# The osmorespiratory compromise in rainbow trout (*Oncorhynchus mykiss*): The effects of fish size, hypoxia, temperature and strenuous exercise on gill diffusive water fluxes and sodium net loss rates

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ABSTRACT

In the context of the osmorespiratory compromise, hypoxia and temperature have been little studied relative to exercise, and diffusive water flux rates (as assessed by $^3H_2O$ efflux) have received almost no attention. We investigated the effects of fish size, hypoxia, exercise and acute temperature increase on diffusive water flux rates and net sodium loss rates in juvenile rainbow trout. Trout weighing 13–50 g were used to determine the effects of fish size under normoxia. Thereafter 25–50 g trout were selected to assess the effects of different hypoxia levels (3.15, 5.25 and 8.40 KPa), time course of hypoxia (1 h 8.40 KPa, 3 h 8.40 KPa, 1 h 8.40 KPa + 1 h normoxic recovery, and 1 h 8.40 KPa + 3 h normoxic recovery), strenuous exercise (5 min) and acute temperature challenge (transfer from 8 °C to 13 °C or to 18 °C). Small fish (13 g) had higher diffusive water flux rates than larger fish, turning over > 100% of their fractional body water pool per hour against 34% per hour for 50 g fish. Hypoxic exposure exerted a biphasic effect, increasing the diffusive water flux rate at 8.40 KPa and 5.25 KPa, while returning it to control levels at 3.15 KPa. All the levels of hypoxia increased net $Na^+$ loss. One hour hypoxia (8.40 KPa) increased diffusive water flux rate while prolonged 3 h hypoxia (8.40 KPa), and short or prolonged normoxic recovery returned diffusive water flux rates to control levels. All the treatments over the time course of hypoxia and normoxic recovery increased net $Na^+$ loss rates. Strenuous exercise increased both the diffusive water flux and net $Na^+$ loss rates. Acute temperature rise increased diffusive water flux rates, with $Q_{10}$ values of 4.03 for 8 to 13 °C and 2.16 for 8 to 18 °C, but the net $Na^+$ loss rate did not change. There was no significant correlation between diffusive water flux rate and net $Na^+$ loss rates at different hypoxia levels, over the course of hypoxia and normoxic recovery, or during acute temperature stress. In contrast, we observed a significant correlation between diffusive water flux and net $Na^+$ loss rates following exercise. Overall, diffusive water flux and sodium loss were regulated differently during acute temperature challenge and hypoxia, while following exercise the two parameters were regulated in a similar fashion.

## 1. Introduction

The gill of teleost fish is a multi-functional organ (Evans et al., 2005) that is used not only for respiratory gas exchange (Randall and Daxboeck, 1984; Nikinmaa, 2006) but also as a major site for ionoregulation (Marshall and Grosell, 2006; Hwang and Lee, 2007), nitrogenous waste excretion (Wood, 1993), acid-base balance (Perry and Gilmour, 2006) and water exchange (Evans, 1969; Loretz, 1979; Isaia, 1984). The latter process is perhaps the least well-studied. These functions of the gill require certain conditions for optimal performance that in many cases, differ from one another. For example, conditions enabling osmoregulation would be thick epithelial membranes with reduced functional surface area and permeability so as to minimize

adverse ion and water exchanges (Gonzalez and McDonald, 1992; Gonzalez and McDonald, 1994), whereas conditions facilitating respiratory gas exchange would include increased surface area, thin epithelial membranes, with high blood and water flow rates (Randall et al., 1967; Randall et al., 1972; Sundin and Nilsson, 1997). In order for the gill to balance between these two opposing demands, there has to be a compromise. The osmorespiratory compromise represents this trade-off (Randall et al., 1972; Nilsson, 1986).

Conditions that favour increased oxygen uptake and/or demand such as hypoxia, exercise and temperature stress resulted in an increase in unfavourable net fluxes of osmolytes (e.g. Randall et al., 1972; Wood and Randall, 1973a; Wood and Randall, 1973b; Gonzalez and McDonald, 1992; Gonzalez and McDonald, 1994; Postlethwaite and

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McDonald, 1995; Iftikar et al., 2010; Robertson et al., 2015a; Robertson et al., 2015b; Giacomin et al., 2017). Most of these studies have involved salmonids such as the rainbow trout, and the majority have focused on gas and ion exchange. The evidence for effects on water exchange is far less extensive than that for ion fluxes, which is somewhat surprising considering that water constitutes about 80% of the body of the fish (Isaia, 1984), and the gills account for about 90% of water exchange (Motais et al., 1969; Haywood et al., 1977). Stevens (1972), using tilapia, and Wood and Randall (1973c) and Hofmann and Butler (1979) both using rainbow trout, all concluded, based on measurements of urine flow rate and/or body weight changes, that net water uptake increased with $O_2$ uptake during exercise in freshwater fish. Loretz (1979) reported an increase in $^3H_2O$ flux rate with increase in temperature in goldfish, while Giacomin et al. (2017) observed a decreased $^3H_2O$ flux rate at an acutely reduced temperature (7.5 °C) and an increased $^3H_2O$ flux rate at an acutely elevated temperature (22 °C) in seawater spiny dogfish. Additionally, Loretz (1979) demonstrated that $^3H_2O$ efflux increased during moderate hypoxia, while Giacomin et al. (2017) reported that changes in $^3H_2O$ efflux paralleled those in $O_2$ uptake during temperature challenges.

Unidirectional water flux rates (often called "diffusive water flux"), as measured by $^3H_2O$ fluxes, are approximately 100-fold greater than net water flux rates (often called "osmotic water flux") as estimated from body weight changes and urine flow, and the relationship between the two is complex (see review by Franz, 1968). In part this is because diffusive water flux is a direct measurement, whereas osmotic water flux is an indirect calculation (Loretz, 1979). Furthermore, osmotic water flux is a net flux dependent on the differences between osmotic (and hydrostatic) pressures between external and internal environments, while diffusive water flux is unidirectional. Additionally, diffusive water exchange probably occurs mainly through the cell membrane, at least in part via aquaporins (Evans et al., 2005) in freshwater teleosts, whereas osmotic water flux may additionally involve bulk flow through paracellular pathways (Potts et al., 1967; Evans, 1969; Motais et al., 1969; Loretz, 1979; Isaia, 1984). Diffusive water flux and net water flux may not necessarily change in parallel. For example, in the Amazonian oscar, a teleost with exceptional hypoxia tolerance that shows an unusual decrease in gill ion fluxes during severe hypoxia (Wood et al., 2007; Wood et al., 2009; De Boeck et al., 2013; Robertson et al., 2015b), osmotic permeability to water also declined, but the fall in net water flux (30%) was less than that (70%) in diffusive water permeability (Wood et al., 2009). Interestingly, while ion fluxes decreased during hypoxia, they increased during exercise in oscars (Robertson et al., 2015a), though water fluxes were not measured in the exercise study. This suggests that oscars employed different approaches in dealing with the osmorespiratory compromise during exercise versus hypoxia.

