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PULSATILE UREA EXCRETION IN THE TOADFISH (*OPSANUS BETA*) IS DUE TO A PULSATILE EXCRETION MECHANISM, NOT A PULSATILE PRODUCTION MECHANISM

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Summary

When subjected to a crowding/confinement protocol in the laboratory, toadfish become facultatively ureotelic, excreting approximately 90 % of their nitrogenous waste as urea-nitrogen (urea-N). The great majority of this excretion occurs *via* large, irregular pulses from the head region which occur on average once per day, with a duration of 3 h or less. Pulses measured chemically by the appearance of urea-N in the external water were identical to those measured by assaying [^{14}C]urea appearance in the water from the blood plasma. Individual toadfish maintained plasma urea concentrations over widely differing ranges (6600–39 890 μmol-N l^{-1}). However, independent of absolute levels, both [^{14}C]urea and total urea were distributed at ratios close to unity between the blood plasma and the water compartments of liver and white muscle. At times of pulsatile excretion, plasma urea concentration fell sharply. These decreases, distributed throughout the tissues of the whole body, closely matched the sizes of the measured excretion pulses. Between pulses, plasma urea concentration increased steadily at a much slower rate; the rate of rise, when distributed throughout the tissues of the whole body, corresponded to the time-averaged excretion rate over the whole day. Infusion of a typical pulse amount of urea immediately after the end of a natural pulse event raised plasma urea concentration slightly above the pre-pulse level, but did not induce another pulse event. Plasma cortisol levels declined by approximately 60 % over the 4 h period prior to a natural pulse event and then rose quickly again once the pulse had occurred. These results indicate that urea pulses are due to activation of an excretion mechanism that rapidly clears urea from the blood plasma, thereby lowering stores throughout the whole body. Metabolic production of urea is continuous and is not responsible for pulsatile excretion. The pulse event is not triggered by a specific plasma urea threshold, but may involve the hypothalamo–interrenal axis.

Key words: urea, ureotelism, pulsatile excretion, toadfish, *Opsanus beta*, gills, liver, cortisol, nitrogen metabolism.

Introduction

Nitrogen metabolism in the gulf toadfish *Opsanus beta* is most unusual. Unlike almost all other teleosts, which are obligate ammonioteles and produce only small amounts of urea by uricolysis or arginolysis (Wood, 1993), the toadfish expresses a full complement of ornithine–urea cycle (OUC) enzymes in the liver (Read, 1971; Mommsen and Walsh, 1989). Although normally ammoniotelic, the toadfish becomes facultatively ureotelic under a variety of circumstances. For example, when subjected to crowding or close confinement in the laboratory, *O. beta* greatly reduces ammonia excretion while nitrogen excretion continues, albeit at a reduced level, almost entirely in the form of urea (Walsh *et al.* 1994*a*; Walsh and Milligan, 1995). This transition to ureotely appears to be mediated through the hypothalamo–interrenal 'stress' axis. Upon confinement/crowding, a transient surge in plasma cortisol level triggers an increase in hepatic glutamine synthetase (GNS) activity approximately 24 h later (Hopkins *et al.* 1995). Elevated GNS activity acts to trap ammonia, thereby reducing ammonia excretion, and serves to feed nitrogen from this source into the OUC (Walsh and Milligan, 1995; Anderson and Walsh, 1995).

Recently, we have shown that, under these circumstances, over 90 % of urea excretion occurs in pulses of less than 3 h duration (Wood *et al.* 1995*a*). Although irregular, on average these pulses occur approximately once per day. We have also demonstrated that the site of the pulsatile urea excretion is the

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head end of the fish, presumably the gills, in contrast to an earlier supposition that urea 'pulsing' occurred *via* periodic release of urine or rectal fluid (Walsh *et al.* 1990, 1994*a*; Griffith, 1991; Barber and Walsh, 1993). In separate studies, we detected the presence of a facilitated diffusion type of urea transporter in toadfish liver cells (Walsh *et al.* 1994*b*; Walsh and Wood, 1996). It is conceivable that this transport system may be important in moving urea rapidly from the production site to the blood plasma during pulse events. These observations have led directly to the present study on confined ureotelic toadfish, using fish fitted with chronic indwelling catheters for repetitive blood sampling and injections.

Our first objective was to determine whether this pulsatile urea excretion originated from the blood plasma compartment or at least from a compartment in equilibrium with it. To this end, we labelled plasma urea with [^{14}C]urea and compared chemically measured ('cold') urea pulses with those estimated from the simultaneous appearance of radioactivity in the external water. A second goal, facilitated by the use of [^{14}C]urea in these experiments, as well as by 'cold' urea measurements, was to evaluate the degree of equilibration of urea between key body compartments. Having established that the plasma compartment was the immediate source of urea pulses and was well equilibrated with other compartments, our third objective was to determine whether urea pulses reflected pulsatile metabolic production or pulsatile excretion. We hypothesized that if production were pulsatile, plasma urea levels would surge prior to and/or during pulses; however, if excretion were pulsatile in the face of constant metabolic production, plasma urea levels would fall rapidly during pulses and rise steadily between pulses. The latter hypothesis proved to be correct and led to our fourth objective, to determine whether the pulse event was triggered by a threshold level of urea in the blood plasma. To this end, we monitored toadfish until a natural pulse event occurred, and then immediately reinfused a sufficent dose of urea to raise the plasma concentration back to or slightly above the pre-pulse level. We reasoned that if the pulse event was triggered by a plasma threshold, another pulse should soon occur. Having established that this was not the case and that the pulse event was independent of absolute plasma urea levels, our final objective was to determine whether cortisol was involved in controlling the phenomenon. Measurements of plasma cortisol levels prior to and following natural pulse events point to an involvement of the hypothalamo–interrenal 'stress' axis in mediating pulses, in addition to its previously established role in the original transition to ureotelism (Hopkins *et al.* 1995).

