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ABSTRACT

Gulf toadfish, *Opsanus beta,* are facultatively ureotelic and can excrete the majority of their nitrogenous waste as urea. Urea excretion occurs in "pulses." The hypothesis that pulsatile urea excretion reflects sudden, transient, generalized increases in the branchial conductance was investigated by the simultaneous monitoring of cardiorespiratory variables, oxygen uptake, and whole-body urea, ammonia, and/or ^3H_2O effluxes. The direct monitoring of both expired branchial water and water exiting a respirometer demonstrated that urea pulses were derived from the gills. No significant changes in ventilation or cardiac frequency, oxygen uptake, or ammonia efflux were observed during natural urea pulses, refuting the hypothesis that pulsatile urea excretion reflects pulsatile increases in the generalized diffusive properties of the gill for solute transfer. An alternative model for pulsatile urea excretion postulates that the gill urea permeability is increased periodically by the insertion and/or activation of specific urea transporters into gill cell membranes. Pulsatile urea excretion was abolished by pretreatment with the cytoskeletal-disrupting agent colchicine; colchicine may block trafficking of urea transporter–containing vesicles. Exocytosis of water following the fusion of vesicles with gill cell membranes could explain the significantly elevated ^3H_2O efflux observed during urea pulses.

Introduction

The gulf toadfish, *Opsanus beta,* is unusual among teleost fish in being both facultatively ureotelic and ureogenic. In contrast to most teleost fish, which are obligate ammonioteles (see reviews by Mommsen and Walsh [1992], Wood [1993], and Wright [1995]), the toadfish switches to ureotelism in response to moderate stresses in the laboratory, such as crowding or confinement, excreting the majority of its nitrogenous waste as urea (Walsh et al. 1990, 1994*a*). While the extent to which urea excretion occurs in the wild remains uncertain, the results of a recent field study suggest that toadfish in a subtidal environment may excrete a substantial portion of their waste nitrogen (perhaps >50%) as urea (Hopkins et al. 1997). The toadfish also expresses a full complement of ornithine-urea-cycle enzymes in the liver and synthesizes urea via the ornithine-urea cycle (Mommsen and Walsh 1989; Anderson and Walsh 1995), whereas the small amount of urea excreted by ammoniotelic teleosts is produced by uricolysis or arginolysis (Wood 1993).

An additional, unusual aspect of nitrogenous waste excretion in the gulf toadfish is the discharge of urea in a pulsatile fashion (Walsh et al. 1990; Barber and Walsh 1993); over 90% of the urea excreted has been found to be released in a single "pulse" of less than 3-h duration, approximately once in 24 h (Wood et al. 1995, 1997, 1998). The site of the urea pulse has been localized to the anterior end of the toadfish, probably the gills and/or skin of the head (Wood et al. 1995), eliminating periodic release of urine or rectal fluid as the source of urea pulses (Walsh et al. 1990; Griffith 1991; Barber and Walsh 1993). Recently, Wood et al. (1997) demonstrated that urea pulse

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events in the toadfish are the result of a pulsatile excretion mechanism and not a pulsatile urea-production mechanism. Urea is continuously synthesized in the liver via the ornithine-urea cycle and, owing to the presence of a facilitated diffusion carrier in hepatocyte cell membranes (Walsh et al. 1994*b;* Walsh and Wood 1996), steadily equilibrates into the plasma, such that plasma and body urea stores gradually increase over time (Wood et al. 1997). On activation of the excretion mechanism, the plasma and body urea stores are rapidly lowered.

The nature, and precise location, of the pulsatile urea-excretion mechanism are as yet unclear. One possibility is that pulsatile urea excretion is the result of periodic, sudden increases in the rate of general solute transfer across the gill. Solute transfer across the gill is determined by the convective factors of ventilation and perfusion as well as the diffusive conductance, which is a function of the gill surface area, the diffusion distance, and the permeability of the gill to the solute in question. Periodic, transient changes in one or more of these factors could constitute the mechanism underlying pulsatile urea excretion in the toadfish. Thus, one objective of the present study was to test the hypothesis that pulsatile urea excretion reflects sporadic, generalized increases in gill conductance, that is, a relatively nonspecific effect whereby transfer across the gill is increased not just for urea but for a wide range of other molecules as well. This objective was accomplished by monitoring cardiovascular and ventilatory variables together with oxygen uptake and nitrogen excretion in ureotelic toadfish. A second, related aim, entailed distinguishing between the gills and the skin of the head as the potential site of urea excretion.

Given the low true permeability of cell membranes to urea (Marsh and Knepper 1992; Gillin and Sands 1993), an alternative model for pulsatile urea excretion in the toadfish could involve the periodic insertion and/or activation of specific urea transporters into cell membranes at the excretion site (Walsh 1997; Wood et al. 1997, 1998). For example, a vasopressin-sensitive urea transporter is a key component of the urinary-concentrating mechanism in the mammalian kidney (You et al. 1993), and a similar neurohypophyseal hormone-dependent urea transporter may also exist in the amphibian bladder (Bentley 1987). The nature of this urea transporter, that is, whether it is a channel or a carrier, remains unclear (Sands et al. 1997). Vasopressin-induced increases in urea transport in the collecting duct are thought to be mediated by insertion of urea transporter-containing vesicles into the apical cell membrane (Nielsen et al. 1996). Thus, the third objective of the present study involved examining the potential contribution of vesicle trafficking to pulsatile urea excretion in the toadfish using the cytoskeletal-disrupting agent, colchicine.

The factor or factors triggering the activation of the pulsatile urea-excretion mechanism in toadfish also remain unclear (Walsh 1997; Wood et al. 1997), and therefore the final objective of the present study was to carry out an investigation of the potential involvement of behavioral cues in triggering urea pulses.

Material and Methods

Experimental Animals

Sexually mature gulf toadfish, *Opsanus beta,* were captured with a roller trawl by commercial shrimpers in Biscayne Bay, Florida, in May 1996. In the laboratory, all fish were initially held under noncrowded conditions in 45-L aquariums supplied with a sand substrate, several polyvinylchloride tubes as refuges, and flowing, aerated, sand-filtered seawater from Biscayne Bay (25° ± 1°C; 29‰–34‰ salinity). Stocking density was 3–4 fish per aquarium, or 7–10 g fish L^{-1} seawater. Prophylactic treatments with a combination of malachite green (0.05 mg L^{-1}) and formalin (15 mg L^{-1}) were administered on days 1 and 3 of laboratory holding against the ciliate *Cryptocaryon irritans* (Wood et al. 1995). Toadfish were fed ad lib. with live shrimp on alternate days and were maintained under these conditions for at least 1 wk.

A standardized crowded treatment was used to induce ureotelism (Walsh et al. 1994*a;* Hopkins et al. 1995; Wood et al. 1995). Toadfish (54–230 g, $N = 36$), together with their individual shelters, were placed in 6-L plastic tubs at densities exceeding 80 g fish L^{-1} seawater (i.e., three to six fish per tub) for 48–72 h before experimentation. Water flow and aeration were maintained during crowding, but fish were not fed. Following the crowding protocol, fish were either transferred directly into individual covered plastic 3-L containers for experimentation (for behavioral observations) or were first fitted with catheters for blood and/or water sampling and then placed in individual containers (for respirometry and the colchicine experiment).

