J Exp Biol Advance Online Articles. First posted online on 29 May 2015 as doi:10.1242/jeb.122176
Access the most recent version at http://jeb.biologists.org/lookup/doi/10.1242/jeb.122176

Electrical aspects of the osmorespiratory compromise: TEP responses to hypoxia in the euryhaline killifish (*Fundulus heteroclitus*) in fresh water and sea water

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Key Words: transepithelial potential, gill permeability, P_{Cl}/P_{Na} ratio, diffusion potential, electrogenic potential

Summary

The osmorespiratory compromise, the trade-off between the requirements for respiratory and ionoregulatory homeostasis at the gills, becomes more intense during environmental hypoxia. One aspect which has been previously overlooked is possible change in transepithelial potential (TEP) caused by hypoxia which will influence branchial ionic fluxes. Using the euryhaline killifish, we show that acute hypoxia reduces the TEP across the gills by approximately 10 mV in animals acclimated to both fresh water (FW) and sea water (SW), with a higher PO_2 threshold in the former. TEP becomes negative in FW, and less positive in SW. The effects are immediate, stable for at least 3 h, and reverse immediately upon return to normoxia. Hypoxia also blocks the normal increase in TEP that occurs upon FW to SW transfer, but does not reduce the fall in TEP which occurs in the opposite transfer. These effects may be beneficial in FW but not in SW.

Introduction

The osmorespiratory compromise is a trade-off between the requirements of the gills to promote respiratory gas transfer, yet to restrict the passive fluxes of ions and water. In the traditional compromise, fish increase their effective gill permeability to O_2 during exercise or hypoxia by changing blood perfusion patterns. This results in increases in branchial surface area and/or reductions in the mean blood-to water diffusion distance, both of which favour gas exchange but also exacerbate unfavourable ion and water movements (Randall et al., 1972). The usual interpretation is that these effects result exclusively from changes in gill permeability. However at least for ions, branchial permeability is only one of the three factors affecting both passive and active fluxes; the other two are concentration gradient and electrical gradient, which together dictate the electrochemical gradient on an electrolyte (Kirschner, 1970). However, the electrical gradient is often overlooked. While the impacts of ion concentration gradients on ionic fluxes have received considerable attention , to our knowledge, the influence of electrical gradients has never been examined systematically in the context of the osmorespiratory compromise.

In the present study, we used the Atlantic killifish *Fundulus heteroclitus*, a model species which is both euryhaline and hypoxia-tolerant (Burnett et al., 2007). The origin of the transepithelial potential (TEP) across the gills has been studied extensively in killifish and is relatively well understood in both fresh (FW) and sea water (SW) (e.g. Pic, 1978; Potts, 1984; Wood and Grosell, 2008). To put the importance of this factor in perspective, using data from the FW-acclimated killifish and the Goldman-Hodgkin-Katz equation (Wood and Grosell, 2008), a modest + or - 10mV change in the TEP could result from changing plasma [Na^+] from the normal level of 140 mM to 93 mM or 221 mM respectively, or altering the P_{Cl}/P_{Na} permeability ratio over a 3-fold range.

We hypothesized that changes in blood perfusion patterns during hypoxia, or even hypoxia itself, could alter either electrogenic or diffusive components of the TEP, or both. Therefore in the present study we tested whether acute hypoxia exposure would alter the TEP across the gills of intact killifish in either FW or SW. Having found significant effects which persist for some time, we then examined the influence of hypoxia on the important immediate changes in TEP which help killifish adjust quickly as they move between SW and FW.

Results and Discussion

In *Series 1*, FW- and SW-acclimated killifish were exposed acutely to various levels of hypoxia ranging from 10% saturation (PO_2 = 2.06 kPa, 15.5 torr) to 40% saturation (8.22 kPa, 61.7 torr). The order was randomized to negate any cumulative effects of stress from handling (Pic, 1978; Wood and Grosell, 2008; see Methods). While hypoxia affected TEP in both salinities, the response was clearly more sensitive in FW. The threshold in FW was between 40% and 30% saturation, with TEP (note: reference = water side as 0 mV) falling significantly from the control value of about +5 mV to hypoxic values of -5 to -7mV, at 30%, 20%, and 10% saturation (Fig. 1A). In SW, the control TEP in normoxia was about +13 mV,

and this remained unchanged down to 20% saturation, but dropped significantly to about +8 mV at 10% saturation (Fig. 1B).

