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Nitrogen metabolism and excretion in *Allenbatrachus grunniens* (L): effects of variable salinity, confinement, high pH and ammonia loading

P. J. Walsh*†, Z. Wei‡, C. M. Wood*§, A. M. Loong‡, K. C. Hiong‡, S. M. L. Lee‡, W. P. Wong‡, S. F. Chew¶ and Y. K. Ip‡

**NIEHS Marine and Freshwater Biomedical Sciences Center, Rosenstiel School of Marine and Atmospheric Science, University of Miami, 4600 Rickenbacker Causeway, Miami, FL 33149, U.S.A., ‡Department of Biological Sciences, National University of Singapore, 10 Kent Ridge Road, Singapore 117543, Republic of Singapore, §Department of Biology, McMaster University, Hamilton, Ontario, L8S 4K1, Canada and ¶Natural Sciences, National Institute of Education, Nanyang Technological University, 1 Nanyang Walk, Singapore 637616, Republic of Singapore*

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The nitrogen metabolism and excretion patterns of the grunting toadfish *Allenbatrachus grunniens* and the effects of salinity on these processes were examined. Individuals of *A. grunniens* were subjected to several experimental treatments, including variable salinity (2 to 30), high pH (8·5 compared to 7·0 for controls), high environmental ammonia (10 mM) and confinement to small water volumes, and measurements were made of activities of selected enzymes of nitrogen metabolism, ammonia and urea excretion rates, and tissue and plasma contents of ammonia, urea and amino acids. Activities of key ornithine-urea cycle enzymes were rather low (*e.g.* liver carbamoyl phosphate synthetase III activity was 0·001 μmols min^{-1} g^{-1}), and *A. grunniens* consistently demonstrated a low capacity for urea excretion despite significant elevations of plasma and tissue ammonia contents by the high pH and high ammonia treatments. This species could thus be categorized as ammoniotelic. Total free amino acid contents in plasma and tissues were increased by the high pH and high ammonia treatments, but no patterns were discerned in individual amino acids that would indicate any preferential accumulation (*e.g.* alanine and glutamine) as has been noted previously in several semi-terrestrial fish species. Thus, it appeared that *A. grunniens* was not unusual in its patterns of nitrogen metabolism and excretion in comparison to other 'typical' teleosts. Furthermore, manipulation of salinity had no major effects on nitrogen excretion in either this species or in comparative studies with the ureotelic gulf toadfish *Opsanus beta*. The results are discussed in the context of the broader pattern of nitrogen metabolism and excretion in the Batrachoididae.

Key words: *Allenbatrachus grunniens*; ammoniotely; Batrachoididae; carbamoyl phosphate synthetase; ornithine-urea cycle; ureotely.

†Author to whom correspondence should be addressed. Tel.: +1 305 421 4617; fax: +1 305 421 4001; email: pwalsh@rsmas.miami.edu

INTRODUCTION

The Batrachoididae (toadfish and midshipmen) are comprised of >19 genera and 69 species inhabiting temperate, sub-tropical and tropical environments worldwide (Nelson, 1994). While the family is mostly marine, many species are estuarine, encountering very low salinities (*e.g.* <1), and a few members of the family live in true freshwater habitats. One distinguishing physiological trait of some members of the family is that they can synthesize urea *via* a fully functional ornithine-urea cycle (O-UC) and in some cases will excrete nearly all of their nitrogenous waste as urea (Walsh, 1997; Wood *et al*., 2003) instead of the more typical end product of ammonia seen in other adult teleosts. To date, however, this trait has only been observed within one genus in the family (*Opsanus*) in the form of high O-UC enzyme activities and nearly complete ureotely for the gulf toadfish *Opsanus beta* (Goode & Bean) (Walsh, 1997; Wood *et al*., 2003), moderate O-UC enzyme activities and moderate ureotely for the oyster toadfish *Opsanus tau* (L.) (Read, 1971; Anderson & Walsh, 1995; Wang & Walsh, 2000) and varying degrees of ureotely and O-UC enzyme expression for other *Opsanus* species (J.F. Barimo & P.J. Walsh, unpubl. data). One other batrachoidid species that has been examined in depth, the plainfin midshipman *Porichthys notatus* Girard, has been shown to be much more like other teleosts in lacking significant O-UC enzyme activity (Anderson & Walsh, 1995) and excreting nearly all of its nitrogenous waste as ammonia under a variety of physiological circumstances (Walsh *et al*., 2001).

The ecological and evolutionary significance of urea synthesis and excretion in the *Opsanus* species has been the topic of a number of studies in recent years, and at least four hypotheses have been proposed to explain why these species are ureotelic (Hopkins *et al*., 1997; Walsh, 1997; Wood *et al*., 2003). An understanding of the rationale for why these species invest considerable energy (five ATPs per urea) in this process, however, remains elusive. Therefore, in addition to the many ongoing and published studies on the physiology and ecology of *O. beta*, a broader phylogenetic approach in the study of ureotely within the family Batrachoididae needs to be undertaken. The family is subdivided into three sub-families (Nelson, 1994): Batrachoidinae, which contains fifteen genera including the genus *Opsanus*, Porichthyinae, which contains the midshipman genera *Aphos* and *Porichthys* and Thalassophryninae, which contains the venomous toadfish genera *Daector* and *Thalassophryne*, in which the two freshwater species are also found [*Daector quadrizonatus* (Eigenmann) and *Thalassophryne amazonica* Steindachner] (Collette, 1966). At present only limited molecular phylogenetic information is available (Freshwater *et al*., 2000; Collette, 2001). Since the one enzymatic and physiological finding of a lack of ureotely was for *P. notatus*, which is in a different subfamily from *Opsanus*, it was necessary to examine another species within the same subfamily as *Opsanus*.

The grunting toadfish *Allenbatrachus grunniens* (L) is broadly distributed in the Indo-West Pacific, and is native to the coasts of Myanmar, India, Madagascar, Malaysia, Sri Lanka and Thailand (Greenfield, 1997; www.fishbase.org). As a member of the subfamily Batrachoidinae, it has been confirmed by molecular methods to be more closely related to the genus *Opsanus* than is *P. notatus* (Freshwater *et al*., 2000) and would thus appear to be one good choice for the

phylogenetic study of ureotely. Additionally, this species is known to thrive in extremely dilute environments (Kottelat, 1989) and a source of specimens from a population inhabiting a nearly freshwater system in Thailand was available in suitable numbers for physiological experiments. Members of the family examined to date have been shown to have a morphologically aglomerulate kidney (Marshall, 1929), however, with a limited number of functional glomeruli (McDonald *et al.*, 2000, 2002, 2003). Although the bulk of urea excretion in *O. beta* occurs *via* the gills (Wood *et al.*, 2003), it has been hypothesized that renal urea excretion may be important in water balance (Griffith, 1991). Thus, a second objective of the present study was to examine the effects of salinity variation on ureotely in this species (as well as in *O. beta* for reference to a well-characterized system) to determine if ureotely was correlated in some meaningful way with variations in salinity.

The present study examined several biochemical and physiological measures of nitrogen metabolism and excretion in *A. grunniens* under a variety of conditions known to induce ureotely in other fish species (*i.e.* confinement, ammonia exposure and high pH). Nitrogen metabolism and excretion were also examined in both very dilute and full strength sea water.

MATERIALS AND METHODS

COLLECTION AND MAINTENANCE OF TOADFISH

Allenbatrachus grunniens (*c.* 18 to 45 g, 10–30 cm total length, L_T) were purchased from Thailand (collected from Chao Phrya; *c.* 15° N; 103° E) through a local supplier (Qian Hu Fish Farm Trading Co., Singapore) and transported to the National University of Singapore (NUS). Their identity was confirmed by comparison to type specimens at the Raffles Museum, NUS (K.K.P. Lim, pers. comm.). Prior to experimentation, *A. grunniens* were held for several weeks in aquaria with water at a salinity of 2 [made with dechlorinated, by aeration, Singapore tap water with added sea water (salinity 30) transported to the NUS from the Singapore coast] at 25° C, range ± 2° C and a 12L : 12D photoperiod at a density of *c.* 1 kg fish to 10 l of water. Approximate ionic composition of this water in mmol l^{-1} was: Na^+ 27·4, Cl^- 31·9, Mg^{2+} 3·1, Ca^{2+} 0·6 and pH 7·1. Water in these holding aquaria was changed three times a week, and fish were fed every other day with small guppies *Poecilia reticulata* (Peters). Food was withheld for 48 h prior to, as well as during, experimentation. No attempt was made to distinguish the sex of the fish.

