Histopathological and Enzyme Changes in *Clarias gariepinus* (Burchell 1822) Exposed to Nitrite at Different Water Temperatures

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Abstract: Nitrite is a natural component of the nitrogen cycle in ecosystems.lt is an intermediate in the oxidation of ammonium to nitrate.The elevation of ambient nitrite concentration is a potential problem for freshwater fish. This study was designed to investigate the effect of different water temperatures on the toxic effect of nitrite in a freshwater fish. Sixty *Clarias gariepinus* (300 ± 1.30g), were exposed to nitrite at different water temperatures (27^0C and 35^0C) for 48hours. Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), Aspartate aminotransferase (AST) and total protein levels were assayed in the gill, liver and tissue (skin) of the fish. The statistical analysis was performed using the Statistical Analysis (SPSS 11.0 for Windows). Statistical differences were determined by one-way analysis of variance (ANOVA) and paired-sample t test. ALT and ALP increased significantly ($P<0.05$) in nitrite-intoxicated fish at 35^0C compared to the value obtained at 27^0C in the organs while a significant decrease ($P<0.05$) was observed for the enzyme AST at 35^0C compared to 27^0C. Protein level in all the tissues showed a significant decrease in nitrite-intoxicated fish at higher temperature. The histopathological changes observed in the gills of nitrite-treated fish at 35^0C were that of congestion and vacuolization while the liver showed generalized fatty35^0C were that of congestion and vacuolization while the liver showed generalized fatty degeneration, congestion of central veins and multifocal necrosis. Moderate hydropic degeneration of the epidermal layer was observed in the skin tissue. These results revealed that high temperature can increase the toxic action of nitrite in fish.

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Introduction

Nitrite is a natural component of the nitrogen cycle in ecosystems and its presence in the environment is a potential problem due to its well-documented toxicity to animals (Watenpaugh 1985; Lewis and Morris 1986; Williams 1997 and Jensen 2003). It is an intermediate in the oxidation of ammonium to nitrate. Nitrite is a well-known toxicant for fish as well as a disrupter of multiple physiological functions including ion regulatory, respiratory, cardiovascular, endocrine and excretory processes (Kroupova et al., 2005).

Nitrite accumulates in tissues such as gills, liver, brain and muscle (Margiocco et al., 1983). According to Casillas et al., (1983), enzyme activities are considered as sensitive biochemical indicators before hazardous effects occur in fish and are important parameters for testing water and the presence of toxicants. These biochemical methods has been advocated to serve as an early warning of potentially damaging changes in stressed fish.

Alanine aminotransferase (ALT) is one of the enzymes that catalyze the reactions of transmination of alanine, glutamic and aspartic acids. In conjunction with a co-enzyme, it couple the protein, carbohydrate and fat metabolism and tricarboxylic acid cycle under altered physiological, pathological and induced environmental stress conditions (Murugesan et al., 1999). Aspartate aminotranferase (AST) is also an enzyme found in liver, muscle and heart tissues. An increased level of AST has been associated with heart attack, liver function problems and injury or disease to the muscles.

Alkaline phosphatase (ALP) on the other hand, is a brush border enzyme, which splits various phosphorus esters at an alkaline pH. It is well known that phosphatases are involved in carbohydrate metabolism, growth and differentiation, protein synthesis, synthesis of certain enzymes, secretory activity and transport to phosphorylated intermediates across the cell membranes (Vijayavel et al. 2006). Inhibition and induction of these biomarkers is a good approach to measure potential impacts of pollutants on environmental organisms (El-Shehawi et al. 2007).

There had been various reports on the toxic effect on Nitrite in fish. Kroupova et al. (2006) focused on the haematological and biochemical changes associated

with nitrite intoxication in Common Carp (*Cyprinus carpio* L.) at different water temperature. Velmurugan et al.(2007) also investigated the changes on some tissue enzymes of *Clarias gariepinus* fingerlings exposed to sublethal concentrations of Cadmium Chloride, a heavy metal but there is little information on the histopathological and enzyme changes of *C. gariepinus* exposed to nitrite at higher temperatures. This study is quite necessary as *C. gariepinus* is a hardy fish and is the most cultured species in Nigeria and the temperature during the growing season of this fish in Nigeria vary between 27^0C and 35^0C.

Materials and methods

Experimental Fish

Sixty pieces C. *gariepinus* of mean weight 300±1.30g (mean ±SD) and mean total length of 60.0±1.0cm were used for the study. The fish were collected in the morning between 8 – 9a.m. They were obtained from Bowen fish farm and maintained for 2 weeks in 4 circular plastic tanks (300L) with dechlorinated tap water. Four days before the start of the experiment, the fish where divided into four groups and acclimated to 27^0C and 35^0C. During acclimation and experiment period, fish were not fed.