With this background in mind, we used $^3H_2O$ to measure diffusive water flux rates across the gills of juvenile rainbow trout (*Oncorhynchus mykiss*). Our first objective was to determine if the size of the fish affects their water flux rate under resting normoxic control conditions, before addressing the possible influences of experimental treatments. Therefore, our first hypothesis was that diffusive water flux would scale with body mass in a similar fashion to that traditionally found for $O_2$ consumption (i.e. a mass scaling allometric coefficient in the range of 0.6–0.9; Clarke and Johnson, 1999). This proved not to be the case. After sorting out the issues of fish size, our second objective was to determine the effects of acute temperature stress, hypoxia and exercise on both diffusive water flux and net sodium loss rates. These treatments were designed to invoke the osmorespiratory compromise. The trout is an energetic, highly aerobic species, living in a variable habitat (Hardy, 2002), and it is constantly under threat by such environmental stressors. In the present experiments, the effect of these treatments on net $Na^+$ loss rates was also examined, to assess the component that has been traditionally measured in osmorespiratory compromise studies. Our second hypothesis was that all three treatments would increase both net sodium loss and diffusive water flux rates. Our third hypothesis was that diffusive water flux rates and net sodium loss rates would be correlated on an individual basis, as they are likely affected in the same manner by changes in gill permeability.

## 2. Materials and methods

### 2.1. Experimental fish

Rainbow trout (*Oncorhynchus mykiss*) were obtained from Miracle Springs Inc. (Fraser Valley, BC, Canada), and held for a minimum of two weeks prior to experiments in a 500-L flow-through holding tank at 8 °C. Dechlorinated Vancouver tap water (in mM: $Na^+$ .0.06; $Cl^-$, 0.05; $Ca^{2+}$, 0.03; $Mg^{2+}$, 0.007; $K^+$, 0.004; and in mg/L $CaCO_3$, alkalinity, 3.0; hardness 3.3; pH 7.0) was used for acclimation and all experiments. Fish were maintained in 12 L:12D photoperiod and fed every day at 1% of their body weight with commercial pellets (EWOS, Surrey, BC, Canada). On the day of experiment, fish were first netted individually from the holding tank and put through the tritiated water loading protocol as described in Section 2.2. The remaining fish in the holding tank were then fed as described above, so experimental fish had been fasted for about 24 h. All experimental procedures were approved by the University of British Columbia Animal Care Committee (certificate A14-0251) in accordance with the Canadian Council on Animal Care guidelines. None of the fish died as a result of the experimental treatments.

### 2.2. Experimental protocol

All procedures were carried out in covered containers which were shielded in black plastic to minimize visual disturbance and fitted with aeration devices and sampling ports. Containers were submerged in a table-trough that served as a water bath, maintained at 8 °C by a flow-through of Vancouver dechlorinated tap water. In order to avoid the stress of injection, fish were loaded with tritiated water ($^3H_2O$, Perkin Elmer, Woodbridge, ON, Canada) by external incubation for 6 h; preliminary experiments demonstrated that equilibration was complete within this time. Adequate measures (tank shielding, minimal movement by the experimenter, quiet) were ensured so that fish were not stressed before and during the experimental period as stress has been shown to affect rainbow trout sodium loss rates (Postlethwaite and McDonald, 1995). For each experimental run, 5 fish were loaded simultaneously in a 2-L water volume labelled with 40 μCi of $^3H_2O$. After the equilibration period, fish were individually netted from the loading container, quickly rinsed with dechlorinated water to remove any external $^3H_2O$ on the body surface, and then gently transferred to the experimental containers. Each held 1 L of dechlorinated water devoid of $^3H_2O$. Then 5 mL of water was sampled at time zero and thereafter every 5 min for 60 min, with a final sample taken after 6 h when washout was complete. The fish was weighed at this time, and then returned to its holding tank. The 0–60 min samples were used for diffusive water flux measurements, and the 6-h sample was used to calculate the original dose of $^3H_2O$ in the fish at time 0 (see Calculations). Additional 10-mL samples were taken at 0 min and 60 min for net $Na^+$ flux rates, but some samples for $Na^+$ determinations were lost, so net $Na^+$ flux rates were measured in most but not all individuals. Note that because the efflux of $^3H_2O$ from the fish is rapid, it was necessary to make all experimental measurements during the first 1-h period after the fish was removed from the loading medium. Therefore, in the prolonged hypoxia experiments, it was necessary to start the hypoxia treatment during the loading period, as outlined below. In the acute hypoxia trials, the fish were added directly to water already set to the desired $O_2$ level (e.g. 8.40 KPa), and this was always the case during $^3H_2O$ efflux trials, whereas in the longer-term hypoxia pre-exposures the water $PO_2$ was brought to the desired $PO_2$ over 1–2 min. Fluctuations in $PO_2$ during hypoxia were about ± 0.4 KPa. This fluctuation in

PO$_2$ occurred mainly because the fish were consuming oxygen during the trial.

### 2.3. Effects of fish size on diffusive water flux rate

To test the hypothesis that fish size affects diffusive water flux rate, we sampled 27 fish ranging from 13 to 50 g body weight. Five fish were sampled daily and loaded with $^3$H$_2$O as outlined above, and then the experiment was done in the standard fashion. Normoxic conditions ($> 16.79$ KPa) were maintained during both the loading and experimental periods. Following the finding that the size of the fish affects the diffusive water flux rate, we then used fish only within the size range of 25–50 g to probe subsequent questions.

### 2.4. Effects of different levels of hypoxia on diffusive water and net Na$^+$ flux rates

In this investigation, we addressed the effects of different oxygen levels [normoxia ($> 16.79$ KPa, $N = 10$), 8.40 KPa ($N = 7$), 5.25 KPa ($N = 7$) and 3.15 KPa ($N = 10$)] on diffusive water flux and on net Na$^+$ flux rates. Fish were loaded with $^3$H$_2$O in the standard fashion under normoxic conditions ($> 16.79$ KPa) and then acutely transferred to the hypoxic condition for experimental measurements, which were also done in the standard fashion. To achieve reduction in dissolved O$_2$, N$_2$ gas was bubbled into the 1 L water until the desired saturation was reached (8.40 KPa, 5.25 KPa, or 3.15 KPa), and thereafter to sustain this level a mixture of air and N$_2$ was used with constant monitoring with a portable O$_2$ electrode (Accument Ap84, Fisher Scientific, Singapore). The electrode was calibrated daily (i.e. before each experiment) using two-point calibration with N$_2$-equibrated water (0%) and air-equilibrated water (100%); in practice we found that calibration drift was negligible from day to day. The electrode could be inserted into the experimental chamber through a hole in the lid, thereby avoiding disturbance to the fish.

