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# THE EFFECTS OF SOFTWATER ACCLIMATION ON GILL MORPHOLOGY AND RESPIRATORY GAS TRANSFER IN THE RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

by

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A thesis submitted to the School of Graduate Studies and Research
in partial fulfillment for the Degree
Master of Science

University of Ottawa/Université d'Ottawa
The Ottawa-Carleton Institute of Biology

ISBN 0-612-04943-4

Canada

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

Master of Science (1995) University of Ottawa
Biology Université d'Ottawa

Title: The effects of softwater acclimation on gill morphology and respiratory gas transfer in the rainbow trout, *Oncorhynchus mykiss*.

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## ACKNOWLEDGEMENTS

I would like to take this opportunity to thank all the people who helped me accomplish the work necessary to complete this thesis.

Foremost on my list are my supervisors Dr James Fenwick and Dr Steve Perry, without whom this feat would not have been possible. Their support, advice, and willingness to help me whenever necessary is sincerely appreciated. Thanks!

My committee members Dr Tom Moon and Dr Ken Storey also deserve credit as they offered helpful ideas and criticisms throughout the years; along with Bea Valentine who patiently labored to cultivate my microscopy skills.

But what made this experience so unforgettable are all my fellow colleagues, comprised of both cubites and D'Iorioites. Special thanks to Kathleen Gilmour, Shawn Bindon, Steve Reid, Gary Sullivan, Adriana Gambarotta, Diana Otto, Marosh Furimsky, Jean-Michel Weber, Randy Fournier, François Haman, Tim Cano, Kathleen Stritch, Wendy Mackenzie, Nicholas Bernier, Stephen Dugan and Lars Juergensen, for offering encouragement and smiles when times were tough, for being great buddies, and for giving me an excuse to bake! Best of luck to you all!

Particular gratitude is extended to my family, Mom, Dad, and Nicholas. Thanks for your undying support and patience.

# THE EFFECTS OF SOFTWATER ACCLIMATION ON GILL MORPHOLOGY AND RESPIRATORY GAS TRANSFER IN THE RAINBOW TROUT, *ONCORHYNCHUS MYKISS*

## SUMMARY

Exposure of rainbow trout, *Oncorhynchus mykiss*, to ion-poor water or softwater elicits several morphological and physiological adaptations. This thesis examines these changes.

The effects of a naturally-induced chloride cell proliferation on gill morphology was investigated (Chapter 2) by acclimating fish to softwater ($[Na^+]$ = 0.055 mmol $l^{-1}$; $[Cl^-]$ = 0.029 mmol $l^{-1}$; $[Ca^{2+}]$ = 0.059 mmol $l^{-1}$; $[K^+]$ = 0.007 mmol $l^{-1}$). This study was performed to test the hypothesis that chloride cell proliferation associated with softwater exposure will cause an increase in the blood-to-water diffusion distance and thus impede gas transfer. Following 1, 2, and 4 weeks exposure to softwater, the results of scanning electron microscopy revealed a doubling of the chloride cell fractional area (CCFA: percentage of gill epithelium surface covered by chloride cells) due to both an increase in the number of chloride cells and the apical exposed surface area of individual chloride cells. The increase in fractional area was most pronounced after 2 weeks of acclimation. A concomitant increase in the blood-to-water diffusion distance was measured on lamellar transverse sections viewed utilizing transmission electron microscopy. Thus the chloride cell fractional area was positively correlated to the blood-to-water diffusion distance. The thickening of the lamellar epithelium also resulted in a reduction in the area of the inter-lamellar water channels, with the possible consequence of a decrease in ventilatory water flow.

Gill $O_2$ uptake, $CO_2$ excretion, ventilation and blood respiratory/acid-base variables were evaluated in control and softwater acclimated trout to test the hypothesis that gill chloride cell proliferation and increased blood-to-water diffusion distance, elicited by 2 weeks of softwater exposure, impairs the diffusion of respiratory gases across the gills (Chapter 3). The proliferation of chloride cells 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 $PO_2$ ($PaO_2$), $PCO_2$ ($PaCO_2$) and pH (pHa) using an extracorporeal blood circulation set-up under conditions of normoxia and graded hypoxia.

During normoxia, ventilation frequency and opercular displacement were significantly higher in the softwater trout; opercular displacement was similar in both groups. $PaCO_2$ and plasma $HCO_3^-$ concentrations 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 in the 2 groups. $CO_2$ excretion and $O_2$ uptake during normoxia were unaffected by acclimation to softwater.

During hypoxia the ventilation frequency and amplitude increased in the control trout, whereas only the ventilation amplitude increased in the softwater acclimated fish. The rate of $PaO_2$ reduction during hypoxia was significantly greater in the softwater fish and at the most severe level of hypoxia $PaO_2$ was significantly lower in the softwater fish. The rate of $PaCO_2$ reduction (caused by hyperventilation) was significantly lower in the softwater acclimated fish 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 pHa was statistically lower in the softwater acclimated fish.

This thesis demonstrated that respiratory gas transfer is impaired by chloride cell proliferation during hypoxia and that hyperventilation is a compensatory physiological adjustment for softwater fish. Further, the results of this thesis illustrate the compromises which occur when both ionoregulatory and respiratory functions of the gill are challenged simultaneously.

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## List of Abbreviations

| | |
|---|---|
| CC | chloride cell |
| CCFA | chloride cell fractional area |
| °C | degrees Celsius |
| DAP | dorsal aortic blood pressure |
| h | hour |
| kg | kilogram |
| kPa | kilopascal |
| μm | micron |
| MC | mucous cell |
| mg | milligram |
| mmol | millimoles |
| P | pillar cell |
| PC | pavement cell |
| pHa | arterial pH |
| $PaCO_2$ | arterial partial pressure of carbon dioxide |
| $PwO_2$ | water partial pressure of oxygen |
| $PaO_2$ | arterial partial pressure of oxygen |
| S.E.M. | standard error of the mean |
| SEM | scanning electron microscopy |
| τ | harmonic mean distance |
| TEM | transmission electron microscopy |
| $V_f$ | ventilation frequency |
| R.O. | reverse osmosis |

# CHAPTER 1

# GENERAL INTRODUCTION

The fish gill is a multifunctional structure that is involved in such diverse physiological functions as osmotic and ionic regulation (Avella and Bornancin, 1990), acid-base balance (Goss *et al.* 1992a, b) and gas exchange (Leino *et al.* 1987). Because of the remarkable capacity of these structures to integrate various functions and adjust them to the needs of the organism, there are teleost species which can inhabit a wide range of aquatic environments from softwater to full strength seawater and higher.

The gill epithelium forms a thin barrier which is located between two liquid compartments, the external water and the internal blood of the fish, and these compartments typically have very different ionic compositions. To perform its numerous roles, the teleost gill is very complex in organization and is composed of a variety of cell types, each with its own unique morphology suited to its specific role. Additionally, the various functions of the gill are linked to one another in such a way that a modification to the gill to enhance one aspect of function may affect another.

The large rounded chloride cells are primarily associated with ion transport but there is a link between this function and that of pH regulation. As a consequence of their thickness, chloride cells are inefficient sites for gas exchange. On the other hand, the expansive and thin-bodied pavement cells are ideally suited for the diffusion of respiratory gases. But the chloride and pavement cells must share the available surface area of the gill. Any adaptation which uses an increase in the apical surface area of the ion-transporting chloride cells to enhance ionic uptake could compromise gas transfer by simultaneously causing a thickening of the blood-to-water diffusion distance and consequently a reduction in diffusive capacity of the gill.

To test if such compromises occur, this thesis focused on the effects of a naturally induced chloride cell proliferation on gas transfer in the rainbow trout, *Oncorhynchus mykiss*. The hypothesis tested was that *the proliferation of chloride cells elicited by softwater acclimation will result in an increase in the blood-to-water diffusion distance of the lamellar epithelium and impair respiratory gas exchange.*

Softwater, a natural condition in which fish may find themselves in the wild, results in a compensatory proliferation of chloride cells (Laurent *et al.* 1985; Perry and Wood, 1985; Avella *et al.* 1987; Leino *et al.* 1987; Spry and Wood, 1989; Laurent and Hebibi, 1989), presumably to enhance ion uptake. This increase in number and size of the chloride cells was assessed using scanning electron microscopy (Chapter 2). Also, an ultrastructural analysis was performed using transmission electron microscopy, where the mean blood-to-water diffusion barrier width was measured (Chapter 2). The effects of this chloride cell proliferation on several aspects of respiratory physiology ($O_2$ uptake, $CO_2$ excretion, arterial blood $O_2$, $CO_2$ and pH levels, along with ventilatory parameters) were investigated in Chapter 3.

## THE GILL AND OSMOREGULATION

Teleost species are often faced with significant ionic changes in their aquatic environments, and fish which are able to iono- and osmo-regulate under these conditions are termed "euryhaline". In order to meet the demands for internal ionic homeostasis maintenance these fish have evolved mechanisms for adjustment which largely operate via the gills.

Freshwater fish are hyper-osmotic relative to their environment and are subject to the continuous influx of water together with the diffusive loss of ions. To partially compensate for these effects, fish adjust the permeability of their gills to water by increasing the concentration of prolactin in their systems. Prolactin secretion reduces the conductance of epithelial pathways and favors electrolyte retention (see review by Hirano, 1986). Freshwater fish drink little if any water. By producing a large volume of very dilute urine they are able to offset the substantial osmotic uptake of water. The urine flow volume is only slightly smaller than the total water entry across the body surfaces. To compensate for the ions lost in the urine, these fish absorb ions from their ambient medium by active uptake across the branchial epithelium.

Conversely, marine teleosts are hypo-osmotic to their environment and are faced with the opposite problems to that of freshwater fish. Marine fish experience an osmotic loss of water and a continual salt influx. To compensate, marine teleosts must ingest sufficient seawater to balance the osmotic loss of water across the gills. The water and salts are then absorbed from the gut correcting the water shortage but exacerbating the problems of ion loading. The kidney is, however, very efficient at excreting divalent ions but cannot excrete all of the excess monovalent ions. Thus an extrarenal excretion of $Na^+$ and $Cl^-$ must exist, and this occurs across the gills.

The branchial epithelium is made up of a mosaic of cell types: the chloride cells, the respiratory or pavement cells, and the mucous cells. Chloride cells were first described by Keys and Willmer (1932) in the branchial epithelium of seawater adapted eels and were referred to as "chloride secreting cells" by analogy with the oxyntic cells of the stomach.

These large, spherical, granular cells which comprise less than 10% of the total surface area of the gill epithelium of healthy freshwater fish, have been studied extensively (Evans, 1979; Karnaky, 1980, 1986; Girard and Payan, 1980; Laurent and Dunel, 1980; Laurent and Perry, 1990). In freshwater, chloride cells are normally found sparsely distributed on the filament, in inter-lamellar regions, and on the bases of the lamellae. Ultrastructurally, the chloride cell is characterized by a cytoplasm which has abundant, uniformly-spaced, mitochondria. It is believed that these supply the ATP which is necessary for the numerous ion pumps which are located in the invaginated membranes of the chloride cell ($Na^+/K^+$-ATPase (Philpott, 1980); $Ca^{2+}$-ATPase (see review by Fenwick, 1989)). Chloride cells also have an elaborate tubulovesicular reticulum which is continuous with the cell's basal and lateral plasmalemma (Laurent and Dunel, 1980). The large single nucleus spans the epithelium from the apical surface to the basal lamina of the chloride cell (Karnaky, 1980). Figure 1.1a depicts a representative transmission electron micrograph of a chloride cell. The chloride cell apical surface morphology is characterized by the presence of microvilli which serve to maximize the apical surface area of the cell exposed to the ambient water. Figure 1.2a shows a representative scanning electron micrograph of a chloride cell.

