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Na^+ versus Cl^- transport in the intact killifish after rapid salinity transfer

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Abstract

Much of the early research elucidating the general mechanisms of euryhalinity was performed on the common killifish. More recently, its opercular epithelium with abundant mitochondria-rich cells has proven to be a powerful model for analyzing the mechanisms of active NaCl transport under Ussing conditions in vitro (i.e., with isotonic saline on both surfaces, at short-circuit). However, it is unclear whether this preparation duplicates the gill under real world conditions—i.e., at open-circuit, with real seawater (SW) or freshwater (FW) on the mucosal surface. There have been only limited studies, mostly about 35 years ago, on ion transport in the intact killifish. Therefore, using radioisotopes (^{22}Na, ^{36}Cl), we developed and evaluated methods for the independent measurement of unidirectional Na^+ and Cl^- influx and efflux rates and internal pools in intact killifish acclimated to 10% SW and abruptly transferred to either 100% SW or FW. Internal Na^+ pools were disturbed less than internal Cl^- pools by transfer, and were corrected after 3 days in 100% SW or 7 days in FW. Influx and efflux rates in 10% SW were about 3000 μmol kg^{-1} h^{-1} and increased to 15,000–18,000 μmol kg^{-1} h^{-1} after transfer to 100% SW, remaining approximately equal and equimolar for Na^+ and Cl^-, and stable from 0.5 to 7 days post-transfer. After transfer to FW, Na^+ influx and efflux rates dropped to 1000–1500 μmol kg^{-1} h^{-1}, with efflux slightly exceeding influx, and remained approximately stable from 0.5 to 7 days. However, while Cl^- efflux responded similarly, Cl^- influx rate dropped immediately to negligible values (20–50 μmol kg^{-1} h^{-1}) without recovery through 7 days. These results differ from early ion transport data in 100% SW, and demonstrate that fluxes stabilize quickly after salinity transfer. They also show that the intact animal responds more quickly than the epithelium, provide qualitative but not quantitative support for the opercular epithelium as a model for the gill under real world SW conditions, and no support for its use as a gill model under real world FW conditions, where branchial Cl^- uptake is negligible.

Keywords: Killifish; Euryhalinity; Na^+ transport; Cl^- transport; Gill; Opercular epithelium

1. Introduction

The common killifish or mummichog, *Fundulus heteroclitus*, lives in tidal marshes and estuaries throughout the eastern coast of North America. The species has a powerful capacity for euryhalinity, tolerating abrupt seawater–freshwater transfer (and vice versa) and constant salinities ranging from 0 to 120 ppt (i.e., freshwater to ~360% seawater; [1]). As such, *F. heteroclitus* and some of its close relatives (e.g., *F. kansae*, *F. similis*, *F. chrysotus*) were studied extensively in the 1940s–1970s, revealing many of the basic principles which we now accept as fundamental to euryhaline osmoregulation in teleosts (reviewed by Wood and Marshall [2]).

These early key findings include: drinking in seawater and its virtual suspension in freshwater [3]; a marked increase in glomerular filtration rate and altered tubular processing, which transform renal function from water conservation in seawater to Na^+ and Cl^- conservation and water excretion in freshwater [4–7]; the key role of increased prolactin secretion in facilitating "Na^+ retention" (i.e., marked decreases in branchial and renal Na^+ and Cl^- losses) after transfer from seawater to freshwater [8–13]; the key role of increased cortisol secretion in upregulating branchial Na^+K^+ATPase activity for active salt excretion after transfer from freshwater to seawater [14]; the more rapid, almost instantaneous modulation of branchial Na^+K^+ATPase activity upon salinity transfer, originally reported by Towle et al. [15] and recently confirmed by Mancera and McCormick [16]; the dramatic reduction in

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unidirectional Na^+ influx and efflux rates, measured radioisotopically, which occurs soon after seawater to freshwater transfer [3,10,17,18]; and the marked changes in the ultrastructure and histochemistry of the mitochondria-rich cells (MRCs, often called "chloride cells") in the gills which are thought to reflect the changeover of their function from salt secretion in seawater to salt uptake in freshwater [19–22].

In 1950, Burns and Copeland [23] reported that the skin lining the opercular bone of seawater-adapted *F. heteroclitus* was richly endowed with MRCs. In 1977, the whole focus of killifish ionoregulatory physiology changed markedly when Karnaky et al. [24] mounted this "opercular epithelium" in an Ussing chamber with isotonic saline on both sides and demonstrated active NaCl transport from serosal (blood) to mucosal (water) surfaces. Since that time, the killifish opercular epithelium and analogous preparations from other euryhaline species (e.g., *Oreochromis mossambicus*, *Gillicthys mirabilis*, *Blennius pholis*) have been the subject of hundreds of studies that have capitalized on their flat, thin morphology, which is ideal for radioisotopic flux measurements, electrophysiological analyses, and immunohistochemical and ultrastructural studies (for reviews, see Refs. [2,25–32]). Indeed, it is fair to say that studies on the opercular epithelium have provided the bulk of the evidence supporting the mechanism which is now almost universally accepted as the process for active NaCl extrusion across the seawater gill, plus most of the detailed information on its extracellular regulatory and intracellular signaling pathways. In brief, this "Silva" model (originally proposed by Silva et al. [33]), for equimolar Na^+ and Cl^- "co-transport" envisages transcellular, electrogenic secondary active Cl^- secretion through the MRCs, energized by basolateral Na^+K^+ATPase, which creates an electrochemical gradient for Na^+ to diffuse passively outward through the paracellular channels in a 1:1 ratio with Cl^-.

The overwhelming success of studies with the opercular epithelium have led to a decline in physiological investigations on the intact killifish in the last 20 years, apart from a few notable exceptions [34–39]. Most workers make the implicit assumption that the opercular epithelium accurately mimics the transport functions of the gills in the live animal. Unfortunately, the assumption that the opercular epithelium is an accurate surrogate for the intact gill has never been critically evaluated, and there are good reasons to question its validity. One difficulty in relating opercular epithelia flux data to intact killifish flux data is the paucity of the latter. There were a few studies about 35 years ago [3,10,17,18], but these generally concentrated on Na^+ and were complicated by methodological issues (see Discussion). Nevertheless, based on such studies, plus their own data on the opercular epithelium, Degnan and Zadunaisky [40] concluded that this skin could account for only about 1% of the unidirectional Na^+ and Cl^- efflux rates in the whole seawater-adapted killifish.

The majority of studies have employed the opercular epithelia from seawater-adapted killifish set up under "Ussing conditions"—i.e., with identical isotonic saline on both surfaces, and at short-circuit so as to set the transepithelial potential (TEP) to zero, as illustrated by the data of Degnan et al. [41] in Fig. 1B. When unidirectional ion fluxes are measured with radioisotopic tracers under such circumstances, the preparations actively transport Cl^- from serosa to mucosa, as shown by significant exceedance of the Ussing flux ratio criterion [42] for passive transport alone; the short-circuit current is equivalent to the active Cl^- transport. In contrast, the observed Na^+ flux ratio is identical to the Ussing prediction, indicating that Na^+ passively follows the electrogenic Cl^- transport. This supports the standard seawater Silva model. However, the presence of mucosal saline (equivalent to approximately 35% seawater) and a TEP artificially set to 0 mV is not a "real world condition" for a seawater-adapted killifish, where transport has to occur against much greater electrochemical gradients.

Only rarely has the seawater-adapted preparation been set up under "real world conditions", with 100% seawater on the mucosal surface, physiological saline on the serosal surface, and a naturally developed, highly serosal-positive TEP of electrogenic origin. In a review paper, Péqueux et al. [29] reported data indicating that the preparation could achieve a net excretion of Cl^- (Na^+ movement not reported) when the NaCl content of outside saline was raised to typical seawater levels. However, Degnan and Zadunaisky [40] appear to be the only workers to employ real seawater on the mucosal surface. Under such circumstances, the observed Cl^- flux ratio again exceeds the Ussing prediction, whereas Na^+ is again moving passively, as illustrated by the data of Degnan and Zadunaisky [40] in Fig. 1C. However, for both ions, the observed unidirectional flux ratios do not exceed 1.0, so there is no net extrusion of NaCl, in contrast to the situation under Ussing conditions, and likely in contrast to the situation in the gills of the intact seawater animal.

