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THE FUNCTION OF THE URINARY BLADDER *IN VIVO* IN THE FRESHWATER RAINBOW TROUT

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Summary

Two different approaches were employed to examine the possible urine storage and ionoregulatory functions of the urinary bladder *in vivo* in the freshwater trout. An indirect approach, using non-catheterized fish, involved 'spot sampling' from the bladder to determine urine composition and measurement of PEG-4000 release into surrounding water to quantify urination events. The direct approach employed a new external catheterization technique to collect naturally discharged urine. Both methods demonstrated that trout urinate in intermittent bursts at 20–30 min intervals, and that natural urine flow rate is at least 20 % lower and urinary Na^+ and Cl^- excretion rates at least 40 % lower than those determined by the traditional internal bladder catheter technique. The urine is stored for approximately 25 min prior to discharge, and significant ionic reabsorption *via* the bladder epithelium occurs during this period; a small residual volume is always likely to be retained. We conclude that previous studies using internal bladder catheterization have underestimated the ionoregulatory effectiveness of the entire renal system by preventing the function of the urinary bladder.

Introduction

In most teleosts, the paired mesonephric ducts leave the kidney, unite and open into a widened duct commonly termed the urinary bladder (Goodrich, 1931; Bell and Bateman, 1960; Hickman and Trump, 1969). The outlet to and through the urogenital papilla is guarded by one or more sphincters, so urine may be stored in the bladder for some time prior to natural discharge. Cameron and Wood (1978) obtained some preliminary data suggesting that urination was indeed periodic in two Amazonian erythrinid teleosts. *In vitro*, a substantial mucosal to serosal transport has been described in the isolated urinary bladder epithelium from the freshwater rainbow trout (Lahlou and Fossat, 1971; Fossat *et al.* 1974; Fossat and Lahlou, 1977, 1979*a*,*b*, 1982; Demarest and Machen, 1982; Harvey and Lahlou, 1986; Marshall, 1988). However, it remains unknown whether such bladder transport processes occur *in vivo*, and thereby influence the volume or composition of naturally voided urine.

Key words: urinary bladder, urine composition, glomerular filtration, renal function, ionoregulation, rainbow trout, *Oncorhynchus mykiss*.

Up to now, virtually all data about urine flow and composition *in vivo* in the rainbow trout have been collected using internal bladder catheters (e.g. Holmes and Stainer, 1966; Hunn, 1969; Hunn and Willford, 1970; Hofmann and Butler, 1979; Elger and Hentschel, 1983; Wheatley *et al.* 1984; Oikari and Rankin, 1985; Elger *et al.* 1986; Erickson and Gingerich, 1986; Wood, 1988). It has long been recognized that urine collected by this method has no residence time in the bladder (Lahlou, 1967; Beyenbach and Kirschner, 1975; Marshall, 1988), since the catheter drains the urine by siphon as soon as it enters from the mesonephric ducts. Any ion transport role of the bladder would be prevented.

The aim of the present study was to establish the possible storage and ionoregulatory roles of the urinary bladder *in vivo* in the freshwater rainbow trout. In particular, we wished to quantify the natural pattern of urination, and the volume and composition of naturally vented urine in comparison to that collected by the traditional internal bladder catheter. Two separate approaches were used and compared. The first, a combination of 'spot sampling' from the bladder and measurement of the appearance of a glomerular filtration marker in the external water in different groups of fish, was chosen for its non-invasive qualities, but involved a number of assumptions. The second, a new external catheterization method to collect naturally discharged urine, involved very few assumptions but was a more invasive technique. Using both methods, natural urination patterns were recorded and bladder ion transport abilities were assessed.

Materials and methods

Experimental animals

Adult rainbow trout (*Oncorhynchus mykiss* Walbaum=*Salmo gairdneri* Richardson; 200–425 g) were obtained from Spring Valley Trout Farm, Petersburg, Ontario. The fish were acclimated to 15±1°C and fasted for at least 7 days prior to experiments. Both acclimation and experimentation were carried out in dechlorinated Hamilton tapwater [Ca^{2+}=1.8; Cl^-=0.8; Na^+=0.6; Mg^{2+}=0.5; K^+=0.04; titration alkalinity (to pH 4.0)=1.9; total hardness ≈140 mg l^{-1} as $CaCO_3$; pH 8.0]. Urinary catheterization (see below) was performed under MS-222 anaesthesia (100 mg l^{-1}; Sigma). Following surgery, fish were placed in darkened Perspex 'flux' boxes (McDonald and Rogano, 1986) served with vigorous aeration and a flowing water supply. During the 48 h recovery period urine was collected continuously, with the catheter emptying into a collecting vial approximately 3.0 cm below the box water level. Non-catheterized fish intended for urine spot sampling and/or [^{3}H]PEG-4000 flux measurements (see below) were similarly placed in their Perspex boxes 48 h prior to experiments.

Experimental techniques

Internal urinary catheter

This was similar to the technique described by many previous workers (e.g. Holmes and Stainer, 1966; Wood and Randall, 1973; Beyenbach and Kirschner,

1975; Oikari and Rankin, 1985), and was designed to collect urine directly from the bladder. The catheter was made of 45 cm of Clay–Adams PE-60 with one end heat-flared to 1.5 times its original diameter. The flared end of the water-filled catheter was advanced dorsally 1.0–1.5 cm through the urogenital papilla into the urinary bladder. The integrity of the seal (two 2–0 silk ligatures around the papilla) was tested by injecting 0.5–1.0 ml of water, and the catheter was then secured with three purse-string ligatures, one just anterior to, and two to the side of, the anal fin.

