The Journal of Experimental Biology 205, 651–659 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
JEB3854

Accumulation of ammonia in the body and NH_3 volatilization from alkaline regions of the body surface during ammonia loading and exposure to air in the weather loach *Misgurnus anguillicaudatus*

T. K. N. Tsui[1], D. J. Randall[2,*], S. F. Chew[3], Y. Jin[1], J. M. Wilson[4] and Y. K. Ip[1]

[1]*Department of Biological Sciences, National University of Singapore, 10 Kent Ridge Road, Singapore 117543, Republic of Singapore,* [2]*Department of Biology and Chemistry, City University of Hong Kong, Tat Chee Avenue, Kowloon, Hong Kong, China,* [3]*Department of Natural Sciences, National Institute of Education, Nanyang Technological University, 1 Nanyang Walk, Singapore 637616, Republic of Singapore* and [4]*Centro Interdisciplinar de Investigacao Marinha e Ambiental-CIIMAR, Rua do Campo Alegre 823, 4150-180 Porto, Portugal*

*Author for correspondence (e-mail: bhrand@cityu.edu.hk)

Accepted 18 December 2001

Summary

The weather loach *Misgurnus anguillicaudatus* inhabits rice fields that experience drought in summer and ammonia loading during agricultural fertilisation. Exposure of specimens to ammonia led to the accumulation of ammonia in muscle, liver and blood. The level of ammonia reached in the plasma was the highest reported among fishes. Ammonia was not detoxified to urea, and urea excretion rate was unaffected by ammonia exposure. Fish acidified the water to reduce ammonia loading. Ammonia loading, unlike aerial exposure, did not induce glutamine synthesis, and there was no accumulation of glutamine. This is a unique observation different from those reported for other fishes in the literature. An initial switch to partial amino acid catabolism led to the accumulation of alanine and was probably associated with a decreased rate of ammonia production. Aerial exposure led to decreases in rates of ammonia and urea excretion, as well as the accumulation of tissue ammonia. As the internal ammonia levels increased, *M. anguillicaudatus* was able to excrete some ammonia in the gaseous form (NH_3). The percentage of ammonia excreted as NH_3 increased with time of exposure and with increasing temperature. It appears that air-breathing through the gut is involved, with the anterior portion of the digestive tract playing a central role: it became significantly more alkaline in fish exposed to air or to environmental ammonia. The skin, which also became more alkaline during air exposure, may also be involved in ammonia volatilization in air-exposed fish. This represents the first report of a fish using volatilization of NH_3 as part of a defence against ammonia toxicity. It can be concluded that the main strategy adopted by *M. anguillicaudatus* confronted with ammonia loading or air exposure is to tolerate high ammonia levels in the tissues. During periods of elevated tissue ammonia levels, some ammonia is lost by volatilization *via* air-breathing using the gut. In addition, some ammonia may be lost across the skin during air exposure.

Key words: ammonia, ammonia loading, urea, air exposure, volatilization, weather loach, *Misgurnus anguillicaudatus*.

Introduction

The weather loach *Misgurnus anguillicaudatus* is a small freshwater fish that lives in rice fields. It is capable of using its intestine as an additional respiratory organ (McMahon and Burggren, 1987) to breathe air. It can gulp air, pass it through the digestive tract and release it from the vent. This enables it to live in oxygen-poor waters or to bury itself in mud during long-lasting droughts in the summer. When there is a lack of water, it will bury and position itself in the soft mud such that the mouth has access to air through a small opening, the fish gulping air occasionally and respiring through its intestine. By doing so, this loach can survive for more than a month until the drought is over.

Chew et al. (2001) reported that *M. anguillicaudatus* was capable of partial amino acid catabolism leading to a slight accumulation of alanine in the first 24 h of aerial exposure. Thus, it is possible that this loach is able to use certain amino acids as an energy source to sustain muscular activities on land, as has been suggested for the mudskipper *Periophthalmodon schlosseri* (Ip et al., 1993, 2001a,b). After 24 h of aerial exposure, glutamine accumulated in the muscle and liver of *M. anguillicaudatus* (Chew et al., 2001). Simultaneously, ammonia accumulated to very high levels in all the tissues examined. The present study was undertaken to determine whether *M. anguillicaudatus* could excrete NH_3 by

volatilization during air exposure. Attempts were made to evaluate the roles of the skin and the digestive tract in NH_3 volatilization. The effects of placing *M. anguillicaudatus* in concentrated ammonia solutions on ammonia volatilization from the gut and on tissue ammonia levels were also determined.

Materials and methods

Collection and maintenance of specimens

Mature *Misgurnus anguillicaudatus* (8–15 g) were obtained from the Yuen Long wet-market in Hong Kong and transferred to Singapore by air. They were kept in dechlorinated tap water at 25 °C in the laboratory. No attempt was made to separate the sexes. Fish were acclimated to laboratory conditions for at least 48 h before experimentation. During this period, half the water was changed daily and the fish were fed with commercial fish food. Fish were fasted for 24 h prior to experiments and were not fed during the experimental period.

Exposure of fish to experimental conditions

Ammonia exposure

Fish were exposed to 30 mmol l^{-1} NH_4Cl in 20 vols (w/v) of water, adjusted to pH 7.2 using NaOH, at 25 °C for various periods (12, 24 or 48 h). Those collected at the beginning of the experiment and those exposed to dechlorinated tap water (pH 7.2) for 48 h served as controls.

Aerial exposure

Fish exposed to terrestrial conditions were placed in individual plastic boxes (20.5 cm×14.5 cm×6.0 cm) containing a thin film of dechlorinated tap water (50 ml). The boxes were, in turn, placed in a fibreglass humidity chamber for the entire period of aerial exposure.

Determination of ammonia gas excreted by fish

Ammonia exposure

Fish were kept individually in conical flasks containing 100 ml water or NH_4Cl (10 mmol l^{-1} or 20 mmol l^{-1}) solution. The flasks were tightly sealed such that they were both airtight and watertight, except for the air inlet and outlet. Air was bubbled through the water in the flask at a constant rate of 20 l h^{-1}. The flask was connected to two test tubes. The first test tube served as a water trap. The second, containing 3 ml of freshly prepared 0.5 mmol l^{-1} H_2SO_4, served to trap any ammonia gas present in the air stream, which was bubbled through the acid. At the end of each 24 h period, the acid in the trap was collected and analysed for ammonia as described above. Blank control experiments were also run in which animals were not present in the flasks to account for volatilization of ammonia from the bathing solutions. In one experiment, fish were placed in small net cages before being submerged in the flask to determine whether preventing the fish from gulping air at the water surface would affect ammonia volatilization.