### 2.5. Effects of the time course of hypoxia on diffusive water and net Na$^+$ flux rates

Here we measured the effects of short term (1 h, $N = 8$ for diffusive water flux, and $N = 5$ for net Na$^+$ loss) and longer-term exposure (3 h, $N = 10$ for diffusive water flux, and $N = 10$ for net Na$^+$ loss) to hypoxia (8.40 KPa), as well as the effects of subsequent return to normoxia for 1 h ($N = 10$ for diffusive water flux, and $N = 10$ for net Na$^+$ loss) and 3 h ($N = 9$ for diffusive water flux, and $N = 9$ for net Na$^+$ loss) after 1 h of hypoxia. The 1-h hypoxia treatment followed the same method as described above. For 3-h hypoxia, fish were incubated with $^3$H$_2$O in the standard 6-h loading period followed by 2 h at 8.40 KPa in the same media. The fish were then rinsed with clean water before transfer to individual 1-L chambers containing water at 8.40 KPa for flux measurements over the following hour, which represented the 3rd h of hypoxia. These trials unavoidably subjected the fish to a longer loading period (up to 9 h vs. the standard 6 h). As $^3$H$_2$O loading occurs to equilibrium, this would not affect the loading, but it may have subjected the fish to higher ammonia levels during the loading period. The possible influence of this procedural difference could not be evaluated because the flux period must start immediately after the end of the loading period. For the 1 h at 8.40 KPa + 1 h normoxia recovery trial, the loading of $^3$H$_2$O was done for 6 h plus 1 h at 8.40 KPa in the same media while the washout count was done under normoxia ($> 16.79$ KPa) for 1 h. For the 1 h at 8.40 KPa + 3 h normoxia recovery trial, loading was done for 6 h plus 1 h at 8.40 KPa plus 2 h at normoxia, all in the same media, before transfer of the fish to an individual container for the washout count under normoxia for 1 h. Flux measurements were performed in the standard fashion.

### 2.6. Effects of exercise on diffusive water and net Na$^+$ flux rates

We determined the effects of strenuous exercise on the diffusive water flux with an $N = 8$ for non-exercised and $N = 6$ for exercised and net Na$^+$ flux rates with an $N = 6$ for non-exercised and $N = 6$ for exercised fish. Briefly, at the end of 6 h of loading with $^3$H$_2$O in the standard fashion under normoxic conditions, the trout were strenuously exercised as a group for 5 min. The exercise was achieved by vigorously stirring the water pool. At the end of the 5 min of exercise, fish were quickly rinsed with clean water and then transferred to the 1-L water volume for individual flux measurements in the standard fashion.

### 2.7. Effects of acute temperature challenge on diffusive water and net Na$^+$ flux rates

For this investigation, fish were first loaded with $^3$H$_2$O in the standard fashion under normoxic conditions at 8 °C. They were then acutely transferred to either 8 °C [acclimation control ($N = 15$ for diffusive water flux and $N = 7$ for net Na$^+$ loss)], 13 °C ($N = 15$ for diffusive water flux and $N = 15$ for net Na$^+$ loss), or 18 °C ($N = 10$ for diffusive water flux and $N = 10$ for net Na$^+$ loss) for flux measurements in the standard fashion.

### 2.8. Analytical techniques and calculations

The concentration of $^3$H$_2$O in water samples was analyzed using a scintillation counter (LS6500, Beckman Coulter, Fullerton, CA, USA). Briefly, 10 mL of Optiphase 3 fluor (Perkin-Elmer, Wellesley, MA, USA) was added to the 5-mL water sample. Our internal standardization tests demonstrated that quenching was constant, so no correction was necessary. The Na$^+$ concentration was determined using flame atomic absorption spectrophotometry (AAnalyst 800, Perkin-Elmer, Wellesley, MA, USA). Certified reference materials (CRM) BURTAP-05 (Environment Canada, Burlington, ON) and blanks were analyzed together with Na$^+$ water samples. Na$^+$ was not detected in the blanks and our recovery rate for Na$^+$ from the BURTAP-05 range was 96–104%. The limit of quantification was 0.5 µmol L$^{-1}$.

The net Na$^+$ flux rate (µmol g$^{-1}$ h$^{-1}$) was calculated by subtracting the final water sodium concentration from the initial water sodium concentration (µmol L$^{-1}$), and factoring in the known fish weight (g), volume (L, mean volume over the experimental period) and time (h).

$$\text{Na net} = [(\text{initial} - \text{final}) \times \text{volume}]/[\text{time} \times \text{weight}] \qquad (1)$$

Thus, positive values represent net gains, and negative values represent net losses by the fish.

The rate constant of $^3$H$_2$O efflux, representing the unidirectional diffusive efflux of water expressed as a decimal fraction of the total body water pool per hour (h$^{-1}$) was calculated by determining the rate of decline in the $^3$H$_2$O in the fish, which is known to be approximately exponential with time (Evans, 1967).

$$k = (\ln \text{ CPM}_1 - \ln \text{ CPM}_2)/(\text{time}_1 - \text{time}_2) \qquad (2)$$

where k is the rate constant of the efflux (in h$^{-1}$), CPM$_1$ = total $^3$H$_2$O radioactivity (in cpm) in the fish at time$_1$ (in h), and CPM$_2$ = total $^3$H$_2$O radioactivity (in cpm) in the fish at time$_2$ (in h). The product of $k \times 100\%$ yields the percentage of body water turned over per hour.

In practice, by measuring the $^3$H$_2$O radioactivity in the water after 6 h, when complete equilibration between the fish and the known volume of water had occurred, it was possible to calculate accurately the total amount of $^3$H$_2$O radioactivity (CPM$_{total}$) in the system – i.e. the total amount that had been taken up by the fish during the loading period. Therefore, from CPM$_{total}$ and from measurements of $^3$H$_2$O radioactivity appearance in the water at each sampling time, it was possible to back-calculate the CPM in the fish at each time during the efflux experiment. In these calculations, it was important to use the volume present at each sample time, and to take into account

cumulative $^3H_2O$ cpm removed during sampling. $^3H_2O$ efflux rates were calculated by regressing the natural logarithm of CPM in the fish against time over the 1-h measurement period to yield the slope k.

The rate constants of water efflux (k) were converted to actual diffusive water flux rates in mL/h by assuming that the water space is 80% of the body mass of the fish (Holmes and Donaldson, 1969; Isaia, 1984; Olson, 1992).

$$\text{Diffusive water flux (mL/h)} = M^* k^* 0.8 \qquad (3)$$

where M = fish weight, k = rate constant, 0.8 = fractional water pool.

The logarithm of diffusive water flux (Y = mL/h) was plotted against the logarithm of fish weight (M) with intercept (a) and slope (b), the latter representing the allometric mass scaling coefficient:

$$Y = aM^b \qquad (4)$$

The allometric mass scaling coefficient (b) determined from this plot was then used to correct the observed flux rate of an individual fish ($X_{obs}$, mL/h) of mass $M_{obs}$ (g) to that ($X_{corr}$) of a 35 g fish which was in the midrange of the fish used in the experimental tests:

$$X_{corr} = X_{obs}*10^{b*\log(35g/Mobs)} \qquad (5)$$

The value obtained was then divided by 35 to obtained the flux rate in mL/g/h.

The temperature coefficients ($Q_{10}$ values) for diffusive water flux rates were calculated for the temperature ranges 8–13 °C, 13–18 °C and 8–18 °C using the van't Hoff equation:

$$Q_{10} = (R_2/R_1)^{[10/(T_2-T_1)]} \qquad (6)$$

where $R_2$ and $R_1$ represent diffusive water flux rates at two temperatures $T_2$ and $T_1$, and where $T_2 > T_1$. Because the measurements done at each temperature involved a different set of fish, we used mean values in calculating the $Q_{10}$ for diffusive water flux rates.

