Interrenal response in climbing perch (*Anabas testudineus* Bloch) to nitrate exposure: Hydromineral and metabolic considerations

A.S. Vijayasree[1], V. Rejitha[1], Valsa S. Peter [2], Oommen V. Oommen [1] and M.C. Subhash Peter [1]*

[1] Department of Zoology, University of Kerala, Kariavattom, Thiruvananthapuram 695581, India
[2]Department of Zoology, Fatima Mata National College, Kollam 691001, India

SUMMARY

The physiological response of climbing perch to water-borne nitrate, an important component of the effluents of coconut husk retting, was examined to identify the mechanism of nitrate tolerance in fish. Indices of interrenal function, and metabolic and osmoregulatory homeostasis were analyzed in fish treated with potassium nitrate. Nitrate loading in water for 48 h produced a significant increase in the plasma cortisol by a low dose (247 μ*M*), whereas a higher dose (494 μ*M*) had little effect. A remarkable cortisol surge was found in the nitrate-treated fish kept for recovery in clean water for 96 h, which correlated with the rise in the plasma Na^+. Glucose, lactate and Na^+ concentrations in the plasma showed reduction in the nitrate-exposed fish, whereas plasma urea increased. Nitrate exposure had little influence on the gill and kidney Na^+, K^+-ATPase activities but had a stimulatory effect on liver Na^+, K^+-ATPase activity, indicating a major role of liver in nitrate tolerance. Overall, the present data indicate that nitrate exposure induces an integrated stress response in climbing perch as a result of an activated interrenal axis and disturbed metabolic and hydromineral regulations. This suggests a protective role of cortisol in the regulation of nitrate tolerance in this fish.

Key words: *Anabas testudineus*, fish, interrenal, nitrate, Na^+, K^+-ATPase, metabolism, osmoregulation, stress.

Introduction

The dynamic nature of aquatic environment induces stress on its biota due to the presence of various stressors of either natural or anthropogenic origin (Leji *et al.*, 2007; Peter and Peter, 2007). Increasing concentrations of nitrate in surface water and groundwater are becoming a worldwide concern, yet there is little information on the toxicity of nitrate in fishes (Scott and Crunkilton, 2000; Smith, 2003; Camargo, 2005). In freshwater or estuarine system close to land nitrate can reach a high level, particularly in coconut retting grounds (Madhukumar and Anirudhan, 1996). Nitrate appears to be much less toxic than ammonia or nitrite and it has been shown that above 30 ppm it may inhibit growth and impair the immune system causing toxic effects in some aquatic species (Camargo, 2005). Accumulation of excess nitrate could lead to imbalance of ecosystem and, thus, nitrate level in water is widely used as an indicator of water quality. Nitrate toxicity to aquatic animals increases with increasing nitrate concentrations and exposure times, which depends on the body size, water salinity and environmental adaptation (Camargo, 2005).

Retting of coconut husk, an essential step in the coir production, in the saline stretches of backwaters of Kerala in Southern India, poses a threat to the life of aquatic organisms due to the release of toxic effluents as by-products, including nitrate (Madhukumar and Anirudhan, 1996; Leji *et al.*, 2007). In fish, stressors evoke a complex neuroendocrine response and it is generally accepted that fish depend on the release of cathecholamines (Perry and Reid, 1993) and corticosteroids (Sumpter, 1997; Wendelaar Bonga, 1997; Iwama *et al.*, 2006) to cope with stressful challenges. It is generalized that in fish the hypothalamo-pituitary-interrenal axis responds to a number of environmental variables (Wendelaar Bonga, 1997; Peter, 2007). As the product of this axis, cortisol plays a decisive role in hydromineral and metabolic regulation in fish (Mommsen *et al.*, 1999; Babitha, 2008). Furthermore, it is known that stressors may influence the rate of energy utilization, thus affecting growth and metabolism (Wendelaar Bonga, 1997; Barton, 1997; Peter *et al.*, 2004). Studies in perch have demonstrated that exposure to stressors alters the metabolic and hydromineral regulation and affect the thyroid activity (Peter *et al.*, 2004, 2007). The alterations in energy metabolism, one of the main outputs of secondary stress responses (Barton, 1997), could thus be immediately beneficial to the fish under stress (Brown, 1993).

