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METHODS & TECHNIQUES

Measuring gill paracellular permeability with polyethylene glycol-4000 in freely swimming trout: proof of principle

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ABSTRACT

The influence of swimming activity on gill paracellular permeability has not been measured previously in fishes. We critically assessed the use of tritium-labeled polyethylene glycol ([^{3}H]PEG-4000) for this purpose, a substance that is also a classic marker for extracellular fluid volume, glomerular filtration rate and drinking rate. Tests (8 h) on resting freshwater trout showed that when measuring [^{3}H]PEG-4000 clearance from the plasma in the efflux direction, correction for a large excretion via glomerular filtration was essential, necessitating urinary catheterization. When measuring [^{3}H]PEG-4000 clearance from the water in the influx direction, correction for a significant uptake by drinking was essential, necessitating terminal gut removal, whereas glomerular filtration losses were minimal. After correction for these alternate routes of loss and uptake, [^{3}H]PEG-4000 clearance rates by efflux from the plasma and by influx from the water were identical, showing that gill paracellular permeability is not rectified, and can be measured in either direction. The influx technique with terminal gut removal was used to assess gill paracellular permeability in trout without urinary catheters freely swimming at 1.2 body lengths s^{-1} for 8 h. Branchial [^{3}H]PEG-4000 clearance rate (by influx from the water) increased significantly by ~80% in accord with a similar measured increase in O_2 consumption rate. Thus in trout, gill paracellular permeability does increase during exercise, in accord with the traditional concept of the osmorespiratory compromise.

KEY WORDS: Osmorespiratory compromise, Branchial fluxes, Drinking rate, Glomerular filtration rate, Exercise

INTRODUCTION

Recent studies (Sollid and Nilsson, 2006; Henriksson et al., 2008; Wood et al., 2009; Iftikar et al., 2010) have demonstrated renewed interest in the 'osmorespiratory compromise' (Randall et al., 1972; Wood and Randall, 1973a; Wood and Randall, 1973b; Wood and Randall, 1973c; Stevens, 1972; Hofmann and Butler, 1979; Gonzalez and McDonald, 1992; Gonzalez and McDonald, 1994; Postlethwaite and McDonald, 1995). Under conditions requiring improved oxygen uptake ($\dot{M}_{O_2}$; e.g. exercise, hypoxia), the permeability of the teleost gill to respiratory gases increases via elevated functional surface area and/or decreased mean water-to-blood diffusion distance, resulting in unfavourable net ion and water fluxes. It is commonly assumed that these passive fluxes occur via the paracellular pathway (i.e. tight junctions in the branchial epithelium), but gill paracellular permeability has never been measured in exercising fish *in vivo*. One experimental assessment in hypoxia (Wood et al., 2009) found no change in branchial permeability (measured in the efflux direction) to polyethylene glycol MW-4000 [PEG-4000, a classic gill paracellular marker (e.g. Wood and Part, 1997; Chasiotis and Kelly, 2011)]. Two recent zebrafish studies (Kumai et al., 2011; Kwong et al., 2013) reported conflicting relationships between Na^+ fluxes and gill permeability (in the influx direction) to a smaller molecule (PEG-400). PEG-4000 is also a classic marker of drinking rate (e.g. Shehadeh and Gordon, 1969) and glomerular filtration rate (GFR) (e.g. Beyenbach and Kirschner, 1976), both of which can confound gill permeability measurements. In the present study, we critically assess the handling of tritium-labeled polyethylene glycol ([^{3}H]PEG-4000) in resting rainbow trout *in vivo* in order to address these complications, determine whether gill paracellular permeability is the same in influx and efflux directions, and test whether exercise increases gill paracellular permeability in freely swimming trout.

