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EFFECTS OF FEEDING AND CONFINEMENT ON NITROGEN METABOLISM AND EXCRETION IN THE GULF TOADFISH *OPSANUS BETA*

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Summary

In order to elucidate further the cues for, and the biochemical mechanisms of, the transition to ureogenesis in the gulf toadfish *Opsanus beta*, experiments on the effects of feeding (i.e. nitrogen loading) were carried out. Baseline nitrogen excretion rates were first measured on solitary toadfish in large water volumes (i.e. unconfined conditions). These nitrogen excretion rates were higher, and had a higher proportion as ammonia (61 %), than previously published 'control' measurements. Feeding of unconfined toadfish elevated total nitrogen excretion approximately threefold, with little change in the proportion of urea *versus* ammonia. During the first 24 h of confinement of unfed toadfish, absolute levels of urea excretion remained constant while ammonia excretion rates fell to near zero, so that toadfish became 90 % ureotelic. When fed prior to confinement, urea excretion rates remained constant for the first 24 h, and the bulk of the nitrogen was excreted as ammonia (80 %); excretion of the excess dietary nitrogen took up to 48 h to complete. If pre-adapted to confinement and then fed, toadfish excreted only about 55 % of their nitrogenous waste as ammonia, and excretion of excess dietary nitrogen was completed by 24 h. Elevations of hepatic glutamine synthetase (GNS) activities accompanied confinement and were shown to be almost exclusively in the cytosolic compartment and to be correlated with a decrease in the ratio of hepatic levels of glutamate:glutamine. These GNS activity increases also appear to account in part for the decrease in the percentage of ammoniotely in toadfish under conditions of nitrogen loading after confinement. However, additional means of regulating total nitrogen excretion (e.g. changes in protein turnover rates) and the degree of ureogenesis *versus* ammoniogenesis (e.g. *N*-acetylglutamate stimulation of carbamoylphosphate synthetase) must be postulated to account fully for changes in nitrogen excretion rates and activation of ureogenesis under some circumstances.

Key words: ornithine–urea cycle enzymes, glutamine synthetase, ureogenesis, amino acids, feeding, stress, gulf toadfish, *Opsanus beta*.

Introduction

The vast majority of teleost fish excrete their waste nitrogen principally as ammonia (ammoniotely); however, recent studies have expanded the list of teleosts that excrete the bulk of their waste nitrogen as urea (ureotely) (Wood, 1993). Ureotely in teleosts can be obligate or facultative, with the gulf toadfish *Opsanus beta* being an example of facultative ureotely (Barber and Walsh, 1993; Walsh *et al.* 1990, 1994). Recent evidence suggests that under some circumstances gulf toadfish can be predominantly ureotelic in nature (T. E. Hopkins and P. J. Walsh, unpublished data), but the ecological/evolutionary significance of ureotely in this species remains to be determined. Ureogenesis does not appear to be important for osmoregulation (Walsh *et al.* 1990) or acid–base balance (Walsh *et al.* 1989; Barber and Walsh, 1993) in this species. In the laboratory, ureogenesis by toadfish appears to function when normal pathways of ammonia excretion are blocked (Walsh *et al.* 1990; Barber and Walsh, 1993), presumably to detoxify ammonia, and ureotely can be activated by air exposure and NH_4Cl exposure (up to 150 μmol l^{-1}) (Walsh *et al.* 1990). Most recently we reported that, even in the absence of an increase in the ammonia concentration in the water, confinement and crowding can cause a switch to ureotely in toadfish in as little as 24 h; furthermore, this transition is accompanied by substantial activation of hepatic glutamine synthetase (Walsh *et al.* 1994), the enzyme believed to be responsible for trapping ammonia for the ornithine–urea cycle (O-UC) in fish (Campbell and Anderson, 1991; Mommsen and Walsh, 1991).

For the sake of simplicity, these initial laboratory studies have concentrated solely on *baseline* nitrogen excretion in unfed (i.e. post-absorptive) toadfish. Since total nitrogen excretion in fish increases several-fold following a meal as a

result of the deamination of excess amino acids (Wood, 1993), the digestive and absorptive period may be the most challenging one for fish in terms of potential ammonia toxicity. With this background information available, the primary aim of the present study was to investigate the effects of nitrogen loading (*via* feeding) on nitrogen metabolism and excretion in this facultatively ureotelic species, in order to determine whether the nature or time course of the transition to ureotely is altered. Furthermore, this study pursues some other issues raised by a previous study (Walsh *et al.* 1994). First, can the switchover to ureotely be induced in solitary fish, or are pheromonal, visual or other 'social' cues necessary? Second, we wished to subject the preliminary observation of low hepatic glutamine levels (Walsh *et al.* 1994) to further methodological scrutiny. Third, in view of our recent observation of the apparent importance of mitochondrial glutamine synthetase to ureogenesis in this family of fish (Batrachoididae) (Anderson and Walsh, 1995), we wished to examine the effects of feeding and confinement on subcellular compartmentalization of this pivotal enzyme.