### 2.9. Statistical analyses

All data were first checked for normality and homogeneity of variances and where data failed the test, data were transformed. Some data passed after square root transformation and the remaining data were analyzed using Kruskal-Wallis One Way Analysis of Variance on Ranks. All data are expressed as the mean ± SEM (N). Data were compared using a one-way ANOVA with size, $O_2$ level, time, exercise and temperature as the independent variables in different series. Significantly different means were separated using Tukey's post hoc test at $p < 0.05$. Linear regression analysis, curve fitting and statistical analysis were performed using SigmaPlot 11 (Systat Software, San Jose, CA, USA).

## 3. Results

### 3.1. Effects of fish size on diffusive water flux rates

Fish size significantly affected ($p < 0.001$) the fraction of the body water pool that the fish turned over per hour. Specifically, fish of 13 g body weight had the highest fractional turnover, > 100% of the body water pool per hour. In contrast, fish weighing 50 g had the lowest fractional turnover, only 34% of their water body pool per hour (Fig. 1A). While all the data could be fitted with a single exponential relationship (not shown, $R^2 = 0.94$ and $p < 0.0001$), the data of very small fish appeared to be discontinuous with a different slope from those of the larger fish (25–50 g), and the data of the larger fish were fitted better by a linear relationship (Fig. 1A, $R^2 = 0.85$ and $p < 0.0001$). As only larger fish in the latter weight range were used in the experimental tests, the allometric scaling analysis was restricted to 25–50 g fish (Fig. 1B, C). For analysis of allometric scaling, the fractional turnover (k, Eq. 2) was converted to an absolute efflux rate in mL/h by Eq. 3. Fig. 1B illustrates that absolute flux rate in the 25–50 g size range depended significantly ($R^2 = 0.54$, $p < 0.001$) on body size,
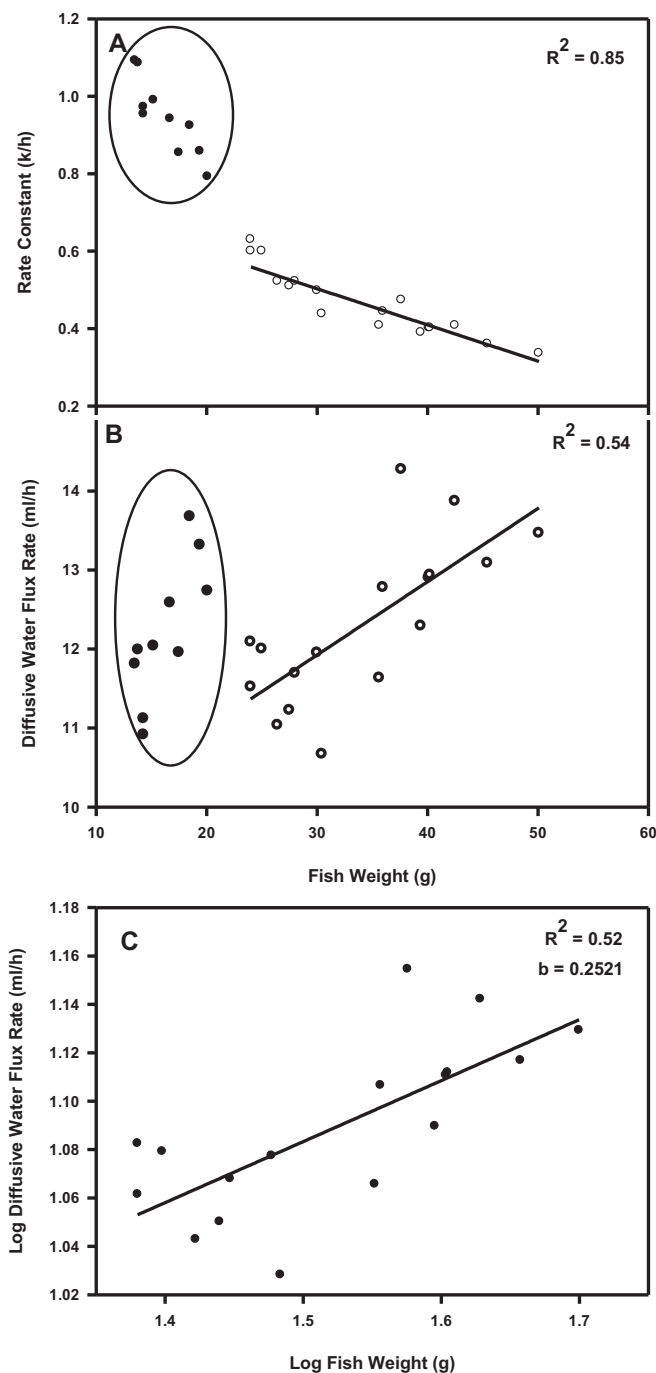


**Fig. 1.** Effects of fish size on diffusive water flux rate (A) rate constant [decimal fraction of the total body water pool effluxed per hour (h$^{-1}$)] (N = 27), (B) diffusive water flux rate (mL/h) (N = 27), and (C) log diffusive water flux vs log fish weight $N = 17$ (The slope from this regression was used to calculate the allometric scaling; see Materials and methods). Note that the values for very small fish have been circled to highlight their deviation from the relationship for larger fish. The circled points were not used in subsequent calculations, and have been omitted from panel C (see Materials and methods).

but with a low slope (i.e. absolute efflux rate in mL/h exhibited only a small increase with absolute body mass). When these data were transformed into a log-log plot, the relationship remained significant ($R^2 = 0.52$, $p < 0.001$), and the slope (b = 0.252) represented the allometric mass scaling coefficient (Fig. 1C). This value was then used to scale all experimental data to that of a 35 g fish by Eq. 5. The result was then divided by 35 to yield a flux rate in mL/g/h. [Note that including the smaller fish in the scaling analysis would have biased the outcome, resulting in a much lower allometric mass scaling coefficient (0.021)].
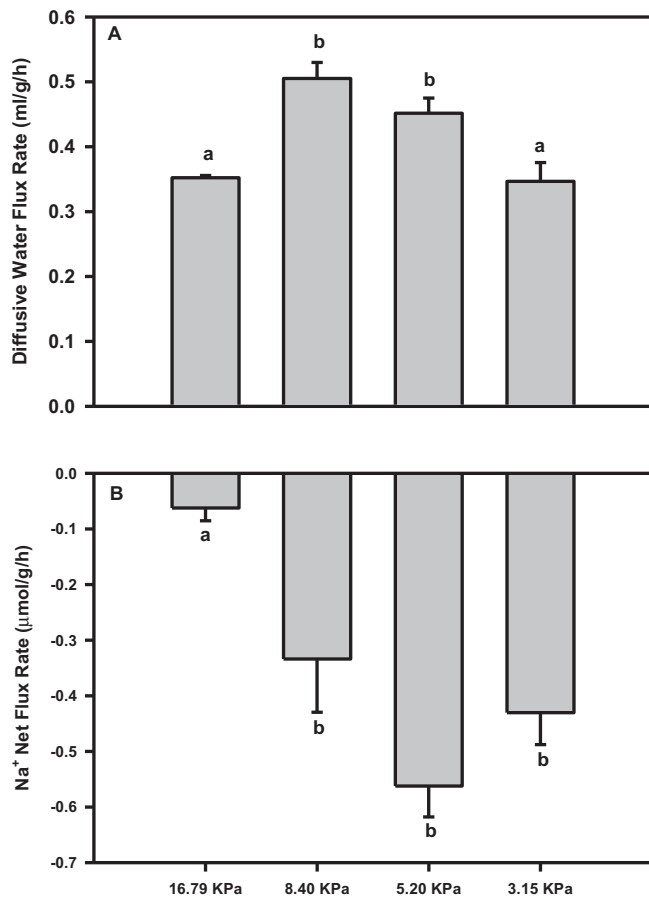
**Fig. 2.** Effects of different levels of hypoxia on (A) diffusive water flux rate (mL/g/h) of rainbow trout exposed to normoxia (> 16.79 KPa, $N = 10$), 8.40 KPa ($N = 7$), 5.25 KPa ($N = 7$) and 3.15 KPa ($N = 10$) and (B) net $Na^+$ loss rate (μmol/g/h) of rainbow trout exposed to normoxia (> 16.79 KPa, $N = 7$), 8.40 KPa ($N = 5$), 5.25 KPa ($N = 5$) and 3.15 KPa ($N = 10$). Different sets of fish were used for each treatment (see Materials and methods). Values are means ± SEM. Means not sharing the same letter are significantly different from one another at $p < 0.05$.

