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EFFECTS OF AMMONIA ON DIFFERENT BIOLOGICAL TRAITS OF THE ORANGE MUD CRAB (*Scylla olivacea*)

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Abstract
The orange mud crab (*Scylla olivacea*) is currently one of the most emerging crustacean species for coastal aquaculture, particularly in the Asian region. This species is sensitive to various aspects of water quality parameters. Ammonia is considered as one of the most common pollutants in crab farms that adversely affect overall production. Therefore, the present study observed the effects of different experimental doses of ammonia (0PPM, 5 PPM and 10 PPM) to investigate the effects on various aspects of cellular, physiological, biochemical and genetic alterations in the orange mud crab (*Scylla olivacea*). Significantly lower hemocyte counts were observed for the 5 PPM and 10 PPM ammonia treatments compared to the control group while no significant difference was detected between the two treatment groups. Ammonia treatments significantly altered expression pattern of the selected set of candidate genes. The α-amylase (growth gene) showed reduced expression (1.5–2 fold lower) in treatments while Toll like receptor (immune response gene) and Acetyl-CoA Carboxylase (metabolic gene) also showed significantly lower expression levels in treatment groups compared to the control. Significantly higher ($P < 0.05$) and constant rates of O_2 consumption in the control group throughout the experiment indicate that these crabs faced no stress and performed faster growth. Therefore, crabs in the control group (0 PPM) showed almost double growth (increase in body weight) compared to treatment groups (5 PPM and 10 PPM). Results indicate that different experimental doses of ammonia significantly altered the expression of candidate genes together with changes in physiological (O_2 consumption rates, growth), biochemical (total hemocyte counts, glucose and serotonin levels) and cellular (gill ultrastructure) parameters that adversely affected growth and mortality of crabs. Therefore, attempts are encouraged to maintain minimum or zero ammonia levels for sustainable mud crab farming across the coastal regions of Bangladesh.
Keywords: Ammonia concentrations, crab, physiological variations, biochemical alterations

Introduction

The coastal regions of Bangladesh provide ideal environmental and ecological conditions for open water fisheries as well as for farming of commercially important aquatic species. Shrimp farming is the major aquaculture activity across the coastal belt of Bangladesh but the farming of shrimp has been impeded due to outbreaks of various bacterial and viral diseases for a considerable time period (Rahi et al., 2022). The orange mud crab (*Scylla olivacea*) has shown enough promise to consider it as the ideal replacement species in this regard. Mud crab farming has gained considerable attention across the coastal regions of Bangladesh because of its high market price in domestic and international markets, ease of culture, acceptability to low cost feed and being less prone to disease outbreaks (Rahi et al., 2020). Initially, mud crab farming in Bangladesh was completely dependent on the supply of wild seed collected from the coastal rivers (particularly around the Sundarbans region) causing severe loss of biodiversity. Recent success in hatchery seed production has opened a new dimension to expand the farming of orange mud crab at an industrial scale (Rahi et al., 2020). Therefore, mud crab has emerged as an alternative livelihood, income and food source, and an innovative way to help vulnerable coastal communities to adapt to projected climate change scenario. Mud crab has already gained popularity in coastal communities in the greater Khulna region (Azam et al. 1998) and is recognized as candidate species for farming in brackish water environments (Ballao et al. 1999). Approximately 100,000 people directly or indirectly related to mud crab sector in Bangladesh including seed collection, farming and marketing. In 2021-2022, Bangladesh exported 7707 tons of live hard-shell crabs to international markets and earned US $ 21.1 million (FRSS, 2023). Over the past decade, production from the mud crab fishery has gradually increased, and more coastal communities becoming interested in crab farming. A healthy environment with adequate water quality and the biological characteristics of coastal water bodies are advantageous for the productive cultivation and farming of any commercially important aquatic species (Rahi and Shah, 2012; Afroz et al., 2021) including the mud crab. The availability of adequate water resources, human resources, great interest, and demand for exports accelerate its expansion. Furthermore, the mud crab is less susceptible to disease, easier to cultivate, more resistant to harsh environmental conditions, and can even live without water for a specified time (Salam et al., 2012; Rahi et al., 2020). Its short production cycle, increasing demand and price in the international market, and low investment requirement make this sector a fast-growing industry in Bangladesh. In an attempt to produce larger quantities of orange mud crab, improved farming systems are practiced (e.g., practicing higher stocking densities, application of more feed etc.). This higher density and excess food particles produce different types of toxic gases (i. e., ammonia) in the farming systems that can cause severe mortality and production failure. Ammonia is known as a common environmental toxicant in closed water bodies specially, in ponds that can build up rapidly following inhibition of denitrifying bacteria and reach dangerous concentrations (Anthonisen et al., 1976; Russo, 1985). Many human factors, including industrial wastes, municipal wastewater treatment plants, and farming contribute to increased ammonia concentration (Augspurger et al., 2003; Shah et al., 2011; Islam et al., 2014). Compared to ionized ammonia, un-ionized ammonia is more toxic for aquatic animals (Russo, 1985). In an intensive farming system, ammonia is a common pollutant resulting from the excretion of cultured animals and uneaten food particles (Lin and Chen, 2003). Accumulation of ammonia in the crab farms or ponds can reduce growth, increase oxygen consumption, alter protein concentrations of hemolymph and metabolic activity, causes cellular damage, increase susceptibility to diseases and also causes high mortality (Arana, 1997; Barbieri, 2009).

Considering the importance of crab culture and the harmful effects of ammonia, the study was conducted to investigate the effects of different doses of ammonia on the growth performance,

O_2 consumption rates, hemocyte counts, hemolymph glucose and serotonin (known as crustacean stress hormone), gill ultra-structure and expression pattern of three candidate genes (Alpha-amylase, Toll-like receptor and Acetyl-CoA Carboxylase) in the orange mud crab. This study will act as a foundation to take necessary precautions against the consequences of toxic ammonia which could be an obstacle for sustainable production of the orange mud crab.

Material and Method

Experimental tank preparation, sample collection and maintenance

In total, 18 experimental glass tanks (25 L each) were prepared for this experiment for three different ammonia levels (6 replicated tanks for each experimental group including 0 PPM as control, 5 PPM and 10 PPM). Juvenile crabs (crablets: ≈1.5 g body weight) were collected from a commercial crab hatchery located at Shyamnagar, under Satkhira district of Bangladesh. Crablets were brought in the Wet laboratory of Fisheries and Marine Resource Technology (FMRT) Discipline, Khulna University, Bangladesh and were acclimated in the experimental tanks for 5 days. The crablets were acclimated in 10‰ salinity (the same salinity maintained in the hatchery). A total of 180 crablets were reared in the experimental tanks. In each tank, 10 crabs were allocated randomly and maintained with aeration. Each tank was partitioned with PVC sheets to create 10 individual chambers (1 crablet was stocked in each chamber to avoid cannibalism among them during molting). All the experimental crabs were fed with Tilapia fish meat twice daily (at 07.00 am and 07.00 pm) at the rate of 5% of their total body weight. Uneaten food materials and other nitrogenous wastes were siphoned out daily (with an exchange of 20% of total tank water) to maintain water quality as optimum as possible. In addition, crab shells were removed regularly using scoop net as mud crab undergo regular moulting for growth. Ammonia (NH_3) stock solution was prepared by mixing the salt NH_4Cl with tap water. NH_3 solution was then added slowly in the replicated tanks to achieve the two treatment conditions. NH_3 level was increased in the treatment groups at the rate of 2.5 PPM per day to impose minimum stress level during the acclimation phase. Firstly, 1.5 PPM NH_3 was added in tanks at 7.00 am in the morning and the remaining 1 PPM was added at 7.00 pm.

Evaluation of growth performance

Crablets were maintained in the experimental tanks for two months (under three different ammonia levels: 0 PPM, 5 PPM and 10 PPM) for checking their growth performance. Crablets were sampled fortnightly to investigate the effects of ammonia stress in growth performance (by measuring their total body weight) of mud crab. For growth measurement, 30 crabs were collected randomly from each ammonia level (5 individuals from each of the replicated tanks) and weighed on a digital balance, then released in the tanks. Survival rates were estimated by counting the number of crabs at the beginning and at the end of the experiment. Different growth parameters were estimated according to the following equations:

$$\text{DWG (\%)} = ((BW_f - BW_i) / (BW_i \times t) \times 100$$

$$\text{SGR (\%)} = ((\ln BW_f - \ln BW_i) / t) \times 100$$

$$\text{Feed intake (FI)} = \text{(feed given – feed wastage) / number of individuals}$$

$$\text{Feed conversion ratio (FCR)} = \text{feed intake (g) / weight gain (g)}$$

$$\text{Protein efficiency ratio (PER)} = \text{(Weight gain / Protein intake) x 100}$$

Here, BW_f = Body weight final, BW_i=Body weight initial, t = 60 (total experimental time)

Measuring the rates of O_2 consumption

For measuring O_2 consumption rate, 250 ml flow respirometric chamber (Q-Box Aqua Respiratory System, Qubit, Canada) was used. O_2 consumption rates were measured by deducting the O_2 concentration during entry (O_2 en) to exit (O_2 ex) from each chamber. Rate of water flow (FR) was 1.5 Lh/l. Finally, ammonia induced alterations in the rate of O_2 consumption was estimated according to Rosas et al. (2001) using the following equation:

$$O_2 \text{ consumption} = [O_2\text{en} - O_2\text{ex}] \times FR$$

Crab specimens were collected over 11 different time intervals (1H, 12H, 24H, Day 2, Day 3, Day 4, Day 5, Day 10, Day 20, Day 30 and Day 60) for this measurement.

