Role of ureogenesis in tackling problems of ammonia toxicity during exposure to higher ambient ammonia in the air-breathing walking catfish *Clarias batrachus*

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In the present study, the possible role of ureogenesis to avoid the accumulation of toxic ammonia to a lethal level under hyper-ammonia stress was tested in the air-breathing walking catfish *Clarias batrachus* by exposing the fish at 25 mM NH_4Cl for 7 days. Excretion of ammonia by the NH_4Cl-exposed fish was totally suppressed, which was accompanied by significant accumulation of ammonia in different body tissues. The walking catfish, which is otherwise predominantly ammoniotelic, turned totally towards ureotelism from ammoniotelism with a 5- to 6-fold increase of urea-N excretion during exposure to higher ambient ammonia. Stimulation of ureogenesis was accompanied with significant increase of some of the key urea cycle enzymes such as carbamyl phosphate synthetase (urea cycle-related), argininosuccinate synthetase and argininosuccinate lyase both in hepatic and non-hepatic tissues. Due to this unique physiological strategy of turning towards ureotelism from ammoniotelism via the induced urea cycle, this air-breathing catfish is able to survive in very high ambient ammonia, which they face in certain seasons of the year in the natural habitat.

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1. Introduction

The vast majority of teleost fishes are ammoniotelic excreting ammonia as the major nitrogenous end product in response to their aquatic habitat (for reviews, see Wood 1993; Saha and Ratha 1998). However, under certain circumstances such as high ambient ammonia or aerial exposure, ammonia excretion is inhibited, and toxic ammonia becomes concentrated in blood and body tissues. Fishes are generally known to tolerate relatively higher accumulation of ammonia than mammals (for reviews, see Wood 1993; Saha and Ratha 1998). Plasma total ammonia ($NH_3 + NH_4^+$) normally remains between 0·05 to 2 mM in most teleost fishes (Campbell and Anderson 1991; Wood 1993). In contrast, blood ammonia levels greater than 0·05 mM can be toxic to the central nervous system of most mammals (Meijer *et al* 1990). It is, therefore, interesting to study the different mechanism(s) of how fish manage accumulated ammonia especially in those teleosts that are regularly faced with ammonia loading situations as part of their life cycle.

More recently, the expression of high urea cycle enzymes with an accompanying active urea cycle has been reported in several teleost species as an adaptation to unique environmental circumstances. Examples include the marine toadfishes, *Opsanus beta* and *Opsanus tau*

Keywords. Ammoniotelism; ammonium chloride; hyper-ammonia stress; urea cycle; ureogenesis

Abbreviations used: ARG, Arginase; ASL, argininosuccinate lyase; ASS, argininosuccinate synthetase; CPS, carbamyl phosphate synthetase; GDH, glutamate dehydrogenase; GS, glutamine synthetase; NAG, N-acetyl glutamate; OTC, ornithine transcarbamylase.

(Read 1971; Mommsen and Walsh 1989), the alkaline lake-adapted tilapia *Alcolapia grahami* (Randall *et al* 1989), some Indian freshwater air-breathing fishes (Saha and Ratha 1987, 1989; Saha *et al* 1999), and the gobiid fish, *Mugilogobius abei* (Iwata *et al* 2000). Furthermore, some of these species have been reported to excrete significant amounts of urea in response to various adverse environmental conditions such as confinement (stress), severely alkaline water, ammonia loading and exposure to air (Randall *et al* 1989; Walsh *et al* 1990, 1994; Saha and Ratha 1998; Saha and Das 1999; Saha *et al* 2001, 2002b). Accordingly, interest in the study of the urea cycle during early embryonic developmental stages, regulation of expression of the urea cycle enzymes, and nitrogen excretion patterns under different environmental constraints in different teleosts has recently increased.

The freshwater amphibious air-breathing walking catfish (*Clarias batrachus*) that are found predominantly in the Indian subcontinent, spend a substantial part of their lives on mudflats in response to habitat drying and are observed to migrate terrestrially during wetter periods (Liem 1987). This facultative air-breather usually inhabits stagnant, slow-flowing swampy water bodies or wetlands, which are often covered with macrovegetation, such as water hyacinth, and these waters are also characterized by low dissolved oxygen, and high bicarbonate and ammonia levels (for review, see Saha and Ratha 1998). During summer, when the swamps dry up, they usually burrow inside the mud to avoid total dehydration. Another unique characteristic reported in this catfish and in the singhi catfish (*Heteropneustes fossilis*) is the extreme tolerance to a high concentration of ambient ammonia, with a capacity to survive an exposure of 75 mM NH_4Cl for several weeks (Saha and Ratha 1994, 1998; Saha *et al* 2002a). This tempted us to investigate the possible physiological and biochemical adaptive mechanisms to tolerate such a high level of ambient ammonia by this group of air-breathing catfishes. One such adaptation, which has already been reported, is the presence of a functional urea cycle with relatively high levels of activity of all the urea cycle enzymes both in the walking and singhi catfishes (Saha and Ratha 1987, 1989; Saha *et al* 1999).