External urinary catheter

This new technique was designed to collect urine as it was naturally discharged from the urogenital papilla. The catheter was made from a 40 cm Bard 'All-Purpose Urethral Catheter' (size 12-French; elastic rubber), with the dilated end cut off to form a funnel. This funnel was stitched to the ventral surface of the fish around the urogenital papilla and anus using 16 equally spaced purse-string ligatures (2–0 silk). The intestine was ligated with 2–0 silk through a small incision in the ventral surface immediately anterior to the anus. This served to prevent blockage of the catheter by expelled faecal sacs, which occurred in preliminary experiments without ligation. During comparisons of internal and external catheters (series iii, below), fish fitted with internal catheters were also ligated intestinally.

Once the catheter had been secured, the area around the stitches was thoroughly wiped to remove mucus and a thin film of tissue cement (3M Vetbond) was applied. The integrity of the seal was tested by injecting water into the funnel with a 26 gauge needle while holding the open end vertically above the fish, thereby applying a pressure head of 3.9 kPa. This test was adequate since, in the Perspex box, pressure on the catheter was only −0.29 kPa (the open end being 3 cm below the water level) to ensure the unimpeded functioning of the direct recording devices (see below).

Urine spot sampling

To assess urine composition in non-catheterized trout, spot sampling was performed as described by Shehadeh and Gordon (1969). The fish was rapidly immobilized by a high dose of MS-222 (300 mg l^{-1}) added to the box, then quickly transferred to the operating table where it was artificially ventilated. The flared tip of an internal catheter (see above) was inserted through the papilla into the urinary bladder, and mild suction applied with a 1 ml syringe to drain the bladder as completely as possible; the volume obtained was recorded.

PEG-4000 flux

Polyethylene glycol (PEG, M_r 4000) was employed as a marker for glomerular filtration rate (GFR) and for the appearance of urine in the external water. A dose of 17 μCi of [1,2-^{3}H]polyethylene glycol (PEG-4000; New England Nuclear) in 0.66 ml of Cortland saline (Wolf, 1963) was injected into the caudal vein using a

22 gauge needle. Measurements of [^{3}H]PEG-4000 appearance in the water and/or urine began either 14 h (series ii) or 48 h (series iii) after injection to allow equilibration of the label throughout the extracellular space. [^{3}H]PEG-4000 was chosen in preference to commonly used inulin derivatives or other labels because in our experience it is resistant to radioautolysis and degradation by micro-organisms and the fish's own metabolism. Furthermore, it undergoes less post-filtration reabsorption, is less permeable across the bladder, and therefore yields higher GFRs in seawater-adapted rainbow trout (Beyenbach and Kirschner, 1976). PEG-4000 has been used previously to measure GFR in freshwater-adapted rainbow trout (Erickson and Gingerich, 1986).

Direct recording of urination

The quantity and pattern of urination from fish fitted with either external or internal urinary catheters (series iii) were measured by passing the freely venting catheter outflow across an infra-red optical switch (Clarex CL1–375) connected to a Harvard 508184 oscillograph. A drop passing through the beam was shown as a single spike on the chart record. Urine flow rate (UFR) was calculated from the cumulative number of drops over time.

Maximum bladder volume determination in vitro

Fish were fitted with an internal catheter and then killed by severing the spinal cord. A longitudinal incision was made in the ventral surface, the ureteral entrances were ligated and the bladder was removed. The bladder was filled with water through the catheter to the maximum volume that could be achieved without bursting, then weighed, cut in half to ensure complete drainage, and reweighed.

Experimental series

Series i

This series tested whether there were differences in composition between urine collected by spot sampling (presumably more representative of naturally vented urine) and urine collected by the traditional internal urinary bladder catheter. Trout fitted with internal catheters (N=19) and non-catheterized trout for spot sampling (N=16) were placed simultaneously in Perspex boxes. After 48 h recovery, urine flow was collected over a 3 h period from each catheterized fish, and a urine sample was taken from each non-catheterized fish by spot sampling. The fish were allowed to recover for another 48 h, and the procedure was repeated. Samples from the two times were pooled for individual fish and stored frozen for later determination of ammonia, urea, Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}, Cl^{-}, $NO_3{}^{2-}$, $SO_4{}^{2-}$ and total phosphate concentrations.

Series ii

This series assessed the natural pattern and volume of urination in non-

catheterized trout (N=12) by monitoring the appearance of [^{3}H]PEG-4000 in the water. Water flow to the flux boxes was stopped 14 h post-injection and the flux boxes thereafter operated as closed systems with initial volumes of approximately 3 l. Water samples (5 ml) were taken without replacement every 5 min for 8 h. At the end, the fish were rapidly immobilized with MS-222 (300 mg l^{-1}) and weighed; residual water volume was measured. Spot urine samples and caudal vein blood samples were taken; plasma was extracted by centrifugation (10 000 g for 2 min). Water, urine and plasma were analysed for [^{3}H]PEG-4000 by scintillation counting to determine urination pattern, UFR and GFR (see below).

Series iii

This series directly compared the pattern of urination, UFR, GFR, urine composition and urinary ion excretion rates between trout fitted with internal (N=9) and external (N=10) urinary catheters. Fish were fitted with catheters, injected with [^{3}H]PEG-4000 and allowed to recover for 48 h. Water flow was then stopped and the box operated as a closed system, with an initial volume of 3.5 l. Water samples were taken at 2 h intervals for 8 h to monitor extra-renal [^{3}H]PEG-4000 excretion. Throughout this period, urination was directly recorded in both groups using the infra-red optical switches, and UFR calculated from the chart recordings. Urine was frozen for later analysis of ammonia, urea, Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}, Cl^{-}, $NO_3{}^{2-}$, $SO_4{}^{2-}$, total phosphate and PEG-4000 concentrations.