Correspondence to be addressed to: Dr M. C. Subhash Peter, Ph.D., E-mail: subashpeter@yahoo.com

Gills and kidneys, the primary sites for maintaining water and mineral balance, are sensitive to the action of pollutants. Na^+/K^+-ATPase is an important energizer for ion transport in epithelial tissue and a number of hormones have been shown to influence its activity (Leena and Oommen, 2000; McCormick *et al.*, 2001). It is comprehensible that toxicants of various origins disturb osmoregulatory potential and other physiological processes of fish (Wendelaar Bonga, 1997; Peter *et al.*, 2004). Chemical stressors have been shown to disturb water and ion regulation in fishes (Engelhardt, 1981; Snell and Persoone, 1989) and impair metabolic and endocrine functions since toxicants reach the body through the branchial and oral surfaces (Brown, 1993; Wendelaar Bonga, 1997; Peter *et al.*, 2004).

It is likely that certain degree of compensatory and adaptive modifications may occur in the physiological response of fish to nitrate contamination. The interrenal function, and metabolic and osmoregulatory activities in climbing perch were examined and the indices including plasma cortisol of these integrated processes were quantified in the nitrate-challenged fish to address the mechanism of nitrate tolerance.

Materials and methods

Fish

The climbing perch, *Anabas testudineus* of approximately 50 g body weight were collected and acclimated in tap water at 28 ± 1°C under natural photoperiod (12 L/12 D) for three weeks prior to the experiment. Fish were fed with commercial fish feed at a ration of 1.5% of body weight per day.

Experimental protocol

The climbing perch were divided into four groups of six each. The untreated group 1 fish was taken as control. Fish in groups 2 and 3 were kept in water rich in concentrations (247 µ*M* and 494 µ*M*) of nitrate for 48 hr. These doses were derived from KNO_3 (Spectrum Chemicals, Cochin, India), which was used as the source of nitrate. Fish in group 4 were first kept at 494 µ*M* nitrate for 48 hr. and later kept for recovery in clean freshwater for another 48 hr.

Sampling and analyses

After the treatment, the experimental fish were anesthetized in 2-phenoxyethanol (SRL, Mumbai, India) and blood was taken from the caudal vessels using a heparinized syringe. The fish were then sacrificed by spinal transsection, and gills, kidney and liver tissues were excised, washed in ice-cold 0.25 M SEI buffer (pH 7.1) and stored at -20°C.

Plasma cortisol, glucose, lactate and urea

The total plasma cortisol was quantified by an ELISA method with a commercial corisol kit (DiaMetra, Foligno, Italy, Catalog No. DKO 001) as described elsewhere (Babitha, 2009). The concentration of plasma glucose (GOD/POD method; Span Diagnostics, Surat, India), lactate (PAP method; Radiant Diagnostics, GmbH/ Germany) and urea (DAM method; Span Diagnostics, Surat) were measured using test kits in a UV spectrophotometer 2202 (Systronics, New Delhi).

Na^+, K^+-ATPase activity

The ouabain-sensitive Na^+, K^+ dependent adenosine triphosphatase (Na^+, K^+-ATPase, E.C. 3.6.3.9) specific activity was measured in tissue homogenates as described elsewhere (Verbost,1994; Peter et al., 2000). Saponin (0.2 $mg.mg^{-1}$ protein) was routinely added to optimize substrate accessibility. Tissues were homogenized in 0.25 *M* SEI buffer (pH 7.1) and the supernatant obtained was used to measure the specific activity of Na^+, K^+-ATPase. The liberated inorganic phosphate, P*i*, in the assay mixture was determined spectrophotometrically (Systronics 2202, New Delhi, India) and expressed as µmol. P*i*. h. mg $protein^{-2}$.

Plasma nitrate and minerals

The concentration of nitrate in the plasma was determined spectrophotometrically (Goldman and Jacob, 1961) and the plasma Na^+ and K^+ were measured in a flame photometric analyzer (Systronics 129, New Delhi, India) using standards (Remedix Diagnostics, Palakkad, India).

Statistical analysis

Before statistical analyses, the data were checked for normal distribution and variance homogeneity. One-way analysis of variance, (ANOVA) followed by Student-Newman-Keul's test, was employed to test the significant difference between the treatment groups using Instat-3 Software (GraphPad Software Inc., San Digeo, California, USA). Significant difference between groups was accepted if $P < 0.05$ and the values are in mean ± SEM (n = 6).