In the present study, we report the effects of higher ambient ammonia on the patterns of uptake/excretion of ammonia and urea-N, changes in the tissue levels of ammonia and urea-N and also changes in the activity of urea cycle enzymes in the walking catfish while exposed to 25 mM NH_4Cl for seven days with the intention of determining the role of ureogenesis in ammonia detoxification processes. Although the walking catfish can survive at 75 mM NH_4Cl, we decided to expose the fish to 25 mM NH_4Cl, the concentration they may face in their natural habitat in some seasons of the year (Saha and Ratha 1998).

2. Materials and methods

2.1 *Animals*

C. batrachus, weighing 85 ± 15 g were purchased from commercial sources and acclimatized in the laboratory for approximately one month at constant room temperature (28 ± 2°C) in a 12 h : 12 h light and dark photoperiod before being used for the experiments. The sex of the fish was not a factor in these studies. Minced pork liver and rice bran (5% of the body wt.) were given as food, and the water, collected from a nearby natural stream, was changed on alternate days. Food was withdrawn 24 h prior to each experiment.

2.2 *Experimental protocol*

Nine fishes of similar sizes were weighed and placed individually in plastic buckets containing 2 l of 25 mM NH_4Cl solution (pH 6·95 ± 0·11) prepared in bacteria-free filtered stream water. Fishes were exposed to the NH_4Cl solution for seven days. Another nine *C. batrachus* were kept individually in plastic buckets containing 2 l of bacteria-free filtered stream water (pH 7·04 ± 0·10), which served as controls. Both the NH_4Cl solution and the water from each bucket were replaced with a fresh medium every day at a fixed time after collection of some medium from each bucket for the measurement of ammonia and urea-N concentrations. On day 1, 3 and 7, three fishes from each treatment were removed, and killed immediately by decapitation after collecting blood from the caudal vein with a heparinized syringe. Liver, kidney, muscle and brain were dissected out and tissue samples were plunged into liquid nitrogen before storing at – 60°C for analysis of ammonia and urea, and also for assaying the activity of the urea cycle enzymes. All enzyme assays and analysis were completed within two weeks of collecting the tissue. Blood, collected from each fish, was centrifuged at 10,000 *g* for 10 min, and the plasma was processed for estimation of ammonia and urea as described by Saha and Ratha (1989).

2.3 *Estimation of ammonia and urea-N*

Levels of ammonia and urea-N excreted or taken up by the fish, kept either in water or in 25 mM NH_4Cl, were measured enzymatically (Kun and Kearney 1974). Ammonia and urea levels in organ tissue and blood plasma were also measured by the same enzymatic methods after processing the tissue as described in Saha and Ratha (1989).

2.4 *Enzyme assay*

A 10% homogenate (w/v) of different tissues was prepared in a homogenizing buffer containing 100 mM Tris-HCl buffer (pH 7·5), 50 mM KCl, 1 mM ethylene diamine tetra acetic acid (EDTA) and 1 mM dithiothreitol (DTT) using a motor-driven Potter-Elvehjem glass homogenizer with a Teflon pestle. The homogenate was treated with 0·5% Triton X-100 in 1 : 1 ratio for 30 min. The homogenate was then subjected to mild sonication for proper breakage of mitochondria and centrifuged at 10,000 *g* for 10 min. The supernatant was used for assaying the enzymes. All steps were carried out at 4°C. The five enzymes of the urea cycle, carbamyl phosphate synthetase (CPS), ornithine transcarbamylase (OTC), argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL), and arginase (ARG) were assayed following the method described in Saha *et al* (1995). However, for the assay of urea cycle-related CPS activity, 1 mM of uridine-5′-triphosphate (UTP) was also added in the reaction mixture to inhibit the pyrimidine synthesis-related CPS II activity (Saha *et al* 1997). It should be noted that the assay method used here for CPS activity does not distinguish between the two different forms of urea synthesis-related enzymes namely CPS I (ammonia- and N-acetyl-L-glutamate-dependent, mitochondrial) and CPS III (glutamine- and N-acetyl-L-glutamate-dependent, mitochondrial). This is because a part of ammonia (as NH_4Cl) taken as a nitrogen donating substrate in the reaction mixture might be converted to glutamine in the presence of glutamine synthetase enzyme, thus serving as a nitrogen donating substrate for CPS III. The reaction for all the enzymes was stopped by adding 0·5 ml of 10% perchloric acid per 1 ml of reaction mixture after a specific time of reaction, followed by centrifugation to precipitate out the protein. Citrulline formed in the case of CPS and OTC, citrulline used in the case of ASS, and urea formed in the case of ASL and ARG were measured spectrophotometrically (Beckman, DU 640) in the supernatant (Moore and Kauffman 1970) and expressed as enzyme activity. All the enzyme assays were carried out at 30°C. One unit of enzyme activity is defined as that amount that catalyzes 1 µmol of product formed or substrate used per h at 30°C.

