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Defecation and the fate of dietary sodium in the common killifish (*Fundulus heteroclitus macrolepidotus* Walbaum, 1792)

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ABSTRACT

While uptake of Na^+ from the water is well-characterized in fish, little is known about the uptake of Na^+ from the diet. A method involving radiolabeling of the diet with [$^{22}Na^+$] and [^3H] PEG-4000 has been developed to study this process, and to separate the systemic efflux of absorbed [$^{22}Na^+$] *via* gills and kidney from the potential efflux of [$^{22}Na^+$] into the water *via* defecation. Killifish were acclimated to 10% sea water, a typical salinity for this estuarine fish where they are hyperosmotic to the environment. The fish were fed a single meal (0.81% ration, containing 5.09 μmol total Na^+ g body weight^{-1}) of re-pelleted food labeled with both radiotracers. Effluxes into the water were monitored for 48 h. Sharp increases in [^3H] PEG-4000 appearance provided a clear definition of defecation events, which started at about 7 h post-feeding and finished by 16 h, with the midpoint at 11.5 h. In contrast, the evolution of [$^{22}Na^+$]-radioactivity in the water showed a smooth curvilinear relationship starting at 3 h, with a gradually declining slope through 48 h. There was no efflux of [$^{22}Na^+$] associated with defecation events, showing that all [$^{22}Na^+$] was quickly absorbed from the meal. By 48 h, about 50% of the consumed [$^{22}Na^+$] had been excreted to the external water by systemic efflux. The efflux relationship was best described by a two-phase exponential relationship, with a breakpoint at about 13 h post-feeding. The first phase (rate constant 0.0309 h^{-1}) corresponded to branchial efflux of dietary [$^{22}Na^+$] equilibrated with the exchangeable whole body Na^+ pool, while the second phase was much slower (0.0114 h^{-1}). These results emphasize the importance of dietary Na^+ when the killifish is hyper-regulating, and provide a method applicable to a wide range of conditions, ions, and species.

1. Introduction

An elegant "thought experiment" by Smith et al. (1989), based mainly on calculations from literature values, concluded that freshwater fish in the wild may acquire more than half of their Na^+ uptake from the food consumed. The situation is less clear for fish living in sea water or intermediate salinities. However, the vast majority of experimental studies on ionoregulation in fish have been performed on fasted animals, an approach often justified on the basis of standardizing metabolic rate, or avoiding the annoying problem of defecation into the water as a confounding factor in ion flux rate measurements. Recently, we have argued that this has created a rather biased or abnormal view of ionoregulation, particularly for freshwater fish, because in nature, they may take up ions from both the water and the diet, whereas only the waterborne route has been considered in most laboratory investigations (Wood and Bucking, 2011). Indeed, when the gastro-intestinal route has been considered at all, it is usually from the point of view of understanding ionic uptake from water that has been drunk (*e.g.* Grosell, 2006; Loretz, 1995), rather than from food which has been consumed. This biased view is the picture which is presented in most standard comparative physiology textbooks. However, a recent text devoted entirely to the gastro-intestinal tract of fish as a multifunctional organ (Grosell et al., 2011) has re-emphasized the need to integrate feeding into our understanding of ionoregulation in fish.

In practice, this is quite difficult. While it is relatively easy to measure the ion content of food, it is more challenging to measure how much is actually taken up from the food, and how that fraction is handled internally by the fish. Because of their high solubility, some of the electrolytes may be lost to the water before the food is consumed, and some may not be absorbed but rather pass out in the feces and rectal fluid in the process of defecation. Yet at the same time some of the absorbed ions may also pass out across the gills or kidney, even before absorption is completed. Can these two routes be separated? And do ions absorbed from the diet enter the same internal pool as ions absorbed across the gills? Radiotracer techniques have been fundamental to quantifying ionoregulatory processes in the much simpler situation of ion exchange with the water alone (Evans et al., 2005; Marshall and Grosell, 2006). In the present study we apply radiotracer methods to successfully address these problems in the study of dietary ion uptake and fate. In particular, we use this approach to describe the fate of ^{22}Na uptake from the diet, and separate the appearance of ^{22}Na in the water

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from the possible efflux of dietary ^{22}Na by defecation. For the latter, we have exploited [^{3}H] polyethylene-glycol ([^{3}H] PEG-4000), a high molecular weight, non-toxic marker of the fluid phase that is often used to quantify drinking rate in fish (Wood and Bucking, 2011). Earlier we demonstrated that this compound moved through the digestive tract of trout at a comparable rate to a solid phase marker (Bucking and Wood, 2006), but to our knowledge it has never been used before to detect defecation events in fish.