For a limited set of comparison experiments, gulf toadfish were obtained and held in flowing sea water at the Rosenstiel School at the University of Miami as previously described by Wood *et al.* (1995).

EXPOSURE OF FISH TO EXPERIMENTAL CONDITIONS

Series I. Baseline experiments and effects of high pH on A. grunniens

Preliminary measurements of nitrogen excretion in *A. grunniens* showed relatively low levels of urea excretion relative to ammonia excretion at a salinity of 2 and pH 7·0. Since a proton-trapping model for ammonia excretion is believed to play an important role in ammonia excretion in freshwater fishes (Wright *et al.*, 1989), raising the pH of the water may inhibit ammonia excretion (Wilkie, 2002) and activate or enhance ureotely in *A. grunniens*. For urea and ammonia excretion measurements, fish ($n = 5$ for each treatment) were weighed and placed individually in plastic tanks (25 × 14 × 12 cm) containing a measured volume of water *c.* ×60 the mass of the fish. The two groups for this series were a 'buffer control' (salinity 2 water with 10 mmol l^{-1} Tris adjusted to pH 7·0, with

2 mol l^{-1} HCl) and a 'high pH' (10 mM Tris adjusted to pH 8·5, with 2M NaOH); increases in Cl^- and Na^+ were insignificant relative to the background. pH was confirmed using an ATI Orion model 420A pH meter (Boston, MA, U.S.A.) and a Corning Combo w/RJ Tris-electrode (Halstead, Essex, U.K.). Excretion was monitored in the buffer control for 4 days, and in the high pH group for 1 day in the same control water, then 2 days at high pH, and then 1 day recovery in control water. Water was sampled for ammonia and urea determinations at the start and end of each 24 h flux period and then water was changed every 24 h for 4 days. For determinations of plasma and tissue metabolites and enzyme activities, two additional groups of fish were exposed to buffer control and high pH treatments at an approximate ratio of 100 for water to fish mass for 48 h (with one water change) and sacrificed.

Series II. Effect of high ammonia exposure on A. grunniens

Since external ammonia is an effective inhibitor of ammonia excretion and inducer of ureotely in other selected fish species, high ammonia conditions may induce or enhance ureotely in *A. grunniens*. Fish were set up in tanks for flux measurements (4 days) and metabolite and enzyme (2 days) determinations as in series I, but conditions in series II were water of salinity 2 (control) and water of salinity 2 plus 10 mM NH_4Cl, both with no added buffer and adjusted to pH 7·0. Ammonia concentrations were confirmed by assay. This concentration of ammonia was chosen since it represents approximate LC_{50} (concentration which is lethal to 50% of the test population) values for other batrachoidids at pH 8·0 in sea water (Wang & Walsh, 2000), and while not lethal at pH 7·0 it still represents a significant ammonia challenge to these fish.

Series III. Effect of confinement and high salinity on A. grunniens

Since confinement to small water volumes has been shown to induce glutamine synthetase (GSase) activity and to convert ammoniotelic gulf toadfish to ureotely, does a similar transition occur in *A. grunniens*? Furthermore, since *A. grunniens* is primarily marine, does a typical marine salinity activate or enhance ureotely? Two groups of six fish were kept under the same conditions as those used for acclimation one in water of salinity 2 and the other in water of salinity 30 for a total of 4 weeks. After this time, six fish from each group were crowded in groups (Wood *et al.*, 1995) in 4 l of either salinity 2 or 30 water, and not fed, and water was sampled and changed every 24 h for 3 days. Subsequently, fish were placed in individual aquaria (as in series I with 1 l of either salinity 2 or 30 water) and the water was sampled and changed every 24 h for an additional 2 days. An assay of ammonia and urea on sea water only confirmed that bacterial action on these compounds was minimal (*e.g.* <3% decrease in urea content was observed). Fish were then sacrificed for plasma and tissue analysis. Parallel groups of unconfined salinity 2 and 30 water fish were sacrificed at this time for plasma and tissue analysis.

Series IV. Effect of low and high salinity on O. beta

Since virtually all prior publications on ureotely with *O. beta* have been conducted in full strength sea water, even though the species tolerates low salinity well (Serafy *et al.*, 1997), the effect of salinity on ureotely in this reference species was tested. After crowding for 48 h to ensure ureotely (Wood *et al.*, 1995), individual gulf toadfish ($n = 6$–9 per group) were placed in plastic aquaria containing 2 l of filtered Biscayne Bay (BB) water (salinity 34), and water was collected at 0 and 24 h. Water was then changed with one group receiving salinity 34 water, one group receiving 20% strength sea water (salinity 34 water diluted with reverse osmosis prepared deionized water) or 200% strength sea water [prepared by adding an appropriate amount of 'Instant Ocean' (Marineland, Moorpark, CA, U.S.A.) to BB water] (pH was 8·1 in all cases). Water was sampled and changed every 24 h for 6 days. At the end of the experiment, fish were netted and blood was sampled by caudal puncture, and plasma used to determine total osmolality (Wescor Vapor Pressure Osmometer), urea and protein. Additionally fish were anaesthetized and sacrificed and livers frozen for analysis of GSase activity.

COLLECTION OF TISSUES AND PLASMA AND METABOLITE ANALYSIS

For the collection of tissues, fish were killed by a blow to the head. The lateral muscle, liver and brain were excised and immediately freeze-clamped in liquid nitrogen with pre-cooled aluminum tongs and wrapped in aluminum foil. The procedure was carried out within several minutes. Samples were stored at −80° C until analysis. For the collection of plasma, fish were stunned with a light blow to the head after which the caudal peduncle was severed and the blood was collected into heparinized (sodium) micro-haematocrit capillary tubes. The blood was then transferred to microcentrifuge tubes and centrifuged at 5000*g* for 5 min at 4° C (Beckman CS-15R). The supernatant was decanted and transferred to a fresh tube to which an equal volume of 6% trichloroacetic acid (TCA) was added. This mixture was centrifuged at 10 000*g* for 15 min at 4° C, and these deproteinized supernatants re-centrifuged for another 10 min, then decanted and frozen at −80° C until analysis. Frozen tissues were weighed and powdered at 0° C after which five volumes of ice-cold 6% TCA were added and the samples homogenized three times using an Ikawerk Staufen Ultra-Turrax homogenizer (Janke & Kunkel Grnbh & Co, Staufen, Germany) at maximum speed (24 000 rpm) for 20 s with off intervals of 10 s each. The samples were then centrifuged for 10 min at 10 000*g* and supernatants kept at −37° C until analysis up to 1 month later.

Tissue, plasma and water samples were analysed for total ammonia using the enzymatic method of Kun & Kearney (1974) using freshly prepared ammonium chloride as a standard. Change in absorbance was monitored at 340 nm using a Shimadzu UV-1601 spectrophotometer. Urea was determined spectrophotometrically by the diacetyl monoxime method of Rahmatullah & Boyde (1980) using urea as a standard.

For the analysis of free amino acids (FAAs) deproteinized samples were adjusted to pH 2·2 with 4M LiOH and diluted with an equal volume of 0·2M Li citrate buffer (pH 2·2). The samples were filtered and stored frozen at −35° C until analysis. Amino acids were quantified with a Shimadzu LC-6A Amino Acid Analyzer with Shimpack ISC-07/S1504 Li type column. An analytical grade amino acid standard (Sigma) was used for comparison.