2.5 *Calculation for nitrogenous balance sheet*

The calculation for the nitrogenous balance sheet was made assuming the total fish body weight as 100 g. The total excretion/uptake of ammonia and urea-N by the fish was calculated from the average rate of excretion of ammonia and urea-N for a period of seven days, and then finally converted to 100 g fish body weight (average rate of excretion per 100 g fish per h × 24 × 7). The uptake of ammonia by the fish exposed to 25 mM NH_4Cl was calculated by subtracting the concentration of ammonia present in the medium after exposing the fish for 24 h from the concentration of ammonia in the control bucket containing only 25 mM NH_4Cl [(total ammonia in control bucket – total ammonia left after 24 h of keeping the fish in 25 mM NH_4Cl solution) × 7 × 100/g fish weight]. The extra accumulation of ammonia and urea-N by day 7 in major fish tissues such as the muscle, liver, kidney, brain and blood plasma were calculated taking the average weight of each tissue as 50, 2, 1, 0·5 and 4 g, respectively, for 100 g fish (Saha *et al* 2002a).

2.6 *Chemicals*

Enzymes, co-enzymes and substrates were purchased from Sigma Chemicals (St. Louis. MO, USA). The other chemicals used were of the analytical grade and obtained from local sources. Deionized and double-glass-distilled water was used in all preparations.

2.7 *Statistical analysis*

Data collected from 3–9 replicates were statistically analysed and presented as mean ± SE. Comparisons of the unpaired mean values between the experimental and respective controls were made using unpaired Student's *t*-test and differences with $P < 0{\cdot}05$ were regarded as statistically significant.

3. Results

3.1 *Excretion/uptake of ammonia and urea-N*

The rates of ammonia and urea-N excretion of the control fish were relatively stable over time and showed no significant variation with duration of starvation (figure 1). The rates of excretion of ammonia and urea-N averaged to 227 and 78·5 µmol/kg/h, respectively, over the experimental periods of 7 days, with the percentage of nitrogen excreted as urea-N accounting for 25·6% of the total nitrogen excretion (ammonia-N + urea-N). Ammonia excretion by the fish exposed to 25 mM NH_4Cl was totally inhibited from the first day itself, which was accompanied with significant uptake of ammonia from the external media (calculated by subtracting the real amount of ammonia present in the media at 24 h interval from the initial concentration of ammonia) (figure 1A). Maximum uptake of ammonia was seen on the third day (245 µmol/kg/h), followed by gradual decrease, but the uptake of ammonia dominated over its excretion by the NH_4Cl-exposed fish throughout the period of experiment.

The rate of urea-N excretion of the fish exposed to 25 mM NH_4Cl increased significantly ($P < 0{\cdot}001$) within first day (about 2·5-fold), followed by further increase up to day 3 (figure 1B). Thereafter, urea-N excretion remained approximately six times higher than the control values.

3.2 *Changes of tissue levels of ammonia and urea-N*

The physiological concentration of ammonia in different tissues and in plasma almost remained stable till day 7 of the experiment (table 1). However, there was significant increase in the concentration of ammonia in all the tissues (except in brain) and in plasma of the NH_4Cl-exposed fish within the first day, which increased further after 7 days. The concentration of ammonia increased maximally in the liver (23·2 µmol/g wet wt.), followed by the kidney (22·6 µmol/g wet wt.), muscle (12·2 µmol/g wet wt.), and plasma (2·47 µmol/ml) after 7 days of exposure, with an increase of 116%, 123%, 106% and 280%, respectively, compared to respective controls.