Analytical methods and calculations

Ammonia was determined by a micro-modification of the salicylate assay (Verdouw *et al.* 1978), and urea by the diacetyl monoxime method (Crocker, 1967). All cation concentrations were determined by atomic absorption (Varian AA-1275); 0.2 % $LaCl_3$ was used to eliminate Na^{+} interference in the Ca^{2+} and Mg^{2+} measurements. All anion concentrations were determined using high pressure liquid chromatography (IC-Pak anion exchange column, Waters 510 pump, Waters 430 conductivity detector) on urine samples which had first been passed through a 0.2 μm nylon filter (Schleicher & Schuell Ltd). [^{3}H]PEG-4000 (counts min^{-1} ml^{-1}) in water (counts min^{-1}(w)), urine (counts min^{-1}(u)) and plasma (counts min^{-1}(p)) was determined by scintillation counting (LKB Rack-beta 1217).

In series ii, [^{3}H]PEG-4000 radioactivity in the external water of non-catheterized fish underwent periodic rapid increases ('steps') with intervening periods of relative constancy (see Fig. 1). These steps were interpreted as bouts of urination. Preliminary experiments demonstrated that a rise of 20 counts min^{-1} (in a 5 ml water sample) was the minimum step that could be detected reliably. Any increase of more than 20 counts min^{-1} in a sample (t_n) compared with one taken 5 min earlier (t_{n-1}) was considered to be produced by fish urination. Total [^{3}H]PEG-4000 activity in the water (total counts min^{-1}(w)) at each time was calculated by

multiplying by the appropriate flux box volume. Whenever a step occurred, individual urination burst volumes (V_{burst}) were calculated as:

$$V_{burst} = \frac{(\text{total counts min}^{-1}(\text{w}))_n - (\text{total counts min}^{-1}(\text{w}))_{n-1}}{\text{counts min}^{-1}(\text{u})}. \quad (1)$$

UFR and GFR were then calculated as:

$$\text{UFR} = \frac{\Sigma V_{burst}}{\text{mass} \times \text{time}}, \quad (2)$$

$$\text{GFR} = \frac{\text{UFR} \times \text{counts min}^{-1}(\text{u})}{\text{counts min}^{-1}(\text{p})}. \quad (3)$$

The extra-renal clearance rate (ECR) was estimated as:

$$\text{ECR} = \frac{\text{total counts min}^{-1}(\text{w}) - \Sigma V_{burst} \times \text{counts min}^{-1}(\text{u})}{\text{counts min}^{-1}(\text{p}) \times \text{mass} \times \text{time}}, \quad (4)$$

which assumed that any appearance of [^{3}H]PEG-4000 that did not occur in steps (i.e. the overall rate of slow rise during plateau phases – see Fig. 1) represented extra-renal excretion. In series i, urine was collected volumetrically from trout with internal urinary catheters, while in series iii the pattern of urination was recorded directly using optical switches in fish fitted with either internal or external catheters. In both cases, UFR was calculated from the cumulative collected volume:

$$\text{UFR} = \frac{\Sigma V_{total}}{\text{mass} \times \text{time}} \quad (5)$$

and the urinary excretion rate (U) of any substance (X) was given by:

$$U_x = [X]_u \times \text{UFR}. \quad (6)$$

The UFR values calculated in series ii using equation 2 were similarly applied to the urine ionic concentration values ($[X]_u$) determined by spot sampling in series i in order to estimate urinary excretion rates in non-catheterized fish.

In series iii, burst volumes (V_{burst} in ml) were calculated by multiplying the number of drops in a burst by the average drop volume, as detected by the optical switches. The percentage of UFR that occurred in bursts was calculated as:

$$\text{percentage in bursts} = \frac{\Sigma V_{burst}}{\Sigma V_{total}} \times 100\,\%. \quad (7)$$

In this series, GFR was calculated directly from the appearance of PEG-4000 in the urine using equation 3 and ECR was calculated directly from the appearance of PEG-4000 in the water:

$$\text{ECR} = \frac{\text{total counts min}^{-1}(\text{w})}{\text{counts min}^{-1}(\text{p}) \times \text{mass} \times \text{time}} \quad (8)$$

Statistical analysis

Results were expressed as means±1 S.E.M. (N) throughout. Statistical significance (P≤0.05) of differences was assessed using Student's two-tailed paired or unpaired t-tests as appropriate.

Results

Urine composition in non-catheterized vs *internally catheterized trout*

Series i demonstrated that the concentrations of the two major urinary electrolytes, Na^+ and Cl^-, were significantly lower, by 70 % and 35 %, respectively, in non-catheterized (spot-sampled) fish (Table 1). Ammonia concentration was also reduced by 40 %. Other substances were not significantly affected. Largely as a result of the differences in Na^+ and Cl^-, the total concentration of all measured substances was significantly lower in urine obtained by spot sampling. Total charge balance (i.e. Σcations−Σanions) was significantly positive for urine obtained by internal catheterization, suggesting the presence of 4–5 mequiv l^{-1} of unmeasured anions. In contrast, charge balance was not significantly different from zero for urine obtained by spot sampling. These results indicate that the composition of urine is modified during its normal residence in the urinary bladder, a process that is prevented by internal catheterization.

