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PULSATILE UREA EXCRETION IN GULF TOADFISH (*OPSANUS BETA*): EVIDENCE FOR ACTIVATION OF A SPECIFIC FACILITATED DIFFUSION TRANSPORT SYSTEM

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Summary

When toadfish are made ureotelic by a crowding/confinement protocol, they excrete approximately 90 % of their urea nitrogen (urea-N) production in large, irregular pulses (1–2 pulses per day) from the gill region. We investigated three hypotheses as to the mechanism of pulsatile excretion: (i) the presence of an active reabsorptive 'back-transport' mechanism that is periodically inhibited to allow urea-N excretion to occur; (ii) the periodic occurrence of a generalized, non-specific increase in gill permeability; and (iii) the presence of a specific facilitated diffusion transport system that is periodically activated. Exposure of toadfish during non-pulse periods to treatments designed to block a 'back-transport' mechanism (Na^+-free sea water or the urea analogues 30 mmol l^{-1} thiourea or 30 mmol l^{-1} acetamide in the external water) did not stimulate a leakage of urea-N, thereby opposing the first hypothesis. The second hypothesis was opposed by several results. Neither injection of the potent branchial vasodilator L-isoprenaline (10^{-5} mol l^{-1}) nor infusion of NH_4Cl, the latter at levels known to stimulate urea-N efflux in perfused gills, had any effect on urea-N excretion. Furthermore, during natural pulse events, when the normally very low gill permeability to urea (3×10^{-7} cm s^{-1}) increased at least 35-fold, there was no accompanying increase in permeability to either ^3H_2O (1.5×10^{-5} cm s^{-1}) or the paracellular marker [^{14}C]PEG-4000 (10^{-8} cm s^{-1}). However [^{14}C]thiourea permeability (1.5×10^{-7} cm s^{-1}) increased approximately fivefold, in support of the third hypothesis. Furthermore, when 30 mmol l^{-1} urea was placed in the external water, a concentration (60 000 μmol-N l^{-1}) approximately three times that of blood (20 000 μmol-N l^{-1}), each efflux pulse event (measured with [^{14}C]urea) was accompanied by a net uptake, such that blood urea-N levels rose rather than fell. A proportional 1:1 relationship between influx per unit external concentration and efflux per unit internal (i.e. plasma) concentration indicated a fully bidirectional transport system. The simultaneous presence of 60 mmol l^{-1} thiourea in the external water inhibited the influx component by 73 %, further supporting this conclusion. These data, together with recent molecular, morphological and endocrinological evidence, strongly suggest that pulsatile urea-N excretion is caused by the periodic activation of a facilitated urea transporter in the gills, similar to the vasopressin-regulated urea transporter in the mammalian kidney.

Key words: urea, thiourea, ureotelism, pulsatile excretion, toadfish, *Opsanus beta*, gills, facilitated diffusion, nitrogen excretion.

Introduction

The gulf toadfish (*Opsanus beta*) is unusual amongst teleost fish in expressing a full complement of ornithine–urea cycle enzymes in its liver as an adult (Read, 1971; Mommsen and Walsh, 1989; Anderson and Walsh, 1995). The toadfish is normally ammoniotelic when first collected from the wild, but becomes facultatively ureotelic (>70 % urea-N, <30 % ammonia-N excretion) when subjected to crowding and/or confinement in the laboratory (Walsh *et al.* 1994*a*; Walsh and Milligan, 1995; Hopkins *et al.* 1995). Under these conditions, the toadfish demonstrates a remarkable pattern of urea-N excretion, with over 90 % appearing in pulses of less than 3 h duration which emanate from the head region, presumably from the gills (Wood *et al.* 1995*a*). These pulses are irregular in time and amongst individuals, but occur on average approximately once per day. The phenomenon is not due to pulsatile production by the liver, which actually synthesizes

urea at a steady rate (Wood *et al.* 1997). Rather, urea-N pulses are due to the periodic activation of an excretion mechanism that rapidly clears urea from the blood plasma, thereby lowering stored levels throughout the whole body (Wood *et al.* 1997). The adaptive significance of this behaviour remains unknown, but its existence presents a fascinating physiological problem as to the mechanism and control of pulsatile excretion.

Classically, urea has been viewed as an unreactive molecule which moves passively across biological membranes by simple diffusion. Indeed, the branchial fluxes of urea have often been used as a general index of gill surface area or permeability (e.g. Bergman *et al.* 1974; Haywood *et al.* 1977). However, recent research in higher vertebrates has suggested a much more complex picture, with a variety of facilitated diffusion and secondary active transport mechanisms possible (e.g. Marsh and Knepper, 1992; Dytko *et al.* 1993; Gillin and Sands, 1993). For example, in the mammalian kidney, a hormonally controlled facilitated diffusion carrier has been pharmacologically characterized (Chou and Knepper, 1989; Knepper and Star, 1990; Knepper and Chou, 1995), cloned in two species (You *et al.* 1993; Smith *et al.* 1995) and localized subcellularly in the tubule cells of the terminal inner medullary collecting duct (IMCD; Nielsen *et al.* 1996). Strong evidence also exists for the presence of a Na^+-coupled active urea transporter which may be experimentally induced in the initial portion of the IMCD by dietary protein restriction (Isozaki *et al.* 1993, 1994*a*,*b*). There is precedent for such transporters in fish. Active, Na^+-coupled urea reabsorption has long been recognized in the elasmobranch kidney (Schmidt-Nielsen and Rabinowitz, 1964; Schmidt-Nielsen *et al.* 1972; Hays *et al.* 1977). Recently, pharmacological evidence has been obtained for a facilitated-diffusion-type transporter in hepatocytes of the toadfish itself, which apparently serves to equilibrate urea rapidly between the liver and blood (Walsh *et al.* 1994*b*; Walsh and Wood, 1996).

With this background in mind, the goal of the present study was to test three general hypotheses about the mechanism of pulsatile urea excretion in the toadfish. The first hypothesis (Fig. 1A) was that there is a reabsorptive 'back-transporter' in the gills, normally pumping urea back into the blood as it continually leaks across the gill cells. Periodic inhibition of such a transporter would lead to pulsatile excretion. This idea was tested by placing putative inhibitors of such a transporter in the external water. The second hypothesis (Fig. 1B) was that pulsatile urea excretion occurs because of a periodic generalized increase in gill permeability, i.e. a relatively non-specific effect such as gill vasodilation, whereby permeability is increased not just to urea but to a wide range of other molecules as well. This model was evaluated by the use of agents thought to increase general gill surface area or permeability and by testing for possible changes in the permeability of the gills to other substances during natural pulse events. The third hypothesis (Fig. 1C) was that pulsatile excretion occurs because of periodic insertion or activation of urea transport proteins in the gills. This explanation, which proved to be the most probable, was evaluated by pharmacological studies and by testing whether bidirectional urea transport capability, a key property of facilitated diffusion urea carriers, could be seen at the time of natural pulses.

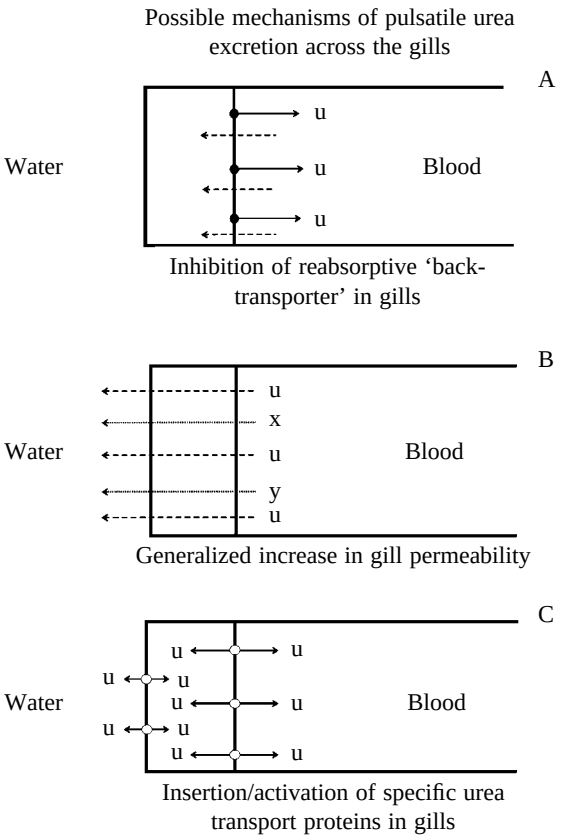

Fig. 1. Three hypotheses for the mechanism of pulsatile urea-N excretion in the toadfish tested in the present study. (A) A reabsorptive 'back-transport' mechanism (filled arrows) in the gill cells which continually pumps urea (u) back into the blood as it leaks out passively (dashed arrows) through the cells. The mechanism is shown on the basolateral membrane, but alternatively could occur on the apical or both membranes. Periodic inhibition of the 'back-transporter' would allow urea-N pulses. (B) A generalized increase in passive gill permeability which occurs periodically, resulting in leakage of urea (u, dashed arrows) as well as other substances (x, y, etc., dotted arrows). (C) The insertion and/or activation of specific facilitated diffusion transport proteins for urea (u, bidirectional arrows) in the gill cells which occurs periodically. The mechanism is shown on both apical and basolateral membranes, but might only occur on whichever membrane is limiting for urea-N diffusion.

