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# A Multi-Biomarker Approach in Assessing the Growth and Physiological Stress in *Pangasianodon hypophthalmus* under Acute Ammonia Exposure

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***Authors' contributions***

*This work was carried out in collaboration between all authors. Author FF designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors AB, TVR and RRA managed the literature searches and corrected the manuscript. Author MR managed the analyses of the study. Authors RSS and NMS helped in the study. All authors read and approved the final manuscript.*

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## ABSTRACT

Fish plays a crucial role in the nutritious diet around the world and aquaculture has rapidly grown to meet increasing demand, becoming the fastest-growing food industry. Ammonia is a major toxin in fish production systems. This study was designed to evaluate the impact of ammonia on the

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growth, haematology and biochemical profile of *Pangasianodon hypophthalmus*. A total of 96 fish of *P. hypophthalmus* having total length (24.63±0.94 cm) and weight of (163.43±8.90g) were stocked in 300 litres collapsible tarpaulin tanks with various TAN levels (60,70,80 ppm) and fed with commercial pellet feed @ 2.5% body weight twice a day. In the control, the highest length and weight recorded were 26.12±1.014 cm and 166.82±2.602g, while the lowest were 25.14±0.476 cm and 163.74±0.286g in the 80 ppm. The sub lethal test revealed that SGR and PER decreased with increasing TAN concentration. In contrast, the FCR increased with increasing concentration of TAN from 0 to 80 ppm. Similarly, haematological parameters decreased in fish exposed to ammonia, particularly in the 80 ppm group compared to levels of 70 and 60 ppm. In contrary, TLC showed gradual increase from 60 to 80 ppm compared to control fish. MCV and MCHC also showed an irregular pattern with changes in the Hb and Ht. As the TAN level increased, biochemical parameters were also increased and showed higher value at 80 ppm compared to 60 and 70 ppm. It is concluded that the *P. hypophthalmus* showed good survival and growth rate up to 60 ppm of TAN.

*Keywords: Pangasianodon hypophthalmus; haematology; TAN; biochemical parameters; biomarker.*

## 1. INTRODUCTION

Fish makes a vital contribution to the survival and health of a significant portion of the world's population. There is a global need for cheap sources of protein to meet the world's over-population. Ever-growing population, fish takes an important lead in transforming food systems and eliminating hunger and malnutrition. Exotic fish species introduction is a standard tool worldwide for improving local fishery potential, as biological controlling agents, expanding species diversity in aquatic systems, increasing aquarium trade, and promoting sport fishing [1 and 2]. Striped catfish (*Pangasianodon hypophthalmus*), commonly known as pangasius or locally as pangas or Thai pangas, is an essential exotic species. It is a fast-growing aquaculture commodity, especially in the Asian region; Vietnam, Thailand, India, Myanmar, Indonesia, and Bangladesh [3 and 4]. One of the most common stressors to fish health and production is ammonia. The primary source of ammonia is the fish excreta, and excretion is directly related to the feeding rate and the protein levels. In water, ammonia occurs in two forms as free ammonia and Total Ammonia Nitrogen (TAN) which includes free ammonia ($NH_3$) and ionized ammonia ($NH^{4+}$) [5].

The ammonia toxicity induces nervous disorders such as convulsion, lethargy, loss of equilibrium, increasing mucus in both gills and the body surfaces, sideway swimming, gill hemorrhage, and necrosis, kidney damage and circling and spiralling movements, and finally death. The haematological parameters are an essential tool of diagnosis that reveals fish health [6 and 7]. Knowledge of haematology is critical since it deals with morphology, physiology, and blood biochemistry. Generally analysis of blood cell characteristics indicates the disease status of the fish and haematological indices aid in diagnosing and assessing fish disease [8]. Any stress factor may create an internal physiological imbalance in fish that appears through disorder hormones and enzymes functions and changes in some blood picture characteristics like Red Blood cell (RBC), White blood cell (WBC), Haemoglobin etc, which need a systematic physiological response by fish against stressors returning to homeostasis [9].

