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Stress responses of red tilapia (*Oreochromis* spp.) to high ammonia levels

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Abstract
Red tilapia is becoming a popular species in aquaculture but few studies are available regarding its responses to environmental stress. In this study, 16 pieces of Red tilapia was subjected to high ammonia levels. Stress level was monitored by means of observing the changes in ventilation rate (VR), eye color pattern (ECP) and skin color of the fish before the introduction of stressor and immediately after 2, 4, 6, 8, 10 and 30 min later. Three treatments were assessed: T1 (control); T2 (0.046-0.05 ppm of NH_3-N); T3 (0.06-0.10 ppm of NH_3-N) and T4 (0.11-0.15 ppm of NH_3-N). Results showed that the mean VR, ECP and skin color of the Red tilapia exposed to ammonia stress (T2, T3 and T4) were significantly higher ($P<0.05$) compared to control (T1). Faster VR, darker ECP and intensified skin color were observed in all treatments with high ammonia levels.

Keywords: Red tilapia, stress responses, environmental stress, ammonia

Introduction
Red tilapia is one of aquaculture's most adaptive species which is abundantly found in the wild and known to be cultured in several parts of the world [1]. This fish is suitable for intensive and extensive conditions and have high consumer acceptance in several Asian countries because of their resemblance to premium marine species [2]. It has been gaining popularity nowadays in the market because of its enticing color, increased marketability and high salinity tolerance in some strains. An efficient production of nice-looking, well-tasting red tilapia requires a simultaneous genetic improvement of all economically important traits and a good handling and welfare technique [3].

Water quality of the aquatic ecosystem is important to maintain a suitable environment for fish culture. Of all the water quality parameters that affect fish, ammonia is the most important after dissolve oxygen especially in intensive culture systems [4]. It is the major end product in the breakdown of feed in ponds which is excreted through the gills and feces of fish. Higher ammonia levels may inhibit the fish from extracting energy from feed efficiently and may cause stress and damages in gills and other tissues [5]. Total ammonia nitrogen (TAN) in water is composed of toxic (un-ionized) ammonia (NH_3) which predominates when pH is high and nontoxic (ionized) ammonia (NH_4^+) which predominates when pH is low [6]. The concentration of ammonia in fish pond should be closely monitored to avoid both chronic acute toxic effects which may lead to fish mortality and unexplained production losses.

Stress is described as chemical and physical factors causing body reactions that may contribute to disease and/or death [7]. The response to stress in fish is characterized by the stimulation of the hypothalamus, which results in the activation of the neuroendocrine system and a subsequent cascade of metabolic and physiological changes [8]. In order to avoid stress-related problems, and to improve fish quality, thus, optimizing productions, studies regarding stress in fish is necessary [9]. In the study of El-sherif and El-feky [10], fish exposed to environmental stress, specifically ammonia stress affects the hematocrit and hemoglobin parameters of fish and may cause anemia to fish. In this study, Red tilapia was subjected to high ammonia levels to determine its stress responses by observing the physiological and morphological changes in fish such as Ventilation Rate (VR), Eye Color Pattern (ECP) and Skin Color.

Materials and Methods

Experimental Fish

Fifty Red tilapia averaging 76.61±2.36 g in weight were acclimated for one week in concrete indoor tanks supplied with well-aerated water. After the isolation, 16 pro-active fish were chosen and to be subjected to high ammonia levels. The fish were obtained from Freshwater Aquaculture Center (FAC), Central Luzon State University (CLSU), Science City of Muñoz, Nueva Ecija, Philippines.

Experimental Procedure

There were four treatments used in this study (Table 1). Each treatment was replicated four times. Sixteen glass aquaria with a size of 10 x 10 x 10 inches were used on this experiment and fish were reared for 15 days at a rate of one fish per aquarium.

Table 1: Experimental treatments.

Treatments	Descriptions
1 (Control)	No addition of ammonium chloride
2	0.046-0.05 ppm of NH_3-N
3	0.06-0.10 ppm of NH_3-N
4	0.11-0.15 ppm of NH_3-N

The fish were fed 4 % of their body weight twice daily, morning and afternoon. Fish were transferred to aquaria with high ammonia concentrations for 60 minutes and changes in VR, ECP and skin color were monitored. Physiological changes were observed 0 minutes before introduction of stressors and immediately 2, 4, 6, 8, 10, 30, and 60 minutes after the introduction of stressors. Each fish was transferred back to its original aquaria after the observation period. Completely Randomized Design (CRD) was used on this experiment.