Materials and methods

Experimental animals, holding conditions and cannulation

Sexually mature gulf toadfish (*Opsanus beta* Goode and Bean; 108±7 g, range 47–202 g, *N*=45) were captured with a roller trawl by commercial shrimp fisherman in Biscayne Bay, Florida, USA, and held in the laboratory at 25±1 °C, 31±2 ‰ salinity, under natural photoperiod in April and May 1996. During the first 7–10 days, they were maintained in groups of 3–4 fish in 45 l aquaria supplied with a bed of beach sand,

flowing sea water and individual polyvinylchloride (PVC) pipes for shelter ('non-crowded' conditions, less than 12 g fish l^{-1} seawater). During this period, the fish were subjected to two treatments with Malachite Green plus formalin as prophylaxis against the ciliate *Cryptocaryon irritans* (see Wood *et al.* 1997) and were fed live shrimp on alternate days.

A standardized crowding and confinement protocol was used to induce and maintain ureotelic behaviour (see Walsh *et al.* 1994*a*; Wood *et al.* 1995*a*, 1997; Hopkins *et al.* 1995). In brief, toadfish together with their PVC shelters, were transferred in groups of 3–6 into covered 6 l tubs supplied with flowing sea water ('crowded conditions', greater than 80 g fish l^{-1} seawater) for 48–72 h prior to surgery. They were not fed during this period. Following cannulation, fish were placed individually into covered rectangular 3 l containers made of opaque plastic and allowed to recover for 24 h prior to the start of experiments. Each container was supplied with a PVC shelter, aeration and flowing sea water,

Surgery was performed under MS-222 anaesthesia (0.67 g l^{-1}, adjusted to pH 8.0 with NaOH). Each fish was fitted with a single catheter (Clay-Adams PE50) filled with toadfish saline containing sodium heparin (100 i.u. ml^{-1}). Catheters were placed, *via* the haemal arch, into either the caudal artery or the caudal vein exactly as described by Wood *et al.* (1997). The arterial or venous location of the catheter had no detectable influence on the results.

General experimental design

Following recovery, each toadfish was first monitored for 24–48 h to ensure that it was exhibiting ureotelic 'pulsing' behaviour. The water flow was shut off, the container volume was set to 2.0 l, the aeration was set to provide good mixing and a peristaltic pump plus fraction collector system started to continually collect hourly 5 ml water samples for colorimetric assay of urea-N and ammonia-N. In some cases, hourly water samples were drawn by hand because of a shortage of fraction collectors. Throughout this initial monitoring period and the subsequent experiments, the water was changed at 24 h intervals or more frequently, depending on the experimental design. The containers were bathed externally with flowing sea water on a wet-table which maintained the experimental temperature at 25±1 °C.

In many of the experiments, it was necessary to measure urea-N excretion rate under conditions in which the chemistry of the external water was altered (e.g. by addition of urea analogues, high levels of urea itself) in such a way as to prevent accurate colorimetric analysis of urea by the normal diacetyl monoxime assay (Rahmatullah and Boyde, 1980; Price and Harrison, 1987). In these experiments, the procedure developed by Wood *et al.* (1997) was employed, in which the fish was loaded with [^{14}C]urea, the specific activity of urea in the plasma was measured periodically and urea-N excretion was determined by hourly monitoring of the appearance of ^{14}C radioactivity in the water. Wood *et al.* (1997) demonstrated that more than 95 % of the ^{14}C counts in plasma and water remained as urea (i.e. no degradation). More importantly, there was a 1:1 relationship between urea-N pulses measured colorimetrically and urea-N pulses measured radioactively, demonstrating that urea-N pulses originate from the plasma or from a compartment in equilibrium with plasma. Typically, fish in these experiments were injected *via* the catheter with 400 μCi kg^{-1} of [^{14}C]urea (New England Nuclear, specific activity 8.60 mCi $mmol^{-1}$; 1 mCi=3×10^7 Bq) in 2 ml kg^{-1} saline, washed in with a further 2 ml kg^{-1} toadfish saline. After a 3 h equilibration period, the experiment was started. In longer-term experiments, the plasma specific activity was 'topped up' at 48 h intervals by additional injections of 200 μCi kg^{-1} [^{14}C]urea.

Periodic blood sampling was used not only to monitor plasma specific activity, but also to follow changes in absolute plasma urea-N concentrations accompanying pulsatile excretion events. Long catheters allowed blood sampling without disturbance. Blood samples were drawn at 2 h or 4 h intervals with some breaks, depending on the experimental design; 200 μl was first drawn to clear the catheter, then 50 μl was taken as the true sample, and then the first 200 μl was returned, together with sufficient saline to flush the catheter and replace the sampled volume. Approximately 25 blood samples were drawn from each fish (maximum 36). Haematocrits typically declined from 25–30 % to 5–12 %, but this had no detectable influence on the excretion patterns or plasma levels of urea-N.

Testing the back-transport hypothesis (Fig. 1A)

Series 1

Na^+-free sea water was used to block possible Na^+-coupled urea transport (Schmidt-Nielsen *et al.* 1972; Isozaki *et al.* 1994*a*), while thiourea and acetamide were chosen as urea analogues which are known to act as competitive inhibitors of carrier-mediated urea transport in many systems (Marsh and Knepper, 1992). A potential limitation of this approach should be noted which relates to the location of putative 'back-transporters'. If they are located on the apical membranes of the gill cells, then these agents applied in the external water would undoubtedly have access to them. However, if they are located on the basolateral membranes, low permeability of the apical membranes could impede access.

The rationale behind these experiments was to wait until a natural urea-N pulse had occurred and finished, and then to change the external water to a treatment medium containing the inhibitor of the putative back-transport mechanism for a further 3 h period. On the basis of past experience (Wood *et al.* 1995*a*, 1997), we knew that a second natural pulse would rarely occur within 8 or 9 h of the first one. Therefore, a significant appearance of urea-N into the external medium during this 3 h period would indicate that the reabsorptive back-transporter had been pharmacologically inhibited, allowing urea-N to leak out from the blood. Finally, the external medium was changed back to ordinary sea water ('washout') for a further 3 h. This was performed as a safeguard against non-specific stress effects of the treatment,

which would probably be manifested as a continuation of urea-N leakage after removal of the inhibitor.

The [^{14}C]urea technique was used. Hourly water samples were analysed immediately following collection. Once the occurrence and completion of a natural pulse had been detected, the external sea water was changed to (a) synthetic Na^+-free sea water (N=6); (b) sea water containing 30 mmol l^{-1} thiourea (N=5); or (c) sea water containing 30 mmol l^{-1} acetamide (N=9). There was a time lag of approximately 2 h between the end of the natural pulse and the changeover. In (a), the container and fish were first flushed four times with distilled water to ensure complete removal of Na^+, following which the synthetic Na^+-free sea water was added: 400 mmol l^{-1} N-methyl-D-glucamine, 10 mmol l^{-1} $CaCl_2{\cdot}2H_2O$, 33 mmol l^{-1} $MgSO_4{\cdot}7H_2O$, 20 mmol l^{-1} $MgCl_2{\cdot}6H_2O$, 2.5 mmol l^{-1} $KHCO_3$, 6.8 mmol l^{-1} KCl, 0.5 mmol l^{-1} KBr, adjusted to pH 8.0 by titration with HCl. Measured mean Na^+ concentrations (Varian 1275 atomic absorption spectrophotometer) were 0.35±0.08 and 0.68±0.10 mmol l^{-1} at the start and end of the 3 h period, respectively, relative to a normal seawater concentration of approximately 450 mmol l^{-1}. In (b) and (c), 30 mmol l^{-1} thiourea and acetamide were employed so as to give a level approximately three times higher than urea levels (approximately 10 mmol l^{-1}=20 000 μmol-N l^{-1}) in the body fluids (Wood *et al.* 1995*a*, 1997).

