Ammonia tolerance in the slender lungfish (*Protopterus dolloi*): the importance of environmental acidification

Chris M. Wood, Patrick J. Walsh, Shit F. Chew, and Yuen K. Ip

Abstract: *Protopterus dolloi* Boulenger, 1900 is an obligate air-breather and exhibits ammoniotely (88% ammonia-N excretion, 12% urea-N excretion) under normal aquatic conditions, but tolerates 7 days of exposure to 30 mmol·L^{-1} NH_4Cl, a treatment fatal to most other fish. Internal N accumulation is minimal and the subsequent washout of ammonia-N and urea-N after return to control conditions is negligible, indicating that N excretion continues and (or) that N metabolism is markedly depressed. Exposure to 30 mmol·L^{-1} NH_4Cl in a closed system without aeration results in depressed urea-N excretion. The lungfish greatly acidifies the external water, a volume 25-fold greater than its own volume. The extent of this acidification increases with time. After several days, the external pH falls from about 7.0 to below 5.0 over a 24-h period, thereby markedly reducing the concentration of NH_3 (the form that diffuses across biological membranes). CO_2 excretion is partially responsible for this acidification, because vigorous water aeration reduces but does not eliminate the acidification, and urea-N excretion increases moderately. However, a substantial excretion of titratable acid (non-CO_2 acidity) also occurs. One exceptional lungfish was able to maintain its aerated environment at a stable pH of 3.7. Environmental acidification may be a less costly strategy for avoiding toxicity than detoxifying ammonia by increasing urea production.

Résumé : *Protopterus dolloi* Boulenger, 1900 a une respiration aérienne obligée et il est ammoniotélique (88 % de l'excrétion en azote ammoniacal, 12 % en azote urique) dans les conditions aquatiques normales; il tolère, cependant, une exposition de sept jours à 30 mmol·L^{-1} de NH_4Cl, un traitement fatal à la plupart des autres poissons. L'accumulation azotée interne est minimale et le lessivage d'azote ammoniacal et d'azote urique après le retour aux conditions témoins est négligeable, ce qui indique que l'excrétion azotée continue et (ou) que le métabolisme de l'azote est fortement réduit. Une exposition à 30 mmol·L^{-1} de NH_4Cl en système fermé sans aération cause une réduction de l'excrétion d'azote urique; de plus, le protoptère acidifie fortement l'eau environnante, un volume 25 fois supérieur à son propre volume. L'importance de cette acidification augmente en fonction du temps. Après plusieurs jours, le pH externe baisse d'environ 7,0 à moins de 5,0 en 24 h, réduisant ainsi de façon marquée les concentrations de NH_3 (la forme perméable dans les membranes biologiques). L'excrétion de CO_2 est partiellement responsable, parce qu'une agitation vigoureuse de l'eau réduit, mais n'élimine pas, l'acidification et que l'excrétion d'azote urique augmente modérément. Il se produit aussi une importante excrétion d'acide titrable (non dû au CO_2). Un protoptère exceptionnel a réussi à maintenir son environnement aéré à un pH stable de 3,7. L'acidification du milieu peut être une stratégie moins coûteuse pour éviter la toxicité que la détoxification de l'ammoniaque par une production accrue d'urée.

[Traduit par la Rédaction]

Introduction

Like other African lungfish, the slender lungfish, *Protopterus dolloi* Boulenger, 1900, which is endemic to the Congo River drainage, is an obligate air-breather with very small internal and external gills but a prominent lung. *Protopterus dolloi* expresses a full complement of ornithine–urea cycle (OUC) enzymes in its liver but appears to be ammoniotelic under normal aquatic conditions (Chew et al. 2003*b*). In Zaire, it is reported to dig a burrow in the raft-like substrate of floating swamps, using this as a combined wet-season breeding nest and dry-season burrow, in which aestivation may occur without cocoon formation (Brien et al. 1959). However, when desiccated, it exhibits the unusual ca-

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pacity to secrete a mucus cocoon and aestivate on the surface of the substrate (Poll 1961); it does not have to bury in the mud like other lungfish. Recently, Chew et al. (2004) were able to induce this condition in the laboratory and reported that after 40 days there was a significant upregulation of hepatic OUC activity and a reduction in ammonia production. Similarly, during 6 days of terrestrialization (the precursor to aestivation, in which the lungfish is air-exposed but not allowed to dry out), the same phenomenon occurred, and there was a marked washout of urea-N but not ammonia-N upon return to aquatic conditions (Chew et al. 2003*b*). Thus ureagenesis, an expensive mechanism that requires 2.0–2.5 ATP per unit of N fixed (Wood 1993), appears to become important to survival during air exposure and aestivation.

Another situation in which increased urea synthesis may become important is when the lungfish becomes trapped in small ponds during droughts prior to full desiccation, and ammonia builds up in the water owing to the lungfish's own metabolism as well as external biodegradation and evaporation. Alternatively, ammonia may accumulate for similar reasons in the combined breeding nest and dry-season burrow (Brien et al. 1959). Ammonia is a weak base with a pK of about 9.3, and acute toxicity is thought to be mainly attributable to the NH_3 fraction in the environment (e.g., Haywood 1983; United States Environmental Protection Agency 1999; Ip et al. 2001) because of its ability to diffuse across biological membranes (Jacobs and Stewart 1936; Wood 1993) and be fixed internally as NH_4^+, an ion that has a multitude of toxic effects on neural and metabolic functions (Ip et al. 2001). Chew et al. (2005) found that *P. dolloi* exhibits remarkable ammonia tolerance, withstanding exposure to 30–100 mmol·L^{-1} NH_4Cl around pH 7.0 (or about 0.3–1.0 mmol·L^{-1} NH_3) without apparent ill effect. These waterborne concentrations are one to two orders of magnitude higher than those that would kill most fish (Haywood 1983; Tomasso 1994; United States Environmental Protection Agency 1999). This puts the slender lungfish in the same "league" of high ammonia tolerance as several teleosts that express the OUC, i.e., the Lake Magadi tilapia (*Alcolapia grahami* Seegers and Tichy, 1999; Randall et al. 1989; Wood et al. 1989; Walsh et al. 1993), the Indian catfish (*Heteropneustes fossilis* (Bloch, 1794); Saha and Ratha 1994), and the Gulf and oyster toadfishes (*Opsanus beta* (Goode and Bean, 1880) and *O. tau* (L., 1766); Wang and Walsh 2000). Chew et al. (2005) reported only minimal internal ammonia buildup but substantial internal urea accumulation and elevated urea-N excretion during 6 days of exposure to high NH_4Cl concentrations, plus a large washout of urea-N over the 2 days following return to control conditions. While OUC activity was not measured, all of these observations suggest that *P. dolloi* detoxifies ammonia-N to urea-N via the OUC, as occurs in the ammonia-tolerant teleosts listed above.