Materials and methods

Experimental animals, holding conditions and cannulation

Sexually mature gulf toadfish (*Opsanus beta*, Goode and Bean; 90–203 g) were captured by roller trawl in Biscayne Bay, Florida, USA, in March 1995. In the laboratory, the fish were held in 45 l or 80 l aquaria supplied with a bed of beach sand and served with flowing, sand-filtered seawater from Biscayne Bay (20–24 °C; 31 ‰ salinity) under a natural photoperiod. Stocking density was less than 12 g fish l^{-1} seawater, and each fish was provided with an individual polyvinylchloride pipe for refuge. On days 1 and 3 of holding, 2 h static prophylactic treatments with Malachtite Green (0.05 mg l^{-1}) plus formalin (15 mg l^{-1}) were administered to eliminate potential infection by the ciliate *Cryptocaryon irritans*. Experiments were performed on days 6–12 on unfed fish.

A 'crowding/confinement' procedure with a stocking density greater than 80 g l^{-1} was used to induce ureotelism (see Walsh *et al.* 1994*a*; Hopkins *et al.* 1995). At 48–72 h prior to cannulation, groups of 3–6 toadfish, together with their individual polyvinylchloride shelters, were transferred to small 6 l plastic tubs (30 cm×25 cm×8 cm deep) again supplied with sand and flowing seawater. Following cannulation, fish were placed in individual covered plastic 3 l containers, fitted with polyvinylchloride shelters, aeration and flowing seawater, and allowed to recover for 24 h. This individual confinement is effective in maintaining ureotelic 'pulsing' behaviour in most fish (Wood *et al.* 1995*a*).

For surgery, fish were anaesthetized in MS-222 (0.67 g l^{-1} adjusted to pH 7.8 with NaOH). The caudal vertebrae were exposed by a 2 cm lateral incision between the epaxial and hypaxial muscle masses. The haemal arch was cannulated with Clay-Adams PE 50 polyethylene tubing filled with toadfish saline, 100 i.u. ml^{-1} sodium heparin, as described by Barber and Walsh (1993). The catheter was placed in the caudal artery in the majority of fish, but in a few the caudal vein was cannulated; the location of the catheter had no detectable influence on the results and the data were combined. The catheter was secured by means of a heat-flared PE 160 sleeve glued in place with cyanoacrylate tissue cement (Vetbond, 3M Corporation) and sutured at the site of exit from the muscle masses. The wound was dusted with oxytetracycline and closed with silk suture.

Experimental design

Following recovery, each fish 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 set to 2.0 l, the aeration set to provide good mixing, and a peristaltic pump plus fraction collector system started continuously to collect hourly water samples for urea and ammonia analyses. Throughout the subsequent experiment, the water was changed at 24 h intervals.

Following this initial screening, blood sampling commenced while automated hourly water sampling continued. Typically, approximately 20 blood samples (maximum 26) were drawn from each fish at 2 h intervals, with some breaks, over the next 2–4 days. Our goal was to monitor changes in blood composition accompanying natural pulse events. Haematocrits typically declined from 25–30 % to 10–15 %. Long catheters were used to ensure that the fish were not disturbed during sampling. For blood sampling, 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

clear the catheter and replace the sampled volume. Blood samples were centrifuged at 10 000 ***g*** for 2 min, and the plasma was divided into aliquots for subsequent analyses.

In the first series, at 3 h prior to the start of blood sampling, each fish was injected *via* the catheter with 100 μCi of [^{14}C]urea (Amersham, Arlington Heights, Illinois, 2.04 GBq mmol^{-1}) in 200 μl of saline, which was washed in with a further 200 μl of saline. In this series, plasma samples were analyzed for urea (2×10 μl) and ^{14}C radioactivity (1×10 μl), and water samples were analyzed for urea, ammonia and ^{14}C radioactivity. At the end of the experiment, a final blood sample was drawn, and then fish were rapidly killed by an overdose of neutralized MS-222 (3 g l^{-1}). Liver and white muscle samples were dissected out for ^{14}C radioactivity analyses.