### 3.2. Effects of different levels of hypoxia on diffusive water and net $Na^+$ flux rates

Diffusive water flux rate responded differentially to the level of hypoxia. Overall, there was a significant influence ($p < 0.001$) among the various levels of oxygen on the diffusive water flux rates of rainbow trout (Fig. 2A). Flux rate increased significantly by 43.5% when trout were acutely exposed to 8.40 KPa hypoxia. Acute exposure to 5.25 KPa hypoxia increased the flux rate by 28.3%, not significantly different from that of 8.40 KPa hypoxia. In contrast to both 8.40 KPa and 5.25 KPa hypoxia exposure, 3.15 KPa hypoxia decreased flux rate by 1.5% to a value that was statistically similar to the normoxia control treatment (Fig. 2A). Overall, oxygen levels significantly ($p < 0.001$) affected net $Na^+$ loss rates (Fig. 2B). All the hypoxia levels resulted in significant increases in net $Na^+$ loss rates. Specifically, 5.25 KPa hypoxia caused the highest elevations in $Na^+$ loss of 8-fold, while 3.15 KPa and 8.40 KPa hypoxia induced 6- and 4-fold increases respectively, though they were all statistically similar. There were no significant correlations between net $Na^+$ loss rates and diffusive water flux rates in individual fish following the exposure of trout to hypoxia, either within one hypoxia treatment, or when all the hypoxia treatments were combined.
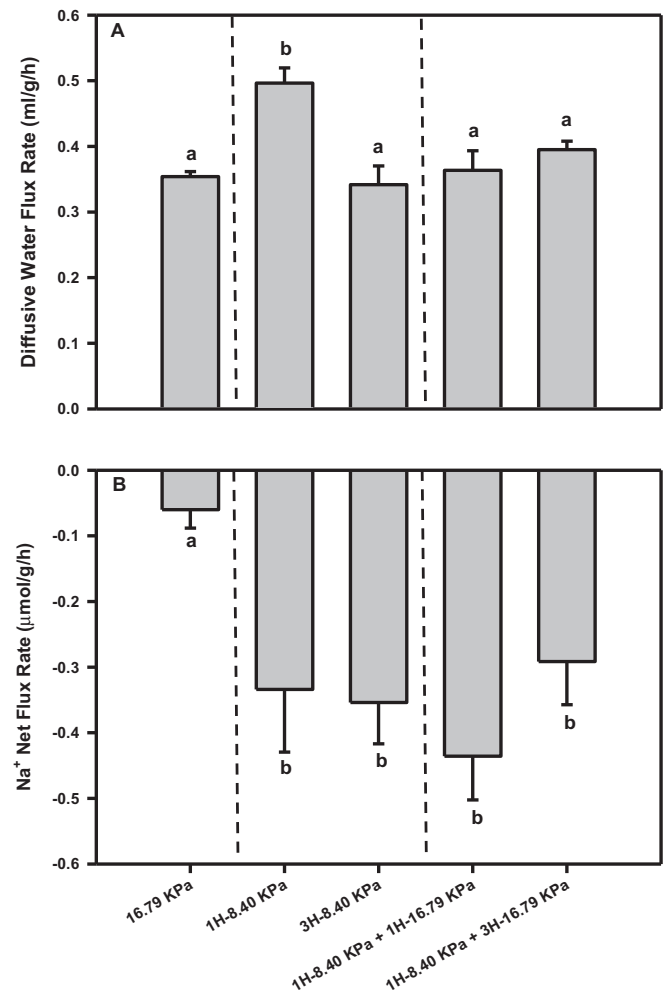


**Fig. 3.** Effects of time course of hypoxia and recovery on (A) diffusive water flux rate (mL/g/h) of rainbow trout exposed to normoxia (> 16.79 KPa, $N = 10$), 1-h 8.40 KPa ($N = 8$), 3-h 8.40 KPa ($N = 10$), 1-h 8.40 KPa + 1-h normoxia ($N = 10$) and 1-h 8.40 KPa + 3-h normoxia ($N = 9$) and (B) net $Na^+$ loss rate (μmol/g/h) of rainbow trout exposed to normoxia (> 16.79 KPa, $N = 5$), 1-h 8.40 KPa ($N = 5$), 3-h 8.40 KPa ($N = 10$), 1-h 8.40 KPa + 1-h normoxia ($N = 10$) and 1-h 8.40 KPa + 3-h normoxia ($N = 9$). Different sets of fish were used for each treatment (see Materials and methods). Values are means ± SEM. Means not sharing the same letter are significantly different from one another at $p < 0.05$.

