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RESEARCH ARTICLE

The osmorespiratory compromise in the euryhaline killifish: water regulation during hypoxia

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ABSTRACT

Freshwater- and seawater-acclimated *Fundulus heteroclitus* were exposed to acute hypoxia (10% air saturation, 3 h), followed by normoxic recovery (3 h). In both salinities, ventilation increased and heart rate fell in the classic manner, while $\dot{M}_{O_2}$ initially declined by ~50%, with partial restoration by 3 h of hypoxia, and no O_2 debt repayment during recovery. Gill paracellular permeability (measured with [14C] PEG-4000) was 1.4-fold higher in seawater, and declined by 50% during hypoxia with post-exposure overshoot to 188%. A similar pattern with smaller changes occurred in freshwater. Drinking rate (also measured with [14C] PEG-4000) was 8-fold higher in seawater fish, but declined by ~90% during hypoxia in both groups, with post-exposure overshoots to ~270%. Gill diffusive water flux (measured with ^3H_2O) was 1.9-fold higher in freshwater fish, and exhibited a ~35% decrease during hypoxia, which persisted throughout recovery, but was unchanged during hypoxia in seawater fish. Nevertheless, freshwater killifish gained mass while seawater fish lost mass during hypoxia, and these changes were not corrected during normoxic recovery. We conclude that this hypoxia-tolerant teleost beneficially reduces gill water permeability in a salinity-dependent fashion during hypoxia, despite attempting to simultaneously improve $\dot{M}_{O_2}$, but nevertheless incurs a net water balance penalty in both freshwater and seawater.

INTRODUCTION

Traditionally, the osmorespiratory compromise in fish gills has been interpreted as the trade-off between low permeability, to limit unfavourable ion and water fluxes, and high permeability, to promote respiratory gas exchange (Randall et al., 1972; Nilsson, 1986; Sardella and Brauner, 2007). When one of these requirements increases (e.g. greater O_2 uptake), the other is impacted (e.g. greater osmotic and ionic leakiness). The compromise has been documented most extensively with respect to increased ion loss

accompanying exercise in freshwater fish, when O_2 demand increases (e.g. Wood and Randall, 1973a,b; Gonzalez and McDonald, 1992, 1994; Wood, 1988; Postlethwaite and McDonald, 1995; Robertson and Wood, 2014; Robertson et al., 2015a; Onukwufor and Wood, 2018). There have been fewer studies on the compromise with respect to increased water exchange during exercise (Stevens, 1972; Wood and Randall, 1973c; Hofmann and Butler, 1979; Wood, 1988; Onukwufor and Wood, 2018), and almost none on seawater fish (Farmer and Beamish, 1969; Stevens, 1972).

Environmental hypoxia is another circumstance where there is a need to improve the ability of the gills to take up oxygen. Here, the story appears to be more complex. Some very hypoxia-tolerant species, such as the Amazonian oscar, are able to actually reduce ion and water permeability during hypoxia, without sacrificing the ability to improve respiratory gas exchange (Wood et al., 2007, 2009; Scott et al., 2008; Matey et al., 2011; De Boeck et al., 2013; Robertson et al., 2015b). This pattern contrasts with hypoxia-intolerant species (e.g. salmonids) that exhibit the traditional compromise, resulting in increased ion losses and water fluxes (Swift and Lloyd, 1974; Kobayashi and Wood, 1980; Thomas et al., 1986; Iftikar et al., 2010; Onukwufor and Wood, 2018).

A common finding of many studies, both with exercise and hypoxia, has been that branchial ion and/or water permeability is not a fixed function of gas-exchange capacity, but can be independently regulated over time, in a manner favourable to ionic and osmotic homeostasis (e.g. Wood and Randall, 1973b,c; Swift and Lloyd, 1974; Gonzalez and McDonald, 1992, 1994; Postlethwaite and McDonald, 1995; Wood et al., 2007, 2009; Matey et al., 2011).

Following the discovery of aquaporin proteins as the major water-conductive channels in cell membranes, by Agre and colleagues (Preston et al., 1992), it is now generally recognized that there can be two separate pathways (transcellular and paracellular) for water movement across epithelia such as gills. There are also two different ways of assessing gill water permeability. Diffusive water flux rates can be determined directly by measuring the exchange rate of ^3H_2O. This yields the unidirectional flux rate of water. In contrast, net water flux rates (often called 'osmotic' water flux rates), can be measured only indirectly in intact fish from changes in urine flow rate, drinking rate and body mass (Loretz, 1979). Diffusive water flux rates may be many-fold greater than osmotic flux rates. For example, a resting freshwater rainbow trout in normoxia at 13°C exchanges approximately 80% of its body water pool per hour by diffusion (Onukwufor and Wood, 2018), yet has a net osmotic water entry, as estimated from urine flow rate, of only approximately 0.3% of its pool per hour (Wheatly et al., 1984). Diffusive water flux rates are quantitatively very similar in the influx and efflux directions, so it is impossible to precisely calculate net water flux rate from their difference. Thus, they can be measured in either direction; the efflux method (used in the present study) is easier, more accurate and non-destructive.

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List of symbols and abbreviations	
A	ventilatory buccal pressure amplitude
BL	body length
C_{PEG}	[14C]PEG-4000 influx clearance rate
[14C]PEG-4000	polyethylene glycol-4000 labelled with radioactive carbon
D	drinking rate
f	ventilatory frequency
^3H_2O	tritiated water
HNO_3	nitric acid
J_{H_2O}	net whole body water flux rate
$k_{^3H_2O}$	rate constant of ^3H_2O turnover in h^{-1}
M_b	body mass
$\dot{M}_{O_2}$	rate of oxygen consumption on a molar basis
MS-222	tricaine methanesulfonate
NaOH	sodium hydroxide
P_{crit}	critical oxygen tension
PEG-4000	polyethylene glycol, molecular weight=4000 Da
P_{O_2}	partial pressure of oxygen
R	radioactivity
t	time
V	volume
VI	ventilatory index

While both diffusive and osmotic flux rates are functions of gill water permeability, the relationship between the two is complex (see Potts et al., 1967; Franz, 1968; Motais et al., 1969; Isaia, 1984; Evans et al., 2005; Kwong et al., 2013). In short, it is speculated that diffusive flux occurs through the entire gill surface, so may be dominated by the transcellular pathway (i.e. including aquaporin-mediated flux), whereas osmotic flux may be dominated by bulk flow through the paracellular pathway. [14C]Polyethylene glycol M.W. 4000 (PEG-4000) has been proposed as a marker of paracellular permeability as this molecule is thought to be too large to pass through the transcellular pathway (Pappenheimer and Reiss, 1987; Wood and Pärt, 1997; Watson et al., 2001). In the hypoxia-tolerant freshwater oscar, exposure to severe hypoxia caused a directly measured 70% decrease in diffusive water permeability and an indirectly calculated 30% decrease in osmotic water permeability, while the PEG-4000 clearance rate of the gills did not change (Wood et al., 2009). Nevertheless, oscars exhibited a modest net gain of water (as evidenced by a small mass gain) because the volume output of the kidney was severely inhibited by hypoxia.

Our objective was to improve mechanistic understanding of the osmorespiratory compromise during hypoxia, with respect to water balance in both freshwater and seawater fish. As a model species, we selected the common killifish (Fundulus heteroclitus), renowned for both its exceptional euryhalinity (Wood and Marshall, 1994) and its extraordinary hypoxia tolerance (Burnett et al., 2007). The same severe hypoxia treatment (10% air saturation, over a 3 h period) as used in the oscar (Wood et al., 2009) was utilized as a standard challenge, though in one trial, steady-state exercise was employed for comparative purposes. Hypoxia experiments focused on oxygen consumption rates ($\dot{M}_{O_2}$), diffusive water flux rates measured with tritiated water ^3H_2O (Onukwufor and Wood, 2018), gill paracellular permeabilities and drinking rates measured by the PEG-4000 method (Robertson and Wood, 2014), whole body mass changes as an indicator of changes in net whole body water content (Stevens, 1972; Wood and Randall, 1973c; Wood et al., 2009), ventilation measured by buccal pressure recording, and heart rates measured by impedance recording.

Our major hypotheses were: (i) killifish, as a species with exceptional hypoxia tolerance, would reduce diffusive water permeability, without changing gill paracellular permeability, while simultaneously attempting to improve branchial O_2 uptake by increased ventilation and bradycardia, similar to the oscar (Scott et al., 2008; Wood et al., 2009); (ii) as in the oscar, net water gains (in freshwater) or losses (in seawater) would be relatively small; (iii) the relative changes during hypoxia would be greater in freshwater than in seawater killifish because diffusive water permeability is generally higher in freshwater than seawater teleosts (Evans, 1967; Potts and Fleming, 1970; Isaia, 1984); (iv) drinking rates would also be reduced during hypoxia, especially in seawater killifish, as drinking is energetically expensive (Takei et al., 2009; Grosell, 2011); and (v) exercise, unlike hypoxia, would not result in decreases in diffusive water exchange rates.

