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ORIGINAL PAPER

A critical analysis of transepithelial potential in intact killifish (*Fundulus heteroclitus*) subjected to acute and chronic changes in salinity

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Abstract We investigated the in vivo salinity-dependent behavior of transepithelial potential (TEP) in *Fundulus heteroclitus* (3–9 g) using indwelling coelomic catheters, a technique which was validated against blood catheter measurements in a larger species (*Opsanus beta*; 35–70 g). In seawater (SW)-acclimated killifish, TEP was +23 mV (inside positive), but changed to −39 mV immediately after transfer to freshwater (FW). Acute transfer to dilute salinities produced a TEP profile, which rapidly attenuated as salinity increased (0, 2.5, 5 and 10% SW), with crossover to positive values between 20 and 40% SW, and a linear increase thereafter (60, 80 and 100% SW). TEP response profiles were also recorded after acute transfer to comparable dilutions of 500 mmol L^{-1} NaCl, $NaNO_3$, Na gluconate, choline chloride, *N*-methyl-D-glutamate (NMDG) chloride, or 1,100 mosmol kg^{-1} mannitol. These indicated high non-specific cation permeability and low non-specific anion permeability without influence of osmolality in SW-acclimated killifish. While there was a small electrogenic component in high salinity, a Na^+ diffusion potential predominated at all salinities due to the low P_{Cl}/P_{Na} (0.23) of the gills. The very negative TEP in FW was attenuated in a linear fashion by log elevations in $[Ca^{2+}]$ such that P_{Cl}/P_{Na} increased to 0.73 at 10 mmol L^{-1}. SW levels of $[K^+]$ or $[Mg^{2+}]$ also increased the TEP, but none of these cations alone restored the positive TEP of SW-acclimated killifish. The very negative TEP in FW attenuated over the first 12 h of exposure and by 24–30 h reached +3 mV, representative of long-term FW-acclimated animals; this reflected a progressive increase in P_{Cl}/P_{Na} from 0.23 to 1.30, probably associated with closing of the paracellular shunt pathway. Thereafter, the TEP in FW-acclimated killifish was unresponsive to $[Ca^{2+}]$ (also to $[K^+]$, $[Mg^{2+}]$, or chloride salts of choline and NMDG), but became more positive at SW levels of $[Na^+]$. Killifish live in a variable salinity environment and are incapable of gill Cl^- uptake in FW. We conclude that the adaptive significance of the TEP patterns is that changeover to a very negative TEP in FW will immediately limit Na^+ loss while not interfering with active Cl^- uptake because there is none. Keeping the shunt permeability high for a few hours means that killifish can return to SW and instantaneously re-activate their NaCl excretion mechanism.

Keywords Gill permeability · P_{Cl}/P_{Na} ratio · TEP · Diffusion potential · Electrogenic potential · Calcium-dependent potential · *Opsanus beta*

Introduction

The common killifish (*Fundulus heteroclitus*) lives in estuaries and tidal flats, and tolerates direct transfer between seawater (SW) and freshwater (FW) (Griffith 1974). Thanks to its opercular epithelium, which can easily be removed and studied in vitro as a surrogate model for the gills, this species has played a fundamental role in elucidating the mechanisms of active salt excretion across the gills in SW, as well as the mechanisms by which this salt excretion is turned off during exposure to dilute

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salinities (reviewed by Karnaky 1980; Zadunaisky 1984; Marshall and Bryson 1998; Marshall 2003; Marshall and Grosell 2006). There has been less work on the intact animal in vivo, but in general this has supported findings from in vitro studies of the opercular epithelium in SW and intermediate salinities, but not in FW (reviewed by Wood and Marshall 1994; Wood and Laurent 2003). A critical difference in FW is that the opercular epithelium in vitro actively takes up small amounts of Cl^- but not Na^+ from the external medium (Marshall et al. 1997; Burgess et al. 1998), whereas the intact killifish exhibits a vigorous uptake of Na^+, but negligible uptake of Cl^- across the gills (Patrick et al. 1997; Patrick and Wood 1999; Wood and Laurent 2003).

An important parameter in understanding transport mechanisms at the gills is the transepithelial potential (TEP), because this is one of the two factors (the other is the concentration gradient of the relevant ion) determining the true electrochemical gradients against which active transport occurs and internal ionic homeostasis is maintained. In most FW teleosts, the TEP is negative (expressed as inside potential relative to outside potential as zero), whereas in most SW teleosts the TEP is positive, with the majority of measurements falling in the −15 to +25 mV range (reviewed by Potts 1984; Potts and Hedges 1991; Marshall and Grosell 2006). There have been many measurements of TEP across the opercular epithelium in vitro, and these suggest that the TEP in *Fundulus* may exhibit a much wider range, especially in FW. For example, when opercular epithelia from SW-acclimated killifish were mounted with 100% SW on the apical surface and physiological saline on the basolateral surface, TEPs of +29 to +37 mV have been reported (Degnan and Zadunaisky 1979, 1980; Pequeux et al. 1988). Conversely, epithelia from FW-acclimated killifish exposed to apical FW exhibited TEPs of −40 to −65 mV (Wood and Marshall 1994; Marshall et al. 1997; Burgess et al. 1998).

Are these extreme TEP values seen in the isolated opercular epithelium in vitro representative of those in the intact animal in vivo? To our knowledge, there has been only one study of TEP across the gills of *Fundulus* in vivo: Pic (1978) reported TEPs of about +23 mV in SW-acclimated animals in 100% SW, and about −28 mV immediately after acute transfer of these same animals to FW. These data suggest that the TEP range between SW and FW might be greater than in most teleosts, but less than that seen with isolated opercular epithelia, and are not conclusive. For example, Pic (1978) did not measure TEPs in FW-acclimated killifish, and emphasized that TEP measurements in intact killifish were very sensitive to attenuation by handling stress, i.e. they became less positive in SW and less negative in FW if the animals were stressed.

In this collaborative study, we addressed this issue of the true in vivo TEP in *Fundulus* to answer questions of mutual concern. One of our laboratories has an ongoing interest in the mechanisms of ionoregulation in *Fundulus*, especially in FW (e.g. Patrick et al. 1997; Patrick and Wood 1999; Scott et al. 2004b, 2005, 2006; Laurent et al. 2006). Extreme TEPs might help explain the very different handling of Na^+ versus Cl^- observed in the gills of the intact animal (Wood and Laurent 2003). The other laboratory has an ongoing interest in the mechanisms of toxicity of copper, a Na^+ antagonist, in *Fundulus* (e.g. Blanchard and Grosell 2005, 2006) and has recently related the degree of sensitivity to copper at different salinities to the electrochemical driving force for Na^+ across the gills at these salinities (Grosell et al. 2007). However, this analysis used only the Nernst equilibrium potential for Na^+; if large differences in TEP exist across salinities, the interpretation of this relationship would be altered.

The goals of the present study were therefore to measure TEP in intact, unrestrained *Fundulus heteroclitus*. The small size of the animals precludes blood vessel cannulation, so we validated the approach employed (intraperitoneal coelomic catheters) by comparing TEP measurements made with this method versus measurements made in the traditional fashion with indwelling blood vessel catheters in a larger species, the gulf toadfish, *Opsanus beta*. Killifish were acclimated and tested in both 100% SW and FW, and after acute transfer from 100% SW to FW. A very negative TEP was found in the latter circumstance, and therefore its nature and time course were investigated.

Methods

Experimental animals

Common killifish of the northern subspecies (*Fundulus heteroclitus macrolepidotus*; 3–9 g) were purchased from Aquatic Research Organisms (ARO) Ltd. (Hampton, New Hampshire); the animals had been collected by beach-seining of local tidal flats in early spring, 2007. At the University of Miami, they were acclimated to either 100% SW (from Biscayne Bay) or FW (dechlorinated City of Miami tapwater; see Table 1 for composition) under flow-through conditions at the experimental temperature (24–26°C), and were fed a daily 3% body mass ration of commercial pellets. Gulf toadfish (*Opsanus beta*; 35–70 g) were obtained as bycatch from local shrimp fishermen using roller trawls, and held in flowing 100% SW. The toadfish were fed to satiation with squid every 2nd day. All procedures were approved by the University of Miami Animal Care and Use Committee.

Table 1 Ionic composition (mmol L^{-1}) of the Miami freshwater (FW), synthetic Ca^{2+}-free FW and 100% seawater (SW) used in the experiments

	Miami FW	Synthetic Ca^{2+}-free FW	100% SW
Na^+	1.06	1.70	485
K^+	0.08	0.08	10.6
Ca^{2+}	0.43	–	10.7
Mg^{2+}	0.13	0.14	59.5
Cl^-	1.21	1.00	569
SO_4^{2-}	0.14	0.14	31.6
$HCO_3^- + CO_3^{2-}$	0.85	0.78	2.30
pH	7.4	7.4	8.0

Cannulation

Fish were anesthetized in MS-222 (200 mg L^{-1}) in their appropriate acclimation water, held briefly in air (5–10 min) and wrapped in wet cloth during surgery. In toadfish, a saline-filled PE50 catheter (Clay-Adams) was first inserted into the caudal artery or vein as described by Wood et al. (1997). Results from the two vessels were identical. In both species, a saline-filled PE50 catheter was inserted through the peritoneal wall into the coelom via a puncture site (made with a 19 gauge needle) just lateral and anterior to the rectum. The catheter was inserted about 1–2 cm in killifish, and about 3–4 cm in the larger toadfish. A 1 cm PE160 sleeve, heat-flared at both ends, was glued to the PE50 with cyanoacrylate resin and anchored to the body wall with several silk sutures; this prevented the catheter from changing depth in the coelom. The saline used was Cortland saline (Wolf 1963) with the [NaCl] raised by 20 mM for marine fish; sodium heparin (50 i.u. ml^{-1}) was added to the saline for the blood vessel catheters. The catheters were temporarily heat-sealed and cut to a length so as to minimize any disturbance to the movement of the fish. The animals were then allowed to recover for 24 h in their experimental chambers, which were continually flushed with aerated SW or FW, as appropriate. The chambers were polyethylene food containers of approximately 3 L volume for toadfish and 150 ml for killifish.