We have attacked this problem in the common killifish or mummichog (*Fundulus heteroclitus*), a small, strongly euryhaline fish native to the Atlantic coast of North America (Griffith, 1974). This killifish lives predominantly in waters of intermediate salinity in tidal flats, marshes, and estuaries, moving in and out on the tide to feed (Marshall, 2003). It has been the subject of both early classic radiotracer studies (Maetz et al., 1967; Motais et al., 1966; Pic, 1978; Potts and Evans, 1967), as well as more recent ones (Patrick and Wood, 1999; Patrick et al., 1997; Prodocimo et al., 2007; Wood, 2011; Wood and Laurent, 2003) focused on waterborne ionoregulation, and indeed the killifish has become a model organism for this topic (Burnett et al., 2007; Marshall, 2003; Marshall et al., 1999; Wood and Marshall, 1994). However, again the role of ion uptake from the diet has been almost completely overlooked. Our objective was to develop a radiotracer-based method to describe the uptake and handling of ^{22}Na from the diet, and to separate its systemic efflux (*i.e. via* gills and kidney) from efflux through defecation.

2. Methods and materials

Procedures were approved by the McMaster University Animal Care Committee and complied with the regulations of the Canada Council for Animal Care.

2.1. Animals

Common killifish of the northern subspecies (*F. heteroclitus macrolepidotus*; 3.30–10.22 g) were collected by beach seining from tidal flats close to Hampton, New Hampshire by Aquatic Research Organisms (ARO) Ltd. At McMaster University, they were held for several months prior to experiments in 500-l fiberglass tanks containing recirculated, charcoal-filtered 10% sea water (SW) at 18–20 °C, the subsequent experimental temperature. The measured composition of this 10% sea water used for acclimation and experimentation was $Na^+ = 48–52$, $Cl^- = 56–60$, $Ca^{2+} = 1.1–1.6$, $Mg^{2+} = 4.9–5.5$ mmol l^{-1}, pH = 7.7–8.1. The fish were fed once daily to satiation with a mix of commercial flakes (Wardley Total Tropical Gourmet Flake Blend, Hartz Mountain Corp., Secausus, NJ, U.S.A.) and frozen brine shrimp (San Francisco Bay Brand, Newark, CA, U.S.A.).

2.2. Diets

The experimental diet was made by grinding the commercial flakes into a fine powder using a mortar and pestle, and then compounding the powder (60% by weight) with a slurry of defrosted brine shrimp (40% by weight). The paste was then extruded through a 20-ml syringe into long thin ropes of food paste, dried in an oven (60 °C) for 24 h and then crumbled by hand to form food pellets. This diet was used during the pre-experimental training program. The diet used for the actual experiment was made from a 30-g subsample of the same batch of food paste which was spiked with 17.5 μCi of ^{22}Na (as NaCl, NEN-Dupont, Boston, MA, U.S.A.) and 100 μCi of [^{3}H] polyethylene glycol-4000 (PEG-4000; NEN-Dupont, Boston, MA, U.S.A.). The spiking had no effect on the measured Na^+ concentration of the diet which was 627.1 ± 2.5 μmol g^{-1} ($N = 7$). The accompanying [$^{22}Na^+$]-radioactivity was $449{,}034 \pm 1924$ cpm g^{-1} and the [^{3}H] PEG-4000 radioactivity was $767{,}947 \pm 4379$ cpm g^{-1}.

2.3. Experiments

Three experimental series were performed. In each, the fish were first trained to eat the diet by hand-feeding. Groups of 8 fish were isolated in a single 15-l bucket of 10% SW, with daily water changes. Each morning, the fish were fed by hand as a group for 7–10 days. Thereafter, each fish was transferred to a separate bucket, and training continued for another 3–5 days with individual feeding. The goal was to have each fish consume the diet immediately, without time for any leaching or dispersal of the pellets. On the day of the actual experiment, the fish were fed the amount of radio-labeled food (exact weight recorded) that the experimenter knew they would consume immediately. They were then transferred to an individual polyethylene chamber containing 200 ml of 10% SW and fitted with a lid, sampling port, and individual aeration; the aeration served to provide good mixing in the chamber. Experimental sampling commenced immediately. In each series, 5–6 animals met the criterion of immediate feeding with no regurgitation of radioactivity into the flux chamber, and were used in the analyses.

In *series 1*, 5-ml water samples for measurements of [$^{22}Na^+$] and [^{3}H] PEG-4000 radioactivities were taken at one-hour intervals from 0 to 9 h, and then at 24 and 48 h, with water changes at 9 h and 24 h. Based on the results of *series 1*, we focused on later time points in *series 2* and *3*, with samples taken at 0 h, 7–24 h (hourly intervals), then at 32, 36, 40, 44, and 48 h, with water changes at 12, 24, and 32 h. In *series 3*, the fish were watched carefully for defecation throughout the experiment, particularly in the 6–18 h period, and feces were removed within 30 min for separate counting. In addition, at 48 h the fish were rapidly rinsed in non-radioactive media, euthanized in neutralized MS-222 (0.5 g l^{-1}; Syndel Laboratories, Vancouver, Canada), blotted, weighed, and the gut was carefully dissected out after ligating it at both ends with silk suture. The feces, gut, and carcass were counted separately for [$^{22}Na^+$] and [^{3}H] PEG-4000 radioactivities.