MATERIALS AND METHODS
Experimental animals
Experiments were performed in February–May over four years (2015–2017, 2019). Adult common killifish of the northern subspecies [Fundulus heteroclitus macrolepidotus (Walbaum 1792); 3–7 g, mixed sex] were collected the preceding October by beach-seining of local tidal flats by Aquatic Research Organisms (ARO) Ltd (Hampton, NH, USA) and held in their facility in 65% seawater at 22°C for several months. After shipment to the University of Miami, they were held at 23–25°C under flow-through conditions in either dechlorinated freshwater or full-strength seawater at a density of 30–50 animals per 50 litre tank for at least 1 month prior to experiments. During this time, they were fed at a daily ration of 1.5% body mass with sinking pellets (50% protein; Purina Aquamax, Shoreview, MN, USA). The ionic composition of the food (Wood et al., 2010) and of Miami freshwater and seawater (Wood and Grosell, 2008) have been reported earlier. Fish were fasted for 24 h prior to experiments, which were performed at the acclimation temperature. All procedures followed an approved University of Miami Animal Care Protocol (IACUC no. 13-225).

Experimental series
All experiments were performed with freshwater and seawater treatments in parallel. In Series 1 and 2 (diffusive water flux measurements), handling of the fish was unavoidable, because measurements had to be made immediately after the fish were transferred from a radioactivity loading vessel, in which they had been confined for 6–8 h, to a radioisotope-free individual vessel (see Series 1). Therefore, to control for the possible disturbance associated with this confinement and transfer, fish in all but two subsequent series were subjected to an identical pre-treatment and transfer protocol, but without radioactivity. In Series 6 and 7, this was not possible because of the need for prior surgical preparation.

For all experimental measurements, fish were held in individual 250-ml Erlenmeyer flasks served with aeration tubing and shielded with black plastic; the flasks were partially submerged in a wet table to maintain the experimental temperature. Normoxia (>80% air saturation=>16.5 kPa=>124 Torr) or hypoxia (10% air saturation=2.1 kPa=15 Torr) was achieved by bubbling with air or nitrogen, respectively; hypoxia could be maintained within ±2% saturation during experiments. Water changes (to hypoxia or normoxia) were performed by flowing pre-equilibrated water through the 250 ml flasks so that the desired partial pressure of O_2 (P_{O_2}) could be achieved within 3 min. All fish were weighed after completion of the procedures.

Series 1: diffusive water flux rates during normoxia, hypoxia and recovery

Treatments included normoxia, hour 1 of hypoxia, hour 3 of hypoxia, hour 1 of normoxic recovery after 3 h of hypoxia, and hour 3 of normoxic recovery after 3 h of hypoxia. Different fish (N=8) were used for each treatment. To avoid the disturbance of injection, fish were loaded with ^3H_2O by incubation in 2 litres of freshwater or seawater labelled with 20 µCi l^{-1} of ^3H_2O (Amersham Pharmacia Biotech, Little Chalfont, UK) for 6–8 h; preliminary experiments demonstrated that equilibration was complete within this time. For uniformity, all fish in each experimental treatment (N=8) were loaded with ^3H_2O simultaneously in the same incubation medium in a single 1.5 litre Erlenmeyer flask. The flask was shielded, held on the wet table for temperature control, and gassed appropriately (see below).

Because the efflux of ^3H_2O from the fish is rapid, it was critical to make all experimental measurements during the 1 h period immediately after the fish were transferred out of the loading medium. Therefore, in the prolonged hypoxia and normoxic recovery experiments, it was necessary to start the hypoxia treatment during the incubation in the loading vessel. Thus, for the 3 h hypoxia treatment, hypoxia was started 2 h before the end of loading, for the 1 h normoxic recovery treatment, hypoxia was started 3 h before the end of loading, and for the 3 h normoxic recovery treatment, hypoxia was started 5 h before the end of loading followed by normoxia in the last 2 h of loading. At the start of each 1 h measurement period, the fish were quickly rinsed, and then added to 220 ml of radioisotope-free freshwater or seawater in the individual 250 ml Erlenmeyer flasks. The water P_{O_2} had been pre-set to the desired level (hypoxia or normoxia). Water samples (5 ml) were taken for scintillation counting at 0, 10, 20, 30, 40, 50 and 60 min, with a final sample at approximately 24 h, by which time the ^3H_2O in the fish had completely equilibrated with the external water. This was used for calculating the total amount of radioactivity loaded into each fish (see Calculations).

Series 2: diffusive water fluxes and oxygen consumption rates during steady-state aerobic exercise

Treatments included exercise [swimming at 2 body lengths (BL) s^{-1}=14–17 cm s^{-1}] for 1 h in a shielded Brett-style swimming respirometer, and rest (~0.5 BL s^{-1}=4 cm s^{-1}) for 1 h in the same respirometer, both under normoxic conditions. In preliminary experiments, we found that the former was the maximum speed that killifish could sustain for 1 h, and the latter was the minimum current needed to keep the fish oriented without bouts of spontaneous activity. Different fish were used for each treatment (N=8). Because the volume of the swimming respirometer (5000 ml) was many-fold greater than the 220 ml used in Series 1, a much higher concentration (200 µCi l^{-1}) of ^3H_2O was used during the 6–8 h pre-experimental loading period. At the start of the experiment, the fish was rinsed, measured for length and placed in the respirometer; because killifish are generally passive in air, anaesthesia was unnecessary. A water sample (5 ml, 0 h) was taken immediately, and the current speed was set to 4 cm s^{-1}. The speed was gradually increased from 0.5 to 2 BL s^{-1} over 4 min, and kept at the latter speed for the duration of the hour, during which time the fish swam continuously. Additional water samples (5 ml) were taken at 10, 20, 30, 40, 50, 60 min, and immediately thereafter the fish was removed and placed in the standard 220 ml flask for 24 h equilibration of the remaining ^3H_2O burden with the external water. Measurements of $\dot{M}_{O_2}$ were taken from 10 min onwards and averaged over the hour using the on-board respirometry system (see Analytical techniques). Water P_{O_2} stayed above 80% air saturation during all tests, and there was no detectable blank $\dot{M}_{O_2}$.

Series 3: oxygen consumption rates during normoxia, hypoxia and recovery

The treatments were exactly parallel to the five treatments of Series 1, with $\dot{M}_{O_2}$ measurements during normoxia, the first and third hours of hypoxia, and the first and third hours of normoxic recovery after 3 h of hypoxia. The only difference was that no ^3H_2O was used during the sham 6–8 h loading period, and during measurements, the individual 250 ml flasks were filled to capacity so that they could be sealed. $\dot{M}_{O_2}$ was measured over a 15–20 min period (in normoxia, exact time noted) or a 5–10 min period (in hypoxia) in the middle of each hour by stopping the gassing, taking an initial P_{O_2} reading, then sealing the flask until the final P_{O_2} reading was taken, after which gassing was resumed. The optical P_{O_2} probe fit snugly into the neck of the flask. Blanks with no fish were run with every treatment, but background $\dot{M}_{O_2}$ was negligible. Different fish (N=8) were used for each treatment.

Series 4: gill paracellular permeability and drinking rate during normoxia, hypoxia and recovery

The methodology developed by Robertson and Wood (2014) was used in which the clearance of radiolabelled polyethylene glycol (M.W. 4000) from the external water is used to measure both drinking rate (by appearance in the gut) and gill paracellular permeability (by appearance in the rest of the carcass). In preliminary experiments, we found that the minimum period over which reliable measurements could be made was 3 h, and therefore three experimental treatments (N=8 each) were used for each salinity: normoxia (3 h), hypoxia (3 h) and normoxic recovery (3 h) after 3 h of hypoxia. As in Series 3, these followed a sham 6–8 h loading period. After this pre-treatment, the fish were placed directly in the freshwater or seawater at the appropriate P_{O_2}, labelled with 25 µCi l^{-1} [^{14}C] PEG-4000 (Amersham Pharmacia Biotech, Little Chalfont, UK). Water samples (2×5 ml) were taken at 0, 1.5 and 3 h from each flask for scintillation counting. At 3 h, the fish were killed by overdose (0.8 g l^{-1}) with MS-222 (Syndel Labs, Parksville, BC, Canada) neutralized with NaOH and pre-equilibrated to the appropriate P_{O_2}, rinsed quickly in radioisotope-free water and blotted dry. The body cavity was opened by dissection and the entire gastrointestinal tract (ligated at both ends to prevent content loss) was removed. The gut and carcass were weighed separately, and incubated in sealed plastic tubes with an equal volume of 2 mol l^{-1} HNO$_3$ for 72 h at 37°C. The digest was then centrifuged (5000 g for 5 min) and the clear supernatant was aliquoted in triplicate for scintillation counting.