For surgery, fish were anesthetized in a solution of MS-222 (0.67 g L^{-1}), adjusted to pH 8.0 with NaOH, and then transferred to an operating table where anesthesia was maintained for the duration of the surgery (usually less than 30 min) by wrapping the fish in paper towels soaked with anesthetic solution. Toadfish are tolerant of air exposure, and it was not necessary to irrigate the gills during surgery. The haemal arch was cannulated with polyethylene tubing (Clay Adams PE 50) as described by Wood et al. (1997). In the majority of fish, the cannula was placed in the caudal artery. For the few fish cannulated in the caudal vein, cardiovascular variables could not be monitored, but the location of the cannula had no other discernible influence on the results, and the data were combined (see also Wood et al. 1997). For respirometry, fish were additionally fitted with catheters (PE 160) inserted into each gill pouch and secured with ligatures for the collection of expired water and, in some cases, the monitoring of ventilation. Finally, small diameter plastic-coated wires (28–30 A.W.G.) were sutured to each operculum for the monitoring of ventilation by the measurement of impedance changes associated with movements of the opercula.

Following surgery, fish for the colchicine experiment were placed in individual covered 3-L plastic containers supplied

with polyvinylchloride shelters, aeration, and flowing seawater, while for respirometry experiments, toadfish were transferred to individual respirometers (see below) supplied with aerated, flowing seawater. Spontaneous breathing recommenced within a few minutes of reimmersion, and fish were allowed to recover for 24 h before experimentation.

Experimental Design—Respirometry

The basic protocol for respirometry experiments involved monitoring cardiorespiratory variables (arterial blood pressure, cardiac frequency, ventilation frequency, ventilation amplitude, and oxygen uptake [$\dot{M}O_2$]), nitrogenous waste excretion (urea-N and ammonia-N), and tritiated water efflux for toadfish ($N = 20$) held undisturbed in the respirometers for extended periods of time (5–24 h). Respirometers were manufactured from either 500- or 1,000-mL plastic graduated cylinders. The ends of the graduated cylinder were removed to leave cylinders of either 400 or 800 mL volume, a baffle was glued a few centimeters from the inflow end of the respirometer, and plastic stoppers fitted with either an inflow or an exit tube were used to render the respirometer watertight. A small hole was made approximately halfway along the length of the respirometer for the passage of the cannulae and impedance leads. Respirometers were supplied with flowing, aerated seawater from a head tank, and the rate of flow of water through the respirometer could be adjusted as required; during the recovery period, a flow rate of at least 100 mL min^{-1} was used. Before initiating an experiment, the water flow to the respirometer was adjusted to the mass of the fish to achieve a measurable inflow-outflow O_2 partial pressure (PO_2) difference (see below); flow rates during respirometry experiments ranged from 49.5 to 210 mL min^{-1}. Respirometers were covered with black plastic during experiments to minimize disturbance to the fish.

Cardiorespiratory Variables. Arterial blood pressure was measured by connecting the saline-filled caudal artery cannula to a pressure transducer (Model 1050BP, BioPac Systems, St. Laurent) that, in turn, was linked to a data acquisition system (BioPac Systems) and computer. In a few fish, a second pressure transducer (Model EM751, Elcomatic with a Harvard Transducer Amplifier) was used to monitor ventilation by measuring the pressure changes in the gill pouches associated with the breathing cycle, using a seawater-filled catheter attached to the operculum. The pressure transducers were calibrated against a static column of water. Ventilation in most fish was assessed using an impedance converter (Model 2991, Transmed Scientific, San Luis Obispo, Calif.) to measure the changes in impedance between the two wire leads sutured to the opercula. The output of the impedance converter was collected by the data acquisition system. Cardiac frequency and ventilation frequency were determined automatically using a rate function feature of the data acquisition system; ventilation amplitude data were not compiled because difficulties were experienced in obtaining amplitude recordings that were stable over the course of an entire respirometry experiment (5–24 h).

$\dot{M}O_2$ was determined using flow-through respirometry. The PO_2 of water flowing into the respirometer was measured continuously by siphoning water from the inflow tube into a thermostatted cuvette containing a PO_2 electrode (Model E-5046, Radiometer, Copenhagen) linked to a double O_2 meter (Cameron Instruments, Port Aransas, Tex.) and the data acquisition system. Similarly, the PO_2 of water exiting the respirometer was measured continuously by siphoning water from a point near the outflow tube into a second, temperature-controlled measuring cell housing a second, identical PO_2 electrode connected to the double O_2 meter. For some fish, the PO_2 of expired water was determined by siphoning water from one of the gill-pouch catheters into a third thermostatted cuvette containing a third, identical PO_2 electrode, which was connected to a second O_2 meter (Cameron Instruments) linked to the data acquisition system. Calibration of the PO_2 electrodes was carried out using air-equilibrated seawater. $\dot{M}O_2$ (mmol kg^{-1} h^{-1}) was then calculated as $\dot{M}O_2 = \alpha O_2 \times \text{flow} \times (P_{in}O_2 - P_{out}O_2)/\text{mass}$, where $P_{in}O_2$ and $P_{out}O_2$ are the PO_2 values of water flowing into and out of the respirometer, respectively, αO_2 is the solubility coefficient (mmol L^{-1} $Torr^{-1}$, where 1 Torr = 133.322 Pa) of O_2 in seawater at 25°C (Boutilier et al. 1984), "flow" was the water-flow rate through the respirometer (L h^{-1}), and "mass" was the mass of the fish (kg). Where the PO_2 of expired water (P_eO_2) was also measured, ventilation volume ($\dot{V}w$; mL min^{-1} kg^{-1}) was calculated according to the Fick equation: $\dot{V}w = 1{,}000 \times \dot{M}O_2/[60 \times \alpha O_2(P_{in}O_2 - P_eO_2)]$.

Nitrogen Excretion. Urea and ammonia excretion were assessed by measuring the urea and ammonia levels in water flowing into and out of the respirometer. Outflowing and expired water were sampled continuously with a peristaltic pump and fraction collector system set to collect half-hourly samples. Samples of inflowing water were collected periodically by hand. Approximately 2.5 h before initiating a respirometry experiment, fish were injected via the arterial cannula with 11.1–14.8 MBq kg^{-1} of [^{14}C]urea (Du Pont New England Nuclear, Boston; specific activity, 318 MBq $mmol^{-1}$) in 200 μL toadfish saline. Wood et al. (1997) demonstrated that under these conditions, the appearance of ^{14}C activity in the external water provides an accurate measure of urea excretion. All water samples were analyzed for ^{14}C radioactivity and total ammonia ($NH_3 + NH_4^+$). Urea excretion (μmol N kg^{-1} h^{-1}) was calculated from the ^{14}C activity in the water sample (cpm mL^{-1}) multiplied by the rate of water flow through the respirometer (mL h^{-1}) and factored by the fish mass (kg) and the plasma urea specific activity, which was determined from the urea concentration (μmol N mL^{-1}) and ^{14}C activity (cpm mL^{-1}) of a plasma sample. Similarly, ammonia excretion (μmol N kg^{-1} h^{-1}) was calculated from the difference in total ammonia con-

centration between the outflowing and inflowing water samples (μmol N L^{-1}) multiplied by the rate of water flow through the respirometer (L h^{-1}) and factored by the fish mass (kg). For the few fish for which estimates of ventilation volume were obtained, urea excretion based on expired water samples was calculated, in addition to the standard determination of urea excretion from the outflowing water samples, using an analogous equation in which the rate of water flow through the respirometer was replaced with ventilation volume. Urea pulses were identified on the basis of the measurement in a sample of outflowing water of a ^{14}C count that was at least double the baseline value.

