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Histomorphometric Analysis of Skin and Stress Indices of Nile Tilapia (*Oreochromis niloticus*) Exposed to Different Concentrations of Ammonia

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Abstract

BACKGROUND: Intensive aquaculture, which is always associated with high concentrations of nitrogen pollutants, can lead to increased economic damage and energy loss, and increased fish mortality.

OBJECTIVES: This study aimed to investigate the histomorphometry of the skin and stress indices of the Nile tilapia on exposure to different concentrations of ammonia.

METHODS: In this study, Nile tilapia fingerlings were prepared and kept in the tub for adaptation, oxygenation, and feeding. A Range Finding test was performed on the fishes to find the ammonia killing range. Then, an acute toxicity test was performed on tilapia fish for 96 hours. For sub-lethal toxicity studies, 120 Nile tilapia fry were divided into 4 groups, including three groups based on different percentages of LC_{50} 96 h (10, 20, and 30% LC_{50} 96h) and a control group. The groups were exposed to ammonia for 14 days in an environment with constant temperature and pH control. Finally, histological examination and stress indices were performed on fish.

RESULTS: The results showed that a higher concentration of ammonia resulted in tissue damage, including discoloration and skin blackening, and behavioral changes, including swallowing air from the water surface, decreased appetite and decreased mobility which finally resulted in death in these animals ($P \leq 0.05$). The highest and lowest glucose levels were reported for the control group and 2.7 mg/L. In addition, stress indices were significantly increased in experimental groups ($P \leq 0.05$).

CONCLUSIONS: The data obtained from this study showed that increasing the amount of ammonia can lead to irreversible tissue damage to the structure of the skin and other tissues. In addition, the increase in oxidative stress in the Nile tilapia was due to the increase in ammonia, which led to lesions in these fish.

KEYWORDS: Ammonia, Histomorphometry, Nile tilapia, Skin, Stress

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Introduction

Fish is one of the most important sources of the human food chain. Due to climate change and the environmental effects of fishing in the ocean, fish farming is a priority for human societies (Adeli *et al*., 2011). Ammonia poisoning is one of the leading causes of fish mortality on farms. Ammonia with the chemical formula NH_3 is a combination of nitrogen and hydrogen. Ammonia is a stable binary hydride, and its simplest hydride is a colorless gas with a distinct pungent odor (Carlozzi and Padovani, 2016). Ammonia is a common nitrogenous waste, especially among aquatic organisms, and by acting as a precursor and in fertilizers, it significantly contributes to the nutritional needs of terrestrial organisms. Ammonia, directly or indirectly, is also a component of many pharmaceutical products and is used in many commercial cleaning products. This substance mainly accumulates by moving air and water downwards (Ogbonna and Chinomso, 2010). Because ammonia is one of the most significant excreted substances in aquatic environments produced by protein digestion, its toxicity has been studied extensively (Basir *et al*., 2012; Suliman *et al*., 2021).

Studies have shown that ammonia damages the skin, gills, and other tissues, leading to a variety of symptoms that may include respiratory problems such as rapid gill breathing, shortness of breath, swallowing air from the water surface, irregular swimming, and sudden darting movement, bruising of the skin (bleeding) and excessive production of mucus (dark spots on the skin), lethargy, loss of appetite, fish lying in bed, red streaks on the fins, body, and red or purple gills. Once the injury continues with ammonia poisoning, streaks or bloodstains appear on the body and fins. Internal damage also occurs to the brain, organs, and central nervous system. Bleeding occurs on the inner and outer surfaces of the fish and eventually leads to its death. Ammonia toxicity increases with acidity and water temperature (Morovvati *et al*., 2017; Dastan *et al*., 2017). Ammonia levels are usually controlled by biological filters that contain bacteria that break down ammonia into less toxic substances. Here, excreted ammonia is broken down by bacteria into nitrite and then nitrate (Jumaa *et al*., 2018). In addition, ammonia poisoning can be prevented mainly by routinely monitoring its levels using assay kits and avoiding high density of fish aqua culturing and overfeeding. Dangerous ammonia levels must be dealt with quickly, for example, by changing large amounts of water or using an ammonia-removing agent such as zeolite and new solutions (Morovvati *et al*., 2017).