Testing the generalized permeability increase hypothesis (Fig. 1B)

Series 2

This experiment (N=5) was based on findings in parallel experiments on an isolated, perfused toadfish head preparation (P. Part, unpublished results) that high levels of total ammonia (T_{Amm}) in the perfusate would greatly elevate the permeability of the gills to urea. Hourly urea-N excretion was monitored until a natural urea-N pulse had occurred and finished as in series 1, except that measurements were performed colorimetrically. Rates of ammonia-N and urea-N excretion were then monitored for a further 3 h prior to and 5 h following infusion *via* the catheter with 2000 μmol kg^{-1} T_{Amm} (11.4 ml kg^{-1} of 175 mmol l^{-1} NH_4Cl, washed in with a further 2 ml kg^{-1}, delivered over 20 min). Blood samples for the measurement of plasma T_{Amm} were drawn 1 h before and at 1, 3 and 5 h after the infusion.

Series 3

The synthetic β-adrenergic agonist L-isoprenaline, a powerful branchial vasodilating agent (Wood, 1974), was administered to induce a generalized increase in gill permeability. High circulating levels of catecholamines appear to elevate functional gill surface area in all teleosts including *Opsanus beta* (Oduleye and Evans, 1982; Oduleye *et al.* 1982). An identical protocol to that for series 2 was followed, except that plasma T_{Amm} was not measured and toadfish (N=6) were injected with a dose of L-isoproterenol-D-bitartate calculated to yield an extracellular concentration of approximately 10^{-5} mol l^{-1} (0.3 ml kg^{-1} of a 10 mmol l^{-1} stock, stabilized with 10^{-3} mol l^{-1} ascorbic acid in saline, and washed in with a further 2 ml kg^{-1} of saline).

Series 4

This series examined whether the increases in urea-N permeability that occurred during natural pulse events were correlated with simultaneous increases in the permeability to other molecules. Substances tested were [^{14}C]polyethylene glycol, M_r=4000 (PEG-4000, a paracellular diffusion marker; Wood and Part, 1997), tritiated water (^3H_2O, a transcellular diffusion or water-channel marker; Oduleye and Evans, 1982; Part *et al.* 1998) and [^{14}C]thiourea (a urea analogue that is not only a competitive blocker but also a substrate transported by certain facilitated diffusion carriers; Marsh and Knepper, 1992). Radiotracers allowed the use of trace amounts so that blocking or non-specific effects would be negligible.

Toadfish were injected *via* the catheter with (a) 400 μCi kg^{-1} [^{14}C]PEG-4000 (NEN, specific activity 0.80 mCi $mmol^{-1}$) in 4.8 ml kg^{-1} saline (N=6); (b) 800 μCi kg^{-1} ^3H_2O (NEN, specific activity 1 mCi g^{-1}), i.e. 0.8 ml kg^{-1} tritiated water (N=4); or (c) 400 μCi kg^{-1} [^{14}C]thiourea (NEN, specific activity 58.0 mCi $mmol^{-1}$) in 1.8 ml kg^{-1} saline (N=6). In each case, the injection was washed in with a further 2 ml kg^{-1} saline. Six different fish were used for each of the [^{14}C]PEG-4000 and [^{14}C]thiourea experiments, whereas two each of the fish injected with these two radiolabels were also injected with ^3H_2O. After a 3 h post-injection equilibration period, water samples were taken hourly and blood samples every 2 h over the following 20–36 h, with some breaks, for the measurement of plasma and water radioactivities and urea-N concentrations. Urea excretion was measured colorimetrically. These measurements allowed calculation of urea, PEG-4000, tritiated water and thiourea permeabilities in common units (cm s^{-1}, see below) during natural pulse events and the intervening non-pulse periods.

Testing the specific urea transporter hypothesis (Fig. 1C)

Series 5

This experiment (N=8) evaluated whether pulsatile urea-N excretion could be inhibited by systemic administration of phloretin, a potent competitive blocker of many facilitated diffusion urea transporters in higher vertebrates (Marsh and Knepper, 1992). Because phloretin must be solubilized in dimethylsulphoxide (DMSO), the possible effects of this vehicle alone were evaluated on each fish during the initial 24 h period prior to the administration of phloretin.

During this initial 24 h monitoring period, DMSO (1 ml kg^{-1} dissolved in 4 ml kg^{-1} saline and washed in with a further 2 ml kg^{-1} saline) was injected at 0, 8 and 16 h. Water samples were taken hourly for colorimetric assay of urea-N and ammonia-N, and blood samples every 4 h for assay of plasma urea-N concentrations. At 24 h, the containers were flushed, 250 μmol kg^{-1} phloretin in the above vehicle was injected, and sampling commenced again. Further doses of 100 μmol kg^{-1} phloretin were injected at 8 h and 16 h after the initial injection, and the experiment was terminated at 24 h.

Series 6

An important property of most facilitated diffusion urea carriers is that they are bidirectional, transporting urea in either direction with equal efficiency in proportion to the relevant concentrations (Chou *et al.* 1990). We therefore reasoned that, if a pulsatile urea excretion event occurred because of activation/insertion of such a carrier in the gills, then net *inward* urea transport should take place at this time if the external water contained a high level of urea. The *efflux* component could be measured by the appearance of [^{14}C]urea radioactivity in the water, and the *net* flux could be calculated from the change in plasma urea-N levels (see below). The *influx* component could then be calculated by difference.

After the initial 24 h monitoring period, toadfish (N=14) were loaded with [^{14}C]urea, and the external sea water was replaced with sea water containing 30 mmol l^{-1} urea (60 000 μmol l^{-1} urea-N, i.e. approximately three times higher than blood levels). After a 3 h equilibration period, water samples were taken hourly and blood samples every 2 h for the measurement of plasma and water radioactivities and urea-N concentrations. Sampling was continued for a further 20–30 h (with some breaks) or until at least one natural urea-N pulse event occurred.

As the results confirmed the occurrence of bidirectional transport during pulsatile events, the experiment was extended in some of the toadfish (N=7) to test whether the influx component in the presence of 30 mmol l^{-1} urea could be inhibited by the simultaneous presence of a high concentration of external thiourea (60 mmol l^{-1}), a competitive inhibitor in many facilitated diffusion transport systems (Chou and Knepper, 1989; Marsh and Knepper, 1992). At the end of the monitoring period in 30 mmol l^{-1} urea alone, the containers were flushed and refilled with normal sea water for approximately 12 h. The plasma specific activity was then 'topped up' by an additional injection of 200 μCi kg^{-1} [^{14}C]urea, and the external medium was changed to one containing 30 mmol l^{-1} urea (60 000 μmol urea-N l^{-1}) plus 60 mmol l^{-1} thiourea. After a 3 h equilibration period, hourly water sampling and blood sampling every second hour were resumed for another 30 h, with some breaks, to examine the unidirectional flux components during natural pulses in the presence of the putative inhibitor.

Analytical techniques

Water and plasma samples for analysis of urea-N, ammonia-N or radioactivity were assayed immediately, stored at 4 °C for no more than 24 h prior to assay, or frozen at −20 °C pending later analysis. Standard chemical colorimetric assays on a micro-plate reader (Thermomax; Molecular Devices) were employed for urea-N (Rahmatullah and Boyde, 1980; Price and Harrison, 1987) and ammonia-N (Ivancic and Deggobis, 1984) in sea water, while ammonia-N (T_{Amm}) in plasma was measured enzymatically using a Sigma kit (Mondzac *et al.* 1965). For analysis of ^{14}C and ^{3}H activity, 1 ml of radioactive sea water, or 10 μl of radioactive plasma plus 1 ml of 'cold' sea water, was added to 10 ml of ICN Ecolume. Single-labelled samples were counted on a TmAnalytic 6895 BetaTrac liquid scintillation counter, while dual-labelled samples were counted on a Beckman LS1801 liquid scintillation counter, employing inbuilt quench correction programs.

