Ammonia-induced and Withdrawal-dependent Neurotoxic (Acetylcholinesterase) and Antioxidative (Catalase) Responses in Catfish, *Clarias batrachus* (Linnaeus, 1758)

**A. SHIWANAND and G. TRIPATHI***

Department of Zoology, J.N.V. University, Jodhpur-342 033, India

**Abstract**

Ammonia-induced and its withdrawal-dependent changes in acetylcholinesterase (AChE) and catalase (CAT) of liver, gill and skeletal muscle of *Clarias batrachus* (Linnaeus, 1758) were investigated. AChE activity declined gradually in these tissues up to 35 days of ammonia exposure. In contrast, CAT activity increased up to 7 days and thereafter, it declined to some extent. Maximum effects of ammonia on CAT and AChE were on 7th and 35th day of treatment respectively. AChE activity declined maximally in gills (61%), but CAT activity enhanced maximally in liver (1.6 fold). Withdrawal of ammonia from water for 14 days after 35 days of exposure indicated incomplete recovery in AChE activity. However, withdrawal of ammonia showed complete reversal in CAT. Removal of ammonia from water may help in damage control. Since ammonia-induced neurotoxic (AChE) effect was more pronounced than antioxidative (CAT) response, AChE may be used as an indicator of ammonia toxicity in aquaculture.

**Introduction**

Freshwater bodies are polluted by extensive use of agrochemicals. Intense application of nitrogen fertilisers can increase ammonia concentration in aquatic ecosystem (Gangbazo et al. 1995; Palanivelu et al. 2005; Bobmanuel et al. 2006). High concentration of ammonia can reduce fish growth (EPA 1998; Wicks and Randall 2002). Ammonia can impair the tricarboxylic acid (TCA) cycle in fish, which is responsible for acetyl-CoA oxidation to CO$_2$, so increased NH$_3$ can lead to a build-up of acetyl-CoA (Kaizer et al. 2009). Acetylcholinesterase (AChE) is responsible for the hydrolysis of the acetylcholine to choline and acetate in cholinergic synapses. In fish, AChE is enriched in brain and muscle tissues and is considered as an indicator of aquatic pollution. Tham et al. (2009) assessed *C. batrachus* as a source of AChE for the detection of insecticides. AChE inhibition as a result of phosphorylation leads to accumulation of acetylcholine which triggers the release of catecholamines (Reddy et al. 1990). Torre et al. (2002) demonstrated the high sensitivity of AChE activity as an exposure biomarker. Low oxygen level increase ammonia toxicity in silver catfish, *Rhamdia quelen* (Quoy and Gaimard 1824) (Kaizer et al. 2009). Falfushynska and Stolyar (2009) showed that the AChE activity decrease in liver of *Cyprinus carpio* Linnaeus, 1758 in summer and it is the most sensitive biomarker of pollution. Bervoets et al. (2009) reported a significant relationship between metal load in tissues and AChE activity.
Catalase (CAT) is an antioxidant enzyme and also considered as an adaptation enzyme in fish (Orbea et al. 2000; Sole et al. 2004; Fernandez-Diaz et al. 2006). Ammonia has been shown to induce oxidative stress in gill and brain of the mudskipper *Boleophthalmus boddarti* (Pallas, 1770)(Ching et al. 2009). It also induces antioxidant defenses of juvenile crucian carp *Carassius auratus* (Linnaeus, 1758) (Yang et al. 2010). Hegazi et al. (2010) described ammonia associated increase in the activities of xanthine oxidase, aldehyde oxidase, superoxide dismutase, catalase, glutathione peroxidase, glutathione S-transferase, glutathione reductase, γ-glutamyl cys-teinyl synthetase and γ-glutamyl transpeptidase in liver and white muscle of Nile tilapia (*Oreochromis niloticus*) (Linnaeus, 1758). Long-term ammonia exposure significantly impairs the feeding and growth of juvenile crucian carp and also induces various antioxidant enzymes in liver of the fish (Yang et al. 2011). Chen et al. (2011) reported that oxidative stress and lipid peroxidation occur in the larvae of grass carp, *Ctenopharyngodon idella* (Valenciennes, 1844) exposed to a combination of ammonia and microcystin. Ammonia has been found to significantly increase body weight but affect antioxidant system in the larvae of bighead carp, *Hypophthalmichthys nobilis* (Richardson, 1845) (Sun et al. 2012).