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RESULTS AND DISCUSSION

The Efflux experiment refers to trials in which the fish were caudally injected with [^{3}H]PEG-4000, and branchial and renal effluxes of [^{3}H]PEG-4000 were monitored, whereas Influx experiments were those in which the uptake of [^{3}H]PEG-4000 from the external water into the fish was measured. All experiments lasted 8 h, based on preliminary trials in which this period offered the best resolution of gill permeabilities, urinary excretion rates, and drinking rates. A key objective was to establish whether [^{3}H]PEG-4000 permeability was rectified in resting trout, i.e. whether it exhibited the same clearance rate in the influx and efflux directions. A second goal was to determine to what degree it was necessary to correct for fluxes of [^{3}H]PEG-4000 that did not cross the gills but rather passed by renal excretion (assessed in Efflux and Influx-1 experiments) or drinking uptake (assessed in the Influx-2 experiment).

Urine flow rates (UFR) and GFR measured via bladder cannulation were not significantly different between the Efflux and Influx-1 experiments (Table 1), and were very similar to previous rates reported in larger trout (e.g. Wood and Randall, 1973c; Hofmann and Butler, 1979; Curtis and Wood, 1991) when appropriately scaled by Eqn 6 (see Materials and methods). In the Efflux experiment, approximately equal rates of [^{3}H]PEG-4000 excretion occurred via the branchial route to the water, and via glomerular filtration to the urine over 8 h (Fig. 1A), demonstrating the need to correct for renal excretion. Curtis and Wood (Curtis and Wood, 1991) and Scott et al. (Scott et al., 2004) reached similar conclusions using indirect methods. In the Influx-1 experiment, where the external water was dosed with [^{3}H]PEG-4000, only ~13% of the influxed counts per minute (cpm) appeared in the urine, while 87% stayed in the fish at 8 h (Fig. 1B), so the need for urinary correction is much smaller. The Influx-2 experiment evaluated whether part of the measured [^{3}H]PEG-4000 uptake into the body occurred via the gut. These fish were not catheterized, to minimize stress-induced drinking (Fuentes and Eddy, 1997). Radioactivity

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List of symbols and abbreviations

[^{3}H]PEG-4000	tritium-labeled polyethylene glycol, molecular weight = 4000
ECFV	extracellular fluid volume
GFR	glomerular filtration rate
$\dot{M}_{O_2}$	oxygen consumption rate
P_{O_2}	partial pressure of oxygen
UFR	urine flow rate

found in the gastrointestinal tract after 8 h was surprisingly high (24.2% of the whole-body uptake; Fig. 1C), demonstrating the need to correct for uptake by drinking; a similar conclusion was reached by Kwong et al. (Kwong et al., 2013).

These three experiments yielded three separate estimates of gill PEG-4000 permeability in resting trout, expressed as clearance rates (Fig. 2). The mean value determined in the Efflux experiment did not differ significantly from those measured in the opposite direction in the Influx-1 and Influx-2 experiments, averaging ~0.230 ml 20 g^{-1} h^{-1} or 11.5 ml kg^{-1} h^{-1} overall, demonstrating that [^{3}H]PEG-4000 permeability is not rectified. Note that in the Efflux experiment, efflux permeability was measured directly by branchial [^{3}H]PEG-4000 clearance from plasma to water in urinary-catheterized fish. In the Influx-1 experiment, influx permeability was calculated from the sum of appearance rates of [^{3}H]PEG-4000 from water into body and urine, and then corrected by subtracting the estimated uptake rate via drinking. This was done by reducing the whole-body [^{3}H]PEG-4000 component by 24.2% for each fish, based on the distribution of cpm found in the Influx-2 experiment (see Fig. 1C), or by subtracting the mean drinking rate measured in Influx-2 from the total clearance rate. The results differed by less than 3%. In the Influx-2 experiment, the gut was removed prior to analysis (see Materials and methods), so influx permeability was calculated from the appearance of [^{3}H]PEG-4000 in the remaining carcass over 8 h plus the estimated loss of cpm to the urine, based on the distribution of cpm determined in the Influx-1 experiment (see Fig. 1B).