The original intention of the present study was to exploit this high NH_4Cl tolerance to learn more about the mechanisms of elevated urea-N excretion in the slender lungfish, for comparison with our findings on facilitated urea-N transporters in the Gulf toadfish (Wood et al. 1998, 2003; Walsh et al. 2000) and the Lake Magadi tilapia (Walsh et al. 2001). However, in the course of the study, we found another potentially less costly mechanism for high ammonia tolerance that may well occur in nature: an impressive ability of *P. dolloi* to acidify its own environment, thereby minimizing the availability of NH_3. The present report documents this phenomenon and analyzes its causation.

Materials and methods

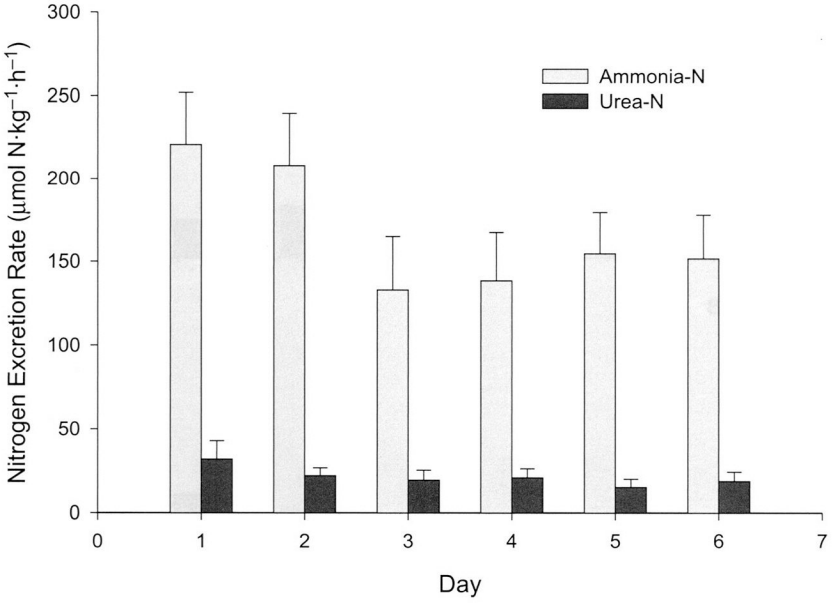
Experimental animals

Specimens of *P. dolloi* weighing about 40 g (range 15–95 g) that had been collected in Zaire or Nigeria were purchased from a local tropical fish dealer in Singapore. The animals were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care. They were held at 25–27 °C under a natural photoperiod in the wet laboratory at the National University of Singapore for several weeks prior to experimentation. All experiments were performed in the wet laboratory under these conditions. During the holding period, lungfish were confined individually in small plastic aquaria containing approximately 2 L of dechlorinated tapwater that was not aerated but supplemented with seawater to a salinity of 2 ppt (parts per thousand), pH about 7.1–7.2. This was found to prevent fungal infections and keep the animals very healthy. Approximate water composition was as follows (mmol·L^{-1}): Na^+, 30; Cl^-, 36; Mg^{2+}, 3.1; and Ca^{2+}, 2.6; titration alkalinity (to pH = 4.0) was 0.64 mmol·L^{-1}. The lungfish were fed bloodworms every second day and the water was changed the day after feeding. Feeding was suspended 48 h prior to experimentation.

Experimental treatments

For experimental exposures and measurements of N waste excretion, lungfish were transferred with minimal disturbance to new plastic containers containing 25× their body mass of the experimental solution, which in all cases was made up in 2 ppt water. Water samples (2 × 5 mL for urea-N and ammonia-N) and pH measurements were taken at the start and end of each 24-h period, after which the water was renewed. Flux rates (μmol·kg^{-1}·h^{-1}) were calculated from changes in concentration (μmol·L^{-1}) using the known mass (kg), volume (L), and time (h). More frequent measurements were taken during the first few hours after a return to control conditions to document any rapid washout of urea-N and (or) ammonia-N. In one series, pH measurements were taken every 2–4 h over the first 24 h of experimental exposure, and additional water samples (4 × 10 mL) were taken at the beginning and end of this 24-h period for measurements of titratable acid and ammonia excretion. In another experiment, titratable acid fluxes were measured over 24-h periods on days 1, 4, and 7 of exposure to 30 mmol·L^{-1} NH_4Cl to evaluate whether there was a progressive change with time. We also used this experiment to determine whether a potential progressive buildup of microbial flora on the walls of the container contributed to the environmental acidification and (or) resulted in depressed urea-N excretion owing to microbial conversion of urea-N to ammonia-N. In this experiment, the lungfish were transferred to fresh containers every 24 h rather than being subjected to water renewal every 24 h in the same container.

Fig. 1. Rates of ammonia-N and urea-N excretion over 6 successive days in slender lungfish (*Protopterus dolloi*) (N = 7) kept under control conditions (30 mmol·L^{-1} NaCl in 2 ppt water). Values are means ± 1 SE. There were no significant differences ($P > 0.05$) among days.



The lungfish were too small to cannulate, and we did not wish to sacrifice these valuable animals. Therefore, blood samples were taken by caudal puncture after the animal had been anesthetized for 15 min by addition of 2-phenoxyethanol (Sigma) to a concentration of 1% in the experimental chamber. It was necessary to monitor the lungfish closely during anesthetization and recovery to ensure they did not drown. Blood samples, typically 200 μL, were drawn though a 23-gauge needle into a 1-ml syringe that had been rinsed with a 5000 IU·mL^{-1} lithium heparin solution (Sigma) made up in 140 mmol·L^{-1} NaCl. Plasma was separated by rapid centrifugation (10 000 *g* for 30 s), decanted, and frozen immediately in liquid N_2 for later analysis of ammonia-N and urea-N concentrations.

Analytical techniques

Urea-N concentrations in water and plasma were measured by the diacetyl monoxime method of Rahmahtullah and Boyde (1980). Ammonia-N concentrations in water were measured by the indophenol blue method of Ivancic and Degobbis (1984). For all water samples, freshly prepared urea and NH_4Cl standards were made up in the appropriate test water. Ammonia in plasma was measured by the enzymatic method of Kun and Kearney (1971). A Shimadzu UV-1601 spectrophotometer was used for all measurements. Water pH was measured using a Thermo Orion Model 410 meter and Trizma combination electrode. Titratable alkalinity was measured by titration of 10-mL water samples to pH 4.0 using the same electrode system, a Gilmont microburette, and the general methods outlined by McDonald and Wood (1981). Start and end values over 24 h were measured in duplicate; a third pair of water samples was titrated if the delta values in the first two pairs did not agree. Titratable acid flux was calculated from the delta values (McDonald and Wood 1981). Because standardized acid was not available for the titrations, a nominal 0.02 mol·L^{-1} HCl solution was made up by dilution of 12 mol·L^{-1} HCl and later standardized both by measurement of its Cl^- concentration (Radiometer CMT10 coulometric titrator) and by titration against standardized NaOH (Sigma). The two measurements agreed that the concentration of HCl was exactly 0.016 mol·L^{-1}, and this value was used in all calculations.