ANALYSIS OF ENZYME ACTIVITIES AND PROTEIN

Frozen tissue samples were homogenized three times in five volumes of ice-cold buffer containing 50 mM Imidazole (pH 7·4), 50 mM NaF, 3 mM EGTA, 3 mM EDTA and 0·5 mM phenylmethylsulphonylfluoride (PMSF) in the Ultra-Turrax as above. Homogenates were centrifuged at 10 000*g* for 15 min at 4° C in a Beckman J2-21M/E centrifuge. The supernatant was passed through a 10 ml BIORAD desalting column (BIORAD Laboratories, Inc, CA, U.S.A.) equilibrated with buffer without PMSF. Enzyme activities were measured on a Shimadzu UV-160 UV-VIS spectrophotometer at 25° C, using the methods of Ip *et al*. (1993) for glutamate dehydrogenase (GDH), Shankar & Anderson (1985) for GSase and Peng *et al*. (1994) for alanine amino transferase (ALT), aspartate amino transferase (AST), malate dehydrogenase (MDH) and malic enzyme (ME). The methods of Anderson & Walsh (1995) were used for the O-UC enzymes carbamoyl phosphate synthetase (CPSase), ornithine transcarbamoylase (OTC), argininosuccinate synthase and lyase (AS/AL) and arginase (ARG). Protein was measured by the method of Bradford (1976) using bovine serum gamma globulin as a standard.

STATISTICAL ANALYSIS

Results are presented as means + S.E. *t*-tests and one-way ANOVA with Student–Newman–Keul multiple range tests were used to compare difference between means where applicable, with $P < 0{\cdot}05$ level used for significance.

RESULTS

This study began with an initial screen for O-UC activity in liver and muscle in *A. grunniens*. While values of OTC, AS/AL and ARG were reasonably high, activities of CPSase were either not detectable or extremely low (CPSase III) relative to those previously reported in *O. beta* (Table I) and appeared not able to support *de novo* urea synthesis *via* an O-UC (Table I). In contrast, arginase activity in *A. grunniens* was higer than in *O. beta*.

Fasting for 4 days had no significant effects on the rates of ammonia and urea excretion in *A. grunniens* (unpubl. data). In the experiments of series I, ammonia excretion rates outweighed urea excretion rates in *A. grunniens* under both control and high pH conditions (Fig. 1). Although high pH markedly depressed ammonia excretion rates, there was not a corresponding increase in urea excretion rates (Fig. 1). Notably, ammonia excretion remained depressed during the 1 day of recovery at low pH.

High pH treatment had no effect on urea concentrations in the plasma, muscle and liver, but marked increases on ammonia concentrations were seen (Fig. 2). Notably, the brain appeared to be somewhat protected from these increases in that the elevation was not statistically significant (Fig. 2). There were numerous significant effects on both individual and total amino acid concentrations in muscle, liver, brain and plasma (Table II). The most interesting changes were as two and a half to five-fold increases in muscle amino acids, including both essential and non-essential ones, most notably in the large increases in alanine and total FAAs.

The data for nitrogen excretion and nitrogenous metabolites were used to create an approximate nitrogen balance sheet for a hypothetical 30 g *A. grunniens* (Table III) according to the methods of Ip *et al.* (2001*a*, *b*). It was estimated that a 30 g fish would have 18 g of muscle and 0·8 g of liver. Based on the fasting control (unpubl. data), the amount of ammonia excreted for a hypothetical 30 g fish was estimated to be 441 μmol N (Tables III and IV). This analysis indicates that while a significant portion of the reduction in ammonia excretion caused by

TABLE I. Mass specific activities (mean ± S.E., $n = 3$) of enzymes involved in urea synthesis *de novo* from the liver of *Allenbatrachus grunniens* (in salinity 2 fresh water) in comparison with those from *Opsanus beta* (in salinity 34 sea water)

	Activities ($\mu mol\,min^{-1}\,g^{-1}$ wet mass)	
Enzymes	*A. grunniens*	*O. beta**
Glutamine synthetase	0·69 ± 0·07	6·97 ± 3·10
Carbamoyl phosphate synthetase (Glutamine + NAG + UTP)	0·001 ± 0·001	0·50 ± 0·16
Ornithine transcarbamoylase	37·4 ± 2·4	72·3 ± 23·1
Argininosuccinate synthetase + lyase	0·39 ± 0·02	0·60 ± 0·11
Arginase	240 ± 14	12·3 ± 3·0

*, results obtained from Anderson & Walsh (1995). NAG, N-acetyl-L-glutamate; UTP, uridine triphosphate.

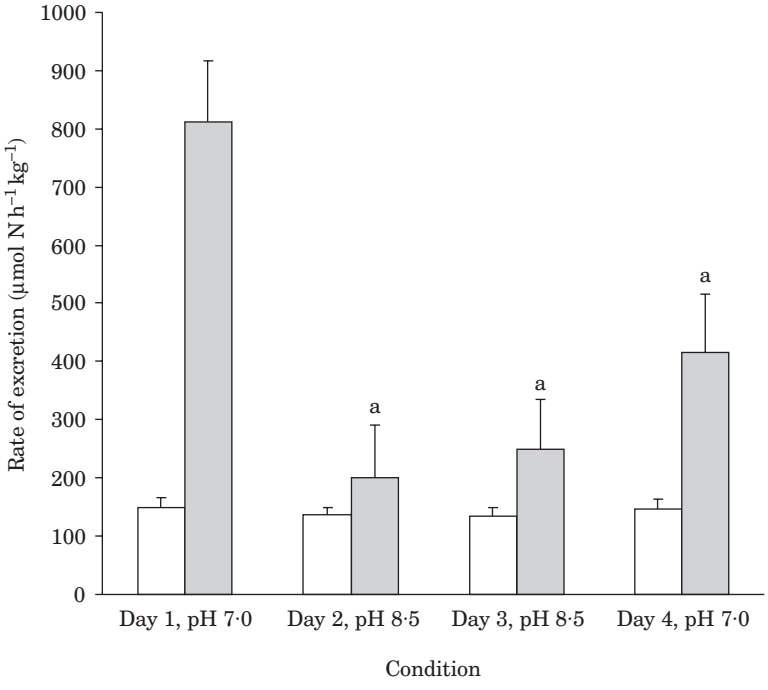

FIG. 1. Effects of 2 days of exposure to alkaline pH (salinity 2 water containing 10 mmol l^{-1} Tris-HCl at pH 8·5) followed by 1 day of recovery in water of neutral pH (salinity 2 water containing 10 mmol l^{-1} Tris-HCl at pH 7·0) on the rates of urea (□) and ammonia (■) excretion in *Allenbatrachus grunniens*. Values are means + S.E. ($n = 5$). a, significantly different from the day 1 control value, $P < 0{\cdot}05$.

high pH was compensated by an increase in amino acids accumulated in the muscle, a significant portion went uncompensated, which amounted to ($-203 + 123$ μmol N) or -80 μmol N for a 30 g fish over 2 days at high pH.

High pH treatment led to an increase in liver GSase activity, and a reduction in muscle ALT activity, but no other changes in muscle or liver enzyme activities (Table V). The GSase activities in the brain of specimens exposed to high pH ($143{\cdot}2 \pm 5{\cdot}1$ μmol min^{-1} g^{-1}) was not significantly different from that of the control ($148{\cdot}5 \pm 5{\cdot}3$ μmol min^{-1} g^{-1}).

Exposure of *A. grunniens* to 10 mM ammonia levels in series II led to a significant depression in urea excretion during day 2 and 3 which returned to the prior level during recovery in day 4 (Fig. 3). Notably, control rates of urea excretion in series II fish were not different from rates in series I, suggesting that there was no effect of buffering alone on urea excretion. Ammonia excretion levels were not determined in the ammonia-exposed fish due to the inherent difficulties of measuring a small increase in water ammonia against the large background concentration of 10 mM. It is reasonable to assume, however, that ammonia excretion would have been nearly completely impeded; the amount of ammonia, which would theoretically be excreted by a hypothetical 30 g specimen within a 48 h period, was estimated to be 441 μmol N (calculated from Fig. 1).