In *Series 2*, FW- and SW-acclimated killifish were exposed to 10% saturation (PO_2 = 2.06 kPa, 15.5 torr) for 3 h, together with a comparable control series, where the animals were handled identically but maintained at greater than 80% saturation (normoxia). In FW, the rapid decrease in TEP by about 8 mV to negative values was stable over 3 h, and immediately reversed back to the positive control value when the animals were returned to normoxic conditions (Fig. 2A). TEP in the controls did not change significantly. In SW, the higher normoxic TEP decreased significantly by about 10 mV immediately upon exposure to hypoxia, and remained stable at a lower but still positive value for 3 h (Fig. 2B). Upon return to normoxia, this decrement was quickly reversed, and again there were no significant changes in the controls.

Series 3 examined whether hypoxia (10% saturation, PO_2 = 2.06 kPa, 15.5 torr), either in the environment of origin, or in the environment of destination, affected the large changes in TEP which normally occur when killifish move acutely between FW and SW, and *vice versa*. When FW-acclimated killifish were tranferred from normoxic FW to normoxic SW, TEP increased by about 11 mV (Fig. 2C). However, when the transfer was from normoxic FW to hypoxic SW, the increment was essentially abolished. The same lack of response occurred when the transfer was from hypoxic FW to hypoxic SW; thus hypoxia in the destination environment, and not hypoxia in the originating environment was responsible for this blockade (Fig. 2C). Indeed, transfer from hypoxic FW to normoxic SW was accompanied by an overshoot, an increase in TEP which was about 75% greater than in the normoxia-to-normoxia control, although this difference was not quite significant (P = 0.07) (Fig. 2C). In contrast, when SW-acclimated killifish were transferred to FW, neither hypoxia in the destination environment , hypoxia in the originating environment, nor hypoxia in both had any significant effect on the approximate 45 mV drop in TEP which occurred (Fig. 2D).

The present TEP values measured under normoxic conditions are very similar to those reported in previous studies on similarly handled killifish in FW, SW, and abruptly transferred between the two (Pic, 1978; Wood and Grosell, 2008). Current understanding of the TEP has been summarized in the latter study. In brief, in 100% SW, killifish gills exhibit high cation and low anion permeability. As a result, there is a positive Na^+ diffusion potential, upon which is superimposed a positive electrogenic potential due to the secondary active excretion of Cl^-; the positive TEP facilitates the passive extrusion of Na^+ through the paracellular "shunt" pathway. Upon acute exposure to FW, the Na^+ diffusion potential immediately reverses to very negative values, and the electrogenic component is abolished. The resulting very negative TEP is beneficial in immediately limiting Na^+ loss, and does not impact active Cl^- uptake because there is none in this species in FW (Patrick et al., 1997). The shunt permeability stays high for a few hours, allowing the killifish to return to higher salinity and instantaneously re-activate NaCl excretion. However, after acclimation to FW (24 h+), the paracellular shunt pathway closes, anion permeability becomes slightly greater than

cation permeability, and the TEP becomes slightly positive. The animals are now well-acclimated for life in FW, but not to move rapidly back and forth between salinities.

In the present study, exposure to severe hypoxia (10% saturation) decreased the TEP by similar amounts in both FW and SW killifish (Fig. 2A,B), confirming that there is an important electrical element to the osmorespiratory compromise. This has been overlooked in previous analyses of the phenomenon, and as illustrated by the simulations below, it is a quantitatively significant factor in the trade-off. A fall in TEP of 10 mV is used in these calculations for the hypoxia effect in both FW and SW, representative of the data in Figs. 1 and 2. This similarity may be happenstance, because the absolute TEP values in normoxia and hypoxia were very different in the two salinities, as were the threshold levels for the response (40-30% saturation in FW versus 20-10% in SW; Fig. 1), and perhaps also the mechanisms, as suggested subsequently.

