

An in vitro investigation of gastrointestinal Na⁺ uptake mechanisms in freshwater rainbow trout

Sunita R. Nadella · Dhanisha Patel · Allen Ng ·
Chris M. Wood

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Abstract In vitro gut-sac preparations of all four sections (stomach, anterior, mid, and posterior intestine) of the gastrointestinal tract (GIT) of freshwater rainbow trout, together with radiotracer (²²Na) techniques, were used to study unidirectional Na⁺ uptake rates (UR, mucosal → blood space) and net absorptive fluid transport rates (FTR) under isosmotic conditions (mucosal = serosal osmolality). On an area-specific basis, unidirectional Na⁺ UR was highest in the mid-intestine, but when total gut area was taken into account, the three intestinal sections contributed equally, with very low rates in the stomach. The theoretical capacity for Na⁺ uptake across the whole GIT is sufficient to supply all of the animal's nutritive requirements for Na⁺. Transport occurs by low affinity systems with apparent K_m values 2–3 orders of magnitude higher than those in the gills, in accord with comparably higher Na⁺ concentrations in chyme versus fresh water. Fluid transport appeared to be Na⁺-dependent, such that treatments which altered unidirectional Na⁺ UR generally altered FTR in a comparable fashion. Pharmacological trials (amiloride, EIPA, phenamil, bafilomycin, furosemide, hydrochlorothiazide) conducted at a mucosal Na⁺ concentration of 50 mmol L⁻¹ indicated that GIT Na⁺ uptake occurs by a variety of apical mechanisms (NHE, Na⁺ channel/H⁺ ATPase, NCC, NKCC) with relative contributions varying among sections. However, at a mucosal Na⁺ concentration of 10 mmol L⁻¹, EIPA, phenamil, bafilomycin, and hydrochlorothiazide were no

longer effective in inhibiting unidirectional Na⁺ UR or FTR, suggesting the contribution of unidentified mechanisms under low Na⁺ conditions. A preliminary model is presented.

Keywords Na⁺ · Diet · Gut sacs · Transport kinetics · Mucus · Low-affinity transport · Pharmacological agents

Introduction

Freshwater fish osmoregulate by intake of ions from their environment, through branchial or dietary pathways to compensate for the ion losses to their hypoosmotic surroundings. Despite the presence of the above two distinct routes of uptake, most studies in the past have focused on branchial uptake, usually in the absence of feeding. Indeed, the traditional view has been that the gills and kidney control salt and water balance in freshwater fish, while the gastrointestinal tract (GIT) becomes important for ion and water balance only in seawater fish (reviewed by Wood and Bucking 2011, and Grosell 2011). Even in the latter, the emphasis has been on electrolyte uptake from ingested water, rather than uptake from the ingested food. However, this general belief was first challenged when Smith et al. (1989) provided evidence for the diet as an important source of ions for wild freshwater fish. Later, D'Cruz and Wood (1998) demonstrated that freshwater trout survived chronic exposure to low pH by utilizing the NaCl content of the diet to maintain ionic homeostasis. Consequently, the role of the GIT in ionoregulation and osmoregulation has gained importance, and several more recent studies have investigated its contribution to ion and water uptake in freshwater fish at the whole animal level (e.g., Bucking and Wood 2006a, b, 2007, 2009; Cooper and Wilson 2008).

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S. R. Nadella (✉) · D. Patel · A. Ng · C. M. Wood
Department of Biology, McMaster University, 1280 Main Street
West, Hamilton, ON L8S4K1, Canada
e-mail: nadellsr@mcmaster.ca

In the intestinal epithelium of seawater teleosts, there is firm evidence for apical Na^+ entry via co-transporters for Na^+ , K^+ , 2Cl^- (NKCC2; Musch et al. 1982; Halm et al. 1985; O'Grady et al. 1986;) and Na^+ , Cl^- (NCC; Frizzell et al. 1979; Halm et al. 1985). On the apical membrane, there is also molecular evidence for the expression of a Na^+/H^+ exchanger (NHE3), a V-type proton pump (H^+ ATPase), and a favorable electrochemical gradient for Na^+ uptake through epithelial channels, but the potential involvement of these mechanisms in Na^+ uptake is unclear at present (reviewed by Grosell 2011). Nevertheless, in the intestine of seawater-acclimated tilapia, brush border membrane vesicle experiments suggested that apical Na^+/H^+ exchange, in concert with $\text{Cl}^-/\text{HCO}_3^-$ exchange, plays a major role in Na^+ and Cl^- uptake (Howard and Ahearn 1988). Na^+ exit across the basolateral membrane via Na^+ , K^+ ATPase (NKA; Grosell et al. 2007) completes the transepithelial Na^+ movement. Basolateral Na^+ , HCO_3^- co-transport (NBC1) also occurs, but is probably secretory rather than absorptive for Na^+ (Grosell and Genz 2006).

Analogous processes in freshwater teleosts have yet to be studied in comparable mechanistic detail. In vitro studies on freshwater GIT preparations have indicated that Na^+ uptake is often associated with the uptake of nutrient compounds such as glucose (Smith 1966) or amino acids (Ferraris and Ahearn 1984). More recently, Nadella et al. (2006, 2007) found evidence for L-histidine-facilitated and phenamil-sensitive Na^+ transport in rainbow trout intestinal sacs. The mRNA expressions for two isoforms of NKA have been identified in the GIT with the $\alpha 3$ isoform localized to the stomach and $\alpha 1c$ isoform ubiquitously distributed through the entire tract (Nadella et al. 2011). NHE3 (Grosell et al. 2007) and NBC1 (Buckling and Wood 2012) are also expressed in all sections of the tract. Clearly, there is a need to follow-up on these initial findings to better understand the mechanisms of gastrointestinal Na^+ uptake in freshwater fish.

In contrast to the GIT, Na^+ uptake mechanisms in the gills of freshwater fish have been thoroughly investigated. From this vast body of research (reviewed by Flik et al. 1997; Marshall 2002; Kirschner 2004, Hwang and Lee 2007; Evans 2011, Kumai and Perry 2012), three mechanisms for apical Na^+ uptake have emerged: (1) an apical V-type proton pump (H^+ ATPase) linked to Na^+ absorption via an as yet unidentified epithelial Na^+ channel; (2) an NHE; and (3) an NCC. Basolateral Na^+ transport into the blood occurs via NKA and/or NBC1.

In the present study, our overall working hypotheses were: (1) that Na^+ uptake in the various sections of the GIT in the freshwater rainbow trout would occur by some or all of the same processes known to occur in the freshwater gills and/or in the seawater GIT; and (2) that the GIT Na^+ transport processes would operate with much lower affinity

than those at the gills, because the apical GIT transporters normally encounter a much higher Na^+ concentration in the chyme than occurs in fresh water (by 2–3 orders of magnitude; Wood and Buckling 2011). This difference has recently been seen for Ca^{2+} transport process in the GIT versus gills of freshwater trout (Klinck et al. 2012).

Therefore, our objectives were to characterize the relative rates and concentration-dependent kinetics of Na^+ uptake in the various sections of the GIT, and to pharmacologically identify and quantify the relative importance of mechanisms involved. The Na^+ concentration used in the pharmacological trials was chosen based on the results of the kinetics experiments, and then was reduced in some subsequent tests to evaluate possible higher affinity sites. We employed the in vitro gut-sac technique which has been successfully used in the past to study gut transport of other substances (e.g., Grosell and Jensen 1999; Bury et al. 2001; Nadella et al. 2006, 2007; Ojo and Wood 2007; Klinck and Wood 2011; Klinck et al. 2012). The technique is advantageous as it allows the study of individual gut sections which can be exposed to specific treatments on both the mucosal and serosal surfaces. As our focus was on the active uptake process(es) for Na^+ , rather than net Na^+ transport, we used a radiotracer (^{22}Na) to measure the unidirectional uptake flux, and applied all blocking agents to the apical (mucosal) surface.

Methods

Experimental organisms

Animal handling was in compliance with the regulations of the Canada Council for Animal Care under McMaster University Animal Utilization Protocol 09-04-10. Adult rainbow trout (*Oncorhynchus mykiss*) weighing approximately 200–300 g were kept in 500-L tanks and supplied with aerated dechlorinated Hamilton tap water [Na^+ 0.5, Cl^- 0.7, Ca^{2+} 1.0 mmol L^{-1} , hardness ~140 ppm as CaCO_3 , pH ~ 8.0, temperature 12 ± 2 °C]. They were fed a commercial 5-point dried pellet diet every alternate day (with 41.0 % crude protein, 11.0 % crude fat, 3.5 % crude fiber, 1.0 % Ca, 0.85 % total P, and 0.45 % Na^+) at a ration of 2 % body mass. The fish were not fed for 24 h prior to experiments. Fish were euthanized with an overdose of neutralized MS-222 (Sigma-Aldrich, St. Louis, MO, USA) and the entire gastrointestinal tract (stomach to posterior intestine) was excised.

Gastrointestinal sacs

The gastrointestinal sac technique (Nadella et al. 2006, 2007) was used to measure unidirectional Na^+ transport.

The tract was excised from the fish and divided into four sections: stomach, anterior, mid and posterior intestine, using the co-ordinates described in these papers.

Each section was first separated and cleaned using Cortland saline [NaCl 123 mM, KCl 5 mM, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 1 mM, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.9 mM, NaHCO_3 11.9 mM, $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ 2.8 mM, glucose 5.5 mM, pH 7.4 (Wolf 1963)]. Once cleaned, each section was tied at the distal end using a 2-0 silk thread (Ethicon, Johnson & Johnson; Skillman, NJ, USA). A heat-flared PE50 (for intestinal sections) or PE160 (for stomach) cannula tube (Intra-medical Clay Adams, Becton–Dickinson; Mississauga, ON, Canada) was inserted into each sac with the flared ends inside. Finally, the proximal end was closed using another silk thread. Cortland saline appropriately amended for each experiment (see below) containing radiolabeled Na^+ (^{22}Na ~ 0.1 $\mu\text{Ci}/\text{mL}$; Amersham Pharmacia Biotech, GE. Health Care Baied’Urfe, QC, Canada) was injected through the cannula, filling the mucosal compartment, and the tube was then sealed. Each preparation was blotted dry and the mass of the sac determined to the nearest 0.1 mg. Subsequently, the sac preparation was placed in a fixed volume (40 ml for stomach and anterior intestine and 12 ml for mid and posterior intestine) of the Cortland saline serving as the serosal bath, which was constantly aerated with a 99.7 % O_2 and 0.3 % CO_2 gas mixture for an appropriate incubation period (see below and “Results”). In all experiments, the osmolality of the internal (mucosal) saline had been adjusted by the addition of mannitol, an inert sugar, to equal that of the external (serosal) saline, as checked by a vapor pressure osmometer (Wescor 5100C; Logan, UT, USA). In those experimental treatments where the osmolality of the mucosal solution had been raised above that of the regular Cortland saline, mannitol was added to the serosal saline so as to achieve osmotic balance. To account for seasonal and temporal variations, separate simultaneous controls were used for every treatment.