2.4. Analyses

Water (5 ml-samples), food pellets, feces, gut, and carcass samples were first counted for [$^{22}Na^+$]-radioactivity on a Minaxi-γ Auto gamma 5530 counter (Canberra Packard, Mississauga, Ontario, Canada) (note: [^{3}H] PEG-4000 produces only beta-radioactivity so is not detected by gamma counting). Water samples were then recounted for combined [$^{22}Na^+$] and [^{3}H] PEG-4000 radioactivities on a Tri-Carb 2900TR liquid scintillation counter (Perkin Elmer, Waltham, MA, U.S.A.) using 10 ml ACS fluor (Amersham, Oakville, ON, Canada). The food pellets, feces, gut, and carcass samples were digested in 4 volumes of analytical grade 1 N HNO_3 in a sealed tube, and baked in an oven at 60 °C for 48 h with periodic vortexing. The digest was then cooled, centrifuged at 500 G for 5 min; 1 ml of supernatant was added to 5 ml of Ultima Gold AB (Packard Bioscience, Groningen, Netherlands), a scintillation fluor specifically designed for the counting of acidic digests. The counting windows on the scintillation counter were set so as to minimize the spillover of [$^{22}Na^+$] radioactivity into the [^{3}H]-window, and the counting efficiency of [$^{22}Na^+$] in the gamma counter relative to that in the [^{3}H]-window of the scintillation counter (generally 5–8%) was measured on every run. This ratio was used to correct the [^{3}H] cpm for the small contribution of [$^{22}Na^+$] cpm. Internal standardization was employed to correct for [^{3}H] quenching in the digest samples and for minor differences in counting efficiency between the ACS and Ultima Gold AB fluors. All efficiencies were corrected to those of the water samples in ACS fluor. Total sodium concentrations in acid digests of food pellets and in water samples were measured by atomic absorption spectrophotometry (AAS; Varian Australia Model 220FS, Mississauga, ON, Canada).

2.5. Statistics

Data have been expressed as means ± 1 SEM (N). Differences between [$^{22}Na^+$] and [^3H] PEG-4000 appearance in the water at the same times were assessed by Student's two tailed paired *t*-test. Percentage data were arc-sin transformed prior to statistical test. Rate constants (fractional loss h^{-1}) quantifying the efflux of $^{22}[Na^+]$-radioactivity were determined by fitting two-phase exponential models to the time-series data using Sigmaplot™ V.10; the break-point between the two phases was chosen so as to maximize the r^2 value for the first phase(Causton, 1983). A significance level of $P<0.05$ was employed throughout.

3. Results

Killifish consumed a mean ration of 0.811 ± 0.065% (17) of body weight, corresponding to 5.09 ± 0.41 (17) μmol Na^+ g body weight^{-1}.

The first appearance of [^3H] PEG-4000 radioactivity in the water occurred at 1 h post-feeding, though it remained very low (<10% of the consumed total) until between 6 and 11 h in different fish. Fig. 1A illustrates an example from *series 1* where a precipitous increase started after 6 h, and Fig. 1B an example from *series 2* where the appearance was slower, a marked increase occurring only after 10 h, and the precipitous rise only after 12 h. These sharp rises are interpreted as defecation.

The appearance of [$^{22}Na^+$]-radioactivity in the water exhibited a very different pattern. The first detection of [$^{22}Na^+$] in the water was not until 2 h (3 h in most fish), and thereafter appearance occurred at a steady rate through 9–15 h, gradually slowing thereafter.