In the second series, ^{14}[C]urea was not used, so plasma samples could be analyzed for cortisol (1×15 μl) in addition to urea (2×10 μl). Water samples were analyzed for urea and ammonia. Typically, the first 24 h of blood monitoring focused on describing the changes in plasma urea and cortisol levels during natural pulse events, whereas the remainder of the experiment focused on the question of whether the pulse event was triggered by a plasma urea threshold. To this end, hourly water samples were drawn by hand around the clock and analyzed immediately for urea during this part of the experiment. Once a natural pulse event had been detected and had finished, the fish was immediately injected with 2000 μmol-N kg^{-1} of urea, an amount equal to a typical natural pulse. The urea was delivered as a 3.125 ml kg^{-1} injection of 320 mmol l^{-1} urea (=640 mmol l^{-1} urea-N, iso-osmotic to plasma), washed in with 200 μl of saline. In control experiments, an equal total volume of toadfish saline alone was injected. Blood samples were drawn at 1 and 2 h after injection, and then at the regular 2 h intervals thereafter. At the end of the experiment, a final blood sample was drawn, the fish was rapidly killed as above, and terminal liver and white muscle samples were taken for 'cold' urea analyses.

Analytical techniques

Water and plasma samples were analyzed for urea and ^{14}C radioactivity either immediately or after storage at 4 °C for less than 24 h. Water samples for ammonia analysis and tissue samples for urea, [^{14}C]urea and water content analyses were frozen at −20 °C. Plasma samples for cortisol assay were frozen at −80 °C.

Standard chemical assays were employed for the analysis of urea in seawater and blood plasma (Price and Harrison, 1987) and ammonia in seawater (Ivancic and Deggobis, 1984). Tissue water content was determined by drying to a constant mass at 65 °C. Urea in tissue samples was determined by homogenizing approximately 1 g of wet tissue in 10 ml of ice-cold 8 % perchloric acid (PCA) and applying the same assay to the supernatant, using standards appropriately diluted in 8 % PCA. The amount of [^{14}C]urea radioactivity was determined by liquid scintillation counting of water (1 ml plus 10 ml of ICN Ecolume fluor) and plasma samples (10 μl in 1 ml of seawater plus 10 ml of fluor). Tissue samples (approximately 100 mg) for [^{14}C]urea radioactivity determinations were digested in 1 ml of NCS digest medium (Amersham), neutralized with 30 μl of glacial acetic acid, then counted in 10 ml of ICN Cytoscint fluor. A Beckman LS1801 counter with an inbuilt quench correction program was employed. In separate tests, small subsamples (N=3) of water and plasma from the experiments were treated with urease, and the $^{14}CO_2$ counts so generated were collected into hyamine hydroxide filter traps, as described by Wood and Perry (1991). These tests confirmed that more than 95 % of the ^{14}C counts were urea.

Cortisol was measured using a commercial ^{125}I radioimmunoassay kit (ICN Immuno Corporation) with standards diluted to the same protein range as toadfish plasma; a Canberra-Packard Minaxi gamma counter was used.

Calculations

All urea and ammonia concentrations and fluxes have been expressed in units of nitrogen for comparative purposes. 'Pulses' of urea-N excretion were identified from step-wise increases in water urea concentration seen in hourly samples taken by the fraction collector system or manually (see Wood *et al.* 1995*a*). Excretion rates (in μmol-N kg^{-1} h^{-1}) of urea-N and ammonia-N were calculated from changes in concentration in the water (μmol-N l^{-1}) multiplied by the volume (l) and factored by time (h) and mass (kg).

In experiments which tested whether the blood plasma was the compartment of origin for urea pulses, excretion events quantified in the traditional way by chemical assay were compared with values calculated from simultaneous measurements of ^{14}[C]urea appearance in the external water. For these calculations, the total radioactivity (cts min^{-1}) appearing in the water was divided by the measured specific activity (cts min^{-1} μmol^{-1} urea-N) of the plasma sample immediately preceding the pulse event.

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 assessed. The significance of differences between means was evaluated using Student's two-tailed t-test, unpaired or paired as appropriate. For multiple comparisons, the t value was adjusted *via* the Bonferroni procedure. A fiducial level of $P \leqslant 0.05$ was employed throughout.

Results

Degree of ureotely

In the initial monitoring period prior to the start of blood sampling, the toadfish (N=16) used in this study were predominantly ureotelic, excreting 94 % urea-N (127.6±33.6 μmol-N kg^{-1} h^{-1}) and only 6 % ammonia-N (8.6±2.3 μmol-N kg^{-1} h^{-1}) when averaged over periods of 24–48 h. Of the total urea-N excretion, 86.3±4.1 % occurred in discrete pulse events of 3 h duration or less. Mean urea-N pulse