Results

Plasma cortisol, glucose, lactate and urea

The plasma cortisol increased three-fold ($P < 0.01$) in the fish exposed to a low concentration (247 µ*M*) of nitrate, whereas the high concentration (494 µ*M*) did not

produce any significant effect (Fig. 1). However, the nitrate-treated fish kept for recovery showed a remarkable increase (P <0.05) in the level of cortisol. The plasma glucose showed a substantial decease (P <0.01) upon exposure to low concentration of nitrate, whereas the high concentration or recovery did not alter its level (Fig. 2a). The plasma lactate decreased significantly in both low (P <0.01) and high concentration (P <0.05) nitrate-exposed fish. Allowing the fish for 96 h to recover in freshwater after nitrate exposure did not reverse this effect (Fig. 2b). The fish treated with a low concentration of nitrate showed elevated plasma urea (Fig. 2c). Neither the high concentration nor the recovery altered the plasma urea (Fig. 2c).

Plasma cortisol

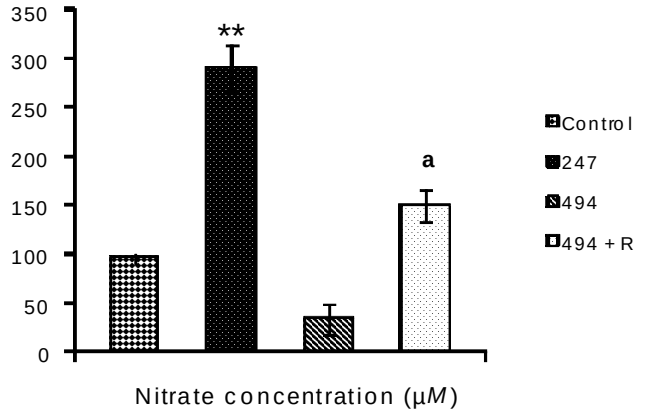


Fig.1 Plasma cortisol in *A. testudineus* loaded with nitrate for 48 h with or without 96 h recovery (R). Each column represents mean ± SEM (n = 6).

** P <0.01 compared with untreated control. a: P <0.05 when compared with 494 µ*M* KNO_3-treated fish.

Na*$^+$*, K*$^+$*-ATPase specific activity

The hepatic Na^+, K^+-ATPase activity increased (P <0.05) in the fish when treated with 494 µ*M* of nitrate (Fig. 3b). This increase was reversed (P <0.05) in the fish kept for recovery in freshwater. The branchial and renal Na^+, K^+-ATPase activities did not respond to nitrate exposure or its withdrawal (Fig. 3a and 3c).

Plasma nitrate and ions

The nitrate concentration of the plasma did not change in the fish treated with both concentrations of nitrate (Table 1). The plasma Na^+ significantly declined in fish treated with both low (P <0.05) and high (P <0.01) concentrations of nitrate, while the plasma K^+ concentration was not altered upon nitrate exposure (Table 1).

Table 1: Changes in the plasma sodium, potassium (mM/L) and nitrate (ìg/dL) in freshwater climbing perch loaded with varied concentrations of nitrate for 48 h with or without 96 h recovery (R).[Values are mean ± SEM for six fish each].

Status	Plasma Na^+	Plasma K^+	Plasma Nitrate
Control (Untreated)	168.0 ± 4.38	4.7 ± 0.67	184.1 ± 18.96
Nitrate (247 µM)	121.8 ± 9.08*	2.8 ± 0.33	134.5 ± 22.13
Nitrate (494 µM)	102.9 ± 6.03**	3.9 ± 0.66	227.2 ± 11.83
Nitrate (494 µM + R)	143.7 ± 17.99*	3.8 ± 0.25	155.0 ± 26.98

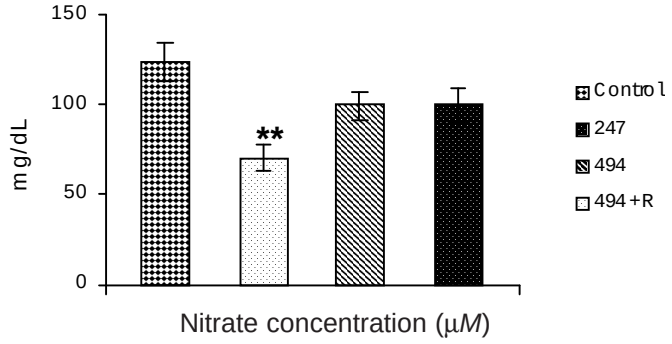
*P< 0.05 ** P< 0.01 compared with untreated controls (ANOVA followed by SNK test).