Ammonia Stress

Ammonia stress was induced by adding ammonium chloride to each aquarium [11]. The ammonium chloride was obtained from the Water Quality Laboratory of the College of Fisheries, Central Luzon state University and sourced out from Chemline Scientific Corporation. The absorbance of the water samples was measured using HITACHI U-5100 Spectrophotometer. The total ammonia nitrogen (TAN) was computed using the formula (Equation 1):

$$\frac{\text{Concentration of the TAN}}{\text{Concentration of TAN in the sample}} = \frac{\text{Absorbance of the TAN standard}}{\text{Absorbance of the sample}} \quad (1)$$

The percentage of un-ionized ammonia added in the water was calculated following the equation of Emerson, Russo, Lund, and Thurtson (1975) (Equation 2):

$$NH_3\text{-N} = \frac{\text{TAN} \times (NH_3\text{-N as a function of pH and temperature})}{100} \quad (2)$$

The temperature of water in each aquarium was maintained to 28°C by the use of heater and the pH was monitored. The un-ionized ammonia (NH_3-N) concentration was calculated by based on the method used in the study of Thurston, Russo & Emerson [12]. The amount of ammonium chloride added on the aquaria also depends on water temperature and pH. The water with ammonia was renewed every 5 days.

Ventilation Rate (VR)

The VR was visually estimated by counting (i.e. seconds) for 20 successive opercular or buccal movements (adapted from Alvarenga and Volpato, [13]). The VR was measured daily 0 minute before introduction of stressor and immediately after 2, 4, 6, 8, 10, 30, and 60 minutes of introduction of stressor.

Eye Color Pattern (ECP)

The ECP was monitored daily during the entire experiment and is quantified as darkened area of both the iris and sclera. Both pigments present in the eyes of the fish (red and black) were recorded. The circular area of the eye is divided into eight equal parts using imaginary diameter lines and each part is equal to 12.5% [14].

Skin Color

Skin color of the red tilapia was analyzed using a color chart (Figure 1) from pale pink (1) to intense orange (13). It was also recorded daily along with VR and ECP.

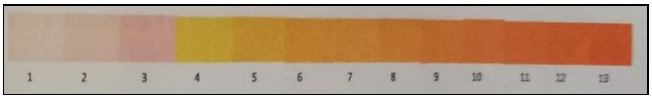

Fig 1: Skin color chart

Statistical Analyses

The correlation between VR and ECP was assessed using Pearson correlation coefficient. The data obtained in this study were analyzed by one-way analysis of variance (ANOVA) procedure of statistical package for social sciences (SPSS) version 16 for windows. Means were compared by Duncan's new multiple range test.

Results and Discussion

Ventilation Rate

The ventilation rate of the fish significantly increased 2, 4, 6, 8, 10, 30, and 60 minutes after subjecting to high ammonia levels. It shows that fish undergone a stressful condition since immediately after 2 minutes of subjecting to stressor, VR of T2, T3 and T4 were all significantly faster compared to T1 which is the control group (Table 2). It is proven that VR is an indicator of stress in fish since it changes quickly in response to the disturbance imposed [15]. In a situation that increases alertness, it is expected that there is an adjustment of ventilatory response, since one of the functions of the ventilatory system is to supply the body with enough oxygen for all behavioral tasks such as quick action or quick escape.

Highest mean of VR was observed on the fish subjected to 0.06-0.10 ppm of NH_3-N for 30 minutes given that this concentration is considered to be lethal to fish. Furthermore, fish in T4 died after 60 minutes of exposing to 0.11-0.15 ppm of NH_3. According to Robinette [16], the toxic levels for un-ionized ammonia for short-term exposure usually lie between 0.6 and 2.0 mg L-1 for pond fish, and sub-lethal effects may occur at 0.1 to 0.3 mg L-1. It was also revealed in the study of Emerson *et al.* [17], that a concentration of 0.6 mg/L of un-ionized ammonia, is capable of killing fish even if exposed briefly while chronic exposure to levels as low as 0.06 mg/L can cause gill and kidney damage and reduction in growth.

Table 2: The VR of the fish subjected to different levels of un-ionized ammonia.