When exposed to a stressor, recognizing a real or perceived threat by the central nervous system (CNS) gets the physiological response initiated in fish. The most widely used indicator of stress in fish is the steroid hormone cortisol, which increases in response to various physical stressors and mediates elevated plasma levels of oxidative fuels such as glucose by suppressing appetite [10, 11, 12 and 13].

Changes in enzyme activities in fish have been used frequently as biomarkers of intoxication and water pollution [14]. ALT catalyzes the transfer of amino groups from alanine to α-ketoglutarate to form glutamate and pyruvate. In contrast, aspartate aminotransferase (AST) catalyzes the transfer of the amino group from aspartate to α-ketoglutarate to form glutamate and oxaloacetate [15]. The increment of Alanine transaminase (ALT) and Aspartate transaminase (AST) levels occurs during nervous shock, hypoxia, and stress where their standard plasma levels would be affected by several factors such as pollution, ammonia and nitrite toxicities and other environmental parameters like temperature and salinity [16]. Creatinine is a reliable kidney function indicator as the kidneys maintain blood creatine in a normal range. Increased creatinine level signifies kidney disease or impaired kidney function. The changes in the blood components, hormones and enzymes act as biomarkers in assessing the physiological stress of the fish. Thus, the study was designed to determine the growth and physiological stress in *Pangasianodon hypophthalmus* using various biomarkers when exposed to various TAN levels.

## 2. MATERIALS AND METHODS

### 2.1 Fish and Rearing Conditions

For conducting the experiment, the fish were procured from local farmers and brought to the wet laboratory of Department of Fisheries Resource Management, College of Fishery Science, Muthukur. After disinfecting the seeds, they were acclimatized to laboratory conditions for one week in fresh tap water in 500 litres collapsible tarpaulin tanks. Later, initial length and weight of the fish were recorded as 24.63 ± 0.94 cm and weight 163.43 ± 8.90 g respectively. Experimental fish were fed with commercial pellet feed @ 2.5% body weight twice a day. The experiment was carried out for a period of 30 days from April, 2021 to May, 2021 to assess the growth performance, survival, haematology and biochemistry of the fishes at various TAN levels.

### 2.2 Preparation of Experimental Media

#### 2.2.1 Ammonia medium

1000 ppm stock ammonia solution (TAN) was prepared by dissolving 3.819 g laboratory reagent grade Ammonium Chloride ($NH_4Cl$) in 1000 mL distilled water. Similarly the other required concentration of ammonia solution viz 60 ppm, 70 ppm and 80 ppm were prepared.

### 2.3 Lethal Studies

The fishes were exposed to various ammonia (TAN-N) levels ranging from 0 to 90 ppm at an interval every 5 ppm in triplicates for about 96 h with an observation for every 12 h interval. After 12 h of exposure 60% mortality was observed in 90 ppm tank and 100% mortality was observed after 24 h of exposure. 30% and 70% mortality was observed in 85 ppm after 24 h and 48 h respectively and fishes in other 9 tanks were observed with reduced swimming behaviour and less feed intake after 36 and 72 h of exposure respectively. Hence, upper ammonia (TAN) levels ranging from 60 to 80 ppm were opted for the present experiment, taking ‘0’ ppm as control and other chosen treatment levels were 60, 70 and 80 ppm.

### 2.4 Estimation of Growth Parameters

Growth performance of the experimental fish was assessed by estimating various parameters like Weight Gain (WG), Specific Growth Rate (SGR), Protein Efficiency Ratio (PER), and Food Conversion Ratio (FCR) using standard formulae [17].

WG (g) = Final mean weight – Initial mean weight

DWG (g/day) = WG / Total days of experiment

SGR (%/day) = [ln (final weight) – ln (initial weight)] x 100 / total days of experiment

FCR (g/g) = [Dry weight of feed given /wet weight of fish gained]

Protein Efficiency Ratio (PER) = Increased body weight (g) / Protein consumed (g)

Survival (%) = Final number of fish x 100 / Initial number of fish

### 2.5 Estimation of Haematological Parameters

Blood samples from randomly chosen fish from all experimental units were collected during the morning hours to evade diurnal variations in the blood components without sacrificing the animals. The fish were anesthetized using clove oil and caught gently with most care to avoid additional stress while handling. The fish were held firmly on a sponge bed and blood was collected from the caudal peduncle using 1 ml syringe rinsed with anticoagulant (Heparin) fitted with 26G needle. Collected blood was immediately flushed into the K2 EDTA coated blood collection vials of 2 ml capacity and EDTA coated vial of 4 ml for haematological and biochemical studies respectively.