Materials and methods

Experimental organisms

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 1993 and September 1994. Sexually mature toadfish (30–210 g) were held in an outdoor tank at the shrimpers' holding facility with running sea water (and ambient seasonal conditions) for less than 24 h following capture. Toadfish were then transferred to the laboratory, where they were kept in 45 or 80 l glass aquaria, with a bed of beach sand and polyvinylchloride tubes for shelter, and supplied with flowing, aerated sea water (sand-filtered and ultraviolet-light-sterilised) for 6–10 days prior to the start of an experiment. Stocking densities were never more than 5 g of fish per liter of sea water and, for some experiments, solitary fish were isolated in individual 45 l aquaria. Temperatures in these holding tanks and during the experiments described below were 26±2°C. Fish were treated with a 2 h static prophylactic dose of Malachite Green (final concentration 0.05 mg l^{-1}) in formalin (15 mg l^{-1}) (AquaVet, Hayward CA) on days 1 and 2 following transfer to the laboratory to prevent infection by a common ciliate (*Cryptocaryon irritans*) (Stoskopf, 1993). Prior to experimentation, fish were fed a single meal on day 3 of laboratory acclimation (and again on day 7 if they were held this long before being used in experiments). Experiments were typically begun 72 h post-feeding, i.e. on day 6 or 10 following introduction to the laboratory. Each meal, including experimental meals below, was of live pink shrimp, *Penaeus duorarum*, also from Biscayne Bay, at a ration of approximately 2.5 % of the fish's body mass. Although gender was recorded when fish were killed, fish were randomly assigned to control or test groups without regard for gender. No significant differences between sexes was seen in any of our results.

Experimental design

Three experimental series were undertaken. In series I, *solitary* toadfish were allowed to remain in 45 l glass aquaria (referred to as *unconfined*) and either fed again or not fed at the end of a 72 h post-feeding period during laboratory acclimation outlined above. Seawater flow was then turned off for 24 h and mixing was achieved by aeration. After the flow had been turned off, water samples (10 ml) were taken at 0, 2, 4, 8 and 24 h (unfed fish at 24 h only), with minimal disturbance to the fish, and were frozen (−20 °C) for subsequent analysis of ammonia and urea concentrations. In parallel control tanks without fish, no significant accumulation of ammonia or urea was observed. At 24 h, fish were anesthetised in 1.0 g l^{-1} tricaine methanesulfonate (buffered with sodium bicarbonate), weighed and killed. In additional fish, measurements were extended to a 24–48 h period after feeding by waiting until 24 h to stop the water flow, followed by terminal sampling at 48 h.

In series II, toadfish were held in groups of 3–5 and either fed or not fed in their glass tanks at time zero. Then some fish of each group were allowed to remain in their glass holding tanks (*unconfined*), while others of each group were transferred to small plastic tubs (30 cm long × 25 cm wide × 10 cm high) containing 6 l of aerated filtered sea water, without flow. The water in these tubs was changed every 24 h (referred to as *confined* below and virtually identical to the *confined-static water* conditions described in Walsh *et al.* 1994). In contrast to Walsh *et al.* (1994), however, only one fish was put in each tub. Transfers of fish were made by gently netting the fish and exposing them to air for less than 5 s. Fish were similarly transferred at water changeover every 24 h. (Note that we have shown previously that this netting transfer *per se* does not seem to be the causative agent in provoking a transition to ureogenesis, Walsh *et al.* 1994.) At various time intervals up to 96 h, 10 ml of sea water was withdrawn from the tubs and frozen (−20 °C) for later analysis of ammonia and urea. At 96 h, fish from all four treatments (*unconfined fed*; *unconfined unfed*; *confined fed*; *confined unfed*) were killed and their livers frozen at −80 °C for enzyme analyses, or they were used fresh for analyses of enzyme subcellular compartmentalization. Additionally, at 48 h, a subset of fish from all four treatments was killed, their blood was sampled by caudal puncture and liver samples were frozen in tongs precooled in liquid nitrogen. Blood was centrifuged at 13 000 ***g*** for 1 min, and plasma was removed and frozen in liquid nitrogen. Plasma and livers were then stored at −80 °C for amino acid analyses.

In the experiments of series III, toadfish were transferred to tubs as above at 72 h following feeding in glass tanks. After an additional 72 h in these tubs (i.e. pre-adaptation to confined conditions), half the toadfish were fed and half were not, and

sampling continued for up to an additional 96 h. At the end of an experiment, fish were killed and processed as detailed above.

Analytical methods

Water samples were analyzed for ammonia by the method of Ivancic and Deggobis (1984), and for urea by the method of Price and Harrison (1987), both of which have an approximate detection limit of 1–2 μmol l^{-1} as previously applied to toadfish (Walsh *et al.* 1994). For the analysis of amino acids, frozen livers were pulverized in a mortar and pestle precooled with liquid nitrogen and then homogenized on ice with 5 vols of chilled acetone plus 1 vol of 10 μmol l^{-1} α-aminobutyric acid (α-ABA) in 0.1 mmol l^{-1} HCl (as an internal standard) using a Brinkman polytron. Homogenates were centrifuged at 10 000 ***g*** for 5 min to remove debris. 50 μl of plasma was combined with 75 μl of acetone and 20 μl of α-ABA in HCl and centrifuged as for tissues. Tissue or plasma supernatants were then derivatized with phenylisothiocyanate (PICT) by drying 25 μl of the above supernatant under nitrogen, followed by addition of 20 μl of 1:1:3 triethylamine:methanol:water and redrying, followed by addition of 20 μl of freshly prepared 1:1:1:7 triethylamine:water:PICT:methanol and incubation at room temperature for 20 min. Samples were then dried under a nitrogen stream, dissolved in 1 ml of 5 mmol l^{-1} sodium phosphate, pH 7.4, with 5 % acetonitrile. Samples (20 μl) were injected onto a reverse phase column (CSC.sil, 80A/ODS2, 25 cm) at 40 °C and separated using a 7 % to 60 % acetonitrile gradient. Derivatized amino acids were detected at 254 nm with a Beckman ultraviolet/visible light detector.