Aerial exposure

Experiments in which fish were air-exposed were performed at 20, 25 and 30 °C. Fish were kept initially as described above in conical flasks containing 100 ml of dechlorinated water. After 24 h, the acid in the trap was analysed for ammonia content. The water in the flask was collected for ammonia assays, and the flask was rinsed. The same fish was then immediately exposed to terrestrial conditions for 24 h in the same apparatus, but with only 1 ml of dechlorinated water present in the flask. At the end of the second 24 h period, the acid in the trap was collected for ammonia assay and totally replenished. The small volume of water inside the flask was collected, and the flask was rinsed and refilled with 1 ml of fresh water. Ammonia production under terrestrial conditions was then monitored for 72 h. To evaluate the effects of a larger volume of water on NH_3 volatilization, an experiment was performed at 25 °C with 3 ml, instead of 1 ml, of water in the flask.

Determination of blood pH, P_{CO_2} *and* P_{O_2}

Specimens were anaesthetised in 0.02 % 3-aminobenzoic acid ethyl ester (MS222; Sigma, USA). The tail was cut, and arterial blood was collected in heparinised capillary tubes. Blood variables (pH, P_{CO_2} and P_{O_2}) were determined using an IL 1306 blood gas analyzer.

Collection and preparation of muscle, liver and plasma samples

To collect muscle and liver tissue, specimens were killed by a blow to the head. The lateral muscle and liver were excised and immediately freeze-clamped in liquid nitrogen with pre-cooled aluminium tongs (Faupel et al., 1972). The entire procedure was carried out within 1 min. Samples were stored at –80 °C until analysis.

The frozen samples were powdered in liquid nitrogen, weighed and homogenized three times in 5 vols of 6 % trichloroacetic acid (TCA) using an Ika-werk Staufen Ultra-Turrax homogenizer (Janke & Kunkel, Germany) at a maximum speed of 24 000 revs min^{-1} for 20 s each, with 10 s intervals. The homogenate was centrifuged at 10 000 ***g*** at 4 °C for 15 min in a Beckman J2-21M/E refrigerated centrifuge (Beckman Instruments Inc., USA).

A second group of specimens was used for the collection of blood samples. The tail of the fish was cut, and blood exuding from the severed caudal artery was collected in heparinized (sodium heparin) micro-haematocrit capillary tubes. The blood samples were centrifuged at 5000 ***g*** at 4 °C for 5 min. The resulting plasma was collected and deproteinised immediately by adding an equal volume of 6 % TCA, followed by centrifugation at 10 000 ***g*** for 10 min. The supernatant fluid was kept at –80 °C until analysis.

Determination of ammonia, urea and free amino acid levels

For ammonia and urea analyses, the deproteinized samples were neutralised to pH 5.5–6.0 with 2 mol l^{-1} $KHCO_3$. Ammonia content was determined by the method of Kun and

Table 1. *Effects of 48 h of ammonia exposure (30 mmol l^{-1} NH_4Cl) and air exposure on the pH,* P_{O_2} *and* P_{CO_2} *of the blood of* Misgurnus anguillicaudatus

	Control	Ammonia-exposed	Air-exposed
pH	7.285±0.051 (6)	7.329±0.028 (6)	7.528±0.017(11)*
P_{O_2} (mmHg)	33.5±4.3 (6)	33.5±4.5 (6)	34.4±2.0 (11)
P_{CO_2} (mmHg)	30.1±1.1 (6)	27.4±2.8 (6)	34.5±3.1 (11)

1 mmHg=0.133 kPa.

Values are means ± S.E.M. with number of determinations in parentheses.

*Significantly different from the corresponding control, $P<0.05$.

Kearney (1974). The change in absorbance at 340 nm (25 °C) was monitored using a Shimadzu UV-160A spectrophotometer. Freshly prepared ammonium chloride (Merck) was used as a standard.

The urea content in 0.2 ml of deproteinised sample was analysed colorimetrically according to the method of Jow et al. (1999). The difference in absorbance of the sample in the presence and absence of urease was used as an estimate of the urea content of the sample.

To measure free fatty acid (FAA) levels, the deproteinised samples were diluted with an equal volume of 0.2 mol l^{-1} lithium citrate buffer (Pierce, USA), and the pH was adjusted to 2.2 with 4 mol l^{-1} lithium hydroxide. FAA levels were measured using a Shimadzu LC-6A amino acid analysis system with a Shim-pack ISC-07/S1504 Li type column. Analytical-grade FAA standards purchased from Sigma Chemical Co. served as references. Results were expressed as μmol g^{-1} wet mass tissue or μmol ml^{-1} plasma.

Determination of urea excretion rates

Specimens were exposed to 200 ml of 30 mmol l^{-1} NH_4Cl solution (pH 7.2) at 25 °C for 48 h. The external medium was changed daily. At 24 h and 48 h, a water sample was collected and analysed for urea content. Preliminary studies revealed that urea excretion rate was linear for at least 24 h. Urea content was analysed colorimetrically as described above. The rate of urea excretion was expressed as μmol day^{-1} g^{-1} fish.

Determination of the pH of water samples, the skin and the digestive tract

The pH of water samples and the surface of the skin of *M. anguillicaudatus* was monitored using a PHR-146 micro combination pH electrode (Lazar Research Laboratories) and an Orion 720A pH meter. Preliminary results indicated that the pH over different regions of the body differed by less than 0.1 unit. Subsequently, it was decided to report the pH obtained from the side of the fish just above the pectoral fins. The specimen was then killed, and the digestive tract was dissected out. The digestive tract was divided into three regions: anterior, middle and posterior. Each region was cut open longitudinally, and the pH of the mucosal surface was measured using the micro combination pH electrode.