Calculations

All urea and ammonia concentrations and fluxes have been expressed in units of nitrogen for comparative purposes and consistency with previous studies (Wood *et al.* 1995*a*, 1997). Pulses of urea-N excretion were identified from stepwise increases in seawater urea-N concentration or [^{14}C]urea radioactivity recorded in hourly water samples. When colorimetric methods were employed, excretion rates (in μmol-N kg^{-1} h^{-1}) of urea-N and ammonia-N were calculated from changes in concentration in the external sea water (μmol-N l^{-1}) multiplied by the volume (l) and factored by time (h) and mass (kg). When the [^{14}C]urea method was used, the total radioactivity appearing in the water in each hour (cts min^{-1}) was divided by the measured specific activity (cts min^{-1} μmol^{-1} urea-N) of the preceding plasma sample, again factored by time (h) and mass (kg). Total urea-N pulse sizes were calculated in the same way, but not factored by time.

In experiments designed to measure bidirectional urea-N fluxes with 60 000 μmol urea-N l^{-1} in the external water, the *efflux* component was calculated exactly as outlined above from the appearance of [^{14}C]urea radioactivity in the water. The *net flux* component was calculated from the measured change in plasma urea-N concentration accompanying the pulse event, divided by 0.75. Wood *et al.* (1997) demonstrated that, on average, the change in plasma urea-N concentration (μmol l^{-1}) was 75 % of the measured urea-N pulse (μmol kg^{-1}). The *influx* was then calculated as the difference between the net flux and the efflux. Influx and efflux per unit concentration were calculated by dividing the influx (μmol-N kg^{-1} pulse^{-1}) by the external concentration (60 000 μmol-N l^{-1}) and the efflux (μmol-N kg^{-1} pulse^{-1}) by the measured plasma concentration (μmol-N l^{-1}).

Permeabilities (p, cm s^{-1}) to urea, [^{14}C]PEG-4000, $^{3}H_2O$ and [^{14}C]thiourea were calculated during natural pulse events and during the periods immediately preceding and immediately following the pulse events. In each case, a 3 h period was employed, recognizing that the ability of the peristaltic pump/fraction collector system to resolve complete urea-N pulses was limited to 3 h (see Wood *et al.* 1995*a*, 1997).

$$p = \frac{\Delta X_w \times V}{T \times X_p \times A},$$

where ΔX_w is the increase in the concentration of the substance in the water (μmol l^{-1} or cts min^{-1} l^{-1}) in the 3 h period, X_p is the concentration of the substance in the blood plasma (in the same units) at the start of the period, V is volume in cm^3, T is time (10 800 s=3 h) and area (A) was taken as 1.92 cm^2 g^{-1} body mass, the value reported by Hughes and Gray (1972) for the gill surface area of the related *Opsanus tau* (see Discussion).

Data have been expressed as means ± 1 S.E.M. (N) where N represents either the number of fish or the number of pulse

events, as specified. Regression lines were fitted by the method of least squares, and the significance of Pearson's correlation coefficient *r* was assessed. Student's two-tailed *t*-test, unpaired or paired as appropriate, was employed to evaluate the significance of differences between means. For multiple comparisons, the *t* value was adjusted using the Bonferroni procedure (Nemenyi *et al.* 1977). A fiducial limit of $P \leqslant 0.05$ was employed throughout.

Results

Patterns of nitrogen metabolism

During the initial 24–48 h monitoring period, only two of the 45 toadfish studied did not exhibit pulsatile urea-N excretion; they were rejected. Excluding the fish treated with DMSO for series 5 (see below), the other 35 toadfish were predominantly ureotelic, excreting 77 % urea-N (220.2±23.9 μmol-N kg^{-1} h^{-1}) and only 23 % ammonia-N (66.2±11.0 μmol-N kg^{-1} h^{-1}). Of the total urea-N excretion, 89.7±1.7 % occurred in discrete pulse events of 3 h or less. Mean urea-N pulse size was 3150±391 μmol-N kg^{-1}, and mean frequency was 1.67±0.12 pulses day^{-1}. Ammonia-N excretion was never pulsatile. Mean plasma urea-N concentration was 19 562± 2714 μmol-N l^{-1} (*N*=35) and mean plasma ammonia-N (T_{Amm}) concentration, measured only in the fish of series 2, was 124±22 μmol-N l^{-1} (*N*=5).

Testing the back-transport hypothesis (Fig. 1A)

Averaged over 3 h, urea-N excretion rate during natural pulse events in series 1 was 600–1000 μmol-N kg^{-1} h^{-1} (Fig. 2). After completion of the pulses, the external sea water was exchanged for a medium containing one of three potential inhibitors of a possible reabsorptive back-transport mechanism in the gills: Na^+-free sea water (Fig. 2A), 30 mmol l^{-1} thiourea in normal sea water (Fig. 2B) or 30 mmol l^{-1} acetamide in normal sea water (Fig. 2C). None of these agents resulted in any significant 'leakage' of urea-N into the external media above that normally seen (approximately 20 μmol-N kg^{-1} h^{-1}), despite the fact that measured blood plasma urea-N levels remained in the range 15 000–26 000 μmol-N l^{-1} in these fish. There was also no significant leakage upon return to normal sea water ('washout'). These results argue against the hypothesis that pulsatile urea-N excretion occurs because of periodic inhibition of a back-transport mechanism that normally prevents urea-N leakage.

Testing the generalized permeability increase hypothesis (Fig. 1B)

When toadfish of series 2 were infused with 2000 μmol kg^{-1} NH_4Cl a few hours after completion of a natural pulse, plasma T_{Amm} levels were significantly elevated approximately 3.5-fold to 425 μmol-N l^{-1}, measured at 1 h post-infusion (Fig. 3A). Although declining thereafter, plasma T_{Amm} remained significantly elevated at 5 h. This treatment did not cause any stimulation of urea-N excretion, which remained at the normal basal rate of approximately 20 μmol-N kg^{-1} h^{-1} for the next 5 h

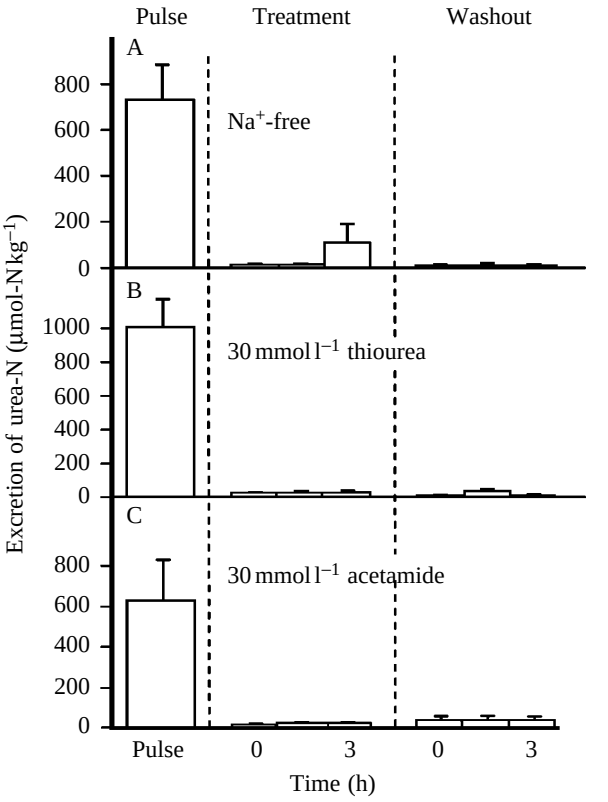

Fig. 2. The influence of treatments designed to inhibit a possible branchial 'back-transport' mechanism on urea-N excretion (amount excreted per hour) in toadfish. Immediately after a natural urea-N pulse had occurred (shown averaged over 3 h, the minimum resolution time of the recording system), the external sea water was changed to a 'treatment' of (A) Na^+-free sea water (*N*=6), (B) normal sea water containing 30 mmol l^{-1} thiourea (*N*=5) or (C) normal sea water containing 30 mmol l^{-1} acetamide (*N*=9). A final 3 h 'washout' period represented a return to normal sea water. The small, non-significant elevation at 2–3 h of the Na^+-free treatment was due to a small apparent natural pulse (527 μmol-N kg^{-1}) in a single fish only. Values are means + 1 S.E.M.

(Fig. 3C). Interestingly, there was a marked stimulation of ammonia-N excretion, significant for the first 2 h after infusion, but only approximately 50 % (1000 μmol kg^{-1}) of the infused ammonia load was excreted before ammonia-N excretion returned to control levels (Fig. 3B).