Statistical analyses

Unless otherwise noted, data have been expressed as means ± 1 SE (N), where N represents the number of lungfish sampled. For comparisons within a treatment, either a Student's paired t test (two-tailed) for single comparisons or a one-way ANOVA followed by Dunnett's test for multiple comparisons against a single reference value was employed. For comparisons between treatments at the same time, a Student's unpaired t test (two-tailed) was used. A significance level of $P \leq 0.05$ was used throughout.

Results

Because our routine experimental treatment was exposure to 30 mmol·L^{-1} NH_4Cl (in 2 ppt water), we used 30 mmol·L^{-1} NaCl (in 2 ppt water) as the control treatment so as to eliminate any osmotic differences. Under this control condition, lungfish exhibited relatively stable ammonia-N and urea-N excretion rates over 6 days; the small decrease in ammonia-N excretion after day 2 was not significant. These lungfish were clearly ammoniotelic, ammonia-N accounting for about 88% of total N excretion and urea-N about 12% (Fig. 1).

Contrary to expectation, when a separate set of lungfish was simultaneously exposed to 30 mmol·L^{-1} NH_4Cl over the same time course, urea-N excretion was not elevated, but rather significantly depressed relative to the controls on days 1, 2, and 3 before returning to control rates on days 4–6 (Fig. 2). Ammonia-N fluxes could not be reliably detected

Fig. 2. Rates of urea-N excretion over 6 successive days in slender lungfish (A) kept in 30 mmol·L^{-1} NaCl (control) (N = 7) or (B) exposed to 30 mmol·L^{-1} NH_4Cl (N = 7), both in 2 ppt water, without aeration. Values are means ± 1 SE. An asterisk indicates a significant difference ($P \leq 0.05$) from the control rate on the same day.

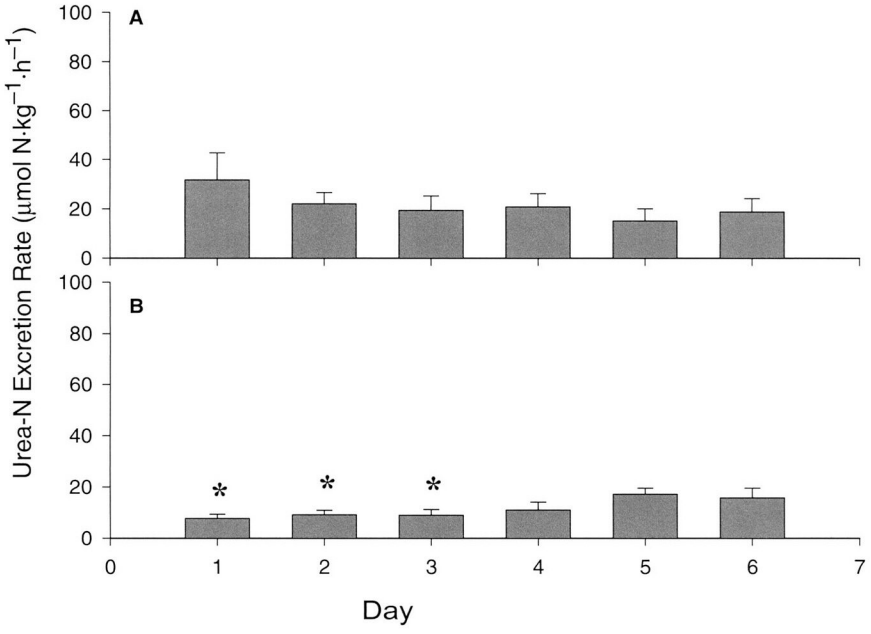
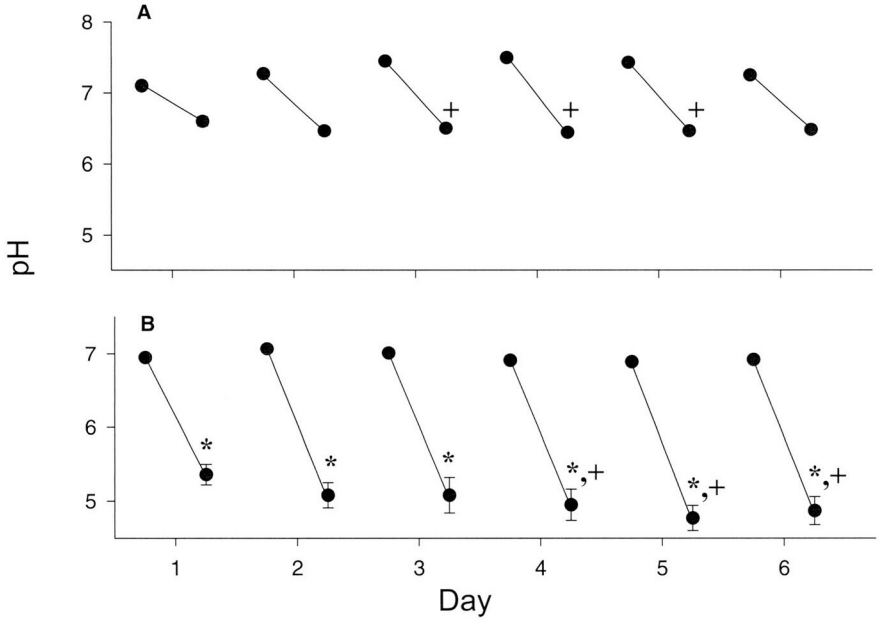


Fig. 3. Changes in water pH over each 24-h period of the experiments presented in Fig. 2: slender lungfish (A) kept in 30 mmol·L^{-1} NaCl (control) (N = 7) or (B) exposed to 30 mmol·L^{-1} NH_4Cl (N = 7), both in 2 ppt water, without aeration. The water was renewed every 24 h; the measured pH values at the start and end of each 24-h period are shown. An asterisk indicates a significantly greater ($P \leq 0.05$) decline in pH relative to the control data on the same day. A plus sign indicates a significantly greater ($P \leq 0.05$) decline in pH (ΔpH) relative to day 1 within a treatment group.



against the high background ammonia-N concentration (note: had ammonia-N flux remained unchanged, it would have altered water ammonia concentration by only about 170 μmol·L^{-1}, against a background of 30 000 μmol·L^{-1}).