Haematological Analysis like Total Erythrocyte Count (TEC), Total Leukocyte count (TLC), Haemoglobin Content (Hb), Haematocrit Value (Ht), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin Concentration (MCHC) were analysed according to the standard procedure. Total erythrocyte count (TEC) and Total Leukocyte count (TLC) of the fish blood were estimated using Neubauer's haemocytometer [18]. Sahli's method was followed for determination of haemoglobin content of the blood [19]. The Wintrobe tube was used for the estimation of haematocrit value. MCV and MCHC were determined by using the following formula;

$$\mathrm{MCV} = \frac{\mathrm{Haematocrit}\ (\%) \times 10}{\mathrm{RBC\ Counr\ in\ Million}}$$

$$MCHC = \frac{Haemoglobin\ (g/dl)}{Haematocrit\ (\%)}$$

## 2.6 Estimation of Biomarkers

The estimation of biomarkers like Cortisol, Glucose, Alanine aminotransferase (ALT), Aspartate Aminotransferase (AST) and creatinine were carried out by collecting blood from all experimental fishes including control unit in the ammonia stress study. Cortisol was estimated by Chemiluminescent Immunoassay (CLIA) method [20]. Trinder method was used for the estimation of glucose present in the blood samples of experimental fish [21]. The ALT and AST were done by using method described by [22] and Jaffe's reaction method was used to determine serum creatinine [23].

## 2.7 Statistical Analysis

The data were analysed using software SPSS 16.0 choosing the Duncan's test for comparing multiple variables. The Two-way Analysis of Variance (Univariate) was also applied to assess the effect of interaction between different TAN levels and their respective time intervals on all the haematological parameters and biochemical parameters.

# 3. RESULTS AND DISCUSSION

## 3.1 Effect of TAN on Growth Parameters

The growth parameters of experimental fish such as length-weight, SGR, PER and FCR are shown in Table 1. The growth in terms of length and weight, SGR and PER were found high in control unit (0 ppm) followed 60 ppm, 70 ppm and 80 ppm whereas FCR showed significant ($p<0.05$) increase from 60 ppm to 80 ppm. Estimation of SGR, PER and FCR are useful tool for the comparison of growth among different sizes of fishes [24]. The finding of the present study is in agreement with the finding obtained by [25] reported that growth parameters decreased significantly as total ammonia nitrogen increased in Chinese sturgeon (*Acipenser sinensis*) exposed to various level of ammonia. Increase of ammonia concentration decreased the specific growth rate and protein efficiency ratio of many fishes viz., channel catfish [26]. In the present study, the growth of fish which exposed to 80 ppm TAN was significantly retarded. It reveals that some of energy derived from feed used for the metabolic maintenance rather than growth.

## 3.2 Effect of TAN on Haematological Parameters

The haematological parameters of experimental fishes when exposed to different TAN level such as Total Leukocyte Count (TLC), Total Erythrocyte Count (TEC), Haemoglobin (Hb), Haematocrit (Ht), Mean Corpuscular Volume (MCV) and Mean Corpuscular Haemoglobin Concentration (MCHC) are shown in Table. 2. The haematological parameters such as Erythrocyte Count (TEC), Haemoglobin (Hb), Haematocrit (Ht) showed a significant ($p<0.05$) decrease from 60 ppm to 80 ppm while compared to control.

### 3.2.1 Total Leukocyte Count (TLC)

Mean count of total leukocyte in *P. hypophthalmus* exposed to different TAN levels was high in 80 ppm and significant difference ($p<0.05$) could be observed between treatments despite no difference between control and 60 ppm. [27 and 28] recorded an increase in leukocyte counts (WBCs) in tilapia exposed to ammonia. Thus, it is opined that an increase or decrease in the number of white blood cells is a normal reaction to toxicant exposure [29].

