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Rapid regulation of Na^+ and Cl^- flux rates in killifish after acute salinity challenge

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A R T I C L E I N F O

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The common killifish lives in tidal marshes and estuaries where it encounters hourly fluctuations in salinity. Radiotracers (^{22}Na, ^{36}Cl) were employed to examine its ability to rapidly adjust unidirectional influx and efflux rates of Na^+ and Cl^- in the 8-h period after acute transfer from the acclimation medium (10% sea water, SW) to either 100% SW or fresh water (FW). Flux rates were measured over 0.5–1.0 h intervals, and compared with stable rates measured at 12 h–7 d post-transfer under identical conditions in an earlier study. After transfer to 100% SW, Na^+ and Cl^- influx rates increased 7-fold with large overshoots in the first hour, but thereafter decreased by 35–50%, reaching levels at 1–2 h not significantly different from 12 h–7 d values. Na^+ and Cl^- efflux rates increased by 40–100% in the first hour post-transfer but thereafter rose gradually, reaching the 12 h–7 d values only by 5.0–5.5 h for Na^+, and remaining significantly lower even at 7.5–8.0 h for Cl^-. After transfer to FW, Na and Cl influx rates dropped by 97–99% in the first hour. Na^+ influx recovered to a level equal to about 40% of the 12 h–7 d rate by 1–2 h, thereafter not changing through 8 h. Cl^- influx exhibited no recovery, and remained negligible even at 12 h–7 d. Na^+ and Cl^- efflux rates decreased by 26–27% in the first 0.5 h after transfer to FW, and reached levels comparable to the 12 h–7 d values by 1.0–1.5 h for Na^+ and 2.5–3.0 h for Cl^- Overall, there were two clear trends. The passive components (Na^+ and Cl^- influxes in 100% SW, Na^+ and Cl^- effluxes in FW) were regulated more rapidly than the active fluxes (Na^+ and Cl^- effluxes in 100% SW, Na^+ influx in FW), and Na^+ balance was regulated more quickly and precisely than Cl^- balance, for transfers in both directions. Rapid adjustments of both active and passive fluxes suggest that they involve direct responses of channels and tight junctions to salinity changes, as well as post-translational mechanisms of transporter regulation. These short-term responses are effective in changing ion flux rates long before “structural” re-organizations of the gill transport machinery occur by genomic mechanisms. This strategy, combined with an ability to tolerate internal ionic disturbances, is of obvious benefit in a species which routinely encounters hourly salinity fluctuations in its natural environment.

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1. Introduction

The common killifish or mummichog (*Fundulus heteroclitus*) is renowned for its euryhalinity, tolerating abrupt sea water (SW) to fresh water (FW) transfer, and *vice versa* (Griffith, 1974). This species, which lives in marshes and estuaries on the Atlantic coast of North America, moves in and out on the tide to feed, and undoubtedly experiences daily salinity challenges (Marshall, 2003). One somewhat unusual aspect of its physiology is that the killifish exhibits a negligible capacity to take up Cl^- at its gills in FW, despite a vigorous Na^+ uptake mechanism (Patrick et al., 1997; Patrick and Wood, 1999; Wood and Marshall, 1994); Cl^- must therefore be taken up from the diet via the intestine when the fish resides in FW (Scott et al., 2006; Wood et al., 2010). The only other euryhaline fish known to exhibit this characteristic in FW is the eel, *Anguilla sp.* (Grosell et al., 2000; Kirsch, 1972). In the FW-acclimated killifish, this trait appears to be associated with the presence of “cuboidal cells” (putative Na^+-transporting cells) in the gill epithelium, different from the standard “chloride cells” or mitochondria-rich cells found in the gills of most FW teleosts (Laurent et al., 2006).

Much of our work on the killifish in the past decade has focussed on the changes in ion fluxes, gill morphology, enzyme activities, cortisol, drinking rate, intestinal function, and associated molecular responses which occur after the fish are transferred from brackish water to either FW (moderately hard Hamilton water from Lake Ontario) or 100% SW (Laurent et al., 2006; Scott et al., 2005, 2006, 2008; Wood and Laurent, 2003). In these studies, the first sampling point was at 12 h after transfer. In general, massive changes in unidirectional Na^+ and Cl^- flux rates (decreases after transfer to FW, increases after transfer to SW) were complete by 12 h with negligible further changes at 3 d and 7 d post-transfer. In contrast, enzymatic, endocrine, morphological, intestinal, and molecular responses appeared to be still in transition at 12 h, especially after transfer to FW. Many other studies using different salinity transfer protocols or different FW qualities have reached similar conclusions (Katoh and Kaneko, 2003; Mancera and McCormick, 2000; Marshall et al.,

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1999; Scott et al., 2004a,b; Scott and Schulte, 2005; Shaw et al., 2007) — i.e. that a variety of compensations were still in transition for at least several days post transfer.

These observations raised the possibility that the killifish may recruit very rapid non-genomic mechanisms to adjust ion flux rates almost immediately, long before systemic and molecular mechanisms of homeostasis are implemented to deal with longer term salinity acclimation. Such a strategy would be useful for a species subject to daily, even hourly, salinity fluctuations in its environment. This idea is supported by our recent finding that transepithelial potential (TEP) across the gills changes almost instantaneously in a manner which would be adaptive for branchial Na^+ balance when killifish are moved acutely between salinities, and that these responses are then further adjusted over the following 24 h (Wood and Grosell, 2008, 2009). It is also supported by measurements of rapid changes in Na^+ influx and efflux rates in killifish subjected to a progressive salinity increase over 6 h (Prodocimo et al., 2007). Furthermore, several early Na^+ and Cl^- flux studies on *F. heteroclitus* all reported a small instantaneous drop in unidirectional efflux rates (phase I), followed by a larger progressive drop (phase II) which started at about 30 min after transfer from SW to FW (Maetz et al., 1967; Motais et al., 1966; Pic, 1978; Potts and Evans, 1967); Wood and Marshall (1994) provide a summary diagram.

With this background in mind, the focus of the present study was on the rapid changes in the unidirectional influx and efflux rates of Na^+ and Cl^- that occur after killifish are transferred from brackish water (10% SW) to either 100% SW or FW (moderately hard Hamilton water from Lake Ontario). Techniques for radiotracer flux measurements were based on those developed in an earlier study (Wood and Laurent, 2003) of longer-term responses (12 h, 3 d, 7 d), and were appropriately modified so as to measure fluxes over 0.5–1 h intervals in the first 8 h after transfer. The data could then be compared with these earlier longer-term measurements (Wood and Laurent, 2003). Our specific hypothesis was that killifish would exhibit almost instantaneous changes (i.e. first 0.5–2 h) in unidirectional flux rates that would be of adaptive significance, and that these would be followed by further progressive changes so as to become close to the 12 h—7 d flux rates by 8 h post-transfer.

