The Journal of Experimental Biology 216, 3925-3936

doi:10.1242/jeb.084228

RESEARCH ARTICLE

Uptake, handling and excretion of Na^+ and Cl^- from the diet *in vivo* in freshwater- and seawater-acclimated killifish, *Fundulus heteroclitus*, an agastric teleost

Carol Bucking[1,2], Chris M. Wood[1,2] and Martin Grosell[1]

[1]Rosenstiel School of Marine and Atmospheric Sciences, University of Miami, Miami, FL 33149, USA and [2]Department of Biology, McMaster University, 1280 Main St West, Hamilton, ON, Canada, L8S 4K1

*Author for correspondence (carolbucking@gmail.com)

SUMMARY

A radiotracer approach using diets labelled with $^{22}Na^+$, $^{36}Cl^-$ and [^{14}C]polyethylene-4000 (PEG-4000) was employed to investigate the role of intestinal uptake from the food in ion homeostasis in the killifish *Fundulus heteroclitus*. This euryhaline teleost lacks both a stomach and the capacity for Cl^- uptake at the gills in freshwater. PEG-4000 appearance in the water was minimal up to 10–11 h post-feeding, indicating the virtual absence of Na^+ and Cl^- loss in the faeces up until this time. Rapid uptake of dietary Na^+ and Cl^- occurred and more than 88% of $^{22}Na^+$ and $^{36}Cl^-$ were absorbed in the intestine by 3 h post-feeding; excretion rates of Na^+ and Cl^- originating from the food were greatest during this period. Uptake and excretion of Cl^- from the diet was fivefold to sixfold greater than that of Na^+ in freshwater, and approximately threefold greater in seawater. Excretions of dietary Na^+ and Cl^- by seawater-acclimated killifish were far greater than by freshwater-acclimated killifish in this time frame, reflecting the much greater branchial efflux rates and turnover rates of the internal exchangeable pools. At both 3 and 9 h post-feeding, the largest fraction of dietary Na^+ was found in the carcass of freshwater-acclimated fish, followed by the external water, and finally the digestive tract. However, in seawater-acclimated fish, more was excreted to the water, and less was retained in the carcass. For Cl^-, which was taken up and excreted more rapidly than Na^+, the majority of the dietary load had moved to the external water by 9 h in both freshwater and seawater animals. After 7 days training on a low-salt natural diet (live *Lumbriculus variegatus* worms; 31.5 μmol Na^+ g^{-1} wet mass) *versus* a high-salt synthetic pellet diet (911 μmol Na^+ g^{-1} dry food mass), freshwater killifish exhibited a lower absolute excretion rate of Na^+ from the low-salt diet, but relative uptake from the intestine and retention in the carcass were virtually identical from the two diets. Seawater killifish excreted relatively more Na^+ from the low-salt diet. Overall, our results emphasize the importance of dietary Na^+ and Cl^- in the electrolyte economy of the killifish, particularly in freshwater, and especially for Cl^-.

Key words: intestine, gills, ion flux rates, chloride uptake, ionoregulation, feeding, defaecation.

Received 14 December 2012; Accepted 11 July 2013

INTRODUCTION

The comparison of osmoregulation and ionoregulation in the diametrically opposed environments of freshwater and seawater has been the subject of several excellent reviews (e.g. Evans et al., 2005; Hwang and Lee, 2007; Evans, 2008). Branchial and renal processes are now relatively well understood. Typically, in freshwater fish, active uptake of Na^+ and Cl^- occurs at the gills, while the kidney excretes a high volume of dilute urine to compensate for osmotic influx of water from the hypotonic environment. In contrast, in seawater teleosts, the gills actively excrete excess Na^+ and Cl^- gained from the hypertonic environment. Much of this Na^+ and Cl^- load originates from the gastrointestinal (GI) tract as marine fish must drink seawater to replace water lost by osmosis across the gills and body surface. In fact, the role of the intestine in seawater ion and water balance is much more apparent compared with in freshwater fish. Again the subject of several reviews (e.g. Wilson et al., 2002; Grosell, 2006; Grosell, 2011a), the intestine is essential for homeostasis in seawater fish through desalination of ingested water, solute-coupled fluid absorption and HCO_3^- secretion, which leads to more water uptake through $CaCO_3$ precipitation.

The role of the diet in overall piscine ion regulation has been generally overlooked, and until recently, there has been little activity in this area, with most ionoregulatory studies being carried out on fasted fish. In general, the diet is thought to present a salt load, especially commercial diets and salt-rich invertebrates, which may be beneficial for freshwater fish and potentially compromising in seawater fish (for a review, see Wood and Bucking, 2011). However, recent studies on one model species, the rainbow trout (*Oncorhynchus mykiss*), suggest that physiological processes associated with digestion may influence the outcome of dietary ion effects. For example, biliary secretion of Na^+ in freshwater trout (Grosell et al., 2000) appears to eliminate any net advantage of dietary Na^+ uptake (Bucking and Wood, 2006b). A surprising finding in this species is that most of the GI ion uptake occurs in the stomach, and not in the intestine (Bucking and Wood, 2006a; Bucking and Wood, 2006b; Bucking and Wood, 2007). How universal these patterns are across the multitude of teleost species is unknown.

However, not all fish ion- and water-regulate in the same manner. For example, another model species, the common killifish (*Fundulus heteroclitus*) lacks a stomach (Babkin and Bowie, 1928). Furthermore, the killifish is unusual in that the gills actively take up only Na^+, and not Cl^-, in freshwater, in contrast to most other species (Wood and Marshall, 1994; Patrick et al., 1997; Patrick et al., 1999; Wood and Laurent, 2003; Tomasso and Grosell, 2005;

Wood, 2011). This has led researchers to surmise that killifish must rely on the diet for overall Cl^- uptake in freshwater. In fact, experiments using *in vitro* gut sac preparations demonstrated that intestinal Cl^- uptake was upregulated in killifish during acclimation to freshwater (Scott et al., 2006), and that intestinal uptake of Cl^- was greater in freshwater-acclimated killifish than in seawater-acclimated specimens (Wood et al., 2010). This is in contrast with the trout (Bucking et al., 2009) and most other species (Grosell, 2006; Grosell, 2011b), where intestinal Cl^- uptake *in vitro* is increased with increasing salinity. However, it remains to be proven whether these patterns occur *in vivo*, where the gut is processing true chyme rather than saline.

Recently, we have developed a radiotracer method to study the uptake and fate of ^{22}Na from the diet in the killifish *in vivo* (Wood and Bucking, 2012). The inert marker polyethylene glycol-4000 ([^{14}C]-PEG-4000) is used to detect defaecation events, so as to separate systemic efflux of absorbed $^{22}Na^+$ into the external water from rectal efflux *via* the faeces. In the present study, we have utilized this technique to study the intestinal uptake, subsequent internal distribution and excretion of both $^{22}Na^+$ and $^{36}Cl^-$ from the diet in the killifish. We have compared the handling of $^{22}Na^+$ and $^{36}Cl^-$ from a high-salt pellet diet in both freshwater- and seawater-acclimated animals, and we have further compared the handling of $^{22}Na^+$ from a low-salt natural diet (live oligochaetes) in both freshwater and seawater killifish. Radiotracer experiments of this nature are complex, so we have also critically assessed the methodology and theory of this approach.

Our working hypotheses were that Cl^- would be taken up from the diet at a greater rate than Na^+, and that this difference would be especially prominent in freshwater killifish. We also predicted that retention of both ions from the diet would be greater in freshwater animals than in seawater animals, because the former have a greater need to acquire and conserve ions. Therefore, loss rates by systemic efflux across the gills and kidney would be much lower in freshwater killifish. Finally, we postulated that relative uptake and retention of Na^+ would be greater from the low-salt natural diet in freshwater killifish for the same reasons, but that this difference would not occur in seawater killifish.

MATERIALS AND METHODS

Experimental procedures were approved by institutional animal care committees at the University of Miami and McMaster University.

Animals

Killifish [*Fundulus heteroclitus* (Linnaeus 1766); both sexes, 2–6 g] were captured from the wild and held by Aquatic Research Organisms (Hampton, NH, USA) in quarantine before being sent to the Rosenstiel School of Marine and Atmospheric Sciences, where they were acclimated to laboratory conditions for at least 4 weeks before experimentation. Animals were either maintained in flow-through seawater [Na^+=485, Cl^-=569, K^+=10.6, Ca^{2+}=10.7, Mg^{2+}=59.5 and SO_4^{2-}=31.6 mmol l^{-1}; 22–24°C, 37.5 ppt, pH 8.0 (Wood and Grosell, 2008)] or freshwater (dechlorinated Miami City tap water; Na^+=1.06, Cl^-=1.21, K^+=0.08, Ca^{2+}=0.43, Mg^{2+}=0.13 and SO_4^{2-}=0.14 mmol l^{-1}; 22–24°C, pH 7.4 (Wood and Grosell, 2008)] in 50 l glass aquaria. Fish were fed commercial pellets (Na^+=230, Cl^-=170, Ca^{2+}=628, Mg^{2+}=77 and K^+=326 μmol g^{-1}) every 24 h and subjected to a 16 h:8 h light:dark cycle.

Diet preparation

Several radiolabelled diets were prepared, with either high or low salt content. Both high- and low-salt diets were also made in an identical fashion, but without the incorporation of radiotracers, and used for pre-experimental training of the fish.

High-salt diets (Na^+=911 μmol g^{-1} dry food mass; Cl^-=918 μmol g^{-1} dry food mass) were made by grinding commercial fish flakes (TetraMin, Spectrum Brands, Blacksburg, VA, USA) into a fine powder using a mortar and pestle. Brine shrimp (San Francisco Bay Brand, Newark, CA, USA) were then mixed with the powder (40% w/w) to form a paste. Subsequently, radiotracers were added (17.5 μCi of $^{22}Na^+$or $^{36}Cl^-$, 100 μCi [^{14}C]PEG-4000 per 30 g food) to the paste. Radiotracers (^{22}Na, ^{36}Cl, both as NaCl and [^{14}C]PEG-4000) were obtained from Amersham (Little Chalfont, UK) or NEN-Dupont (Boston, MA, USA). The isotopes were then thoroughly incorporated by mixing, and the paste was extruded through a 20 ml syringe (without a needle attached) to form long, thin ropes. The diets were then dried in an oven (60°C) for 24 h and crumbled by hand to form food pellets. The diets were stored at –20°C before use.