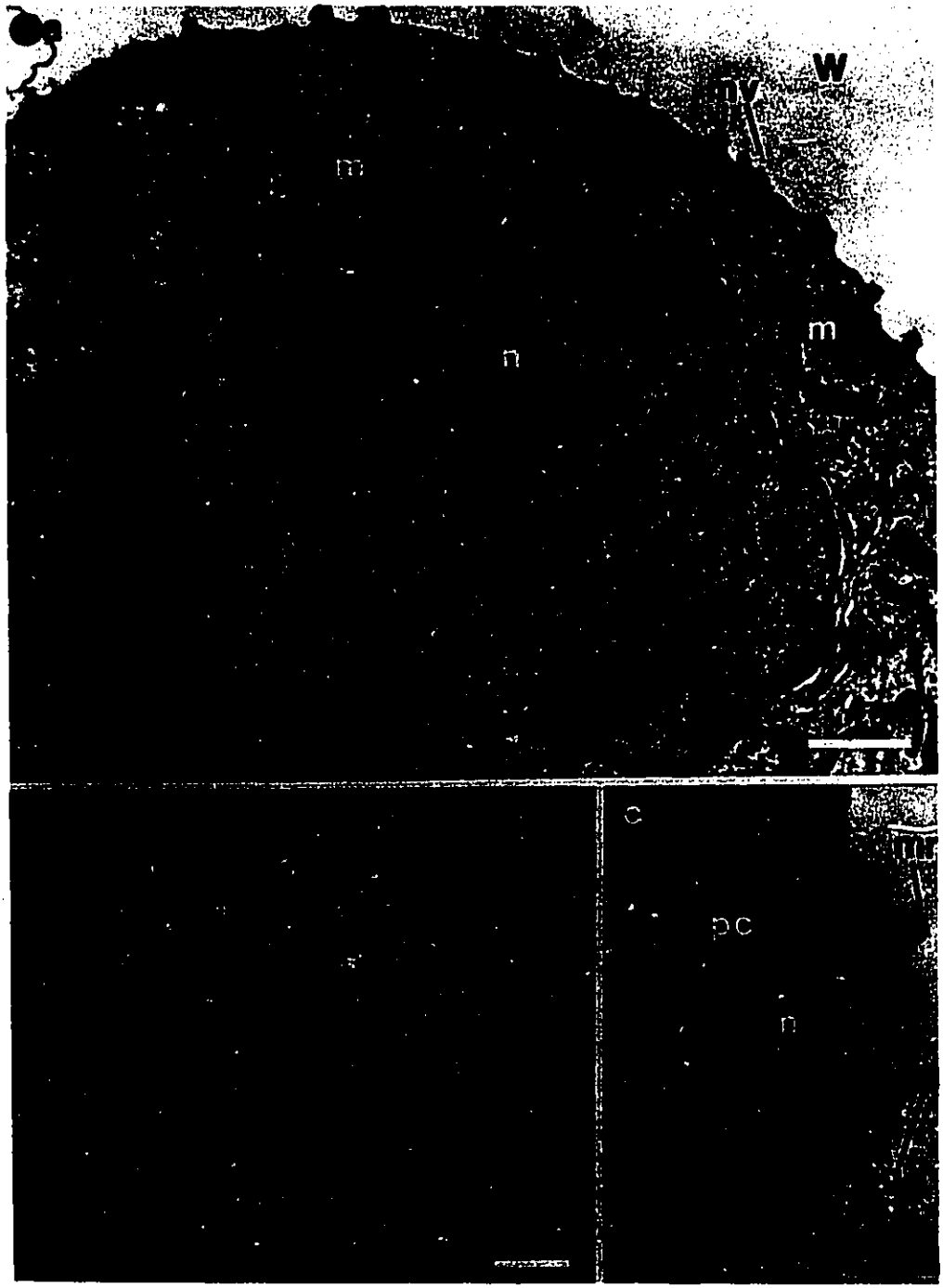
The pavement cell is the most abundant cell type in the gill epithelium and covers much of both the gill's lamellar and filamental surfaces. These cells form an extensive, thin, flat layer through which most of the respiratory gas exchange occurs ($CO_2$ and $O_2$). Ultrastructurally, these cells have a large, elongated nucleus (contributing to a particularly high nucleus/cytoplasm ratio), and few mitochondria (Figure 1.1c). The apical surface of

these pavement cells is characterized by an extensive maze of microridges which also probably serve to increase the gas exchange surface area of the gill.

The third main cell type is the mucous cell (Figures 1.1b, 1.2b, 1.2c). The mucous cell functions by secreting a polyanionic glycoprotein mucus coat over the surface of the epithelial cells. Mucus may impede diffusive ion loss from the fish to the environment and may actually assist active ion uptake by acting as an ion-concentrating layer (Handy, 1989; Perry and Laurent, 1993). This less prominent cell-type can be distinguished by intact mucus globules (Figure 1.2b), or if mucus has already been expelled, empty dark pits (Figure 1.2c).

On the basis of these characteristics, it is possible to readily discern one cell type from another and thereby quantify the surface area of the gill epithelium occupied by exposed chloride cells. This was accomplished in Chapter 2.

**Figure 1.1:** Transmission electron micrographs of trout gill epithelium showing cross-sections of the three main cell types: **a)** mitochondria (m) -rich chloride cell (CC) with an extensive network of tubules (t), large nucleus (n), vesicles (v), rough endoplasmic reticulum (rer), and microvilli (mv) in contact with the ambient water (w), × 20 000; **b)** a mucous cell (mc) filled with intact globules (g), × 18 000; **c)** a pavement cell (pc), showing its expansive nucleus (n) and microridges (mr) in contact with the external environment, glycocalyx (g), × 17 000, *bars*: 1 μm .


w
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c
pc
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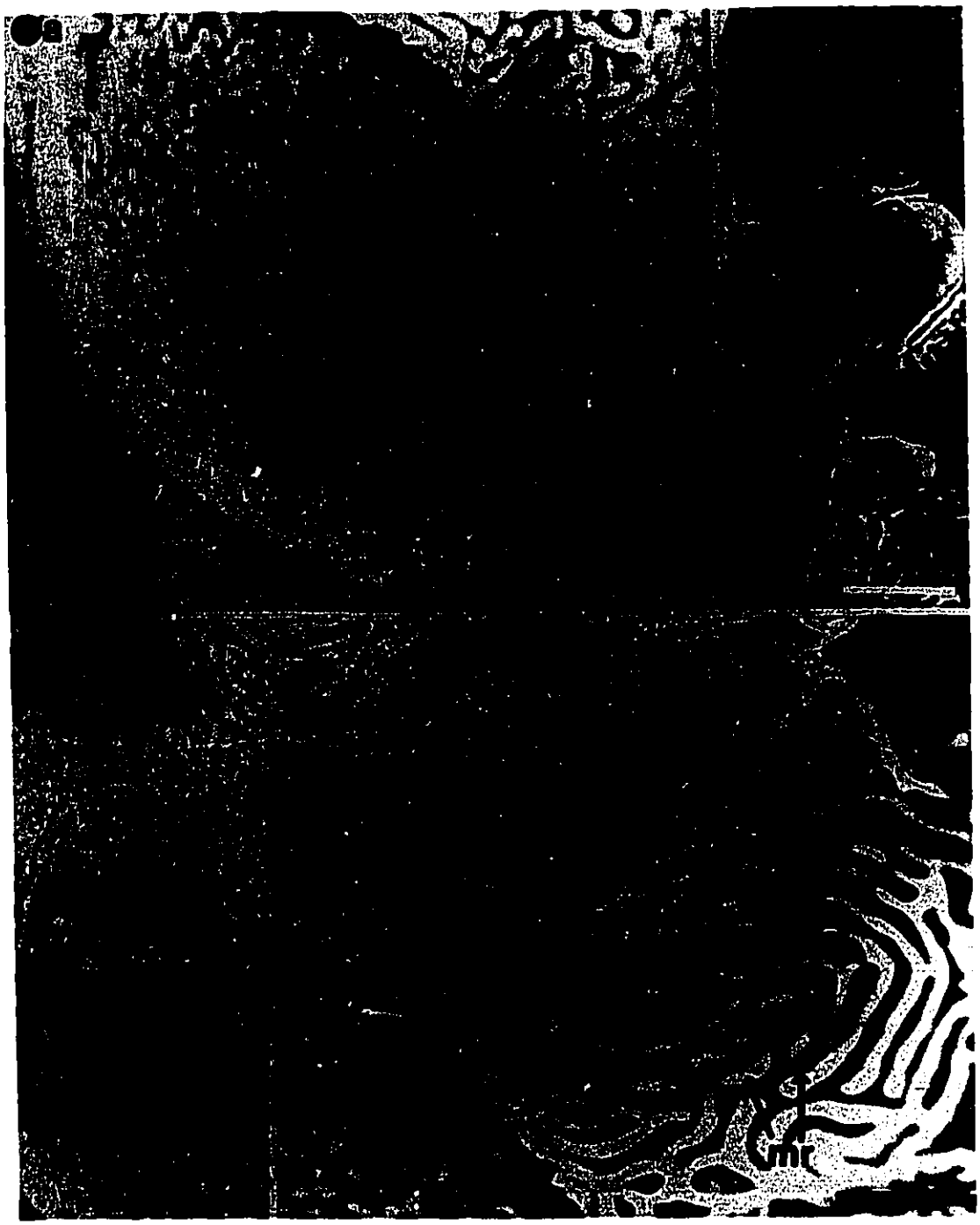
**Figure 1.2:** Scanning electron micrographs of trout gill epithelium showing surface morphology of the three main cell types: **a)** chloride cell (CC) seen with its projecting microvilli (mv), *bar*: 1 μm, × 11 000; **b)** mucous cell (mc) with intact globules (g) filled with mucus, *bar*: 5 μm, × 6 500; **c)** mucous cell which has expelled its globules of mucus (arrows indicate dark empty pits), *bar*: 5 μm, × 8 500; **d)** pavement cell (PC) with is maze-like array of microridges (mr), *bar*: 1 μm, × 13 000.



## ION REGULATION AND ACID-BASE BALANCE

It is clearly established that the chloride cell is responsible for much of the transepithelial calcium uptake in freshwater fish (Flik and Perry, 1989; Marshall *et al.* 1992, McCormick *et al.* 1992). $Ca^{2+}$ diffuses passively from the water into the cell via a calcium channel. When in the cell, $Ca^{2+}$ becomes bound to calcium-binding proteins which transport it to the basolateral membrane. $Ca^{2+}$-ATPases, located on this membrane, actively transport $Ca^{2+}$ against the electrochemical gradient existing between the cytoplasm of the chloride cell and the blood of the fish (Perry and Flik, 1988). The chloride cells are also responsible for branchial salt extrusion in saltwater fish. Indeed, Foskett (1987) provided direct evidence of this as the electrical current characteristic of salt transport was found to be localized over these cells in the killifish operculum epithelium.

The situation in freshwater regarding $Na^+$ and $Cl^-$ uptake, however, is currently debated. On the basis of correlation, several studies (Perry and Laurent, 1989; Laurent and Perry, 1990; Perry *et al.* 1992b) implicated the chloride cell in both $Na^+$ and $Cl^-$ uptake. Positive correlations exist between chloride cell surface area and the rates of $Na^+$ and $Cl^-$ uptake. As the surface area of the chloride cells increases, so too does access to the ion-transporting sites, enhancing the NaCl transporting capacity (Perry and Wood, 1985; McDonald and Rogano, 1986; Avella *et al.* 1987; Bindon *et al.* 1994a). Conversely, other studies (Goss *et al.* 1992a; Laurent *et al.* 1994; Morgan *et al.* 1994) suggest that the chloride cell is the site of $Cl^-$ uptake while the pavement cell is responsible for $Na^+$ uptake.

According to the first model for $Na^+$ and $Cl^-$ uptake, the apical membrane of the chloride cell has either a $Na^+/H^+$ ($NH_4^+$) exchanger (Wright and Wood, 1985) (where $Na^+$

enters the cell and is exchanged for an acidic equivalent), or an electrogenic $H^+$-pump (which uses ATP and excretes protons, contributing a negative charge inside the cell and facilitating a favorable movement of positive $Na^+$ ions into the cell through $Na^+$ channels) (Lin and Randall, 1991). Sodium is then pumped into the blood in exchange for $K^+$ via the $Na^+/K^+$-ATPase located on the basolateral membrane. $Cl^-/HCO_3^-$ exchangers are also found on the apical membrane of the chloride cell (Perry *et al.* 1981; Perry and Randall, 1981; Perry and Laurent, 1989) where chloride ions are taken up from the environment in exchange for basic equivalents ($HCO_3^-$).

The second model for $Na^+$ and $Cl^-$ uptake proposes the involvement of the pavement cell as well. It is suggested that the $Na^+/H^+(NH_4^+)$ exchanger or $H^+$- pump/$Na^+$ channel is located on the apical surface of the pavement cell. As in the previous model, the $Cl^-/HCO_3^-$ exchanger is found on the apical membrane of the chloride cell. It is obvious from these models that salt regulation and acid-base regulation are intimately linked. For example, if $Cl^-/HCO_3^-$ exchange is increased, blood pH will fall, becoming acidic; if the $Na^+/H^+$ exchangers or $H^+$ pumps/$Na^+$ channels are increased, the blood pH will increase, causing the blood to become alkaline. Alternatively, a pH drop will result in a stimulation of the $Na^+/H^+$ exchanger or $H^+$ pump/$Na^+$ channel activity and an inhibition of $Cl^-/HCO_3^-$ exchange to compensate; a pH rise will result in a stimulation of the $Cl^-/HCO_3^-$ exchanger and an inhibition of the work by the $Na^+/H^+$ exchanger or $H^+$ pump/$Na^+$ channel.

Morphological changes in chloride cells and pavement cells often occur during acid-base disturbances. During acidosis, the chloride cells become almost completely

covered over by pavement cells (Goss *et al.* 1992a) and an increase in $Na^+$ uptake is observed. This supports the suggestion that the $Na^+/H^+$ ($NH_4^+$) exchanger or $H^+$ pump/$Na^+$ channel is located on the pavement cells. More hydrogen pumps are needed to remove acidic equivalents from the blood. During alkalosis a huge increase in the surface area of the chloride cell occurs allowing an increased release of $HCO_3^-$ which accumulates in the blood. The pavement cells and thus the $Na^+/H^+$ exchangers or $H^+$ pumps/$Na^+$ channels are not as abundant and $Na^+$ uptake is reduced.

## GAS EXCHANGE AND THE GILL

The teleost gill is a gas exchange apparatus whose overall organization permits the exchange of $O_2$ and $CO_2$ between the cells and the surrounding milieu. The gill consists of four pairs of gill arches, each equipped with many lamellae which arise in a perpendicular direction from the upper and lower surfaces of the filaments. The gill surface area is 2 × as large as the external surface area of the rest of the fish (Hughes and Morgan, 1973) and this inevitably leads to exchanges between the ambient environment and the body tissues of the fish, including gaseous $CO_2$ and $O_2$ transfer. Fish gills, with their high surface to volume ratio and thin diffusive barrier separating the blood from the surrounding medium are ideal structures for respiration.