Experimental procedure and fish sampling

The test was performed in a semi-static assay for 48 hours.12 fish were kept in 4 thermostat-controlled water bath, each containing 40 liters of test solution. The nitrite concentration was obtained by adding $NaNO_3$ to dechlorinated tap water. The dose of nitrite represented the median lethal concentration (LC_{50}) for *Clarias gariepinus* at a similar chloride water concentration and similar relative weight of fish according to Ajani (2006).

During the acclimation and experimental period, the basic chemical indices of water taken were acid neutralization capacity 42mg/l, total ammonia 60mg/l, phosphate 32mg/l, hardness 69.2mg/l, chloride 19.0mg/l, Oxygen saturation of the water 8.0mg/l and the pH 7.4 using HACH freshwater aquaculture test kit(FF – 1A).Four groups each containing 8 specimens of four-month old *C. gariepinus* were exposed to nitrite at different water temperature (27^0C and 35^0 C).

Nitrite and chloride content were checked twice during the test and the measured values did not differ from the nominal value by more than 7 percent. After treatment, both the experimental and control fishes were killed after 48hours. Gill, liver and tissue were removed out of each fish and frozen at -20^0C until analysis.

Biochemical Examinations

Tissues were homogenized in 3 vol (v/w) of 10mM Tris-HCI, 0.25M sucrose**.** Adequate measures were taken to minimize pain or discomfort.

After treatment, both the experimental and control fishes were sacrificed after 48hours. Gill, liver and tissue were obtained and frozen at -20^0C until analysis.

Tissues were homogenized in 3 vol (v/w) of 10mM Tris-HCI, 0.25M sucrose buffer (pH 7.4) at 4^0C, using a homogenizer equipped with a Teflon pestle. Homogenates were centrifuged at 1600 x g for 20minutes at 4^0C to remove cell debris. The resulting supernatant was collected and used in the estimation of AST, ALT, ALP and protein.

Measurement of enzyme activities and protein were performed using semiautoanalyser [Microlab-200 (Merck)]. The protein content of the enzyme source was estimated by the Biuret method (Gormall et al., 1949), using bovine serum albumin as standard. The protein values are expressed mg/g.

Histopathology

Samples from the gill, liver and tissue obtained were collected in 10% buffered formalin, routinely processed and stained with haematoxylin and eosin for histological examination using x 40 of light microscope.

Statistical Analysis

The statistical analysis was performed using the Statistical Analysis (SPSS 11.0 for Windows). Statistical differences were determined by one-way analysis of variance (ANOVA) and paired-sample t test. The significance of test results was ascertained at $P<0.05$.

RESULTS

Significant changes of ALT, AST, ALP and protein were observed in nitrite-intoxicated fish at 35^0C when compared to that at 27^0C. They are presented in tables I-III.

The protein levels in the nitrite-intoxicated fish at higher temperature (35^0C) decreased significantly ($P<.05$) to that at lower temperature (27^0C) in the tissue, liver and gill. However, drastic decline in the levels was observed in the tissue.

ALT and ALP increased significantly ($P<0.05$) in nitrite-treated tissue at 35^0C compared to that at 27^0C while a significant decrease was observed for the enzyme AST at 35^0C compared to 27^0C.

The value obtained for ALT and ALP at 35^0C nitrite-intoxicated fish is 3 folds and more than 6 folds when compared to the value obtained at 27^0C in the liver of *C. gariepinus*. The highest increase in ALT and ALP found in the liver of *C. gariepinus* reflects the level of damage nitrite can inflict on fish at higher temperature.

Table 1: Enzyme activities and protein levels in tissue (skin) of *C. gariepinus* after 48-h nitrite exposure

Enzyme metabolite	27^0C		35^0C	
	Nitrite-free	Nitrite	Nitrite-free	Nitrite
ALT(mg/l)	54.10 ± 8.05^b	38.31 ± 6.02^a	52.62 ± 9.01^b	54.63 ± 7.01^b
AST(mg/l)	362.00 ± 14.01^c	181.0 ± 10.11^b	338.10 ± 8.62^c	146.0 ± 8.62^a
ALP(mg/l)	18.08 ± 11.21^c	1.00 ± 9.21^a	16.10 ± 11.81^c	11.30 ± 10.12^b
Protein(mg/l)	4.80 ± 0.50^c	4.00 ± 0.04^b	3.51 ± 0.01^b	1.63 ± 0.15^a

The values are expressed as means ± SD for n = 3.
Mean followed by different superscript in the same row are significantly different (P<0.05)

Table 2: Enzyme activities and protein levels in liver of *C. gariepinus* after 48-h nitrite exposure

