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INTERACTIONS OF ACID–BASE STATUS AND NITROGEN EXCRETION AND METABOLISM IN THE UREOGENIC TELEOST *OPSANUS BETA*

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Summary

The interactions of acid–base status, waste nitrogen excretion and metabolism in the gulf toadfish *Opsanus beta*, a ureogenic teleost, were examined by exposing toadfish to a variety of water treatments. Our measurements show that, like other marine teleosts, toadfish rapidly regulate acid–base status by manipulating blood [HCO_3^-]. Several treatments affected nitrogen excretion. The initial stages of hypercapnia (1% CO_2) led to significant reductions in ureogenesis, and the later compensated stages (with elevated plasma [HCO_3^-]) led to significant increases in ureogenesis. Treatment of water with HCl (which lowered pH and reduced the carbonate content) substantially inhibited ureogenesis. Subsequent experiments with NH_4Cl loading and several other treatments suggest that this depression is less likely to be the result of acid–base perturbations, but is probably an enhancement of the fish's ability to excrete waste nitrogen as ammonia, thereby decreasing the drive for ureogenesis. Enzyme activities and hepatocyte ureogenic potential were unaffected by various acid–base treatments, but a significant depletion of plasma amino acid levels was associated with the increase in plasma [HCO_3^-] induced by hypercapnia. Changes in ureogenesis associated with our treatments appear to be due primarily to changes in substrate levels, rather than to wholesale changes of the biochemical machinery. Our results are discussed in the context of the hypothesis of Atkinson and colleagues, that ureogenesis is a means for acid–base regulation *via* bicarbonate consumption.

Introduction

The effects of hypercapnia on the acid–base physiology and metabolism of fishes have been studied reasonably well. The initial response to elevated inspired water P_{CO_2} is a corresponding rapid increase in plasma P_{CO_2} and a depression of pH due to this entry of CO_2 and subsequent hydration and dissociation of carbonic acid; the acidification is followed by a slower recovery to normal acid–base status *via* accumulation of HCO_3^- by ion-exchange processes at the gill (e.g. Claiborne and Heisler, 1984, 1986; Heisler, 1982;

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Hyde and Perry, 1989; Perry *et al.* 1987; Toews *et al.* 1983). Furthermore, changes in these acid–base variables have been shown to exert substantial effects on hepatic carbohydrate metabolism in the rainbow trout both *in vivo* and *in vitro* (Mommsen *et al.* 1988; Perry *et al.* 1988; Walsh *et al.* 1988).

A second hepatic metabolic process believed to be markedly influenced by acid–base variables is urea synthesis *via* the ornithine–urea cycle (O-UC). For over a decade, Atkinson and co-workers have championed the hypothesis that ureogenesis plays a major role in overall acid–base homeostasis in terrestrial vertebrates in that the pathway consumes base as bicarbonate that is liberated from normal protein catabolism (see Atkinson, 1992). Experimental testing of this hypothesis has received the most attention in mammalian systems, where different observations have both supported and refuted the hypothesis. A balanced summary of the experimental evidence and status of the hypothesis is presented by Meijer *et al.* (1990).

Regardless of the applicability of the hypothesis of Atkinson and colleagues to mammals, the importance of ureogenesis to acid–base balance in fish has been much less studied and is an unresolved issue. The ureogenic Lake Magadi tilapia (*Oreochromis alcalicus grahami*) lives at extremely high pH (10) and carbonate concentrations (nearly 200 $mmol\,l^{-1}$), conditions which probably impose a very high external base load on the organism and which may have played an important role in the evolution of ureogenesis in this species *via* acid–base effects; however, alternative explanations based solely on adaptations for waste nitrogen excretion are at least equally plausible (Randall *et al.* 1989; Wood *et al.* 1989). The marine teleost toadfishes (genus *Opsanus*) also have a complete O-UC and are ureotelic under a variety of conditions (Mommsen and Walsh, 1989; Walsh *et al.* 1990). However, *in vitro* experiments with toadfish hepatocytes have failed to demonstrate that this pathway can influence acid–base balance in the manner predicted by Atkinson and colleagues; ureogenesis in toadfish hepatocytes was pH-insensitive *in vitro* (Walsh *et al.* 1989). In order for hepatic ureogenesis to be most effective at consuming base and thereby correcting alkalosis, they predict pathway flux to be maximal at high pH and minimal at low pH.

Recognizing the inherent limitations of *in vitro* studies, we decided to re-examine the interactions of acid–base balance and ureogenesis more directly through *in vivo* studies of the effects of hypercapnia and other treatments on ureogenesis in the gulf toadfish *Opsanus beta*. Recovery from acidosis in other teleosts exposed to hypercapnia relies intimately on bicarbonate *accumulation/retention*, a situation which would appear to be directly at odds with the *consumption* of bicarbonate by ureogenesis. Therefore, these experiments should shed light on the applicability of the Atkinson hypothesis to ureogenic marine teleost fish, as well as on the underlying mechanisms regulating ureogenesis in these species. Our results demonstrate that ureogenesis does not impart a substantial impact on acid–base balance in *Opsanus beta.* It appears that, when faced with the apparently opposing needs to excrete waste nitrogen as urea *versus* defending acid–base status from an acid-challenge, this species does both equally well. In the toadfish, the drive to make and excrete urea appears to be more related to whether the fish can excrete ammonia rather than to acid–base status *per se.*

Materials and methods

Experimental organisms and surgical procedures

Individuals of the gulf toadfish, *Opsanus beta* (Goode and Bean), were captured with a roller trawl by commercial shrimpers in Biscayne Bay, Florida, USA, between September 1991 and January 1993. Toadfish were held in an outdoor tank with running sea water for 24–48h, after which they were treated with Prefuran (a broad-spectrum antibacterial, antifungal and antiparasitic agent, Argent Chemical Co., Seattle, WA) for 30min at 0.15g in 5l of sea water. The fish were then transferred to indoor glass aquaria with flowing, aerated, filtered sea water at ambient photoperiod. Fish were not fed for a minimum of 72h before the start of each experiment, but were typically held in captivity for no longer than 2 weeks. They were used without regard for gender.

Fish were fitted with cannulae of PE-50 tubing surgically implanted in the caudal vein through a lateral incision approximately 2cm caudal to the anus (Walsh, 1989). They were allowed to recover for 48h in 10l plastic tubs supplied with flowing sea water, during which time the cannulae were periodically flushed with 0.1ml of Hanks balanced salts solution (HBSS, as in Walsh, 1989) containing 100i.u.ml^{-1} sodium heparin. Temperature was maintained at 24.0±1.0˚C.