Because the slender lungfish is an obligate air-breather, no aeration was used in the experiments presented in Fig. 2. Figure 3 illustrates the changes in water pH over each 24-h period. In both control and experimental treatments, there was a significant fall in water pH each day, but the drop in pH was about 3-fold greater in the 30 mmol·L^{-1} NH_4Cl treatment than in the control treatment. This acidification amounted to a decline of >2 pH units over 24 h (in a volume of water equal to 25× that of the lungfish) in the later days of the experiment. Thus, the acidification tended to increase

over time and was significantly greater, relative to day 1, on days 4–6 in the experimental group and on days 3–5 in the control treatment.

A drop in water pH from about 7.0 to below 5.0 (Fig. 3) would reduce NH_3 concentration in the water by more than 100-fold and could be a potent mechanism for reducing ammonia loading and avoiding toxicity (see Discussion). Once the potential importance of this phenomenon was realized, we started vigorously aerating the experimental chambers to drive off CO_2 and thereby evaluate how much of the acidification was due to CO_2 accumulation in the water. In a second experimental series (data not shown) and a third experimental series (Fig. 4A), which were already ongoing, aeration was started on day 4 and day 2, respectively. In both series, the aeration initially blocked or reversed the acidification, but the phenomenon resumed to a reduced extent on subsequent days (e.g., Fig. 4A). In a fourth series, vigorous aeration was employed throughout, but acidification still occurred to a modest extent and again increased over time (Fig. 4C). Importantly, in all of these series, urea-N excretion rates increased significantly over time and became greater than the corresponding rates in the non-aerated NH_4Cl series of Fig. 2B. Increases were significant only on day 6 in the second series (data not shown), from day 4 onwards in the third series (Fig. 4B), and from day 3 onwards in the fourth series (Fig. 4D) (i.e., 1–2 days after aeration was started). This suggests that ammonia loading and associated increases in urea production occur when environmental acidification is partially blocked by preventing CO_2 buildup in the water.

To evaluate the extent of internal N accumulation during exposure to 30 mmol·L^{-1} NH_4Cl, plasma samples were obtained by caudal puncture on day 7. The animals were allowed to recover for about 12 h under the same conditions and then the washout of urea-N and ammonia-N was followed for 24 h after return to control conditions (30 mmol·L^{-1} NaCl) on day 8. Another plasma sample was obtained from some of these fish after 24 h in control conditions. Control lungfish kept in 30 mmol·L^{-1} NaCl throughout (N = 5–7) were similarly sampled and monitored for N washout to check for any disturbance or fasting effects. In the experimental treatments, data were obtained from six lungfish for which there had been no aeration, six lungfish for which there had been aeration throughout, and five to six lungfish for which aeration had started at intermediate times. Although there was a tendency for greater ammonia-N washout from the aerated fish and greater plasma urea-N concentrations in these animals, there were no significant differences among treatments, so the data were combined.

Relative to day 7 control values, there was a modest rise (50%) in plasma urea-N concentration in lungfish exposed to 30 mmol·L^{-1} NH_4Cl for 7 days (Table 1). After return to control conditions for 24 h, plasma urea-N had fallen significantly and was comparable to that in animals maintained in control conditions throughout, which remained unchanged (Table 1). Measurements of ammonia-N concentrations in plasma obtained by caudal puncture were considered unreliable (see Discussion) but remained less than 0.3 mmol·L^{-1} in all samples. Overall, these data suggest that internal N accumulation was low.

This conclusion was reinforced by the N washout measurements. In the experimental fish that had been exposed to 30 mmol·L^{-1} NH_4Cl for 7 days, ammonia-N excretion was elevated relative to that in control fish only during the first 2 h after return to 30 mmol·L^{-1} NaCl (Fig. 5). There was no change in the controls. Urea-N excretion was modestly elevated in both treatments in these first 2 h, but there were no significant differences between control and experimental treatments (Fig. 6). Overall, the extra N washout after 7 days of exposure to 30 mmol·L^{-1} NH_4Cl amounted to about

Fig. 4. The effect of vigorous aeration of the experimental chambers, to prevent CO_2 buildup, on (A, C) changes in water pH over each 24-h period and (B, D) accompanying rates of urea-N excretion, in two experimental series in which slender lungfish were exposed to 30 mmol·L^{-1} NH_4Cl in 2 ppt water over 6 successive days. Values are means ± 1 SE. In the series shown in panels A and B (N = 7), aeration was started after day 1, while in the series shown in panels C and D (N = 7), aeration was employed throughout. Asterisks indicate significantly different ($P \leq 0.05$) pH changes (ΔpH) or urea-N excretion rates relative to the corresponding means (from Figs. 2B and 3B, respectively) on the same day from lungfish exposed to 30 mmol·L^{-1} NH_4Cl without aeration. Plus signs indicate significantly different ($P \leq 0.05$) pH changes (ΔpH) or urea-N excretion rates relative to day 1 values for the same lungfish within an experimental series.