Following these quick steps makes more time until the underlying factor is identified (Jumaa *et al*., 2018). In the inland waters of Iran, there are 174 species of fish, some of which have economic value. Nile tilapia was first introduced to Iran from Indonesia and now forms an essential part of the fish farming basket in Iran (Azari *et al*., 2020; Khanjani and Sharifinia, 2021). The appearance of tilapia is very similar to that of sunfish or crappie. Still, its disconnected lateral line can easily be identified, which is characteristic of the cichlid family. Their body shape is compressed on the side, and they have long dorsal fins. The front of their dorsal fins is full of sharp bumps. These razors are visible on the ends of the anal fins and the anterior fins of the fish. Wide vertical lines are usually seen on both sides of the fingerlings and sometimes the body of the adult fish (Amin *et al*., 2019; El Asely *et al*., 2020). This species is an economically valuable fish for breeders. They reproduce easily, using a wide range of natural and artificial foods (Ibrahim *et al*., 2020). They cope with low water quality and grow rapidly in warm water. In addition to the low initial cost, these features make tilapia the most widely grown fish in the tropics and subtropics. This study aimed to study the effect of toxicity of different concentrations of ammonia on the skin and stress indices of the Nile tilapia.

Materials and Methods

Fish Farming and Adaptation

In this study, 120 juvenile Nile tilapia fishes (35±1 g) were transferred to the laboratory to adapt to environmental conditions. The prepared fish was kept in a 500-liter tub for 14 days. During this period, fish were fed commercial food (Bio mar, France daily for 2% of the fish's body weight by a plate under proper aeration. During the experiment, the average temperature of breeding water was 27±1°C, the oxygen concentration was 6.2 ±1 mg/L, and water hardness was 269 ±3 mg/L.

Acute Toxicity Test

In order to find the effect of acute ammonia toxicity, a range-finding test was performed (Adeli *et al*., 2011) by stationary method and according to standard instructions for 96 hours. Ammonia (Merck, Germany) was prepared as an ammonium chloride solution. The feeding of fish was stopped 24 hours before the acute toxicity test. All effective physicochemical parameters of water (pH, dissolved oxygen, and temperature) were monitored daily. The final test for acute ammonia toxicity was completed after determining the lethal range. For this purpose, 3 treatments with control groups of skin and tissue specimens were evaluated. The fish in each treatment were placed in a pre-aerated 15-liter aquarium. The dead fish were collected from the aquarium environment as soon as they were observed, and the number of losses was calculated and recorded at 24 hours intervals. After the test, the data obtained for the acute toxicity test were analyzed by Probit Analysis with a 95% confidence level.

Sub-lethal Toxicity Test

To evaluate the lethal toxicity of 120 Nile tilapia fingerlings, they were divided into 4 groups, three of which were selected based on different percentages of LC_{50} (10, 20, and 30% LC_{50} 96 h) and placed with a control group. The fish were exposed to ammonia for two weeks in a temperature and pH-controlled environment (Campos *et al*., 2015; Pinto *et al*., 2016). The temperature and pH of the breeding water were monitored at 3 hours intervals. Therefore, 120 fingerlings were randomly distributed in 100-liter aquariums and subjected to a semi-static toxicity test (daily change of 20% of aquarium water). One hour before changing the water, feeding was done for 2% of the fish's body weight. The remaining food was removed from the aquariums by replacing water. To keep the ammonia concentration constant, an equal amount of water with ammonia was added to the aquarium, equal to the amount of water pouring out of the aquarium.