The lamellae are the most important units of the gill system with regards to gas exchange. Each lamella is made up of 2 epithelia which are separated by a series of pillar cells between which blood is free to flow. The direction of blood flow in the gills is opposite (countercurrent) to that of the water passing over the lamellae. This facilitates gas exchange by helping to maintain sufficient partial pressure differences across the

blood-to-water diffusion barrier so that maximal gas transfer may occur. Theoretically, the most efficient gas exchange possible could occur if complete equilibration of the arterial blood to inspired water and of the expired water to venous blood was accomplished. The capacity/rate for $O_2$ (flow times $O_2$ content) of blood perfusing the gills and for water flowing over the gills are almost equal, conditions in which a counter current arrangement of flow is most advantageous. Deoxygenated blood enters the lamellar capillaries and $O_2$ from the water passing over the gills diffuses in oxygenating it. The oxygen is then transferred to the tissues of the body via the blood.

The transfer of $O_2$ from the environment to the cells is completed in a series of steps as outlined by Perry and McDonald (1993): gill ventilation, branchial diffusion, blood oxygen transport and tissue diffusion.

The maintenance of water flow over the branchial surfaces of teleost fish is accomplished by the complex integration of buccal and opercular cavity muscle pumps (the buccal force pump and the opercular suction pump) which work out of phase. Water is drawn into the mouth, is forced over the gills and out through the opercular clefts. The volumes of the buccal and opercular cavities may be altered by raising and lowering the floor of the mouth (buccal cavity) and by moving the opercula (opercular cavities). A pressure differential is maintained across the gills with the pressure being higher in the buccal cavity compared to that in the opercular cavity thus ensuring a continual unidirectional pulsed flow of water between the gill arches (see review by Milsom, 1989). Also the rhythmic contractions of intrinsic musculature of the gill arches and filaments occurs to position the lamellae in an optimal orientation relative to the water flow stream.

Convection requirements for fish are 4-8 fold higher than for air-breathing vertebrates (Milsom, 1989) because air-saturated water has only about 1/30 of the $O_2$ content of air. There is also a higher cost of breathing in aquatic vertebrates due to the higher viscosity and mass of water compared to air. Ventilatory flow is determined by both the frequency and depth of breathing. It can be increased up to 10-15 fold in active species, but the cost of breathing at high ventilation rates may limit any gains in $O_2$ delivery (Perry and McDonald, 1993).

To circumvent these high costs, ventilation of the gills can also be associated with swimming movements in an activity known as "ram ventilation". In this situation the fish swims with its mouth open and as a result the water enters the mouth and leaves via the opercula. The work of ventilation is shifted from the ventilatory muscles to the locomotion muscles, almost eliminating the cost of ventilation.

The quantitative relationship between the morphometric features of the lamellar surface area and diffusion distance is expressed by Fick's equation:

$$Mx = \frac{(Kx)\,(A)\,(\Delta Px)}{D} \qquad (1.1)$$

where Mx is the rate of gas transfer for a particular gas (x); Kx is Krogh's constant of diffusion (or the permeation coefficient); A is the functional surface area over which gas exchange may take place; $\Delta Px$ is the difference in the partial pressure across the gas exchange barrier; and D is the mean blood-to-water diffusion distance across the epithelium. This equation illustrates how changes in gill morphology (in particular the barrier thickness and the lamellar surface area) can greatly affect gas transfer. Transfer of

$O_2$ across the gill surface is directly proportional to its area and inversely proportional to its thickness.

The actual value for the permeation coefficient (K) has not yet been determined for fish gill epithelia. $O_2$ diffuses much more slowly through tissues than water and the value of 1/3 $K_{water}$ is a reasonable estimate for the fish gill (Randall and Daxboeck, 1984). It is also possible that this K value is different for the various components of the barrier.

A proliferation of large chloride cells on the epithelium of lamellae would be a greater barrier to diffusion than the thin pavement cells. In this thesis, it is hypothesized that a proliferation of chloride cells will cause an increase in the blood-to-water diffusion distance (D),which will result in a decrease in the rate of gas transfer (Mx) according to Fick's law.

Gill diffusion distance not only varies considerably with species, but also within the gill apparatus of a single animal (Hughes and Morgan, 1973). Thus a mean must be measured for the diffusion distance. An appropriate measuring technique using transmission electron microscopy was established by Weibel and Knight (1964) and originally was used to assess the mean harmonic distance of the mammalian lung.

The fish gill is not uniformly perfused with blood or ventilated with water at any one time. In fact, at rest only about 60% of the lamellae (mainly basal and middle lamellar channels) of *Oncorhynchus mykiss* are perfused (Booth, 1978). But as ventilation increases, so too does the recruitment of water channels, and an increase in blood pressure and dilation of lamellar arterioles leads to opening and recruitment of distal lamellae for blood flow (Perry and McDonald, 1993).Thus we should distinguish between total surface

area and functional surface area. The total surface area is the total area that can potentially be used for gas exchange (this parameter can be measured by morphometric studies), while the functional surface area is the portion of the gill actually perfused and ventilated.

Using previous work by Bindon *et al.* (1994a) as reference, it was accepted that the total lamellar surface area does not change with proliferation of chloride cells or with an increase in blood-to-water diffusion distance.

Bindon *et al.* (1994a,b) used chronic treatment with hormones (growth hormone and cortisol) to artificially induce a chloride cell proliferation and study the compromises on gas transport in the rainbow trout. A potential limitation in the study by Bindon *et al.* (1994a,b) was the possibility of non-specific metabolic effects associated with the use of pharmacological levels of hormones. Since hormones may have had other non-desirable effects, this present study was designed to test if chloride cell proliferation resulting from the normal stress of softwater exposure would cause the same response. In this natural progression of the above pharmacological study, the well-documented natural condition of soft-water exposure was utilized to elicit a branchial chloride cell proliferation (Leino et al. 1987; Perry and Wood, 1985; Spry and Wood, 1988; Perry and Laurent, 1989; McDonald and Rogano, 1986) instead of hormonal injections.

A study by Thomas *et al.* (1988) examined the effects of softwater acclimation on gas transfer in the rainbow trout. Using light microscopy, chloride cell proliferation was evident. Also, these softwater fish experienced a lower $PaO_2$ than control fish at the same water $PO_2$. The softwater trout were more sensitive to hypoxia. Although a detailed

morphological analysis was not performed, they speculated that this chloride cell proliferation resulted in an increased blood-to-water diffusion distance resulting in a reduced diffusibility of the gill. In contrast, a study by Laurent and Hebibi (1989) reported that chloride cell proliferation induced by ion-poor water somehow caused a thinning of the blood-to-water diffusion barrier.

In an attempt to resolve these conflicting results, a thorough examination was undertaken in this thesis to determine the effects of a naturally-induced chloride cell proliferation on gill morphology with particular emphasis on the blood-to-water diffusion distance. Also the effects of these gill morphological changes on respiration were examined, as several physiological responses to hypoxia were investigated.

# CHAPTER 2

# THE EFFECTS OF SOFTWATER ACCLIMATION ON GILL MORPHOLOGY IN THE RAINBOW TROUT, *ONCORHYNCHUS MYKISS*.

## INTRODUCTION

As described in Chapter 1, the teleost fish gill separates the ambient water outside from the blood inside by an expansive epithelial barrier comprised principally of three cell types: pavement cells, mucous cells, and chloride cells (reviewed by Laurent and Perry, 1991; Perry and Laurent, 1993).

Previous studies in both this and other laboratories (Perry and Wood, 1985; Spry and Wood, 1988; Thomas *et al.*, 1988; Laurent and Hebibi, 1989; Perry and Laurent, 1989) showed that various ionoregulatory stresses induced a significant increase in the relative proportion of chloride cells on all gill epithelial surfaces and in particular the lamellar surfaces. These chloride cells are substantially thicker than the pavement cells and intuitively pose a greater barrier to gas diffusion. Thus, chloride cell proliferation is likely to cause an increase in the average water-to-blood diffusion distance. Should this occur, compensatory adjustments to ion stress, adjustments which take the form of increased size or number of chloride cells, might at the same time jeopardize the gas transfer function of the gills.

Indeed, it was recently shown in this laboratory (Bindon *et al.*, 1994a, b), that when the chloride cell density and relative surface area are increased by chronic injections of hormones (growth hormone, cortisol), the average blood-to-water diffusion distance increased and that this was associated with a decreased ability of trout to maintain normal blood $PO_2$ levels during hypoxia. That study, however, involved the artificial induction of chloride cell proliferation by the use of injected hormones. In contrast, Laurent and Hebibi

(1989) reported that chloride cell proliferation induced by ion-poor water, caused a reduction of the blood-to-water diffusion distance.

In light of these conflicting results, the goal of this chapter was to conduct a thorough investigation of the effects of naturally-induced chloride cell proliferation on gill morphology with particular emphasis on the blood-to-water diffusion distance. Specifically, we tested the hypothesis that exposure of trout to ion deficient water would induce substantial chloride cell proliferation and that this would be associated with a thickening of the blood-to-water diffusion barrier.

# MATERIALS AND METHODS

*Experimental Animals*

Rainbow trout (*Oncorhynchus mykiss*) of both sexes weighing between 209 g and 495 g (mean weight = 323.1 ± 13.3 g, experimental n = 24) were purchased from Linwood Acres Trout Farm (Campbellcroft, Ontario) 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 (refer to Table 2.1 for water chemistry). The photoperiod was kept constant at 12h light:12h dark. Fish were acclimated to these conditions for at least 5 weeks before any experiments were performed and they were fed to satiation daily with a dried commercial trout diet (Purina Trout Chow).

*Protocol*

Fish were divided among two large fibreglass tanks (Living Stream; Toledo, Ohio). The first tank (control group, n = 12) was supplied with running dechlorinated tapwater. The second tank (softwater acclimated group, n = 12) was supplied with running reverse osmosis (R.O.) water mixed with dechlorinated tapwater at an approximate ratio of 80% R.O. and 20% tapwater (refer to Table 2.1 for water chemistry). Total water flow rates for the tanks were adjusted to 5 litres/min. The experimental group of fish was exposed to the softwater condition by gradually increasing the proportion of R.O. water over a period of 3 days until the final conditions were met (30% R.O:70% tapwater, 60% R.O:40% tapwater, 80% R.O: 20% tapwater). The third day was recorded as day 1 of softwater exposure. 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). At 1, 2, and 4 weeks, fish were killed by spinal transection, a method which minimizes mucus secretion onto the gills (M.D. Powell, unpublished data). The gills were excised and processed for morphological examination. The central portion of the second gill arch (left side) was reserved for the scanning electron microscopy (SEM) study, while the central portion of the second gill arch (right side) was reserved for the transmission electron microscopy (TEM) study.

*Tissue Fixation*

A standard fixation procedure was used to prepare gill tissue for SEM and TEM. Small pieces of gill tissue were excised and quickly rinsed in ice-cold 0.15 M sodium cacodylate buffer (pH 7.4) to remove any excess mucus and blood. They were then fixed using 5% glutaraldehyde in 0.15 M sodium cacodylate buffer (osmotic pressure of final fixative = 292 mOsm) at 4° C for 1 h, rinsed three times in buffer and then post-fixed at room temperature for 1 h (1% osmium tetroxide in distilled water). Glutaraldehyde was used instead of formaldehyde because Mazzone *et al.* (1980) demonstrated that glutaraldehyde causes less shrinkage of respiratory tissue.

*Blood-to-Water Diffusion Distance*

The central portion of the gill was removed from the gill arch tissue and fixed in glutaraldehyde, as described above. The filament pairs were then isolated and separated at the septum prior to post-fixation. Subsequent to dehydration in an ethanol series (40, 70, 80, 95, 2 × 100%), a portion of the filament was removed. The filament pieces (each containing about 20 lamellae) were cut parallel to the lamellae near the septum and about

1 mm towards the distal end. The gill pieces were immersed in a propylene-oxide bath (2 × 15 min). Araldite infiltration was accomplished by exposing the gills to 33% araldite:67% propylene oxide (1 h), 50% araldite:50% propylene oxide (1 h), 70% araldite:30% propylene oxide (overnight) and 100% araldite (8 h). The filament pieces were embedded in flat embedding molds in fresh araldite. They were then oriented so that the lamellae would be cross-sectioned when cut. Fourteen sample blocks were made per fish. The araldite was allowed to harden slowly (24 h at 25° C, 40 h at 60° C).

Five blocks were selected at random per fish and trimmed under a stereomicroscope. Ultrathin (50 - 70 nm) cross-sections were cut across the middle regions of the lamellae. Of these, 10 sections were picked randomly (10 per block; 50 per fish) and mounted on 200-mesh copper grids. Grids were stained with lead citrate and saturated uranyl acetate before being examined under TEM (Philips 500, Amsterdam). Twenty-five randomly selected lamellar regions were photographed per fish (5 photographs per block; 5 blocks per fish), to yield micrographs similar to those shown in Figure 8. Blood-to-water diffusion distance was determined by randomly placing a circular grid (Weibel and Knight, 1964) of equidistant parallel lines over each of these micrographs (Laurent and Hebibi, 1989). A digitizer tablet (Sigmascan, Jandel) was calibrated to account for magnification. Measurement of the intercept length ($l_h$) of several lines randomly crossing the blood space and water were taken. This grid was superimposed ten times per picture and approximately 1200 measurements were made per fish. The harmonic mean blood-to-water barrier thickness ($\tau_h$) was calculated using the equation:

(2.1) $$\tau_h = 2/3\ (l_h)$$

*Epithelial Surface Investigation*

Chloride cell fractional area (percentage of gill epithelium covered by exposed chloride cells), chloride cell density (numbers of chloride cells with apical surfaces exposed to the water) and chloride cell size (surface area of exposed chloride cell apical membranes) were analyzed using SEM.

