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RESEARCH ARTICLE

Effects of ammonia toxicity on growth performance, cortisol, glucose and hematological response of Nile Tilapia (*Oreochromis niloticus*)

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ABSTRACT

Ammonia is a production limiting factor in the aquaculture media affecting fish production. A study was designed to scrutinize effects of ammonia on growth performance, survival, cortisol and hematological parameters of Tilapia fish. The study examined effects of 96 h-incubation of male and female Tilapia with 3 mg ammonium chloride per a liter of water compared to control. The study has been carried out in the physiology laboratory of the department of animal and fish production, Alexandria University. Fourteen aquaria were used (6 control and 8 ammoniated). Each aquarium contained 6 fish (half the population males and the other have females). Duration of the control reared fish was 30 days, however the duration for ammoniated group was 4 days. In all stressed fish, there found decreases in final body weight, average daily gain and specific growth rate as compared to controls. Hematological parameters revealed increases ($P<0.05$) in total leukocyte counts in both males and females exposed to stressors. There were significant decreases ($P<0.05$) in red blood cell, hematocrit value and hemoglobin concentration in both males and females. There were non-significant differences ($P>0.10$) in these parameters between males and females. Exposing both male and female tilapia to ammonia, resulted in increases ($P<0.05$) in mean corpuscular volume (MCV). Mean corpuscular hemoglobin (MCH) didn't change in male tilapia, while females expressed increased MCH values in the ammonia condition. Mean corpuscular hemoglobin concentration (MCHC) decreased ($P<0.05$) under ammonia with no differences between males and females. Differential leukocyte count exhibited increases ($P<0.05$) in neutrophils in ammonia-exposed males and females and decreases ($P<0.05$) in eosinophils and monocytes in males, but not in females. However, lymphocytes decreased ($P<0.05$) in both females and males exposed to ammonia. Cortisol level increased ($P<0.05$) by about 2 folds in both sexes of fish exposed to ammonia (2.95 and 2.72 *vs* 6.40 and 6.48 ng/ml in control male**s** and female**s** *vs* ammonia-exposed males and females).Rearing tilapia fish in media containing high level (3mg/l water) of ammonium chloride not only deteriorated growth rate but it also negatively affected the health wellbeing.

Keywords: Tilapia, physiological responses, ammonia, growth, hematology, cortisol.

INTRODUCTION

There is a global need for cheap sources of protein to meet the world's over-population. More attention is being paid to fish farming and its surrounding media. In the developing countries where the problem is acute, tilapia cultures are believed to offer one of the solutions, especially in view of the depletion of existing fisheries. Tilapias are second only to carps as the most widely farmed freshwater fish in the world. The Nile tilapia (*Oreochromis niloticus*) was one of the first fish species cultured. Biological constraints to the development of commercial tilapia farming are their inability to withstand sustained water temperatures below 25 to 30°C (Popma and Michael, 1999). Tilapia world production has been increased more than six folds between 1986 and 2002. Several stressors negatively affect the intensive tilapia farming. FAO report (FAO, 2006) defined stress as physical or chemical factors that cause body reactions that may result in diseases and deaths.

Physiological responses of fish to environmental stressors grouped as primary, secondary and tertiary. Primary responses, involve the initial neuroendocrine responses, including the release of catecholamine from chromaffin tissue and the stimulation of the hypothalamic-pituitary-interrenal (HPI) axis culminating in the release of corticosteroid hormones into circulation. Secondary responses include changes in plasma and tissue ion and metabolite levels, hematological features, and heat shock or stress proteins (HSPs), all of which relate to physiological adjustments such as in metabolism, respiration, acid-base status, hydromineral balance, immune function and cellular responses. Additionally, tertiary responses, which refer to aspects of

whole-animal performance such as changes in growth, condition, overall resistance to disease, metabolic scope for activity, behavior and ultimately survival (Barton, 2002; Ruane *et al.*, 2002; Iwama *et al.*, 2004). Ammonia is one of the most common stressors to fish health and production. The lethal effects of ammonia toxicity induce nervous disorders such as lethargy, convulsion, loss of equilibrium, side way swimming, increasing mucus in both gills and the body surfaces, gill hemorrhage and necrosis, kidney damage and circling and spiraling movements and finally death. Ammonia causes damage to the gills at a level as low as 0.25 mg/l. Symptoms of ammonia poisoning in fish includes; purple, red or bleeding gills (inflamed), inflamed eyes or anus. Also, fish may clamp and appeared darker in color, red stricken on fins or body, fish may gasp for at the surface of the water tank exhibiting torn and jagged fins (Fernandez and Mazon, 2003).

