

PULSATILE UREA EXCRETION IN THE UREAGENIC TOADFISH *OPSANUS BETA*: AN ANALYSIS OF RATES AND ROUTES

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Summary

This study focused on the rates and routes of urea-N and ammonia-N excretion in the ureagenic toadfish and on the possibility that urea-N excretion occurs in pulses. Experimental approaches included the following: confinement in small individual containers with automated hourly sampling of water to follow temporal excretion patterns; divided chambers to separate excretion from the anterior and posterior parts of the fish; collection of urine and rectal fluid *via* chronic indwelling catheters; and gavage with [¹⁴C]-labelled polyethylene glycol 4000 to detect regurgitation of gastrointestinal fluids. When a standardized 'crowding' pre-treatment was employed to induce ureotelic behaviour, the fish exhibited significant elevations in the activity of glutamine synthetase in liver, kidney and gills, elevated plasma and bile urea-N levels, but unchanged ammonia-N and urea-N levels in most other body fluids. Unencumbered ureotelic fish confined in small containers excreted 82% of their waste-N as urea-N and 18% as ammonia-N; almost all (94%) of this urea-N excretion occurred in a single pulse of less than 3 h duration about once every 24 h. This daily pulse did not occur by regurgitation of gut fluids, by excretion through prominent pores behind the pectoral fins or by discharge of rectal fluid

or urine. Intestinal and urinary excretion accounted for less than 10% of whole-body urea-N excretion and a negligible fraction of ammonia-N excretion. Pulsatile urea-N excretion occurred at the head end across the gills and/or body surface. Ammonia-N excretion, which was not pulsatile, also occurred largely through the head end. However, once the toadfish had been placed in divided chambers, urea-N excretion became continuous rather than pulsatile, and ammonia-N excretion increased greatly. A severe stress response was indicated by high levels of plasma cortisol, and the skin, which lacks scales, became a significant route of both ammonia-N and urea-N excretion. We speculate that the normal adaptive significance is that ureotelism facilitates cryptic behaviour, allowing the toadfish to virtually eliminate N-waste excretion during long periods while it remains sheltered in burrows. However, during severe stress, the effects of extremely high cortisol levels overwhelm the ammonia and urea retention mechanisms, and both substances leak across the general body surface.

Key words: urea, ammonia, *Opsanus beta*, ureotelism, gills, skin, urine, rectal fluid, regurgitation, ornithine-urea cycle, stress.

Introduction

Most teleost fish are obligate ammonioteles excreting the bulk of their waste-N as ammonia, together with only small amounts of urea produced by uricolysis (reviewed by Mommsen and Walsh, 1991; Wood, 1993). The gulf toadfish (*Opsanus beta*) is one of the very few teleosts to exhibit facultative ureotelism, at times excreting more than 50% of its waste-N as urea (Walsh *et al.* 1990). This ability is associated with ureogenesis, the production of urea by an active ornithine-urea cycle in the liver; the cycle is fuelled with nitrogen by the enzyme glutamine synthetase (Mommsen and Walsh, 1989). Amongst teleosts, this characteristic is shared by only a few other species (Saha and Ratha, 1987, 1989, 1994;

Randall *et al.* 1989; Wood *et al.* 1989, 1994; Walsh *et al.* 1993). At least in the toadfish, a marked induction of hepatic glutamine synthetase activity appears to be diagnostic of the facultative transition from ammoniotelism to ureotelism (Walsh *et al.* 1994a; Hopkins *et al.* 1995).

In all teleosts studied to date, the gills appear to be the major site of urea excretion (reviewed by Wood, 1993). However, as pointed out in the latter review, most investigations have ignored a possible role for the skin, while a few studies on marine teleosts have provided direct evidence that the skin can account for over 50% of urea excretion under certain circumstances (e.g. Morii *et al.* 1978; Sayer and Davenport,

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1987). It is noteworthy that the gulf toadfish is a member of the Batrachoididae family, a group characterized by the complete absence of scales from the skin (Hart, 1980). In the toadfish, the gills are clearly important for ammonia excretion (Evans *et al.* 1989), but nothing quantitative is known about the routes of either ammonia or urea excretion, under either ammoniotelic or ureotelic conditions. Nevertheless, there are numerous anecdotal reports that urea excretion appears to be irregular and pulsatile, and suggestions that periodic micturation of urine from the bladder and/or rectal fluid from the intestine serves as the major route of urea excretion in the toadfish (Walsh *et al.* 1990, 1994a; Griffiths, 1991; Mommsen and Walsh, 1992; Barber and Walsh, 1993). The primary objectives of the present study were therefore to partition quantitatively the rates and routes of urea (and ammonia) excretion through the gills, skin, kidney (bladder) and intestinal tract, and to assess whether urea excretion is truly pulsatile in *Opsanus beta*.

Walsh *et al.* (1994a) demonstrated that both the pattern and magnitude of N-waste metabolism in the toadfish can be greatly altered by apparent psychological factors. Therefore, further goals were to characterize the effect of stress on the system and to define experimental conditions under which the condition of ureotelism could be maintained and therefore studied. These responses to disturbance cast light on both the definition and mechanism of ureotelism in the toadfish, especially with regard to the roles of cortisol and hepatic glutamine synthetase, and thereby complement other recent studies on these issues (Walsh and Milligan, 1995; Hopkins *et al.* 1995).

Materials and methods

Experimental animals and holding conditions

The majority ($N=76$) of our experimental animals were sexually mature gulf toadfish (*Opsanus beta*, Goode and Bean; 34–248 g) which had been captured by commercial shrimpers using roller trawls in Biscayne Bay, Florida, USA, between January and March 1994. The divided chambers of series 4 (see below) worked best with large fish, so a few additional large *Opsanus beta* (180–330 g, sexually mature; $N=8$) trawled from Apalachee Bay were purchased from Gulf Specimen Co., Panama, Florida, USA. In our laboratory, all fish were held in running sea water from Biscayne Bay (20–24 °C; 29–33 ‰) under a natural photoperiod. Prophylactic treatments with Malachite Green (0.05 mg l⁻¹) plus formalin (15 mg l⁻¹) were administered on days 1 and 3 of laboratory holding to prevent infection by the ciliate *Cryptocaryon irritans*. Experiments were performed on days 6–10, using unfed fish and a temperature of 20±1 °C.

The standard holding condition ('non-crowded') consisted of 2–5 fish in a large glass aquarium (45 or 80 l; maximum density 12 g fish l⁻¹) served with flowing, sand-filtered aerated sea water. All aquaria were furnished with a bed of sand and gravel, and individual polyvinylchloride (PVC) shelters matched to the size of the fish. A 'crowded' treatment was employed in order to induce ureotelism (see Walsh *et al.*

1994a; Hopkins *et al.* 1995). For this, groups of 3–6 toadfish, each with their individual shelters, were placed for 48–72 h in small 6 l plastic tubs (30 cm×25 cm×8 cm deep) at a density greater than 80 g fish l⁻¹. Water flow and aeration were maintained during crowding.

Experimental series

For all surgical procedures, as well as terminal body fluid and tissue sampling, fish were immersed in MS-222 (1 g l⁻¹, adjusted to pH 7.8 with NaOH), a treatment which rendered them rapidly unconscious. In view of this species' tolerance of air exposure, irrigation of the gills was unnecessary. Ventilation resumed within 15 min of return to anaesthetic-free sea water.

Series 1

This experiment examined possible differences in body fluid composition between 'non-crowded' (i.e. probably ammoniotelic; $N=13$) and 'crowded' (i.e. probably ureotelic; $N=12$) toadfish as a guide to possible routes of urea and ammonia excretion. Blood was sampled anaerobically by blind caudal puncture and heparinized at 100 i.u. ml⁻¹ with sodium heparin. A subsample was used for the measurement of pH, and the remainder was centrifuged at 10 000 g for 2 min. A subsample of plasma was assayed for total CO₂ concentration, and the remaining plasma was frozen at -70 °C for later analysis of total ammonia-N, urea-N and cortisol concentrations. Gentle pressure was applied to the anus, and rectal fluid was withdrawn using a wide-bore catheter to avoid plugging with carbonate deposits. The body cavity was then opened, and fine-bore 26 gauge needles were used to extract bile from the gall bladder, urine from the left (larger) lobe of the asymmetric urinary bladder, and finally fluid from internal cysts. Virtually all fish collected from Biscayne Bay contained variable numbers (3 to more than 20) of fluid-filled cysts clustered around the bladder, gonoducts and posterior portion of the intestine, which are probably granulomas remaining at the sites of earlier parasitic infections (Dr M. C. Schmale, University of Miami, personal communication). In view of their location, they could potentially be involved in nitrogenous waste metabolism. Rectal fluid, bile and cyst fluids were assayed for pH and total CO₂, then frozen for later analysis of total ammonia-N and urea-N. Finally, samples of liver, kidney and parasite cysts were dissected out, blotted and frozen for later analysis of glutamine synthetase activities.