TEP measurements

TEP was measured by means of 3 M KCl-agar bridges connected via Ag/AgCl electrodes to a high impedance electrometer (Radiometer pHM 84 meter, Copenhagen, Denmark). The reference bridge was placed in the water in the fish chamber, and the measurement bridge was connected to either the blood catheter or the coelomic catheter. TEP measurements were expressed relative to the apical (water) side as 0 mV after correction for junction potential, which was less than 2 mV in all cases.

In order to minimize stress, all measurements were made in the fish's holding chamber, and external solutions were changed without air-exposing the fish. TEP measurements were taken 5–10 min after a solution change, but in practice, TEP reached stability in less than 2 min.

Experiments

In different experiments, TEP was determined in 100% SW-acclimated fish, at various times after acute exposure of SW-acclimated fish to FW, and for killifish only, in FW-acclimated fish. In all experiments where the fish was exposed to a range of different concentrations of a particular variable, the order of application of the concentrations was randomized, and the fish was returned to its holding treatment (FW or 100% SW) for 5 min in between tests. The only exception was in the calcium tests, where a sequential addition protocol in a constant background solution of synthetic FW was used, as outlined below.

When experiments involved exposure to various concentrations of SW or a particular compound, the exposure solutions were made by volumetric additions of the stock solutions in the appropriate proportion (0, 2.5, 5, 10, 20, 40, 60, 80 and 100%) to City of Miami FW (composition as in Table 1). These stock solutions included 500 mmol L^{-1} NaCl, 500 mmol $L^{-1}NaNO_3$, 500 mmol L^{-1} Na gluconate ($HOCH_2(CH(OH))_4CO_2Na$), 500 mmol L^{-1} choline chloride ($C_5H_{14}ONCl$), 500 mmol L^{-1} *N*-methyl-D-glutamate chloride ($C_7H_{17}NO_5Cl$, NMDG-chloride), 10.5 mmol L^{-1} KNO_3, 60 mmol L^{-1} $MgNO_3$, and 60 mmol L^{-1} $MgSO_4{\cdot}7H_2O$, all made up in FW, so that only the components of interest would change. The NMDG-chloride stock was made by neutralizing *N*-methyl- D-glutamic acid with HCl, then checking the Cl concentration using a Radiometer CMT 10 chloride titrator (Copenhagen, Denmark). A stock solution of approximately 1,150 mmol L^{-1} mannitol was similarly made up in FW; this concentration was chosen so as to have the same osmolality as the SW (1,100 mosmol kg^{-1}), as measured by vapor pressure osmometry (Wescor 5100C, Logan, UT, USA).

In the calcium tests, the fish were tested in a synthetic FW, which was nominally Ca^{2+}-free (composition in Table 1). This synthetic FW was synthesized by combining 80 μmol L^{-1} $KHCO_3$, 140 μmol L^{-1} $MgSO_4{\cdot}7H_2O$, 700 μmol L^{-1} $NaHCO_3$ and 1,000 μmol L^{-1} NaCl in distilled water. Apart from $[Ca^{2+}]$, the principal difference versus true FW was $[Na^+]$, which was approximately 640 μmol L^{-1} higher (c.f. Table 1). This modest elevation in $[Na^+]$ was an unavoidable consequence of omitting a calcium salt, and was accepted since variation in $[Na^+]$ over this range (1,060–1,700 μmol L^{-1}) had no detectable effect on TEP. In contrast to the other tests, a semi-logarithmic series of $[Ca^{2+}]$ exposures was performed, and this was

achieved by sequential addition to the fish chamber from a 3 M $Ca(NO_3)_2{\cdot}4H_2O$ stock solution made in synthetic FW. Spot samples taken from the fish chambers during the actual tests and analyzed for [Ca^{2+}] by flame atomic absorption spectroscopy (Varian SpectrAA-220FS, Mulgrave, Australia) indicated that the nominally Ca^{2+}-free exposures had actual [Ca^{2+}] levels varying from 3.8 to 15.7 μmol L^{-1}; these points were therefore plotted as nominally 0.01 mmol L^{-1} [Ca^{2+}]. Measured [Ca^{2+}] levels in the other intended exposures (0.032, 0.10, 0.32, 1.00, 3.20 and 10.00 mmol L^{-1}) were within ±15% of nominal values. The sequential additions were made at 5–10 min intervals, so the entire test took about 1 h.

Calculations

Modeling of the data was performed using the Nernst equation (Eq. 1) and the Goldman-Hodgkin-Katz equation (Eq. 2) to estimate the equilibrium potentials (E) across the gills (Goldman 1943; Sten-Knudsen 2002):

$$E = \frac{-RT}{zF} \ln \frac{[C]_{in}}{[C]_{out}}, \quad (1)$$

$$E = \frac{-RT}{F} \ln \frac{P_C[C]_{in} + P_A[A]_{out} + \cdots}{P_C[C]_{out} + P_A[A]_{in} + \cdots} \quad (2)$$

where z is the valence, R, T and F are the standard thermodynamic constants, $[C]_{in}$ is the activity of a particular cation in the blood plasma, $[C]_{out}$ is the activity of the same cation in the environment, P_C is the permeability of the gills to that cation, $[A]_{in}$ is the activity of a particular anion in the blood plasma, $[A]_{out}$ is the activity of the same anion in the environment, P_A is the permeability of the gills to that anion, etc.

Statistics

Data have been expressed as means ± 1 SEM (N). The significance of differences ($P \leq 0.05$) between means was assessed using Student's paired or unpaired two-tailed t-test, as appropriate, with the designated Bonferroni correction when more than one comparison was made. Regression lines were fitted by the method of least squares, and the significance of slopes relative to equality assessed through the t-distribution.

Results

Toadfish: TEP measurements in 100% SW and FW

SW-acclimated toadfish were employed to evaluate whether measurements from intraperitoneal coelomic catheters yielded the same TEP values as those from blood vessel catheters. After the initial recordings in 100% SW, the toadfish, which are only partially euryhaline, were transferred to FW in order to yield a greater range of TEP values. This exercise demonstrated excellent agreement between coelomic catheter and blood catheter measurements of TEP, with an overall 1:1 relationship (slope = 0.946, not significantly different from 1.0, $R^2 = 0.98$; Fig. 1). Based on this validation, we proceeded to make TEP measurements on killifish via coelomic catheters.

In 100% SW, toadfish exhibited a TEP, which was not significantly different from 0 mV overall (Table 2). The effect of acute FW exposure was to greatly increase variability, with some toadfish exhibiting more positive potentials, and others more negative potentials (Fig. 1); the overall mean remained not significantly different from 0 mV (Table 2).

Killifish: TEP measurements in 100% SW and FW

Recordings were made initially on SW-acclimated killifish, revealing a very different pattern from that in toadfish. When the first measurements were made in 100% SW, TEP was highly positive (mean = +22.5 mV, range = +11.5 to +49.0 mV) and when the first measurements were immediately made after acute transfer to FW, TEP was highly negative (mean = −39.4 mV, range = −11.3 to −64.5 mV; Table 2). These data are very similar to those reported by Pic (1978) (which are also summarized in Table 2)

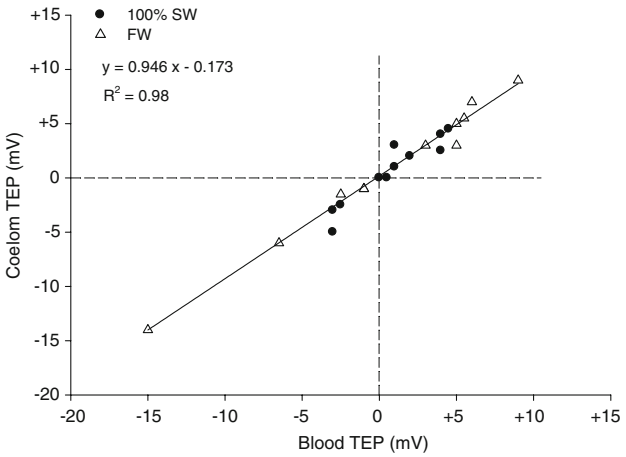

Fig. 1 The relationship between transepithelial potential (TEP) measured via an intraperitoneal coelomic catheter versus TEP measured via a blood catheter in SW-acclimated toadfish. After the SW measurements (*solid circles*), the animals were acutely exposed to FW (*open triangles*) to increase the range of absolute values. Individual *data points* from 13 toadfish are shown (note some points are identical, plotting on top of one another), together with the regression relationship. The *slope* was not significantly different from 1.0 ($P > 0.05$)

Table 2 Transepithelial potentials (mV) of toadfish and killifish measured in 100% seawater (SW) or freshwater (FW) after various acclimation and experimental conditions

Acclimation condition	Experimental 100% SW	Condition FW
SW-acclimated toadfish (first measurement)	+1.7 ± 1.2 (13)	+0.3 ± 1.7 (13)
SW-acclimated killifish (first measurement)	+22.5 ± 3.5 (9)	−39.4 ± 5.9* (8)
SW-acclimated killifish (from experimental series)	+14.1 ± 0.8† (41)	−38.5 ± 1.7* (41)
FW-acclimated killifish (first measurement)	+14.9 ± 1.3 (8)	+3.9 ± 2.9* (8)
FW-acclimated killifish (from experimental series)	+12.7 ± 1.0 (21)	+0.5 ± 1.5* (21)
SW-acclimated killifish (Pic 1978)	+23.3 ± 2.5	−28.3 ± 6.4*

Killifish for which measurements were taken from the experimental series underwent a greater degree of disturbance than killifish for which first measurements were taken. Means ± 1 SEM (N)

* $P \leq 0.05$ of the group in FW experimental condition relative to comparable group in 100% SW experimental condition

† $P \leq 0.05$ relative to values for comparable acclimation condition (first measurement) under the same experimental condition

under apparently comparable exposure conditions, although the present FW potentials were somewhat more negative.