Samples of mucosal and serosal saline were collected at the start and end of the flux period and counted for ^{22}Na activity by gamma counting (1480 Automatic Gamma Counter; Perkin Elmer, Woodbridge, ON, Canada). At the end of the flux period, the sac preparation was removed from the flux chamber, blotted dry and reweighed, to allow calculation of net fluid transport rate.

Radiolabeled Na^+ transport into three fractions was determined, as explained by Nadella et al. (2006, 2007). The gut sacs were cut open, washed in 10 ml (stomach) or 5 ml (intestinal sections) of Cortland saline and then with 10 or 5 ml of 1 mmol L^{-1} EDTA (ethylene diaminetetra acetic acid) solution, and then blotted dry with a small piece of paper towel. All these solutions plus the blotting towel were retained for analysis so as to provide an estimate of

the amount of ^{22}Na adsorbed onto luminal mucus, representing the “mucus-bound” fraction. The “epithelium” (i.e., the enterocytes) was then scraped gently with a glass slide and collected separately, representing Na^+ that had been absorbed across the apical surface of the enterocytes, but not exported to the blood. The remaining tissue, combined with the serosal saline, was considered the “bloodspace”, representing all Na^+ that had been exported across the basolateral membranes of the enterocytes. The gross surface area of the intestinal tissue was determined by tracing its outline onto graph paper (Grosell and Jensen 1999). The tissue, serosal samples, wash solutions and epithelial scrapings were counted separately for gamma ^{22}Na activity.

Time course of Na^+ uptake

To estimate an optimal flux period for Na^+ uptake, a time course series was completed wherein gut sacs ($N = 5$) from each GIT section were filled with 150 mmol L^{-1} Na^+ in Cortland saline (one of the points on the kinetic curve, see below) along with 0.1 $\mu\text{Ci mL}^{-1}$ of ^{22}Na and incubated in the serosal bath for 0.5, 1, and 2 h. Following these flux periods, the preparations were sampled as detailed above.

Kinetic analysis (effect of varying Na^+ concentrations)

The concentration-dependent kinetics of unidirectional Na^+ uptake in each of the four GIT sections was measured to ascertain if transport is a saturable process. Bucking and Wood (2006a, b) measured an Na^+ concentration of approximately 150 mmol L^{-1} in the fluid phase of chyme of newly fed rainbow trout. Using this concentration as a base, six treatment groups were employed with Na^+ concentration as follows: 10, 30, 75, 150, and 200 and 280 mmol L^{-1} ($N = 5$ at each point). In each case except for the 10 mmol L^{-1} point, the Na^+ concentration was manipulated by removing or adding NaCl to the basic Cortland saline of Wolf (1963), then compensating with mannitol, so that there was no difference in osmolality between the mucosal and serosal saline. For the 10 $\text{mmol Na}^+ \text{L}^{-1}$ tests, NaCl was removed, $\text{KH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ was substituted for $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, and the concentration of NaHCO_3 was slightly lowered to 10 mmol L^{-1} , followed by osmotic compensation with mannitol. Considering Na^+ concentration was altered by manipulating NaCl , Cl^- levels in the series were altered as follows to 7, 37, 82, 157, 207 and 287 mmol L^{-1} .

Pharmacological series

Based on the results of the kinetic analysis, 50 $\text{mmol Na}^+ \text{L}^{-1}$ was used in the mucosal saline for all standard treatments, with appropriate osmotic compensation by mannitol

addition. All drugs were dissolved in DMSO (Caledon Labs, Georgetown, ON, Canada) to a final concentration of 0.1 % DMSO in the final mucosal saline. The same concentration of DMSO was used in the controls. Based on the results of the time course study, a uniform 2-h flux period was used for all treatments.

After completion of the standard pharmacological experiments with 50 mmol Na⁺ L⁻¹, a further set of experiments was carried out with some of the drugs using a mucosal Na⁺ concentration of 10 mmol L⁻¹ in a saline identical to that used in the preceding kinetic analysis series. In these tests, the Na⁺ concentration of the serosal saline was also set to 10 mmol L⁻¹ to ensure that mucosal Na⁺ concentration did not rise due to net back-flux.

Based on previous studies with the freshwater gill and seawater GIT (see “Introduction” for references), in the standard pharmacological tests, the following drugs were applied in the mucosal saline in all four sac preparations of the GIT, together with simultaneous controls ($N = 7$ – 10 for each). (1) Amiloride HCl (10^{-4} M) as a joint NHE and Na⁺ channel blocker; (2) 5-(*N*-ethyl-*N*-isopropyl)-amiloride HCl (EIPA; 10^{-4} M) as a more specific NHE blocker; (3) phenamil methane sulfonate (10^{-4} M) as a more specific Na⁺ channel blocker; (4) bafilomycin A1 (10^{-5} M) as a blocker of V-type H⁺ATPase; (5) hydrochlorothiazide (10^{-3} M) as a blocker of NCC; and (6) furosemide (10^{-4} M) as a blocker of NKCC. All drugs were obtained from Sigma-Aldrich (St. Louis, MO, USA) except bafilomycin A1 which was purchased from LC Laboratories (Woburn, MA, USA).

A subsequent set of tests used the same concentrations of EIPA, bafilomycin, hydrochlorothiazide, and phenamil ($N = 9$ for experimentals and controls) in a mucosal Na⁺ concentration of 10 mmol L⁻¹. The EIPA, bafilomycin, and hydrochlorothiazide experiments at 10 mmol Na⁺ L⁻¹ were performed simultaneously, so shared the same control group.

Analytical techniques and calculations

In each experimental study, the sac preparations were blotted in a standardized fashion and weighed to the nearest 0.1 mg on a Sartorius H110 balance (Gottingen, Germany) prior to and after the flux period to allow for calculation of net fluid transport. Net fluid transport rates were determined from the change in total mass of the sac preparation over the experimental period which provided a gravimetric measure of changes in fluid content. This was normalized by taking into account the gross surface area of exposed epithelium and time elapsed.

Rate of fluid transport (FTR) was calculated as follows:

$$\text{FTR} = (\text{IW} - \text{TW})/\text{ISA}/t \quad (1)$$

where IW = initial weight of sac preparation in mg, TW = terminal weight of sac preparation after flux in mg, ISA = intestinal surface area in cm², and t = time in hours. This yielded a rate of net water movement from mucosal to serosal surface, expressed in $\mu\text{l cm}^{-2} \text{h}^{-1}$.

Unidirectional Na⁺ uptake rate (UR) was calculated as follows:

$$\text{UR} = \text{compartment cpm} / (\text{SA} \times \text{ISA} \times t) \quad (2)$$

where compartment cpm = the total ²²Na activity of the compartments measured (blood space, mucus-bound fraction, or mucosal epithelium), and SA = measured specific activity of the mucosal saline (cpm μmol^{-1}). This produced an Na⁺ uptake rate, expressed in $\mu\text{mol cm}^{-2} \text{h}^{-1}$.

The concentrations of Na⁺ in mucosal saline for specific activity calculations were measured by flame atomic absorption spectroscopy (FAAS; Varian SpectrAA-220FS, Mulgrave, Australia).

Statistical analyses

Where appropriate, curvilinear regression analyses of Na⁺ uptake kinetics were performed with a hyperbolic curve fit (single rectangular 2 parameters $y = ax/(x + b)$; Sigma plot Windows version 10.0) in order to fit the parameters of the Michaelis–Menten equation:

$$\text{UR} = (\text{UR}_{\text{max}} \times [X]) / ([X] + K_m) \quad (3)$$

where UR = unidirectional uptake rate, $[X]$ = concentration of substrate, UR_{max} = maximum transport rate when the system is saturated with substrate, and K_m = concentration of substrate which provides a UR of half the UR_{max} value. Linear relationships were fitted by least squares regression. Curvilinear regressions were used when the r^2 was greater than for simple linear regression using a minimum of 5 consecutive data points. When the 5-point criterion was not fulfilled, but the r^2 for a curvilinear fit was still greater than for linear regression, no relationship was plotted through the data points on figures.

A standard one-way analysis of variance (ANOVA) or a Kruskal–Wallis one-way ANOVA on ranks (when data failed normality or equal variance tests) was used to compare multiple treatment groups, followed by LSD post hoc tests to identify individual differences. In the pharmacological trials, effects of drug treatment relative to the control in each GIT section were evaluated by unpaired Student's t test, or by a Mann–Whitney rank sum test in the case of failed normality or equal variance tests. As the a priori prediction was always that the drug treatment would inhibit UR and FTR, one-tailed tests were used. Data have been reported as mean \pm SEM (N) and differences were considered significant at $P < 0.05$.

