioned research were consistent with the results of the present study to a great degree. The reduction of RBCs in the treatment of acute ammonia-N poisoning may be caused by hemolytic anemia and lysis of blood cells.

Environmental stress along with infectious agents can cause many changes in the level of the hormones, glucose, protein and cholesterol in fish blood [52,53]. The serum protein has various functions in the body as indicators of immune system, liver, and kidney disorders [54,55]. Our results showed that, with increasing salinity, the serum protein level decreased significantly compared to the control treatment. Fazio et al. [56], in a research entitled the effect of salinity on serum hematological and biochemical parameters of mullet (*Mugil cephalus*) fish, reported that increasing salinity caused a significant increase in total protein compared to the control treatment. Similar to our findings, Imanpoor et al. [57] who investigated the effect of salinity and different temperatures on growth and blood factors in goldfish (*Carassius auratus*) stated increasing salinity caused a significant decrease in total protein. Árnason et al. [58] observed that increasing salinity did not affect serum protein of cod (*Gadus morhua*) fish. Amino acids play a key role in the adaptation of fish to salinity changes as energy stores or important osmolytes to regulate cell volume [59]. According to our results, it seems that the level of activity and osmotic compatibility of fish is higher in high salinities.

Total protein in the treatment of 50% and 30% of LC_{50}-96 h of ammonia-N had a significant decrease compared to the control. Having been exposed to ammonia-N, the decrease in protein can be related to the decrease in protein synthesis due to liver dysfunction. Reduction of total protein is a characteristic of many diseases and it may occur due to liver disease, reduction of absorption or excretion of protein [60,61]. Das, P. et al. [61] who investigated the effect of 96-h nitrite-N poisoning on *Labeo rohita* fish reported that no significant difference in total protein was observed in the poisoned treatments compared to the control group, and stated a decrease in serum protein may be due to an increase in the excretion of blood protein through the kidneys. Generally, the change in total blood protein is not a specific indicator, but can indicate a metabolic or pathological change [62]. *l*

Cholesterol metabolism takes place primarily in the liver. Exposure to toxic substances may lead to a decrease in blood cholesterol levels which is due to a disturbance in the absorption of cholesterol and triglycerides from the intestines due to the toxicity of intestinal cells or due to liver damage and disturbances in the production of cholesterol and lipoproteins [63]. The results showed that the increase in salinity in the treatment 3 had a significant decrease in cholesterol level compared to control and treatment 4 ($P < 0.05$). The reason for this reduction may be a better adaptation of tilapia fish to low and high salinity. Imanpoor et al. [57] observed that increasing salinity at different temperatures had no significant effect on serum triglycerides of goldfish after 90 days. According to the results, the cholesterol level in 50% of LC_{50}-96 h of ammonia-N (treatment 5) was higher than the treatment of 30% of LC_{50}-96 h of ammonia-N (treatment 9) and the control treatment. Similarly, Metwally and Wafeek [64], in investigating the effects of ammonia-N poisoning on Nile tilapia, showed that the level of serum cholesterol increased significantly in the treatments exposed to ammonia-N compared to the control group. Studies have shown that many pollutants increase the cholesterol level [65,66]. An excessive increase in cholesterol is a sign of irregularities in fat and lipoprotein metabolism, particularly a decrease in physiological liver function [67,68]. An increase of the cholesterol concentration of the serum can be a result of kidney syndrome [69,70]. The results showed that the increasing salinity in interventional (combined) treatments caused a significant increase in the cholesterol of the serum. It is possible that the reason for these results is due to the damage to liver cells and irregular kidney function when salinity increases. As a conclusion, it can be claimed that the interaction between salinity and ammonia in cholesterol is somewhat synergistic.