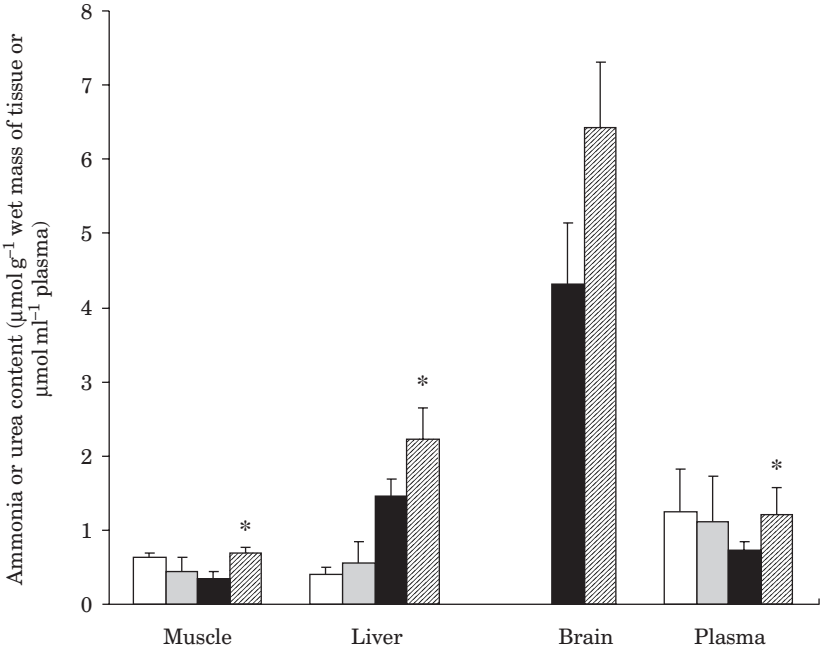

FIG. 2. Contents of urea (□, control; ▨, experimental) and ammonia (■, control; ▨, experimental) in the muscle, liver, brain and plasma of *Allenbatrachus grunniens* exposed to the control condition (salinity 2 water containing 10 mmol l^{-1} Tris-HCl at pH 7·0) or alkaline pH (salinity 2 water containing 10 mmol l^{-1} Tris-HCl at pH 8·5) for 2 days. Values are means + S.E. ($n = 5$). *, significantly different from the corresponding control, $P < 0{\cdot}05$.

Ammonia exposure led to similar significant elevations in tissue and plasma ammonia content (Fig. 4) compared to the high pH treatment, and similarly no significant changes in urea concentrations (Fig. 4). Notably this treatment increased brain ammonia concentration significantly (Fig. 4). Ammonia exposure had similar effects to those of high pH on muscle amino acid concentrations, notably the large increase in muscle alanine and total FAA (Table VI). Unlike alkaline pH, however, ammonia exposure did not exert any significant effect on the total FAA concentration of the liver, in spite of a significant increase in total FAA concentration in the brain and muscle, and a significant decrease in total FAA in plasma. In addition, ammonia exposure led to a significant increase in the glutamine content as well as the total FAA content in the brain (Table VI), which were not observed in specimens exposed to alkaline pH (Table II).

The nitrogen balance sheet approach for high ammonia exposure (Table IV) shows that the elevation of accumulated nitrogen is approximately the same as that for high pH exposure (Table III). The difference between the assumed reduction in N excretion and the measured increase in N accumulation ($-473 + 184 = -289$ μmol N), however, was greater than that of a similar hypothetical specimen exposed to alkaline pH (Table III), which probably explained why more ammonia was accumulated in the tissues, especially the brain, of ammonia-exposed fish.

TABLE II. Effects of 2 days of exposure to alkaline pH (10 mmol l^{-1} Tris-HCl, pH 8·5, in salinity 2 water) on concentrations (mean ± S.E., $n = 4$) of various free amino acids (FAAs) and total FAA in the muscle, liver, brain and plasma of *Allenbatrachus grunniens*. Specimens exposed to salinity 2 water containing 10 mmol l^{-1} Tris-HCl at pH 7·0 for 2 days served as controls

	Muscle (μmol g^{-1})		Liver (μmol g^{-1})		Brain (μmol g^{-1})		Plasma (μmol ml^{-1})	
FAA	Control	pH 8·5	Control	pH 8·5	Control	pH 8·5	Control	pH 8·5
Alanine	0·54 ± 0·08	2·23 ± 0·24*	0·41 ± 0·11	0·11 ± 0·01*	0·10 ± 0·01	0·067 ± 0·006*	0·025 ± 0·006	0·012 ± 0·006
Arginine	0·008 ± 0·005	0·002 ± 0·001	0·006 ± 0·001	0·001 ± 0·001*	0·082 ± 0·010	0·067 ± 0·006	ND	ND
Asparagine	0·040 ± 0·006	0·19 ± 0·02*	ND	ND	ND	ND	ND	ND
Aspartate	0·36 ± 0·09	0·38 ± 0·07	0·29 ± 0·10	0·17 ± 0·01	0·27 ± 0·02	0·16 ± 0·03*	0·009 ± 0·001	0·005 ± 0·001
Glutamate	0·58 ± 0·05	0·52 ± 0·04	2·83 ± 1·41	1·64 ± 0·39	3·72 ± 0·18	3·19 ± 0·21	0·015 ± 0·003	0·010 ± 0·002
Glutamine	0·13 ± 0·02	0·68 ± 0·11*	0·006 ± 0·004	0·070 ± 0·007*	4·13 ± 0·36	4·23 ± 0·18	0·036 ± 0·007	0·026 ± 0·006
Glycine	0·19 ± 0·02	0·80 ± 0·17*	0·32 ± 0·06	0·26 ± 0·04	0·63 ± 0·06	0·62 ± 0·10	0·021 ± 0·005	0·011 ± 0·004
Histidine	0·060 ± 0·011	0·26 ± 0·04*	0·097 ± 0·009	0·059 ± 0·005	0·075 ± 0·014	0·091 ± 0·024	0·006 ± 0·001	0·003 ± 0·001
Isoleucine	0·16 ± 0·03	0·42 ± 0·03*	0·048 ± 0·015	0·007 ± 0·004*	0·023 ± 0·005	0·007 ± 0·002*	0·014 ± 0·004	0·005 ± 0·001*
Leucine	0·26 ± 0·05	0·76 ± 0·03*	0·13 ± 0·03	0·023 ± 0·009*	0·049 ± 0·011	0·020 ± 0·003*	0·028 ± 0·007	0·010 ± 0·002
Lysine	1·13 ± 0·37	0·95 ± 0·21	0·081 ± 0·020	0·011 ± 0·005*	0·068 ± 0·007	0·030 ± 0·018	0·024 ± 0·008	0·010 ± 0·002
Methionine	0·036 ± 0·008	0·24 ± 0·06*	0·017 ± 0·003	ND	0·001 ± 0·001	0·006 ± 0·002*	ND	ND
Phenylalanine	0·064 ± 0·008	0·34 ± 0·08*	0·034 ± 0·010	0·076 ± 0·010*	0·018 ± 0·006	0·049 ± 0·011*	0·041 ± 0·007	0·038 ± 0·008
Proline	0·13 ± 0·02	0·61 ± 0·15*	0·058 ± 0·024	0·21 ± 0·05*	0·045 ± 0·007	0·050 ± 0·010	0·005 ± 0·002	0·002 ± 0·001
Serine	0·094 ± 0·018	0·54 ± 0·10*	0·17 ± 0·02	0·061 ± 0·008*	0·12 ± 0·01	0·080 ± 0·016	0·009 ± 0·002	0·007 ± 0·001
Taurine	9·73 ± 0·91	6·63 ± 1·08	10·2 ± 1·4	14·3 ± 3·2	6·48 ± 0·79	6·83 ± 1·28	0·013 ± 0·003	0·005 ± 0·001*
Threonine	0·12 ± 0·01	0·77 ± 0·22*	0·14 ± 0·04	0·041 ± 0·005*	0·18 ± 0·03	0·21 ± 0·03	0·004 ± 0·001	0·002 ± 0·001
Tryptophan	0·047 ± 0·008	0·35 ± 0·07*	ND	ND	0·012 ± 0·006	0·053 ± 0·011*	0·026 ± 0·007	0·007 ± 0·001*
Tyrosine	0·056 ± 0·009	0·29 ± 0·06*	0·046 ± 0·006	0·035 ± 0·011	0·023 ± 0·013	ND	0·008 ± 0·002	0·002 ± 0·001*
Valine	0·21 ± 0·06	0·65 ± 0·04*	0·081 ± 0·013	0·13 ± 0·02	0·036 ± 0·008	0·022 ± 0·003	0·17 ± 0·03	0·19 ± 0·05
Total FAA (minus taurine)	4·1 ± 0·5	10·6 ± 1·2*	5·3 ± 1·3	2·5 ± 1·3*	9·5 ± 0·9	8·9 ± 1·2	0·46 ± 0·09	0·35 ± 0·06

*, significantly different from the control value; ND, not detected.