Pairs of filaments, still attached at the septum, were separated from the gill arch after fixation (as above). Each filament pair, was dehydrated in ethanol, bathed twice in 1,1,1,3,3,3-hexylmethyldisilizan (Aldrich) and air-dried. This method is as acceptable as critical point drying for SEM (Laurent and Hebibi, 1989). Pairs of filaments were adhered with silver paint to SEM specimen stubs suitable for use on a Philips 500 scanning electron microscope. The tissue was oriented in such a way that the lateral side of the filaments were parallel to the face plane of the stub plate. One photograph from each anterior and posterior filament was taken (total of 10 per fish). At approximately 1000 X magnification the microscope was focused on the trailing edge of the filament epithelium close to where the lamellae meet the filament and about 10 lamellae distal from the septum. This location was chosen as it is the same location from which the TEM results were derived. The apical chloride cell area was measured by tracing the chloride cell perimeters (from the micrographs) onto a calibrated digitizer tablet connected to a microcomputer using commercial software (Sigma Scan, Jandel). Chloride cell fractional area (CCFA) and cell density were determined using the following equations:

(2.2) $$\text{CCFA} = \frac{\text{total area of whole and partial chloride cells}}{\text{picture area} \times 10^{-6}}$$

(2.3) chloride cell density = CCFA/average chloride cell area

*Water Analysis*

Water $[Na^+]$, $[Ca^{2+}]$, 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).

*Statistical Analysis*

Variability of the data is indicated by ± 1 standard error of the mean (S.E.M.). Results were statistically analyzed using 2-sample T-tests between appropriate sample means; the fiducial limit was 5%.

**Table 2.1:** Water chemistry variables for the control (City of Ottawa tapwater), and softwater (artificial softwater) conditions that were utilized for trout acclimation studies.

| | City of Ottawa dechlorinated tap water (n = 28) | Artificial softwater (n = 28) |
|---|---|---|
| $[Ca^{2+}]$ (mmol $l^{-1}$) | 0.469 ± 0.009 | 0.059 ± 0.002 |
| $[Na^{+}]$ (mmol $l^{-1}$) | 0.154 ± 0.002 | 0.055 ± 0.002 |
| $[Cl^{-}]$ (mmol $l^{-1}$) | 0.149 ± 0.001 | 0.029 ± 0.002 |
| $[K^{+}]$ (mmol $l^{-1}$) | 0.023 ± 0.001 | 0.007 ± 0.001 |
| pH | 6.8 | 6.7 |
| temperature (°C) | 9.5 | 10.5 |

Values are means ± 1 SEM.

# RESULTS

*Epithelial Surface Morphology*

Three chloride cell surface morphometric parameters were measured (Figure 2.1). Chloride cell fractional area (CCFA; Figure 2.1A), average chloride cell area (Figure 2.1B), and chloride cell density (Figure 2.1C) were assessed in control and softwater-acclimated trout over a 4 week period. Chloride cell fractional area was significantly elevated by approximately 100% in the softwater acclimated fish at all sampling times (1, 2, and 4 weeks). This increase in chloride cell fractional area was caused by the combined effects of an increase in average chloride cell area (Figure 2.1B) and chloride cell density (Figure 2.1C); except at 2 weeks where chloride cell density was not significantly different from controls.

Representative scanning electron micrographs of control and softwater acclimated rainbow trout are shown in Figures 2.2 and 2.3. These micrographs convey the general morphological appearance of the control and softwater acclimated fish gill filamental and lamellar epithelia. Proliferation of chloride cells was observable over the entire surface of the gill epithelium: the lamellae (Figures 2.2c and 2.2d), the base of the lamellae (Figures 2.3a and 2.3b), and the filament (Figures 2.3c and 2.3d). The proliferation of chloride cells led to a pronounced thickening of the lamella and, in turn, this decreased the width of the interlamellar water channels in the softwater-exposed trout (arrows in Figures 2.2a and 2.2b).

*Blood-to-Water Diffusion Distance*

Figure 2.4 shows the effect of softwater acclimation on the mean harmonic blood-to- water diffusion distance ($\tau_h$). The thickness of the lamellar diffusion barrier increased significantly in trout acclimated to softwater when compared to controls at all time periods. Figure 2.5 shows two representative transmission electron micrographs that illustrate the difference in the blood-to-water diffusion distance between the control and softwater fish. Figure 2.6 shows a magnified view of the difference in barrier thicknesses between the two treatments. Clearly the proliferation of the voluminous chloride cells (which are found but sparsely in control fish) was the cause of the doubling of the diffusion barrier in the softwater trout.

The experimental protocol used in this study in which gill chloride cell fractional area and blood-to-water diffusion distance measurements were performed on the same fish permitted simple correlation analysis. The analysis demonstrated that lamellar blood-to-water diffusion distance for all three acclimation periods was significantly correlated ($r = 0.81$; $p < 0.05$) with chloride cell fractional area (Figure 2.7).

**Figure 2.1:** The temporal effects of softwater-acclimation (1, 2, and 4 weeks) on the surface morphometry of the rainbow trout gill: **a)** chloride cell fractional area; **b)** average chloride cell area; and **c)** chloride cell density. Values shown are means ± 1 S.E.M. (n = 4 for each group). * significantly different from control value ($p<0.05$).

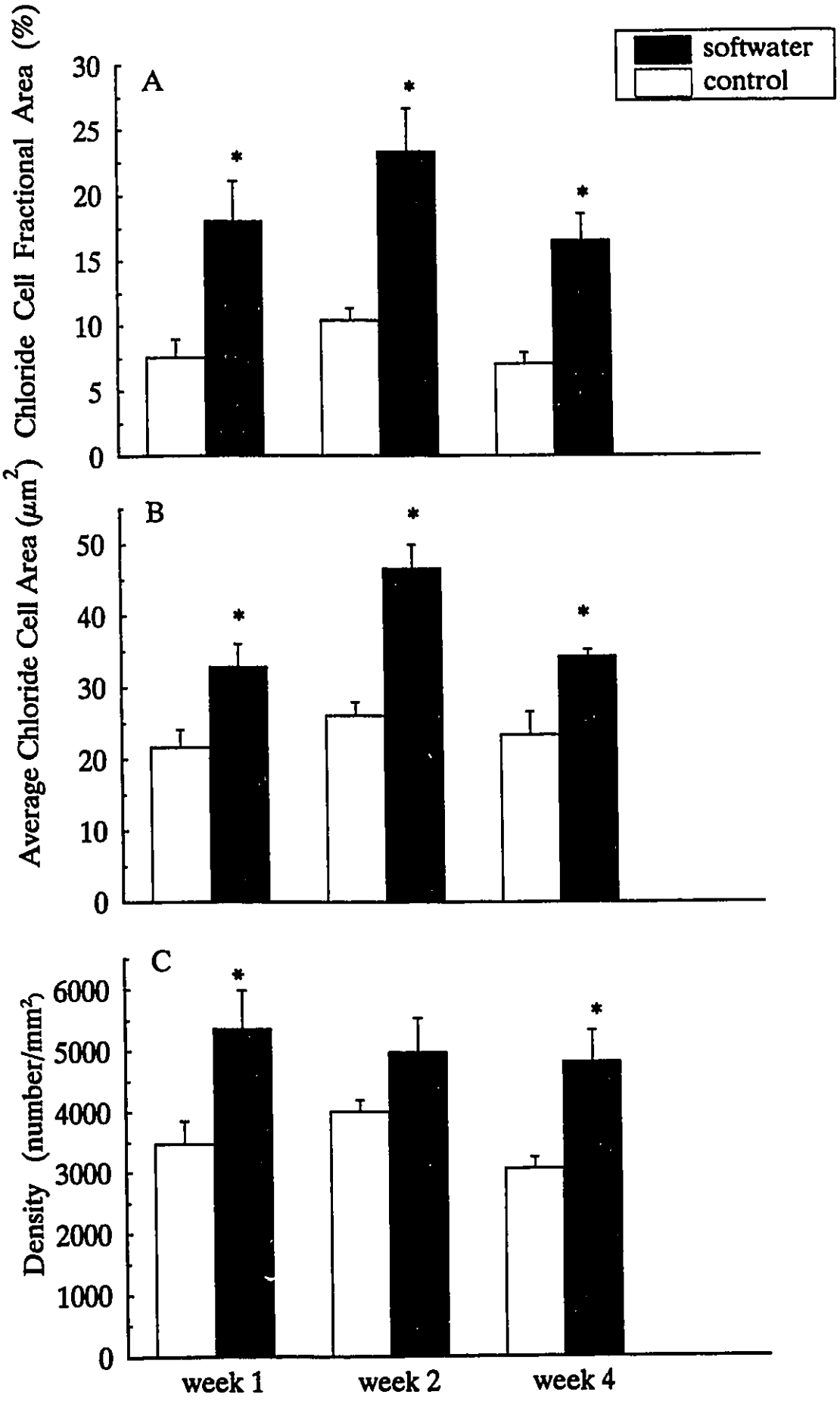

softwater
control
A
B
C
Chloride Cell Fractional Area (%)
Average Chloride Cell Area (μm²)
Density (number/mm²)
0
5
10
15
20
25
30
0
10
20
30
40
50
0
1000
2000
3000
4000
5000
6000
*
week 1
week 2
week 4

**Figure 2.2:** Representative scanning electron micrographs of trout gill lamellar epithelia under control conditions **(a, c)**, and after 2 weeks of softwater exposure **(b, d)** pc = pavement cell, cc = chloride cell, f = filament, *bars*: 10 μm. Observe the obvious proliferation of chloride cells on the lamellae, and the thickening of the lamellar base thus diminishing the width of water channel (arrows) between lamellae. For clarity, only a few chloride cells and pavement cells are labeled. (a, b: × 1 600; c,d: × 2 000).

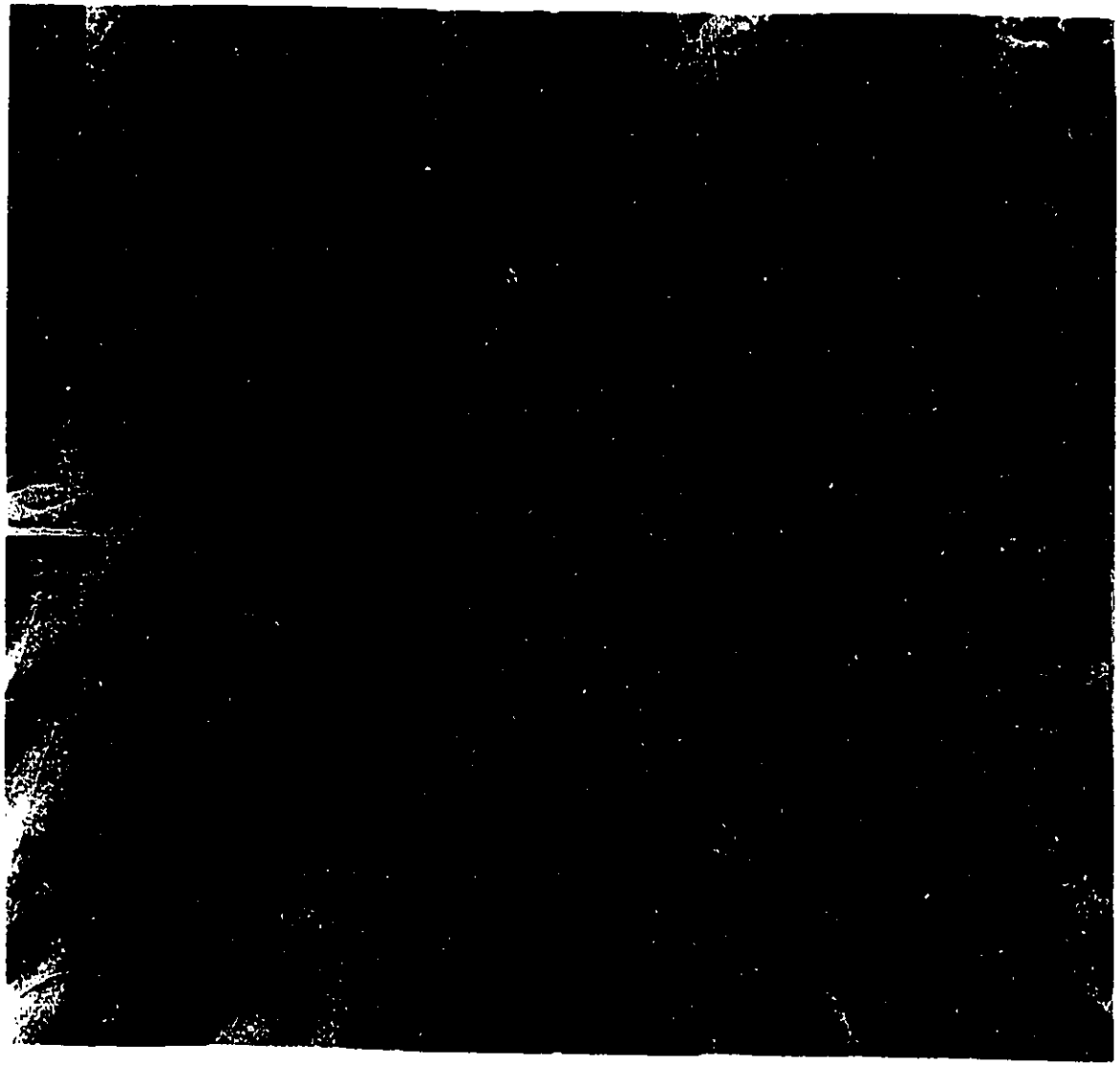


**Figure 2.3:** Representative scanning electron micrographs showing trout gill epithelia at the base of the lamellae **(a, b)** and on the filamental surface **(c, d)** under control conditions **(a, c)** and after 2 weeks of softwater exposure **(b,d)**; cc = chloride cell, pc = pavement cell, *bars*: 10 μm. Note how the changes in filamental chloride cell morphology are mirrored on the lamellae. (a, b: × 3 550; c, d: × 2 500).