Experimental design

At the beginning of an experiment (i.e. at −12h), seawater flow was stopped and water volume was reduced to 4l. Water for each treatment was changed every 12h. In preliminary experiments, these conditions were found to induce measurable urea excretion in the gulf toadfish. Three main experimental series were undertaken.

Effects of hypercapnia and mineral acidosis

In this first series (series I), toadfish were exposed to three treatments: (IA) *control*, 4l of aerated sea water; (IB) *hypercapnia*, 4l of sea water pre-equilibrated with 1% CO_2 in air and subsequently continuously gassed with this same mixture [obtained by mixing pure CO_2 and compressed air (Liquid Carbonics, Miami, FL) with a Cameron Instrument Company gas-mixing flowmeter (Port Aransas, TX)]; (IC) *mineral acidosis*, 4l of sea water treated with 9.5–10.0ml of 1mol l^{-1} HCl [to reduce the seawater pH to roughly the levels seen in treatment IB] and gassed with air. In this series, several samples were taken. Water was sampled (and changed) every 12h, assayed immediately for pH and total CO_2 concentrations, and a sample chilled for subsequent assay of ammonia and urea. Blood (0.7ml) was sampled through the cannula at −12, 0, 3, 6, 12, 24, 36, 48 and 72h, and assayed immediately for pH and total CO_2. The remaining blood was centrifuged at 14800 ***g*** in 1.5ml microcentrifuge tubes and the plasma was decanted. A sample was frozen for lactate analysis and another was refrigerated for analysis of ammonia and urea. The pelleted blood cells were resuspended in a volume of HBSS with an amount of heparin equivalent to that withdrawn, and reinjected into the fish. For the first six sets of fish (*N*=18), at 72h, an additional 1ml blood sample was withdrawn and plasma was separated, frozen in liquid nitrogen and stored at −80˚C for subsequent analysis of plasma amino acid content. These 18 fish were then anaesthetized for 5min in 0.5 g l^{-1}

MS-222 sea water buffered with $NaHCO_3$. Hepatocytes were isolated from one lobe of the liver, as in Walsh (1987), for measurements of urea synthetic capacity, and the remaining liver lobe and a brain and a kidney sample were frozen in liquid nitrogen and stored at −80˚C for subsequent analysis of enzyme activities. The size range of organisms in this series was 120–460g, and the fish were collected between September 1991 and March 1992.

Effects of mineral acidosis and ammonia loading

In this second series (series II), toadfish were exposed to three treatments: (IIA) *control*, as above; (IIB) *mineral acidosis*, as above; (IIC) *mineral acidosis plus NH_4Cl*, as in IIB with the addition of 20ml of $1 mol\,l^{-1}$ NH_4Cl to yield a final concentration of $5 mmol\,l^{-1}$ and (IID) $0.5 mmol\,l^{-1}$ NH_4Cl at normal pH. Note that, at these low pH values (approx. 6.8), the relatively high total ammonia level is necessary to achieve approximately the same water NH_3 concentration in both treatments IIC and IID. Water was changed and sampled every 12h as above and stored for urea analyses, and a terminal blood sample was taken for analysis of pH and total CO_2. The size range in this series was 47–170g, and the fish were collected between November and December 1992.

Effects of carbonate reduction

In this third series (series III), toadfish were exposed to three treatments: (IIIA) *control*, as above; (IIIB) *normal pH with carbonates reduced*, carbonates were reduced in 4l of sea water by the addition of 10ml of $1 mmol\,l^{-1}$ HCl and degassing, followed by back-titration to pH7.9 with $1 mmol\,l^{-1}$ NaOH; (IIIC) *normal pH with carbonates reduced and buffer added*, as in IIIB except that $5 mmol\,l^{-1}$ Hepes was added prior to back-titration. Total CO_2 concentrations in treatments IIIB and IIIC were nominally less than $0.2 mmol\,l^{-1}$. Other aspects were similar to the second experimental series, except that the absence of NH_4Cl treatments permitted ammonia flux measurements, that fish ranged in size from 38 to 157g, and that the experiments were performed in January 1993.

Determination of blood buffer value

For the purposes of constructing a pH–bicarbonate diagram, 2.4ml of blood was collected from each toadfish through a cannula and equilibrated in sequence with 0.261, 0.516, 1.03 and 2.03% CO_2 in air (humidified by gassing through HBSS). Blood from each of three fish was gassed separately for 1h in round-bottomed 50ml flasks (determined to be adequate for equilibration in pilot runs), at which point a sample was withdrawn for measurement of pH and total CO_2, and then the gassing mixture was changed to the next elevated CO_2 level and the procedure was repeated. Temperature was thermostatted to 24.0±0.5˚C.

Hepatocytes

Rates of urea synthesis were measured in isolated hepatocytes exactly as described in Walsh *et al.* (1989) using their control aspartate ($0.25 mmol\,l^{-1}$), ornithine ($1 mmol\,l^{-1}$), pH (7.75) and bicarbonate ($3 mmol\,l^{-1}$) values (except where noted below), but four substrate regimes were used: $5 mmol\,l^{-1}$ glutamine; $5 mmol\,l^{-1}$ glutamine with high bicarbonate ($15 mmol\,l^{-1}$); $0.2 mmol\,l^{-1}$ NH_4Cl; $1.0 mmol\,l^{-1}$ NH_4Cl.

Enzyme activities

Tissues were homogenized on ice in 5 vols of homogenization buffer (20 mmol l^{-1} K_2HPO_4, 10 mmol l^{-1} Hepes, 0.5 mmol l^{-1} EDTA, 1 mmol l^{-1} dithiothreitol, 50% glycerol, adjusted with NaOH to pH7.5 at 24˚C) using a Brinkman polytron. Homogenates were spun at 8000***g*** for 30min at 4˚C in a Jouan CR412 centrifuge. The supernatant or a 1:10 dilution was used directly for the assay at 24˚C of glutamine synthase (GNS), arginase (ARG), ornithine–citrulline transcarbamoylase (OCT), alanine aminotransferase (AlaAT), aspartate aminotransferase (AspAT), glutamate dehydrogenase (GDH), citrate synthase (CS) and malate dehydrogenase (MDH) by previously published methods (Mommsen and Walsh, 1989) using a Perkin–Elmer Lambda 2 recording spectrophotometer.