2. Materials and methods

2.1. Experimental animals

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

Common killifish of the northern strain (*Fundulus heteroclitus macrolepidotus*; 3–8 g) were collected by beach seine from a brackish estuary near Antigonish, Nova Scotia, Canada. At McMaster University, they were held for several months prior to experiments in 500-L fiberglass tanks containing recirculated, charcoal-filtered 10% SW 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, U.S.A) and 50% frozen brine shrimp (San Francisco Bay Brand, Newark, CA, U.S.A.). Fish intended for experiments were removed from the holding tank before the morning feeding, so were fasted for slightly more than 24 h prior to flux measurements. Each fish was placed in an individual polyethylene chamber containing 250 ml of 10% SW, and allowed to settle for 2 h prior to experimentation. Each chamber was fitted with a lid, an aeration line, and a sampling port.

2.2. General radiotracer methods

The ionic composition (means and ranges) of the 10% SW, 100% SW, and FW used in these experiments are summarized in Table 1. Unidirectional flux rates were measured using radiotracers (^{22}Na, ^{36}Cl, as NaCl, NEN-Dupont, Boston, MA, U.S.A.) by the methods outlined below. All approaches used were designed to meet the criteria that fluxes could be measured reliably over intervals of no more than 1 h, and that the specific activity (SA: the ratio of radioisotopic to total Na^+ or Cl^-) in the compartment (fish or external water) in which the appearance of radioactivity was being monitored remained less than 10% of the SA in the compartment (fish or water) from which the radioactivity originated (Kirschner, 1970). This thereby avoided the problem of recycling or "backflux" of the radiotracer. In experiments where it was necessary to load the fish with radiotracer prior to flux measurements, this was done in one of two ways. The first was to incubate the fish for 24 h, in batches of 12, in 1 L of 10% SW (the acclimation medium) spiked with 60 µCi of ^{22}Na or ^{36}Cl. The fish were then quickly rinsed with non-radioactive 10% SW, then transferred to individual polyethylene chambers containing 250 ml of 10% SW. The second method was to inject radioisotope intraperitoneally into the fish (0.25 $\mu Ci\ g^{-1}$ in 2.5 $\mu l\ g^{-1}$ of Cortland saline (Wolf, 1963). This was done using a special 50 µl Hamilton gas-tight syringe, modified with a very short (1 cm) fixed Huber point needle. Our earlier study (Wood and Laurent, 2003) demonstrated that this was less stressful if done quickly without anesthesia, that intraperitoneal injections yielded more uniform data than intravenous injections, and that a 40-min equilibration period was adequate prior to the start of flux measurements. In that earlier study we validated that the two approaches yielded identical unidirectional Na^+ efflux data in 10% SW (Wood and Laurent, 2003).

2.2.1. Unidirectional influx determinations

In 10% and 100% SW, less than 2% of the total Na^+ or Cl^- is inside the fish, so it is impossible to measure influx rates by disappearance of ^{22}Na or ^{36}Cl from the external water. Appearance of radiotracer in the fish is the technique of choice, as long as the problem of radiotracer cycling can be avoided. As worked out in the earlier study (Wood and Laurent, 2003), a flux period of 0.5 h satisfied this criterion. Therefore, after the 2-h settling period, either ^{22}Na (1 µCi) or ^{36}Cl (2 µCi) were added to the external water, and quickly mixed. Duplicate water samples (2×5 ml) were taken at the start and end of the flux period for radioactivity and total Na^+ or Cl^- determinations. The fish was then quickly rinsed in non-radioactive media containing a terminal amount of neutralized MS-222 (0.5 $g\ L^{-1}$), blotted, weighed, and then processed for measurement of whole body radioactivity (^{22}Na or ^{36}Cl). Unidirectional influx rates (J_{in}) were calculated as:

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

where $\Sigma_{(\text{cpm in fish})}$ 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).

This technique was used to measure J_{in}^{Na} and J_{in}^{Cl} in 10% SW and at 0–1 h, 1–2 h, 3–4 h, and 7–8 h after transfer to 100% SW. In each case, the 0.5-h flux period was in the middle of the hour. A minimum of 10 animals was used at each time.

In FW, about 50% of the total Na^+ is inside the fish, so ^{22}Na disappearing from the water is diluted by a large internal "cold" Na^+ pool.

Table 1
Mean and range of ion concentrations (mmol L^{-1}) in the media used in the rapid transfer experiment.

	10% SW	100% SW	FW
Na^+	48 (41–68)	502 (477–535)	0.78 (0.68–1.02)
Cl^-	61 (41–74)	561 (528–599)	0.95 (0.70–1.19)
Ca^{2+}	1.4 (1.2–1.6)	10.6 (8.4–12.2)	0.91 (0.79–1.49)
Mg^{2+}	5.1 (4.8–5.6)	52 (47–57)	0.38 (0.28–0.52)

Note: N>80 for Na^+ and Cl; N=5–12 for Ca^{2+} and Mg^{2+}.

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In theory, J_{in} can be measured by monitoring disappearance of radioactivity from the water or appearance of radioactivity in the fish. However in practical terms, the former is easier, and allows sequential measurements on the same animal for up to 8 h before the 10% SA ratio criterion is reached. This approach was therefore employed to measure J_{in}^{Na}.

The fish was transferred to 250 ml of FW to which ^{22}Na (4 μCi) had been added. Duplicate water samples (2×5 ml) were taken at 0 h, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, and 8 h for radioactivity and total Na^+ determinations. In each 1-h period, the unidirectional influx rate of Na^+ was calculated as:

$$J_{in}^{Na} = \frac{(cpm_i - cpm_f)\ x\ V}{\text{Mean } SA_{ext}\ x\ T\ x\ W} \quad (2)$$

where cpmi and cpmf are the water ^{22}Na radioactivities (per ml) at the start and end of each 1-h period respectively, V is the starting volume (ml) in each period, and mean SA_{ext} is the mean external specific activity of Na^+ (in cpm μmol^{-1}) over the same 1-h period.

In preliminary experiments, we found that mean J_{in}^{Na} values measured by this "disappearance" method were identical to those measured by appearance of ^{22}Na in the fish. Although the variance was slightly greater, the disappearance method offered the great advantage of continuous sequential measurements over 1-h periods on each fish for up to 8 h post-transfer. A total of 10 animals were used.