The overall conclusion from these trials with resting trout was that gill [^{3}H]PEG-4000 permeability was not rectified. It seems unlikely that permeability would become rectified during exercise. Therefore, the influx technique with terminal gut removal was employed to assess gill paracellular permeability in trout without urinary catheters freely swimming at 1.2 body lengths (BL) s^{-1} for 8 h. Preliminary trials determined that at this velocity, trout would swim continuously for 8 h and exhibit a sustained increase in $\dot{M}_{O_2}$.

Continuous exercise elevated branchial [^{3}H]PEG-4000 permeability significantly by 81%, as estimated by PEG-4000 clearance rates from the water, from 0.262 ml 20 g^{-1} h^{-1} (13.10 ml kg^{-1} h^{-1}) to 0.475 ml 20 g^{-1} h^{-1} (23.75 ml kg^{-1} h^{-1}; Fig. 3A). This assumes no change in GFR during exercise, but if GFR had increased by 23.1% as reported by Hofmann and Butler (Hofmann and Butler, 1979) for the same $\dot{M}_{O_2}$ elevation, then permeability would have increased by

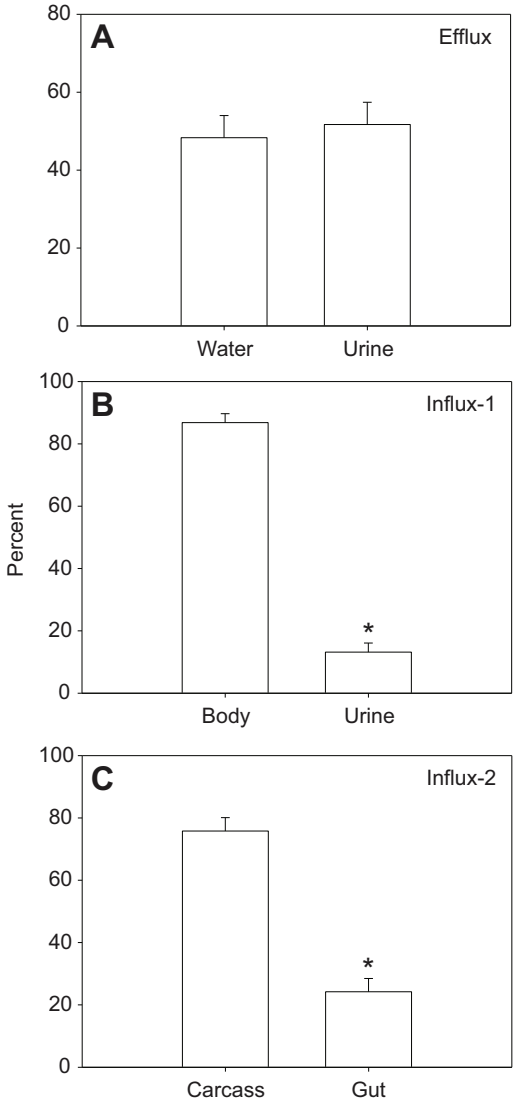

Fig. 1. Relative fate of [^{3}H]PEG-4000 cpm after 8 h in three experimental series on resting trout at 18°C. (A) Efflux series in which [^{3}H]PEG-4000 was injected into fish fitted with urinary bladder cannulae (*N*=12). The percent of the total [^{3}H]PEG-4000 cpm excreted to the water and in the urine is shown. (B) Influx-1 series in which [^{3}H]PEG-4000 uptake from the water into the whole body and urine was monitored in fish fitted with urinary bladder cannulae (*N*=14). The percent of the total [^{3}H]PEG-4000 cpm recovered in the whole body and in the excreted urine is shown. (C) Influx-2 series in which [^{3}H]PEG-4000 uptake from the water into the gastrointestinal tract (gut) and remaining carcass was monitored in non-cannulated fish (*N*=8). The percent of the total [^{3}H]PEG-4000 cpm recovered in the gut and in the remaining carcass is shown. Data are means ± s.e.m. Asterisks indicate significant differences (*P*<0.05), checked both by chi square and paired Student's *t*-tests on arcsin-transformed data.