Tritiated Water Excretion. In several experiments, fish were additionally injected with 22.2–29.6 MBq kg^{-1} of $[^3H]H_2O$ (Du Pont New England Nuclear; specific activity, 37 MBq g^{-1}). The rate of tritiated water efflux (mol kg^{-1} h^{-1}) was calculated from the ^3H activity (cpm mL^{-1}) in the water sample multiplied by the rate of water flow through the respirometer (mL h^{-1}) and factored by the fish mass (kg) and the plasma water specific activity, which was determined from the ^3H activity (cpm L^{-1}) of a plasma sample assuming that the concentration of water in body tissues was 1,000 g L^{-1} or 55.5 mol L^{-1}.

Experimental Design—Colchicine Experiment

A different experimental design, similar to that of Wood et al. (1995, 1997), was used to investigate the effects of colchicine treatment on pulsatile urea excretion because of the requirement to monitor fish for extended periods of time (52 h) and the time and equipment constraints of the respirometry setup (limited to monitoring one fish at a time). The experiment was divided into two periods, an initial 24-h control period in which toadfish ($N = 8$) were monitored to ensure that ureotelic pulsing behavior was being exhibited, followed by 28 h of monitoring of the then colchicine-treated fish to evaluate the pattern of nitrogenous waste excretion.

To begin the control period, water flow to the container was stopped, the container volume was set to 2.0 L, the aeration was adjusted to provide good mixing, and a peristaltic pump plus fraction collector system was started to collect continuous, hourly water samples for urea and ammonia analyses. Blood samples (50 μL) were collected every 4 h for determination of the plasma urea concentration. At 24 h, the water was changed and colchicine treatment was initiated; water and blood sampling were then continued for another 28 h. Fish were injected via the caudal vessel cannula initially with 0.025 mg colchicine kg^{-1} (Sigma, St. Louis) in toadfish saline and subsequently at 8-h intervals with a maintenance dose of 0.012 mg colchicine kg^{-1}.

Hourly excretion rates (μmol N kg^{-1} h^{-1}) of ammonia and urea into the external water in the colchicine experiment were calculated from changes in the concentration of ammonia-N or urea-N in the water (μmol N L^{-1}), multiplied by the volume (L), and factored by time (h) and fish mass (kg). Stepwise increases in the water urea concentration measured in the hourly samples of external water were used to identify pulses of urea excretion.

Experimental Design—Behavioral Observations

As it was not possible to observe fish clearly in the respirometers, an experimental design similar to that of the colchicine experiment was used for behavioral recordings. Following the standardized crowding treatment, ureotelic toadfish ($N = 8$) were placed directly into individual plastic 3-L containers supplied with a polyvinylchloride shelter, approximately 2 L of water, and aeration to ensure mixing. Water samples were collected continuously from the container, on a half-hourly basis, using a fraction collector system, for determination of the water urea concentration. The container was placed in an isolated, darkened room, and the behavior of the fish during a 12-h observation period was recorded (Cohu Solid State Camera, Radio Shack video enhancer, Fisher studio-standard stereo VCR) under infrared light. Tapes were subsequently scored for the time spent in each of three activities during pulse and nonpulse periods: (1) sheltering in the refuge, during which the fish was completely hidden from view in the polyvinylchloride shelter; (2) partial emergence from the refuge, in which the head of the fish was visible, but the majority of the body remained in the refuge; and (3) swimming forays, during which the fish emerged completely from the refuge. Pulse periods were identified on the basis of the appearance of stepwise increases in the concentration of urea in the half-hourly samples of external water. The frequency of occurrence of unusual behaviors, such as eversion of the oesophagus (Wood et al. 1995) or apneic events, during pulse and nonpulse periods was also examined.

Analytical Techniques

Standard chemical methods in combination with a microplate reader (Thermomax, Molecular Devices, Palo Alto, Calif.) were used for the analysis of urea-N in seawater and plasma (Price and Harrison 1987) and total ammonia-N in seawater (Ivancic and Deggobis 1984). ^{14}C radioactivity in single-label experiments was determined by liquid scintillation counting of plasma (10 μL + 1 mL seawater + 10 mL Ecolume fluor) and seawater (1 mL + 10 mL Ecolume fluor) samples in a TmAnalytic 6895 BetaTrac liquid scintillation counter. Samples were prepared for counting in an identical fashion in experiments incorporating $[^{14}C]$urea and ^3H_2O dual labeling, but a Beckman LS 1801 liquid scintillation counter was employed for simultaneous determination of ^{14}C and ^3H radioactivities.

Statistical Analysis

Files from the data acquisition system employed in respirometry experiments, containing the cardiorespiratory and $\dot{M}_{O_2}$

Table 1: Rates and patterns of urea-N and ammonia-N excretion in toadfish held under crowded conditions and then placed in either a respirometry setup (flow-through system, volume = 400–800 mL) or a static setup (volume = 2.0 L)

	Respirometry Setup (N = 20 fish)	Static Setup (N = 7 fish)
Observation time (h)	11.2 ± .9	24*
Urea-N excretion rate (μmol N kg^{-1} h^{-1})	203.7 ± 45.6	163.6 ± 34.9
Ammonia-N excretion rate (μmol N kg^{-1} h^{-1})	121.3 ± 25.1	151.6 ± 21.9
Urea-N pulse rate (pulses h^{-1})	.23 ± .13	.083 ± .024*
Size of urea-N pulse (μmol N kg^{-1} $pulse^{-1}$)	710 ± 149[a]	1,770.2 ± 341.9[b,*]
Percentage of urea-N output as pulses	74.2 ± 5.0	94.2 ± 1.0*
Percentage of total N output as urea-N	63.8 ± 5.2	50.7 ± 5.4
Mean pulse duration (min)	61.6 ± 4.7[a]	<180

Note. Patterns were monitored by means of half-hourly (respirometry) or hourly (static) water sampling with a fraction collector. The collection system used in the static setup precluded a finer time resolution for pulse duration than 180 min (see text). Data for the static setup were collected during the colchicine experiment. Values are means ± 1 SEM.

[a] N = 56 urea pulses (for 20 fish) for the respirometry setup.

[b] N = 14 urea pulses (for 7 fish) for the static setup.

* Indicates a significant difference between the respirometry setup and static setup ($P < 0.05$, Student's t-test or rank sum test, as appropriate).

data, were stored on magnetic tape and subsequently analyzed using the BioPac Acknowledge software (version 3.0.3, BioPac Systems). Mean data were compiled for 30-min intervals corresponding to the water sampling periods. These mean data were used in most statistical analyses and for the construction of most graphs and tables. In a few instances, the continuous data collected by the data acquisition system were used to display representative data recordings.

All urea and ammonia concentrations and fluxes are expressed in units of nitrogen for comparative purposes. Data have been expressed as means ± 1 SEM (N), where N represents either the number of fish or the number of urea pulse events, as specified. Statistical analysis was accomplished using repeated-measures one-way ANOVA followed by a post hoc multiple-comparison test (Bonferroni) for pairwise comparisons among groups, linear regression using the least squares method and with determination of the significance of the correlation coefficient, or Student's two-tailed t-test for paired or unpaired data, as appropriate. Where assumptions of normality or equal variances were violated, equivalent nonparametric statistical tests were used. The fiducial limit of significance in all analyses was 5%.

Results

Cardiorespiratory Variables during Natural Urea Pulses

Toadfish used in respirometry experiments were ureotelic, excreting 64% of their nitrogenous waste as urea-N, and this urea excretion was pulsatile, with 74% of the total urea-N excreted in pulses that typically lasted about 1 h (Table 1). Despite the added encumbrances of opercular catheters and impedance leads and the confined nature of the respirometer, the pattern of urea excretion in the respirometry experiment (Table 1) was similar to that observed in a static setup, as used in the colchicine experiment (Table 1), as well as to that reported in previous studies (Wood et al. 1995, 1997, 1998). The main differences noted in the pattern of urea excretion between these previous studies and the respirometry experiment were the significantly higher frequency and smaller size of urea pulses in the respirometry experiment (Table 1).