Period (minutes)	Concentration of Un-Ionized Ammonia			
	Control (no Addition of Ammonium Chloride)	**0.046-0.05 ppm**	**0.06-0.10 ppm**	**0.11-0.15 ppm**
0	21.95±0.60[a]	22.52±0.08[a]	21.72±1.17[a]	22.50±0.58[a]
2	22.80±0.09[b]	25.30±0.32[a]	24.53±2.38[ab]	24.00±0.82[ab]
4	23.03±0.09[c]	26.12±0.46[b]	27.18±0.90[a]	27.50±0.58[a]
6	24.13±0.32[c]	26.60±0.21[b]	27.00±1.16[ab]	28.25±1.26[a]
8	24.03±0.12[d]	27.77±0.56[c]	28.65±0.81[b]	30.50±0.50[a]
10	23.60±0.21[d]	29.00±0.54[c]	31.07±0.78[b]	34.00±1.41[a]
30	20.78±0.03[c]	32.30±0.87[b]	38.50±0.41[a]	37.50±1.73[a]
60	24.40±0.14[c]	32.80±0.81[b]	37.80±1.40[a]	dead

*Means having different superscripts within a row are significantly different at each other by DMRT at the 5% probability level.

Eye Color Pattern

The ECP of Red tilapia in T2, T3 and T4 were significantly darker compared to T1 after 2, 4, 6, 8, 10, 30 and 60 minutes of subjecting to stressor. The highest ECP was observed in the Red tilapia subjected to 0.06-0.10 ppm NH_3-N for 60 minutes. Results showed that the mean ECP of the Red tilapia gradually increased as the duration of exposure to stress increases. ECP has been proven to be a potentially easy, inexpensive, non-invasive and reliable way to measure conditions of fish stress in Nile tilapia [14, 18] and other fish species [19]. According to Frietas *et al.* [20], eyes of Nile tilapia with greater than 25% darkened area is considered to be in the group with darker ECP. In this study the ECP values of Red tilapia exposed to ammonia stressor were greater than 25% which implies that darkening of the eyes of Red tilapia was a part of stress response in this fish species.

Table 3: The ECP of the fish subjected to different levels of un-ionized ammonia.

Period (minutes)	Concentration of Un-Ionized Ammonia			
	Control (no Addition of Ammonium Chloride)	**0.046-0.05 ppm**	**0.06-0.10 ppm**	**0.11-0.15 ppm**
0	6.64±0.06[a]	6.25±0.06[a]	6.25±0.06[a]	0.00±0.00[a]
2	6.64±0.06[a]	18.36±0.03[a]	15.23±0.07[a]	6.25±0.06[a]
4	6.64±0.06[b]	31.84±0.02[a]	33.01±0.07[a]	25.00±0.03[a]
6	6.64±0.06[b]	32.42±0.02[a]	33.20±0.07[a]	25.00±0.03[a]
8	6.64±0.06[b]	32.62±0.02[a]	33.40±0.07[a]	28.13±0.02[a]
10	6.84±0.06[b]	34.18±0.01[a]	36.72±0.06[a]	31.25±0.02[a]
30	7.42±0.07[b]	36.33±0.01[a]	50.78±0.02[a]	50.00±0.00[a]
60	7.42±0.06[b]	39.45±0.01[a]	58.40±0.01[a]	dead

*Means having different superscripts within a row are significantly different at each other by DMRT at the 5% probability level.

Skin Color

Statistical analysis showed that there was a significant difference in the skin color between T1 (control) and the treatments subjected to high ammonia levels (T2, T3, & T4). Significantly higher scores or darker skin colors were attained by T2, T3 and T4 compared to T1. This suggests that skin color of Red tilapia intensifies and becomes redder when subjected to ammonia stress. Results also showed that skin color intensifies as the duration of time that the fish is exposed to stress increases. Highest rate and change in skin color was attained by T4 after 30 minutes of subjecting to stressor. This may also justify that fish in T4 experienced more stress as compared to other treatments since the fish died after 60 minutes of exposure to ammonia stress.

Color change in animals is fundamental for many situations such as camouflage to avoid predators [21], thermoregulation [22] and sexual selection - to stand out and attract mates [23]. However, rapid change in color is associated with social signaling and breeding [24]. In teleost fishes, skin color can change in response to environmental conditions, physiological challenges, stressful stimuli [25] and cultural condition [26]. In this study, intensifying color of red tilapia can be due to their stress coping style against high ammonia levels (Figure 2).

Table 4: The skin color of the fish subjected to different levels of un-ionized ammonia.