Ammonia is a potent neurotoxicant and may cause oxidative stress. AChE and CAT are the enzymes involved in nerve conduction and protection of cell from oxidative stress respectively. It is the reason why these two enzymes were chosen for study. The toxicological and withdrawal effects of ammonia on AChE and CAT of a freshwater fish were examined in this study. The hypothesis was to investigate whether enzymes can adapt to ammonia and whether its withdrawal from water can revert the neurotoxic and antioxidative functions in fish. The effects of ammonium chloride and its withdrawal-dependent changes in an economically important freshwater fish *C. batrachus* were investigated to test the proposed hypothesis.

**Materials and Methods**

**Fish and chemicals**

Adult and healthy specimens of the air-breathing walking catfish, *C. batrachus* weighing 184±6 g and with body length 23-29 cm were collected from local fish market of Jodhpur, India. This freshwater fish is commonly used for human consumption and is available in almost all fish markets of India. An experimental protocol for its collections was followed. Collections were done from November to February, 2010. This period is the reproductively regressed phase of the fish. The fish were maintained in tap water under laboratory conditions at least 1 week prior to experimentation. They were starved for 35 or 49 days depending on experiments. All chemicals used in this study were of analytical grade and procured from Sisco Research Laboratory (SRL), Mumbai, India. Double glass distilled water was used for preparation of chemicals.
**Ammonia treatment and withdrawal experiments**

Fish were exposed to sublethal concentration (60 mg L\(^{-1}\)) of ammonium chloride for 35 days. Water of container was changed on alternate days and a fresh concentration of ammonium chloride was maintained during ammonia exposure period. The pH of water varied from 7.2-7.6 during the experimentation period. Three separate experiments were carried out as follows:

(A) Ammonia treatment experiment for effects on AChE and CAT

Group 1: Normal individuals were kept untreated for 35 days (Control group).

Group 2: Individuals were exposed to sublethal concentration of ammonium chloride (60 mg L\(^{-1}\)) for 35 days (Treated group).

Liver, gills, and skeletal muscle were removed from untreated and ammonia treated individuals for assay and generating data on both AChE and CAT enzymes as shown in Fig. 1 and 2 respectively.

(B) Ammonia withdrawal experiment for AChE (Fig. 3)

Group 1: Normal individuals were kept untreated for 49 days (Normal/Control 1 group)

Group 2: Individuals were exposed to ammonium chloride (60 mg L\(^{-1}\)) for 49 days (Treated/Control 2 group).

Group 3: Individuals were kept in freshwater for 14 days after 35 days of exposure of ammonium chloride (Withdrawal group).

(C) Ammonia withdrawal experiment for CAT (Fig. 4)

Group 1: Normal individuals were kept untreated for 35 days (Normal/Control 1 group)

Group 2: Individuals were exposed to ammonium chloride (60 mg L\(^{-1}\)) for 21 days (Treated/Control 2 group).
Group 3: Individuals were kept in freshwater for 14 days after 7 days of exposure of ammonium chloride (Withdrawal group).

Samples (each comprising five individuals) were obtained on the day of completion of treatment mentioned against each group.

**Tissue processing and enzyme preparation**

Fish were killed by decapitation and liver, gill and skeletal muscle were removed immediately and rinsed in 0.6% saline. They were properly cleaned and weighed quickly. Tissues were stored at –40°C in Remi Deep Freezer (BDI-8158) until used for analysis. Acetylcholinesterase (AChE, E.C. 3.1.1.7) and catalase (CAT, E.C. 1.11.1.6) activities remained almost unaltered during period of storage for several weeks. Tissues were homogenised (10% w/v) in sodium phosphate buffer (0.1M, pH 7.4) using Potter-Elvehjem homogeniser fitted with a Teflon Pestle (Remi RB1278). Homogenisation was done at about 4 ºC. Homogenates were centrifuged at 12000 g for 15 min in a high-speed refrigerated centrifuge (Remi Cooling Compufuge, CPR 24). The supernatant was collected and used for the assay of AChE and CAT activity. The subcellular fractionation and preparation of enzyme were done according to the method described by Tripathi and Shasmal (2010).