Table 1. *A comparison of ion concentrations in urine collected by internal bladder catheterization with those in urine obtained by spot sampling in series i*

Substance	Internally catheterized	Spot sampled
Na^+ (mmol l^{-1})	7.12±0.79 (16)	2.12±0.41 (15)†
K^+ (mmol l^{-1})	0.92±0.09 (16)	0.81±0.06 (15)
Ammonia (mmol l^{-1})	0.79±0.13 (16)	0.47±0.07 (16)†
Ca^{2+} (mmol l^{-1})	1.35±0.22 (16)	1.35±0.09 (16)
Mg^{2+} (mmol l^{-1})	0.50±0.11 (16)	0.79±0.10 (16)
Cl^- (mmol l^{-1})	5.21±0.34 (16)	3.35±0.23 (16)†
NO_3^{2-} (mmol l^{-1})	0.05±0.02 (16)	0.09±0.02 (16)
SO_4^{2-} (mmol l^{-1})	1.08±0.10 (16)	1.24±0.08 (16)
Phosphate (mmol l^{-1})	0.40±0.23 (16)	0.46±0.23 (16)
Urea (mmol l^{-1})	0.81±0.16 (15)	0.55±0.10 (15)
All measured substances (mmol l^{-1})	17.73±1.27 (15)	11.16±0.43 (14)†
Σcations−Σanions (mequiv l^{-1})*	4.34±1.04 (16)‡	0.69±0.57 (15)

Values are mean±1 S.E.M. (N).

* In this calculation, phosphate (in mequiv l^{-1}) was calculated based on an assumed urine pH of 7.2 (Wood, 1988) and pK of 6.8 (Wheatley *et al.* 1984).

† Significantly different from internally catheterized value (P≤0.05).

‡ Significantly different from zero (P≤0.05).

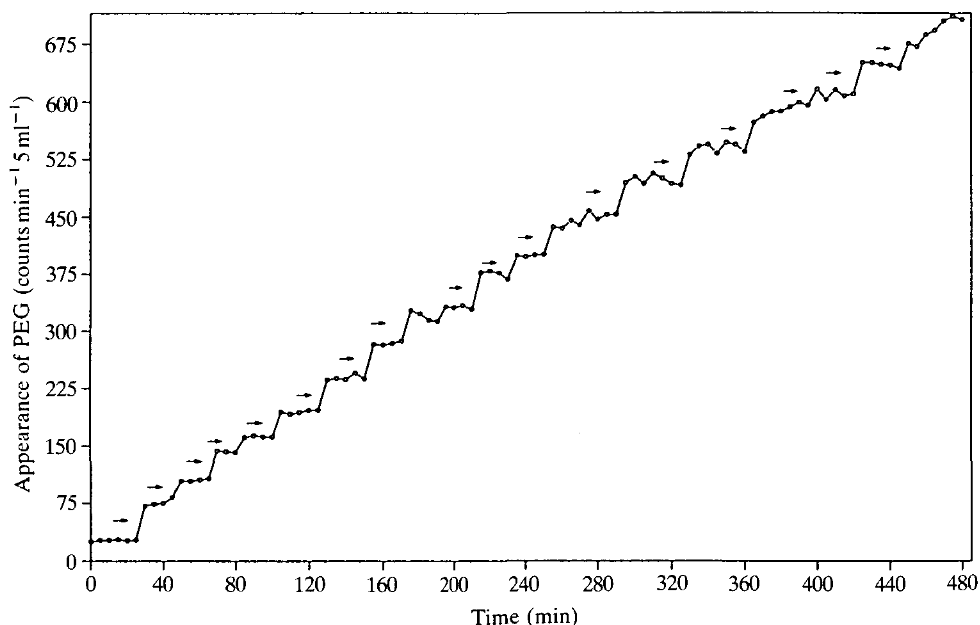

Fig. 1. A typical record of the appearance of PEG-4000 in the external water of a non-catheterized, naturally urinating trout (series ii) over an 8 h period. Sudden 'step' increases in water radioactivity, marked by arrows, were interpreted as bursts of voluntary urination.

Urination patterns of non-catheterized fish

Non-catheterized fish urinated in bursts, indicated by 'step' increases in water PEG-4000 radioactivity (e.g. Fig. 1). These steps were not synchronized in different fish tested simultaneously, indicating that they were not triggered by unknown outside disturbances. On average, fish urinated at very regular intervals of approximately 30 min (Table 2); in 12 trout the range of mean interval was 25.8–34.6 min. Assuming that all urination occurred in bursts, the calculated UFR for the non-catheterized fish of series ii (2.40 ml kg^{-1} h^{-1}) was significantly lower (by 23 %) than that directly measured in the internally catheterized fish of series i (3.11 ml kg^{-1} h^{-1}; N=19) (Table 2). The calculated V_{burst} for naturally urinating fish of 1.20 ml kg^{-1} (range 0.76–1.92) may be compared with a mean maximum volume of the urinary bladder of 2.20±0.19 ml kg^{-1} (range 1.50–2.80; N=6) and a mean urine volume obtained by spot sampling of 0.52±0.11 ml kg^{-1} (range 0.13–1.67; N=14). The maximum urinary volume obtained by spot sampling was 1.67 ml kg^{-1}.