Total hemocyte counts

Hemolymph samples were collected from 3 replicated crabs using 12 ml syringe inserted through the muscle beneath swimming legs over 11 different time intervals (1H, 12H, 24H, Day 2, Day 3, Day 4, Day 5, Day 10, Day 20, Day 30 and Day 60). A total 300 µl hemolymph were collected from each crab (100 µl sample was used for total hemocyte counts and remaining sample was used for measuring glucose and serotonin). Collected hemolymph samples were immediately preserved in equal volumes (300 µl) of 25% Heparin (Zentiva, Czech Republic) as anti-coagulant solution. Crabs were immediately released back to the experimental tanks after hemolymph collection. Hemocytes were counted from the preserved hemolymph samples according to the methods outlined in Song et al. (2003) and Rahi et al. (2021a).

Hemolymph glucose and serotonin levels

To measure glucose and serotonin, 200 µl hemolymph was collected from each crab (three replicates from each treatment). Samples were pipetted well to prevent clotting and then placed in 1.5 ml tubes. Hemolymph glucose levels were measured using 100 µl anticoagulated samples (from 400 µl hemolymph sample), which were separated from the cells by centrifuging at 800g (4°C) for 10 minutes. In the end, a commercial kit used to evaluate the glucose levels of experimental crabs using the cell-free aliquots (Glu L 1000, PLIVA-Lachema, Czech Republic) according to Pravda and Svobodová (2003) and Rahi et al. (2021b).

To estimate serotonin levels, 300 µl of the anticoagulated hemolymph samples were used. To extract cell free hemolymph (CFH) from each of the replicates, samples were centrifuged for two minutes at 16000 g (4°C) for collecting 80 µl CFH that were subsequently subjected to an hour-long heating process at 80°C (Rahi et al., 2021c). After being heat-denatured, CFH samples were thoroughly mixed using a vortex for 20 seconds. They were then diluted with 80 µl (0.05 M) of phosphate-buffered saline (PBS) and mixed again using a vortex for 20 seconds. To extract 50 µl of supernatant from each sample, these solutions were centrifuged at 13,000 g for 20 minutes. Finally, cortisol levels were determined using a competitive ELISA reader (IBL International, Germany) according to Fuchs et al. (2015) and Bögner et al. (2018).

Gill ultra-structural observation

Gill tissue was collected from three crabs (three biological replicates from each experimental group) for investigating gill ultra-structure. Gill sample was collected 30 days after the commencement of NH_3 stress experiment. Gill tissues were transferred in 2.5% Glutaraldehyde immediately after dissection, kept at room temperature for 24 hours and then preserved at 4°C

until use. Gill tissues were completely dehydrated through a series of washing steps in ethanol and hexa-methyl-disilisane (HMDS) according to Figure 1. Finally, the dehydrated gill samples were used for scan electron microscopy (SEM) for ultra-structural imaging.

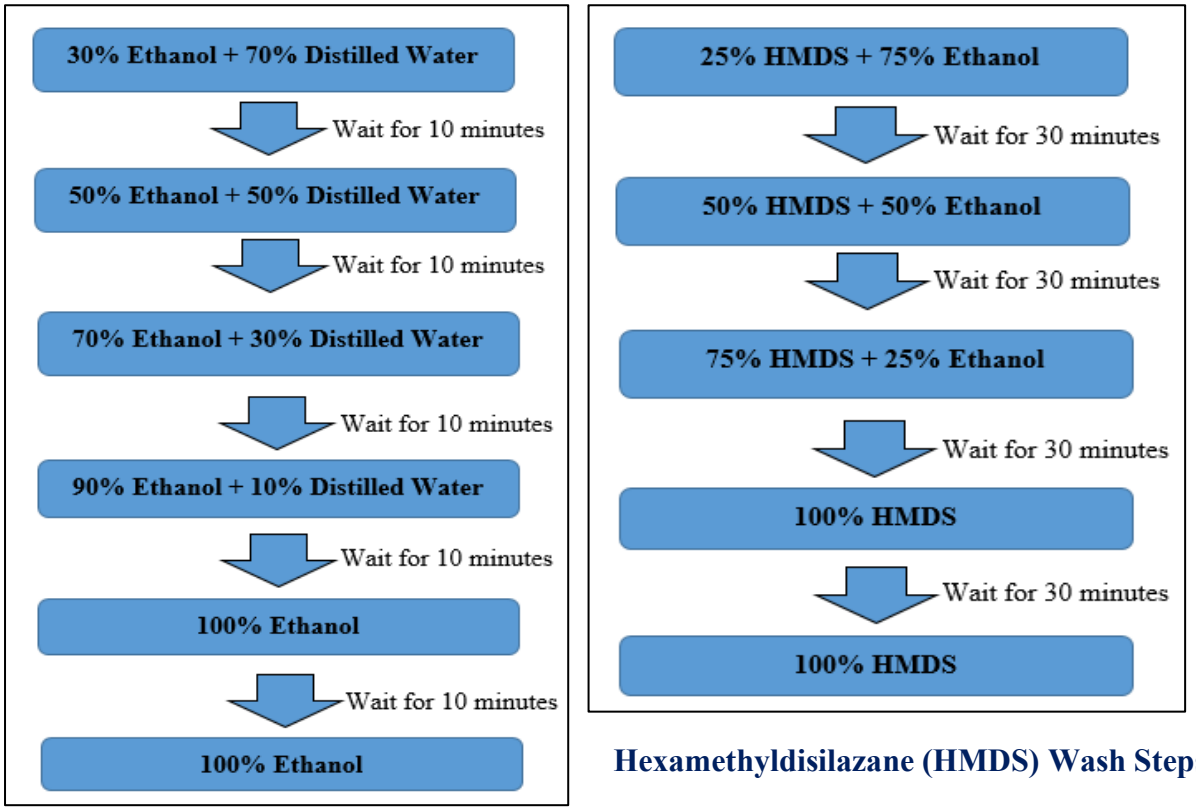

Figure 1: Flow chart for serial washing steps in ethanol and hexa-methyl-disilisane (HMDS) for dehydrating gill tissue of mud crab (*Scylla olivacea*).

Gene expression study

Hepatopancreas tissue was dissected from the experimental mud crabs for gene expression analysis. Dissected tissues were preserved in RNAlaterTM buffer (Ambion, Life Technologies, USA) for maintaining RNA integrity. Three crabs were collected from each experimental condition including the treatments and control (3 biological replicates) for hepatopancreas tissue collection. Hepatopancreas is an ideal tissue to investigate the expression pattern of growth, metabolism and immune related genes. RNAlater preserved tissue samples were kept in room temperature for 12 hr and then maintained at −80°C to ensure the integrity of RNA. Total RNA was extracted from the preserved tissues using commercially available RNA isolation kit (Bioline, UK) following the manufacturer's protocol. RNA samples were converted to mRNA and cDNA, using cDNA synthesis kit (Bioline, UK) according to the manufacturer's protocol. cDNA samples were preserved at −20°C for subsequent analysis involving real time quantitative polymerase chain reaction (RT-qPCR). Sequences of the three selected (growth, immune response and fatty acid metabolism) genes: α-Amylase, Toll-like receptor, Acetyl-CoA Carboxylase and a reference gene 18S were available from some earlier studies (Waiho et al., 2017; Lv et al., 2019; Rahi et al., 2020) to design specific primers for this study. 18S was used as the reference gene in this study to obtain relative gene expression values for each of the genes. Previously, 18S was found to be a suitable reference gene for investigating gene expression pattern in different crustacean species (Havird et al., 2014; Rahi, 2017; Aziz et al.,

2018; Moshtaghi et al., 2018; Rogl et al., 2018; Rahi et al., 2019; Rahi et al., 2020). In the present study, expression pattern of 18S was stable (or constant) in all three experimental ammonia levels that confirm the suitability of this gene as an ideal reference gene.

Specific details of each primer have been presented in the Table 1. RT-qPCRs were performed in 20 µl reaction mixtures that included: 3 µl ultra-pure distilled water (Invitrogen, USA), 5 µl cDNA (template), 1 µl forward primer, 1 µl reverse primer and 10 µl 2x SensiFAST SYBR No-ROX Mix (Bioline, UK) (Moshtaghi et al., 2018). Reaction mixtures were then placed in the real-time thermal cycler (CFX96, Bio-Rad, USA). Gene expression values of the mud crab samples were obtained as Ct (cycle threshold) values. Ct values were then converted to relative gene expression values by using the delta– delta Ct method (Pfaffli, 2001) applying the following equation:

$$R = 2- [\Delta Ct \text{ sample} - \Delta Ct \text{ control}]$$

Here, control represents expression values of 18S.