Histological Examination

Tissue sampling was performed randomly on the day of the experiment and 14 days after exposure. At the end of the experimental period, the fish were randomly selected from each group and anesthetized using 0.5 g/L of clove powder. Then, sampling of the skin tissue of the head and trunk of the fish was performed using a tissue passage device (Automated tissue processors, Leica ASP300 S, Germany). Specimens were subjected to 10% formalin buffer, fixed, and transferred to 70% alcohol overnight (Mohamed *et al*., 2021; Mohamed *et al*., 2020). Tissue dehydration was performed using serial ethanol concentrations of 70, 80, 90, and 100%, and then the tissues were exposed to xylene. The tissues were then molded and paraffinized with paraffin at a melting point of 56-58°C on the tissue embedding console system. Section of 4 to 6 microns was prepared from the molds obtained using a microtome machine, and after placing on a slide, they were placed in an oven at 60°C for half an hour to remove excess paraffin from the tissue. The tissues were deparaffinized in xylene, rehydrated, and stained using hematoxylin and eosin solutions to stain the specimens. The slides were examined by a light microscope attached to a Dinolit lens as well as a computer system equipped with Dinocapture software. For histomorphometric valuation, five microscopic grounds per section and five sections per fish were exhausted.

Results

Survival Rate

The study of survival rate in this study showed that no losses were observed during the adaptation period in different treatments under sublethal ammonia levels (0.9, 1.8, and 2.7 mg / l) and control during 14 days.

Histological Examination of Nile Tilapia Skin

The results of microscopic studies of fish skin exposed to different concentrations of ammonia compared with those studied in the control group showed changes such as an increase in the number of mucosal and caveat cells, an increase in the number of epithelial cells, and also an increase in the thickness of the epidermis, which regulated histological results. No specific cell lesion was seen. Overall, the severity of these complications increased significantly from low to high concentration treatment ($P \leq 0.05$) (Figures 1 and 2).

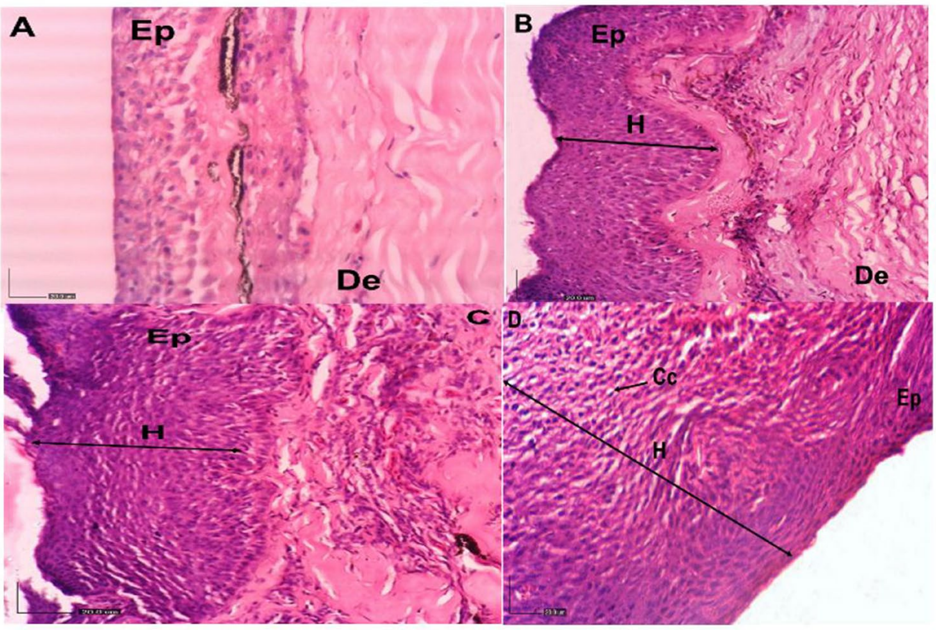

Figure 1. Tissue micrograph of Nile tilapia skins in experimental groups (H&E, x40) A) Epidermis (Ep), dermis (De), caveat cell (Cc), hyperplasia (H) in control groups, B) Exposure to ammonia 0.9 mg/L., C) Exposure to ammonia 1.8 mg/L., D) Exposure to ammonia 2.7 mg/L during 14 days.