For analysis of maximal enzyme activity, tissues were homogenized on ice in 3–4 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 pH 7.5 at 24 °C) using a Brinkman polytron. Homogenates were spun at 8000 ***g*** for 20 min at 4 °C in a Jouan CR412 centrifuge. The supernatant or a 1:10 dilution in homogenization buffer was used directly for the assay at 24 °C of alanine aminotransferase (AlaAT), aspartate aminotransferase (AspAT), glutamate dehydrogenase (GDH), glutamine synthetase (GNS), carbamoylphosphate synthetase (CPSase), ornithine–citrulline transcarbamoylase (OTC), argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL) and arginase (ARG) by previously described methods (Mommsen and Walsh, 1989; Barber and Walsh, 1993; Walsh *et al.* 1994). Subcellular compartmentalization of ARG and GNS activities was assessed by the methods of Anderson and Walsh (1995) using lactate dehydrogenase (LDH) as a marker enzyme for the cytosolic compartment and GDH as a marker enzyme for the mitochondrial compartment.

All biochemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA) and all other chemicals were reagent grade. Values were tested for significance at the 0.05 level using an unpaired Students' *t*-test or analysis of variance (ANOVA) and Student–Neuman–Kuels test (Zar, 1974).

Results

Patterns of nitrogen excretion

In series I, solitary unconfined toadfish allowed to acclimate to large (45 l) glass tanks with a suitable substratum (polyvinylchloride pipes) for cover exhibited 72 h post-feeding nitrogen excretion rates totaling 56.97±10.46 μmol 100 g^{-1} h^{-1} (S.E.M., N=10), with 61.1 % as ammonia and 38.9 % as urea (−24 h to 0 h group in Fig. 1). This control rate was rather consistent over at least an additional 24 h (0–24 h U group in Fig. 1) to 48 h (data not shown). Upon feeding, this rate increased rapidly, even in the first 2 h, to a peak rate of over 250 μmol 100 g^{-1} h^{-1} (4–8 h group in Fig. 1), with a total rate for the first 24 h of 183.8±16.2 μmol 100 g^{-1} h^{-1} (N=15) with 58 % as ammonia and 42 % as urea (0–24 h group in Fig. 1). The calculated total nitrogen excreted in a 24 h period was 4410 μmol N 100 g^{-1} for fed fish *versus* 1367 μmol N 100 g^{-1} for unfed fish. Under these conditions, the fed nitrogen load was cleared quickly in that there was no excess nitrogen excretion over that of unfed fish in the 24–48 h period (24–48 h in Fig. 1). Fish killed at 24 h post-feeding typically had small remnants of shrimp in the lower intestines (very rarely some was still in the stomach), and by 48 h had empty intestines (or rarely an undefined 'paste' remained in the lower intestines).

In series II, solitary post-absorptive unconfined fish transferred to confined conditions exhibited virtually the same pattern of nitrogen excretion as that seen in an earlier study of fish confined in groups (Walsh *et al.* 1994). There was an initial clamp-down on nitrogen excretion in the first 24 h, with

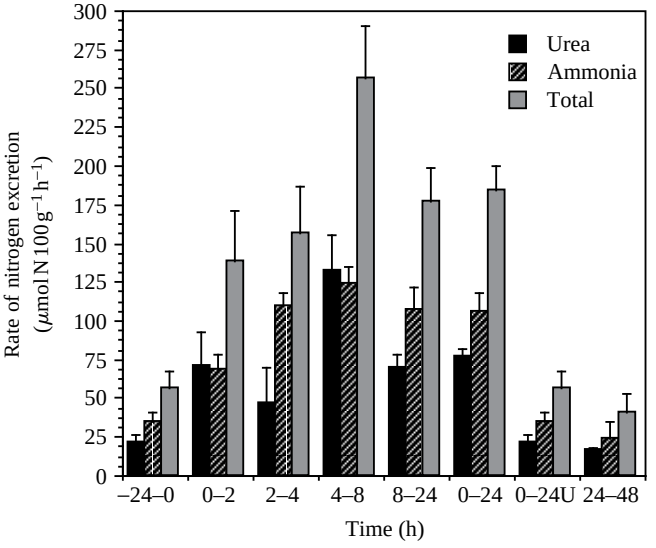

Fig. 1. Series I. Plot of nitrogen excretion rate (urea-N, dark filled bars; ammonia-N, hatched bars; total-N, light filled bars) in unconfined toadfish *versus* time after feeding. Times note the length of a flux period (−24–0 is the 24 h prior to feeding). U refers to unfed, unconfined (control) fish. Values are means + 1 S.E.M. (N=6–15 fish per group). All post-feeding values for 0–24 h are significantly elevated above controls except for the 2–4 h urea excretion rate. There is no significant difference between 0–24 h U and 24–48 h (fed) rates.