In series 3, a similar protocol demonstrated that injection of the powerful branchial vasodilator L-isoprenaline had no effect on urea-N excretion over the following 5 h (Fig. 4B). However, isoprenaline caused an elevation of ammonia-N excretion that was significant at 1 h and 3 h post-injection (Fig. 4A).

In series 4, the permeability of toadfish to several radiolabelled compounds was compared with that for urea during pulsing and non-pulsing periods; a logarithmic scale has been employed in Fig. 5 to encompass the wide absolute range of permeabilities measured. Urea permeability during non-pulsing periods was approximately 3×10^{-7} cm s^{-1} and increased 25- to 50-fold to approximately 10^{-5} cm s^{-1} (minimum estimate;

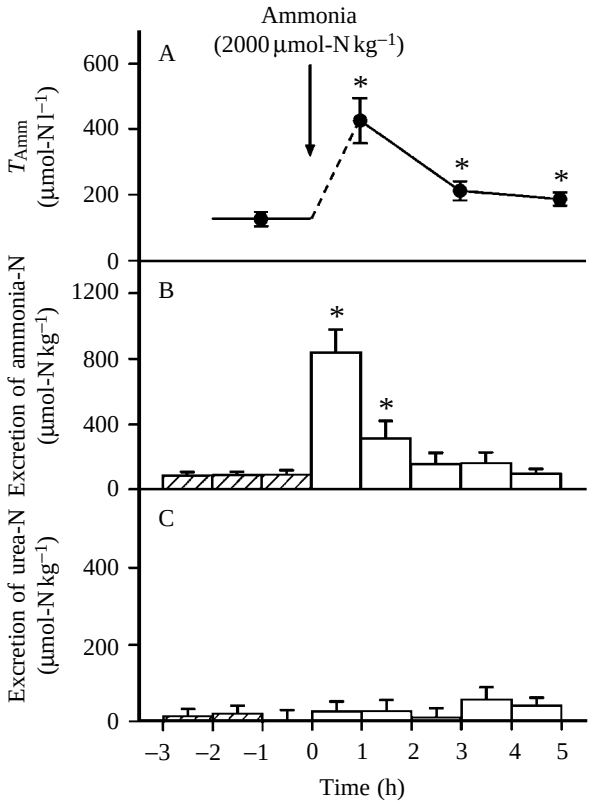

Fig. 3. The influence of an infusion (over 20 min, starting at time zero) of 2000 μmol kg^{-1} NH_4Cl into toadfish during non-pulsing periods on (A) plasma total ammonia-N concentration, (B) ammonia-N excretion and (C) urea-N excretion. Values are means ± 1 S.E.M. (N=5). Asterisks indicate means significantly different ($P \leqslant 0.05$) from the pre-infusion control mean.

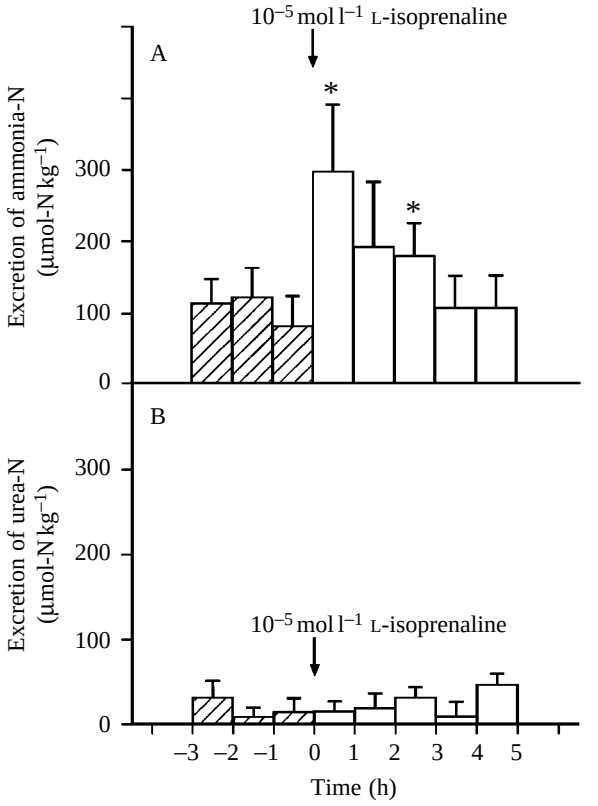

Fig. 4. The influence of an injection (at time zero) of a dose of L-isoprenaline calculated to yield an extracellular concentration of approximately 10^{-5} mol l^{-1} into toadfish during non-pulsing periods on (A) ammonia-N excretion and (B) urea-N excretion. Values are means ± 1 S.E.M. (N=6). Asterisks indicate means significantly different ($P \leqslant 0.05$) from the pre-injection control mean.

see Discussion) during natural pulses. Permeability to the paracellular permeability marker PEG-4000 (approximately 10^{-8} cm s^{-1}) was more than one order of magnitude lower than that to urea during non-pulsing periods and exhibited no increase during natural pulses (Fig. 5A). Permeability to ^3H_2O (approximately 1.5×10^{-5} cm s^{-1}) was almost two orders of magnitude higher than that to urea during non-pulsing periods, but again did not increase during natural pulses (Fig. 5B). During non-pulsing periods, permeability to the urea analogue thiourea (approximately 1.5×10^{-7} cm s^{-1}) was similar to, but slightly lower than, that to urea (difference not significant). Unlike the other markers, permeability to thiourea increased significantly during urea pulses, but the elevation was only fivefold in contrast to the 35-fold increase in urea permeability in this group of fish (Fig. 5C). Thiourea permeability was therefore significantly lower than urea permeability during pulses. Fig. 6 shows an example of an original record of the concentrations of urea-N and [^{14}C]thiourea (cts min^{-1}) during two natural pulse events, illustrating the correspondence in time between thiourea and urea pulses.

Taken together, the ammonia, L-isoprenaline, PEG-4000 and ^3H_2O data argue strongly against any generalized (i.e. non-specific) increase in permeability as the mechanism of pulsatile urea-N excretion. The thiourea data suggest the activation of a selective transport system which recognizes another molecule similar to urea.

Testing the specific urea transporter hypothesis (Fig. 1C)

As a control in series 5, prior to phloretin administration, the DMSO/saline vehicle was administered to the same fish at 8 h intervals over the initial 24 h monitoring period (Table 1). This caused subtle changes in the pattern of nitrogen waste excretion, with somewhat lower urea-N excretion rates, and a lower percentage (approximately 56 %) of nitrogen excretion occurring in the form of urea relative to non-injected animals (see above). However, urea-N excretion remained pulsatile at approximately the same frequency, 90 % of total urea-N excretion still occurred in discrete pulses, and plasma urea-N concentration fell in the predicted manner coincident with each pulse event (cf. Figs 2, 3 of Wood *et al.* 1997).

Phloretin, a potent blocker of many facilitated diffusion systems, proved to be highly toxic when administered systemically. Two toadfish (omitted from the data set of Table 1) died almost immediately after the first injection (250 μmol kg^{-1}). Table 1 summarizes the data for the

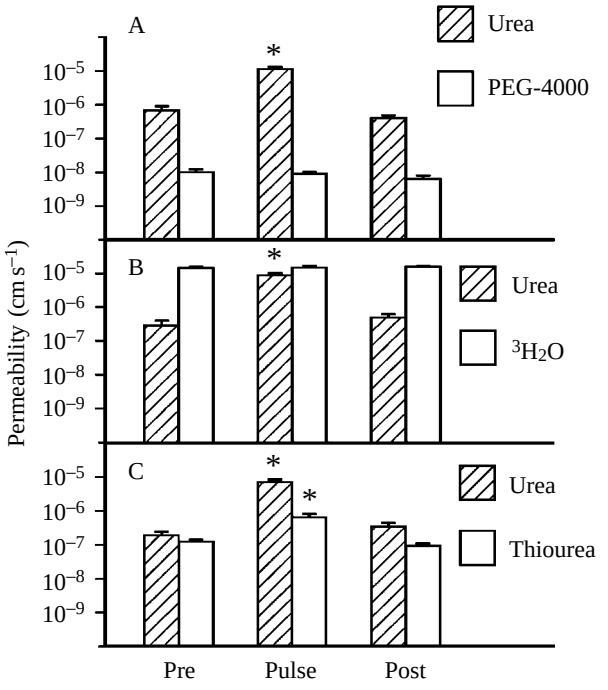

Fig. 5. A comparison of changes in urea permeability of the gills of toadfish occurring during natural urea-N pulses with those of (A) [^{14}C]polyethylene glycol-4000 permeability (PEG-4000; *N*=16 pulses from six fish), (B) tritiated water permeability (^3H_2O, *N*=6 pulses from four fish) and (C) [^{14}C]thiourea permeability (*N*=19 pulses from six fish). Note the logarithmic scale for permeability. Data represent mean values for 3 h periods immediately before ('Pre'), during ('Pulse') and after ('Post') natural urea-N pulses. Values are means + 1 S.E.M. Asterisks indicate means significantly different ($P \leqslant 0.05$) from the 'Pre' values.

remaining six fish for the 0–16 h period. All survived past 17 h (i.e. past the second and third maintenance injections of 100 μmol kg^{-1}), but only one remained alive at 24 h and survived indefinitely. Phloretin treatment had no significant effect on the absolute rates of urea-N or ammonia-N excretion. However, phloretin abolished the pulsatile excretion pattern, such that pulse frequency dropped to zero and urea simply accumulated in the external water at a steady rate from all fish. Interestingly, despite this continuous excretion, plasma urea-N concentration increased steadily and significantly during phloretin treatment (Table 1).