Low-salt diets (live oligochaetes; Na^+=31.5 μmol g^{-1} wet mass) were also prepared. California blackworms (*Lumbriculus variegatus*; Aquatic Foods, Fresno, CA, USA) were acclimated to laboratory conditions for ~5 days before use. Briefly, the blackworms were maintained in high-walled containers with a shallow bath of dechlorinated Miami city tap water (described above). The bath water was changed every 48 h. Before being used as an experimental diet, 10 g of worms were placed in 5 ml of water that contained 6 μCi of $^{22}Na^+$. The worms were incubated with the radioisotope for 24 h. The blackworms were briefly rinsed in clean water to remove loosely bound surface radioactivity before being fed to the killifish.

To determine an appropriate experimental feeding procedure, each diet (except the ^{36}Cl-labelled high-salt diet, because of its expense) was tested for leaching of radioisotope to the water before experimentation began. Briefly, each diet (1 g) was placed in water (100 ml). The water was then sampled at various time points following the addition of the food to the water. There were only small amounts of $^{22}Na^+$ or [^{14}C]PEG-4000 isotope detected in the water up to 1 h following introduction of the dried, high-salt diets. In contrast, $^{22}Na^+$ radioisotope was found in the water immediately following transfer of live worms. These factors were taken into account in the subsequent experimental designs.

Experimental design

Following laboratory acclimation, individual fish were removed from the 50 l glass aquaria, weighed and placed in individual containers (500 ml plastic food containers with lids). Each experimental series involved 12 fish, except in a few tests where a few fish failed to feed satisfactorily (N=10–11). Each series was conducted on a separate set of fish. The containers were blackened on one end to provide shelter for the animals and were supplied with individual water and air lines to supply freshwater/seawater and oxygen, respectively. The containers were also equipped with a removable flap on the top through which water samples could be taken as well as food delivered. The fish were trained to consume the high-salt food pellets or the low-salt blackworms (delivered by hand on forceps through the feeding flap) for ~1 week before experimentation began. The fish generally ate sparingly during the first few days, but built up to the ration used in the actual experiments by the end of the training period.

Series 1

Once training was complete, the animals were fasted for ~36 h to clear the previous meal from the GI tract. The animals were then fed a high-salt diet with [^{14}C]PEG-4000 incorporated

(841,738 dpm g^{-1} dry food mass) until satiated. Feeding took ~30 min, and water flow was continued during feeding. The amount of food ingested by each fish was recorded and an attempt to provide similar rations in all further series was undertaken. Water flow was stopped at the end of feeding, the water volume in the chambers was set to 500 ml, and an initial water sample was taken (5 ml), representing time 0 h. Care was taken to ensure all food was eaten to prevent leaching of the isotope into the water, and any uneaten food was removed within 30 min following the beginning of feeding (i.e. before time 0 h). Water samples (5 ml) were then taken every 60 min for 16 h, and then at 24, 28 and 32 h following feeding. Water was thoroughly flushed from the chambers at 12 and 24 h following feeding. Subsequent to each water change, water flow was again ceased and an initial water sample was taken (5 ml).

Series 2

The experiment was then repeated with a high-salt diet with $^{22}Na^+$ added (287,943 dpm g^{-1} and 911 μmol Na^+ g^{-1} dry food mass) and with a low-salt diet with $^{22}Na^+$ added (66,011 dpm g^{-1} and 31.5 μmol Na^+ g^{-1} wet food mass). When the low-salt diet (live blackworms) was fed, fish were transferred to a new container 30 min after the beginning of feeding, an initial water sample was taken and subsequent water sampling and flushing occurred as before with the high-salt diets. This was done as feeding the worms transferred a significant amount of isotope in the water administered with the worms, contaminating the water found in the containers as described above.

Series 3

A similar experiment was conducted with a high-salt diet with $^{36}Cl^-$ added (190,038 dpm g^{-1} and 918 μmol Cl^- g^{-1} dry food mass). In this experiment, as a prior leaching test was not performed, the fish were transferred to a clean container following a 30 min feeding period with flowing water. Water samples were taken every hour as before. Based on the [^{14}C]PEG-4000 experiment of Series 1, this trial was terminated 9 h after feeding because thereafter the appearance of the isotope in the water was potentially confounded by elimination of faeces. Additionally, it was observed that a larger proportion of the dietary $^{36}Cl^-$ appeared in the water within the first few hours in comparison with the dietary $^{22}Na^+$. Out of concern for a greater possibility of isotope recycling *via* drinking, the experiments were terminated earlier than those of Series 1 and 2.

Series 4

The goal of this series was to quantify the distribution of the ingested Na^+ or Cl^- load between the digestive tract, the carcass (remainder of the fish) and the external water at 3 and 9 h after feeding – i.e. before any complication from defaecation could occur.

Similar to the previous series, trained fish in individual containers supplied with either freshwater or seawater were fed to satiation with one of the three diets (N=12 for each diet in each salinity): low-salt diet with $^{22}Na^+$ added (freshwater=66,011 dpm g^{-1} and 31.5 μmol g^{-1} wet food mass; seawater=198,286 dpm g^{-1} and 31.5 μmol g^{-1} wet food mass), high-salt diet with $^{22}Na^+$ added (287,943 dpm g^{-1} and 911 μmol Na^+ g^{-1} dry food mass for both salinities) and high-salt diet with $^{36}Cl^-$ added (190,038 dpm g^{-1} and 918 μmol Cl^- g^{-1} dry food mass, again for both salinities). Again, care was taken to ensure minimal leaching of the food into the water by removing any uneaten food immediately following the cessation of feeding. To ensure parallelism between the $^{22}Na^+$ and the $^{36}Cl^-$ experiments, once the meal had been ingested (30 min following the beginning of feeding), the fish were transferred to new containers with clean water and an initial water sample (5 ml) was taken. Water samples were then taken every hour following feeding as before. However, in this series, fish were euthanized by an overdose of anaesthesia (MS-222, 0.2 g l^{-1}; Syndel Laboratories, Vancouver, Canada; neutralized with NaOH) at 3 h (N=6 for each salinity) and 9 h (N=6 for each salinity) following feeding. Following euthanization, the GI tract was exposed through a mid-line incision, ligated at both ends with silk suture, and then removed intact. The amount of radioisotope was then determined in the whole body, the GI tract and the external water.

Analysis

$^{36}Cl^-$ and [^{14}C]PEG in the water was determined by placing 5 ml water samples into 10 ml of Ecolume fluor (MP Biomedicals, St Louis, MO, USA) and counting in a Tricarb 2100TR liquid scintillation counter (Packard Instruments, Downers Grove, IL, USA). $^{22}Na^+$ was measured in 1 ml water samples directly on a Cobra II gamma counter (Packard Instruments). To determine the radioactivity of the carcass, the gut and the food, all three were first dissolved in five volumes of 1 mol l^{-1} HNO_3 and heated (60°C) for 24 h in a sealed tube. The resulting extract was then centrifuged, and the supernatant was tested for radioactivity by scintillation counting for $^{36}Cl^-$ (1 ml neutralized extract plus 4 ml water plus 10 ml fluor) and by gamma counting for $^{22}Na^+$ (1 ml directly). Na^+ and Cl^- concentrations in extracts of the diets were measured using atomic absorption spectrophotometry (Model 220FS, Varian Australia, Mulgrave, VIC, Australia) and coulometric titration (CMT-10; Radiometer, Copenhagen, Denmark), respectively. For quench correction of [^{14}C] and [$^{36}Cl^-$] scintillation samples, counting efficiency of tissue and food samples was corrected back to the same efficiency as water samples by internal standardization *via* the constant addition method (Rogers and Moran, 1966).

The amount of radioisotope excreted to the water (dpm_w g^{-1} fish mass) was then determined after accounting for individual fish masses and the volume of the container at each time point. The initial water samples taken immediately following feeding (30 min) and water changes (12 and 24 h) were subtracted from subsequent samples to eliminate background contamination.

Finally, the cumulative amount of Na^+ or Cl^- excreted to the water that originated from the consumed meal (μmol g^{-1} fish mass) was determined as:

$$\text{Ion excretion} = [dpm_w\ g^{-1}\ \text{fish mass}] / SA, \quad (1)$$

where SA was the specific activity of the food (dpm μmol^{-1} Na^+ or Cl^-).

Statistics

JMP (version 8, SAS Institute, Cary, NC, USA) was used to test the data for significance. The raw data were first examined for normality and heterogeneity of variance. Data that failed to meet these conditions as well as all percentage data were transformed as appropriate. In all series, two-way (salinity and time) repeated-measures ANOVA was used to analyze the data initially, and this was collapsed to a one-way repeated-measures ANOVA and the data were combined in Series 1 only, where salinity had no significant effect. Appropriate *post hoc* tests were then used to examine the data for significant changes: Tukey's test in Series 1, where salinity had no effect, and the Holm–Sidak test in Series 2, 3 and 4, where both factors were significant. Differences in radioisotope distribution among the water, carcass and GI tract were compared using paired and unpaired *t*-tests, again as appropriate,

followed by Bonferroni corrections. A significance level of $P<0.05$ was used throughout.

RESULTS

Series 1

On average fish consumed an approximate 5% body mass ration of the [^{14}C]PEG-4000-labelled diet (841,738 dpm g^{-1} dry food mass), regardless of external salinity. Two-way ANOVA demonstrated that while the influence of time was significant, salinity had no effect on the appearance of [^{14}C]PEG-4000 in the water from the ingested diet, so values were combined across salinities (Fig. 1). During the first hour following ingestion, slightly less than 1% of the ingested [14C]-PEG-4000 appeared in the water, thereafter increasing by an additional 4% at 2 h to a total of ~5% of the ingested load. Thereafter, [^{14}C]PEG-4000 radioactivity in the external water remained unchanged for 10 h, suggesting negligible additional excretion. However, at 11 h following meal ingestion, water radioactivity increased dramatically, representing ~26% of the ingested load (Fig. 1). Excretion to the water continued to increase for the remainder of the experiment, and by 24–32 h following feeding, [^{14}C]PEG-4000 appearance in the water had reached ~65% of the ingested amount (Fig. 1).