The assessment of stress caused by various stressors generally relies on the determination of serum glucose, lactate, and cortisol values as reliable indices [71]. Fish under acute stress conditions increases the amount of catecholamines such as adrenaline and noradrenaline and subsequently, the levels of cortisol and corticosteroids are increased [72]. The negative feedback to the increase of catecholamine and corticosteroids is usually an increase of the production of serum glucose [73]. Generally, the release of cortisol is the primary response, on the other hand, glucose and lactate are the secondary responses during a stress [73]. The increase of salinity (treatments 2, 3 and 4) caused a significant increase in glucose and cortisol in these treatments compared to the control treatment. Similar to our findings, Kawamura et al. [74] who conducted a study on TGGG hybrid in salinities of 5, 10, 15, 20, 30 and 35 g/L observed that the measured cortisol was significantly increased in treatments of 5, 15 and 30 g/L. Tsui et al. [75], in the study of the effect of a sudden change in salinity on factors affecting stress in *Epinephelus malabaricus* fish, reported that the level of glucose and cortisol in the group transferred from 29 to 34 g/L of salinity was increased. The cortisol causes the proliferation of chloride cells and activates the Na + -K + ATPase enzyme in the gills [76]. It has been proven that the increase of plasma cortisol and catecholamines create ion balances in fish exposed to high and low salinity [77]. The results of the present study showed that the levels of cortisol and glucose in 50% and 30% of LC_{50}-96 h of ammonia-N treatments were significantly increased compared to the control treatment. Similarly, Shi et al. [78] reported that the ammonia stress caused a significant increase in cortisol and glucose values in black sea bream, *Acanthopagrus schleglii*. Exposure to high levels of ammonia-N resulted in similar results in *Megalobrama amblycephala* [79],

Labeo rohita [80] and *Anoplopoma fimbria* [81] that were consistent with our research results. It is assumed that cortisol facilitate the process of glucogenesis and gluconeogenesis in fish liver under favorable or stressful conditions, consequently the level of serum glucose increases [82]. Based on the findings of the present research, the increase of salinity in 50% of LC_{50}-96 h of ammonia-N (namely treatments 6, 7 and 8) compared to 50% of LC_{50}-96 h of ammonia-N (treatment 5) and the increase of salinity in 30% of LC_{50}-96 h of ammonia-N (namely treatments 10, 11 and 12) compared to 30% of LC_{50}-96 h of ammonia-N (treatment 9) caused the ascending trend in cortisol and glucose levels. It is obvious that the simultaneous increase of ammonia and salinity has a synergistic effect on the increased levels of stress indicators. Relying on the results, it can be stated that the interaction effect of salinity and ammonia poisoning is synergistic to a great extent.

Ammonia and urea excretion can be applied as indicators of nitrogen balance of fish and a sign of the effects of environmental and nutritional factors on protein metabolism [83]. Most bony fish primarily excrete ammonia which is, energetically speaking, more beneficial than converting ammonia to urea [84], nonetheless in fish under stressful situations such as levels of high environmental ammonia, high pH, exposure to air, or high density occurs the augmentation of the urea excretion [85].

According to the results of the present study, with raising salinity, there was a significant increase in serum urea compared to the control treatment, and serum ammonia was increased with increasing salinity up to 8 g/L and decreased at 12 g/L. Shrivastava et al. [86], in a research entitled the effect of different salinities on the osmotic regulation of seabass (*Dicentrarchus labrax*), reported that in salinity of 2.5 and 10 ppt, the ammonia level was increased, yet the blood ammonia level was decreased in salinity of 32 ppt. Altinok and Grizzle [87] pronounced urea and ammonia excretion in channel catfish, goldfish, and rainbow trout with increasing salinity (0, 1, 3, and 9 g/L) was significantly increased compared to fresh water.