In a high density of aquaculture system nitrogen compounds have been identified as major metabolic products in fish culture (Erol *et al.,* 2010). In an intensive farmed freshwater fish culture, the most common pollutant is ammonia and the common sources of ammonia to fish are the unconsumed fish food and/or the fecal matter that constitute a big hazard for fish culture industry as it causes severe respiratory problems as gasping, flaring opercula, asphyxia and death of fish (Lin and Chen, 2003). Recently, Barbieri and Bondioli (2015) found that exposure of Pacu fish to different concentrations of ammonia-N caused an elevation in total hemoglobin and blood glucose. The sub-lethal effects induced decrease in growth rate and resistance to diseases and poor food conversion (Kuttchantran, 2013). Fish excretion is the main source of ammonia in fish body and its excretion is directly related to the feeding rate and the protein levels. Also, organic matter produced by algae or added to ponds as feed is a good source of ammonia. Severe reduction in both growth and survival rates were noticed in *tilapia rendalli* reared in quail manure tanks (Masautso *et al.,* 2014).

Ammonia accumulation may reduce growth, increases oxygen consumption and ammonia-N excretion, altering concentrations of haemolymph protein and free amino acids levels and causes mortality increment (Lin and Chen, 2003; Barbieri and Bondioli, 2015). El-Sayed (2015) studied effects of ammonium nitrate on the hematological parameters and the serum attributes of Nile tilapia (*O. niloticus*) and found a parallel disturbance in all parameters with the increase of ammonia concentration. Therefore, the aim of the present study was to investigate effects of a common stress factor (i.e. hyper-ammonia levels) on growth performance, physiological characteristics and differences between sexes of Nile tilapia.

MATERIALS AND METHODS

Study Location

This study has been performed during October 2011 to May 2012 in the physiology laboratory, Department of Animal and Fish Production, Faculty of Agriculture, University of Alexandria, Egypt.

Experimental Design

The study utilized 14 glass aquaria measuring 70 L× 40 W × 30 H cm with a maximum capacity of 84 liters and each contained 60 liters of de-chlorinated tap water. Eighty four growing Nile tilapia (*Oreochromis niloticus*) fish were used in the experiment. Half of the population was males and the other half were females. The fish were purchased from El-Shark El-Awsat farm in El-Behira governorate. The average initial body weight was 50±5 g. Fish were fed at 3% of mean body weight at three times a day (8.00 AM, 12.00 PM and 18.00 PM.), as adaptation period and the unhealthy fish were removed from experimental aquaria and replaced with healthy ones. The fish were acclimatized for at least 20 days before the beginning of the experiment. Also the aquaria were supplied with a good aeration throughout the experimental period. Water temperature was thermostatically adjusted at 27 ±2C° as an optimum temperature for *O. niloticus* and hydrogen ion concentration (*p*H) was monitored using *p*H meter with a pH range of 7-8. Fish from the same parental stock were randomly distributed (6 fish /aquarium).

Measurement of Growth Performance

Live body weights of each group were recorded pre and post treatments. Body weight gain, average daily gain and specific growth rate were estimated by the following equations:

Body weight gain (g fish^{-1}) = final body weight (g) – initial body weight (g).

$$\text{The average daily gain (ADG) (g day}^{-1}) = \frac{\text{Body weight gain (g)}}{\text{Treatment Period (days)}}$$

$$\text{Specific growth rate (SGR)(\%)} = \frac{\text{Ln final body weight} - \text{Ln initial body weight}}{\text{Treatment period (days)}} \times 100$$

Table 1. Experimental outline of fish feeding and blood sampling

Group	Treatment	Duration (days)	Blood sampling interval (days)
I- Control	6 fish /aquarium, protein level was 30%, without ammonium chloride.(6 aquaria)	30	0, 7, 14, 21and30
II- Ammonia	6 fish/aquarium, diet-protein level was 30%+3mg ammonium chloride/liter water. (8 aquaria)	4	0 and 4

Blood Sampling

Fish were bled from the caudal vessels. Two sets of blood samples were collected one with anti-coagulant (heparin) and the other without coagulant. The whole blood samples were used for the measurements of hematological parameters. On the other hand, the non-coagulated blood samples were centrifuged (3000 rpm for 20 minutes) and sera were harvested and used for the measurements of cortisol and glucose.

