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ORIGINAL PAPER

TEP on the tide in killifish (*Fundulus heteroclitus*): effects of progressively changing salinity and prior acclimation to intermediate or cycling salinity

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Abstract Transepithelial potentials (TEP) were measured in killifish, acclimated to freshwater (FW), seawater (SW), 33% SW or cycling salinities relevant to tidal cycles in an estuary, and subsequently subjected to salinity changes in progressive or random order. Random compared to progressive salinity changes in an upward or downward direction in FW- and SW-acclimated fish, respectively, did not greatly influence responses to salinity change. Fish acclimated to SW or 33% SW as well as those acclimated to cycling salinities behaved similarly (TEP more positive than +15 mV in 100% SW, decreasing to ~0 mV at 20–40% SW, and more negative than −30 mV in FW). In contrast, FW-acclimated fish displayed a less pronounced TEP response to salinity (0 mV in FW through 20% SW, increasing thereafter to values more positive than +10 mV at 100% SW). We conclude that when evaluated under estuarine tidal conditions, the killifish gill exhibits adaptive electrical characteristics, opposing Na^+ loss at low salinity and favouring Na^+ extrusion at high salinity, changes explained at least in part by the Cl^- to Na^+ permeability ratio. Thus animals living in the estuaries can move to lower and higher salinities for short periods with little physiological disturbance, but this ability is lost after acclimation to FW.

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Introduction

In a recent study (Wood and Grosell 2008), we validated an intraperitoneal catheterization technique (Pic 1978) for measuring transepithelial potential (TEP) in the common killifish (*Fundulus heteroclitus*), a small species which has been widely used as an experimental model for the mechanisms of euryhalinity (reviewed by Zadunaisky 1984; Wood and Marshall 1994; Marshall and Bryson 1998; Marshall 2003; Wood and Laurent 2003). Animals acclimated to and tested in 100% seawater (SW) exhibited strongly positive TEP values (greater than +15 mV, external reference). These values decreased when the fish were acutely exposed to lower salinities, falling below 0 mV between 20% SW and 40% SW, and reaching highly negative values (lower than −30 mV) upon acute exposure to FW. After acclimation to FW, much of the sensitivity of TEP to acute salinity change disappeared. In FW through to 20% SW, TEP stayed constant close to 0 mV, whereas abrupt transfer of FW-acclimated fish to higher salinities induced a rise in TEP but not to the levels seen in 100% SW-acclimated animals (Wood and Grosell 2008).

Based on a variety of ion substitution experiments and previously established theory (e.g. Potts and Eddy 1973; Eddy 1975; Potts 1984; Potts and Hedges 1991; Potts et al. 1991), the following interpretation was offered (Wood and Grosell 2008). The gills of 100% SW-acclimated killifish exhibit high non-specific cation permeability and low non-specific anion permeability, largely via the paracellular "shunt" pathway. As a result, Na^+ permeability is much higher than Cl^-

permeability, resulting in a positive diffusion potential at high salinities, superimposed on which is a small positive electrogenic potential due to the secondary active extrusion of Cl^-. As salinity decreases, the electrogenic potential is diminished, and the diffusion potential becomes increasingly negative below the isotonic point (i.e. between 40 and 20% SW). It was argued that the positive TEP at high salinity is adaptive for the passive extrusion of Na^+ through the paracellular "shunt" pathway in the gills (Degnan and Zadunaisky 1980), a model first proposed by Silva et al. (1977). Conversely, the negative TEP upon acute exposure to dilute salinities is adaptive in immediately limiting Na^+ loss, while the negative potential will not impose a challenge to active Cl^- uptake at the gills because there is none in this species in FW (Patrick et al. 1997; Patrick and Wood 1999; Wood and Laurent 2003; Scott et al. 2004). Such a strategy would be adaptive for an animal living in high salinity but making periodic brief feeding forays into dilute salinities. On the other hand, acclimation to FW, a process which takes about 24 h before TEP stabilizes at ~0 mV, necessitates the commitment to structural changes in the gills which tend to shut down the paracellular shunt pathway, such that P_{Cl}/P_{Na} increases to >1.0. These animals are now well-adapted for life in FW, but not to move rapidly back and forth to higher salinities.

A potential problem with these theories is that they are based only on experiments with killifish acclimated to either FW or 100% SW and acutely exposed to different salinities. However, in the real world, killifish live in estuaries and tidal marshes subject to two tides a day on the east coast of North America. Their home range is small, and therefore they will be exposed to tide-related fluctuations in salinity; killifish probably spend the bulk of their time at intermediate salinities and move into very dilute salinities only briefly to feed at the front of the tide (Griffith 1974; Abraham 1985; Kneib 1986; Marshall 2003). Indeed, the preferred salinity of *F. heteroclitus* is reported to lie in the range 22–57% SW (Fritz and Garside 1974). Most salinity changes which they experience will be progressive rather than acute, and are likely reversed on the next tide.