### 3.3. Effects of time course of hypoxia on diffusive water flux and net $Na^+$ flux rates

Overall, there was a significant influence of the time course protocol of hypoxia and recovery ($p < 0.001$) on the diffusive water flux rates (Fig. 3A). In this series, 1 h of 8.40 KPa hypoxia increased the diffusive water flux rate by 40.2% while the prolonged 3 h of 8.40 KPa hypoxia returned the flux rate (measured in the third hour) to the control normoxia (> 16.79 KPa) rate (Fig. 3A). Similarly, both 1 h and 3 h of normoxic recovery after 1 h of 8.40 KPa hypoxia returned the flux rate to the control normoxic rate (Fig. 3A). Overall, there was also a significant effect ($p = 0.021$) of the time course protocol on the net $Na^+$ loss rates (Fig. 3B), but the pattern was very different. Our results revealed a general pattern of increased net $Na^+$ loss in all the experimental treatments (Fig. 3B). Specifically, net $Na^+$ loss rate increased 4.5-fold during exposure to 8.40 KPa hypoxia for 1 h, and this effect was sustained during the prolonged 3 h of 8.40 KPa hypoxia (4.8-fold increase), as well as during both 1 h (6-fold increase) and 3 h (3.8-fold increase) of normoxic recovery. All these experimental values were statistically similar. There were no significant correlations between
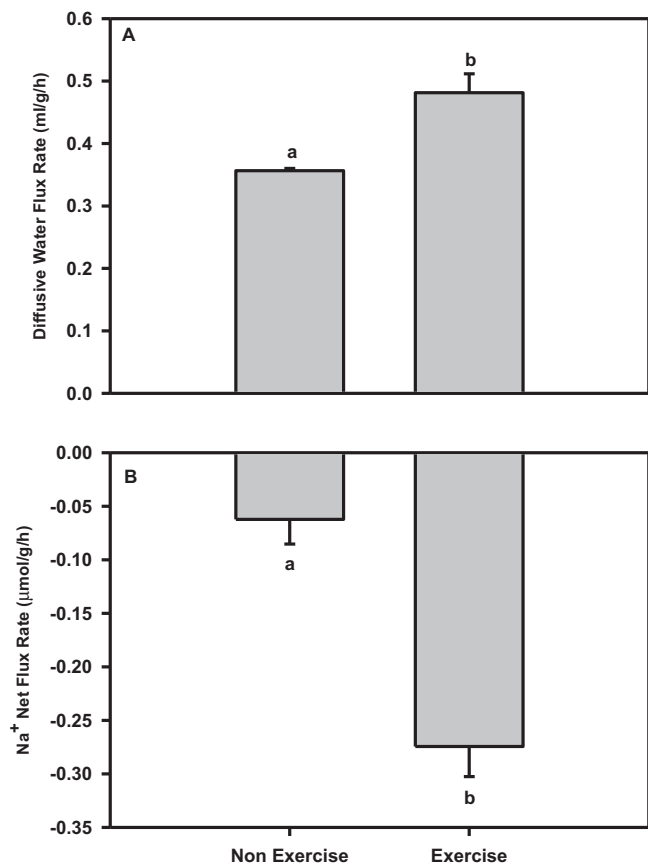
**Fig. 4.** Effects of exercise on (A) diffusive water flux rate (mL/g/h) of rainbow trout exposed to normoxia at rest ($N = 8$) and after 5 min of strenuous exercise ($N = 6$), and (B) net $Na^+$ loss rate (µmol/g/h) of rainbow trout exposed to normoxia at rest ($N = 6$) and after 5 min of strenuous exercise ($N = 6$). Different sets of fish were used for each treatment (see Materials and methods). Values are means ± SEM. Means not sharing the same letter are significantly different from one another at $p < 0.05$.

diffusive water flux and net $Na^+$ loss rates in individual trout in any of the individual treatments, or when all the various treatments were combined for the time course protocol of hypoxia and recovery.

### 3.4. Effects of exercise on diffusive water flux and net $Na^+$ flux rates

There was a significant effect ($p < 0.001$) of strenuous exercise on the diffusive water flux rate, which increased by 35% during the 1-h recovery period (Fig. 4A). Furthermore, we observed a significant 3-fold increase ($p < 0.001$) in net $Na^+$ loss rate following exercise compared to non-exercised trout (Fig. 4B). The correlation between diffusive water flux rate and net $Na^+$ loss rate in individual fish in this exercise protocol was significant ($p < 0.001$) with $R^2 = 0.55$ (Fig. 5).

### 3.5. Effects of acute temperature challenge on diffusive water flux and net $Na^+$ flux rates

Overall, there was a significant effect ($p < 0.001$) of acute temperature challenge on the diffusive water flux rate of rainbow trout (Fig. 6A) Specifically, elevations in temperature from the acclimation temperature of 8 °C to 13 °C or to 18 °C increased the water flux rate by 2.3-fold and 3.3-fold respectively. However, for net $Na^+$ loss rate, our results revealed no significant effect ($p = 0.614$) of temperature challenge (Fig. 6B). The mean $Q_{10}$ effects of acute temperature challenge on diffusive water flux rate were different over the different temperature intervals. Specifically, a higher $Q_{10}$ value occurred for the elevation from 8 to 13 °C (4.03) and a lower value (2.16) for the elevation from 8 to 18 °C. Overall, our results show that there was no significant
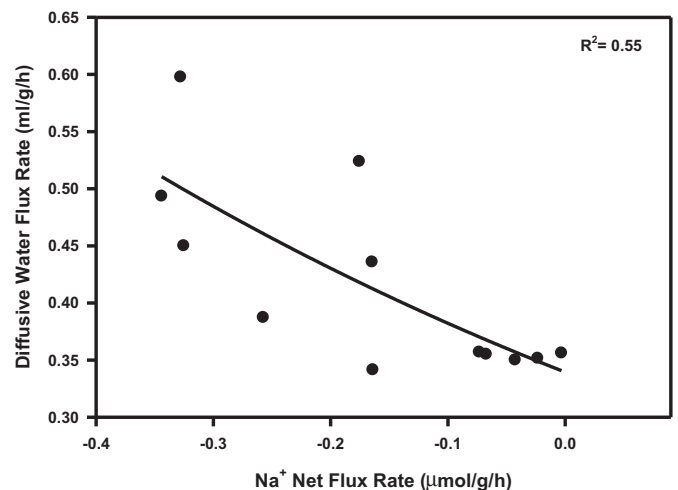


**Fig. 5.** Relationship of diffusive water flux rate (mL/g/h) and net $Na^+$ loss rate (µmol/g/h) in individual fish during the exercise experiments ($N = 12$, [N number is less than in Fig. 4 because $Na^+$ flux was not measured in all fish]). Values are means ± SEM. Significant correlation ($p < 0.05$) with $R^2 = 0.55$.
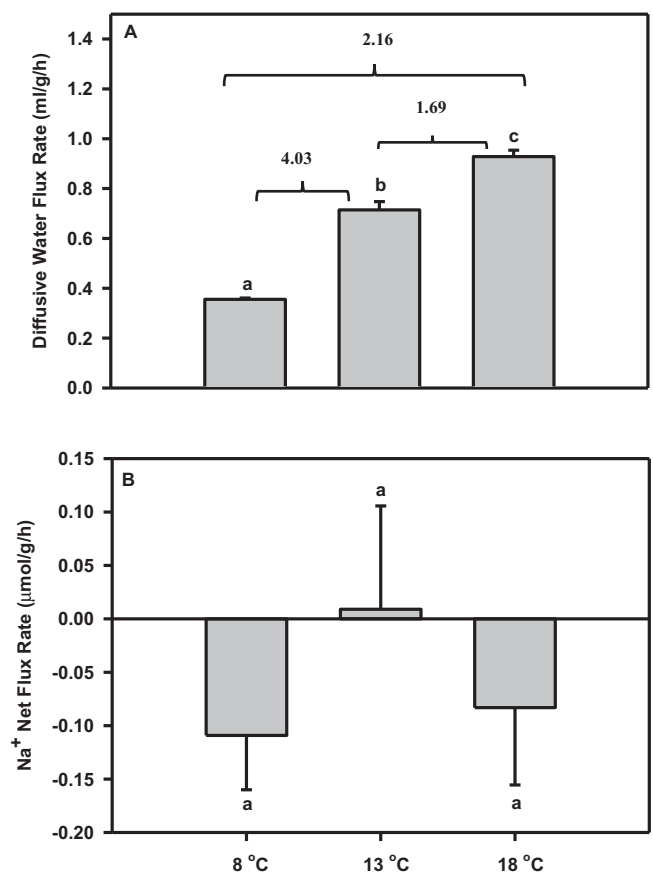


**Fig. 6.** Effects of acute temperature stress on (A) diffusive water flux rate (mL/g/h) and mean $Q_{10}$ value of rainbow trout exposed to 8 °C ($N = 15$), 13 °C ($N = 15$) and 18 °C ($N = 10$), and (B) net $Na^+$ loss rate (µmol/g/h) of rainbow trout exposed to 8 °C ($N = 7$), 13 °C ($N = 15$) and 18 °C ($N = 10$). All tests were performed under normoxia (> 16.79 KPa). Different sets of fish were used for each treatment (see Materials and methods). Values are means ± SEM. Means not sharing the same letter are significantly different from one another at $p < 0.05$.

correlation between diffusive water flux rate and net $Na^+$ loss rates in individual trout following temperature challenge, either within one challenge treatment, or when the two treatments were combined.