Table 1. Primers with specific details used in this study (Rahi et al., 2020).

Name of Gene	Sequence	T_m (°C)	Product Size (bp)
Alpha-amylase	F: ACTGCTCTTGTGGATTGGTG	53	190
	R: GTCTTCTGCCTGCACATTCT		
Toll-like Receptor (TLR)	F: CTTCGCCTTCGGAGTCAC	56	185
	R: GGATTCACCGTCGTCATACC		
Acetyl-CoA Carbo-xylase (ACoAC)	F: ACAACTCGGCTCTGGTGTTC	59	220
	R: CTCGGCCTAGACCCAAGTC		
18S (Reference Gene)	F: GCGGTAATTCCAGCTCCA	58	200
	R: AGCCTGCTTTGAGCACTCTC		

Data analysis

Quantitative analysis of gene expression (α-mylase, Toll-like receptor, Acetyl-CoA Carboxylase), growth, total hemocyte count and rate of O_2 consumption data were used for performing different types of statistical analysis using the software program SPSS (version 23). SPSS was used for the analysis of variance (ANOVA) test followed by Duncan's multiple range test, post hoc test (Tukey's HSD), correlation test and profile plots for the measurement between different types of data (growth, O_2 consumption and gene expression). Data on growth (mean ± S.E., n = 30), O_2 consumption (mean ± S.E., n = 3), hemocyte counts (mean ± S.E., n = 3) and gene expression (mean ± S.E., n = 3) were tested for homogeneity of variance and normality. Both one-way and two-way ANOVA were performed depending on data type and interactions tested. Two-way ANOVA was performed when data sets were compared by considering experimental ammonia levels and sampling times as variables. ANOVA tests between different parameters were performed by applying the 5% level of significance.

Results

Growth and survival performance

Significantly higher ($F_{(5, 125)} = 73.6$, $P = 0.00$) growth (and different growth-related parameters like SGR, DWG, FCR and feed intake) and survival performance were observed for the control group over the treatment groups (5 PPM and 10 PPM) (Table 2 and Figure 2).

Table 2. Effects of different doses of ammonia on specific growth parameters of orange mud crab (*Scylla olivacea*) for a period of 60 days. Different superscript letters indicate significant differences among experimental conditions.

	Control	**5 PPM NH_3**	**10 PPM NH_3**
Initial Weight (BW_i) (g)	$1.5^a \pm 0.21$	$1.52^a \pm 0.26$	$1.54^a \pm 0.25$
Final weight (BW_f) (g)	$5.34^a \pm 0.5$	$3.26^b \pm 0.69$	$3.12^b \pm 0.68$
Daily Weight Gain, DWG (%)	$4.27^a \pm 1.42$	$1.91^b \pm 1.42$	$1.71^b \pm 1.42$
Specific Growth Rate, SGR (%)	$2.12^a \pm 0.52$	$1.27^b \pm 0.52$	$1.18^b \pm 0.52$
Feed Intake (g g^{-1} day^{-1})	0.164^a	0.143^b	0.141^b
Feed Conversion Ratio (FCR)	$0.04^a \pm 0.02$	$0.08^b \pm 0.02$	$0.09^b \pm 0.02$
Survival (%)	87^a	63^b	60^b

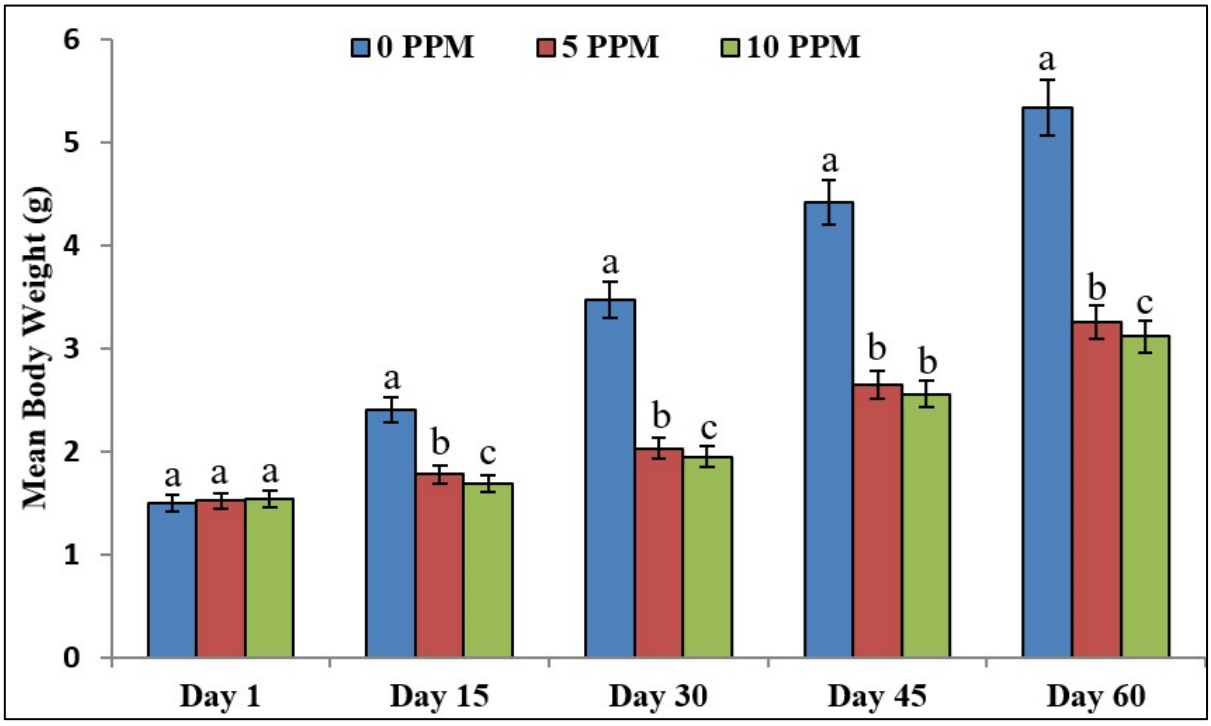

Figure 2. Ammonia induced changes (Mean ±S.E.) in the body weight of experimental mud crab (*Scylla olivacea*) across the sampling times. Different letters above the bar indicate significant difference at 5% level of significance.

The control group showed almost double growth (≈5.5 g body weight) over the treatments (3.1 – 3.3 g). Significant differences ($P < 0.05$) were observed between control and treatment for the entire experimental period (60 days) except 1st day. No significant differences were observed between the two treatments (5 PPM and 10 PPM) for 1st and 45th days while significance differences were observed for 15th, 30th and 60th days.

Rates of O_2 consumption

The O_2 consumption rate was stable throughout the experiment in the control group while it was found to vary significantly for the treatment groups across the sampling times. Significantly higher O_2 consumption rates were obtained for the control group ($F_{(11, 55)} = 96.2$, $P = 0.01$) compared to the treatments (Figure 3). Significant difference in O_2 consumption was observed between the treatments (5 PPM and 10 PPM) at 24H, 2nd, 3rd and 10th days; while no significant differences were observed between the treatments throughout the experimental period. Both the treatment groups showed a general trend in O_2 consumption rates including decreasing trends until the 4th day (the lowest O_2 consumption rates at this time) followed by increasing trends up to 20th day and finally stable trends for the remainder. The lowest O_2 consumption rates among the two treatment groups were observed for the 10 PPM ammonia treated crabs.

