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THE EFFECTS OF SOFTWATER ACCLIMATION ON RESPIRATORY GAS TRANSFER IN THE RAINBOW TROUT *ONCORHYNCHUS MYKISS*

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Summary

Gill O_2 uptake, CO_2 excretion, ventilation and blood respiratory/acid–base variables were evaluated in control and softwater-acclimated trout (*Oncorhynchus mykiss*) to test the hypothesis that gill chloride cell (CC) proliferation, elicited by 2 weeks of softwater exposure, impairs the diffusion of respiratory gases across the gill. The proliferation of CCs in softwater fish was verified using light microscopy, and its impact on respiratory gas transfer was assessed *in vivo* by continuous monitoring of arterial blood P_{O_2} (Pa_{O_2}), P_{CO_2} (Pa_{CO_2}) and pH (pHa) using an extracorporeal blood circulation under conditions of normoxia and graded hypoxia [water P_{O_2} (Pw_{O_2}) was lowered from 20.0 kPa to 5.3 kPa within 20 min].

During normoxia, ventilation frequency was significantly higher in the softwater trout (78±4 *versus* 57±4 breaths min^{-1}; mean ± S.E.M.), while ventilation amplitude was similar in both groups (1.0–1.1 cm opercular displacement). Pa_{CO_2} and plasma HCO_3^- concentration were significantly lower in the softwater fish and the blood acid–base status was characterized by a mixed respiratory alkalosis and metabolic acidosis such that blood pH was not statistically different between the two groups. CO_2 excretion (2.5–2.8 mmol kg^{-1} h^{-1}) and O_2 uptake rates (2.3–5.1 mmol kg^{-1} h^{-1}), as measured during normoxia, were unaffected by acclimation to soft water.

During hypoxia, ventilation frequency and amplitude increased in the control trout, whereas only ventilation amplitude increased in the softwater-acclimated fish. The rate of Pa_{O_2} reduction during hypoxia was significantly greater in the softwater fish (0.84±0.06 *versus* 0.65±0.06 kPa Pa_{O_2} kPa^{-1} Pw_{O_2}) and, at the most severe level of hypoxia (Pw_{O_2}=5.3 kPa), Pa_{O_2} was significantly lower in the softwater fish. The rate of Pa_{CO_2} reduction (caused by hyperventilation) was significantly lower in the softwater-acclimated fish (0.002±0.001 *versus* 0.005±0.001 kPa Pa_{CO_2} kPa^{-1} Pw_{O_2}; mean ± S.E.M.; P<0.06) and, indeed, was not statistically different from zero. Blood pH did not change significantly during hypoxia in either group but, through much of the hypoxic period (7–15 kPa Pw_{O_2}), pHa was statistically lower in the softwater-acclimated fish.

These results demonstrate that exposure of trout to soft water for 2 weeks is associated with proliferation of lamellar CCs and impaired branchial gas transfer. Hyperventilation was identified as a compensatory physiological adjustment.

Key words: *Oncorhynchus mykiss*, rainbow trout, gill, chloride cell, hypoxia, soft water, gas transfer, ventilation.

Introduction

Acclimation of teleost fish to soft water elicits an array of adaptive physiological responses aimed at maintaining ionic homeostasis in an ion-poor environment (McDonald and Rogano, 1986; see reviews by Laurent and Perry, 1991; Perry and Laurent, 1993). One of the best documented responses is the proliferation of chloride cells (CCs) on the lamellar surfaces of the gill (Laurent *et al.* 1985, 1994; Perry and Wood, 1985; Avella *et al.* 1987; Leino *et al.* 1987; Spry and Wood, 1988; Perry and Laurent, 1989; Laurent and Hebibi, 1989). Because of the presumed role of the CC in transbranchial Ca^{2+} uptake (Perry and Flik, 1988; Marshall *et al.* 1992; McCormick *et al.* 1992; Perry *et al.* 1992*a*) and NaCl uptake (Perry and Laurent, 1989; Laurent and Perry, 1990; Perry *et al.* 1992*b*), this proliferation of CCs in soft water is thought to be a strategy to optimize gill ion-transport capacity (Perry and Wood, 1985; Perry and Laurent, 1989).

Recent studies have demonstrated that branchial proliferation of the large spherical CCs, caused either by exogenous cortisol/growth hormone treatment (Bindon *et al.* 1994*a*) or by softwater exposure (A. M. Greco, J. C. Fenwick

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and S. F. Perry, in preparation), causes a marked thickening of the lamellar blood-to-water diffusion distance. In the absence of compensatory adjustment(s), such morphological changes would be expected to impede the diffusion of respiratory gases across the gill (Randall and Daxboeck, 1984). Indeed, Bindon *et al.* (1994*b*) demonstrated experimentally that the thickening of the diffusion barrier caused by the hormone treatment was associated with an elevation of arterial blood P_{CO_2} (Pa_{CO_2}) and a reduction of Pa_{O_2} during severe hypoxia. A potential limitation in the study of Bindon *et al.* (1994*b*) was the possibility of non-specific metabolic effects associated with the use of pharmacological (i.e. non-physiological) levels of hormones to elicit CC proliferation.

The goal of the present study was to evaluate the consequences of naturally induced CC proliferation on respiratory gas transfer. This was achieved by exposing trout to soft water for a period of 2 weeks, after which the lamellar blood-to-water diffusion distance is known to be doubled (A. M. Greco, J. C. Fenwick and S. F. Perry, in preparation). Respiratory function along with ventilation parameters were investigated using an extracorporeal blood circulation set-up under conditions of normoxia and graded hypoxia.

Materials and methods

Experimental animals

Rainbow trout [*Oncorhynchus mykiss* (Walbaum)] of both sexes and weighing between 574 and 964 g (mean mass 727±30 g, experimental N=15; mean ± S.E.M.) were purchased from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada) and transferred to the fish holding facility of the University of Ottawa. Fish were kept indoors in large tanks furnished with flowing, dechlorinated and aerated City of Ottawa tapwater (see Table 1 for water chemistry). The photoperiod was kept constant at 12 h:12 h light:dark. Fish were fed to satiation daily with a dried commercial trout diet (Purina Trout Chow) and were acclimated to laboratory conditions for at least 4 weeks before any experiments were performed.

Acclimation protocol

Fish were divided between two large fibreglass tanks (Living Stream; Toledo, OH, USA). The first tank (control group) was supplied with running dechlorinated tapwater. The second tank (softwater-acclimated group) was supplied with dechlorinated tapwater diluted with running reverse osmosis (RO) water (see Table 1 for water chemistry). Total water flow rates to the tanks were adjusted to 5 l min^{-1}. The experimental group of fish was exposed to the softwater condition by a gradual increase in the proportion of RO water over a period of 3 days until the final conditions were met. The third day was recorded as day 1 of softwater exposure. Fish were acclimated to softwater conditions for 2 weeks. Softwater fish were fed to satiation daily, the quantity of food consumed was recorded, and the control fish were fed accordingly (average= 1 % body mass day^{-1}).