Series 2

This experiment examined the rate and pattern of urea and ammonia excretion in ureotelic fish subjected to a minimum of experimental disturbance and unencumbered by catheters, membranes or restraints. Toadfish ($N=12$) which had been held under 'crowded' conditions were gently transferred, together with their PVC shelters, to individual containers containing a known volume (2.5–4.0 l) of continuously aerated sea water. The water was changed every 24 h for 2–4 days, with 10 ml samples drawn at the beginning and end of each daily period

for analysis of total ammonia-N and urea-N. On day 2 or 3, a peristaltic pump plus fraction collector system was set up to collect hourly water samples (volume 5 ml) continuously for approximately 24 h (range 21–50 h). All samples were analysed for total ammonia-N and urea-N. At the end of the experiment, some of the fish ($N=7$) were terminally sampled for plasma cortisol and urea levels, and glutamine synthetase activity in liver, kidney, gill and skin tissue.

Series 3

This experiment assessed whether the patterns observed in series 2 were an artefact caused by the transfer from 'crowded' to solitary conditions. Three toadfish were allowed to live together for 5 days under the 'crowded' condition in a 6.0 l tub served with flowing sea water and aeration. The water flow was then turned off, the level topped up to maximum, and a peristaltic pump plus fraction collector system set up so as to collect hourly 5 ml water samples as above over the next 49 h.

Series 4

The goal of this experiment was to partition urea and ammonia excretion between anterior (i.e. gills and head region) and posterior routes in ureotelic fish while simultaneously collecting urinary and intestinal excretions separately. Toadfish ($N=8$) which had been held under 'crowded' conditions were fitted with urinary and rectal catheters and placed in divided chambers.

The urinary catheter was implanted first. Urine volume and composition are known to be greatly altered during residence in the bladder in the related *Opsanus tau* (Lahlou *et al.* 1969; Howe and Gutknecht, 1978), so we used a post-bladder catheter to collect naturally vented rather than ureteral urine (Wood and Patrick, 1994). A 1.0 cm length of PE 10 tubing was heat-polished at the tip, connected to 40 cm of PE 50 tubing and filled with physiological saline. The PE 10 tip was inserted a few millimetres into the long thin urinary papilla, which was then tied around the catheter with silk suture. In turn, the catheter was glued to the ventral body wall using a strip of dental dam and cyanoacrylate tissue cement (Vetbond, 3M Corporation).

A wide-bore catheter was used to collect rectal fluid in order to avoid blockage by carbonate pellets which are periodically vented (Walsh *et al.* 1991). The dilated end of a 40 cm Bard all-purpose urethral catheter (size 12 French, elastic rubber; Davol Inc.) was stretched around a 1 cm length of rigid plastic tubing (i.d. 2.5 mm) with a 5 mm o.d. flange at the end. The catheter was filled with physiological saline, passed through another much larger strip of dental dam and inserted 0.5 cm into the anus. The anus was tightly sutured around the flange using a purse-string ligature, and the catheter was glued in place with a second piece of dental dam, thereby overlying the urinary catheter. Additional sutures were used to anchor the edges of the dental dam and to secure the two catheters (tied together) to the posterior ventral body wall. Once the fish had been placed in their experimental chambers, urine and rectal fluid were continuously collected into separate covered vials.

The tips of the catheters were secured approximately 3 cm below the water surface.

The classic 'divided chamber' system of Smith (1929) was employed to separate anterior (i.e. gills and head region) and posterior (i.e. body) routes of urea and ammonia excretion. A hole was cut in a latex membrane (medium burr dental dam) to fit the body of each individual fish snugly immediately behind the pectoral fins and opercular openings. The membrane was secured to the skin with several shallow silk sutures. A neoprene gasket was then used to clamp the membrane firmly between separate front (volume 1.72 l) and rear acrylic compartments (volume 2.32 l) of the chamber. The toadfish head is extremely large, so on average 56.5% of the fish's volume was in the anterior compartment and 43.5% in the posterior compartment. Stainless-steel mesh platforms supported the head and body at the same level so as to avoid tension on the membrane. Water levels were balanced in the two compartments, and the membrane was carefully checked for leaks using dye. Each compartment was separately aerated and mixed with magnetic stirring bars under the mesh platforms. Membranes and catheters routinely remained patent over the 4–5 days of the experiment.

The water in the anterior and posterior compartments was changed every 24 h, with 10 ml samples drawn at the beginning and end of each daily period for analysis of total ammonia-N and urea-N. Urinary and rectal fluid collection vials were also changed every 24 h and preserved for the same analyses. Owing to the presence of the catheters, any urea or ammonia appearing in the water of the posterior compartment represented excretion across the body skin. The peristaltic pump plus fraction collector system for hourly water sampling was used selectively to document excretion patterns in the anterior and posterior compartments.

Series 5

Experimental stress caused either by the divided chamber or by catheterization was suspected to be the cause of the absence of a standard ureotelic pattern in the fish of series 4. To address this issue, the experiment of series 4 was therefore repeated, again with fish ($N=6$) from 'crowded' holding conditions, but now using the membrane and divided chamber only (no catheters). Prior to placement in the divided chamber, the fish (unencumbered) were first subjected to the flux protocol of series 2 for 24 h to ensure that they exhibited the standard ureotelic pattern at the start of the experiment. The fish were then set up with membranes in the divided chambers, and the same protocol as series 4 was followed for 3 days. Terminal samples were taken for plasma cortisol and urea levels and glutamine synthetase activity in liver, kidney and gill tissue. As a further check, a group of fish ($N=8$) from standard 'non-crowded' holding conditions (i.e. probably ammoniotelic) was set up with membranes in the divided chambers and monitored for 4 days.

Series 6

The results of series 5 suggested that the divided chamber

caused the complicating stress. Therefore, this experiment employed only catheters to separate the urinary and intestinal outputs from the remainder of the excretion. Toadfish ($N=8$) from 'crowded' holding conditions were fitted with urinary and rectal catheters and placed in narrow 2.5 l chambers (aluminium loaf pans). The narrow width together with the PVC shelters discouraged the toadfish from turning around and tangling their catheters; aeration ensured mixing. Urine and rectal fluid collection vials were changed at 24 h intervals for 4 days; water sampling and renewal followed the same schedule. Samples were frozen for analysis of total ammonia-N and urea-N. In addition, the peristaltic pump plus fraction collector system was used to document the pattern of excretion for each fish for a period of about 48 h (range 25–76 h) between days 2 and 4. At the end of the experiment, the fish were terminally sampled for plasma cortisol and urea levels and glutamine synthetase activity in liver, kidney and gill tissue.

Series 7

Toadfish possess prominent axillary pores immediately behind each pectoral fin ('finpits'); to our knowledge, their function is unknown. For fish in divided chambers, they would vent into the front compartment. To test whether they contributed to urea excretion, toadfish which had been held under 'crowded' conditions were either fitted with occlusive finpit patches ($N=3$) or sham-operated ($N=3$); they were otherwise unencumbered. In experimental animals, the pore was filled with cyanoacrylate tissue cement, tightly closed using a locking (blanket) suture (Summerfelt and Smith, 1990), and then glued shut with a small patch of dental dam. In sham-operated fish, comparable procedures were used but the pores were not closed. Thereafter, the fish were treated identically to those fish of series 2.

Series 8

[^{14}C]polyethylene glycol 4000 (PEG-4000) (NEN; $10\text{--}20\text{ mCi mmol}^{-1}=370\text{--}740\text{ mBq mmol}^{-1}$), a standard drinking rate marker which undergoes minimal gastrointestinal absorption (Shehadeh and Gordon, 1969), was used to evaluate whether regurgitation through the mouth contributed to urea excretion. Toadfish ($N=6$) from 'crowded' holding conditions were first fitted with rectal and urinary catheters, placed in 2.5 l chambers and followed for 24–36 h as in series 6. Then, for gavage, the fish (non-anaesthetised) was held vertically, the mouth held open, and the isotope solution (259 kBq of PEG-4000 in $300\ \mu\text{l}$ of 33% sea water, ethanol vehicle removed) was introduced directly into the stomach *via* PE 50 tubing. Observations were continued for a further 48 h with continuous hourly sampling by fraction collector. Water, urine and rectal fluid samples were analyzed for total ammonia-N and urea-N levels and [^{14}C] radioactivity, and terminal plasma samples were assayed for [^{14}C] radioactivity.