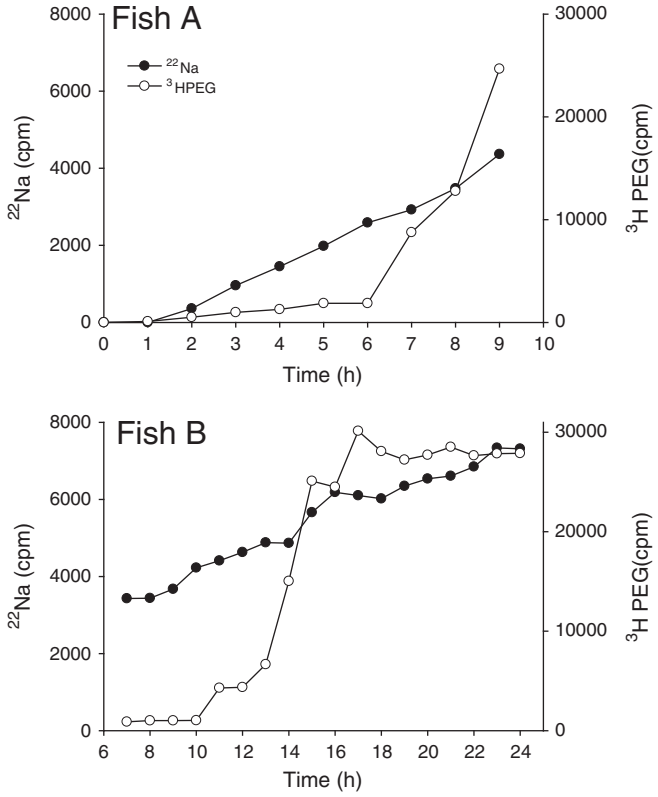

Fig. 1. Examples of the appearance of [^3H] PEG-4000 radioactivity and [$^{22}Na^+$] radioactivity in the water after killifish were fed a single meal spiked with the two radio-labels. (A) Fish A, *series 1*, 9.70 g, 0.526% ration. Note the sharp increase in [^3H] PEG-4000 cpm after 6 h without an accompanying change in the rate of appearance of [$^{22}Na^+$] cpm. (B) Fish B, *series 2*, 5.20 g, 0.962% ration. Note the more delayed increase in [^3H] PEG-4000 cpm after 10–12 h, again without an accompanying change in the rate of appearance of [$^{22}Na^+$] cpm. The sharp increases in [^3H] PEG-4000 cpm are interpreted as defecation events.

Close inspection of individual records indicated that there was little or no change in the slope of the [$^{22}Na^+$] curve associated with clear defecation events (*e.g.* Fig. 1A, B). Thus loss of dietary [$^{22}Na^+$] through the feces appeared to be negligible.

Although the three series covered different sampling times, the [^3H] PEG-4000 and [$^{22}Na^+$]-radioactivity relationships were clearly similar in all fish, so the data were averaged at all sample times (Fig. 2). This illustrates that defecation started at about 7 h post-feeding and was essentially finished by 16 h, with the midpoint at about 11.5 h. In contrast, the evolution of [$^{22}Na^+$]-radioactivity in the water showed a smooth curvilinear relationship with no change in slope at the time of defecation, but a gradually declining slope as time proceeded. At virtually all times, the relative appearance of [$^{22}Na^+$] was significantly different from that of [^3H] PEG, being higher from 4 h to 8 h, and lower from 12 h onwards (Fig. 2). By the end of the experiment (48 h), 81.5 ± 3.1% (17) of the consumed [^3H] PEG-4000 radioactivity had appeared in the water and excretion appeared to have ended. However, only 50.1 ± 3.5% (17) of the consumed [$^{22}Na^+$]-radioactivity had been effluxed by 48 h, and appearance of $^{22}Na^+$ in the water was clearly continuing.

Compartmental analysis was performed on the fish of *series 3* at 48 h post-feeding (Table 1). These animals had a slightly greater total excretion of [^3H] PEG-4000 radioactivity (92.7%) and [$^{22}Na^+$]-radioactivity (57.2%) to the water than in *series 1* and *2*, but the differences were not significant. The analysis demonstrated that there was essentially no [^3H] PEG-4000 (<1%) left in the gut or in the freshly collected feces, whereas a very small amount was in the carcass (1.5%) at 48 h. In contrast, for [^{22}Na], there was a substantial amount (27.3%) left in the carcass, while the amounts in the gut and feces were again negligible. Overall recovery was significantly higher for [^3H] PEG-4000 radioactivity (95.2%) than for [$^{22}Na^+$]-radioactivity (85.4%).

4. Discussion

4.1. Defecation

[^3H] PEG-4000 appearance in the water provided a very clear indication of defecation events in the killifish. Our visual observations of the times at which feces appeared in *series 3* corresponded well to the times of sharp increases in [^3H] PEG-4000 radioactivity in the water. Therefore, this compound, which has been commonly used as both a drinking rate marker (when added to the external water; Scott et al., 2006, 2008) and a glomerular filtration marker (when

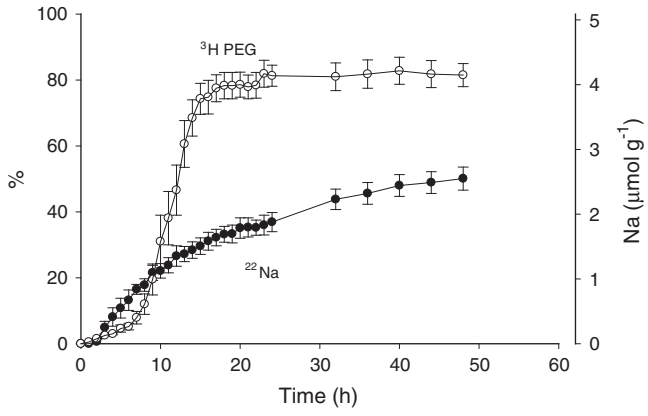

Fig. 2. The mean patterns of appearance of [^3H] PEG-4000 radioactivity and [$^{22}Na^+$] radioactivity in the water for 48 h after killifish were fed a single meal spiked with the two radio-labels. Note the very different appearance profiles of [$^{22}Na^+$] cpm *versus* [^3H] PEG-4000 cpm. On the right-hand axis, the cumulative excretion of the dietary Na^+ load, in μmol Na^+ g body weight^{-1}, has been calculated, based on the measured specific activity of the diet. Data from all three series have been averaged. Means ± 1 SEM (N = 6 at 1–6 h, 17 at 6–9 h, 24 h, and 48 h, and 11 at other time points.