Table 1. *Water chemistry variables for the control (City of Ottawa tapwater) and softwater (artificial soft water) conditions that were utilized for trout acclimation*

	City of Ottawa dechlorinated tapwater	Artificial soft water
$[Ca^{2+}]$ (mmol l^{-1})	0.37±0.01 (N=39)	0.04±0.01* (N=40)
$[Na^{+}]$ (mmol l^{-1})	0.12±0.002 (N=39)	0.05±0.002* (N=40)
$[K^{+}]$ (mmol l^{-1})	0.019±0.001 (N=39)	0.009±0.001* (N=40)
$[Cl^{-}]$ (mmol l^{-1})	0.15±0.001 (N=39)	0.04±0.002* (N=40)
Pw_{O_2} (kPa)	20.2±0.3 (N=7)	20.2±0.2 (N=8)
$[HCO_3^{-}]$ (mmol l^{-1})	0.44±0.01 (N=7)	0.16±0.02* (N=8)
Temperature (°C)	10.5	11.5
pH	6.8	6.6

Values are means ± 1 S.E.M.
*indicates a significant difference from the control value using two-sample t-test, P<0.05.

Surgical techniques

After acclimation for 2 weeks, trout were anaesthetized in a solution of MS-222 (0.125 g l^{-1}) neutralized with $NaHCO_3$ (0.25 g l^{-1}) and placed onto a surgery table. The gills were irrigated continuously throughout the operation with oxygenated anaesthetic solution. The dorsal aorta was cannulated using flexible polyethylene tubing (Clay-Adams PE 50, i.d. 0.580 mm, o.d. 0.965 mm) as described by Soivio *et al.* (1975). The fish was moved onto its left side and an incision was made on the right-hand flank, parallel and 1 cm posterior to the outer edge of the operculum. This allowed access to the coeliac artery, which was cannulated (PE 50) in two directions (orthograde and retrograde) (Thomas and LeRuz, 1982). The cannulae would later form an extracorporeal loop through which arterial blood would be pumped, enabling the measurement of blood respiratory and acid–base variables. After suturing the wound, the cannulae were flushed with heparinized saline (50 i.u. ml^{-1} ammonium heparin) to prevent blood from clotting. Small (1 cm^2) brass plate electrodes were stitched to the epithelium of each operculum to allow the measurement of ventilation amplitude *via* an impedance converter.

The fish were revived after surgery by irrigating the gills with fresh oxygenated water and were then introduced into an experimental chamber furnished with flowing and aerated water. Fish were left to recover for 24 h prior to experimentation; food was withheld for this period.

Experimental arrangement

Fish were held in black Perspex boxes with clear windows

used for observing the interior of the box; windows were normally covered to avoid disturbing the fish. The cannulae and electrode wires were passed through a slit in the chamber lid. The dorsal aortic cannula was connected to a pressure transducer (Bell and Howell 4-327-1) which, in turn, was connected to a recording physiograph. Blood pressure was monitored during the experiment to establish the patency of the extracorporeal preparation. Persistent reduction in blood pressure indicates blood loss and in such cases experiments were terminated. Opercular displacement was monitored using an impedance converter and amplifier. Ventilation frequency was measured periodically by visually counting buccal movements.

The two coeliac cannulae formed a loop which was connected in series with thermostatted (10–12 °C) cells containing P_{O_2} (Radiometer model E-5046), P_{CO_2} (Radiometer model E-5036) and pH (Metrohm combination electrode) electrodes, which were attached to a Radiometer PHM-73. This external loop tubing was flushed with heparinized saline (540 i.u. ml^{-1}) before starting the blood flow. Blood was removed from the 'arterial' (retrograde) cannula at a constant rate (1.2 ml min^{-1}) and passed through the cells by means of a small peristaltic pump before being returned to the fish through the 'venous' (orthograde) cannula. The total volume of blood in the extracorporeal loop was less than 4 % of the total blood volume of the fish. Water P_{O_2} (P_{wO_2}) in the experimental chamber was measured by means of another Radiometer P_{O_2} electrode connected to a PHM-72 meter.

Calibration of the P_{CO_2} and P_{O_2} electrodes was carried out by equilibrating water with gas of the appropriate P_{CO_2} and P_{O_2} using a Wöstoff pump (M301 A/F) and pumping the water across the electrodes with the peristaltic pump. pH electrode calibration was achieved with buffer solutions.

Oxygen consumption ($\dot{M}_{O_2}$) and CO_2 excretion ($\dot{M}_{CO_2}$) were measured using closed system respirometry techniques (Holeton and Randall, 1967). Water flow to the fish box was halted and P_{wO_2} was monitored continuously until it had decreased by about 4 kPa. After each 1 kPa decrease in P_{wO_2}, water samples (1.5 ml) were taken from an area close to the mouth of the fish *via* the output of the P_{wO_2} electrode. Changes in P_{wO_2}, mass of the fish and volume of the box, along with constants from Boutilier *et al.* (1984) were used for calculating $\dot{M}_{O_2}$. Total CO_2 was measured (Cameron Capnicon, model 5) on the withdrawn water samples and used to calculate $\dot{M}_{CO_2}$.

The extracorporeal arrangement allowed the arterial oxygen partial pressure (P_{aO_2}), the arterial carbon dioxide partial pressure (P_{aCO_2}), the water oxygen partial pressure (P_{wO_2}) and the arterial pH (pHa) to be monitored continuously during the experiment. Mean values for each parameter were captured and stored every 5 s. All measuring devices produced analog outputs which were transformed into digital outputs with the aid of an analog–digital interface (Data Translation Incorporated). These output values were transmitted to a microcomputer and the output was recorded using a customized data acquisition software (AD-DATA; P. Thoren, Göteborg, Sweden).

Experimental protocol

The protocol consisted of two separate stages, normoxia and hypoxia. The pumping of the blood to the external loop was started during normoxia and the variables were allowed to stabilize for 20 min. The closed system respirometry was then performed and, following a recovery period with P_{wO_2} levels at normoxia (20 kPa), a pre-hypoxia blood sample of 0.5 ml was taken for the measurement of haematocrit (Hct), haemoglobin concentration ([Hb]), C_{aO_2} and plasma ion concentrations.

During the second stage, fish were subjected to hypoxia by bubbling compressed N_2 through a water equilibration column which supplied the experimental holding box. The flow rate of N_2 was carefully monitored to produce a linear decrease in P_{wO_2}. The hypoxic period was imposed over a 20 min period with P_{wO_2} being reduced at an approximate rate of 0.8 kPa min^{-1} to an end value of 5.3 kPa.