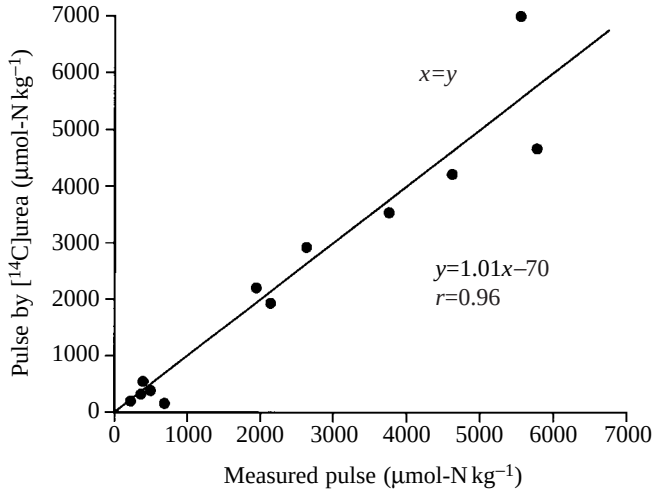

Fig. 1. The relationship between the size of individual urea-N pulses measured chemically from the appearance of urea in the external water (*x*-axis) and the size calculated from simultaneous determinations of the appearance of ^{14}[C]urea radioactivity in the water (*y*-axis). The latter values were calculated using the measured plasma specific activity immediately preceding the pulse event. *x=y* represents the line of equality; the equation for the actual regression line is also given, which was significant at $P \leqslant 0.001$.

size was 2294.1±435.0 μmol-N kg^{-1} and mean frequency was 1.10±0.18 pulses day^{-1}. Ammonia-N excretion was never pulsatile.

During the longer period (72–96 h) of blood sampling, these conditions did not change; urea-N excretion again accounted for 94 % (123.8±29.8 μmol-N kg^{-1} h^{-1}), while ammonia-N excretion accounted for only 6 % (7.3±2.5 μmol-N kg^{-1} h^{-1}) of total nitrogen excretion. Pulses represented 93.5±1.6 % of total urea-N excretion, with a mean size of 2327.9±402.9 μmol-N kg^{-1} and a mean frequency of 1.11±0.16 pulses day^{-1}.

Origin of urea pulses

Twelve pulse events in eight toadfish were monitored simultaneously by chemical assay and by appearance of ^{14}[C]urea radioactivity in the external water. As illustrated in Fig. 1, over a wide range of absolute pulse sizes, the two types of measurements were in 1:1 agreement. The calculation of pulse size by [^{14}C]urea appearance employed the measured plasma specific activity, so the urea pulses clearly originate from the blood plasma or from a compartment in equilibrium with plasma.

Equilibration of urea between body compartments

Terminal plasma urea-N levels varied greatly among individual toadfish (absolute range 6600–39 890 μmol-N l^{-1}), averaging 19 220±2400 μmol-N l^{-1} (N=16) overall. However, within each individual, levels tended to vary by no more than 25 % over time.

Terminal measurements of plasma and tissue ^{14}C radioactivity revealed excellent equilibration of urea-N between the water compartments of plasma, liver and white muscle, with distribution ratios close to unity (Table 1). In the second series, this conclusion was reinforced by measurements of 'cold' urea-N concentrations, which were approximately equal in the three compartments in individual fish, again leading to distribution ratios close to unity (Table 1). However, there were minor but significant deviations above 1.0 for ^{14}C radioactivity in white muscle and for 'cold' urea-N in liver, the importance of which is unclear.

Table 1. *Distribution ratios of [^{14}C]urea and total urea between plasma and liver or white muscle tissue water compartments in individual toadfish*

	Liver	White muscle
$\frac{\text{cts min}^{-1}\,[^{14}\text{C}]\text{urea l}^{-1}\text{ tissue water}}{\text{cts min}^{-1}\,[^{14}\text{C}]\text{urea l}^{-1}\text{ plasma water}}$	1.044±0.062	1.168±0.045*
$\frac{\mu\text{mol urea-N l}^{-1}\text{ tissue water}}{\mu\text{mol urea-N l}^{-1}\text{ plasma water}}$	1.248±0.068*	1.061±0.088
Tissue water content (%)	80.39±0.66	80.59±0.83

Values are means ± S.E.M. (N=8).
*Significantly different (P<0.05) from 1.0.

Pulsatile production or pulsatile excretion

Serial measurements of plasma urea-N levels revealed sharp drops accompanying natural pulse events, followed by slow increases between events. Fig. 2 illustrates a typical example. Fig. 3A summarizes the overall pattern using those events where continuous plasma data were available for at least 4 h

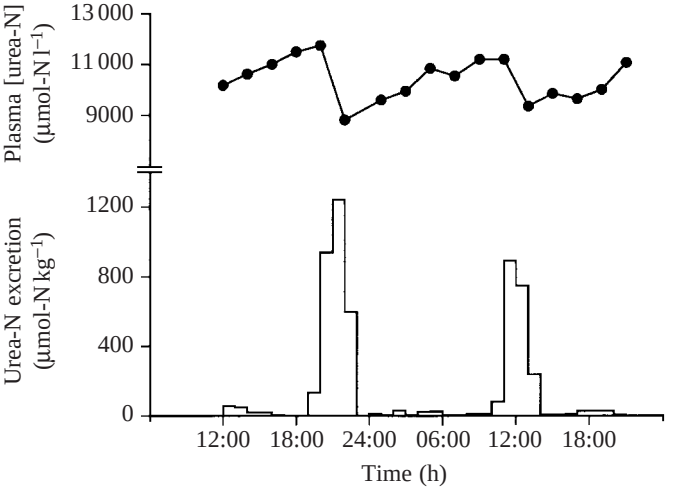

Fig. 2. Typical example of the changes in plasma urea-N concentration accompanying two natural pulsatile excretion events over a 48 h period in an individual toadfish. Note the sharp drop in plasma urea-N level coincident with each event and the slower rise between events.