Finally, there are also few studies [3,43,44] where the opercular epithelia of freshwater-adapted killifish have been set up under "real world conditions", with freshwater on the mucosal surface, physiological saline on the serosal surface, and a naturally developed, highly serosal-negative TEP of diffusive origin. Under such circumstances, as illustrated by the data of Burgess et al. [44] in Fig. 1A, when the observed and Ussing flux ratios are calculated for transport in the "uptake" direction (i.e., transport from mucosa to serosa), only Cl^- is actively taken up, and Na^+ is again passively transported, very different from the "standard" freshwater gill model of active, independent Na^+ and Cl^- uptake (e.g., Refs. [45,46]). Furthermore, the unidirectional flux ratios for both ions are below 1.0, indicating that large net NaCl losses occur, rather than the net uptake anticipated for the gills of the intact freshwater animal.

With this background in mind, we decided to revisit the transport physiology of the intact killifish in this study. Our goal was to obtain accurate, independent measurements of

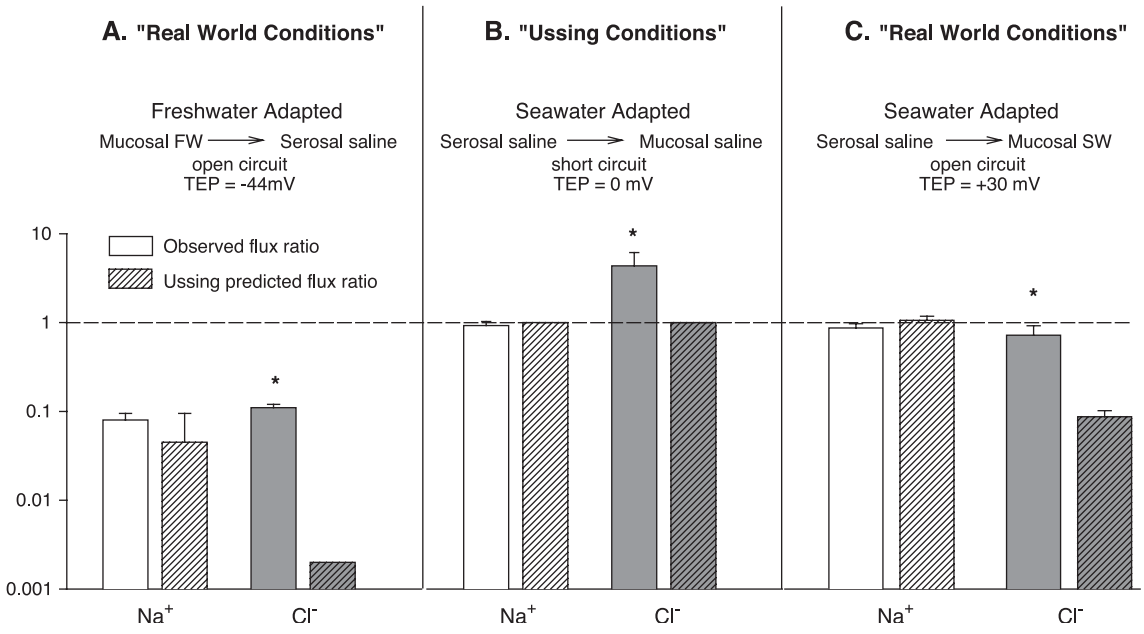

Fig. 1. Representative literature reports of observed and predicted unidirectional flux ratios for Na^+ (clear bars) and Cl^- (shaded bars) for killifish opercular epithelial preparations set up under various conditions. Predicted unidirectional flux ratios for passive transport were calculated using the Ussing equation (cf. [47]). (A) "Real world" freshwater conditions (mucosal freshwater, serosal saline at open circuit). Flux ratios calculated for transport from mucosa to serosa. Note the highly negative inside TEP. Data from Ref. [44]. (B) "Ussing conditions" (mucosal saline, serosal saline, at short-circuit). Flux ratios calculated for transport from serosa to mucosa. Note that TEP is set to zero by the short-circuit current. Data from Ref. [41]. (C) "Real world" seawater conditions (mucosal seawater, serosal saline at open circuit). Flux ratios calculated for transport from serosa to mucosa. Note the highly positive inside TEP. Data from Ref. [40]. Means ± 1 S.E. The dotted line indicates a flux ratio of 1 (no net transport). Asterisks indicate significant difference ($P < 0.05$) between observed and predicted flux ratios.

unidirectional Na^+ and Cl^- influx and efflux rates of the whole animal in seawater and freshwater, for comparison with the opercular epithelial literature. Particular attention was paid to methodological issues. Because the animal normally lives in estuaries and tidal marshes, we acclimated animals to brackish water (10% seawater) as the control condition and measured Na^+ and Cl^- flux rates before and after transfer (at 12 h, 3 days, and 7 days post-transfer) to either freshwater or 100% seawater.

2. Materials and methods

2.1. Experimental animals

Common killifish (*F. heteroclitus*, 3–8 g) were collected by beach seine from a brackish estuary near Antigonish, NS, Canada, and air-shipped to McMaster University, Hamilton, ON, Canada. At McMaster, they were kept for several months prior to experiments in 500-l fiberglass tanks containing recirculated, charcoal-filtered 10% seawater at 18–20 °C, the subsequent experimental temperature. The fish were fed once daily to satiation with a mix of 50% commercial flakes (Wardley Total Tropical Gourmet Flake Blend, Hartz Mountain, Corp., Secausus, NJ, USA) and 50% frozen brine shrimp (San Francisco Bay Brand, Newark, CA, USA).

2.2. Experimental protocols

The basic experimental design involved the collection of independent measurements of unidirectional Na^+ and Cl^- influx and efflux rates in killifish acclimated to 10% seawater, and after transfer to either freshwater or 100% seawater for 0.5 days (12 h), 3 days, or 7 days. Measurements of the water chemistry made during the actual flux determinations are summarized in Table 1. Fish intended for experiments were removed from the holding tank before the morning feeding, and so were starved for slightly more than 24 h prior to flux measurements. In fish transferred to 100% seawater or freshwater for 3 or 7 days as part of the experimental protocol, daily feeding was continued in the new media, except on the day of flux measurement. This feeding was found to be essential to keep the fish healthy after transfer to freshwater.

Table 1
Concentrations of major electrolytes in the various test media, as measured in the flux experiments

	10% Seawater	100% Seawater	Freshwater
Na^+ (mmol l^{-1})	54.2 ± 2.8 (24)	499.8 ± 10.7 (36)	0.82 ± 0.02 (36)
Cl^- (mmol l^{-1})	71.2 ± 3.1 (24)	551.5 ± 1.3 (36)	1.07 ± 0.02 (36)
Ca^{2+} (mmol l^{-1})	1.73 ± 0.02 (8)	10.92 ± 0.14 (8)	0.78 ± 0.01 (8)
Mg^{2+} (mmol l^{-1})	5.40 ± 0.05 (8)	53.5 ± 0.28 (8)	0.44 ± 0.01 (8)

Means ± 1 S.E. (*N*).

Unidirectional fluxes were measured using radiotracers (^{22}Na, ^{36}Cl; NEN, Boston, MA, USA) by the methods outlined below. All measurements were made on fish that had been allowed to settle for 2 h in individual polyethylene chambers fitted with a lid and an aeration line, and containing 250 ml of the appropriate medium. Preliminary experiments with shorter settling times, smaller volumes and/or open chambers produced higher unidirectional flux rates, likely associated with stress as earlier reported by Maetz et al. [10] and Pic [18]. In freshwater, it is possible to determine unidirectional efflux indirectly [47,48] as the difference between unidirectional influx (measured by the uptake of radioisotopically labeled Na^+ or Cl^-) and net flux (measured by the chemical determination of changes in total Na^+ or Cl^- in the external medium). However, in 10% or 100% seawater, the high background levels of total Na^+ and Cl^- make determinations of net changes undetectable in any reasonable time period, and therefore prevent the indirect measurement of efflux. For this reason, and to allow comparability of data across treatments, we elected to make direct efflux and influx measurements in all media, and as far as possible, to use comparable methods in all media.