The collection of both outflowing and expired water samples enabled urea-excretion rates based on the appearance of ^{14}C in the water samples from each source to be compared. Increases in urea excretion indicative of a urea pulse event coincided for urea excretion calculated from the outflowing and the expired water samples. A representative example from an individual fish is presented in Figure 1, and the overall pattern (for N = 8 fish) is summarized by the significant, positive correlation observed in Figure 2. Urea excretion determined from the expired water samples (1,059.6 ± 389.5, N = 8, μmol N kg^{-1} h^{-1} during pulse periods) was generally higher than that calculated on the basis of outflowing water samples (266.0 ± 27.5 μmol N kg^{-1} h^{-1}, N = 8, during pulse periods), although the difference was not significant (paired t-test, $P > 0.05$). The use of ventilation volume (Fig. 1*B*), which was determined using an indirect approach based on the Fick principle (Dejours 1973), as well as the use of inflowing, rather than inspired, values for O_2 tension and [^{14}C]urea counts in the calculation of urea excretion from expired water samples, should be noted.

Monitoring of cardiorespiratory variables (arterial blood

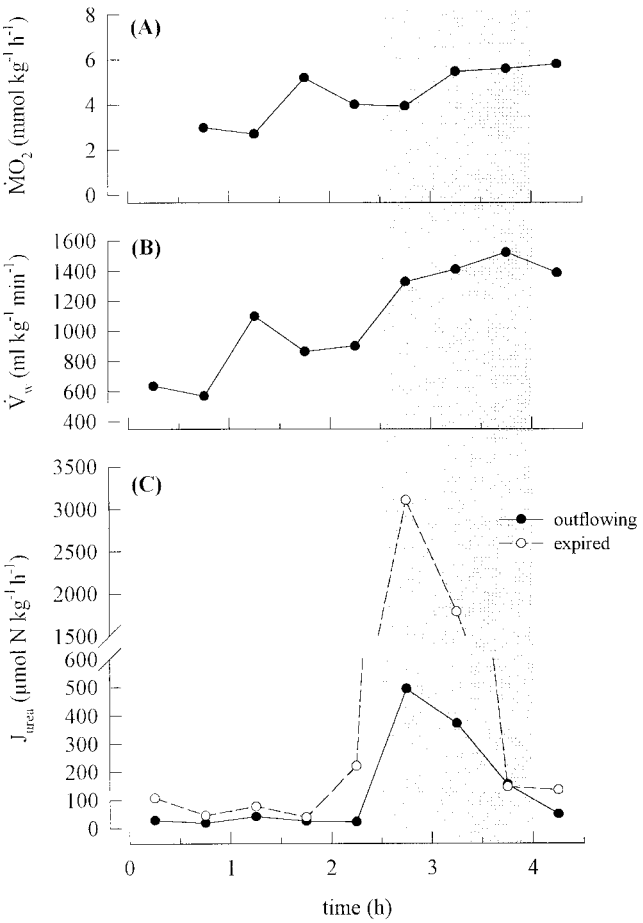

Figure 1. Representative data for a single toadfish in a respirometer illustrating $\dot{M}O_2$ (*A*), ventilation volume ($\dot{V}w$), calculated from the $\dot{M}O_2$ using the Fick principle (*B*), and urea-N excretion rates (J_{urea}) determined from the appearance of [^{14}C]urea in outflowing and expired water samples (*C*) as a function of time. The period during which a natural pulsatile urea-excretion event occurred (based on the outflowing water data) is marked by the grey band. Urea-N excretion rates were determined by the collection of half-hourly water samples using a fraction collector, and the rates shown are instantaneous rates for a half-hour sample period. $\dot{M}O_2$ was monitored using a data acquisition system, and the data shown represent mean values for half-hour sample periods corresponding to the half-hourly water samples.

pressure, cardiac frequency, ventilation frequency, and $\dot{M}O_2$), together with nitrogenous waste excretion and tritiated water efflux during natural pulsatile urea-excretion events, did not reveal any consistent changes in cardiorespiratory variables associated with urea pulses; a representative data set is illustrated in Figure 3. To analyze these data statistically, mean values for each variable for the 1-h period preceding a pulse, the 1-h period following each pulse, and the duration of the pulsatile event itself were tabulated (Table 2). In addition to the significant 14-fold elevation in urea excretion during pulse periods, the tritiated water efflux during urea pulses was slightly (1.5-fold) but significantly increased over the prepulse efflux rate. Neither ammonia excretion nor the cardiorespiratory variables exhibited any significant differences between pulse and nonpulse periods, and furthermore, no significant relationship between urea excretion and $\dot{M}O_2$ was detected (Fig. 4*A*). $\dot{M}O_2$ was significantly and positively correlated with ventilation frequency (Fig. 4*B*) but not with cardiac frequency (data not shown).

One difficulty with the above analysis is the possibility that small, transient changes in cardiorespiratory variables linked to urea pulse events might be masked by the examination of mean values for periods of 30 min or more. Such an effect was of particular concern with the arterial blood-pressure recordings, where the periodic occurrence of blood-pressure events, consisting of a sudden drop in arterial pressure followed by a transient increase above the baseline value, was noted. An example of an arterial blood-pressure event is depicted in the original representative data recording presented in Figure 5. In this particular example, an increase in ventilation amplitude

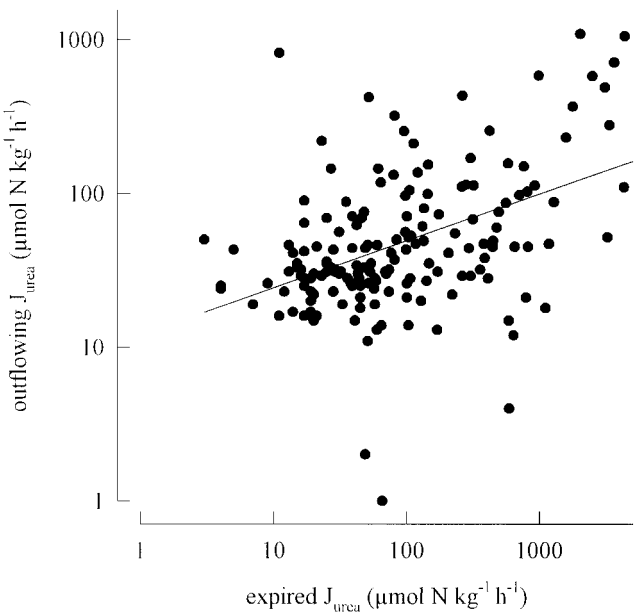

Figure 2. The relationship between the rate of urea-N excretion determined from the appearance of [^{14}C]urea in water leaving the respirometer (outflowing J_{urea}) and that based on the collection of expired water (expired J_{urea}). Expired urea excretion was determined from the appearance of [^{14}C]urea in water samples collected from a catheter placed in the opercular cavity and ventilation volume, which was calculated by means of the Fick principle from $\dot{M}O_2$ and the P_{O_2} values of the inflowing and expired water. A significant linear relationship was observed, and the regression equation is outflowing = 57.3 + 0.10 expired ($r = 0.59$, $P < 0.05$, $N = 177$). Data are values for all half-hour sample periods in a respirometry experiment for individual fish (8 toadfish in total). Note the log scales for both outflowing and expired urea excretion; the regression analysis, however, was performed on the original (linear) data set.