Thus, if the basic ideas laid out in Wood and Grosell (2008) are correct, we hypothesized that the supposed adaptive trends in TEP would be seen to an equal or even greater degree in killifish subjected to more environmentally relevant salinity changes, i.e. either progressively upward or downward over a 6 h tidal cycle, than in killifish step-changed from 100% SW- or FW-acclimation to an intermediate salinity. We further postulated that in killifish acclimated to an intermediate salinity (33%) close to isotonic; the basic TEP response versus salinity profile should be similar to that of a 100% SW-acclimated fish and not to that of a FW-acclimated fish, if the responses were to be of adaptive value. (Note that Na^+ and Cl^- concentrations in 33% SW would be about 160 and 188 mmol L^{-1} respectively, whereas in killifish blood plasma, their concentrations would normally be in the 130–160 mmol L^{-1} range (Scott et al. 2006, 2008, so 33% SW is slightly hypertonic). Furthermore we proposed that the responses of these 33% SW-acclimated fish to progressive upward or downward changes in salinity might well be greater than in their FW-acclimated or 100% SW-acclimated counterparts. In other words, we hypothesized that the TEP's of the 33% SW-acclimated fish would be more positive at high salinities, and more negative at low salinities. Finally, we acclimated killifish to a tidal cycle where salinity changed back and forth between FW and 100% SW over 6 h intervals twice per day in a progressive fashion, as might occur in an estuary. We hypothesized that the basic TEP response versus salinity profiles of these cycling fish would again be more similar to those of 100% SW-acclimated fish than to FW-acclimated fish, and that the absolute changes in TEP in the cycling fish would be greater, and therefore more adaptive, than in either of the comparison groups.

Materials and methods

Experimental animals and acclimation conditions

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. They were held at the University of Miami for approximately 1 year in flowing 100% SW at the experimental temperature (24–26°C), with daily feeding of an approximately 3% body mass ration of commercial pellets. Experiments were performed in spring, 2008. Acclimations to conditions other than 100% SW (i.e. FW, 33% SW, or a tidal cycle) were done under flow-through conditions for 7–10 days prior to experimentation. Daily feeding was continued during acclimation but suspended 1 day prior to experimentation. Compositions of Miami SW (100% = 37.5 ppt) and FW have been reported by Wood and Grosell (2008).

Intraperitoneal catheters for TEP measurements (PE-50 polyethylene tubing with PE-160 sleeves, filled with saline) were implanted under MS-222 anaesthesia (200 mg L^{-1}) exactly as described by Wood and Grosell (2008). The anaesthetic water was appropriate to the acclimation condition (100% SW, 33% SW, FW, or in the case of animals acclimated to the tidal cycle, 50% SW) The animals were then allowed to recover for 24 h in their experimental chambers which were continually flushed with water of the appropriate composition, including fluctuating composition when relevant. These chambers were polyethylene food containers of 150 ml volume, fitted with individual inflows and air-lines.

Simple gravity-fed mixing chambers were fed with Miami SW and FW, and used to establish acclimation conditions of 33% SW and the twice per day tidal cycle. In the former, daily salinity measurements with a refractometer indicated that the actual range was 29–37% SW. The tidal cycle fluctuated from 100% SW at 6:00 am, to FW at 12:00 noon, then to 100% SW at 6:00 pm, and FW again at 12:00 midnight. Within each 6 h segment, the salinity change was approximately linear over time, so that the mean time-averaged exposure salinity was 50% SW.

TEP measurements

Transepithelial potential (TEP) was measured by means of 3 M KCl-agar bridges connected via Ag/AgCl electrodes to a high impedance electrometer (Radiometer pHM 84, Copenhagen, Denmark). The reference bridge was placed in the water in the fish chamber, and the measurement bridge was connected to 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. Stable TEP's were generally recorded within 2 min of attaching the agar bridges.

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. In the case of all progressive salinity change experiments, the water flowed directly from the tidal cycle mixing tank to the fish holding containers. Salinity was checked with a refractometer at the time of TEP recording and manually adjusted if it was not within ±2% SW of the intended value.

Experimental treatments

1. FW-acclimated killifish subjected to acute increases in salinity

FW-acclimated animals ($N = 6$) were exposed to acute changes (increases) in salinity (FW, 10, 20, 40, 60, 80, and 100% SW). The order of application of the step changes was randomized. The TEP electrodes were attached 2–3 min after the salinity change, and stable TEP values were recorded within 2 min, so the total exposure time at each salinity was about 5 min. In between each measurement, the animal was returned to FW for 5 min. The idea was to examine the response to a step change (an increase) in salinity.