Morphology

CC proliferation was confirmed using light microscopy. At the end of the experiment, each fish was returned to normoxia. Fish were killed using a lethal dose of anaesthetic (0.5 g l^{-1} MS-222), and several pairs of filaments still attached at the septum were removed from the left second gill arch. The tissue was immediately immersed in Champ Maillet's fluid, a mixture of 1.2 % ZnI_2 and 0.2 % OsO_4, as described by Garcia-Romeu and Masoni (1970), and left at room temperature for 30 h. This fixative/stain causes a reduction of osmic acid to osmium, which blackens the phospholipids. CCs have an intricate plasma membrane with many invaginations and thus stain strongly. After rinsing in distilled water for 6 h (changed three times), the gills were dehydrated in an ethanol series and embedded in paraffin wax. Sections were cut (8 μm thick) and placed onto gelatin-coated glass slides. Paraffin sections were deparaffinized in three changes of xylene for 3 min each. Sections were then mounted in permount (BDH chemicals) before being viewed using a light microscope (40× objective; Leitz Wetzlar – Dialux 20 EB). Photographs were taken using an attached camera (Wild Heerbrugg Mps 45 Photoautomat).

Water and blood analysis

Plasma total CO_2 and bicarbonate concentration ($[HCO_3^-]$) were calculated using the Henderson–Hasselbalch equation, based on the P_{aCO_2} and pHa values measured with the extracorporeal set-up and constants from Boutilier *et al.* (1984). Haemoglobin content ([Hb]) was determined spectrophotometrically using a commercial kit (Sigma Chemical Company). Total oxygen content ($[O_2]$) was determined on 20 μl samples according to the method of Tucker (1967). Haematocrit was measured in duplicate using microcapillary tubes centrifuged at 10 000 ***g*** for 10 min. The remaining blood was centrifuged at 10 000 ***g*** for 30 s and the plasma was frozen and stored at −80 °C for later ion analysis. Water and plasma $[Na^+]$, $[Ca^{2+}]$ and $[K^+]$ were determined by flame emission spectrophotometry (Varian, model Spectra AA

250 Plus). [Cl^-] was determined by a mercuric thiocyanate spectrophotometric assay method (Zall *et al.* 1956). Acclimation water pH was measured using a pH meter (Ionalyzer, model 407A, Orion Research).

Data and statistical analysis

Data are presented as means ±1 standard error of the mean (S.E.M.). For data obtained from the extracorporeal experiments, statistical analyses were performed on the mean data displayed at intervals of 1.3 kPa $P_{W_{O_2}}$ using one-way analysis of variance (ANOVA). At each point (i.e. at each 1.3 kPa change in $P_{W_{O_2}}$), a two-sample *t*-test was performed to compare softwater with control fish. Also, linear regressions were performed and tested with *t*-tests to determine whether rates of change of blood and ventilation parameters with $P_{W_{O_2}}$ were significantly different in the control and softwater fish. Differences in other measured parameters (e.g. [Hb], [Ca^{2+}], etc.) were analyzed using two-sample *t*-tests. Except when stated otherwise, the fiducial limit of significance was 5 %.

Results

Gill morphology

Representative light micrographs of control (Fig. 1A) and softwater-exposed (Fig. 1B) rainbow trout gills illustrate the general morphological appearance of the filaments and lamellae from the two groups. The black-stained CCs were clearly protruding from and proliferated over the entire surface of the gill epithelium of the softwater fish. CCs were smaller and less numerous on the control fish gills.

Plasma ion levels and respiratory variables during normoxia

After 2 weeks, plasma [Cl^-] was significantly lower in the softwater-acclimated fish, while [Ca^{2+}], [K^+], [Na^+] and osmolarity were unaffected (Table 2). Table 3 shows the blood and gas transfer parameters measured immediately before the start of the hypoxic period. Ventilation parameters were measured before determining rates of O_2 uptake and CO_2 excretion. Mean red blood cell haemoglobin concentration (MCHC) was significantly lower in softwater trout compared with controls, indicating cell swelling. [HCO_3^-] in the