Analytical methods

Standard chemical methods were employed for the analysis of urea-N in sea water and body fluids (Price and Harrison, 1987) and total ammonia-N in sea water and urine (Ivancic and

Deggobis, 1984), and an enzymatic method was employed for analysis of total ammonia-N in proteinaceous body fluids (Mondzac *et al.* 1965; Sigma kit). [^{14}C] radioactivity was determined by liquid scintillation counting of water and body fluid samples (diluted into 5 ml of sea water plus 10 ml of ICN Ecolume) in a Beckman LS1801 liquid counter with quench correction. Urine and rectal fluid volumes were determined gravimetrically. Total CO_2 concentrations in body fluids were measured with a Corning model 965 analyzer, and pH with a Radiometer E5021 capillary electrode assembly and pHM 84 meter at the experimental temperature. Plasma cortisol was measured using a commercial radioimmunoassay kit (ICN Immuno Corporation).

For analysis of enzymes (maximal activities), tissues were homogenized on ice in 3–4 volumes of homogenization buffer ($20\text{ mmol l}^{-1}\ \text{K}_2\text{HPO}_4$, $10\text{ mmol l}^{-1}\ \text{Hepes}$, $0.5\text{ mmol l}^{-1}\ \text{EDTA}$, $1\text{ mmol l}^{-1}\ \text{dithiothreitol}$, 50% glycerol, adjusted with NaOH to pH 7.5 at 24°C) using a Brinkmann polytron. Homogenates were spun at $8000g$ for 20 min 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 alanine aminotransferase (AlaAT), aspartate aminotransferase (AspAT), glutamate dehydrogenase (GDH), glutamine synthetase (GNS), carbamoylphosphate synthetase (CPS), ornithine–citrulline transcarbamoylase (OTC), argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL) and arginase (ARG) by methods described previously (Mommensen and Walsh, 1989; Barber and Walsh, 1993; Walsh *et al.* 1994a).

Calculations

All urea and ammonia concentrations and fluxes are expressed in units of nitrogen for comparative purposes. Excretion rates (in $\mu\text{mol-N kg}^{-1}\text{ h}^{-1}$) of ammonia-N and urea-N into the external water were calculated from changes in concentration in the water ($\mu\text{mol-N l}^{-1}$) multiplied by the volume (l) and factored by time (h) and mass (kg). 'Pulses' of excretion were identified from hourly samples taken by the fraction collector system; the net increase in water concentration factored by volume and mass represented the amount excreted in each 1 h period. Excretion rates of ammonia-N and urea-N through the urine or rectal fluid were calculated from the urine flow rate (UFR; $\text{ml kg}^{-1}\text{ h}^{-1}$) or rectal fluid flow rate (RFR; $\text{ml kg}^{-1}\text{ h}^{-1}$) and the concentration ($\mu\text{mol-N ml}^{-1}$) in the fluid. UFR and RFR were calculated from the volumes of fluid collected, factored by time (h) and mass (kg).

Data are expressed as means ± 1 s.e.m. (N). Statistical significance at $P\leq 0.05$ was assessed using Student's two-tailed *t*-test, paired or unpaired as appropriate, with the *t*-value adjusted for multiple comparisons *via* the Bonferroni procedure.

Results

Series 1

Liver glutamine synthetase (GNS) activity was significantly increased two- to threefold in toadfish held under 'crowded' conditions (Table 1), associated with induction of ureotelism; urea-N levels in blood plasma, bile and cyst fluid were all

significantly elevated by crowding. Small increases in rectal fluid and urine urea-N were not significant. Crowding had no effects on the much lower concentrations of ammonia-N in all fluids, on plasma cortisol level or on kidney GNS activity; GNS activity could not be detected in parasite cysts.

Acid–base status was also unaffected by crowding, but varied greatly amongst body compartments. Ammonia-N concentration was 5- to 25-fold higher in bile than in any other fluid (Table 1), a difference probably reflecting diffusion trapping by the extremely low biliary pH (5.533 ± 0.023 , $N=20$) which, in turn, was associated with an absence of measurable total CO_2 ($0.0 \pm 0.0 \text{ mmol l}^{-1}$) in the bile. In contrast, the extremely high pH of rectal fluid (8.702 ± 0.021 , $N=21$) was associated with a very high concentration of total CO_2 ($68.4 \pm 2.9 \text{ mmol l}^{-1}$; a mixture of HCO_3^- and CO_3^{2-} at this pH). Nevertheless, ammonia-N levels were higher in rectal fluid than in blood or urine (Table 1), perhaps reflecting biliary input

Table 1. Concentrations of urea-N and ammonia-N in various body fluids, glutamine synthetase activity in various tissues and plasma cortisol levels in toadfish held under either 'non-crowded' or 'crowded' conditions

		Non-crowded	Crowded
Urea-N (mmol l^{-1}) ^a	Plasma	10.17 \pm 1.38 (12)	14.18 \pm 1.53* (12)
	Bile	11.86 \pm 2.08 (8)	19.59 \pm 2.22* (12)
	Rectal fluid	10.25 \pm 2.55 (9)	14.16 \pm 1.71 (12)
	Urine	15.26 \pm 2.81 (8)	20.31 \pm 2.53 (9)
	Cyst fluid	12.35 \pm 1.60 (11)	17.57 \pm 2.11* (6)
Ammonia-N ($\mu\text{mol l}^{-1}$) ^a	Plasma	158 \pm 25 (12)	126 \pm 16 (12)
	Bile	3472 \pm 555 (8)	3401 \pm 484 (12)
	Rectal fluid	570 \pm 352 (10)	1066 \pm 490 (11)
	Urine	243 \pm 80 (7)	289 \pm 72 (9)
	Cyst fluid	530 \pm 159 (11)	499 \pm 112 (6)
GNS ($\mu\text{mol g}^{-1} \text{ min}^{-1}$) ^b	Liver	5.73 \pm 1.79 (12)	12.27 \pm 2.10* (12)
	Kidney	4.72 \pm 0.87 (12)	5.44 \pm 1.12 (12)
	Cyst	ND (12)	ND (12)
Plasma cortisol (ng ml^{-1})		15.78 \pm 2.21 (12)	21.43 \pm 5.26 (12)

Values are means \pm S.E.M. (N).

* $P \leq 0.05$ compared with non-crowded value.

^aNote different units for urea-N versus ammonia-N concentrations.

^bEnzyme activities in μmoles of substrate converted to product per gram wet tissue per minute.

ND, not detectable; GNS, glutamine synthetase.

and/or bacterial generation of ammonia in the gastrointestinal tract. The acid–base status of both urine ($\text{pH}=7.688 \pm 0.060$; total $\text{CO}_2=4.1 \pm 0.4 \text{ mmol l}^{-1}$, $N=13$) and cyst fluid ($\text{pH}=7.868 \pm 0.064$; total $\text{CO}_2=5.8 \pm 0.5 \text{ mmol l}^{-1}$, $N=16$) were similar to that of blood plasma (measured $\text{pH}=7.429 \pm 0.025$ but depressed approximately 0.4 units by caudal sampling – Barber and Walsh, 1993; total $\text{CO}_2=4.5 \pm 0.2 \text{ mmol l}^{-1}$, $N=24$). However, ammonia-N levels in cyst fluid were similar to those in rectal fluid and 3–5 times higher than in either plasma or urine (Table 1).

Series 2

Toadfish which had been 'crowded' for 2–3 days and then transferred to individual small containers with shelters were uniformly ureotelic, excreting 82% of their waste-N as urea-N and only 18% as ammonia-N (Table 2). This pattern remained constant over the 2- to 4-day experimental period. Hourly fraction collection revealed that the vast majority (94%) of urea-N excretion occurred in large pulses, with typical pulses lasting 4–5 h (Fig. 1). However, some 'smearing' of the pulse by mixing in the first and last fractions was unavoidable, so the actual pulse event probably lasted 3 h or less. Mean pulse frequency was $1.01 \pm 0.12 \text{ pulses day}^{-1}$ ($N=12$) (Table 2). There was no evidence of a circadian rhythm or tidal/lunar rhythm. Urea-N pulses occurred at different times of the day and night in fish examined simultaneously. The small amount of ammonia-N excretion showed no evidence of pulsatility. Between urea-N pulses, ammonia-N and urea-N excretion occurred either at a very low level (e.g. Fig. 1A,B) or not at all for extended periods (Fig. 1C,D). (Amazingly, one toadfish monitored for 50 h excreted neither ammonia-N nor

Table 2. Rates and patterns of urea-N and ammonia-N excretion in toadfish held under 'crowded' conditions and then placed in individual containers after various treatments

	Series 2, unencumbered ($N=12$)	Series 6, catheters only ($N=8$)	Series 8, catheters and gavage ($N=6$)
Observation time (h)	26.3 \pm 2.2	53.9 \pm 6.3*	48.5 \pm 0.2*
Urea-N excretion rate ($\mu\text{mol-N kg}^{-1} \text{ h}^{-1}$)	103.8 \pm 22.5	82.5 \pm 18.0	130.4 \pm 32.3
Ammonia-N excretion rate ($\mu\text{mol-N kg}^{-1} \text{ h}^{-1}$)	23.2 \pm 10.6	34.4 \pm 10.9	51.8 \pm 13.6
Urea-N pulses (pulses day^{-1})	1.01 \pm 0.12	0.85 \pm 0.17	0.74 \pm 0.17
Ammonia-N pulses (pulses day^{-1})	0	0	0
Size of urea-N pulse ($\mu\text{mol-N kg}^{-1} \text{ pulse}^{-1}$)	2423.4 \pm 491.2	1571.2 \pm 302.0	2578.5 \pm 518.6
% of urea-N output as pulses	94.4 \pm 2.7	76.8 \pm 9.7*	55.3 \pm 7.8*

Patterns were monitored by means of hourly water sampling with a fraction collector.