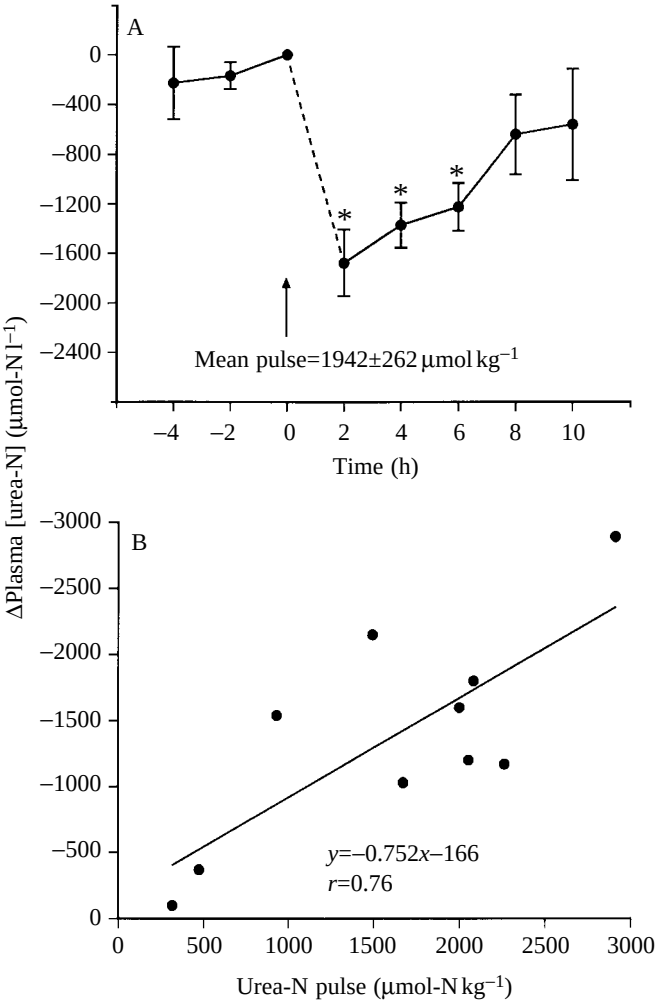

Fig. 3. (A) Changes in plasma urea-N levels for 4 h prior to and 10 h following natural pulsatile excretion events. Values are expressed as differences from the plasma value immediately preceding the pulse event (taken as 0 at time 0 h). Asterisks represent significant differences ($P \leqslant 0.05$) from this reference value (N=6 pulses in five fish). Means ±1 S.E.M. (B) The relationship between the measured drop in plasma urea-N and the size of individual excretion pulses. The fitted regression is significant at $P \leqslant 0.01$ (N=10 pulses in seven fish).

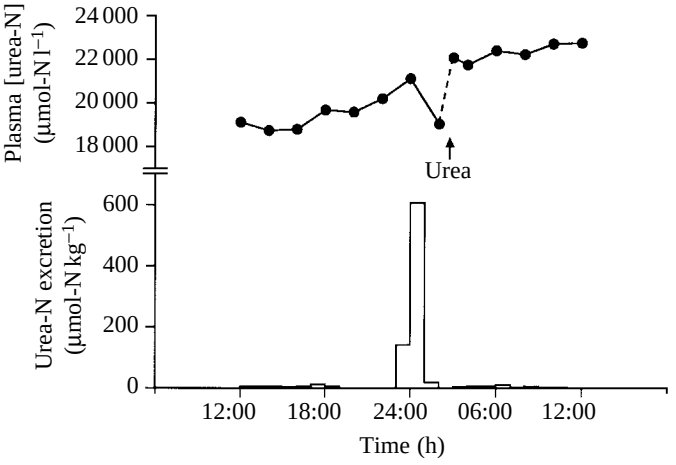

Fig. 4. Typical example of the response to injection (at the arrow) of urea (2000 μmol-N kg^{-1}) immediately after a natural pulse event in an individual toadfish. Note the rapid restoration of plasma urea-N concentration to slightly above the pre-pulse level and the absence of any pulsatile excretion after the injection.

prior to and 10 h after the pulse (N=6 pulses from five fish). The mean decrease in plasma urea concentration (1677±268 μmol-N l^{-1}) was not significantly different from the measured pulse size (1942±262 μmol-N kg^{-1}). Fig. 3B includes several additional data points where the 4 h pre-pulse criterion was not met but the fall in plasma urea-N was still quantified (N=10 pulses from seven fish). Over a wide range of pulse sizes, there was a clear positive relationship (r=0.76, $P \leqslant 0.01$) between the drop in plasma urea-N concentration and size of the measured excretion pulse. On average, the fall in plasma urea (per l) was approximately 75 % of the measured pulse (per kg fish).