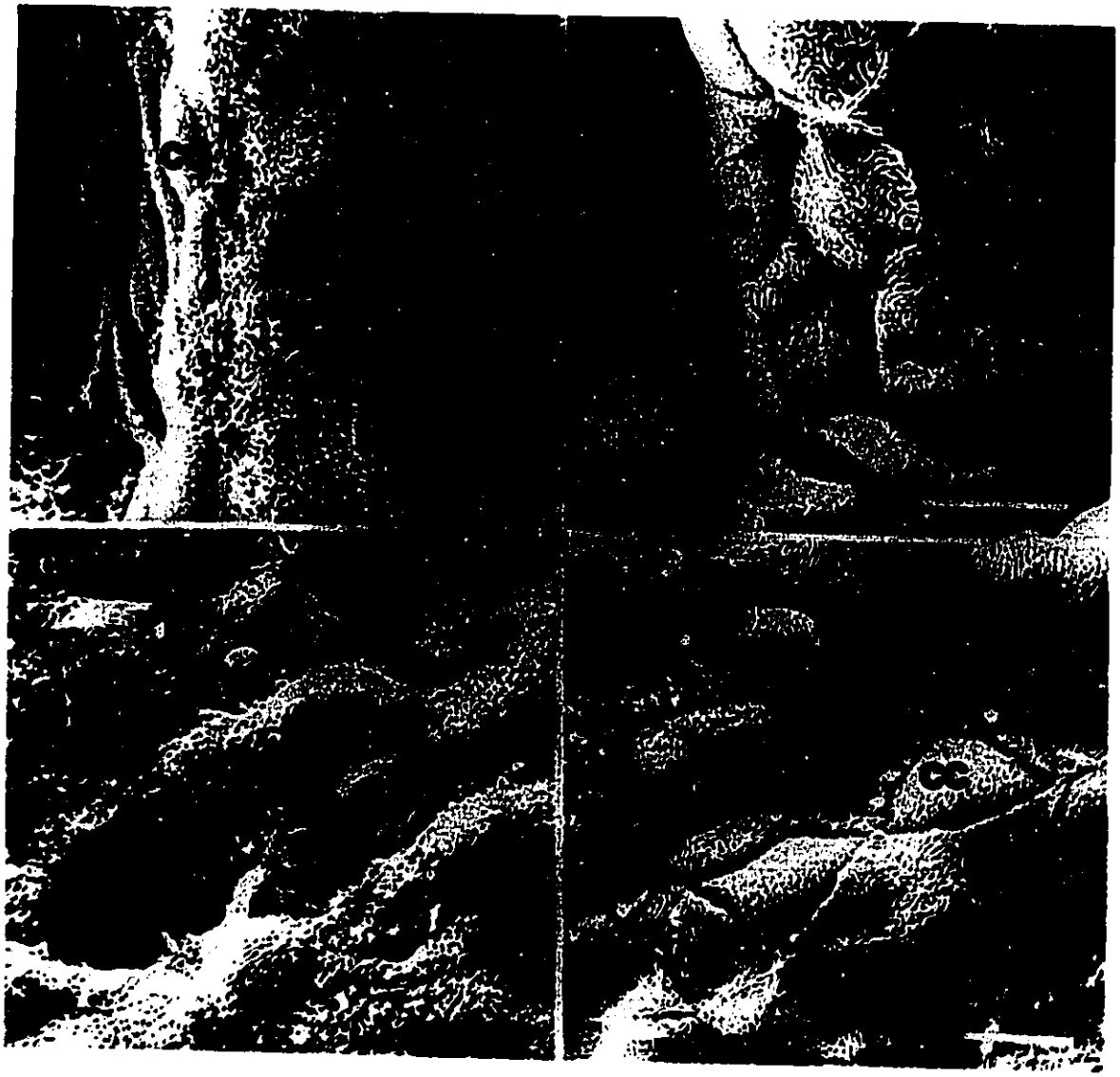


**Figure 2.4:** The temporal effects of softwater acclimation on blood-to-water diffusion distance ($\tau_h$). Values shown are means ± 1 S.E.M. (n = 4 for each group). * significantly different from control value ($p<0.05$).

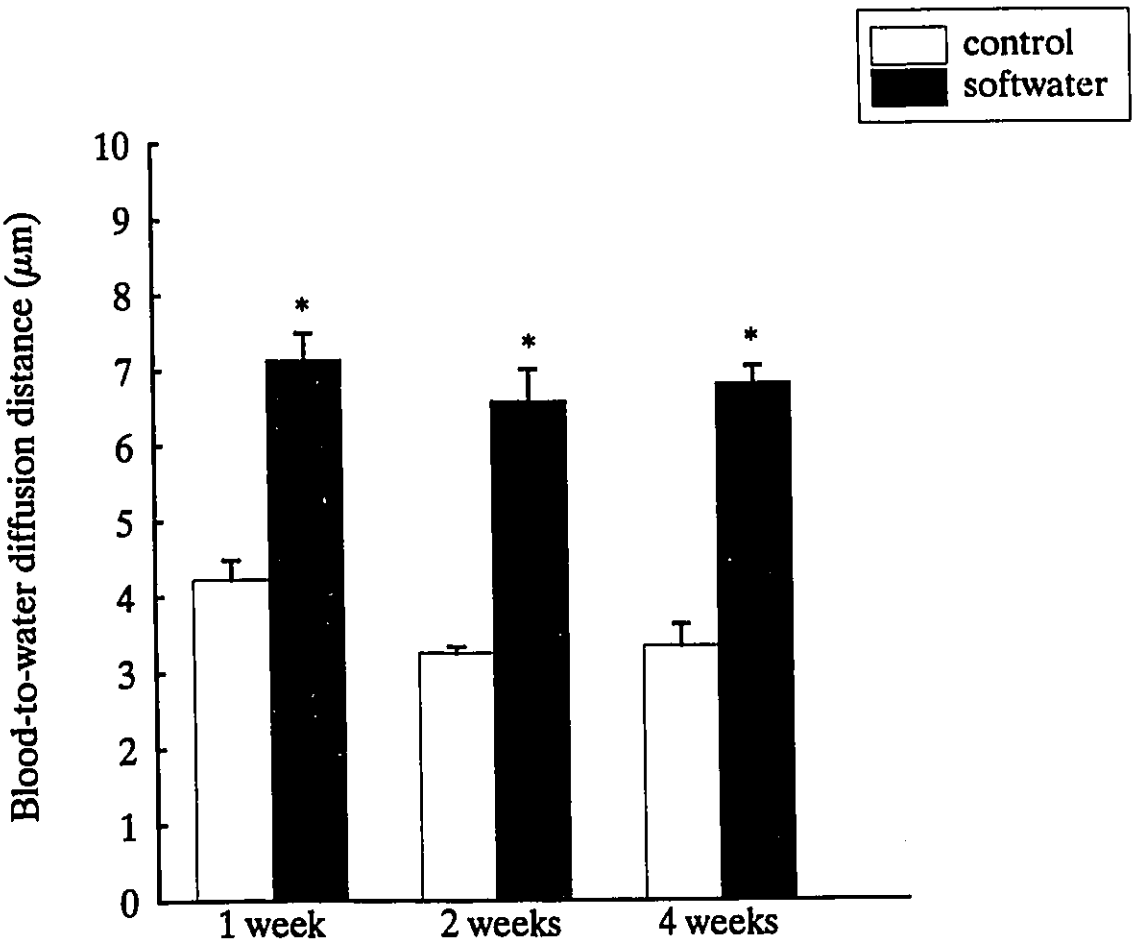

control
softwater
Blood-to-water diffusion distance (μm)
10
9
8
7
6
5
4
3
2
1
0
*
*
*
1 week
2 weeks
4 weeks

**Figure 2.5:** Representative transmission electron micrographs showing cross-sections of trout gill lamellae under control conditions **a)** and after 2 weeks exposure to softwater **b)**. Note the significant thickening of the gill epithelium after exposure to softwater, W = water, p = pillar cell, g = glycocalyx, pc = pavement cell, rbc = red blood cells, cc = chloride cells. The apparent difference in size of the blood channels in the two micrographs is solely a result of the particular plane of sectioning, *bars*: 1 μm. × 5 000.

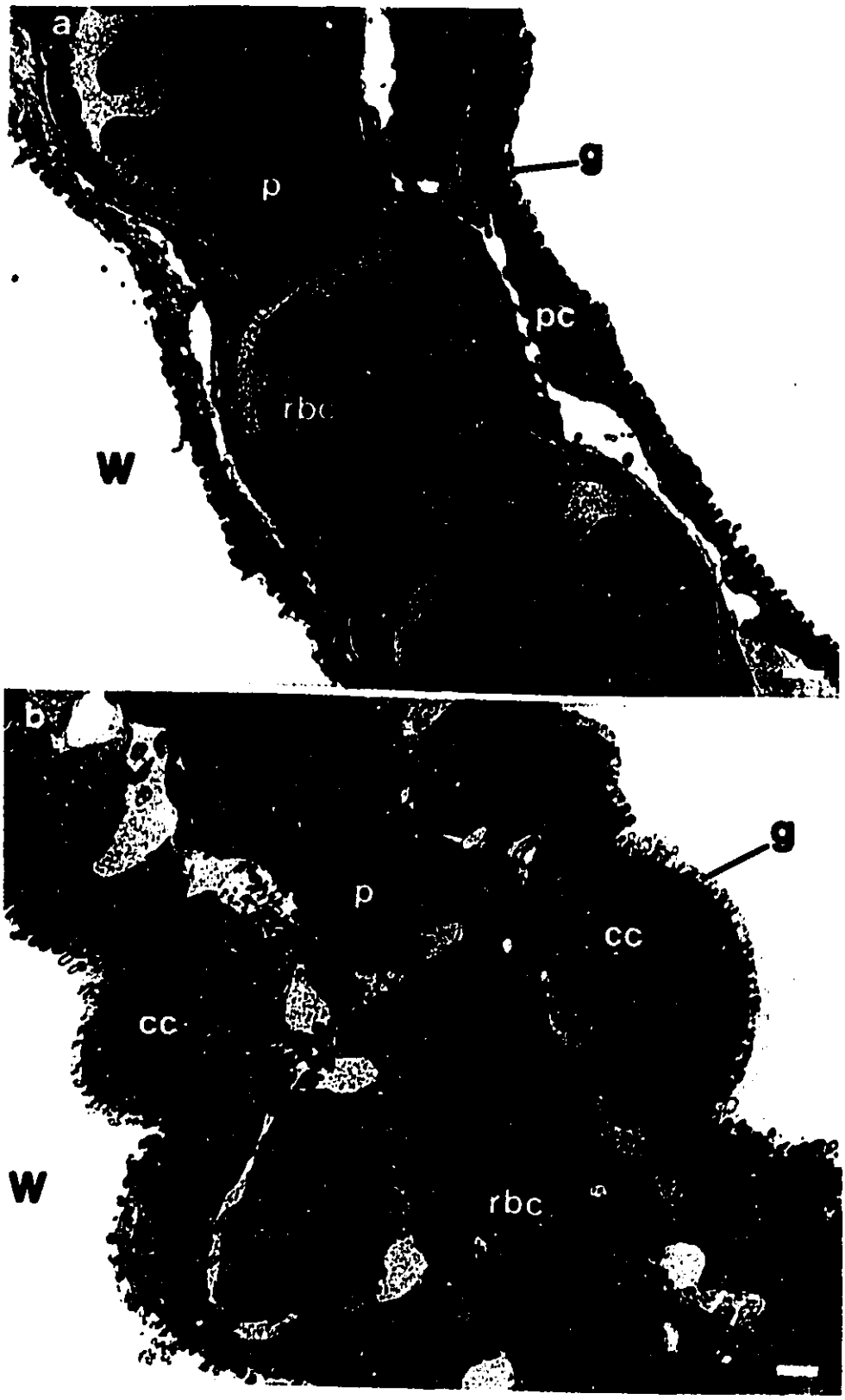

a
g
p
pc
rbc
w
b
g
p
cc
cc
w
rbc

**Figure 2.6:** Representative transmission electron micrographs of lamellar cross-sections in control **a)** and softwater acclimated trout **b).** The double-headed arrows represent possible diffusional paths of $O_2$ and $CO_2$ across the blood-to-water barrier. The path in the softwater trout **b)** is greater than in **a)** as can be explained by the presence of the thicker chloride cell (cc), as opposed to the thin pavement cell (pc) in a). W = water, rbc = red blood cell, p = pillar cell, m = mitochondria, rer = rough endoplasmic reticulum, *bars*:1 μm. × 16 500.