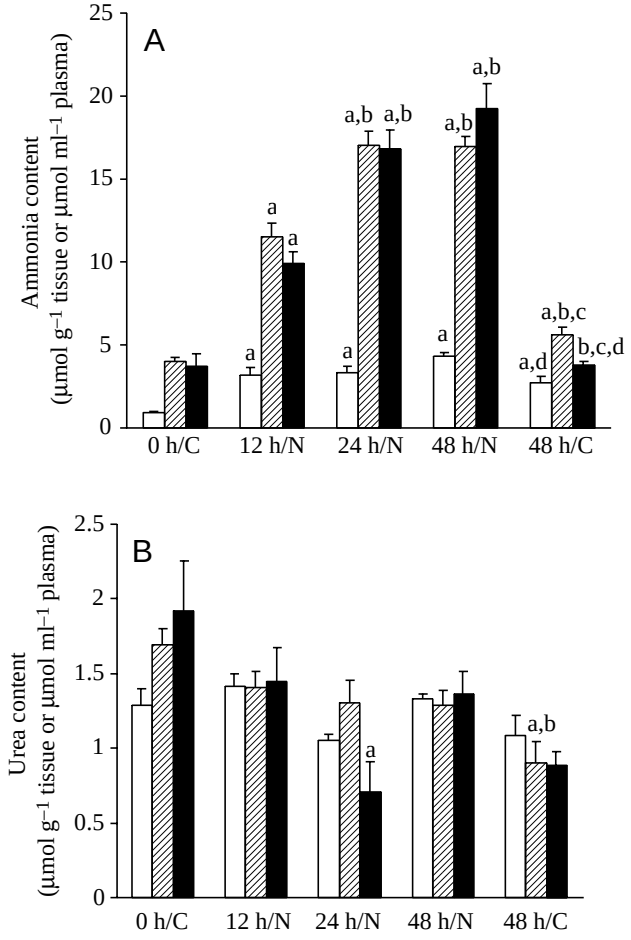


Fig. 1. (A) Effects of various periods of ammonia exposure (30 mmol l^{-1} NH_4Cl) on the concentrations of ammonia (A) and urea (B) in the blood plasma (open columns), liver (hatched columns) and muscle (filled columns) of *Misgurnus anguillicaudatus*. Values are means + S.E.M. (N=6). [a]Significantly different from the 0 h/C condition, $P<0.05$; [b]significantly different from the 12 h/N condition, $P<0.05$; [c]significantly different from the 24 h/N condition, $P<0.05$; [d]significantly different from the 48 h/N condition, $P<0.05$. N, 30 mmol l^{-1} NH_4Cl; C, control.

Statistical analyses

Results are presented as means ± S.E.M. Student's *t*-test and one-way analysis of variance (ANOVA) followed by Student–Neuman–Keul's multiple range test were used to compare differences between means where applicable. Differences at $P<0.05$ were regarded as statistically significant.

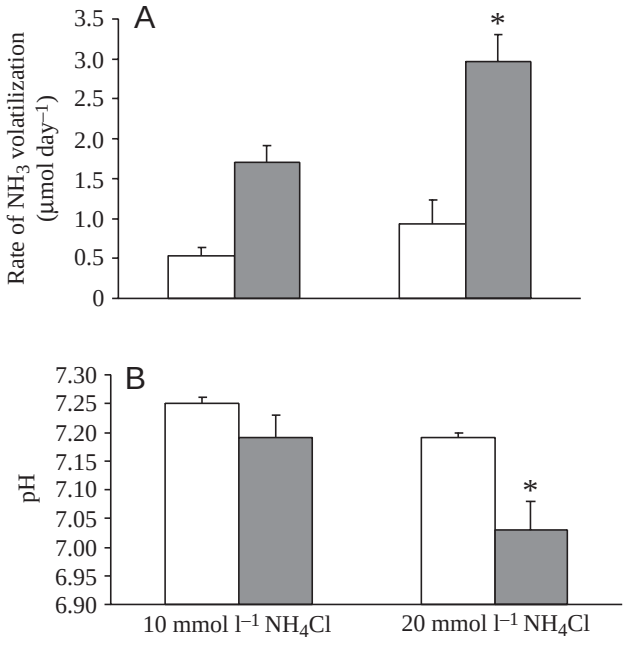
Results

Exposure to 30 mmol l^{-1} NH_4Cl at pH 7.2 did not affect blood pH, P_{O_2} or P_{CO_2}, but aerial exposure resulted in a significant increase in blood pH (Table 1). Ammonia built up progressively in the muscle and liver (Fig. 1A) following ammonia exposure.

Table 2. *Effects of various periods of ammonia exposure on the contents of various free amino acids (FAAs) and the total FAA (TFAAs) in the muscle of* Misgurnus anguillicaudatus

	FAA concentration (μmol g^{-1})				
FAA	0 h/C	12 h/N	24 h/N	48 h/N	48 h/C
Ala	1.3±0.3	5.2±0.8[a]	3.5±0.8[b]	2.0±0.4[b]	1.8±0.3[b]
Arg	0.20±0.06	0.35±0.09	0.20±0.01	0.31±0.15	0.30±0.07
Asn	0.50±0.08	0.65±0.19	0.59±0.15	0.61±0.28	0.59±0.10
Asp	0.10±0.03	0.55±0.19	0.46±0.16	0.14±0.03	0.35±0.14
Cys	0.02±0.02	0.12±0.06	0.05±0.03	0.002±0.002	ND
Gln	1.5±0.2	2.3±0.4	3.2±0.4	4.1±1.4	3.2±0.5
Glu	0.30±0.05	1.3±0.6	1.4±0.1	0.91±0.26	2.0±0.3[a]
Gly	4.7±1.4	3.7±0.7	2.5±0.4	2.4±0.5	3.8±0.4
His	1.9±0.6	2.7±0.7	3.0±0.2	2.3±0.3	0.95±0.06[c]
Ile	0.28±0.01	0.55±0.09[a]	0.25±0.03[b]	0.07±0.02[b]	0.46±0.08[a,c,d]
Leu	0.51±0.04	0.94±0.13[a]	0.55±0.03[b]	0.20±0.04[a,b,c]	0.76±0.13[a,c,d]
Lys	0.99±0.34	2.3±0.5[a]	1.7±0.2	0.77±0.13[b]	1.98±0.36
Met	0.083±0.002	0.12±0.03	0.06±0.01[b]	0.01±0.01[a,b]	ND[a,b,c]
Orn	ND	0.12±0.03[a]	0.07±0.02	0.02±0.02[b]	ND[b]
Phe	0.22±0.01	0.51±0.09[a]	0.21±0.05[b]	0.23±0.02[b]	0.36±0.03[b]
Pro	0.22±0.02	0.57±0.05[a]	0.50±0.04[a]	0.33±0.08[b]	0.47±0.02[a]
Ser	0.56±0.07	1.4±0.2[a]	1.3±0.2[a]	1.1±0.2	0.98±0.11
Tau	5.6±0.2	3.2±0.6[a]	3.2±0.3[a]	2.4±0.5[a]	3.8±0.5[a]
Thr	1.0±0.2	2.3±0.5	2.4±0.4	1.1±0.3	1.5±0.2
Try	0.01±0.01	0.05±0.03	0.02±0.01	0.003±0.003	ND
Tyr	0.13±0.01	0.22±0.05	0.09±0.01[b]	0.08±0.01[b]	0.15±0.02
Val	0.41±0.03	0.79±0.10[a]	0.39±0.03[b]	0.14±0.04[a,b,c]	0.69±0.11[a,c,d]
TFAAs	20.3±2.8	29.7±1.3	25.6±2.2	19.2±4.1	23.6±0.8

Values are means ± S.E.M. (*N*=4).
N, 30 mmol l^{-1} NH_4Cl; C, control.
ND, not detectable.
[a]Significantly different from the 0 h/C value, $P<0.05$; [b]significantly different from the 12 h/N value, $P<0.05$; [c]significantly different from the 24 h/N value, $P<0.05$; [d]significantly different from the 48 h/N value, $P<0.05$.