Series 2

Both freshwater- and seawater-acclimated fish consumed an approximate 5% body mass ration of the $^{22}Na^+$-labelled low-salt diet [31.5 μmol Na^+ g^{-1} wet food mass (live worms)] and statistical testing revealed that both time and salinity were significant factors in Na^+ appearance in the surrounding water. In fish acclimated to freshwater, the amount of ^{22}Na in the external water fluctuated between ~0.1 and 0.2 μmol Na^+ g^{-1} fish mass, or 5 and 13% of the ingested Na^+ load, from 1 to 24 h, suggesting no additional excretion during this period (Fig. 2A). However, at 28 h following the meal, the amount of Na^+ excreted to the water increased sharply (Fig. 2A). The cumulative efflux of dietary Na^+ to the water reached ~0.4 μmol Na^+ g^{-1} fish mass, representing 26% of the ingested Na^+ load, by 32 h. In contrast, seawater-acclimated fish that ingested a comparable low-salt diet of worms exhibited a much more rapid release of Na^+ to the water over the first 3 h following meal ingestion, increasing 1.5-fold from ~0.4 to ~0.7 μmol Na^+ g^{-1} fish mass, or 27 to 42% of the dietary Na^+ (Fig. 2B). The amount of Na^+ excreted to the water subsequently remained stable until 28 h, when it again increased to ~0.9 μmol Na^+ g^{-1} fish mass or 58% of ingested Na^+ for the remainder of the experiment (Fig. 2B).

Overall, the majority of Na^+ excretion occurred during the first hour following the consumption of a meal in freshwater fish, while the majority of Na^+ excretion in marine fish occurred over the first 3 h. The cumulative Na^+ efflux for seawater-acclimated fish over 32 h was more than twofold greater than for freshwater fish fed a comparable diet, a highly significant difference (Fig. 2B *versus* 2A). In fact, the amount of Na^+ excreted to the water by seawater-acclimated fish in the first hour following feeding was similar to the total that was excreted over 32 h by freshwater-acclimated fish (Fig. 2A,B). Additionally, there appeared to be little evidence of faecal loss, as shown by the relatively stable levels of Na^+ excretion 9 h following feeding (see Series 4).

Freshwater- and seawater-acclimated fish again both ate a 5% ration when presented with the high-salt $^{22}Na^+$-labelled diet (911 μmol Na^+ g^{-1} dry food mass). As with the low-salt diet, statistical testing revealed that both time and salinity were significant factors in Na^+ appearance in the surrounding water. At least qualitatively, freshwater-acclimated fish ingesting the high salt diet showed a temporal pattern of $^{22}Na^+$ appearance in the water (Fig. 2C) similar to that of freshwater fish that ingested the low-salt diet (cf. Fig. 2A). The amount excreted fluctuated non-significantly from 0.2 to 0.7 μmol Na^+ g^{-1} fish mass (N=12), ranging from 0.3 to 1.4% of the ingested Na^+ load, from 1 to 24 h, once again suggesting no additional excretion during this period (Fig. 2C). Subsequently, the amount of Na^+ excreted to the water increased greatly at 28 h, to 2.3 μmol Na^+ g^{-1} fish mass (5% of ingested Na^+), and remained essentially unchanged at 32 h (Fig. 2C).

Seawater-acclimated fish ingesting the same 5% ration of a high-salt diet excreted an increasing amount of Na^+ over the first 5 h following meal ingestion, in contrast with freshwater-acclimated fish (Fig. 2C), but qualitatively similar to seawater-acclimated fish that ingested a low-salt diet (Fig. 2B). Initially, ~1.6 μmol Na^+ g^{-1} fish mass (3.5% of ingested Na^+) was excreted 1 h following feeding (Fig. 2D), more than fivefold greater than seen in freshwater fish fed the same meal (cf. Fig. 2C). $^{22}Na^+$ efflux then increased progressively over the next 4 h to peak at ~4.6 μmol Na^+ g^{-1} fish mass (10% of ingested Na^+). Thereafter, there was no further appearance through 24 h (Fig. 2D), with values fluctuating from 8 to 11%. However, at 28 h, Na^+ excretion once again increased 1.3-fold to ~6.2 μmol Na^+ g^{-1} fish mass (14% of ingested Na^+) and by 32 h, the cumulative excretion of Na^+ to the water was threefold higher than seen with freshwater-acclimated fish (Fig. 2C) at the same time.

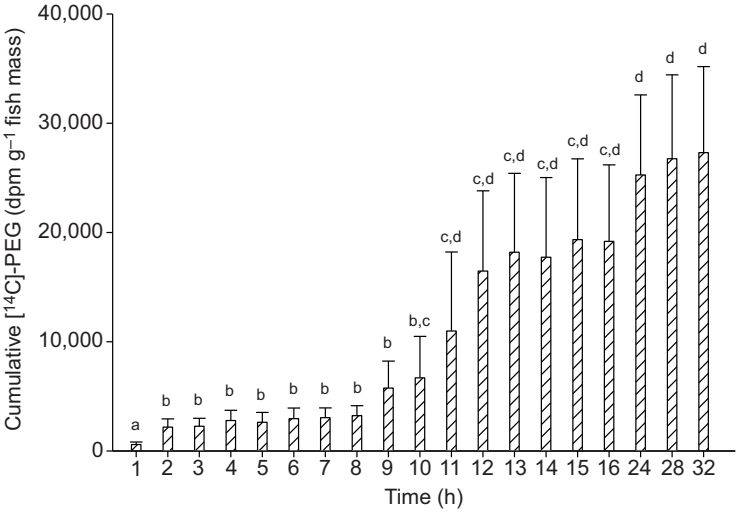

Fig. 1. Cumulative [^{14}C]-PEG-4000 (polyethylene glycol) found in the water (dpm g^{-1} fish mass) following feeding from freshwater- or seawater-acclimated killifish in Series 1. There were no significant differences between freshwater or seawater fish so the data have been combined. Fish consumed a 5% ration (0 h). N=24 (N=12 for each salinity). Different letters represent significant (P<0.05) differences between time points.

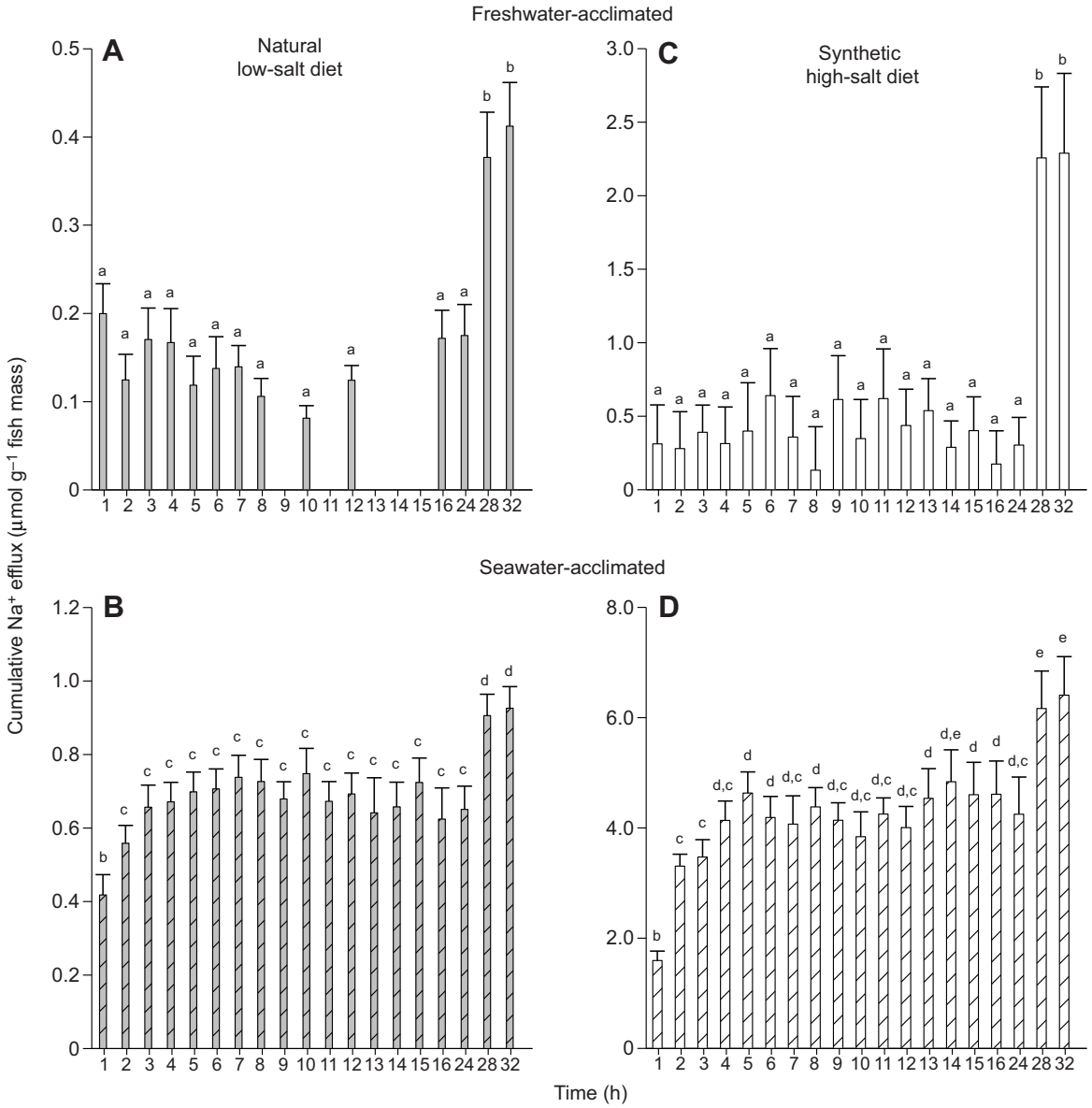

Fig. 2. Cumulative Na^+ excreted to the water (µmol g^{-1} fish mass) originating from the diet in Series 2 for (A) freshwater- and (B) seawater-acclimated killifish fed a natural low-salt diet (66,011 dpm g^{-1}; 31.5 µmol Na^+ g^{-1}; 5% ration), and (C) freshwater- and (D) seawater-acclimated killifish fed a synthetic high-salt diet (287,931 dpm g^{-1}; 911 µmol Na^+ g^{-1}; 5% ration). Feeding occurred at 0 h. Uneaten food was removed from each container but fish were not transferred to another container. *N*=11–12 for each salinity. For each diet, bars that share letters are not significantly different (*P*>0.05).