## 4. Discussion

### 4.1. Critique of methods

A possible confounding problem with our experimental design is handling stress as a result of transferring the fish from the loading container to the washout container. However, our experimental design was constrained by the fact that the flux period must start immediately after transfer to a new medium, or else an unquantifiable amount of $^3H_2O$ cpm would have been lost, so gradual flushing simply would not work. We therefore chose to use step transfer to the measurement chamber and to standardize this across all treatments. Handling stress has been demonstrated to cause alterations in biochemical and physiological parameters such as hyperglycemia, increased cortisol level, ion dysregulation and increased plasma glucose levels in fish (Houston et al., 1971; Wedemeyer, 1972, 1976; Strange et al., 1977; Strange and Schreck, 1978; Pickering et al., 1982; López-Patiño et al., 2014). However, these alterations depend on the severity and duration of the handling stress (Wedemeyer, 1972; Pickering et al., 1982; López-Patiño et al., 2014). Our approach was to minimize the handling stress to a few seconds during which the fish were quickly and gently transferred. Unfortunately, there is no other way to measure the diffusive water efflux rate without transferring the fish to a separate container, as our report and those of others have shown (Potts and Evans, 1967; Evans, 1969; Potts et al., 1970; Loretz, 1979; Wood et al., 2009; Giacomin et al., 2017). This however does not invalidate the results from this investigation because all the fish were handled in similar manner whether they were in experimental treatments or control. In addition, the control net sodium flux rates in our tests were all close to zero (Figs. 2B, 3B, 4B), which suggests that our methods resulted in very minimal stress to the fish.

### 4.2. Fish size affects diffusive water flux rates

Our results clearly revealed that the size of rainbow trout affected the fractional amount of the body water pool exchanged per hour. Specifically, fish weighing about 13 g exchanged over 100% per hour, while 50 g fish exchanged only 34% of their body water pool per hour (Fig. 1A). Qualitatively similar findings have been reported by Potts et al. (1967) and Evans (1969), who noted that water exchange is size-dependent with larger fish having lower relative water exchange rates than smaller fish. The reasons for higher fractional turnover rate of the body water pool in small fish are likely the relatively larger surface area to volume ratio, which is particularly reflected in the gills (Hughes, 1966; Palzenberger and Pohla, 1992), and the higher effective diffusing capacity of the gills which would likely accompany the greater respiratory rate and/or metabolic activity (De Jager and Dekkers, 1975; Hughes, 1984; Palzenberger and Pohla, 1992). The combination of large surface area and high metabolic rate potentially makes the gill epithelial membrane more permeable leading to the higher fractional turnover of the body water pool observed with small fish.

Contrary to our hypothesis that diffusive water flux would scale with body mass in a similar fashion as traditionally found for $O_2$ consumption with mass scaling allometric coefficients in the range of 0.6–0.9 (Clarke and Johnson, 1999), we observed a far lower scaling coefficient of 0.25 over the 25–50 g size range (Fig. 1C), and a coefficient close to zero if the very small fish were included in the analysis (see Section 2.8 and Eq. 4 in Materials and methods). This contrasts with values of 0.85 for brown trout, 0.94 for three-spined stickleback, 0.92 for eel, 0.81 for European flounder and 0.83 for Mozambique tilapia, all in our originally predicted range (Potts et al., 1967; Evans, 1969). The very low scaling coefficient in the present study was unexpected, and the exact reason is unknown. It could be due to the relatively narrow size range, or to the small size of fish and/or to the low temperature at which the measurements were done; both Potts et al. (1967) and Evans (1969) used fish that were acclimated above 20 °C

compared to ours at 8 °C. In support of this view, in a separate study (J.O. Onukwufor and C.M. Wood, unpublished results), we have subsequently measured diffusive water exchange rates in larger rainbow trout (85–120 g) acclimated to 18 °C, and obtained a scaling coefficient (0.87) in the expected range.

### 4.3. Different levels and time courses of hypoxia differentially affect diffusive water and net $Na^+$ flux rates

The different levels of hypoxia exposure had differential effects on diffusive water flux rates in rainbow trout. For example, when trout were exposed to 8.40 KPa hypoxia their water flux rate increased by 43.5% (Fig. 2A), similar to the 57% increase seen in goldfish exposed to 10.50 KPa hypoxia (Loretz, 1979). This would reflect the classic osmorespiratory compromise, the result of a trade-off to improve respiratory gas exchange from the limited $O_2$ environment by increasing the functional surface area and/or decreasing the diffusion distance at the gills – i.e. effectively increasing gill permeability not only to oxygen, but also to ions and water (Randall et al., 1972; Nilsson, 2007). However, surprising to us was the observation that 5.25 KPa hypoxia had no further stimulatory effect (indeed a non-significant attenuation), whereas 3.15 KPa hypoxia had no effect at all on diffusive water turnover (Fig. 2A). Furthermore, when 8.40 KPa hypoxia was extended for 3 h, and when two different recovery time courses were examined, there were no significant effects on diffusive water flux rates, which returned to normoxic control values (Fig. 3A). Thus, the effect appears to be biphasic, and has the hallmarks of a regulated phenomenon. Potentially it may result from a combination of the classic osmorespiratory compromise (increased permeability to oxygen, at the expense of increased permeability to water and ions) at moderate, acute hypoxia (e.g. Iftikar et al., 2010; Robertson et al., 2015b) transitioning to a different compromise (decreased permeability to water and ions, but not necessarily to oxygen) during a more extended time course, and or during more severe hypoxia. The latter was seen in the Amazonian oscar where there was a 70% decrease in diffusive water permeability during 2.10 KPa hypoxia (Wood et al., 2009) while effective oxygen permeability remained high (Scott et al., 2008) which was interpreted as an adaptive response to decrease energy expenditure at a time of severe $O_2$ limitation in a species which lives in a frequently hypoxic environment and is very tolerant of hypoxia (Almeida-Val and Hochachka, 1995; Muusze et al., 1998; Almeida-Val et al., 2000). Yet the rainbow trout is very sensitive to hypoxia (Iftikar et al., 2010; Onukwufor et al., 2014), so potentially it transitions to this other strategy of decreased water permeability at a higher oxygen tension.