Ammonia content in the muscle and liver was 18.9 and 17.5 μmol g^{-1}, respectively, after a 48 h exposure. In comparison, the plasma ammonia level rose abruptly in the first 12 h of ammonia exposure and reached 4.2 μmol ml^{-1} by 48 h (Fig. 1A). There were no increases in urea level in the muscle, liver or plasma (Fig. 1B). The urea excretion rate of the specimen kept under control condition was 0.75±0.12 μmol day^{-1} g^{-1} (*N*=5), which was unaffected by exposure to 30 mmol l^{-1} NH_4Cl.

The total free amino acid (TFAA) concentrations in the muscle (Table 2), liver (Table 3) and plasma (Table 4) were unaffected by ammonia exposure. However, there were minor changes in the levels of some free amino acids in these tissues. In the muscle, there was a transient increase in alanine level from 1.3 to 5.2 μmol g^{-1} after 12 h of ammonia exposure, but

Fig. 2. Effects of ammonia loading (10 mmol l^{-1} or 20 mmol l^{-1} NH_4Cl) on (A) the NH_3 volatilization rate (μmol day^{-1}) and (B) the pH of the surrounding medium of *Misgurnus anguillicaudatus*. Open columns represent the control condition (no fish). Filled columns represent the experimental condition (with fish). Values are means + S.E.M. (*N*=4). *Significantly different from the corresponding control condition, $P<0.05$.

Table 3. *Effects of various periods of ammonia exposure on the contents of various free amino acids (FAAs) and the total FAA (TFAAs) in the liver of* Misgurnus anguillicaudatus

	FAA concentration (μmol g^{-1})				
FAA	0 h/C	12 h/N	24 h/N	48 h/N	48 h/C
Ala	0.59±0.04	0.70±0.02	0.51±0.08	0.69±0.10	0.49±0.10
Arg	0.19±0.03	0.11±0.01	0.07±0.02[a,b]	0.059±0.004[a]	0.10±0.05
Asn	0.48±0.15	0.25±0.03[a]	0.14±0.01[a]	0.15±0.01[a]	ND[a]
Asp	1.4±0.4	1.1±0.1	0.70±0.06	0.65±0.07	ND
Cys	ND	0.03±0.01	0.04±0.01	0.06±0.03	ND
Gln	2.3±0.7	1.8±0.3	2.0±0.1	3.0±0.5	1.4±0.4
Glu	1.8±0.2	3.6±0.2[a]	3.2±0.2[a]	5.5±0.4[a,b,c]	3.3±0.4[a,d]
Gly	0.77±0.13	0.78±0.15	0.38±0.03	0.40±0.11	0.75±0.06
His	0.28±0.05	0.34±0.03	0.16±0.01[a,b]	0.16±0.01[a,b]	0.15±0.05[a,b]
Ile	0.30±0.07	0.27±0.02	0.18±0.02	0.08±0.03	0.23±0.08
Leu	0.63±0.16	0.45±0.03	0.33±0.03	0.21±0.28[a]	0.40±0.14
Lys	0.83±0.10	0.62±0.04[a]	0.27±0.04[a,b]	0.23±0.02[a,b]	0.33±0.09[a,b]
Met	ND	0.07±0.01[a]	0.02±0.01[b]	0.012±0.008[b]	ND[b]
Orn	ND	0.06±0.01[a]	0.021±0.005[a,b]	0.011±0.006[b]	ND[b]
Phe	0.26±0.05	0.20±0.02	0.15±0.02	0.08±0.01[b]	0.30±0.03[c,d]
Pro	0.38±0.03	0.29±0.04	0.19±0.02	0.13±0.01[a]	0.48±0.09[b,c,d]
Ser	0.68±0.16	0.39±0.02[a]	0.20±0.02[a]	0.20±0.01[a]	0.33±0.04[a]
Tau	11.8±2.3	11.4±0.7	10.5±0.7	8.6±0.2	11.5±1.2
Thr	1.2±0.3	0.86±0.10	0.37±0.08	0.28±0.03	0.76±0.35
Try	ND	0.035±0.002[a]	0.008±0.005[b]	0.011±0.004[b]	0.015±0.011[b]
Tyr	0.11±0.01	0.13±0.05	0.087±0.011	0.089±0.029	0.11±0.01
Val	0.53±0.14	0.59±0.02	0.35±0.05	0.31±0.04	0.50±0.10
TFAA	24.4±3.2	24.6±1.0	19.8±0.7	20.9±0.8	21.0±2.4

Values are means ± S.E.M. (N=4).
N, 30 mmol l^{-1} NH_4Cl; C, control.
ND, not detectable.
[a]Significantly different from the 0 h/C value, P<0.05; [b]significantly different from the 12 h/N value, P<0.05; [c]significantly different from the 24 h/N value, P<0.05; [d]significantly different from the 48 h/N value, P<0.05.

it decreased thereafter. This was accompanied by transient increases in the levels of some essential amino acids. Ammonia exposure did not affect glutamine levels in the muscle, liver or plasma, but led to an increase in the glutamate level in the liver and a decrease in the glutamate level in the plasma.