2. FW-acclimated killifish subjected to progressive increases in salinity

FW-acclimated animals ($N = 8$) were exposed to progressive changes (increases) in salinity (FW, 10, 20, 40, 60, 80, and 100% SW) over 6 h. The rate of salinity increase was 15–20% SW per hour. TEP measurements were taken at 0–0.5 h for FW, 0.5–1 h for 10% SW, 1–2 h for 20% SW, 2–3 h for 40% SW, 3–4 h for 60% SW, 4–5 h for 80% SW, and 5–6 h for 100% SW. Note that the exposure to increasing salinity was continuous, and the animals were not returned to FW between tests, in contrast to series 1. To examine the potential for an artifact induced by this procedure of multiple repeat measurements, a separate group ($N = 5$) were subjected to the same progressive salinity increase but TEP recordings were made only once when the highest salinity (100% SW) had been reached at 5–6 h.

3. 100% SW-acclimated killifish subjected to acute decreases in salinity

100% SW-acclimated animals ($N = 8$) were exposed to acute changes (decreases) in salinity (100, 80, 60, 40, 20, 10% SW, and FW). The order of application of the step changes was randomized. As in series 1, the TEP electrodes were attached 2–3 min after the salinity change, and stable TEP values were recorded within 2 min, so the total exposure time at each salinity was about 5 min. In between each measurement, the animal was returned to 100% SW for 5 min in between tests. Again the idea was to examine the response to a step change (a decrease this time) in salinity.

4. 100% SW-acclimated killifish subjected to progressive decreases in salinity

100% SW-acclimated animals ($N = 5$) were exposed to progressive changes (decreases) in salinity (100, 80, 60, 40, 20, 10% SW, and FW) over 6 h. The rate of salinity decrease was 15–20% SW per hour. TEP measurements were taken at 0–1 h for 100% SW, 1–2 h for 80% SW, 2–3 h for 60% SW, 3–4 h for 40% SW, 4–5 h for 20% SW, 5–5.5 h for 10% SW, and 5.5–6 h for FW. Note that the exposure to decreasing salinity was continuous, and the animals were not returned to 100% SW between tests, in contrast to series 3.

5. 33% SW-acclimated killifish subjected to progressive decreases or increases in salinity

TEP's were initially recorded from 33% SW-acclimated animals ($N = 8$) in their acclimation salinity, and then subsequently from the same animals subjected to the relevant downward portion (20% SW, 10% SW, and FW) of progressive change, as in series 4. TEP's were also recorded from a second set of 33% SW-acclimated animals ($N = 8$) in their acclimation salinity, and then subsequently from the same animals subjected to the relevant upward portion (40, 60, 80, and 100% SW) of progressive change, as in series 2.

6. Tidal cycle-acclimated killifish subjected to progressive increases in salinity

TEP measurements were begun on tidal cycle-acclimated killifish ($N = 7$) at 12:00 noon when salinity was at its lowest (i.e. FW). Measurements then continued during progressively increasing salinity (10, 20, 40, 60, 80 and 100% SW) over the ensuing 6 h, as in series 2.

7. Tidal cycle-acclimated killifish subjected to progressive decreases in salinity

TEP measurements were begun on tidal cycle-acclimated killifish ($N = 8$) at 6:00 am when salinity was at its highest (i.e. 100% SW). Measurements then continued during progressively decreasing salinity (80, 60, 40, 20 and 10% SW, FW) over the ensuing 6 h, as in series 4.

Statistics

Data have been expressed as means ±1 SEM (N). The significance of differences ($P \leq 0.05$) between means of different treatments at the same salinity was assessed using Student's unpaired two-tailed t test, with the designated Bonferroni correction when more than one comparison was made.

Results

Responses of FW-acclimated killifish to increases in salinity; progressive versus acute changes

This experiment tested the hypothesis that progressive upward salinity change, as would occur on the incoming tidal cycle, would result in more adaptive TEP changes at higher salinities (i.e. higher TEP's at and above 40% SW) than would randomized acute changes from FW to high salinity. The results indicated the opposite (Fig. 1). While both treatment groups exhibited TEP's which were not significantly different from 0 mV in FW, from 40% SW onwards, the TEP tended to increase to a lesser extent with salinity in the progressive exposure group than in the acute treatment group, with significant differences at 80% and 100% SW.

Earlier we noted that cumulative experimental stress could reduce the TEP at high salinity (Wood and Grosell 2008). Since the progressive exposure group had been subjected to 6–7 successive TEP measurements by the time the highest salinities (80% and 100% SW measurements) were reached, a control experiment was performed to check whether cumulative handling stress was a confounding factor depressing the TEP values in this group. FW-acclimated killifish were subjected to the same progressive salinity increase in the same manner, but TEP was measured only once, when the highest salinity (100% SW) had been reached after 6 h. The measured TEP of +11.9 ± 1.0 (5) mV was not significantly higher than the value of +10.5 ± 0.7 (8) mV recorded in the original progressive series, and was still significantly lower than in the original acute series (+15.5 ± 1.0 (8) mV). Therefore the conclusion remains unchanged.