Table 1. Glomerular filtration (GFR) and urine flow rates (UFR) over 8 h experimental periods at 18°C in resting trout fitted with urinary bladder catheters in Efflux (*N*=12) and Influx-1 (*N*=14) experiments

	ml 20 g h^{-1}		ml kg^{-1} h^{-1}	
	Efflux series	Influx-1 series	Efflux series	Influx-1 series
GFR	0.210±0.028	0.185±0.061	10.50±1.40	9.25±3.05
UFR	0.138±0.018	0.096±0.021	6.90±0.90	4.70±1.04

Data are means ± s.e.m.
No significant differences between comparable Efflux and Influx-1 means (*P*>0.05) by Student's unpaired two-tailed *t*-test.

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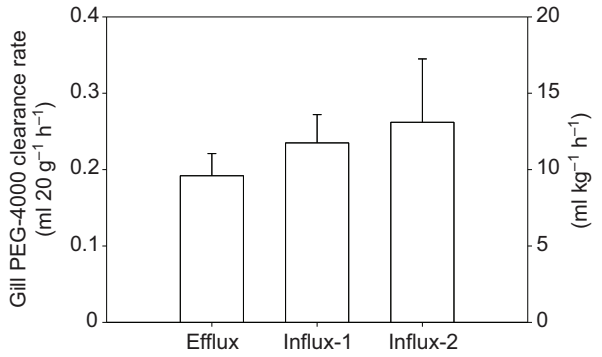

Fig. 2. Branchial [^{3}H]PEG-4000 permeabilities, expressed as gill clearance rates, in resting trout at 18°C in the same three experimental series as in Fig. 1: Efflux (*N*=12), Influx-1 (*N*=14) and Influx-2 (*N*=8). Data are means ± s.e.m. Note that the right-hand *y*-axis translates the units to the more traditional per kilogram basis. There were no significant differences (*P*>0.05, one-way ANOVA), indicating that gill permeability to PEG-4000 is not rectified.

88%. These increments are very similar to the significant 75% increase in mean $\dot{M}_{O_2}$ measured in these same fish (Fig. 3B); metabolic rates were stable in both resting and swimming fish between 0 and 1, 3.5–4.5 and 7–8 h). Drinking rates, as estimated by [^{3}H]PEG-4000 accumulation rates in the gut, were 0.066 ml 20 g^{-1} h^{-1} (3.32 ml kg^{-1} h^{-1}; Fig. 3C) at rest, and were not significantly altered by 8 h of continuous exercise.

In summary, these experiments have shown that gill paracellular permeability can be measured in either the efflux or influx directions, but in the former, urinary catheterization is essential to avoid the confounding effect of glomerular filtration. For freely swimming fish, where it is desirable to minimize the stress and hydraulic drag of catheterization, the influx technique is preferable and only minor correction for glomerular filtration is required in experiments of this duration. [^{3}H]PEG-4000 uptake by drinking does not increase during exercise, but is sufficiently high even at rest that gut removal must be employed to avoid substantial errors. Finally, the current data support the traditional view (see Introduction) that paracellular permeability increases in proportion to $\dot{M}_{O_2}$ during exercise.

MATERIALS AND METHODS

Animals and experiments

Rainbow trout (*Oncorhynchus mykiss* Walbaum; 15–30 g, ~12 cm) from Humber Springs Trout Farm (Orangeville, ON, Canada) were held at ~18°C, 12 h:12 h light:dark photoperiod, in 500 l flow-through tanks containing Hamilton dechlorinated tap water ([Na^+]=0.6 mmol l^{-1}, [Cl^-]=0.8 mmol l^{-1}, [Ca^{2+}]=0.9 mmol l^{-1}, [Mg^{2+}]=0.15 mmol l^{-1}, [K^+]=0.05 mmol l^{-1}; pH~8.0). Fish were fed every other day with a 10% body mass ration of five-point trout pellets (Martin Mills Inc., Elmira, ON, Canada) and fasted for 48 h prior to experimentation. Three experimental series were performed with resting fish at 18°C, each using radiolabeled [^{3}H]PEG-4000 (Sigma-Aldrich, St Louis, MO, USA). In all series, the experimental periods were 8 h, water samples (5 ml) were taken at 2 h intervals throughout for measurement of water [^{3}H]PEG-4000 radioactivity, after which fish were killed by overdose of MS-222 (Syndel Labs, Parksville, BC, Canada). A terminal blood sample taken by caudal puncture was immediately centrifuged (5000 ***g***, 2 min) for measurement of plasma [^{3}H]PEG-4000 concentration. The whole body (after gut removal in Influx-2) was immediately washed in clean water to remove any superficial [^{3}H]PEG-4000, and then processed for [^{3}H]PEG-4000 determination. All procedures were approved by the McMaster University Animal Research Ethics Board and were in accordance with the Guidelines of the Canadian Council on Animal Care.