Values are means \pm 1 S.E.M.

* $P \leq 0.05$ relative to series 2.

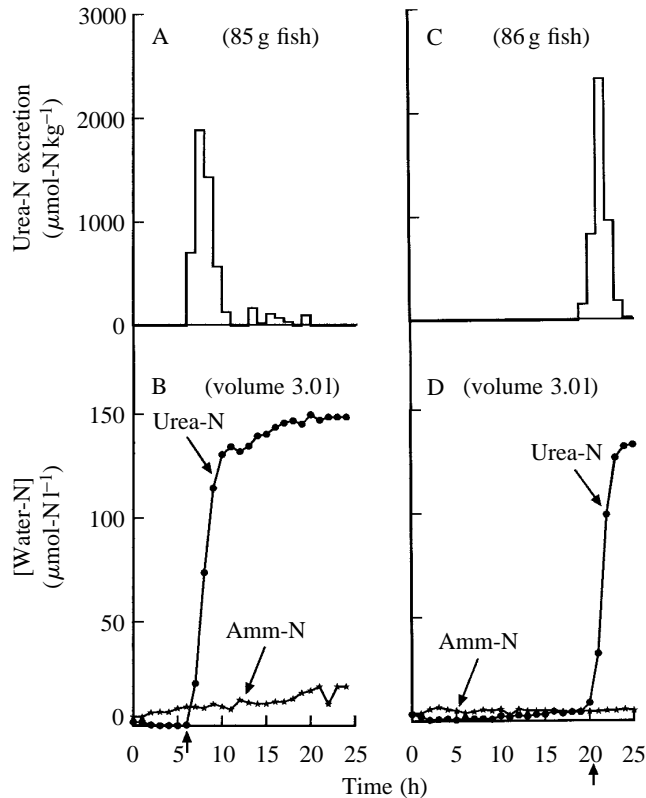


Fig. 1. Typical hourly records of urea-N and ammonia-N (Amm-N) excretion in two toadfish which had been held under 'crowded' conditions and then transferred without disturbance (no catheters or membranes) to individual containers (series 2). The hourly urea-N excretions (A,C) were calculated from the hourly water-N concentrations (B,D). Note the predominant single daily pulses (marked by vertical arrows) of urea-N excretion (A,C), and the low (B) or negligible (D) ammonia-N and urea-N excretion in the intervening periods.

urea-N over this period.) In effect, ureotelic toadfish typically excreted almost their entire daily N-production in a single urea-N pulse of less than 3 h duration!

Calculations based on the mean size of the daily pulse (Table 2), and urine and rectal fluid urea-N concentrations from series 1 (Table 1), proved instructive as to the possible route of excretion. A single daily 'pulse discharge' of 119 ml kg^{-1} of urine or 171 ml kg^{-1} of rectal fluid would be needed to account for the daily urea-N pulse. Neither value seems realistic.

Terminal plasma urea-N, cortisol and liver GNS activity (Table 3) were comparable to those recorded in the 'crowded' fish of series 1 (Table 1). Kidney GNS activity was significantly elevated relative to series 1. Appreciable GNS activity also occurred in skin and gills (Table 3), and the latter was significantly elevated relative to gill samples taken from 'non-crowded' fish ($0.97 \pm 0.04 \mu\text{mol g}^{-1} \text{ min}^{-1}$, $N=3$).

Series 3

The remarkable pattern of daily urea-N pulses documented in series 2 was not a behavioural artefact due to transfer from 'crowded' holding conditions to solitary containers. Three fish

Table 3. Concentrations of urea-N and cortisol in plasma and activities of glutamine synthetase in various tissues of toadfish held under 'crowded' conditions and then subjected to various treatments

	Series 2, unencumbered	Series 5, divided chamber	Series 6, catheters only
Plasma urea-N (mmol l^{-1})	12.77 ± 1.39 (7)	10.13 ± 0.71 (5)	14.66 ± 2.27 (7)
Plasma cortisol (ng ml^{-1})	39.60 ± 11.30 (7)	$103.01 \pm 42.46^*$ (7)	86.24 ± 24.33 (7)
GNS ($\mu\text{mol g}^{-1} \text{ min}^{-1}$) ^a			
Liver	13.71 ± 3.26 (6)	8.68 ± 2.11 (7)	$6.05 \pm 1.16^*$ (7)
Kidney	9.23 ± 2.62 (6)	7.60 ± 1.88 (7)	$4.08 \pm 0.57^*$ (7)
Gill	2.25 ± 0.39 (6)	1.18 ± 0.39 (7)	1.60 ± 0.28 (7)
Skin	0.85 ± 0.28 (4)	—	—

Values are means \pm S.E.M. (N).
^{*} $P \leq 0.05$ relative to series 2.
^aEnzyme activities in μmoles of substrate converted to product per gram wet tissue per minute.

which had been co-existing under 'crowded' holding conditions in one tub for 5 days were monitored without disturbance for a further 49 h, by means of the fraction collector. On the basis of the results of series 2, the three fish would be expected to exhibit about six urea-N pulses in this period. In fact, six or seven (one may have been a composite of two pulses) were observed (Fig. 2); overall urea-N and ammonia-N excretion rates were comparable to those of series 2.

Series 4

In this series, fish were fitted with both urinary and rectal catheters and membranes and were placed in divided chambers. Although the fish had been subjected to the standard 'crowded' holding conditions, the ureotelic pattern seen in series 2 and 3 was not exhibited, and the fish excreted 63–68% ammonia-N throughout the experiment (Fig. 3). Total-N excretion increased significantly with time, and by day 4 was about three times greater than in the ureotelic fish of series 2 and 3, whereas absolute urea-N excretion rates were comparable to those of ureotelic fish. Hourly sampling of water with the fraction collector revealed no evidence of pulsatile excretion in either front or rear compartments. These results suggested that the stress of the experimental procedures prevented expression of the ureotelic condition and characteristic pulsatile pattern.

Nevertheless, these experiments led to several clear conclusions. First, the anterior end was the dominant route for ammonia-N excretion (61–77%) and accounted for about half (41–60%) of urea-N excretion. At the posterior end, the major portion of both ammonia-N and urea-N excretion appeared in

Fig. 2. Hourly records of water urea-N and ammonia-N (Amm-N) concentration (lower panel) and the urea-N excretions calculated from these data (upper panel) for three toadfish (137, 83 and 226 g) which had been kept under 'crowded' conditions in a single tub for 5 days and which were then monitored in this same tub (closed volume) without disturbance for a further 49 h (series 3). Note the clear evidence of urea-N pulsatility and the low rate of ammonia-N excretion. Vertical arrows mark the six or seven urea-N pulses; (?) indicates where one pulse may represent a composite of two.