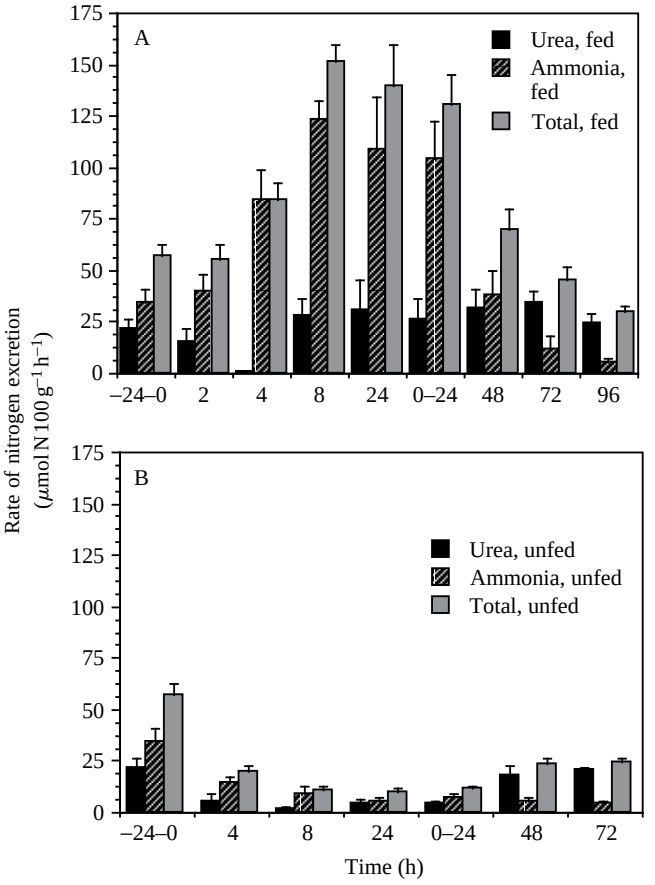

Fig. 2. Series II. Plot of nitrogen excretion rate (urea-N, dark filled bars; ammonia-N, hatched bars; total-N, light filled bars) in toadfish transferred from unconfined to confined conditions and either fed (A) or unfed (B) just prior to transfer. Times as in Fig. 1 except that the initial flux time has been omitted for clarity (e.g. 2=0–2 h, 72=48–72 h, etc.). Values are means + 1 S.E.M. (N=6–9 fish per group). In fed fish (A), all ammonia-N and total-N excretion rates between 2 and 48 h are significantly higher than those for unfed fish (B), as is the total-N excretion rate for the 48–72 h period. In unfed fish (B), all excretion rates for confined fish are significantly lower than those for unconfined fish, with the exception of 48 and 72 h urea-N excretion rates.

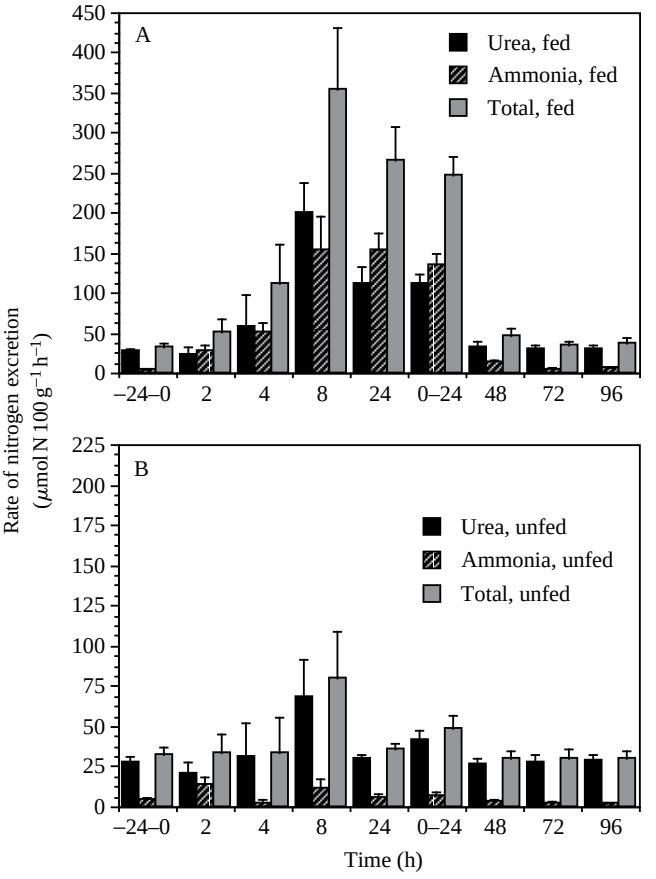

Fig. 3. Series III. Plot of nitrogen excretion rate (urea-N, dark filled bars; ammonia-N, hatched bars; total-N, light filled bars) in confined toadfish that were previously confined for 72 h and then either fed (A) or not fed (B) at time zero. Times as in Fig. 2, except that −24–0 h refers to the last 24 h of the initial 72 h confinement period. Values are means + 1 S.E.M. (N=6–14 fish per group). Both urea-N and ammonia-N excretion rates for fed fish (A) are significantly higher than those for unfed fish (B) for the 8–24 h periods, while only the ammonia-N excretion rate is significantly elevated for the 2–4 h period. Note that the scale on the y-axis in A is double that in B.