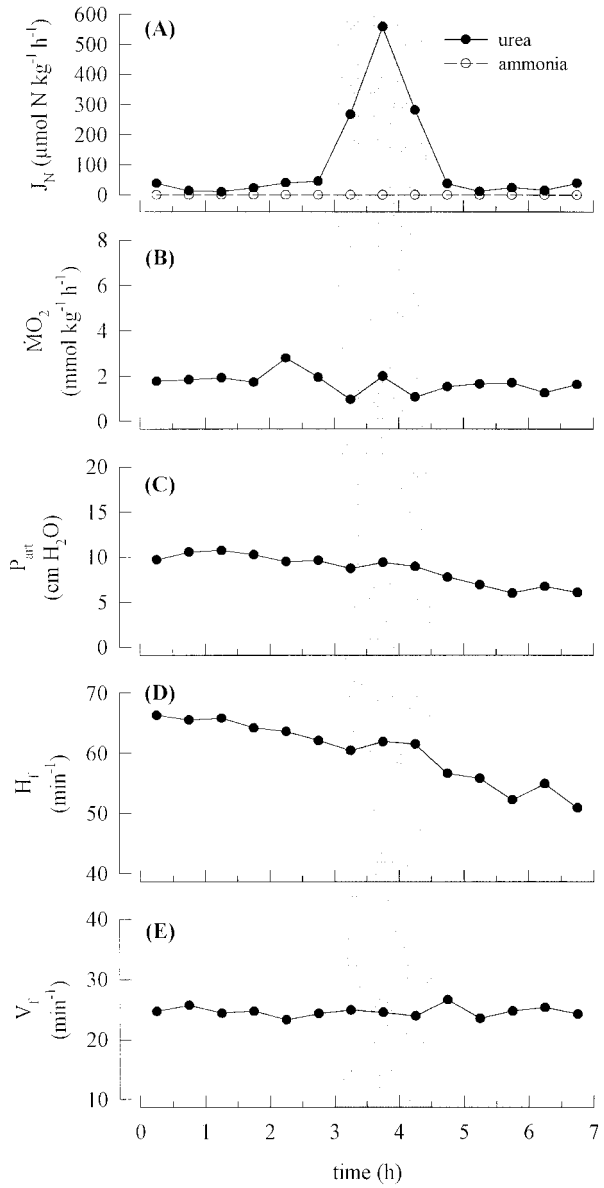

Figure 3. Representative data for one toadfish in a respirometry setup showing urea-N and ammonia-N excretion rates, J_N (*A*); $\dot{M}O_2$ (*B*); arterial blood pressure, P_{art} (*C*); cardiac frequency, H_f (*D*); and ventilation frequency, V_f (*E*) during a respirometry experiment. A natural pulsatile urea-excretion event is marked by the grey band. Urea-N and ammonia-N excretion rates were determined by the collection of half-hourly water samples using a fraction collector, and the rates shown are instantaneous rates for a half-hour sample period. All other variables were monitored using a data acquisition system, and the data shown represent mean values for half-hour sample periods corresponding to the half-hourly water samples.

accompanied the arterial blood-pressure event, but in other cases, no change in ventilation amplitude was observed; overall, no obvious, consistent ventilatory effect was observed during arterial blood-pressure events. The occurrence of these arterial blood-pressure events in relation to the occurrence of urea pulses (30-min urea pulse periods) was evaluated. Of the 173 arterial blood-pressure events observed (using $N = 14$ recordings), only 35% occurred during urea pulse periods. However, the number of urea pulse periods during which an arterial blood-pressure event also occurred was 28 out of 35 (80%), and interestingly, the one fish for which no urea pulses were recorded during the course of the respirometry experiment also did not exhibit any arterial blood-pressure events. Statistical analysis of these data indicated that the frequency of occurrence of arterial blood-pressure events during pulse periods (one per 41.0 ± 7.7 min, $N = 14$ recordings) was significantly higher (paired sign rank sum test, $P < 0.05$) than the overall frequency during all periods (one per 75.4 ± 19.8 min, $N = 14$ recordings).

Effect of Colchicine

Treatment of ureotelic toadfish with colchicine, a blocker of microtubule formation, caused marked and significant reductions in both the rate and frequency of pulsatile urea excretion, in the absence of changes in ammonia excretion (Table 3). During the control period, fish typically pulsed twice, accounting for approximately 94% of their total urea excretion, and distinct decreases in the plasma urea concentration generally accompanied the natural pulse events (Fig. 6). Urea excretion was significantly reduced following the administration of colchicine and appeared in the external water in a continuous rather than a stepwise fashion (Fig. 6*B*); only one colchicine-treated fish pulsed (Table 3), and the single "pulse" occurred during the 2 h immediately following the initial injection of colchicine. While pulsatile urea excretion was blocked by colchicine, the significant increases in the plasma urea concentration (Fig. 6*A;* Table 3) following treatment with colchicine indicated that urea production continued.

Behavioral Observations

The examination of approximately 144 h of recordings of ureotelic toadfish did not reveal any distinctive behaviors that were associated with urea pulses. Eversion of the oesophagus was not necessary for a pulse event, and apneic episodes were not more frequent during pulse than nonpulse periods. One series of recordings ($N = 4$ fish) was scored for the length of time fish spent in each of three activities: sheltering in the refuge, partially emerged from the refuge, or fully emerged from the refuge in a swimming foray (Table 4). Sheltering in the refuge was the most common activity in both pulse and nonpulse periods, accounting for, on average, 88% of the total observation time (12 h). While the time during which toadfish were partially or fully emerged from the shelter in either pulse or nonpulse periods was a relatively small proportion of the total observation time, a tendency for toadfish to engage in partial emergence from the refuge to a

Table 2: Cardiovascular and respiratory variables together with urea, ammonia, and tritiated water flux rates for toadfish during pulse and nonpulse periods

	Before Pulse	Pulse	After Pulse
Urea-N excretion (μmol N kg^{-1} h^{-1})	38.0 ± 5.4 (38)	540.3 ± 118.3 (41)[a,b]	43.0 ± 6.4 (35)
Ammonia-N excretion (μmol N kg^{-1} h^{-1})	107.5 ± 24.6 (38)	148.9 ± 32.1 (41)	126.3 ± 30.5 (35)
Tritiated water efflux (mol kg^{-1} h^{-1})	3.19 ± .41 (12)	4.63 ± .60 (13)[a]	3.44 ± .39 (11)
$\dot{M}_{O_2}$ (mmol kg^{-1} h^{-1})	2.91 ± .28 (34)	3.02 ± .30 (37)	3.11 ± .38 (35)
Arterial blood pressure (cm H_2O)	13.4 ± .9 (32)	13.3 ± 1.0 (35)	12.8 ± 1.1 (33)
Cardiac frequency (min^{-1})	64.3 ± 2.5 (22)	66.2 ± 2.2 (25)	65.6 ± 2.5 (24)
Ventilation frequency (min^{-1})	44.5 ± 2.2 (31)	44.9 ± 2.4 (34)	43.7 ± 2.3 (32)

Note. Before pulse and after pulse represent mean values for the 1-h period preceding and following a pulse, respectively. Urea excretion, ammonia excretion, and tritiated water efflux are instantaneous excretion rates for the sample period. Values are means ± 1 SEM; values in parentheses refer to the number of pulses for 20 fish; these values are variable because measurements for every variable were not available for all pulses.

[a] Indicates a significant difference from the before pulse value ($P < 0.05$, one-way repeated-measures ANOVA followed by Bonferroni *t*-tests for multiple comparisons).