Hematological and Cortisol Analyses

Red and white cells counts were made using Natt and Herrick solution as the diluent and stain (Natt and Herrick, 1952). The red and white blood cells counts were expressed in millions and thousand per mm^3 blood, respectively. Hematocrit value (PCV) was determined using hematocrit capillary tubes after centrifugation at 3000 rpm for 20 minutes in PCV centrifuge.

Hemoglobin (Hb) concentration was measured by colorimetric kits (Diamond, Egypt). The principle of the procedure based on that hemoglobin is first oxidized by potassium ferricyanide into methemoglobin which is converted into cyanomethemoglobin and the color was measured at a wavelength of 540 nm. Cortisol concentration in blood serum was determined by a commercial ELISA (Dima, Germany) kit using the method of Foster and Dunn (1974). Intra-assay CV was 4.3%.

Statistical Analysis

Data for growth, blood attributes, cortisol and glucose were analyzed by the least square analysis of variance (SAS, 2000). Comparisons between treatment means were achieved by the least significant differences (LSD) (Steel and Torrie, 1984). Significant levels were considered at $P<0.05$.

RESULTS AND DISCUSSION

Growth Performance

Initial and final body weights are presented in Table 2. The fish chosen for this study was, to a great extent, similar in size and weight (Figure 1). Final body weight was taken differently among treatments, since duration of the treatment varied according to the experimental outline. Weight gain, average daily gain (ADG, g/fish/day) and specific growth rate (SGR) as estimates of daily growth were reduced ($P<0.05$) in the presence of ammonia. In control media male fish exhibited higher ($P<0.05$) average daily gain and SGR (%) than females. Likewise, in the ammoniated aquaria the negative growth was less in males (-1.88 g/fish/day) than in females (-1.95 g/fish/day). Also, specific growth rate (%) was positive in control and negative in ammonia-exposed fish.

The Female's growth was most likely affected by ammonia (Table 2). Evidently, several investigators reported similar results. Frances *et al.* (2000) reported a decrease in body weight of silver perch as a consequence to elevated ammonia in water. Also, Foss *et al.* (2003) obtained similar trend in juvenile spotted wolfish. Moreover, Dosdat *et al.* (2003) and Lemarie *et al.* (2004) reported such phenomena in European sea bass. Recently, Sakala and Musuka (2014) reported a toxic effect of increased ammonia on tilapia growth rate. The loss of weight in ammonia treatment is more likely due to the inhibition of the fish appetite leading to significant reduction of feed intake and disturbance in metabolism. As shown in Figure 2, there found necrosis and lesions in the body and necrosis and splitting in fins. Ammonia and urea were found to be the

two main nitrogenous products excreted by teleosts, with ammonia usually representing 75-90% of the nitrogenous excretion (Handy and Poxton, 1993).

Ammonia toxicity depends on the concentration of the unionized ammonia (NH_3). Fish branchial membranes are relatively permeable to NH3, but not to $NH4^+$, due to its molecular size. When dissolved in water, ionized and unionized forms of ammonia are in equilibrium, which is affected by water pH, temperature and salinity. High levels of ammonia cause stress and produce harmful physiological response such as osmoregulatory disturbances, kidneys and branchial epithelium damages and retarded growth (Meade, 1989; Soderberg, 1994), inefficient immune response (Cheng *et al.,* 2004; Pinto *et al.,* 2007) and reduced survival (Jobling, 1994).

Table 2. Effect of high level of ammonia on growth of tilapia fish (Mean±SEM)

Parameters	Control		Ammonia	
	Male	Female	Male	Female
Initial Weight (g)	52.07±0.78	47.57±0.67	51.81±0.54	48.8±0.55
Final Weight(g)	68.46±0.50^a	61.0±0.93^b	44.26±1.63^c	40.96±1.23^c
Growth (g)	16.40±0.35^a	13.43±0.35^b	-7.54±0.35^c	-7.83±0.35^c
ADG (g/fish/day)	0.54±0.01^a	0.44±0.01^b	-1.88±0.01^c	-1.95±0.01^d
SGR (%)	0.91±0.02^a	0.82±0.02^b	-5.46±0.03^c	-4.30±0.02^d

^{a,b} Means in the same row with different superscripts differ significantly (P< 0.05).