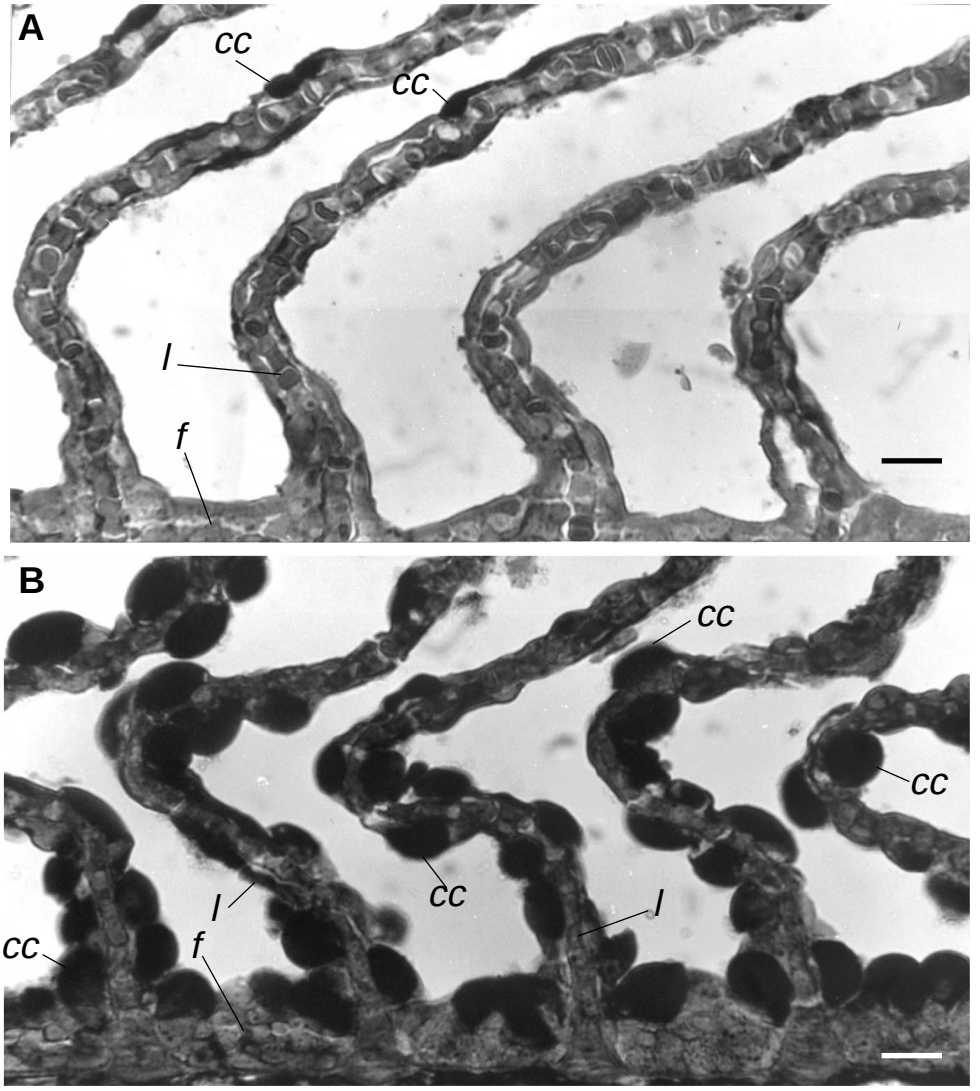

Fig. 1. Representative light micrographs of trout gills showing filament and lamellae from (A) control fish and (B) fish exposed to softwater conditions for 2 weeks. Chloride cells (CCs), which are stained black, have proliferated over the entire surface of the gill epithelium of the softwater fish; *l*, lamellae; *f*, filament. The CCs of the control fish are smaller and far less numerous. Scale bars, 15 μm.

Table 2. *Plasma ion levels measured in control and softwater-acclimated trout*

	Control trout (N=7)	Softwater trout (N=8)
$[Ca^{2+}]$ (mmol l^{-1})	2.4±0.2	1.8±0.3
$[Na^{+}]$ (mmol l^{-1})	141.0±4.6	128.4±7.7
$[K^{+}]$ (mmol l^{-1})	2.7±0.4	2.8±0.5
$[Cl^{-}]$ (mmol l^{-1})	143.3±4.1	123.6±3.8*
Osmolarity (mmol kg^{-1})	288.9±1.5	280.8±3.8

Values are means ± 1 S.E.M.
*indicates a significant difference from the control value using two-sample t-test, P<0.05.

Table 3. *Blood, ventilation and gas exchange variables measured during the normoxia (pre-hypoxia) stage of the extracorporeal experiments in control and softwater-acclimated trout*

	Control trout (N=7)	Softwater trout (N=8)
Haematocrit (%)	22.8±1.5	21.1±1.9
[Hb] (ml 100 ml^{-1})	9.14±1.66	7.02±0.88
MCHC (g ml^{-1})	0.40±0.03	0.30±0.02*
$[O_2]$ (ml 100 ml^{-1})	10.06±0.74	8.96±0.84
$[O_2]$/[Hb] (ml O_2 g^{-1} Hb)	1.08±0.11	1.32±0.17
$[HCO_3^-]$ (mmol l^{-1})	6.71±0.51	4.74±0.58*
Blood pressure (kPa)	3.25±0.20	3.28±0.48
$\dot{M}_{O_2}$ (mmol kg^{-1} h^{-1})	2.28±0.23	5.10±1.80
$\dot{M}_{CO_2}$ (mmol kg^{-1} h^{-1})	2.53±0.52	2.78±0.58
R, $\dot{M}_{CO_2}/\dot{M}_{O_2}$	1.18±0.23	0.86±0.30
Ventilation frequency (min^{-1})	57.4±3.59	78.1±4.43*
Opercular displacement (cm)	1.02±0.09	1.12±0.18

Values are means ± 1 S.E.M.
*indicates a significant difference from the control value using two-sample t-test, P<0.05.
Hb, haemoglobin; MCHC, mean cellular haemoglobin concentration.

softwater trout was significantly lower than in the controls and, together with the reduced blood pH (see Fig. 6), represented a base deficit (metabolic acid load) of approximately 3 mmol l^{-1}. Rates of CO_2 excretion and O_2 uptake were unaffected by acclimation to soft water. The gill respiratory exchange ratio (R: $\dot{M}_{CO_2}/\dot{M}_{O_2}$) showed large variability and was not statistically different between groups. The ventilation frequency was significantly elevated in the softwater-acclimated fish by 21 breaths min^{-1} (36 % increase) compared with the ventilation frequency of the control fish, while ventilation amplitude was not affected by softwater acclimation.

Graded hypoxia

During hypoxia, the control fish increased their ventilation frequency significantly at $P_{W_{O_2}}$ values of 14.8 kPa and below (Fig. 2A). The ventilation frequency of the softwater-acclimated trout remained constant during hypoxia, yet was considerably greater than in the control trout owing to the high initial (normoxia) values. The regression analysis (Fig. 2B) demonstrated a significant difference in the slope of the relationship between ventilation frequency and $P_{W_{O_2}}$ during hypoxia between the control and softwater-acclimated trout.

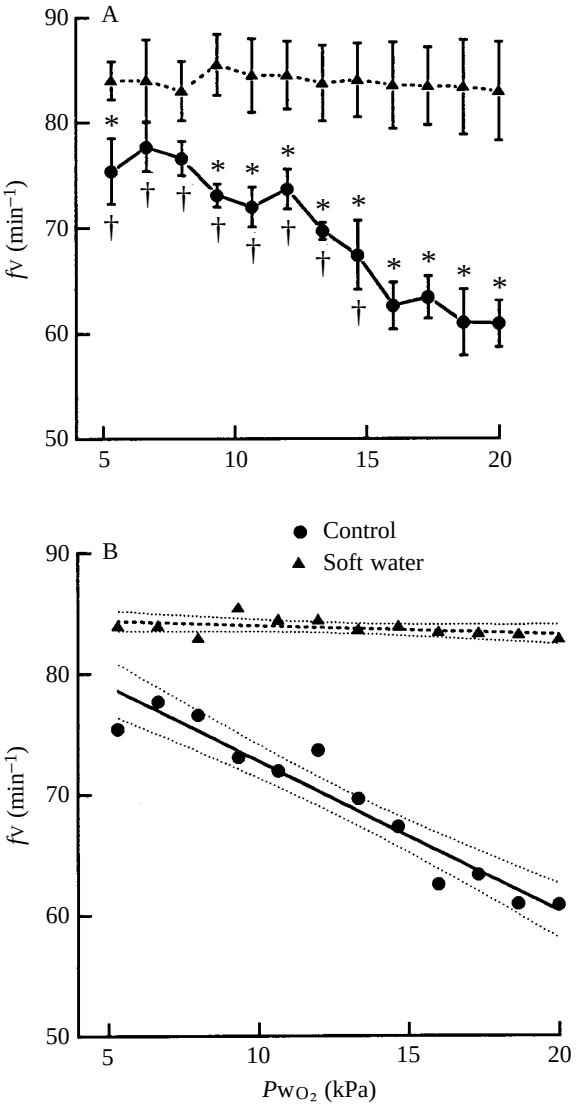

Fig. 2. (A) Effect of environmental hypoxia on ventilation frequency (f_V) in control (solid line; N=7) and softwater-acclimated (dashed line; N=8) rainbow trout. † indicates a significant difference (P<0.05) in f_V from the values measured during normoxia (at $P_{W_{O_2}}$=20 kPa). * indicates a significant difference (P<0.05) between the treatment groups. Error bars represent ±1 S.E.M. (B) Regression analysis showing the mean linear relationships between f_V and $P_{W_{O_2}}$ in the control fish (slope=−1.21±0.15 breaths min^{-1} kPa^{-1} $P_{W_{O_2}}$) and softwater-acclimated rainbow trout (slope=−0.207±0.056 breaths min^{-1} kPa^{-1} $P_{W_{O_2}}$). There is a significant difference (P<0.05) between the two groups in the slopes of the lines relating ventilation frequency and $P_{W_{O_2}}$. 95 % confidence limits are shown as dotted lines.