TABLE III. A 'balance sheet' of nitrogenous accumulation (μmol N) and nitrogenous excretion (μmol N) in a hypothetical 30 g *Allenbatrachus grunniens* exposed to control (pH 7·0) or experimental (pH 8·5) conditions for 2 days

A. grunniens (30 g)	Control	pH 8·5	Difference
Excreted			
Ammonia	441	237	–203
Urea	NS	NS	
	Reduction in nitrogenous excretion		–203 μmol N
Retained in muscle (18 g)			
Ammonia	6·57	12·64	+6·07
Urea	NS	NS	
Free amino acids	73·8	190	+117
Retained in liver (0·8 g)			
Ammonia	NS	NS	
Urea	NS	NS	
Free amino acids	4·24	2·00	–2·24
	Increase in nitrogenous accumulation		+123 μmol N
	Deficit		–80 μmol N

NS, not significantly different between the control and experimental conditions.

Ammonia treatment had similar effects to high pH on enzyme activities in the liver, and also in this case muscle. The GSase activity was significantly elevated, and muscle ALT was again depressed, but no changes in other enzymes were observed (Table VII). The GSase activity in the brain of specimens exposed to high ammonia($148{\cdot}5 \pm 5{\cdot}1\ \mu mol\ min^{-1}\ g^{-1}$) was not significantly different from that of the control animals ($145{\cdot}1 \pm 10{\cdot}7\ \mu mol\ min^{-1}\ g^{-1}$).

The confinement and salt- *v.* freshwater treatments of series III showed that *A. grunniens* was not significantly ureotelic (<25%), nor did urea and ammonia excretion change significantly. The rates in salinity 2 water were $468{\cdot}1 \pm 88{\cdot}0\ \mu mol\ N\ h^{-1}\ kg^{-1}$ for ammonia and $86{\cdot}5 \pm 20{\cdot}8\ \mu mol\ N\ h^{-1}\ kg^{-1}$ ($n = 5$) for urea excretion, respectively. In salinity 30 water the rates for ammonia and urea excretion were $294{\cdot}9 \pm 55{\cdot}5$ and $97{\cdot}0 \pm 17{\cdot}4\ \mu mol\ N\ h^{-1}\ kg^{-1}$ ($n = 6$) respectively. Urea contributed a very small amount to the total osmolality of the tissues and plasma and only slight significant variations in tissue and plasma ammonia and urea concentrations were evident (Table VIII). Far fewer changes in amino acids were observed in the treatment groups of series III, with the most evident being an increase in the total FAA in the liver of confined salt-water *A. grunniens* (unpubl. data).

Transfer of *O. beta* in series IV to either 20 or 200% strength sea water had only slight effects on ammonia and urea excretion rates. Ammonia excretion rates tended to increase immediately post-transfer to 20% strength sea water, with the 24 and 48 h points being significantly different from 100% sea water [Fig. 5(a)]. Urea excretion was significantly lowered in the 200% strength

TABLE IV. A 'balance sheet' of nitrogenous accumulation (μmol N) and nitrogenous excretion (μmol N) in a hypothetical 30 g *Allenbatrachus grunniens* exposed to control or high environmental ammonia (10 mmol l^{-1} NH_4Cl at pH 7·0) conditions for 2 days

A. grunniens (30 g)	Control	NH_4Cl	Difference
Excreted			
Ammonia	441	0	−441
Urea	41	9·2	−31·8
	Reduction in nitrogenous excretion		−472·8 μmol N
Retained in muscle (18 g)			
Ammonia	13·1	36·7	+23·6
Urea	NS	NS	
Free amino acids	68·4	226·8	+158·4
Retained in liver (0·8 g)			
Ammonia	0·8	2·4	+1·6
Urea	NS	NS	
Free amino acids	NS	NS	
	Increase in nitrogenous accumulation		+183·6 μmol N
	Deficit		−289 μmol N

NS, not significantly different between the control and experimental conditions.

seawater fish at 72 h, thereafter increased until it was significantly elevated at 144 h [Fig. 5(b)]. Despite these shifts in nitrogen excretion rates, by the end of the experiment, only modest changes in plasma osmolality and urea content were

TABLE V. Effects of 2 days of exposure to alkaline pH (10 mmol l^{-1} Tris-HCl, pH 8·5, in salinity 2 water) on the mass specific activities (mean ± S.E., $n = 5$) of glutamine synthetase (GSase), alanine aminotransferase (ALT), aspartate aminotransferase (AST), malate dehydrogenase (MDH) and malic enzyme (ME) of *Allenbatrachus grunniens*. Specimens exposed to salinity 2 water containing 10 mmol l^{-1} Tris-HCl at pH 7·0 for 2 days served as controls

	Muscle (μmol min^{-1} g^{-1} wet mass)		Liver (μmol min^{-1} g^{-1} wet mass)	
Enzymes	Control	pH 8·5	Control	pH 8·5
GSase	0·38 ± 0·05	0·26 ± 0·04	2·23 ± 0·32	6·09 ± 1·11*
ALT	1·87 ± 0·28	0·85 ± 0·11*	19·3 ± 2·5	26·8 ± 2·8
AST	11·2 ± 3·1	8·93 ± 2·3	37·5 ± 6·8	39·7 ± 2·1
MDH	28·9 ± 8·7	21·5 ± 4·6	168 ± 7	203 ± 8
ME	0·71 ± 0·05	0·54 ± 0·04	12·6 ± 0·7	10·8 ± 0·5

*, significantly different from the control value ($P < 0·05$).

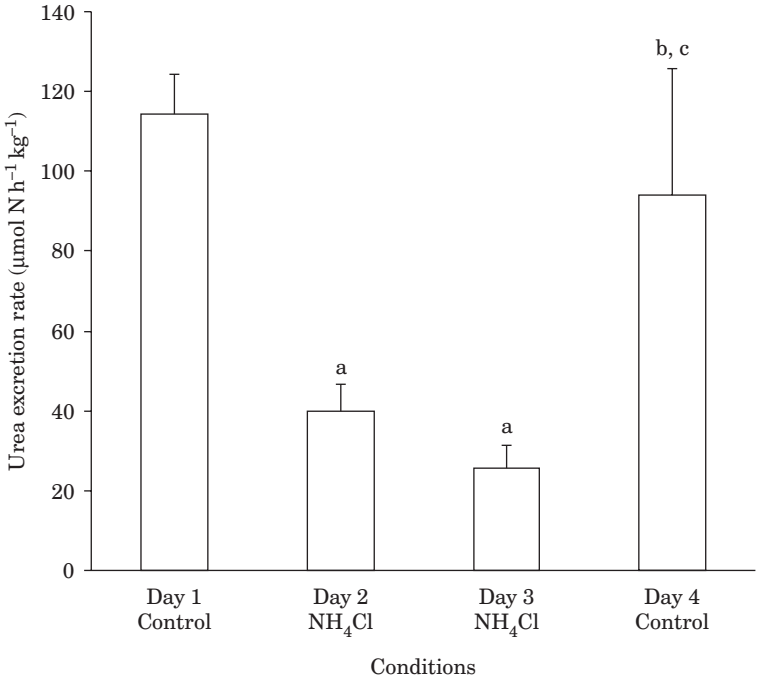

FIG. 3. Effects of 2 days of exposure to environmental ammonia (salinity 2 water containing 10 mmol l^{-1} NH_4Cl at pH 7·0) followed by 1 day of recovery in water of neutral pH (salinity 2 water at pH 7·0) on the rate of urea (□) excretion in *Allenbatrachus grunniens*. Values are means + S.E. ($n = 5$). a, significantly different from the day 1 control value, $P < 0{\cdot}05$; b, significantly different from the day 2 (NH_4Cl) value, $P < 0{\cdot}05$; c, significantly different from the day 3 (NH_4Cl) value, $P < 0{\cdot}05$.

observed with urea never contributing more than *c*. 5% to the total osmolality of the plasma (Table IX). There was a depression of GSase activity in the 20% strength seawater fish (Table IX).