Analytical procedures, calculations and statistics

Blood pH was measured using a Radiometer PHM-84 Research pH meter connected to a G297 microelectrode and a calomel reference electrode, calibrated with Radiometer precision buffers at 24˚C. Seawater pH was measured using an Orion model 601A digital ion analyzer with a combination pH/reference electrode; in several cases the instrument was connected to a chart recorder for continuous 12h measurements. Total CO_2 of both blood and seawater samples was measured using a Corning 965 carbon dioxide analyzer. These values were used to calculate bicarbonate concentrations and carbon dioxide partial pressures utilizing the Henderson–Hasselbalch equation and appropriately determined constants from the general models proposed by Boutilier *et al.* (1984), using previously published values for plasma total molarity, [Na^+] and [protein] in marine fish (toadfish where possible) and our measured values of temperature. Urea and ammonia concentrations in plasma and sea water were determined using the Indophenol Blue method (Ivancic and Deggobis, 1984) as modified by Walsh *et al.* (1990). In experimental series II and III, urea was measured by the diacetylmonoxime-based method of Price and Harrison (1987). Plasma lactate was measured using standard enzymatic test kits (Sigma Chemical Co., St Louis, MO). Plasma amino acid concentrations were determined by the Amino Acid Analysis Facility of the University of California, Davis, on a Beckman model 7300 amino acid analyser, using a three-buffer lithium elution system with ninhydrin detection. All chemicals were reagent grade and biochemicals were purchased from Sigma Chemical Co.

Sample means for all analyses and treatments were analyzed using a single-factor analysis of variance (ANOVA) as described by Zar (1974). The F-statistics were calculated with the assistance of a Macintosh SE computer running Microsoft EXCEL v.3.0, with the probability of committing a type I error maintained at 0.05 for all analyses. If the ANOVA indicated a significant difference between group means, the means were analyzed using the Student–Newman–Keuls multiple-comparisons test to indicate which means were different. These calculations were performed manually and the alpha values were again confined to 0.05.

Results

Acid–base status and nitrogen production

For experimental series I, the acid–base variables for the sea water reached expected levels rapidly such that three conditions were established: control; hypercapnia (elevated

P_{CO_2} and [HCO_3^-], depressed pH); and mineral acidosis (depressed [HCO_3^-] and pH) (Fig. 1). Blood acid–base variables remained virtually constant in control fish, but a significant initial acidosis occurred in both hypercapnia and mineral acid treatments (Fig. 2). In hypercapnia, the acidosis was corrected by about 24h as the fish accumulated bicarbonate (Fig. 2), and a pH–bicarbonate diagram shows that the pattern was typical of other fish studied previously (Fig. 3, i.e. an initial respiratory acidosis along the blood buffer line, followed by an increase in bicarbonate and pH, more or less along a constant P_{CO_2} isopleth). The mean ± S.E.M. (N=3) blood buffering capacity was -19.634 ± 1.842 mmol HCO_3^- pH unit^{-1} l^{-1}. In this first mineral acidosis series, the blood pH remained slightly depressed for the entire 72h period (Fig. 2). In all treatments, plasma lactate remained at resting levels (i.e. <0.3 mmol l^{-1}, results not shown), indicating no adverse effects on blood oxygen delivery.

Nitrogen excretion was rather variable over the various measurement periods, but several significant effects were seen. In the 12–24h flux period, there was a significant reduction in urea excretion in both the hypercapnic and mineral acid treatments (Fig. 4), corresponding with an acidotic period in both groups (Fig. 2). In the 24–36h and 60–72 h flux periods, a significant elevation of urea excretion was noted in hypercapnia relative to controls (Fig. 4), corresponding to normal blood pH, but elevated blood [HCO_3^-] (Fig. 2). The fish exposed to mineral acidosis excreted more ammonia than those given the other treatments in the 60–72h flux period (Fig. 4), a time when blood pH remained depressed (Fig. 2).

Since the nitrogen excretion data for toadfish are rather variable (see Discussion) and significant differences were not noted consistently, and also since plasma urea varied over time and from fish to fish (Fig. 5), we reasoned that another informative representation of the data could be made by summing total ammonia excretion, by summing urea excretion and by computing and summing urea *production.* To do the latter, the changes in plasma urea concentrations between the 0 and 72h samples for each fish were transformed to a whole-body change in concentration. We assumed that the concentration of urea was uniform in all tissues with respect to the measured plasma concentrations. We then multiplied the difference in concentration between 0 and 72h by the body mass (converted to ml) of the fish. These changes in whole-body urea were added to, or subtracted from, the summed excretion values and a mass-specific total production rate was calculated for the entire 72h period (Fig. 6). From these grand means, it can be seen that hypercapnia led to a slight upward trend (P>0.05) in ureogenesis, while ammonia excretion remained unaffected (Fig. 6). Mineral acidosis, in contrast, led to a substantial depression of urea excretion and ureogenesis, and a substantial increase in ammonia excretion (Fig. 6).

To determine whether this depression of ureogenesis by mineral acidosis could be reversed by ammonia stress, experimental series II was conducted. Again, a marked depression of ureogenesis was noted in mineral acidosis *versus* controls, but ureogenesis could be returned to normal levels by raising the ammonia level in the water at the depressed pH; total ammonia was 5 mmol l^{-1}, but NH_3 was only 31.6 μmol l^{-1} (Fig. 7). Exposing toadfish to a comparable NH_3 level at normal seawater pH (i.e. at 0.5 mmol l^{-1} total NH_4Cl) further elevated urea excretion above the values obtained in all other treatments (Fig. 7).

However, despite these significant effects on nitrogen excretion, there were no significant differences in blood acid–base variables at the end of the treatments (Table 1).

To determine whether the depression of ureogenesis by water treated with mineral acid was the result of a pH effect, of removal of carbonates, or of removal of the carbonate