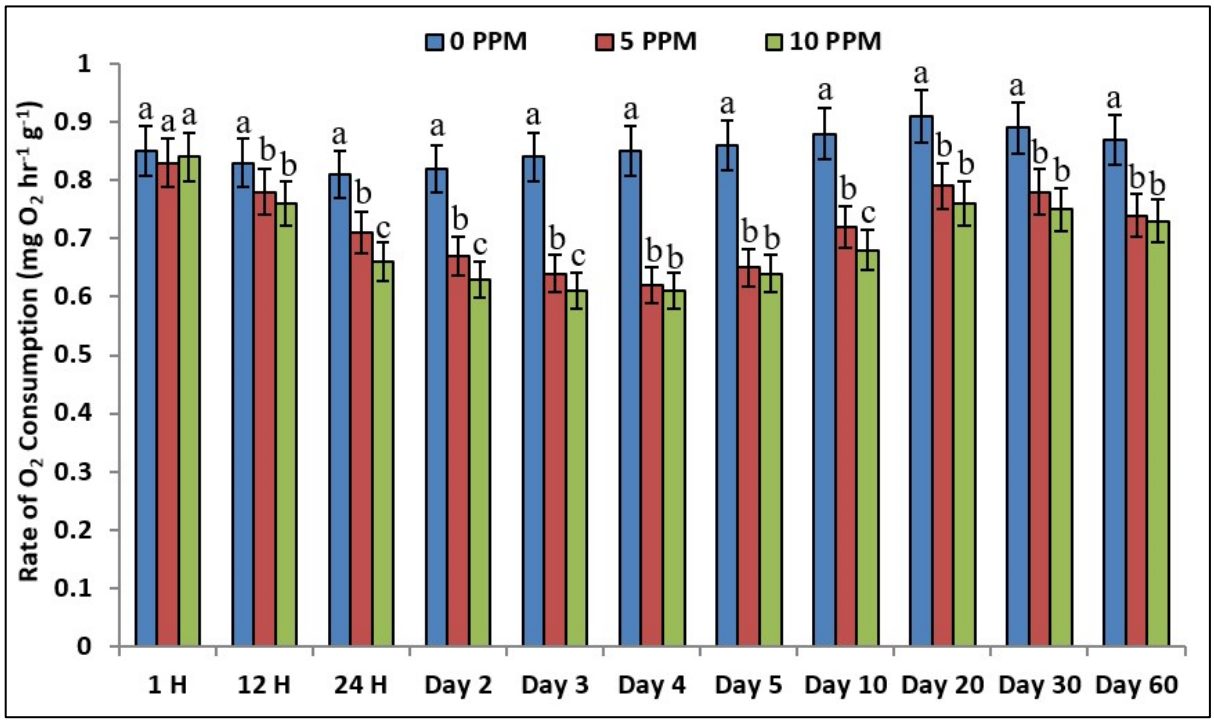

Figure 3. Ammonia induced changes (Mean ±S.E.) in the rate of O_2 consumption in experimental mud crab (*Scylla olivacea*) individuals across the sampling times. Different letters above the bar indicate significant difference at 5% level of significance.

Changes in hemocyte counts

The highest levels of hemocyte counts were observed for the control group throughout the experimental period. Significantly higher ($F_{(11, 55)} = 102.3$, $P = 0.02$) hemocyte counts (45–49 million/ml) were observed in the control group over the two treatment groups (5 PPM and 10 PPM NH_3 doses). At the beginning of this experiment (1H), no significant difference was observed between the control and treatment groups, following which hemocyte counts declined significantly (at different orders of magnitude) in the two treatments. Levels of hemocyte counts were ranked as 0 PPM (45–49 million/ml) > 5 PPM (33–45 million/ml) > 10 PPM (32–45 million/ml) (Figure 4). No significant differences ($P < 0.05$) were observed between the treatment groups from the beginning to the end of this study while 24H, 4th and 30th days showed significance difference. Hemocyte counts sharply declined from 12H to 24H for the two treatments, followed by a gradual decline up to the 2nd day (lowest levels of hemocyte counts were found on the 2nd day), then it followed gradual increasing trend up to the 20th day and finally reached a stable pattern for the remaining time frame.

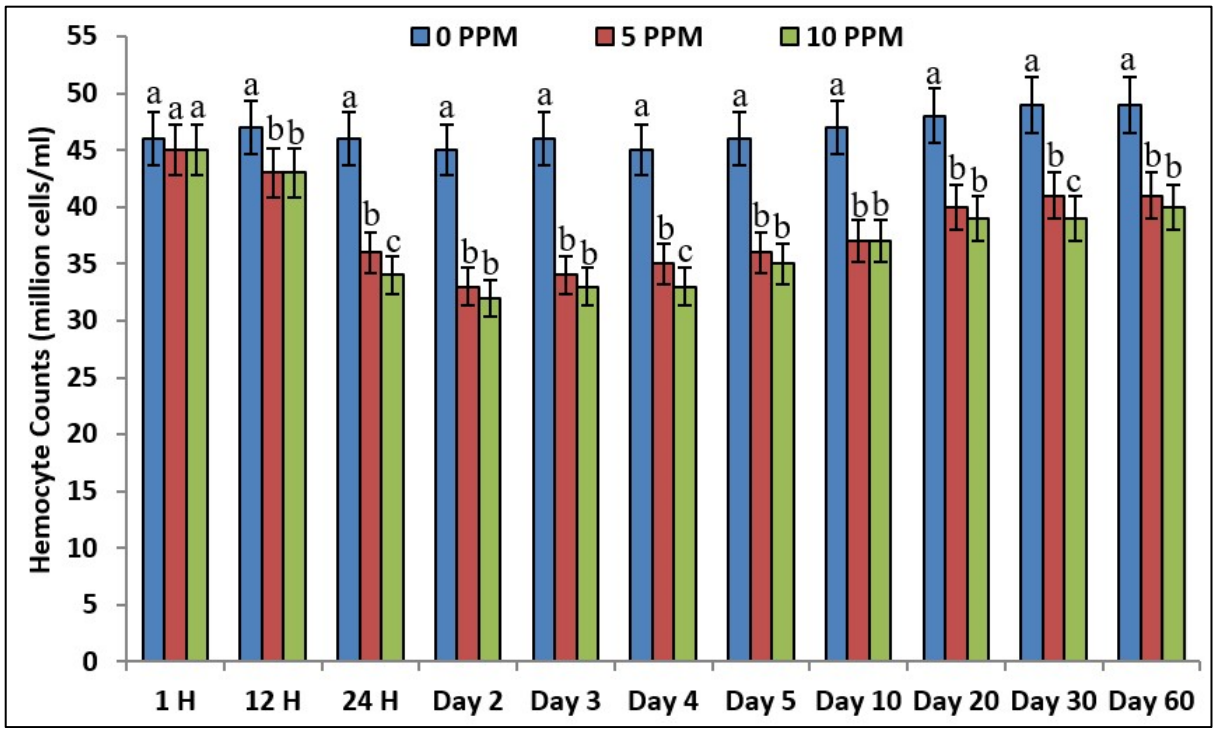

Figure 4. Hemocyte counts (Mean ±S.E.) of experimental mud crabs (*Scylla olivacea*) across the sampling times. Different letters above the bar indicate significant difference at 5% level of significance.

Changes in hemolymph glucose and serotonin levels

Hemolymph glucose levels were also found to be significantly affected by the two ammonia doses ($F(5, 55) = 58.4$, $P = 0.03$). The treatment groups (5 PPM and 10 PPM) showed almost double levels of glucose compared to the control (Figure 5). The generalized pattern in ammonia dose specific glucose level was found as an increasing trend up to 5th day (peak level), followed by a declining phase up to 20th day and then a stable trend for the remainder. All the treatments showed significantly higher glucose levels over the control from beginning to the end. No significant differences ($P < 0.05$) were found between the treatments (5 PPM and 10 PPM) from the beginning to the end of the experiment with some exceptions (significance difference between 5 PPM and 10 PPM on 12H, 24H and 4th day); particularly 10 PPM showed significantly higher glucose levels than the 5 PPM from 12H to the end (Figure 5).

Different doses of ammonia significantly ($F(5, 55) = 86.9$, $P = 0.01$) altered the hemolymph serotonin levels (Figure 6) of experimental mud crabs (*Scylla olivacea*) while dose specific response (higher levels of serotonin with increased ammonia doses) was also observed. Stable and significantly lower levels of serotonin were found throughout the experiment for the control. The treatment groups (5 PPM and 10 PPM) showed significantly higher serotonin levels ($P < 0.05$) over the control from the beginning to the end. The general trend in NH_3 specific serotonin level was found as an increasing trend up to 4th day (the peak level of serotonin for the two treatments), followed by a declining trend up to 10th day and finally a stable trend for the remainder (Figure 6). No significant differences were observed between the treatments (5 PPM and 10 PPM) up to 2nd day following which differences between these two treatments were irregular (significant differences were found only at 2nd, 3rd and 4th days).