2.3. Influx determinations

The uptake of radioisotope from the medium can be measured by its disappearance from the water (the classical "freshwater" indirect technique) or by its appearance in the fish [47,48]. The latter method was used throughout. While the former is easier and non-invasive, the latter is far more accurate. Indeed, it is the only viable method in 10% or 100% seawater unless massive amounts of radioactivity are used, because the high background levels of non-radioisotopic Na^+ or Cl^- in the water drown out detectable changes in the small radioisotopic fraction. However, an additional complication is that in 10% and 100% seawater, only about 0.2–3% of the non-radioactive pool is in the fish (compared to about 50% when the fish is in freshwater), and the absolute influx rates are much higher than in freshwater. As a result, internal specific activity (SA: the ratio of radioisotopic to total Na^+ or Cl^-) can quickly rise to the point where "backflux" of the radioisotope from the fish to the media becomes a significant source of error. We adopted the criterion that SA_{int} (internal) must remain below 10% of the SA_{ext} (external) at the end of the flux determination, and therefore $<5\%$ averaged over the period [47].

Based on a number of preliminary trials designed to maximize the accumulation of radioactivity in the fish, while ensuring that this criterion was met even in 100% seawater, we adopted a flux period of 0.5 h for measurements in all three media, though in freshwater, measurements up to 2 h yielded similar data. Most experiments were performed with dual labeling (^{22}Na and ^{36}Cl) to allow simultaneous measurement of both Na^+ and Cl^- influx rates. The fish were allowed to settle for 2 h in 250 ml of the appropriate media in their individual chambers, and then ^{22}Na (1 μCi) and ^{36}Cl (4 μCi) were added and quickly mixed. Duplicate water samples (2 × 5 ml) were taken at the start and end of the flux period for radioactivity (^{22}Na, ^{36}Cl) and total Na^+ and Cl^- measurements. Immediately following the final water sample, the fish were rinsed in non-radioactive media for 5 min, killed by a cephalic blow, blotted to remove external water, weighed, and then processed for measurements of total body radioactivities (^{22}Na, ^{36}Cl) and total body Na^+ and Cl^- pools. Unidirectional influx rates (J_{in}) in μmol kg^{-1} h^{-1} were calculated as:

$$J_{in} = \frac{\Sigma_{(cpm\ in\ fish)}}{\text{Mean } SA_{ext} \times T \times W} \quad (1)$$

where $\Sigma_{(cpm\ in\ fish)}$ is the total ^{22}Na or ^{36}Cl radioactivity in the fish (in cpm), mean SA_{ext} is the mean external specific activity (in cpm μmol^{-1}), W is body weight (in kg), and T is time (in h).

2.4. Efflux determinations

The loss of radioisotope from the fish to the medium provides a direct measure of the unidirectional efflux rate. Theoretically, this washout will follow one (or more) exponential functions against time which can be linearized by taking the natural logarithm [47]. The slope of this plot of ln radioactivity versus linear time (in hours) provides a rate constant, which when multiplied by 100% yields K, the fraction per hour of the labeled internal pool that is being lost to the media. The labeled internal pool can be determined by chemically measuring the total internal Na^+ or Cl^- pool (Σ_Z) in the animal, and then adjusting it for the fractional labeling (F) of that pool by the radioisotope. Unidirectional efflux rate (J_{out}) in μmol kg^{-1} h^{-1} can then be calculated as:

$$J_{out} = \frac{K \times \Sigma_Z \times F}{W} \quad (2)$$

A number of practical considerations were worked out in preliminary trials. Because the cpm washout into the external media was relatively small, single label experiments provided more reliable data than did dual label tests. Radioisotopes (^{22}Na or ^{36}Cl in separate fish) were administered (0.25 μCi g^{-1} in 2.5 μl g^{-1} of Cortland saline; [49]) using a special 50 μl Hamilton gas-tight syringe, modified with a very short (1 cm) fixed Huber point needle for injection. Intraperitoneal injections proved more reliable than caudal arch injections, and lower, more consistent efflux data were obtained if the fish were not anaesthetized for injection. Therefore, killifish were quickly dried, weighed, injected intraperitoneally with a precisely known amount of radioactivity, and then placed back into their individual chambers containing 250 ml of the appropriate medium. After a further 40-min equilibration period, starting water samples (time zero) were taken with subsequent hourly samples

(2 × 5 ml) up to 5 h. The difference between the known amount of injected radioactivity and the measured loss seen in the start samples provided the known amount of radioactivity in the fish at time zero. Measurements over several subsequent hours were required to obtain a reliable estimate of K. As long as the measurement period was limited to 4–5 h, the efflux data were well described by a single exponential function, and even in freshwater, the 5% specific activity criterion (in this case, mean SA_{ext} remaining < 5% of SA_{int}) was ensured.

In theory, the same rate constant should be obtained from the slope of a plot of ln [Δcpm appearing in the media] in each hour (for example, see Ref. [10]), as from a plot of ln [cpm remaining in the fish] in each hour (for example, see Ref. [3]). In practice, because of the nature of linear regression, an "error" of constant size in a single data point has a much greater influence on the slope of the ln [Δcpm appearing] plot (where values are small) than on the slope of the ln [cpm remaining] plot (where values are much larger), so the latter approach was adopted throughout.

We were concerned that injecting the radioisotope, as also done by previous workers [3,10,17,18] might unduly stress the killifish. Therefore, a preliminary experiment was carried out in which control killifish (acclimated to 10% seawater) were loaded with ^{22}Na in two different ways. One group (N = 6) received the standard intraperitoneal injection of a precisely known amount of ^{22}Na, and the experiment was performed in the normal way. The other group (N = 10) was incubated for 24 h in 1000 ml of 10% aerated seawater containing 60 μCi of ^{22}Na, so as to gradually load from the water. These fish were then quickly rinsed in non-radioactive water, transferred to individual flux chambers containing 250 ml of 10% seawater, allowed to settle, and then the experiment was performed in the normal fashion. However, since the original load of ^{22}Na in each fish was not known, after the 5-h sample, it was necessary to measure total body radioactivity (^{22}Na) in each individual, and then, from the measured losses to the medium over 5 h, to back-calculate to the amount of radioactivity in the fish at time zero.

The fractional labeling (F) of the internal pool can be determined in several ways. The simplest is to extrapolate the ln [cpm remaining] versus linear time plot to time zero, and to compare the resulting estimate of total radioactivity in the fish with the known amount present in the fish at that time. However, a more direct method is to sacrifice a subset of fish at the midpoint of the efflux period, and to compare the chemically measured Σ_Z in the fish with the labeled internal pool calculated from measurements of total radioactivity in the fish and plasma specific activity:

$$\text{Labeled internal pool} = \Sigma_{(\text{cpm in fish})} \times \frac{\text{plasma [Z]}}{\text{plasma [cpm]}} \quad (3)$$

This comparison was made in one control series with killifish acclimated and tested in 10% seawater. F was measured by the extrapolation method in one group (N = 6). In the other (N = 6), at 2.5 h (the midpoint of the normal 5-h efflux period), fish containing a precisely known $\Sigma_{(\text{cpm in fish})}$ of ^{22}Na were anaesthetized with 0.01% MS-222 and bled by caudal puncture into a lightly heparinized Hamilton syringe. The blood was spun in a microhematocrit capillary at 500 × g for 5 min, the plasma decanted and processed for determination of total plasma [Na^+] and plasma [^{22}Na cpm], and the whole body analyzed for ^{22}Na radioactivity and total Na^+.