**Table 1. Growth parameters of *P. hypophthalmus* exposed in different TAN levels**

| Parameters | Control | 60 ppm | 70 ppm | 80 ppm |
|---|---|---|---|---|
| Length (cm) | $26.12^{d}\pm1.01$ | $25.88^{c}\pm0.88$ | $25.51^{b}\pm0.71$ | $25.14^{a}\pm0.47$ |
| Weight (g) | $166.82^{d}\pm2.60$ | $164.47^{b}\pm0.88$ | $164.37^{b}\pm0.75$ | $163.74^{a}\pm0.28$ |
| SGR | $0.16^{d} \pm 0.023$ | $0.04^{c} \pm 0.008$ | $0.03^{b} \pm 0.006$ | $0.011^{a} \pm 0.004$ |
| PER | $1.74^{d} \pm 0.14$ | $1.74^{d} \pm 0.14$ | $0.44^{b} \pm 0.07$ | $0.13^{a} \pm 0.04$ |
| FCR | $2.32^{a} \pm 0.23$ | $7.53^{b} \pm 1.26$ | $9.55^{c} \pm 1.71$ | $32.79^{d} \pm 10.93$ |

***: *Means giving the same superscript do not differ significantly while others differ significantly at P<0.05 level by Duncan's test*

**Table 2. Haematological parameters of *P. hypophthalmus* exposed in different TAN levels**

| Parameters | Control | 60 ppm | 70 ppm | 80 ppm |
|---|---|---|---|---|
| TLC ($10^3$/µl) | $40.27^a \pm 0.50$ | $40.88^a \pm 0.51$ | $43.08^b \pm 3.04$ | $42.08^c \pm 2.73$ |
| TEC ($10^6$/µl) | $2.77^d \pm 0.12$ | $2.67\ ^c\pm 0.09$ | $2.49^b \pm 0.15$ | $2.37^a \pm 0.20$ |
| Hb (g/dL) | $13.92^d \pm 0.24$ | $13.43^c \pm 0.33$ | $12.94^b \pm 0.50$ | $12.65^a \pm 0.41$ |
| Hct (%) | $37.00^c \pm 1.45$ | $36.42^c \pm 0.90$ | $35.72^b \pm 0.94$ | $33.57^a \pm 1.20$ |
| MCV (fL) | $133.22^a \pm 1.13$ | $136.19^b \pm 2.74$ | $143.77^c \pm 6.15$ | $142.23^d \pm 9.88$ |
| MCHC (g/dL) | $37.62^a \pm 0.96$ | $36.86^a \pm 1.20$ | $36.37^a \pm 1.71$ | $37.66^a \pm 1.97$ |

***: *Means giving the same superscript do not differ significantly while others differ significantly at P<0.05 level by Duncan's test*

### 3.2.2 Total Erythrocyte Count (TEC)

Mean counts of total erythrocyte count of *P. hypophthalmus* exposed to different TAN levels showed a significant ($p<0.05$) decrease in 60 ppm to 80 ppm. Similar observation was also made in blood of *Cyprinus carpio* [30]. However, conflicting results have been reported in the haematological parameters of fish exposed to various ammonia levels. The erythrocyte count of *Oreochromis niloticus* increased after a 24 hour exposure to ammonia, and then decreased until the end of the exposure time of seven days [31]. No difference in erythrocyte count in *Labeo capensis* exposed to lethal and sublethal ammonia concentrations [32]. The reduction in TEC in the current study could be attributed to decrease of erythropoietic activity in the kidneys of the experimental fish [33] or might be due to haemodilution caused by impaired osmoregulation across the gill epithelium [34].