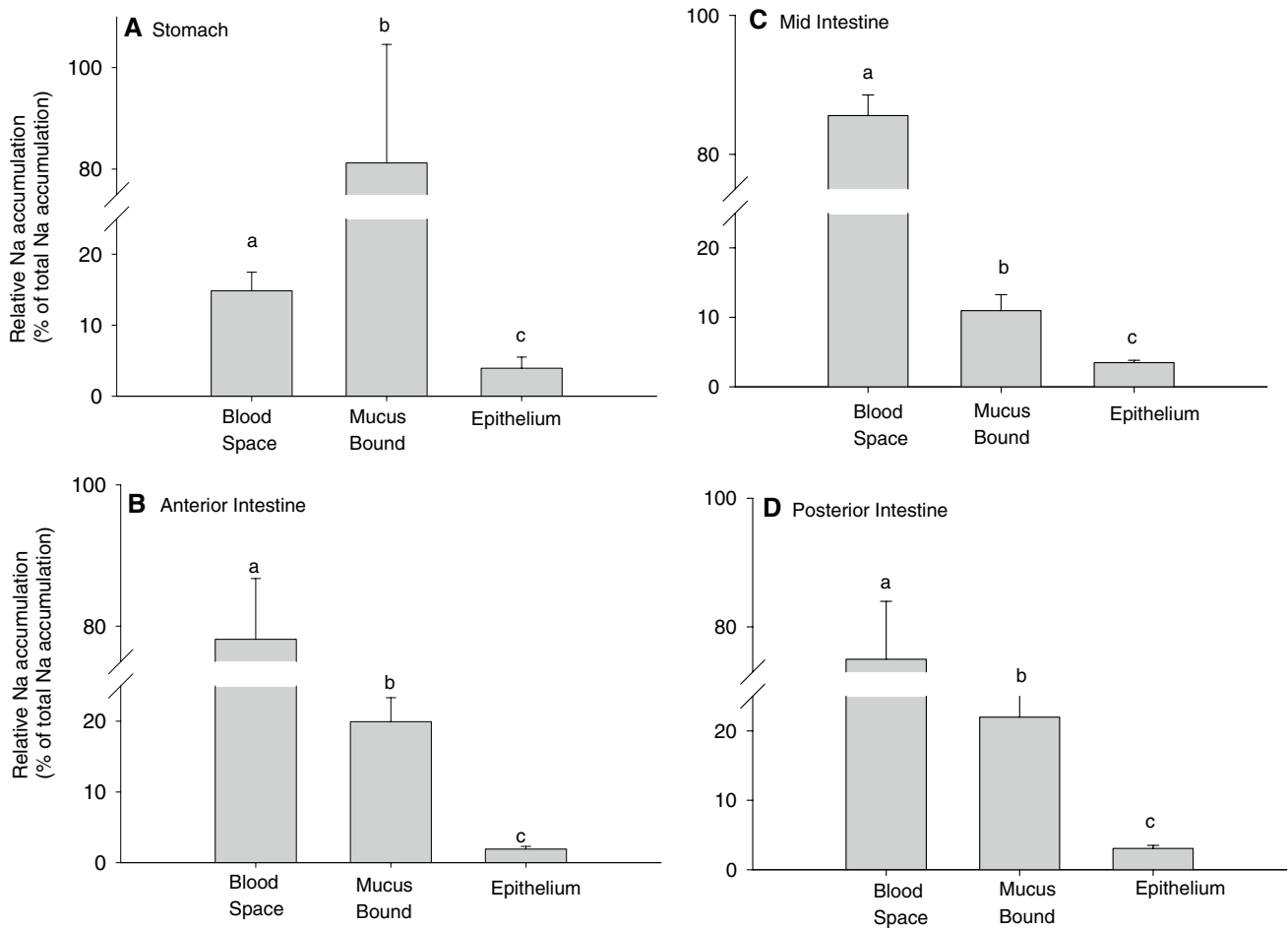


Fig. 1 Relative ^{22}Na accumulation is shown as a percentage of total accumulation in blood space, mucus-bound, and epithelium fractions of **a** stomach, **b** anterior intestine, **c** mid-intestine, and **d** posterior

intestine. Values are mean \pm SEM ($n = 5$ per treatment). Statistical significance was tested by ANOVA followed by LSD tests. Means labeled with different letters are significantly different ($P < 0.05$)

Results

Time course of Na^+ uptake and fluid transport

Unidirectional Na^+ uptake rates into the blood space compartment and fluid transport rates were variable at 0.5 h but remained constant and stable over 1- and 2-h periods (data not shown). Based on these observations, a 2-h flux period was chosen for all subsequent experiments.

Spatial pattern of unidirectional Na^+ uptake and relative Na^+ accumulation

The spatial distributions of $^{22}\text{Na}^+$ in the three measured compartments—mucus-bound, epithelium, and bloodspace—are illustrated in Fig. 1 for the 2-h tests at a mucosal Na^+ concentration of 150 mmol L^{-1} . These data provide a measure of the relative contribution of each compartment to the fate of Na^+ taken up from the lumen of the

GIT. Approximately 80 % could be attributed to the bloodspace (see “Methods” for definitions) in all three intestinal sections with 10–20 % in the mucus-bound compartment and less than 5 % in the epithelium fraction. However, in the stomach, a very different pattern was apparent with ~80 % in the mucus-bound, ~15 % in the bloodspace, and less than 5 % in the epithelium (Fig. 1).

Comparison of absolute rates of unidirectional Na^+ uptake per unit area into the blood space only of GIT sections exposed to $150 \text{ mmol Na}^+ \text{ L}^{-1}$ (Fig. 2) revealed that the mid and posterior intestinal sections had the highest transport rates ($\sim 20 \mu\text{mol cm}^{-2} \text{ h}^{-1}$), the anterior intestine about 50 % of this ($\sim 10 \mu\text{mol cm}^{-2} \text{ h}^{-1}$), with by far the lowest rate in the stomach ($\sim 1 \mu\text{mol cm}^{-2} \text{ h}^{-1}$) (Fig. 2). This same basic pattern was seen in most of the subsequent pharmacological studies performed at $50 \text{ mmol Na}^+ \text{ L}^{-1}$ in the mucosal saline (Figs. 7a, 8a, 9a, 10a, 11a, 12a).

However, in order to assess the total contributions of each section to overall unidirectional Na^+ uptake rate

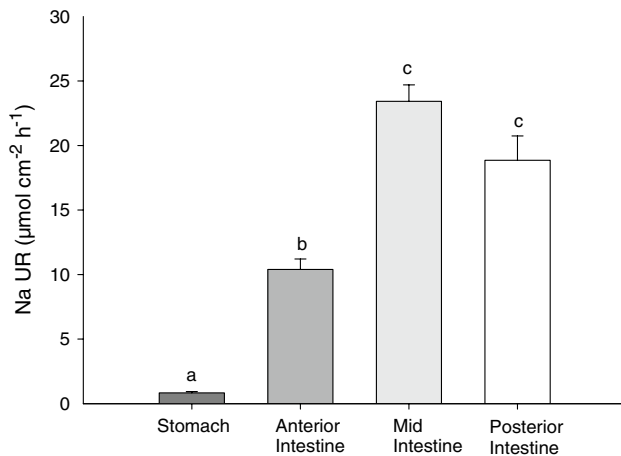


Fig. 2 Unidirectional Na⁺ uptake rates (UR) into the blood space compartment for 2-h flux tests with gut sacs of the four GIT sections at a mucosal Na⁺ concentration of 150 mmol L⁻¹. Values are mean ± SEM ($n = 5$ per treatment). Statistical significance was tested by ANOVA followed by LSD tests. Means labeled with different letters are significantly different ($P < 0.05$)

through the GIT, it is necessary to take into account the total surface area of each section, which scale in the order stomach > anterior > mid > posterior intestine (Table 1; area data from Nadella et al. 2006 for comparably sized trout). On this basis, the individual contributions of the three intestinal sections are about equal, and about 10-fold greater than that of the stomach (Table 1).

Concentration-dependent Na⁺ uptake kinetics

Mucus-bound compartment

In all four GIT sections, unidirectional Na⁺ uptake into the mucus-bound compartment exhibited apparent biphasic

Table 1 Estimated total unidirectional Na⁺ uptake rate into the blood space compartment in four gastrointestinal segments of rainbow trout based on measured in vitro rates and total surface areas

	Unidirectional Na ⁺ uptake rate (µmol cm ⁻² h ⁻¹)	Surface area (cm ²)	Total unidirectional Na ⁺ uptake rate (µmol h ⁻¹)
Stomach	0.84	35	29.4
Anterior intestine	10.39	30	311.7
Mid-intestine	23.43	11	257.7
Posterior intestine	18.85	16	301.6

Total Na⁺ uptake rate for a 250 g fish: 900.4 µmol h⁻¹ = 3,601.6 µmol kg⁻¹ h⁻¹

Total surface area data from Nadella et al. (2006) for comparably sized trout

relationships, with possible curvilinear saturable components at the lower mucosal Na⁺ concentrations, and linear components at higher concentrations (Fig. 3a–d). Curves were not fitted to the possible curvilinear components due to insufficient data points (see “Methods”).

Epithelium compartment

In contrast to the stomach, where there was a single clear Michaelis–Menten relationship (Fig. 4a), unidirectional Na⁺ uptake into the epithelium compartment exhibited a biphasic relationship in the mid-intestine, with a clear saturable relationship at lower concentrations and an apparent linear component at high concentration (Fig. 4c). Km values were higher (i.e., lower affinity) in the mid-intestine (181 mmol L⁻¹) compared to the stomach (102 mmol L⁻¹). UR_{max} was 0.27 µmol cm⁻² h⁻¹ in the mid-intestine indicative of a higher transport capacity in this region compared to the stomach where UR_{max} was 0.11 µmol cm⁻² h⁻¹.

Blood space compartment

Kinetic relationships were linear for unidirectional Na⁺ uptake into the blood space of the anterior and posterior intestines (Fig. 5b, d). For the stomach (Fig. 5a) and mid-intestine (Fig. 5c) there were possible curvilinear relationships at lower concentrations, but there were insufficient data points for reliable curve-fitting (see “Methods”).

Fluid transport rates

In all four GIT sections, despite the absence of an osmotic gradient, net fluid transport rates were always in the mucosal-to-serosal direction and increased progressively with increasing mucosal Na⁺ concentration in the kinetic experiments (Fig. 6). In the anterior and mid-intestine, rates stabilized over the range of 140–280 mmol Na⁺ L⁻¹, but this did not occur in the stomach or posterior intestine. Rates also tended to be highest in the anterior intestine and lowest in the stomach, with comparable intermediate rates in the mid and posterior intestine. A similar pattern was seen in the subsequent pharmacological studies performed at 50 mmol Na⁺ L⁻¹ in the mucosal saline (Figs. 7b, 8b, 9b, 10b, 11b, 12b).

Effects of pharmacological agents on unidirectional Na⁺ uptake and fluid transport rates

From the results of the kinetics experiments, possible saturable relationships indicative of transporter mediation were seen only at lower mucosal Na⁺ concentrations (Figs. 3, 4, 5). Thus a mucosal Na⁺ concentration of 50 mmol L⁻¹ was used for the first series of pharmacological experiments,