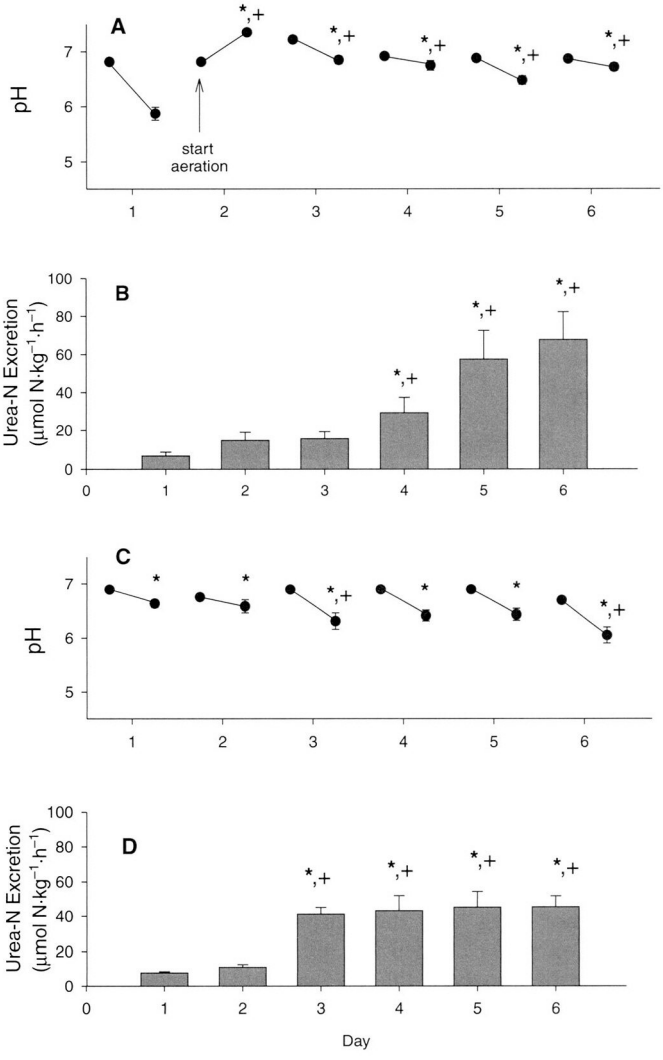
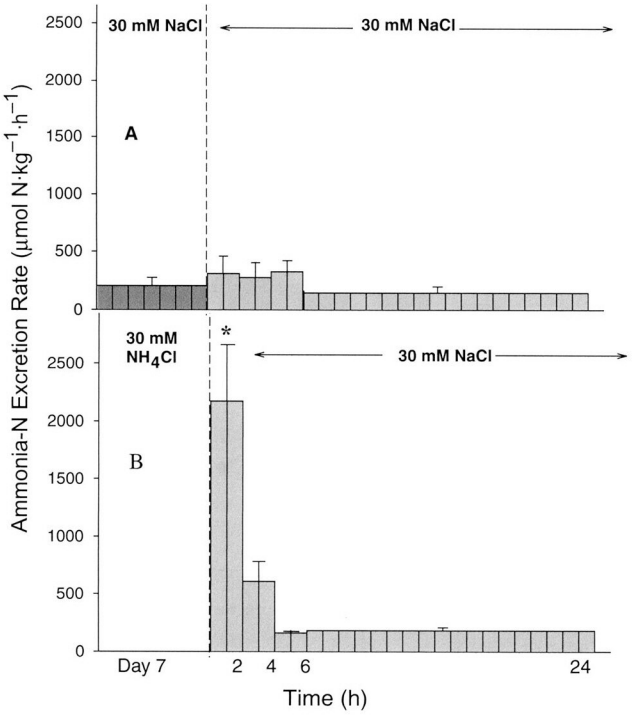


Table 1. Plasma urea-N concentrations in slender lungfish (*Protopterus dolloi*) kept under control conditions in 30 mmol·L^{-1} NaCl for 7 days or exposed to 30 mmol·L^{-1} NH_4Cl for 7 days, both in 2 ppt water, and 24 h after return to control conditions.

	Plasma urea-N (mmol N·L^{-1})	
Treatment	After 7 days	After 24 h control
30 mmol·L^{-1} NH_4Cl	27.84±2.84*a* (18)	15.16±2.91*b* (6)
30 mmol·L^{-1} NaCl	18.34±2.92*b* (7)	15.16±2.67*b* (5)

Note: Values are means ± 1 SE; sample size (*N*) is given in parentheses. Values followed by different letters within a row or within a column are significantly different ($P \leq 0.05$).

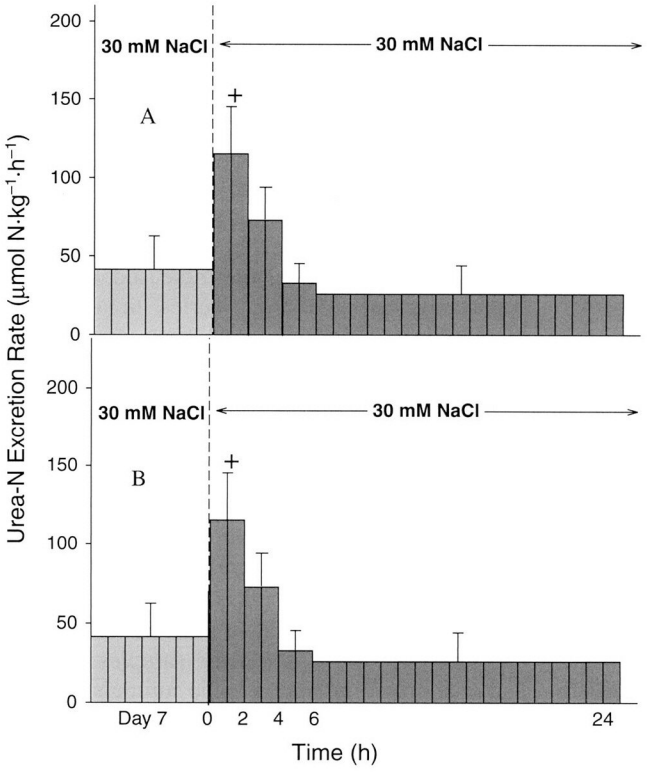
Fig. 5. Excretion rates of ammonia-N over a 24-h period in slender lungfish (A) kept under control conditions for 7 days in 30 mmol·L^{-1} NaCl ($N = 5$) and then transferred to the same condition or (B) returned to 30 mmol·L^{-1} NaCl after 7 days of exposure to 30 mmol·L^{-1} NH_4Cl ($N = 17$), both in 2 ppt water. The asterisk indicates a significant difference ($P \leq 0.05$) relative to control data for the same time point.



4300 µmol N·kg^{-1}, or the equivalent of only one day's N production under control aquatic conditions (cf. Fig. 1).

To further analyze the environmental acidification phenomenon, detailed pH measurements were taken over the first 24 h in separate control and experimental treatments, with or without aeration, with titration measurements taken at the beginning and end. Aeration eliminated the acidification in the control treatment, but in the 30 mmol·L^{-1} NH_4Cl treatment, the drop in water pH was reduced by about half but not eliminated (Fig. 7). Titration measurements (which measure only those acidic equivalent fluxes that are not due to CO_2 excretion) demonstrated that lungfish were in approximate acid–base balance under control conditions regardless of the presence or absence of aeration (Fig. 8A). Note that the standard convention has been used in Fig. 8:

Fig. 6. Excretion rates of urea-N over a 24-h period in slender lungfish (A) kept under control conditions for 7 days in 30 mmol·L^{-1} NaCl ($N = 5$) and then transferred to the same condition or (B) returned to 30 mmol·L^{-1} NaCl after 7 days of exposure to 30 mmol·L^{-1} NH_4Cl ($N = 17$), both in 2 ppt water. A plus sign indicates a significant difference ($P \leq 0.05$) relative to day 7 data for the same treatment group. There were no significant differences ($P > 0.05$) between experimental and control treatments at any time.



fluxes into the animal (i.e., uptake) are plotted as positive values, and fluxes out of the animal (i.e., excretion) are plotted as negative values. Titratable acid uptake just balanced net ammonia-N excretion, so net acid flux was not significantly different from zero in both 30 mmol·L^{-1} NaCl treatment groups (i.e., aerated and non-aerated). In contrast, lungfish exposed to 30 mmol·L^{-1} NH_4Cl exhibited a strong titratable acid excretion of about 200 µmol·kg^{-1}·h^{-1}, which was not significantly different between the aerated and non-aerated treatments (Fig. 8B). Ammonia-N fluxes could not be measured owing to the high background ammonia-N levels (see above), so net acid flux could not be calculated (see Discussion). Nevertheless, these measurements demonstrate a substantial acidic equivalent efflux in addition to that caused by CO_2 excretion in ammonia-exposed lungfish. Urea-N fluxes were slightly depressed (not significant) in both aerated and non-aerated treatments on this first day of exposure to 30 mmol·L^{-1} NH_4Cl (Fig. 8).