### 3.2.3 Haemoglobin and haematocrit

Mean values of total haemoglobin and haematocrit of *P. hypophthalmus* exposed to different TAN levels decreased from 60 ppm to 80 ppm. Significant difference ($p<0.05$) could be observed between treatments of 60 ppm, 70 ppm and 80 ppm in both haemoglobin and haematocrit in the blood of experimental fishes despite no difference was observed between 60 ppm and control in the case of haematocrit. Evidently, several investigators reported similar findings ie., reduction of Hb and Hct in their studies conducted in various species viz., Nile Tilapia [35], mrigal [16]. The decrease of Hb content in blood of the experimental fish might be due to the prevailing anoxic condition created by the depression and exhaustion in the haemopoietic condition occurred when they exposed to various stress factors [36 and 37]. The phenomena of decreasing of Ht in the experimental fishes of the present study after acute exposure to ammonia could be attributed to shrinking of the volume of the erythrocytes due to the concurrent high plasma osmolality or eventually by decreased number of red blood cells due to the acute toxicity of ammonia [38].

### 3.2.4 MCV and MCHC

Mean values of MCV and MCHC of *P. hypophthalmus* exposed to different TAN levels showed irregular trend between treatments. MCV results showed significant difference ($p < 0.05$) between treatments whereas no significant ($p>0.05$) difference of MCHC could be observed between treatments. [39] reported that the values of MCV and MCHC were markedly reduced in blood of the fish when they were exposed to high ammonia and temperature. [40] also observed the similar reduction in MCV and MCHC of *Sebastes schlegelii* when it exposed to ammonia. However, [41] reported that increase in MCV and decease in MCHC in common carp, *C. Carpio* when exposed to ammonia. The increase of MCV in the blood of the experimental fish after ammonia exposure could be attributed to increased water content in RBCs due to chloride shift and a decrease in plasma chloride at the same level of ammonia in water. Furthermore, the decrease in MCHC value might be due to hemodilution and/or a lack of Hb production in circulation.

## 3.3 Effect of TAN on Bio-Chemical Parameters

Thebiochemical parameters such as Cortisol, Glucose, Alanine aminotransferase (ALT), Aspartate aminotransferase (AST) and Creatinine of the experimental fish when exposed to different TAN level are shown in Table. 3.

### 3.3.1 Cortisol

Cortisol level of the experimental fish *P. hypophthalmus* increased along with the increase of TAN (Table 3). Mean values of cortisol rate of the experimental fish exposed to different TAN levels during different periods are presented in Fig. 1. High level of cortisol was observed in 80 ppm and low in control. The level of cortisol production varied significantly ($p < 0.05$) between treatments of 60 ppm, 70 ppm and 80 ppm with control. [42 and 43] also observed increase of cortisol in plasma in channel catfish (*Ictalurus punctatus*) and in the fingerlings of *Labeo rohita* respectively when subjected to various ammonia levels. The stress in the living environment activates the hypothalamus pituitary inter-renal (HPI) axis of the fish and in turn leads to increased secretion of cortisol in blood plasma [10].In the present study also ammonia toxicity caused the stress to the experimental fish *P. hypophthalmus* which might in turn activated hypothalamus pituitary inter- renal axis to stimulate the release of corticosteroid hormones into the blood circulation [44].

**Table 3. Biochemical parameters of *P. hypophthalmus* exposed to different TAN levels**

| Parameters | Control | 60 ppm | 70 ppm | 80 ppm |
|---|---|---|---|---|
| Cortisol (ng/ml) | $3.00^{a} \pm 0.19$ | $3.99^{b} \pm 0.44$ | $5.45^{c} \pm 0.70$ | $6.21^{d} \pm 0.88$ |
| Glucose (mg/dL) | $60.37^{a} \pm 1.64$ | $67.24^{b} \pm 3.45$ | $71.70^{c} \pm 5.51$ | $76.99^{d} \pm 5.62$ |
| ALT(U/L) | $20.59^{a} \pm 1.51$ | $24.49^{b} \pm 3.75$ | $26.95^{c} \pm 1.63$ | $28.55^{d} \pm 2.28$ |
| AST(U/L) | $23.65^{a} \pm 0.92$ | $27.09^{b} \pm 3.21$ | $28.88^{c} \pm 2.89$ | $29.65^{d} \pm 2.52$ |
| Creatinine(mg/dL) | $0.119^{a} \pm 0.02$ | $0.185^{b} \pm 0.03$ | $0.217^{c} \pm 0.04$ | $0.315^{d} \pm 0.06$ |