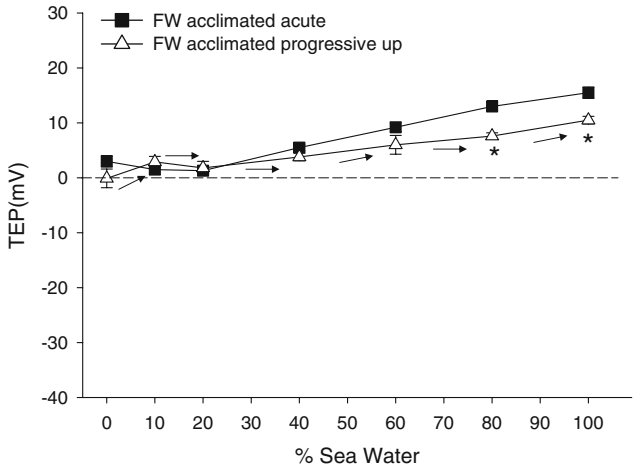

Fig. 1 The TEP response pattern of FW-acclimated killifish to either acute changes in salinity in random order, with intervening return to FW ($N = 6$, *closed squares*) or progressive increases in salinity over 6 h ($N = 8$, *open triangles*) as indicated by the *arrows*. 0% = FW. Means ± 1 SEM. Where *error bars* are not shown, the SEM's lay within the height of the symbol. *Asterisk* indicates significant difference ($P \leq 0.05$) between the two treatments at the same salinity

Responses of SW–acclimated killifish to decreases in salinity; progressive versus acute changes

This experiment tested the reciprocal hypothesis, that progressive downward salinity change, as would occur on the outgoing tidal cycle, would result in more adaptive TEP's at lower salinities (i.e. higher TEP's at and above 40% SW, lower TEP's at and below 20% SW) than would randomized acute changes from 100% SW to low salinity. The results supported this hypothesis, with the progressive treatment group exhibiting a significantly more positive TEP at 40% SW (Fig. 2), and a significantly more negative TEP at 10% SW, though there was no significant difference at 0% SW (i.e. FW; Fig. 2).

Responses of 33% SW-acclimated killifish to progressive increases and decreases in salinity

We had hypothesized that acclimation to this intermediate salinity, close to isotonic to body fluids, would result in a TEP versus salinity response profile more similar to that of

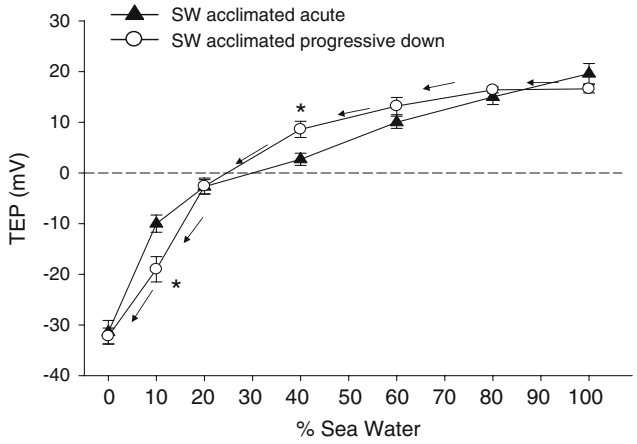

Fig. 2 The TEP response pattern of 100% SW-acclimated killifish to either acute changes in salinity in random order, with intervening return to 100% SW ($N = 8$, *closed triangles*) or progressive decreases in salinity over 6 h ($N = 5$, *open circles*) as indicated by the *arrows*. 0% = FW. Means ± 1 SEM. Where *error bars* are not shown, the SEM's lay within the height of the symbol. *Asterisk* indicates significant difference ($P \leq 0.05$) between the two treatments at the same salinity