The physiological concentration of urea-N in different tissues and in plasma of *C. batrachus*, kept as control, was also found to remain quite stable over the period of 7 days of starvation (table 2). However, the concentration of urea-N increased significantly in all the tissues and in plasma of the NH_4Cl-exposed fish within the first day, which increased further after 7 days. The concentration of urea-N increased maximally in the liver (21·4 µmol/g wet wt.), followed by the kidney (18·6 µmol/g wet wt.), muscle (8·8 µmol/g wet wt.), brain (5·8 µmol/g wet wt.) after 3 days, and in plasma (2·64 µmol/g wet wt.) after 7 days, with an increase of 183%, 138%, 175%, 31% and 257%, respectively, compared to respective controls.

3.3 *Changes in the activities of urea cycle enzymes*

The changes in the activities of different urea cycle enzymes in liver, kidney and muscle tissues of *C. batrachus* during exposure to NH_4Cl are presented in tables 3–5. In liver and kidney (most ureogenic tissues) significant

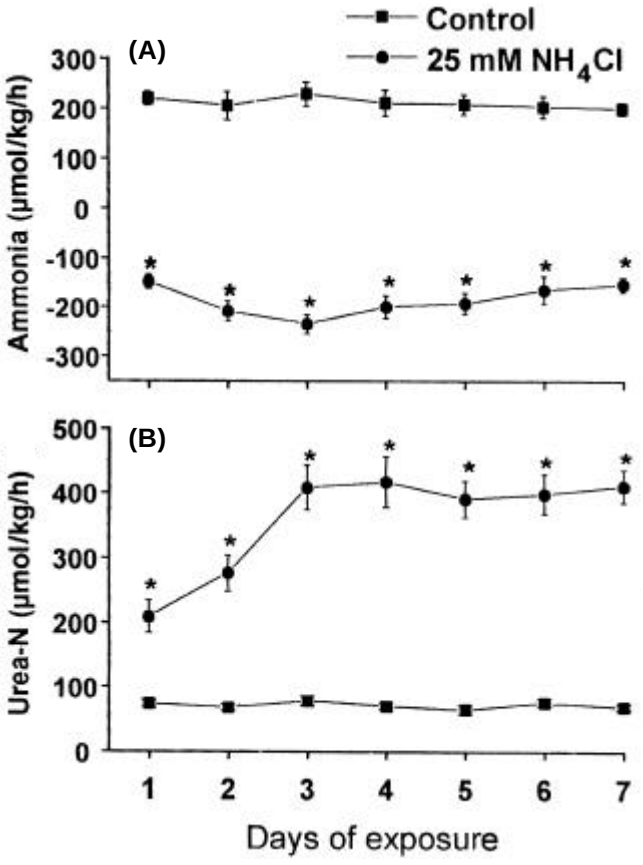

Figure 1. Changes in the excretion rate (µmol/kg/h) of ammonia (**A**) and urea-N (**B**) by *C. batrachus* exposed to 25 mM NH_4Cl. Values are plotted as mean ± SE (n = 4–9). *Significantly different from respective control values ($P < 0{\cdot}001$).

Table 1. Changes in the concentration of ammonia in different tissues (µmol/g wet wt.) and in plasma (µmol/ml) of *C. batrachus* during exposure to 25 mM NH_4Cl.

	Days of exposure		
Tissues	1	3	7
Liver			
Control	10·8 ± 0·92	10·7 ± 0·62	10·7 ± 0·5
Treated	14·4 ± 1·09[a]	19·7 ± 1·15[b]	23·2 ± 2·02[c]
	(33)	(84)	(116)
Kidney			
Control	10·6 ± 0·54	9·6 ± 0·74	10·1 ± 0·67
Treated	15·9 ± 1·12[b]	20·4 ± 1·54[c]	22·6 ± 1·84[c]
	(50)	(112)	(123)
Muscle			
Control	5·5 ± 0·21	5·1 ± 0·92	5·9 ± 0·38
Treated	8·2 ± 0·54[a]	11·8 ± 0·88[c]	12·2 ± 0·84[c]
	(49)	(131)	(106)
Brain			
Control	3·2 ± 0·24	3·4 ± 0·22	3·3 ± 0·22
Treated	3·8 ± 0·32	4·1 ± 0·27	3·9 ± 0·34
	(19)	(20)	(18)
Plasma			
Control	0·66 ± 0·09	0·69 ± 0·08	0·65 ± 0·08
Treated	1·01 ± 0·14[a]	2·21 ± 0·18[c]	2·47 ± 0·25[c]
	(53)	(220)	(280)

Values are expressed as mean ± SE (n = 3).
Percent increase of ammonia concentration compared to respective controls are given in parentheses.
[a,b,c]P values significant at < 0·05, < 0·01 and < 0·001, respectively.

Table 2. Changes in the concentration of urea-N in different tissues (μmol/g wet wt.) and in plasma (μmol/ml) of *C. batrachus* during exposure to 25 mM NH_4Cl.