Discussion

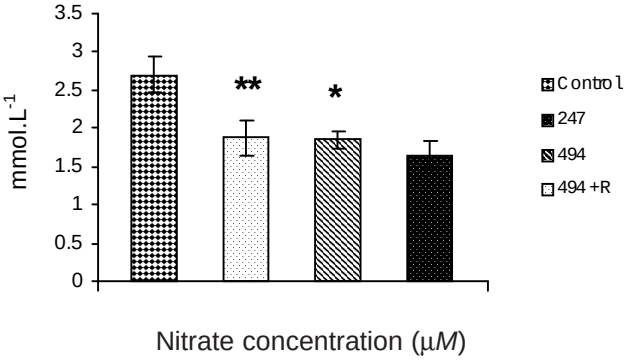
Cortisol, the final link in the hypothalamo-hypophysial-interrenal axis, is often considered as the indicator of stress in fishes. A three-fold increase in the plasma cortisol in the nitrate-treated perch indicates an induction of stress response in this fish. This is consistent with the earlier studies which showed a high cortisol release as one of the main endocrine responses to stress (Wendelaar Bonga, 1997; Flik *et al.*, 2006). For example, elevated plasma cortisol has been reported in tilapia after Cu exposure (Dang et al., 2000) and in climbing perch after treatment of effluent of coconut husk retting (Peter and Peter, 2007). The rise in plasma cortisol in the recovery fish indicates a protective role of cortisol in the nitrate-induced tolerance in fish. Similar protective effect of cortisol on stress-induced apoptosis has also been documented in tilapia (Nolan *et al.*, 1999).

Plasma glucose exceeding the basal level is an indicator of sympathetic activation during stress (Randall and Perry, 1992; Peter and Peter, 2007). The decrease in plasma glucose probably rules out the involvement of chromaffin axis in the nitrate-treated fish. A plausible explanation for the drop in blood glucose might be due to a high utilization of glucose for oxidation, which correlates well with the decreased plasma lactate. It is known that diversified metabolic responses occur in fish depending on the nature of stressors. For example, exposure of perch to

A. Plasma Glucose



B. Plasma lactate



C. Plasma urea

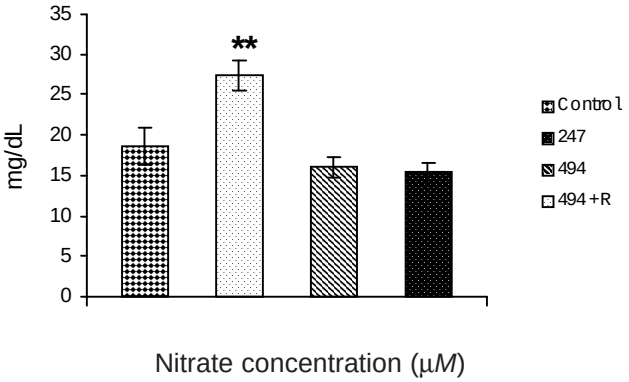
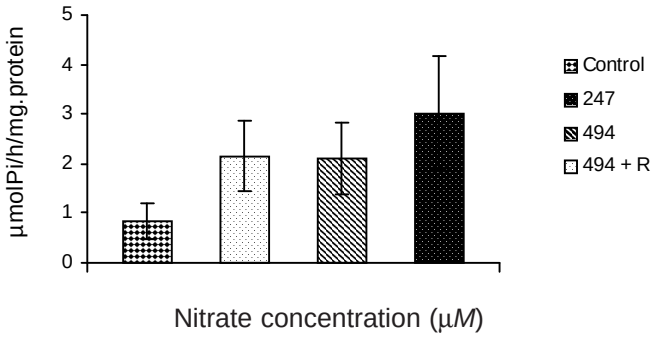


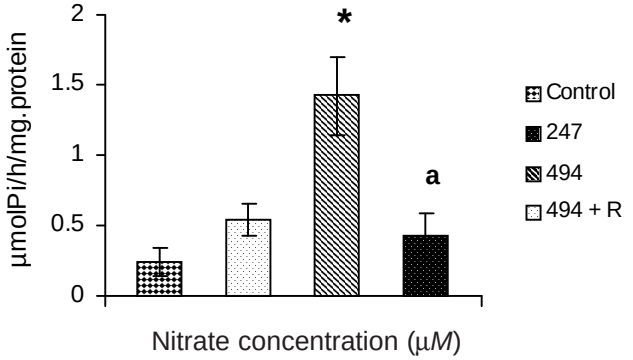
Fig.2 Plasma glucose (a), lactate (b) and urea (c) in *A. testudineus* treated with nitrate as KNO_3 for 48 h with or without 96 h recovery (R). Each column represents mean ± SEM (n = 6).

* *P* <0.05 compared with untreated control. ** *P* <0.01 when compared with untreated control.