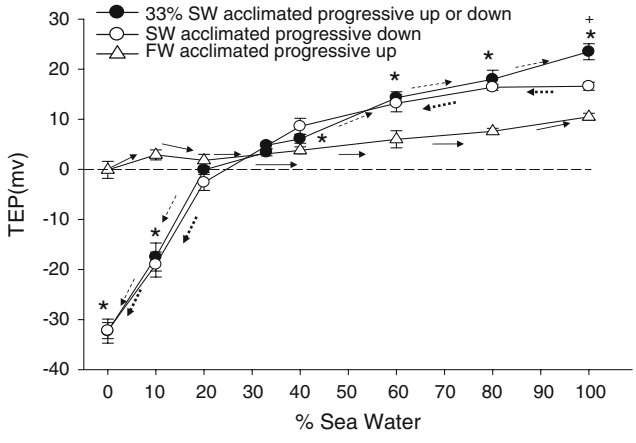

Fig. 3 The TEP response patterns of two groups of 33% SW-acclimated killifish (*closed circles*, $N = 8$ in both groups) to either progressive decreases in salinity or progressive increases in salinity as indicated by the *thin broken arrows*. The initial recordings from both groups were taken at 33% SW, and were not significantly different ($P > 0.05$). The response patterns of FW-acclimated killifish ($N = 8$, *open triangles*) to progressive increases in salinity over 6 h, as indicated by *closed arrows*, and of 100% SW-acclimated killifish ($N = 5$, *open circles*) to progressive decreases in salinity over 6 h, as indicated by the *thick broken arrows*, are also illustrated. 0% = FW. Means ± 1 SEM. Where *error bars* are not shown, the SEM's lay within the height of the symbol. *Asterisk* indicates significant difference ($P \leq 0.05$) between the 33% SW-acclimated fish and the FW-acclimated fish at the same salinity; *Plus* indicates significant difference ($P \leq 0.05$) between the 33% SW-acclimated fish and the 100% SW-acclimated fish at the same salinity

a SW-acclimated animal than to that of a FW-acclimated animal. Furthermore we proposed that the responses of these 33% SW-acclimated fish to progressive upward or downward changes in salinity would be greater than in their FW-acclimated or 100% SW-acclimated counterparts.

The first hypothesis was strongly confirmed. At their acclimation salinity, both of the 33% SW-acclimated groups exhibited TEP values (+3.4 ± 0.7 (8) mV, +4.8 ± 0.7 (8) mV) which were indistinguishable from the lines of either the FW-acclimated or 100% SW-acclimated groups at comparable salinity (Fig. 3). However, when subjected to progressively lower salinities, the 33% SW-acclimated fish tracked the response profile of the 100% SW-acclimated fish to downward salinity change. When subjected to progressively higher salinities, they again tracked the response profile of the 100% SW-acclimated group, even though the latter were exposed to downward rather than upward changes. At salinities less than 20% SW, TEP's were significantly more negative than in the FW-acclimated group, and in salinities greater than 33% SW, TEP's were significantly more positive than in the FW-acclimated group (Fig. 3).

With respect to the second hypothesis, it was certainly confirmed for progressive upward changes in salinity above 33% SW, where TEP's were significantly higher than in the FW-acclimated fish (Fig. 3). The greatest difference was at 100% SW. Indeed, here, the TEP in the 33% SW-acclimated group (+23.5 ± 1.6 (8) mV) was far above that in the FW-acclimated group (+10.5 ± 0.7 (8) mV) and even significantly greater than the starting value in the 100% SW-acclimated group (16.6 ± 0.8 (5) mV). However, the hypothesis was not confirmed for progressive downward changes in salinity below 33% SW, where the response profile of the 33% SW-acclimated killifish was identical to that of the 100% SW-acclimated group, as noted above.

Responses of tidal cycle-acclimated killifish to progressive increases and decreases in salinity

A first experiment tested the hypothesis that acclimation to a regime which cycled between FW and 100% SW at 6 h intervals would result in TEP's which were more adaptive to progressive change across the whole salinity range than those exhibited by FW-acclimated animals. Thus TEPs would be more negative at or below 20% SW at lower salinities and more positive at or above 40% SW in the cycling fish than in the FW-acclimated fish. Note that for both groups, the progressive salinity change was in the upward direction (FW to 100% SW over 6 h), and for the cycling group started at its nadir in salinity (i.e. FW) at 12:00 noon. The overall hypothesis was strongly confirmed. TEPs were significantly lower in FW and in 10% SW, and significantly higher in 40, 60, 80, and 100% SW in the cycling group (Fig. 4a).

A second experiment tested the hypothesis that acclimation to the cycling salinity regime would also result in