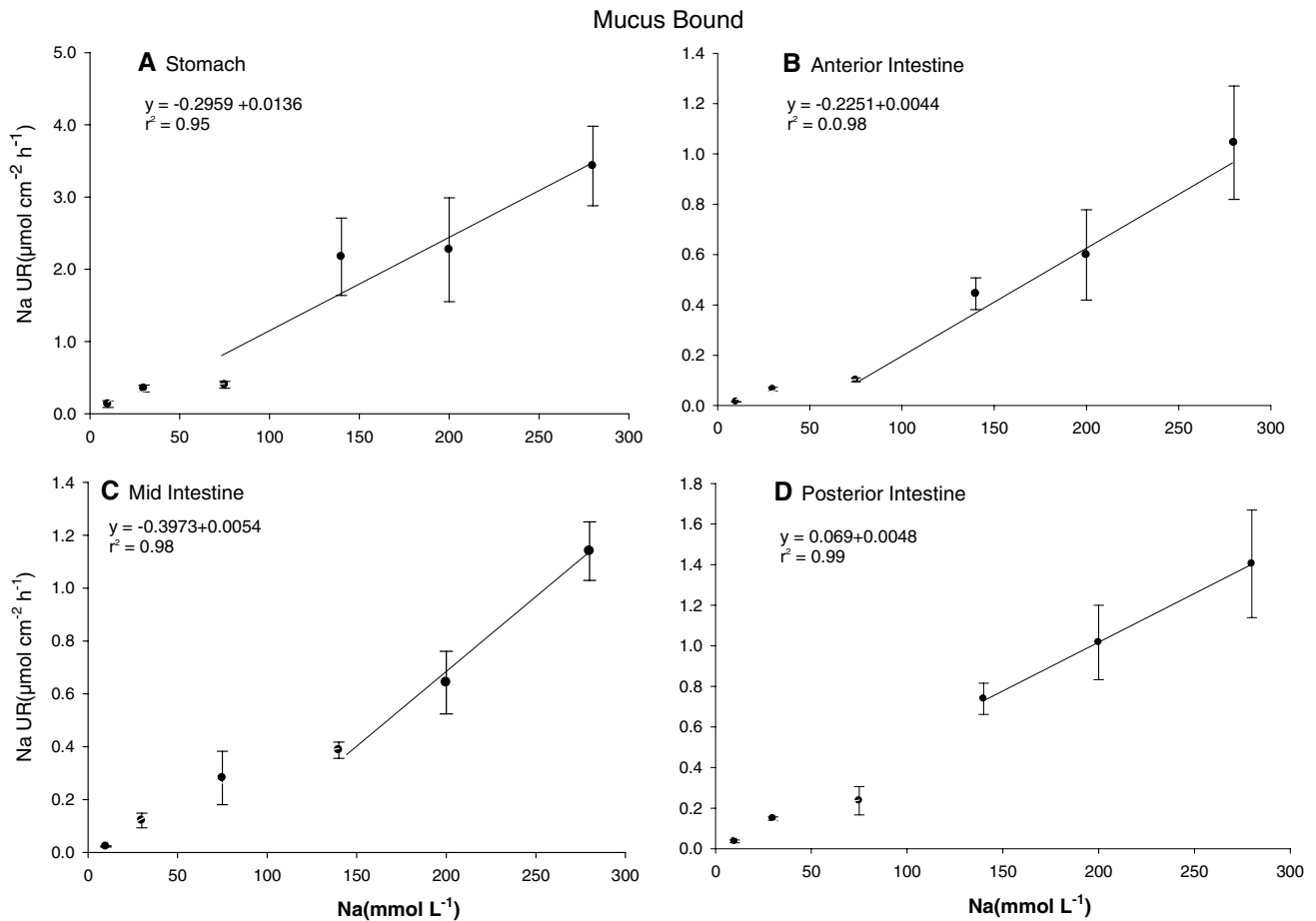


Fig. 3 Concentration-dependent kinetics of unidirectional Na⁺ uptake rate (UR) into the mucus-bound compartment of gut sacs from the four sections of the GIT: **a** stomach; **b** anterior intestine; **c** mid-intestine; and **d** posterior intestine. Six different mucosal Na⁺ concentrations were used: 10, 30, 75, 150, 200, and 280 mmol L⁻¹. Values are mean ± SEM (*n* = 5 per treatment). Linear relationships were fitted by least squares regression

centrations were used: 10, 30, 75, 150, 200, and 280 mmol L⁻¹. Values are mean ± SEM (*n* = 5 per treatment). Linear relationships were fitted by least squares regression

based on the premise that this would yield the best chance of targeting carrier-mediated processes. This ensured that the uptake measurements were performed below possible substrate saturation for all segments and compartments, because in the saturation plateau region, the reserve capacity of the system makes it much harder to reveal specific pharmacological effects. Figures 7, 8, 9, 10, 11, 12 summarize the observed effects on unidirectional Na⁺ uptake rates (UR) into the blood space compartment only and fluid transport rates (FTR). Effects in the mucus-bound and epithelium compartments are not shown because they were absent, apart from one significant decrease as noted in the text below.

Amiloride (10⁻⁴ M) significantly reduced Na⁺ UR into the blood space compartment by 35–50 % in the stomach and in the mid-intestine (Fig. 7a), but had no significant effect in the anterior or posterior intestine. There was an accompanying large reduction (70 %) in FTR in the stomach, but not in other sections (Fig. 7b).

EIPA (10⁻⁴ M) significantly reduced Na⁺ UR into the blood space compartment by about 35 % only in the anterior and mid-intestine. A large 75 % reduction in the stomach was not significant (*P* = 0.085). Notably there were significant decreases in FTR in all three intestinal sections, but not in the stomach (Fig. 8b). There was also a significant 70 % reduction in Na⁺ transport into the epithelium in the anterior intestine only (data not shown) which was the only significant effect of any of the tested drugs in this compartment in any of the GIT sections.

Phenamil (10⁻⁴ M) significantly reduced Na⁺ UR into the blood space compartment by about 30 % and FTR by about 20 % in all three intestinal segments (Figs. 9a, b). A 50 % reduction in FTR in the stomach was also significant, but a corresponding 50 % reduction in Na⁺ UR in the stomach was not quite significant (*P* = 0.056).

Bafilomycin (10⁻⁵ M) significantly inhibited Na⁺ UR into the blood space compartment by ~50 % in the stomach, anterior and mid-intestine, but had no effect on the

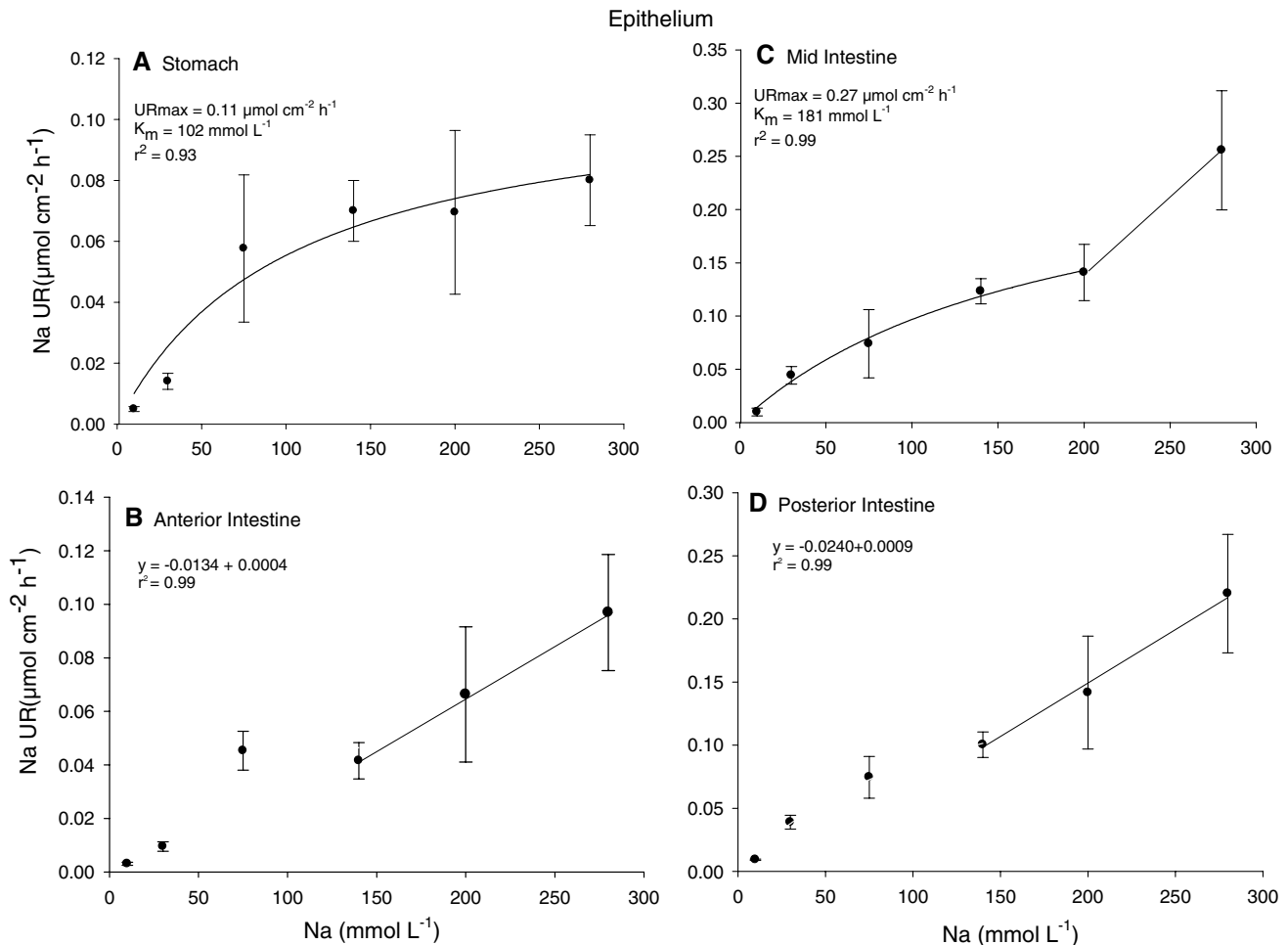


Fig. 4 Concentration-dependent kinetics of unidirectional Na^+ uptake rate (UR) into the epithelium compartment of gut sacs from the four sections of the GIT: **a** stomach; **b** anterior intestine; **c** mid-intestine; and **d** posterior intestine. Six different mucosal Na^+ concentrations were used: 10, 30, 75, 150, 200, and 280 mmol L^{-1} .

posterior intestine (Fig. 10a). Bafilomycin also caused comparable significant declines in FTR in the anterior and mid-intestine, but not in the stomach ($P = 0.135$) (Fig. 10b). There were no effects on either parameter in the posterior intestine.

Hydrochlorothiazide (10^{-3} M) significantly decreased Na^+ UR into the blood space compartment by 35–40 % in both the mid and posterior intestine, but was ineffective on this parameter in the stomach and anterior intestine (Fig. 11a). In marked contrast, there were significant reductions of FTR in the stomach and anterior intestine, but not in the mid or posterior intestine, though the small decrease in the latter was close to significance ($P = 0.070$) (Fig. 11b).

Furosemide (10^{-4} M) was effective only in the anterior intestine where it decreased both Na^+ UR into the blood

space compartment and FTR by ~30 % (Fig. 12a, b). There were no changes in other sections.