In FW–acclimated killifish, there is no electrogenic component, so all acute effects were likely attributable to decreases in the P_{Cl}/P_{Na} ratio caused by hypoxia, because the fall in TEP is immediate but stable for at least 3 h (Fig. 2A), while changes in plasma ion concentrations take some time to develop. This effect on the P_{Cl}/P_{Na} ratio could result either from blood flow changes in the gills during hypoxia, or effects of low O_2 itself on permeability; other environmental factors such as water pH and calcium concentration are well known to alter P_{Cl}/P_{Na} (Potts, 1984; Wood and Grosell, 2008). The 10 mV drop in TEP in FW (Fig. 2A) would reflect a change in the P_{Cl}/P_{Na} from about 1.30 to 0.82. In SW-acclimated killifish acutely transferred to FW, the P_{Cl}/P_{Na} ratio, which is 0.36 under normoxia, would decrease further to 0.22 under hypoxia, based on the data in Fig. 2D. In both cases, the more negative potentials under hypoxia would favour the retention of Na^+, reducing the electrochemical gradient driving Na^+ loss by about 9% in FW-acclimated animals, and by about 12% in animals acutely transferred from SW to FW. Paradoxically, by changing the P_{Cl}/P_{Na} ratio, hypoxia may actually help Na^+ balance in FW, though from this analysis, we have no way of knowing if hypoxia affects active Na^+ uptake, which is electroneutral in FW fish (e.g. Kirschner, 1970; Potts, 1984).

In SW, the situation is more complicated because both electrogenic and diffusive components could be affected by hypoxia. Assuming a P_{Cl}/P_{Na} ratio of 0.36 again for the SW-acclimated killifish, then this would predict a diffusion potential of +10 mV, about 2/3 of the total observed TEP, meaning that the electrogenic component is about +5mV. However if P_{Cl}/P_{Na} dropped to 0.22 again under hypoxia, the diffusion potential would actually increase to +16 mV, whereas a decrease in TEP was observed. Indeed when FW-acclimated animals were transferred to hypoxic SW, they were not able to increase TEP at all (Fig. 2C). The more likely explanation is that the inhibitory mechanism is different in SW, and the drop in TEP is mainly due to inhibition of the electrogenic component by hypoxia. Thus active Cl^- extrusion would be reduced. To our knowledge, there is no published information on the effect of severe hypoxia on active ion transport rates in killifish *in vivo*, but in the isolated opercular epithelium of SW-acclimated killifish studied *in vitro*, complete anoxia reduces the active Cl^- current by 75-83% (Karnaky et al., 1977; Barnes et al., 2014). This effect, together with the fact that the reduced TEP in the current study results in about a 70% increase in the

electrochemical gradient driving Na^+ entry, suggests that the electrical response to hypoxia would be deleterious to ionoregulatory homeostasis in SW-acclimated killifish. As SW fish also drink as part of their osmoregulatory strategy, there may also be direct or indirect effects on gut function.

Interestingly, a fish would occasionally escape during TEP measurement under hypoxia, reaching the better oxygenated surface layer. In SW-acclimated killifish, TEP would rise immediately, but this did not happen in FW-acclimated animals, supporting the interpretation of a different etiology for the hypoxia-induced TEP reduction in the two media. In future studies, it will be of interest to evaluate these ideas by actually measuring the effects of acute severe hypoxia on unidirectional ion flux rates *in vivo* in FW- and SW- acclimated killifish. It would also be instructive to look at a freshwater fish with active Cl^- uptake mechanisms, as well as stenohaline species in FW as well as in SW.

Materials and Methods

Adult killifish (*Fundulus heteroclitus macrolepidotus* (Walbaum 1792) (5-12 g) were purchased from Aquatic Research Organisms (ARO) Ltd. (Hampton, New Hampshire, USA), and acclimated for at least 1 month to either FW or 100% SW (24-26^{O}C, composition as in Wood and Grosell, 2008) at the University of Miami. All procedures followed an approved University of Miami Animal Care Protocol (IACUC no. 13-225, renewal 02). The fish were fitted with saline-filled intraperitoneal catheters under anaesthesia as described by Wood and Grosell (2008) and allowed to recover for 24 h in shielded 800-mL beakers served with airlines and flowing SW or FW. TEP measurements were performed as in that paper, using 3M KCl-agar bridges connected via Ag/AgCl electrodes to a high impedance voltmeter (Radiometer pHM84, Copenhagen, Denmark). TEP was expressed relative to the apical (water) side as 0 mV after correction for junction potential. Water O_2 levels (as % air saturation) were monitored with a YSI Optical Probe and meter (Digital Professional Series, Yellow Springs, OH, USA), ensuring that measured values were within ± 2% of nominal. Different water O_2 levels were achieved by volumetric mixing of 100% and 0% (N_2-equilibrated) water, and adjusted as necessary by air or N_2 gassing.