resumption of nitrogen excretion in the 24–48 h period, although at a much reduced total rate (Fig. 2B). Beyond 24 h, over 90 % of nitrogen excretion was as urea (48 and 72 h in Fig. 2B), but note that the total urea excretion rate is not significantly different from that in unconfined controls (−24 h to 0 h in Fig. 2B).

In contrast, when solitary toadfish were fed just prior to confinement in series II, the nitrogen excretion rate did not decrease, but remained constant in the 0–2 h period, then the ammonia-N excretion rate increased as early as the 2–4 h period (Fig. 2A). The urea-N excretion rate returned to preconfinement levels by the 4–8 h period, but was greatly exceeded by the ammonia-N excretion rate (80 % of the total) in the first 24 h (Fig. 2A). Total nitrogen excretion remained elevated until the 72–96 h period (Fig. 2A), relative to that of unfed confined fish (Fig. 2B). Furthermore, compared with that of fed unconfined toadfish, individuals that were fed and then confined reduced the total amount of nitrogen excreted and spread out their excess nitrogen excretion over a longer period. The total feeding-related nitrogen excretion by these two groups (i.e. minus the baseline level of 1367 μmol N 100 g^{-1} seen in series I and II) was higher in series I (3043±242 μmol N 100 g^{-1}) than in series II (2063±238 μmol N 100 g^{-1}) (P<0.05), and faeces were not usually observed in the water of series II confined fish until 48–72 h compared with 24–48 h for series I fish.

In series III, when toadfish were pre-adapted for 72 h to confined conditions, the effect of feeding on nitrogen excretion patterns was altered compared with that of toadfish which were first fed and then confined (series II). As in prior experiments (Fig. 2; and Walsh *et al.* 1994), toadfish pre-adapted to confined conditions exhibited much reduced rates of nitrogen

excretion, with the bulk of this being excreted as urea (−24 h to 0 h period in Fig. 3A,B). For unfed toadfish kept in continued confinement, this pattern did not change with the exception of a statistically insignificant peak at 4–8 h (Fig. 3B). When toadfish were fed after pre-adaptation to confined conditions, the proportion of nitrogen excreted as urea and as ammonia was much more balanced (Fig. 3A) compared with those of fish not pre-adapted (Fig. 2A), and the excess nitrogen load was cleared in 24 h as in unconfined fish (Fig. 1), which is faster than in fish that had first been fed and then confined (Fig. 2A). However, total feeding-related nitrogen excretion (5222±540 μmol N 100 g^{-1} after subtraction of nitrogen excretion by non-fed controls) was also much higher than in either series I or series II (P<0.05).

Metabolic aspects

Confinement for 96 h (series II) had little effect on activities of the O-UC and related enzymes, with the exception of GNS where confinement resulted in a four- to fivefold increase in activity (Table 1); these results were similar to those from our earlier study (Walsh *et al.* 1994). Feeding did not appear to modify this response (Table 1). The effect of these treatments on compartmentalization of two enzymes involved in ureogenesis, ARG and GNS, which are known to exist in both the cytosolic and mitochondrial compartments (Anderson and Walsh, 1995), was also examined. Good separation of these compartments was routinely obtained, as indicated by enrichment of the marker enzymes GDH in the mitochondrial fraction and LDH in the soluble fraction (Table 2). Confinement did not change the relative distribution of ARG activities (Table 2). However, the confinement-induced increase in GNS activity was nearly all in the soluble fraction (Table 2). Feeding did not modify either ARG or GNS compartmentalization (data not shown). At the end of this 96 h post-feeding period in series III, the hepatic GNS values did not differ between the fed and unfed groups [13.67±1.55 units g^{-1} for the fed group and 16.12±3.48 units g^{-1} for the unfed group; mean ± 1 S.E.M. (N=6); units of enzyme activity are defined in Table 2] and

Table 1. *Hepatic enzyme activities of gulf toadfish* Opsanus beta *following 96 h of exposure to different confinement and feeding regimes (series II)*

Enzyme	Unconfined, unfed (N=9) (units g^{-1})	Unconfined, fed (N=8) (units g^{-1})	Confined, unfed (N=9) (units g^{-1})	Confined, fed (N=7) (units g^{-1})
AlaAT	37.92±2.09	33.93±1.75	43.48±1.77	38.64±2.07
AspAT	66.5±5.7	55.3±2.4	66.2±2.2	63.3±3.5
GDH	35.4±3.1	28.2±1.4	32.4±2.6	29.8±2.1
GNS	2.34±0.20	3.14±0.59	10.4±1.9*	11.1±0.9*
CPSase	0.21±0.04	0.15±0.02	0.19±0.03	0.19±0.03
OTC	45.0±4.1	39.9±3.5	43.8±1.9	42.8±3.1
ASS	0.04±0.01	0.03±0.01	0.04±0.01	0.04±0.01
ASL	0.69±0.05	0.71±0.07	0.64±0.05	0.65±0.06
ARG	78.1±3.0	66.1±5.7	72.9±2.6	69.0±5.2

Activities are expressed as μmol substrate utilized per minute per gram fresh liver mass (units g^{-1}).