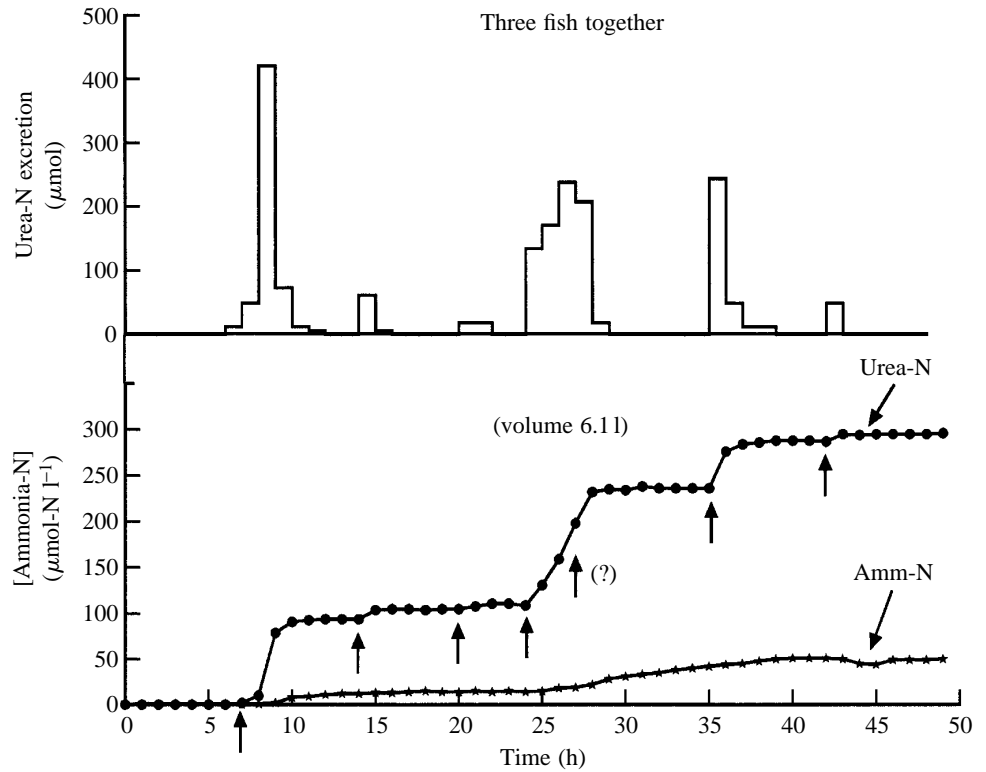
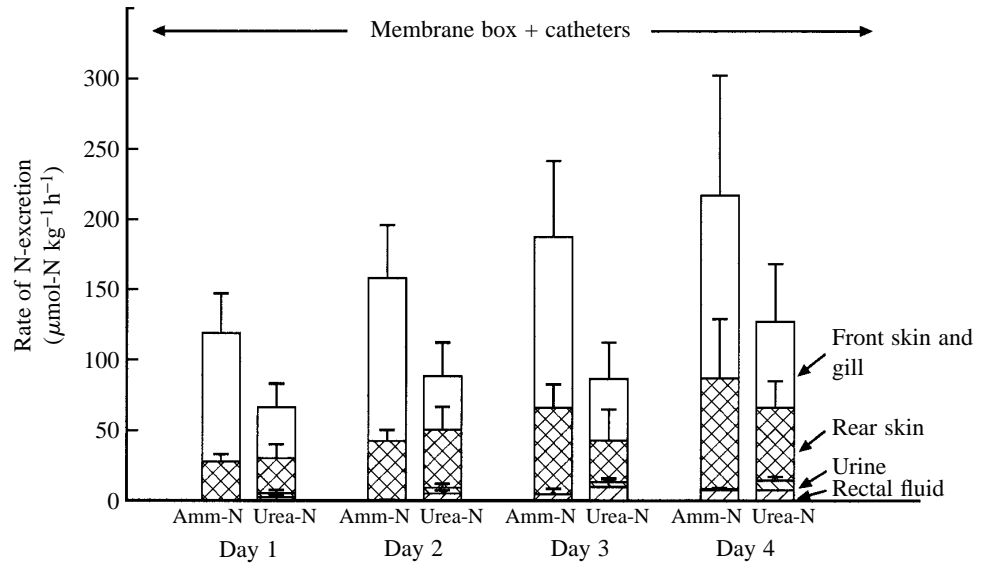


Fig. 3. Partitioning of total-N excretion between ammonia-N (Amm-N) and urea-N, between front and rear compartments, and in the latter amongst skin, urine and rectal fluid routes in eight toadfish which had been held under standard 'crowded' conditions (series 4). Fish were fitted with membranes and catheters and placed in divided chambers for 4 days; fluxes were measured over successive 24 h intervals. Means +1 S.E.M. Increases in total-N, ammonia-N and urea-N excretion in both front and rear compartments were significant ($P \leq 0.05$) between days 1 and 4.



the water, rather than in the voided urine and rectal fluid, indicating that the skin can serve as an important excretory route. Indeed, measured urinary and rectal excretion accounted for only about 2% and 5%, respectively, of whole-body urea-N excretion, and even less for ammonia-N excretion (0.5% and 1.5% respectively). Urea-N concentrations in urine and rectal fluid collected by chronic catheterization (Table 4) were similar to, or slightly lower than, those measured by spot sampling in uncatheterized toadfish of series 1 (Table 1). Both urine and rectal fluid flow appeared to be intermittent as a result

of periodic micturation, although the pattern was not documented. Measured flow rates over 24 h intervals (Table 4) were less than 10% and 5%, respectively, of the rates needed to explain pulsatile urea-N excretion in series 2.

Series 5

This experiment demonstrated that the membranes and divided chamber alone were a sufficient stress factor to prevent expression of the ureotelic condition and pulsatile excretion pattern. On day 1, prior to membrane placement, all

unencumbered fish from 'crowded' holding conditions exhibited the typical pulsatile ureotelic pattern (e.g. Fig. 4, cf. Fig. 1). Total-N excretion rate was $111.7 \pm 26.2 \mu\text{mol kg}^{-1} \text{h}^{-1}$ ($N=6$) and the proportion excreted as urea-N was 89%, very similar to the values of series 2 and 3 (cf. Table 2). On the first day (day 2) after placement in the divided chambers (no catheters), total-N excretion increased 2.5-fold, and by day 4

Table 4. Flow rates and urea-N and ammonia-N concentrations of rectal fluid and urine collected by catheter in toadfish held under 'crowded' conditions and then subjected to various treatments

	Series 4, divided chamber and catheters	Series 6, catheters only	Series 8, catheters and gavage
Rectal fluid			
Flow rate ($\text{ml kg}^{-1} \text{h}^{-1}$)	0.700 ± 0.175 (8)	0.362 ± 0.082 (8)	0.582 ± 0.038 (6)
Urea-N (mmol l^{-1})	8.64 ± 1.29 (8)	9.97 ± 3.13 (8)	7.37 ± 1.75 (6)
Ammonia-N (mol l^{-1})	2.25 ± 1.10 (8)	2.16 ± 0.59 (8)	1.01 ± 0.20 (6)
Urine			
Flow rate ($\text{ml kg}^{-1} \text{h}^{-1}$)	0.287 ± 0.196 (5)	0.260 ± 0.096 (8)	0.341 ± 0.160 (5)
Urea-N (mmol l^{-1})	18.87 ± 2.45 (5)	17.50 ± 2.34 (8)	15.61 ± 2.38 (5)
Ammonia (mmol l^{-1})	1.16 ± 0.52 (5)	0.56 ± 0.14 (8)	0.77 ± 0.21 (5)

Values are means \pm S.E.M. (N) for days 2–4 of the experiment.

There were no significant differences ($P > 0.05$) for any variable amongst the three series.

the increase was 3.5-fold, entirely as a result of elevated ammonia-N excretion; urea-N excretion remained unchanged (data not shown). Both the increase (which occurred in all fish) and the absolute rates were very similar to those seen in series 4 (cf. Fig. 3). Terminal sampling on day 4 revealed a substantial elevation of plasma cortisol level (Table 3). Liver GNS activity was somewhat lower than in the typical ureotelic fish of series 3, although the difference was not significant.

For two fish, continuous hourly records were made throughout the first day (day 2) in the divided chamber (e.g. Fig. 4). In both animals, ammonia-N excretion was negligible initially (as on day 1), but started abruptly in the front compartment about halfway through day 2 (at hour 13 in the example of Fig. 4). This suggests the sudden expression of a stress response. Thereafter, ammonia-N excretion continued at a gradually increasing rate. Urea-N excretion was no longer pulsatile but occurred at a steady rate.