Using this same data set (N=10 pulses from seven fish), the mean rate of increase in plasma urea-N concentration over the 10 h following the pulse was 118.4±48.1 μmol-N l^{-1} h^{-1}. This value was very similar to the time-averaged overall urea-N excretion rate of 123.8±29.8 μmol-N kg^{-1} h^{-1} (N=16 fish) reported above.

These results clearly indicate that the pulse event represents pulsatile excretion rather than pulsatile production. When taken together with the data of Table 1 showing equilibrium of plasma urea with the largest body compartment (white muscle) and the major production site (liver), the data suggest that steady metabolic production gradually increases plasma and tissue stores between pulse events. Activation of the excretion mechanism quickly clears urea-N from the plasma, thereby decreasing plasma and tissue stores.

Plasma urea threshold

Injection of a typical pulse amount of urea-N (2000 μmol-N kg^{-1}) immediately after a natural pulse raised plasma urea-N concentrations to slightly above the pre-pulse level, but did not stimulate another pulse event. Fig. 4 illustrates a typical example. Plasma urea-N levels (Fig. 5) and excretion (Fig. 6) were monitored for 8 h after the injection of either a dose of urea (N=6 different fish) or an equivalent volume of saline (N=6 different fish), as well as in the non-injected fish described in the previous section. On average, the injection of 2000 μmol-N kg^{-1} significantly raised plasma urea-N levels by approximately 2500 μmol-N l^{-1}, measured 1 h after injection (Fig. 5). This elevation was maintained over the following 8 h. Saline infusion caused a small but significant decrease in plasma urea-N at 1 h, presumably a dilution effect. In all three groups, plasma levels increased gradually with time, reflecting the steady rate of production. In none of the groups was there any pulsatile urea-N excretion over the following 8 h (Fig. 6). These results show clearly that the pulse event is not directly triggered by a plasma urea-N threshold.

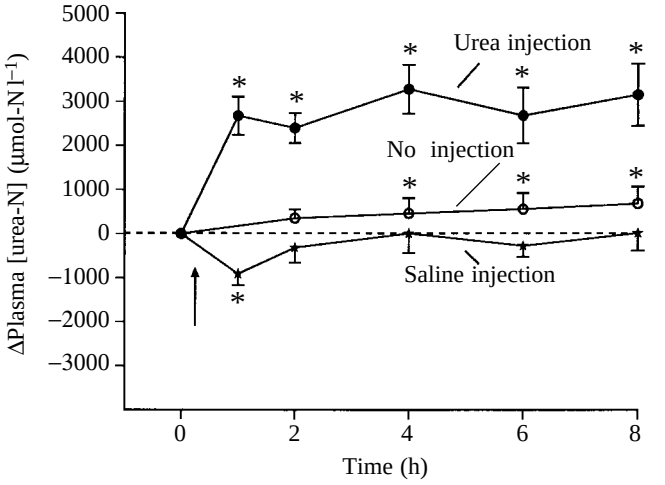

Fig. 5. Changes in plasma urea-N concentrations over the 8 h period following the end of a natural pulse event in toadfish injected (at the arrow) with either 2000 μmol-N kg^{-1} of urea-N (*N*=6 fish) or the same total volume of saline only (3.125 ml kg^{-1} plus wash-in; *N*=6 fish), or not injected (*N*=10 pulses in seven fish). Values are expressed as differences from the plasma value immediately following the pulse event, prior to injection (taken as 0 and plotted at time 0 h). Asterisks represent significant differences ($P \leq 0.05$) from this reference value. Means ±1 S.E.M.

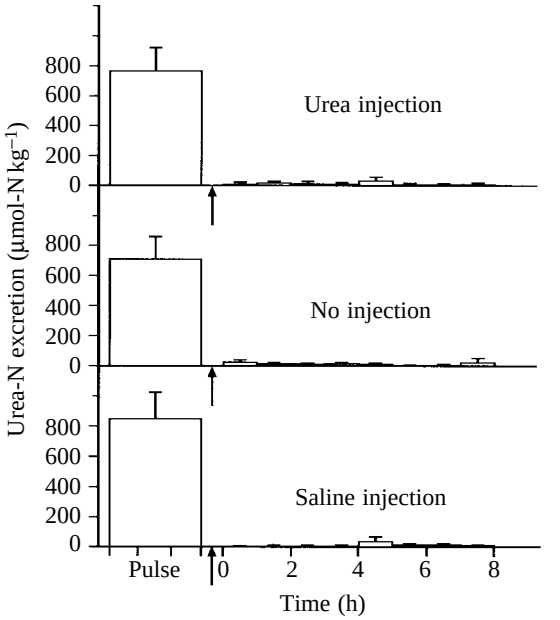

Fig. 6. Urea-N excretion, plotted at hourly intervals, in the 8 h period after the end of a natural pulse event in toadfish injected (at the arrow) with either 2000 μmol-N kg^{-1} of urea-N (*N*=6 fish) or the same total volume of saline only (3.125 ml kg^{-1} plus wash-in; *N*=6 fish), or not injected (*N*=10 pulses in seven fish). The pulse event itself is shown as an average for 3 h (e.g. 800 μmol kg^{-1} for 3 h would represent a total pulse size of 2400 μmol kg^{-1}). Note the complete lack of pulse events in the post-injection period. Means +1 S.E.M.