Effects of pharmacological agents at lower mucosal Na^+

A subset of the same drugs was tested in separate experiments with a mucosal Na^+ concentration of 10 mmol L^{-1} , in order to target possible higher affinity transporters. EIPA (10^{-4} M), bafilomycin (10^{-5} M), and hydrochlorothiazide (10^{-3} M) were tested simultaneously against the same controls. There were no significant effects of any of these agents on either unidirectional Na^+ UR into the blood space compartment or FTR in any of the four sections (Fig. 13a, b). In a separate experiment performed at a different time of year with phenamil (10^{-4} M) and a mucosal Na^+ concentration of 10 mmol L^{-1} , absolute values of Na^+ UR and

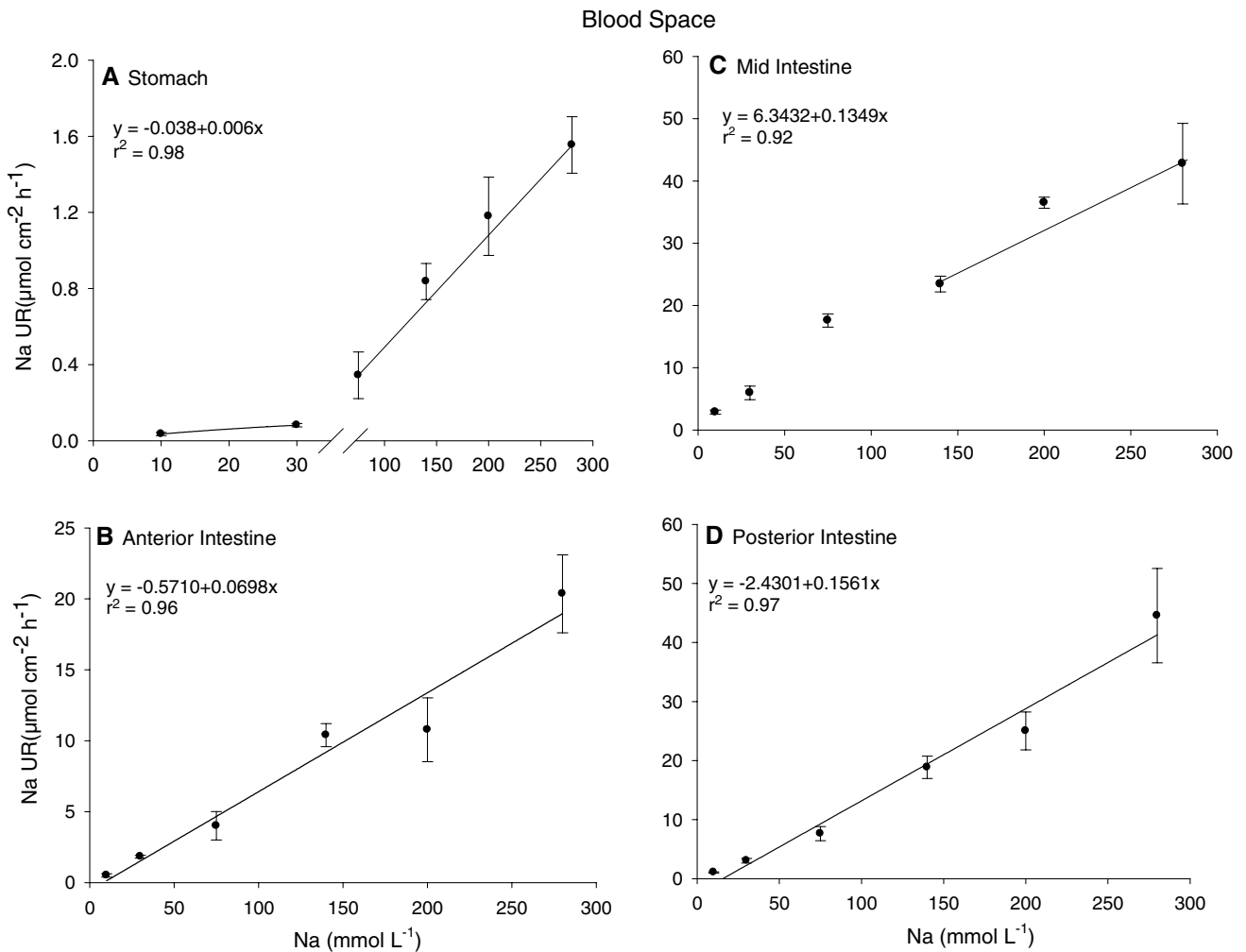


Fig. 5 Concentration-dependent kinetics of unidirectional Na⁺ uptake (UR) into the blood space compartment of gut sacs from the four sections of the GIT: **a** stomach; **b** anterior intestine; **c** mid-intestine; and **d** posterior intestine. Six different mucosal Na⁺ con-

centrations were used: 10, 30, 75, 150, 200 and 280 mmol L⁻¹. Mean ± SEM (*n* = 5 per treatment). Linear relationships were fitted by least squares regression

FTR were higher, but phenamil had no significant effects on either parameter in any of the four sections (Table 2).

Discussion

Dietary versus branchial Na⁺ uptake

It is important to note that in this study, unidirectional Na⁺ uptake flux rates (UR) were measured, not net Na⁺ uptake flux rates, so these GIT transport determinations are somewhat analogous to the unidirectional Na⁺ influx determinations commonly made at freshwater fish gills. At the latter, unidirectional Na⁺ influx (and efflux) rates are usually many-fold greater than net flux rates, which are often close to zero—i.e., net balance. We are aware of only three comparable fish GIT studies where both unidirectional and net

Na⁺ fluxes have been measured (Gibson et al. 1987; Grosell et al. 2005; Scott et al. 2006), and a similar situation occurred in all—unidirectional flux rates were many-fold greater (5–20×) than net flux rates. Therefore, it is likely that this is the case both in the present study and in vivo, especially since the GIT is generally considered to be a ‘leaky epithelium’ (Loretz 1995; Grosell 2011). Nevertheless, under all experimental conditions, there was always a net fluid transport (FTR) in the mucosal-to-serosal direction despite the absence of an osmotic gradient in our experiments. As argued subsequently, this FTR appeared to be coupled to net Na⁺ and Cl⁻ flux. Making a conservative assumption that the fluid flux was isotonic (see below), then the net mucosal-to-serosal flux rates of Na⁺ would have been about 15 % of the unidirectional Na⁺ UR shown in Fig. 2, in accord with previous reports. It is noteworthy, however, that both the unidirectional UR and estimated

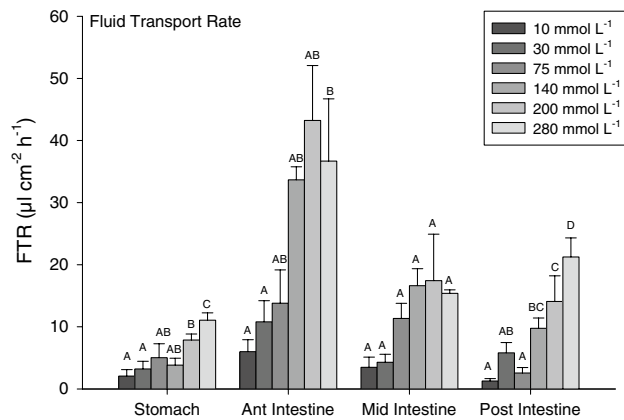


Fig. 6 Fluid transport rates (FTR) with different mucosal Na^+ concentrations (10, 30, 75, 150, 200 and 280 mmol L^{-1}) in the kinetics experiments on gut sacs from all four sections of the GIT. Mean \pm SEM ($n = 5$ per treatment). Statistical significance within sections was tested by ANOVA followed by LSD tests. Within the same section, bars sharing the same letter are not significantly different ($P \geq 0.05$)

net Na^+ flux rates in these trout freshwater preparations are several-fold higher than the previous measurements on GIT sacs from seawater flounder (Grosell et al. 2005) and brackish water killifish (Scott et al., 2006), attesting to the importance of dietary Na^+ uptake in freshwater trout.

Total unidirectional Na UR into the blood space compartment measured *in vitro*, calculated from rates of Na^+ transport (Fig. 2), and taking into account relevant surface area of the four GIT sections in a 250 g trout, was approximately $3,600 \mu\text{mol kg}^{-1} \text{h}^{-1}$ (Table 1), which might yield a net uptake flux rate (15 %) of about $540 \mu\text{mol kg}^{-1} \text{h}^{-1}$. In trout of approximately similar size, in identical water quality, Wood (1988) reported unidirectional and net gill Na^+ uptake rates of 350 and $65 \mu\text{mol kg}^{-1} \text{h}^{-1}$, respectively. Clearly, the dietary source could potentially provide about 8 times higher unidirectional and net Na^+ uptake rates than the branchial. Fish can likely derive adequate quantities of Na^+ from either source, but may choose the one which is energetically less costly based on physiological and seasonal constraints, such as food availability and ion content (see below).

Spatial patterns of gastrointestinal Na^+ uptake: *in vitro* versus *in vivo*

In the present *in vitro* experiments, the three intestinal sections were clearly the major sites of unidirectional Na^+ UR in the trout, each accounting for about one-third of the total, while only about 3 % occurred in the stomach (Table 1), though the latter value would increase to about 15 % if the substantial binding by gastric mucus were taken into account. This may appear surprising given the

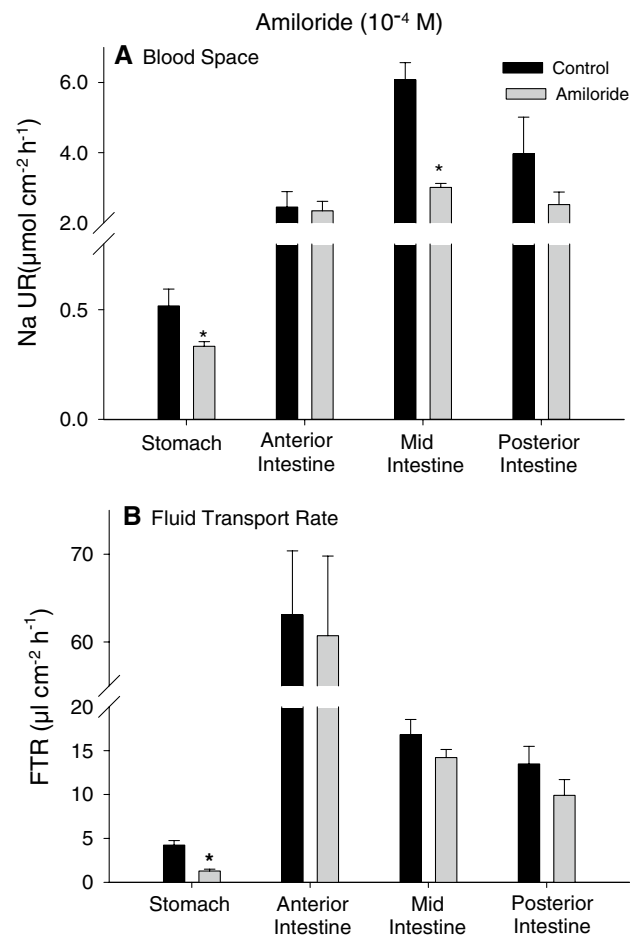


Fig. 7 Effect of amiloride (10^{-4} M) administered in mucosal saline with an Na^+ concentration of 50 mmol L^{-1} on **a** unidirectional Na^+ uptake rate (UR) into the blood space compartment and **b** fluid transport rate (FTR) in gut sacs from all four sections of the GIT. Values are mean \pm SEM ($n = 10$ per treatment). Within each section, statistical significance of the drug's effect in decreasing the rates was tested using unpaired Student's *t* tests (one-tailed). Means labeled with asterisks are significantly different ($P < 0.05$)

growing body of literature detailing the importance of stomach in ionic (Bucking and Wood 2006b, 2007) and metal uptake (e.g., Ojo and Wood 2007; Klinck and Wood 2011, 2013; Nadella et al. 2011). The mammalian stomach is capable of active Na^+ uptake (Machen et al. 1978). Indeed, Bucking and Wood (2006b) found 90 % of the net Na^+ content from a single meal in trout was removed from the chyme in the stomach of freshwater rainbow trout, yet this was put back again by secretions into the intestinal segments so that net uptake was negligible overall. These *in vivo* data seem to directly contradict the present *in vitro* data. However, it must be realized that *in vitro* data only reflect the capacity of the system under the conditions applied, not what actually happens *in vivo*. In the present study, all the GIT sections were provided with identical