The GFR determined from plasma PEG-4000 clearance through the urine in non-catheterized fish (4.52 ml kg^{-1} h^{-1}) was about twice the UFR (Table 2). This calculation assumed that all urination occurred in bursts; GFR thereby accounted for about 80 % of the total clearance, the remaining 20 % comprising the ECR. If some fraction of urine discharge did not occur in bursts, but continuously,

Table 2. *A comparison of urine flow rates (UFR) measured by internal bladder catheterization (series i) with those measured by the appearance of PEG-4000 in the water from non-catheterized fish (series ii). Glomerular filtration rate (GFR), urination pattern and extra-renal clearance rate (ECR) of PEG-4000 are also shown for the latter.*

	Internally catheterized (N=19)	Non-catheterized (N=12)
Mass (g)	320.8±17.4	324.4±9.6
UFR ($ml\,kg^{-1}\,h^{-1}$)	3.11±0.22	2.40±0.29*
GFR ($ml\,kg^{-1}\,h^{-1}$)	–	4.52±0.50
Burst volume ($ml\,kg^{-1}$)	–	1.20±0.14
Interval (min)	–	29.82±0.90
ECR ($ml\,kg^{-1}\,h^{-1}$)	–	1.15±0.20

Values are means±1 S.E.M.
* Significantly different from internally catheterized value ($P \leq 0.05$).

UFR and GFR will have been underestimated and ECR proportionately overestimated. However, the present figures are supported by the direct measurements of UFR, GFR and ECR in the externally catheterized fish of series iii (see below).

Urinary excretion rates in non-catheterized vs *internally catheterized trout*

Excretion rates were estimated for non-catheterized fish by applying the mean UFR value obtained from PEG-4000 fluxes in series ii to the measured ion concentrations of urine obtained by spot sampling in series i. The resulting estimates were compared to the directly measured excretion rates for internally catheterized fish in series i (Fig. 2). Urinary excretion rates were significantly lower in non-catheterized fish for Na^+ (by 79 %), K^+ (by 35 %), ammonia (by 58 %), Cl^- (by 51 %) and urea (by 43 %). The total excretion rate of all measured substances in non-catheterized trout (26.8±1.0 $\mu mol\,kg^{-1}\,h^{-1}$; N=14) was less than half (47 %) that (57.5±6.9 $\mu mol\,kg^{-1}\,h^{-1}$; N=15) in fish fitted with internal bladder catheters, despite only a 23 % difference in UFR. The urinary bladder clearly plays a significant role in reabsorption *in vivo*.

Urine composition in externally catheterized vs *internally catheterized trout*

In series iii, external catheters were employed to collect ‘naturally’ vented urine in one group, while internal bladder catheters were used in a second group. In agreement with series i (see Table 1), urinary Cl^- concentration was significantly lower in externally catheterized fish (Table 3). However, urinary Na^+ concentration was not significantly lower ($0.10>P>0.05$), and there were also no differences in ammonia, the total concentration of measured substances, or Σcations$-\Sigma$anions. Except for a higher NO_3^{2-} concentration, the urine composition of the internally catheterized fish of series iii was virtually identical to that of

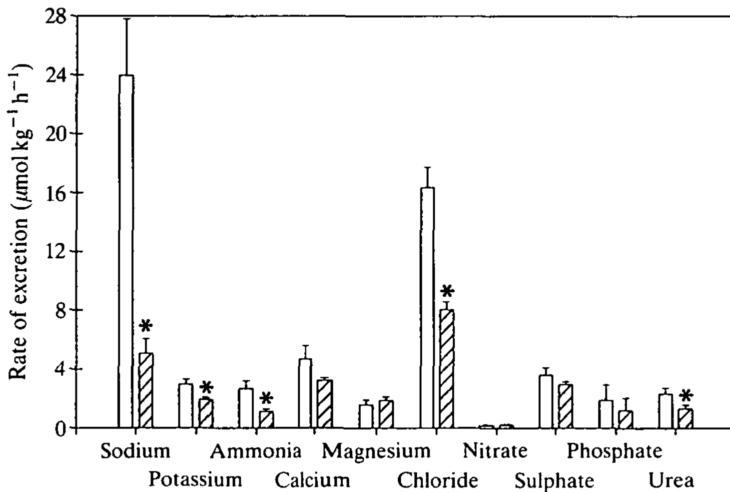

Fig. 2. A comparison of the measured rates of urinary ion excretion from fish fitted with internal bladder catheters (open columns; N=16; series i) with the estimated rates for non-catheterized fish (cross-hatched columns; N=16; data from series i and ii combined; see text for details). Means±1 S.E.M. An asterisk indicates a significant difference between internally catheterized and non-catheterized values ($P \leq 0.05$).

Table 3. *A comparison of ion concentrations in urine collected by internal bladder catheterization with those in urine obtained by external catheterization in series iii*

Substance	Internally catheterized	Externally catheterized
Na^+ (mmol l^{-1})	6.73±0.66 (9)	4.84±0.74 (9)
K^+ (mmol l^{-1})	0.69±0.09 (10)	0.56±0.08 (9)
Ammonia (mmol l^{-1})	0.72±0.13 (10)	0.67±0.07 (9)
Ca^{2+} (mmol l^{-1})	1.09±0.13 (10)	1.45±0.13 (8)
Mg^{2+} (mmol l^{-1})	0.48±0.11 (10)	0.46±0.04 (9)
Cl^- (mmol l^{-1})	6.46±0.38 (9)	4.79±0.44 (10)†
$NO_3{}^{2-}$ (mmol l^{-1})	0.28±0.04 (9)	0.23±0.03 (10)
$SO_4{}^{2-}$ (mmol l^{-1})	0.99±0.11 (9)	0.92±0.07 (10)
Phosphate (mmol l^{-1})	0.25±0.05 (9)	0.17±0.05 (10)
Urea (mmol l^{-1})	0.92±0.08 (10)	0.92±0.06 (9)
All measured substances (mmol l^{-1})	18.61±1.34 (9)	15.72±1.08 (8)
Σcations$-\Sigma$anions (mequiv l^{-1})*	1.90±0.63 (9)	2.27±0.60 (8)

Values are means±1 S.E.M. (N).