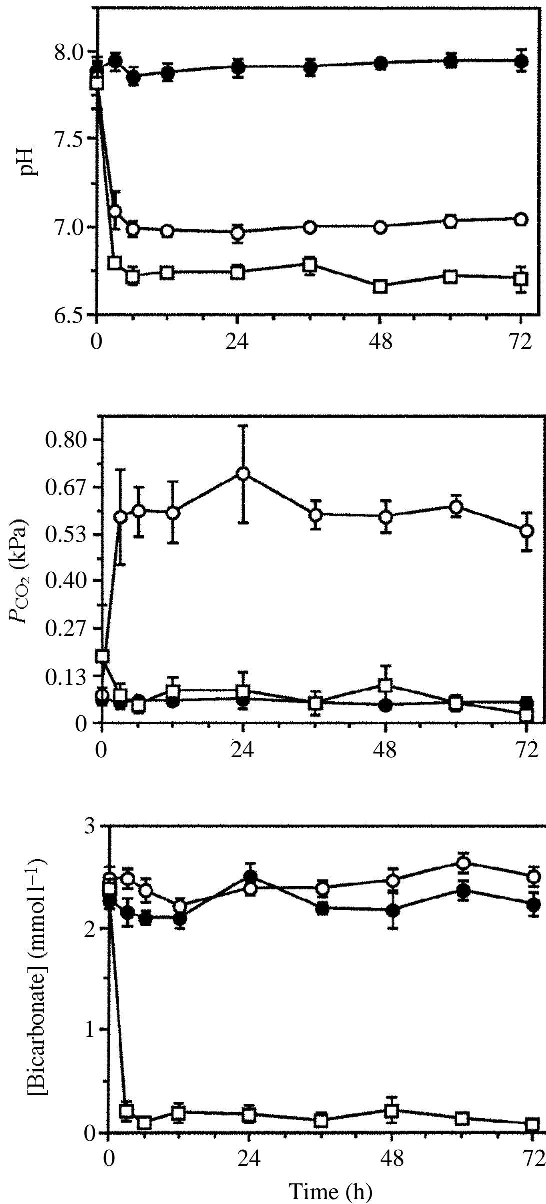

Fig. 1. The effects of hypercapnia (1% CO_2) and mineral acid (2.5mmol l^{-1} HCl) treatment on seawater acid–base variables, pH, P_{CO_2} and [HCO_3^-] (experimental series I). Filled circles, control; open circles, hypercapnia; open squares, mineral acidosis. Values are means ± 1 S.E.M. (some error bars are hidden by the symbol), with *N* ranging from 6 to 9.

buffering capacity, experimental series III was undertaken. Although removal of carbonates and buffering with Hepes (including back-titration to normal pH) reduced

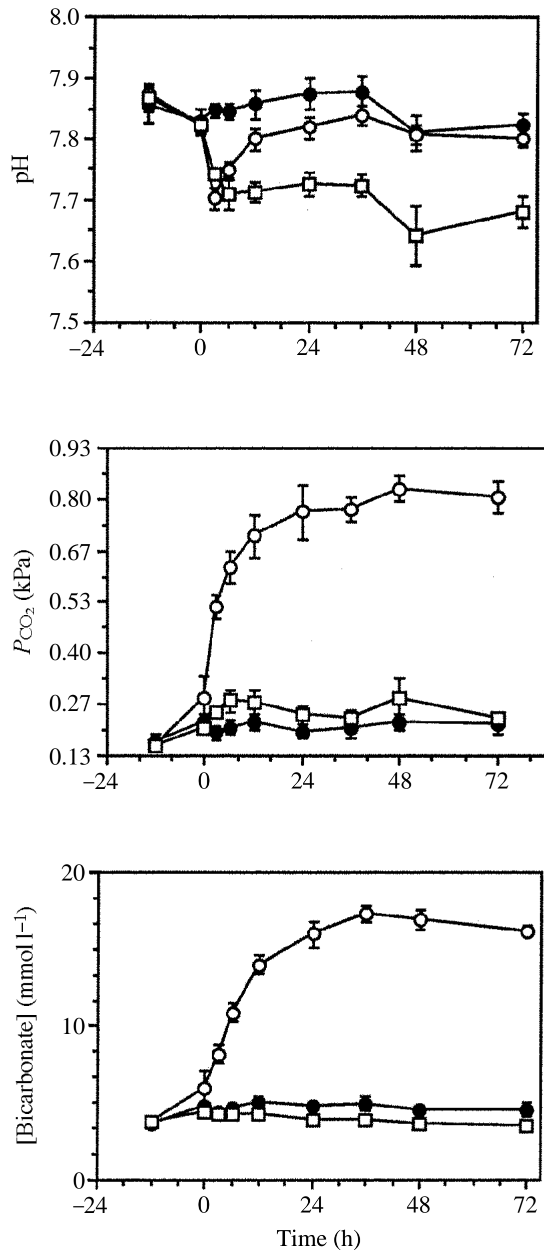

Fig. 2. The effects of hypercapnia and mineral acid treatment on blood acid–base variables, pH, P_{CO_2} and [HCO_3^-], in the gulf toadfish (experimental series I). Symbols and values as in Fig. 1. For pH, hypercapnia 3, 6 and 12h values and mineral acidosis 3–72h values are significantly different from control values; for P_{CO_2} and [HCO_3^-], hypercapnia 3–72h values are significantly different from control values, P<0.05.

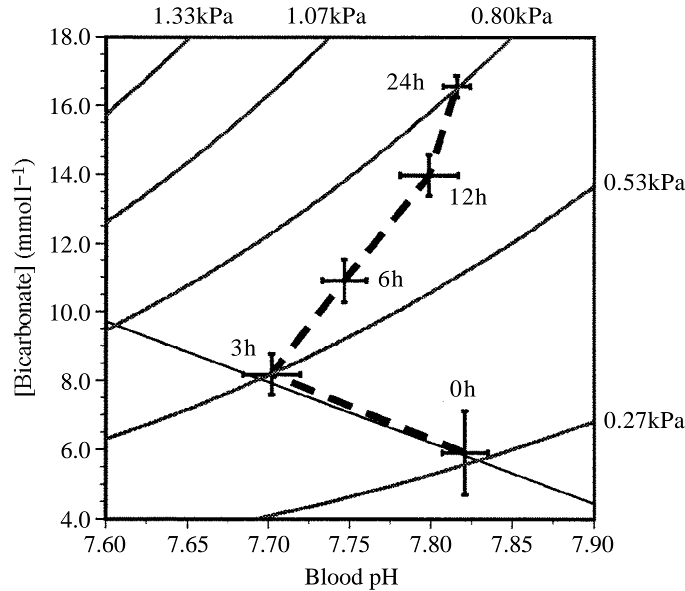

Fig. 3. pH–bicarbonate diagram of blood acid–base status for gulf toadfish exposed to hypercapnia (experimental series I). The buffer line is the mean of buffer lines obtained *in vitro* from three control toadfish. Values as in Fig. 1.

ammonia excretion (Fig. 8), none of the treatments altered urea excretion. Furthermore, only the Hepes treatment produced a significant change in blood acid–base status, namely a slight reduction in bicarbonate concentration and reduction of P_{CO_2} (Table 1).