Period (minutes)	Concentration of Un-Ionized Ammonia			
	Control (No Addition of Ammonium Chloride)	**0.046-0.05 ppm**	**0.06-0.10 ppm**	**0.11-0.15 ppm**
0	4.45±0.92[a]	5.50±0.35[a]	5.10±0.99[a]	5.50±0.58[a]
2	4.45±0.92[b]	5.50±0.35[ab]	5.10±0.99[ab]	6.00±0.00[a]
4	4.45±0.92[b]	5.52±0.33[ab]	5.10±0.99[ab]	6.00±0.00[a]
6	4.45±0.92[b]	5.53±0.33[a]	5.35±0.70[ab]	6.00±0.00[a]
8	4.45±0.92[b]	5.57±0.35[a]	5.35±0.70[ab]	6.25±0.50[a]
10	4.45±0.92[b]	5.57±0.35[a]	6.37±0.48[a]	6.50±0.58[a]
30	4.47±0.94[c]	5.77±0.38[b]	6.40±0.49[ab]	7.00±0.00[a]
60	4.47±0.94[c]	6.02±0.46[b]	6.92±0.17[a]	N/A

*Means having different superscripts within a row are significantly different at each other by DMRT at the 5% probability level.

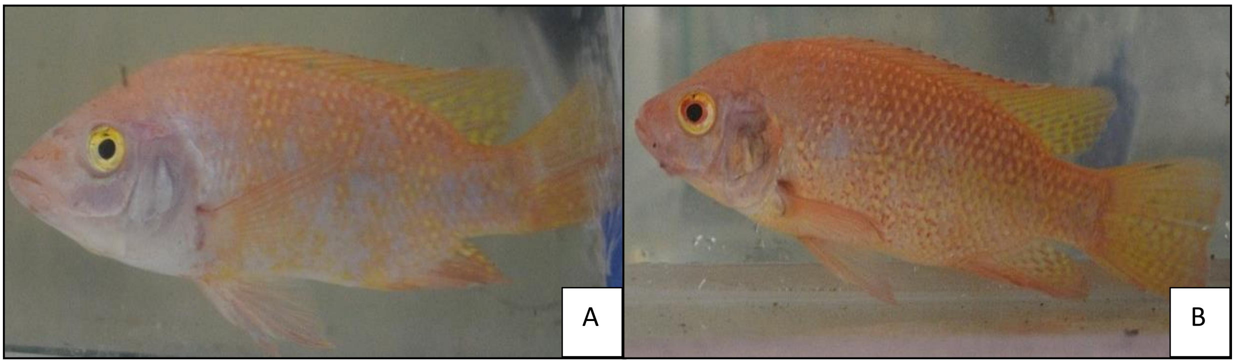

Fig 2: Skin color of red tilapia subjected to 0.11-0.15ppm of un-ionized ammonia: (a) before introduction to stressor; (b) after introduction to stressor

Water Quality Parameters

The dissolved oxygen, temperature and pH shows no significant differences (Table 5) in all treatments since the continuous supply of oxygen was provided during the experiment and the temperature was maintained using heater. The TAN and NH_3 shows significant difference between control and all treatments since ammonium chloride was added in the water. The TAN and NH_3 of the water was monitored daily using spectrophotometer and water was changed every five days to maintain the desired concentration of NH_3 in each treatment.

Table 5: Mean water quality parameters observed in the study.

Water Quality Parameters	Concentration of Un-Ionized Ammonia			
	Control (no Addition of Ammonium Chloride)	0.046-0.05 ppm	0.06-0.10 ppm	0.11-0.15 ppm
DO	6.402±0.230[a]	6.532±0.126[a]	6.540±0.166[a]	6.413±0.228[a]
Temperature	28.148±0.017[a]	28.143±0.013[a]	28.060±0.145[a]	28.175±0.050[a]
pH	8.152±0.017[a]	8.097±0.032[a]	8.138±0.045[a]	8.075±0.050[a]
TAN	0.046±0.004[d]	0.635±0.037[c]	1.074±0.099[b]	1.720±0.230[a]
NH_3	0.004±0.001[d]	0.050±0.001[c]	0.091±0.002[b]	0.135±0.006[a]

Conclusion

Physiological and morphological aspects of Red tilapia such as ventilation rate, eye color pattern and skin color changed as stress response to high ammonia levels. A large variation between pre-stress period and post-stress period value of the stress indicators were observed. High levels of un-ionized ammonia such as 0.046-0.05, 0.06-0.10 and 0.11-0.15 ppm were considered to be stressful since faster VR, darker ECP and intensified skin color were observed in the fish samples. Moreover, concentration such as 0.06-0.10 and 0.11-0.15 ppm of un-ionized ammonia is considered to be lethal and can cause death to Red tilapia if prolonged.

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