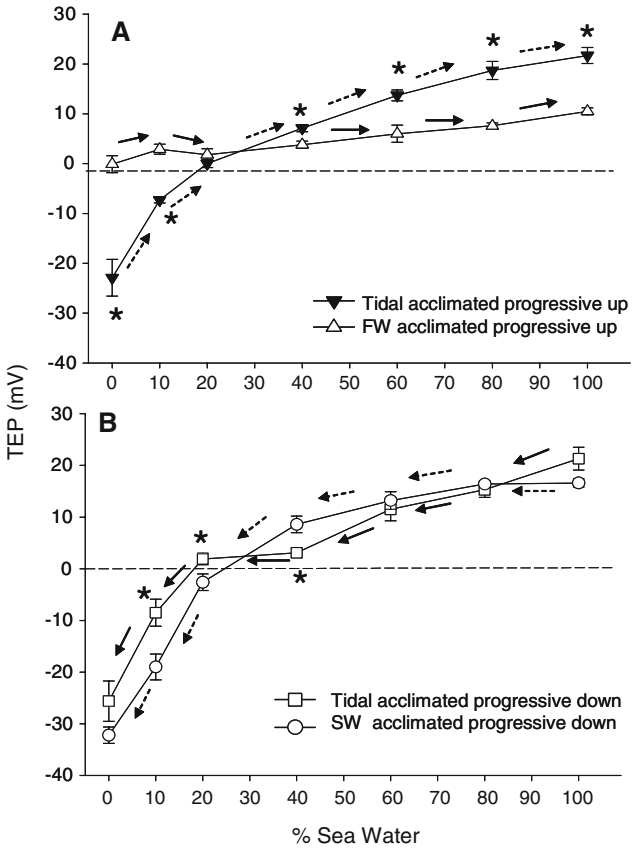

Fig. 4 **a** The TEP response pattern of killifish acclimated to a tidal cycle in salinity (FW to 100% SW to FW to 100% SW every 24 h) to progressive increases in salinity over 6 h ($N = 7$, *closed triangles*), as indicated by the *broken arrows*, starting from the nadir in salinity (FW) at 12:00 noon. The TEP response pattern of FW-acclimated killifish ($N = 8$, *open triangles*) to progressive increases in salinity over 6 h, as indicated by *solid arrows*, is also shown. 0% = FW. **b** The TEP response pattern of killifish acclimated to a tidal cycle in salinity (FW to 100% SW to FW to 100% SW every 24 h) to progressive decreases in salinity over 6 h ($N = 8$, *open squares*), as indicated by the *solid arrows*, starting from the zenith in salinity (100% SW) at 6:00 am. The TEP response pattern of 100% SW-acclimated killifish ($N = 5$, *open circles*) to progressive decreases in salinity over 6 h, as indicated by *broken arrows*, is also shown. 0% = FW. Means ± 1 SEM. Where *error bars* are not shown, the SEM's lay within the height of the symbol. *Asterisk* indicates significant difference ($P \leq 0.05$) between the two treatments at the same salinity

TEP's which were again more adaptive to progressive change across the whole salinity range than those exhibited by 100% SW-acclimated animals. Note that for both groups, the progressive salinity change was in the downward direction (100% SW to FW over 6 h), and for the cycling group started at its zenith in salinity (i.e. 100% SW) at 6:00 am. This hypothesis was not confirmed. Indeed TEP in the cycling fish was significantly lower than in the 100% SW-acclimated fish when exposed to 40% SW, yet was significantly higher than in this group at 20% SW and 10% SW (Fig. 4b). At other salinities there were no significant differences. Interestingly, there was virtually no hysteresis in the responses profiles of the cycling fish, which were similar for progressive salinity changes in both directions (Fig. 4a vs. b); the only significant difference was minor, a higher TEP in the upward regime (7.1 ± 0.7 (7) mV) than in the downward regime (3.1 ± 0.9 (8) mV) at 40% SW.

In accord with an additional hypothesis, the response profiles of the cycling fish to progressive salinity changes in both the upward (Fig. 4a) and downward directions (Fig. 4b) were generally similar to those of 100% SW-acclimated fish, and very different from those of FW-acclimated fish. In this respect, killifish acclimated to cycling salinity behaved very similarly to those acclimated to 33% SW (Fig. 3). Indeed in relevant comparisons (i.e. the progressive downwards profile in the cycling group compared against the progressive downwards profile in the 33% SW group, and progressive upwards versus progressive upwards), there were no significant differences between the cycling fish and the 33% SW-acclimated fish.

Discussion

There are two important overall conclusions of the present study. The first is that when killifish were tested under conditions closer to those which occur in their natural habitat (i.e. exposure to progressive rather than acute changes in salinity) and were acclimated to conditions closer to those which occur in their natural habitat (i.e. either 33% SW, or cycling salinity rather than to FW or 100% SW), their TEP responses were basically similar to those seen under less realistic conditions. Therefore the arguments presented earlier (Wood and Grosell 2008) as to the adaptive value of these changes, as outlined in the Sect. "Introduction", appear to remain valid. To our knowledge, there have been no previous studies on the TEP responses of euryhaline teleosts to progressive salinity change, or on the influence of prior acclimation to intermediate or cycling salinity on the response pattern. However Prodocimo et al. (2007) have demonstrated that *F. heteroclitus* acclimated to intermediate salinity successfully maintain Na^+ balance over a cycling salinity regime similar to that used here. TEP likely plays a key role.

The second important conclusion is that killifish acclimated to either 33% SW or to a salinity which cycled between FW and 100% SW at 6 h intervals displayed a TEP versus salinity response profile basically similar to that of a 100% SW-acclimated animal, and very different from that of a FW-acclimated animal. The response pattern of the 100% SW-acclimated animal appears to be the default pattern for intermediate and cycling salinities, and only if the FW exposure is prolonged (i.e. 24 h+) does the FW response pattern set in (Wood and Grosell 2008). Nevertheless, it should be noted that 33% SW is actually slightly hypertonic to killifish

blood plasma (see Introduction). Had a lower intermediate acclimation salinity (e.g. 20% SW) been used which was hypotonic to blood plasma, it is possible that a more FW-like TEP pattern would have been observed.