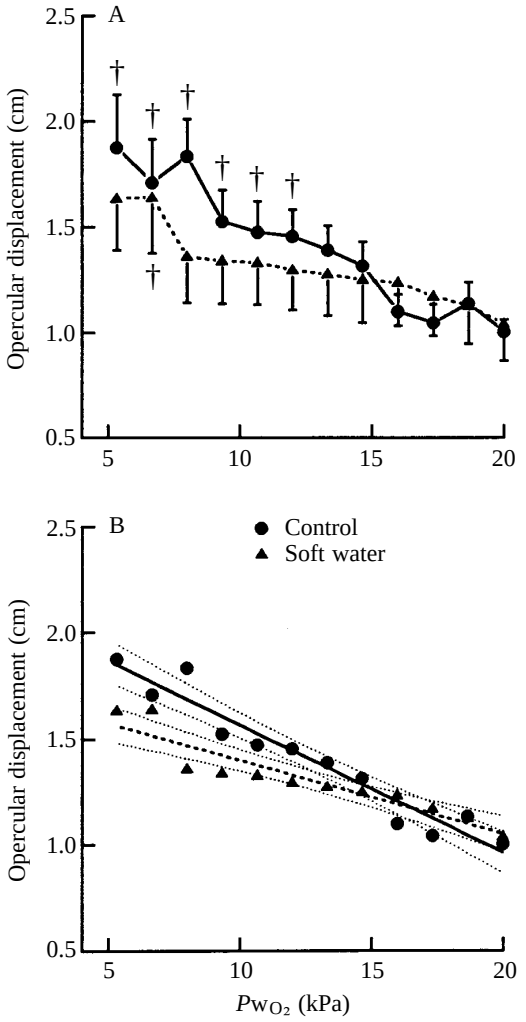

Fig. 3. (A) Effect of environmental hypoxia on mean opercular displacement in control (solid line; N=7) and softwater-acclimated (dashed line; N=8) rainbow trout. † indicates a significant difference (P<0.05) in opercular displacement from the values measured during normoxia ($P_{W_{O_2}}$=20 kPa). Error bars represent 1 S.E.M. and are shown in only one direction for clarity. (B) Regression analysis showing the mean linear relationships between opercular displacement and $P_{W_{O_2}}$ in the control (slope=-0.061 ± 0.012 cm kPa^{-1} $P_{W_{O_2}}$) and softwater-acclimated trout (slope=-0.026 ± 0.005 cm kPa^{-1} $P_{W_{O_2}}$). There is a significant difference (P<0.05) between the two groups in the slopes of the lines relating opercular displacement and $P_{W_{O_2}}$. 95% confidence limits are shown as dotted lines.

The effects of graded hypoxia on opercular displacement are shown in Fig. 3A. Both the control and the softwater-acclimated fish increased their opercular displacement when exposed to hypoxia, but in the case of the softwater fish this increase was not apparent until a greater level of hypoxia had been imposed (6.7 kPa). Furthermore, a linear regression analysis (Fig. 3B) revealed a significant difference in the slope of the relationship between opercular displacement and $P_{W_{O_2}}$ between the two groups, with the slope for the control group being steeper than that of the softwater group.

The effects of graded hypoxia on $P_{a_{O_2}}$ are shown in Fig. 4A. Both groups experienced significant decreases in $P_{a_{O_2}}$ compared with normoxia values at all levels of $P_{W_{O_2}} \leqslant 16$ kPa. The softwater-acclimated fish were able to maintain $P_{a_{O_2}}$ values comparable to those in the control fish, except at the lowest $P_{W_{O_2}}$ of 5.3 kPa, at which point the $P_{a_{O_2}}$ of the softwater trout was significantly lower than in the controls. Fig. 4B reveals that the slope of the relationship between $P_{a_{O_2}}$ and $P_{W_{O_2}}$ was significantly steeper in the softwater-acclimated trout. Fig. 4C illustrates the relationship between $P_{W_{O_2}}$ and the difference between $P_{W_{O_2}}$ and $P_{a_{O_2}}$ ($P_{W_{O_2}}-P_{a_{O_2}}$), providing an estimate of O_2 transfer effectiveness [effectiveness=$(P_{a_{O_2}}-P_{v_{O_2}})/(P_{W_{O_2}}-P_{v_{O_2}})$; see Cameron, 1989] during progressive hypoxia.

The absolute values of $P_{a_{CO_2}}$ were similar in control and softwater-acclimated fish at all levels of hypoxia (Fig. 5A). The slope of the relationship between $P_{a_{CO_2}}$ and $P_{W_{O_2}}$ during hypoxia was significantly less steep (P<0.06) in the softwater-acclimated fish than in the control fish (0.002 ± 0.001 *versus* 0.005 ± 0.001 kPa $P_{a_{CO_2}}$ kPa^{-1} $P_{W_{O_2}}$; Fig. 5B).

Blood pH did not change significantly during hypoxia in either group (Fig. 6A), but through much of the hypoxic period (7–14.8 kPa $P_{W_{O_2}}$), pHa was statistically lower in the softwater-acclimated fish. The regression analysis (Fig. 6B) demonstrated that the slopes of the relationships between pHa and $P_{W_{O_2}}$ in the two groups of fish were not different from one another during hypoxia.

Discussion

Gill morphology and ionic regulation in softwater-acclimated fish

In the present study, trout were maintained for 2 weeks in soft water. After this period, plasma Cl^- levels were significantly depressed and, although not significant, there was a trend towards reduced plasma levels of Ca^{2+} and Na^+ (Table 2). Thus, despite the proliferation of chloride cells and the possible activation of other regulatory mechanisms (McDonald and Rogano, 1986), the ionoregulatory capability appeared to remain impaired. It is not unreasonable to suggest, however, that the plasma ion levels would have been reduced still further in the absence of chloride cell proliferation.