However, when the first measurements were made on FW-acclimated killifish in FW, the highly negative potential was no longer seen, and the TEP was not significantly different from 0 mV (mean = +3.9 mV, range = −9 to +16 mV; Table 2). After acute transfer to 100% SW, these FW-acclimated killifish exhibited a positive TEP (mean = +14.9 mV, range = +9 to +22 mV) in comparison to a more positive mean TEP = +22.5 mV in SW-acclimated killifish (not significantly different, $P = 0.07$).

In this initial series, we noted that if the killifish struggled during the set-up procedure, the absolute magnitude of the TEP of SW-acclimated killifish, in either 100% SW or FW, tended to be lower, in agreement with the original observations of Pic (1978) on the effects of stress. Therefore, data from such fish were excluded and subsequent fish, which struggled were not put through the protocols. Also, in subsequent experimental series, the order of measurements, including the SW and FW measurements, was randomized in order to negate any bias due to unavoidable cumulative handling of stress, as some series involved as many as ten separate measurements in different solutions. The mean data for the measurements in SW and FW for the two acclimation groups from these experimental series are included for comparison in Table 2. These reveal that in SW-acclimated killifish, experimental disturbance (cumulative handling of stress) significantly attenuated the positive TEP measured in 100% SW by about one-third (+14.1 versus +22.5 mV), but did not alter the negative TEP measured immediately after acute exposure to FW (−38.5 versus −39.4 mV, not significantly different). There was also no substantive effect of experimental disturbance on the TEP values measured in FW-acclimated killifish in either FW (+0.5 versus +3.9 mV, not significantly different), or after acute exposure to 100% SW (+12.7 versus +14.9 mV, not significantly different).

Killifish: time course of the negative FW TEP in SW-acclimated animals

The very negative potential seen immediately after transfer to FW (mean = −38.9 mV in this series) progressively attenuated with time (Fig. 2); by 24 h, the TEP reached a value (+1.2 mV) that was not significantly different from 0 mV, and also not significantly different from that in FW-acclimated killifish (Table 2). There were no further changes at 4 days (96 h) and 7 days (168 h). All TEP values after FW transfer remained significantly lower than the original measurements in 100% SW (Fig. 2), in accordance with comparable data in Table 2.

Subsequent experiments involved manipulation of the composition of the external medium in order to characterize the nature of the very negative TEP seen after acute transfer of SW-acclimated killifish to FW, as well as the near 0 mV TEP seen in FW-acclimated killifish. At the same time, these trials also served to characterize the nature of the TEP in 100% SW.

Killifish: the influence of SW dilutions on TEP in SW- and FW-acclimated animals

In the first experiment, killifish acclimated to either 100% SW or FW were acutely transferred to a range of salinities in random order. The salinities (0% = FW, 2.5, 5, 10, 20, 40, 60, 80 and 100% SW) were achieved by volumetric mixing of 100% SW and FW, so that all the components were diluted equally. In SW-acclimated animals, the very negative TEP in FW (mean = −31.4 mV in this series) was progressively attenuated as salinity increased, with the greatest increases occurring in the low salinity range (Fig. 3). A cross-over to positive values occurred between 20 and 40% SW, and thereafter TEP continued to increase with salinity in a linear fashion, reaching a typical value in 100% SW for SW-acclimated fish (mean = +19.6 mV). By traditional theory, this pattern has been explained as largely due to a varying Na^+ diffusion potential (negative in FW and positive in SW; Eq. 1), with a small

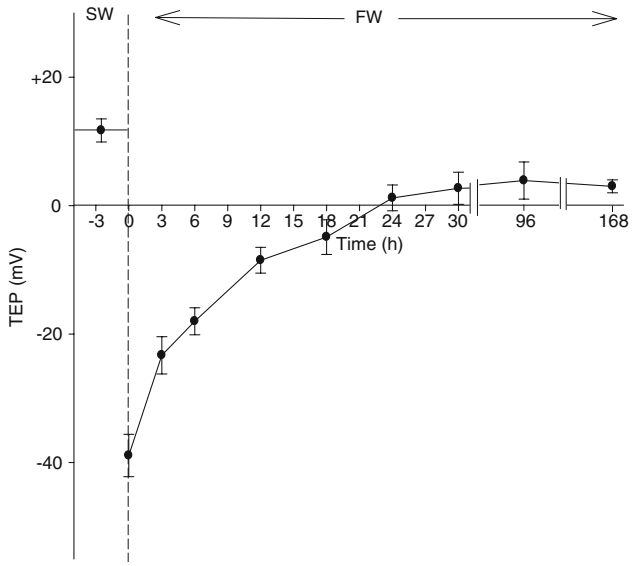

Fig. 2 The time course of changes in transepithelial potential (TEP) when SW-acclimated killifish were transferred to FW. Means ± 1 SEM ($N = 6$–8). From 24 h onwards until 168 h (7 days), there was no further significant change ($P > 0.05$) in TEP relative to the 24 h value. All TEP values after FW transfer remained significantly lower ($P \leq 0.05$) than the original measurements in 100% SW through 168 h

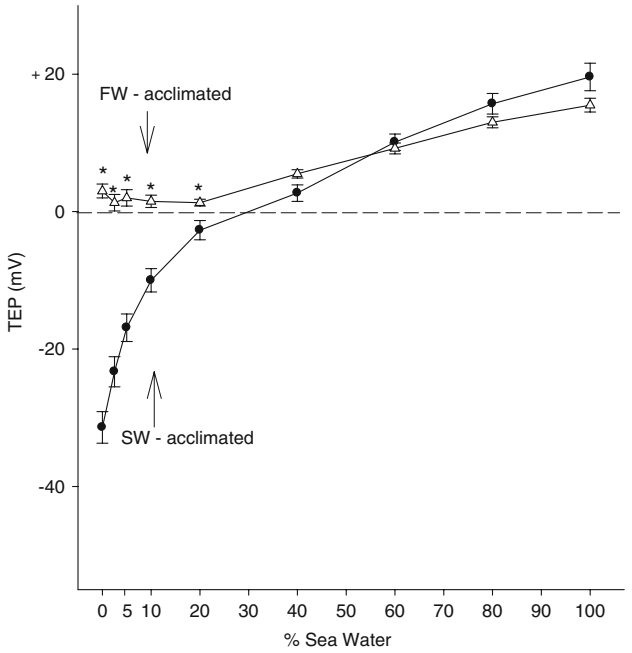

Fig. 3 The influence of acute exposure to various volumetric dilutions of 100% SW on TEP in SW-acclimated (*solid circles*) and FW-acclimated killifish (*open triangles*). 0% = FW. Means ± 1 SEM ($N = 7$ for SW-acclimated fish, $N = 6$ for FW-acclimated fish). * Significant difference ($P \leq 0.05$) from the comparable value in SW-acclimated animals

electrogenic component superimposed on the higher salinities due to active Cl^- extrusion (see "Discussion").

In FW-acclimated animals, acute transfer to various salinities had much less influence on TEP. The slightly positive TEP in FW (mean = +3 mV) did not change until 20% SW, but at higher salinities increased linearly with a slightly lower slope than seen in SW-acclimated killifish. By 100% SW, the TEP (mean = +15.5 mV) was not significantly different from that seen in SW-acclimated animals (Fig. 3). These results indicate that as a result of FW-acclimation, TEPs become far less sensitive to acute variations in salinity, i.e., the putative negative Na^+ diffusion potential at low salinities is eliminated.

Killifish: the influence of $[Ca^{2+}]$ on TEP in SW- and FW-acclimated animals

Na^+ diffusion potentials in teleost gills are typically calcium-sensitive (see "Discussion"), so the next experiment focused on the role of environmental $[Ca^{2+}]$. When SW-acclimated killifish were transferred to synthetic FW, which was nominally Ca^{2+}-free (point plotted as 10 μmol L^{-1} in Fig. 4), the very negative TEP (mean = −34.7 mV) was typical of that seen after acute transfer to natural FW. Progressive elevation of environmental $[Ca^{2+}]$ (as $Ca(NO_3)_2{\cdot}4H_2O$) in half-log unit steps caused an approximately log-linear attenuation of this very negative TEP (Fig. 4). However, even when $[Ca^{2+}]$ was raised to 10 mmol L^{-1} (representative of the calcium concentration in 100% SW, Table 1) over a period of about 1 h, the TEP had increased by only about 25 mV and remained negative (mean = −9.6 mV). When the same experiment was performed on FW-acclimated killifish, the TEP was completely independent of $[Ca^{2+}]$ from nominally 0 through 10 mmol L^{-1} (Fig. 4). These data illustrate that the very negative potential seen after acute transfer to FW is calcium-sensitive, in accordance with the properties of Na^+ diffusion potential. Furthermore, in the FW-acclimated killifish, the TEP became calcium-insensitive, suggesting that the phenomenon was abolished by FW-acclimation. Thus, the massive reduction in environmental $[Ca^{2+}]$ upon acute FW transfer may account in part for the negative TEP. However, it is not a complete explanation because a $[Ca^{2+}]$ typical of 100% SW cannot completely eliminate the negative potential. Furthermore, this $[Ca^{2+}]$ in itself cannot support the positive TEP seen in 100% SW.