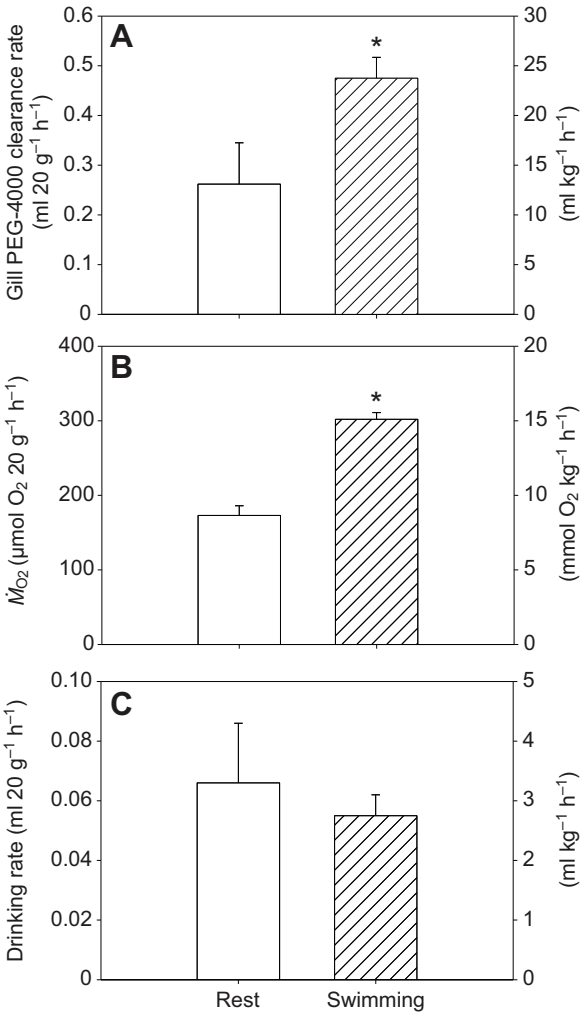

Fig. 3. Gill PEG-4000 clearance and oxygen consumption significantly increase with swimming, whereas drinking rate remains unchanged. The influence of rest (*N*=8) versus continuous swimming (*N*=15) over an 8 h period in trout at 18°C on: (A) branchial [^{3}H]PEG-4000 permeabilities, expressed as gill clearance rates in the influx direction; (B) oxygen consumption rates ($\dot{M}_{O_2}$); and (C) drinking rates. Data are means ± s.e.m. Note that the right-hand *y*-axis translates the units to the more traditional per kilogram basis. Asterisks indicate significant differences (*P*<0.05) assessed by Student's two-tailed unpaired *t*-tests.

The Efflux experiment focused on [^{3}H]PEG-4000 permeability of the gills in the efflux direction, and the extent of urinary PEG-4000 losses with this approach. Fish were fitted with internal urinary bladder catheters while under anaesthesia (0.1 g l^{-1} MS-222 at circumneutral pH) on an operating table as described by Wood and Patrick (Wood and Patrick, 1994), with modifications for their small size (~20 g). Catheters were fashioned from PE10 tubing, with PE60 sleeves (Clay-Adams™, Becton Dickinson, Franklin Lakes, NJ, USA) attached by veterinary cyanoacrylate glue (Vetbond™, 3M Corporation, London, ON, Canada) and anchored to the body wall by silk sutures. Trout were transferred to custom-made plastic chambers containing 2440 ml of continuously aerated water for a 24 h recovery; urine flow (siphon head = 3 cm H_2O) was monitored throughout to ensure catheter patency. Movement was restricted by an internal plastic enclosure which prevented the fish from tangling its catheter.