Overall, as with the low-salt diet, the majority of Na^+ excretion occurred within the first hour following the consumption of a meal by freshwater-acclimated fish, while seawater-acclimated fish exhibited a gradual excretion over the first 4 h. Once again, the amount of Na^+ excreted by seawater-acclimated fish within the first 1 h following feeding (Fig. 2D) was similar to the total amount of Na^+ excreted over 32 h following feeding in freshwater-acclimated fish fed the same high-salt meal (Fig. 2C).

Series 3

Freshwater- and seawater-acclimated killifish consumed approximately a 3.4% ration of the $^{36}Cl^-$-labelled high-salt diet (918 µmol Cl^- g^{-1} dry food mass). Statistical analysis revealed that both salinity and time were significant factors. Specifically, freshwater-acclimated fish excreted ~1.6 µmol Cl^- g^{-1} fish mass to the water during the first hour following feeding, or 5% of the total Cl^- consumed with the diet (Fig. 3A). This excretion was more than fivefold higher than the Na^+ excretion with the same diet in the first hour (cf. Fig. 2C). Cumulative Cl^- efflux to the water continued to increase steadily over the next 8 h to ~4.9 µmol Cl^- g^{-1} fish mass (16% of the ingested Cl^- load; Fig. 3A), in contrast to the pattern of stability seen in cumulative Na^+ appearance over the same period (cf. Fig. 2C), such that total Cl^- efflux was more than fivefold greater than total Na^+ efflux.

Fish acclimated to seawater that consumed a high-salt diet excreted ~2.8 µmol Cl^- g^{-1} fish mass (9% of the ingested $^{36}Cl^-$) to the water within the 1 h following feeding (Fig. 3B). Cl^- efflux to the water proceeded to increase over the next 8 h to result in a cumulative excretion of ~17.8 µmol Cl^- g^{-1} fish mass (56%; Fig. 3B), a value that was approximately threefold higher than Na^+ excreted to the water (Fig. 3B *versus* Fig. 2D) from the same diet and approximately fourfold higher then seen with freshwater-acclimated fish consuming the same meal (Fig. 3A).

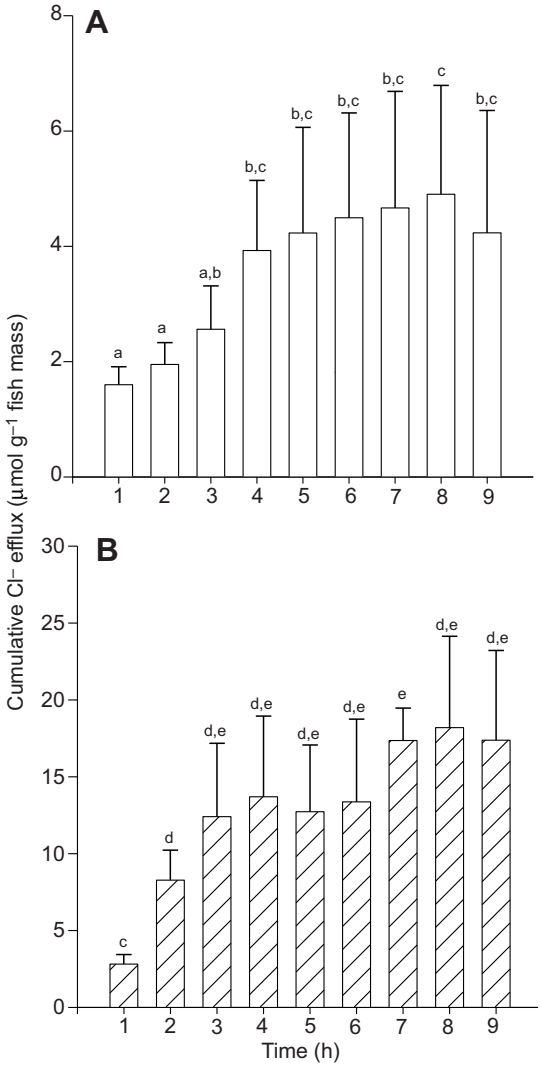

Fig. 3. Cumulative Cl^- excreted to the water (μmol g^{-1} fish mass) originating from the diet in Series 3 for (A) freshwater- and (B) seawater-acclimated killifish fed a synthetic high-salt diet (190,038 dpm g^{-1}; 918 μmol $Cl^- g^{-1}$; 3.4% ration). Feeding occurred at 0 h and fish were transferred to another container after 0.5 h. *N*=11–12 for each salinity. Bars that share letters are not significantly different (*P*>0.05).

Overall, freshwater- and seawater-acclimated killifish showed a similar temporal pattern of Cl^- excretion following the ingestion of a meal; however, it was quantitatively higher in seawater-acclimated fish.

Series 4

In this series, fish were euthanized at 3 and 9 h post-feeding to quantify the distribution of the ingested Na^+ or Cl^- load between the GI tract, the carcass (remainder of the fish) and the external water. Additionally, the water was exchanged following feeding, allowing the more subtle changes in radioisotope concentration to be observed (compared with Series 2). Fish consumed a 4.9% ration of the low-salt diet (31.5 μmol $Na^+ g^{-1}$ wet food mass, live worms) in both freshwater and seawater, and once again, both salinity and time were significant factors. However, in contrast to freshwater-acclimated fish fed the low-salt diet in Series 2 (cf. Fig. 2A), freshwater fish fed the same diet of worms in Series 4 showed a gradual increase in the amount of Na^+ excreted to the water over the first 3 h (Fig. 4A) and 9 h (Fig. 4B) following feeding. In freshwater, 5% of ingested Na^+ had been excreted 1 h following feeding (~0.1 μmol $Na^+ g^{-1}$ fish mass; Fig. 4A). This increased 1.4-fold to 9% at 3 h and to ~11% by 7 h, after which it remained stable (~0.2 μmol $Na^+ g^{-1}$ fish mass; Fig. 4B).

Seawater-acclimated fish exhibited a similar gradual excretion of Na^+. At 1 h following the consumption of a meal, appearance in the external water was ~0.4 μmol $Na^+ g^{-1}$ fish mass or 24% of ingested Na^+ (Fig. 4D), and this had increased 1.6-fold to 40% by 3 h (~0.6 μmol $Na^+ g^{-1}$ fish mass; Fig. 4D) and twofold to 49% by 5 h (~0.8 μmol $Na^+ g^{-1}$ fish mass; Fig. 4E).

Compartmental analysis revealed rapid absorption of dietary $^{22}Na^+$ from the digestive tract in both freshwater and seawater. In freshwater-acclimated killifish, by 3 h post-feeding, the fraction found in the tract was only 7% of the ingested $^{22}Na^+$ load, and this fell to 3% at 9 h. The proportions in the water increased from 9% at 3 h to 11% at 9 h, whereas the fractions in the carcass decreased from 63% at 3 h to 56% at 9 h (Fig. 4C). The unaccounted-for portions of $^{22}Na^+$ were 22% at 3 h and 29% at 9 h. In seawater-acclimated killifish, absorption from the digestive tract was again rapid, with only 8 and 4% remaining at 3 and 9 h post-feeding, respectively (Fig. 4F). The fraction of dietary $^{22}Na^+$ that had been eliminated to the external seawater was 45% by 3 h, and significantly increased to 66% by 9 h (Fig. 4F). The proportion of dietary $^{22}Na^+$ in the carcass correspondingly decreased from 31% at 3 h to 18% at 9 h. The unaccounted for proportion of $^{22}Na^+$ was 15% at 3 h and 12% at 9 h (Fig. 4F).

Fish fed the high-salt diet (911 μmol $Na^+ g^{-1}$ dry food mass) in this series consumed only a 2.8% ration. In contrast to fish fed the low-salt diet (cf. Fig. 4), fish fed the high-salt diet showed a gradual increase in the amount of $^{22}Na^+$ excreted to the water in seawater only (Fig. 5D,E), and not in freshwater (Fig. 5A,B). Despite this, both salinity and time were significant factors. In freshwater, ~7% of the ingested load had been excreted 1 h following feeding (~1.8 μmol $Na^+ g^{-1}$ fish mass; Fig. 5A,B), and this remained stable for the duration of the experiment. In seawater, fish excreted 8% by 1 h (~2.2 μmol $Na^+ g^{-1}$ fish mass); however, this increased to 20% by 3 h and remained relatively stable thereafter (~5.6 μmol $Na^+ g^{-1}$ fish mass; Fig. 5D,E).

Despite the 16-fold higher dietary Na^+ load relative to the fish fed the low-salt diet, fractional absorption of dietary $^{22}Na^+$ from the digestive tract was again rapid. In freshwater fish, the digestive tract contained only 7% at 3 h and 11% of ingested Na^+ at 9 h (Fig. 5C); only the latter value was significantly higher than in the comparable low-salt diet treatment. The majority of $^{22}Na^+$ was in the carcass, 67% at 3 h and 59% at 9 h, comparable to the percentages in the carcass at these times in the fish fed the low-salt natural diet. Similarly, the relative amounts excreted to the external freshwater, 10% at 3 h and 12% at 9 h, were comparable to those seen in killifish fed the low-salt diets (Fig. 5C *versus* Fig. 4C). This distribution was not significantly different between 3 and 9 h (Fig. 5C). The fractions of Na^+ that could not be accounted for were 16% at 3 h and 19% at 9 h. In seawater-acclimated fish, the fraction of $^{22}Na^+$ found in the GI tract was again low and did not significantly change between 3 h (12%) and 9 h (8%). The majority of $^{22}Na^+$ was found in the carcass despite decreasing from 47% at 3 h to 36% at 9 h (Fig. 5F). The amount of $^{22}Na^+$ in the water did not change from 3 h following feeding (22%) to 9 h (20%; Fig. 5F). The fractions of Na^+ that could not be accounted for were 21% at 3 h and 37% at 9 h.