2.5. Whole-body Na^+, Cl^-, and radioactivity measurements

When ^{22}Na was to be measured, the whole fish was first counted for gamma radioactivity, which could be done without killing the fish if required. The carcass was then placed in four volumes of 1 N HNO_3 (trace metal grade) in a sealed tube, and baked in an oven at 60 °C for 48 h with periodic vortexing. Thereafter, the digest was cooled, centrifuged at 500 × g for 5 min, and then 5 ml of supernatant were added to 10 ml of UltimaGold AB (Packard Bioscience, Groningen, Netherlands), a scintillation fluor specifically designed for the counting of acidic digests. This sample was then scintillation counted for the sum of ^{36}Cl and ^{22}Na radioactivity, and gamma counted for ^{22}Na radioactivity (see below). Additional aliquots of supernatant were taken for total Na^+ (by atomic absorption) and Cl^- measurements (by coulometric titration).

2.6. Analytical techniques

^{22}Na radioactivities in injection stock, water, plasma, and whole-body samples were determined on a Minaxi Autogamma 5000 counter (Packard Instruments, Downers Grove, IL, USA). ^{36}Cl radioactivities in comparable samples were determined on a Rackbeta 1217 liquid scintillation counter (LKB-Wallac, Turku, Finland) using either the UltimaGold AB counting system (see above) for tissue digests or 10 ml ACS fluor (Amersham, Oakville, ON, Canada) plus 5 ml water (or sample made up to 5 ml with water) for the other samples. Internal standardization was employed to check for quenching (there was none) and to correct for minor differences in counting efficiency between the ACS and UltimaGold fluors. ^{36}Cl is a pure beta-emitter detectable only by scintillation counting, whereas ^{22}Na is a both a gamma- and beta-emitter detectable by either gamma or scintillation counting. In dual label experiments, the same samples were gamma-counted for ^{22}Na radioactivity alone and then scintillation counted for combined ^{22}Na and ^{36}Cl radioactivity. ^{36}Cl cpm were obtained by subtraction after correcting for the difference in counting efficiency of ^{22}Na between the two counters.

Total Ca^{2+} and Mg^{2+} levels in water samples were measured by atomic absorption spectrophotometry (AAS; Varian Australia Model 220FS, Mississauga, ON, Canada)

Table 2
A comparison of unidirectional Na^+ efflux rates and fractional labeling (F) of the whole-body pool yielded by two different methods of administering the radioisotope

	Na^+ efflux rate (μmol kg^{-1} h^{-1})	F (%)
Injection method	3083 ± 337 (6)	96.75 ± 0.74 (6)
Incubation method	3202 ± 649 (10)	96.77 ± 0.51 (10)

Means ± 1 S.E. (N).
There were no significant differences.

using certified standards (Fisher Scientific, Fair Lawn, NJ, USA.). Total Na^+ concentrations in water, plasma, and tissue digests were also measured by AAS. Cl^- concentrations in digests and in 10% and 100% seawater samples were measured by coulometric titration using a CMT-10 chloridometer (Radiometer, Copenhagen, Denmark), and in freshwater samples by the colorimetric method of Zall et al. [50]. The same Radiometer NaCl certified standard stock was used for both Na^+ and Cl^- measurements.

2.7. Statistics

Data have been generally expressed as means ± 1 S.E. (N). Regression lines were fitted by the method of least squares. Comparisons between measurements of the same parameter after different times in 100% seawater or freshwater were evaluated by ANOVA, followed by the Bonferroni test. Comparisons between different fluxes at the same time were made by Student's t-test, either paired or unpaired as appropriate. Percentage data were arcsine-transformed prior to the test. A significance level of $P<0.05$ was used throughout.

3. Results

3.1. Control condition (10% seawater) and methodology tests

In control killifish that had been acclimated for several months to 10% seawater, unidirectional Na^+ efflux rates and fractional labeling of the internal Na^+ pool (F determined by the extrapolation method) were the same, regardless of whether the radioisotope was administered by intraperitoneal injection or 24 h incubation (Table 2). In the comparison of the two methods for measuring F, the extrapolation method yielded a value of 97.50 ± 0.33% ($N=6$), while the direct method yielded a value of 98.28 ± 0.90% ($N=6$). These were not significantly different, and were comparable to other extrapolation estimates of F for 10% seawater-acclimated killifish in Table 2. The direct estimate of F was based on a radiolabeled internal pool of 66.32 ± 3.42 mmol kg^{-1} ($N=6$) and a chemically measured total internal pool of 68.47 ± 2.86 mmol kg^{-1} ($N=6$); plasma Na^+ was 126.8 ± 5.8 mmol l^{-1} ($N=6$) in these fish. Based on these results, the injection method, which is simpler, quicker, and uses much less radioisotope, was used throughout, as was the extrapolation method for estimating F, as these methods do not necessitate blood sampling or counting the fish.

For control killifish in 10% seawater, the overall total Na^+ pool (67.41 ± 2.80 mmol kg^{-1}, $N=22$) in the whole body was substantially greater than the Cl^- pool (43.99 ± 3.63 mmol kg^{-1}, $N=12$). For both ions, F was >96%, while K was about 4.4% h^{-1} for Na^+ and 6.7% h^{-1} for Cl^-, though these values were not significantly different (Table 3). Control killifish exhibited unidirectional flux rates of Na^+ that were approximately equal to those of Cl^- at around 3000 μmol kg^{-1} h^{-1} (e.g., Fig. 3). For both ions, separately measured unidirectional influx rates slightly exceeded efflux rates (by about 15%) but again there were no significant differences. Thus under control conditions, killifish were in approximate balance with equimolar Na^+ and Cl^- flux rates.

3.2. Transfer to 100% seawater

Transfer from 10% to 100% seawater actually represented about a nine-fold increase in environmental Na^+ levels and an eight-fold increase in Cl^- levels (Table 1). In contrast, the internal Na^+ pool of killifish rose by only 20% at 12 h, had returned to control values at 3 days, and was actually 20% below control values at 7 days (Fig. 2).

Table 3
Changes in K (the percentage per hour of the labeled internal pool which is lost to the media) and F (the fractional labeling of the whole-body pool) after transfer of killifish from 10% seawater to 100% seawater for various periods

	Na^+		Cl^-	
	K	F	K	F
10% Seawater (control)	4.36 ± 0.53 (22)	97.19 ± 0.33 (22)	6.68 ± 0.92 (12)	96.44 ± 0.34 (12)
100% Seawater (12 h)	21.57 ± 1.35*(6)	97.34 ± 0.85 (6)	37.65 ± 3.58*,†(6)	87.65 ± 4.83 (6)
100% Seawater (3 days)	26.51 ± 1.25* (12)	92.88 ± 0.97* (12)	54.01 ± 3.08*,† (12)	75.82 ± 2.73*,† (12)
100% Seawater (7 days)	34.11 ± 4.11* (6)	100.16 ± 2.27 (6)	44.96 ± 4.60* (11)	89.24 ± 1.87*,† (11)

Means ± 1 S.E. (N).
* Significantly different ($P<0.05$) from comparable value in 10% seawater control group.
† Significantly different ($P<0.05$) from comparable Na^+ value.

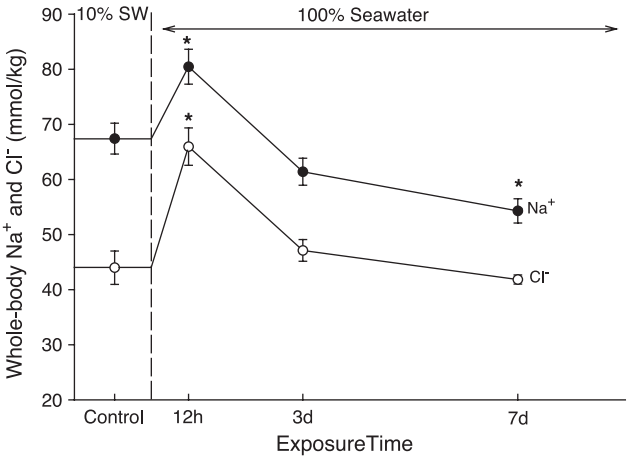

Fig. 2. Responses in killifish of the internal whole-body Na^+ (closed circles) and Cl^- pools (open circles), measured chemically, after transfer from the control condition (10% seawater) to 100% seawater at time zero (dashed line). Means ± 1 S.E. $N=22$, 6, 6, and 6 for Na^+, and $N=12$, 6, 6, and 6 for Cl^- at control, 12-h, 3-day and 7-day time points, respectively. Asterisks indicate significant difference ($P<0.05$) from control value. Cl^- pools were significantly lower than Na^+ pools at all times.