Regardless of its significance in ionoregulation, chloride cell proliferation is a well-established response of trout and other teleosts to soft water exposure and must markedly influence the gill blood-to-water diffusion barrier at such times. Recently A. M. Greco, J. C. Fenwick and S. F. Perry (in preparation), using fish of the same stock and almost identical acclimation conditions as in the present study, reported a twofold increase in the lamellar diffusion distance (from 3.26 ± 0.08 to 6.58 ± 0.43 μm) based on detailed analysis of transmission electron micrographs. Using a similar analytical procedure, Bindon *et al.* (1994*a*) observed a comparable thickening of the lamellar diffusion barrier associated with the chloride cell proliferation accompanying chronic treatment of trout with

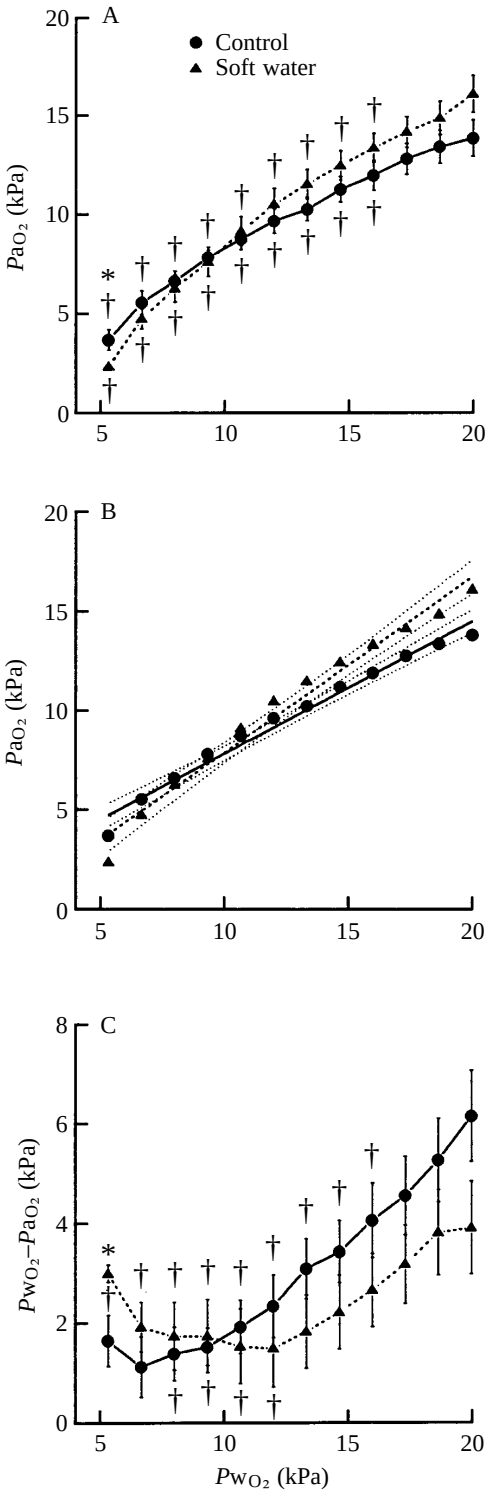

Fig. 4. (A) Effect of environmental hypoxia on arterial oxygen partial pressure (P_{aO_2}) in control (solid line; N=7) and softwater-acclimated rainbow trout (dashed line; N=8). † indicates a significant difference (P<0.05) in P_{aO_2} from the values measured during normoxia (P_{wO_2}=20 kPa). * indicates a significant difference (P<0.05) between the treatment groups. Error bars represent ±1 S.E.M. (B) Regression analysis showing the mean linear relationships between P_{aO_2} and P_{wO_2} in the control fish (slope=0.84±0.06 kPa P_{aO_2} kPa^{-1} P_{wO_2}) and softwater-acclimated fish (slope=0.65±0.06 kPa P_{aO_2} kPa^{-1} P_{wO_2}). There is a significant difference (P<0.05) between the two groups in the slopes of the lines relating P_{aO_2} and P_{wO_2}. 95 % confidence limits are shown as dotted lines. (C) Effect of environmental hypoxia on the difference between P_{wO_2} and P_{aO_2} ($P_{wO_2}-P_{aO_2}$). Symbols as in A.

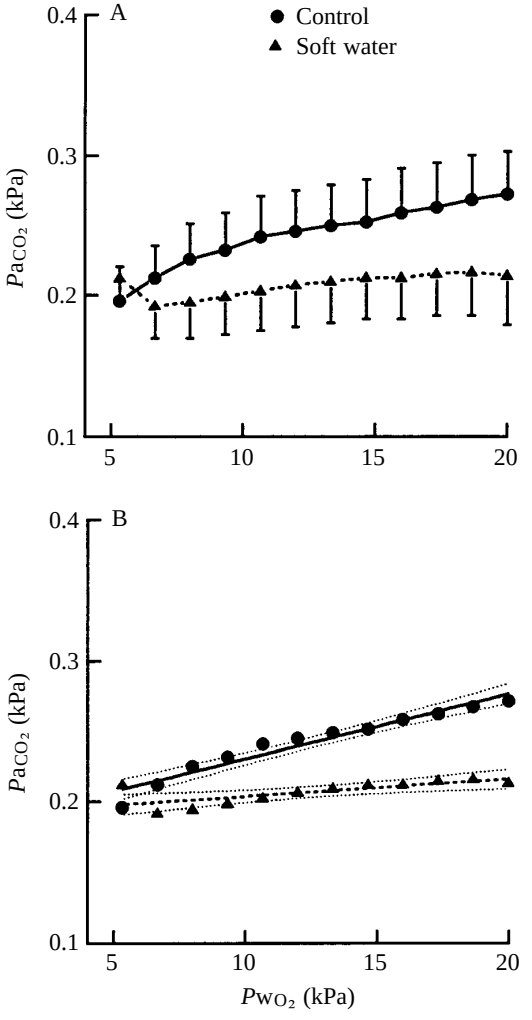

Fig. 5. (A) Effect of environmental hypoxia on arterial carbon dioxide partial pressure (P_{aCO_2}) in control (solid line; N=7) and softwater-acclimated rainbow trout (dashed line; N=8). Error bars indicate 1 S.E.M. and are shown in only one direction for clarity. (B) Regression analysis showing the mean linear relationships between P_{aCO_2} and P_{wO_2} in control fish (slope=0.005±0.001 kPa P_{aCO_2} kPa^{-1} P_{wO_2}) and softwater-exposed rainbow trout (slope=0.002±0.001 kPa P_{aCO_2} kPa^{-1} P_{wO_2}). There is a significant difference (P<0.06) between the two groups in the slopes of the lines relating P_{aCO_2} and P_{wO_2}. 95 % confidence limits are shown as dotted lines.

cortisol and/or growth hormone. On the basis of the positive correlations between gill chloride cell fractional surface area and the blood-to-water diffusion distance established previously in this laboratory (Bindon *et al.* 1994*a*; A. M. Greco, J. C. Fenwick and S. F. Perry, in preparation), it is reasonable to assume that the thickness of the diffusion barrier was increased markedly in the present experiments.