To evaluate whether the rate of titratable acid excretion increased with time during exposure to 30 mmol·L^{-1} NH_4Cl, as suggested by the data presented in Figs. 3 and 4, a separate series was performed. Titratable acid fluxes were measured over 24-h periods on days 1, 4, and 7. The results confirmed a significant increase in the rate of titratable acid

Fig. 7. Water pH measured at frequent intervals in containers in which slender lungfish were exposed for the first 24 h to either control (30 mmol·L^{-1} NaCl) or experimental conditions (30 mmol·L^{-1} NH_4Cl) in the presence or absence of vigorous aeration to prevent CO_2 buildup. Values are means ± 1 SE (N = 7–8 per treatment). Note that acidification was eliminated by aeration in the control treatment but was only attenuated by aeration in the 30 mmol·L^{-1} NH_4Cl treatment.

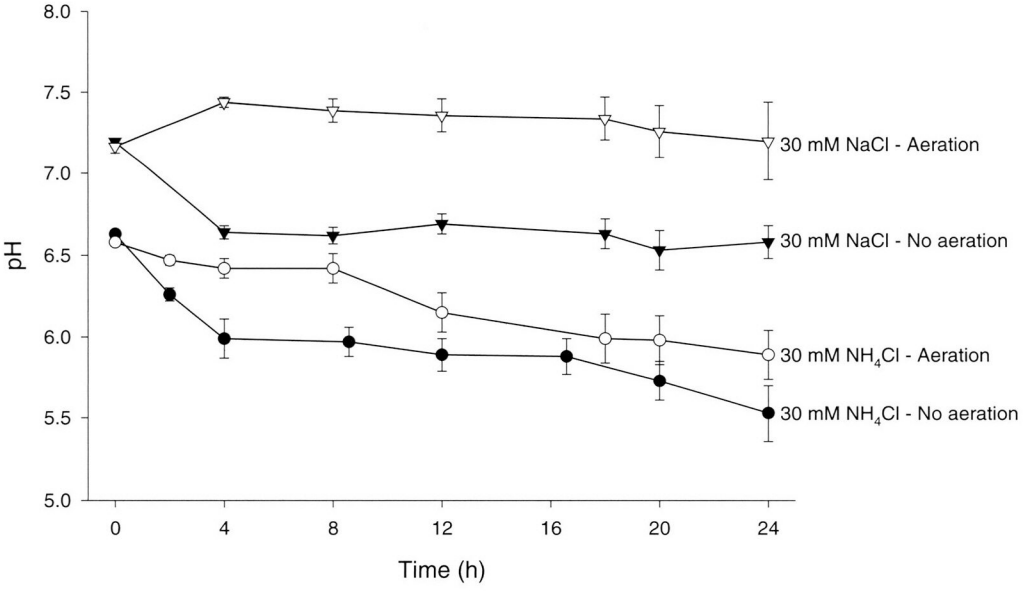
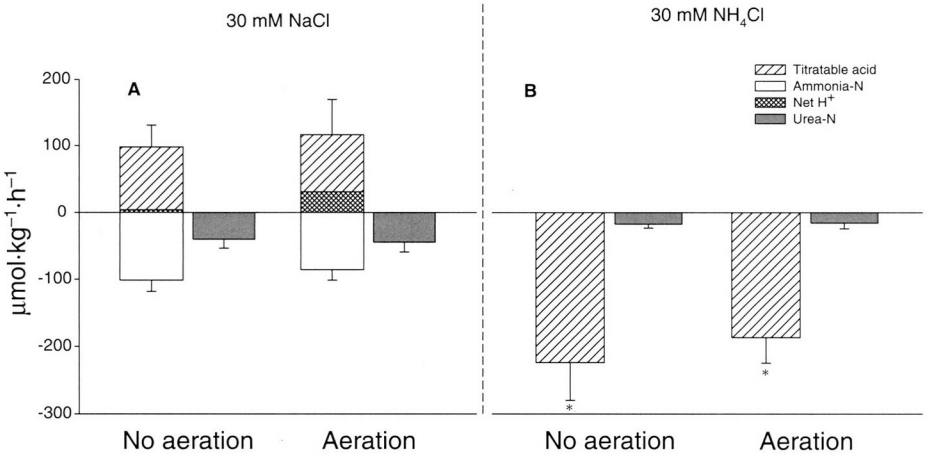


Fig. 8. Acid–base and N fluxes of slender lungfish over the first 24 h of exposure to either (A) control (30 mmol·L^{-1} NaCl) or (B) experimental conditions (30 mmol·L^{-1} NH_4Cl) in the presence or absence of vigorous aeration to prevent CO_2 buildup. Data are from the same experiment shown in Fig. 7. The standard convention is used such that fluxes into the animal (i.e., uptake) are positive values and fluxes out of the animal (i.e., excretion) are negative values. Ammonia-N fluxes could not be measured and therefore net acid flux could not be calculated in the 30 mmol·L^{-1} NH_4Cl treatments (see text). Values are means ± 1 SE (N = 7–8 per treatment). Asterisks indicate significant differences ($P \leq 0.05$) relative to the corresponding parameter in the control treatment. There were no significant differences ($P > 0.05$) attributable to aeration.



excretion from day 1 to day 4 (Table 2). The rate on day 7 was also significantly higher than that on day 1 but not significantly different from the rate on day 4. Note that the absolute values of titratable acid flux (Table 2) were about twice those of the series shown in Fig. 8. This may reflect the fact that a different freshwater source was used in this series to make up the experimental media (30 mmol·L^{-1} NH_4Cl in 2 ppt water); this freshwater had a titratable alkalinity approximately 2.5-fold higher than that used in other experiments. The pattern of increasing then stabilizing urea-N excretion in this series was virtually identical to that of other series with aeration throughout (e.g., Fig. 4D).