***: *Means giving the same superscript do not differ significantly while others differ significantly at P<0.05 level by Duncan's test*

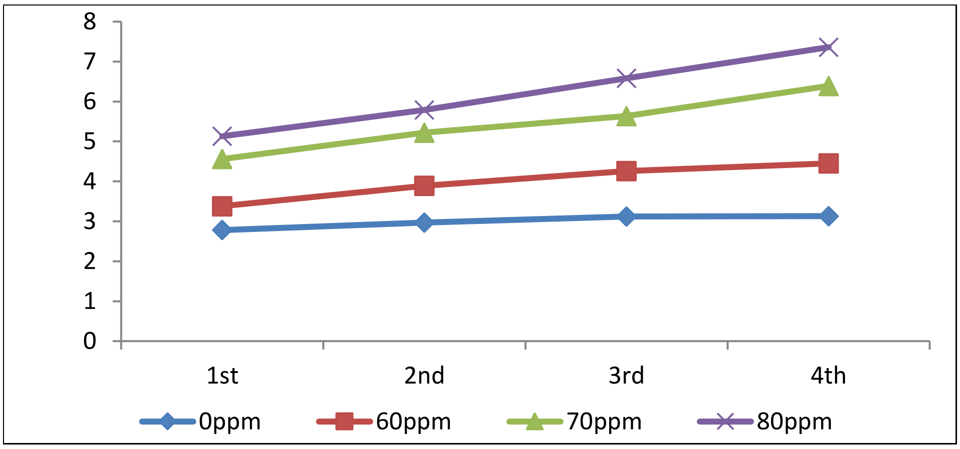


**Fig. 1. Mean of Cortisol (ng/ml) observed in *P. hypophthalmus* obtained in different TAN levels during the successive weeks**

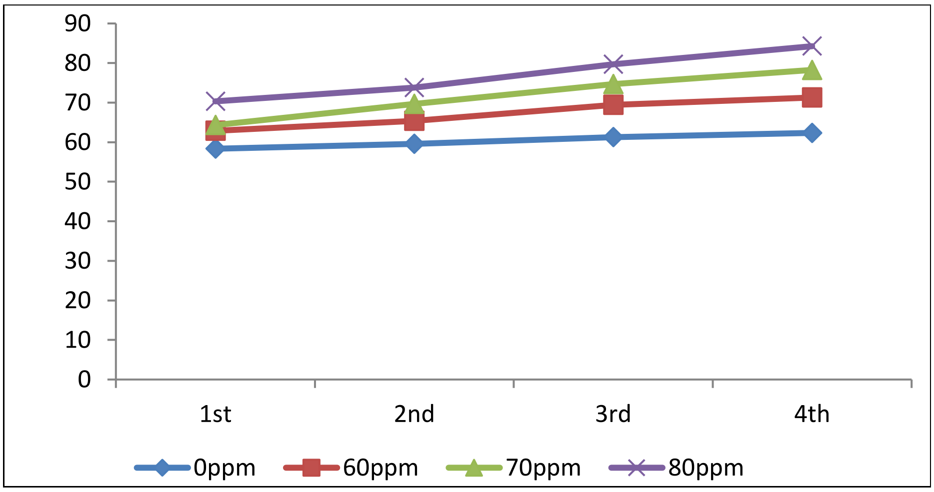


**Fig. 2. Mean of glucose (mg/dL) observed in *P. hypophthalmus* in different TAN levels during the successive weeks**

### 3.3.2 Glucose

Glucose level in the blood plasma of the experimental fish *P. hypophthalmus* increased with TAN levels from 60 to 80 ppm (Table 3.). Mean values of Glucose of *P. hypophthalmus* exposed to different TAN levels in different week periods are presented in Fig. 2. Further, significant difference in serum glucose level ($p < 0.05$) was observed between the treatments and control. The glucose in the blood has become a common method for estimating the effect of stress [45 and 46]. The plasma glucose of the fish is found as important biomarker which would be more sensitive to ammonia exposure [47]. [48] reported that serum blood glucose was significantly increased after exposure to ammonium chloride compared to control group. The increase in glucose in the present study might be due to the glycolytic activity of catecholamines and the effect of glucocorticoids by stress response under exposure to toxic ammonia substances [49].