* In this calculation, phosphate (in mequiv l^{-1}) was calculated based on an assumed urine pH of 7.2 (Wood, 1988) and pK of 6.8 (Wheatley *et al.* 1984).

† Significantly different from internal value ($P \leq 0.05$).

Table 4. *A comparison of urine flow rates (UFR), glomerular filtration rates (GFR), urination patterns and extra-renal clearance rates (ECR) of PEG-4000 measured in internally and externally catheterized fish of series iii*

	Internally catheterized	Externally catheterized
Mass (g)	340.0±18.7 (11)	330.0±14.4 (10)
UFR ($ml\,kg^{-1}\,h^{-1}$)	2.53±0.12 (11)	2.01±0.17 (10)*
GFR ($ml\,kg^{-1}\,h^{-1}$)	4.40±0.27 (8)	4.05±0.48 (7)
Burst volume ($ml\,kg^{-1}$)	–	0.45±0.06 (10)
Burst %	11.8±4.5 (11)	64.7±5.6 (10)*
Interval (min)	–	21.0±2.4 (10)
ECR ($ml\,kg^{-1}\,h^{-1}$)	0.61±0.17 (8)	0.68±0.16 (6)

Values are means±1 S.E.M.
* Significantly different from internally catheterized value ($P \leq 0.05$).

the comparable group in series i (cf. Tables 1, 3), indicating that the intestinal ligation and other differences in procedure between series i and iii did not confound the results.

Urination patterns of externally vs *internally catheterized fish*

The UFR ($2.01\,ml\,kg^{-1}\,h^{-1}$) of externally catheterized trout in series iii was significantly lower (by 21 %) than that ($2.53\,ml\,kg^{-1}\,h^{-1}$) of internally catheterized fish (Table 4). This compared well with the difference (23 %) between non-catheterized fish and internally catheterized fish in series i and ii (Table 2). GFR ($4.0–4.5\,ml\,kg^{-1}\,h^{-1}$) was not significantly different between externally and internally catheterized trout in series iii (Table 4) or non-catheterized trout in series ii (Table 2). Thus, differences in UFR between groups were not due to differences in GFR. GFR accounted for 86 % of the total PEG-4000 clearance in externally catheterized fish, not significantly different from 88 % in internally catheterized fish (Table 4) and 80 % in non-catheterized trout (Table 2). ECR values were therefore comparable between groups.

Fig. 3 displays typical records of urination pattern from internally and externally catheterized trout of series iii, as directly recorded *via* infra-red optical switches. Only 12 % (0–10 % in most fish) of the total UFR occurred in bursts in internally catheterized trout (Table 4). Urine simply dripped from the catheter at an even rate for most of the time. In contrast, 65 % (range 35–92 % in 10 trout) of the total UFR occurred in discrete bursts in externally catheterized trout. As in series ii, these bursts were asynchronous in different trout tested at the same time. Average V_{burst} was $0.45\,ml\,kg^{-1}$ (range 0.25–0.85), considerably lower than that ($1.20\,ml\,kg^{-1}$; Table 2) estimated in non-catheterized fish of series ii. The mean interval was about 21 min (range 9.7–31.3), again significantly lower than the 30 min measured in series ii. Thus, relative to the PEG-4000 flux approach, the external catheterization technique suggested that urination occurred more frequently, in bursts of smaller volume, with some non-burst urination.

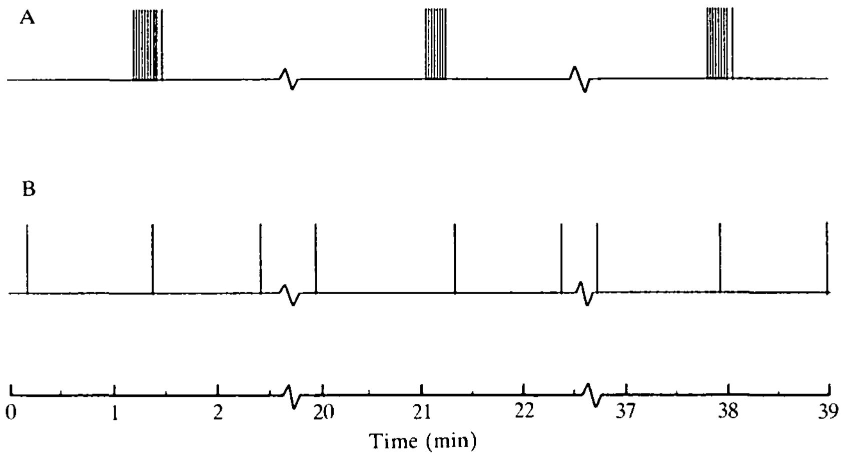

Fig. 3. A typical record of the urlnation pattern of an externally catheterized fish (A) and an internally catheterized fish (B). Each drop of urine was recorded as a spike on the recording (see text for details). During the first and second breaks in the record, there was no urination in A, but in B there were 12 and 11 widely spaced drops, respectively.

