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Effect of Ammonia Stress on the Genomic DNA of *Macrobrachium nipponense* Collected from Al Qurna City, Iraq.

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ABSTRACT

Macrobrachium nipponense, decapod crustaceans belonging to Palaemonidae family, is an important nutritionally economically species. Ammonia is a major pollutant in the aquatic environment, and adversely affect the health of *M. nipponense* and other associated commercial species. Here, the animal of interest was exposed to acute toxicity levels of ammonia at 3, 3.5, 4, 4.5, 5, and 6mg/ L for a duration of 72 hours. DNA damage served as an indicator for ammonia toxicity in the current study. Fragmentation occurring in DNA of the animal as a result of ammonia toxicity was substantiated using the comet assay and expressed as a percentage of tail DNA. The results showed an increase in the DNA damage at ammonia concentrations of 4.5 and 5mg/ L for a duration of 72 hours, with the tail DNA percentage reaching 14 and 19%, respectively. While the highest DNA damage occurred at ammonia concentration of 6mg/ L for a duration of 72 hours, the tail DNA recorded 23%. These findings suggested that exposure to ammonia caused damage in the DNA of *M nipponense* which reflects its effect on the physiological functions and threaten this economically important animal.

INTRODUCTION

The origin of *M. nipponense* (De Hann, 1849) is China, and it has been observed in Taiwan and Japan **(Cai & Ng, 2002)**, Ozbakstan **(Mirabdullaev & Niyazov, 2005)**, Singapore and the Philippines **(Cai & Shokita, 2006)**, as well as North Iran **(De Grave & Ghane, 2006)**. Its first recorded presence in Iraq was at Garmmat Ali River, near the Al-Hammar Marshes **(Salman *et al*., 2006)**. This species is economically important and cultured worldwide **(Fu *et al*., 2012)**. The animal is sensitive to ammonia, which is a major environmental pollutant. Increasing concentrations of ammonia may include toxic influences. For instance, elevated ammonia can cause susceptibility to pathogens, leading to inhibited growth, decreased osmoregulation, increased molting frequency, and mortality **(Jiang *et al*., 2004)**. Rapid elevation or transient increases in ammonia levels cause toxic effects on shrimp **(Chen *et al*., 1990)**. The detoxificantion of ammonia by molecular mechanisms is still unclear. Few studies have inestigated the molecular and

biological responses of *M. nipponense* to ammonia toxicity **(Ding *et al*., 2017)**. According to **Cheng and Chen (2002)** and **Cheng *et al*. (2003)**, the susceptibility to pathogens increases in *M. rosenbergii* when exposed to ammonia. Moreover, the molecular mechanism underlying ammonia stress in *M. rosenbergii* are still not understood. Several studies revealed that the exposure to ammonia could lead to increasing levels of ROS or reactive oxygen species, resulting in an oxidative stress **(Halliwell, 1999; Cheng *et al*., 2015)**. The bio-macromolecules, such as DNA, are damaged by ROS overproduction, leading to cell dysfunction. In response to this stress, cells develop antioxidant defense system, which includes non enzymatic antioxidants (such as thioredoxine, glutathion) and enzymatic antioxidants (such as SOD, CAT) **(Sies, 1991)**. Studies by **Zhang *et al*. (2015)**, **Li *et al*. (2018)** and **Jiang *et al*. (2019)** mentioned that antioxidant enzymes activities were affected in crustaceans exposed to ammonia. **Liang *et al*. (2016)** revealed that *Litopenaeus vannamei* exposed to ammonia (20mg/ L) resulted in a decrease in hepatopancreas SOD enzyme activity. While **Jiang *et al*. (2019)** showed that *L. vannamei* exposed to 15mg/ L ammonia for 6- 24h resulted in increased levels of total-antioxidant-capacity (TAC), as well as activity levels of antioxidant enzymes in both intestine and hepatopancreas tissues. In this article, we have employed the comet assay technique to investigate the potential DNA damage caused by different different concentrations of ammonia in the hemolymph of *M. nipponense* sampled from Shatt al- Arab River at Basrah province, Iraq.

MATERIALS AND METHODS

1. Sampling

The adult specimens of *M. nipponense* were obtained from the Shatt Al- arab River at Al Qurna City. The specimens were collected during the spring of 2022. The sampling site is situated at approximately 30.999562 and 47.460719 latitude and longitude, respectively (30° 59' 58.4232" N and 47° 27' 38.5884" E GPS) (Fig. 1). The trawl net of 1cm was used for sample collection. The animal specimens were transported to the laboratory in freshwater collected from the site of collection.