Table 1
Compartmental analysis of the distribution of [^{3}H] PEG-4000 radioactivity and [$^{22}Na^+$] radioactivity at 48 h post-feeding in the killifish of *series 3*. Values are expressed as a percentage of the amount consumed. Means ± 1 SEM (N = 6).

	[^{3}H] PEG-4000	[$^{22}Na^+$]
Water	92.7 ± 3.2	57.2 ± 4.7*
Feces	0.6 ± 0.1	0.0 ± 0.0*
Gut	0.4 ± 0.2	0.9 ± 0.2*
Carcass	1.5 ± 0.2	27.3 ± 4.8*
Total	95.2 ± 3.3	85.4 ± 2.9*
Unknown	4.8 ± 3.3	14.6 ± 2.9*

* $P<0.05$.

injected into the bloodstream; Scott et al., 2004) in killifish, has yet another use as a defecation marker. This conclusion fits well with the findings of Bucking and Wood (2006) that [^{3}H] PEG-4000 radioactivity (as a fluid phase marker) moves through the digestive tract of freshwater trout at about the same rate as ballotini beads, a solid phase marker. Although [^{3}H] PEG-4000 was incorporated into the solid phase of the food in the present study, it is highly soluble. After ingestion, it was likely distributed mainly in the fluid phase of the chyme in the intestinal tract, and within the slurry of feces and rectal fluid at the time of defecation. This is reinforced by the finding that very little of the [^{3}H] PEG-4000 could be recovered in the feces (Table 1); most was probably lost to the external water within the period of up to 30 min during which the feces sat in flux chamber before collection.

It is notable that a very small but detectable percentage (1.5%) of the total dietary load of [^{3}H] PEG-4000 was found in the carcass at 48 h (Table 1). This fits with recent findings, based on *in vitro* gut sac preparations, that PEG-4000 has a slight but finite permeability ($\sim 2\times10^{-7}$ cm s^{-1}) across the killifish intestinal tract (Wood and Grosell, 2012) and an older observation by Bogé et al. (1988) that it was absorbed at a very slow rate from the intestinal perfusate in intact rainbow trout. Thus PEG-4000 cannot be considered as a "non-absorbable" marker but rather a poorly absorbed one that is a very useful label for tracking the movement and evacuation of intestinal tract contents in fish.

Overall the recovery of [^{3}H] PEG-4000 radioactivity was very high (95.2 ± 3.3%; Table 1) indicating that losses due to leaching prior to consumption or to counting errors were negligible. Recovery of [^{22}Na]-radioactivity was also high (85.4 ± 2.9%; Table 1), but there was clearly a small fraction which could not be accounted for. We suspect that this was mainly due to blood loss upon dissection, as blood plasma has a high concentration of Na^+.

We are aware of no previous measurements of gut transit time in the killifish. It is well known that gut transit time in fish is influenced by many factors, decreasing with greater temperature, smaller body size, and greater feeding frequency (Fange and Grove, 1979; Stevens and Hume, 1996). Taking these factors into account, our measurement of a mean time of 11.5 h at 18–20 °C in the killifish, with defecation starting at 7 h and finishing at 16 h after a single discrete meal, appears to be fast relative to other teleosts (see Tables in these reviews). This may relate to morphology of the gut in the killifish which lacks a stomach and has a short simple intestine (Babkin and Bowie, 1928).

4.2. The fate of dietary Na^+

Our concern that significant [$^{22}Na^+$] efflux might occur through defecation proved unfounded. All of the [$^{22}Na^+$] present in the diet was absorbed prior to defecation, such that the evolution of [$^{22}Na^+$] into the water exhibited a smooth curvilinear relationship over time, with no change in slope at the time of defecation. Prior to interpreting this relationship, it is important to consider specific activity relationships.