For net $Na^+$ loss, the observation that all three levels of hypoxia increased net $Na^+$ loss (Fig. 2B) supported our original hypothesis. Others have also reported increased net $Na^+$ loss during exposure of trout to hypoxia (Thomas et al., 1986; Iftikar et al., 2010; Robertson et al., 2015b). Specifically, the increased net $Na^+$ loss could be due to the increased surface area and decreased diffusion distance that potentially accompanies increased branchial perfusion and the recruitment of lamellae (Steen and Kruysse, 1964; Richards and Fromm, 1969; Booth, 1978; Loretz, 1979). Holeton and Randall (1967) observed increased lamellar perfusion in trout during exposure to hypoxia, leading to an increase in gill $O_2$ transfer factor (Randall et al., 1967). However, with increasing severity of hypoxia, the elevation in water flux rate was attenuated (Fig. 2A) yet elevated $Na^+$ loss rates were maintained (Fig. 2B), as well as with increasing duration of hypoxia (Fig. 3B), and with increasing duration of normoxic recovery (Fig. 3B). Clearly the two processes are very different. In support of this view were our findings of no significant correlations between water flux and net $Na^+$ loss rates in individual fish at different levels of hypoxia and during different durations of hypoxia and normoxic recovery. This lack of significant correlation was true both when each experimental treatment was examined separately, and when all the treatments were grouped together. Net $Na^+$ loss reflects the balance between active uptake and

passive diffusive loss, whereas tritiated water exchange should reflect only a simple unidirectional process. Although, our study did not probe these pathways it is possible that diffusive water flux is mediated at least in part by aquaporins (Evans et al., 2005), while diffusive $Na^+$ efflux is traditionally thought to occur via paracellular pathways (Evans et al., 2005; Marshall and Grosell, 2006), though we are aware of no conclusive evidence on this issue. The regulation of net $Na^+$ loss rate will likely be much more complex than that of diffusive water exchange rate. For example, our results on trout were quite different from those on oscars where there were negligible changes in net $Na^+$ flux rates during severe hypoxia, because both active $Na^+$ influx and passive $Na^+$ efflux rates were reduced to the same extent (Wood et al., 2007; Wood et al., 2009; De Boeck et al., 2013; Robertson et al., 2015b). In future studies with trout, it will be of interest to use radioactive $Na^+$ to separate the unidirectional flux components of net $Na^+$ balance in experimental treatments similar to those of the present study. In one previous study on trout employing unidirectional flux measurements (Iftikar et al., 2010), complex time-dependent triphasic changes in both influx and efflux rates (initial increases, then decreases, then increases again in both fluxes) occurred at less severe hypoxia levels than those used in the present study.

### 4.4. Exercise alters both diffusive water flux and net $Na^+$ flux

As expected, exercise increased diffusive water flux rate (by 35%, Fig. 4A). This result aligned with the earlier conclusions (Wood and Randall, 1973c; Stevens, 1972; Hofmann and Butler, 1979) based on measurements of urine flow rate and/or body weight changes, that net water uptake ("osmotic water flux") increases with $O_2$ uptake during exercise in freshwater fish. The plausible explanation for the increased diffusive water flux with exercise is the increase in the functional surface area and/or decreased diffusion distance of the fish gill (Holeton and Randall, 1967; Richards and Fromm, 1969; Booth, 1978). This would make the gill more permeable to not only gas but also water flux.

In addition to increased water flux, net $Na^+$ loss also increased following exercise (Fig. 4B). Wood and Randall (1973a, 1973b) had observed increased net $Na^+$ loss during exercise that returned to net $Na^+$ gain during the recovery period, or during prolonged exercise. Gonzalez and McDonald (1992) reported increased $Na^+$ loss following exposure of rainbow trout to 5 min of exercise with a 6 h recovery period. Postlethwaite and McDonald (1995) observed increased $Na^+$ loss when trout were exercised for 0.5 h that returned to pre-exercise level after 7 h. In general, all these changes reflected increases in unidirectional $Na^+$ efflux rate. This suggests that $Na^+$ loss during exercise is a regulated phenomenon and is in part due to increased $Na^+$ permeability that is facilitated by the increase in functional surface area and/or decreased diffusion distance of the gill epithelium. Furthermore, our observation of a significant correlation between diffusive water flux and net $Na^+$ loss (Fig. 5) suggests that the two parameters may be regulated by the same pathway during exercise.

### 4.5. Acute temperature challenge alters diffusive water flux but not net $Na^+$ flux

Our hypothesis that acute temperature rise would increase diffusive water flux and net $Na^+$ loss was partially true. Specifically, our results revealed 2.3- and 3.3-fold increases in diffusive water flux from trout acclimated to 8 °C then transferred to either 13 °C or to 18 °C respectively (Fig. 6A). Similar reports have been observed for painted comber (Isaia, 1972), goldfish (Evans, 1969; Isaia, 1972; Loretz, 1979), eel (Motais and Isaia, 1972), and spiny dogfish (Giacomin et al., 2017) suggesting that the acute response to temperature challenge cuts across species. Future experiments should investigate whether the increased water flux observed here upon acute temperature challenge is a regulated phenomenon which attenuates upon acclimation to the new temperatures.

The $Q_{10}$ value of 4.03 observed for the 8 to 13 °C transfer compared to 2.16 for the 8 to 18 °C challenge suggests that the lower region is more sensitive to acute temperature rise (Fig. 6A). The $Q_{10}$ values above 2.0 reported here are generally within the ranges observed by others for diffusive water fluxes in fish, for example Motais and Isaia (1972) reported a $Q_{10}$ value of 3.32 for 5–25 °C in freshwater eel and Isaia (1972) reported a $Q_{10}$ value of 2.40 for 5–25 °C in goldfish, in accord with other reports for the same species (e.g. 2.48 for 8.5–30 °C by Loretz, 1979, and 2.14 by Evans, 1969). Thus, most values are above 2.0, though there are several exceptions (e.g. 1.77 for 10–20 °C in minnow reported by Evans, 1969, and 1.96 for 7.5–22 °C reported by Giacomin et al., 2017 in spiny dogfish). Generally, $Q_{10}$ values that are > 2.0 suggest that unidirectional water flux is biologically mediated, possibly involving proteins such as aquaporins rather than reflecting simple physicochemical processes such as temperature effects on membrane lipids and tight junctions where $Q_{10}$ values are closer to 1.0.

Our observation that the net $Na^+$ loss did not change with acute temperature rise is similar to the findings of Motais and Isaia (1972) who observed no net changes in $Na^+$ loss in the freshwater eel. The finding of no significant correlation between diffusive water flux and net $Na^+$ loss in individual fish following acute temperature rise confirms that these parameters are regulated separately. Overall, the net $Na^+$ loss that was sustained during prolonged hypoxia and recovery but remained unchanged during acute temperature rise needs further investigation. Specifically, measurement of unidirectional (influx and efflux) rates using radiolabelled $Na^+$ under similar experimental condition would assist in clarifying this observation. Finally, future studies should investigate the actual pathways of diffusive water flux and $Na^+$ loss, to identify whether these fluxes occur through paracellular, transcellular or both pathways during conditions of high $O_2$ demand.

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