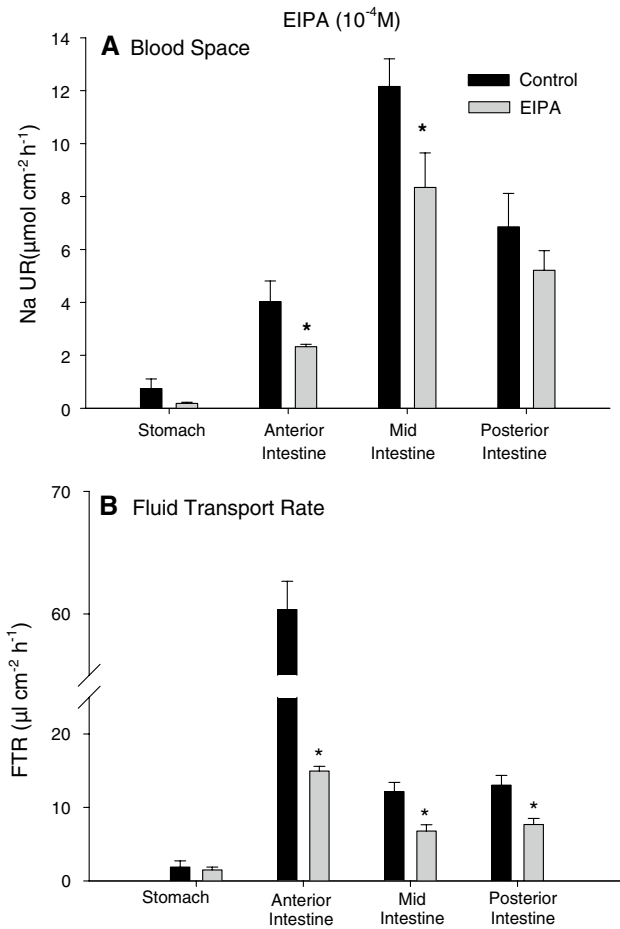


Fig. 8 Effect of EIPA (10^{-4} M) administered in mucosal saline with an Na^+ concentration of 50 mmol L^{-1} on **a** unidirectional Na^+ uptake rate (UR) into the blood space compartment and **b** fluid transport rate (FTR) in gut sacs from all four sections of the GIT. Values are mean \pm SEM ($n = 10$ per treatment). Within each section, statistical significance of the drug's effect in decreasing the rates was tested using unpaired Student's *t* tests (one-tailed). Means labeled with asterisks are significantly different ($P < 0.05$)

Na^+ concentrations of 150 mmol L^{-1} in the mucosal saline, while in vivo, chyme, not saline is processed: the stomach receives the first bolus of food and therefore the highest concentration of Na^+ and as digestion progresses, the intestinal sections downstream come in contact with relatively lower Na^+ levels in the chyme (Bucking and Wood 2006b), and the quality of the chyme changes [discussed by Wood and Bucking (2011)]. Furthermore, the distribution of Na^+ between fluid phases where it is dissolved and solid phases where it is adsorbed to fibrous and particulate material also changes (Bucking and Wood 2009). Finally, it should also be noted that the two studies differ in that Bucking and Wood (2006b, 2009) measured net Na^+ transport while the present study measured only unidirectional Na^+ UR. These rates can vary seasonally

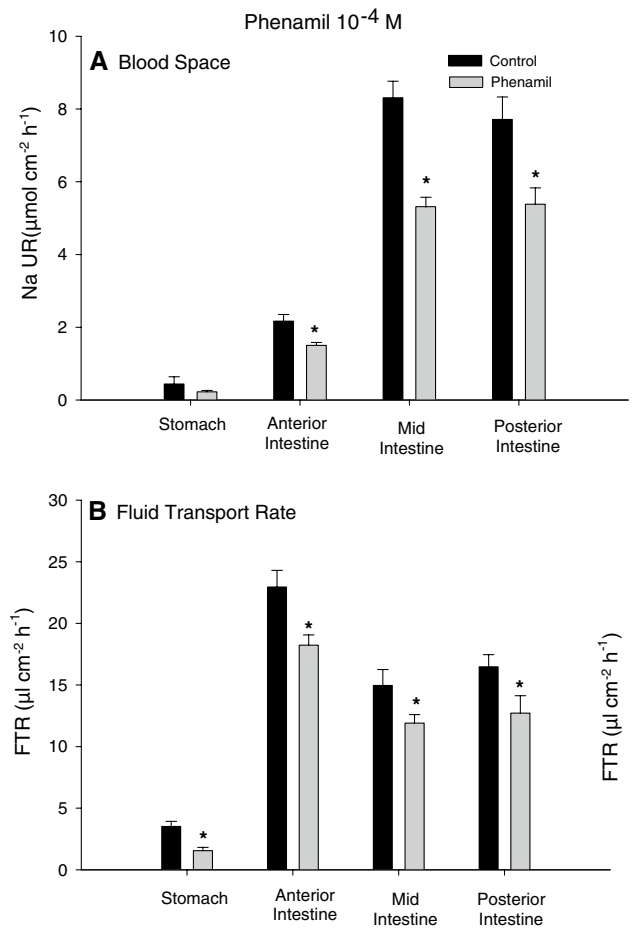


Fig. 9 Effect of phenamil (10^{-4} M) administered in mucosal saline with an Na^+ concentration of 50 mmol L^{-1} on **a** unidirectional Na^+ uptake rate (UR) into the blood space compartment and **b** fluid transport rate (FTR) in gut sacs from all four sections of the GIT. Values are mean \pm SEM ($n = 10$ per treatment). Within each section, statistical significance of the drug's effect in decreasing the rates was tested using unpaired Student's *t* tests (one-tailed). Means labeled with asterisks are significantly different ($P < 0.05$)

and spatially as seen in the differences among the various control groups (Figs. 7, 8, 9, 10, 11, 12).

Fractional distribution of Na^+ uptake

In contrast to the stomach, unidirectional Na^+ UR for all the intestinal segments was highest into the blood space (fully transported across the enterocytes into the muscle layer and serosal fluid), accounting for 80 % of total uptake (Fig. 1). Na^+ bound to mucus (loosely bound Na^+ in the lumen) was 6- to 8-fold higher in the stomach compared to the intestinal sections, and less than 5 % of Na^+ uptake was measured in the epithelial scrapings (Na^+ transported across the mucous layer into enterocytes). Conspicuously high rates of Na^+ uptake into the gastric mucus probably

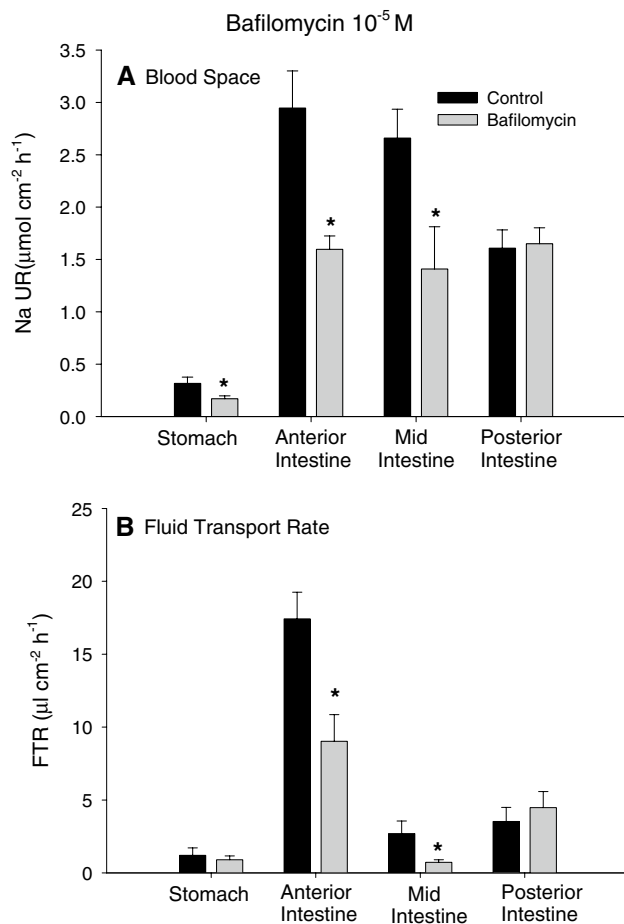


Fig. 10 Effect of bafilomycin (10^{-5} M) administered in mucosal saline with an Na^+ concentration of 50 mmol L^{-1} on **a** unidirectional Na^+ uptake rate (UR) into the blood space compartment and **b** fluid transport rate (FTR) in gut sacs from all four sections of the GIT. Values are mean \pm SEM ($n = 10$ per treatment). Within each section, statistical significance of the drug's effect in decreasing the rates was tested using unpaired Student's *t* tests (one-tailed). Means labeled with asterisks are significantly different ($P < 0.05$)

reflect much higher mucous secretion rates in the stomach, necessary to protect against acid and pepsin secretion in this segment. The apparent curvilinear data in the stomach mucus at lower concentrations suggest a high affinity for Na^+ (Fig. 3a) which may contribute to the ability of the stomach to absorb much of the ingested Na^+ in vivo (Bucking and Wood 2006b, 2007). A similar dominance of accumulation in the mucus-binding compartment of the stomach has been seen for a range of other cationic metals, with dominance of transport into the blood space for these same metals in the three intestinal segments (Ojo and Wood 2007; Nadella et al. 2011; Klinck et al. 2012).