Metabolic aspects

The treatments in experimental series I had no significant effects on the activities of a variety of nitrogen metabolism enzymes in toadfish kidney, brain and liver (Table 2). *In vitro* rates of ureogenesis in toadfish hepatocytes (Table 3) were lowest with ammonia as

Table 1. *Effect of several water treatments on terminal blood acid–base status in the gulf toadfish*

Water treatment	pH	$[HCO_3^-]$ (mmol l^{-1})	P_{CO_2} (kPa)
Mineral acid	7.799±0.025	4.7±0.5	0.23±0.03
Mineral acid + ammonia	7.780±0.037	4.0±0.4	0.21±0.03
Ammonia	7.751±0.019	3.5±0.2	0.20±0.01
Reduced carbonates	7.743±0.024	3.6±0.3	0.20±0.03
Reduced carbonates + Hepes	7.745±0.034	2.6±0.2*	0.16±0.01*
Control	7.774±0.015	3.8±0.2	0.20±0.01

The first set of three treatments corresponds to experimental series II (fish in Fig. 7), the second set of two treatments to experimental series III (fish in Fig. 8). The controls are pooled from the two experiments.

Values are means ± 1 S.E.M. (N=6–9).

* indicates a value significantly different from the control, P<0.05.

the primary nitrogen donor and highest when glutamine was the primary nitrogen donor: this is consistent with the requirement of carbamoylphosphate synthetase III for glutamine (Mommsen and Walsh, 1989). Rates were elevated when bicarbonate was elevated, which is consistent with previous results (Walsh *et al.* 1989). However, none of the *in vivo* treatments had a significant effect on *in vitro* rates of ureogenesis in toadfish hepatocytes (Table 3). Finally, *in vivo* hypercapnia caused a number of significant reductions in plasma amino acid concentrations (Table 4).

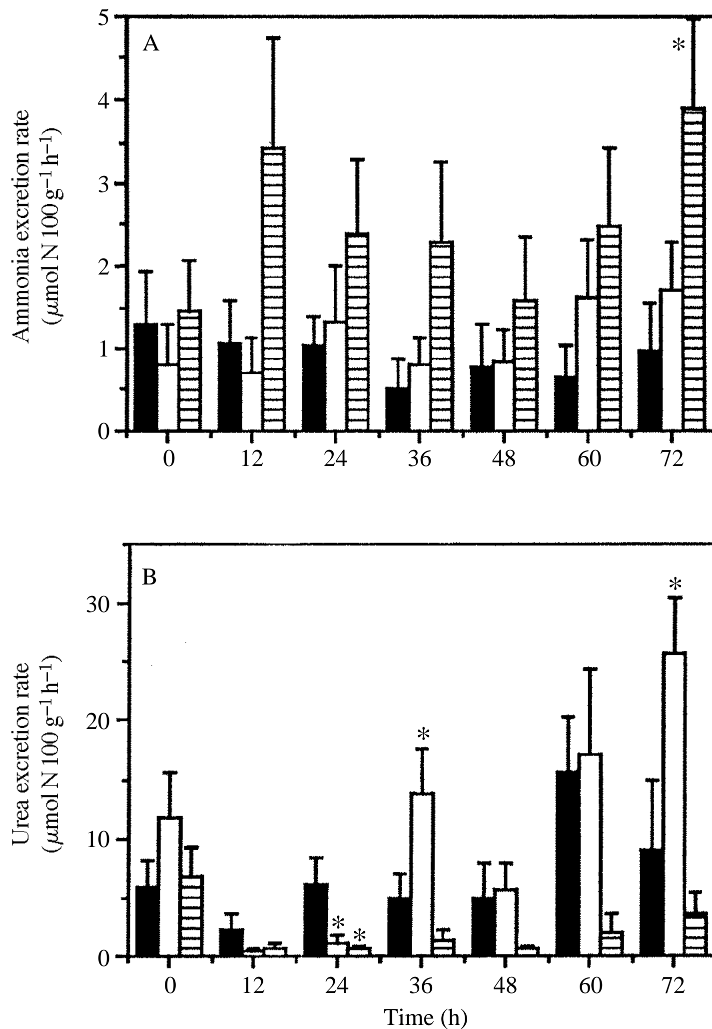

Fig. 4. Mass-specific excretion rates of ammonia-nitrogen (A) and urea-nitrogen (B) in gulf toadfish exposed to control (filled bars), hypercapnia (open bars) and mineral acidosis (hatched bars) conditions (experimental series I). Time indicated is that of the end of each 12 h flux period (e.g. 0=−12 to 0h). Values are mean ± 1 S.E.M., N=6–9; * indicates a value significantly different from the control value in a given period, P<0.05.

Discussion

Acid–base regulation and nitrogen excretion

In designing experimental series I, we reasoned that if ureogenesis were to play an important role in whole-body acid–base balance through consumption of bicarbonate, then exposure of fish to hypercapnia could potentially expose toadfish to conflicting needs. On the one hand, the acidosis caused by elevated P_{CO_2} would require the fish to correct pH by accumulation of base (bicarbonate) *via* branchial ionic exchange and/or a reduction in rates of hepatic ureogenesis. On the other hand, the need to excrete waste nitrogen as urea imposed by the closed nature of the experiments, and the accompanying

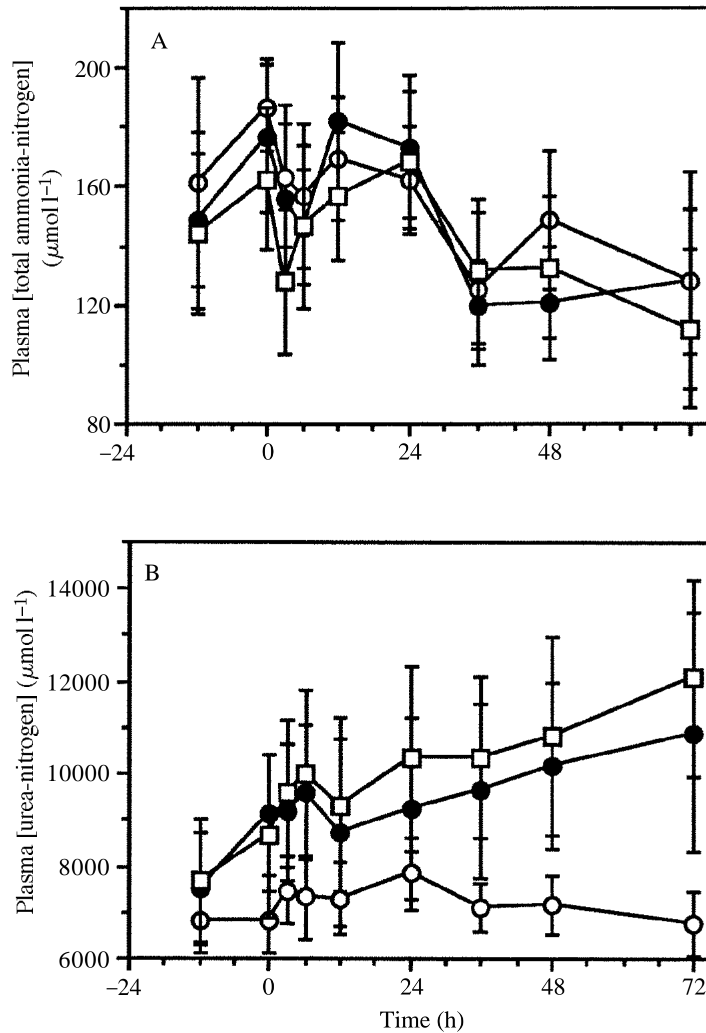

Fig. 5. Plasma ammonia-nitrogen (A) and urea-nitrogen (B) concentrations over time in toadfish exposed to control, hypercapnia and mineral acidosis conditions (experimental series I). Values and symbols as in Fig. 1.