A variety of hormonal, neural, molecular, and transport-protein level responses undoubtedly contribute to overall ion homeostasis of the killifish in the highly variable estuarine environment (reviewed by Marshall 2003). However the fact that the TEP versus salinity response profile remains similar to that of a 100% SW-acclimated killifish over the range of acclimation conditions studied here most likely relates to gill structure. Killifish gill and opercular epithelial structure has been very well studied, and some controversies exist. However there is general agreement that epithelial morphology differs greatly between FW- and SW-acclimated animals, and that the SW-type gill structure persists down to acclimation salinities as low as 1% SW (Copeland 1950; Karnaky 1986; Philpott and Copeland 1963; Lacy 1983; Hossler et al. 1985; Marshall et al. 1997; Daborn et al. 2001; Katoh et al. 2001; Katoh and Kaneko 2003; Katoh et al. 2003; Laurent et al. 2006). Key features include the presence of SW-type mitochondrial-rich cells (MRC's or "chloride cells"), with neighbouring accessory cells, with shallow paracellular tight junctions between the two providing the "shunt" pathway. Killifish acclimated to the tidal cycle in the present study experienced a time-averaged mean salinity of 50% SW, and would have been exposed to a salinity <1% SW for only about 2 h per day.

Why this SW-type gill structure which is ostensibly set up for Na^+ and Cl^- extrusion should persist at or below isotonic salinities has previously been unclear. It may well be that killifish rarely have to spend extended periods in FW, and that the normal situation is a life spent in intermediate salinities with occasional feeding excursions down to dilute SW or FW. The TEP characteristics provided by this gill morphology and described here will serve the killifish well on these short-term forays, the highly negative TEP serving to limit Na^+ losses to tolerable levels. Permanent life in FW demands a changeover to a fundamentally different cell type in the gill for active Na^+ uptake, achieved through a burst of cell division, though it is controversial whether this new cell type is a cuboidal cell (Laurent et al. 2006) or a FW-type MRC (Katoh et al. 2001, 2003; Katoh and Kaneko 2003; Scott et al. 2004). There is general agreement that there also occurs a "paving" over (by pavement cells) and degeneration of the SW-type MRC's, a loss of accessory cells, and a deepening of the tight junctions so as to close the "shunt" pathway and reduce overall gill permeability (Karnaky 1991). An intestinal mechanism for increased Cl^- (and Na^+) acquisition from the food also appears to be activated (Scott et al. 2006). All of these changes may be very costly. Evolution may have selected for a strategy which allows the fish to survive for a reasonable length of time in FW (<24 h) without additional metabolic cost by maintaining the SW-type gill structure and function as long as there is a reasonable chance of return to higher salinity. Upon return to higher salinity, the largely unchanged structure will allow instantaneous re-activation of SW Na^+ and Cl^- secretion.

Other euryhaline species generally show positive TEP's in 100% SW and negative TEP's upon acute challenge with FW, but the magnitude of the changes is generally lower than in killifish, and there is little information on how they respond over time during acclimation to constant, cycling or intermediate salinities (Potts 1984). The killifish is one of a small number of species discovered so far that appear to have relinquished active Cl^- uptake in FW; others are the euryhaline eel, *Anguilla anguilla* (Kirsch 1972, Grosell et al. 2000) and the stenohaline bluegill, *Lepomis macrochirus* (Tomasso and Grosell 2005). It is interesting therefore to note that eels, adapted to 100% SW, show large TEP changes (+22.5 mV in 100% SW, −34.2 mV after acute transfer to FW; House and Maetz 1974) almost identical in magnitude to those of killifish (cf. Fig. 2). However, many euryhaline species (e.g. salmonids, flounder; Grosell et al. 2000; Taylor et al. 2007) retain active Cl^- uptake in FW, so it is unlikely that the lack of Cl^- transport in FW is an adaptation for euryhalinity. Rather, we believe that species such as killifish, eels and also bluegills gave up, or never developed gill Cl^- transport in FW for some other reason (e.g. energy saving, or to avoid nitrite poisoning, as shown by Tomasso and Grosell 2005). The euryhaline ones then developed and/or exploited a more marked TEP response pattern in support of their euryhalinity, because it helps retain Na^+ in FW without hindering the non-existent Cl^- uptake.