At a ration of 0.811% of body weight, the total Na^+ content of the meal was 5.1 μmol Na^+ g body weight^{-1}. The total exchangeable pool of internal Na^+ in killifish acclimated to 10% seawater has been measured at 65.5 μmol Na^+ g body weight^{-1} (Wood and Laurent, 2003). With an outside water volume of about 30 ml g body weight^{-1} and a Na^+ concentration of 50 μmol ml^{-1} in 10% SW, the external Na^+ pool was about 1500 μmol Na^+ g body weight^{-1}. Thus the internal Na^+ pool was 13-fold greater than the dietary Na^+ load, and the external Na^+ pool was 23-fold greater than the internal Na^+ pool, and 300-fold greater than the dietary Na^+ load. The normal criterion for radiotracer flux measurements is that specific activity (*i.e.* ratio of [$^{22}Na^+$]-to-total Na^+) should be kept 10-fold higher on the flux-generating side than on the flux-receiving side, so as to avoid significant recycling of the radiotracer (Kirschner, 1970). This criterion appears to have been comfortably met in the current experimental setup. Even at the end of the experiment (48 h) when 57% of the [$^{22}Na^+$]-radioactivity had moved into the external water (Table 1), the internal specific activity would still have been 11-fold higher than the external specific activity even if we had not changed the water. In practice it was closer to 50-fold higher because of the water replacement. Thus radio-isotopic backflux was not the explanation for the gradually declining slope of the [$^{22}Na^+$] efflux relationship over time. Rather, the data are suggestive of exponential washout.

Using the criteria of Causton (1983) for curve-fitting, these data were best described by a two-phase exponential relationship, with a breakpoint at 10 h (Fig. 3). The rate constant of the first phase (3–9 h) was 0.0309 ± 0.0011 h^{-1} ($r^2 = 0.994$, $P<0.0001$), while that of the second phase (11–48 h) was much slower, 0.0114 ± 0.0004 h^{-1} ($r^2 = 0.983$, $P<0.0001$). Applying the first rate constant to the sum of the dietary and internal Na^+ pools (5.1 + 65.5 = 70.6 μmol Na^+ g body weight^{-1}) yields a unidirectional Na^+ efflux rate of 2182 nmol Na^+ g body weight^{-1} h^{-1}. This is reasonably close to the unidirectional Na^+ efflux rate of about 3000 nmol Na^+ g body weight^{-1} h^{-1} measured in earlier studies on killifish acclimated to 10% SW (Wood, 2011; Wood and Laurent, 2003). This unidirectional efflux is thought to occur mainly across the gills. The clear conclusion is that the dietary Na^+ load is rapidly equilibrated with the whole body Na^+ pool, such that its [$^{22}Na^+$] can serve as a valid marker of unidirectional Na^+ efflux across the gills. This rapid equilibration fits with findings that unidirectional Na^+ flux rates across the intestinal wall

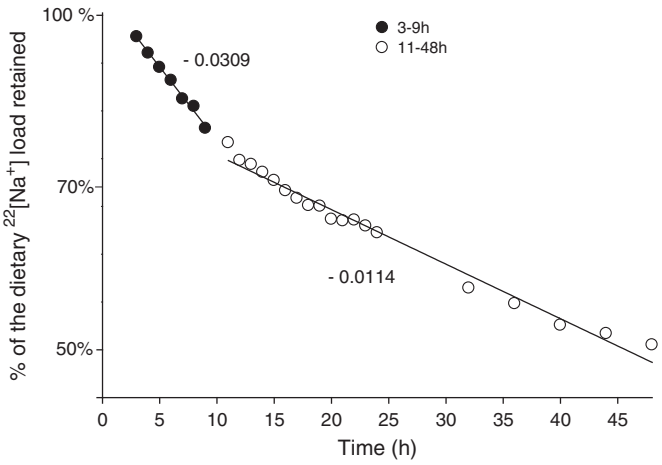

Fig. 3. Analysis of the appearance pattern of [$^{22}Na^+$] radioactivity in the water as a two phase exponential washout with a breakpoint at 10 h, applying the criteria of Causton (1983) for curve-fitting. Note that the Y axis is on a natural logarithm scale. The rate constants for the two phases are given. The equations are: Phase 1: Ln Y = −0.0309 X (±0.0011) + 4.6463 (±0.0069) ($r^2 = 0.994$, $P<0.0001$). Phase 2: Ln Y = −0.0114 X (±0.0004) + 4.4217 (±0.0098) ($r^2 = 0.982$, $P<0.0001$) where Y is the % of the dietary ^{22}[Na$^+$] load retained, X is time in h, and errors are SEM.

of *in vitro* gut sac preparations in killifish acclimated to 10% SW are 10–25 fold greater than net intestinal Na^+ absorption rates (Scott et al., 2006, 2008).

The explanation for the slow phase of [$^{22}Na^+$] efflux is less clear. Possibly, the remaining [$^{22}Na^+$] labels an internal Na^+ pool which exchanges much more slowly, such as the bile, urine, scales, bone *etc*. This raises the prospect that ions absorbed from the diet may to some extent enter a different pool than ions exchanged across the gills. For example, intestinal blood flow proceeds directly to the liver through the hepatic portal system, and the liver synthesizes a new batch of bile once digestion has started.