As the uptake of Cl^- is extremely low in FW, the disappearance method could not reliably detect J_{in}^{Cl}. Instead, the appearance method was used (Eq. (1)) using 4 μCi ^{36}Cl added to 250 ml FW for separate determinations over 1-h periods at 0–1 h, 3–4 h, and 7–8 h post-transfer. A minimum of 6 fish were used at each time.

2.2.2. Unidirectional efflux determinations

Under all conditions (10% SW, 100% SW, FW), unidirectional efflux rates (J_{out}) of Na^+ and Cl^- were measured by monitoring the appearance of radiotracers into the external water at 0.5-h intervals, allowing sequential measurements on the same fish. However, as outlined below, the method of prior loading of the fish with radiotracer varied for ^{22}Na *versus* ^{36}Cl. Duplicate water samples (2×5 ml) for radioactivity determination were taken at the start and end of each flux period. At the end of the experiment, the fish was quickly rinsed in non-radioactive media containing a terminal amount of neutralized MS-222 (0.5 g L^{-1}), blotted, weighed, and then processed for measurement of whole body radioactivity (^{22}Na or ^{36}Cl).

The 24-h pre-incubation method was used for loading ^{22}Na into the killifish for all efflux measurements. Unidirectional Na^+ efflux rates (J_{out}^{Na}) were measured for 5×0.5-h periods in 10% SW (i.e. a 2.5 h pre-transfer interval), followed by 16×0.5-h periods (i.e. 8 h) after transfer to 100% SW or FW. Values for the 5 periods in 10% SW did not vary significantly, and therefore have been presented as means. By this method, J_{out}^{Na} could be monitored continuously in individual fish for the entire experimental period. A total of 16 fish were used in each series.

In preliminary experiments, we found that the 24-h pre-incubation method with ^{36}Cl did not work well for unidirectional Cl^- efflux measurements. The problem encountered was that because the internal radiolabeled pool is about 40% smaller for Cl^- than for Na^+, yet the exchange rate of the pool is about 50% higher, the fish quickly "run out" of ^{36}Cl during the efflux measurements. The total working period was limited to about 5 h before precision became unsatisfactory, and there was no mechanism for loading fish again at this time.

Therefore we chose to load the fish with ^{36}Cl by the injection method, which allowed us to overlap groups to cover the entire experimental period. Two batches of fish (N=9–10) were run for each of the Cl^- efflux series — after transfer to 100% SW, and after transfer to FW. In each case, the first batch covered 5×0.5-h periods in 10% SW (i.e. a 2.5-h control interval, with values again averaged) followed by 7×0.5-h periods after transfer to 100% SW or FW (i.e. the first 3.5 h post-transfer). The second batch was injected at 1.5–2 h post-transfer and provided flux data for 11×0.5 h periods from 2.5 h to 8 h post-transfer. The two groups overlapped at the 2.5–3.0 and 3.0–3.5 h determinations. The J_{out}^{Cl} data were averaged at these times as they were not significantly different between the batches.

As more than 98% of the total Na^+ and Cl^- was in the external water when the fish were in 10% or 100% SW, dilution by this large "cold" pool kept SA_{ext} suitably low in both the ^{22}Na and ^{36}Cl efflux expriments. However, after transfer to FW, it was necessary to renew the external water at 2-h intervals to keep the SA_{ext} below 10% of SA_{int}.

In these experiments, unidirectional efflux rates (J_{out}) were calculated as:

$$J_{out} = \frac{= (cpm_f - cpm_i)\ x\ V}{\text{Mean } SA_{int}\ x\ T\ x\ W} \quad (3)$$

where cpm_f and cpm_i are the water ^{22}Na or ^{36}Cl radioactivities (per ml) at the end and start of each 0.5-h period respectively, V is the starting volume (ml) in each period, and mean SA_{int} is the mean internal specific activity of Na^+ or Cl^- (in cpm μmol^{-1}) over the same 0.5-h period. In order to calculate mean SA_{int} for each flux period, it was necessary to know the total amount of radioactivity (cpm) in the fish at each time, as well as the total internal pool (μmol) of Na^+ or Cl^- that was labeled in the fish . The former could be precisely calculated by first adding the measured total amount (cpm) of ^{22}Na or ^{36}Cl effluxed over the entire experiment to the measured amount remaining in the whole body at the end of the experiment, so as to yield the total radioactivity (cpm) in the fish at the start. Then by subtracting the amount lost in each period, it was possible to back-calculate the total amount (cpm) remaining in the fish at the start and end of each flux period. The total internal pools of Na^+ and Cl^- (μmol) that were radiolabelled were measured in 10% SW and at 12 h after transfer to FW or 100% SW under identical conditions in our earlier study (Wood and Laurent, 2003). For the purposes of calculating SA_{int} at each time in the present experiments, it was assumed that the pool size changed linearly with time after transfer. A sensitivity analysis using the alternate assumption of an exponential change with time illustrated that this introduced negligible error into the estimates of J_{out}.

2.2.3. Analytical techniques

^{22}Na radioactivities in water samples and fish bodies were measured by gamma counting using a Minaxi Autogamma 5000 counter (Packard Instruments, Downers Grove, IL, U.S.A). ^{36}Cl radioactivities in water samples and fish bodies were measured by scintillation counting using a Rackbeta 1217 liquid scintillation counter (LKB-Wallac, Turku, Finland). Water samples (5 ml) were counted using 10 ml ACS fluor (Amersham, Oakville, ON, Canada). Whole bodies were processed by placing the carcass in 4 volumes of 1 N HNO_3 in a sealed tube, and baking in an oven at 60 °C for 48 h with periodic vortexing. The digest was then cooled and centrifuged at 500 G for 5 min; 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. 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.

Na^+ concentrations in all water samples were measured by atomic absorption spectrophotometry (AAS; Varian Australia Model 220FS, Mississauga, ON, Canada). Cl^- concentrations in 10% SW and 100% SW samples were measured by coulometric titration using a CMT-10 chloridometer (Radiometer, Copenhagen, Denmark), and in FW samples by the colorimetric method of Zall et al. (1956). The same Radiometer NaCl certified standard was used for both Na^+ and Cl^- measurements.