In *Series 1*, preliminary experiments showed that there was no effect of O_2 on TEP down to 50% air saturation. Therefore each fish was acutely transferred, without air exposure, from normoxia (80-100%) to 40%, 30%, 20%, and 10% O_2 in the same medium (FW or SW) in random order. Measurements were made after 2 min in the new environment. Because it was necessary to handle the fish during measurements, and TEP is known to be affected by handling stress (Pic, 1978; Wood and Grosell, 2008), the order was randomized. Between each measurement, the fish was returned to normoxia for 5-10 min, and the TEP was recorded again prior to the next transfer. Thus the normoxic value for each fish represents the mean of 5 measurements, while the experimental values are single measurements.

Series 2 examined whether TEP changes under hypoxia (10% saturation) were stable over time, in both FW and SW fish. In the experimental series, TEP measurements were made under normoxia (P) and then at 2 min (0 h), 1 h, 2 h, and 3 h of hypoxia, and finally

after 2 min recovery (R) in normoxia. A simultaneous control series of fish kept in normoxia throughout but handled identically was also performed. During the 3-h hypoxia exposure, a subsurface screen was necessary to prevent the fish performing aquatic surface respiration.

Series 3 examined whether hypoxia (10% saturation) in the originating environment, the destination environment, or both altered the acute TEP response to transfer between FW and SW, and *vice versa*. Fish were kept for 30 min in one medium under normoxia or hypoxia, and then transferred to the other medium under either normoxia or hypoxia (4 combinations for each acclimation salinity). TEP measurements were made before and 2 min after transfer.

Data were evaluated using Nernst and Goldman-Hodgkin-Katz (GHK) equations as in Wood and Grosell (2008), and the same plasma and water ion concentrations and activity coefficients were used (see Supplementary Material -Table S1). The GHK equation was solved for P_{Cl}/P_{Na} (permeability ratio) under various treatments. The net electrochemical gradient on Na^+ was calculated as the difference between the Nernst potential and the TEP, signs considered (Kirchner, 1970). Data have been expressed as means ± 1 SEM (N). A one-way repeated measures ANOVA followed by Dunnett's test was used to detect significant differences from the 100% value (*Series 1*) or from the pre-exposure normoxia value (*Series 2*). A one-way ANOVA followed by Tukey's test was used to differentiate treatment effects in *Series 3*. Unpaired two-tailed Student's t-tests with the Bonferroni correction were used to evaluate simultaneous control *versus* experimental differences in *Series 2*; $P < 0.05$ was employed throughout.

Acknowledgements

We thank Dr. W.S. Marshall for helpful advice and Kevin Schauer for aquatic system maintenance.

Competing Interests

None.

Author Contributions

CMW performed all experiments, analysed the data, and wrote the MS. CMW and MG jointly contributed to conceptual design. MG provided advice and facilities, and edited the manuscript.

Funding

MG is Maytag Professor of Ichthyology and is funded by an NSF (USA) Grant (IOS 1146695) while CMW held a Canada Research Chair and is funded by an NSERC (Canada) Discovery grant.