Series 6 employed an alternative approach to test for a facilitated diffusion transport system. Urea-N concentration in the water was elevated to 60 000 μmol-N l^{-1} (30 mmol l^{-1} urea) in order to detect whether bidirectional transport occurred at the time of natural pulse events. Efflux pulse events were quantified by the appearance of [^{14}C]urea radioactivity in the external water. Fig. 7 depicts a typical record. Under these conditions, plasma urea-N concentration increased in coincidence with each large pulse event, such as the first and third pulses in Fig. 7, or remained more or less unchanged after small pulses, such as the second one in Fig. 7. Fig. 8 summarizes mean data for nine efflux pulses (1350–5730 μmol-N kg^{-1}) in eight toadfish chosen

Table 1. *The influence of phloretin treatment on the rate and pattern of urea-N and ammonia-N excretion in toadfish*

	DMSO control	Phloretin
Ammonia-N excretion rate (μmol-N kg^{-1} h^{-1})	95.9±24.0	91.7±22.3
Urea-N excretion rate (μmol-N kg^{-1} h^{-1})	115.3±27.1	154.0±47.8
Urea-N pulse frequency (pulses day^{-1})	1.6±0.3	0±0*
Percentage of urea-N output as pulses	90.1±5.0	0±0*
Plasma urea-N concentration (μmol-N l^{-1})	18 041±433	21 291±384*

The DMSO/saline vehicle was administered systemically *via* the catheter at 8 h intervals during the initial 24 h control period (see text for details).

Phloretin data were averaged over the first 16 h of treatment. Phloretin, in the DMSO/saline vehicle, was administered systemically in an initial dose of 250 μmol kg^{-1}, followed by a maintenance dose of 100 μmol kg^{-1} at 8 h.

Values are means ± 1 S.E.M. (*N*=6).

*$P \leqslant 0.05$ relative to DMSO control value by paired *t*-test.

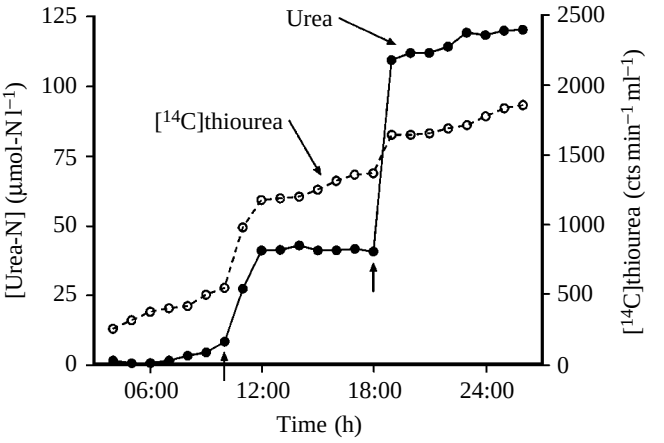

Fig. 6. An original record over a 23 h period of the concentrations of urea-N and [^{14}C]thiourea (cts min^{-1}) in the external water (2 l) of a toadfish (mass 101 g) during two natural pulse events (indicated by arrows), illustrating the correspondence in time between thiourea and urea pulses.

on the basis of the joint criteria of typical pulse size (1000–6000 μmol-N kg^{-1}) plus the availability of continuous plasma data for at least 4 h prior to and 10 h after a pulse event. The patterns depicted in Figs 7 and 8 contrast markedly with the patterns reported in Figs 2 and 3 of Wood *et al.* (1997) for normal pulse events of toadfish in the absence of high external urea levels. Thus, toadfish exposed to high external concentrations became loaded with urea-N by inward transport every time the efflux mechanism was activated, confirming the presence of a bidirectional transport system.

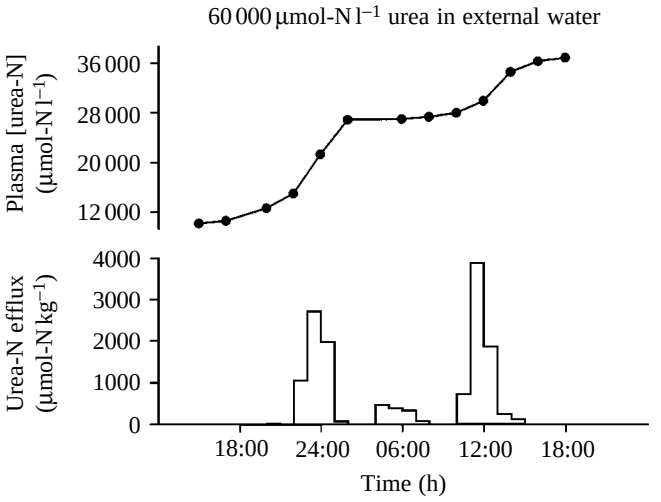

Fig. 7. Typical example of changes in plasma urea-N concentration in an individual toadfish (mass 96 g, over a 28 h period) accompanying natural pulsatile urea-N excretion events when 60 000 µmol l^{-1} urea-N was present in the external water. Urea-N efflux was measured by the appearance of [^{14}C]urea in the external water. Note the clear increases in plasma urea-N concentration accompanying the first and third (large) pulses, and the relative stability during the second (smaller) pulse. In the absence of high external urea concentration, marked decreases in plasma urea-N concentration would be expected at these times.

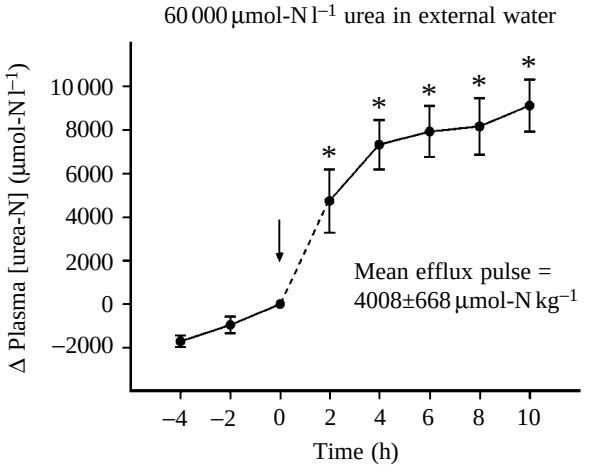

Fig. 8. Changes in plasma urea-N levels for 4 h prior to and 10 h following natural urea-N efflux pulse events in toadfish exposed simultaneously to 60 000 µmol l^{-1} urea-N in the external water. Urea-N efflux pulses were measured by the appearance of [^{14}C]urea in the water. Note the rise in plasma urea-N coincident with pulses. Values are expressed as differences from the plasma urea-N level immediately preceding the pulse event (taken as 0 at time zero). Values are means ± 1 S.E.M. (N=9 pulses in eight fish). Asterisks indicate means significantly different ($P \leqslant 0.05$) from this reference value.