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2.4. Statistics

Data have been expressed as means ± 1 SEM (N). The focus of this study was to evaluate how quickly unidirectional flux rates changed after transfer from 10% SW to either FW or SW. Therefore, each flux rate measurement post-transfer was compared in sequence back to the mean level in 10% SW using a paired or unpaired Student's two-tailed *t*-test as appropriate, so as to find the first time of significant change. Similarly, each flux rate measurement was compared to rates measured at 12 h–7 d post-transfer under identical conditions in our earlier investigation (Wood and Laurent, 2003) working in reverse sequence from 8 h backwards. As rates were not significantly different at 12 h, 3 d, or 7 d post-transfer in that study (with one exception — see Results), the comparison was with the mean of measurements at these three times. The goal was to find the first time post-transfer at which rates were not significantly different from the mean 12 h–7 d values. After this threshold was identified, additional comparisons of the same parameter after different times in 100% SW or FW were evaluated by ANOVA, followed by Dunnett's test. Comparisons between different fluxes at the same time were made by Student's unpaired *t*-test. A significance level of $P<0.05$ was used throughout.

3. Results

3.1. Transfer to 100% SW

3.1.1. Unidirectional influx rates

In the first hour after transfer, J_{in}^{Na} immediately increased approximately 7-fold from ~3450 μmol kg h^{-1} in 10% SW to ~25,100 μmol kg h^{-1} in 100% SW. This was a significant overshoot relative to the 12 h–7 d value of ~16,100 μmol kg h^{-1} (Fig. 1A). However in the second hour, J_{in}^{Na} decreased by almost 50%, and was no longer significantly different from the 12 h–7 d rate at 1–2 h, 3–4 h, or 7–8 h post-transfer, indicating a very rapid regulation.

J_{in}^{Cl} exhibited a very similar pattern of rapid regulation, with an immediate 7-fold increase from ~3300 μmol kg h^{-1} in 10% SW to ~24,200 μmol kg h^{-1} in 100% SW in the first hour after transfer, a significant overshoot relative to the 12 h–7 d value of ~16,500 μmol kg h^{-1} (Fig. 1B). This was followed by an immediate 35% decline in the second hour; these and subsequent rates (1–2 h, 3–4 h, and 7–8 h) of J_{in}^{Cl} were not significantly different from the 12 h–7 d value.

3.1.2. Unidirectional efflux rates

In 10% SW, J_{out}^{Na} and J_{out}^{Cl} were not significantly different from the respective values of J_{in}^{Na} and J_{in}^{Cl} (Fig. 2 *versus* Fig. 1), indicating that the fish were in approximate balance in their acclimation medium. In the first hour after transfer to 100% SW, J_{out}^{Na} increased significantly by about 40% (Fig. 2A), far less than the 7 fold increase in J_{in}^{Na} at this time (Fig. 1A). J_{out}^{Na} continued to increase in a hyperbolic pattern with time, reaching a value not significantly different from the 12 h–7 d rate (~16,950 μmol kg h^{-1}) at about 5.0–5.5 h post-transfer (Fig. 2A). This was far slower than adjustment of J_{in}^{Na}, which was complete by 1–2 h (Fig. 1A).

J_{out}^{Cl} was also regulated more slowly than J_{in}^{Cl}. J_{out}^{Cl} approximately doubled in the first hour after transfer to 100% SW, thereafter increasing progressively in a hyperbolic fashion (Fig. 2B). However, even by 7.5–8.0 h, the rate remained only about 65% of the 12 h–7 d rate (19,300 μmol kg h^{-1}), a highly significant difference. Clearly, unidirectional efflux rates of both Na^{+} and Cl- were adjusted more slowly than unidirectional influx rates.

3.2. Transfer to FW

3.2.1. Unidirectional influx rates

J_{in}^{Na} fell by more than 97% (to ~90 μmol kg h^{-1}) in the first hour after transfer from 10% SW to FW (Fig. 3A). In the second hour, J_{in}^{Na} increased almost 4-fold, but thereafter stabilized at about 400 μmol kg h^{-1} in sequential measurements through until 8 h. This was significantly less than the 12 h–7 d rate of ~940 μmol kg h^{-1}. However, it should be noted that the 12 h rate was only ~700 μmol kg h^{-1} whereas the 3 d and 7 d rates were 950–1170 μmol kg h^{-1} (Wood and Laurent, 2003), suggesting that the slow recovery of J_{in}^{Na} continued beyond 12 h.

For J_{in}^{Cl}, transfer from 10% SW to FW resulted in almost complete elimination, an immediate 99%+ reduction to 28 μmol kg h^{-1} (Fig. 3B). There was no recovery at 3–4 h, 7–8 h, or 12 h–7 d (45 μmol kg h^{-1}). Thus unidirectional Cl^{-} uptake rate remained at negligible levels in FW (Fig. 3A), only 5–10% of unidirectional Na^{+} uptake rate (Fig. 3A).

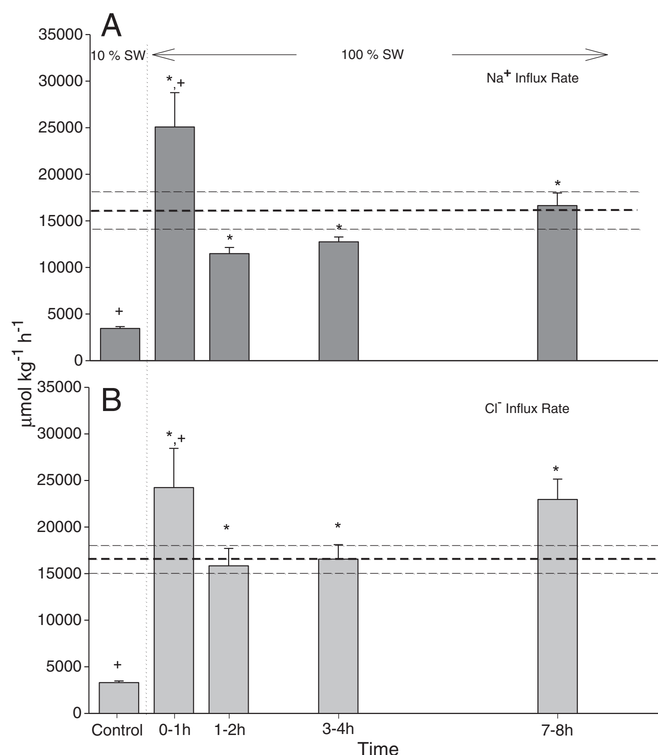

Fig. 1. The influence of transfer from 10% SW to 100% SW on (A) unidirectional sodium influx rate (J_{in}^{Na}) and (B) unidirectional chloride influx rate (J_{in}^{Cl}) in *Fundulus heteroclitus* during the first 8 h post-transfer. Means + 1 SEM (N = 10–12 for both J_{in}^{Na} and J_{in}^{Cl}) . The thick and thin dotted lines illustrate the means ± 1 SEM of the rates at 12 h, 3 d, and 7 d post-transfer (N = 18) reported by Wood and Laurent (2003). Asterisks indicate means which are significantly different ($P<0.05$) from the pre-transfer rate in 10% SW. Daggers indicate means which are significantly different ($P<0.05$) from the 12 h–7 d mean.