Table 3. Effect of high level of ammonia on the hematological parameters of tilapia fish (Mean±SEM)

Parameters	Control		Ammonia	
	Male	Female	Male	Female
WBCs($\times10^3$/mm^3)	24.07±0.13^a	23.09±0.13^a	26.17±0.12^b	25.55±0.13^c
RBCs ($\times10^6$/mm^3)	2.24±0.02^a	2.11±0.03^a	1.17±0.02^b	1.76±0.02^c
PCV (%)	25.09±0.14^a	25.12±0.13^a	21.92±0.12^b	21.91±0.14^b
Hb (g/dl)	7.80±0.06^a	7.78±0.05^a	6.41±0.06^b	6.41±0.05^b
MCV (µm3)	112.93±0.81^a	119.39±0.79^b	131.44±0.78^c	127.81±0.81^d
MCH (pg)	35.15±0.24^a	37.00±0.25^a	37.46±0.24^a	36.66±0.26^a
MCHC (%)	31.11±0.16^a	30.99±0.17^a	28.93±0.16^b	28.96±0.17^b

^{a,b} Means in the same row with different superscripts differ significantly (P< 0.05).

Table 4. Differences of leukocytes differential count (%) between males and females of tilapia fish

Sex	Neutrophil	Eosinophil	Basophil	Monocyte	Lymphocyte
Male	27.76±0.23^b	8.78±0.06^a	8.64±0.06^a	1.94±0.05^b	52.87±0.2^a
Female	30.63±0.23^a	8.62±0.06^a	8.17±0.06^a	2.20±0.05^a	50.38±0.2^b

^{a,b} Means in the same column with different superscripts differ significantly (P< 0.05).

Table 5. Effect of high level of ammonia on blood cortisol of tilapia fish (Mean±SEM)

Metabolite	Control		Ammonia	
	Male	Female	Male	Female
Cortisol (ng/ml)	2.95±0.24^a	2.72±0.3^a	6.40±0.65^b	6.48±0.70^b
Glucose (mg/dl)	52.58±1.45a	54.80±1.45a	83.72±1.45b	85.26±1.45b

^{a,b} Means in the same row with different superscripts differ significantly (p< 0.05).

Changes in the Hematological Parameters

WBCs

Data of the effect of ammonia on WBCs, RBCs, PCV and Hb are presented in Table3. Results show that there were significant increases ($P<0.05$) of leukocyte counts (WBCs) in tilapia exposed to ammonia. This finding agrees with others who recorded an increase of WBCs in red tilapia after exposure to 3 mg NH4Cl/ l for 3 days (Bonnie and Liu, 2004). Moreover, Das *et al.* (2004) obtained similar trend concluding that the increase in WBCs as a result of exposure to stresses is involved in the regulation of immunological function of fish. Such an increase in total leukocyte occurs by the increase in lymphopoies is and/or enhanced release of lymphocytes from lymphoid tissues. The increase in WBCs in males was evidently higher than females supporting the idea of more adaptation of males than females to the surrounding media.

RBCs and PCV

Relative to erythrocyte counts the results show significant ($p<0.05$) decrease in RBCs in ammonia-exposed fish. The decrease of RBCs in ammonia condition was higher in males than in females. Overall, the decrease of RBCs approached 33% of the control value. Since, red blood cells of teleost produced from hematopoietic tissue of the kidney, spleen and liver the stress impact of ammonia might damage such organs to the degree that they caused reduction of erythrocytes (Das *et al.*, 2004). El-Sherif and El-Feky (2008) demonstrated histopathological damage to the kidneys of tilapia fish expose to ammonia. Kidney damages were marked hyaline droplet degeneration and swelling of renal tubules, majority of renal tubules showed vacuolations, necrotic epithelia, necrosis in melanomacrophages center and increased thrombus formation and infiltration of melanomacrophage cells between the renal tubules. These tissue damages might contribute to the decreased ability of renal hematopoietic function.