With 20 mmol l^{-1} NH_4Cl in the external medium, the amount of ammonia recovered from the acid trap in the presence of *M. anguillicaudatus* after a 24 h period was significantly higher than that of the blank without a fish (Fig. 2A). However, this difference disappeared when the fish was prevented from accessing air (data not shown). With a fish in the medium, the pH recorded in the presence of 20 mmol l^{-1} NH_4Cl after 24 h was significantly lower than that of the blank without fish (Fig. 2B). For fish exposed to 30 mmol l^{-1} NH_4Cl, the mucosal surface pH of the anterior portion of the digestive tract was

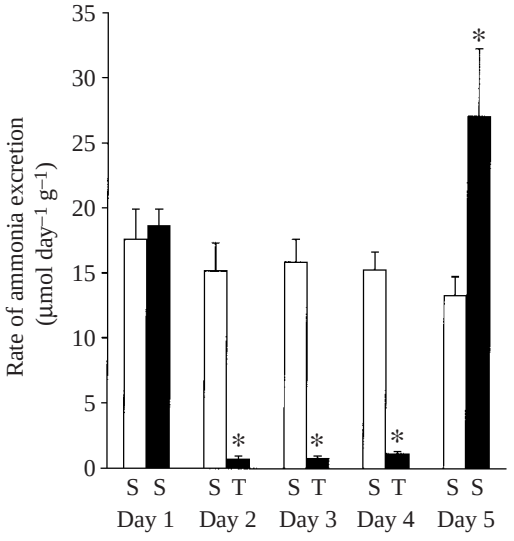


Fig. 3. Effects of aerial exposure on the ammonia excretion rate (μmol day^{-1} g^{-1}) of *Misgurnus anguillicaudatus*. Open columns represent the control condition. Filled columns represent the experimental condition. S, submerged; T, aerial exposure. Values are means + S.E.M. (N=4). *Significantly different from the corresponding control condition, P<0.05.

Table 4. *Effects of various periods of ammonia exposure on the contents of various free amino acids (FAAs) and the total FAA (TFAAs) in the blood plasma of* Misgurnus anguillicaudatus

	FAA concentration (μmol ml^{-1})				
FAA	0 h/C	12 h/N	24 h/N	48 h/N	48 h/C
Ala	0.29±0.04	0.27±0.02	0.25±0.04	0.28±0.02	0.22±0.03
Arg	0.10±0.01	0.083±0.004	0.07±0.01	0.065±0.005[a]	0.09±0.01[d]
Asn	0.10±0.01	0.094±0.004	0.09±0.01	0.060±0.004[a,b]	0.13±0.01[a,b,c,d]
Asp	0.05±0.01	0.027±0.002[a]	0.022±0.002[a]	0.032±0.003[a]	0.008±0.004[a,b,d]
Cys	0.009±0.001	0.013±0.001	0.010±0.001	0.011±0.002	0.009±0.001
Gln	0.23±0.02	0.48±0.02[a]	0.44±0.03[a]	0.38±0.03[a]	0.57±0.08[a,d]
Glu	0.16±0.02	0.08±0.01[a]	0.06±0.01[a]	0.09±0.01[a]	0.08±0.01[a]
Gly	0.34±0.03	0.28±0.03	0.24±0.02[a]	0.21±0.01[a]	0.25±0.01[a]
His	0.27±0.02	0.14±0.01[a]	0.11±0.01[a]	0.114±0.004[a]	0.16±0.01[a]
Ile	0.30±0.02	0.29±0.04	0.35±0.05	0.34±0.04	0.29±0.03
Leu	0.49±0.03	0.52±0.07	0.63±0.10	0.58±0.06	0.41±0.06
Lys	0.30±0.03	0.15±0.01[a]	0.16±0.01[a]	0.18±0.02[a]	0.15±0.02[a]
Met	0.05±0.01	0.047±0.002	0.039±0.002[a]	0.030±0.002[a,b]	0.042±0.003
Orn	0.05±0.02	0.023±0.004[a]	0.019±0.003[a]	0.019±0.002[a]	0.015±0.002[a]
Phe	0.10±0.01	0.073±0.004[a]	0.074±0.006[a]	0.066±0.005[a]	0.066±0.007[a]
Pro	0.078±0.006	0.056±0.007	0.062±0.003	0.055±0.002	0.088±0.008[b,c,d]
Ser	0.20±0.04	0.12±0.01	0.132±0.004	0.093±0.005[a]	0.15±0.02
Tau	0.47±0.08	0.42±0.04	0.27±0.03[a]	0.36±0.03	0.35±0.01
Thr	0.40±0.05	1.11±0.75	0.28±0.03	0.17±0.02	0.43±0.04
Try	0.043±0.002	0.074±0.019	0.052±0.004	0.082±0.018	0.054±0.007
Tyr	0.076±0.007	0.071±0.005	0.068±0.008	0.10±0.02	0.080±0.007
Val	0.49±0.03	0.50±0.06	0.58±0.08	0.48±0.09	0.48±0.04
TFAA	4.6±0.4	4.9±0.8	4.0±0.3	3.8±0.3	4.1±0.2

Values are means ± S.E.M. (N=4).

N, 30 mmol l^{-1} NH_4Cl; C, control.

[a]Significantly different from the 0 h/C value, P<0.05; [b]significantly different from the 12 h/N value, P<0.05; [c]significantly different from the 24 h/N value, P<0.05; [d]significantly different from the 48 h/N value, P<0.05.

significantly higher (more alkaline) than that of the control (Table 5); however, the pH values of the middle and posterior regions of the intestine were unaffected by ammonia exposure (Table 5). Air exposure caused a larger increase in pH of the anterior intestine than ammonia exposure in water. The pH of the skin surface during aerial exposure was significantly higher than that of submerged fish (Table 5).

Aerial exposure led to a marked decrease in the rate of ammonia excretion (Fig. 3). At the same time, there was a progressive increase in the amount of NH_3 volatilized (Fig. 4). The pH of the film of water underlying the fish in air increased from an initial value of 7.2 to between 7.9 and 8.1. Increasing the environmental temperature to 30 °C increased the rate of NH_3 volatilization by *M. anguillicaudatus* exposed to terrestrial conditions (Table 6).

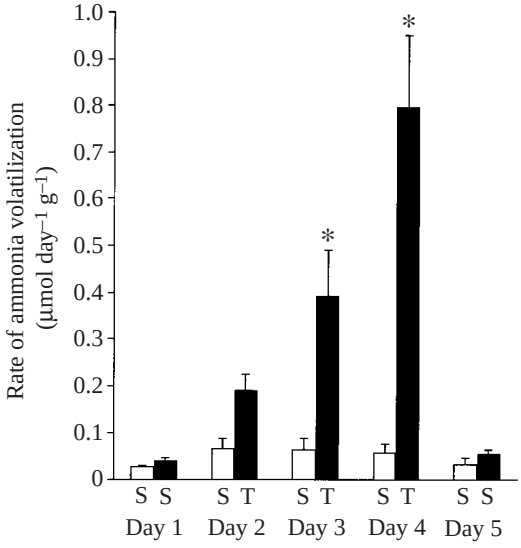


Fig. 4. Effects of aerial exposure on the NH_3 volatilization rate (μmol day^{-1} g^{-1}) of *Misgurnus anguillicaudatus*. Open columns represent the control condition. Filled columns represent the experimental condition. S, submerged; T, aerial exposure. Values are means + S.E.M. (N=4). *Significantly different from the corresponding control condition, P<0.05.