Values are mean ± 1 S.E.M.

*Significantly differs from unconfined treatments; $P \leqslant 0.05$.

AlaAT, alanine aminotransferase; AspAT, aspartate aminotransferase; GDH, glutamate dehydrogenase; GNS, glutamine synthetase; CPSase, carbamoylphosphate synthetase; OTC, ornithine–citrulline transcarbamoylase; ASS, argininosuccinate synthetase; ASL, argininosuccinate lyase; ARG, arginase.

Table 2. *Compartmentalization of hepatic enzymes in unfed gulf toadfish* Opsanus beta *following 96 h of unconfined or confined treatment (series II)*

Enzyme Treatment group	Total activity (units g^{-1})	Debris (% total activity)	Soluble (% total activity)	Mitochondrial (% total activity)
LDH	3.53±0.20 (16)	13.6±1.1	68.8±1.4	17.6±1.5
GDH	6.29±0.65 (16)	19.1±2.0	13.7±1.2	67.3±1.7
ARG				
Unconfined	78.1±3.0 (8)	14.7±0.6	44.6±2.1	40.9±2.7
Confined	72.9±2.6 (9)	14.7±2.2	48.8±6.2	36.4±4.2
GNS				
Unconfined	2.34±0.20 (17)	20.0±2.1	37.0±2.5	43.0±2.7
Confined	10.8±0.8 (7)*	8.0±0.6*	77.3±1.6*	14.7±1.4*
Δunits g^{-1}	+8.43	+0.39	+7.46	+0.58
Percentage change	100.0	4.6	88.5	6.9

Values are means ± 1 S.E.M. (N).

*Significantly differs from unconfined treatments; $P \leqslant 0.05$.

LDH, lactate dehydrogenase; GDH, glutamate dehydrogenase; ARG, arginase; GNS, glutamine synthetase.

Units of enzyme activity are defined in Table 1.

Table 3. *Plasma amino acid concentrations in toadfish* Opsanus beta *subjected to different confinement and feeding regimens for 48 h (series II)*

Amino acid	Unconfined, unfed (N=9) (μmol l^{-1})	Unconfined, fed (N=8) (μmol l^{-1})	Confined, unfed (N=8) (μmol l^{-1})	Confined, fed (N=8) (μmol l^{-1})
Asp	3.19±2.14	1.05±1.05	3.39±1.98	1.93±1.04
Glu	2.91±1.74	5.64±2.43	2.20±1.56	ND
Ser	44.94±4.30	42.34±8.48	30.57±6.56	32.91±6.71
Asn	26.59±1.60	45.64±12.78	29.91±5.76	20.05±6.49
Gln	294.14±57.54	493.69±74.05	379.70±86.60	409.10±121.30
Gly	89.30±7.06	115.15±22.69	72.93±7.38	118.05±19.65
His	33.98±20.71	18.47±6.76	18.43±6.47	11.58±5.70
Tau	53.74±17.44	96.09±17.19	61.73±20.90	63.23±15.51
Thr	ND	ND	ND	ND
Ala	59.78±6.55	106.69±35.37	51.51±9.25	52.65±7.88
Arg	52.45±8.22	48.64±16.10	44.49±6.70	24.09±3.63
Pro	7.63±7.25	34.34±12.62	7.36±2.97	12.81±8.93
Tyr	33.60±4.80	40.70±14.10	16.00±7.80	7.30±4.98
Val	115.22±12.82	205.27±35.14*	110.9±15.16	81.58±14.48
Met	3.66±2.51	24.70±14.7	1.20±1.20	1.07±1.07
Ile	52.77±9.40^{a}	75.75±24.37^{a}	21.16±7.44^{b}	26.77±6.97^{b}
Leu	243.65±51.19^{a}	191.09±55.21^{a}	63.40±8.97^{b}	59.26±13.80^{b}
Phe	18.16±4.47	42.93±15.98*	4.96±3.25	11.64±6.06
Trp	ND	0.22±0.22	ND	ND
Lys	41.75±13.75	101.50±24.55	93.1±13.6	55.44±13.14
Orn	24.08±12.34^{a}	152.49±17.30^{b}	57.78±8.65^{c}	67.62±11.20^{c}
Cit	ND	5.77±5.77	ND	ND

Values are mean ± 1 S.E.M.
ND, not detected.
*Significantly differs from other treatments.
^{a,b,c}Values with same superscript not significantly different; values with different superscripts are significantly different; $P \leqslant 0.05$.

were not significantly higher than values for the other experimental series (e.g. confined value in Table 2).