A subsequent experiment was performed to check that the apparent effect of environmental $[Ca^{2+}]$ was not due to the NO_3^- anion in $Ca(NO_3)_2$. The NO_3^- anion concentration was acutely elevated in the Ca^{2+}-free synthetic FW by the same amount (20 mmol L^{-1}, equivalent to the 20 mmol L^{-1} added by 10 mmol L^{-1} calcium nitrate) using $NaNO_3$ rather than $Ca(NO_3)_2$. In SW-acclimated killifish, this resulted in a significant elevation in potential

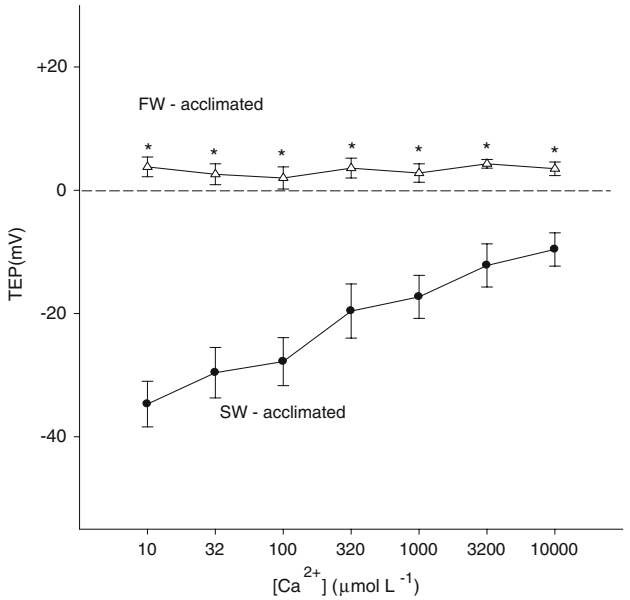

Fig. 4 The influence of progressive elevation of environmental $[Ca^{2+}]$ (as $Ca(NO_3)_2{\cdot}4H_2O$) in a semi-logarithmic series on transepithelial potential (TEP) in SW-acclimated (*solid circles*) and FW-acclimated killifish (*open triangles*) acutely exposed to FW. The experiments were performed using a synthetic FW (composition as in Table 1) that was nominally Ca^{2+}-free. Means ± 1 SEM ($N = 9$ for SW-acclimated fish, $N = 6$ for FW-acclimated fish). * Significant difference ($P \leq 0.05$) from the comparable value in SW-acclimated animals

Table 3 Responses in transepithelial potential (mV) to acute elevation in $NaNO_3$, Na gluconate and NaCl concentrations relative to pre-treatment values in the same fish in Ca^{2+}-free synthetic FW (composition as in Table 1)

	Synthetic Ca^{2+}-free FW pre-treatment	Treatment
SW-acclimated killifish ($n = 9$)		
+20 mmol L^{-1} $NaNO_3$	−32.4 ± 3.9	−22.1 ± 3.7*
+20 mmol L^{-1} Na gluconate	−30.6 ± 3.7	−22.0 ± 2.3*
+500 mmol L^{-1} NaCl	−29.1 ± 2.0	+0.2 ± 1.3*†
FW-acclimated killifish ($n = 8$)		
+20 mmol L^{-1} $NaNO_3$	+2.7 ± 1.3$^{\Delta}$	+0.5 ± 1.7$^{\Delta}$
+500 mmol L^{-1} NaCl	+1.3 ± 1.5$^{\Delta}$	+8.0 ± 1.9*$^{\dagger\Delta}$

Responses of SW-acclimated killifish and FW-acclimated killifish, both tested in Ca^{2+}-free synthetic FW, are shown separately. Means ± 1 SEM (N)

* $P \leq 0.05$ relative to comparable pre-treatment value in Ca^{2+}-free synthetic FW

† $P \leq 0.05$ relative to comparable value for 20 mmol L^{-1} $NaNO_3$ treatment within the same acclimation group

$^{\Delta}$ $P \leq 0.05$ relative to comparable value for SW-acclimated killifish

of about 10 mV (Table 3), but the response was considerably less than the 25 mV elevation caused by 10 mmol L^{-1} $Ca(NO_3)_2$ (c.f. Fig. 3), and the TEP remained negative (mean = −22.1 mV). To confirm that this modest response was due to $[Na^+]$, and not to $[NO_3^-]$, a parallel experiment was performed with the addition of 20 mmol L^{-1} Na gluconate, and an identical response was seen (Table 3). When the same experiment (addition of 20 mmol L^{-1} $NaNO_3$ to Ca^{2+}-free synthetic FW) was performed with FW-acclimated killifish, there was no response, the TEP staying close to 0 mV (Table 3). Finally, to check that the killifish remained responsive to $[Na^+]$ under these Ca^{2+}-free conditions, the response to acute addition of 500 mmol L^{-1} NaCl (similar to 100% SW) was checked. In SW-acclimated killifish, this raised the negative TEP dramatically (by 29 mV) to a value not significantly different from 0 mV (Table 3). In FW-acclimated killifish, this raised the TEP significantly to a positive value (mean = +8.0 mV). Thus, both groups remained responsive to $[Na^+]$, though to differing extents.

Killifish: the influence of $[Na^+]$ on TEP in SW-acclimated animals

To more fully explore the influence of $[Na^+]$ alone, full series were performed on SW-acclimated killifish acutely exposed to volumetric dilutions (0, 2.5, 5, 10, 20, 40, 60, 80 and100%) of each of 500 mmol L^{-1} NaCl, 500 mmol L^{-1} $NaNO_3$, and 500 mmol L^{-1} Na gluconate (Fig. 5). The responses to the three sodium salts were virtually identical and broadly similar to those seen earlier with volumetric dilutions of 100% SW (Fig. 3). However, relative to the SW responses, the curves were down-shifted so that the cross-over points to positive values did not occur until between 40 and 60% mixtures, and the TEPs at 100% were not as positive as in 100% SW (Fig. 5). In general, these support the concept that the negative TEP in dilute solutions is largely a varying Na^+-diffusion potential, though modulated by other aspects of water chemistry. Interestingly, in concentrated solutions (80–100%), the positive TEP in Na gluconate (means = +7.2 to +11.0 mV; Fig. 5) was significantly higher than in NaCl or $NaNO_3$ (+4.0 to +6.0 mV; Fig. 5), though not as high as in 80–100% SW (+15.7 to +19.6 mV; Fig. 3), suggesting that other factors come into play here.

Killifish: the influence of choline chloride and NMDG chloride on TEP in SW- and FW-acclimated animals

Comparable experiments were performed on SW-acclimated killifish acutely exposed to the same volumetric dilution series of 500 mmol L^{-1} choline chloride and 500 mmol L^{-1} NMDG chloride. The objective of these trials was to eliminate any substantive role for $[Cl^-]$, at least in the negative TEP seen in dilute solutions. However, contrary to our expectation of invariant or more negative

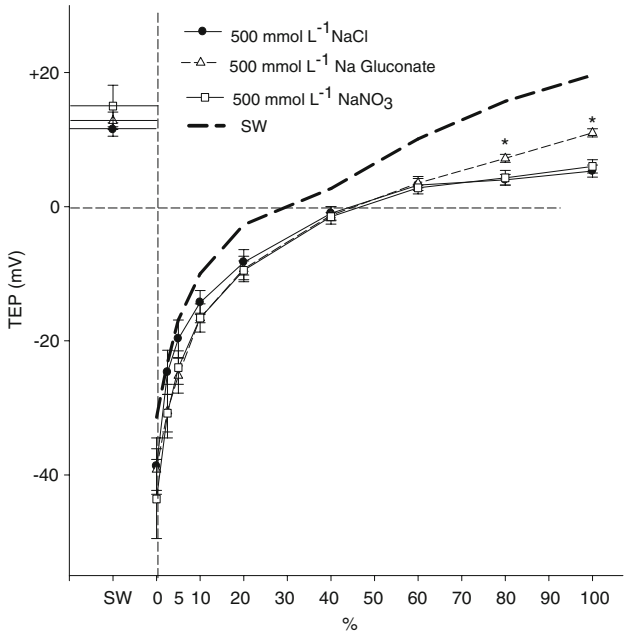

Fig. 5 The influence of acute exposure to various volumetric dilutions of 500 mmol L^{-1} NaCl (*solid circles*), 500 mmol L^{-1} Na gluconate (*open triangles*), and 500 mmol L^{-1} $NaNO_3$ (*open squares*) on TEP in SW-acclimated killifish. 0% = FW. Means ± 1 SEM ($N = 6$ for all treatments). For all three treatments, all values remained significantly below ($P \leq 0.05$) the respective original TEP values measured in these fish in 100% SW. *Significantly higher TEP ($P \leq 0.05$) in the 500 mmol L^{-1} sodium gluconate treatment at these two points relative to the other two treatments; there were no other significant differences among the three treatments. The comparable relationship for various volumetric dilutions of 100% SW is shown as a *dashed line* (data from Fig. 3, lower curve)

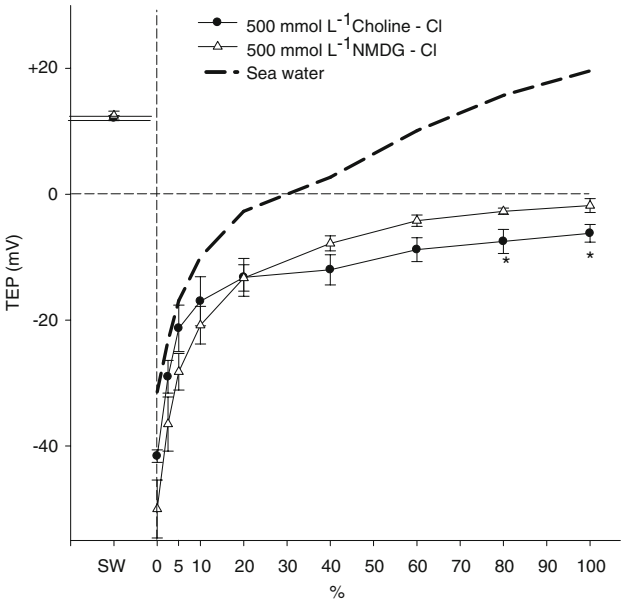

Fig. 6 The influence of acute exposure to various volumetric dilutions of 500 mmol L^{-1} choline chloride (*solid circles*) and 500 mmol L^{-1} *N*-methyl-D-glutamate chloride (NMDG-Cl, *open triangles*) on TEP in SW-acclimated killifish. 0% = FW. Means ± 1 SEM ($N = 6$ for both treatments). For both treatments, all values remained significantly below ($P \leq 0.05$) the respective original TEP values measured in these fish in 100% SW. *Significantly lower TEP ($P \leq 0.05$) in the 500 mmol L^{-1} choline-Cl treatment at these two points relative to the NMDG-Cl treatment; there were no other significant differences among the two treatments. The comparable relationship for various volumetric dilutions of 100% SW is shown as a *dashed line* (data from Fig. 3, lower curve)

potentials in these solutions, increasing concentrations of both chloride salts progressively attenuated the negative TEPs (Fig. 6). Nevertheless, the potentials remained negative, even in 500 mmol L^{-1} (i.e., 100%) solutions of each salt. Interestingly, in 80–100% solutions, TEPs were significantly more negative in choline chloride (means = −6.2 to −7.5 mV) than in NMDG-chloride (−1.8 to −2.7 mV; Fig. 6).