3.2.2. Unidirectional efflux rates

Very different patterns were seen, with more rapid and effective regulation of J_{out}^{Na} and J_{out}^{Cl}. After transfer from 10% SW to FW, the first significant reduction in J_{out}^{Na} occurred at 0–0.5 h, a 26% drop. By 1.0–1.5 h, the decline reached 50%, and rates were not significantly different from the 12 h–7 d mean (~1280 μmol kg h^{-1}). This decline continued in an inverse hyperbolic fashion, and by 6.5–7.0 h, J_{out}^{Na} values were reduced to 660–820 μmol kg h^{-1}, significantly below the 12 h–7 d mean, suggesting that an over-compensation occurred.

J_{out}^{Cl} exhibited an equally rapid adjustment, but without the over-compensation (Fig. 4B). The 27% decline at 0–0.5 h was not quite significant ($P=0.07$), but was significant at all times thereafter, reaching 50% at 1.0–1.5 h. Thereafter, J_{out}^{Cl} continued to fall in a similar fashion to J_{out}^{Na}. Rates not significantly different from the 12 h–7 d mean (~950 μmol kg h^{-1}) were achieved by 2.5–3.0 h.

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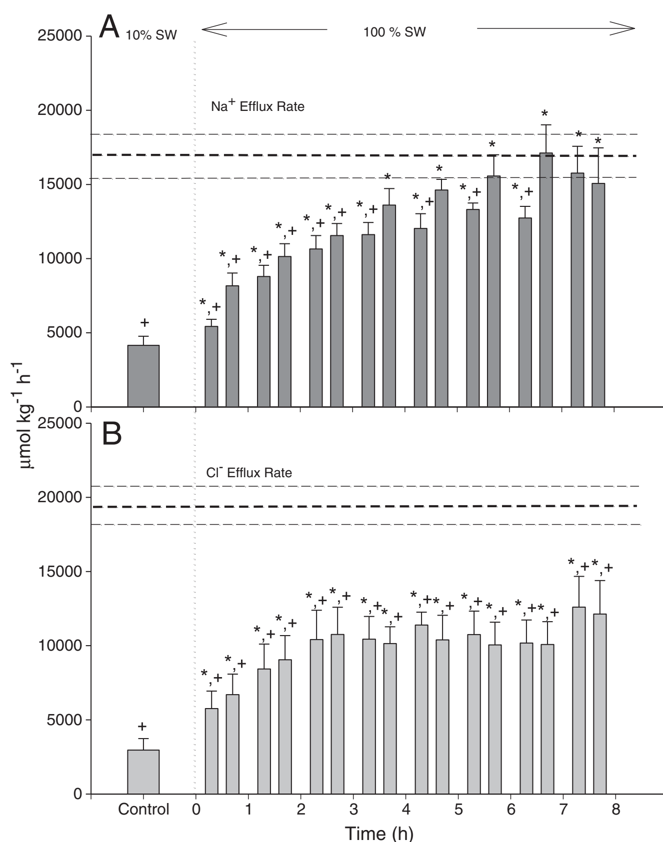

Fig. 2. The influence of transfer from 10% SW to 100% SW on (A) unidirectional sodium efflux rate (J^{Na}_{out}) and (B) unidirectional chloride efflux rate (J^{Cl}_{out}) in *Fundulus heteroclitus* during the first 8 h post-transfer. Means + 1 SEM (N = 16 for J^{Na}_{out}, N = 9–10 for J^{Cl}_{out} except at 2.5–3.0 and 3.0–3.5 h, where N = 18). The thick and thin dotted lines illustrate the means ± 1 SEM of the rates at 12 h, 3 d, and 7 d post-transfer (N = 18) reported by Wood and Laurent (2003). Asterisks indicate means which are significantly different (P < 0.05) from the pre-transfer rate in 10% SW. Daggers indicate means which are significantly different (P < 0.05) from the 12 h–7 d mean.

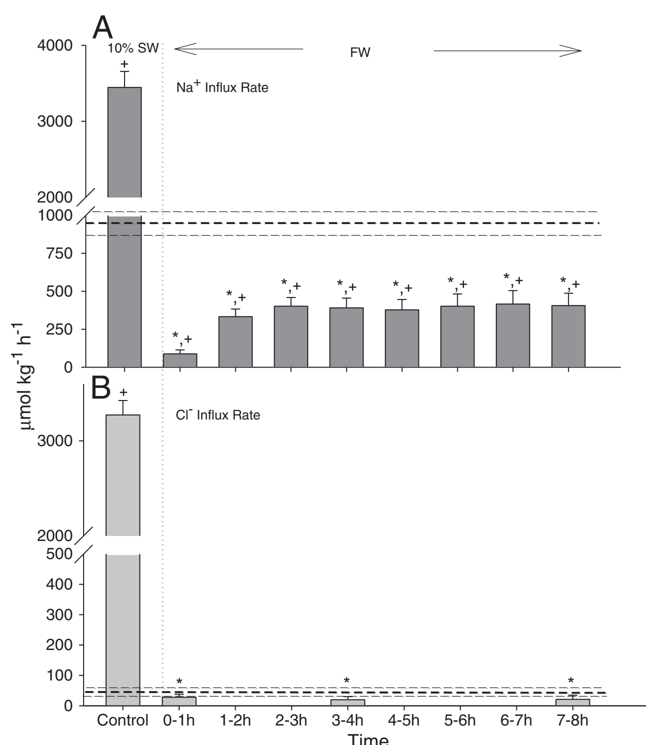

Fig. 3. The influence of transfer from 10% SW to FW on (A) unidirectional sodium influx rate (J^{Na}_{in}) and (B) unidirectional chloride influx rate (Jin^{Cl}) in *Fundulus heteroclitus* during the first 8 h post-transfer. Means + 1 SEM (N = 10 for J^{Na}_{in}, N = 6–8 for J^{Cl}_{in}) . The thick and thin dotted lines illustrate the means ± 1 SEM of the rates at 12 h, 3 d, and 7 d post-transfer (N = 18) reported by Wood and Laurent (2003). Asterisks indicate means which are significantly different (P < 0.05) from the pre-transfer rate in 10% SW. Daggers indicate means which are significantly different (P < 0.05) from the 12 h–7 d mean.