Additional information was obtained by performing the same divided chamber experiments (membranes only, no catheters) with toadfish from 'non-crowded' holding. We predicted that these fish should be ammoniotelic initially and would switch over to ureotelism as a result of confinement (Walsh and Milligan, 1995) unless the response was overridden by the stress of the experimental conditions. In fact, on day 1, five of the six fish were predominantly ammoniotelic, whereas by day 4, only three remained predominantly ammoniotelic, indicating individual variation in the responses. Furthermore, total nitrogen excretion did not change in a consistent fashion ($156.7 \pm 36.7 \mu\text{mol kg}^{-1} \text{h}^{-1}$ on day 1 versus $223.8 \pm 20.9 \mu\text{mol kg}^{-1} \text{h}^{-1}$ on day 4). The fraction collector system was not available, but samples were taken manually at 12 h intervals. Over time, urea-N excretion was clearly much more variable than ammonia-N excretion, exceeding the latter in 18 of 50 such 12 h periods. The major portions of both

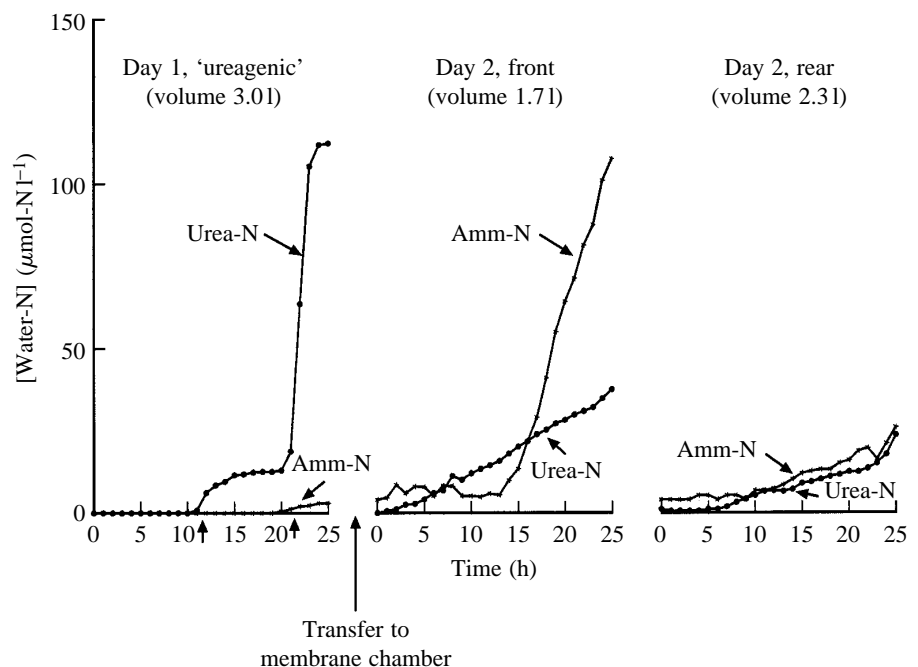


Fig. 4. Typical hourly records of water urea-N and ammonia-N (Amm-N) concentrations in a toadfish of series 5, which had been held under 'crowded' conditions. On day 1, the fish (unencumbered) was placed in an individual container. At the start of day 2, the fish was fitted with a membrane and placed in a divided chamber. Note the typical ureotelic pattern of pulsatile urea-N excretion (vertical arrows mark pulses) and the almost complete absence of ammonia-N excretion on day 1. On day 2, note the sudden start of continuous ammonia-N excretion in the front compartment at 13 h, and the non-pulsatile nature of urea-N excretion.

ammonia-N (78–82%) and urea-N (62–89%) were excreted into the anterior compartment irrespective of whether a fish was exhibiting ammoniotelism or ureotelism in an individual period (Fig. 5). In ureotelic periods, urea-N excretion into the anterior compartment increased dramatically (sixfold relative to ammoniotelic periods), while urea-N excretion into the posterior compartment did not change significantly. The decline in ammonia-N excretion was significant only for the

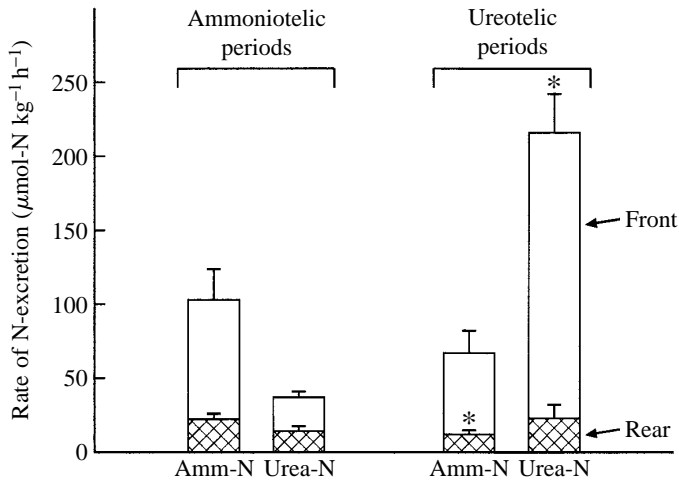


Fig. 5. Partitioning of total-N excretion between front and rear compartments and between ammonia-N (Amm-N) and urea-N in the eight toadfish of series 5, which had been held under standard 'non-crowded' conditions (in contrast to the fish of Fig. 4). Fish were fitted with membranes and placed in divided chambers for up to 4 days; fluxes were measured over successive 12h intervals (50 periods in total). For each fish, mean values were first tabulated separately for all ammoniotelic periods (i.e. total ammonia-N excretion > total urea-N excretion) and for all ureotelic periods (total urea-N excretion > total ammonia-N excretion); grand means ± 1 S.E.M. ($N=8$) were then calculated and are shown here. Asterisks indicate a significant difference ($P \leq 0.05$) from the corresponding mean in ammoniotelic periods.

small posterior component. These results indicated that, for both non-pulsatile, predominantly ammoniotelic excretion and pulsatile, predominantly ureotelic excretion, the head end (probably the gills) was the major route of efflux.

Series 6

This approach (no membranes, catheters only, confined conditions) successfully maintained the pulsatile ureotelic pattern and conclusively eliminated rectal and urinary routes as the source of nitrogen excretion. Urine was collected outside the fish's container by catheterization and so could not contribute to excretion into the water. The size and frequency of urea-N pulses to the water remained the same as in series 2, as did the total ammonia-N and urea-N excretion rates (Fig. 6; Table 2). The percentages of total-N output as urea-N (71% versus 82%) and of total urea-N output occurring as pulses (77% versus 94%) were only moderately lower than those of series 2 (Table 2). These patterns remained fairly stable over the 4 days of the experiment (Fig. 6). Rectal fluid and urinary flows were again clearly periodic; flow rates and N-compositions were comparable to those of series 4 (Table 4) and again contributed only small fractions (urea-N < 10%; ammonia-N < 5%) of measured whole-body excretion rates (Fig. 6).

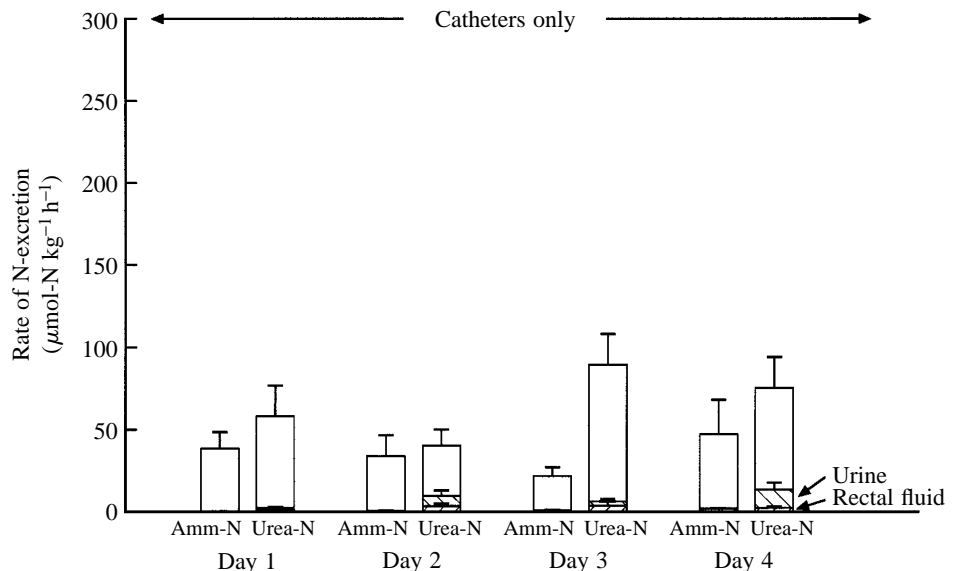
Series 7

Occlusion of the axillary pores ('finpits') had no effect on the pattern or rate of N-waste excretion in toadfish which had been held under 'crowded' conditions. Total urea-N excretion rates (averages over 4 days) were $110.0 \pm 23.0 \mu\text{mol kg}^{-1} \text{h}^{-1}$ ($N=3$) in occluded fish versus $107.0 \pm 30.4 \mu\text{mol kg}^{-1} \text{h}^{-1}$ ($N=3$) in sham-operated fish, while total ammonia-N excretion rates were $24.0 \pm 5.7 \mu\text{mol kg}^{-1} \text{h}^{-1}$ ($N=3$) and $16.1 \pm 3.1 \mu\text{mol kg}^{-1} \text{h}^{-1}$ ($N=3$) respectively. These rates were typical of ureotelic fish (cf. Table 2).