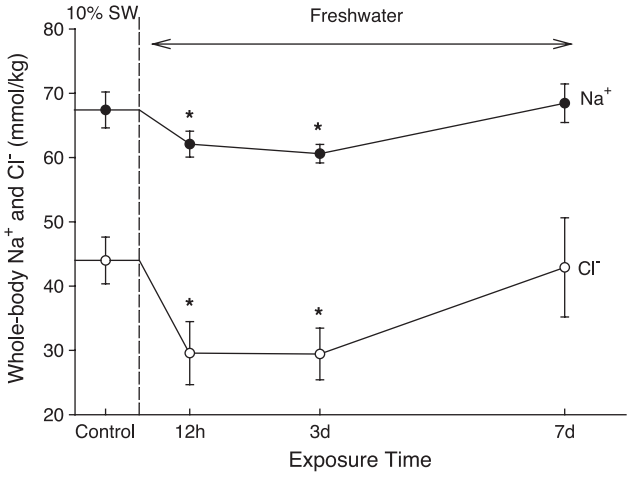

Fig. 4. Responses in killifish of the internal whole-body Na^+ (closed circles) and Cl^- pools (open circles), measured chemically, after transfer from the control condition (10% seawater) to freshwater at time zero (dashed line). Means ± 1 S.E. $N=22$, 6, 6, and 6 for Na^+, and $N=12$, 6, 6, and 6 for Cl^- at control, 12-h, 3-day, and 7-day time points, respectively. Asterisks indicate significant difference ($P<0.05$) from control value. Cl^- pools were significantly lower than Na^+ pools at all times except 7 days.

The internal Cl^- pool rose to a greater relative extent (by about 50%) at 12 h, but returned to control values at 3 and 7 days (Fig. 2).

After transfer, K values for both ions exhibited substantial five- to eight-fold increases (Table 2). In general, the increases were larger for Cl^- than for Na^+, reaching over

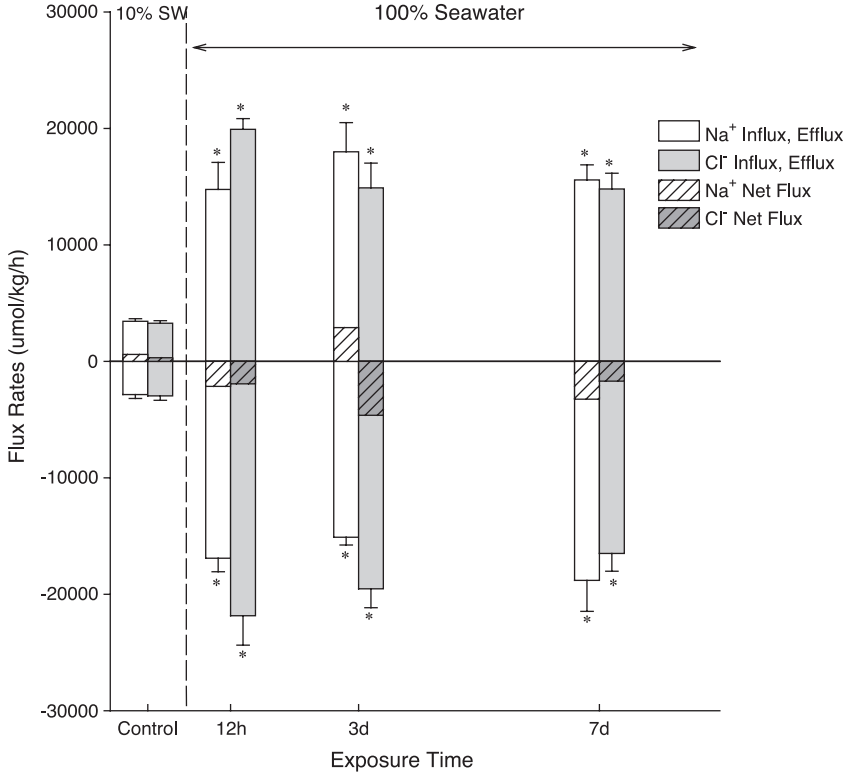

Fig. 3. Responses in killifish of unidirectional influx (upward bars) and efflux rates (downward bars) for Na^+ (clear bars) and Cl^- (shaded bars) after transfer from the control condition (10% seawater) to 100% seawater at time zero (dashed line). The hatched bars represent the arithmetic difference (net flux rates) between the mean influx (positive) and efflux (negative) rates. Means ± 1 S.E. $N=12$, 6, 6, and 6 for Na^+ and Cl^- influx, $N=22$, 6, 12, and 6 for Na^+ efflux, and $N=12$, 6, 12, and 11 for Cl^- efflux at control, 12-h, 3-day, and 7-day time points, respectively. Asterisks indicate significant difference ($P<0.05$) from control values. There were no significant differences between influx and efflux rates at the same time, or between Na^+ and Cl^- flux rates at the same time.

Table 4
Changes in K (the percentage per hour of the labeled internal pool which is lost to the media) and F (the fractional labeling of the whole-body pool) after transfer of killifish from 10% seawater to freshwater for various periods

	Na^+		Cl^-	
	K	F	K	F
10% Seawater (control)	4.36 ± 0.53 (22)	97.19 ± 0.33 (22)	6.68 ± 0.92 (12)	96.44 ± 0.34 (12)
Freshwater (12 h)	1.56 ± 0.17*(6)	98.90 ± 0.21 (6)	3.62 ± 0.61*,†(6)	98.15 ± 0.47 (6)
Freshwater (3 days)	2.43 ± 0.20* (12)	99.21 ± 0.13 (12)	3.04 ± 0.42* (12)	97.76 ± 0.31† (12)
Freshwater (7 days)	2.12 ± 0.27* (6)	99.62 ± 0.10 (6)	2.17 ± 0.28* (6)	99.25 ± 0.30* (6)

Means ± 1 S.E. (N).
* Significantly different ($P<0.05$) from comparable value in 10% seawater control group.
† Significantly different ($P<0.05$) from comparable Na^+ value.

50% h^{-1} for the former at 3 days, but were somewhat compensated by significant falls in F. Nevertheless, even at their lowest point (for Cl^- at 3 days), F values remained relatively high at >75% despite the intense exchanges occurring.

Unidirectional influx and efflux rates for Na^+ increased sharply from control values of 3000 μmol kg^{-1} h^{-1} in 10% seawater to around 15,000–18,000 μmol kg^{-1} h^{-1} after only 12 h in 100% seawater (Fig. 3). These values remained unchanged at 3 and 7 days, and there was never a significant difference between influx and efflux rate at the same time. Very similar changes were seen for unidirectional Cl^- flux rates, in both absolute magnitude and pattern (Fig. 3). There were no significant differences between influx and rates at the same time, or between comparable Na^+ and Cl^- flux rates at the same time. In general, the net NaCl balance was negative after transfer—i.e., efflux rates slightly exceeded influx rates (the one exception was for Na^+ at 3 days), but none of the differences were significant. Thus killifish appear to quickly re-establish ionic homeostasis after transfer to 100% seawater with approximately equimolar Na^+ and Cl^- flux rates.