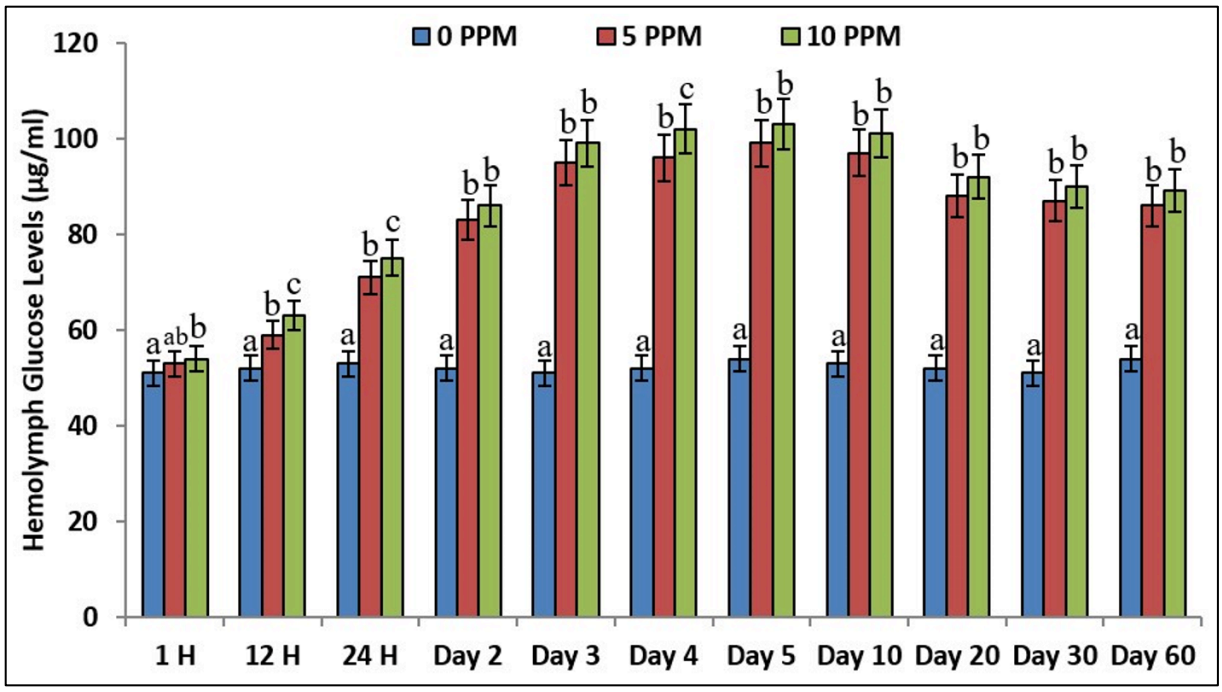

Figure 5. Ammonia induced changes (Mean ±S.E.) in the hemolymph glucose levels in experimental mud crabs (*Scylla olivacea*) across the sampling times. Different letters above the bar indicate significant difference at 5% level of significance.

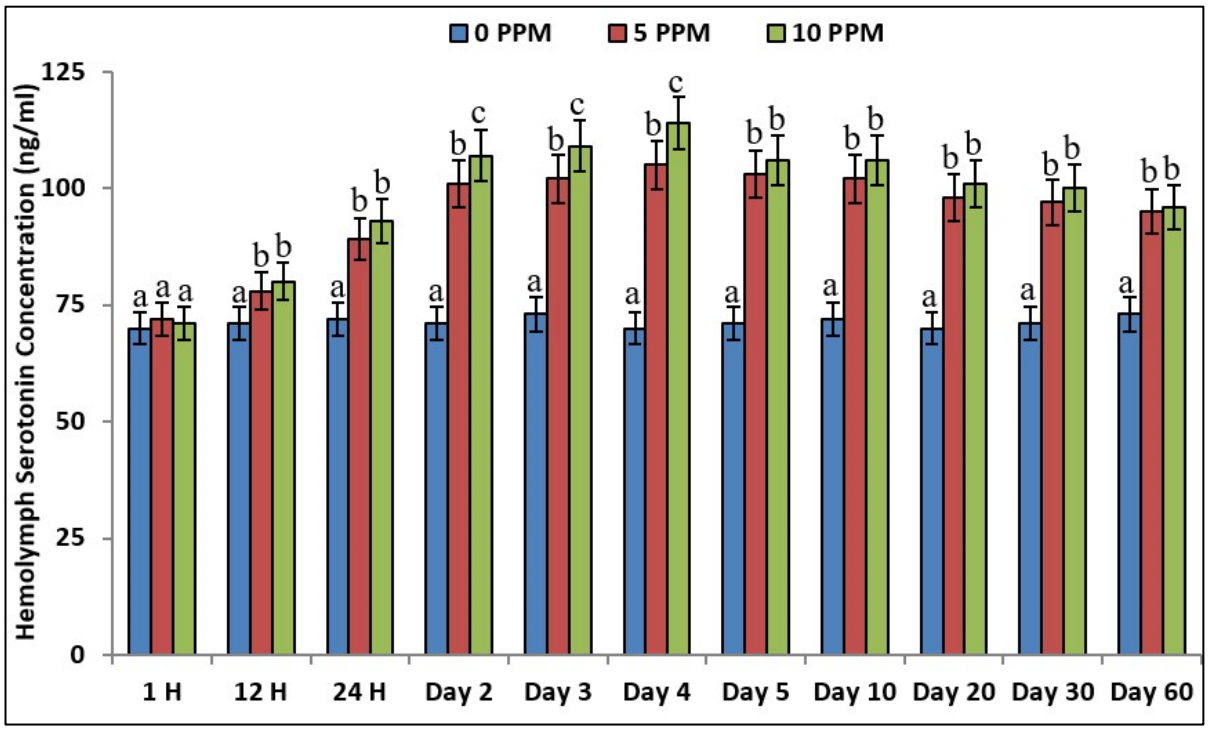

Figure 6. Changes (Mean ± S.E.) in the hemolymph serotonin levels of experimental mud crabs (*Scylla olivacea*). Different letters above the bar indicate significant difference at 5% level of significance.

Gill ultra-structure

Scan electronic microscopic (SEM) view of gill tissue clearly showed ammonia specific differential changes in experimental crabs (Figure 7). Clear structure of gill was observed for

the control group while at 5 PPM blocked condition was observed but at 10 PPM damaged gill structure was observed. Different doses of ammonia were found to remarkably change the gill ultra-structure of experimental mud crab individuals.

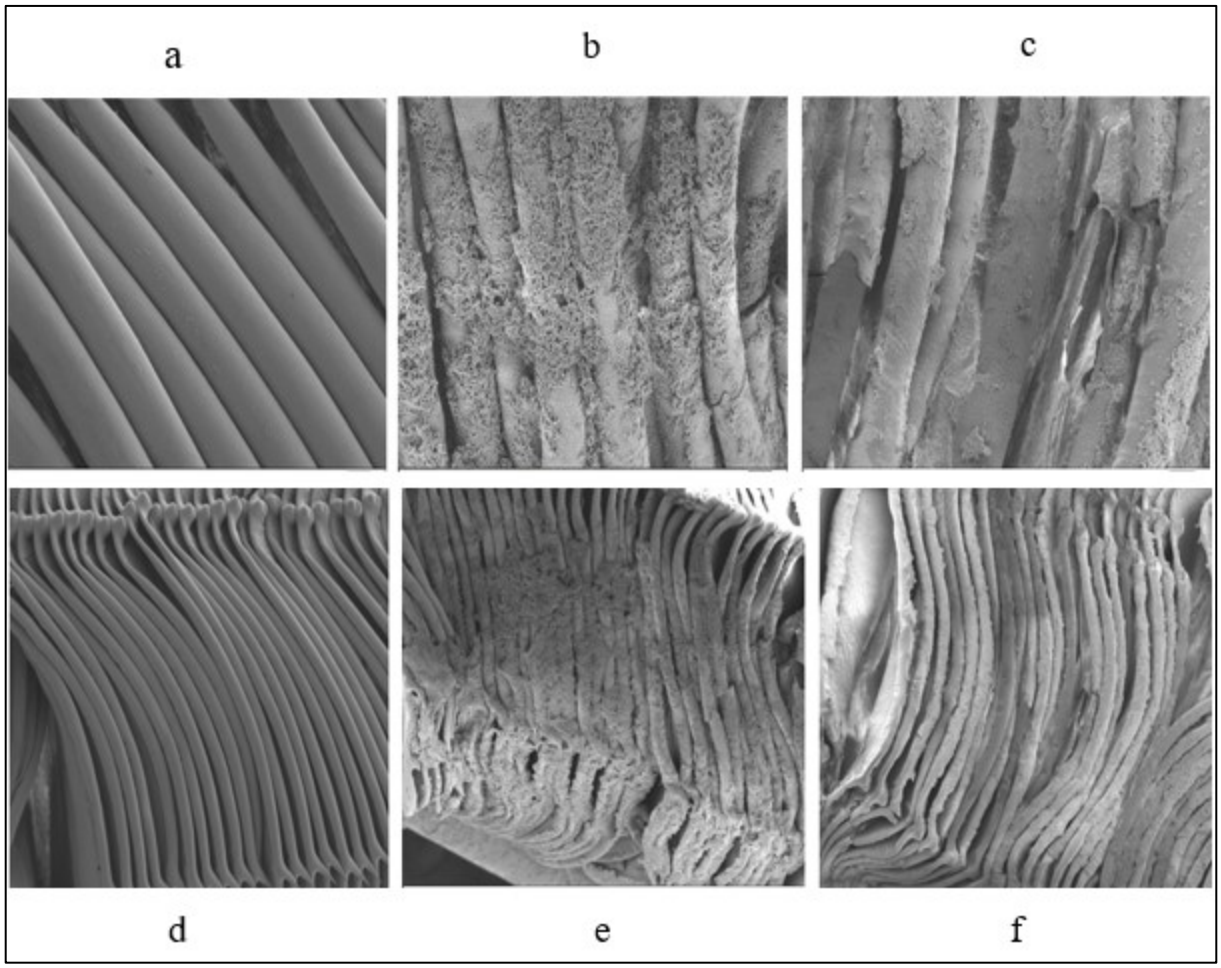

Figure 7. Gill ultra-structure (SEM imaging) of the orange mud crab (*Scylla olivacea*) at three different experimental conditions: a) 0 PPM NH_3 (control) b) 5 PPM NH_3, c) 10 PPM NH_3, d) 0 PPM NH_3 (control), e) 5 PPM NH_3 and f) 10 PPM NH_3. Images for a – c were taken at 500x magnification (50 µm area) while 100x magnification (200 µm area) for d – f.