DISCUSSION

Initial measurements of O-UC enzyme activities in *A. grunniens* indicated that this species has a low capacity for *de novo* urea synthesis, notably in having much lower levels of CPSase III (Table I) than other ureotelic batrachoidids (Anderson & Walsh, 1995). Nonetheless, since enzyme activity measurements only reflect potential maximal rates *in vivo*, and since urea can in fact be produced by other degradative pathways in fishes (*e.g.* arginolysis and purine degradation, Wood, 1993), urea and nitrogen excretion was directly examined under several conditions known to decrease ammonia excretion and activate or enhance urea production and excretion. These measurements seemed especially important in light of the high arginase levels observed (Table I). Both high pH and ammonia exposure led to increased tissue ammonia content (Figs 2 and 4) in *A. grunniens*, and ammonia excretion was depressed, at least at high pH (Fig. 1). Despite these clear effects, urea content in tissues, and urea excretion rates were not enhanced (Figs 1–4) and urea excretion was actually reduced by >50% by ammonia exposure (Fig. 3).

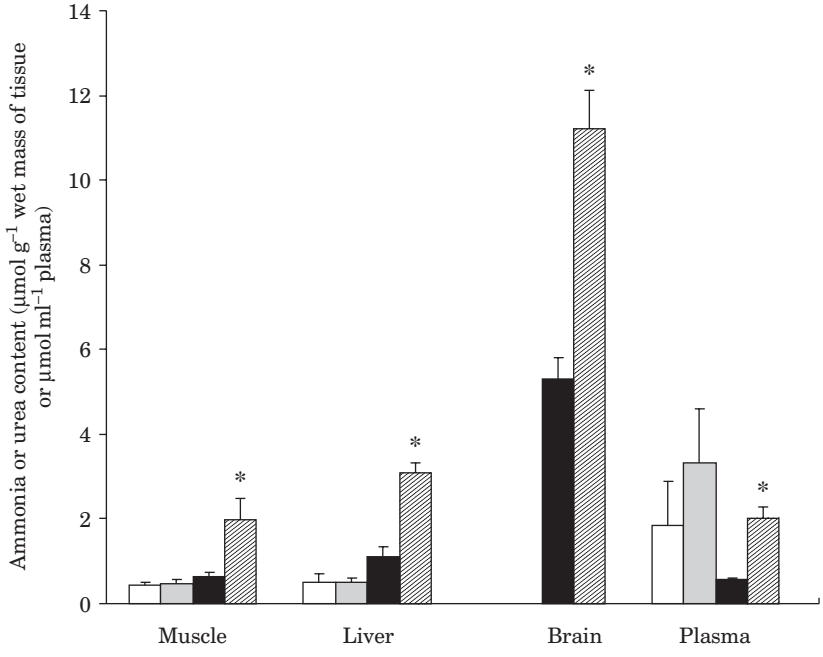

FIG. 4. Contents of urea (□, control; ▨, experimental) and ammonia (■, control; ▨, experimental) in the muscle, liver, brain and plasma of *Allenbatrachus grunniens* exposed to the control condition (salinity 2 water at pH 7·0) or high environmental ammonia (salinity 2 water containing 10 mmol l^{-1} NH_4Cl at pH 7·0) for 2 days. Values are means + S.E. ($n = 5$). *, significantly different from the corresponding control, $P < 0{\cdot}05$.

If the O-UC does not play a major role in ammonia detoxification in *A. grunniens*, does this species undergo other metabolic changes to detoxify ammonia? The largest compartment of the fish is the muscle, and amino acid data for it during alkaline pH exposure (Table II) and ammonia exposure (Table VI) initially suggest that this species can partially offset ammonia accumulation by increasing amino acid contents, especially alanine. Alanine has been shown to be an important end-product in selected fish species (Ip *et al.*, 2001*a*). It is important, however, to bear in mind that an increase in a given tissue amino acid content reflects the sum of both its production (*via* proteolysis or *de novo* synthesis) and its consumption (*via* oxidation and protein synthesis).

While there is no direct proof that exogenous ammonia is being made into amino acids in *A. grunniens*, it is clear at least in *O. beta* that exogenous ammonia labelled with ^{15}N can be incorporated into a variety of metabolites including protein (Rodicio *et al.*, 2003), showing that *de novo* synthesis can trap exogenous ammonia. Since the *A. grunniens* in the present study were not fed, however, it was possible to compare changes in the levels of the non-essential amino acid alanine (which by definition can be synthesized endogenously) to changes in levels of essential amino acids (*i.e.* which cannot be synthesized *de novo* and must be acquired by the diet). Increases in essential amino acids were *c.* seven and a half-fold for tryptophan and *c.* six and a half-fold for methionine in both treatments, which exceeded or were similar to the increases for alanine

TABLE VI. Effects of 2 days of exposure to high environmental ammonia (10 mmol l^{-1} NH_4Cl in salinity 2 water at pH 7·0) on concentrations (mean ± S.E., $n = 4$) of various free amino acids (FAAs) and total FAA in the muscle, liver, brain and plasma of *Allenbatrachus grunniens*. Specimens exposed to salinity 2 water at pH 7·0 for 2 days served as controls