Confinement and feeding had relatively small effects on plasma amino acid levels at 48 h post-treatment (Table 3). At 48 h, plasma levels of valine, ornithine, phenylalanine and citrulline were significantly elevated in unconfined fed toadfish relative to the other groups (Table 3). Furthermore, confinement led to significant decreases in plasma levels of isoleucine and leucine, but to an increase in ornithine level in unfed fish (Table 3). Notably, glutamate was not detectable in the plasma of the confined fed group (Table 3). Liver levels of amino acids were even more resistant to change by these treatments. Liver proline concentration was elevated in the unconfined fed group (Table 4). We have previously reported negligible liver glutamine levels in toadfish when an extraction method with an acidic protein precipitation step was used (Walsh *et al.* 1994). An acetone extraction was used in the present study, and significant levels of liver glutamine were found (Table 4). It is notable that the ratio of glutamate:glutamine was nearly reversed in the unconfined unfed group (2.29) compared with the confined fed group (0.54). Citrulline level was also elevated in this last group. More generally, there are a number of differences in absolute levels of plasma and liver amino acids between the present study and a prior one (Walsh *et al.* 1994); we attribute these differences mainly to the different methodologies used.

Discussion

The results of the present study allow a number of new conclusions regarding the significance of, and mechanisms underlying, the transition from ammoniotely to ureotely in the gulf toadfish. The first notable finding is that 'control' total nitrogen excretion rates in solitary unconfined post-absorptive toadfish (series I; Fig. 1) are two- to threefold higher, with a higher percentage being excreted as ammonia (mean of 61 % ranging up to 88 %), than we have observed in the past (e.g. Barber and Walsh, 1993; Walsh *et al.* 1994). Given the rapidity with which toadfish can clamp down on nitrogen excretion (Fig. 2B; Walsh *et al.* 1994) and the apparent requirement for unconfined (solitary) conditions in order for these fish to exhibit ammoniotely, it is now clear that the transition from high levels of ammoniotely to low total nitrogen excretion/ureotely is an *extremely* sensitive one. On the basis of plasma cortisol measurements in fish treated identically to those in the current study (T. E. Hopkins, C. M. Wood and P. J. Walsh, unpublished data), we believe that the current control values reflect those of

Table 4. *Liver amino acid contents in toadfish* Opsanus beta *subjected to different confinement and feeding regimens for 48 h (series II)*

Amino acid	Unconfined, unfed (*N*=9) (mmol kg^{-1})	Unconfined, fed (*N*=7) (mmol kg^{-1})	Confined, unfed (*N*=9) (mmol kg^{-1})	Confined, fed (*N*=7) (mmol kg^{-1})
Asp	ND	ND	ND	ND
Glu	6.19±0.56*****	4.81±0.45	4.01±0.46	3.13±0.29
Ser	0.33±0.05	0.42±0.09	0.27±0.05	0.25±0.06
Asn	0.05±0.01	0.10±0.04	0.04±0.01	0.03±0.01
Gln	2.70±0.68	4.99±1.01	2.71±0.47	5.80±1.28*****
Gly	1.11±0.03	0.69±0.12	1.01±0.08	1.12±0.11
His	0.11±0.01	0.10±0.01	0.07±0.01	0.09±0.01
Tau	18.75±1.08	16.22±0.79	16.69±1.11	18.88±0.68
Thr	ND	0.18±0.15	ND	ND
Ala	0.12±0.03	0.07±0.02	0.20±0.05	0.11±0.04
Arg	0.33±0.09	0.35±0.16	0.11±0.02	0.19±0.06
Pro	0.13±0.02	0.35±0.13*****	0.09±0.02	0.19±0.07
Tyr	0.08±0.02	0.10±0.1	0.02±0.01	0.03±0.02
Val	0.08±0.02	0.14±0.04	0.08±0.02	0.09±0.03
Met	0.01±0.01	0.03±0.01	0.02±0.01	0.03±0.02
Ile	0.04±0.01	0.05±0.01	0.02±0.01	0.02±0.01
Leu	0.88±0.03	0.90±0.02	0.81±0.05	0.81±0.06
Phe	0.01±0.01	0.01±0.01	ND	ND
Trp	ND	ND	ND	ND
Lys	ND	0.01±0.01	ND	ND
Orn	0.01±0.01	0.04±0.03	ND	ND
Cit	ND	0.01±0.01	ND	0.05±0.01*****

Values are mean ± 1 S.E.M.
ND, not detected.
*Significantly differs from other treatments.

relatively unstressed fish, whereas prior control fish (e.g. Barber and Walsh, 1993; Walsh *et al.* 1994) were probably moderately stressed, although no cortisol data are available from these prior studies to test this speculation.