The relationship between salinity, ammonia excretion and osmotic regulation in buffer fish (*Sphoeroides annulatus*) was done by Pérez-Robles et al. [88] who reported that with increasing salinity up to 40 ppt, the level of excreted ammonia was not significantly different among groups. The results of Zhang et al. [89], on *Miichtys miiuy*, and Gracia-López et al. [90], on *Centropomus undecimalis*, did not show a significant effect on ammonia excretion with increasing salinity. The results in some euryhaline fish such as *Oligocottus maculosus* and *Centropomus parallelus* have expressed increasing salinity has a decreasing effect on the level of excreted ammonia [85, 91]. It has been pointed out that the metabolic changes of those species in salinity changes are short and they adapt to the conditions in a short period of time [92]. Urbina and Glover [93], in investigating the effect of salinity on osmotic regulation and secretion of nitrogenous substances in Inanga fish (*Galaxias maculatus*), observed that with the increase of salinity up to 20 ppt, the level of ammonia secretion was decreased and increased up to 30 ppt, and they, about the ammonia secretion in this fish, proposed the U pattern, which decreased the level of catabolism of amino acids with the increase of salinity up to 20 ppt. The level of cost of osmotic regulation in Inanga fish is low, which was somehow consistent with the results of the present research. Generally, it is assumed that the increase of ammonia excretion rate in high and low salinities be related to the amount of energy spent on protein catabolism [94]. The level of serum urea increased significantly in the treatment of 50% of LC_{50}-96 h of ammonia-N (treatment 5) and 30% of LC_{50}-96 h of ammonia-N (treatment 9) compared to the control treatment in our study. Furthermore, the serum ammonia of 50% of LC_{50}-96 h of ammonia-N, treatment 5, was higher than the control treatment. Peyghan and Takamy [95], in investigating the effect of acute ammonia poisoning on urea changes in common carp, reported that high ammonia levels had an increasing effect on serum urea levels. They believed that the increase of the urea level after acute ammonia poisoning is one of the diagnosis of ammonia poisoning.

According to the results obtained in the present study, the level of serum urea increased significantly with increasing salinity in treatments of 50% of LC_{50}-96 h of ammonia-N (namely 6, 7 and 8) compared to 50% of LC_{50}-96 h of ammonia-N (namely treatment 5), and increasing salinity in the treatment of 30% of LC_{50}-96 h of ammonia-N (9, 10, 11 and 12) have no significant effect on the serum urea. The inverted U pattern was observed in interference treatments of 50% of LC_{50}-96 h of ammonia-N (treatments 5, 6, 7 and 8) and 30% of LC_{50}-96 h of ammonia-N (treatments 9, 10, 11 and 12). In general, the interaction effect of salinity and ammonia-N on the level of serum urea showed an increasing pattern. The reason for this result may be due to the increase of cortisol in these treatments, as such the increase of cortisol has probably caused an increase of the protein catabolism. Considering the reduction of serum protein in the treatment of 50% of LC_{50}-96 h of ammonia-N, probably, the increase of urea and ammonia was due to the stress effect of ammonia-N poisoning and increased salinity, as a result the increase of protein catabolism has been dealing with this stress.

The regulation of vertebrate physiology is greatly influenced by thyroid hormones [96,97]. It has been established that thyroid hormones regulate growth and development, differentiation, immune response, metabolism, behavior, and reproduction [98–103]. The thyroid gland has sensitivity to both biotic and abiotic factors. Salmon and sea bream's thyroid hormone production has been impacted by changes in deiodization activity in the kidney and gill, as well as salinity changes [104–106]. Our results showed that with increasing salinity, the amounts of T_3 and T_4 hormones decreased significantly, and TSH levels in these treatments, namely 2, 3 and 4, were not significantly different compared to the control treatment. McCormick and Saunders [107] who investigated the effect of salinity on the thyroid hormones of Atlantic salmon (*Salmon salar*) reported that the level of T_4 increased in the smolt stage of salmon in the first 6 h in water, 32 g/L of salinity, and after 24 h, it returned to its original value. They stated the level of T_4 in Atlantic salmon was not affected by salinity [107]. Young et al. [108] observed that T3 levels in coho salmon (*Oncorhynchus kisutch*) decreased after 24 h of seawater exposure, but they returned to their baseline levels after 96 h. The type of fish, the duration of exposure and the power of osmotic adjustment can be the possible reasons for the difference in the results. According to the results, following the increase of the ammonia-N dose (so to speak treatmets 5 and 9), the level of T3, T_4, and TSH decreased significantly compared to the control treatment. Gao et al. [109], in investigating the effect of the nitrite –N poisoning on the change of thyroid hormones in *Takifugu rubripes*, reported a decrease in T_4 level and an increase in T_3 amount after long-term exposure to nitrite. Xiao et al. [110] showed that high concentrations of nitrite reduced the amount of T_3 and T_4 in grass carp, *Ctenopharyngodon idella*. Yu et al. [111] pronounced the hexaconazole, a broad-spectrum systemic triazole fungicide, and the tebuconazole, a triazole fungicide used agriculturally to treat plant pathogenic fungi, decreased T_4 and increased T_3. The reason of this result was considered to be a dysfunction of the thyroid gland.