Uptake rates into the blood space were constant at 1 and 2 h, but fell progressively with time in the mucus-bound and epithelium compartments (data not shown). Furthermore, rates in these latter two compartments were generally

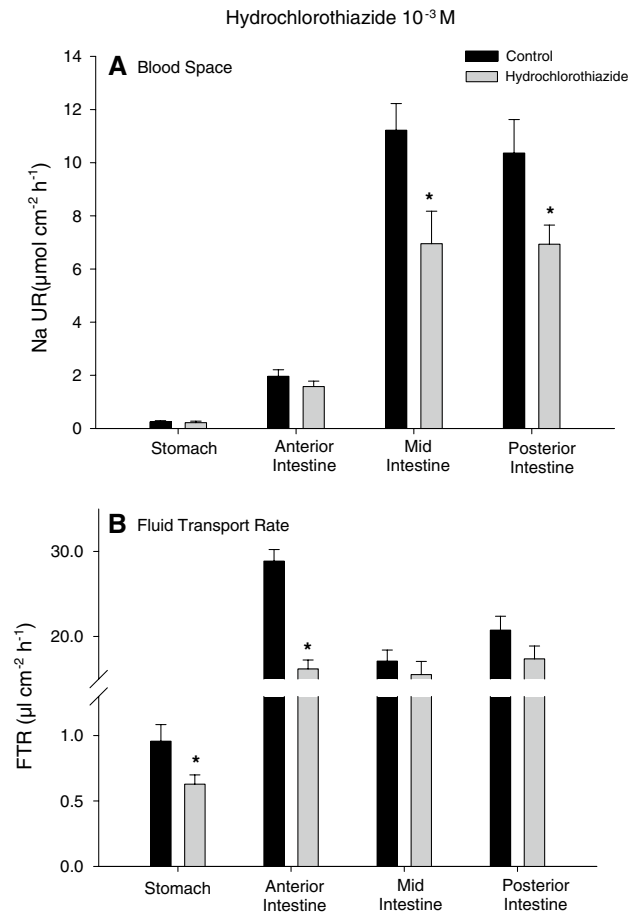


Fig. 11 Effect of hydrochlorothiazide (10^{-3} M) administered in mucosal saline with an Na^+ concentration of 50 mmol L^{-1} on **a** unidirectional Na^+ uptake rate (UR) into the blood space compartment and **b** fluid transport rate (FTR) in gut sacs from all four sections of the GIT. Values are mean \pm SEM ($n = 10$ per treatment). Within each section, statistical significance of the drug's effect in decreasing the rates was tested using unpaired Student's *t* tests (one-tailed). Means labeled with asterisks are significantly different ($P < 0.05$)

unresponsive to the variety of pharmacological agents employed, yet highly responsive in the blood space compartment. Given that the mucus probably saturates quickly, these patterns clearly indicate that it is the apical entry step into the epithelium rather than the basolateral export step which is rate-limiting. If basolateral export had been rate-limiting, then changing apical entry would not have affected the Na^+ transport rate into the blood space, but would have had substantial effects on transport into the epithelium. This was not seen. However, if apical uptake were limiting, then changing apical entry would be reflected in the blood space rate, as seen (Figs. 7, 8, 9, 10, 11, 12), with negligible impact on the epithelium rate. Because of the high transport capacity in the basolateral membranes, regardless of the apical entry rate, the basolateral export rate always reduces intracellular $[\text{Na}^+]$ to a very low

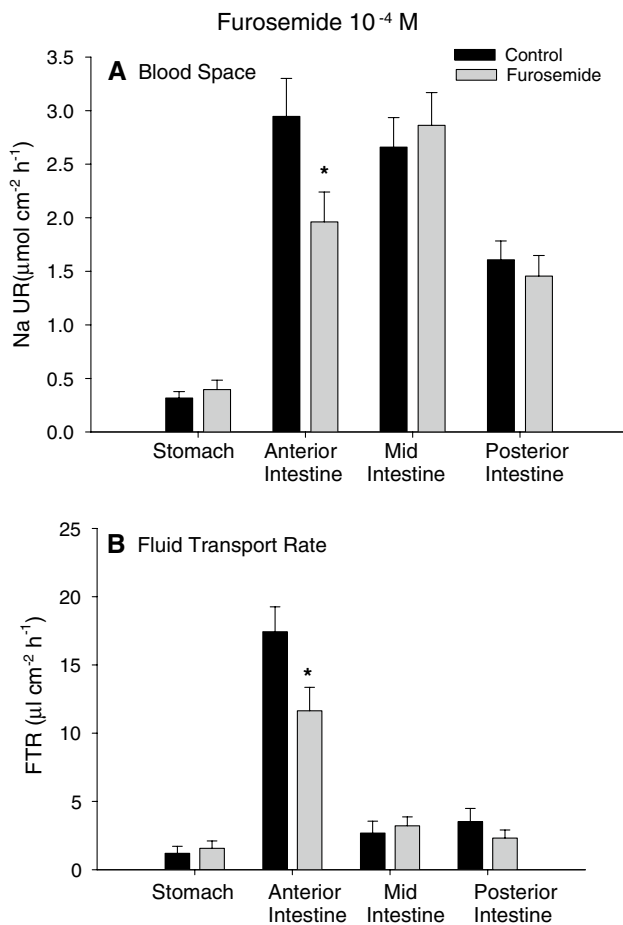


Fig. 12 Effect of furosemide (10^{-4} M) administered in mucosal saline with an Na^+ concentration of 50 mmol L^{-1} on **a** unidirectional Na^+ uptake rate (UR) into the blood space compartment and **b** fluid transport rate (FTR) in gut sacs from all four sections of the GIT. Values are mean \pm SEM ($n = 10$ per treatment). Within each section, statistical significance of the drug’s effect in decreasing the rates was tested using unpaired Student’s t tests (one-tailed). Means labeled with asterisks are significantly different ($P < 0.05$)

concentration—indeed this is how virtually all transporting cells work so as to maintain low stable intracellular Na^+ concentrations. Interestingly, a similar apical limitation has been documented for unidirectional Na^+ and Cl^- uptake rates at the gills of freshwater trout (Morgan et al. 2004).

Fluid transport rate

In marine teleosts, it is well established that transcellular Na^+ and Cl^- transport and associated anion exchange drive water absorption across the intestinal epithelium; in most studies, the transported fluid is isotonic or hypertonic to the body fluids (Loretz 1995; Grosell et al. 2005; Grosell 2011). Accordingly, in the present study on the GIT of a freshwater fish, FTR proceeded in the absence of an osmotic gradient, and progressively increased with increasing

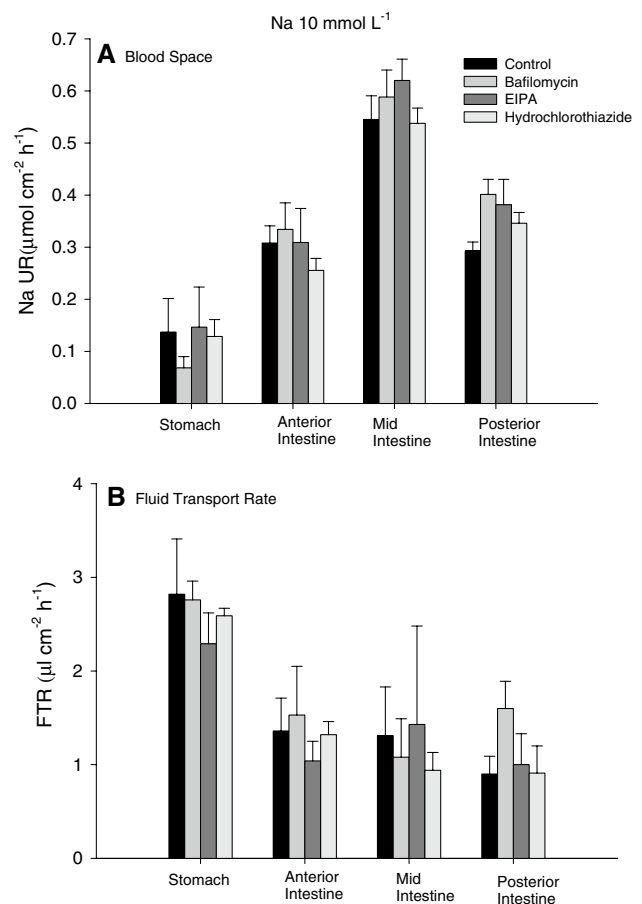


Fig. 13 Effects of bafilomycin (10^{-5} M), EIPA (10^{-4} M), and hydrochlorothiazide (10^{-3} M) administered in mucosal saline at low Na concentration (10 mmol L^{-1}) on **a** unidirectional Na^+ uptake rate (UR) into the blood space compartment and **b** fluid transport rate (FTR) in gut sacs from all four sections of the GIT. Values are mean \pm SEM ($n = 9$ per treatment). There were no significant differences as evaluated by ANOVA

Na^+ and Cl^- concentrations in the present study (Fig. 6). In two of the segments, the relationships were clearly hyperbolic and well-described by the Michaelis–Menten relationship (anterior intestine: $K_m = 169 \text{ mmol L}^{-1}$, $\text{FTR}_{\text{max}} = 30 \mu\text{l cm}^{-2} \text{ h}^{-1}$, $r^2 = 0.88$; mid-intestine: $K_m = 73 \text{ mmol L}^{-1}$, $\text{FTR}_{\text{max}} = 22 \mu\text{l cm}^{-2} \text{ h}^{-1}$, $r^2 = 0.91$), whereas in the stomach and posterior intestine, the relationships were close to linear. At the higher NaCl substrate concentrations, the high NaCl on the luminal side and the added mannitol on the serosal side raised osmolality well above normal. We cannot eliminate the possibility that this caused some tissue damage, but the fact that FTR continued to either increase or plateau, rather than decline, suggests that transport function was not affected. The linkage to Na^+ transport is further reinforced by the finding that most of the pharmacological treatments which inhibited unidirectional Na^+ UR also inhibited FTR (Figs. 7, 8, 9,

Table 2 Effect of phenamil (10^{-4} M) administered in mucosal saline at low Na concentration (10 mM) on Na uptake rate into the blood space (UR) and fluid transport rate (FTR) in all four sections of the trout gut

	Na UR ($\mu\text{mol cm}^{-2}\text{h}^{-1}$)		FTR ($\mu\text{l cm}^{-2}\text{h}^{-1}$)	
	Control	Phenamil (10^{-4} M)	Control	Phenamil (10^{-4} M)
Stomach	0.14 ± 0.02	0.23 ± 0.65	3.78 ± 0.62	9.41 ± 4.54
Anterior intestine	1.60 ± 0.41	2.09 ± 0.31	10.22 ± 6.9	11.79 ± 8.9
Mid-intestine	0.64 ± 0.12	0.44 ± 0.12	2.95 ± 1.07	3.24 ± 0.95
Posterior intestine	0.41 ± 0.07	0.27 ± 0.03	10.91 ± 2.86	8.93 ± 1.38

Values are mean \pm SEM ($n = 9$ per treatment)

10, 11, 12). Note however, that theoretically FTR would be coupled to net Na^+ and Cl^- transport rate, not necessarily to unidirectional Na^+ UR, which may explain the discrepancy between highest FTR values in the anterior intestine (Figs. 6, 7, 8, 9, 10, 11, 12, 13) yet highest unidirectional Na^+ UR values in the mid-intestine (Fig. 2, 7, 8, 9, 10, 11, 12, 13) seen in most experiments.