In contrast to SW-acclimated killifish, FW-acclimated animals exhibited no response to either 500 mmol L^{-1} choline chloride and 500 mmol L^{-1} NMDG-chloride, the TEP staying close to 0 mV, far below the = +15.5 mV seen when the same animals were exposed to 100% SW (Table 4).

Killifish: the influence of osmolality on TEP in SW-acclimated animals

One possible interpretation of these results with SW-acclimated killifish is that the similar attenuation of the negative TEP in dilute solutions seen with mixtures of either SW (Fig. 3), or NaCl (Fig. 5), or chloride-free sodium salts (Fig. 5), or sodium-free chloride salts (Fig. 6) is a non-specific response to osmolality. To evaluate this possibility, an experiment was performed on SW-acclimated killifish acutely exposed to the same volumetric dilution series of 1,100 mosm kg^{-1} solution of mannitol. This concentration was chosen to match the measured osmolality of 100% SW. However, there was no significant attenuation of the negative potential on increase of osmolality, the TEP remaining unchanged up to 100% (1,100 mosm kg^{-1} of mannitol; Fig. 7). The small non-significant variations may have reflected stress, because the fish appeared to be uncomfortable at the higher concentrations of mannitol.

Killifish: the influence of $[K^+]$ and $[Mg^{2+}]$ on TEP in SW- and FW-acclimated animals

An alternate interpretation of the effectiveness of such a wide range of salt solutions in attenuating the negative TEP (in dilute solutions) of SW-acclimated animals is that there is simply high non-specific cation permeability in the gills of these fish. If this is the case, then the reduction of other cations normally present in 100% SW may also play a role

Table 4 Responses in transepithelial potential (TEP, mV) of FW-acclimated killifish to acute exposure to either 500 mmol L^{-1} choline-Cl or 500 mmol L^{-1} NMDG-Cl, relative to TEPs in the same fish in FW or 100% SW

FW-acclimated killifish ($N = 6$)	TEP(mV)
FW	−0.2 ± 0.4
FW + 500 mmol L^{-1} choline-Cl	−0.3 ± 0.4
FW + 500 mmol L^{-1} NMDG-Cl	+1.0 ± 0.6
100% SW	+15.5 ± 1.7*

Means ± 1SEM (N)

* $P \leq 0.05$ relative to the FW value

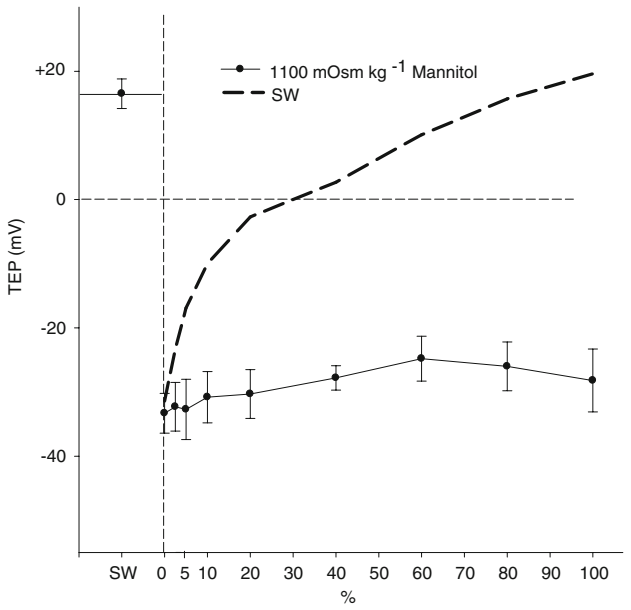

Fig. 7 The influence of acute exposure to various volumetric dilutions of 1,100 mosmol kg^{-1} mannitol (*solid circles*) on TEP in SW-acclimated killifish. 0% = FW. Means ± 1 SEM ($N = 6$). There was no significant effect ($P > 0.05$) of increasing osmolality, and all values remained significantly below ($P \leq 0.05$) the respective original TEP value measured in these fish in SW. The comparable relationship for various volumetric dilutions of 100% SW is shown as a *dashed line* (data from Fig. 3, lower curve)

in the negative TEP developed upon acute FW exposure. Therefore, an experimental series was performed in which $[K^+]$ (as KNO_3) in FW was acutely elevated to 10.5 mmol L^{-1} or $[Mg^{2+}]$ (as either $MgNO_3$ or $MgSO_4$) in FW was elevated to 60 mmol L^{-1}, i.e., to approximate the levels of these cations in 100% SW (c.f. Table 1). The two different Mg^{2+} salts were employed to check whether SO_4^{2-}, another important constituent of SW (c.f. Table 1), played any role.

All three salts (10.5 mmol L^{-1} KNO_3, 60 mmol L^{-1} $MgNO_3$, and 60 mmol L^{-1} $MgSO_4$) were equally effective in partially attenuating the negative TEP (−40.8 mV in this series) developed when SW-acclimated animals were acutely exposed to FW (Table 5). However the TEPs remained highly negative (−21.5 to −25–7 mV), and so these cations cannot support the positive TEP (+22.5 mV in this series) seen in 100% SW. The similarity in response between 60 mmol L^{-1} $MgSO_4$ and 60 mmol L^{-1} $MgNO_3$ suggests that the influence of SO_4^- is unimportant.

In contrast to SW-acclimated killifish, FW-acclimated animals exhibited no response to either 10.5 mmol L^{-1} KNO_3 or 60 mmol L^{-1} of either magnesium salt, the TEP staying in the +2 to +6 mV range, far below the +16.0 mV seen when the same animals were exposed to 100% SW (Table 5).

Discussion

TEP measurements in toadfish

The experiments with *Opsanus beta* demonstrated that TEP values measured with intraperitoneal coelomic catheters were almost identical to those made in the traditional fashion with indwelling blood vessel catheters (Fig. 1). In this regard, they are in agreement with the findings of Potts and Eddy (1973) who obtained similar TEPs from cannulae placed either in caudal blood vessels or in the peritoneal cavity of the European flounder *Platichthys stellatus*. Apparently, the conductance of the extracellular fluid is so high relative to that of the barrier epithelia that a measurement made anywhere in the extracellular compartment will yield the same result. This therefore facilitates TEP

Table 5 Responses in transepithelial potential (TEP, mV) to acute elevation in KNO_3, $Mg(NO_3)_2$ and $MgSO_4$ concentrations, relative to TEPs in the same fish in FW or 100% SW

SW-acclimated killifish ($n = 6$)	TEP (mV)
FW	−40.8 ± 4.4
FW + 10.5 mmol L^{-1} KNO_3	−25.7 ± 6.5*
FW + 60 mmol L^{-1} $Mg(NO_3)_2$	−21.5 ± 3.3*
FW + 60 mmol L^{-1} $MgSO_4$	−23.5 ± 4.7*
100% SW	+22.5 ± 1.1*
FW-acclimated killifish ($n = 6$)	
FW	+4.0 ± 0.5$^{\Delta}$
FW + 10.5 mmol L^{-1} KNO_3	+6.3 ± 1.1$^{\Delta}$
FW + 60 mmol L^{-1} $Mg(NO_3)_2$	+2.4 ± 1.6$^{\Delta}$
FW + 60 mmol L^{-1} $MgSO_4$	+6.3 ± 0.9$^{\Delta}$
100% SW	+16.0 ± 1.2*$^{\Delta}$

Responses of SW-acclimated fish and FW-acclimated fish, both tested in FW, are shown separately. Means ± 1 SEM (N)

* $P \leq 0.05$ relative to the FW value within the same acclimation group

$^{\Delta}$ $P \leq 0.05$ relative to the comparable value in SW-acclimated group

studies on small species such as *Fundulus heteroclitus* where blood vessel cannulation is impossible.

Notably, while tolerant of a wide salinity range (Wood et al. 2004; McDonald and Grosell 2006), the toadfish is not completely euryhaline and does not survive direct transfer to FW for more than about a day. The variable changes in TEP (Fig. 1, Table 2) likely reflected this pathological response. These very small absolute TEP values and inconsistent changes with salinity in *Opsanus beta* are in accordance with data reported for the same species by Kormanik and Evans (1979).

TEP measurements in intact killifish versus isolated opercular epithelia

In general, the present measurements of TEP in intact killifish, both in SW-acclimated animals and after acute transfer of the SW-acclimated animals to FW, were in good agreement with those of Pic (1978), although the present acute FW TEPs were somewhat more negative (Table 2). Pic (1978) used a similar measurement approach, intraperitoneal catheters, but implanted under cold-water anesthesia, with a shorter post-operative recovery period. Together, these two studies demonstrate that there is a wide range in TEP between SW-acclimated (positive) and animals acutely exposed to FW (negative). However, the range is less than that seen when the isolated opercular epithelium is mounted in vitro (see "Introduction"). Furthermore, an important new finding of the present study is that the highly negative potential seen upon acute FW exposure rapidly attenuates over the first 12 h of exposure, and by 24−30 h reaches a slightly positive value (Fig. 2), which is only about 10–20 mV less that seen in SW-acclimated animals (c.f. Table 2). In this respect, the intact killifish appears to be very different from the isolated opercular epithelium, which exhibits a highly negative TEP in vitro even when the donor animal has been acclimated to FW for an extended period (Wood and Marshall 1994; Marshall et al. 1997; Burgess et al. 1998). One possible explanation may be that the electrical properties of the relatively small opercular epithelia, which represent only a small percentage of the conductive surface area relative to the gills (Degnan et al. 1977; Degnan and Zadunaisky 1979), are different from those of the gills, and do not change in the same way with FW acclimation. Another is that the damage associated with dissection of the opercular epithelia away from the underlying bone becomes the dominant influence on their electrical properties when mounted in vitro, and/or that isolation from the nervous and humoral controls normally present in vivo alters their electrical properties. Regardless, one cannot base conclusions about TEP in vivo simply on the behavior of TEP in the isolated opercular epithelium. In the current study, we have assumed, in common with most authors, that the TEP in the whole animal in vivo reflects the properties of the gills, which constitute the largest conductive surface area contacting the external medium. However, it is important to keep in mind that other barrier epithelia (e.g., body or fin skin) may also make a significant contribution.