The low proportion of dietary Na^+ in the intestinal tract within all above treatments (ranging from 3 to 12%) may explain the

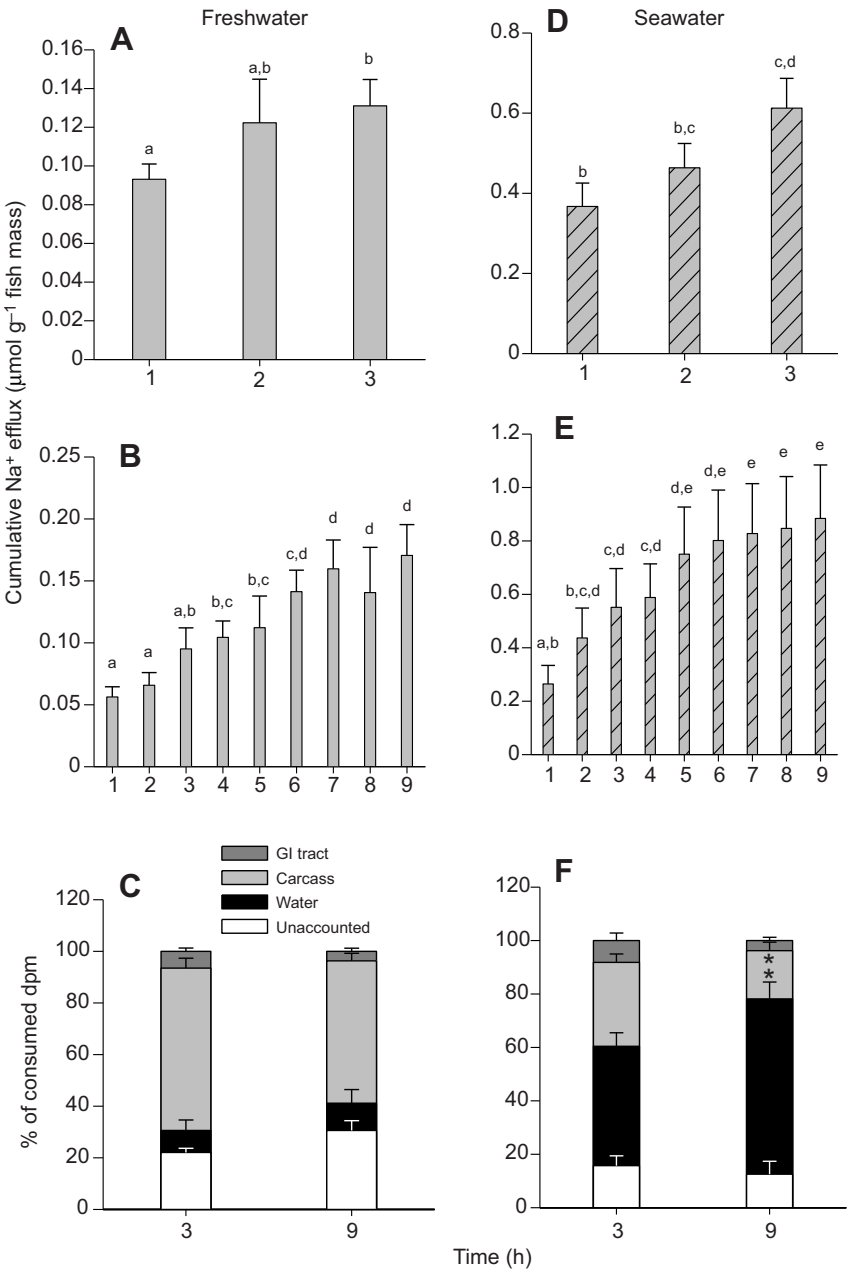

Fig. 4. Cumulative Na^+ excreted to the water (µmol g^{-1} fish mass) originating from the diet in Series 4 by freshwater-acclimated killifish fed a natural low-salt diet (66,011 dpm g^{-1}; 31.5 µmol Na^+ g^{-1}; 4.9% ration) over (A) 3 h post-feeding (N=6) and (B) 9 h post-feeding (N=6), and by seawater-acclimated killifish fed a natural low-salt diet (198,286 dpm g^{-1}; 31.5 µmol Na^+ g^{-1}; 4.9% ration) over (D) 3 h post-feeding (N=6) and (E) 9 h post-feeding (N=6). Percentage of total dpm found in the water, carcass or GI tract of (C) freshwater or (F) seawater fish. Feeding occurred at 0 h and fish were transferred to another container after 0.5 h. For each salinity, bars that share letters are not significantly different (P>0.05). Asterisks indicate a significant change in the proportion of $^{22}Na^+$ from 3 to 9 h.

relative absence of faecal Na^+ excretion observed in Series 2. As a result, no corrections were made for faecal losses in Series 2.

Fish consumed a 3.2% ration of the $^{36}Cl^-$-labelled diet in this series, and again both salinity and time were significant factors. In freshwater-acclimated killifish, the excretion of $^{36}Cl^-$ increased from 10% at 1 h following feeding to 27% at 3 h (from ~3.0 to 7.1 µmol Cl^- g^{-1} fish mass; Fig. 6A), and finally to ~43% at 9 h (~13.5 µmol Cl^- g^{-1} fish mass; Fig. 6B). The fractional amount of Cl^- excreted by freshwater-acclimated fish was higher (10–43%; Fig. 6A,B) than that seen with Na^+ when fish were fed the same diet (~7%; Fig. 5A,B). In contrast, fish in seawater excreted ~9.2 µmol Cl^- g^{-1} fish mass (31% of ingested) 1 h after feeding, which increased to 60% at 3 h (~17.8 µmol Cl^- g^{-1} fish mass; Fig. 6D). The cumulative efflux of Cl^- remained unchanged thereafter (Fig. 6E), accounting for ~45–80% of the ingested Cl^-. By way of contrast, fish in seawater excreted between 20 and 40% of Na^+ from the same diet over the same period (cf. Fig. 5E).

Fractional absorption of dietary $^{36}Cl^-$ from the digestive tract was even more rapid than the fractional absorption of dietary $^{22}Na^+$. In freshwater fish, only 4 and 3% of ingested $^{36}Cl^-$ were left in the tract at 3 and 9 h after the meal, respectively (Fig. 6C), significantly lower than 7–11% of dietary $^{22}Na^+$ (Fig. 5C). The fraction in the carcass was higher compared with the water at 3 h in freshwater-acclimated fish (46% *versus* 26%; Fig. 6C). However, by 9 h this pattern was reversed and the proportion of ingested $^{36}Cl^-$ in the water had increased to 47%, while it had decreased in the carcass to 25% (Fig. 6C). The unaccounted-for proportion of $^{36}Cl^-$ was 25% at 3 h and 26% at 9 h. In seawater-acclimated fish, the absorption and redistribution of $^{36}Cl^-$ was exceedingly fast. The majority of $^{36}Cl^-$ was found in the water in seawater-acclimated fish at both 3 h (60%, N=6) and 9 h (79%) post-feeding (Fig. 6F). Negligible fractions were left in the digestive tract: 1% at 3 h and 0.2% at 9 h (Fig. 6F). The amount in the carcass significantly decreased from 7% at 3 h to 1% at 9 h, and the unaccounted-for proportion of $^{36}Cl^-$ was 30% at 3 h and 20% at 9 h.

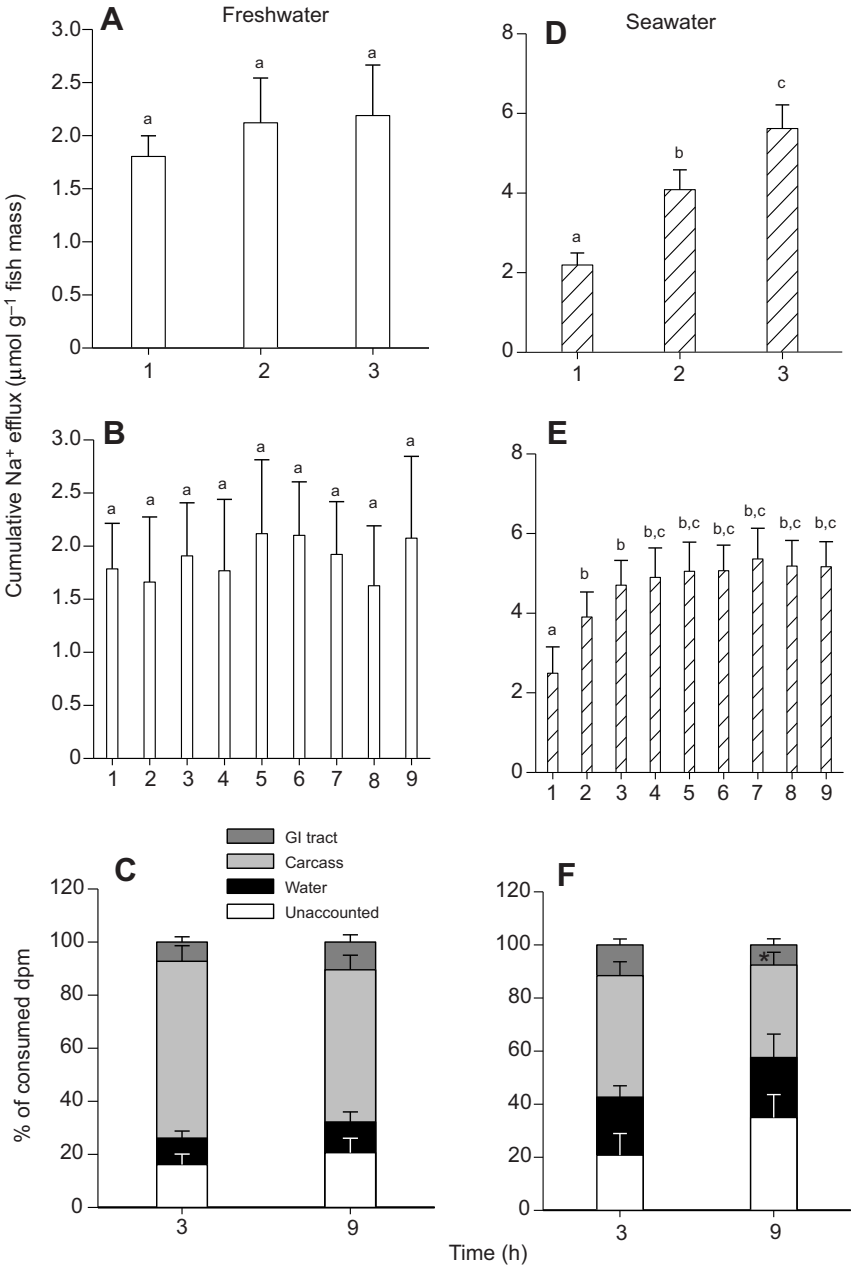

Fig. 5. Cumulative Na^+ excreted to the water (µmol g^{-1} fish mass) originating from the diet in Series 4 by freshwater-acclimated killifish fed a synthetic high-salt diet (287,931 dpm g^{-1}; 911 µmol Na^+ g^{-1}; 2.8% ration) over (A) 3 h post-feeding (*N*=6) and (B) 9 h post-feeding (*N*=6), and by seawater-acclimated killifish fed the same synthetic high-salt diet over (D) 3 h post-feeding (*N*=6) and (E) 9 h post-feeding (*N*=6). Percentage of total dpm found in the water, carcass or GI tract of (C) freshwater or (F) seawater fish. Feeding occurred at 0 h and fish were transferred to another container after 0.5 h. Across salinities, bars that share letters are not significantly different (*P*>0.05). Asterisk indicates a significant change in the proportion of $^{22}Na^+$ from 3 to 9 h.