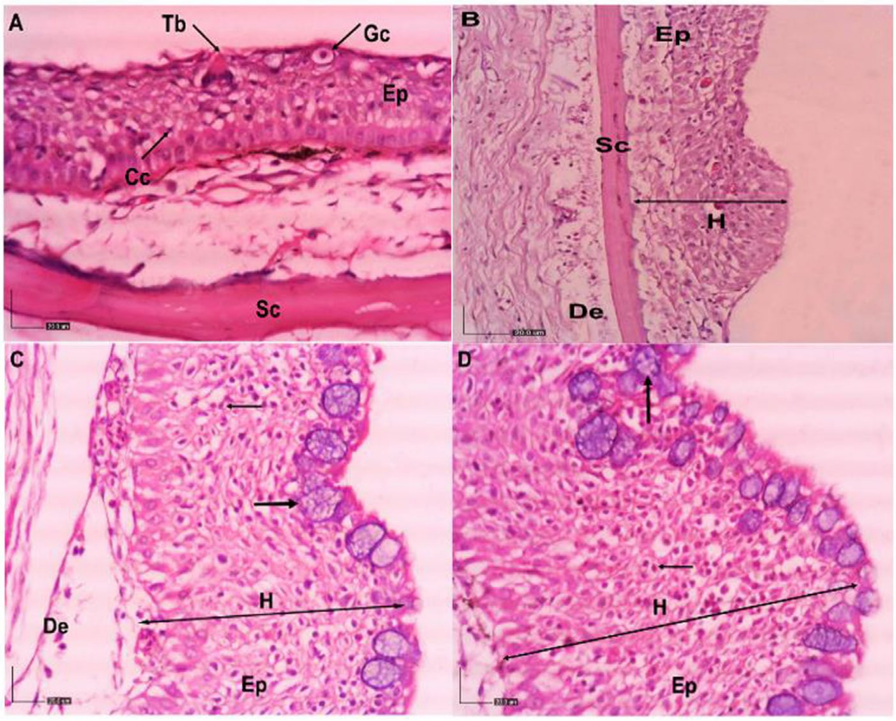

Figure 2. Tissue micrograph of the middle skin of the Nile tilapia trunk of experimental groups (H&E, x40). Epidermis (Ep), Dermis (De), Caveat cell (Cc), Hyperplasia (H), Melanin pigment (Mp), Scales (Sc), Mucosal cell (Gc), Collagen filament (Co), Mucosal cell proliferation (Thick arrows) and proliferation of caveat cells (thin arrows) in control groups, A) Exposure to 0.9 mg/L ammonia, B) Exposure to ammonia 1.8 mg/L, C) Exposure to 2.7 mg/L ammonia during two weeks.

Level of Stress Indices during Adaptation Period

The results of measuring glucose and cortisol levels during the adaptation period in different treatments are shown in the following diagrams. Results showed a significant increase in cortisol and glucose levels in almost all treatments compared to the control group ($P \leq 0.05$). Also, after exposure of fish to ammonia, the maximum and minimum glucose levels were reported as 27.19 ±1.52 mg/dL and 50.35 ±3.19 mg/dL for the control specimens, respectively.