Abbreviations

FW fresh water

GHK Goldman-Hodgkin-Katz

P probability

P_{Cl}/P_{Na} permeability ratio of the gills to Cl^- relative to Na^+

PO_2 partial pressure of O_2

N number of experimental animals

SW sea water

SEM standard error of the mean

TEP transepithelial potential

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Figures

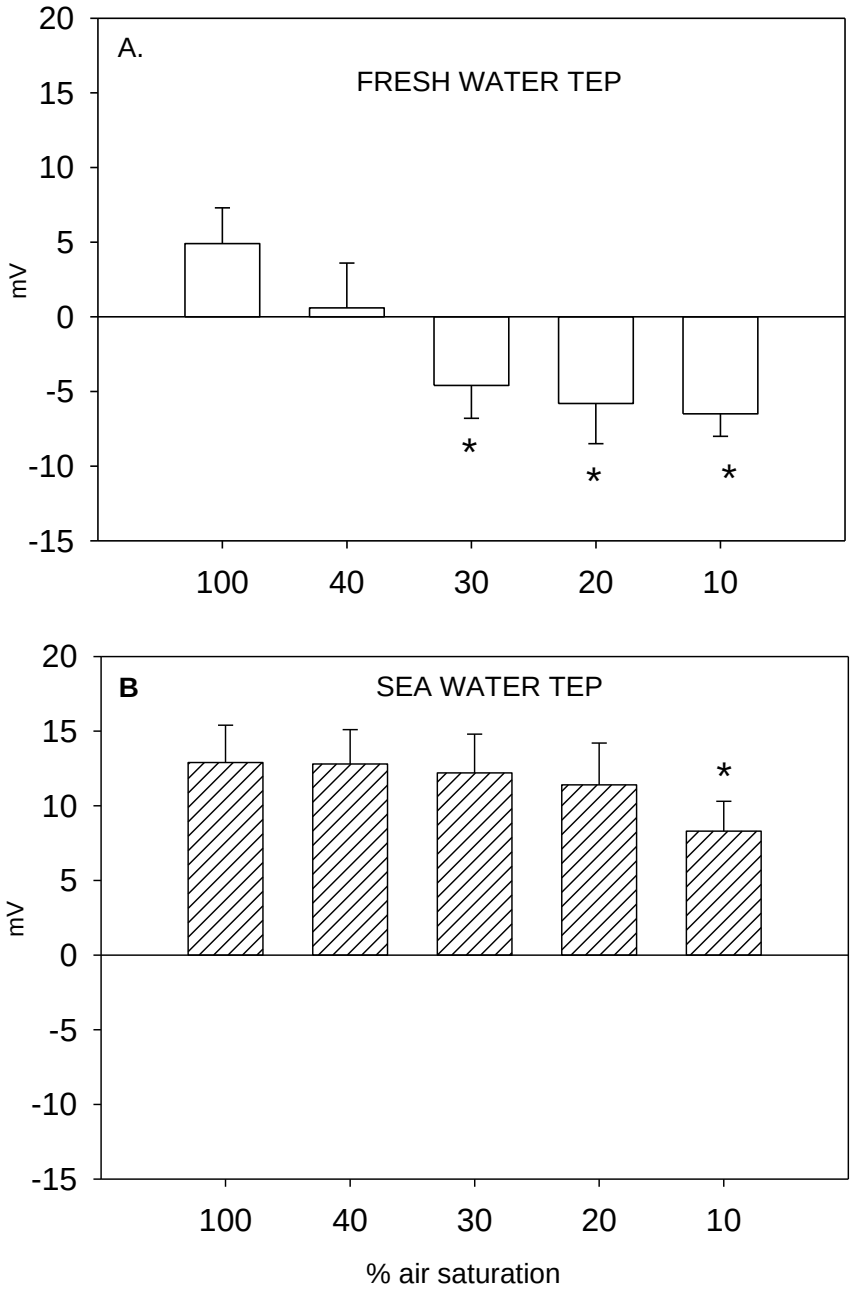

Fig. 1 Tests to determine O_2 threshold for effects of hypoxia on TEP in killifish. (A) FW-acclimated killifish (N = 8) and (B) SW-acclimated killifish (N = 6). The different O_2 levels were presented in random order to the same fish to control for the effects of cumulative handling stress on TEP (see text for details). Means ± 1 SEM. Asterisks indicate means significantly different ($P < 0.05$) from TEP at nominal 100% (80-100%) air saturation. The threshold for TEP decline appears to be between 30 and 40% in fresh water, and between 10 and 20% O_2 in sea water.