[b] Indicates a significant difference from the after pulse value ($P < 0.05$, one-way repeated-measures ANOVA followed by Bonferroni *t*-tests for multiple comparisons).

greater extent in pulse over nonpulse periods was observed but was not significant (Table 4).

Discussion

The pulsatile pattern of urea excretion demonstrated by Wood et al. (1995, 1997, 1998) for ureotelic toadfish held under confined conditions was also observed in the present study. Minor differences in the rates and patterns of nitrogenous waste excretion were, however, apparent between the present study and those of Wood et al. (1995, 1997, 1998) and also between the colchicine and respirometry experimental setups. The degree of ureotely observed in the present study for toadfish held in either experimental setup was relatively low (Table 1), even though urea-excretion rates exceeded those reported in previous work, owing to a substantially higher ammonia-excretion rate (120–150 μmol N kg^{-1} h^{-1}; Table 1) than had been observed previously (10–50 μmol N kg^{-1} h^{-1}; Wood et al. 1995, 1997). While nitrogen excretion rates of ammoniotelic toadfish were not measured, it seems likely that the overall greater rate of nitrogenous waste excretion in ureotelic toadfish in the present study was a result of the warmer water temperatures (25°C versus 20°–24°C). In addition, toadfish in the present study were fed during the initial holding period in the laboratory under noncrowded conditions, a factor that was found previously to elevate the overall rate of nitrogenous waste excretion and favor persistence of significant ammonia excretion in predominantly ureotelic toadfish (Walsh and Milligan 1995). Despite these differences in the rates of urea and ammonia excretion, the proportion of excreted urea eliminated in pulsatile events (74%–94%; Table 1) in the present study was essentially identical to that measured in earlier studies (55%–94%; Wood et al. 1995, 1997, 1998), as was the size of individual urea pulses for toadfish held under static conditions (approximately 1,800 μmol N kg^{-1} $pulse^{-1}$ [Table 1] vs. 1,600–3,150 μmol N kg^{-1} $pulse^{-1}$ [Wood et al. 1995, 1997, 1998]). A 2.8-fold greater frequency of urea pulse events was observed for toadfish held in the flow-through respirometry setup, and correspondingly, the size of individual urea pulses was reduced (Table 1). This more frequent pattern of urea pulses may have reflected the greater extent to which fish in the respirometry setup were encumbered with catheters. Nevertheless, a distinctly pulsatile pattern of urea excretion was observed, with a mean pulse duration of approximately 60 min. The collection of water samples on a half-hourly basis and the flow-through nature of the respirometry setup provided improved time resolution for the detection of pulsatile events over the static setup used in the colchicine experiment and previous studies (Wood et al. 1995, 1997, 1998). In the latter cases, the use of an hourly sampling frequency and the potential for "smearing" of the urea pulse by mixing in the first and last water samples collected resulted in estimates of less than 180 min for the pulse duration.

The site of urea excretion was investigated by Wood et al. (1995), who used a divided-chamber protocol to localize urea pulses to the anterior end of the toadfish but could not distinguish between the gills and the skin of the head as the site of urea excretion. In the present study, a significant, positive correlation was demonstrated in the urea-excretion rates determined from the collection of expired and outflowing water samples (Fig. 2), a result that argues strongly that natural urea pulses are derived from the gills. The rate of urea excretion determined from expired water samples was generally higher than that calculated on the basis of outflowing samples, probably owing to the use of a ventilation volume evaluated by means of an indirect approach based on the Fick principle

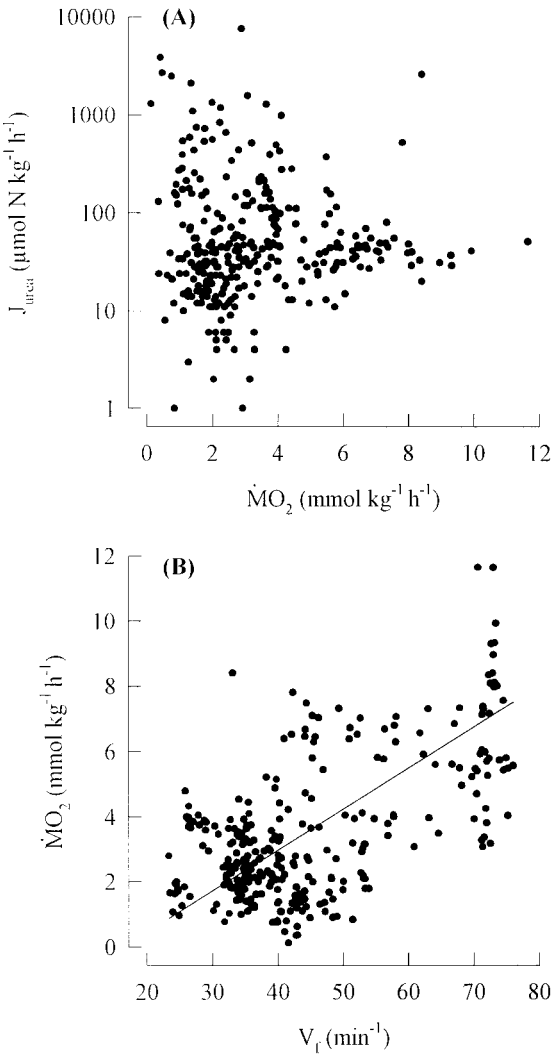

Figure 4. The relationships between urea excretion (J_{urea}) and $\dot{M}O_2$ (*A*), and $\dot{M}O_2$ and ventilation frequency (V_f; *B*). No significant correlation was found between the urea-excretion rate and $\dot{M}O_2$ for toadfish in respirometers ($r = -0.09$, $P = 0.11$, $N = 317$). Note the log scale for urea excretion; the regression analysis, however, was carried out on the original (linear) data set. A significant correlation was observed between $\dot{M}O_2$ and ventilation frequency with a regression equation: $\dot{M}O_2 = -0.87 + 0.094V_f$ ($r = 0.61$, $P < 0.05$, $N = 299$). Data are values for all half-hour sample periods in a respirometry experiment for individual fish (20 toadfish in total).

(Dejours 1973). Measurement of ventilation volume by the indirect method is known to yield estimates that are high relative to direct measurements (see Wood and Munger [1994] for a discussion of the difficulties associated with the indirect measurement of ventilation volume). In addition, inspired P_{O_2} and [^{14}C]urea counts were approximated by the use of inflowing values, which probably contributed to the overestimation of expired urea excretion. The possibility that urea excretion could occur across the inner opercular membrane should also be considered since the opercular membrane in toadfish is well vascularized (P. Laurent, personal communication) and urea excretion from this site would be detected in the expired water (but see below).