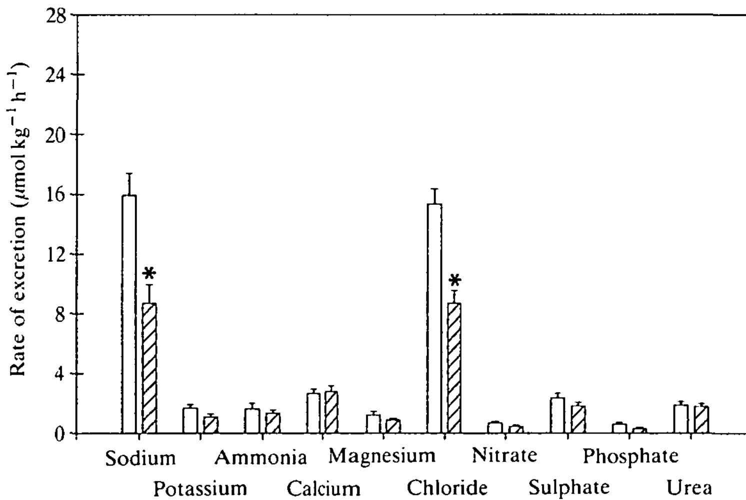

Fig. 4. A comparison of the measured rates of urinary ion excretion from fish fitted with internal bladder catheters (open columns; N=10) with those from fish fitted with external catheters (cross-hatched columns; N=9) in series iii. Values are means±1 S.E.M. An asterisk indicates a significant difference between internally and externally catheterized fish ($P\leq 0.05$).

Urinary excretion rates in externally catheterized vs *internally catheterized trout*

Directly measured excretion rates in externally catheterized trout of series iii were significantly lower for both Na^+ (by 45 %) and Cl^- (by 43 %) (Fig. 4).

Excretion rates of other substances were not significantly different. The total excretion rate of all measured substances in externally catheterized fish ($33.3 \pm 2.8\,\mu mol\,kg^{-1}\,h^{-1}$; N=8) was only about 75% of that ($44.3 \pm 3.3\,\mu mol\,kg^{-1}\,h^{-1}$; N=9) of internally catheterized fish. Qualitatively, these results are in broad agreement with those of series i and ii (see Fig. 2) and reinforce the conclusion that the urinary bladder plays a significant role in reabsorption *in vivo*.

Discussion

Internal catheter measurements

Direct recording demonstrated that urine was drained more or less continuously by the internal catheter, and only occasionally appeared in bursts (Fig. 3, Table 4). In general, the present urinary flow values (Tables 2, 4) and compositions (Tables 1, 3) compared favourably with the results of previous researchers using the same methods (Holmes and Stainer, 1966; Hunn, 1969; Hunn and Willford, 1970; Hofmann and Butler, 1979; Elger and Hentschel, 1983; Giles, 1984; Wheatley *et al.* 1984; Oikari and Rankin, 1985; Elger *et al.* 1986; Erickson and Gingerich, 1986; Wood, 1988). One exception was SO_4^{2-}, which was present at about $1\,mmol\,l^{-1}$ compared to a reported value of $2.9 \pm 0.4\,mmol\,l^{-1}$ (Oikari and Rankin, 1985); the reason for this difference is unknown.

[^{3}H]PEG-4000 was a highly reliable GFR marker, with at least 80% of its excretion occurring through the urinary system, supporting the findings of Beyenbach and Kirschner (1976) obtained on seawater trout. This reliability was further demonstrated by almost identical GFR values in internally, externally and non-catheterized fish (Tables 2, 4); PEG-4000 was probably neither reabsorbed nor secreted by the bladder epithelium. The present mean GFR values ($4.40 \pm 0.27\,ml\,kg^{-1}\,h^{-1}$) were generally lower than those ($5-11\,ml\,kg^{-1}\,h^{-1}$) recorded in previous studies on freshwater rainbow trout using a variety of different GFR markers (Holmes and Stainer, 1966; Elger and Hentschel, 1983; Oikari and Rankin, 1985; Elger *et al.* 1986) including PEG-4000 (Erickson and Gingerich, 1986). These lower values are attributed to relatively lower levels of stress, resulting from generally longer recovery times (48 h) and the absence of arterial catheterization in the present study (B. J. Curtis and C. M. Wood, unpublished results).

GFR values in the present study were calculated using [^{3}H]PEG-4000 concentrations in plasma extracted at the end of each 8 h experiment. However, this did not overestimate GFR, since more recent experiments have shown that plasma [^{3}H]PEG-4000 concentrations drop by less than 3.5% over an 8 h period (3.52 and 3.44% in internally and externally catheterized fish, respectively; B. J. Curtis and C. M. Wood, unpublished results).

Indirect and direct measurements of natural urination

The two methods were in broad general agreement. Both demonstrated that

rainbow trout urinate intermittently in bursts at 20–30 min intervals, that the natural UFR is at least 20 % lower, and the natural urinary excretion rates of Na^+ and Cl^- at least 40 % lower, than those determined by the traditional internal catheter technique. Thus, urine is clearly stored in the bladder for some time prior to discharge, and significant reabsorption occurs during this period. As the relative reduction in ion excretion rates is larger than that in UFR, the effectiveness of the entire renal system in freshwater ionoregulation is improved when the bladder is allowed to function normally. Previous studies using the traditional internal catheter method, which prevents this normal function of the bladder, have therefore underestimated the ionoregulatory effectiveness of the entire renal system.

The indirect approach (combination of spot sampling and PEG-4000 appearance in the water; Fig. 2) indicated a greater effectiveness of the bladder in ion conservation than did the direct approach (the new external catheterization method; Fig. 4). Which technique produced the more accurate picture of the natural situation is unknown, though we favour the indirect approach for the following reasons.