Implications of TEP measurements for Cu toxicity in killifish

The present results have implications for the analysis of Grosell et al. (2007), which related the degree of sensitivity of *Fundulus heteroclitus* to copper at different salinities to the electrochemical driving force for Na^+ across the gills at these salinities (see "Introduction") because copper appears to specifically target Na^+ transport (Blanchard and Grosell 2006). An important finding of the present experiments is that the normal TEP in vivo, when killifish are fully acclimated to their respective media, exhibits only modest variation with salinity, from about +3 mV in FW to +23 mV in 100% SW (means; Figs. 2, 3, Table 2). While this difference is not negligible, it is small compared to the wide range (0–120 mV) of absolute Nernst potentials calculated by Grosell et al. (2007) in their analysis. However, their analysis took into account only the Nernst equilibrium potential for Na^+ (Eq. 1) and not the TEP, whereas the true driving force is the difference between these two parameters, signs considered (Kirschner 1970); if large differences in TEP were to exist across salinities, the interpretation of this relationship would be altered. Since large differences do not appear to exist for killifish, which are fully acclimated to their salinities, the interpretation is supported, i.e., the greatest absolute driving force for Na^+ (Nernst potential; Eq. 1) and greatest Cu toxicity occur in FW, the next greatest in 100% SW, with the lowest values for both around isosmotic salinities (20–40%), in correlation ($r^2 = 0.93$) with Cu toxicity data (Grosell et al. 2007). When corrected for the TEP measurements of the present study, we calculate that these electrochemical driving forces for Na^+ (as absolute values) would amount to about 121 mV in FW, 6 mV in 100% SW and close to 0 mV at 20–40% SW, in accord with greater than 50-fold variation in 96 h LC50s for Cu measured by Grosell et al. (2007). However, it should be noted that killifish in their natural environment may experience rapid salinity fluctuations, and that acute Cu toxicity under such conditions may not adhere to the relationship between salinity and Cu sensitivity seen in animals acclimated to constant salinities.

Theoretical origin of TEP

Classic work several decades ago (e.g., Kerstetter et al. 1970; Potts and Eddy 1973; House and Maetz 1974; Eddy 1975; McWilliams and Potts 1978; Potts 1984; Potts and

Hedges 1991; Potts et al. 1991) has laid down the framework for most of our current understanding of TEP in fish in vivo. In brief, the TEP in FW (usually negative) is essentially a diffusion potential based on the differential permeability of the gill (see Eq. 2) to Na^+ (P_{Na} higher) versus Cl^- (P_{Cl} lower). Almost invariably, this TEP is sensitive to environmental Ca^{2+} concentration, such that a rise in [Ca^{2+}], while reducing both P_{Na} and P_{Cl}, has a greater effect on the former, such that P_{Cl}/P_{Na} increases, and TEP becomes less negative and sometimes even slightly positive. In SW, overall permeability appears to be much higher, but again P_{Na} is much greater than P_{Cl}, to such an extent that TEP (now positive due to the threefold higher [Na^+] in SW than in the blood plasma) is often close to the Nernst potential for Na^+ alone (Eq. 1). However, in addition to this diffusion potential, there is a potential of electrogenic origin due to the active outward transport of Cl^- ions. In isosmotic salinities, the Na^+ (and Cl^-) gradients essentially disappear, so the diffusion potential drops to 0 mV, and any remaining potential is associated with remnant electrogenic components.

The TEP in SW-acclimated killifish at different salinities

At least qualitatively, the present data for SW-acclimated killifish fit this framework reasonably well. Thus, the TEP was positive in SW, close to 0 mV in isosmotic salinities, and very negative after acute transfer to dilute salinities (Figs. 2, 3; Table 2). Under the latter circumstances, the TEP responded to increasing [Ca^{2+}] by becoming increasingly less negative (Fig. 4). The Na^+ diffusion potential alone provides only a qualitative description of these trends. For example, using present measurements of ion concentrations in FW and SW (Table 1), data from Scott et al. (2006) for corresponding plasma ion concentrations of *Fundulus heteroclitus*, and activity coefficients from Robinson and Stokes (1959; c.f. Potts et al. 1991), the Na^+ diffusion potential alone (E_{Na}; Eq. 1) would be +29.1 mV in 100% SW (versus a measured TEP of +22.5 mV), + 8.5 mV in 40% SW (versus a measured TEP of +4.0 mV), and −118.4 mV in FW (versus a measured TEP of − 39.4 mV). Clearly, this is an oversimplification, and some permeability to Cl^- must also be present. Assuming that these are the only two ions involved, the Goldman-Hodgkin-Katz equation (Eq. 2) can be used to estimate the permeability ratios (P_{Cl}/P_{Na}) necessary to describe this data set (e.g., Potts and Eddy 1973; Eddy 1975).

For SW-acclimated killifish, this analysis yields P_{Cl}/P_{Na} ratios of 0.09 in 100% SW, and 0.23 immediately after transfer to 40% SW or FW. If one-third of the positive TEP in 100% SW were due to the positive contribution of the electrogenic component (see below), then the true P_{Cl}/P_{Na} ratio would be 0.23 in 100% SW as well. Acutely raising the [Ca^{2+}] in synthetic FW progressively increases P_{Cl}/P_{Na} ratios from 0.26 at 10 μmol L^{-1} Ca^{2+} to 0.73 at 10,000 μmol L^{-1} Ca^{2+} (Fig. 8a). While this analysis based only on P_{Na} and P_{Cl} ignores any other possible contributors to the diffusion potentials (such as K^+ and Mg^{2+}), it provides a reasonable description of the TEP response to acute variations in salinity (Fig. 3) and [Ca^{2+}] (Fig. 4) in light of previously established theory. Indeed the similarity in the response profiles after transfer to dilutions of SW versus equivalent dilutions of 500 mmol L^{-1} NaCl (Fig. 5) lends further support to this analysis, suggesting that other constituents normally present in SW play only a small role. The moderate depression of this profile below the SW profile, especially at higher salinities, likely reflected the low level of Ca^{2+} (which would lower TEP; Fig. 4) in the dilutions of 500 mmol L^{-1} NaCl. Additionally, the lack of K^+ and Mg^{2+} concentrations normally present in SW would also tend to lower the TEP profile (Table 5). Based on the data in Fig. 4 and Table 5, the absence of each factor (Ca^{2+}, Mg^{2+}, K^+) by itself could explain about 15–25 mV of TEP depression in the 100% treatment, though the effects are unlikely to be independent of one another.

In SW-acclimated killifish, the TEP response profiles to dilutions of 500 mmol L^{-1} $NaNO_3$ or 500 mmol L^{-1} Na gluconate were somewhat surprising (Fig. 5). In the absence of external Cl^-, a substantial upward shift of the profile to more positive values than with 500 mmol L^{-1} NaCl was expected. This did not occur at all with $NaNO_3$, suggesting that the permeability of the SW-acclimated gill to NO_3^- is comparable to that of Cl^-, whereas the modest upward shift with Na gluconate suggests that the permeability to this larger molecule is less than to NO_3^- as expected, but is not negligible. Interestingly, similar results were reported by Potts and Eddy (1973) on SW-acclimated flounder when the Cl^- in 100% SW was replaced by SO_4^{2-}, succinate or benzenesulphonate, indicating that a broad range of anions have similar gill permeabilities to that of Cl^- in these SW-acclimated fish. Alternately, it is possible that these anions adsorb in some way to the gill surface, leading to an alteration in the charge at the surface, though we are aware of no evidence that this may occur. Regardless, it is notable that this did not occur when increasing concentrations of Na benzenesulphonate were tested in the FW-acclimated goldfish (Eddy 1975) or when $NaNO_3$ was tested in FW-acclimated killifish of the present study (Table 3).

Even more surprising were the TEP response profiles of SW-acclimated killifish to dilutions of 500 mmol L^{-1} choline chloride and 500 mmol L^{-1} NMDG chloride; these cations are traditionally considered to have very low permeability through biological epithelia. For example, substitution of choline for Na^+ lowered the TEP from +19

to −46 mV in SW-acclimated flounder (Potts and Eddy 1973). Contrary to our expectation of invariant or more negative potentials as the concentrations of these solutions were increased, a progressive attenuation of the negative TEP occurred (Fig. 6). Indeed, the profiles were similar to those with dilutions of 100% SW, 500 mmol L^{-1} $NaNO_3$, 500 mmol L^{-1} NaCl, or 500 mmol L^{-1} Na gluconate (Fig. 5), though they were depressed somewhat below all of these at higher concentrations (Fig. 6). These results raised the possibility that this was a general response to increasing osmolality, but the lack of response to dilutions of 1,100 mOsm kg^{-1} mannitol (Fig. 7) eliminated this explanation. Potts and Eddy (1973) similarly reported that osmolality had negligible influence on TEP in SW-acclimated flounder. Clearly, these results suggest that there is simply high non-specific cation permeability in the gills of these SW-acclimated killifish. While conductance through this pathway is normally dominated by the high Na^+ concentration in SW, a broad range of cations can be accommodated when substitutions are made.

The effects of stress on TEP in the killifish

In agreement with the original report of Pic (1978), the TEP values in SW-acclimated killifish were very sensitive to attenuation by handling stress, both in 100% SW and after transfer to FW. For this reason, data were not taken from fish which struggled (see "Results"). As originally suggested by Potts and Eddy (1973), stress may tend to abolish the selectively low permeability of the gills to anions (Cl^-), which would tend to reduce the absolute values of diffusion potentials both in SW and after acute transfer to FW. The TEPs appeared to be particularly sensitive to disturbance in 100% SW, because even the mild disturbance of repeated handling in the experimental protocol depressed TEP by about 33% in this medium, whereas there was no significant effect in FW (Table 2). A probable explanation for this additional effect in 100% SW is the influence of catecholamines released from sympathetic neurons and/or chromaffin tissue during stress. These may induce an alpha-adrenergic inhibition of the electrogenic component (active Cl^- efflux), an effect that has been well documented in the opercular epithelia of SW-acclimated killifish (e.g., Mendelsohn et al. 1981; May and Degnan 1985; Marshall et al. 1998). If this explanation is correct, then at least one-third of positive TEP in SW-acclimated animals may be of electrogenic origin.