In Fig. 8, note that the mean rise in plasma urea concentration (in the 3 h period coincident with a pulse event, starting at the arrow) was approximately 6000 µmol-N l^{-1}, or

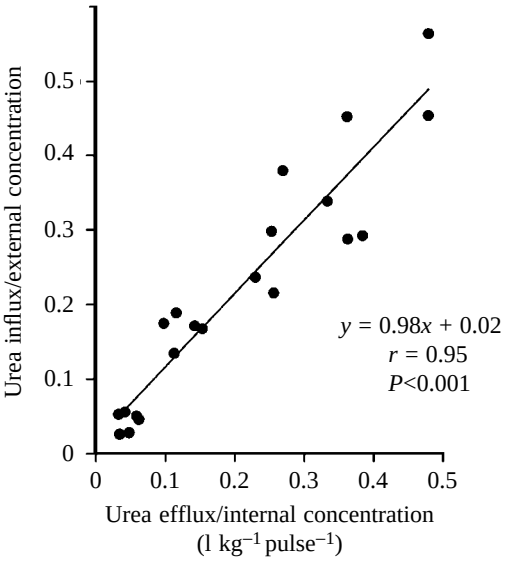

Fig. 9. The relationship between between urea-N influx per unit external concentration and urea-N efflux per unit internal (i.e. plasma) concentration in 21 natural pulse events in 14 toadfish. Note the 1:1 relationship. Urea-N efflux was detected by the appearance of [^{14}C]urea in the water, net flux by the change in plasma urea-N concentration and influx by difference (see text for details).

1.5 times the mean *efflux* pulse of approximately 4000 µmol-N kg^{-1}. A net pulse flux (uptake) of approximately 8000 µmol-N kg^{-1} is obtained by dividing this change in plasma urea-N concentration by 0.75 (see Materials and methods) and, by difference, the mean *influx* pulse must have been approximately 12 000 µmol-N kg^{-1}. Thus, influx was approximately three times greater than efflux. This is exactly the situation predicted for a bidirectional facilitated diffusion transporter with equal efficiency in both directions, because the water concentration (60 000 µmol-N l^{-1}) was almost three times greater than mean plasma urea-N concentration (21 830±2835 µmol-N l^{-1}, N=9) at the time of these pulses.

A more extensive analysis of 21 pulse events from 14 toadfish confirmed this conclusion. Over a wide range of efflux pulse sizes (620–15 870 µmol-N kg^{-1}), there was a highly significant, proportional 1:1 relationship between influx per unit external concentration and efflux per unit internal (i.e. plasma) concentration (Fig. 9).

Interestingly, during exposure to high external urea-N concentrations, the size of urea-N efflux pulses increased significantly compared with the control day (5084±926 *versus* 3450±674 µmol kg^{-1}) in the same fish (N=14), but there was no change in pulse frequency (1.31±0.31 *versus* 1.41±0.19 pulses day^{-1}). This 47 % increase in pulse size probably reflected the proportional 45 % increase in plasma urea-N concentration, which rose significantly during exposure to high urea-N levels (23 960±2219 µmol-N l^{-1} during the experimental period *versus* 16 520±1795 µmol-N l^{-1}, N=14, on the control day).

The experiment was extended in seven toadfish to evaluate

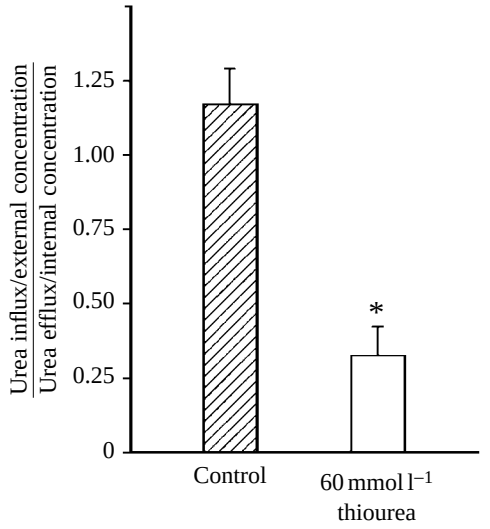

Fig. 10. The influence of 60 mmol l^{-1} thiourea in the external water on the ratio of urea-N influx per unit external concentration to urea-N efflux per unit internal (i.e. plasma) concentration when 60 000 µmol l^{-1} urea-N (30 mmol l^{-1} urea) was present simultaneously in the external water. Values are means + 1 S.E.M. (N=11 pulses in the same seven fish in both the control and 60 mmol l^{-1} thiourea periods; 30 mmol l^{-1} urea was present in both). An asterisk indicates a significant difference from the control value ($P \leqslant 0.05$).

the potential inhibitory effect of high external thiourea concentration. The ratio of urea-N influx per unit external concentration to urea-N efflux per unit internal concentration was significantly reduced by 73 % in the presence of 30 mmol l^{-1} urea plus 60 mmol l^{-1} thiourea (11 pulses) compared with the ratio in the presence of 30 mmol l^{-1} urea alone (11 pulses) in these same fish (Fig. 10).

Discussion

The overall pattern of N-waste excretion in these ureotelic toadfish was similar but not identical to that reported previously (Wood *et al.* 1995*a*, 1997). As in the earlier studies, the present toadfish excreted urea-N in discrete pulses which accounted for approximately 90 % of total urea-N excretion. However, they exhibited somewhat higher absolute rates of both urea-N and ammonia-N excretion, slightly larger urea-N pulse size and frequency, and a slightly lower overall percentage of total-N excretion in the form of urea. These differences were probably the result of two factors: (i) the present experiments were run at a slightly higher temperature (25 °C *versus* 20–24 °C); (ii) the present toadfish were fed during the initial holding period in the laboratory under 'non-crowded' conditions, a factor that is known to elevate overall N-excretion and favour the persistence of significant ammonia-N excretion in predominantly ureotelic fish (Walsh and Milligan, 1995).

An assumption throughout the present paper is that the gills are the site of pulsatile urea-N excretion, whereas our previous study had localized the phenomenon only to the head region (Wood *et al.* 1995*a*). In addition to the molecular and ultrastructural evidence for branchial involvement summarized below, parallel studies have now detected urea-N pulses using an opercular catheter to siphon water coming directly off the gills (K. M. Gilmour, unpublished results) and an absence of urea movement through cephalic skin (P. Part, unpublished results). However, it remains possible that the well-vascularized opercular epithelium may also make a contribution.

This study evaluated three hypotheses to explain the mechanism of pulsatile urea-N excretion (Fig. 1). The results obtained argue against the first two (inactivation of a reabsorptive 'back-transporter' mechanism, or a generalized permeability increase) and strongly favour the third (insertion/activation of a facilitated diffusion carrier).

The back-transport hypothesis was laid out in detail by Wood *et al.* (1997). In brief, the idea is based on findings of secondary active Na^+-coupled urea-N reabsorption in the kidneys of both elasmobranchs (Schmidt-Nielsen *et al.* 1972; Hays *et al.* 1977) and mammals (initial inner medullary collecting ducts of protein-restricted rats; Isozaki *et al.* 1993, 1994*a*,*b*), various other types of urea-N reabsorptive systems in amphibian skin (Dytko *et al.* 1993) and circumstantial evidence for urea-N reabsorption in the elasmobranch gill (Wood *et al.* 1995*b*; Part *et al.* 1998). Na^+-free conditions were tested for obvious reasons, whereas high levels of thiourea and acetamide were tested because both analogues appear to be effective in the elasmobranch gill (Wood *et al.* 1995*b*), while only the latter is effective in elasmobranch kidney (Schmidt-Nielsen and Rabinowitz, 1964) and the skin of the toad *Bufo viridis* (Garcia-Romeu *et al.* 1981; Katz *et al.* 1981). The complete failure of all three treatments, when placed in the external environment of the toadfish, to cause any 'leakage' of urea-N (Fig. 2) argues against the presence of a back-transport system in the gills of *Opsanus beta*. However, one reservation must be kept in mind, as noted earlier. While these results logically eliminate an apical 'back-transporter', a basolaterally located mechanism remains possible, if apical permeability to these externally applied blocking agents was limiting.

All approaches employed to test the idea that a generalized permeability increase constitutes the mechanism for urea-N pulses yielded results that opposed the hypothesis. NH_4Cl infusions were performed because of the results of experiments on an isolated perfused toadfish head preparation (P. Part, personal communication). These *in vitro* tests demonstrated that raising perfusate T_{Amm} from 100 to 400–1600 µmol l^{-1} immediately increased the urea permeability of the gills four- to 32-fold, with minimal effect on ^3H_2O or ammonia-N permeability itself. While the mechanism is unknown, the idea is attractive because it might help coordinate pulsatile events with the internal build-up of a more dangerous N-waste product, which could subsequently be detoxified by conversion to urea. However, an increase in plasma T_{Amm} *in vivo* to 425 µmol l^{-1} (measured at 1 h, undoubtedly higher earlier) caused by exogenous NH_4Cl had no effect on urea-N excretion

(Fig. 3). Presumably the *in vitro* response is in some way associated with the special conditions of perfusion. *In vivo*, approximately 50 % of the exogenous ammonia-N load was not excreted and was probably detoxified through glutamine and/or urea synthesis (Walsh *et al.* 1990; Barber and Walsh, 1993).