Tissues	Days of exposure		
	1	3	7
Liver			
Control	7·2 ± 0·44	9·2 ± 0·74	7·2 ± 0·54
Treated	16·2 ± 1·14^c (125)	21·4 ± 1·74^c (132)	20·8 ± 1·20^c (183)
Kidney			
Control	7·2 ± 0·54	7·8 ± 0·48	8·4 ± 0·56
Treated	16·0 ± 1·04^c (122)	18·6 ± 1·84^c (138)	17·2 ± 1·24^c (104)
Muscle			
Control	2·8 ± 0·24	3·2 ± 0·28	3·8 ± 0·24
Treated	5·8 ± 0·46^c (107)	8·8 ± 0·68^c (175)	7·2 ± 0·68^b (100)
Brain			
Control	1·9 ± 0·24	2·2 ± 0·20	2·3 ± 0·14
Treated	4·8 ± 0·30^a (26)	5·8 ± 0·36^a (31)	6·8 ± 0·63^a (48)
Plasma			
Control	0·76 ± 0·08	0·80 ± 0·10	0·73 ± 0·08
Treated	1·10 ± 0·13^a (44)	2·04 ± 0·25^c (155)	2·64 ± 0·34^c (257)

Values are expressed as mean ± SE (n = 3).
Percent increase of urea-N concentration compared to respective controls is given in parentheses.
$^{a,b,c}P$ values significant at < 0·05, < 0·01 and < 0·001, respectively.

increase of activity of CPS, ASS and ASL were observed after the first day of exposure, followed by a further increase after 7 days for most enzymes. The CPS activity increased maximally by 124% after 7 days, and also by 124% after 3 days in the kidney. The ASS activity increased maximally by 60% in liver and 80% in kidney after 7 days. The ASL activity increased maximally by 59% in liver after 7 days, and 78% in kidney after 3 days. In muscle, which constitutes the major body mass (50% of the total body wt.), there was significant increase of CPS and ASS enzymes activity within the first day of exposure. The activities of CPS and ASS enzymes in the muscle of NH_4Cl-exposed fish increased maximally by 129 and 65%, respectively after 7 days. However, the activities of OTC and ARG enzymes remained unaltered in all the tissues studied in the NH_4Cl-exposed fish. The ASL activity could not be detected in the muscle of both control as well as NH_4Cl-exposed fish by the assay method used in the present study.

4. Discussion

The total nitrogen excretion (ammonia-N + urea-N) by the walking catfish under controlled conditions averaged to about 305 μmol/kg/h over a period of 7 days, out of which 75% (225 μmol/kg/h) was excreted as ammonia and 25% (78·5 μmol/kg/h) as urea-N, suggesting that the walking catfish is primarily ammoniotelic excreting nitrogen mostly through the gills as suggested in other teleosts (for review, see Wilkie 1997) while living in

Table 3. Changes of tissue activity (units/g wet wt.) of urea cycle enzymes in the liver of *C. batrachus* during exposure to 25 mM NH_4Cl.

Days of exposure		Enzymes				
		CPS	OTC	ASS	ASL	ARG
1	Control	3·12 ± 0·25	102·4 ± 6·5	56·1 ± 4·4	60·6 ± 6·8	4024 ± 134
	Treated	5·85 ± 0·47^b (88)	119·4 ± 6·4 (17)	76·0 ± 6·2^a (35)	82·1 ± 7·9^a (35)	4117 ± 200 (3)
3	Control	3·19 ± 0·24	102·3 ± 8·2	57·3 ± 3·4	60·9 ± 4·7	4100 ± 171
	Treated	6·85 ± 0·47^c (115)	115·0 ± 5·9 (12)	86·1 ± 4·4^a (50)	92·5 ± 3·4^a (52)	4107 ± 205 (0)
7	Control	3·32 ± 0·34	107·1 ± 6·8	57·1 ± 3·2	61·7 ± 5·8	4112 ± 152
	Treated	7·42 ± 0·52^c (124)	119·9 ± 7·9 (11)	91·2 ± 7·4^b (60)	98·2 ± 6·5^b (59)	4144 ± 198 (3)

Values are expressed as mean ± SE (n = 3).
Percent increase of enzyme activity compared to respective controls is given in parentheses.
$^{a,b,c}P$ values significant at < 0·05, < 0·01 and < 0·001, respectively.
CPS, Carbamyl phosphate synthetase; OTC, ornithine transcarbamylase; ASS, argininosuccinate synthetase; ASL, argininosuccinate lyase; ARG, arginase.
1 unit of enzyme was defined as that amount which catalyzed 1 μmol of product formed or substrate used per h at 30°C.