4. Discussion

4.1. Overview and comparison to earlier investigations

There has been an immense amount of work on the molecular, cellular, enzymatic, electrical, and morphological mechanisms in the gills responsible for changes in ion flux rates following salinity challenge in *Fundulus* (e.g. Jacob and Taylor, 1983; Katoh et al., 2001; Katoh and Kaneko, 2003; Laurent et al., 2006; Mancera and McCormick, 2000; Marshall, 2003; Marshall et al., 1999, 2002, 2005; Scott et al., 2004a,b, 2005, 2006, 2008; Scott and Schulte, 2005; Shaw et al., 2007, 2008; Towle et al., 1977; Wood and Grosell, 2008, 2009). However, there have been surprisingly few measurements in intact killifish of the ion fluxes themselves, most of them 30–40 years ago (Maetz et al., 1967; Motais et al., 1966; Pic, 1978; Potts and Evans, 1967). This likely reflects both the expense (especially for ^{36}Cl) and the difficulties of making such measurements under conditions of dynamic change after transfer. The present and previous investigation (Wood and Laurent, 2003) aimed to correct this deficiency by directly recording unidirectional ion flux rates, rather than surrogate measures. In general, the results agree with some aspects of the early flux studies, and confirm our hypothesis that there is rapid adaptive regulation of unidirectional flux rates in the first 0.5–2.0 h post-transfer, followed by progressive changes up to, and in some cases beyond 8 h. A very clear result of the present study is that the passive or leakage components (J_{in} in 100% SW, J_{out} in FW) for both Na^+ and Cl^- are regulated more rapidly and effectively than the active components (J_{out} in 100% SW, J_{in} in FW).

The early radiotracer flux studies on *F. heteroclitus* cited above reported an instantaneous 15–30% drop (phase I) in J^{Na}_{out} and J^{Cl}_{out}, in the first 0.5 h after transfer from 100% SW to FW, followed by a larger progressive decline (phase II) over the following 24–48 h (summarized by Wood and Marshall, 1994). Although our transfers were from 10% SW (not 100% SW) to FW, our finding of approximate 25% declines in both J^{Na}_{out} and J^{Cl}_{out} at 0–0.5 h post-transfer (Fig. 4A,B) are in good agreement. However, subsequent declines, presumably the phase II effect, occurred more rapidly, being complete before 8 h. The only study (Maetz et al., 1967) to monitor unidirectional influx rates after transfer to FW reported a large immediate fall in J^{Na}_{in} , but no subsequent recovery. This contrasts with the results of the present study where some recovery occurred (Fig. 3A), but Maetz et al. (1967) did not present a detailed time course analysis. None of these investigation examined the responses of this species following transfer to 100% SW.

4.2. Net effects of changes in unidirectional influx and efflux rates after salinity transfer

The net effect of the slower response of the active components relative to the passive components was that the killifish transiently lost Na^+ and Cl^- after transfer to FW, and loaded with Na^+ and Cl^- after transfer to 100% SW. Fig. 5 illustrates the net flux rates (J_{net}) calculated from the unidirectional flux measurements (J_{in}, J_{out}) of Figs. 1–4.

After transfer to FW, the net losses of Na^+ and Cl^- were approximately the same in the first hour, but thereafter J^{Cl}_{net} was persistently more negative than J^{Na}_{net} (Fig. 5A).Both loss rates attenuated in an exponential fashion, but by 12 h, the integrated net loss of Cl^- (−13.7 mmol kg^{-1}) was about 65% greater than the integrated net loss of Na^+ (−8.3 mmol kg^{-1}). These values compare reasonably well with independent whole body measurements: whole body Cl^-

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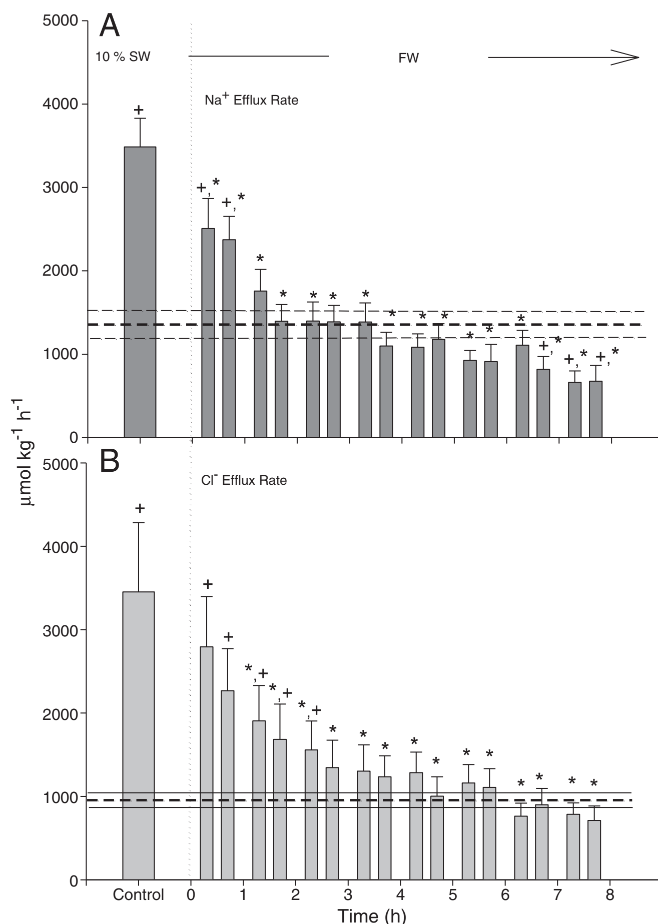

Fig. 4. The influence of transfer from 10% SW to FW on (A) unidirectional sodium efflux rate (J^{Na}_{out}) and (B) unidirectional chloride efflux rate (J^{Cl}_{out}) in *Fundulus heteroclitus* during the first 8 h post-transfer. Means + 1 SEM (N = 16 for J^{Na}_{out}, N = 9–10 for J^{Cl}_{out} except at 2.5–3.0 and 3.0–3.5 h, where N = 18). The thick and thin dotted lines illustrate the means ± 1 SEM of the rates at 12 h, 3 d, and 7 d post-transfer (N = 18) reported by Wood and Laurent (2003). Asterisks indicate means which are significantly different (P<0.05) from the pre-transfer rate in 10% SW. Daggers indicate means which are significantly different (P<0.05) from the 12 h–7 d mean.