Data of hematocrit value (PCV) are shown in Table 3.There exist a significant ($p<0.05$) decrease in PCV in ammonia-exposed fish. The decrease of PCV in ammonia-exposed fish approached 13 % of the control. There were no statistical differences due to gender in this parameter. Similar finding was attained by El-Sherif and El-Feky (2008) even though they tested lower levels of ammonia.

Hb Concentration

Similar trend to what was found with PCV, hemoglobin concentration was lower ($P<0.05$) in ammonia-exposed fish than control by about 18%. There were no obvious differences due to gender of fish. The decreases in RBCs, PCV and Hb concentration obtained in the present study might be attributed to the ammonia toxicity in the media which might have caused damages to the vital organs (i.e. gills, liver, spleen, kidneys).As it has been well established that liver and kidneys are the sites of erythrocyte production, the damage caused by ammonia might suppressed this process. Additionally, the increase of NH3 in the blood circulation might ruptured high percent of RBCs and/or caused hemodilution resulting in a disturbance of osmoregulation across gill epithelium (Vosyliene *et al.,* 2003). Similar results were recorded in red tilapia (Bonnie and Liu, 2004) and in mrigal fish (Das *et al.*, 2004). Surprisingly, there found a species differences on the tolerance of different fish to ammonia level. This has been shown by Dosdat *et al.* (2003) who reported no changes in hematocrit value in European sea bass exposed to 0.014- 0.493 mg NH_3-N /l for 61 days. Greater reduction (≈ 45%) in Hb was recorded in tilapia fish exposed to 0.15 mg NH3/l for 60 days (El-Sherif and El-Feky 2008). Differential leukocytes count revealed increases of neutrophils and monocytes in females, while in males the increase was found in lymphocytes only (Table 4).

Changes in the Erythrocyte Indices (MCV, MCH and MCHC)

There exists a significant increase ($p<0.05$) in MCV values (Table 3) in fishes (males & females) exposed to ammonia compared with control. The increase in the mean corpuscular volume relative to the control attained 12 % higher than control. There were no changes ($P>0.10$) in MCH values due to treatment. Gender didn't express changes in MCH. Contrariwise, percentage of MCHC exhibited significant decreases ($p<0.05$) in ammonia-exposed compared with control fish. No differences were found in MCHC between males and females. The increase in corpuscular volume (MCV) as a consequence to ammonia exposure might be ascribed to the increased water content in red blood cells resulting of chloride shift and the decreased of plasma chloride at the same state of high ammonia in water. Moreover, the decrease of MCHC might be attributed to the hemodilution and/or the lack of production of hemoglobin in circulation. Toxicity of ammonia, nitrate and nitrite was found to impair the oxygen uptake by carp fish resulting in hazardous consequences on red blood cells production and their components (Tilak *et al.,* 2007). More recently, Shokr (2015) obtained similar results when he exposed Nile tilapia to ascending levels of ammonium nitrates.

Figure 1. Healthy control tilapia fish (*Oreochromis niloticus*)

Changes in Serum Cortisol and Glucose

As shown in Table 5, there found a massive increase in serum cortisol in the ammonia-exposed fish. The increase represents 127% more than control values. Likewise, glucose levels increased ($P<0.05$) by about 57%. Recently, Metwally and Wafeek (2014) reported vast increases of cortisol and glucose in fish exposed to ammonia. Evidently, hypothalamo-pituitary interrenal axis, stimulated by ammonia as a stressor elevated blood levels of cortisol which in turn leads to lipolysis, glycogenolysis and gluconeogenesis to provide energy under stress conditions (Hontela *et al.,* 1992). The hyperglycemic condition observed in many teleost release condition is mainly mediated by effect of catecholamine on glucose release from liver which is considered the main carbohydrate store in fish(Van den Thillart and Van Raaji, 1995).

Figure 2. Tilapia exposed to ammonia showing body lesions and necrosis, split and lesioned fins

CONCLUSIONS

One of the most risky stressors to fresh water fish is the elevation of the ammonia and its derivatives in the aquaculture environment. The main negative consequences of the elevated ammonia in Tilapia culture are the sharp decrease in body growth rate, change in hematological traits, increased cortisol and glucose in the blood to cope with ammonia toxic effects.

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