Series 8

As toadfish are capable of everting the oesophagus and vomiting food, we checked whether regurgitation of

Fig. 6. Partitioning of total-N excretion amongst pathways through urine, rectal fluid and other body surfaces, and between ammonia-N (Amm-N) and urea-N, in the eight toadfish of series 6. The fish had been held under standard 'crowded' conditions, fitted with urinary and rectal catheters only and confined in narrow individual containers for 4 days. Fluxes were measured over successive 24h intervals. The open bars represent excretion into the water (i.e. non-urinary, non-rectal). Means ± 1 S.E.M. There were no significant differences in any variable over time ($P > 0.05$).



gastrointestinal fluids contributed to pulsatile urea-N excretion. The results eliminated this possibility and demonstrated that pulsatile urea-N excretion must occur across the gills and/or skin. Fish were set up initially with urinary and rectal catheters as in series 6 and exhibited the typical ureotelic pattern on day 1 (see Fig. 1). At the end of day 1, [^{14}C]PEG-4000 was administered by gavage into the stomach. The procedure caused some disturbance, since both urea-N and ammonia-N excretion increased significantly on days 2 and 3 (data not shown). Nevertheless, the fish remained ureotelic, excreting 72% of their waste-N as urea-N, and their absolute ammonia-N and urea-N excretion rates (Table 2) were not significantly different from those of other ureotelic series. The size and frequency of the urea-N pulses were also similar to those of other series, but the percentage of total urea-N output as pulses was significantly reduced to 55% (Table 2). However, the key point is that typical pulses of urea-N excretion into the water still occurred in all fish, and these were never associated with pulses of [^{14}C]PEG-4000 radioactivity in the water (e.g. Fig. 7). Conversely, there were several instances of the pulsatile appearance of radioactivity (presumably regurgitation events) and these were not associated with urea-N pulses. Overall, up to 30% of the administered dose was recovered in the water and 45–85% in the collected rectal fluid. Radioactivity in terminal plasma samples and collected urine was negligible. Rectal fluid and urine flow rates and excreted nitrogen compositions were similar to those of previous series (Table 4) and again accounted for only very minor fractions of measured whole-body excretion rates.

Discussion

Pulsatile urea-N excretion

The present results clearly demonstrate that urea-N excretion in the ureotelic toadfish is pulsatile, thereby confirming previous anecdotal observations (Walsh *et al.* 1990, 1994a; Griffiths, 1991; Mommsen and Walsh, 1992; Barber and Walsh, 1993).

On average, in unencumbered resting toadfish (series 2 and 3), over 90% of the total daily urea-N excretion was released in a single pulse of less than 3 h duration. However, in contrast to the assumptions of earlier reports, the pulsatility is not due to periodic micturation of urine from the bladder and/or rectal fluid from the intestine (as shown by series 6). Furthermore, pulsatile urea-N excretion does not occur by discharge through the pore-like finpits behind the pectoral fins (see series 7) or by regurgitation of gut fluids through the mouth (see series 8). Overall, the results indicate that pulsatile urea-N excretion must occur through the general body surface and/or gills and point strongly to the head end (gills?) as the major site.

What is the adaptive advantage of venting a whole day's urea-N excretion in a single, short-lasting pulse? We speculate that this strategy may relate to the cryptic behaviour of *Opsanus beta*. In the wild, the species is subject to intense predation by seabirds, sharks and dolphins (Schmidt, 1986; Cummings, 1987; Barros, 1987), and individuals typically spend much of their time sheltered in burrows and crevices (T. E. Hopkins, personal observations). This is especially true of males defending the nest (Breder, 1941). Our observations in the laboratory indicate that they typically orient with their heads towards the entrance of the shelter and withdraw deeper into it when they sense disturbance. We suggest that the ability to release most of a whole day's waste N-production in a single pulse of urea-N excretion, perhaps in a short feeding foray, or just by poking the head out of the burrow, will avoid fouling of the burrow and at the same time minimize any olfactory signal for predators. Observational, behavioural and physiological studies in the field will be required to test this hypothesis fully. Interestingly, the blenny *Blennius pholis* is also reported to release urea-N in a semipulsatile fashion (Sayer, 1988). However, the blenny pattern differs from that of *Opsanus beta* in that the frequency of pulses is much greater (minutes to hours) and larger ammonia-N pulses occur simultaneously.

What is the mechanism of pulsatile urea-N excretion in the toadfish? In contrast to the standard textbook view that urea is

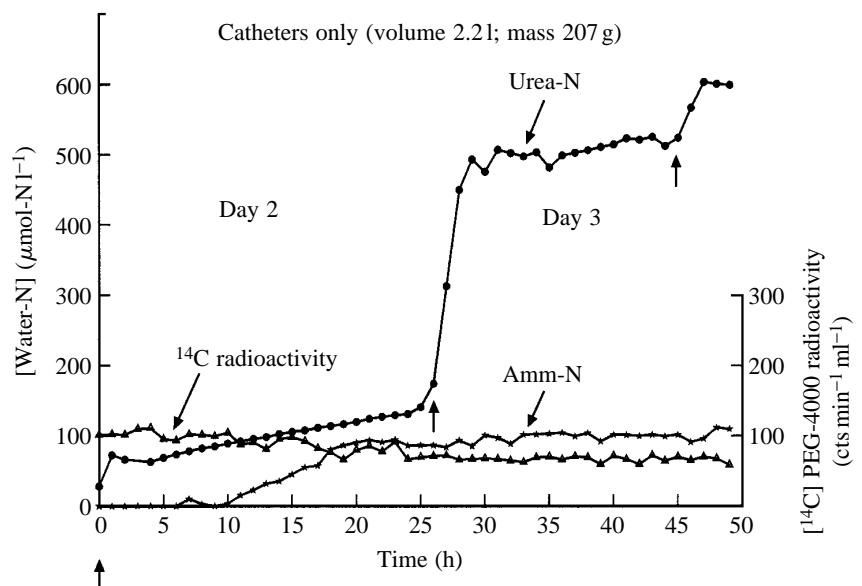


Fig. 7. Typical hourly records of concentrations of urea-N, ammonia-N (Amm-N) and [^{14}C]PEG-4000 radioactivity in the water of a toadfish of series 8. The fish had been fitted with urinary and rectal catheters, so water concentrations reflect excretion across non-urinary, non-rectal pathways. Approximately 1 h before the start of this record, at the end of day 1, the fish was loaded with (259 kBq) of [^{14}C]PEG-4000 (in 300 μl of 33% sea water) by gavage into the stomach. Note the three pulses of urea-N excretion (marked by vertical arrows), at hours 1, 26 and 45, and the absence of coincident increases in water radioactivity.

freely permeable across cell membranes, it is now recognized that urea permeability through lipid bilayers is low and that high urea permeabilities often reflect the presence of specific urea transporters (Marsh and Knepper, 1992; Gillin and Sands, 1993; Wood, 1993). Recently we have demonstrated the presence of a phloretin-sensitive, facilitated-diffusion-type urea transporter in hepatocytes of *Opsanus beta* (Walsh *et al.* 1994b; P. J. Walsh and C. M. Wood, in preparation). Therefore, one possibility is that ureagenesis itself (i.e. the rate of urea production in the liver), rather than the excretion mechanism *per se*, is pulsatile and that the presence of such transporters in liver cell membranes facilitates rapid efflux of urea to the blood at times of pulsatile production. A second possibility is that similar transporters on the gills and/or body surface are normally inactivated, but are subject to rapid activation at times of pulsatile excretion. A third possibility is that an active urea transporter is present at the excretion site(s) which normally transports urea back into the bloodstream ('urea reabsorption'), thereby preventing urea excretion. Wood *et al.* (1995) have provided indirect evidence for such an inwardly directed transporter at the gills in the urea-retaining elasmobranch *Squalus acanthias*. Periodic inactivation of such a transporter, or the elevation of unidirectional efflux rates above reabsorption rates, could explain pulsatile urea excretion. None of these hypotheses is mutually exclusive. Their validity can be tested by careful time course sampling of blood urea levels relative to the excretion cycle, by urea infusion experiments, and by molecular and pharmacological studies on the possible transport mechanisms.

All of these hypotheses assume that urea is synthesized in the liver and transported through the blood to the excretion site(s). To check whether other sites might be involved in urea production, we have surveyed the activities of ornithine-urea cycle and related enzymes in gills, kidney and intestine of *Opsanus beta* (Table 5). Clearly, all necessary enzymes appear to be present in all tissues, although generally at lower levels than in liver. The kidney, in particular, appears to have ureogenic potential, similar to the situation in several ureogenic air-breathing teleosts of the Indian subcontinent (Saha and Ratha, 1987, 1989, 1994). Note, however, that our assay does not discriminate between the CPS III of the teleost ornithine-urea cycle and other forms of CPS, and that enzyme activities are measured as maximum rates under optimal conditions. Nevertheless, future studies should not ignore the possibility of significant extra-hepatic production of urea.