L-Isoprenaline is a powerful synthetic β-adrenergic agonist; at the dose chosen (circulating levels of approximately 10^{-5} mol l^{-1}), it would undoubtedly have caused a maximal vasodilation and increase in surface area of the gills (Wood, 1974; Oduleye and Evans, 1982; Oduleye *et al.* 1982), together with probable increases in cardiac output and ventilatory flow (Randall and Perry, 1992). Marked vasodepressor and cardio-acceleratory actions of L-isoprenaline *in vivo* in *Opsanus beta* have been recorded (S. F. Perry, unpublished results). In toadfish perfused gill preparations, β-adrenergic stimulation has been shown to increase ^3H_2O permeability (Oduleye *et al.* 1982; P. Part, unpublished results), and in perfused gills from trout (*Oncorhynchus mykiss*), increases in [^{14}C]urea fluxes have even been reported (Bergman *et al.* 1974; Haywood *et al.* 1977). The absence of any detectable effect of isoprenaline on urea-N excretion in the present toadfish, even though there was substantial stimulation of ammonia-N excretion (Fig. 4), is a strong argument against the generalized permeability increase hypothesis.

Permeability calculations assumed a pulse period of 3 h, the minimum resolution time of the recording system. Recent experiments employing a monitoring system with a much finer time resolution recorded a mean pulse duration of only 62 min (K. M. Gilmour, unpublished results), but the mean size of the urea-N pulses in that study was only approximately 25 % of the size measured in the toadfish used in the present study. Permeability calculations also assumed a gill area equivalent to that measured in the closely related *Opsanus tau* (Hughes and Gray, 1972). While this choice is probably not a significant source of error, the assumption that fluxes occur only through the gills for all the measured labels is problematic, because the skin of *Opsanus beta* lacks scales and is well-vascularized. Fortunately, at least for urea and ^3H_2O, skin permeabilities and gill permeabilities measured separately *in vitro* were almost identical to one another (P. Part, unpublished results) and were also very similar to the values calculated for the gills of intact, non-pulsing toadfish in the present study (Fig. 5).

Urea permeabilities during non-pulse periods in toadfish (3×10^{-7} cm s^{-1}) were approximately one order of magnitude lower than in other teleosts (see summaries in Isaia, 1984; Part *et al.* 1998), confirming that urea-N excretion is tightly restricted in this species. Paracellular permeability (10^{-8} cm s^{-1}), as typified by PEG-4000 fluxes, appeared to be similarly low (see summary in Wood and Part, 1997), while ^3H_2O permeability (1.5×10^{-5} cm s^{-1}) was fairly typical of other teleosts (Isaia, 1984; Part *et al.* 1998). The key point, however, is that neither PEG-4000 nor ^3H_2O permeabilities increased during natural urea-N pulses, at a time when urea-N permeability increased at least 35-fold (or up to 100-fold if pulse duration was really 1 h rather than 3 h). These results rule out generalized increases in either paracellular or transcellular/water channel permeability as the explanation for natural urea-N pulse events. The relative increase in urea permeability of the gills at these times in the present study was comparable to or greater than that seen in toad bladder (Maffly *et al.* 1960; Eggena, 1973; Levine *et al.* 1973) or mammalian IMCD (Sands *et al.* 1987; Knepper and Star, 1990; Ashkar *et al.* 1995) when neurohypophyseal peptides activate facilitated diffusion carriers in these tissues.

Of the molecules tested, only the analogue thiourea caused a permeability increase during natural pulse events (Fig. 6) and the elevation was only approximately 15 % compared with that for urea permeability (Fig. 5C). This result provided the first evidence that a specific facilitated diffusion carrier might be involved in the toadfish gill because, in three such transport systems for urea which have been characterized (toad bladder carrier, Levine *et al.* 1973; mammalian erythrocyte carrier, Mayrand and Levitt, 1983; mammalian kidney vasopressin-regulated carrier, Chou *et al.* 1990), thiourea is similarly transported with much lower efficiency (<25 % of urea permeability).

Phloretin was evaluated because it is a potent inhibitor of all these carriers and, indeed, sensitivity to phloretin has become a standard test for the involvement of facilitated diffusion in urea transport (Marsh and Knepper, 1992). Unfortunately, the toxicity and non-specificity of phloretin are well recognized: it also inhibits glucose, chloride and other transport systems (Levine *et al.* 1973; Knepper and Star, 1990). In the present study, systemic phloretin administration at the commonly used extracellular level (250 μmol l^{-1}) was highly toxic, but the results, while equivocal, did not oppose the facilitated diffusion hypothesis (Table 1). Phloretin did eliminate pulsatile urea-N excretion and caused an internal accumulation of urea-N, but did not reduce the overall excretion rate of urea-N, which became continuous. We have demonstrated previously that, in highly stressed toadfish, urea production is elevated and urea-N leaks across the general body surface in a continuous fashion (Wood *et al.* 1995*a*), which provides a likely explanation for our observations. In the light of the results discussed below, it might be useful in the future to apply phloretin in the external sea water to achieve a more specific effect, although such experiments will be extremely expensive.

The strongest evidence in favour of facilitated diffusion as the mechanism of urea-N pulses was provided by the elevation of urea and thiourea concentrations in the external sea water. When external urea-N was set to 60 000 μmol-N l^{-1}, approximately three times plasma levels, each natural efflux pulse event was accompanied by an influx of urea-N (Figs 7, 8). Influx pulses per unit external concentration were exactly equal to efflux pulses per unit internal concentration (Fig. 9). This bidirectional transport capability, moving urea in both directions with equal efficiency in proportion to the relevant concentrations, is a key property of facilitated diffusion urea carriers such as UT-2 in the mammalian kidney (Chou *et al.* 1990). It is questionable whether such inward transport would ever occur across the gills of toadfish in nature, but the results clearly show that the capability is there.

A twofold higher concentration (60 mmol l^{-1}) of thiourea relative to urea (30 mmol l^{-1}) in the external sea water blocked the influx component by 73 % (Fig. 10). This inhibition by thiourea is comparable to that seen for the toad bladder carrier (Eggena, 1973), the mammalian erythrocyte carrier (Mayrand and Levitt, 1983; Olives *et al.* 1994), and the mammalian kidney vasopressin-regulated carrier (Chou and Knepper, 1989; You *et al.* 1993) and, indeed, appears to be a common property of facilitated diffusion urea transporters (Knepper and Star, 1990; Marsh and Knepper, 1992).

Four other pieces of evidence acquired in parallel studies from our laboratory also argue in favour of the hypothesis that urea-N pulses are due to the insertion or activation of a facilitated diffusion transport protein in the gills of the toadfish. First, using northern blot analysis, Walsh (1997) and colleagues have detected a 2 kilobase mRNA which hybridizes with the cDNA for the rat vasopressin-regulated transporter (Smith *et al.* 1995). Using rt-PCR with degenerate primers based on the mammalian sequence, they have amplified a 484 base pair fragment with 65 % homology to the rat carrier at the amino acid level. Second, injection of physiological doses of arginine vasotocin (AVT) activates pulsatile urea-N excretion in the toadfish gill (S. F. Perry, unpublished results). This parallels the way in which AVT increases urea-N permeability *via* the facilitated diffusion transporter in the toad bladder and the way in which the mammalian homologue arginine vasopressin increases urea-N permeability *via* the transporter in the mammalian IMCD (see above). Third, ultrastructural studies of toadfish gill pavement cells have shown that, during both natural pulse events and AVT-induced pulses, vesicles emanating from the Golgi bodies appear to fuse with the apical membranes, and the normally smooth apical surface is thrown into ridges (P. Laurent, unpublished results). This is clearly reminiscent of the situation in the ICMD, where vasopressin treatment causes the insertion of vesicles containing the transport protein into the apical membranes (Nielsen *et al.* 1996). Finally, treatment of toadfish with colchicine, an inhibitor of microtubule assembly and therefore of vesicle traffic, completely blocks pulsatile urea-N excretion (K. M. Gilmour, unpublished results). Converging physiological, pharmacological, molecular and ultrastructural evidence therefore all strongly supports the facilitated diffusion hypothesis (Fig. 1C).

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