Cortisol

Between pulses, plasma cortisol levels were typically in the range 80–120 ng ml^{-1}. However, in all fish, there was a consistent fall in plasma cortisol levels prior to the pulse event. Fig. 7 combines data on cortisol levels from *N*=8–11 pulse events in eight fish for the 6 h prior to and 10 h after natural pulses. This summary illustrates that plasma cortisol level declined significantly by approximately 60 % over the 4 h period prior to a pulse and then rose quickly again once the pulse had occurred. The same pattern of a post-pulse rise in plasma cortisol level (data not shown) was seen in fish injected with 2000 μmol-N kg^{-1} (*N*=6) or saline (*N*=5). These observations suggest some association between variations in plasma cortisol level and pulsatile excretion events.

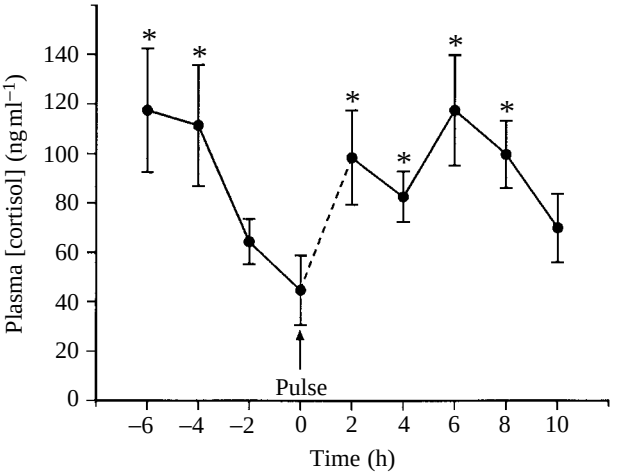

Fig. 7. Changes in plasma cortisol concentrations over the 6 h period prior to and the 10 h period following a natural pulse event (*N*=8–11 pulses in eight fish). Note the fall in cortisol levels prior to the pulse and the rapid rise thereafter. Asterisks represent significant differences ($P \leq 0.05$) from the 0 h value. Means ± 1 S.E.M.

Discussion

Our earlier study (Wood *et al.* 1995*a*) first demonstrated the unusual phenomenon of pulsatile urea-N excretion from the head end of confined toadfish. The present experiments were conducted on a separate batch of toadfish, collected a year later. However, rates and patterns of urea-N excretion in the present ureotelic toadfish were essentially identical in terms of overall excretion rate (approximately 125 μmol-N kg^{-1} h^{-1}), pulse size (approximately 2300 μmol-N kg^{-1}), pulse frequency (approximately 1.1 day^{-1}), percentage of total urea excretion occurring in pulses (approximately 90 %) and percentage of total-N excretion as urea (approximately 94 %). The blenny *Blennius pholis* also appears to release urea-N in a semipulsatile fashion, but these pulses are smaller and of greater frequency (minutes to hours), and larger ammonia-N pulses occur simultaneously (Sayer, 1988). To our knowledge, this pulsatile phenomenon has not been detected in any other teleost, but a cautionary note is that most 'standard' experimental protocols, involving intermittent water sampling

over long intervals, would probably not detect pulsatile excretion, especially if the frequency was high.

The present study has demonstrated that the pulse event really does represent a pulsatile excretion mechanism and not a pulsatile production mechanism. Plasma and body urea-N stores build up gradually over time as production continues at a steady rate and then are rapidly lowered once the excretion mechanism is activated. The liver, which contains a full complement of OUC enzymes (Read, 1971; Mommsen and Walsh, 1989), is probably the major site of urea-N production. Hepatocyte urea-N production rates measured *in vitro* (Barber and Walsh, 1993; Walsh and Wood, 1996) are more than sufficient to explain the present whole-animal rates when scaled through a typical hepato-somatic index. Nevertheless, the possible contribution of other tissues such as gills, kidney and intestine which also express OUC enzymes, albeit at lower levels (Wood *et al.* 1995*a*), cannot be eliminated.

Earlier, we had speculated that the presence of a facilitated diffusion carrier in hepatocyte cell membranes (Walsh *et al.* 1994*b*; Walsh and Wood, 1996) served to facilitate rapid efflux of urea to the blood at times of pulsatile production (Wood *et al.* 1995*a*). The present results demonstrate that this is clearly not the case. Instead, the presence of the carrier apparently ensures that urea steadily equilibrates into the plasma as it is produced, so that concentration gradients between the major production site and the immediate 'reservoir' for excretion (the plasma compartment) remain small or negligible.