DISCUSSION

Overview

To our knowledge, this is the first study to directly compare the uptake and fate of dietary Na^+ and Cl^- in the same euryhaline species in freshwater *versus* seawater. Overall, the present data provide strong support for the importance of dietary Na^+ and especially Cl^- in the electrolyte economy of the killifish, mainly in freshwater. This adds to the growing evidence that dietary ions play a key role in ionoregulatory homeostasis in fish (reviewed by Wood and Bucking, 2011).

The results provide clear answers to two of our initial three working hypotheses. Firstly, in accordance with predictions, Cl^- was taken up from the diet at a greater rate than Na^+, and the difference was more prominent in freshwater killifish. Thus, in the high-salt diet experiments of Series 2 and 3, the appearance in the water of Cl^- from the diet was approximately fivefold faster than Na^+ in freshwater killifish, and approximately threefold faster than Na^+ in seawater killifish. This was confirmed by similar sixfold (freshwater) *versus* threefold (seawater) differences in Series 4. These differences are much larger than can be explained by the ~1.5-fold differences expected as a result of exchangeable pool sizes alone, as discussed subsequently. They were also confirmed by direct measurements of unabsorbed radioactivity in the intestinal tract at 9 h in Series 4. For example, in freshwater killifish, the relative amount of $^{36}Cl^-$ left in the tract at 9 h post-feeding was only 2.5% of the ingested load, whereas the relative amount of $^{22}Na^+$ was 10.5%. Secondly, the hypothesis that retention of both ions from the diet would be greater in freshwater animals than in seawater animals was confirmed. For example, in the high-salt diet experiments of Series 2 and 3, by 9 h only ~1.5% of the $^{22}Na^+$ load and 16% of the $^{36}Cl^-$ load had been excreted by freshwater fish, whereas in seawater fish the values were 10% ($^{22}Na^+$) and 43% ($^{36}Cl^-$). In Series 4, the relative values for freshwater killifish were 7% ($^{22}Na^+$) and 47% ($^{36}Cl^-$), and for seawater killifish 18% ($^{22}Na^+$) and 79% ($^{36}Cl^-$). Finally, we

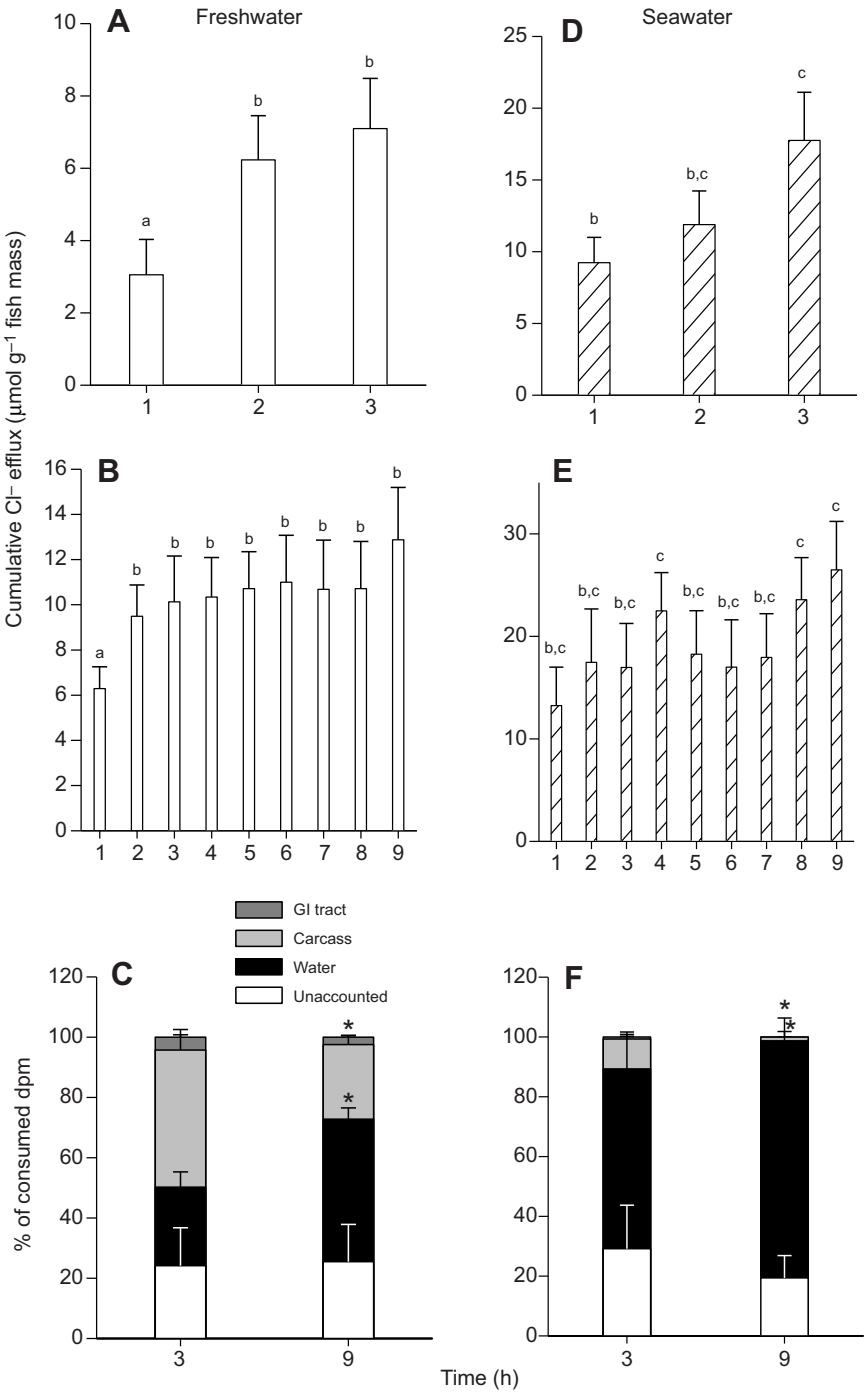

Fig. 6. Cumulative Cl^- excreted to the water (μmol g^{-1} fish mass) originating from the diet in Series 4 by freshwater-acclimated killifish fed a synthetic high-salt diet (190,038 dpm g^{-1}; 918 μmol $^{36}Cl^-$ g^{-1}; 3.2% ration) over (A) 3 h post-feeding (*N*=6) and (B) 9 h post-feeding (*N*=6) and by seawater-acclimated killifish fed the same synthetic high-salt diet over (D) 3 h post-feeding (*N*=6) and (E) 9 h post-feeding (*N*=6). Percentage of total dpm found in the water, carcass or GI tract of (C) freshwater or (F) seawater fish. Feeding occurred at 0 h and fish were transferred to another container after 0.5 h. Across salinities, bars that share letters are not significantly different (P>0.05). Asterisks indicate a significant change in the proportion of $^{36}Cl^-$ from 3 to 9 h.

had predicted that relative uptake and retention of Na^+ would be greater from the low-salt natural diet than from the high-salt synthetic diet in freshwater killifish, but that this difference would not occur in seawater killifish. As discussed subsequently, the hypothesized difference was not detected in freshwater killifish, and it certainly did not occur in seawater killifish.

Patterns of [^{14}C]PEG-4000 appearance

The pattern of defaecation revealed by the [^{14}C]PEG-4000-labelled diet was the same in freshwater and seawater (Fig. 1), and very similar to that reported earlier in killifish acclimated to 10% seawater and fed a much smaller ration (Wood and Bucking, 2012). Thus significant defaecation did not occur until 10–11 h post feeding, as revealed by the significant increases in ^{14}C]PEG-4000 dpm in the water at this time. Based on this result, subsequent analyses (Figs 3–6) focused on the first 9 h post-feeding, so that the appearance of non-absorbed $^{22}Na^+$ or $^{36}Cl^-$ radioactivity *via* faecal discharge would not confound interpretation of true excretion patterns (i.e. *via* gills and urine). Regardless, these later analyses (Fig. 4F, Fig. 5F, Fig. 6F) demonstrated that there was only a very small percentage (<11%, and often lower) of the dietary load of $^{22}Na^+$ or $^{36}Cl^-$ radioactivity that remained unabsorbed in the digestive tract at 9 h. The small amount of ^{14}C]PEG-4000 radioactivity appearing in the external water prior to 9 h averaged ~5% of the total ingested load, and probably has several origins such as leaching from tiny non-eaten food particles, regurgitation

and early small defaecation events; it therefore represents the error in the system. In consequence, appearances of $^{22}Na^+$ and $^{36}Cl^-$ over this time frame that were ≤5% of the ingested load should be interpreted cautiously, as they are close to 'background' – i.e. the resolution limit of the system in quantifying dietary ion excretion to the water.

Radiotracer recovery considerations

In Series 4, the fraction of consumed radioactivity that could not be accounted for at the time of euthanization ranged from 12 to 37% in the various experiments. In a similar experiment, Wood and Bucking (Wood and Bucking, 2012) reported a 15% discrepancy. Subsequent tests revealed that more than half of this discrepancy was accounted for by blood loss upon dissection, as blood plasma has high concentrations of Na^+ and Cl^-, whereas the remainder likely resulted from errors in counting, estimated food consumption, chamber water volumes and losses to the water during the first 30 min prior to transfer. As all fish were treated the same, we elected not to correct the data.

Specific activity and exchangeable pool size considerations

Interpretation of radiotracer experiments of this nature can easily be confounded by specific activity issues (i.e. the ratio of 'hot' to total Na^+ or Cl^-) and differences in exchangeable pool sizes. The first of these issues is whether significant recycling of radioisotope (i.e. 'backflux') occurs between compartments, and the second is the interpretation of radiotracer appearance in the water from the diet, when the radiotracer has moved through an intermediate compartment (the exchangeable whole-body Na^+ or Cl^- pool), which was initially unlabelled. The third is the effect that possible differences in pool size will have on radiotracer appearance rates in the water.