After 24 h, trout that had working catheters were lightly anesthetized again, and injected via the caudal haemal arch with 1 µCi [^{3}H]PEG-4000 dissolved in 0.1 ml Cortland saline (Wolf, 1963). Another 16 h period, with continuous urine collection, allowed sufficient time for [^{3}H]PEG-4000 to equilibrate throughout the extracellular space (Munger et al., 1991). After 16 h, the 2440 ml was

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replaced with 500 ml of fresh water, after which an 8 h experimental period was immediately begun, together with a new urine collection.

The Influx-1 experiment focused on gill [^{3}H]PEG-4000 permeability in the influx direction, and the extent of urinary PEG-4000 losses with this approach. The initial protocol was identical to the Efflux experiment. Trout were fitted with urinary catheters, but were not injected with [^{3}H]PEG-4000. Instead, at the start of the 8 h experiment, the 2440 ml was replaced with 500 ml of fresh water containing 1 μCi [^{3}H]PEG-4000. After a 15 min mixing period, water sampling commenced.

The Influx-2 experiment again focused on gill [^{3}H]PEG-4000 permeability in the influx direction, and assessed whether any of the influx occurred via drinking. Non-cannulated fish were used to avoid stress-induced drinking (Fuentes and Eddy, 1997). The protocol was similar to that of the Influx-1 experiment. However, at the end of 8 h, a period short enough to avoid rectal excretion of ingested PEG-4000 (Wilson et al., 1996), the body cavity of the euthanized fish was opened, and the entire gastrointestinal tract (ligated at both ends to prevent content loss) was removed and analyzed for [^{3}H]PEG-4000 radioactivity versus the remaining carcass.

Based on results of these experiments, an influx approach was adopted to measure gill [^{3}H]PEG-4000 permeability in non-cannulated, freely swimming trout. Fish were transferred to custom-made 3.2 l Blazka swimming respirometers (Beamish et al., 1989) and left to settle with aeration for 30 min before 1 μCi [^{3}H]PEG-4000 was added to the water. Fish were then either swum at 1.2 BL s^{-1} or left stationary for 8 h. An initial water sample was taken 15 min after [^{3}H]PEG-4000 addition and then at 2 h intervals, with terminal plasma, gut and carcass sampling at 8 h. $\dot{M}_{O_2}$ was measured during the first, middle and last hour by stopping aeration, briefly sealing the respirometer, and monitoring the depletion of the partial pressure of oxygen (P_{O_2}).

Analyses and calculations

Water P_{O_2} was measured using a Clarke-type electrode (Cameron Instruments, Port Aransas, TX, USA) connected to a 1900 Polarographic Amplifier (A-M Systems, Carlsborg, WA, USA). $\dot{M}_{O_2}$ was calculated in the usual manner, taking into account fish mass, time, chamber volume and O_2 solubility (Boutilier et al., 1984) at 18°C.

Whole bodies and carcasses (15 ml of 1 mol l^{-1} nitric acid) and gastrointestinal tracts (5 ml of 2 mol l^{-1} nitric acid) were digested at 65°C for 48 h in sealed tubes. One milliliter supernatant was added to 5 ml Ultima Gold AB scintillation fluid (Perkin-Elmer, Waltham, MA, USA). Opti-phase scintillation fluid (Perkin-Elmer) was added to water (5 ml fluor:5 ml water) and plasma and urine samples (5 ml fluor:100 μl sample + 4.9 ml water). [^{3}H]-PEG-4000 radioactivity was determined using a Tri-Carb 2900TR Liquid Scintillation Analyzer (Perkin-Elmer). All results were quench-corrected to the same counting efficiency as water samples by the external standard ratio method, using quench curves constructed from tissue digests or plasma.