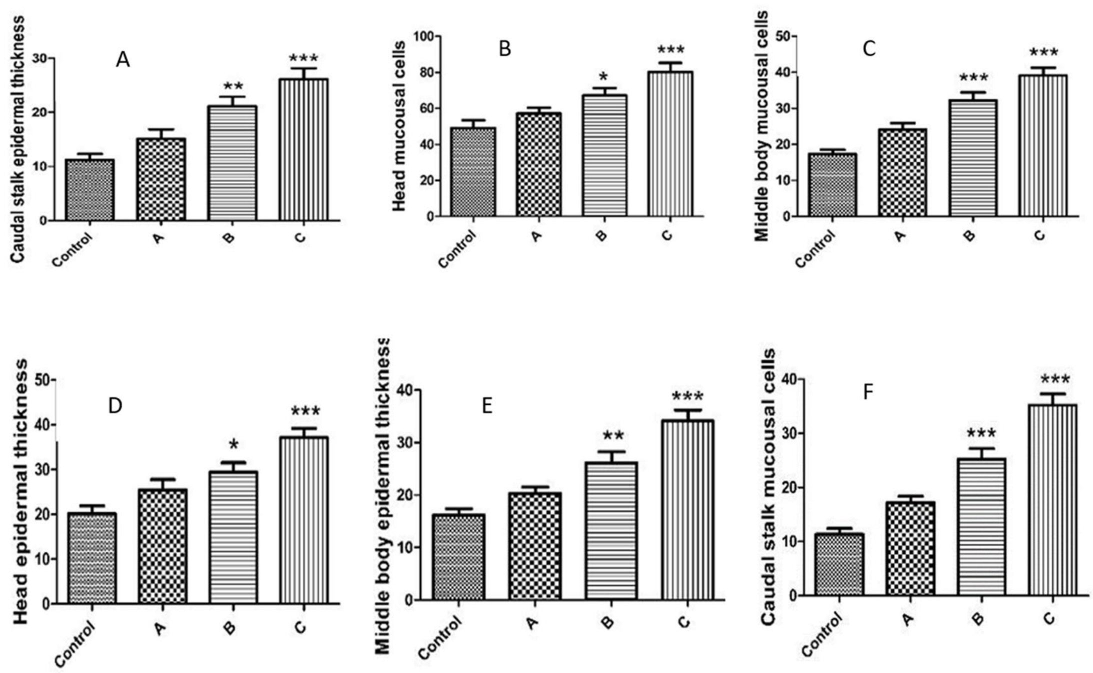

Figure 3. Figure A shows the increase in the thickness of the caudal stalk epidermis, Figure B shows the increase in the mucosal cells of the head specimens, Figure C shows the increase in the thickness of the epidermis of the middle body skin specimens, Figure D shows the increase in the thickness of the epidermis of the head specimen, Figure E shows the increase of the middle body epidermal thickness, and Figure F shows the increase of the mucosal cells of the caudal stalk epidermis, (Different symptoms indicate a significant difference in the level of $P<0.05$).

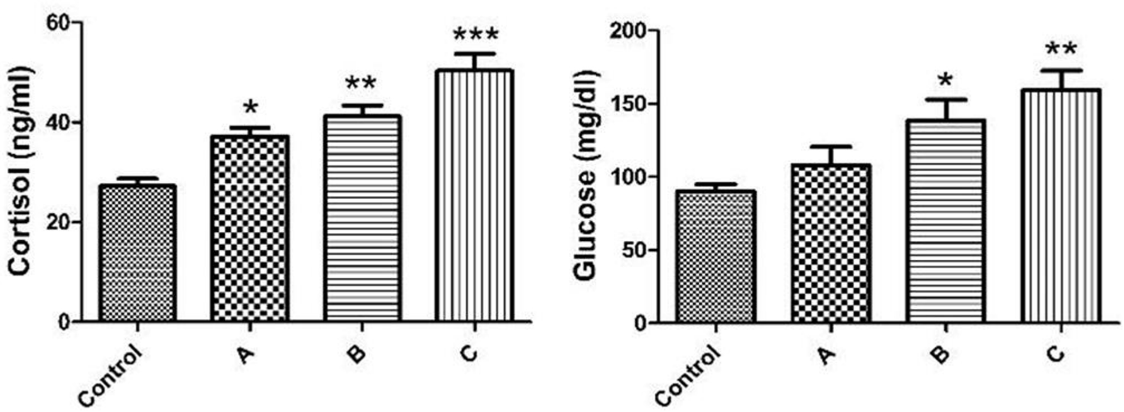

Figure 4. Measurement of cortisol (left chart) and glucose (right chart) levels in ammonia-treated groups 0.9 mg/L (A), 1.8 mg/L (B), and 2/7 mg/L (C) compared to control during two weeks were shown (Different symptoms indicate a significant difference in level of $P<0.05$).