**Enzyme assay**

AChE activity was determined spectrophotometrically following the method of Ellman et al. (1961) using acetylthiocholine iodide as the substrate. Extinction coefficient was 13600 M⁻¹cm⁻¹. Volume of reaction mixture was 3 mL absorbance was recorded at 412 nm. Reading of blank without enzyme was substracted to yield the enzymatic activity. Reaction mixture contained 2.7 mL phosphate buffer (0.1 M, pH 7.4), 0.05 mL DTNB (5,5’- DithioBis (2- Nitrobenzoic acid) (0.18 mM), 0.10 mL enzyme sample and 0.15 mL acetylthiocholine iodide. Acetylthiocholine iodide is broken down to thiocholine and acetate by AChE and thiocholineacted with DTNB to produce a yellow colour. Enzyme activity was expressed in µmol min⁻¹g⁻¹ wet mass. The procedure adopted for the assay of catalase was that of Aebi (1984). Catalase was assayed in a medium containing 50-150 mM H₂O₂ and 300 mM phosphate buffer (pH 7). Total volume of reaction mixture was 3 mL. The decrease in absorbance was noted at 240 nm using Cintra 5 UV-VIS spectrophotometer (GBC, Germany). The enzyme activity was expressed in mmol min⁻¹g⁻¹ wet mass.

**Statistical analysis**

A one-way analysis of variance (ANOVA) followed by Duncan’s multiple range test were used to analyse the data. The level of significance was P<0.05. Computations were done with the help of a computer and statistical package.
Results

ANOVA showed significant changes in the activities of AChE and CAT of liver, gill and skeletal muscle of *C. batrachus*. Higher values of SEM at certain places may be due to individual genetic differences in the expression of enzymes. The impacts of ammonium chloride and its withdrawal-dependent alterations in these enzyme activities were as follows:

**Effects of ammonium chloride on AChE and CAT**

Activity of AChE of liver, gill and skeletal muscle of *C. batrachus* varied significantly (P<0.05) with respect to changes in duration of exposure of ammonium chloride. Treatment of ammonium chloride for 35 days significantly (P<0.05) decreased the activity of AChE in all tissues (Fig. 1). Maximum decline in the enzyme activity was in gill (61.03%) followed by liver (55.11%) and skeletal muscle (51.03%). Activity of CAT from liver, gill and skeletal muscle also varied significantly (P<0.001) with increasing period of exposure of ammonium chloride (Fig. 2). In contrast to AChE, the CAT activity increased gradually and significantly in different tissues in response to exposure of ammonia. Maximum elevation in the enzyme activity was obtained on the 7th day. It was maximally enhanced in liver (1.59 fold) followed by gill (1.52 fold) and skeletal muscle (1.36 fold). Thereafter, it declined gradually and showed incomplete recovery at 35 days. CAT activity did not differ significantly between the 28th and 35th day.

**Effects of withdrawal of ammonium chloride on AChE and CAT**

Withdrawal of ammonium chloride from water for 14 days after its exposure for 35 days showed a gradual recovery in AChE activity in liver, gill and skeletal muscle of the fish, but recovery was not complete (Fig. 3). The pattern of depurative response was similar in different tissues of catfish. Withdrawal of ammonium chloride for 14 days after 7 days of its exposure showed complete reversal in CAT activity of liver, gill and skeletal muscle of the fish (Fig. 4). The trend of depurative response was similar in different tissues in response to ammonia withdrawal after 7 days of exposure.

Discussion

AChE activity of liver, gill and skeletal muscle of *C. batrachus* declined gradually up to 35 days of ammonia exposure. Maximum decline was in gill followed by liver and skeletal muscle (Fig. 1). Decline in AChE activity can affect locomotion and equilibrium in aquatic organisms and may impair feeding, escape and reproductive behaviour (Habig and Di Gulio 1991; Saglio and Trijasse 1998; Bretaud et al. 2000). Activity of AChE has also been reported to decline in the brain of *Oreochromis mossambicus* (Peters, 1852) due to ammonia stress (Babu and Neeraja 2000). Modesto and Martinez (2010) studied AChE inhibition by subjecting it with anti-AChE action.
Fig. 1. Effects of exposure of ammonium chloride on activity (μmol min⁻¹ g⁻¹ wet mass) of acetylcholinesterase (AChE) from liver, gill and skeletal muscle of the freshwater catfish, *C. batrachus*. Each datum represents the mean ±SEM of five individuals (n=5).
Fig. 2. Effects of exposure of ammonium chloride on activity (mmol min\(^{-1}\) g\(^{-1}\) wet mass) of catalase (CAT) from liver, gill and skeletal muscle of the freshwater catfish, *C. batrachus*. Each datum represents the mean ±SEM of five individuals (n=5).
Fig. 3. Effects of withdrawal of ammonium chloride from water after 35 days of its exposure on activity (µmol min\(^{-1}\) g\(^{-1}\) wet mass) of acetylcholinesterase (AChE) from liver, gill and skeletal muscle of the freshwater catfish, *C. batrachus*. Each datum represents the mean ±SEM of five individuals (n=5).