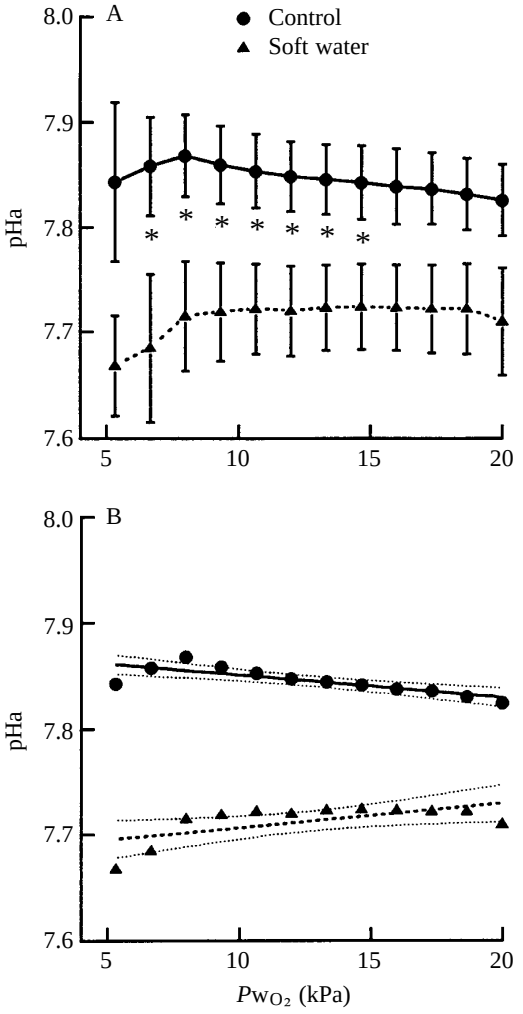

Fig. 6. (A) Effect of environmental hypoxia on arterial pH (pHa) in control (solid line; N=7) and softwater-acclimated rainbow trout (dotted line; N=8). * indicates a significant difference (P<0.05) between the treatment groups. Error bars indicate ±1 S.E.M. (B) Regression analysis showing the mean linear relationships between pHa and $P_{W_{O_2}}$ in the control fish (slope=−0.0026±0.0025 pH units kPa^{-1} $P_{W_{O_2}}$) and softwater-acclimated fish (slope= 0.0021±0.0035 pH units kPa^{-1} $P_{W_{O_2}}$). 95% confidence limits are shown as dotted lines. The slopes did not differ.

Respiratory gas transfer during normoxia

A striking feature in the softwater-acclimated fish was the approximately 36% increase in ventilatory frequency. Given that the opercular displacement (a measure of ventilatory stroke volume) was unchanged, it is likely that the softwater-acclimated fish were experiencing marked hyperventilation. In theory, hyperventilation in the absence of other adjustments will increase the transbranchial diffusion gradients for O_2 and CO_2 (Wood and Perry, 1985) and thereby serve to elevate $P_{a_{O_2}}$ and to lower $P_{a_{CO_2}}$. Thus, hyperventilation can be viewed as a compensatory mechanism to counteract the potential loss of gas transfer effectiveness associated with a thickened diffusion barrier. However, the consequences for O_2 and CO_2 transfer are likely to differ. According to theoretical models (e.g. Malte and Weber, 1985), CO_2 transfer across the fish gill is strongly diffusion-limited whereas O_2 transfer is predominantly perfusion-limited (see also Daxboeck *et al.* 1982). Therefore, under normal conditions, CO_2 transfer is expected to be more sensitive than O_2 transfer to increases in the diffusion barrier thickness. Indeed, in the absence of an obvious hyperventilatory response as was noted in this study, Bindon *et al.* (1994*b*) observed a marked elevation of $P_{a_{CO_2}}$ with no change in $P_{a_{O_2}}$ in trout displaying a hormone-induced increase in the blood-to-water diffusion distance. Thus, in the present study, we speculate that the hyperventilation in the softwater-acclimated fish served to maintain CO_2 excretion in the face of increased diffusion limitations imposed by the chloride cell proliferation. Given the absence or minimal contribution of diffusion limitations to O_2 transfer under normoxic conditions (Daxboeck *et al.* 1982; Malte and Weber, 1985), it is perhaps surprising that $P_{a_{O_2}}$ did not increase with increasing ventilation frequency. Two explanations are offered. First, the proliferation of chloride cells in the softwater-acclimated fish may have thickened the diffusion barrier to the extent that significant diffusion limitations were incurred for O_2 transfer. Second, the localized consumption of O_2 by the metabolically active chloride cells (Perry and Walsh, 1989) on the gill epithelial surfaces may have prevented the rise in $P_{a_{O_2}}$.

In softwater-acclimated fish, rates of O_2 uptake and CO_2 excretion were not significantly different from levels in the control fish. The apparent (not statistically significant) increase in O_2 uptake rate in soft water was the result of very high values in three of the eight fish, with the other five fish displaying values essentially identical to the controls; this was also the cause of the high degree of variability in these data. Certainly there is no theoretical basis for an increased O_2 uptake arising solely from hyperventilation under conditions of normoxia, except for the increased metabolic requirements of breathing itself (see below). Owing to the increased ventilation rate, it is reasonable to assume that the ventilatory convection requirement for CO_2 excretion and O_2 uptake were significantly increased in the softwater-acclimated fish.

Although the hyperventilatory response in softwater-acclimated fish should be viewed as a compensatory mechanism to aid CO_2 excretion (and to a lesser extent O_2 uptake), it will nevertheless increase the overall metabolic cost of breathing. There is considerable debate concerning the energetic requirements associated with breathing water (e.g. see Jones and Schwarzfeld, 1974), with estimated values ranging between 4 and 40% of overall metabolic rate. Regardless, it seems likely that a significant component of the overall metabolic rate in the softwater-acclimated fish was used to fuel the additional breathing movements. In addition, owing to the high metabolic activity of the chloride cell relative to that of the other cell types of the gill (Perry and Walsh, 1989), a component of the additional O_2 uptake (as measured by disappearance of O_2 from the water) may have reflected localized use by the more abundant, and potentially more metabolically active, chloride cells.

In contrast to the results of the present study, Bindon *et al.* (1994*b*) noted that the proliferation of chloride cells induced by cortisol and growth hormone treatment did not elicit an increase in breathing frequency or amplitude under normoxic conditions. While the absence of hyperventilation probably explains the increased $P_{a\mathrm{CO_2}}$ in the hormone-treated fish (Bindon *et al.* 1994*b*), the reasons for the discrepant effects of chloride cell proliferation on gill ventilation are unknown but may reflect the different protocols used to elicit chloride cell proliferation.