Enzyme metabolite	27^0C		35^0C	
	Nitrite-free	Nitrite	Nitrite-free	Nitrite
ALT(mg/l)	60.32 ± 7.10^b	40.36 ± 3.18^a	56.31 ± 8.21^b	120.00 ± 3.01^c
AST(mg/l)	368.38 ± 1.10^c	200.81 ± 5.31^a	340.00 ± 3.87^c	232.43 ± 4.01^b
ALP(mg/l)	21.00 ± 0.51^b	3.01 ± 2.43^a	18.16 ± 6.00^b	20.61 ± 2.80^b
Protein(mg/l)	4.61 ± 3.10^c	3.95 ± 2.41^b	3.40 ± 0.35^b	2.03 ± 0.08^a

The values are expressed as means ± SD for n = 3.
Mean followed by different superscript in the same row are significantly different (P<0.05)

Table 3: Enzyme activities and protein levels in gills of *C. gariepinus* after 48-h nitrite exposure

Enzyme metabolite	27^0C		35^0C	
	Nitrite-free	Nitrite	Nitrite-free	Nitrite
ALT(mg/l)	52.08 ± 6.01^b	39.00 ± 4.02^a	53.00 ± 6.02^b	65.30 ± 4.81^b
AST(mg/l)	261.00 ± 8.11^b	248.00 ± 8.61^b	244.01 ± 7.11^b	198.00 ± 6.31^a
ALP(mg/l)	20.18 ± 3.00^b	3.00 ± 0.04^a	15.06 ± 8.17^b	16.00 ± 6.04^b
Protein(mg/l)	3.81 ± 0.00^b	3.28 ± 2.10^b	3.21 ± 0.81^b	2.00 ± 1.00^a

The values are expressed as means ± SD for n = 3.
Mean followed by different superscript in the same row are significantly different (P<0.05)

Histopathology

The photomicrograph of the nitrite-intoxicated gill,liver and skin of *C.gariepinus* at 27^0C and 35^0C are reflected in figures 1-6.

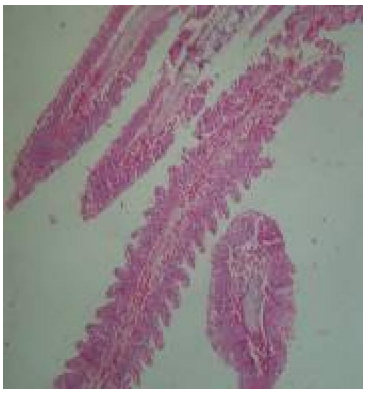

Figure 1: The photomicrograph of the gills of nitrite-intoxicated fish at 35^0C showing congestion and vacuolisation.HXE 40

Figure 2: The photomicrograph of the gills of nitrite-intoxicated fish at 27^0C .HXE 40

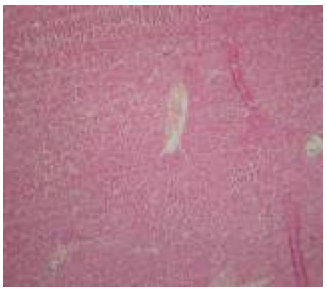

Figure 3: The photomicrograph of the liver of nitrite –intoxicated fish at 35^0C showing generalised fatty degeneration, congestion of central veins and multifocal necrosis. HXE 40

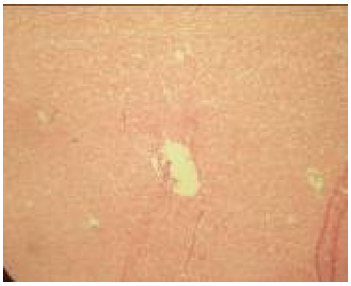

Figure 4: The photomicrograph of the liver of nitrite –intoxicated fish at 27^0C showing no congestion. HXE 40

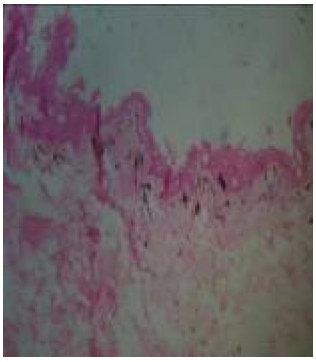

Figure 5: The photomicrograph of the skin of nitrite-intoxicated fish at 35^0 C showing moderate hydropic degeneration of the epidermal layer.HXE 40

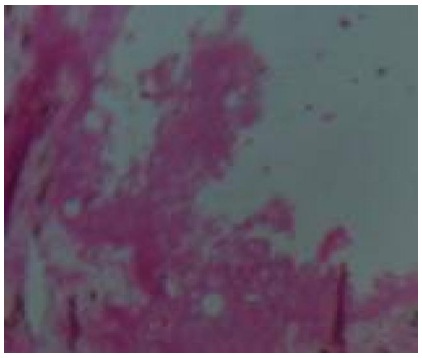

Figure 6: The photomicrograph of the skin of nitrite-intoxicated fish at 27^0C showing no degeneration of the epidermal layer HXE 40