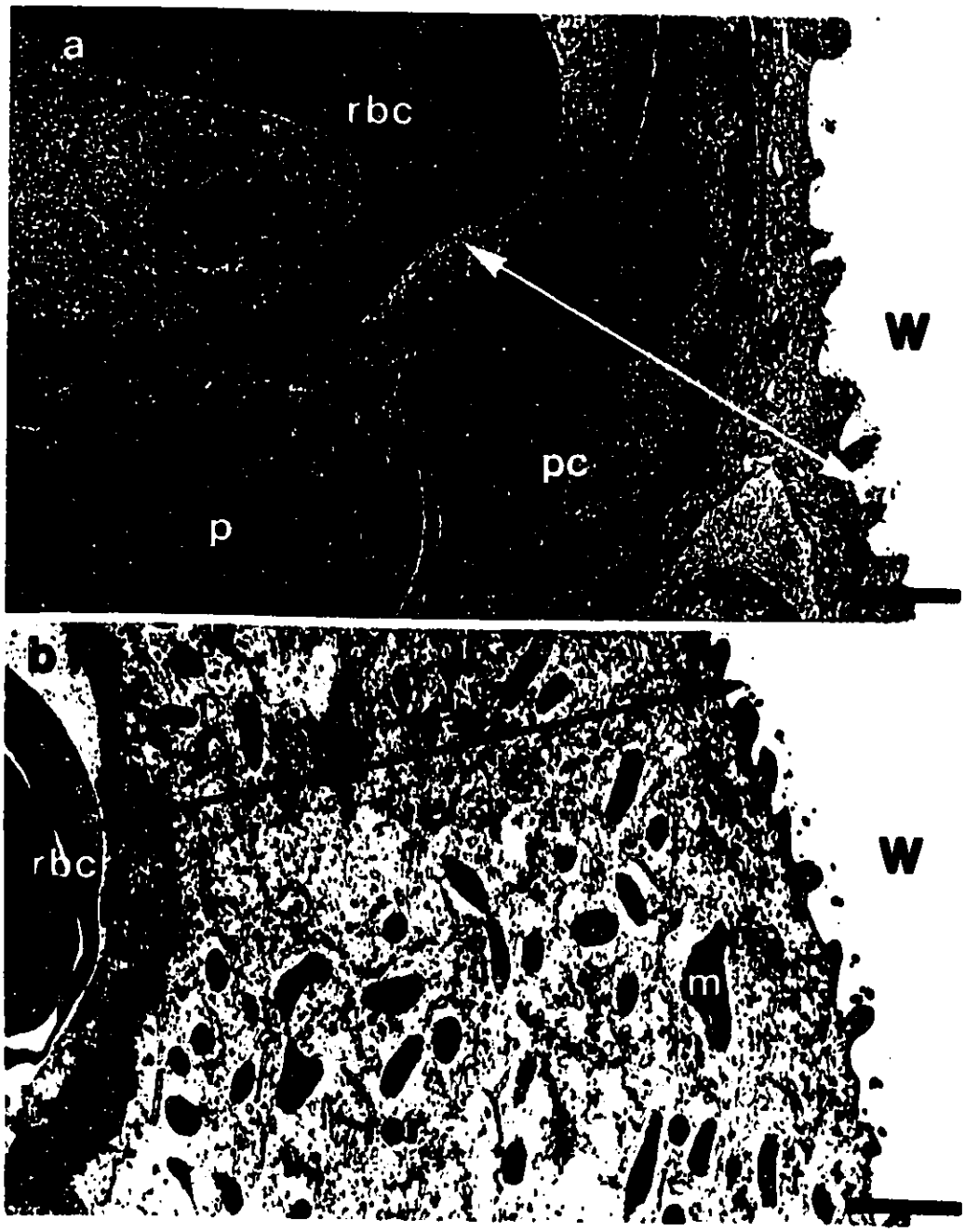
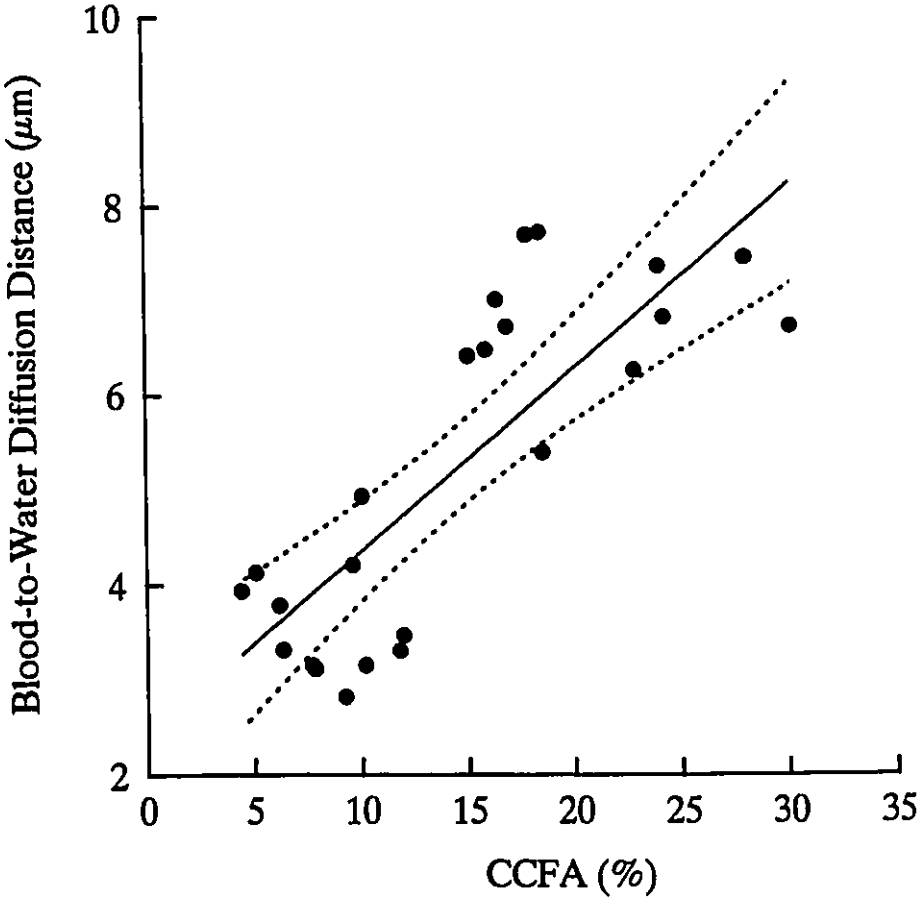

a
rbc
w
pc
p
b
rbc
w
m

**Figure 2.7:** Correlation between chloride cell fractional area (CCFA) and the blood-to-water diffusion distance, n = 24. Values shown are the individual data points obtained from control and softwater fish at all sampling times. R = 0.81; y = 0.19x + 2.42; dotted lines indicate 95% confidence interval.


Blood-to-Water Diffusion Distance (μm)
10
8
6
4
2
0
5
10
15
20
25
30
35
CCFA (%)

## DISCUSSION

Previous work in this and other laboratories has shown that acclimation of freshwater fish to water low in NaCl (Perry and Laurent, 1989), $Ca^{2+}$ (Perry and Wood, 1985), or to both (Laurent *et al.*, 1985; Avella *et al.*, 1987; Leino *et al.*, 1987; Spry and Wood, 1988; Laurent and Hebibi, 1989) causes pronounced morphological changes of the branchial chloride cells. The predominant response is chloride cell proliferation such that the lamellar surfaces, normally populated only sparsely with chloride cells, become inundated with these cells. Apparently fish use this response as an adaptive strategy to optimize ion uptake from the dilute environments. Owing to the dual role of the teleost gill in gas exchange and ionic regulation, such a strategy conceivably could negatively affect gas transfer. Thus, the goal of this study was to investigate the effects of exposure to an ion-deficient medium on chloride cell morphology with particular emphasis on the relationship between chloride cells and the blood-to-water diffusion distance.

The chloride cell fractional area (CCFA - percentage of gill epithelium covered by chloride cells) in control and softwater-acclimated fish was measured by scanning electron microscopy. Such a technique allows the observation of surface morphological structures. Thus the exposed cells, the sites that are believed to function in ionic uptake, are readily distinguished. The filament epithelium is flat, and because of this property it can easily be oriented parallel to the specimen support of the microscope and photographed without distortion. For this reason, the present study of chloride cell morphology was restricted to the filament. Even though gas transfer occurs predominantly over the lamellae, and not over the filament, the method is justified as Laurent and Perry (1990) reported that

increases in chloride cells on the filament were mirrored by similar increases on the lamellae. This is also clearly evident in the present results (Figures 2.4 and 2.5) which demonstrate the simultaneous proliferation of chloride cells on the filamental and lamellar epithelia.

Exposure of fish to ion-poor water elicits a variety of compensatory physiological adjustments. Wendelaar Bonga and van der Meij (1981) found that softwater exposure leads to increased prolactin secretion which reduces the conductance of epithelial paracellular pathways. This lowers the osmotic permeability of the gill and favors electrolyte retention (see review by Hirano, 1986). McDonald and Rogano (1986) reported reductions in gill ionic permeability. Specifically, there was a significant reduction of both $Na^+$ and $Cl^-$ efflux in rainbow trout during the first three days of exposure to softwater.

Positive correlations between chloride cell surface area and the rates of $Na^+$ and $Cl^-$ uptake exist (Perry and Laurent, 1989; Laurent and Perry, 1990; Perry *et al.*, 1992b). As the apical surface area of chloride cell increases, access to the ion-transporting sites on the chloride cell is improved and this is believed to enhance branchial NaCl transporting capacity (Perry and Wood, 1985; McDonald and Rogano, 1986; Avella *et al.* 1987; Bindon *et al.*, 1994a). Perry and Laurent (1989) reported that after 4 days exposure of rainbow trout to softwater there was a stimulation of $Na^+$ and $Cl^-$ transport which was accompanied by a 4-fold increase in plasma cortisol levels during the first 12 - 48 hours. Cortisol is implicated as a key hormone acting in the early stages of exposure to low [NaCl] water and is thought to mediate the compensatory proliferation of chloride cells.

Laurent and Perry (1990) reported that experimental treatment of trout with cortisol caused hypertrophy and a proliferation of chloride cells that was associated with the stimulation of $Cl^-$ and $Na^+$ uptake. Evidence has recently emerged showing that the pavement cell, not the chloride cell, is the site of $Na^+$ uptake (Morgan *et al.*, 1994; Laurent *et al.*, 1994). Further, Goss *et al.* (1992b) found that $Cl^-$ uptake was positively correlated to CCFA, but that $Na^+$ uptake was not. A possible explanation for this phenomenom is that $Cl^-$ uptake stimulation in the chloride cell may in turn trigger $Na^+$ uptake by the PC. Regardless, the proliferation of branchial chloride cells is an important physiological response of the teleost fish to ion-poor water.

*Chloride Cell Morphology and Gas Transfer*

In this study, the chloride cell proliferation induced by softwater acclimation caused a marked increase in the blood-to-water diffusion distance and also, by consequence, a narrowing of the interlamellar water channels (Figure 2.4b). Bindon *et al.* (1994a) reported a similar reduction in the width of interlamellar water channels associated with chloride cell proliferation induced by growth hormone and/or cortisol. Such reductions in inter-lamellar water channel area certainly would be expected to restrict the flow of ventilatory water near the lamellae. This narrowing, coupled with the increased diffusion barrier, would contribute to an impairment of $O_2$ uptake and $CO_2$ excretion. Indeed, during periods of increased ionoregulatory demand caused by softwater, Thomas *et al.* (1988) noted that these fish experienced a lower $PaO_2$ than controls at the same water $PO_2$. A loss of resistance to hypoxia in these softwater-acclimated rainbow trout could correspond to the decrease in the diffusing capacity

observed in this present study. In contrast to these results, Laurent and Hebibi (1989) reported a decrease in the blood-to-water diffusion distance in response to proliferation of chloride cells induced by softwater exposure. Given the protruding nature of lamellar chloride cells and the obvious positive correlations between chloride cell surface area and blood-to-water diffusion distance reported here and elsewhere (Bindon *et al.*, 1994 a), it is difficult to explain the surprising results of Laurent and Hebibi (1989). Indeed these authors themselves did not suggest a mechanism that could explain a thinning of the diffusion barrier concomitant with chloride cell proliferation. Thus, at present we are unable to reconcile the differences among the studies.

The thickening of the diffusion barrier and the apparent reduction of interlamellar channels associated with this naturally-occurring chloride cell proliferation would be expected to limit gas transfer in the absence of compensatory adjustments. Several such possible physiological adjustments include hyperventilation, increased cardiac output, an increase in blood $O_2$ carrying capacity, and an increase in Hb-$O_2$ binding affinity.

Further studies on physiological compensatory adjustments are warranted to directly address the impact of this softwater acclimation-induced chloride cell proliferation on gas exchange in the rainbow trout and form the basis for Chapter 3.

# CHAPTER 3

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

# INTRODUCTION

Acclimation of teleost fish to softwater 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 on the lamellar surfaces of the gill (Laurent *et al.* 1985; Perry and Wood, 1985; Leino *et al.* 1987; Spry and Wood, 1988; Perry and Laurent, 1989; Laurent and Hebibi, 1989) as seen in Chapter 2. Because of the presumed role of the chloride cell in trans-branchial $Ca^{2+}$ uptake (Perry and Flik, 1988; Marshall *et al.* 1992; McCormick *et al.* 1992; Perry *et al.* 1992a) and NaCl uptake (Perry and Laurent, 1989; Laurent and Perry, 1990; Perry *et al.* 1992b), this proliferation of chloride cells in softwater 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 chloride cells, caused either by exogenous cortisol/growth hormone treatment (Bindon *et al.* 1994a) or by softwater exposure (Chapter 2) 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.* (1994b) demonstrated experimentally, using pharmacological levels of hormones to elicit chloride cell proliferation, that the thickening of the diffusion barrier caused by the hormone

treatment was associated with an elevation of arterial blood $PCO_2$ ($PaCO_2$), and a reduction of $PaO_2$ during severe hypoxia.