Discussion

M. anguillicaudatus inhabits muddy swamps and ponds and rice fields subject to periodic drying and has to solve problems of ammonia toxicity under two situations. First, to survive drought, *M. anguillicaudatus* burrows into the mud and breathes air through a hole in the surface of the mud. In the

Table 5. *Effects of 48 h of ammonia exposure (30 mmol l^{-1} NH_4Cl) and air exposure on the pH of the skin and the different parts of the digestive tract of* Misgurnus anguillicaudatus

	Control	Ammonia-exposed	Air-exposed
Skin pH	6.65±0.09 (5)	6.61±0.12 (5)	8.23±0.18 (6)*
Anterior pH	7.40±0.03 (4)	7.93±0.08 (5)*	8.23±0.10 (6)*
Middle pH	7.32±0.05 (4)	7.55±0.06 (5)†	7.68±0.10 (6)†
Posterior pH	7.33±0.05 (3)	7.41±0.11 (5)†	7.70±0.13 (6)†

Values are means ± S.E.M. with number of determinations in parentheses.

*Significantly different from the corresponding control, $P<0.05$; †significantly different from the anterior digestive tract in the same condition, $P<0.05$.

absence of water, ammonia excretion may be impeded, leading to the accumulation of ammonia in its tissues. Second, many farmers add fertilizers to the rice paddies, which may release ammonium salts into the environment and expose the loach to high levels of environmental ammonia.

It is well known that the primary route of ammonia excretion in fish is through the gills (Wood, 1993; Wilkie, 1997). The principal mechanism of ammonia excretion across the gill epithelia is the non-ionic diffusion of NH_3 down a favourable transbranchial gradient of NH_3 from blood to water (Randall and Wright, 1987). An increase in external NH_3 concentration, if large enough, will result in the reversal of the blood-to-water NH_3 gradient, leading to net NH_3 influx, an inhibition of excretion of endogenous ammonia and a rise in the total plasma ammonia level in the body (Wood, 1993; Wilkie, 1997).

Although the ammonia level in the plasma of *M. anguillicaudatus* in the submerged condition falls within the range of those of other facultative air-breathers, it was unexpectedly high in ammonia-exposed (4.2 μmol ml^{-1}) and air-exposed (5.09 μmol ml^{-1}) individuals (Chew et al., 2001) after 48 h of exposure. The ammonia levels in the plasma of *Amphipnous euchia*, *Clarias batrachus*, *Heteropneustes fossilis*, *Anabas testudineus* and *Channa punctatus* range from 0.43 to 0.89 μmol ml^{-1} (Saha and Ratha, 1989). The plasma of the tilapia *Oreochromis nilotica* (in ammonia-loading conditions), the climbing gourami *Anabas scandens* and the snakehead *Channa gachua* (in terrestrial conditions) contained 1.0, 1.6 and 1.6 μmol ml^{-1} ammonia, respectively (Randall et al., 1989; Ramaswamy and Reddy, 1983). To our knowledge, no other fish have been reported to exhibit such a high ammonia concentration in the plasma under ammonia-loading or terrestrial conditions.

Tissue ammonia levels increased markedly in *M. anguillicaudatus* exposed to NH_4Cl in water or in air. The levels in the muscle and liver reached 18.9 μmol g^{-1} and 17.5 μmol g^{-1}, respectively, following NH_4Cl exposure, and 11.08 and 14.52 μmol g^{-1}, respectively, after 48 h of aerial exposure (Chew et al., 2001). In the mudskipper *Periophthalmodon schlosseri*, 3.46 and 1.64 μmol g^{-1} ammonia were accumulated in the liver and muscle, respectively, after 24 h of aerial exposure (Ip et al., 1993) and 7.24 μmol g^{-1} and 14.8 μmol g^{-1}, respectively, after 6 days of exposure to 100 mmol l^{-1} NH_4Cl (Randall et al., 1999). In the portunid crab *Necora puber*, the muscle ammonia content was 10.42 μmol g^{-1} after 12 h of aerial exposure (Durand and Regnault, 1998). Clearly, *M. anguillicaudatus* can tolerate much higher levels of ammonia in its tissues and organs than can other animals, and future research on these ammonia-tolerant processes may increase our understanding of the cellular mechanisms of ammonia toxicity. For most vertebrates, such high levels of ammonia would disturb intracellular pH and membrane integrity (Campbell, 1991), leading to convulsions and death. Working on mammals, Marcaida et al. (1992) proposed that ammonia toxicity was due to the activation of *N*-methyl-D-aspartate (NMDA)-type glutamate receptors, leading to elevated intracellular Ca^{2+} concentrations and cell death. Perhaps *M. anguillicaudatus* has modified NMDA receptors.

Levels of free amino acids (FAAs) were variable in both the ammonia-exposed and control groups. The fish were not fed during the experiment, and this may explain the significant increases in levels of a number of FAAs observed after 48 h of

Table 6. *Effects of temperature on the ammonia excretion rate, NH_3 volatilization rate, proportion of NH_3 volatilized and pH of the ambient water of* Misgurnus anguillicaudatus *on the third day of air exposure*

Temperature (°C)	Volume of water (ml)	Rate of excretion of NH_4^+ into the water (μmol day^{-1} g^{-1})	Rate of NH_3 volatilization (μmol day^{-1} g^{-1})	(NH_3 volatilized × 100)/ (NH_4^+ in water+ NH_3 volatilized)	pH of water
20	1	1.13±0.31 (4)	0.429±0.036 (4)	31.8±7.7 (4)	8.05±0.24 (4)
25	1	1.14±0.14 (8)	0.795±0.155 (8)	41.0±6.3 (8)	8.07±0.08 (8)
30	1	1.15±0.04 (4)	1.67±0.43 (4)[a]	56.8±5.6 (4)	8.01±0.13 (4)
25	3	2.34±0.19 (4)[a,b,c]	0.867±0.259 (4)	26.2±6.5 (4)[c]	7.85±0.05 (4)

Values are means ± S.E.M. with number of determinations in parentheses.