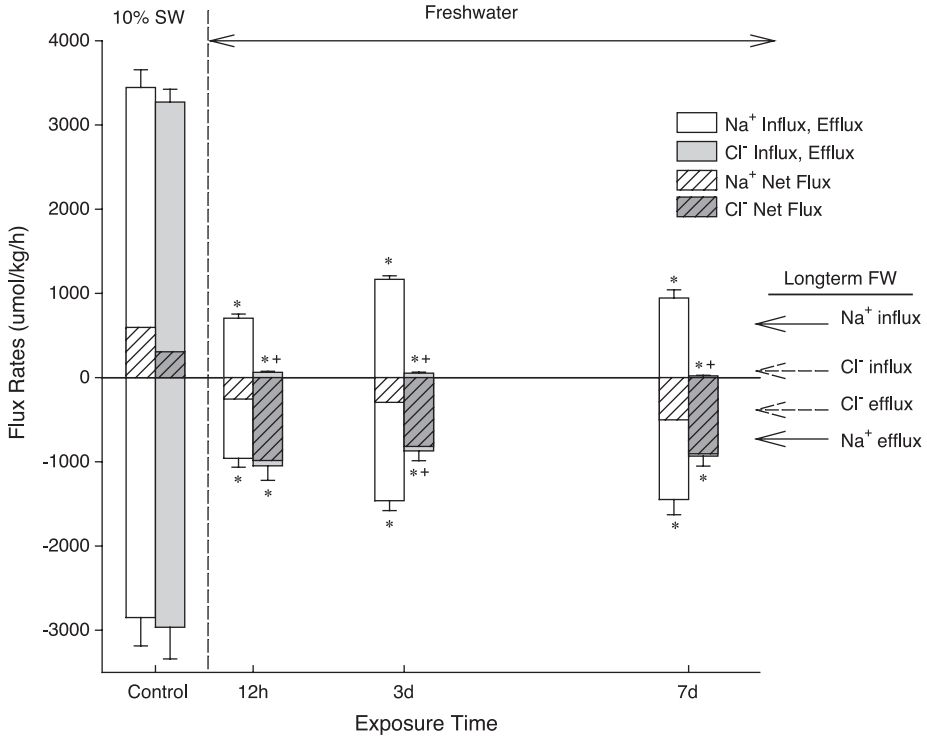

Fig. 5. Responses in killifish of unidirectional influx (upward bars) and efflux rates (downward bars) for Na^+ (clear bars) and Cl^- (shaded bars) after transfer from the control condition (10% seawater) to freshwater at time zero (dashed line). The hatched bars represent the arithmetic difference (net flux rates) between the mean influx (positive) and efflux (negative) rates. Means ± 1 S.E. $N=12$, 6, 6, and 6 for Na^+ and Cl^- influx, $N=22$, 6, 12, and 6 for Na^+ efflux, and $N=12$, 6, 12, and 6 for Cl^- efflux at control, 12-h, 3-day, and 7-day time points, respectively. Asterisks indicate significant difference ($P<0.05$) from control value. Crosses indicate significant difference ($P<0.05$) between Na^+ and Cl^- flux rates at the same time. At every time point in freshwater, Cl^- efflux rates were significantly greater than Cl^- influx rates. There were no individual differences between Na^+ influx and Na^+ efflux rates at the same times, but overall, Na^+ effluxes were significantly greater than Na^+ influxes in freshwater. The values of unidirectional Na^+ and Cl^- fluxes reported for killifish after long-term freshwater acclimation [35] are indicated by arrows on the right-hand side.

3.3. Transfer to freshwater

Transfer from 10% seawater to freshwater represented about a 98.5% drop in environmental Na^+ and Cl^- concentrations (Table 1). In contrast, the internal Na^+ pool of killifish fell by only 8–11% at 12 h and 3 days, and had returned to control values by 7 days (Fig. 4). Changes in the internal Cl^- pool were much larger, with declines of about 33% at both 12 h and 3 days. However, again, full recovery was seen by 7 days (Fig. 4).

At all times after transfer, K values for both ions were approximately halved (Table 4). In general, K values remained higher for Cl^- than for Na^+, but the difference was significant only at 12 h (3.6% h^{-1} versus 1.6% h^{-1}). F values remained close to 100% for both ions at all times in freshwater (Table 4).

In contrast to the responses after transfer to 100% seawater (cf. Fig. 3), unidirectional Na^+ and Cl^- flux rates exhibited very different patterns from each other after transfer to freshwater (Fig. 5). At 12 h, Na^+ influx rate had dropped by 80% to about 700 μmol kg^{-1} h^{-1}, followed by a slight recovery to around 1000 μmol kg^{-1} h^{-1} at 3 and 7 days. Unidirectional Na^+ efflux rates exhibited a similar trend, but with efflux being consistently greater than influx (by about 300 μmol kg^{-1} h^{-1}) at all times in freshwater. This difference was significant overall, but not at any one time. Unidirectional Cl^- influx virtually stopped after transfer to freshwater, falling to less than 2% (62 μmol kg^{-1} h^{-1}) of the control rate in 10% seawater (Fig. 5). There was no recovery thereafter; indeed at 7 days, the Cl^- influx rate was only 20 μmol kg^{-1} h^{-1}. Cl^- efflux, on the other hand, was stable at around −900 μmol kg^{-1} h^{-1} at all times after transfer, such that net Cl^- balance was highly negative. The differences between Cl^- influx and Cl^- efflux were significant at all times in freshwater, as were those between Cl^- influx and Na^+ influx. Cl^- efflux rates were similar to Na^+ efflux rates, though significantly smaller at 3 days.

Overall, these responses reveal a complete dissociation between Na^+ and Cl^- regulation after freshwater transfer. While ionic homeostasis, in terms of whole-body Na^+ and Cl^- pools was restored by 7 days (Fig. 4), net balance, especially for Cl^-, remained highly negative. In this regard, it is important to remember that the fish were fed during the experiment.

4. Discussion

4.1. Overview

The present study is the first to measure the unidirectional influx and efflux rates of both Na^+ and Cl^- in intact *F. heteroclitus* after transfer to both seawater and freshwater. Three main conclusions may be drawn. The first, and most important, is that in 100% seawater, the intact animal appears to behave qualitatively, but not quantitatively, like the opercular epithelial model so widely studied in the literature, whereas in freshwater, the intact animal and the opercular model appear to behave entirely differently. Secondly, unidirectional flux rates of both ions change rapidly (by 12 h) upon salinity transfer, stabilizing thereafter, at least through 7 days post-transfer. Lastly, the unidirectional flux measurements of the present study do not entirely agree with measurements made 30–40 years ago in the same species, probably because of methodological improvements. These points are dealt with in reverse order below.

4.2. Comparisons with earlier unidirectional flux measurements

The unidirectional influx and efflux rates of Na^+ and Cl^- in 100% seawater were in balance at around 15,000–18,000 μmol kg^{-1} h^{-1} in this study (Fig. 3). The most detailed and careful early study appears to be that of Potts and Evans [3], who reported a similar Na^+ influx rate (20,500 μmol kg^{-1} h^{-1}) but a Na^+ efflux rate of −33,600 μmol kg^{-1} h^{-1} and a Cl^- efflux rate of −53,600 μmol kg^{-1} h^{-1}; Cl^- influx rate was not measured. Potts and Evans [3] recognized that the discrepancy of Na^+ influx versus Na^+ efflux was unlikely for a fish "in equilibrium" and attributed it to radioisotopic equilibration problems; however, they thought the Na^+ influx value was probably too low, and did not comment on the Na^+ efflux versus Cl^- efflux discrepancy. Notably, both their K values and chemically measured internal Na^+ and Cl^- pools were substantially greater than those of the present study. Pic [18] also reported high efflux rates for both Cl^- (−37,700 μmol kg^{-1} h^{-1} in one series, −26,500 μmol kg^{-1} h^{-1} in another series) and Na^+ (about −45,000 μmol kg^{-1} h^{-1} in both series), though here the discrepancy was reversed. Methods were not fully described, and influx rates were not determined. However, Motais et al. [17], again without methodological detail, reported a Na^+ efflux rate of about −24,000 μmol kg^{-1} h^{-1}. More convincingly, Maetz et al. [10] reported mean Na^+ influx (18,500 μmol kg^{-1} h^{-1}) and efflux rates (−20,200 μmol kg^{-1} h^{-1}) which were close to balance and much closer to both the present data and to the lower estimate of Potts and Evans [3], though the sample size was small ($N=3$) and the efflux methods rather different from those of our study. Notably, their K values and internal Na^+ pools were also much closer to the present observations (Table 3, Fig. 2). Cl^- exchanges were not measured.