The goal of the present study was to evaluate the consequences of naturally-induced chloride cell proliferation on respiratory gas transfer. This was achieved by exposing trout to softwater for a period of two weeks after which time the lamellar blood-to-water diffusion distance is known to be doubled (Chapter 2). Respiratory function along with ventilation parameters were investigated using an extracorporeal blood circulation setup under conditions of normoxia and graded hypoxia.

# MATERIALS AND METHODS

*Experimental Animals*

Rainbow trout (*Oncorhynchus mykiss*) of both sexes and weighing between 574 and 964 g (mean mass = 727 ± 30 g, experimental n = 15) were used. For more information, please refer to Chapter 2.

*Acclimation Protocol*

An identical acclimation protocol and care of experimental animals as that described in Chapter 2 was followed. For water chemistry, please refer to Table 3.1.

*Surgical Techniques*

After acclimation for two 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. Gills were irrigated continuously throughout the operation with oxygenated anaesthetic solution. The dorsal aorta was cannulated using flexible polyethylene tubing (Clay-Adams PE 50, ID = 0.580 mm, OD = 0.965 mm) as described by Soivo *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, cannulae were flushed with heparinized saline (50 units $ml^{-1}$ 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 Set-up*

Fish were held in black Perspex boxes with clear windows used for observing the interior of the box without 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. Persistant 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 $PO_2$ (Radiometer model E-5046), $PCO_2$ (Radiometer model E-5036) and pH electrodes (Metrohm combination electrode), which were attached to a Radiometer PHM-73. This external loop tubing was flushed with heparinized saline (540 IU $ml^{-1}$) 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 $PO_2$ ($PwO_2$) in the experimental chamber was measured by means of another Radiometer $PO_2$ electrode connected to a PHM-72 meter.

Calibration of the $PCO_2$ and $PO_2$ electrodes was achieved by equilibrating water with gas of the appropriate $PCO_2$ and $PO_2$ 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 ($MO_2$) and $CO_2$ excretion ($MCO_2$) were measured using closed system respirometry techniques (Holeton and Randall, 1967). Water flow to the fish box was halted and $PwO_2$ was monitored continuously until it had decreased by about 4 kPa. After each 1 kPa decrease in $PwO_2$, water samples (1.5 ml) were taken from the area close to the mouth of the fish, from the output of the $PwO_2$ electrode located in the fish box. Changes in $PwO_2$, mass of the fish and volume of the box, along with constants from Boutilier *et al.* (1984) were used for calculating $MO_2$. Total $CO_2$ (Cameron Capnicon Model 5) was measured on the withdrawn water samples and used to calculate $MCO_2$.

The extracorporeal setup (Figure 3.1) allowed the arterial oxygen partial pressure ($PaO_2$), the arterial carbon dioxide partial pressure ($PaCO_2$), the water oxygen partial pressure ($PwO_2$) and the arterial pH (pHa) to be monitored continuously during the experiment. Mean values for each parameter were captured and stored every 5 seconds.

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 $PwO_2$ levels at normoxia (20 kPa), a pre-hypoxia blood sample of 0.5 ml was taken for the measurement of hct, Hb, $CaO_2$, and plasma [ions].

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 $PwO_2$. The hypoxic period was imposed over a 20 min period with the $PwO_2$ being reduced at an approximate rate of 0.8 kPa $min^{-1}$ to an end value of 5.3 kPa.

*Morphology*

Chloride cell 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 (MS 222; 0.5 g $l^{-1}$), 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. Chloride cells have an intricate plasma membrane with many invaginations and thus stain strongly. After rinsing in distilled water for 6 h (changed 3 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 3 baths of xylene for 3 min each. Sections were then mounted in permount (BDH chemicals) before being viewed using a light microscope (40X 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 $PaCO_2$ and pHa values measured with the extracorporeal setup, 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 000g for 10 min. The remaining blood was centrifuged at 10 000g for 30 sec 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 analysis was performed on the mean data displayed at intervals of 1.3 kPa $PwO_2$ using one way ANOVA. At each point (i.e. at each 1.3 kPa change in $PwO_2$), a two sample t-test was performed to compare softwater to control fish. Also, linear regressions were performed and tested with t-tests to see if rates of change of blood and ventilation parameters with $PwO_2$ 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%.

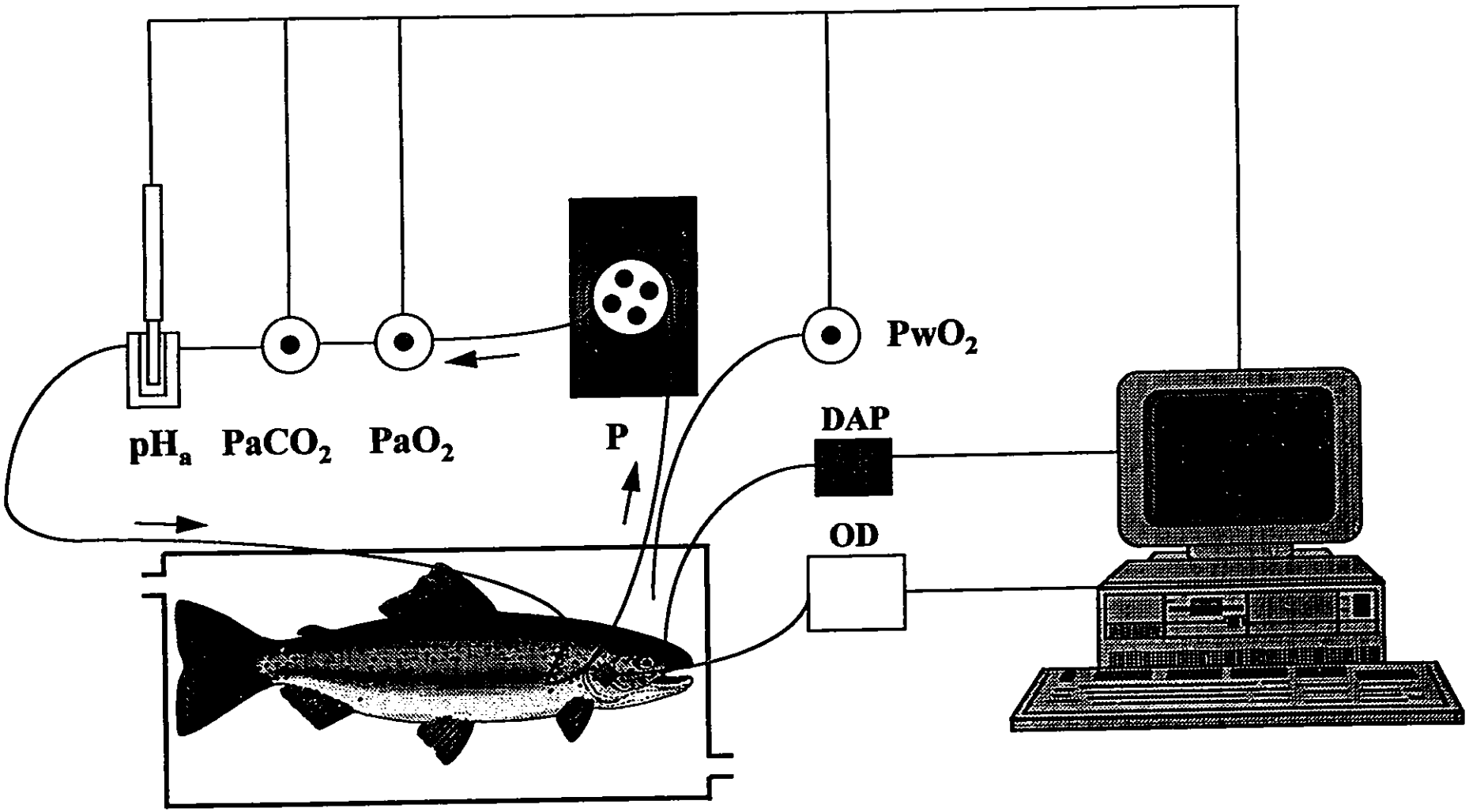
**Table 3.1:** Water chemistry variables for the control (City of Ottawa tapwater) and softwater (artificial softwater) conditions that were utilized for trout acclimation studies.

| | City of Ottawa dechlorinated tapwater | Artificial softwater |
|---|---|---|
| $[Ca^{2+}]$ (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) |
| $PwO_2$ (kPa) | 20.2 ± 0.3 (n = 7) | 20.2 ± 0.2 (n = 8) |
| $CwCO_2$ (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 SEM.

* shows significant differences from controls using 2 sample t-test, $p < 0.05$.

**FIGURE 3.1:** Diagram showing the extracorporeal blood circulation set-up. Blood is pumped from the celiac artery of the fish using a peristaltic pump (P). The blood then flows through an external loop and through several electrodes where $PaO_2$ (arterial partial pressure of oxygen); $PaCO_2$ (arterial partial pressure of carbon dioxide); and pHa (arterial pH) are measured continuously. DAP (dorsal aortic blood pressure), OD (opercular displacement), along with $PwO_2$ (water partial pressure of oxygen) are also measured. All data is collected by a computer data acquisition program.


$PwO_2$
$pH_a$
$PaCO_2$
$PaO_2$
P
DAP
OD

# RESULTS

*Gill Morphology*

Representative light micrographs of control (Figure 3.2A) and softwater exposed (Figure 3.2B) rainbow trout illustrate the general morphological appearance of the gill filaments and lamellae from the two groups. The black-stained chloride cells were clearly protruding from and proliferated over the entire surface of the gill epithelium of the softwater fish. Chloride cells 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 3.2). Table 3.3 shows the blood and gas transfer parameters measured immediately before the exposure to hypoxia. Ventilatory parameters were measured pre-respirometry. Mean red blood cell haemoglobin concentration (MCHC) was significantly lower in softwater trout compared to controls, indicating cell swelling. [$HCO_3^-$] in the softwater trout was significantly lower than that of controls and together with the reduced blood pH represented a base deficit (metabolic acid load) of approximately 3 mmol $l^{-1}$. $CO_2$ excretion and $O_2$ uptake were unaffected by acclimation to softwater. The gill respiratory exchange ratios ($R:MCO_2/MO_2$) showed large variability and were not statistically different. The ventilation frequency was significantly elevated in the softwater acclimated fish by 21 breaths/min (36% increase) compared to 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 $PwO_2$ values of 14.8 kPa and below (Figure 3.3A). The ventilation frequency in 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 (Figure 3.3B) demonstrated a significant difference in the rate of change of ventilation frequency during hypoxia between the control and softwater acclimated trout.

The effects of graded hypoxia on opercular displacement are shown in Figure 3.4A. 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 was imposed (6.7 kPa). Furthermore, a linear regression analysis (Figure 3.4B) revealed a significant difference in the rate of change of opercular displacement 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 $PaO_2$ are shown in Figure 3.5A. Both groups experienced significant decreases in $PaO_2$ compared to normoxia values at all levels of $PwO_2 \leq 16$ kPa. The softwater acclimated fish were able to maintain $PaO_2$ values comparable to the control fish, except at the lowest $PwO_2$ of 5.3 kPa, at which point the $PaO_2$ of the softwater trout was significantly lower than in the controls. Figure 3.5B illustrates the rate of change of $PaO_2$ during hypoxia and reveals that the slope of the relationship between $PaO_2$ and $PwO_2$ was significantly steeper in the softwater acclimated

trout, which is indicative of less efficient gas transfer in these fish. Figure 3.5C illustrates the relationship between $PwO_2$ and the difference between $PwO_2$ and $PaO_2$ ($PwO_2$ - $PaO_2$), providing an estimate of $O_2$ transfer efficiency during progressive hypoxia.

The absolute values of $PaCO_2$ were similar in control and softwater acclimated fish at all levels of hypoxia (Figure 3.6A). The rate of change of $PaCO_2$ during hypoxia was significantly less ($p < 0.06$) in the softwater acclimated fish than in the control fish ($0.002 \pm 0.001$ versus $0.0047 \pm 0.001$ kPa $kPa^{-1}$ $PwO_2$; Figure 3.6B).

Blood pH did not change significantly during hypoxia in either group (Figure 3.7A) but through much of the hypoxic period (7 - 14.8 kPa PwO2), pHa was statistically lower in the softwater acclimated fish. The regression analysis (Figure 3.7B) demonstrated that the rates of change of pHa in the two groups of fish were not different from one another during hypoxia.

**Table 3.2:** 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 significant difference from controls using 2 sample t-test, $p < 0.05$.

**Table 3.3:** Blood, ventilation, and gas exchange parameters 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) |
|---|---|---|
| Ht (%) | 22.8 ± 1.5 | 21.1 ± 1.9 |
| Hb (ml $100ml^{-1}$) | 9.14 ± 1.66 | 7.02 ± 0.88 |
| MCHC (g $ml^{-1}$) | 0.40 ± 0.03 | 0.30 ± 0.02* |
| $[O_2]$ (ml $100ml^{-1}$) | 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 |
| $MO_2$ (mmol $kg^{-1}$ $h^{-1}$) | 2.28 ± 0.23 | 5.10 ± 1.80 |
| $MCO_2$ (mmol $kg^{-1}$ $h^{-1}$) | 2.53 ± 0.52 | 2.78 ± 0.58 |
| R ($MCO_2/MO_2$) | 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 SEM.

* indicates significant differences from controls using 2 sample t-test, $p < 0.05$.

**FIGURE 3.2:** 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, which are stained black, have proliferated over the entire surface of the gill epithelium of the softwater fish. The chloride cells of the control fish are smaller and far less numerous. Scale bar: 15 μm.