[a]Significantly different from value at 20 °C with 1 ml of water, $P<0.05$; [b]significantly different from value at 25 °C with 1 ml of water, $P<0.05$; [c]significantly different from value at 30 °C with 1 ml of water, $P<0.05$.

fasting (Table 2). This somewhat obscures the FAA response to ammonia exposure. However, it is clear that there was a transient increase in alanine level in the muscle after 12 h of exposure to 30 mmol l^{-1} NH_4Cl. *M. anguillicaudatus* might have resorted to partial amino acid catabolism at the onset of exposure to NH_4Cl to slow down the rate of internal ammonia production. Ip et al. (2001a,b) proposed partial amino acid catabolism as a strategy for fish to reduce the rate of production of endogenous ammonia during aerial exposure. The amino groups of certain amino acids are transferred directly or indirectly to pyruvate to form alanine. The resulting carbon chain enters the Krebs cycle and is partially oxidised to malate, which replenishes pyruvate through the reaction catalysed by malic enzyme. In this way, ATP can be derived from amino acids without producing ammonia.

Glutamine has been found to play a role in ammonia detoxification in fish in response to high environmental ammonia concentrations (Arillo et al., 1981; Dabrowska and Wlasow, 1986; Mommsen and Walsh, 1992; Peng et al., 1998). In this regard, *M. anguillicaudatus* is atypical, for although glutamine accumulated in its muscle after 48 h of aerial exposure, accumulation of glutamine did not occur in muscle and liver when specimens were exposed to ammonia. Hence, even if the mechanisms to detoxify endogenous ammonia were present, they were not utilised when the fish were confronted with an influx of exogenous ammonia.

The levels of urea in various tissues and the urea excretion rate of *M. anguillicaudatus* exposed to ammonia remained unchanged during ammonia loading. The activities of glutamine synthetase and ornithine–urea cycle enzymes were too low to render them effective in detoxifying ammonia (Chew et al., 2001). The production of urea as a strategy to detoxify ammonia in response to aerial exposure or ammonia loading does not appear to be a common phenomenon in teleosts (Ip et al., 2001a). This is probably because glutamine and urea production are energy-expensive processes, involving 4 and 5 moles of ATP (or equivalent), respectively, per mole of product formed (Ip et al., 2001a). It would appear that *M. anguillicaudatus* simply tolerated high tissue ammonia levels. In addition, it lowered the pH of the medium during ammonia exposure. This would reduce the NH_3 concentration in the external medium and decrease the rate of ammonia influx.

Aerial exposure resulted in a marked reduction in the rate of ammonia excretion. Presumably, ammonia is excreted across the gills into the water, and this is inhibited during air exposure. A small amount of ammonia is volatilized into the air when the animal is submerged (see Fig. 4). The amount excreted by volatilization increases when ammonia is added to the water and during aerial exposure. NH_3 volatilization has also been observed in the mangrove killifish *Rivulus marmoratus* in response to prolonged air exposure (Frick and Wright, 2002). *M. anguillicaudatus* can breathe air using its intestine (McMahon and Burggren, 1987), gulping air with its mouth and blowing gas out of the vent. Our results indicate that *M. anguillicaudatus* was capable of excreting some ammonia in gaseous form *via* the gut during ammonia-loading. A small quantity of ammonia was volatilized from the NH_4Cl solution in the absence of a fish. This is understandable because a small fraction (0.6 %) of ammonia was present as NH_3 even at pH 7, and it would be carried over by the air current into the acid trap. The fraction of NH_3 present in solution decreases with decreasing pH. Since there was a significant reduction in the pH of the external medium in the presence of a fish, the amount of ammonia volatilized should theoretically be smaller if it was due only to volatilization from the water. However, that was not the case: volatilization increased in the presence of the fish. Furthermore, *M. anguillicaudatus* stopped volatilizing NH_3 when it was prevented from accessing the water surface to gulp air. Hence, it can be concluded that the excess NH_3 volatilized represented ammonia excreted from the fish into the air passing through its gut and blown out of the vent. If it had been excreted across the skin into the water, it would have appeared even when the fish was forcibly prevented from breathing air.

In *M. anguillicaudatus*, the anterior portion of the digestive tract became more alkaline in ammonia-loaded and air-exposed fish. Alkalization would raise the fraction of NH_3 in the ammonia excreted into that region of the digestive tract, and this would augment NH_3 volatilization. When *M. anguillicaudatus* was exposed to terrestrial conditions, the surface of the skin also became significantly more alkaline, and the pH of the water underneath the fish was significantly higher than that of the submerged control. Thus, the skin of *M. anguillicaudatus* may also be a site of NH_3 volatilization during aerial exposure. Similarly, Wieser (1972b) suggested that, in isopods, ammonia diffuses into the film of water retained between the pleopods and is volatilized as a result of alkalization of the solution. Some terrestrial crabs have also been reported to volatilize NH_3 by alkalization of urine (De Vries and Wolcott, 1993; Varley and Greenaway, 1994). Although the skin and digestive tract may both be involved in NH_3 volatilization when the fish is moving on land, excretion of ammonia through the skin would be ineffective when the fish burrows into the mud. It seems probable that, when the fish is surrounded by mud, the digestive tract might become the only avenue for NH_3 volatilization.

For certain isopods (Wieser, 1972a) and for *M. anguillicaudatus* exposed to terrestrial conditions, the rate of NH_3 volatilization increases with temperature. Although the amount of ammonia excreted into the water remained the same at different temperatures, the increase in the rate of NH_3 volatilization led to increases in total ammonia excretion at higher temperatures (Table 6). The observed effects are probably related to the direct effect of temperature on the process of volatilization.

The progressive increase in the amount of NH_3 volatilized by *M. anguillicaudatus* over a 3-day period was apparently related to the increased internal ammonia levels. Besides building up ammonia to a very high level in the plasma during aerial exposure, the blood pH simultaneously became more alkaline. This would lead to a high level of NH_3 in the blood. The high P_{NH_3} gradient would facilitate the efflux of ammonia onto the body surface. Thus, the increase in ammonia

volatilization seen during periods of elevated ammonia level in the water and aerial exposure are related to the alkalization of the gut and blood and the increase in total ammonia concentrations in the body, all raising NH_3 levels and, therefore, ammonia volatilization. This is in agreement with the observation of Wright and O'Donnell (1993) on the isopod *Porcellio scaber* that periodic volatilization of NH_3 was correlated with a large increase in ammonia levels in the haemolymph and pleon fluid.

The loach *Misgurnus anguillicaudatus* is unusual in that it tolerates very high ammonia levels in its tissues, but in turn these high levels, together with alkaline body surfaces, permit a significant ammonia excretion by volatilization during aerial exposure.

References

Arillo, A., Margiocco, C., Medlodia, F., Mensi, P.and Schenone, G. (1981). Ammonia toxicity mechanisms in fish: studies on rainbow trout (*Salmo gairdneri* Richardson). *Ecoltoxicol. Environ. Saf.* **5**, 316–328.