The TEP in FW-acclimated killifish at different salinities

In contrast to SW-acclimated killifish, the situation is clearly very different in FW-acclimated killifish where the TEP is close to 0 mV. It shows negligible responses to stress, only small responses to acute variations in salinity (Fig. 3, Table 2), and no response at all to acute variations in $[Ca^{2+}]$ (Fig. 4). It is also unresponsive to acute variations in most other ions (Tables 3, 4, 5), a point to be discussed subsequently. These observations are in accord with the absence of a significant electrogenic component, and more importantly, a fundamental alteration in the permeability properties of the gills induced by FW-acclimation, which appears to take place within about 24–30 h (Fig. 2). If we assume, as before, that Na^+ and Cl^- are the principal ions involved, the Goldman-Hodgkin-Katz equation (Eq. 2) can be used to describe the effect of time in FW on the P_{Cl}/P_{Na} permeability ratios. This ratio progressively increases from 0.23 immediately after transfer, but stabilizes at about 1.3 after 24–30 h in FW (Fig. 8b). Thus, at least in a qualitative sense, the effect of FW acclimation is similar to that of acutely increasing environmental $[Ca^{2+}]$. Upon acute FW exposure, $[Ca^{2+}]$ is greatly reduced from SW levels, yet the effect of time is to raise the P_{Cl}/P_{Na} ratio back to and indeed above that dictated by SW $[Ca^{2+}]$ ($\sim$10 mmol L^{-1}; Table 1). Once this process is complete, the influence of $[Ca^{2+}]$ on P_{Cl}/P_{Na} ratio, and therefore on TEP, is lost. In this regard, the FW-acclimated killish is very different from FW-acclimated flounder (Potts and Eddy 1973), goldfish (Eddy 1975), rainbow trout (Kerstetter et al. 1970; Perry and Wood 1985) and brown trout (McWilliams and Potts 1978) where TEP and P_{Cl}/P_{Na} ratio remain highly responsive to $[Ca^{2+}]$ in the same concentration range.

The significance of the cation-selective pathway in the ionoregulatory strategy of the killifish

Fundulus heteroclitus is similar to most SW teleosts in having a gill diffusive permeability, which is cation-selective (i.e., low P_{Cl}/P_{Na}), a trait that is thought to facilitate the well-described NaCl excretion mechanism (the "Silva" model, originally proposed by Silva et al. 1977). Here electrogenic, secondary active Cl^- excretion through the transcellular pathway energizes passive Na^+ efflux through the paracellular "shunt" pathway (Wood and Marshall 1994; Marshall 2003). It is likely that the increase in P_{Cl}/P_{Na} ratio (Fig. 8b), as well as an overall reduction in gill permeability during FW-acclimation reflects a closing of the shunt pathway through the paracellular junctions between the gill cells. However, the killifish is different from many other teleosts, both in having a shunt pathway in SW which is highly permeable to a broad range of cations, as well as in rendering this pathway unresponsive to $[Ca^{2+}]$ after acclimation to FW (Fig. 4).

Many aspects of gill physiology and structure in the killifish are known to change after transfer from SW to FW,

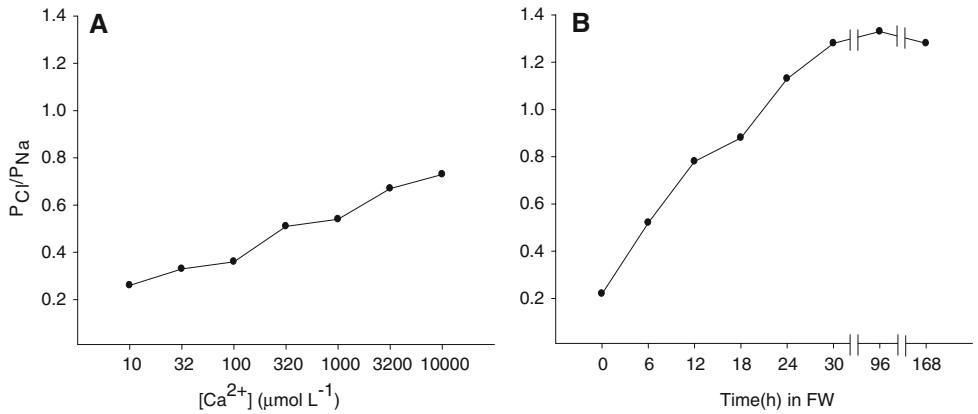

Fig. 8 Changes in P_{Cl}/P_{Na} permeability ratios in SW-acclimated killifish (**a**) after acute transfer to synthetic FW with progressive elevations of environmental $[Ca^{2+}]$ (as $Ca(NO_3)_2{\cdot}4H_2O$) in a semi-logarithmic series and (**b**) at various times after acute transfer to FW. P_{Cl}/P_{Na} ratios were calculated using the Goldman-Hodgkin-Katz equation (Eq. 2) assuming permeability to only these two ions, and using measured transepithelial potential (TEP) values in (**a**) from Fig. 4 and in (**b**) from Fig. 2. See text for other details

and could be responsible for the closing of the shunt pathway and its insensitivity to environmental $[Ca^{2+}]$ after FW acclimation. These include hormonal status (Marshall 2003), expression of transport and signaling genes (Singer et al. 1998; Kultz et al. 2001; Scott et al. 2004a, 2005; Marshall et al. 2005; Choe et al. 2006), alterations in transport protein distribution and activities (Towle et al. 1977; Marshall et al. 2002; Katoh and Kaneko 2003), increases in the depth of tight junctions accompanied by disappearance of accessory cells (Karnaky et al. 1991; Marshall et al. 1997; Katoh and Kaneko 2003; Laurent et al. 2006), a paving over of SW-type mitochondria-rich cells ("chloride cells") by pavement cells (Daborn et al. 2001; Laurent et al. 2006), increased mitotic activity in the gill epithelium (Katoh and Kaneko 2003; Laurent et al. 2006), and the emergence of mitochondria-rich cells with a very different appearance (Copeland 1950; Philpott and Copeland 1963; Marshall et al. 1997; Katoh et al. 2001; Laurent et al. 2006). It remains controversial whether these new cells are FW-type chloride cells (Katoh and Kaneko 2003), or a different cell type, which is thought to be specialized for active Na^+ uptake from dilute solutions ("cuboidal cells", Laurent et al. 2006). Regardless, it is likely that the structural changes (paving over, increasing depth of tight junctions, cell replacement) allow the progressive closing of the shunt pathway over the first 24–30 h during acclimation to FW (Figs. 2, 8b).

However, killifish live in salt marshes and estuaries where salinity may change very rapidly with the tidal cycle. They are known to make daily feeding forays into dilute salinities at the front of the tide (Griffith 1974; Abraham 1985; Marshall 2003). Structural changes for each tidal cycle would be costly. The low P_{Cl}/P_{Na} permeability of the cation-selective shunt pathway is a valuable property for an animal making frequent temporary forays into FW, especially one that is incapable of active gill Cl^- uptake in FW (Patrick et al. 1997; Patrick and Wood 1999; Wood and Laurent 2003). The change-over to a very negative TEP will immediately limit Na^+ loss in FW, while the negative potential will not interfere with active Cl^- uptake in FW because there is none! A number of other teleosts are now known to employ the strategy of no active Cl^- uptake in FW (Tomasso and Grosell 2005) offering a comparative opportunity to examine relationships between branchial TEP and ion homeostasis strategies. Regardless of the generality of the present observations, keeping the shunt permeability high for the first few hours in FW means that killifish can go back to SW and instantaneously re-activate their NaCl excretion mechanism. Only if the salinity change is relatively longlasting may it be worthwhile to invest in the structural changes, which may close the shunt. The instantaneous reduction in unidirectional Na^+ outflux in the first 30 min after transfer of killifish to FW (Motais et al. 1966; Potts and Evans 1967; Maetz et al 1967; Pic 1978) likely reflects this TEP effect, whereas the slower further reduction over 24 –48 h reported in some of these same studies likely reflects structural changes in the gill epithelium. Prodocimo et al. (2007) have recently reported changes in unidirectional Na^+ flux rates in intact killifish, which maintain internal Na^+ balance during a changing salinity regime. In future studies, it will be of interest to similarly monitor changes in TEP in *Fundulus heteroclitus* during fluctuations in salinity representative of a normal tidal cycle

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References

Abraham BJ (1985) Species profiles: life histories and environmental requirements of coastal fishes and invertebrates (mid-Atlantic)—mummichog and striped killifish. US Fish Wild Serv Biol Rep 82(11.40). U.S. Army Corps of Engineers, TR EL-82-4, p 23

Blanchard J, Grosell M (2005) Effects of salinity on copper accumulation in the common killifish (*Fundulus heteroclitus*). Environ Toxicol Chem 24:1403–1413

Blanchard J, Grosell M (2006) Copper toxicity across salinities from freshwater to seawater in the euryhaline fish, *Fundulus heteroclitus*: is copper an ionoregulatory toxicant in high salinities? Aquat Toxicol 80:131–139

Burgess DW, Marshall WS, Wood CM (1998) Ionic transport by the opercular epithelia of freshwater acclimated tilapia (*Oreochromis niloticus*) and killifish (*Fundulus heteroclitus*). Comp Biochem Physiol A 121:155–164

Choe KP, Havird J, Rose R, Hyndman K, Piermarini P, Evans DH (2006) COX2 in a euryhaline teleost, *Fundulus heteroclitus*: primary sequence, distribution, localization, and potential function in the gills during salinity acclimation. J Exp Biol 209:1696–1708

Copeland DE (1950) Adaptive behaviour of the chloride cell in the gill of *Fundulus heteroclitus*. J Morph 87:369–379

Daborn K, Cozzi RRF, Marshall WS (2001) Dynamics of pavement cell-chloride cell interactions during abrupt salinity change in *Fundulus heteroclitus*. J Exp Biol 204:1889–1899