	Muscle		Liver		Brain		Plasma	
FAA	Control	High Ammonia	Control	High Ammonia	Control	High Ammonia	Control	High Ammonia
Alanine	0·44 ± 0·05	3·20 ± 0·59*	0·15 ± 0·05	0·20 ± 0·07	0·14 ± 0·02	0·12 ± 0·02	0·049 ± 0·010	0·023 ± 0·011
Arginine	0·010 ± 0·006	0·004 ± 0·003	0·005 ± 0·002	0·003 ± 0·001	0·066 ± 0·003	0·11 ± 0·03	ND	ND
Asparagine	0·044 ± 0·005	0·15 ± 0·05	ND	ND	ND	ND	0·010 ± 0·002	0·001 ± 0·001*
Aspartate	0·23 ± 0·04	0·38 ± 0·20	0·17 ± 0·03	0·19 ± 0·03	0·35 ± 0·01	0·48 ± 0·10	0·014 ± 0·003	0·002 ± 0·001*
Glutamate	0·46 ± 0·07	0·59 ± 0·10	1·41 ± 0·28	1·24 ± 0·33	5·22 ± 0·23	4·11 ± 0·53	0·051 ± 0·010	0·008 ± 0·003*
Glutamine	0·21 ± 0·01	70·50 ± 0·05*	0·045 ± 0·008	0·12 ± 0·03*	2·63 ± 0·11	5·54 ± 1·02*	0·074 ± 0·023	0·011 ± 0·003*
Glycine	0·22 ± 0·02	0·78 ± 0·11*	0·29 ± 0·05	0·38 ± 0·20	0·66 ± 0·04	0·85 ± 0·10	0·043 ± 0·003	0·012 ± 0·005*
Histidine	0·060 ± 0·003	0·26 ± 0·04*	0·094 ± 0·007	0·095 ± 0·040	0·10 ± 0·02	0·059 ± 0·003*	0·010 ± 0·001	0·004 ± 0·001
Isoleucine	0·16 ± 0·03	0·58 ± 0·05*	0·081 ± 0·007	0·077 ± 0·060	0·037 ± 0·003	0·016 ± 0·007*	0·032 ± 0·010	0·004 ± 0·001*
Leucine	1·13 ± 0·37	0·91 ± 0·08	70·16 ± 0·01	0·13 ± 0·09	0·078 ± 0·005	0·034 ± 0·010*	0·063 ± 0·020	0·008 ± 0·001*
Lysine	0·40 ± 0·06	1·82 ± 0·28	0·042 ± 0·010	0·13 ± 0·10	0·16 ± 0·02	0·047 ± 0·03*	0·042 ± 0·009	0·009 ± 0·004*
Methionine	0·038 ± 0·004	0·24 ± 0·03*	0·002 ± 0·002	0·002 ± 0·002	ND	ND	ND	ND
Phenylalanine	0·045 ± 0·003	0·28 ± 0·04*	0·083 ± 0·020	0·040 ± 0·009*	0·016 ± 0·002	0·013 ± 0·002	0·009 ± 0·004	0·005 ± 0·001
Proline	0·12 ± 0·02	0·54 ± 0·09*	0·020 ± 0·006	0·15 ± 0·06*	0·028 ± 0·002	0·074 ± 0·010*	0·010 ± 0·002	0·003 ± 0·001*
Serine	0·076 ± 0·006	0·56 ± 0·08*	0·096 ± 0·020	0·14 ± 0·05*	0·15 ± 0·04	0·084 ± 0·020*	0·014 ± 0·002	0·008 ± 0·001*
Taurine	9·71 ± 1·72	9·18 ± 0·29	11·2 ± 1·3	12·4 ± 1·3	7·23 ± 0·71	8·34 ± 1·88	0·27 ± 0·03	0·11 ± 0·03*
Threonine	0·14 ± 0·02	0·57 ± 0·08*	0·12 ± 0·02	0·14 ± 0·09	0·15 ± 0·01	0·17 ± 0·05	0·035 ± 0·004	0·008 ± 0·003*
Tryptophan	0·040 ± 0·008	0·31 ± 0·04*	0·006 ± 0·003	0·025 ± 0·030	0·008 ± 0·003	0·033 ± 0·007*	0·011 ± 0·003	ND
Tyrosine	0·063 ± 0·006	0·28 ± 0·03*	0·015 ± 0·008	0·007 ± 0·007	ND	ND	0·010 ± 0·002	0·004 ± 0·001*
Valine	0·17 ± 0·02	0·72 ± 0·05*	0·21 ± 0·04	0·18 ± 0·09	0·058 ± 0·008	0·028 ± 0·010	0·053 ± 0·018	0·006 ± 0·002
Total FAA (minus taurine)	3·8 ± 0·8	12·6 ± 1·2*	3·4 ± 0·5	4·0 ± 1·0	6·9 ± 0·8	11·7 ± 1·7*	0·54 ± 0·06	0·12 ± 0·05*

*, significantly different from the control value; ND, not detected.

TABLE VII. Effects of 2 days of exposure to high environmental ammonia (10 mmol l^{-1} NH_4Cl in salinity 2 water at pH 7·0) on the mass specific activities (mean ± S.E., $n = 5$) of glutamine synthetase (GSase), alanine aminotransferase (ALT), aspartate aminotransferase (AST), malate dehydrogenase (MDH) and malic enzyme (ME) of *Allenbatrachus grunniens*. Specimens exposed to salinity 2 water containing 10 mmol l^{-1} Tris-HCl at pH 7·0 for 48 h served as controls

	Muscle (μmol min^{-1} g^{-1} wet mass)		Liver (μmol min^{-1} g^{-1} wet mass)	
Enzymes	Control	NH_4Cl	Control	NH_4Cl
GSase	0·22 ± 0·01	0·73 ± 0·23*	2·85 ± 0·25	4·76 ± 1·14*
ALT	1·87 ± 0·23	0·74 ± 0·08*	48·2 ± 6·71	45·0 ± 11·5
AST	10·1 ± 0·4	5·43 ± 0·14	59·5 ± 10·3	68·4 ± 11·6
MDH	23·6 ± 8·5	27·8 ± 5·7	228 ± 36	315 ± 39
ME	0·73 ± 0·06	0·78 ± 0·05	8·42 ± 1·51	15·6 ± 2·7*

*, significantly different from the control value ($P < 0·05$).

(*c*. four- to seven-fold) (Tables II and VI). If alanine was an important sink for exogenous ammonia, its increase should greatly exceed that of essential amino acids. This coupled with the observation that alanine accumulation in other species can be as high as 10- to 20-fold (Ip *et al*., 2001*b*; Chew *et al*., 2003) lead to the conclusion that *A. grunniens* is not relying on wholesale amino acid accumulation *via de novo* synthesis as a means of direct ammonia detoxification. Probably the increased tissue amino acid content represents an attempt to ameliorate the exposure to elevated ammonia by decreasing the oxidative production of ammonia; amino acids liberated by proteolysis are probably accumulating, rather than being deaminated and oxidized.

This conclusion is also supported by the calculations in Table III and IV showing a significant decrease in endogenous ammonia production for both treatments. The deficit of −80 μmol N in a hypothetical 30 g fish (Table III) indicates that a reduction in ammonia production had occurred during 2 days

TABLE VIII. Effects of confinement on the contents (mean ± S.E.) of ammonia and urea in the liver and muscle of *Allenbatrachus grunniens*. The number of samples is given in parentheses

	Urea		Ammonia	
Condition/salinity	Liver	Muscle	Liver	Muscle
Unconfined/2	2·24 ± 0·60 (6)	2·21 ± 0·48 (5)	7·00 ± 1·06 (6)	7·27 ± 0·22 (6)
Confined/2	2·86 ± 0·60 (5)	3·63 ± 0·20 (4)	7·48 ± 0·95 (4)	8·17 ± 0·55 (5)
Unconfined/30	3·45 ± 0·87 (6)	3·87 ± 0·56 (5)	7·59 ± 0·51 (6)	6·90 ± 0·07[a] (6)
Confined/30	1·65 ± 0·52 (6)	2·10 ± 0·34[b] (6)	8·47 ± 0·92 (6)	7·96 ± 0·11[b] (6)

[a]Significantly different from the corresponding unconfined and salinity 2 value ($P < 0·05$).
[b]Significantly different from the corresponding unconfined and salinity 30 value ($P < 0·05$).