Liang et al. [112] observed that the difenaconazole decreased serum T_4 levels in zebrafish. Changes in TSH level are an indicator of thyroid axis activity, and the analysis of those is effective for measuring the effect of external environmental factors on thyroid function [113,114]. The stimulation of T4 secretion by TSH occurs exclusively in fish [115]. After being exposed to 1 and 3 mg/L of nitrite, *T. rubripes* fish showed a significant increase in their TSH levels [109]. Previous studies reported that T_4 modulates TSH values through a negative feedback mechanism. A decrease of T_4 values, along with an increase in TSH transcription, has been observed in zebrafish larvae when exposed to environmental chemicals [116–118]. It seems that exposure to levels of ammonia like the nitrite damage the follicular structure of the thyroid gland and that lead to thyroid dysfunction [109].

According to the results, increasing salinity in 50% of LC_{50}-96 h of ammonia-N (namely treatments 6, 7 and 8) had no significant effect on the level of T_3 and T_4 compared to the treatment of 50% of LC_{50}-96 h of ammonia-N (treatment 5). The level of TSH in the mentioned treatments decreased significantly with increasing salinity. The results obtained from this research, unlike the hypothesis of the effect of T_4 on TSH level, enunciated that TSH levels were not decreased along with increasing salinity, treatment 2, 3 and 4, without the influence of T_4, and those can be a proof of the independence of TSH changes against environmental fluctuations. In T_3 hormone, the increase of salinity in 30% of LC_{50}-96 h of ammonia-N (namely treatments 9, 10, 11 and 12) showed a sinusoidal pattern ambiguous for us. Increasing the salinity in these treatments (namely 10, 11 and 12) compared to treatment 9 (only 30% of LC_{50}-96 h of ammonia-N) caused an increase in the T_4 level, and it is possible that increasing salinity at lower levels of ammonia-N create a beneficial effect on this hormone, T_4. Increasing salinity in 30% of LC_{50}-96 h of ammonia-N (namely treatments 10, 11 and 12) compared to 30% of LC_{50}-96 h of ammonia-N (that is treatment 9) caused an increase of T_4 concentration and a decrease of TSH level at the salinity of 12 ppt. The research has shown that T_4 can modulate TSH secretion by its negative feedback [116–118]. Taking into results, it can be pointed out that the interaction of salinity and ammonia poisoning has an antagonistic effect on TSH levels unlike T4 levels.

5. Conclusions

The present study elicited the interference effect of salinity and ammonia-N levels on some hematological and biochemical factors of blood in Nile tilapia. Results showed that although the salinity has a reducing effect on the amount of ammonia in the water, the salinity caused stress and fluctuations in blood biochemical factors during the 96 h of testing. On the other hand, the levels of ammonia-N, as well as the interaction effects of salinity levels and ammonia-N level increased stress (cortisol and glucose) factors in fish. Decrease of blood factors, increase of stress and decrease of thyroid hormones show that the salinity and ammonia caused adverse effects on fish health during the 96 h of testing. In order to extract more detailed information about the interaction effect of salinity and ammonia on fish physiology, we suggest to measure organoleptic properties, body composition (Protein, lipid, ash and moisture) and fatty acid profile along with increasing the duration of the test.

Data availability

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

CRediT authorship contribution statement

Javad Motamedi-Tehrani: Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Data curation. **Rahim Peyghan:** Funding acquisition, Investigation, Methodology, Project administration, Supervision. **Ali Shahriari:** Funding acquisition, Investigation, Methodology, Supervision. **Mohammad Razijalali:** Investigation, Supervision, Methodology. **Eisa Ebrahimi:** Investigation, Resources.

Declaration of competing interest

The authors confirm that they have no known competing financial interests or personal relationships that could have appeared to affect the research reported in the present paper.

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