On a quantitative basis, the absolute amount of dietary Na^+ which was excreted can be calculated from the specific activity of the diet. As illustrated in the right-hand axis of Fig. 2, about 2.55 μmol Na^+ g body weight^{-1} had been excreted by 48 h, so 50% was still retained upon a net basis. To put this in perspective, the net amount retained from the diet at 48 h post-feeding was about 4% of the internal Na^+ pool, and approximately equivalent to the amount passing unidirectionally across the gills in only 1 h, by the preceding calculations. While extremely high, unidirectional Na^+ influx and efflux rates at the gills are approximately equal in the two directions in 10% SW, so there is almost no net flux (Wood, 2011; Wood and Laurent, 2003). Therefore, dietary Na^+ acquisition is clearly an important component of the killifish's net ion budget under the present experimental conditions, where the animal is hyper-regulating relative to the environment.

4.3. Perspectives

The methodology developed in this study has been applied to only one electrolyte (Na^+) in killifish acclimated to only one salinity condition (10% SW), but in future it could be applied to other ions, other species, and other salinities. In *F. heteroclitus* in particular, it will be of interest to apply this approach to compare animals acclimated to freshwater (FW) *versus* 100% SW. Drinking rates *in vivo* are more than 7-fold higher in 100% SW than in FW, and unidirectional Na^+ exchange rates and net Na^+ uptake rates of gut sac preparations *in vitro* are about 2-fold higher (Scott et al., 2006, 2008), so rates of defecation and ion absorption across the intestine *in vivo* may also be very different. Furthermore in FW, unidirectional Na^+ flux rates at the gills are about 30-fold lower than in 100% SW, and the FW-acclimated killifish is unusual inasmuch as there is no active uptake of Cl^- from the water at all across the gills (Patrick and Wood, 1999; Patrick et al., 1997; Wood, 2011; Wood and Laurent, 2003). *In vitro* evidence strongly suggests that Cl^- acquisition occurs entirely from the diet in FW-acclimated killifish, and at a higher rate than Na^+ acquisition (Scott et al., 2006; Wood et al., 2010). However these conclusions remain to be proven and quantified *in vivo*. In future investigations, the present methodology can be used to address these interesting issues.

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References

Babkin, B.P., Bowie, D.J., 1928. The digestive system and its function in *Fundulus heteroclitus*. Biol. Bull. 54, 254–277.

Bogé, G., Lopez, L., Pérès, G., 1988. An *in vivo* study of the role of pyloric caeca in water absorption in rainbow trout (*Salmo gairdneri*). Comp. Biochem. Physiol. A 91, 9–13.

Bucking, C., Wood, C.M., 2006. Water dynamics in the digestive tract of freshwater rainbow trout during the processing of a single meal. J. Exp. Biol. 209, 1883–1893.

Burnett, K.G., Bain, L.J., Baldwin, W.S., Callard, G.V., Cohen, S., Di Giulio, R.T., Evans, D.H., Gomez-Chiarri, M., Hahn, M.E., Hoover, C.A., Karchner, S.I., Katoh, F., MacLatchy, D.L., Marshall, W.S., Meyer, J.N., Nacci, D.E., Oleksiak, M.F., Rees, B.B., Singer, T.D., Stegeman, J.S., Towle, D.W., Van Veld, P.A., Vogelbein, W.K., Whitehead, A., Winn, R.N., Crawford, D.L., 2007. *Fundulus* as the premier teleost model in environmental biology: opportunities for new insights using genomics. Comp. Biochem. Physiol. D 2, 257–286.

Causton, D.R., 1983. A Biologist's Basic Mathematics. Arnold, London.

Evans, D.H., Piermarini, P.M., Choe, K.P., 2005. The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid–base regulation, and excretion of nitrogenous waste. Physiol. Rev. 85, 97–177.

Fange, R., Grove, D., 1979. Digestion. In: Randall, D.J., Hoar, W.S. (Eds.), Fish Physiology, Vol. 8. Academic Press, New York, pp. 161–260.

Griffith, R.W., 1974. Environment and salinity tolerance in the genus *Fundulus*. Copeia 1974, 319–331.

Grosell, M., 2006. Intestinal anion exchange in marine fish osmoregulation. J. Exp. Biol. 209, 2813–2827.

Grosell, M., Farrell, A.P., Brauner, C.J. (Eds.), 2011. The Multifunctional Gut of Fish. : Fish Physiology, Vol. 30. Academic Press, San Diego.

Kirschner, L.B., 1970. The study of NaCl transport in aquatic animals. Am. Zool. 10, 365–376.

Loretz, C.A., 1995. Electrophysiology of ion transport in teleost intestinal cells. In: Wood, C.M., Shuttleworth, T.J. (Eds.), Cellular and Molecular Approaches to Fish Ionic Regulation. : Fish Physiology, Vol. 14. Academic Press, San Diego, pp. 25–56.

Maetz, J., Sawyer, W.H., Pickford, G.E., Mayer, N., 1967. Evolution de la balance minerále du sodium chez *Fundulus heteroclitus* au cours du transfert d'eau de mer d'eau douce: effets du hypophysectomie et de la prolactine. Gen. Comp. Endocrinol. 8, 163–176.