Table 4. Changes of tissue activity (units/g wet wt.) of urea cycle enzymes in the kidney of *C. batrachus* during exposure to 25 mM NH_4Cl.

Days of exposure		Enzymes				
		CPS	OTC	ASS	ASL	ARG
1	Control	2·53 ± 0·12	59·3 ± 2·62	42·0 ± 4·2	42·1 ± 2·7	918 ± 48
	Treated	4·97 ± 0·36^b	65·5 ± 4·35	65·1 ± 6·9^a	54·3 ± 7·5^a	1024 ± 57
		(96)	(10)	(55)	(32)	(12)
3	Control	2·63 ± 0·24	58·5 ± 4·22	42·4 ± 4·8	35·5 ± 3·4	973 ± 64
	Treated	5·92 ± 0·52^c	67·7 ± 4·45	73·9 ± 6·1^b	63·3 ± 4·6^b	1001 ± 88
		(124)	(16)	(74)	(78)	(3)
7	Control	2·63 ± 0·24	58·3 ± 5·23	42·4 ± 3·3	39·6 ± 4·2	904 ± 58
	Treated	5·46 ± 0·43^c	64·7 ± 6·43	76·1 ± 5·6^b	68·5 ± 6·2^b	1024 ± 65
		(107)	(11)	(80)	(73)	(13)

Values are expressed as mean ± SE (n = 3).
Percent increase of enzyme activity compared to respective controls is given in parentheses.
$^{a,b,c}P$ values significant at < 0·05, < 0·01 and < 0·001, respectively.

Table 5. Changes of tissue activity (units/g wet wt.) of urea cycle enzymes in the muscle of *C. batrachus* during exposure to 25 mM NH_4Cl.

Days of exposure		Enzymes				
		CPS	OTC	ASS	ASL	ARG
1	Control	1·07 ± 0·08	11·74 ± 0·67	9·24 ± 0·61	BLD	130 ± 12
	Treated	1·68 ± 0·16^a	12·00 ± 1·12	13·64 ± 0·87^a	BLD	124 ± 10
		(+ 57)	(+ 2)	(+ 48)		(– 5)
3	Control	0·99 ± 0·12	10·64 ± 0·66	9·57 ± 0·71	BLD	125 ± 11
	Treated	2·11 ± 0·32^c	11·61 ± 1·07	15·64 ± 1·12^b	BLD	132 ± 13
		(+ 113)	(+ 9)	(+ 63)		(+ 6)
7	Control	1·02 ± 0·14	11·24 ± 0·68	9·78 ± 0·51	BLD	137 ± 14
	Treated	2·34 ± 0·25^c	12·76 ± 0·51	16·17 ± 1·23^b	BLD	132 ± 12
		(+ 129)	(+ 13)	(+ 65)		(– 4)

Values are expressed as mean ± SE (n = 3).
Percent increase (+)/decrease (–) of enzyme activity compared to respective controls are given in parentheses.
$^{a,b,c}P$ values significant at < 0·05, < 0·01 and < 0·001, respectively.

normal aquatic habitat. However, the most notable observation in the present study is that the walking catfish produced and excreted a large amount of urea-N during exposure to 25 mM NH_4Cl for a period of 7 days. Furthermore, the excretion of ammonia by the NH_4Cl-exposed fish was stopped totally and the total nitrogen was excreted in the form of urea-N. The urea-N excretion averaged to about 405 µmol/kg/h between 3 to 7 days of exposure with an overall 33% increase of total nitrogen excretion. Approximately 5 to 6-fold increase of urea-N excretion rate was observed during exposure to high concentration of external ammonia (figure 1B). This pattern of changes in nitrogen excretion strongly suggests that the walking catfish turned totally towards ureotelism from ammoniotelism during exposure to higher ambient ammonia. This has been possible mainly because this fish has an efficient urea-producing ability via the functional urea cycle with relatively high levels of activity of all the key urea cycle enzymes in hepatic as well as in some extrahepatic tissues (Saha and Ratha 1989; Saha *et al* 1999). In several teleosts, including largemouth bass (*Micropterus salmoides*), carp (*Cyprinus carpio*) and bowfin (*Amia calva*), low levels of CPS III and other urea cycle enzymes are known to be present mainly in muscle (Kong *et al* 1998; Felskie *et al* 1998). Despite the expression of urea cycle enzymes in these species, it seems unlikely that they have a functional urea-producing ability, i.e. functional ureogenesis. As a result, when exposed to

elevated external ammonia levels, largemouth bass and bowfin showed only a limited enhancement of urea excretion (McKenzie and Randall 1990; Kong *et al* 1998). Like the walking catfish, few other ureogenic teleost species, such as the singhi catfish (Saha and Ratha 1990, 1994), toadfish (*Opsanus beta*) (Walsh *et al* 1994), and gobiid fish (Iwata *et al* 2000) also have the potential of synthesizing significant amount of urea during exposure to high external ammonia.