Expression pattern of candidate genes

Significantly higher ($F_{(5, 55)} = 92.5$, $P = 0.00$) expression patterns of α-amylase, Toll like receptor (TLR) and Acetyl-CoA Carboxylase (ACAC) genes were observed in control groups compared to the treatments (Figures 8, 9 and 10). For all three candidate genes, the highest levels of expression pattern (≈1.5 – 2 fold) were observed for the control group compared to the treatments. Significantly higher ($P < 0.05$) expression levels of α-amylase were observed for the control group (0 PPM NH_3) over the two treatment groups (5 PPM and 10 PPM NH_3 doses) for the entire experimental period. The control group showed stable expression levels for α-amylase throughout the experiment (Figure 8). No significant difference was observed among the three experimental conditions initially (1H), following which control group showed significantly higher expression levels over the treatments from 12H to the end. No significant differences were observed between the treatments up to the 3rd day, then significant differences ($P < 0.05$) were observed between the two treatments from 4th day to the end of this experiment (Figure 8).

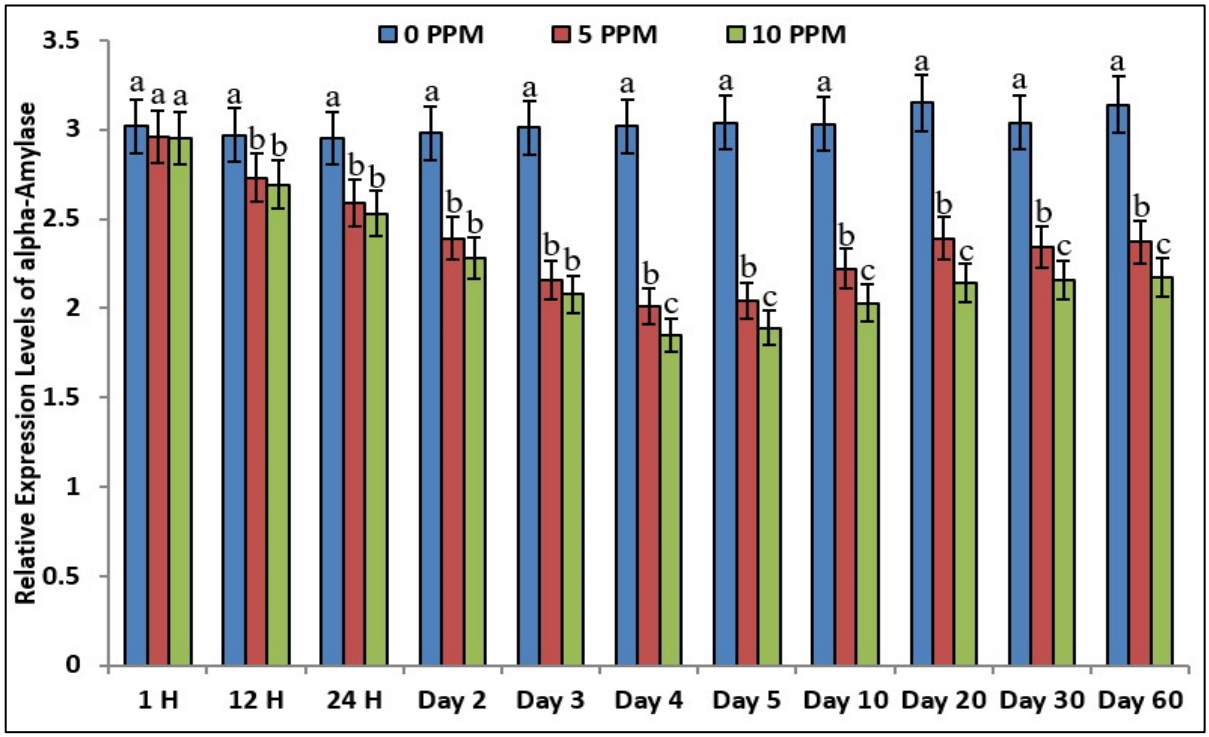

Figure 8. Changes (Mean ± S.E.) in the relative expression levels of alpha-Amylase in experimental mud crabs (*Scylla olivacea*) across the sampling times. Different letters above the bar indicate significant difference at 5% level of significance.

Toll like receptor (TLR) also showed similar trends in expression levels like α-amylase. General pattern in TLR expression includes a general declining trend up to 5th day, followed by an inclining phase up to 20th day and finally a stable trend from 20th day to the end (Figure 9) for the treatments group; while the control group showed a stable trend from the beginning to the end. No significant difference was observed initially (1H), the control group showed significantly higher expression levels ($F(5, 55) = 91.4$, $P = 0.02$) over the treatments from 12H to the end. No significant differences were observed among the treatment groups (5 PPM and 10 PPM NH_3 levels) up to 24H following which significant differences were observed among these two groups from 2nd day to the end of this experiment.

Acetyl-CoA Carboxylase showed different expression pattern compared to the other two genes where no significance differences ($F(5, 55) = 48.4$, $P = 0.05$) were observed among the two treatment groups (5 PPM and 10 PPM NH_3 doses) throughout the course of this experiment with some exceptions (significance differences for 10th and 20th days) (Figure 10). No significant difference was observed among the control and treatment groups at the beginning (1H), following which significantly higher expression levels were observed for the control over the treatments from 12H to the end.

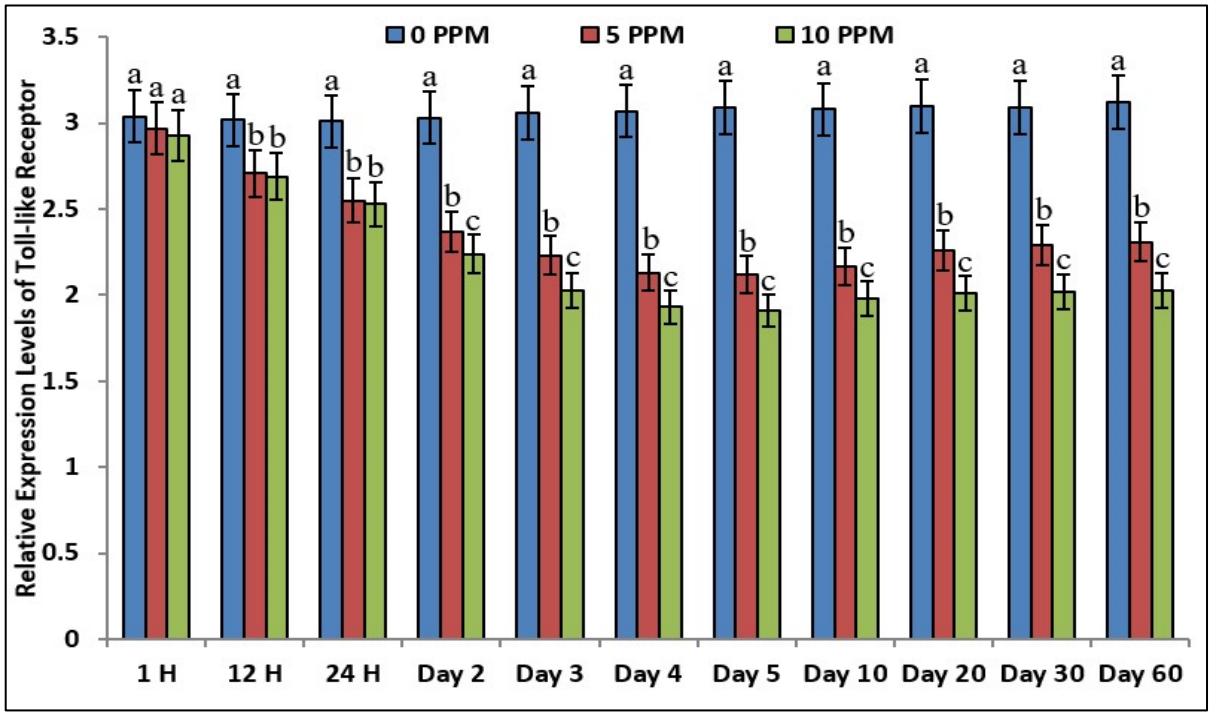

Figure 9. Relative expression levels (Mean ± S.E.) of Toll-like Receptor in experimental *Scylla olivacea* individuals across the sampling times. Different letters above the bar indicate significant difference at 5% level of significance.