Other routes of nitrogen excretion

The measured concentrations of urea-N and ammonia-N in urine and rectal fluid obtained by spot-sampling from the bladder and anus were similar to those reported earlier by Walsh *et al.* (1990) and were not greatly affected by the ammoniotelic or ureotelic condition of the toadfish (Table 1). Catheterization yielded similar or slightly lower urea-N concentrations in collected urine (Table 4). Measured contributions of both intestinal and urinary effluxes to ammonia-N and urea-N excretion were small (<10%), irrespective of whether the toadfish were exhibiting ureotelic

Table 5. Activities of ornithine-urea cycle and related enzymes in various tissues of toadfish held under 'non-crowded' conditions

Enzyme ^a	Liver ^b (N=6)	Gills (N=3)	Kidney (N=4)	Intestine (N=3)
AlaAT	32.86±4.99	3.23±0.01*	6.18±1.59*.b	25.83±3.23
AspAT	42.79±4.84	18.31±1.08*	28.57±7.58 ^b	23.48±0.81*
GDH	42.55±11.21	1.62±0.00*	14.58±3.88 ^b	6.46±0.00*
GNS	3.21±0.63	0.97±0.04	7.79±2.78	7.36±1.37*
CPS	0.16±0.02	0.05±0.01*	0.07±0.01*	0.17±0.04
OTC	44.34±3.23	1.58±1.00*	56.42±5.98	3.41±1.39*
ASS	0.22±0.13	0.01±0.00	0.10±0.01	0.04±0.00
ASL	0.51±0.05	0.08±0.01*	0.40±0.02	0.23±0.02*
ARG	36.92±8.10	34.51±3.76	37.12±3.65	94.83±2.10*

Values are means ± S.E.M.

* $P \leq 0.05$ relative to liver.

^aSee Materials and methods for abbreviations. Enzyme activities are in μ moles of substrate converted to product per gram wet tissue per minute.

^bLiver data and selected kidney enzymes (N=6) reproduced from Walsh *et al.* (1993).

or ammoniotelic behaviour (Figs 3, 6). We are aware of no previous measurements of urine or rectal fluid flow rates in *Opsanus beta*. However, urine flow rates (Table 4) in the present study were comparable to or higher than those determined in the closely related *Opsanus tau* (Lahlou *et al.* 1969; Howe and Gutknecht, 1978) and similar to those in other aglomerular or pauciglomerular marine fish (reviewed by Hickman and Trump, 1969). Measured rectal fluid flow rates (Table 4) were 2–3 times higher than those estimated by an indirect technique in *Opsanus tau* (Howe and Gutknecht, 1978) but very similar to direct measurements in other marine teleosts (Hickman, 1968; Shehadeh and Gordon, 1969). In the toadfish, as in other marine teleosts (Wood, 1993), urine and rectal fluid flow are not important routes of ammonia-N and urea-N excretion, contrary to previous speculation (see Introduction).

In all divided chamber experiments, the anterior end of the toadfish was the dominant route of ammonia-N excretion, in accord with the conventional view that the gills perform this function in all teleosts (reviewed by Wood, 1993), including this species (Evans *et al.* 1989). Urea-N excretion was generally greater into the front chamber, but the trend was not as pronounced as for ammonia-N. Note in particular that in series 4, which was probably the most stressful experiment owing to the use of both dividing membranes and catheters, the rear skin accounted for 40–50% of urea-N excretion and up to 35% of ammonia-N excretion (Fig. 3). These fractions were much greater than urinary and rectal fluid excretions. In view of the massive head of the toadfish (56.5% of the fish's volume), there were approximately equal amounts of skin in the front and rear compartments in these experiments. Therefore, it is probable that the largest fractions of both ammonia-N and urea-N excretion occur across the skin at times

of high stress. In the case of *Opsanus beta*, this could be due to a high blood flow and/or high blood ammonia-N and urea-N levels perfusing the capillaries of a scaleless skin (Hart, 1980). However, the general conclusion is in accord with a number of studies (reviewed by Wood, 1993) which suggest that the skin is relatively more important in this regard in seawater fish, both scaled and scaleless, than in freshwater fish (e.g. Morii *et al.* 1978; Sayer and Davenport, 1987).

We should not necessarily conclude that significant excretion through the skin occurs normally, only that the potential is present. All previous divided chamber studies probably imposed similar stress levels to those of the present study. Under less stressful conditions, particularly when toadfish are exhibiting the standard ureotelic pattern of pulsatility, there are long periods when little or no ammonia-N or urea-N is excreted to the water (e.g. Figs 1, 2, 4, 7).

Ammoniotelism versus ureotelism and the role of stress

The present results cast some light on the complex role of 'stress' itself, and the 'stress hormone' cortisol, in mediating transitions between ammoniotelic and ureotelic behaviour in toadfish. They further suggest that our understanding of the transition between ammoniotelism and ureotelism should focus on the change in ammonia-N excretion, rather than the change in urea-N excretion.

The synthetic corticosteroid dexamethasone will induce elevated hepatic GNS activity in *Opsanus beta* (Mommsen *et al.* 1992), and a marked induction of hepatic GNS (e.g. Tables 1, 3) is a diagnostic correlate of the transition to ureotelism (Mommsen *et al.* 1992; Walsh *et al.* 1994a). Recently, we have shown that, when toadfish are experimentally 'crowded', a rapid surge (peak at 2 h) in plasma cortisol precedes the induction of hepatic GNS (after 24 h), and both responses are blocked by pre-treatment with the cortisol-synthesis antagonist metyrapone (T. E. Hopkins, C. M. Wood and P. J. Walsh, in preparation). The cortisol surge is of moderate size (to about 30–40 ng ml⁻¹) and, more importantly, it is transient (declining by 24 h). In accord with this pattern, there was only a small non-significant elevation in plasma cortisol level (range 20–40 ng ml⁻¹) in animals sampled either immediately after 48–72 h of 'crowding' (series 1, Table 1) or when exhibiting standard ureotelic pulsatility several days later (series 2, Table 2). Very recent results demonstrate that the GNS induction occurs in the cytosolic compartment rather than in the mitochondrion, so it cannot feed nitrogen directly to the ornithine-urea cycle (Walsh and Milligan, 1995). Flux measurements in that study suggest that the transition to ureotelism during confinement involves a *marked reduction* in ammonia-N excretion rather than an increase in urea-N excretion.

In the present study, the transition *in the opposite direction*, from ureotelism to ammoniotelism, appears to be associated with extreme stress (such as that imposed by the divided chamber of series 4 and 5) and extreme elevations in plasma cortisol concentration (range 100 ng ml⁻¹; Table 3). However, as with the transition *towards* ureotelism, it is the rate of ammonia-N excretion (a *marked increase*), rather than the rate of urea-N excretion, which changes greatly (e.g. Figs 3, 4). Indeed,

regardless of treatment, the rate of urea-N excretion (though not the pattern) was relatively constant in all experimental series at around 50–140 μmol-N kg⁻¹ h⁻¹. It may be questioned why urea-N excretion rates were higher (approximately 220 μmol-N kg⁻¹ h⁻¹) in the ureotelic periods of Fig. 5 in comparison with rates in all other series. The explanation is that the measurement and tabulation periods for this experiment were only 12 h, while urea-N pulses occur only about once every 24 h (Table 2). Averaged over 24 h periods, urea-N excretion rates in this experiment were normal (approximately 120 μmol-N kg⁻¹ h⁻¹).

We speculate that whole strategy of ureotelism is to manage N-excretion so that it can occur as rapid pulses of urea-N during times when cryptic behaviour is required. Induction of GNS by a modest transient rise in plasma cortisol level on first perception of the appropriate cues ('crowding', confinement) serves mainly to *prevent ammonia-N excretion* by trapping it as glutamine. Any increase in urea-N excretion is a secondary consequence. Indeed, GNS induction to trap ammonia may occur not only in the liver but throughout the peripheral tissues. Thus, GNS activity was elevated in both the kidney and gills of the ureotelic fish of series 2 relative to that of 'uncrowded' animals, and GNS activity was detectable even in the skin (Table 3). When cortisol levels rise to extreme, non-physiological values under severe stress, its well-known proteolytic effect (reviewed by van der Boon *et al.* 1991) swamps these ammonia-trapping mechanisms and ammonia-N excretion resumes at an accelerating rate (Figs 3, 4). The skin becomes highly permeable to both ammonia-N and urea-N. The ability to control, and indeed to virtually eliminate, ammonia-N excretion is lost. This ability may be the most important adaptive aspect of ureotelic behaviour in *Opsanus beta*.

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