### 3.3.3 Alanine aminotransferase (ALT), Aspartate aminotransferase (AST)

Mean values of ALT and AST rate of *P. hypophthalmus* exposed to different TAN levelsare presented in Table 3. The alterations in the ALT and AST level in the blood serum of the experimental fish in different week periods are given in Figs. 3 & 4. In the present study, the level of ALT and AST measured in the serum of experimental fish of *P. hypophthalmus* increased from TAN value of 60 ppm to 80 ppm. Further, the AST in the serum of experimental fish varied significantly ($p<0.05$) between the treatments of 60 ppm, 70 ppm and 80 ppm. AST and ALT are serum components, can generally be used to assess tissue damage to the liver, kidneys [50], muscle, and gills [51].

[52] demonstrated the effect of ammonia toxicity in *Labeo rohita* that ALT and AST were increased when they exposed to acute toxic dose of unionised ammonia. The negative effect of ambient ammonia concentrations on ALT and AST level has been noted in several species, such as Nile Tilapia [53]. Observed increase in the level of ALT and AST in the blood serum of present experimental fish might be due to the accumulation of ammonia in the body of fish [54] and the process of either deamination or transamination [55].

### 3.3.4 Creatinine

Creatinines level measured at experimental fish *P. hypophthalmus* at different TAN level are presented in Table 3. Mean values of Creatinine rate of *P. hypophthalmus* exposed to different TAN levels at different week periods are presented in Fig. 5. The increasing trend of creatinine was observed in the experimental fish *P. hypophthalmus* from 60 ppm to 80 ppm and higher value was observed in 80 ppm. Creatine accounts for more than 50% of the nitrogenous waste excreted through the kidneys of fish and it act as good indicator for assessing overall health of the gills and kidneys [56]. Increasing level of creatinine with increasing ammonia toxicity was observed in various fishes such as rainbow trout [57], *Lophiosilurus alexandri* [58] and common carp [59]. The increase of creatinine in the experimental fish *P. hypophthalmus* could be attributed to the action of ammonia on glomeruli filtration [60].

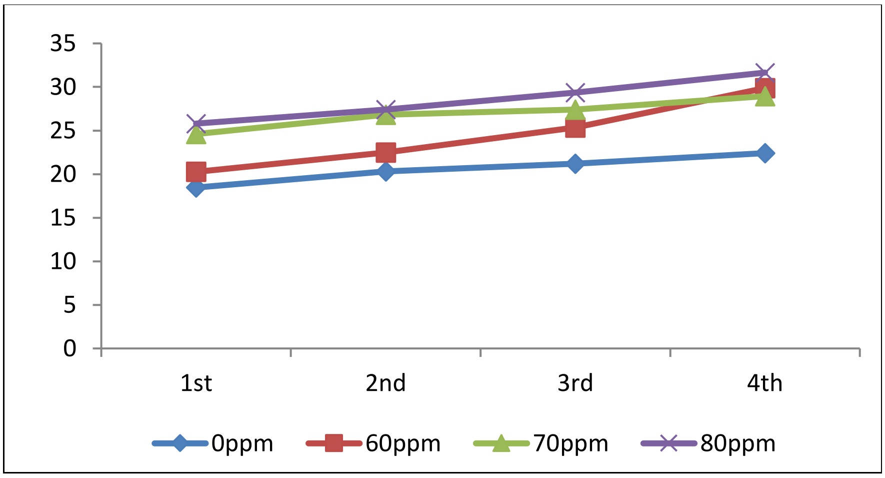


**Fig. 3. Mean of ALT (Alanine aminotransferase) observed in *P. hypophthalmus* in different TAN levels during the successive weeks**