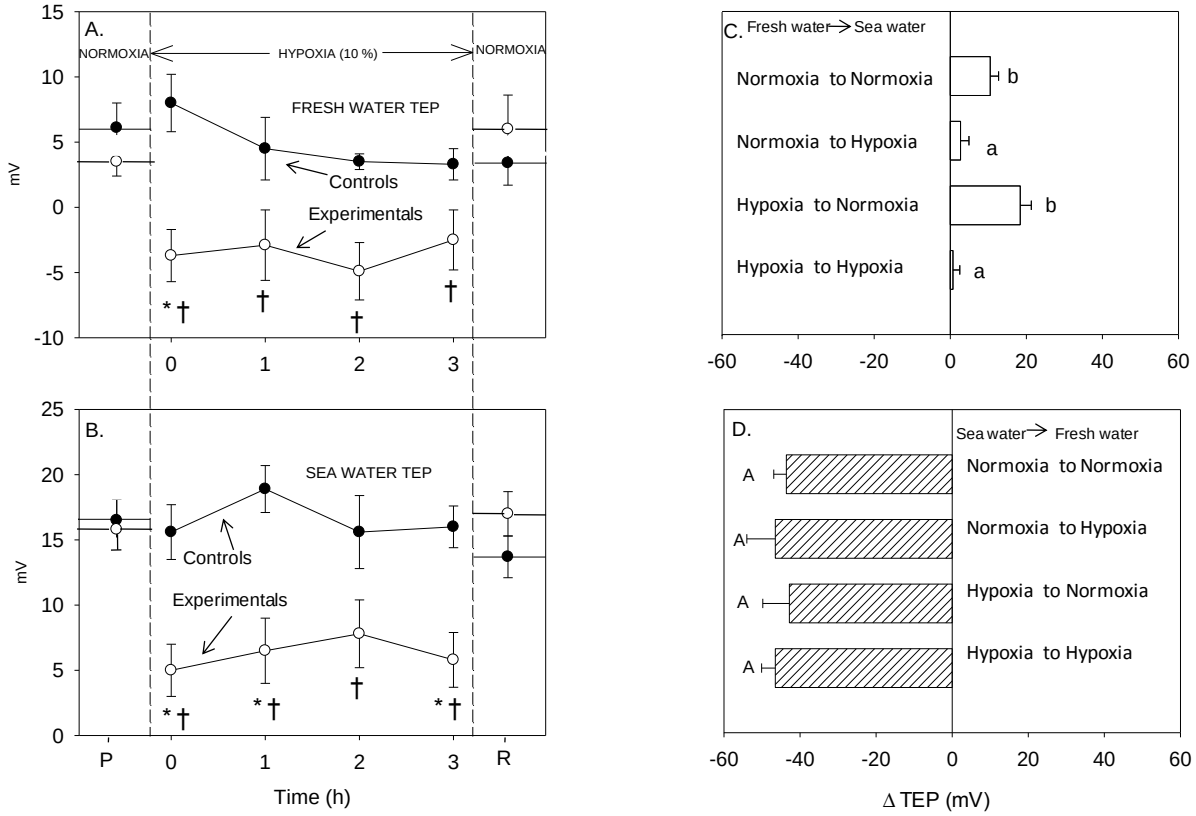

Fig. 2 Tests to determine the stability over time of the effects of hypoxia (10% air saturation) on TEP (A, B), and on the change in TEP upon acute transfer between salinities (C, D) in killifish.

TEP stability tests in (A) FW–acclimated killifish (experimental N = 14; control N = 6) and (B) SW-acclimated killifish (experimental N = 7; control N = 7). Experimental animals were sampled under normoxia ("P" = pre-exposure, 80 – 100%), then acutely subjected to hypoxia (10%) at time 0, held in hypoxia for 3 h, then acutely returned to normoxia ("R") . Control animals were held in normoxia throughout but handled identically to the experimental animals and sampled at the same times. Means ± 1 SEM. Asterisks indicate experimental means significantly different ($P < 0.05$) from control means; daggers indicate experimental means significantly different ($P < 0.05$) from the pre-exposure ("P") measurement.

Changes in TEP upon acute transfer of (C) FW-acclimated killifish to SW (N = 8-13), and (D) SW-acclimated killifish to FW (N =6-7). Means ± 1 SEM. Means sharing the same letters are not significantly different ($P > 0.05$). The fish were transferred under four regimes (normoxia to normoxia, normoxia to hypoxia, hypoxia to normoxia, and hypoxia to hypoxia).