Series 5: net mass changes during normoxia, hypoxia and recovery

A standard weighing protocol, modelled after earlier studies (Stevens, 1972; Wood and Randall, 1973c; Wood et al., 2009) was used to measure changes in body mass. As in Series 4, preliminary experiments revealed that the minimum period over which reliable measurements could be made was 3 h, and therefore three experimental treatments (N=7–8 each) were used for each salinity: normoxia (3 h), hypoxia (3 h) and normoxic recovery (3 h) after 3 h of hypoxia, with different fish in each treatment. At the start of the 3 h treatment, each fish was weighed to an accuracy of 0.1 mg on a model CP2245 microbalance (Sartorius AG, Göttingen, Germany). This was accomplished by blotting it on a soft cotton towel for 30 s; as in Series 2, anaesthesia was unnecessary. The procedure was repeated at the end of the 3 h period, and the net whole body water flux rate (J_{H_2O}) was calculated from the change in body mass. All tests followed the 6–8 h sham-loading pre-treatment.

Series 6: ventilation during normoxia, hypoxia and recovery

Killifish were anaesthetized with neutralized MS-222 (0.3 g l^{-1} in freshwater or seawater) and implanted with buccal catheters for recording of ventilatory pressure amplitude and frequency. The operation took only 3 min, and was performed in air as the killifish is very hypoxia tolerant. A hole was punched in the rostrum using a 19 gauge hypodermic needle, taking care to avoid the nares, and a short length (2 cm) of Clay-Adams PE 160 tubing (Becton, Dickinson and Co., Franklin Lakes, NJ, USA), heat-flared on the buccal side, was threaded through the hole, and then a slightly longer length (4 cm) of PE50, again heat-flared on the buccal side, was threaded through the PE160 sleeve. The two tubes were pulled snug to the roof of the buccal cavity, cemented together with a drop of cyanoacrylate glue, and then anchored with silk suture at the point of exit from the rostrum. After overnight recovery (there was no mortality) in individual darkened chambers served with flowing normoxic freshwater or seawater, the water-filled internal PE50 catheter was joined via the shaft of a 22 gauge needle to a longer (50 cm) PE50 tubing connected to a pressure transducer system. The fish were then placed in the individual 250 ml flasks, allowed to settle for 1 h under normoxic conditions, and then normoxic control ventilation data were collected for a 5 min period. The animals were subsequently subjected to 3 h of hypoxia followed by 3 h of normoxic recovery. Ventilation data were collected for 5 min periods at 0.5, 1.5 and 2.5 h of each experimental period. Thus, the same fish (N=8) were followed through normoxia, hypoxia and normoxic recovery.

Series 7: heart rate during normoxia, hypoxia and recovery

Killifish were anaesthetized with neutralized MS-222 (0.3 g l^{-1}) as in Series 5 and implanted with electrodes for recording cardiac frequency by impedance. Laminated copper wires (50 cm, AWG #32, Belden, Chicago, IL, USA), stripped at the implantation end, were inserted ventrally as fish hook electrodes on either side of the heart. Externally, the two wires were sutured to the ventral skin with surgical silk, and pigtailed together with light tape. The fish were allowed to recover overnight (there was no mortality though several fish pulled out their wires) in individual darkened chambers served with flowing normoxic freshwater or seawater, then transferred to the individual 250 ml flasks. The wires were connected to an impedance converter. After a 1 h settling period under normoxic conditions, normoxic control heart rates were recorded for a 5 min period. The animal was then subjected to 3 h of hypoxia followed by 3 h of normoxic recovery. Heart rate data were collected for 5 min periods at 0.5, 1.5 and 2.5 h of each experimental period. As in Series 6, the same fish (N=7) were followed through normoxia, hypoxia and normoxic recovery.

Radioactivity measurements

All ^{3}H$_2$O radioactivity measurements were made with a Packard Tri-Carb 4910TR scintillation counter (Perkin-Elmer, Wellesley, MA, USA) using 5 ml water plus 10 ml of Ecolume fluor (MP Biomedicals, St Louis, MO, USA). Tests showed that quench was constant so no correction was made. All [^{14}C]PEG-4000 radioactivity measurements were made with a Beckman LS6500 scintillation counter (Beckman Coulter, Fullerton, CA, USA) using 2 ml of aqueous sample (water, tissue digest or tissue digest made up to 2 ml with 2.0 mol l^{-1} HNO$_3$) plus 10 ml of Packard Ultima Gold AB fluor (Perkin-Elmer, Wellesley, MA, USA). Tissue digests were quench-corrected to the same counting efficiency as water samples using a quench curve constructed with various amounts of tissue digest.

Physiological recordings

All oxygen measurements were made with a YSI Optical Probe and Digital Professional Series meter (YSI, Yellow Springs, OH, USA), except in Series 2, in which swimming experiments were performed in a Loligo SW10060 swim tunnel respirometer (Loligo Systems, Viborg, Denmark) fitted with a fibre optic dipping probe (PreSens Precision Sensing, Regensburg, Germany) connected to a Witrox minisensor oxygen meter running on AutoResp™ 2.1.0 software (Loligo Systems). The system was calibrated as described by Stieglitz et al. (2016). Recordings of ventilatory pressure were made with DA100C transducers connected to an MP150 data acquisition system (Biopac Systems Inc., Goleta, CA, USA). The transducers were calibrated against a column of water. Recordings of heart rate were made with a 2991 impedance converter (Transmed Corporation, Fullerton, CA, USA).

Calculations

In Series 1 and 2, the rate constant of ^{3}H$_2$O turnover was calculated from the exponential rate of decline in total ^{3}H$_2$O radioactivity in the fish (Evans, 1967):

$$k_{3H_2O} = \frac{\ln R_1 - \ln R_2}{t_1 - t_2},\qquad(1)$$

where k_{3H_2O} is the rate constant of the efflux (h^{-1}), and R_1 and R_2 are total ^{3}H$_2$O radioactivities (cpm) in the fish at times t_1 and t_2 (h).

In practice, the rate constant k_{3H_2O} was calculated by regression of the natural logarithm of R against time over the range of linearity (generally 10 to 40 or 50 min after transfer to the experimental chambers). By measuring the ^{3}H$_2$O radioactivity in the water after 24 h, when complete equilibration between the fish and the water had occurred, it was possible to accurately calculate the total amount of ^{3}H$_2$O radioactivity (R_{total}) in the system, taking into account radioactivity removed in sampling. The volume of the system was taken as the known volume of external water plus the volume of the fish. Therefore, from R_{total} and from measurements of ^{3}H$_2$O radioactivity appearance in the water at each time interval, it was possible to back-calculate the total R in the fish at each time during the experiment.

In Series 2 and 3, P_{O_2} measurements were converted to O$_2$ concentrations in the water using solubility constants from Boutilier et al. (1984). $\dot{M}_{O_2}$ (µmol O$_2$ kg^{-1} h^{-1}) was calculated as:

$$\dot{M}_{O_2} = \frac{(O_{2\text{-}1} - O_{2\text{-}2}) \times V}{M_b \times (t_1 - t_2)},\qquad(2)$$

where V is the volume of the respirometer (l), $O_{2\text{-}1}$ and $O_{2\text{-}2}$ are oxygen concentrations in the water (µmol l^{-1}) at times t_1 and t_2 (h), and M_b is body mass (g).

In Series 4, gill [^{14}C]PEG-4000 influx clearance rate (C_{PEG}, µl g^{-1} h^{-1}), as an index of paracellular permeability, was calculated as:

$$C_{PEG} = \frac{PEG_{carcass}}{PEG_{water} \times M_b \times t},\qquad(3)$$

where $PEG_{carcass}$ is the PEG in the carcass (cpm) and PEG_{water} is the mean water PEG (cpm ml^{-1}) and t is 3 h. Drinking rate (D, µl g^{-1} h^{-1}) was calculated as:

$$D = \frac{PEG_{gut}}{PEG_{water} \times M_b \times t},\qquad(4)$$

where PEG_{gut} is the total PEG in the gut (cpm) and t is 3 h.

In Series 5, the net whole body water flux rate (J_{H_2O}, µl g^{-1} h^{-1}) relative to initial body mass (g) was calculated from the initial (M_i)

and final (M_f) body masses (mg), assuming 1 mg=1 µl:

$$J_{H_2O} = \frac{M_i - M_f}{M_i \times t},\qquad(5)$$

where t is 3 h.