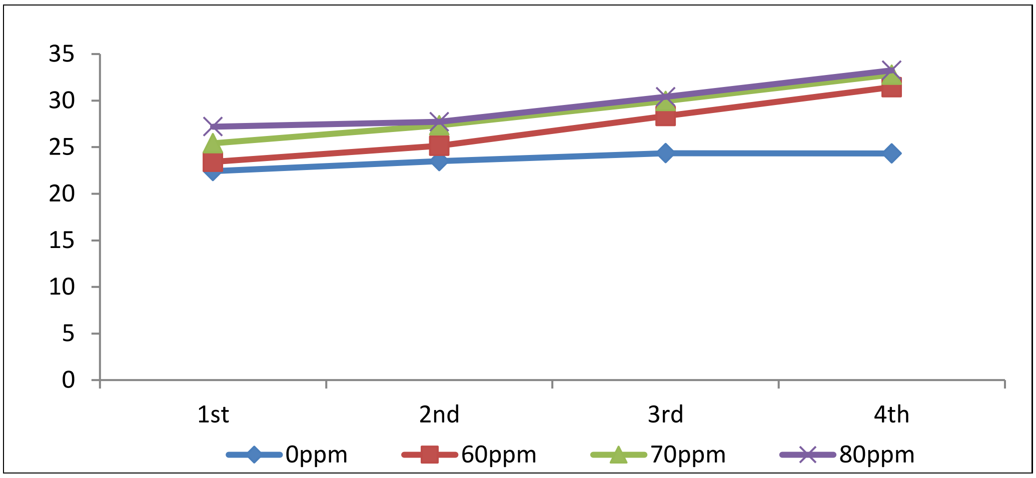


**Fig. 4. Mean of AST (Aspartate aminotransferase) observed *P. hypophthalmus* in different TAN levels during the successive weeks**

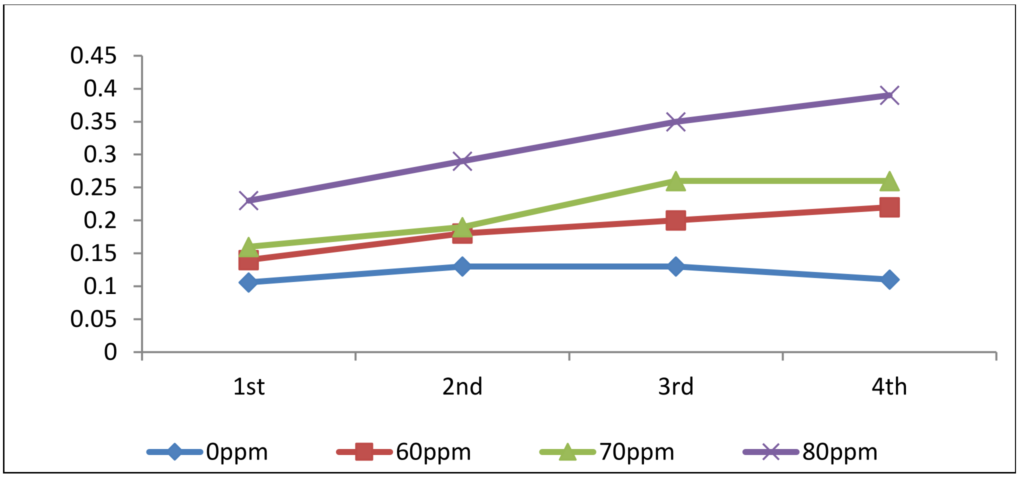


**Fig. 5. Mean of Creatinine (mg/dL) observed in *P. hypophthalmus* in different TAN levels during the successive weeks**

## 4. CONCLUSION

Despite the fact that many studies have been conducted to demonstrate the relationship between stress and fish physiological state. To the best of our knowledge, the current study investigated the effect of stress-induced changes in haematological and biochemical parameters of *Pangasianodon hypophthalmus* at various ammonia levels. As demonstrated in this study, ammonia has a negative impact on both the haematological and biochemical profiles of fish. Nevertheless, the high level of ammonia altered the haematological and biochemical profiles of the experimental fish heavily; the candidate species *Pangasianodon hypophthalmus* proved that it is capable of withstanding high TAN level and attaining good growth up to 60 ppm.

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## DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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