Therefore, in general the present unidirectional flux measurements for intact killifish in 100% seawater (Fig. 3) differ from earlier determinations by being lower, in balance (i.e., influx = efflux), and approximately equimolar for Na^+ and Cl^-. None of the earlier measurements appear to have taken F into account (cf. Table 3), and therefore may have overestimated efflux, particularly Cl^- efflux, but by a relatively small percentage (<30%). More importantly, all

of the earlier studies reported that "stress" could result in greatly elevated and/or more variable flux rates, associated with elevations of *K* values and internal pools. We noted the same in preliminary trials, and our methods were designed to minimize stress as far as practical.

We are aware of no previous unidirectional flux measurements for killifish in 10% seawater, where our Na^+ and Cl^- flux rates were around 3000 μmol kg^{-1} h^{-1}, again close to balance and equimolar. In killifish exposed to freshwater for 7 days, we recorded Na^+ influx and Na^+ efflux rates of around 1000–1500 μmol kg^{-1} h^{-1}, and similar Cl^- efflux rates, whereas Cl^- influx was negligible (Fig. 5). These influx data are in close agreement with more recent measurements of Na^+ and Cl^- influx rates by indirect methods in long-term freshwater-acclimated killifish [35,36]. Using methods similar to the present study, Potts and Evans [3,9] reported that Na^+ influx rate was about 600 μmol kg^{-1} h^{-1} while Na^+ and Cl^- efflux rates were around −800–1000 μmol kg^{-1} h^{-1}. Considering that Na^+ influx will be critically dependent on the exact freshwater Na^+ concentration (cf. Fig. 6, discussed below), agreement is again reasonable. The whole-body Na^+ and Cl^- pool sizes and *K* values in freshwater killifish reported by Potts and Evans [3,9] were also close to the present determinations (Table 4, Fig. 4). However, Maetz et al. [10], using indirect methods, recorded Na^+ influx and efflux rates of only about 20% of the present values in killifish kept for 2–5 weeks at the same freshwater Na^+ level as used in the present study; the reason for this discrepancy may be the much higher Ca^{2+} concentration (2 mM) in the freshwater used by Maetz et al. [10] (cf. Table 1).

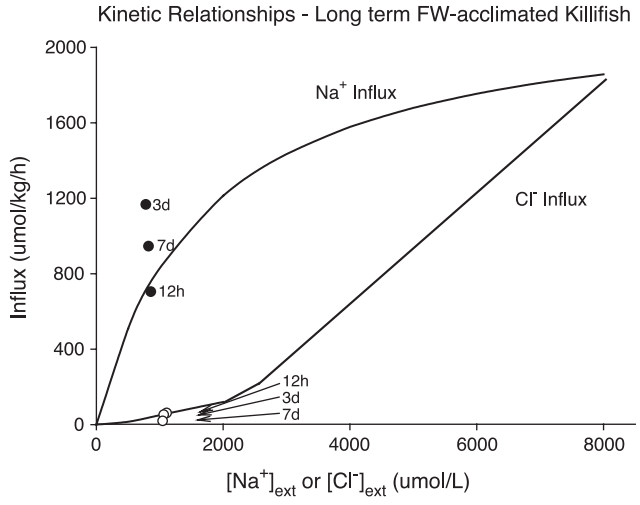

Fig. 6. The mean Na^+ (closed circles) and Cl^- influx rates (open circles) in killifish at 12 h, 3 days, and 7 days after transfer to freshwater plotted on the kinetic curves reported by Ref. [35] (redrawn from that source) for long-term freshwater-acclimated killifish. These curves represent the empirically determined relationships between influx rates and environmental sodium ($[Na^+]_{ext}$) or chloride ($[Cl^-]_{ext}$) levels, which followed a standard Michaelis–Menten relationship for Na^+ but not for Cl^-.

4.3. The responses of unidirectional fluxes to salinity transfers

The flux responses to abrupt salinity transfer were rapid, and approximately stable from 12 h through 7 days (Figs. 3 and 5). However, as discussed subsequently, it is still possible that subcomponents of the fluxes (e.g., gills versus gut in 100% seawater, gills versus kidney in freshwater) may have been changing over this period. Furthermore, in 100% seawater, the flux rates were so high that it would not be possible to resolve a small but important difference between unidirectional influx and efflux. For example, there were no significant differences between Cl^- influx and efflux rates in 100% seawater (Fig. 3); however, if efflux slightly exceeded influx to produce a net flux rate of only −500 μmol kg^{-1} h^{-1} from 12 h through 3 days, this would have been sufficient to account for the return of the internal Cl^- pool to the control level (Fig. 2) over this period.

Previous studies [34,39,51] indicate that after killifish are transferred to 100% seawater, plasma Na^+, Cl^-, and/or osmolality take about 3 days to return to normal. Our measurements of whole-body Na^+ and Cl^- pools (Fig. 2) are in accord with this conclusion. To our knowledge, there have been no previous flux measurements on intact killifish upon transfer to 100% seawater. However, in contrast to the rapid activation of Na^+ and Cl^- efflux seen in our experiments (Fig. 3), several studies have demonstrated that activation of secondarily active Cl^- secretion across the opercular epithelium takes at least 24–48 h for any change to occur, and transport increases progressively for several days thereafter [39,52]. The possible explanations for this difference may be several fold. Most importantly, the opercular epithelia were taken from fish initially acclimated to true freshwater, not 10% seawater, so the transition to 100% seawater was a larger one than in the present studies, and the preparations may have started with a different population of MRCs (see below). Secondly, in these experiments [39,52], the serosal saline was held constant, whereas in vivo, internal Na^+ and Cl^- levels clearly rise after transfer to 100% seawater (e.g., Fig. 2). It is now known that the associated rise in osmolality is a critical factor in the activation of Cl^- secretion by the opercular epithelium [51,52,55]. Thirdly, the isolated opercular epithelium may lack key hormonal and/or neural controls. Certainly, in the gills, mRNA for the apical Cl^- channel increases by 8 h and reaches a maximum by 24 h [53], but in the opercular epithelium, immunofluorescence studies indicate that the expression and targeting of the Cl^- channel and the Na^+K^+, $2Cl^-$ co-transporter (two key elements of secondarily active Cl^- secretion) change much more slowly [54]. In several reports, gill Na^+K^+ATPase activity (another key component) increases rapidly (0.5–3 h) after transfer, probably by a non-genomic mechanism [15,16], a change which precedes a secondary rise at 24–72 h [16,34], though the literature is inconsistent on this point (e.g., [39]). Na^+K^+ATPase

responses to salinity transfer do not appear to have been measured in the opercular epithelium.

After transfer to freshwater, whole-body Na^+ and Cl^- pools remained depressed through 3 days, but were corrected at 7 days (Fig. 4). This contrasts with the report of Marshall et al. [55] that complete recovery of plasma Na^+ occurs within 12 h but agrees with the slower time course (>2 days) reported by Jacob and Taylor [34]; regardless, it is likely that the tissue pools tend to buffer the plasma concentrations. Marked reductions in the unidirectional flux rates of Na^+ and Cl^- were essentially complete by 12 h, although net balance remained negative for both ions, especially Cl^-, through 7 days (Fig. 5). Therefore, the recovery of whole-body Na^+ and Cl^- pools was very probably attributable to acquisition of electrolytes from food; indeed, we found that feeding was essential to keep the animals healthy in freshwater.