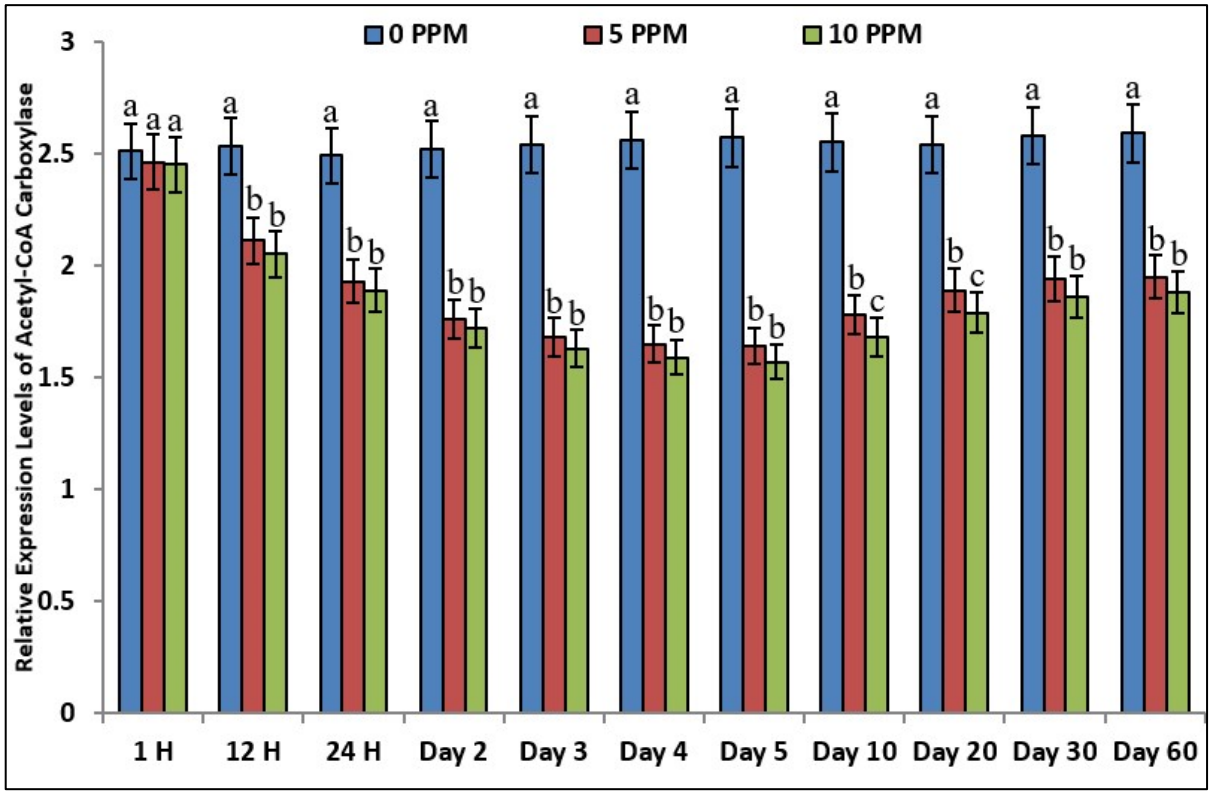

Figure 10. Relative expression levels of Acetyl-CoA Carboxylase (Mean ± S.E.) in experimental mud crab (*Scylla olivacea*) individuals across the sampling times. Different letters above the bar indicate significant difference at 5% level of significance.

Discussion

Ammonia is considered as one of the most dangerous pollutants causing severe toxic effects on the farmed aquatic species (i.e., crustaceans are the most vulnerable candidates to this pollutant) (Wajsbrot et al., 1993; Sabbir et al., 2010; Rahi et al., 2021a). Ammonia is also known to cause severe cellular damage in the gill tissue or creates blockage around the gill that reduce O_2 absorption ability of the affected species (Shin et al., 2016; Lu et al., 2022; Rahi et al., 2022). Previous investigations report that ammonia negatively affects entire biological organizations of farmed aquatic organisms including reduced growth coupled with increased mortality, metabolic alterations, hormonal imbalance, hemato-biochemical imbalance, susceptibility to diseases (lower immunity) and cellular damage (Bouwman et al., 2011; Romano and Zeng, 2013; Zhao et al., 2020; Kim et al., 2022; Rahman et al., 2022). In the current study, two different doses of ammonia significantly altered all of the tested biological parameters of orange mud crab (*Scylla olivacea*) over the control.

Generally, a survival rate of ≥80% is considered to be very good for studies involved with crustacean culture/farming (Rahman et al., 2022). In the present study, 87% survival rate was obtained for the control after 60 days of experimental period (Table 2) indicating experimental crabs were acclimated well with the tank environment. Therefore, any deviation or change from this level would indicate ammonia treatment specific changes not only for the survival rate but also for the other biological parameters tested in this study. Significantly lower growth and survival performance ($P < 0.05$) of ammonia treated crabs compared to the control (Table 2 and Figure 2) clearly indicate adverse role of ammonia on production performance (as well as overall biology) of orange mud crab. No Significant differences for growth and survivability between the treatments (5 PPM and 10 PPM ammonia levels) imply that these two ammonia doses imposed similar scale of stress on experimental crabs. Measuring O_2 consumption rate provides a reliable means of predicting physiological status of crustaceans; under stressful condition O_2 consumption is increased while it is decreased if the stressor blocks the O_2 transportation pathway (e.g., gill) (Rahi et al., 2021b; Lu et al., 2022). Increased O_2 consumption also indicate increased metabolic rate that potentially leads to faster growth (Rahi et al., 2020; Zhang et al., 2021). The higher growth performance in the control group likely supported by the increased O_2 consumption while reduced O_2 consumption provided slower growth in the ammonia treated crabs (Figures 1 and 3). The lower O_2 consumption in the ammonia treated crabs is most likely due to the gill tissue damage (Figure 3) that reduced the efficiency of experimental crabs to absorb required levels of O_2 through the gill tissue.

Counting the number of hemocyte cells in the crustacean body fluid (hemolymph) is an important method for evaluating health and immunity status (Shirangi et al., 2016; De et al., 2019; Rahi et al., 2022). Higher hemocyte counts indicate better immunity and health status while lower counts indicate poor health condition (Rahi et al., 2021b; Seibel et al., 2021). Significantly higher hemocyte counts ($P < 0.05$) of the control group (Figure 4) indicate better immunity/health status while lower levels of hemocyte counts of ammonia treated crabs indicate reduced immunity. Results also imply that both the 5 PPM and 10 PPM ammonia doses imposed similar levels of stress on the experimental crabs (as no significant differences were observed for the hemocyte counts between the treatments).

Measuring hemolymph glucose and serotonin (stress hormone) levels in crustaceans indicates the magnitude of stress on experimental individuals (Rajendiran et al., 2016; Jaffer et al., 2020). Both glucose and serotonin levels are known to negatively affect crustacean growth (Chang et al., 1999; Ali et al., 2015; Rajendiran et al., 2016; Rahi et al., 2022). Crustaceans usually release more glucose and serotonin under stressful conditions, by using protein reserves for

gluconeogenesis in the liver to minimize stress levels (Wu et al., 2015; Rahi et al., 2021b). Significantly higher hemolymph glucose and serotonin levels ($P < 0.05$) of ammonia treated crabs (*Scylla olivacea*) across the entire experimental timeframe (Figures 5 and 6) demonstrate the negative effects of ammonia. Significantly lower and stable levels of glucose and serotonin levels (with superior growth) of mud crab under the control condition throughout the experiment indicate no imposed stress. Therefore, lower growth and survival performance were obtained for ammonia treated crabs due to increased intensity of stress compared to the control. Higher serotonin and glucose levels in treatment groups up to the end of this study (60th day) also indicate ammonia treated crab were under persistent stress.

α-Amylase is a potent growth regulator in crustaceans with increased expression of this gene potentially providing faster growth (Jung et al., 2013; Moshtaghi et al., 2016; Aziz et al., 2017; Moshtaghi et al., 2018; Rahi et al., 2020). Higher expression of this gene was previously found in fast-growing prawn *(Macrobrachium rosenbergii*) and orange mud crab (*Scylla olivacea*) (Aziz et al., 2018; Rahi et al., 2020). Superior growth coupled higher expression of α-amylase in the control group compared to the ammonia treated crabs (5 PPM & 10 PPM) (Figure 8) clearly indicate the important role of this gene for growth regulation in *Scylla olivacea*. In the treatment groups, expression of α-amylase was in a declining trend up to 4th day, increasing trend up to 20th day and stable pattern up to the end implying that ammonia treated crabs were trying to overcome the stress and become stable from 20th day. Strong and significant correlation pattern ($R^2 = 0.76$, $P < 0.05$) between growth and expression of α-Amylase (Figure 11) further validate the important functional role of this gene for growth in the orange mud crab.