Degnan KJ, Karnaky KJ, Zadunaisky JA (1977) Active chloride transport in the in vitro opercular skin of a teleost (*Fundulus heteroclitus*), a gill-like epithelium rich in chloride cells. J Physiol 271:155–191

Degnan KJ, Zadunaisky JA (1979) Open-circuit sodium and chloride fluxes across isolated opercular epithelium from the teleost *Fundulus heteroclitus*. J Physiol 294:483–495

Degnan KJ, Zadunaisky J A (1980) Passive sodium movement across the opercular epithelium: the paracellular shunt pathway and ionic conductance. J Membr Biol 55:175–185

Eddy FB (1975) The effect of calcium on gill potentials and on sodium and chloride fluxes in the goldfish, *Carassius auratus*. J Comp Physiol 96:131–142

Goldman DE (1943) Potential, impedance, and rectification in membranes. J Gen Physiol 27:37–60

Grosell M, Blanchard J, Brix KV, Gerdes R (2007) Physiology is pivotal for interactions between salinity and acute toxicity to fish and invertebrates. Aquat Toxicol 82:162–172

Griffith RW (1974) Environment and salinity tolerance in the genus *Fundulus*. Copeia 1974:319–331

House CR, Maetz J (1974) On the electrical gradient across the gill of the seawater adapted eel. Comp Biochem Physiol 47A:917–924

Karnaky KJ (1980) Ion-secreting epithelia: chloride cells in the head region of *Fundulus heteroclitus* Am J Physiol 238:R185–R198

Karnaky KJ (1991) Teleost osmoregulation: changes in the tight junction in response to the salinity of the environment. In: Cereijido M (ed) Tight junctions. CRC Press, Boca Raton, pp 175–185

Katoh F, Hasegawa S, Kita J, Takagi Y, Kaneko T (2001) Distinct seawater and freshwater types of chloride cells in killifish, *Fundulus heteroclitus*. Can J Zool 79:822–829

Katoh F, Kaneko T (2003) Short-term transformation and long-term replacement of branchial chloride cells in killifish transferred from seawater to freshwater, revealed by morphofunctional observations and a newly established "time-differential double fluorescent staining' technique. J Exp Biol 206:4113–4123

Kerstetter TH, Kirschner LB, Rafuse LB (1970) On the mechanisms of sodium ion transport by the irrigated gills of rainbow trout (*Salmo gairdneri*). J Gen Physiol 56:342–359

Kirschner LB (1970) The study of NaCl transport in aquatic animals. Am Zool 10:365–376

Kormanik GA, Evans DH (1979) HCO_3^--stimulated Cl^- efflux in the gulf toadfish acclimated to sea water. J Exp Zool 208:13–16

Kültz D, Chakravarty D, Adilakshmi T (2001) A novel 14–3-3 gene is osmoregulated in gill epithelium of the euryhaline teleost *Fundulus heteroclitus*. J Exp Biol 204:2975–2985

Laurent P, Chevalier C, Wood CM (2006) Appearance of cuboidal cells in relation to salinity in the gills of *Fundulus heteroclitus*, a species exhibiting branchial Na^+ but not Cl^- uptake in freshwater. Cell Tissue Res 325:481–492

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

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

Marshall WS, Bryson SE, Darling P, Whitten C, Patrick M, Wilkie M, Wood CM, Buckland Nicks J (1997) NaCl transport and ultrastructure of opercular epithelium from a freshwater-adapted euryhaline teleost, *Fundulus heteroclitus*. J Exp Zool 277: 23–37

Marshall WS, Bryson SE (1998) Transport mechanisms of seawater teleost chloride cells: an inclusive model of a multifunctional cell. Comp Biochem Physiol A119:97–106

Marshall WS, Duquesnay RM, Gillis JM, Bryson SE, Liedtke CM (1998) Neural modulation of salt secretion in teleost opercular epithelium by α-adrenergic receptors and inositol 1,4,5-trisphosphate. J Exp Biol 201:1959–1965

Marshall WS, Lynch EA, Cozzi RRF (2002) Redistribution of immunofluorescence of CFTR anion channel and NKCC cotransporter in chloride cells during adaptation of the killifish *Fundulus heteroclitus* to sea water. J Exp Biol 205:1265–1273

Marshall WS, Ossum CG, Hofmann EK (2005) Hypotonic shock mediation by p38 MAPK, JNK, PKC, FAK, OSR1, and SPAK in osmosensing chloride secreting cells of killifish opercular epithelium. J Exp Biol 208:1063–1077

Marshall WS, Grosell M (2006) Ion transport, osmoregulation, and acid–base balance. In: Evans DH, Claiborne JB (eds) Physiology of fishes. CRC Press, Boca Raton, pp 177–230

May SA, Degnan KJ (1985) Converging adrenergic and cholinergic mechanisms in the inhibition of Cl^- secretion in fish opercular epithelium. J Comp Physiol B156:183–189

McDonald MD, Grosell M (2006) Maintaining osmotic balance with an aglomerular kidney. Comp Biochem Physiol A143:447–458

McWilliams PG, Potts WTW (1978) The effects of pH and calcium concentrations on gill potentials in the brown trout, *Salmo trutta*. J Comp Physiol 126:277–286

Mendelsohn SA, Cherksey BD, Degnan KJ (1981) Adrenergic regulation of chloride secretion across the opercular epithelium: the role of cyclic AMP. J Comp Physiol B145:125–135

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

Patrick ML, Pärt P, Marshall WS, Wood CM (1997) Characterization of ion and acid–base transport in the fresh-water adapted mummichog (*Fundulus heteroclitus*). J Exp Zool 279:208–219

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

Péqueux A, Gilles R, Marshall WS (1988) NaCl transport in gills and related structures. In: Gilles R (ed) Advances in comparative and environmental physiology, vol 1. Springer, Heidelberg, pp 1–73

Perry SF, Wood CM (1985) Kinetics of branchial calcium uptake in the rainbow trout: effects of acclimation to various external calcium levels. J Exp Biol 116:411–433

Philpott CW, Copeland DE (1963) Fine structure of chloride cells from three species of *Fundulus*, J Cell Biol 18:389–404

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

Potts WTW (1984) Transepithelial potentials in fish gills. In: Hoar WS, Randall DJ (eds) Fish physiology, vol 10B. Academic, Orlando, pp 105–128

Potts WTW, Evans DH (1967) Sodium and chloride balance in the killifish *Fundulus heteroclitus*. Biol Bull 133:411–425

Potts WTW, Eddy FB (1973) Gill potentials and sodium fluxes in the flounder *Platichthys stellatus*. J Comp Physiol 87:20–48

Potts WTW, Hedges AJ (1991) Gill potentials in marine teleosts. J Comp Physiol B161:401–405

Potts WTW, Fletcher CR, Hedges AJ (1991) The in vivo transepithelial potential in a marine teleost. J Comp Physiol B161:393–400

Prodocimo V, Galvez F, Freire CA, Wood CM (2007) Unidirectional Na^+ and Ca^{2+} fluxes in two euryhaline teleost fishes, *Fundulus heteroclitus* and *Oncorhynchus mykiss*, acutely submitted to a progressive salinity increase. J Comp Physiol B177:519–528

Robinson RA, Stokes RH (1959) Electrolyte solutions. Butterworth, London, p 559

Scott GR, Richards JG, Forbush B, Isenring P, Schulte PM (2004a) Changes in gene expression in gills of the euryhaline killifish *Fundulus heteroclitus* after abrupt salinity transfer. Am J Physiol 287:C300–C309

Scott GR, Rogers JT, Richards JG, Wood CM, Schulte PM (2004b) Intraspecific divergence of ionoregulatory physiology in the euryhaline teleost *Fundulus heteroclitus*: possible mechanisms of freshwater adaptation. J Exp Biol 207:3399–3410

Scott GR, Claiborne JB, Edwards SL, Schulte PM, Wood CM (2005) Gene expression after freshwater transfer in gills and opercular epithelia of killifish: insight into divergent mechanisms of ion transport. J Exp Biol 208:2719–2729

Scott GR, Schulte PM, Wood CM (2006) Plasticity of osmoregulatory function in the killifish intestine: drinking rates, salt and water transport, and gene expression after freshwater transfer. J Exp Biol 209:4040–4050

Silva P, Solomon R, Spokes K, Epstein FH (1977) Ouabain inhibition of gill Na^+-K^+-ATPase: relationship to active chloride transport. J Exp Zool 199:419–426

Singer TD, Tucker SJ, Marshall WS, Higgins CF (1998) A divergent CFTR homologue: highly regulated salt transport in the euryhaline teleost *F. heteroclitus*. Am J Physiol 43:C715–C723

Sten-Knudsen O (2002) Biological membranes: theory of transport, potentials, and electrical impulses. Cambridge University Press, Cambridge

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

Towle DW, Gilman ME, Hempel JD (1977) Rapid modulation of gill $Na^+ + K^+$-dependent ATPase activity during acclimation of killifish *Fundulus heteroclitus* to salinity change. J Exp Zool 202:179–185

Wolf K (1963) Physiological salines for fresh-water teleosts. Progr Fish Cult 25:135–140

Wood CM, Hopkins TE, Walsh PJ (1997) Pulsatile urea excretion in the toadfish (*Opsanus beta*) is due to a pulsatile excretion mechanism, not a pulsatile production mechanism. J Exp Biol 200:1039–1046

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

Wood CM, Marshall WS (1994) Ion balance, acid–base regulation, and chloride cell function in the common killifish, *Fundulus heteroclitus*–a euryhaline estuarine teleost. Estuaries 17:34–52

Wood CM, McDonald MD, Walker P, Grosell M, Barimo JF, Playle RC, Walsh PJ (2004) Bioavailability of silver and its relationship to ionoregulation and silver speciation across a range of salinities in the gulf toadfish (*Opsanus beta*). Aquat Toxicol 70:137–157

Zadunaisky JA (1984) The chloride cell: the active transport of chloride and the paracellular pathways. In: Hoar WS, Randall DJ (eds) Fish physiology, vol 10B. Academic, Orlando, pp 130–176