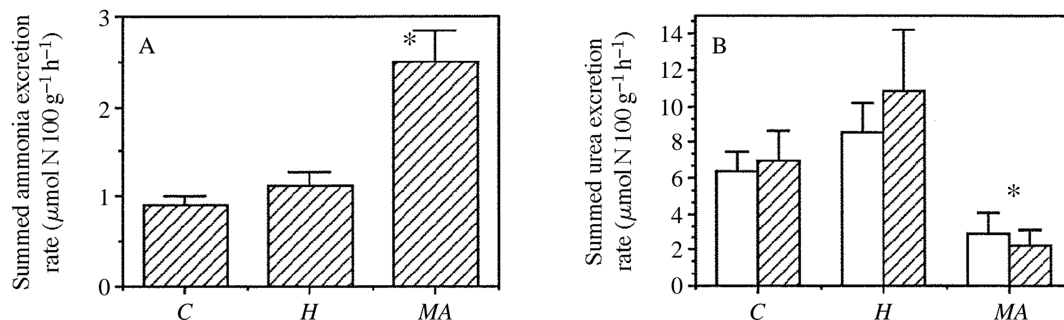

Fig. 6. Mass-specific summed ammonia-nitrogen excretion rates (A) and summed urea-nitrogen excretion rates (hatched bars) and production rates (open bars) (B) for toadfish exposed to control (*C*), hypercapnia (*H*) and mineral acidosis (*MA*) conditions (experimental series I). Values as in Fig. 1; * indicates a value significantly different from the control value at $P<0.05$.

consumption of bicarbonate, would be at odds with correction of the acidosis. The hypercapnic toadfish were able to correct acid–base status within 24h (Fig. 2), and urea excretion was depressed during the 12–24h period (Fig. 4). Additionally, urea excretion was significantly depressed in this same period in the fish exposed to mineral acid, and was always lower than in controls in the other periods, although these other differences were not significant (Fig. 4). (Note that, in this treatment, ammonia excretion was always higher than in the controls and the hypercapnic fish, significantly so in the 60–72h period; we will return to this point below.) Both of these observations are consistent with the view of Atkinson and colleagues, that acidosis should inhibit ureogenesis. However, once pH had recovered in the hypercapnic fish, urea excretion increased, and it was significantly elevated above control levels in the 24–36h and the 60–72h flux periods

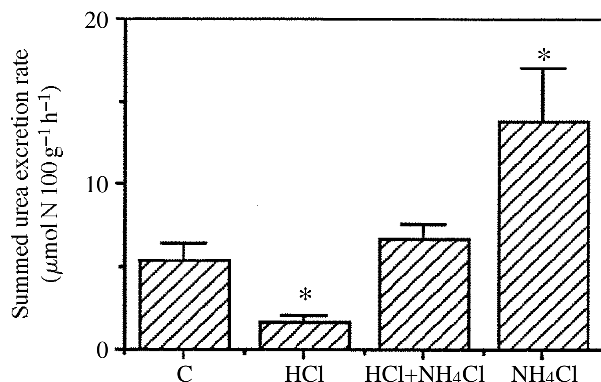

Fig. 7. Mass-specific summed urea-nitrogen excretion rates for toadfish exposed to control (C, normal sea water), mineral acidosis (HCl), mineral acidosis plus $5\,\text{mmol}\,l^{-1}$ NH_4Cl and $0.5\,\text{mmol}\,l^{-1}$ NH_4Cl conditions (experimental series II). Values as in Fig. 1; * indicates values significantly different from the control value at $P<0.05$.

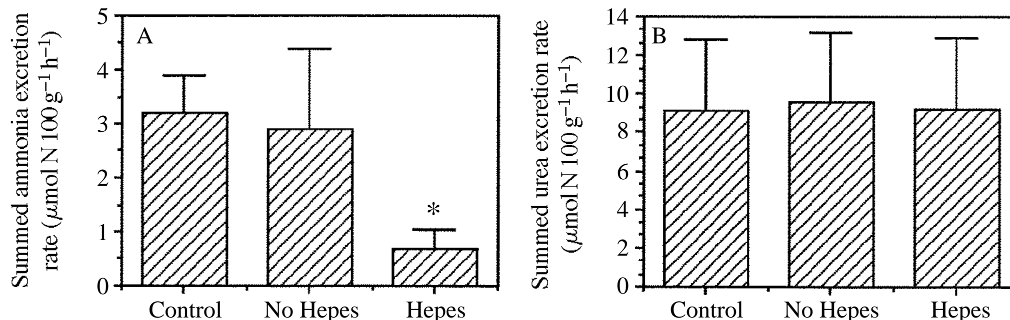

Fig. 8. Mass-specific summed ammonia-nitrogen (A) and urea-nitrogen (B) excretion rates for toadfish exposed to control, sea water with carbonates reduced (to less than 0.5mmol$\,l^{-1}$, no Hepes) and sea water with carbonates reduced and 5mmol$\,l^{-1}$ Hepes added (Hepes) (experimental series III). Values and symbols as in Fig. 7.

(Fig. 4). In order to be consistent with the views of Atkinson and colleagues, it would seem more appropriate if urea excretion returned to control rates once pH had been readjusted (i.e. from 24h onward). It could be argued that urea excretion rates are elevated after 24h to make up for the depression that occurred previously. However, plasma ammonia levels did not increase during the 0–24h period (Fig. 5), indicating that the toadfish did not incur an ammonia load, although this could reflect a decrease in rates of ammoniagenesis.