Kinetics of Na^+ uptake

Na^+ uptake into both the mucus-bound (Fig. 3) and the epithelium compartments (Fig. 4) demonstrated possible saturable relationships at lower Na^+ concentrations, but except for the epithelial compartments of the stomach and mid-intestine (Figs. 4a, c), there were insufficient data points to describe these with confidence in most of the cases. At higher concentrations, the mucus-bound and epithelial relationships were linear, whereas relationships were generally linear over most of the Na^+ concentration range in the blood space compartment (Fig. 5). Smith et al. (1975) used a very different technique to measure Na^+ and Cl^- uptake kinetics in the epithelium compartment of the anterior intestine of both freshwater and seawater flounder, and found similar relationships to those of Fig. 4. These patterns are also very similar to those seen for unidirectional Ca^{2+} uptake kinetics in similar GIT sac experiments on freshwater trout (Klinck et al. 2012). Overall they suggest the presence of both higher affinity and lower affinity binding sites; the former can be saturated, whereas the latter, dominating at higher Na^+ concentrations, may be non-specific and not easily saturated.

In accord with our original hypothesis that the GIT Na^+ transport processes would operate with much lower affinity than those at the gills, the apparent K_m values for these “higher affinity sites” in the GIT would lie in the range of about 100–185 $\text{mmol Na}^+ \text{L}^{-1}$, which is 2–3 orders of magnitude greater (i.e., 2–3 orders of magnitude lower affinity) than K_m values recorded in the gills of similarly sized freshwater trout acclimated to the same Hamilton tapwater (Goss and Wood 1990). This is again very similar to the situation reported for unidirectional Ca^{2+} uptake at the GIT versus the gills in freshwater

trout (Klinck et al. 2012) and reflects the fact that the apical GIT transporters normally encounter Na^+ and Ca^{2+} concentrations which are 2–3 orders of magnitude higher in chyme than in fresh water (Wood and Bucking 2011).

As argued previously, both the gills and the GIT have adequate capacity to fulfill the total uptake needs of the fish for Na^+ (also for Ca^{2+} , see Klinck et al. 2012). However, from an energetic viewpoint, it is likely that high-affinity gill transport is more costly than low-affinity gut transport, as the branchial transporters must work harder to efficiently extract Na^+ from an ion-poor freshwater environment. Indeed, it is well-established that branchial uptake rates of both Na^+ and Ca^{2+} are down-regulated when the dietary supply of these ions is experimentally increased (reviewed by Wood and Bucking 2011).

In the case of transport into the blood space, the general linearity throughout the Na^+ concentration range (except in the mid intestine, Fig. 5c) may have several possible explanations—multiple transporters with a range of different affinities, complex concentration-dependent interactions between apical limitation and basolateral export, or a dominance of non-specific transport mechanisms (e.g., paracellular transport) into this space.

Pharmacological analysis of transporters involved in GIT Na^+ uptake and fluid transport

In tests run at a mucosal Na^+ concentration of 50 mmol L^{-1} , a variety of pharmacological agents, applied in the mucosal saline so as to target apical uptake processes, were effective in partially inhibiting unidirectional Na^+ UR into the blood space compartment and/or FTR in various segments of the GIT (Figs. 7, 8, 9, 10, 11, 12). In general, these results support our original hypothesis that some or all of the same processes are involved as are known to occur in freshwater gills and/or in seawater GIT (see “Introduction”). Notably none of the drugs resulted in complete blockade, their effectiveness varied among segments, and only one drug (phenamil) was effective in all four segments (Fig. 9), suggesting the presence of several different types of transporters whose relative importance varies among different sections of the tract.

Amiloride reduced unidirectional Na^+ uptake rate and/or FTR only in the stomach and mid-intestine (Fig. 7). Amiloride blocks both NHEs and Na^+ channels, but is not a sensitive tool for discriminating between the two (Benos 1982; Kleyman and Cragoe 1988). EIPA, however, preferentially blocks NHEs (26-fold more potent than amiloride against NHEs, <0.035-fold less potent against Na^+ channels; Kleyman and Cragoe 1988). EIPA reduced unidirectional Na^+ UR and/or FTR in the three intestinal segments (Fig. 8), suggesting that this mechanism is present throughout the intestine, but not in the stomach. In contrast, phenamil (17-fold more potent than amiloride against Na^+ channels, <0.008-fold less potent against NHEs; Kleyman and Cragoe 1988) inhibited unidirectional Na^+ UR and/or FTR in all four segments (Fig. 9). This result corroborates our earlier report of partial blockade by phenamil in the mid- and posterior intestine (Nadella et al. 2007), and suggests that some type of Na^+ channel is involved in all sections. Na^+ uptake through epithelial channels is often energized by a V-type proton pump (H^+ ATPase), for which bafilomycin is a specific blocker (Beyenbach and Wieczorek 2006). Its inhibitory actions in three of the four sections suggest that this mechanism occurs in the stomach, anterior, and mid-intestine, but not in the posterior intestine (Fig. 10). There was also evidence for the involvement of co-transport mechanisms. Furosemide, a specific blocker of NKCC (Giménez 2006), was effective in reducing unidirectional Na^+ UR and FTR only in the anterior intestine (Fig. 12), whereas hydrochlorothiazide, an antagonist of NCC (Stokes et al. 1984), was effective on UR only in the mid and posterior intestine, yet curiously effective on FTR only in the stomach and anterior intestine (Fig. 13). The explanation for this latter discrepancy is not apparent.

Figure 14 provides a general model for Na^+ uptake in the GIT of the freshwater rainbow trout, incorporating all the apical mechanisms for which evidence has been garnered in the present study. On an overall basis, these results fit with previous studies on the GIT of seawater teleosts that have provided firm evidence for NKCC2 (Musch et al. 1982; Halm et al. 1985; O’Grady et al. 1986) and NCC (Frizzell et al. 1979; Halm et al. 1985) as apical Na^+ uptake processes. Furthermore, the amiloride, phenamil, bafilomycin, and EIPA data provide direct pharmacological and transport evidence for contributions by NHE and Na^+ channel/ H^+ ATPase systems to Na^+ uptake in the freshwater GIT. The ubiquity of phenamil sensitivity (Fig. 9), suggesting the involvement of Na^+ channels in all four sections, is particularly noteworthy. Previously, there had been molecular evidence that these mechanisms occur in the GIT of seawater fish, but no direct evidence that they contributed to Na^+ uptake (reviewed by Grosell 2011). Notably, however, Howard and Ahearn (1988) reported Na^+ and Cl^- uptake by Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ exchange mechanisms (and curiously no evidence for NCC

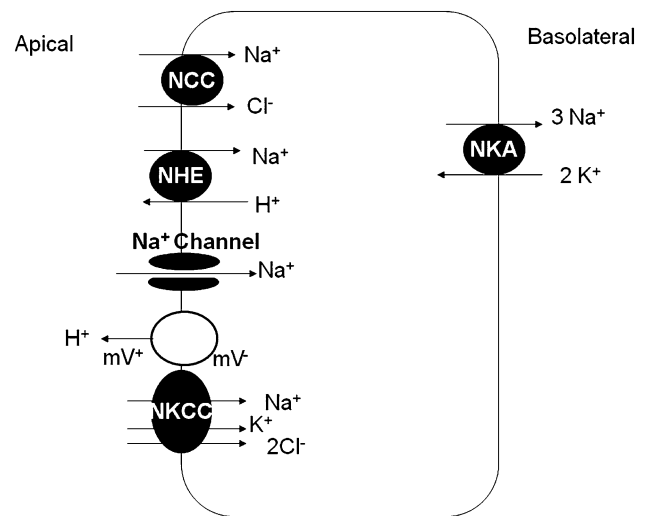


Fig. 14 A general model for the uptake of Na^+ in the GIT of the freshwater rainbow trout, incorporating all the apical processes for which evidence was garnered in the present study

or NKCC mechanisms) in brush border membrane vesicles from the intestine of seawater-acclimated tilapia. In the gills of freshwater fish, there is direct evidence for the involvement of NHEs, Na^+ channel/ H^+ ATPase systems, and NCC, but not of NKCC in active Na^+ uptake (reviewed by Flik et al. 1997, Marshall 2002, Kirschner 2004, Hwang and Lee 2007, Evans 2011, and Kumai and Perry 2012).

NHE systems are thermodynamically challenged to transport Na^+ against large concentration gradients, so they are often replaced by Na^+ channel/ H^+ ATPase systems when there is a need to actively take up Na^+ from dilute solutions (e.g., Avella and Bornancin 1989; Kirschner 2004; Parks et al. 2008). The same may be true of NCC systems which depend on the Na^+ gradient. Therefore, our purpose in running a subset of tests at a mucosal Na^+ concentration of 10 mmol L^{-1} was to discern whether NHE-mediated uptake (targeted by EIPA) and NCC-mediated uptake (targeted by hydrochlorothiazide) would be relatively diminished, and Na^+ channel/ H^+ ATPase transport (targeted by phenamil and bafilomycin) relatively augmented under these conditions. None of these agents had any effect on unidirectional Na^+ UR or FTR (Fig. 13; Table 2). This indicates that none of these mechanisms are active at low mucosal Na^+ concentrations which are well below the apparent K_m values of the overall transport systems. Yet unidirectional Na^+ UR and FTR continued. The implication is that there are other, as yet unidentified Na^+ transport systems which function at low Na^+ concentrations, and/or that FTR is driven by the active uptake transport of other moieties [e.g., glucose and amino acid transporters (Smith 1966; Ferraris and Ahearn 1984)], some of which are known to be Na^+ -coupled entraining Na^+ uptake by solvent drag.

Conclusions

Based on these *in vitro* tests, the theoretical capacity for Na^+ uptake across the GIT of freshwater rainbow trout is sufficient to supply all of the animal's nutritive requirements for Na^+ . Transport occurs by low affinity systems with K_m values 2–3 orders of magnitude higher than those in the gills, in accord with comparably higher Na^+ concentrations in chyme versus fresh water. Na^+ transport and fluid transport appear to be coupled, such that treatments which reduce unidirectional Na^+ uptake rate generally reduce FTR. Pharmacological trials indicate that GIT Na^+ uptake occurs by a variety of apical mechanisms (NHE, Na^+ channel/ H^+ ATPase, NCC, NKCC) with relative contributions varying among sections. In future, it will be useful to substantiate these findings using *in vivo* preparations where hormonal and neuronal signals can play their customary role. Additional evidence of mRNA and protein expression for the transporters will validate their presence in the freshwater trout GIT.

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