For cannulated fish, urinary [^{3}H]PEG-4000 excretion was calculated as the product of the 8 h urine volume times the measured urine cpm ml^{-1}, and branchial [^{3}H]PEG-4000 excretion as the product of the external water volume times the 8 h increment in water cpm ml^{-1}.

Urinary [^{3}H]PEG-4000 clearance rates (equivalent to GFR) were calculated as:

$$\text{GFR} = \frac{\text{PEG cpm 8 h loss to urine}}{\text{Mean 0–8 h plasma cpm/ml}} \times \frac{1}{M} \times \frac{1}{\Delta t}, \quad (1)$$

where M is mass (g), t is time (h), and the PEG cpm 8 h loss to urine and mean 0–8 h plasma cpm ml^{-1} ([initial + final] × 0.5) were based upon measured sample radioactivity.

Gill [^{3}H]PEG-4000 efflux clearance rates (i.e. plasma clearance rates) were calculated in an analogous manner as:

$$\text{Gill efflux clearance rate} = \frac{\text{PEG cpm 8 h loss to water}}{\text{Mean 0–8 h plasma cpm/ml}} \times \frac{1}{M} \times \frac{1}{\Delta t}. \quad (2)$$

Although plasma data were available only from terminal blood samples, initial plasma [^{3}H]PEG-4000 concentrations were estimated using the validated assumption (see below) that this marker is restricted to an extracellular fluid volume (ECFV) of 193 ml kg^{-1} (Munger et al., 1991).

$$\text{Initial plasma concentration} = \frac{\left[\text{PEG cpm 8 h loss to urine}\right] + \left[\text{PEG cpm 8 h loss to water}\right] + \left[\text{ECFV} \times \text{terminal plasma} \frac{\text{cpm}}{\text{ml}}\right]}{\text{ECFV}}. \quad (3)$$

In influx experiments, gill [^{3}H]PEG-4000 influx clearance rates (i.e. water clearance rates) were calculated as:

$$\text{Gill influx clearance rate} = \frac{\text{PEG cpm 8 h gain from the water}}{\text{Mean 0–8 h water cpm/ml}} \times \frac{1}{M} \times \frac{1}{\Delta t}. \quad (4)$$

Water cpm ml^{-1} were based on measured sample radioactivity. [^{3}H]PEG-4000 uptake from the water was based on appearance of cpm in the fish, because cpm disappearance from the high volume of external water could not be detected reliably. Appearance was determined by measuring total cpm in the carcass and also by multiplying the terminal plasma cpm ml^{-1} by the assumed ECFV of 193 ml kg^{-1}. The two estimates never differed by more than 10%, and mean values using the two approaches have been reported. Calculated gill influx clearance rates were then corrected for cpm uptake by drinking and losses to the urine (see Results and Discussion).

Drinking rates were calculated based on total gut [^{3}H]PEG-4000 cpm measured in the excised gastrointestinal tract:

$$\text{Drinking rate} = \frac{\text{Gut PEG cpm 8 h gain from the water}}{\text{Mean 0–8 h water cpm/ml}} \times \frac{1}{M} \times \frac{1}{\Delta t}. \quad (5)$$

All data have been expressed as means ± s.e.m. (N) with statistical tests (P<0.05) reported in the figure legends. All rate functions ($\dot{M}_{O_2}$, gill clearance rates, GFR, UFR, drinking rates) were scaled to a 20 g fish prior to averaging by multiplying by the mass scaling coefficient (S_{Cc}) from Clarke and Johnson (Clarke and Johnson, 1999):

$$S_{Cc} = 10^{0.79 \log\left(20/M\right)}. \quad (6)$$

Plots revealed that this removed the dependence of all rate functions on body mass. These scaled data have also been converted to the more common per kilogram basis.

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Competing interests

The authors declare no competing financial interests.

Author contributions

The study was jointly conceived by L.M.R. and C.M.W., and the experiments were performed by L.M.R. C.M.W. and L.M.R. jointly analyzed the data and wrote the manuscript.

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