Liver

Gill

Skeletal muscle

Days of ammonia withdrawal after 35 days of exposure
Fig. 4. Effects of withdrawal of ammonium chloride from water after 7 days of its exposure on activity (mmol min$^{-1}$ g$^{-1}$ wet mass) of catalase (CAT) from liver, gill and skeletal muscle of the freshwater catfish, *C. batrachus*. Each datum represents the mean ±SEM of five individuals (n=5).
When the AChE activity is inhibited, acetylcholine is not hydrolysed in nerve synapses and neuromuscular junctions, leading to overstimulation of brain and muscular tissue (Roex et al. 2003). In contrast to AChE, CAT activity initially enhanced up to 7 days and thereafter, declined gradually and reached below the control level at 35 days of ammonia treatment (Fig. 2). It means CAT tried to cope up with the oxidative stress initially but failed to do so later on. Ammonia associated changes in CAT activity may be due to allosteric modification in the enzyme molecule. Though the exact mechanism is not known, elevated activities of antioxidant and associated enzymes in fish in response to environmental stress have been observed by many workers (Winston 1991; Valavanidis et al. 2006; Craig et al. 2007). Activities of antioxidant enzymes are often enhanced in situations of high oxidative stress as an adaptive response to help detoxify oxygen free radicals and prevent cellular damages. Ching et al. (2009) demonstrated ammonia induced decrease in catalase from gills and brain of B. boddarti. Like the present observations, ammonia associated fluctuation in activity of CAT in liver of C. auratus with time has been reported (Yang et al. 2010). Ammonia significantly affects the antioxidant system (Sun et al. 2012) and the CAT of fish is highly sensitive to ammonia intoxication (Chen et al. 2011). Faria et al. (2010) studied the responses of biomarkers to pollutant concentrations and differences were reflected in high activities and levels of antioxidant enzymes. Hegazi et al. (2010) suggested that the levels of oxidative stress biomarkers and the activities of the enzymes significantly increased in liver and white muscle of fish exposed to ammoniacal nitrogen.

The withdrawal of ammonia from water for 14 days after 35 days of its exposure showed incomplete recovery in AChE activities of liver, gill and skeletal muscle (Fig. 3). Lack of recovery in activity of AChE might be due to ammonia-induced irreversible changes in the enzyme molecules and damages in the tissues. Therefore, AChE may be accepted as a potent indicator of ammonia toxicity in the catfish, C. batrachus. Withdrawal of ammonium chloride from water for 14 days after 7 days of exposure presented complete recovery in CAT activity of C. batrachus (Fig. 4). It showed reversible effect of ammonia on catalase of the catfish. The present investigations demonstrated inhibitory effect of ammonia on AChE and CAT reflecting neurotoxic and antioxidative responses in fish. Withdrawal of ammonia from water showed complete recovery in CAT but not in AChE. Lack of ammonia withdrawal-dependent complete recovery in AChE activity disclosed the possibility for this enzyme being an indicator of ammonia toxicity in fish. Therefore, it can be suggested that ammonia is irreversibly neurotoxic but reversibly antioxidative in the freshwater fish. The findings may have some ecotoxicological and pisciculture relevance in aquatic biology.

**Conclusion**

AChE activity declined gradually in liver, gill and skeletal muscle of C. batrachus after ammonia exposure. In contrast, CAT activity increased up to 7 days and then declined. Withdrawal of ammonia from water exhibited incomplete/complete recovery in AChE and CAT activity respectively. Removal of ammonia from water may help in damage control. Since ammonia-induced
neurotoxic effect was more pronounced than antioxidative response, AChE may be used as biomarker of ammonia toxicity in fish.

Acknowledgements

The authors gratefully acknowledge the University Grants Commission (UGC), New Delhi for providing financial support in the form of UGC research project (No. 36-104/2008(SR). One of us, A. Shiwanand is thankful to UGC for providing a project fellowship.

References


Received: 22/04/2012; Accepted: 10/10/2012 (MS12-29)