In Series 6, the ventilatory index (VI, cm H_2O min^{-1}) was calculated as the product of ventilatory rate (frequency, f, min^{-1}) and buccal pressure amplitude (A, cm H_2O).

$$VI = f \times A.\qquad(6)$$

Statistical analyses

Data are reported as means±1 s.e.m. (N=number of fish). In most experiments, data were analysed by two-way ANOVA, and specific differences were identified by either Dunnett's test (difference from control) or Tukey's test (differences among treatments). One-way repeated-measures ANOVA was employed in Series 6 and 7. All data were checked for normality (Shapiro–Wilk test) and homogeneity (Bartlett's chi square), and where necessary were subjected to logarithmic or square root transformations. Pair-wise comparisons were made with Student's unpaired t-test or one-sample t-test, as appropriate. All tests were two-tailed and significance was accepted at $P<0.05$.

RESULTS

Diffusive water fluxes, oxygen consumption, paracellular permeability and drinking rates during normoxia, hypoxia and recovery (Series 1, 3 and 4)

In seawater killifish, the rate constant k for ^3H_2O exchange (k_{3H_2O}, Series 1) was approximately 0.6 h^{-1} (i.e. 60% of the body water pool per hour, or approximately 480 µl g^{-1} h^{-1}) under normoxia, and did not change during either hour 1 or hour 3 of hypoxia (Fig. 1A). Upon return to normoxia, k_{3H_2O} fell significantly by approximately one-third during the first hour of recovery, but was restored to
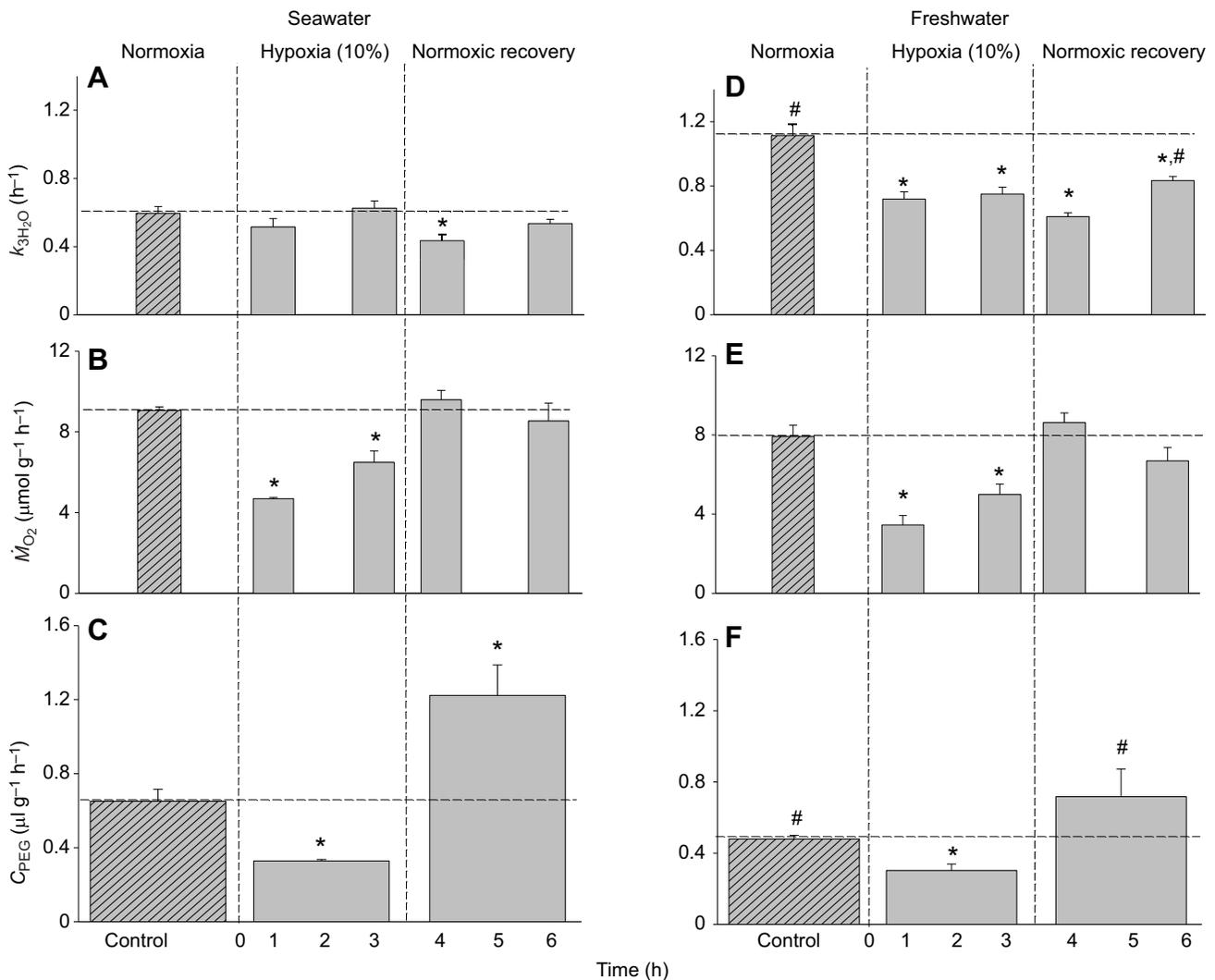
Fig. 1. The effects of hypoxia and normoxic recovery on rates of diffusive water exchange (k_{3H_2O}, Series 1), oxygen consumption ($\dot{M}_{O_2}$, Series 3) and [^{14}C]PEG-4000 clearance (C_{PEG}, Series 4) of killifish acclimated to either seawater or freshwater. (A) Diffusive water exchange in seawater. (B) Oxygen consumption in seawater. (C) [^{14}C]PEG-4000 clearance in seawater. (D) Diffusive water exchange in freshwater. (E) Oxygen consumption in freshwater. (F) [^{14}C]PEG-4000 clearance in freshwater. Fish were exposed to normoxic control conditions, severe hypoxia (10% air saturation) for 1 or 3 h, followed by normoxic recovery for 1 or 3 h. For C_{PEG}, measurements were made over 3 h. Data are means±1 s.e.m. (N=8 different fish in each treatment, for each measured parameter). Two-way ANOVA with Dunnett's test (* represents significant differences from respective normoxic control means) or Tukey's test (# represents significant differences between salinities at the same time). For $\dot{M}_{O_2}$, there were overall significant effects of treatment only, whereas for k_{3H_2O} and C_{PEG} there were overall effects of both salinity and treatment.

normoxic control values during the final hour. $\dot{M}_{O_2}$ (Series 3) followed a very different pattern, dropping significantly by approximately 50% from the normoxic control rate of 9 μmol g^{-1} h^{-1} during hour 1 of hypoxia, with partial restoration to approximately 70% by hour 3 (Fig. 1B). Upon return to normoxia, control normoxic rates were re-established during both the first and third hours, with no evidence of overshoot. Paracellular permeability (Series 4), as measured by gill [^{14}C]PEG-4000 influx clearance rate (C_{PEG}), exhibited yet a different pattern (Fig. 1C). C_{PEG} was approximately 0.65 μl g^{-1} h^{-1} under normoxia, and decreased significantly by 50% over the 3 h period of hypoxia. However, during the 3 h period of normoxic recovery, there was a massive overshoot of C_{PEG} to 188% of control values.

In freshwater killifish, k_{3H_2O} (Series 1) was approximately 1.15 h^{-1} (i.e. approximately 920 μl g^{-1} h^{-1}) (Fig. 1D) under normoxia, significantly higher than in seawater under the same conditions (see Fig. 1A). During both hours 1 and 3 of hypoxia, k_{3H_2O} was significantly depressed to approximately 65% of control values, and this depression was maintained during both the first and third hours of recovery. Unlike the very different pattern of k_{3H_2O} response, absolute rates and changes in $\dot{M}_{O_2}$ (Series 3) during hypoxia and normoxic recovery (Fig. 1E) were very similar to those in seawater animals (see Fig. 1B). In contrast to diffusive water permeability (i.e. k_{3H_2O}), control paracellular permeability (C_{PEG}, Series 4) was approximately 0.48 μl g^{-1} h^{-1} (Fig. 1F), significantly lower than the comparable value in seawater (see Fig. 1C). However, the pattern of response to hypoxia was qualitatively similar, with a significant

37% decrease during hypoxia, and a non-significant overshoot to 149% during normoxic recovery (Fig. 1F).

Drinking rates (D), which were measured in the same Series 4 experiments, were substantially higher in seawater killifish (~1.61 μl g^{-1} h^{-1}) than in freshwater animals (~0.20 μl g^{-1} h^{-1}) under normoxia (Fig. 2). Thus, D was lower than C_{PEG} in freshwater, and much greater than C_{PEG} in seawater (see Fig. 1C,F). Nevertheless, in both salinities, 3 h of hypoxia had a qualitatively similar effect, virtually obliterating the D (89–91% inhibition), whereas an overshoot to 252–289% occurred during the 3 h recovery period.