The present results also demonstrate that plasma urea levels are broadly representative of whole-body levels during both the slow rise between pulse events and the rapid decreases accompanying pulse events. The white muscle, accounting for approximately 60 % of the body mass (C. J. Kennedy and P. J. Walsh, unpublished data), is the largest storage compartment, and urea distribution ratios were always close to unity between muscle water and plasma water. We speculate, therefore, that the same facilitated diffusion carrier may be present in white muscle cell membranes and, indeed, other tissues of *O. beta*.

How does the pulsatile excretion mechanism work? At times of pulse events, urea is rapidly cleared from the blood plasma, whereas in the intervening periods urea excretion is close to zero. In recent years, it has been recognized that true urea permeability through cell membranes may in fact be quite low, in contrast to the standard textbook view of high urea permeability across lipid bilayers. High urea permeabilities often reflect the presence of specific urea transporters (Marsh and Knepper, 1992; Gillin and Sands, 1993). At least two general families of urea carriers have now been identified, the facilitated diffusion type (Knepper and Star, 1990) and a Na^+-dependent secondary active transport process (Isozaki *et al.* 1994). The urea carrier present in toadfish hepatocytes appears to belong to the former type (Walsh and Wood, 1996). With this background in mind, several possible models can be proposed.

The first involves the presence of Na^+-dependent secondary active transport carriers for urea at the excretion site (probably the gills/skin of the head region). If this model is correct, such carriers would probably be located on basolateral cell membranes and would normally prevent excretion by actively 'reabsorbing' urea from the cytosol as it moved out from the blood across the basolateral membrane by passive diffusion. Periodic inactivation of these inwardly directed transporters would result in pulsatile excretion events. Such an active transport system for urea reabsorption has been identified in the inner medullary collecting duct (IMCD) of mammalian kidney (Isozaki *et al.* 1994) and appears to be similar to an active transport system reported previously in elasmobranch kidney (Schmidt-Nielsen and Rabinowitz, 1964; Schmidt-Nielsen *et al.* 1972; Hays *et al.* 1977). Some indirect evidence for an analogous system in elasmobranch gills, which are similarly 'impermeable' to urea, has been gathered recently (Wood *et al.* 1995*b*; Pärt *et al.* 1997).

The second model proposes that facilitated diffusion carriers of the type seen in hepatocytes are normally absent or inactive at the excretion site in toadfish, resulting in very low urea permeability. Periodic activation of nascent transporters, or insertion of new transporters into cell membranes, would result in pulsatile excretion. Such a system is also present in the IMCD of mammalian kidney and can be activated hormonally in a matter of minutes (Knepper and Star, 1990; Marsh and Knepper, 1992).

Other possibilities include a combination of these two models, which are not mutually exclusive, or something entirely different. The latter could include periodic sloughing or bursting of urea-rich cells or granules at the excretory surfaces, periodic diversion of blood flow to a specific secretory site in the head region, or periodic general increases in gill permeability by the opening of transcellular pores or paracellular junctions. All of these possibilities can be addressed in future work by a combination of physiological, morphological, pharmacological and molecular techniques. For example, the Na^+-dependent secondary active urea transporter and the facilitated diffusion urea transporter of mammalian IMCD exhibit very different pharmacologies (Isozaki *et al.* 1994), and the latter has now been cloned (You *et al.* 1993).

The present urea infusion experiments have demonstrated that the pulsatile excretion event is not triggered by a specific threshold concentration of urea in the blood plasma. This is further supported by the observation that individual toadfish have 'normal' plasma urea-N levels ranging from 6600 to 39 890 μmol-N l^{-1}. What then triggers the event? The marked decrease in plasma cortisol concentration prior to a pulse and the sharp rise thereafter suggest some involvement of the hypothalamo–interrenal axis, although this may not necessarily be directly causative. In the mammalian kidney, for example, glucocorticoids play a role in regulating urea reabsorption, but other hormones such as glucagon and arginine vasopressin (AVP) are also involved (Marsh and Knepper, 1992). In particular, AVP directly activates the facilitated diffusion transporter of the IMCD (Knepper and Star, 1990). In the toadfish, the drop in cortisol level could be a necessary requirement for the action of another hormone or

neurotransmitter, such as arginine vasotocin (AVT), the teleostean homologue of AVP. Alternatively, the drop in cortisol level could merely be correlated with behavioural cues that allow a pulse to occur. There is a clear need for more detailed endocrinological and behavioural studies on this phenomenon.

Finally, it should be noted that the absolute levels of cortisol monitored in the periods between pulses (typically 80–120 ng ml^{-1}) in the ureotelic fish used in the present study were much higher (3–5 times) than in our previous study, and indeed were in the range that we had previously considered 'pathological' (Hopkins *et al.* 1995). However, this was clearly not the case as all fish remained healthy throughout the present experiments. The most obvious difference between the two studies is that the fish used in the present study were repetitively sampled by cannulation, whereas in the earlier study the fish were sampled only once by terminal anaesthesia and caudal puncture. In rainbow trout, repetitive blood sampling by cannulation similarly elevates plasma cortisol levels (Bry, 1982; Brown *et al.* 1986). With respect to urea pulsing, the significance of this discrepancy is that cortisol turnover rates may be more important than absolute levels of cortisol.

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