We made two additional observations relevant to the environmental acidification phenomenon. We noted that one exceptional lungfish exposed to 30 mmol·L^{-1} NH_4Cl was able to routinely acidify its environment to about 5.3 over 24 h, even in the presence of vigorous aeration. For interest, at the end of an experiment, we left the water unchanged to see whether the fish would acidify the water further if given the opportunity. This fish reduced its water pH to about 3.7 and

Table 2. Rate of titratable acid fluxes in slender lungfish exposed to 30 mmol·L^{-1} NH_4Cl in 2 ppt water for 7 days.

Day	Rate (μmol·kg^{-1}·h^{-1})
1	–447±33*a*
4	–555±86*b*
7	–590±103*b*

Note: Values are means ± 1 SE (N = 6). Negative values denote excretion by the animal. Values followed by different letters are significantly different ($P \leq 0.05$).

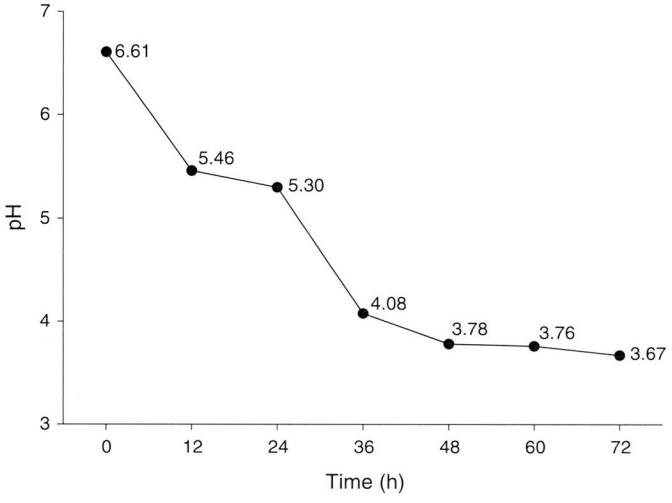
held it there for 72 h in the presence of aeration, without any apparent ill effect (Fig. 9). In an attempt to block environmental acidification and thereby increase internal ammonia loading, we exposed seven lungfish to 30 mmol·L^{-1} NH_4HCO_3 (pH 8.15) in 2 ppt water, a solution they were unable to acidify. All seven of these lungfish became comatose within 3 h (when the experiment was terminated), and only two could be revived, illustrating the importance of environmental acidification to survival.

Discussion

In attempting to exploit the activation of ureagenesis, which *P. dolloi* may use to avoid ammonia toxicity (Chew et al. 2005), we inadvertently discovered a second, potentially less expensive mechanism that may reduce or obviate the need to activate costly ureagenesis in circumstances where the external medium is not renewed by water inflow. This mechanism, acidification of the local environment so as to convert NH_3 to NH_4^+ and thereby minimize the P_{NH_3} for diffusive entry, avoids the consumption of 2.0–2.5 ATP per urea-N fixed (Wood 1993) by the OUC and the associated input of ammonia-N into the cycle. Indeed, part of the mechanism is clearly due to CO_2 input into the surrounding water (Figs. 4, 7) and is therefore essentially free, as metabolism produces CO_2 as a by-product. Given an effective pK around 6.1, and the well-known log-linear relationship between P_{CO_2} and pH (Davenport 1974), acidification by CO_2 will be most effective in the pH range of 6.0–7.0. This alone will decrease ambient P_{NH_3} 10-fold. We are aware of no studies in which CO_2 excretion by *P. dolloi* has been partitioned between aerial (lungs) and aquatic routes (internal or external gills, skin), but in other African lungfish, aquatic CO_2 excretion is predominant (Lenfant and Johansen 1968; McMahon 1970; Burggren and Johansen 1986). It seems quite possible that a lungfish under stress from high environmental ammonia might "shunt" a greater percentage of CO_2 excretion to the aquatic pathway by reorganizing blood flow (Szidon et al. 1969; Laurent et al. 1978) or modifying local carbonic anhydrase activity. This is an interesting topic for future research.

However, during exposure to high ambient ammonia concentrations, *P. dolloi* also activates a non-CO_2 acidic equivalent secretion mechanism (Fig. 8), which can acidify the environment to pH 5.0 or below (Fig. 3B) and thereby achieve yet another 10-fold (or greater) reduction in P_{NH3}. This mechanism appears to become more effective with time

Fig. 9. Water pH changes over 72 h in a container in which one exceptional slender lungfish was exposed to 30 mmol·L^{-1} NH_4Cl in 2 ppt water with vigorous aeration but without daily changes of water.



(Fig. 3B, Table 2), and this can be seen clearly when the CO_2 contribution to acidification is prevented by vigorous aeration (Figs. 4A, 4C). Indeed, in the exceptional example shown in Fig. 9, acidification of the environment progressed to an apparent stabilization point at pH 3.7, which would have achieved a more than 1000-fold reduction in ambient P_{NH_3}. We made no attempt to characterize the mechanism, but this pattern of progressive activation suggests that a transporter is involved, one which can sustain a pH gradient between blood and environment of about 4 units, because normal blood pH in *Protopterus* sp. is about 7.6 (Lahiri et al. 1970; DeLaney et al. 1974, 1977). Clearly, this points to a V-type H^+-transporting ATPase (Merzendorfer et al. 1997). The most likely location for such a mechanism would be the putative ionocytes, rich in mitochondria and abundant on both gills and skin in other *Protopterus* species (Laurent and Dunel 1980; Sturla et al. 2001), but at present we cannot eliminate other possibilities such as the kidney or even the digestive tract (e.g., reflux of stomach acid into the ambient water). Such a mechanism would obviously have a cost, but it may be less than the activation of ureagenesis. Ip et al. (2001) have argued that active NH_4^+ excretion, as seen in the giant mudskipper, *Periophthalmodon schlosseri* (Pallas, 1770) (Randall et al. 1999; Ip et al. 2004), may have less than half the expenditure of ATP per unit of N "detoxified" relative to OUC-based ureagenesis. If H^+ excretion occurs by a comparable mechanism, the same cost saving would apply.