The control measurements of the present study confirm that toadfish become primarily ureotelic during confinement. Interestingly, this is accomplished by lowering the total nitrogen excretion rate and eventually allowing the absolute rate of urea excretion to rise to pre-confinement levels (Figs 2B, 3B). These findings indicate that the transition to ureotely in toadfish represents a shutting down of ammoniotely, rather than a massive *de novo* activation of ureogenesis *per se*. This interpretation fits well with the lack of changes in *maximal* activity in the O-UC enzymes noted (Table 1). Thus, the four- to fivefold activation of hepatic glutamine synthetase (Table 1) in the cytosolic compartment (Table 2) probably represents one simple mechanism of turning off ammoniotely by trapping ammonia in the conversion of glutamate to glutamine. One fate of this glutamine produced in the cytosol may be to enter the mitochondria for urea synthesis by CPSase III (Anderson and Walsh, 1995). It is not likely that a substantial proportion of this hepatic glutamine is used for pyrimidine nucleotide biosynthesis since levels of CPSase II are quite low (Anderson and Walsh, 1995), nor is it likely that significant amounts of urea are generated by the uricolytic pathway since levels of these enzymes are relatively low in confined toadfish [uricase: 0.85±0.13; allantoicase: 0.61±0.09; allantoinase: 0.06±0.01 units g^{-1}, mean ±1 S.E.M. (*N*=7), P. J. Walsh, unpublished data] in comparison with levels in other species where uricolysis is activated (e.g. Wright, 1993).

This study further demonstrates that, following confinement, *solitary* fish are able to make the transition to an increased percentage of nitrogen excretion as urea (series II, Fig. 2B), with virtually the same kinetics and pattern as crowded fish (Walsh *et al.* 1994), which we believe rules out the necessity for conspecific communication (e.g. by pheromones or auditory or visual cues) in this transition.

The central findings of this study are that feeding enhances nitrogen excretion and, more importantly, that confinement modifies how toadfish deal with this increased nitrogen load. Unconfined toadfish appear to process a meal rapidly, clearing the excess nitrogen load in 24 h or less (series I, Fig. 1). Interestingly, this is accomplished by substantially increasing not only ammonia excretion rates, but urea excretion rates as well, in spite of the observation that these unconfined fish have characteristically low hepatic GNS values (Table 1). This finding would suggest that, at least in unconfined fish, elevation of maximal GNS activity is not necessary to stimulate urea production. When unconfined fed toadfish are initially exposed to confinement (which appears to be a stressful condition invoking release of cortisol, T. E. Hopkins, C. M. Wood, and P. J. Walsh, unpublished data), urea excretion rates are not elevated, most of the nitrogen is excreted as ammonia, and excretion (and apparently digestion) is spread over a longer period (Fig. 2). These observations are consistent with the lack of GNS activation that exists for at least part of the first 24 h (Walsh *et al.* 1994) (such that ammonia excretion occurs unchecked) and the observations in other species that stressful conditions shunt blood away from digestive organs (e.g. Axelsson and Fritsch, 1991), possibly prolonging the digestive process. It is not clear, however, why ureogenesis is not activated as it is in the unconfined fed toadfish (Fig. 1). These observations for fed unconfined and fed then confined toadfish, taken together, further support the hypothesis that elevated GNS activities do not necessarily support elevated ureogenesis *per se*, but may be more important in regulating the degree of ammoniotely. In support of this interpretation, when GNS activity is elevated to levels characteristic of confined fish in other treatments by pre-adaptation to confined conditions (series III), and *basal* ammonia excretion is clamped off (Fig. 3B), less of the nitrogen load induced by feeding is excreted as ammonia (55 %, Fig. 3A) compared with that in toadfish not pre-adapted to confinement (80 %, Fig. 2A). However, the nitrogen load induced by feeding is much higher in series III and apparently exceeds the capacity for ammonia trapping allowed by even the elevation of maximal GNS activity (Fig. 3A). In these preconfined fish, the percentage of nitrogen

excreted by ureotely following feeding is higher (Fig. 3A) in the first 24 h than in fish not preconfined (Fig. 2A), but absolute levels of ammonia excretion are about the same. Thus, the importance of GNS in clamping down on ammonia excretion may be less relevant during feeding.

A final important potential explanation for the differences in the patterns of nitrogen excretion in series I, II and III is that protein synthesis rates may differ in these groups. While a decrease was observed in the ratio of hepatic glutamate:glutamine in the most nitrogen-loaded toadfish (confined and fed) compared with the least nitrogen-loaded (unconfined and unfed) toadfish in series II (Table 4), this difference cannot account for all the differences in the ammonia excreted; perhaps protein synthesis rates differ in the two groups. Furthermore, one could explain the rapid apparent clearance of a larger amount of excess nitrogen in series III by a much reduced incorporation of dietary amino acids into protein. Measurements of protein synthesis and turnover rates (see, for example, Houlihan *et al.* 1995) would be informative in this regard.

The lack of changes in the *maximal* activities of the O-UC enzymes (Table 1) and the discussions above on GNS activation point towards additional biochemical mechanisms for activation of ureogenesis in the toadfish. Allosteric activation of CPSase III by the potent activator *N*-acetylglutamate could contribute to elevated ureogenic rates. These potential *in vivo* changes are not reflected by the assays used to measure maximal activities, which contain sufficient quantities of *N*-acetylglutamate to induce maximal CPSase III activity. Furthermore, since toadfish liver mitochondria appear to contain sufficient glutaminase activity (Anderson and Walsh, 1995) to deaminate glutamine to glutamate, shunting it away from CPSase III, the absolute levels of hepatic GNS may be less important in regulating rates of ureogenesis than the ratio of GNS to glutaminase. Since cytosolic glutamine production may predominate, transport of glutamine into the mitochondria could be a third potential regulatory point. Examination of these possibilities will provide fruitful avenues for further investigation.

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