Discussion

Today environmental problems, inefficiency, and the high cost of fishing in the sea have led to increased fish farming as a suitable alternative. Providing inexpensive and valuable food is one of the most important goals of the food industry worldwide (Wanka *et al*., 2018). In recent years, Nile tilapia has become one of the most common fish for breeding and consumption worldwide, ranking second after carp species (Abdi *et al*., 2011). Asia is the top tilapia producer and accounts for more than 70% of global tilapia production. Many diseases in aquatic life cause a loss of capital and energy and increase the risk of fish farming (Føre *et al*., 2018). Ammonia toxin is one of the most important chemicals that destroy aquatic tissues, which is produced due to protein digestion by these organisms and can lead to increased mortality among them (Zuffo *et al*., 2021). Ammonia in aqueous media can be detected in both ionized and non-ionized forms. The non-ionized type of which has higher toxicity due to its high permeability through the epithelium of aquatic organisms (Abdel-Tawwab *et al*., 2020; Nogueira *et al*., 2018). So far, the effects of different concentrations of water-soluble ammonia on the Nile tilapia have not been studied, so this study aimed to investigate the effect of this toxin on the skin histomorphology and stress indices of this fish. The present study's data showed that increasing ammonia concentration in the experimental group results in appetite and mobility decrease, skin discoloration and blackening, significant histomorphologic responses, and an increase in stress indices in aquatic animals (Deswati *et al*., 2018). In this study, poisoning symptoms appeared in high concentrations of ammonia and the last days, followed by nervous behavioral symptoms. On the skin's outer surface, pallor and bleeding mucus accumulation were evident on the body's surface. The skin tissue of the fish changed, such as an increase in the number of mucus cells and caveat cells, which led to an increase in the thickness of the epidermis. In general, the severity of these factors was higher from low concentration treatment to high concentration treatment and had a statistically significant difference compared to control. These experiments also showed that increasing ammonia caused a significant increase in cortisol and glucose in most treatments compared to the control group. El Shafi *et al.,* in a study, examined the chronic effects of ammonia on the Nile tilapia. This experiment was performed for 75 days using fish with an average body weight of 20 grams. Five nominal concentrations of total ammonia nitrogen (control, 2.5, 5, 7.5, and 10 mg) were generated as treatment groups. The data showed that increasing the ammonia concentration increased the mortality rate.

This study concluded that for the cultivation of Nile tilapia in duckweed-fed ponds, due to the negative effect of ammonia on growth performance, its level should be maintained below 0.1 mg/L (El-Shafai *et al*., 2014). In another study, Aysel *et al.* examined the lethal effects of ammonia on Nile tilapia. Their study aimed to determine the mean lethal concentration (LC50) of ionized ammonia for larvae and juveniles of Nile tilapia. The mean 48-hour LC50 values for larvae and juveniles were 1.009±0.02 mg/L and 7.40±0.01 mg /L, respectively. Larvae exposed to different ammonia concentrations are motivated very quickly to lose their balance in the water and swim sideways. In fingerlings, increased movements, ventilation, seizures, spiral swimming, attempts to swallow air from the water surface, increased mucous secretions in the gills and body surface, bleeding in the gills, and skin darkness were observed. Histological studies showed hyperplasia and lamella fusion in gills (Benli and Köksal, 2005). A study by Benli *et al.* also confirmed the negative effects of high ammonia concentrations on the liver, gills, and kidneys of Nile tilapia (Benli *et al*., 2008). The findings of the present study in the field of behavioral changes as well as the toxic effects of ammonia were following the findings of the mentioned studies. In addition, the study results by Jian *et al.* confirmed the effects of ammonia on the incidence of behavioral responses in Nile tilapia (Jian-yu *et al*., 2005), which is in agreement with the present study. Another study by Yilmaz et al. (2020) confirms the data of the Magouz (Magouz *et al*., 2021) study and the present study on the occurrence of behavioral changes in the presence of high ammonia levels in the water. Mercante *et al.* (2018) also confirmed the increase in tissue problems due to the increase in the duration of exposure of fish to aquatic ammonia. They showed that this exposure could increase cortisol and oxidative stress leading to death in studied aquatic animals. The results of Yousefi *et al.* (2020) research showed that after 12 hours of exposure to ammonia, the fish changed the initial treatments of stress factors. Cortisol increased in the first two treatments and then decreased in the following three treatments. But it was still higher than control cortisol in all treatments. The glucose levels reached their lowest level in the first treatment and showed a significant increase in other treatments (Yousefi *et al*., 2020). In the present study, increasing the ammonia concentration led to a significant increase in stress factors.