This latter point raises the interesting question whether *P. dolloi* activates NH_4^+ excretion (or coupled $NH_3 + H^+$ excretion) as well as titrable acid excretion during exposure to high NH_4Cl concentrations. The present experiments do not answer this question because we could not resolve net ammonia-N fluxes against the 30 mmol·L^{-1} background. If net ammonia-N excretion still occurred during exposure to 30 mmol·L^{-1} NH_4Cl, then in terms of traditional acid–base balance (McDonald and Wood 1981), the actual net acid excretion would have been greater than the titratable acid effluxes

measured (Fig. 8B). If net ammonia-N uptake occurred, the reverse would have been true. In view of the very limited internal N accumulation that was observed (see below), we believe the former is far more likely and that some continued ammonia-N excretion was facilitated by the low external pH created by the elevated titratable acid excretion. If we assume that the typical rate of titratable acid excretion during high environmental NH_4Cl exposure is 200 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ (Fig. 8B), then in our test water (titratable alkalinity to pH 4.0 = 0.64 $mmol \cdot L^{-1}$) it would take only about 80 h for a lungfish to acidify (to pH 4.0) a fully aerated environment 25-fold greater in volume than its body if the water were not changed every day, and the exceptional lungfish (Fig. 9) did this in less than half that time. If aeration were not employed and CO_2 were allowed to accumulate, these times would be reduced by about 50%, so it is clearly an effective strategy. We believe that this environmental acidification by CO_2 and titratable acid excretion to prevent ammonia loading and facilitate ammonia excretion may well occur in nature. For example, Greenwood (1986) noted that water sampled from *P. dolloi* burrows in Zaire "had a pH between 4.7 and 6.5, was rich in organic matter, and had a high CO_2 and low O_2 content".

The importance of environmental acidification to facilitate ammonia excretion and (or) minimize ammonia uptake has been known for many years (e.g., Lloyd and Herbert 1960), and the ability of standard teleosts to acidify the boundary water layer at the gill surface, thereby facilitating ammonia excretion, is well documented (Wright et al. 1986, 1989; Playle and Wood 1989; Wilson et al. 1994). However, *P. dolloi* appears to be one of the few fish for which activation of an acid secretion mechanism for the purpose of large-scale environmental acidification has been reported. Recently, this phenomenon was documented in the giant mudskipper, which exhibits a capacity for high ammonia tolerance comparable to that of *P. dolloi* and appears to be able to actively excrete both NH_4^+ (Randall et al. 1999) and titratable acid (Ip et al. 2004). In the mudskipper, the acid secretion was activated by both high environmental NH_4Cl exposure and high environmental pH, occurred in the head region (including gills) rather than the body, and was inhibited by bafilomycin A1 (Ip et al. 2004), indicating involvement of a V-type H^+-ATPase (Merzendorfer et al. 1997). Acidification was studied in buffered media only over the pH range 7.0–9.0 (Chew et al. 2003*a*; Ip et al. 2004), so it is not known whether the mudskipper can drive environmental pH below 5, as the slender lungfish does (Figs. 3, 9). Interestingly, the mudskipper also exhibited an unusually low permeability of the skin to NH_3, and it increased its cholesterol and saturated fatty acid content after 6 days of exposure to 30 $mmol \cdot L^{-1}$ NH_4Cl (Ip et al. 2004). It would be interesting to determine whether the same characteristics are present in *P. dolloi* as additional mechanisms to minimize diffusive NH_3 entry.

The fact that the same patterns of increasing titratable acid excretion with time and increasing, then stable, urea-N excretion were seen when lungfish were transferred to fresh containers alleviates worries that the microbial buildup on the walls of the containers in the water renewal experiments contributed to the observed phenomena. However, it certainly remains possible that microbial flora on the surface of the lungfish itself contributes to the observed phenomena; future experiments with antibiotics would be instructive.

In the present study, urea-N excretion of *P. dolloi* was either depressed (Fig. 2) or moderately elevated (Fig. 4) during 6 days of exposure to 30 $mmol \cdot L^{-1}$ NH_4Cl, depending on the extent to which aeration attenuated environmental acidification. In the experiment without aeration (Fig. 2), the cumulative depression of urea-N excretion relative to the control rate was about −1500 $\mu mol\ N \cdot kg^{-1}$, whereas in both experiments where aeration was employed (Fig. 4), the cumulative elevation was about +1500 $\mu mol\ N \cdot kg^{-1}$. *Protopterus dolloi* exhibited surprisingly little washout (equivalent to about one day of N production at the control rate; Fig. 1) of either ammonia-N (+4300 $\mu mol\ N \cdot kg^{-1}$; Fig. 5) or urea-N (negligible; Fig. 6) and only a small accumulation of urea-N in the plasma (Table 1) after 7 days of exposure to 30 $mmol \cdot L^{-1}$ NH_4Cl. Determinations of ammonia-N concentrations in plasma obtained by caudal puncture are usually unreliable overestimates (for discussion, see Wood 1993), but we observed no increase over time, and the highest values recorded were less than 0.3 $mmol \cdot L^{-1}$. All these observations point to very limited internal N accumulation during high environmental ammonia exposure. Either ammonia-N excretion continued or N metabolism was greatly reduced during this period; very likely, both adjustments occurred.

Chew et al. (2005) similarly reported only minimal accumulation of ammonia-N in plasma (0.29 ± 0.02 $mmol \cdot L^{-1}$, measured by terminal caudal severance) in *P. dolloi* during 6 days of exposure to high NH_4Cl concentration, but in many other respects our results differ substantially from those of that study. Chew et al. (2005) observed substantial internal accumulation of urea-N (about 80 $mmol\ N \cdot L^{-1}$ in blood plasma) and elevated urea-N excretion (about 4000 $\mu mol\ N \cdot kg^{-1}$) during the exposure period plus a massive washout of urea-N (about 16 000 $\mu mol\ N \cdot kg^{-1}$) over the 2 days following a return to control conditions. All of these observations indicated that *P. dolloi* retained N and activated the OUC substantially as a detoxification mechanism in the study of Chew et al. (2005), in contrast to the present results.

In part, the differences between the two studies may relate to the aeration regime (Chew et al. (2005) aerated vigorously throughout), but we believe a far more important difference is the ionic composition of the external environment. Chew et al. (2005) used true freshwater, whereas we used freshwater amended to 2 ppt with seawater, raising the background Na^+ and Cl^- levels to 30–36 $mmol \cdot L^{-1}$. This would have provided far more substrate for any ion-coupled V-type H^+-ATPase (e.g., Na^+-H^+) (Merzendorfer et al. 1997), facilitating environmental acidification and thereby minimizing ammonia-N entry or facilitating continued ammonia-N excretion in the present study. The addition of 30 $mmol \cdot L^{-1}$ NH_4Cl also further raised the osmolality to about 120 $mosmol \cdot kg^{-1}$. Recently, Y.K. Ip et al. (unpublished data) found that a salinity of 4 ppt (osmolality about 120 $mosmol \cdot kg^{-1}$) causes a marked reduction of urea-N production and excretion and an overall down-regulation of N metabolism in *P. dolloi*. In nature, evaporation of the pools or burrows in which lungfish become trapped during the dry season could well create similar conditions. We speculate

that the combination of an increased capacity for environmental acidification and decreased N metabolism would help the slender lungfish survive this transient period and preadapt them for the ensuing aestivation when water disappears entirely.

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