A. Branchial Na^+, K^+-ATPase activity



B. Hepatic Na^+, K^+-ATPase activity



C. Renal Na^+, K^+-ATPase activity

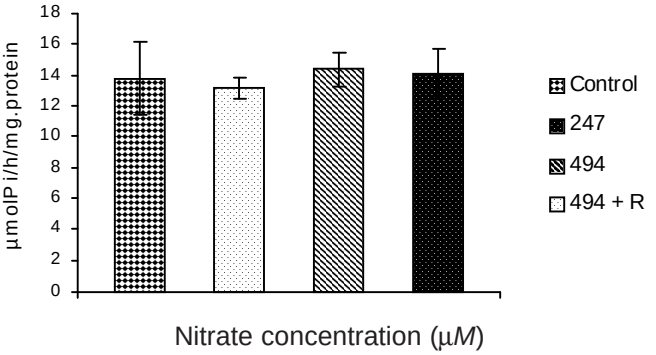


Fig.3 Branchial (a), hepatic (b) and renal (c) Na^+, K^+-ATPase activity in *A. testudineus* treated with nitrate as KNO_3 for 48 h with or without 96 h recovery (R). Each column represents mean ± SEM (n = 6).

***** *P* <0.05 compared with untreated control. a: *P* <0.05 when compared with 494 µ*M* KNO_3-treated fish.

kerosene-rich water caused hyperglycaemia (Peter et al., 2007), whereas glucose remained unchanged in perch exposed short-term to husk retting effluents (Leji et al., 2007). The same stressor produced hyperglycaemia when treated for five days (Peter and Peter, 2007). Plasma glucose, an indicator of stress, thus, showed a negative correlation with the cortisol. On the other hand, a persistent hyperglycaemia was reported in the tench (*Tinca tinca* L.) kept in potassium nitrate-enriched water (Demaal et al., 1980). Increased plasma glucose and cortisol have been recorded in the common carp *Cyprinus carpio* exposed to sub-lethal concentrations of lead nitrate for fourteen days (Zare et al., 2007). Similar results were reported in rainbow trout (*Oncorhynchus mykiss*) exposed to silver nitrate for six days (Webb and Wood, 1998).

The reduced plasma lactate in the nitrate-loaded fish indicates a high turnover of pyruvate oxidation associated with increased mitochondrial respiration. It has been reported that the plasma lactate concentration increased in rainbow trout after exposure to $AgNO_3$ (Rose-Janes and Playle, 2000). On the contrary, the increased plasma urea turnover indicates an increased ureogenic potential in the nitrate-treated fish. The unaffected plasma nitrate even after loading high dose of ambient nitrate indicates a tight regulation of nitrate availability in the plasma as a consequence of tolerating excess nitrate.

The declined plasma glucose and lactate and the elevated urea thus may point to an enhanced metabolic cost required to maintain the energy homeostasis in the nitrate-loaded fish. It is likely that the cortisol is essential for the unique metabolic adaptation which could help the fish to tolerate excess nitrate. Similar metabolic reallocations supported by the interrenal and thyroid hormones have been demonstrated in fish during tolerance to many stressors (Peter et al., 2004; Peter et al., 2007; Leji et al., 2007). In addition, cortisol has been shown to initiate ureogenesis at low concentrations while high cortisol may override the ureogenic response presumably by mobilizing energy substrates such as amino acids and glucose (Hopkins et al., 1995).

Na^+, K^+-ATPase, an index of hydromineral regulation, is abundant in the osmoregulatory tissues including gills and kidney. The Na^+, K^+-ATPase activities in these tissues were not affected by nitrate loading, implying that the osmoregulatory potential in these tissues is less affected by excess nitrate. However, a reduction in the plasma Na^+ in the nitrate-treated fish indicates a disturbed hydromineral balance and may be partly due to the influx of K^+ from the ambient medium. Alternatively, the increased gill permeability may also be attributed to the loss of plasma Na^+. In contrast to the unresponsive Na^+, K^+-ATPase in gills and kidney tissues to nitrate loading, an increased hepatic Na^+, K^+-ATPase activity occurred, which showed a reversal in the fish kept for recovery. A major role of liver in nitrate handling is indicated as it correlates with metabolic turn over.

Overall, the present data indicate that water-borne potassium nitrate induces an integrated stress response in the climbing perch with increase in the interrenal function and disturbance in the metabolic and hydromineral regulations. Our data also illustrate that cortisol has a protective role in the regulation of tolerance mechanism in the post-stress fish.

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