The significant increase in the concentration of ammonia, observed in various tissues of walking catfish during exposure to higher ambient ammonia (25 mM NH_4Cl), was tissue-specific with a maximum concentration in liver, followed by kidney, muscle and plasma. But the total accumulation of ammonia would be much higher in the muscle, since it constitutes the major part of the body mass (50% of the total body weight) (table 6). The accumulation of ammonia possibly resulted due to total inhibition of ammonia excretion generated endogenously, accompanied with the uptake of ammonia by the fish gills possibly by simple diffusion from the external medium containing higher concentration of ammonia as against blood ammonia (figure 1A). The capacity of accumulation of ammonia in this catfish was found to be much higher than many teleosts (Dabrowska and Wlasow 1986; Iwata 1988; Jow *et al* 1999; Iwata *et al* 2000), but similar to the situation observed in singhi catfish (*H. fossilis*) (Saha and Ratha 1994) and loach (*Misgurnus anguillicaudatus*) (Tsui *et al* 2002). This could be one of the reasons for tolerating such high ambient ammonia. The extreme sensitivity of the brain to ammonia toxicity (Cooper and Plum 1987), it does not allow any extra accumulation of ammonia. However in this fish brain, ammonia accumulation is possible mainly because of the presence of a very high activity of glutamine synthetase (GS) and glutamate dehydrogenase (GDH, reductive amination direction) activity (Saha *et al* 2002a). Ammonia might detoxified very efficiently to glutamate and subsequently converted to glutamine in the brain by the coupled steps of GDH and GS as suggested in some other teleosts (Mommsen and Walsh 1991). Furthermore, the *in vivo* accumulation of ammonia to a lethal concentration might prevented in this catfish by converting a part of the accumulated ammonia to urea through the already

Table 6. A balance sheet of nitrogenous excretion/uptake and accumulation (all as mmol N) for 100 g body weight of *C. batrachus* during exposure to water and 25 mM NH_4Cl for a period of 7 days.

	Nitrogen excretion/uptake/accumulation (mmol N)		
	Control	25 mM NH_4Cl	Difference
Total excretion/uptake from 100 g fish for 7 days*			
Ammonia	+ 3·81	– 3·19	– 7·00
Urea-N	+ 1·32	+ 6·01	+ 4·69
Total reduction in nitrogenous excretion in 7 days			– 2·31
Total retention in different tissues in 7 days†			
Liver (2 g)			
Ammonia	0·021	0·046	+ 0·025
Urea-N	0·014	0·042	+ 0·028
Muscle (50 g)			
Ammonia	0·295	0·610	+ 0·315
Urea-N	0·190	0·360	+ 0·170
Kidney (1 g)			
Ammonia	0·010	0·023	+ 0·013
Urea-N	0·008	0·017	+ 0·009
Brain (0·5 g)			
Ammonia	0·0016	0·002	+ 0·0004
Urea-N	0·0011	0·003	+ 0·002
Plasma (4 g)			
Ammonia	0·0016	0·002	+ 0·0004
Urea-N	0·0011	0·003	+ 0·0002
Increase in nitrogenous accumulation			+ 0·578

*Calculated from the rate of excretion/uptake (figure 1) (see, § 2).
†Amount present in tissues on day 7 of the experiment.

existing functional urea cycle, thus causing higher accumulation of urea in different tissues and in the plasma of the NH_4Cl-exposed fish (table 2). The accumulation of urea-N was also tissue specific with a maximum increase in concentration in the liver, followed by the kidney, muscle, brain and plasma. However, if we calculate the total accumulation of urea-N in respective tissues, maximum accumulation was seen in muscle, since muscle constitutes the major potion of the body mass (table 6).