DISCUSSION

The response to pollution is reflected as changes in some enzyme activities, especially key enzymes of biotransformation systems of organisms which can be used as biomarkers that are sensitive to pollution, of note are ALT, ALP and AST. These biomarkers, therefore, provide a tool for specific early warning sign for aquatic pollution also in fish species (Sirmac and Braunbeck 2000). ALT is an enzyme frequently used in the diagnosis of damage caused by pollutants in various tissues such as liver, muscle and gills (De la Torre et al. 1999; 2000). This enzyme is known to play a key role in mobilizing L-amino acids for gluconeogenesis and function as links between carbohydrate and protein metabolism under altered physiological, pathological and induced environmental conditions (Nichol and Rosen 1963; Knox and Greengard 1965; Victor 1985; and Velmurugan et al. 2007).

Significant ($P<0.05$) elevation in the level of AST and ALT in the liver, gill, tissue of the fish in this study is similar to that obtained in Cadmium Chloride toxicity (Velmurugan et al. 2007) as this response as been reported to be stress induced since nitrite generates ketoacid-like: ketoglutarate and oxaloacetate necessary to meet the excess energy demand under the toxic situation. The elevations in ALT activity were also observed in *C. carpio* and *Oreochromis niloticus* exposed to cadmium (De La Torre et al. 2000; De Smet and Blust 2001; Almeida et al. 2002).

ALP is a membrane-bound enzyme related to the transport of various metabolites (Lin et al 1976; Wahwon et al. 1992). Various membrane functions such as permeability, the activity of bound enzymes and hormone receptors and the efficiency of transport systems are controlled by membrane dynamics (Shinitzky,1984). ALP is an integral enzyme known to be intimately associated with the hydrophobic core of

the intestinal microvillus membrane (Brasitus et al.1979). The enzyme AST activity at higher temperature in nitrite water increased when compared with lower temperature (27^0C).

The increase in ALP activity at higher temperature in nitrite water supports earlier findings. Sastry and Subhadra (1985) recorded elevation in ALP in ovary and muscles of freshwater catfish, *Heteropneustes fossilis* exposed to cadmium. Also, the ALP activity showed an increased in all the organs liver, gills, gut and gonads examined in Rosy barb (*Puntius conchonius*) exposed to mercuric chloride (Gill et al. 1990).

Accumulation of the environmental pollutants and toxicants has been shown to cause alterations in the activities of many enzymes concerning cellular energy metabolism (Sebert et al. 1993).

The decrease in protein content of fish at 35^0C nitrite water indicates the physiological adaptability of the fish to compensate for the stressful condition. Protein catabolism must have occurred to compensate for the high energy demand in order to adjust/overcome the toxic stress of nitrite.Sancho et al. (1997) corroborated this. Also, the utilization of protein in cell repair and organization cause the depletion in the tissues at 35^0C nitrite water. Bradbury et al. (1987) pointed out that the decreased protein content might also be attributed to the destruction or necrosis of cellular function and consequent impairment in protein synthesis.

Histopathological studies of fish exposed to pollutants revealed that fish organs were efficient indicators of water quality (Cardoso et al. 1996; Barlas 1999; Cengiz et al. 2001). The gills are important organs in fish for respiration, osmotic regulation, acid base balance and nitrogenous waste excretion (Heath 1987). Michael et al. (1987) also observed hyperplasia and hypertrophy in the gills of *Clarias lazera* chronically exposed to nitrite.

From this study, the histopathological changes observed in the gills of nitrite – treated fish at 35^0C showed congestion and vacuolization. This is well in line with earlier findings of Svobodova et al. (2005b). They recorded hyperplasia, vacuolization and elevated number of chloride cells as the main histological lesions that occurred in the gills of nitrite treated carp (*Cyprinus carpio*).

The high demand on the liver due to the stressful condition is which and is what is revealed in the histopathology. The pathology of the liver of nitrite – intoxicated fish at 35^0C was that of generalized fatty degeneration, congestion of central veins and multifical necrosis. The main functions of the liver are to process nutrients from food, make bile, remove toxins from the body and build proteins. According to Daniel (2009), interference with these important functions can lead to poor health.

The tissue of the skin of nitrite-intoxicated fish at 35^0C showed moderate hydropic degeneration of the epidermal layer while at 27^0C, there was no significant lesion.

CONCLUSION

Nitrite has deleterious effect in *C. gariepinus* fish at higher temperature (35^0C). Significant alterations in the levels of AST, ALP, ALT and protein in different tissues of this fish reflect this. Thus, nitrite build-up in culture systems should attract prompt attention.

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