Overall, these experiments indicated that changes in diffusive water permeability and paracellular permeability did not parallel one another during hypoxia and normoxic recovery. Changes in D and C_{PEG} were qualitatively similar, decreasing during hypoxia and increasing during normoxic recovery in both seawater and freshwater fish.

Diffusive water fluxes and oxygen consumption rates during steady-state aerobic exercise (Series 2)

Diffusive water exchange rates (Fig. 3), measured in fish at rest but orienting into a gentle current in the 5 litre swim tunnel, were similar to those measured in the 250 ml flasks (see Fig. 1A,D). Again, k_{3H_2O} was significantly lower in seawater (Fig. 3A) than in freshwater (Fig. 3C), whereas $\dot{M}_{O_2}$ values at rest did not differ between the two salinities (Fig. 3B,D). During steady-state swimming at 2 BL s^{-1},
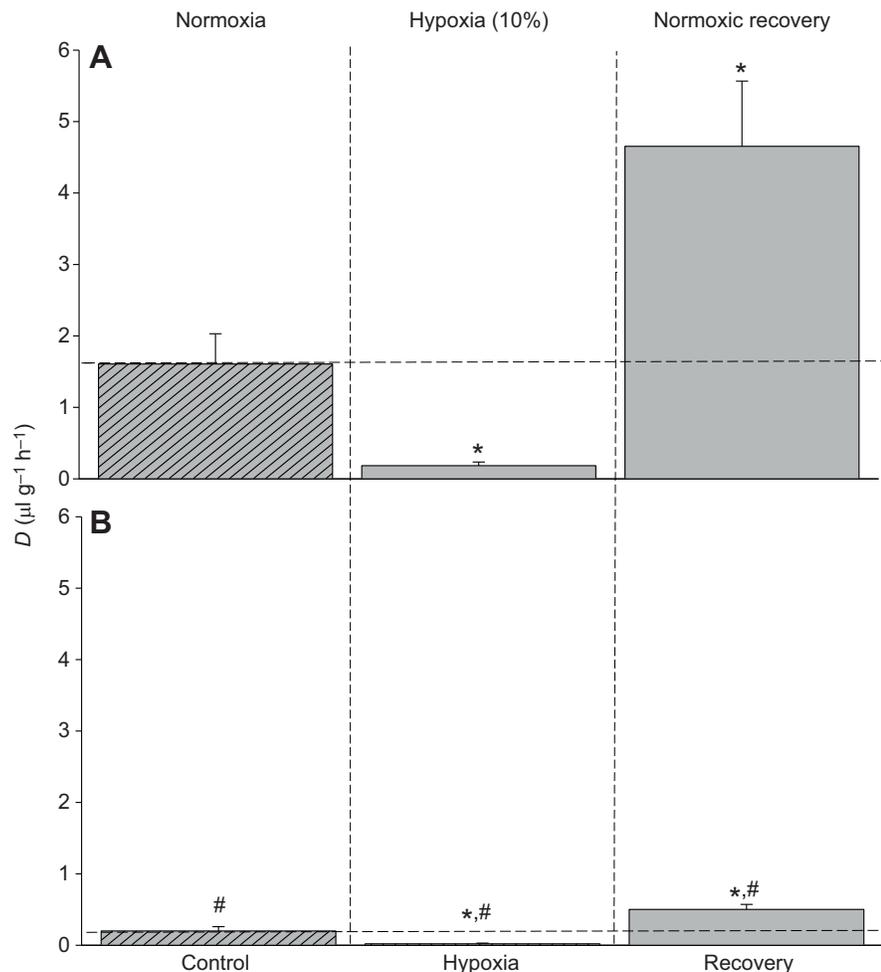
Fig. 2. The effects of hypoxia and normoxic recovery on drinking rates (D, Series 4) of killifish acclimated to either seawater or freshwater. (A) Drinking rate in seawater. (B) Drinking rate in freshwater. Data are means±1 s.e.m. (N=8 different fish in each treatment). Other details as in legend of Fig. 1. The overall effects of both salinity and treatment were significant.
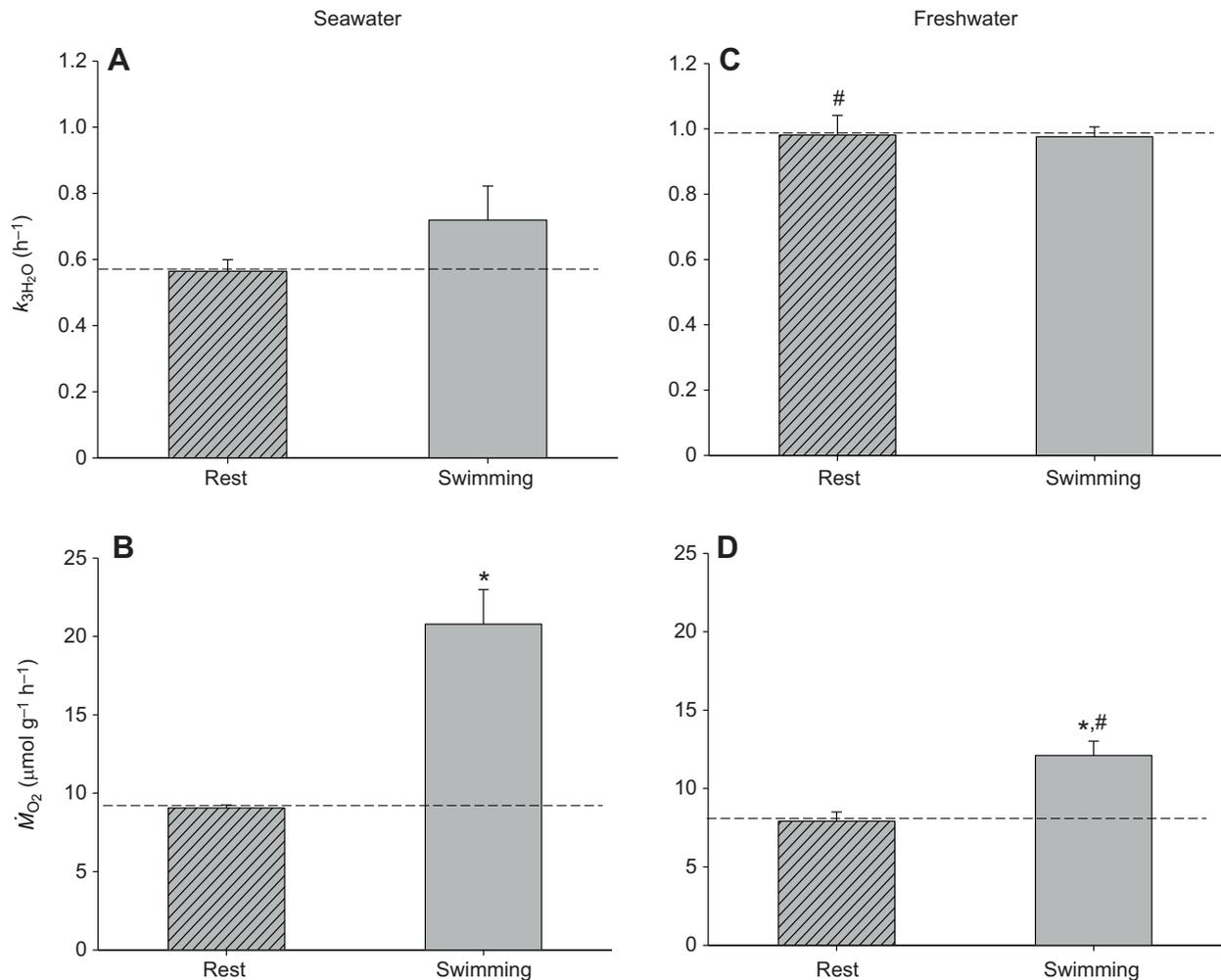
Fig. 3. The effects of steady-state exercise on rates of diffusive water exchange (k_{3H_2O}, Series 2) and oxygen consumption ($\dot{M}_{O_2}$) of killifish acclimated to either seawater or freshwater. (A) Diffusive water exchange in seawater. (B) Oxygen consumption in seawater. (C) Diffusive water exchange in freshwater. (D) Oxygen consumption in freshwater. Measurements were made at rest or during 1 h of swimming at 2 body lengths s^{-1}. Data are means±1 s.e.m. (N=8 different fish in each treatment, for each measured parameter). Other details as in legend of Fig. 1. For k_{3H_2O}, there were overall significant effects of salinity but not swimming, whereas for $\dot{M}_{O_2}$ there were overall significant effects of swimming but not salinity, as well as significant interaction.

there was no significant change in k_{3H_2O}, despite significant increases in $\dot{M}_{O_2}$ to 230% of resting rates in seawater and 150% in freshwater. The swimming $\dot{M}_{O_2}$ values were significantly higher in the seawater killifish, suggesting a higher cost of transport than in freshwater.