Given that urea pulses occur across the gills of the toadfish and that simultaneous measurements of the plasma urea concentration and urea excretion have clearly demonstrated that it is urea excretion that occurs in a pulsatile fashion in the face of continuous metabolic urea production (Wood et al. 1997), a number of potential pulsatile excretion mechanisms can be conjectured (Walsh 1997; Wood et al. 1997, 1998). One such model, which the present study was designed to test, postulates rapid, periodic, generalized increases in the branchial conductance, that is, a relatively nonspecific effect in which urea move-

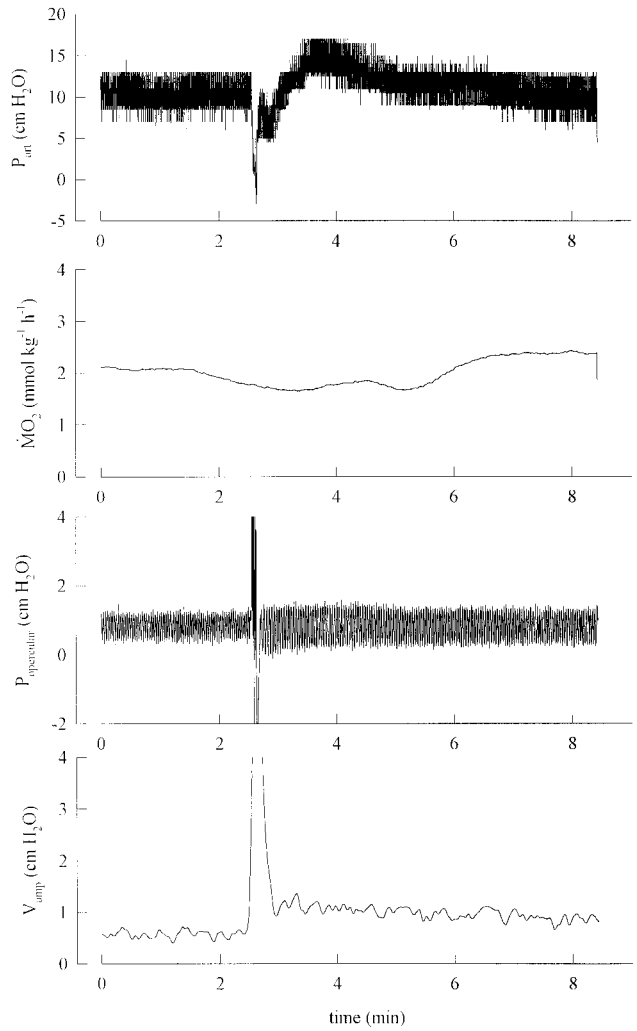

Figure 5. A representative original data recording illustrating a typical arterial blood-pressure event (P_{art}), together with $\dot{M}O_2$, the opercular pressure ($P_{opercular}$), and ventilation amplitude (V_{amp}; determined from opercular pressure). The arterial blood-pressure event in this particular case occurred approximately 1 h before a natural urea pulse.

Table 3: The effect of colchicine treatment on the rates and patterns of urea-N and ammonia-N excretion in toadfish ($N = 7$ fish)

	Control	Colchicine Treated
Urea-N excretion (μmol N kg^{-1} h^{-1})	163.6 ± 34.9	55.8 ± 13.4*
Ammonia-N excretion (μmol N kg^{-1} h^{-1})	151.6 ± 21.9	126.1 ± 19.5
Urea-N pulses (pulses d^{-1})	2.0 ± .2	.1 ± .1*
Size of urea-N pulses (μmol N kg^{-1} $pulse^{-1}$)[a]	1,770.2 ± 341.9	1,461.6
Percentage of urea-N in pulses	94.2 ± 1.0	6.0 ± 6.0*
Plasma urea-N (mmol N L^{-1})	12.5 ± 1.5	16.7 ± 1.6*

Note. Patterns were monitored by means of hourly water sampling with a fraction collector. Values are means ± 1 SEM.

[a] $N = 14$ urea pulses (for 7 fish) during the control period, and one urea pulse during colchicine treatment.

* Indicates a significant difference between colchicine treated and control ($P < 0.05$, Student's paired t-test).

ment across the gill as well as that of a wide range of other molecules is increased. Both convective and diffusive components contribute to branchial conductance, specifically, ventilation, perfusion, gill surface area, diffusion distance, and gill permeability, and therefore detectable changes in one or more of these variables would be expected to occur during pulsatile urea events if the excretion mechanism involves generalized increases in solute transfer. In the present study, ventilation and perfusion variables were measured directly, while changes in branchial conductance resulting from alterations of gill surface area, diffusion distance, and gill permeability were monitored indirectly through measurements of $\dot{M}O_2$, ammonia excretion, and tritiated water excretion. However, no significant changes in mean values of ammonia excretion, $\dot{M}O_2$, arterial blood pressure, cardiac frequency, or ventilation frequency were associated with natural pulsatile urea-excretion events (Table 2; Fig. 3). The absence of a significant difference between pulse and nonpulse periods in the measured ventilation and cardiovascular variables indicates that sudden changes in convective factors likely do not account for pulsatile urea-excretion events. Furthermore, generalized increases in gill permeability by the opening of transcellular pores or paracellular junctions seem an unlikely candidate for the pulsatile mechanism in view of the absence of any correlative relationship between $\dot{M}O_2$ and urea excretion (Fig. 4*A*). Thus, the experimental evidence obtained from monitoring cardiorespiratory variables and nitrogen excretion in ureotelic toadfish does not support the hypothesis that pulsatile urea excretion reflects pulsatile generalized increases in gill conductance.

In addition to refuting the hypothesis of generalized increases in branchial conductance as the mechanism of pulsatile urea excretion, the respirometry experiments generated a detailed data set on the cardiovascular and respiratory characteristics of *Opsanus beta.* These data can be used to investigate the relationships between $\dot{M}O_2$ and ventilation or cardiac frequencies. Such relationships are of interest because of the opportunity afforded for the prediction of metabolic rate in a natural habitat, using telemetry or other relatively noninvasive techniques (Hawkins and Urquhart 1983; Priede 1985; Lucas et al. 1993; van Rooij and Videler 1996). For example, ventilation frequency was found to be a useful predictor of metabolic rate in the reef fish *Sparisoma viride* (van Rooij and Videler 1996), while reliable correlations between heart rate and $\dot{M}O_2$ have been reported for several teleosts, including pike, rainbow trout, cod, sole, and bass (Priede and Tytler 1977; Armstrong 1986; Sureau and Lagardère 1991; Thorarensen et al. 1996). Ventilation frequency may be of limited value as a predictor of metabolic rate, however, since one or more of ventilation amplitude, oxygen extraction from the ventilatory water, and ventilation frequency may be varied to meet metabolic demands (e.g., Randall 1982; Randall and Daxboeck 1984; Wood and Perry 1985); similar considerations apply to the use of cardiac frequency (Thorarensen et al. 1996). Thus, it is necessary to examine different species on an individual basis. The significant positive correlation measured between ventilation frequency and $\dot{M}O_2$ under resting conditions for *O. beta* in the present study (Fig. 4*B*) indicates that ventilation frequency may be a useful index of metabolic rate in this species; oxygen uptake and ventilation frequencies were similar to, but slightly higher than, those reported for the related toadfish *Opsanus tau* (Ultsch et al. 1981), probably owing to the use of lower temperatures and the measurement of the standard rate of oxygen uptake in the latter study. Cardiac frequency, on the other hand, does not appear to be a reliable predictor of $\dot{M}O_2$ in *O. beta.*

An alternative model for the pulsatile mechanism of urea excretion in the toadfish gill postulates a urea-specific transporter that is activated periodically in response to a neural or hormonal signal (Walsh 1997; Wood et al. 1997, 1998). Periodic activation could be accomplished through either insertion of urea transporter-containing vesicles into gill cell membranes or posttranslational modification of gill cell membrane consti-