With respect to the first issue (possible backflux), calculations were carried out based on measured radioactivity levels in the diet, carcass and water, measured Na^+ and Cl^- concentrations in the water and diet, and previous determinations of exchangeable whole-body pool sizes for Na^+ and Cl^- in freshwater- and seawater-acclimated *F. heteroclitus* (Wood and Laurent, 2003). The normal criterion for radiotracer flux measurements is that specific activity on the flux-generating side should be kept 10-fold higher than on the flux-receiving side so as to avoid significant recycling of the radiotracer (Kirschner, 1970). This criterion was met for fluxes from diet to body and body to water up to 9 h in all experiments with the low-salt natural diet. However, in trials with the high-salt synthetic diet, while this criterion was met for fluxes from body to water throughout the experiment, it was not met for fluxes from diet to body after ~3 h, and by 9 h the specific activity ratio was only twofold to fivefold. Thus significant radiotracer backflux likely occurred from the body pool to the digestive tract contents after 3 h, resulting in an overestimate of the amount of Na^+ or Cl^- remaining in the tract by the end of the experiment. Nevertheless, as by 3 h, more than 88% of the dietary Na^+ and Cl^- had already been absorbed from the tract in all experimental conditions, the influence of this problem on the overall conclusions of this study were negligible.

With respect to the second and third issues, interpretation of radiotracer appearance in the water from the diet, it should be emphasized that the measured flux represents only the Na^+ or Cl^- that originated from the diet, because it is based on the specific activity of the diet. It does not represent the total flux of Na^+ or Cl^- out of the animal, which will depend on the specific activity of the exchangeable Na^+or Cl^- pool of the animal. For example, for a given constant whole-body Na^+ efflux rate and a given constant Na^+ absorption rate from the diet, less of the total efflux rate will originate from the diet if the internal pool size is big than if it is small. Fortunately, these pool sizes were measured in an earlier study on *F. heteroclitus* (Wood and Laurent, 2003), and they are similar in freshwater *versus* seawater animals for both Na^+ (actually 18% lower in seawater animals after 7 days acclimation) and Cl^- (no difference), which simplifies interpretation of freshwater *versus* seawater differences. Nevertheless, the exchangeable whole-body Cl^- pool is only 65% of the exchangeable whole-body Na^+ pool (Wood and Laurent, 2003), so all other factors being equal, we would expect dietary Cl^- efflux into the water to occur at 1.53 times the rate of dietary Na^+ efflux. The actual differences observed were far greater (threefold to sixfold), reinforcing the conclusion that dietary Cl^- is absorbed from the diet and excreted to the water at a higher rate than dietary Na^+, especially in freshwater-acclimated killifish.

The importance of dietary Na^+ and Cl^- in *F. heteroclitus*

Despite the lack of a stomach (Babkin and Bowie, 1928), which appears to be the major ion-absorptive site in rainbow trout (Bucking and Wood, 2006b; Wood and Bucking, 2007), killifish clearly absorb most (≥88%) of the dietary Na^+ and Cl^- through their digestive tract within 3 h post-feeding, regardless of whether the diet is high or low in salt. Indeed, the rapid absorption of ions speaks to a high-capacity absorptive epithelium in the proximal portion of the digestive tract, although this requires further investigation. Furthermore, much of this absorbed Na^+ and Cl^- enters the exchangeable whole-body pools. Wood and Laurent (Wood and Laurent, 2003) measured the rate constants for turnover (*K*; the percentage per hour of the exchangeable whole-body pool that is lost to the external water) in freshwater- and seawater-acclimated *F. heteroclitus*. *K* values were ~2% for both Na^+ and Cl^- in freshwater animals, *versus* 34% (Na^+) and 45% (Cl^-) in seawater animals, in accord with the large freshwater *versus* seawater differences seen in the present study. It is possible to make a rough estimate of the fractions of total effluxes to the water that originate from the food. Using values averaged over the first 3 h after the meal when efflux rates were greatest, Na^+ efflux originating from the meal in freshwater ranged from ~50 $\mu mol\,kg^{-1}\,h^{-1}$ from the low-salt natural diet to ~700 $\mu mol\,kg^{-1}$ from the high-salt synthetic diet, and dietary Cl^- efflux (measured only from the high-salt diet) ranged from 900 to 2300 $\mu mol\,kg^{-1}\,h^{-1}$. To put this in perspective, reported unidirectional Na^+ and Cl^- efflux rates originating from the whole body of freshwater-acclimated killifish averaged ~800 $\mu mol\,kg^{-1}\,h^{-1}$ [200 to 1500 $\mu mol\,kg^{-1}\,h^{-1}$ in various studies (Potts and Evans, 1966; Potts and Evans, 1967; Maetz et al., 1967; Patrick et al., 1997; Patrick and Wood, 1999; Wood and Laurent, 2003; Wood, 2011)]. Thus dietary Na^+ can account for 6–87% of unidirectional whole-body Na^+ efflux, and dietary Cl^- can account for up to 100% of unidirectional whole-body Cl^- efflux.

In seawater-acclimated killifish, Na^+ efflux rates from the diet ranged from ~200 (low-salt diet) to 1800 $\mu mol\,kg^{-1}\,h^{-1}$ (high-salt diet) and Cl^- efflux rates from 4000 to 6000 $\mu mol\,kg^{-1}\,h^{-1}$ (high-salt diet only). Unidirectional whole-body Na^+ and Cl^- efflux rates have been measured in several studies on *F. heteroclitus* (Motais et al., 1966; Maetz et al., 1967; Potts and Evans, 1967; Pic, 1978; Wood and Laurent, 2003; Wood, 2011), with reported values ranging from 17,000 to 45,000 $\mu mol\,kg^{-1}\,h^{-1}$. Thus at most, dietary Na^+ accounts for ~10% and dietary Cl^- accounts for 35% of unidirectional whole-body efflux rates. Again, we emphasize that these figures apply to unidirectional Na^+ and Cl^- flux rates originating from the diet, not the total (net) flux rates from the whole

animal, which are thought to be approximately equal by the classic Silva model (Silva et al., 1977) as elaborated below.

Furthermore, the present study provides direct *in vivo* evidence of the critical importance of Cl^- uptake from the diet in freshwater killifish, to support conclusions based previously on indirect evidence. Earlier gill ion flux measurements in *F. heteroclitus* had shown that Cl^- uptake *via* the gills was negligible in freshwater whereas Cl^- loss rates to the water were not (Wood and Marshall, 1994; Patrick et al., 1997; Patrick and Wood, 1999; Wood and Laurent, 2003; Wood, 2011), despite a lower gill permeability to Cl^- than to Na^+, as revealed by transepithelial potential experiments (Wood and Grosell, 2008). This contrasts with Na^+ flux rate measurements in the same studies, where vigorous active Na^+ uptake at the gills and passive Na^+ loss occurred at similar rates. Furthermore, earlier *in vitro* gut sac experiments (Scott et al., 2006; Wood et al., 2010) had indicated that Cl^- uptake across the gut was upregulated in freshwater killifish. The present data confirm that the rate of Cl^- uptake from the diet is greater than that of Na^+, and that this dietary Cl^- is lost to the external water at a faster rate, especially in freshwater animals. This is most likely similar in other fish lacking active branchial Cl^- uptake in freshwater such as the eel and bluegill (e.g. Tomasso and Grosell, 2005); however, this remains to be confirmed.

The mechanisms of intestinal uptake and transepithelial excretion of Na^+ and Cl^- were not examined in the present study. With respect to the latter, in seawater animals, the gills are undoubtedly the major route of efflux, and the classic Silva model (Silva et al., 1977) of equimolar transcellular Cl^- extrusion and paracellular Na^+ extrusion energized by basolateral Na^+/K^+-ATPase probably applies (Evans et al., 2005; Hwang and Lee, 2007; Evans, 2008). In freshwater animals, the branchial efflux mechanisms are less clear, though branchial losses are generally thought to be passive, *via* channels and tight junctions, and Na^+ and Cl^- excretion in the urine becomes a minor but significant component [see calculations in Wood and Laurent (Wood and Laurent, 2003) and Wood (Wood, 2011)]. Interestingly, branchial acid-base regulation in freshwater killifish seems to rely mainly on differential modulation of Na^+ and Cl^- efflux rates, i.e. strong ion difference (SID) effects (Stewart, 1981), rather than on manipulation of Na^+ (*versus* H^+) and Cl^- (*versus* HCO_3^-) uptake rates, in view of the absence of the latter (Wood and Marshall, 1994; Patrick et al., 1997).

With respect to intestinal uptake mechanisms, earlier we reported that Cl^- uptake *in vitro* was greater in freshwater killifish gut sac preparations than seawater preparations, was partially coupled to greater HCO_3^- secretion, and was upregulated by feeding (Wood et al., 2010). Net Cl^- uptake also tended to be greater than net Na^+ uptake (Scott et al., 2006). This fits with the general view that intestinal Cl^- uptake occurs partially in exchange for HCO_3^- and partially coupled to Na^+ uptake (Grosell, 2006; Grosell, 2011a; Grosell, 2011b), but the killifish is unusual in having greater intestinal Cl^- uptake in freshwater than seawater. Nevertheless, this coincides with the unusual absence of an active Cl^- uptake mechanism at the gills in this euryhaline species when it is in freshwater, and the fact that branchial acid-base regulation depends on the differential regulation of Na^+ *versus* Cl^- efflux, as discussed earlier. In turn, this also coincides with the lack of HCl secretion in the digestive tract because the stomach is absent (Babkin and Bowie, 1928). Indeed, because of the elevated intestinal HCO_3^- secretion following feeding, the freshwater-acclimated killifish is the only teleost known to exhibit a postprandial 'acidic tide' (Wood et al., 2010) rather than the standard 'alkaline tide' seen in most fish (Wood et al., 2005; Bucking and Wood, 2008; Cooper and Wilson, 2008; Bucking et al., 2009; Li et al., 2010). An increase in branchial Cl^- excretion (representing a strong anion) over Na^+ excretion (representing a strong cation) would help to relieve the acid load in the blood through the SID theory (Stewart, 1981), as suggested by Wood and Marshall (Wood and Marshall, 1994) and Patrick et al. (Patrick et al., 1997).