The objective of this experiment was to evaluate whether a very different demand on $\dot{M}_{O_2}$ (exercise rather than hypoxia) would cause similar changes in diffusive water permeability. Clearly, it did not in freshwater (see Fig. 1D), though the lack of change in k_{3H_2O} during swimming in seawater (Fig. 3A) was consistent with the lack of response to hypoxia (cf. Fig. 1A).

### Net mass changes during normoxia, hypoxia and recovery (Series 5)

In Series 5, changes in body mass were used as a measure of net whole body water flux rate over 3 h. In the control normoxic period, changes were not significantly different from zero in either seawater (Fig. 4A) or freshwater (Fig. 4B), indicating the lack of a handling effect. During hypoxia in freshwater, killifish gained approximately 60 mg g^{-1} of original body mass, whereas during hypoxia in seawater, they lost an almost identical amount. These changes were significant relative both to zero and to the control normoxic

values. During the normoxic recovery period, in both salinities, changes were not significantly different from zero. Therefore, when these data were converted to net whole body water flux rates (J_{H_2O}; Fig. 4), this parameter exhibited a pattern which differed from those of both ^3H_2O exchange (Fig. 1A) and paracellular permeability (Fig. 1C).

During 3 h of hypoxia, seawater fish lost approximately 21 µl g^{-1} h^{-1}, and during normoxic recovery, J_{H_2O} returned to net balance, but the net loss of body water was not restored (Fig. 4A). Similarly, freshwater fish gained approximately 21 µl g^{-1} h^{-1} during hypoxia, with a return to zero balance upon restoration of normoxia (Fig. 4B). Thus the net gain of body water was not excreted during the first 3 h of recovery.

Ventilation and heart rate during normoxia, hypoxia and recovery (Series 6 and 7)

In Series 5, control ventilatory rate in seawater killifish was approximately 82 min^{-1} (Fig. 5A), buccal pressure amplitude was approximately 0.5 cm H_2O (Fig. 5B), and therefore the ventilatory index (VI, the product of frequency×amplitude) was approximately 41 cm H_2O min^{-1} (Fig. 5C). Frequency increased significantly by approximately 35% (Fig. 5A), amplitude by approximately 140%
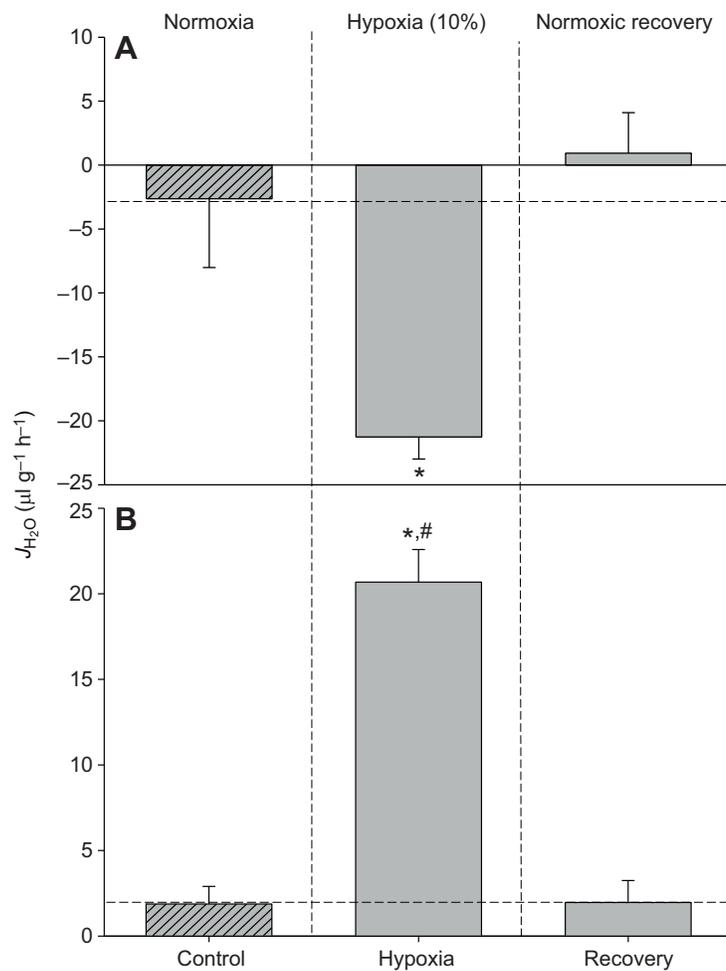
Fig. 4. The effects of hypoxia and normoxic recovery on net whole body water flux rates (J_{H_2O}) (Series 5) of killifish acclimated to either seawater or freshwater. (A) Net whole body water flux rates in seawater. (B) Net whole body water flux rates in freshwater. Data are means±1 s.e.m. (N=7–8 different fish in each treatment). J_{H_2O} was calculated from changes in body mass. Student's one-sample t-test demonstrated that mass changes were not significantly different from zero during normoxic control and normoxic recovery periods in both salinities. Other details as in legend of Fig. 1. The overall effects of both salinity and treatment, as well as their interaction, were significant.

(Fig. 5B) and VI by approximately 3.2-fold (Fig. 5C) throughout the 3 h of hypoxia, and then returned to control levels throughout the normoxic recovery period. In Series 6, the accompanying cardiac response was a classic bradycardia throughout hypoxia, a slowing of the heart rate by approximately 35% from the control value of approximately 70 min^{-1}, with a return to control values throughout normoxic recovery (Fig. 5D).

Patterns in freshwater fish were qualitatively similar, with significant increases in ventilation and decreases in heart rate throughout hypoxia, and a return to control values throughout recovery (Fig. 5E,F,G,H). However, the control ventilation frequency in normoxia (~104 min^{-1}) was significantly higher than for seawater-acclimated fish, whereas the control pressure amplitude (~0.85 cm H$_2$O) and VI (88 cm H$_2$O min^{-1}) were non-significantly higher, and the control heart rate (~60 min^{-1}) was non-significantly lower. There were no significant differences between the two salinities in any of these parameters during hypoxia, but a significantly lower heart rate during normoxic recovery in the freshwater-adapted animals (Fig. 5H).

These ventilatory and cardiac measurements confirmed that killifish were indeed attempting to improve O$_2$ uptake during severe hypoxia rather than shutting down. The qualitatively similar patterns between seawater and freshwater further demonstrated that differences in diffusive water exchange responses between salinities (Fig. 1A versus 1D), and particularly the persistence of reduced exchange throughout normoxic recovery in freshwater (Fig. 1D) could not be explained by differences in cardio-ventilatory responses.