Campbell, J. W. (1991). Excretory nitrogen metabolism. In *Experimental and Metabolic Animal Physiology. Comparative Animal Physiology*, fourth edition (ed. C. L. Prosser), pp. 277–324. New York: Wiley-Interscience.

Chew, S. F., Jin, Y. and Ip, Y. K. (2001). The loach *Misgurnus anguillicaudatus* reduces amino acid catabolism and accumulates alanine and glutamine during aerial exposure. *Physiol. Biochem. Zool.* **74**, 226–237.

Dabrowska, H. and Wlasow, T. (1986). Sublethal effect of ammonia on certain biochemical and haematological indicators in common carp (*Cyprinus carpio* L.). *Comp. Biochem. Physiol.* **83C**, 179–184.

De Vries, M. C. and Wolcott, D. L. (1993). Gaseous ammonia evolution is coupled to reprocessing of urine at the gills of ghost crabs. *J. Exp. Zool.* **267**, 97–103.

Durand, F. and Regnault, M. (1998). Nitrogen metabolism of two portunid crabs, *Carcinus maenas* and *Necora puber*, during prolonged air exposure and subsequent recovery: a comparative study. *J. Exp. Biol.* **201**, 2515–2528.

Faupel, R. P., Seitz, H. J., Tarnowsk, W., Thiemann, V. and Weiss, C. (1972). Problem of tissue sampling from experimental animals with respect to freezing techniques, anoxia, stress and narcosis – new method for sampling rate liver tissue and physiological values of glycolytic intermediates and related compounds. *Arch. Biochem.* **148B**, 509–522.

Frick, N. T. and Wright, P. T. (2002). Nitrogen metabolism and excretion in the mangrove killifish *Rivulus narmoratus.* I. The influence of environmental salinity and external ammonia. *J. Exp. Biol.* **205**, 79–89.

Ip, Y. K., Chew, S. F. and Randall, D. J. (2001a). Ammonia toxicity, tolerance and excretion. In *Fish Physiology*, vol. 19 (ed. P. A. Wright and P. M. Anderson), pp. 109–148. New York: Academic Press.

Ip, Y. K., Lee, C. Y., Chew, S. F., Low, W. P. and Peng, K. W. (1993). Differences in the responses of two mudskippers to terrestrial exposure. *Zool. Sci.* **10**, 511–519.

Ip, Y. K., Lim, C. B., Chew, S. F., Wilson, J. M. and Randall, D. J. (2001b). Partial amino acid catabolism leading to the formation of alanine in *Periophthalmodon schlosseri* (mudskipper): a strategy that facilitates the use of amino acids as an energy source during locomotory activity on land. *J. Exp. Biol.* **204**, 1615–1624.

Jow, L. Y., Chew, S. F., Lim, C. B., Anderson, P. M. and Ip, Y. K. (1999). The marble goby *Oxyeleotris marmoratus* activates hepatic glutamine synthetase and detoxifies ammonia to glutamine during air exposure. *J. Exp. Biol.* **202**, 237–245.

Kun, E. and Kearney, E. B. (1974). Ammonia. In *Methods of Enzymatic Analysis*, vol. IV (ed. H. U. Bergmeyer and K. Gawehn), pp. 1802–1806. New York: Academic Press.

Marcaida, G., Felipo, V., Hermenegildo, C., Minana, M. D. and Grisolia, S. (1992). Acute ammonia toxicity is mediated by NMDA type of glutamate receptors. *FEBS Lett.* **296**, 67–68.

McMahon, B. R. and Burggren, W. W. (1987). Respiratory physiology of intestinal air breathing in the teleost fish *M. anguillicaudatus*. *J. Exp. Biol.* **133**, 371–393.

Mommsen, T. P. and Walsh, P. J. (1992). Biochemical and environmental perspectives on nitrogen metabolism in fishes. *Experientia* **48**, 583–593.

Peng, K. W., Chew, S. F., Lim, C. B., Kuah, S. S. L., Kok, W. K. and Ip, Y. K. (1998). The mudskippers *Periophthalmodon schlosseri* and *Boleophthalmus boddaerti* can tolerate environmental NH_3 concentrations of 446 and 36 μM, respectively. *Fish Physiol. Biochem.* **19**, 59–69.

Ramaswamy, M. and Reddy, T. G. (1983). Ammonia and urea excretion in three species of air-breathing fish subjected to aerial exposure. *Proc. Ind. Acad. Sci.* **92**, 293–297.

Randall, D. J., Wilson, J. M., Peng, K. W., Kok, T. W. K., Kuah, S. S. L., Chew, S. F., Lam, T. J. and Ip, Y. K. (1999). The mudskipper, *Periophthalmodon schlosseri*, actively transports NH_4^+ against a concentration gradient. *Am. J. Physiol.* **46**, R1562–R1567.

Randall, D. J., Wood, C. M., Perry, S. F., Bergman, H. L., Maloiy, G. M. O., Mommsen, T. P. and Wright, P. A. (1989). Urea excretion as a strategy for survival in a fish living in a very alkaline environment. *Nature* **337**, 165–166.

Randall, D. J. and Wright, P. A. (1987). Ammonia distribution and excretion in fish. *Fish Physiol. Biochem.* **3**, 107–120.

Saha, N. and Ratha, B. K. (1989). Comparative study of ureogenesis in freshwater air-breathing teleosts. *J. Exp. Zool.* **252**, 1–8.

Varley, D. G. and Greenaway, P. (1994). Nitrogenous excretion in the terrestrial carnivorous crab *Geograpsus grayi*: site and mechanism of excretion. *J. Exp. Biol.* **190**, 179–193.

Wieser, W. (1972a). O/N ratios of terrestrial isopods at two temperatures. *Comp. Biochem. Physiol.* **43A**, 859–868.

Wieser, W. (1972b). Oxygen consumption and ammonia excretion in *Ligia beaudiana* M.-E. *Comp. Biochem. Physiol.* **43A**, 869–876.

Wilkie, M. P. (1997). Mechanisms of ammonia excretion across fish gills. *Comp. Biochem. Physiol.* **118A**, 39–50.

Wood, C. M. (1993). Ammonia and urea metabolism and excretion. In *The Physiology of Fishes* (ed. D. H. Evans), pp. 379–425. Boca Raton: Cleveland Rubber Company Press.

Wright, J. C. and O'Donnell, M. J. (1993). Total ammonia concentration and pH of haemolymph, pleon fluid and maxillary urine in *Porcellio scaber* Lattreille (Isopoda, Oniscidea): relationships to ambient humidity and water vapour uptake. *J. Exp. Biol.* **176**, 233–246.