In both the present and previous experiments (Walsh *et al.* 1990) on nitrogen metabolism and excretion in the toadfish, we have noted substantial variability in urea excretion rates. This variability appears to be due to several factors. First, urea excretion occurs sporadically (e.g. often in only one of four 6h measurement periods in a 24 h cycle), probably because of irregular voiding of the urinary bladder and/or gut fluids, rather than by a constant flux through, for example, the gills (Walsh *et al.* 1990). Second, plasma urea concentrations appear not to be regulated very closely (Fig. 5), such that the urea excretion observed in a given period may not actually correlate in the short term with urea production in that period. Third, there may be a strong seasonal component to ureogenesis. In the present experiments, variability was lessened when experimental series were completed in a short period; note the reduction in variation in the results from the second series, which was carried out over the course of 2 months, compared with the results of the first series, which were obtained over 7 months (e.g. compare Fig. 6 with Fig. 4). Furthermore, we have extended the hepatocyte ureogenesis data set (Table 3) to toadfish collected over an annual cycle, and a distinct peak is evident in May–July, with a nadir in November–January (P. J. Walsh, unpublished data). Finally, preliminary data indicate that the transition between ammoniagenesis and ureogenesis in the toadfish appears to be extremely sensitive to confinement, to container size and to handling history (i.e. stress-related factors, B. Tucker and P. J. Walsh, unpublished data).

Given that several of these factors could be contributing to variability in our experiments, that we only observed significant differences in ammonia and urea excretion in three of the flux periods in experimental series I (Fig. 4) and that these excretion data

Table 2. *Effects of hypercapnia and mineral acidosis on enzyme activities in toadfish brain, liver and kidney*

Tissue Enzyme	Treatment: Control	Hypercapnia	Mineral acidosis
Brain			
GDH	1.605±0.286	1.892±0.382	1.945±0.230
AspAT	39.94±4.13	43.55±6.88	40.91±2.96
AlaAT	2.485±0.285	2.779±0.442	2.409±0.178
GNS	21.95±2.81	31.34±5.00	25.54±1.80
MDH	14.99±1.73	19.46±2.79	16.47±0.88
CS	4.512±0.590	5.356±0.826	5.061±0.462
Liver			
GDH	72.5±8.9	76.0±17.4	111.5±25.5
AspAT	188.3±18.3	175.1±7.0	186.7±20.0
AlaAT	60.75±5.61	51.15±5.22	61.22±5.86
GNS	21.62±4.86	24.84±4.93	24.50±5.42
OCT	114.0±9.4	110.4±10.9	114.5±20.6
ARG	109.5±15.3	112.5±19.5	113.6±16.3
MDH	330.0±30.5	330.1±12.7	347.4±31.4
CS	1.526±0.308	1.741±0.448	1.326±0.249
Kidney			
GDH	11.88±1.62	10.93±1.02	13.71±1.38
AspAT	19.81±2.21	21.48±1.09	22.57±2.05
AlaAT	4.283±0.541	4.537±0.597	4.126±0.353
GNS	12.07±1.70	15.15±2.80	13.50±2.58
MDH	80.01±4.70	114.9±20.3	91.73±6.06
CS	3.708±0.249	4.209±0.221	4.176±0.260

Units are μmol of substrate converted to product $min^{-1} g^{-1}$ wet tissue mass and values are means ± 1 S.E.M. (N=6).

GDH, glutamate dehydrogenase; AspAT, aspartate aminotransferase; Ala, alanine aminotransferase; GNS, glutamine synthase; MDH, malate dehydrogenase; CS, citrate synthase; OCT, ornithine–citrulline transcarbamoylase; ARG, arginase.

were inconclusive, we elected to compare our experimental treatments based on longer-term averages of ammonia excretion and urea excretion and production rates (Figs 6 and 7). Urea production during the entire hypercapnic period was not statistically different from control levels, but marginally elevated (Fig. 6). These results have two possible interpretations: (1) that ureogenesis is not affected by acid–base state and does not affect bicarbonate concentration enough, in relation to other processes, to play a significant role in the acid–base balance of the toadfish; or (2) that ureogenesis is depressed during acidosis, but that, in these experiments, the hypercapnic acidosis was corrected too rapidly (in less than 24h) to have a notable effect on total ureogenesis.

The remaining results shed light on these alternative interpretations. The mineral acid treatment was initially designed as a control; in the event that we observed a depression of

Table 3. In vitro *rates of ureogenesis (μmol urea g^{-1} hepatocyte wet mass h^{-1}) under four substrate regimes ([HCO_3^-] = 3 mmol l^{-1} unless noted, see Materials and methods for other details) in hepatocytes isolated from toadfish exposed to hypercapnia and mineral acidosis*

	Substrate			
Treatment	NH_4Cl (0.2 mmol l^{-1})	NH_4Cl (1.0 mmol l^{-1})	GLN (5 mmol l^{-1})	GLN/High [HCO_3^-] (15 mmol l^{-1})
Control	0.861±0.309	0.849±0.306	2.684±0.875	5.129±1.344
Hypercapnia	1.067±0.409	1.109±0.429	3.126±0.977	5.407±1.374
Mineral acidosis	0.922±0.323	1.011±0.336	2.640±0.756	4.101±0.901

Values are means ± 1 S.E.M. (*N*=6 fish per treatment).
GLN, glutamine.

ureogenesis by the respiratory acidosis of hypercapnia, we wished to be able to determine which acid–base variable was responsible. However, this treatment was interesting in its own right. In experimental series I, depression of seawater pH (and removal of its carbonate buffering system) led to a slight but chronic depression of pH in the toadfish (Fig. 2) and a marked depression of total ureogenesis (Fig. 6). There are two probable interpretations to these results as well. The first interpretation is that ureogenesis is depressed because the chronic reduction in pH requires bicarbonate retention in order to elevate pH. This interpretation is consistent with the views of Atkinson and colleagues, but clearly at odds with our prior observations on the pH-insensitivity of ureogenesis in toadfish hepatocytes *in vitro*. It is also at odds with the observation that blood pH remains depressed. A second interpretation, however, based on one current model of ammonia excretion (Wright *et al.* 1989), is that the reduced water pH and/or the reduced carbonate buffer content of the water (Fig. 1) facilitated ammonia excretion by diffusive trapping of NH_3, either directly by excess protons, or indirectly by allowing enhanced acidification of the gill–water boundary layer by CO_2 excretion and subsequent carbonic-anhydrase-mediated hydration and dissociation. Either of these scenarios would decrease the need for ureogenesis.