The accumulation of ammonia in different body tissues was accompanied with a significant stimulation of the activity of certain key enzymes of the urea cycle such as CPS (urea cycle-related), ASS and ASL in both hepatic and extra-hepatic tissues after the first day of exposure, followed by a further increase of activity at later stages of NH_4Cl exposure (tables 3–5). The CPS activity, which is considered a critical enzyme or rate regulatory enzyme of the urea cycle (Anderson 2001), increased by 2 to 2·5-fold in liver, kidney and muscle of NH_4Cl-exposed fish. Therefore, this catfish might have acquired a chronic adaptation by elevating the rate of urea-N excretion, synthesized via the induced urea cycle, for long-term maintenance of nitrogen waste excretion during exposure to high ambient ammonia. Active involvement of extra-hepatic tissues for the synthesis of urea via the urea cycle has been emphasized recently in various fish species, which includes the walking catfish (Saha *et al* 1999), toadfish (Wood *et al* 1995), rainbow trout (Korte *et al* 1997), and the alkaline lake tilapia (Lindley *et al* 1999). Although ASL activity could not be detected in the muscle of the walking catfish, it may so happen that the argininosuccinate formed in muscle is transported to liver and kidney for further conversion to urea-N, thereby involving more than one tissue in the process of urea synthesis in the same species as suggested earlier by Saha *et al* (1999) and Iwata *et al* (2002). Although the activities of different urea cycle enzymes in muscle were comparatively low, the total units of different enzymes in muscle could be physiologically significant in this fish since muscle represents a high percentage of the body mass. The possible mechanism(s) of stimulation of ureogenesis during exposure to high ambient ammonia could be via changes in cellular events, such as enzyme phosphorylation, allosteric effects or in the concentration of low molecular weight effectors like N-acetyl glutamate (NAG) (a positive allosteric modulator of CPS III and I). Increase in the concentration of NAG is known to enhance flux through the CPS III enzyme in the gulf toadfish during confinement stress (Julsrud *et al* 1998). Recently, the presence of both the urea cycle-related CPSs, CPS I-like and CPS III (most predominant in teleost species) have been reported in the walking catfish (Saha *et al* 1999). But the assay method used here for CPS activity does not distinguish between these two types of CPSs. Therefore, it is not possible at present to identify whether both the CPSs or only one type was induced in the NH_4Cl-exposed fish. Release of certain stress-related hormones, such as cortisol, could be another means of stimulation of ureogenesis under hyper-ammonia stress in this fish as shown by Hopkins *et al* (1995) in the gulf toadfish (*Opsanus beta*). As such high accumulation of ammonia (substrate) *in vivo*, observed in different tissues including the plasma in the NH_4Cl-exposed fish, could be another means of stimulation of ureogenesis, since some of the urea cycle enzymes were stimulated in the perfused liver of the walking catfish by infusing NH_4Cl (Saha and Das 1999). A detailed investigation of these aspects would possibly clarify the mechanism(s) of stimulation of ureogenesis in this walking catfish under various environmental constraints faced regularly by them.

To assess the importance of urea synthesis in the process of ammonia detoxification, the nitrogenous balance sheet was prepared from the total accumulation/uptake of ammonia and urea-N, and also from the total amount of ammonia and urea-N excreted for a period of 7 days assuming the total fish body weight as 100 g (table 6). In the NH_4Cl-exposed fish, there was a total inhibition of ammonia excretion. This was accompanied by more uptake of ammonia from the external medium, thus causing a total uptake of 7·0 mmol of ammonia in 7 days assuming that the production rate of ammonia remained the same as in the control fish. Out of this total uptake of ammonia, 4·69 mmol was excreted as urea-N, and 0·35 and 0·21 mmol was accumulated in the form of ammonia and urea-N, respectively, in 7 days, leaving a balance of 1·75 mmol of ammonia. This suggests that the enhanced rate of ureogenesis by the induced urea cycle due to accumulation of ammonia during exposure to high ambient ammonia plays a critical role in the process of ammonia detoxification in this walking catfish. From the nitrogenous balance sheet, it is also evident that there was an overall decrease in the nitrogen excretion by the NH_4Cl-exposed fish. There could be two possible causes of decreased nitrogenous waste excretion by the NH_4Cl-exposed fish: (i) decrease in amino acid catabolism rate as suggested in mudskippers (Lim *et al* 2001) and loach (Chew *et al* 2001) during aerial exposure mainly to avoid ammonia toxicity, and (ii) excretion of nitrogenous wastes in some form other than ammonia and urea such as amino acids. Conversion of some part of accumulated ammonia to non-essential free amino acids could be another possible means of detoxification of ammonia recently shown in this walking catfish (Saha *et al* 2002a).

In conclusion, it appears that *C. batrachus*, unlike other typical teleosts, is capable of stimulating ureogenesis by inducting the already existing functional urea cycle both in hepatic as well as in some non-hepatic tissues, thus turning from ammoniotely to ureotely as one of the

major unique physiological strategies to avoid the problem of accumulation of toxic ammonia to a lethal level during exposure to higher ambient ammonia, and also possibly while living inside the mud peat during the summer.

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