Wood and Marshall [2] synthesized a number of early flux studies on intact killifish [3,10,17], which demonstrated that after transfer to freshwater, unidirectional Na^+ efflux dropped in two phases, a small, almost instantaneous (<0.5 h) fall reflecting the reduction in external Na^+ concentration (“exchange diffusion effect”, phase I) and a larger but slower fall (phase II) complete by 12 h, and likely of humoral origin. Pic [18] reported a relatively larger reduction in Cl^- efflux than Na^+ efflux during the slower phase. These observations agree with our data (Fig. 5). At least in the opercular epithelium, α-adrenergic stimulation via neural or blood-borne sympathetic agents could contribute to the rapid effects, acting to turn off secondarily active Cl^- secretion, and thereby Na^+ secretion [41,56,57]. In addition, retraction of the MRCs in response to hypotonic shock with consequent covering by the pavement cells may also play a role in the rapid reduction of Na^+ efflux in the opercular epithelium [58]. Prolactin also probably plays an important role, particularly in the slower response (see Introduction). Maetz et al. [10] presented evidence, at least for Na^+, that there was a further reduction in efflux, beyond the phase II effect, seen only after long-term acclimation to freshwater. Based on the Na^+ and Cl^- efflux rates measured by Patrick et al. [35] in long-term freshwater-acclimated killifish (arrows in Fig. 5), efflux rates would be reduced eventually by >50% from the day 7 values to about -700 and -300 μmol kg^{-1} h^{-1} for Na^+ and Cl^- efflux, respectively.

However, the reduction of efflux is only part of the story of freshwater adaptation; influxes from the dilute external concentration must also be activated. As discussed subsequently, the pattern of ion transport in the opercular epithelium in freshwater differs greatly from that in the whole animal. Nevertheless, it is interesting that activation of the freshwater transport pattern in the intact killifish appears to be almost complete by 12 h, whereas in the opercular epithelium, this process takes >48 h [43], perhaps because of the factors discussed earlier which are different in opercular epithelium studies (different starting media, constant serosal osmolality, lack of neural/hormonal input). The unidirectional Na^+ and Cl^- influx rates at 12 h in the whole animal (Fig. 5) were essentially identical to those measured by Patrick et al. [35] in long-term freshwater-acclimated killifish (arrows in Fig. 5). Maetz et al. [10], who measured only Na^+ influx, reached the same conclusion, that the adjustment of influx was complete within a few hours after transfer to freshwater. Patrick et al. [35] demonstrated that Na^+ influx in long-term freshwater-acclimated killifish exhibited classical Michaelis–Menten kinetics as a function of the external Na^+ concentration (Fig. 6). The present Na^+ influx data at 12 h to 7 days are in accord with this relationship. Interestingly, Patrick et al. [35] also reported the curious relationship depicted in Fig. 6 whereby measurable Cl^- influx did not occur until external Cl^- concentration surpassed 2000 μmol l^{-1}. Clearly, the very small, almost undetectable rates of Cl^- influx recorded at 12 h to 7 days from freshwater with $[Cl^-]_{ext}$ at about 1000 μmol l^{-1} agree with this relationship.

4.4. Fluxes in the intact killifish versus the opercular epithelium

Our results indicate that caution should be used in considering the opercular epithelium, which has proven to be such a useful model under “Ussing conditions”, as a model for the intact gill of *F. heteroclitus* under “real world conditions” (cf. Fig. 1). Firstly, there is the problem of much slower time course of changes of the active components of opercular epithelial transport, to transfers in both directions. As discussed earlier, these may have been due, at least in part, to methodological problems, as well as to the fact that neural and humoral agents that support rapid responses in the whole animal are lost when the preparation is mounted in vitro.

Secondly, the present flux data provide qualitative but not quantitative support for the transport behavior of the opercular epithelial model mounted at open circuit with mucosal seawater and serosal saline (Fig. 1C). Under these conditions, unidirectional fluxes of both Na^+ and Cl^- across the epithelium are the same in both directions (i.e., the observed flux ratio is not significantly different from 1.0), so there is no net extrusion of either ion [40]. Ostensibly the same pattern is seen in the whole organism (Fig. 3). Inasmuch as our data indicate equimolar effluxes of Na^+ and Cl^-, in contrast to earlier in vivo reports of Cl^- efflux >Na^+ efflux [3] or Na^+ efflux >Cl^- efflux [18], they support the co-transport of Cl^- and Na^+ envisaged by the Silva model [33], and seen in the opercular epithelium [40]. However, it must be remembered that in 100% seawater, a substantial portion of the influx in vivo occurs by drinking. Drinking rate estimates for killifish in 100% seawater vary from 9.4 to 23.5 ml kg^{-1} h^{-1} [3,13], so 5000–12000 μmol kg^{-1} h^{-1} of the measured Na^+ and Cl^- influx rates (15,000–18,000 μmol kg^{-1} h^{-1}) would be across the gut, rather than across the gills. In contrast, less than -1000

μmol kg^{-1} h^{-1} of Na^+ and Cl^- efflux would pass out via the urine [4–6], so clearly the gills are achieving net NaCl excretion in vivo. Likely, in the face of the strong electrical and chemical gradients opposing outward Cl^- movement across the opercular epithelium under "real world conditions", Cl^- transport from serosa to mucosa is less efficient in vitro than in vivo, and/or diffusive influxes are artificially elevated by damage when the epithelium is removed from the fish.

Finally, our flux data provide no support for the transport behavior of the opercular epithelial model mounted at open circuit with mucosal freshwater and serosal saline (Fig. 1A). In vitro, the opercular epithelium exhibits only tiny influxes of Na^+ and Cl^-, amounting to about 10% of the efflux rates, so net balance is highly negative; only Cl^- uptake satisfies the Ussing [42] criterion for active transport [2,43,44]. However, in vivo, the intact killifish in freshwater exhibits a vigorous Na^+ influx of comparable magnitude to Na^+ efflux, but negligible Cl^- influx (Figs. 5 and 6). Assuming an opercular epithelial area of about 200 cm^2 kg^{-1} for killifish of the size used here [40], the small Cl^- influx in vivo (20–50 μmol kg^{-1} h^{-1}) could be entirely accounted for by the opercular epithelium! By way of contrast, the opercular influx of Na^+ could account for less than 5% of the Na^+ influx measured in the whole animal in vivo. This raises two interesting possibilities. Firstly, the opercular epithelium in freshwater may behave as a fundamentally different organ from the gills, accounting for all of the very slight Cl^- uptake which occurs, as well as the major portion of Ca^{2+} uptake (see Ref. [59] for calculations). Secondly, since the gills of freshwater killifish are rich in MRCs yet no Cl^- uptake is occurring, perhaps the MRCs are performing Na^+ uptake. This idea runs contrary to present views that gill MRCs perform Cl^- and Ca^{2+} uptake in freshwater, while both pavement cells and MRCs may contribute to Na^+ uptake [45,46]. However, as detailed by Patrick and Wood [36], ion and linked acid–base transport in the freshwater killifish certainly do not adhere to "standard" models. Indeed, the recent finding by Katoh et al. [60] that vacuolar H^+-ATPase is localized to the basolateral rather than the apical membranes of MRCs in freshwater killifish suggests that this species operates a fundamentally different transport system from that of most other fish living in freshwater that have been studied to date.

4.5. Concluding remarks

The very rapid flux responses of the intact killifish raise interesting questions as to how gill transport processes can be fundamentally reorganized in less than 12 h. Can the same cells do two very different tasks in freshwater versus seawater, or is rapid cell renewal necessary? The phenomenon of two very different morphological types of MRCs in the gills of *F. heteroclitus* in freshwater versus seawater is well documented. However, it remains unclear whether these are "re-engineered" versions of the same cell, or different cells. The seawater-type MRCs are reported to persist down to a salinity of only about 1% seawater [20,26,60–63]. Future flux studies should focus on the time course of responses within this 12-h window, on the morphological responses of the gills, and on the mitotic rates of these cells. As the seawater-type MRCs persist down to 1% seawater, we might predict that transfer from 10% to 100% seawater would elicit little cell renewal, whereas transfer from 10% seawater to freshwater might elicit rapid renewal of the MRCs. Preliminary immunocytochemical results using the BRDU technique to detect mitotic events [64] are in accord with this hypothesis (P. Laurent and C.M. Wood, unpublished data).

Acknowledgments

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