As outlined in the Sect. "Introduction", the TEP of the killifish in natural waters is interpreted as a diffusion potential dictated by the relative permeability of the gills to the two dominant ions (Na^+ and Cl^-) in extracellular fluid and the external environment. At higher salinities, a positive electrogenic component may be superimposed on the diffusion potential, complicating interpretation. However at intermediate salinities, this electrogenic component is probably shut down, so under these conditions the P_{Cl}/P_{Na} ratio can be estimated from the measured TEP using the Goldman–Hodgkin–Katz equation (Goldman 1943; Sten-Knudsen 2002), as detailed by Wood and Grosell (2008). The estimates are summarized in Table 1 for two diagnostic salinities, 10% SW and 60% SW at which most of the notable differences between treatments occur and the influence of an electrogenic component is likely to be negligible.

This analysis indicates that a key difference of the two FW-acclimation treatments versus all others was the much higher (i.e. reversed) P_{Cl}/P_{Na} ratios (Table 1) which prevented the occurrence of a negative TEP in 10% SW in the former (Fig. 1). Even in 60% SW, the P_{Cl}/P_{Na} ratio in these two treatments remained higher than in the other groups.

Table 1 Relative permeability ratio of the gills of killifish to Cl^- versus Na^+ (P_{Cl}/P_{Na}) under different treatment conditions at two different salinities as calculated from the mean measured TEP values, using the Goldman–Hodgkin–Katz equation

Acclimation and test condition	Measurement 10% SW	Salinity 60% SW
FW-acclimation–acute change	1.56	0.23
FW-acclimation–progressive upward change	1.87	0.36
100% SW-acclimation–acute change	0.43	0.20
100% SW-acclimation–progressive downward change	0.08	0.10
33% SW-acclimation–progressive upward change	–	0.07
33% SW-acclimation–progressive downward change	0.13	–
Tidal acclimation–progressive upward change	0.59	0.09
Tidal acclimation–progressive downward change	0.52	0.15

The electrogenic component of TEP is assumed to be negligible at these salinities. Other details as in Wood and Grosell (2008)

The analysis also indicates that when 100% SW-acclimated killifish were subjected to progressive rather than acute reductions in salinity, they maintained lower P_{Cl}/P_{Na} ratios (Table 1), thereby explaining the TEP differences (Fig. 2) which are thought to be of adaptive value. Note that the 100% SW-acclimated fish had been acclimated to full-strength seawater in the laboratory for about a year. This may be an extreme situation relative to conditions in the field.

The higher P_{Cl}/P_{Na} ratios in the FW-acclimation group exposed to the progressive upward salinity change may have contributed to the surprising observation that for this acclimation treatment, progressive increases in salinity were less effective than acute changes in raising TEP at high salinity (Fig. 1). However, the likely contribution of an electrogenic component at the salinities (80–100% SW) at which this difference became significant is a confounding factor. Perhaps there is also a reduced ability to activate electrogenic Cl^- extrusion at high salinity? Whatever the explanation, this finding reinforces the conclusion that once fully acclimated to FW, these animals are not well set up to move rapidly into SW. Indeed several studies have shown that when FW-acclimated *F. heteroclitus* are acutely transferred to 100% SW, plasma (Na^+) and/or osmolality are significantly elevated within 1 h, and are not restored until 3–5 days post-transfer (Jacob and Taylor 1983; Zadunaisky et al. 1995; Marshall et al. 1999).

The analysis also showed that 33% SW-acclimated fish were able to maintain a very low P_{Cl}/P_{Na} ratio in 60% SW (Table 1). This could explain the more positive TEP's observed at higher salinity in these fish (Fig. 3), but again an enhanced electrogenic component may be an alternate or additional explanation. Finally, the analysis indicated that when killifish were acclimated to a tidal cycle of fluctuating salinity, they exhibited somewhat higher P_{Cl}/P_{Na} ratios than did either 100% SW-acclimated or 33% SW-acclimated animals, and therefore TEP's which appear to be somewhat less adaptive at lower salinities (e.g. Fig. 4b). Perhaps the short twice-daily exposures to FW induced a slight change towards the FW characteristic. Nevertheless, these fish remain basically similar to the 33 and 100% SW-acclimation treatments in their response patterns, and very different from the FW-acclimation groups.

To conclude, when evaluated in vivo under conditions simulating a more realistic environment of an estuary (progressively changing salinity, prior acclimation to either cycling salinity or fixed intermediate salinity), the killifish gill exhibits electrical characteristics which appear to be adaptive, opposing Na^+ loss at low salinity and favouring Na^+ extrusion at high salinity. These properties are similar to those seen in killifish acclimated to 100% SW, but not after acclimation to FW. Thus animals living in the estuaries can move to lower and higher salinities for short periods with little physiological disturbance, but this ability is lost after acclimation to FW. These differences are thought to relate to differences in gill morphology and changes in the nature of the paracellular shunt pathway in the gills. In the past, quantitative studies of branchial morphology and permeability to the paracellular marker PEG-4000 have proven helpful in understanding differences in FW adaptation ability between different races of *F. heteroclitus* (Scott et al. 2004). In the future, use of similar approaches may help cast further light on the mechanisms proposed in the present study.

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