Conclusion

The present study's data indicate an increase in the negative effects of ammonia on the skin and the level of stress indices in Nile tilapia. These results emphasize the monitoring of ammonia levels in the breeding environment of these fish and prove that increasing the concentration of ammonia can lead to increased behavioral responses by these organisms in addition to causing tissue problems.

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Conflict of Interest

The authors declared that there is no conflict of interest.

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تجزیه و تحلیل هیستومورفومتریک پوست و شاخص‌های استرس ماهی تیلاپیا نیل (*Oreochromis niloticus*) در معرض غلظت‌های مختلف آمونیاک

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زمینه مطالعه: آبزی‌پروری به صورت فشرده همواره با غلظت بالای آلاینده‌های نیتروژن همراه است، که این امر می‌تواند منجر به افزایش خسارت اقتصادی و اتلاف انرژی و افزایش مرگ و میر ماهی‌ها شود.

هدف: این مطالعه با هدف بررسی هیستومورفومتری پوست و شاخص‌های استرس ماهی تیلاپیای نیل طی مواجهه با غلظت‌های مختلف آمونیاک انجام شد.

روش کار: در این مطالعه ماهیان جوان تیلاپیا نیل تهیه و برای سازگاری، هوادهی و تغذیه در وان نگهداری شدند. آزمایش محدوده یابی بر روی ماهی‌ها برای یافتن محدوده کشنده آمونیاک انجام شد. سپس آزمایش سمیت حاد بر روی ماهی تیلاپیا به مدت ۹۶ ساعت انجام گرفت. برای مطالعات سمیت تحت کشنده، ۱۲۰ قطعه بچه ماهی تیلاپیا نیل به ۴ گروه شامل: سه گروه بر اساس درصدهای مختلف LC_{50} 96h (۱۰، ۲۰ و ۳۰ درصد LC_{50} 96h) و یک گروه کنترل تقسیم شدند. گروه‌ها به مدت ۱۴روز در محیطی با دمای ثابت و pH کنترل شده در معرض آمونیاک قرار گرفتند. در نهایت مطالعات بافتی و فاکتورهای استرس بر روی ماهیان انجام گرفت.

نتایج: نتایج نشان داد که غلظت بالاتر آمونیاک منجر به آسیب بافتی از جمله تغییر رنگ و سیاه شدن پوست و تغییرات رفتاری از جمله بلع هوا از سطح آب، کاهش اشتها،کاهش تحرک و در نهایت منجر به مرگ ماهی شد ($P\leq$۰/۰۵). بالاترین و کمترین میزان گلوکز به ترتیب برای گروه کنترل و تیمار ۲/۷ میلی‌گرم در گزارش شده که پس از مواجهه با آمونیاک به‌طور معنی‌داری افزایش یافت ($P\leq$۰/۰۵). همچنین شاخص‌های استرس در گروه‌های آزمایشی به‌ویژه در مقادیر بالا به‌طور معنی‌داری افزایش یافت ($P\leq$۰/۰۵).

نتیجه‌گیری نهایی: داده‌های به‌دست‌آمده از این مطالعه نشان داد که افزایش میزان آمونیاک می‌تواند منجر به آسیب غیرقابل برگشت بافتی به ساختار پوست و سایر بافت‌ها شود. علاوه بر این، افزایش استرس در تیلاپیا نیل به دلیل افزایش آمونیاک که منجر به ضایعاتی در این ماهی‌ها شد.

واژه‌های کلیدی: آمونیاک، هیستومورفومتری، تیلاپیا نیل، پوست، استرس

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