Low-salt *versus* high-salt diets

The fish had been trained on the low-salt natural diet and the high-salt synthetic diet for ~7 days prior to the experiment. We had therefore predicted that in the freshwater killifish, the relative uptake and retention of Na^+ would be greater from the former, reflecting the scarcity of Na^+ in freshwater, but that this difference would not occur in seawater killifish, because of the excess of Na^+ in the marine environment. This hypothesis was based on previous studies showing that high-salt diets increased branchial Na^+ efflux across the gills in freshwater fish (Smith et al., 1995; Niyogi et al., 2006), and that acclimation to ion-poor waters decreased branchial Na^+ efflux rate (McDonald and Rogano, 1986). However, the results did not detect the predicted effect in freshwater killifish, and confirmed that it was absent in seawater killifish. Thus, in Series 4, for freshwater killifish, excretion of Na^+ from the low-salt diet up to 9 h was much lower on an absolute basis than from the high-salt diet, but by this time, almost identical percentages (~11%) had been excreted to the water and retained (~56%) in the carcass, regardless of the dietary salt load. Percentages remaining in the digestive tract at 3 h (before radiotracer recycling in the high-salt diet became a potential problem) were also similar (~7%). In seawater killifish, much more of the dietary Na^+ load was excreted to the water and much less was retained in the carcass from the low-salt diet, whereas the relative amounts remaining in the digestive tract were similar. The explanation for the marginal effect in freshwater killifish may be a matter of threshold. In critically reviewing a number of salt-feeding papers on salmonids (Smith et al., 1995; Kamunde et al., 2003; Pyle et al., 2003; Niyogi et al., 2006), Wood and Bucking (Wood and Bucking, 2011) concluded that the threshold for increases in branchial Na^+ efflux rate lay above a salt concentration of 1270 μmol Na^+ g^{-1} dry food mass. The high-salt diet used in the present experiments contained only 911 μmol Na^+ g^{-1} dry food mass. It would be interesting to test whether the same considerations apply to uptake and retention of Cl^- from high- and low-salt diets in the freshwater killifish. However, cost considerations precluded repeating the experiment with low-salt diets labelled with $^{36}Cl^-$.

Concluding remarks

Relative to the immense amount of knowledge on ionoregulation in relation to waterborne ions, our understanding of ionoregulation with respect to dietary ions remains in its infancy. The present study has highlighted the differential handling of dietary Na^+ and Cl^-, and the impact of external salinity, but many other factors remain unexplored, such as the interactive impacts of drinking, exercise, environmental O_2 and other ions in the diet. One particularly important factor may be behavioural changes associated with different ionic composition of the diet. For example, the high-salt synthetic diet is also high in Ca^{2+}, and in a companion study using the same high-salt synthetic diet, we found that killifish voluntarily choose a higher salinity after eating this diet, apparently to help their Ca^{2+} homeostasis (Bucking et al., 2012). The interaction among waterborne, dietborne and behavioural aspects of ionoregulation is a rich area for future investigation.

ACKNOWLEDGEMENTS

We thank Ora Johannsson for help with the experiments.

AUTHOR CONTRIBUTIONS

All authors contributed significantly to the experimental design and execution, as well as the interpretation of the results and the preparation of the manuscript.

COMPETING INTERESTS

No competing interests declared.

FUNDING

Supported by a National Sciences and Engineering Council (NSERC) Discovery Grant to C.M.W. and National Science Foundation (NSF) grant IOS 1146695 to M.G. C.M.W. is supported by the Canada Research Chair Program. C.B. was supported by an NSERC Canada Graduate Scholarship.

REFERENCES

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

Bucking, C. and Wood, C. M. (2006a). Gastrointestinal processing of Na^+, Cl^-, and K^+ during digestion: implications for homeostatic balance in freshwater rainbow trout. *Am. J. Physiol.* **291**, R1764-R1772.

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

Bucking, C. and Wood, C. M. (2007). Gastrointestinal transport of Ca^{2+} and Mg^{2+} during the digestion of a single meal in the freshwater rainbow trout. *J. Comp. Physiol. B* **177**, 349-360.

Bucking, C. and Wood, C. M. (2008). The alkaline tide and ammonia excretion after voluntary feeding in freshwater rainbow trout. *J. Exp. Biol.* **211**, 2533-2541.

Bucking, C., Fitzpatrick, J. L., Nadella, S. R. and Wood, C. M. (2009). Post-prandial metabolic alkalosis in the seawater-acclimated trout: the alkaline tide comes in. *J. Exp. Biol.* **212**, 2159-2166.

Bucking, C., Wood, C. M. and Grosell, M. (2012). Diet influences salinity preference of an estuarine fish, the killifish *Fundulus heteroclitus*. *J. Exp. Biol.* **215**, 1965-1974.

Cooper, C. A. and Wilson, R. W. (2008). Post-prandial alkaline tide in freshwater rainbow trout: effects of meal anticipation on recovery from acid-base and ion regulatory disturbances. *J. Exp. Biol.* **211**, 2542-2550.

Evans, D. H. (2008). Teleost fish osmoregulation: what have we learned since August Krogh, Homer Smith, and Ancel Keys. *Am. J. Physiol.* **295**, R704-R713.

Evans, D. H., Piermarini, P. M. and 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.

Grosell, M. (2006). Intestinal anion exchange in marine fish osmoregulation. *J. Exp. Biol.* **209**, 2813-2827.

Grosell, M. (2011a). Intestinal anion exchange in marine teleosts is involved in osmoregulation and contributes to the oceanic inorganic carbon cycle. *Acta Physiol.* **202**, 421-434.

Grosell, M. (2011b). The role of the gastrointestinal tract in salt and water balance. In *The Multifunctional Gut of Fish, Fish Physiology,* Vol. 30 (ed. M. Grosell, A. P. Farrell, and C. J. Brauner), pp. 136-164. Amsterdam: Academic Press.

Grosell, M., O'Donnell, M. J. and Wood, C. M. (2000). Hepatic versus gall bladder bile composition – *in vivo* transport physiology of the gallbladder in rainbow trout. *Am. J. Physiol.* **278**, R1674-R1684.

Hwang, P.-P. and Lee, T.-H. (2007). New insights into fish ion regulation and mitochondrion-rich cells. *Comp. Biochem. Physiol.* **148A**, 479-497.

Kamunde, C. N., Pyle, G. G., McDonald, D. G. and Wood, C. M. (2003). Influence of dietary sodium on waterborne copper toxicity in rainbow trout, *Oncorhynchus mykiss*. *Environ. Toxicol. Chem.* **22**, 342-350.

Kirschner, L. B. (1970). The study of NaCl transport in aquatic animals. *Am. Zool.* **10**, 365-376.

Li, K.-G., Cao, Z.-D., Peng, J.-L. and Fu, S.-J. (2010). The metabolic responses and acid-base status after feeding, exhaustive exercise, and both feeding and exhaustive exercise in Chinese catfish (*Silurus asotus* Linnaeus*). J. Comp. Physiol. B* **180**, 661-671.

Maetz, J., Sawyer, W. H., Pickford, G. E. and 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.

McDonald, D. G. and Rogano, M. S. (1986). Ion regulation by the rainbow trout, *Salmo gairdneri*, in ion-poor water. *Physiol. Zool.* **59**, 318-331.

Motais, R., Romeu, F. G. and Maetz, J. (1966). Exchange diffusion effect and euryhalinity in teleosts. *J. Gen. Physiol.* **50**, 391-422.

Niyogi, S., Kamunde, C. N. and Wood, C. M. (2006). Food selection, growth and physiology in relation to dietary sodium chloride content in rainbow trout (*Oncorhynchus mykiss*) under chronic waterborne Cu exposure. *Aquat. Toxicol.* **77**, 210-221.

Patrick, M. L. and 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.* **122**, 445-456.

Patrick, M. L., Pärt, P., Marshall, W. S. and 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. and Evans, D. H. (1966). The effects of hypophysectomy and bovine prolactin on salt fluxes in fresh-water-adapted *Fundulus heteroclitus*. *Biol. Bull.* **131**, 362-368.

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

Pyle, G. G., Kamunde, C. N., McDonald, D. G. and Wood, C. M. (2003). Dietary sodium inhibits aqueous copper uptake in rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* **206**, 609-618.

Rogers, A. W. and Moran, J. F. (1966). Evaluation of quench correction in liquid scintillation counting by internal, automatic external, and channels' ratio standardization methods. *Anal. Biochem.* **16**, 206-219.

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

Silva, P., Solomon, R., Spokes, K. and Epstein, F. H. (1977). Ouabain inhibition of gill Na-K-ATPase: relationship to active chloride transport. *J. Exp. Zool.* **199**, 419-426.

Smith, N. F., Eddy, F. B. and Talbot, C. (1995). Effect of dietary salt load on transepithelial Na+ exchange in freshwater rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* **198**, 2359-2364.

Stewart, P. A. (1981). *How to Understand Acid-Base, a Quantitative Acid-Base Primer for Biology and Medicine*. New York, NY: Elsevier.

Tomasso, J. R., Jr and Grosell, M. (2005). Physiological basis for large differences in resistance to nitrite among freshwater and freshwater-acclimated euryhaline fishes. *Environ. Sci. Technol.* **39**, 98-102.

Wilson, R. W., Wilson, J. M. and Grosell, M. (2002). Intestinal bicarbonate secretion by marine teleost fish – why and how? *Biochim. Biophys. Acta* **1566**, 182-193.

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

Wood, C. M. and Bucking, C. (2012). Defecation and the fate of dietary sodium in the common killifish (*Fundulus heteroclitus macrolepidotus* Walbaum 1972). *J. Exp. Mar. Biol. Ecol.* **434-435**, 53-57.

Wood, C. M. and Grosell, M. (2008). A critical analysis of transepithelial potential in intact killifish (*Fundulus heteroclitus*) subjected to acute and chronic changes in salinity. *J. Comp. Physiol.* **178**, 713-727.

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

Wood, C. M. and 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., Kajimura, M., Mommsen, T. P. and Walsh, P. J. (2005). Alkaline tide and nitrogen conservation after feeding in an elasmobranch (*Squalus acanthias*). *J. Exp. Biol.* **208**, 2693-2705.

Wood, C. M., Bucking, C. and 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.