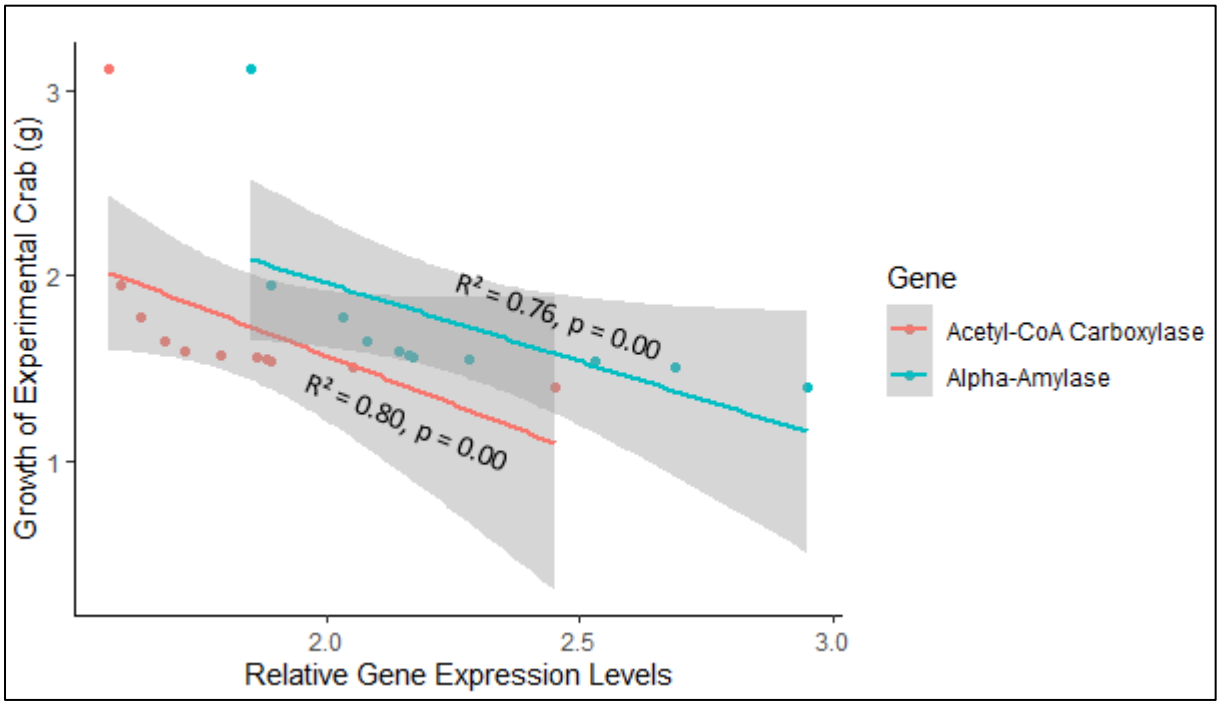

Figure 11. Correlation plot of growth (Y axis) with Alpha amylase and Acetyl CoA Carboxylase (X axis) of *Scylla olivacea* at 3 different experimental conditions.

Acetyl CoA Carboxylase (ACoAC) gene is known to be involved with fatty acid metabolism (an important metabolic or metabolism related gene), play important role in growth regulation in different crustaceans (Jung et al., 2013; Aziz et al., 2017; Moshtaghi et al., 201; Waiho et al.,2017). Ammonia treatments significantly reduced the expression levels of ACoAC of experimental mud crabs while no significant differences were detected among the treatments

(Figure 10). Declining expression of this gene up to 4th day indicate imposing stress on ammonia treated crabs while increasing expression from 5th day indicate the attempts of experimental crabs to eliminate the effects of ammonia stressor (Figure 10). Significantly strong and positive correlation (R^2 = 0.80, $P < 0.05$) between growth and ACoAC (Figure 11) implying the importance of this metabolic gene for growth regulation in orange mud crab.

Toll-like receptor (TLR) is an important immune stimulant gene that triggers signal transduction to induce the synthesis of immune molecules and antimicrobial peptides in crustacean hemolymph to detect and kill microbial pathogens (Deris et al., 2020; Rahi et al., 2021b). Higher expression levels of TLR throughout the experiment at control condition (coupled with higher hemocyte counts) (Figures 9) indicate better immunity of the crabs. Reduced expression of TLR and lower hemocyte counts in ammonia treated crabs clearly indicate poor immunity. Significant ($P < 0.05$) and strong correlation (R^2 = 0.84) pattern between TLR and total hemocyte counts (Figure 12) implying the important role of this gene in immunity for the orange mud crab.

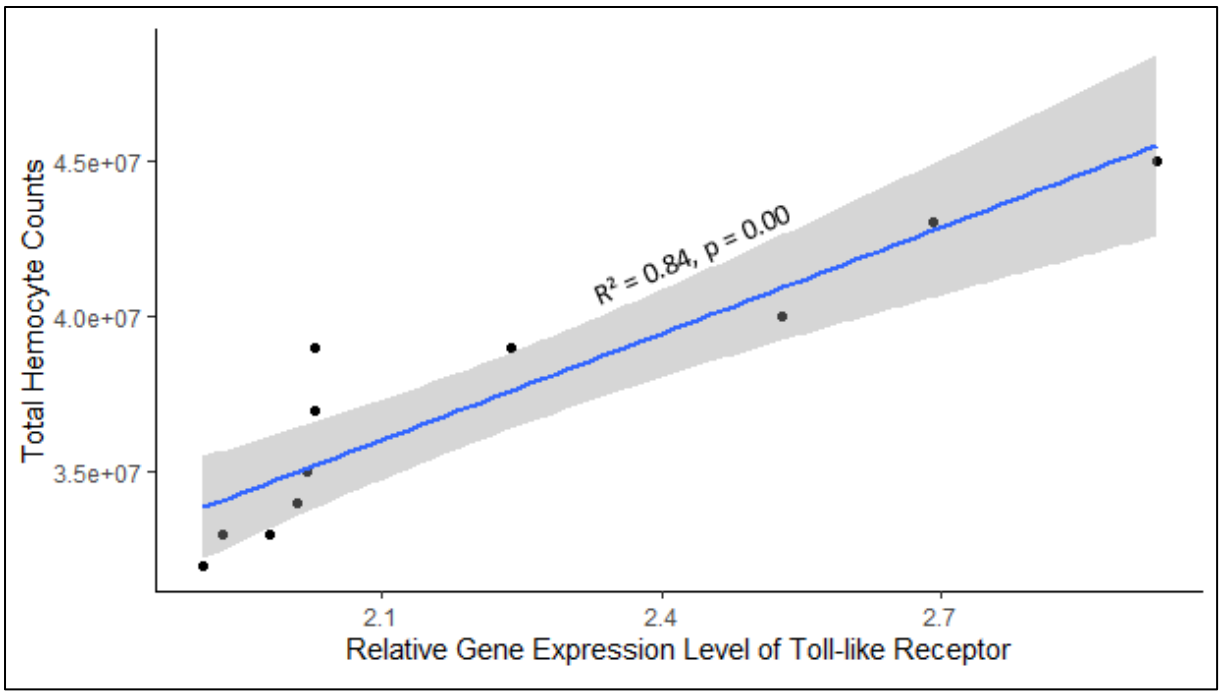

Figure 12. Pattern of relationship between Total Hemocyte Count (million cells/ml) (Y axis) and Toll-Like Receptor gene (X axis) of *Scylla olivacea* at 3 different experimental conditions.

Conclusion

The present study investigated the physiological (growth and O_2 consumption), cellular (gill ultra-structure), biochemical (total hemocyte counts, glucose and serotonin levels) and genetic (expression pattern of three selected genes) alterations of orange mud crab (*Scylla olivacea*) exposed to three different doses of ammonia levels (0 PPM, 5 PPM and 10 PPM). Experimental ammonia doses significantly altered the growth, feed intake, FCR, O_2 consumption, survival, gill ultra-structure, hemolymph parameters and expression pattern of three selected candidate genes (alpha amylase, acetyl Co-A carboxylase and toll like receptor). Results of this study imply that presence of ammonia in farming system can cause severe damage to crustaceans (i. e., crabs). The damage starts with internal biological processes (cellular physiological, biochemical and genetic damages) first and then reflected to the phenotypic levels in the form of disease or slower growth or mortality. As mud crab farming is practiced with low cost and low quality feeds, this species is always under severe risk of being exposed to ammonia.

Therefore, special care must be taken to maintain the farming environment ammonia free (or at least within the tolerance limit) for mud crab and broadly for other crustaceans. For sustainable production, high quality feed, aeration and hygiene/biosecurity should be maintained to keep mud crab out of any stress for maximizing production and profit.

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Ethical approval
Not applicable.
According to the 'Animal Welfare Act 2019' approved by the 'National Parliament of Bangladesh', crabs (and other crustaceans) are not considered as animals. So, the authors confirm that animal ethics clearance was not required for this study.

Informed consent
Not available.

Data availability statement
The authors confirm that we do not have any data to share. All of the generated from the experiments conducted under this study are presented in the form of tables and figures.

Conflicts of interest
The authors confirm that there is no conflict of interests for publishing this study.

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Dania Aziz: Conceptualization, Methodology, Resources, Review and Editing
Md. Lifat Rahi: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Review and Editing
"All authors have read and agreed to the published version of the manuscript."

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