DISCUSSION
Overview
Our results show that the osmorespiratory compromise during severe hypoxia in the killifish is complex, multi-faceted and salinity-dependent, with important differences from the previously studied trout and oscar. With respect to our original hypotheses, we had predicted that, as a species with exceptional tolerance to hypoxia, the killifish would reduce diffusive water permeability during hypoxia, without changing gill paracellular permeability, while simultaneously making ventilatory and cardiac adjustments to improve $\dot{M}_{O_2}$. Only part of this hypothesis was confirmed. The expected ventilatory and cardiac changes occurred, and gill diffusive water fluxes were decreased in freshwater killifish during hypoxia, as in the oscar but in contrast to the trout. However, the reduction was maintained during normoxic recovery, unlike the oscar. In both freshwater and seawater, paracellular permeability also decreased, again unlike the oscar. Despite these apparent homeostatic responses, killifish exhibited a net mass loss during hypoxia in seawater, and a net mass gain during hypoxia in freshwater, changes that were not corrected during normoxic recovery. The net whole body water flux rates calculated from these data were much higher than in the oscar, again contrary to one of our original hypotheses. However, as predicted, drinking rates were substantially reduced during hypoxia. Our hypothesis that diffusive water exchange would not decrease during exercise (in contrast to hypoxia) was also confirmed. Furthermore, the diffusive water permeability under normoxia was higher in freshwater than in
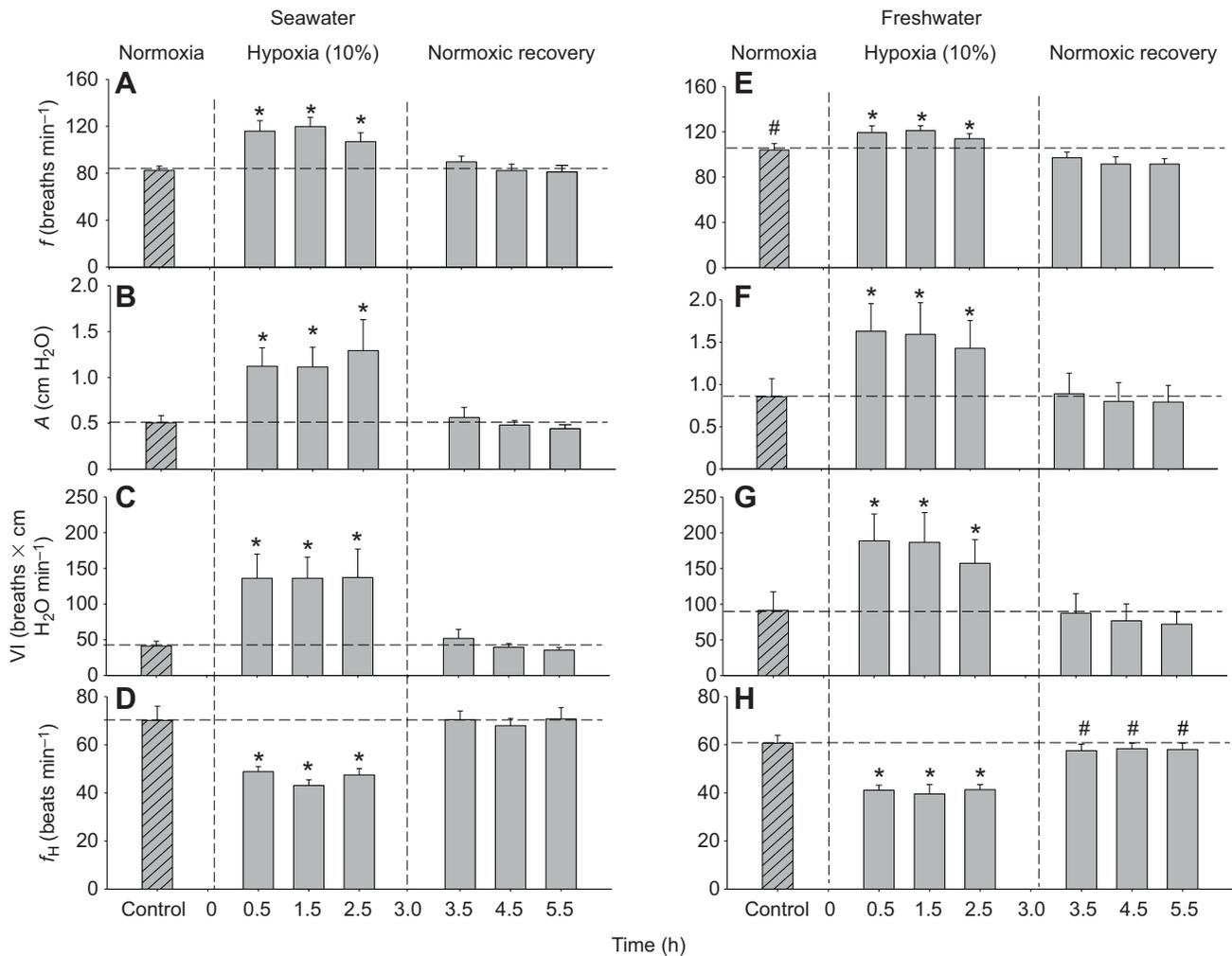
Fig. 5. The effects of hypoxia and normoxic recovery on ventilatory parameters (Series 6) and heart rate (Series 7) of killifish acclimated to either seawater or freshwater. (A,E) ventilation rate (f); (B,F) buccal pressure amplitude (A); (C,G) ventilatory index (VI) and (D,H) heart rate (f_H). Means±1 s.e.m. (N=8 in Series 5, N=7 in Series 6; in both, the same fish were followed throughout normoxia, hypoxia and normoxic recovery). One-way repeated-measures ANOVA with Dunnett's test [* represents significant differences (P<0.05) from normoxic control mean] and unpaired Student's test (# represents significant differences between salinities at the same time).

seawater killifish and changed to a greater extent during hypoxia, all in accord with predictions. While answering our hypotheses, our data highlight several paradoxes in our current understanding of water regulation in fish.

The cardio-respiratory response to hypoxia and normoxic recovery

The level of hypoxia used (10% air saturation=2.1 kPa=15 Torr) was below the critical O_2 tension (P_{crit}) reported in several recent studies on *F. heteroclitus* (reviewed by Wood, 2018). This was confirmed by the 50% fall in $\dot{M}_{O_2}$ during the first hour (Fig. 1B,E). Ventilation and heart rate (Fig. 5) were recorded to ensure that killifish were not simply shutting down during hypoxia, as seen in some species (Richards, 2009). Clearly, this was not the case. Rather, killifish exhibited a vigorous hyperventilation, in which elevations in ventilatory amplitude dominated, and were able to increase $\dot{M}_{O_2}$ by the third hour of maintained hypoxia (Fig. 1B,E). These ventilatory changes, as well as the observed bradycardia, are typical adaptive responses of many teleosts to hypoxia (Perry et al., 2009; Farrell and Richards, 2009). Routine activity (Chapman and McKenzie, 2009) and tissue O_2 demand (Richards, 2009) may also

have been suppressed, as there was no evidence of a post-hypoxia oxygen debt. Interestingly, freshwater killifish appeared to exhibit higher ventilation and lower heart rates than seawater animals, both at rest and during hypoxic challenge (Fig. 5), yet consumed less O_2 during steady-state swimming (Fig. 3B,D). A detailed examination of salinity effects on cardio-respiratory function in *F. heteroclitus* would be informative. Other adaptive responses to hypoxia likely involved improvement in the functional permeability of the gills to O_2 (e.g. increased lamellar perfusion and functional surface area, thinning of diffusion distance), as well as improvements in blood–O_2 affinity and capacity (Perry and Wood, 1989; Wells, 2009; Farrell and Richards, 2009).

Diffusive water fluxes, branchial [14C]PEG-4000 clearance rates and drinking rates as indicators of the osmorespiratory compromise during hypoxia

As outlined in the Introduction, diffusive water flux probably occurs through the entire gill surface, so may be dominated by the transcellular pathway, whereas osmotic water flux is thought to be dominated by bulk flow through the paracellular pathway. We therefore measured ^3H_2O water exchange (k_{3H_2O}) as a direct measure

of diffusive water flux, and PEG-4000 clearance (C_{PEG}) as a proxy for the latter. As a point of clarification, C_{PEG} values are not a measure of water flux, but rather an indicator of the permeability of the paracellular pathway. With a molecular weight 250-fold smaller than PEG-4000 and a prevailing osmotic gradient, water would be expected to move through the pathway at a much greater rate than PEG-4000.

Under control conditions, k_{3H_2O} was much lower in seawater than in freshwater (Fig. 1A,D), in general agreement with previous studies (Evans, 1969; Motais et al., 1969; Potts and Fleming, 1970; Isaia, 1984). This difference correlates with the lower expression of aquaporins in the gills reported in many studies, and may be an adaptation to counter the higher osmotic gradient between the environment and plasma in seawater (Isaia, 1984; Lignot et al., 2002; Cutler et al., 2007; Tipsmark et al., 2010; Madsen et al., 2015; Breves et al., 2016). The absolute values of k_{3H_2O} measured in freshwater and seawater killifish were reasonably similar to values recorded in a variety of teleost species when adjusted for size and temperature (Potts et al., 1967; Evans, 1969; Motais et al., 1969; Loretz, 1979; Onukwufor and Wood, 2018). In contrast to k_{3H_2O}, C_{PEG} was higher in seawater killifish under normoxia than in freshwater. This agrees with the general belief that the tight junctions in the branchial epithelium in seawater teleosts are 'leaky' to facilitate paracellular Na^+ efflux between the chloride and accessory cells (e.g. Wood and Marshall, 1994; Wilson and Laurent, 2002) and the observation that the expression levels of claudins are generally lower in the gills of seawater-adapted fish (Chasiotis et al., 2012), including *F. heteroclitus* (Whitehead et al., 2010, 2011). There are only a few previous measurements of PEG-4000 permeability in fish gills, all in freshwater (Scott et al., 2004; Sloman et al., 2004; Robertson and Wood, 2014; Robertson et al., 2015a). The present C_{PEG} values are the lowest yet recorded, indeed approximately 65% below the earlier low values of Scott et al. (2004) for the same species.