It is possible that small bursts or slow seepage of urine may have been missed in the non-catheterized fish, leading to an underestimate of UFR and ion excretion rates. This could account for the larger average V_{burst} and interval seen in non-catheterized than in externally catheterized trout (cf. Tables 2, 4). Nevertheless, estimated UFR was certainly not lower in non-catheterized fish, and the larger average V_{burst} appeared more reasonable relative to other indices of bladder storage capacity (see below). Furthermore, any underestimate of ion excretion rates was probably counteracted by the fact that spot sampling collects urine at random times during the urination cycle. Since the overall trend is for ion reabsorption during the residence period, true concentrations in vented urine must be even lower than indicated in Table 1. On balance, we feel this latter factor, combined with the more reasonable size for V_{burst}, means that the indirect approach is quantitatively more realistic. In the direct method, the slight negative pressure (−0.29 kPa) applied to the external catheter may have interfered with the competence of the urinary sphincters, causing them to open at a lower internal bladder pressure. This would explain the 35 % of UFR that did not occur in bursts, the much smaller average V_{burst} and the shorter interval seen in externally catheterized fish. The net effect would be to over-estimate UFR and ion excretion rates by reducing urine residence time in the bladder.

Ion reabsorption by the urinary bladder

The net rates of Na^+ reabsorption by the bladder *in vivo* calculated using data from the indirect (Fig. 2) and direct approaches (Fig. 4) were approximately 19 and 7 $\mu mol\,kg^{-1}\,h^{-1}$, respectively. The corresponding values for Cl^- absorption were about 8 and 5 $\mu mol\,kg^{-1}\,h^{-1}$. Assuming a bladder surface area of about 7.5 $cm^2\,kg^{-1}$ (based on a filled volume of about 2 $ml\,kg^{-1}$), these rates would

translate to 0.7–2.5 μmol $cm^{-2} h^{-1}$. *In vitro*, reported net mucosal-to-serosal Na^+ and Cl^- transport rates (with isotonic conditions on both sides) were 1.5–2.8 μmol $cm^{-2} h^{-1}$ (Fossat *et al.* 1974; Fossat and Lahlou, 1979*a*; Marshall, 1988). Thus, despite the large urine-to-plasma concentration gradients opposing transport *in vivo*, reabsorption is almost as effective as in the absence of gradients *in vitro*.

Urine residence time in the bladder

The significance of the mean residence time (T_r) lies in the kinetics of the transport mechanism. In a static system, clearly the longer the T_r, the greater the net reabsorption. However, the bladder is a dynamic system, with continuous entry of new urine from the ureteral ducts and periodic discharge of processed urine. Here the advantages of longer T_r are more subtle. The greater the T_r, the larger will be the mean operating volume of the bladder and, therefore, the surface area involved in transport. Furthermore, for a given net reabsorption rate, the longer the T_r, the lower the fluctuation in concentration to which the transport sites are exposed and, presumably, the more efficient the system.

It is possible to estimate T_r assuming a constant flow rate of urine down the mesonephric ducts, assuming that all discharge of urine from the bladder occurs in bursts, and using average values for burst volume (V_{burst}) and interval between urinations (T_i):

$$T_r = \frac{T_i}{2} \times \frac{(V_r + V_t)}{(V_{burst})}, \quad (9)$$

where V_r is the average residual volume left in the bladder after a bout of urination and V_t ($=V_r+V_{burst}$) is the average total volume in the bladder immediately before each bout.

Although the present study provides measurements of T_i and V_{burst} measured by both indirect (Table 2) and direct methods (Table 4), the difficulty lies in determining the correct value for V_r and/or V_t. However, the measurements of maximum urinary bladder volume (2.20 ml kg^{-1}) and volumes obtained by spot-sampling provide some guidance. V_t must clearly be less than 2.20 ml kg^{-1}, for it is unlikely that this maximum possible distension would ever occur *in vivo*. A more reasonable estimate would be 1.67 ml kg^{-1} (the largest volume obtained by spot sampling); the difference between this figure and V_{burst} measured by the indirect method (1.20 ml kg^{-1}; Table 2) would yield V_r=0.47 ml kg^{-1}. Applying these figures, plus values for T_i and V_{burst} obtained by the indirect method (Table 2), to equation 9 yields an estimate of 26.6 min for T_r. This suggests that mean residence time is approximately the same as the burst interval (29.8 min). If the values obtained by the direct method from externally catheterized fish (Table 4) were used instead, T_r would be much longer, 67.4 min, but this seems unreasonable because the small V_{burst} would result in a value for V_r of 1.22 ml kg^{-1}, considerably greater than the mean volume obtained by spot sampling (0.52 ml kg^{-1}).

The significance of urinary bladder function in vivo

To conclude, in resting freshwater rainbow trout, the urinary bladder stores urine for 25–30 min prior to discharge in bursts, and probably always retains at least a small residual volume. Periodic, rather than continuous, discharge of urine may be advantageous in avoiding olfactory signals to potential predators. Intermittent urination clearly also plays a significant role in normal ionoregulation. During the holding period, the bladder actively reabsorbs Na^+ and Cl^- and possibly other substances, thereby aiding the function of the kidney in salt conservation. On a quantitative basis, this reabsorption by the bladder ($<20\,\mu mol\,kg^{-1}\,h^{-1}$) amounts to only a small percentage of the total NaCl reabsorption by the kidney (approx. $500\,\mu mol\,kg^{-1}\,h^{-1}$). Nevertheless, the net result of this final 'scavenging' is to cut urinary NaCl losses by at least 40 %. In turn, this will reduce by a comparable amount the need for net Na^+ and Cl^- uptake from the water at the gills, where the gradients opposing uptake, and therefore the costs, are undoubtedly greater than in the bladder.

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