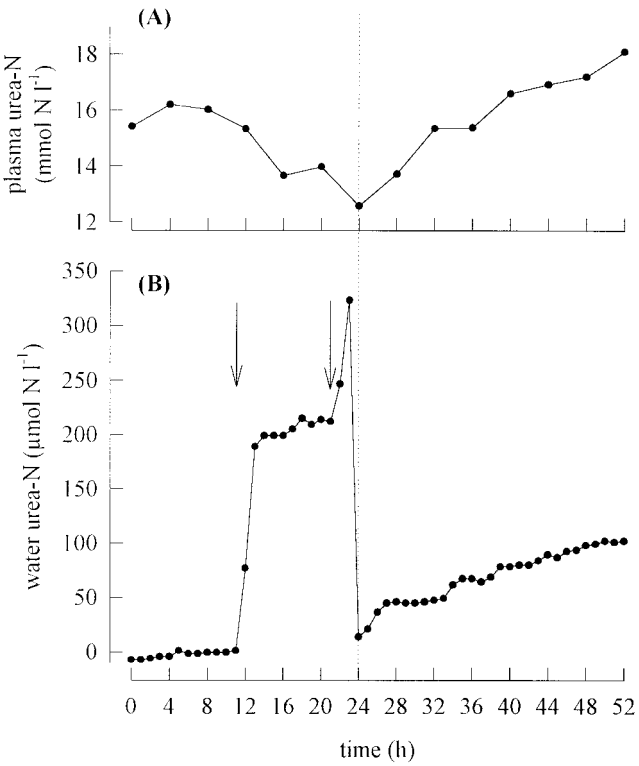

Figure 6. Representative data for one toadfish in a static setup showing the effect of colchicine treatment (0.025 mg kg^{-1}) on the pattern of urea-N excretion. Plasma urea-N concentrations (*A*) and the concentration of urea-N (measured chemically; *B*) in hourly samples of external water obtained using a fraction collector were monitored for 24 h before and following the administration of colchicine. Note the loss of pulsatile urea-N excretion events following colchicine treatment. The dotted line marks the initiation of the colchicine treatment, at which point the water in the static bath was changed, resulting in the return of the water urea-N concentration to zero. The vertical arrows mark urea pulse events.

tutive urea transporters. Vesicle trafficking is dependent on cytoskeletal function, and therefore the inhibition of pulsatile urea excretion by treatment with the microtubule-disrupting agent colchicine (Table 3; Fig. 6) is consistent with an activation mechanism in which urea transporters are inserted into the gill cell membranes. The steady increase in the plasma urea concentration following colchicine administration (Table 3; Fig. 6) argues against an inhibitory effect of colchicine on urea synthesis in the liver or equilibration into the blood; the movement of urea from hepatocytes to plasma occurs continuously via a constitutive facilitated-diffusion carrier located at the hepatocyte membrane and would not be expected to be dependent on vesicle trafficking (Walsh et al. 1994*b;* Walsh and Wood 1996). Furthermore, a transmission electron microscopy examination of gill tissue obtained from toadfish during natural pulsatile excretion events yielded morphological evidence for vesicle fusion with the apical membrane of gill cells (P. Laurent, personal communication). Exocytosis of water from urea transporter-containing vesicles following vesicle fusion with gill-cell apical membranes could also account for the significant elevation of the tritiated water efflux that was observed in pulse over prepulse periods (Table 2).

Additional evidence in favor of the idea that urea pulses are due to periodic insertion or activation of a facilitated-diffusion transporter in the gills was obtained in a recent study by Wood et al. (1998). Specifically, the presence of a high urea concentration in the external seawater results in a net uptake of urea, rather than a net excretion, each time the excretion mechanism is activated, demonstrating that the transport system is capable of bidirectional transport. Furthermore, the influx component is potently inhibited when high levels of the urea analogue thiourea are presented simultaneously in the external medium. Thiourea itself is transported by the system, but to a much smaller extent than urea. All of these observations are diagnostic of a facilitated diffusion system similar to those identified in other tissues of higher vertebrates, such as toad bladder and mammalian red-cell membranes and kidney (reviewed by Marsh and Knepper [1992]). One notable difference between the present study and that of Wood et al. (1998) was the lack of change in tritiated water permeability during natural pulses reported in the latter study. The resolution of the recording system employed by Wood et al. (1998), which was similar to that used in the present colchicine experiment, was probably insufficient to detect the small increase in tritiated water flux observed in the present study.

A mechanism for the pulsatile urea excretion of toadfish constituting a periodically activated urea-specific transporter at the gill is also supported by recent work by Walsh and colleagues (see Walsh 1997), in which reverse transcription-PCR was used to amplify a 484-bp cDNA fragment from toadfish gill. The resultant PCR product was found to encode a protein exhibiting high homology with the vasopressin (AVP)-sensitive renal urea transporter cloned from the mammalian collecting duct (You et al. 1993). In this respect, it is notable that intravascular injection of toadfish with the fish neurohypophysial homologue of AVP, arginine vasotocin (AVT), at physiologically relevant doses has been found to elicit marked, transient increases in urea excretion similar to those observed during natural urea pulses (Perry et al. 1998). It should also be noted that gill was the only tissue of several tested (skin, liver, kidney, red blood cells) to express the renal urea transporter-like message (see Walsh 1997), which, while not excluding the inner opercular membrane (see above), does implicate the gill as a major contributor to urea excretion.

The neural or hormonal signal involved in triggering the pulsatile excretion mechanism in toadfish has not yet been identified. It appears to be a relatively specific signal in that urea pulses occurred in the absence of significant changes in ventilation or cardiovascular function. In this context, it is interesting to consider the apparent association between the occurrence of transient, small arterial blood-pressure "events" (Fig. 5) and the occurrence

Table 4: Summary of time (min) spent in various activities for ureotelic toadfish ($N = 4$ fish) during 12-h observation periods

Activity	Pulse Periods	Nonpulse Periods
Swimming forays	2.7 ± 1.0	50.0 ± 16.7
	(5.6 ± 1.0)	(7.4 ± 2.4)
Partial emergence	8.9 ± 5.3	25.8 ± 14.6
	(18.7 ± 9.3)	(3.7 ± 2.1)
Sheltering in refuge	38.9 ± 15.2	593.6 ± 23.6
	(75.7 ± 10.3)	(88.9 ± 4.3)

Note. Pulse vs. nonpulse periods were identified on the basis of the concentration of urea in half-hourly water samples obtained from the experimental chamber using a fraction collector. Values are means ± 1 SEM; values in parentheses refer to the percentage of a pulse or nonpulse period occupied by the activity. No significant differences in the percentage values were found between pulse and nonpulse periods (paired Student's *t*-test, $P > 0.05$) for swimming forays or partial emergence; sheltering in refuge was not tested to avoid statistical difficulties associated with total time being a constraining variable.

of urea pulses. The origin of these blood-pressure events is difficult to ascertain. However, taking into account the AVT-induced pressor effect, which has been documented in toadfish (Lahlou et al. 1969; Perry et al. 1998), and the potential involvement in the pulsatile urea-excretion mechanism of a transporter analogous to the AVP-sensitive mammalian renal urea transporter (Walsh 1997), it is attractive to speculate that the increased frequency of blood-pressure events during urea pulses reflected the AVT-mediated activation of a specific urea transporter-excretion mechanism. Measurements of plasma AVT concentrations in ureotelic toadfish during both urea pulse and nonpulse periods clearly would be useful in establishing whether AVT is involved in triggering urea pulses.

It is likely, however, that even if AVT is the proximate stimulus for activation of the pulsatile urea-excretion mechanism, other factors are also involved in triggering a urea pulse. For example, circulating cortisol levels were found to decrease markedly immediately before a urea pulse and increase sharply following the pulse, implying a role for the hypothalamic-pituitary interrenal axis (Wood et al. 1997). The correlation between plasma cortisol concentration and the occurrence of a urea pulse does not necessarily indicate causality. Wood et al. (1997) suggested that one possible explanation is that the decrease in plasma cortisol concentration is associated with a behavioral cue that allows a pulse to occur. Although the preliminary observations obtained in the present study did not reveal any distinct behaviors that were associated with the occurrence of urea pulses (Table 4), more detailed studies on both the behavioral and endocrinological events associated with pulsatile urea excretion in toadfish are clearly required.

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