The lower bicarbonate concentration in the soft water (see Table 1) may also have contributed to the maintenance of CO_2 excretion independently of ventilation adjustments. Adjacent to the gill epithelium is a micro-environment or boundary layer, the chemistry of which can vary markedly from that of the bulk water flowing over the gill (Playle and Wood, 1989; Randall *et al.* 1991). The excretion of CO_2 acidifies the boundary layer (Wright *et al.* 1986) owing to the presence of carbonic anhydrase on the external surface of the gill (Rahim *et al.* 1988). The acidification of the boundary layer promotes CO_2 excretion because the rapid hydration of CO_2 to HCO_3^- and H^+ lowers the $P_{\mathrm{CO_2}}$ of the boundary layer contributing to the maintenance of a favourable $P_{\mathrm{CO_2}}$ gradient across the gill. The reduced concentration of HCO_3^- in the soft water (see Table 1) would be expected to enhance the acidification process and thereby accelerate CO_2 excretion.

The blood acid–base status of softwater fish was characterized by a metabolic acidosis superimposed upon a slight respiratory alkalosis. The metabolic acidosis may have arisen from differential effects of softwater acclimation on net Na^+ and Cl^- fluxes across the gill. On the basis of a current model of branchial ion transfer in freshwater teleosts (e.g. Morgan *et al.* 1994), chloride cell proliferation might be expected to increase Cl^- uptake, *via* Cl^-/HCO_3^- exchange, without influencing Na^+ uptake. Such a response would reduce branchial net acid excretion and thereby induce metabolic acidosis.

Respiratory gas transfer during hypoxia

During hypoxia, the water-to-blood O_2 diffusion gradient is reduced and, consequently, the likelihood of diffusion limitations increases with decreasing water $P_{\mathrm{O_2}}$. Furthermore, the hyperventilatory response known to accompany hypoxia (e.g. see review by Randall, 1982) lowers $P_{a\mathrm{CO_2}}$. The extent of the reduction in $P_{a\mathrm{CO_2}}$ will vary according to the extent of the hyperventilation and the prevailing conditions for CO_2 diffusion across the gill. Thus, hypoxia was used in the present study as a probe to investigate further possible diffusion limitations on gas transfer imposed by the proliferation of lamellar chloride cells. The results demonstrated that both O_2 and CO_2 transfers were impaired during hypoxia in the softwater-acclimated fish. In particular, the greater, more rapid decline of $P_{a\mathrm{O_2}}$ during progressive hypoxia in the softwater fish provided evidence of impaired O_2 uptake and suggested a loss of O_2 transfer effectiveness in these fish during hypoxia. This view was reinforced by graphically relating the water−arterial blood $P_{\mathrm{O_2}}$ difference ($P_{w\mathrm{O_2}}-P_{a\mathrm{O_2}}$) to $P_{w\mathrm{O_2}}$ (Fig. 4). Generally, during hypoxia the effectiveness of gas transfer increases owing to a variety of factors including hyperventilation and lamellar recruitment. Consequently, the difference between $P_{w\mathrm{O_2}}$ and $P_{a\mathrm{O_2}}$ is diminished. In the present study, the apparent O_2 uptake effectiveness as estimated by $P_{w\mathrm{O_2}}-P_{a\mathrm{O_2}}$ increased with the onset of hypoxia in both the control and softwater fish. As the severity of the hypoxia increased, however, two distinctly different patterns emerged. In the control fish, the apparent effectiveness continued to increase throughout most of the hypoxic period, whereas in the softwater fish, an obvious inflection was observed at 12 kPa $P_{w\mathrm{O_2}}$, at which point the apparent effectiveness of O_2 uptake began to decrease ($P_{w\mathrm{O_2}}-P_{a\mathrm{O_2}}$ increased). The simplest explanation for these data is that diffusion limitations imposed by chloride cell proliferation became more evident as the diffusion gradient for O_2 transfer was reduced during hypoxia. An additional explanation for the diminished ability of the softwater fish to maintain $P_{a\mathrm{O_2}}$ during hypoxia is their blunted hyperventilatory response (Figs 2, 3). Although ventilation volume was not measured directly, the absence of any changes in breathing frequency, coupled with an attenuated ventilatory amplitude response, indicates that ventilation volumes during hypoxia were reduced in the softwater fish in comparison with the control fish. The lack of a change in breathing frequency may simply have reflected the fact that breathing frequencies were already maximal (under this particular set of conditions) prior to hypoxia. Alternatively, the breathing frequency response to hypoxia may have been somehow otherwise modified by softwater exposure. In general, the ventilatory response of trout to hypoxia is variable, with some fish responding solely by changing stroke volume (Smith and Jones, 1982) whereas others alter both frequency and stroke volume (e.g. Davis and Cameron, 1971). The attenuated ventilation amplitude response in the softwater-acclimated fish in the present study may have been related to the high breathing frequency, as high frequencies are likely to constrain the amplitude of opercular displacement.

Unlike in the control fish, $P_{a\mathrm{CO_2}}$ remained constant during hypoxia in the softwater-acclimated fish. It seems likely that the effectiveness of CO_2 transfer was already maximal prior to hypoxia and that further increases were precluded by the thickened diffusion barrier. It would have been informative to measure both CO_2 excretion and O_2 uptake rates during hypoxia at the lowest $P_{w\mathrm{O_2}}$ values (5.3 kPa). However, there are several problems associated with the closed system respirometry when used at such severe levels of hypoxia. In particular, a reduction in $P_{w\mathrm{O_2}}$ of only 0.5 kPa when the flow of water is stopped is often sufficient to promote struggling by the fish and the release of catecholamines into the circulation. At such times, the interpretation of O_2 consumption and CO_2 excretion data becomes problematic.

Thomas *et al.* (1988) compared the hypoxic responses of trout acclimated naturally to waters containing approximately 1.0 or 0.1 mmol l^{-1} NaCl. In agreement with the present results, Thomas *et al.* (1988) reported that the fish acclimated

to ion-poor water were less resistant to hypoxia, and these authors attributed the difference to proliferation of lamellar chloride cells induced by the dilute environment. In apparent contrast with the results of the current study and other studies reporting impaired gas transfer (Bindon *et al.* 1994*b*) or a thickening of the diffusion barrier (Bindon *et al.* 1994*a*; A. M. Greco, J. C. Fenwick and S. F. Perry, in preparation) associated with chloride cell proliferation, Laurent and Hebibi (1989) reported a reduction in the blood-to-water diffusion distance in trout exposed to soft water. Presently, we are unable to provide an explanation for the different results. Most of the available evidence supports the view that chloride cell proliferation, while presumably benefiting certain aspects of ionic regulation, may have detrimental consequences for respiratory gas transfer.

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