2. Experiment

Before the experiment, the animals were acclimated to laboratory conditions in containers filled with stored tap water for 3 days. The temperature was maintained at 27°C, pH at 8, and the shrimp were fed three times daily with commercial feed. Additionally, water was exchanged daily at a rate of 20%. A stock of ammonia exposure solution, 1000mg/ L of (NH_4Cl), was set by using distilled water. From this stock solution, a series of ammonia concentrations (3, 3.5, 4, 4.5, 5 and 6mg/ L) were prepared using stored tap water in 5L exposure container. The experiment began by transferring 10 acclimated animals into each exposure container, maintaining the same acclimation conditions. For the control, it underwent the same subjected conditions of the experiment

as the other groups, except that no ammonia was added. After 72h of exposure, animals of each treatment were taken out and anaesthetized on ice. The hemolymph was then obtained from the animal heart by using an anti-coagulant sterilized syringe. The hemolymph was kept frozen until it was used in the tests.

3. Comet assay

To assess the DNA damage, Oxiselect comet assay kit (Cell Biolabs, USA) was used. The procedure was performed depending on the instructions provided by the producer company. the principle involves mixing the cells sample (hemolymph in this study) with a low melting point agarose, which is then applied on an Oxiselect comet slide covered with regular point agarose. After that, the mixed cells were exposed to a lysis buffer to lyse the cells and remove proteins, cytoplasmic and nucleoplasmic constituents, RNA, and cellular membranes. Then, an alkaline solution was applied on the slides to denature and relax the DNA. Subsequently, slides were electrophoresed to distinguish between the intact DNA and the damaged DNA. Vista green DNA dye was used to stain the samples, which were then visualized under a microscope connected to an image analysis system. The damage in DNA was scored according to **Duthie and Collins (1997)**.

Fig. 1. Site of sample collection

RESULTS

The comet assay is a useful biomarker for early detection of a genotoxicity, changes in integrity of DNA, and various cellular parameters. In addition, it provides insights into the presence of disturbances in the environment before they escalate to affect higher biological organizations **(Shugart, 1990; Clements, 2000)**. In the current study, *M. nipponense* (De Hann, 1849) collected from Shatt al- Arab River (Fig. 1) was exposed to several concentrations of ammonia for 72 hours. The results indicated that DNA was not affected at the concentrations of 3 and 3.5mg/ L of ammonia exposure for 72 hours ($P > 0.05$). While, the damage in the DNA appeared clearly at the concentrations of 4, 4.5, 5 and 6mg/ L of ammonia exposure for 72 hours ($P \leq 0.01$). Furthermore, the highest DNA damage occurred at a concentration of 6mg/ L, with the DNA tail percentage recorded at 23%. In addition, the results revealed that there are no significant differences ($P > 0.05$) in the percentage of DNA tail between concentrations of 4 and 4.5mg/ L of ammonia exposure (Fig. 2). Fig. (3) displays the disparity between the hemolymph DNA of *Macrobrachium nipponense* affected by ammonia exposure compared to unaffected DNA, as determined by the comet assay.

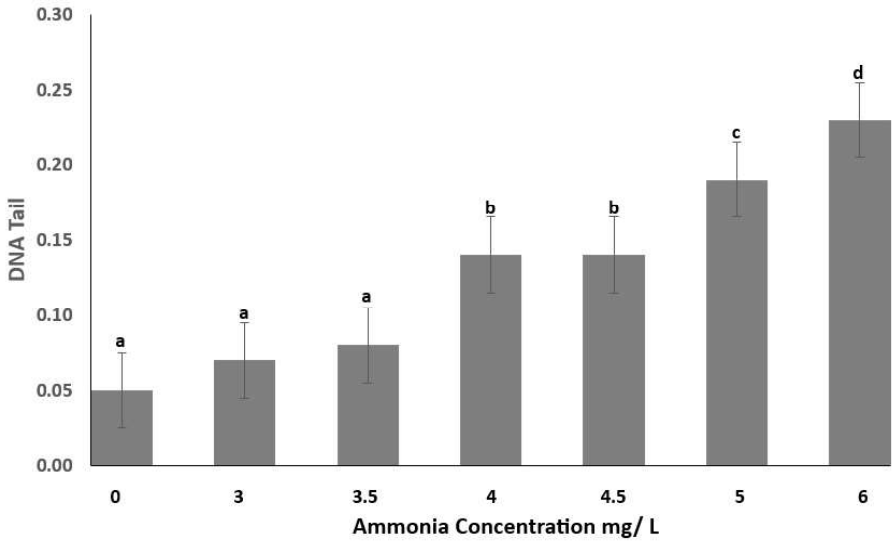

Fig. 2. Damage in hemolymph DNA of *Macrobrachium nipponense* resulting from exposure to ammonia at different concentrations for 72h. The values of the DNA tail reflect the extent of DNA damage using comet assay. Duncan's multiple test range was applied, and same letters indicate no significant differences