The rapid reduction of k_{3H_2O} during severe hypoxia in freshwater killifish (Fig. 1A) was similar to that of another hypoxia-tolerant fish, the stenohaline freshwater oscar (Wood et al., 2009), but differed in persisting throughout 3 h of normoxic recovery. This suggests that the reduction was a regulated phenomenon. However, another hypoxia-tolerant fish, the stenohaline goldfish, exhibited an increase in k_{3H_2O} during hypoxia (Loretz, 1979). In seawater killifish, k_{3H_2O} was not reduced in the face of the same hypoxic challenge though it was slightly lower during the first hour of recovery (Fig. 1D). Perhaps seawater animals were already close to their lower limit for diffusive water permeability. In the oscar, decreased k_{3H_2O} was accompanied by reduced ion fluxes; both were attributed to a morphological covering of chloride cells by pavement cells (Wood et al., 2009; Matey et al., 2011; De Boeck et al., 2013; Robertson et al., 2015b). Interestingly, neither the killifish (Fig. 3) nor the oscar (Robertson et al., 2015a) showed this osmorespiratory compromise during exercise, suggesting that the response is specific to hypoxia in these hypoxia-tolerant species. In the hypoxia-intolerant trout, exactly the opposite occurred during hypoxia: increased k_{3H_2O}, increased ion fluxes and uncovering of the chloride cells (Iftikar et al., 2010; Matey et al., 2011; Robertson et al., 2015b), though the increased k_{3H_2O} was attenuated over time (Onukwufor and Wood, 2018). In the future, examination of gill ion fluxes, morphology, and aquaporin expression during and after hypoxia, across a range of salinities, will be informative. The limited information available suggests that aquaporin 3 expression patterns in the gills may be unusual in killifish, not changing in protein content between freshwater and seawater acclimation, but the effects of hypoxia exposure were not examined (Jung et al., 2012).

Another important difference between killifish and oscar was the marked reduction in C_{PEG} during hypoxia, followed by compensatory overshoot during recovery, in both freshwater and seawater (Fig. 1C,F). In the oscar, C_{PEG} remained unchanged during these treatments. Clearly, C_{PEG} reflects a different pathway from k_{3H_2O}. To our knowledge, the present data provide the first evidence that gill paracellular permeability can be altered by acute changes in O_2 availability, though it increases with exercise in the trout (Robertson and Wood, 2014), but not in the oscar (Robertson et al., 2015a). One possible control is the stress hormone cortisol, which is known to increase during acute hypoxia (Mommsen et al., 1999) and to decrease gill paracellular permeability through effects on tight junctions (Chasiotis et al., 2012).

Seawater teleosts ingest water to counter osmotic losses and thereby maintain internal fluid volume. The process is costly, especially because it necessitates active absorption of NaCl as well as active HCO_3^- secretion by the gastrointestinal tract and excretion of the absorbed NaCl at the gills (Takei and Balment, 2009; Grosell, 2011). In freshwater, drinking is essentially counterproductive to fluid volume homeostasis. Therefore, as expected, drinking rates in freshwater killifish were very low relative to seawater individuals (Fig. 2), in accord with many previous observations (Takei and Balment, 2009). Even in seawater, control normoxic rates were lower than previously reported for this species (Potts and Evans, 1967; Malvin et al., 1980; Scott et al., 2006). Seawater killifish stopped drinking almost entirely during hypoxia, with a compensatory rebound during recovery (Fig. 2A). This response would help minimize metabolic costs at a time of O_2 limitation. Interestingly, the same pattern was seen with the very low drinking rate in freshwater (Fig. 2B). To our knowledge, these responses have not been documented previously, though many other gastrointestinal processes are inhibited by hypoxia (Wang et al., 2009). Drinking and volume regulation are under complex control by angiotensin II and a variety of other dipsogenic, as well as antidipsogenic, hormones (Takei and Balment, 2009). Future research should probe the proximate mechanism by which hypoxia inhibits drinking.

To ensure that the degree of disturbance was the same for all fish, so as to allow comparisons among different parameters, animals were subjected to the same prior confinement and handling necessitated by the ^3H_2O loading procedure that was unavoidable for the diffusive water flux measurements. This prior treatment did not appear to affect routine $\dot{M}_{O_2}$ values (Fig. 1B,E) that were well within the ranges reported in other studies on resting killifish under normoxia at comparable temperature (summarized by Wood, 2018), but it is possible that this disturbance was responsible for the relatively low values of paracellular permeabilities (Fig. 1C,F) and drinking rates (Fig. 2) under normoxia discussed earlier.

The overall responses in net water balance

The benefit of decreasing both the transcellular (Fig. 1D) and paracellular permeabilities (Fig. 1F) of the gills in freshwater, and the paracellular permeability in seawater (Fig. 1C) during hypoxia is obvious – decreased water entry in the former, and decreased water loss in the latter. They may also help reduce passive losses (in freshwater) and gains (in seawater) of major ions. This will minimize osmoregulatory and ionoregulatory costs, at a time when the effective O_2 permeability of the gills is elevated, and the now-limited $\dot{M}_{O_2}$ must be prioritized to increased cardio-ventilatory demands. In seawater, it allows the killifish to reduce costly drinking (Fig. 2A), and in freshwater, it likely allows the kidney to reduce its output, as in the oscar (Wood et al., 2009). If these adjustments are beneficial to osmoregulation and ionoregulation, why are they not

present all the time (i.e. during normoxia)? It may be because they cause other unfavourable effects, such as reductions in nitrogenous waste excretion, as seen in the oscar in freshwater (Wood et al., 2007, 2009), and changes in transepithelial potential, which have been documented in the killifish in both freshwater and seawater (Wood and Grosell, 2015). Potentially, they may also interfere with the ability to acid–base regulate, though to our knowledge, this has not been tested.

In light of these permeability reductions, we might have expected a strong homeostasis of water content during hypoxia, especially in freshwater, similar to that observed in the stenohaline oscar during a comparable hypoxic exposure. However, our measurements of net whole body water flux rates (J_{H_2O}) demonstrated that this was not the case (Fig. 4B). Freshwater killifish exhibited a rate of net water gain of about $21\,\mu l\,g^{-1}\,h^{-1}$, 10-fold greater than the $2.1\,\mu l\,g^{-1}\,h^{-1}$ observed in the oscar subjected to an identical hypoxia treatment (Wood et al., 2009). We are aware of no other data on J_{H_2O} in hypoxia-exposed fish. However, the rate is identical ($21\,\mu l\,g^{-1}\,h^{-1}$) to that measured when freshwater-acclimated killifish were acutely exposed to seawater (Kidder et al., 2006), and much lower than measured in two other euryhaline species in freshwater during various enforced exercise regimes – the hypoxia-intolerant rainbow trout ($64\,\mu l\,g^{-1}\,h^{-1}$, Wood and Randall, 1973c; $201\,\mu l\,g^{-1}\,h^{-1}$, Stevens, 1968) and the tilapia ($87\,\mu l\,g^{-1}\,h^{-1}$, Stevens, 1972). Perhaps euryhalinity constrains the ability to limit net water flux. Comparable data are needed on a range of species varying in hypoxia and salinity tolerance to evaluate this hypothesis.

Interestingly, in seawater killifish, absolute J_{H_2O} during hypoxia exposure was identical to that in freshwater killifish, but of opposite sign (i.e. net water loss at a rate of $21\,\mu l\,g^{-1}\,h^{-1}$; Fig. 4A). This occurred despite the approximately two-fold greater osmotic gradient between the environment and body fluids in seawater. Similarly, tilapia exercised in seawater suffered net water loss at the same rate as they gained it during exercise in freshwater (Stevens, 1972). Overall, these data are in accord with lower diffusive water permeability as an adaptation to the higher osmotic gradient in seawater, but not easy to reconcile with the idea that osmotic water flux occurs mainly through the paracellular pathway, which paradoxically must be kept high for Na^+ efflux in seawater. It is also difficult to understand how net water loss can occur during hypoxia in seawater (Fig. 4A), despite unchanged diffusive permeability (Fig. 1A) and decreased paracellular permeability (Fig. 1C), and even more difficult to understand how net water gain can occur during hypoxia in freshwater (Fig. 4B) when both permeabilities are reduced (Fig. 1A,C). Normal urine flow is reported to be approximately $10\,\mu l\,g^{-1}\,h^{-1}$ in resting freshwater killifish, and approximately $0.6\,\mu l\,g^{-1}\,h^{-1}$ in resting seawater killifish (Stanley and Fleming, 1964; Fleming and Stanley, 1965). Even if hypoxia completely stopped urine output in freshwater and greatly stimulated it in seawater, simple calculations based on these values and on measured drinking rates (Fig. 4) indicate that changes in these parameters cannot fully explain the discrepancies. Perhaps it is time to re-assess our understanding of diffusive water exchange and paracellular permeability in fish gills. Considering that fish live in water, it is surprising how little we yet know about how they regulate this substance.

Competing interests
The authors declare no competing or financial interests.

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