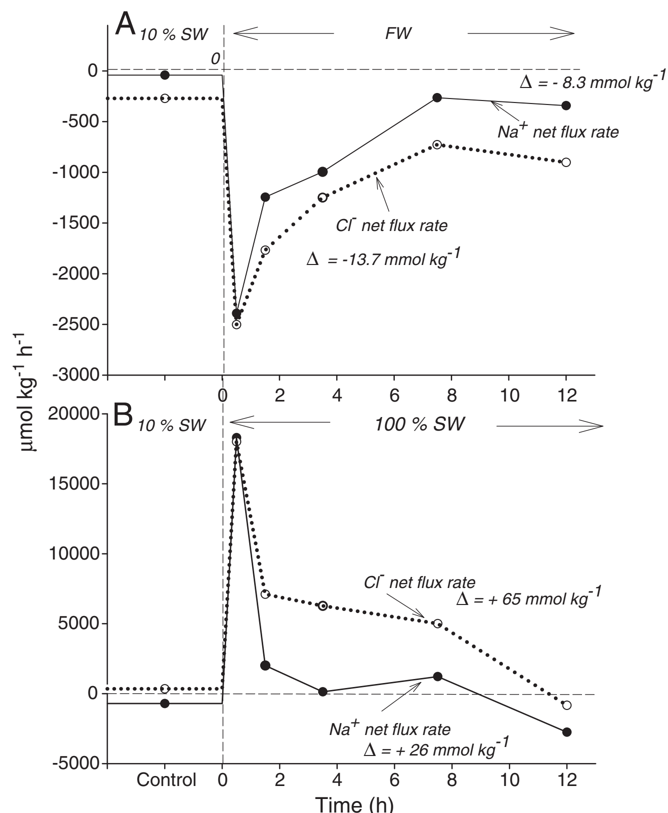

Fig. 5. The estimated net flux rates of sodium (J^{Na}_{net}) and chloride (J^{Cl}_{net}) in *Fundulus heteroclitus* during the first 12 h after transfers from 10% SW to (A) FW and (B) 100% SW. Rates were calculated from mean values of unidirectional influx and efflux rates reported in Figs. 1–4. The delta values represent the cumulative addition to or loss from the fish.

dropped by −14.4 mmol kg^{-1} and whole body Na$^+$ by −5.3 mmol kg^{-1} at 12 h after transfer to FW (Wood and Laurent, 2003). The greater net Cl$^-$ loss was not due to differences in J^{Cl}_{out} *versus* J^{Na}_{out}, which were very comparable (Fig. 4), but rather to the failure of the killfish to activate any meaningful J^{Cl}_{in} after transfer to FW (Fig. 3B), in contrast to J^{Na}_{in} (Fig. 3). Fig. 5A suggests that the fish continued in negative Cl$^-$ balance after 12 h, but it must be remembered that the fish had not been fed, and Cl$^-$ is supplied by the diet when the killifish is in FW (Wood et al., 2010).

After transfer to 100% SW, net flux rates were in the opposite direction and initially much higher; note the different scale in Fig. 5B *versus* Fig. 5A. In the first hour, the net gains of Na$^+$ and Cl$^-$ were approximately the same, but thereafter J^{Cl}_{net} was persistently more positive than J^{Na}_{net} until 12 h, though both declined (Fig. 5B). By 12 h, the fish were exhibiting a slight net excretion of both ions. Over 12 h, the integrated net gain of Cl$^-$ (+65 mmol kg^{-1}) was about 2.5 fold greater than the integrated net gain of Na$^+$ (+26 mmol kg^{-1}). These different net loads agree qualitatively but not quantitatively with independent whole body measurements: whole body Cl$^-$ increased by +22 mmol kg^{-1} and whole body Na$^+$ by +13 mmol kg^{-1} at 12 h after transfer to 100% SW (Wood and Laurent, 2003). The reason for the quantitative discrepancy is not clear, though Potts and Evans (1967) reported differences of +30 mmol kg^{-1} for both Na$^+$ and Cl$^-$ in long term acclimated killifish. In our experiments, the greater loading of Cl$^-$ than Na$^+$ reflected the slower regulation of J^{Cl}_{out} (Fig. 2B) which remained about 35% below the 12 h–7 d value at 8 h, and also below the simultaneous J^{Cl}_{in}, in contrast to J^{Na}_{out} (Fig. 2A). Patterns of J^{Cl}_{in} and J^{Na}_{in} were very similar (Fig. 1A,B). Thus after transfer to both FW and 100%SW, the regulation of Cl$^-$ was slower than that of Na$^+$, and in both cases the active component was the culprit.

A probable consequence of the imbalance of Cl$^-$ *versus* Na$^+$ loads and losses would be a transient metabolic acidosis after transfer to 100% SW and a transient metabolic alkalosis after transfer to FW due to decreases in the "strong ion difference" (SID) in the former and increases in the SID in the latter (Stewart, 1981). These acid–base disturbances have been documented in numerous salinity transfer studies with other species (e.g. Maxime et al., 1990; Walker et al., 1989), and indeed, even after long term acclimation, blood plasma pH and HCO_3^- levels were lower in 100% SW *Fundulus* than in FW *Fundulus* (Wood et al., 2010).

4.3. The role of extra-branchial sites of Na$^+$ and Cl$^-$ fluxes

Simple calculations indicate that the majority of the influxes measured in this study occur at the gills, rather than the gut. Measured drinking rates in killifish under virtually identical conditions to those of the present experiments were 2.9 ml kg^{-1} h^{-1} after transfer to 100% SW (Scott et al., 2008), and 0.4 ml kg^{-1} h^{-1} at 12 h after transfer to FW (Scott et al., 2006). Based on the water ion concentrations of Table 1, in 100% SW, drinking would have accounted for about 1500 μmol kg h^{-1} of Na$^+$ and Cl$^-$ uptake, relative to measured values in the 10,000–25,000 μmol kg h^{-1} (Fig. 1A,B). However it is possible that the initial overshoots at 0–1 h post-transfer could have been due to excessively high drinking rates induced by the stress of