Marshall, W.S., 2003. Rapid regulation of NaCl secretion by estuarine teleost fish: coping strategies for short-duration freshwater exposures. Biochim. Biophys. Acta 1618, 95–105.

Marshall, W.S., Grosell, M., 2006. Ion transport, osmoregulation, and acid–base balance. In: Evans, D.H., Claiborne, J.B. (Eds.), The Physiology of Fishes. CRC Press, Boca Raton, pp. 177–230.

Marshall, W.S., Emberley, T.R., Singer, T.D., Bryson, S.E., McCormick, S.D., 1999. Time course of salinity adaptation in a strongly euryhaline estuarine teleost, *Fundulus heteroclitus*: a multivariable approach. J. Exp. Biol. 202, 1535–1544.

Motais, R., Garcia-Romeu, F., Maetz, J., 1966. Exchange diffusion effect and euryhalinity in teleosts. J. Gen. Physiol. 50, 391–442.

Patrick, M.L., Wood, C.M., 1999. Ion and acid–base regulation in the freshwater mummichog (*Fundulus heteroclitus*): a departure from the standard model for freshwater teleosts. Comp. Biochem. Physiol. A 122, 445–456.

Patrick, M.L., Pärt, P., Marshall, W.S., Wood, C.M., 1997. Characterization of ion and acid–base transport in the freshwater adapted mummichog (*Fundulus heteroclitus*). J. Exp. Zool. 279, 208–219.

Pic, P., 1978. A comparative study of the mechanisms of Na^+ and Cl^- excretion by the gill of *Mugil capito* and *Fundulus heteroclitus*: effects of stress. J. Comp. Physiol. 123, 155–162.

Potts, W.T.W., Evans, D.H., 1967. Sodium and chloride balance in the killifish *Fundulus heteroclitus*. Biol. Bull. 133, 411–425.

Prodocimo, V., Galvez, F., Freire, C.A., Wood, C.M., 2007. Unidirectional Na^+ and Ca^{2+} fluxes in two euryhaline teleost fishes, *Fundulus heteroclitus* and *Oncorhynchus mykiss*, acutely submitted to a progressive salinity increase. J. Comp. Physiol. B 177, 519–528.

Scott, G.R., Rogers, J.T., Richards, J.G., Wood, C.M., Schulte, P.M., 2004. Intraspecific divergence of ionoregulatory physiology in the euryhaline teleost *Fundulus heteroclitus*: possible mechanisms of freshwater adaptation. J. Exp. Biol. 207, 3399–3410.

Scott, G.R., Schulte, P.M., Wood, C.M., 2006. Plasticity of osmoregulatory function in the killifish intestine: drinking rates, water transport, and gene expression after freshwater transfer. J. Exp. Biol. 209, 4040–4050.

Scott, G.R., Baker, D.W., Schulte, P.M., Wood, C.M., 2008. Physiological and molecular mechanisms of osmoregulatory plasticity in killifish after seawater transfer. J. Exp. Biol. 211, 2450–2459.

Smith, N.F., Talbot, C., Eddy, F.B., 1989. Dietary salt intake and its relevance to ionic regulation in freshwater salmonids. J. Fish Biol. 35, 749–753.

Stevens, C.D., Hume, I.D., 1996. Physiology of the Vertebrate Digestive System, Second Edition. Cambridge University Press, Cambridge, U.K.

Wood, C.M., 2011. Rapid regulation of Na^+ and Cl^- flux rates in killifish after acute salinity challenge. J. Exp. Mar. Biol. Ecol. 409, 62–69.

Wood, C.M., Bucking, C., 2011. The role of feeding in salt and water balance. In: Grosell, M., Farrell, A.P., Brauner, C.J. (Eds.), The Multifunctional Gut of Fish. : Fish Physiology, Vol. 30. Academic Press, San Diego, pp. 165–212.

Wood, C.M., Grosell, M., 2012. Independence of net water fluxes from paracellular permeability in the intestine of *Fundulus heteroclitus*, a euryhaline teleost. J. Exp. Biol. 215, 508–517.

Wood, C.M., Laurent, P., 2003. Na^+ versus Cl^- transport in the intact killifish after rapid salinity transfer. Biochim. Biophys. Acta 1618, 106–119.

Wood, C.M., Marshall, W.S., 1994. Ion balance, acid–base regulation, and chloride cell function in the common killifish, *Fundulus heteroclitus* — a euryhaline estuarine teleost. Estuaries 17, 34–52.

Wood, C.M., Bucking, C., Grosell, M., 2010. Acid–base responses to feeding and intestinal Cl^- uptake in freshwater- and seawater-acclimated killifish, *Fundulus heteroclitus*, an agastric euryhaline teleost. J. Exp. Biol. 213, 2681–2692.