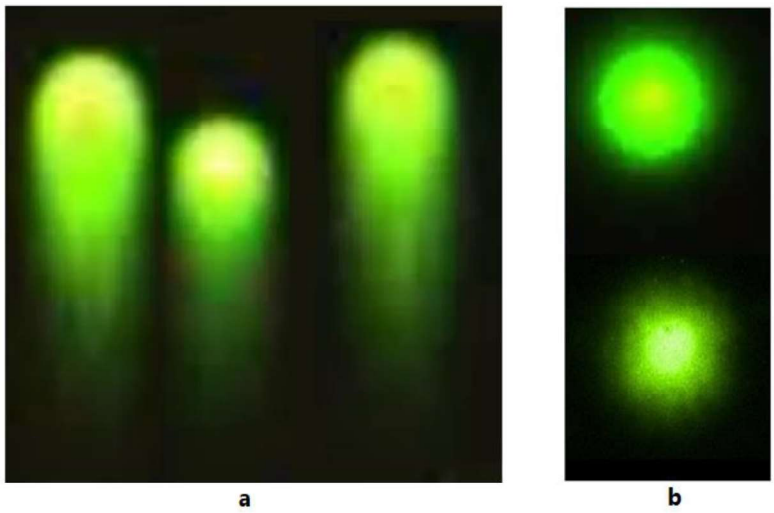

Fig. 3. Comet assay showing hemolymph DNA of *Macrobrachium nipponense*: **a)** DNA damaged by ammonia looks like comet, head plus tail. **b)** Intact DNA unaffected by ammonia (control)

DISCUSSION

There are heavy noxious effects of ammonia elevating in water on prawns. It was reported that ammonia increases the concentration of reactive oxygen species in aquatic animals, causing high oxidative stress. Moreover, studies have demonstrated the adverse effect of ammonia on antioxidant enzymes in several organs of crustacean **(Cheng *et al*., 2015; Liang *et al*., 2016; Jiang *et al*., 2019)**. In the current study, we investigated the effect of ammonia stress on the genomic DNA in hemolymph of *M. nipponense* collected from Shatt al- Arab River by using the comet assay. The results revealed that the genomic DNA was affected at ammonia concentrations of 4, 4.5, 5 and 6mg/ L for an exposure period of 72 hours. However, no effect of ammonia was observed at lower concentrations. This suggests that antioxidant systems may have attempted to eliminate the noxious effect of ammonia. Several studies mentioned the effect of ammonia on the genetic material of shrimp which aligns with the results of the current study. **Yu *et al.* (2019)** demonstrated that ammonia causes alterations in the expression patterns of numerous immune genes, such as alpha-2-macroglobulin, serine protease inhibitor and lectin 3 in shrimp *M. nipponense* collected from the lake of Weishan in the province of Shandong, China. Moreover, **Dong *et al.* (2021)** revealed that the expression of mRNA relative to heat shock proteins, Caspase 3 and antioxidant enzymes were affected in both the hepatopancreas and muscles by exposure to ammonia in *Macrobrachium rosenbergii* from a farm in Yancheng City, China. Furthermore, a study by **Liu *et al.* (2020)** suggested that the induction of ammonia stress experienced by the shrimp of *Litopenaeus*

vannamei caused apoptosis of hemocytes and affected the immunity of the shrimp, leading to increased susceptibility to pathogens.

CONCLUSION

This study provides an impression about the effect of ammonia stress on the genetic material. It was clear that, at low concentrations of ammonia, there were no effect on hemolymph DNA of *M. nipponense* compared to the control. This can be attributed to the different physiological mechanisms that protect genetic material and other molecules from ammonia stress such as antioxidant enzymes. However, when ammonia concentration reached a rate of 4mg/ L and above, the damage in hemolymph DNA clearly appeared. Additionally, the DNA tail, represented by damage value, recorded the highest percentage (23%) at the ammonia concentration of 6mg/ L. As ammonia concentration increased, the protecting mechanisms lose their ability to protect the DNA and other molecules from the damage caused by ammonia stress. In summary, elevated levels of ammonia can pose a threat to economically important species like *M. nipponense*.

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