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acute transfer, because some early measurements in SW killifish were as high as 9.4 to 23.5 ml kg h^{-1} (Potts and Evans, 1967; Potts and Fleming, 1970). These could provide Na^+ and Cl^- influx rates in the 5000–12,000 μmol kg h^{-1} range. In FW, drinking would have accounted for Na^+ and Cl^- influxes of only about 0.35 μmol kg h^{-1}, negligible even relative to the very low measured J_{in}^{Cl} of 20–45 μmol kg h^{-1} (Fig. 3B). Wood and Laurent (2003) provided data indicating that this very small J_{in}^{Cl} occurs entirely at the opercular epithelia, which unlike the gills, do actively take up Cl^- in FW (Burgess et al., 1998; Wood and Marshall, 1994). With respect to efflux rates, the kidney may play a significant role in FW animals. Early studies on the related *Fundulus kansae* (Fleming and Stanley, 1965; Stanley and Fleming, 1964, 1966) actually measured urine production in these very small killifish, and found that it accounted for about 20% of the whole animal Na^+ and Cl^- efflux rates in FW-acclimated fish, but a negligible fraction in SW-acclimated fish. Progressive reduction of urinary Na^+ and Cl^- losses in the first 12 h post-transfer may contribute to the observed reductions in J_{out}^{Na} and J_{out}^{Cl} (Fig. 4A,B).

4.4. Mechanisms of rapid modulation of ion fluxes

It seems likely that the very short term modulation of ion flux rates (0.5–2 h post-transfer) occurs mainly by non-genomic mechanisms (but see Shaw et al., 2008, as discussed below), whereas thereafter there is an increasing contribution of changes resulting from transcription and translation of new mRNA, as well as from hormonal modulation. Originally, the almost instantaneous decreases in J_{out}^{Na} and J_{out}^{Cl} upon transfer to FW ("phase I") were interpreted as exchange diffusion effects reflecting the reduction of external counter-ions for self-exchange. As discussed by Wood and Marshall (1994), this explanation cannot be completely eliminated, but it appears more likely that such very rapid effects may be direct responses of channels and tight junctions to the salinity change itself.

One of the most important of these direct responses in the very short term may be the transepithelial potential (TEP), which changes almost instantaneously after salinity transfer in the killifish (Wood and Grosell, 2008, 2009). Upon transfer to FW, the TEP becomes much more negative inside; the accompanying change in the electrochemical gradient will retard Na^+ loss by reducing J_{out}^{Na} and favoring J_{in}^{Na}. Upon transfer to 100% SW, the TEP becomes positive inside, thereby retarding net Na^+ gain. Of course, in both instances, the effects on Cl^- balance will be opposite, but of lesser consequence, because gill permeability to Cl^- is lower than that to Na^+ (Wood and Grosell, 2008), and J_{in}^{Cl} is virtually nonexistent in FW anyway (Fig. 3B). Nevertheless, this differential influence of the TEP change may explain the more rapid and effective reduction of J_{out}^{Na} relative to J_{out}^{Cl} upon FW transfer (Fig. 4A,B). These TEP effects are superimposed on the general phenomenon whereby decreasing salinity reduces both Na^+ and Cl^- permeability of the ionocytes (Degnan and Zadunaisky, 1980). Changes in the structure of the tight junctions also occur very rapidly upon salinity transfer so as to "tighten" the gill epithelium in more dilute environments; again these may be, at least in part, direct responses to salinity change (Karnaky, 1991).

While our focus in these experiments has been on external salinity, there is abundant evidence that changes in internal salinity (i.e. plasma osmolality) can rapidly modulate active Cl^- secretion in killifish opercular epithelial preparations by inducing changes in cell volume (Hoffmann et al., 2002; Marshall et al., 1999, 2000; Zadunaisky et al., 1995). Decreases in basolateral osmolality inhibit secretion, and *vice versa*. Such changes in osmolality occur within a few hours of salinity transfer (Marshall et al., 1999; Zadunaisky et al., 1995). Another rapidly acting influence may be neural activity. Neural inhibition of active Cl^- secretion has been demonstrated in the SW killifish opercular epithelium preparation (Marshall et al., 1998); it is possible that similar neural controls could rapidly change ion transport at the gills. In the original Silva model (Silva et al., 1977) for NaCl transport in the SW teleost gill, changes in active Cl^- secretion through the transcellular pathway of ionocytes are accompanied by equimolar changes in the movement of Na^+ through the paracellular pathway. However in the present experiments, when killifish were transferred to 100% SW, a situation which would be expected to activate Cl^- secretion, J_{out}^{Cl} increased more slowly than J_{out}^{Na} (Fig. 2A,B), resulting in the loading of far more Cl^- than Na^+ into the animal (Fig. 5). The reason for this discrepancy is unknown, but it does suggest that other transport mechanisms may be involved.

Finally, the present data showing that these "active" components (J_{out}^{Cl} , J_{out}^{Na}) of ionic regulation increased significantly in the first 0.5 h after transfer to 100% SW (Fig. 2A,B) coincide well with observations of Towle et al. (1977) that gill Na^+,K^+, ATPase activity can double within 0.5 h in killifish undergoing a similar transfer protocol. Conversely, Na^+,K^+, ATPase activity can fall by 50% within 1 h after transfer to FW, which could contribute to the observed rapid reductions in J_{out}^{Cl} and J_{out}^{Na} (Fig. 4A,B). Changes in such a short time frame likely represent post-translational effects such as changes in phosphorylation. In this regard, Flemmer et al. (2010) have recently reported significant increases and decreases in the fractional level of phosphorylation of another key gill transporter (Na^+,K^+,2 Cl^- co-transporter, NKCC1) within 1 h of transfer of killifish to higher and lower salinities respectively. Earlier, Marshall et al. (2005) found evidence implicating several phosphorylation pathways in even more rapid modulation of NKCC1 and the Cl^- channel protein (CFTR) in the killifish opercular epithelium subjected to acute salinity transfer *in vitro*. Clearly, the present data show that such rapid modulations occurring long before "structural" re-organizations of genomic origin are effective in changing ion flux rates. This strategy, combined with an ability to tolerate internal disturbances in electrolyte levels, is of obvious benefit in a species which routinely encounters hourly salinity fluctuations in its natural environment. Nevertheless, there is some evidence that genomic effects can occur in the killifish within this time frame. Shaw et al. (2008) reported that serum-and glucocorticoid-inducible kinase (SGK1) mRNA increased within 1 h of transfer from FW to 100% SW, and they associated this with an increase in CFTR abundance in the apical cell membrane. Many of the genes for transporters and regulatory factors thought to be involved in gill ion transport in this species have now been cloned. Clearly the next step is a detailed time course analysis of gene and protein expression in the gills, the tissue which accounts for the great majority of the ion fluxes.

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