To distinguish between these two possibilities, experimental series II and III were undertaken. In addition to the control and mineral acid treatments, we exposed the fish to a combined mineral acidosis and external NH_3 load, and an equivalent NH_3 load under control conditions. We reasoned that if the depression of ureogenesis by mineral acidosis were simply a reflection of an increased ability to excrete ammonia, reduction of this ability to excrete ammonia should return ureogenesis to control values if the acid–base disturbance caused by ureogenic bicarbonate consumption was not very consequential. This treatment restored ureogenesis to normal levels, but values were still depressed relative to those in fish exposed only to an ammonia load (Fig. 7). This depression relative to the ammonia-only treatment could reflect the difference in the buffering capacity of the two waters, i.e. that it may still be more difficult to excrete ammonia into normally buffered sea water than into poorly buffered sea water, even at equivalent NH_3

Table 4. *Plasma amino acid concentrations (μmol l^{-1}) in toadfish exposed to control hypercapnia or mineral acidosis conditions for 72h.*

Amino acid	Treatment		
	Control	Hypercapnia	Mineral acidosis
Taurine	24.62±10.08	18.52±8.73	15.00±3.73
Aspartate	11.33±3.20	7.62±0.76	7.78±0.48
Threonine	100.69±11.92	66.87±5.84*	105.84±13.01
Serine	52.21±10.43	32.98±2.74*	47.52±4.10
Asparagine	16.45±2.19	9.93±0.77*	21.88±4.05
Glutamate	14.99±3.58	9.67±1.74	10.01±1.20
Glutamine	41.20±5.24	27.88±5.01*	51.60±5.21
Proline	18.11±2.65	10.41±0.58*	18.80±1.48
Glycine	83.87±5.87	67.91±5.84	99.42±13.95
Alanine	67.24±7.54	38.15±4.47*	79.35±13.06
Citrulline	4.27±1.11	3.88±0.59	3.22±0.48
Valine	79.49±5.48	59.06±9.32*	98.68±16.20
Methionine	17.54±3.01	11.10±1.08*	20.82±2.80
Isoleucine	52.90±5.25	43.38±7.53	69.84±12.41
Leucine	90.90±8.66	70.53±12.20*	119.97±19.64
Tyrosine	26.10±3.47	20.48±2.15	31.65±5.02
Phenylalanine	24.83±3.98	17.36±1.03	24.27±2.84
Tryptophan	15.30±3.35	11.92±3.56	14.38±1.87
Ornithine	121.34±7.08	98.02±9.00	113.97±12.29
Lysine	139.52±10.57	130.10±7.71	141.39±19.42
Histidine	24.35±3.61	19.95±1.09	26.24±1.50

Values are means ± 1 S.E.M. (*N*=6 per treatment).
*Significantly different from control at $P<0.05$ level.

levels. Experimental series III demonstrated that buffering has some effect on ammonia excretion, but that reduction of carbonate content and carbonate buffering capacity had no effect on ureogenesis (Fig. 8). We conclude that the majority of the effects of mineral-acid-treated water on ureogenesis are based on water pH effects. In addition, the depression of ureogenesis in the combined mineral acid/NH_4Cl treatment, relative to the NH_4Cl treatment, probably reflects some effect of pH on the ability of ammonia to enter the fish, even at equivalent NH_3 levels.

The most germane result of experimental series II and III is that fish in all treatments regulated acid–base status at levels equivalent to controls, with one slight exception (the pH of the Hepes-buffered fish was maintained despite a reduction in [HCO_3^-]) (Table 1). Interestingly, the slight depression in blood pH noted in the mineral acid treatment compared with controls in experimental series I was absent in experimental series II (Table 1). One potential explanation for this difference is that experimental series II lacked the potential acid–base stress associated with serial blood sampling (although haematocrits never fell below an acceptable limit in series I, results not shown). Furthermore, experimental series II was conducted over a short period, rather than over 7

months, and with a smaller size range of fish, either of which may have contributed to these differences. Regardless of the slight differences in acid–base status between experimental series I and II, the fish in mineral acidosis, combined mineral acidosis/ammonia-loaded and ammonia-loaded treatments displayed the *same* acid–base state, which, taken together with our other results, led us to conclude tentatively that the primary factor influencing rates of ureogenesis in the toadfish is the drive to excrete nitrogen. Acid–base consequences, if any, are probably secondary. Recently, similar experiments with the Lake Magadi tilapia led to the same conclusion (C. M. Wood, H. L. Bergman, P. Laurent, J. N. Maina, A. Narahara and P. J. Walsh, unpublished results). The emerging picture for control of ureogenesis in teleost fish could be tested further by experiments with the toadfish involving direct infusion into the bloodstream of acid, base, bicarbonate and ammonia where potential changes in the nitrogen excretion mechanisms might be less of a confounding factor.

Metabolic aspects

Interestingly, our treatments had no discernible effects on enzyme activities (Table 3) and hepatocyte ureogenic potential *in vitro* (Table 2). In some respects, this result is not surprising since fish in most treatments (except mineral acidosis) produced urea at significant and comparable rates. The reduction in urea synthesis rates in the mineral-acid-treated fish appears to have been achieved not by gross changes in the synthetic machinery, but rather at the level of substrate (specifically nitrogen) supply. Since ammonia excretion appears to be elevated in this group (Fig. 6), the rate of nitrogen supply to the O-UC may be lower. Finally, the decrease in several plasma amino acid levels at the end of 72h of hypercapnia is interesting (Table 4). One potential explanation for this observation is that, although the slight total increase in ureogenesis in this group was not significant, it may have been elevated enough during the 60–72h period (Fig. 4) to bring about a reduction in these amino acid nitrogen donors to the O-UC. Our prior *in vitro* measurements with hepatocytes suggest that the elevated plasma bicarbonate levels may be responsible for this increase in rates of ureogenesis and subsequent reduction of plasma amino acids. These experiments showed that $[HCO_3^-]$ was the only acid–base variable to which ureogenesis responded, with the K_m for bicarbonate being $1.3\,mmol\,l^{-1}$ and with saturation occurring at about $9\,mmol\,l^{-1}$ (Walsh *et al.* 1989). This observation was substantiated in the present study (Table 3), and so it appears that increased levels of bicarbonate might stimulate ureogenesis enough to effect a decrease in plasma amino acid levels.

Our results indicate that, in the ureogenic teleost toadfish, the primary driving force for urea synthesis and excretion appears to be the need to detoxify ammonia, and that acid–base consequences, if any, are probably secondary. The toadfish is an interesting and convenient model for the study of the evolution of ureogenesis in vertebrates. Additional studies on the control of flux through the O-UC in the ureogenic teleost fishes are sure to generate interesting insights into the evolution and regulation of waste nitrogen excretion in vertebrates.

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