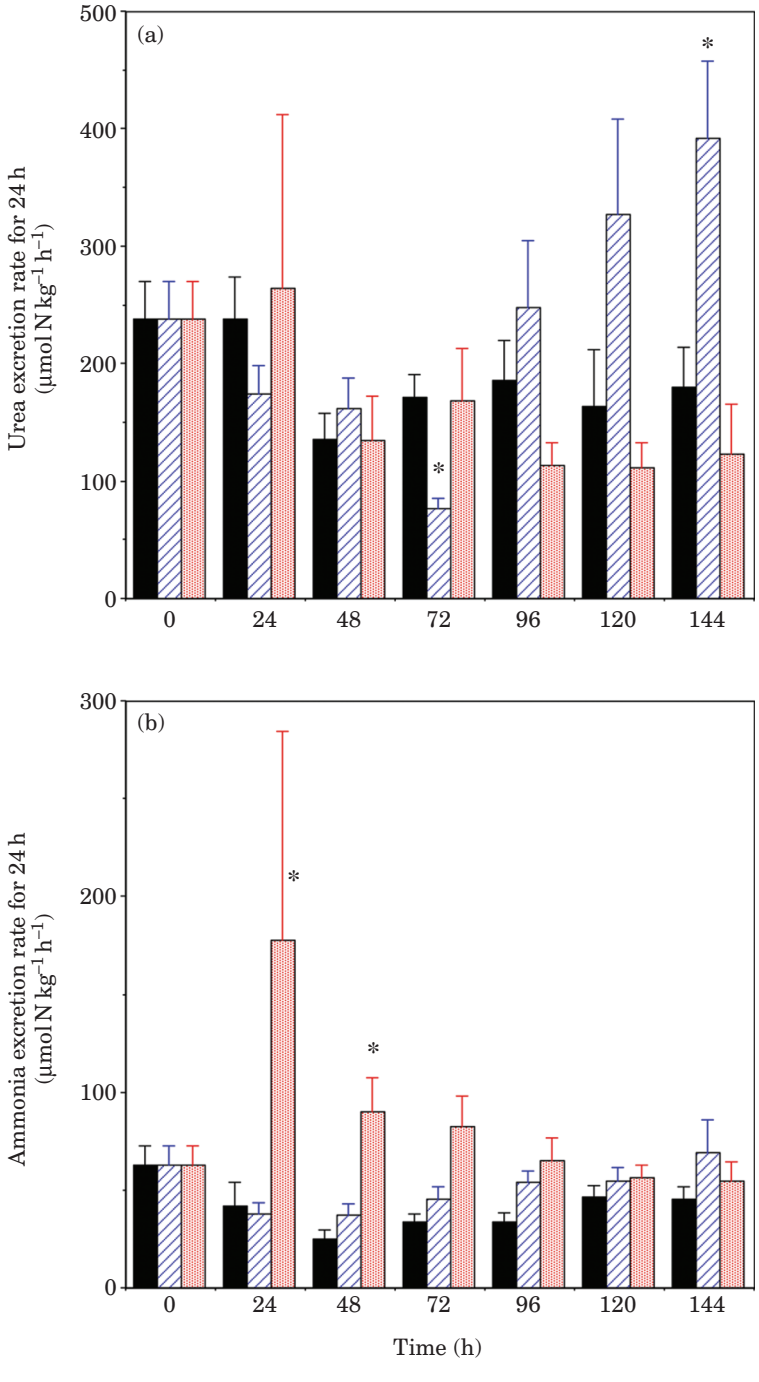

FIG. 5. The effects of transfer from 100% Biscayne Bay sea water (■, $n=9$) to 200% (▨, $n=8$) or 20% strength sea water (▩, $n=6$) on excretion of (a) urea or (b) ammonia in *Opsanus beta*. Control period (time 0 h) represents means of three group fluxes of 23 animals. *, significantly different from the corresponding control, $P<0{\cdot}05$.

TABLE IX. Effects of salinity acclimation on plasma urea, protein and osmolality, and hepatic GSase activity (mean + S.E.) in *Opsanus beta*. The number of samples is given in parentheses

Salinity	Osmolality (mosmol kg^{-1})	Plasma urea (mmol l^{-1})	Plasma protein (mg ml^{-1})	GSase activity (units g^{-1})
20% strength sea water	310·8 + 2·6* (6) (−4·6%)	7·45 + 0·99 (6) (2·4%)	24·4 + 0·9 (6)	8·88 + 1·98* (6)
100% strength sea water	325·1 + 4·1 (9)	8·47 + 0·80 (9) (2·6%)	26·4 + 1·5 (6)	19·03 + 2·00 (6)
200% strength sea water	395·7 + 7·3* (8) (+21·7%)	20·50 + 3·07* (8) (5·2%) (+242%)	28·9 + 1·2 (6)	15·40 + 3·13 (6)

*, significantly different from 100% strength sea water ($P < 0{\cdot}05$).
+ or −%, change from 100% strength sea water.
%, per cent of total osmolality made up by urea.

of exposure to pH 8·5. This reduction is equivalent to 1·67 μmol N h^{-1} 30 g^{-1}. Since the rate of N produced is equal to the rate of N (ammonia-N + urea-N) excreted in order for the internal ammonia and urea contents to be maintained at steady state levels in a control fish, the rate of N production in a 30 g control *A. grunniens* can be estimated to be 19 μmol N h^{-1} 30 g^{-1} (from Table III). Thus, 1·67 μmol N h^{-1} 30 g^{-1} represents a reduction of 8·8% in rate of N production in fish exposed to pH 8·5. In comparison, ammonia-loading exerted a greater effect, leading to a deficit of −289 μmol N in a 30 g fish during the 2 day experimental period. This is equivalent to 6·0 μmol N h^{-1} 30 g^{-1}, or 31·6% reduction in rate of N production in fish exposed to 10 mmol l^{-1} NH_4Cl. The excess ammonia retained in the muscle of fish exposed to ammonia-loading (23·6 μmol N, Table IV) was 3·9-fold greater than that of fish exposed to alkaline pH (6·07 μmol N, Table III), but the excess FAA in the former (158·4 μmol N) was only 1·35-fold greater than that of the latter (117 μmol N). Since excess ammonia accumulated in the muscle during ammonia loading did not lead to a proportional increase in excess N retained as FAA, these results support the proposition stated above that *de novo* synthesis of amino acids was not a major means of ammonia detoxification in *A. grunniens*.

The liver showed a different pattern of changes in total FAA profiles than muscle, notably a decrease in total FAA (high pH, Table II) or no effect on total FAA (high ammonia, Table VI), generally indicating a greater decrease in proteolysis than in deamination in this tissue. Additionally, since liver is generally viewed as a central co-ordinating organ of nitrogen metabolism, but not an especially large body compartment (at least compared to muscle), changes in amino acid contents in this tissue probably reflect specialized metabolic aspects. For example, noting the increase in liver GSase activity following high pH (Table V) and the decrease in glutamate and increase in glutamine in liver (Table II), and the liver and muscle increase in GSase following high ammonia exposure (Table VII) (and similar metabolite changes, Table VI), it is tempting to conclude that an important route for ammonia scavenging is *via* glutamine. These and other potential pathways of ammonia scavenging have

been speculated to contribute to the rather high tolerance to ammonia that the family appears to have in general, even amongst non-ureotelic members (*e.g. P. notatus*, Wang & Walsh, 2000). Interestingly, however, brain ammonia concentrations are observed to rise in *A. grunniens* during high ammonia exposure (Fig. 4) despite the increase in glutamine (Table VI), suggesting that the glutamine detoxification route is inadequate and more direct brain tolerance mechanisms may be involved since brain is an important target tissue for the toxic effects of ammonia.

Both confinement and increases in salinity appeared to have little effect on urea or ammonia excretion or tissue metabolites (Table VIII) in *A. grunniens*. Salinity also has little effect on nitrogen excretion in the ureotelic *O. beta* (Fig. 5), where osmolality of the plasma is reasonably well defended from 20% to 200% strength sea water (Table IX). In both species, tissue and plasma urea contents do not appear to account for a large proportion of osmolyte balance, although there is some evidence in *O. beta* for urea content to change in the appropriate direction to more or less match the direction of change in total osmolality (Table IX). A similar pattern was noted in the osmoregulatory strategy of the Lake Magadi tilapia *Oreochromis grahami* (Boulenger) (Wood *et al.*, 2002). It is not clear how to interpret the increase in urea excretion data in 200% strength sea water at 144 h [Fig. 5(b)], although one simple explanation for this increase is that, since urea excretion is *via* facilitated diffusion and is strongly linked to the concentration gradient (Wood *et al.*, 1997), the increased excretion simply reflects the elevated plasma urea concentration (Table IX). The lack of effect of confinement on nitrogen metabolism and excretion in *A. grunniens* is in marked contrast to the response of *O. beta*. Confinement and other stressors appear to activate a psychological response in *O. beta* involving cortisol, serotonin and probably other neuro or humoral factors with the effect of changing the pattern of nitrogen excretion to predominantly ureotely (Walsh, 1997; Wood *et al.*, 2003).

It is clear from examining *A. grunniens* that there is at least one member of the same subfamily as *O. beta* (Batrachoidinae) that does not appear capable of synthesizing urea *de novo via* a functional O-UC. It will be instructive to examine other members of this family, from both similar and dissimilar habitats to those occupied by *O. beta*, and to ultimately 'overlay' the phylogenetic distribution of the trait of ureotely onto an independent molecular phylogeny for the group. Such analyses may contribute to conclusions regarding the evolutionary or ecological rationale for why some members of Batrachoididae invest such a large amount of energy into the synthesis of urea.

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