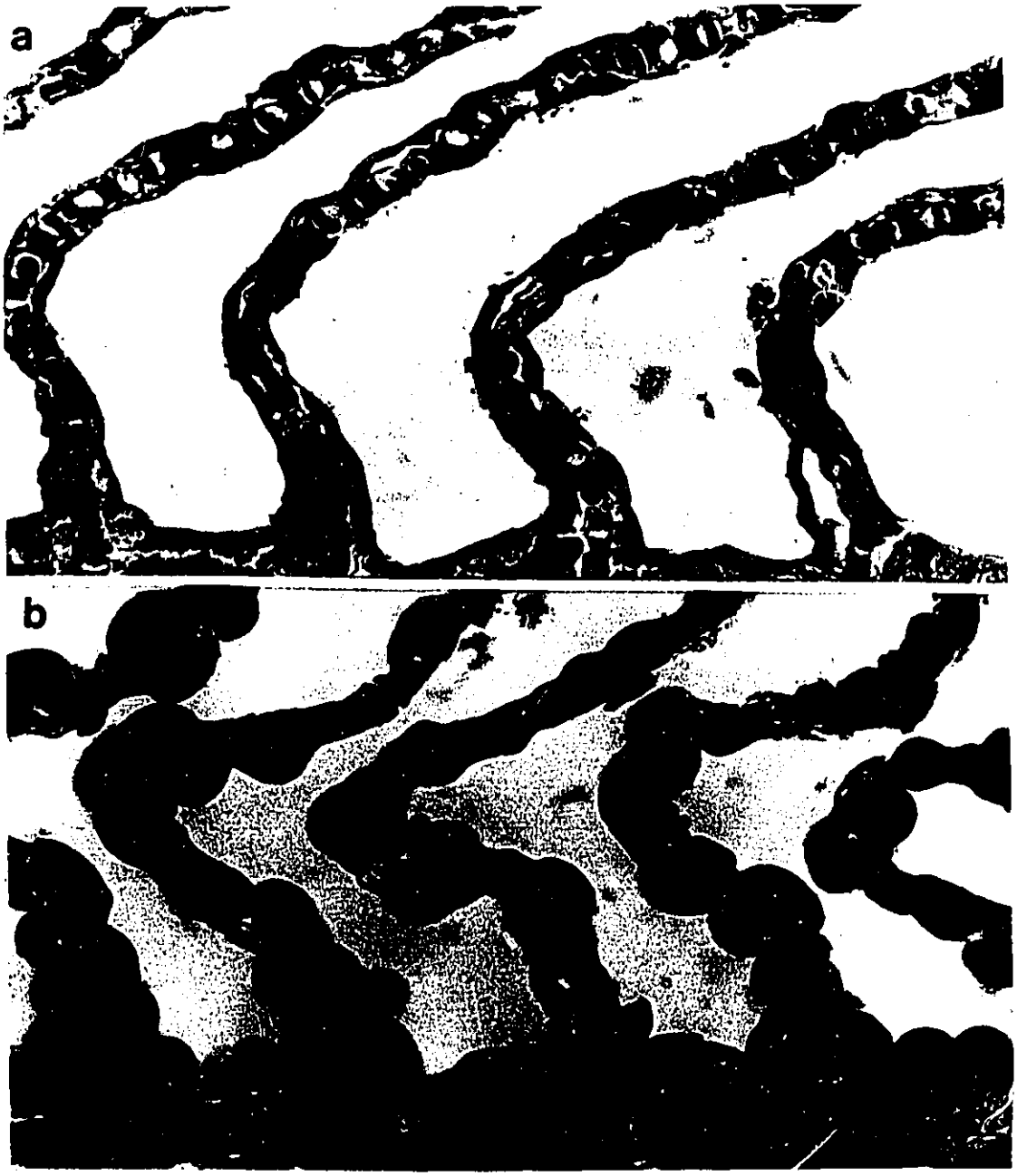
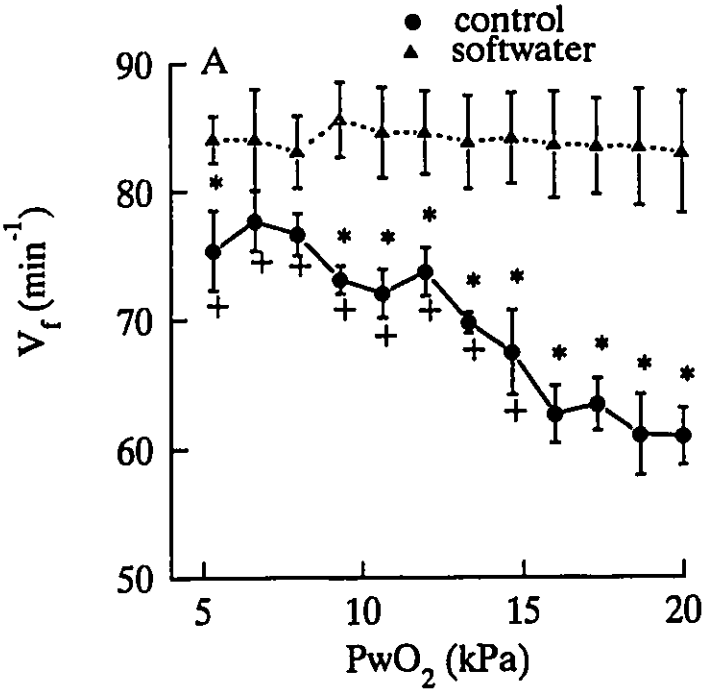

a
b

**FIGURE 3.3:** A) Effect of environmental hypoxia on ventilation frequency ($V_f$) in control (solid line; n = 7) and softwater acclimated (dotted line; n = 8) rainbow trout. + indicates a significant difference ($p < 0.05$) of $V_f$ from the values measured during normoxia (at $PwO_2$ = 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 $V_f$ and $PwO_2$ in the control fish (slope = -1.21 ± 0.15 breaths $kPa^{-1}$ $PwO_2$) and softwater acclimated rainbow trout (slope = -0.207 ± 0.056 breaths $kPa^{-1}$ $PwO_2$). # indicates a significant difference ($p < 0.05$) in the rate of change of ventilation frequency between the two groups.


control
softwater
A
90
80
70
60
50
5
10
15
20
$V_f$ (min$^{-1}$)
$PwO_2$ (kPa)

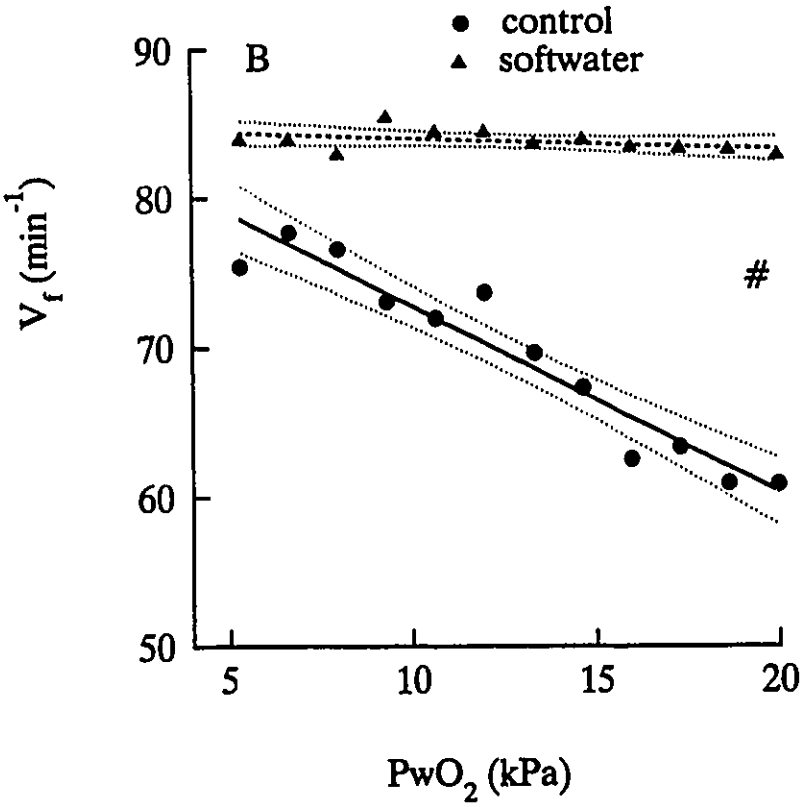
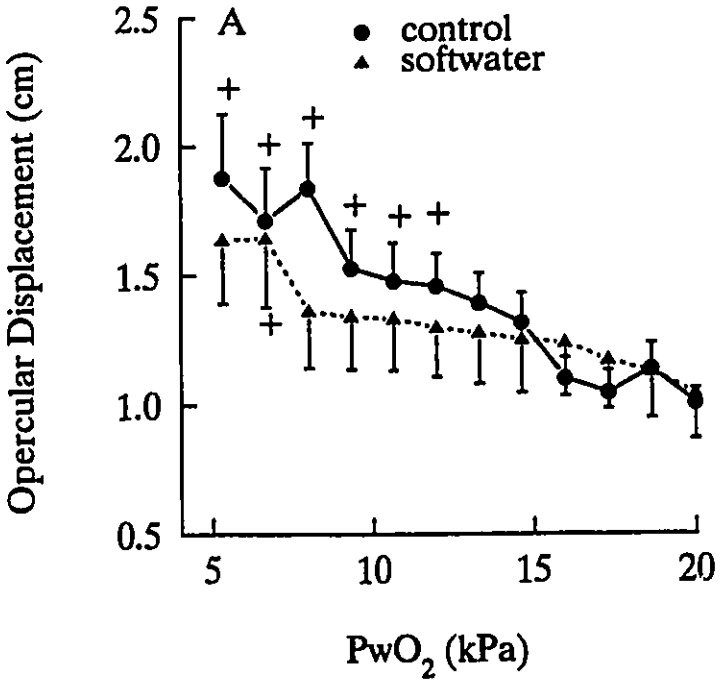

control
softwater
B
90
80
70
60
50
5
10
15
20
#
$V_f$ (min$^{-1}$)
$PwO_2$ (kPa)

**FIGURE 3.4:** A) Effect of environmental hypoxia on mean opercular displacement in control (solid line; n = 7) and softwater acclimated (dotted line; n = 8) rainbow trout. + indicates a significant difference ($p < 0.05$) in opercular displacement from the values measured during normoxia ($PwO_2$ = 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 $PwO_2$ in the control (slope = -0.061 ± 0.012 cm $kPa^{-1}$ $PwO_2$) and softwater acclimated trout (slope =-0.026 ± 0.005 cm $kPa^{-1}$ $PwO_2$). # indicates a significant difference ($p < 0.05$) in the rate of change of opercular displacement between the two groups. 95 % confidence limits are shown as dotted lines.


A
control
softwater
Opercular Displacement (cm)
2.5
2.0
1.5
1.0
0.5
5
10
15
20
PwO2 (kPa)

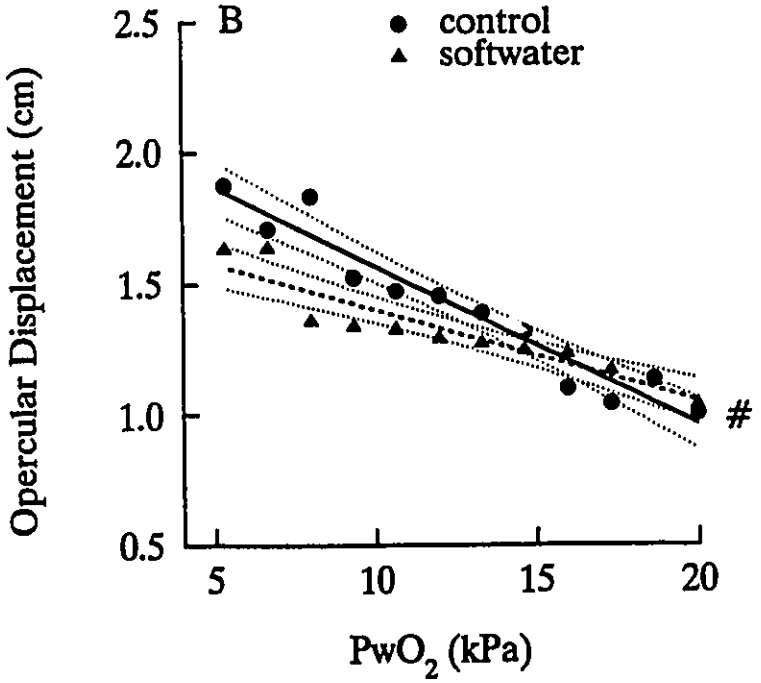

B
control
softwater
Opercular Displacement (cm)
2.5
2.0
1.5
1.0
0.5
#
5
10
15
20
PwO2 (kPa)

**FIGURE 3.5:** A) Effect of environmental hypoxia on arterial oxygen partial pressure ($PaO_2$) in control (solid line; n = 7) and softwater acclimated rainbow trout (dotted line; n = 8). + indicates a significant difference ($p < 0.05$) in $PaO_2$ from the values measured during normoxia ($PwO_2$ = 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 $PaO_2$ and $PwO_2$ in the control fish (slope = 0.84 ± 0.06 kPa $PaO_2$ $kPa^{-1}$ $PwO_2$) and softwater acclimated fish (slope = 0.65 ± 0.06 kPa $PaO_2$ $kPa^{-1}$ $PwO_2$). # indicates a significant difference in the rate of change of $PaO_2$ between the two groups ($p < 0.05$). 95 % confidence limits are shown as dotted lines.

C) Effect of environmental hypoxia on the difference between $PwO_2$ and $PaO_2$ ($PwO_2$ - $PaO_2$). Symbols as in (A).

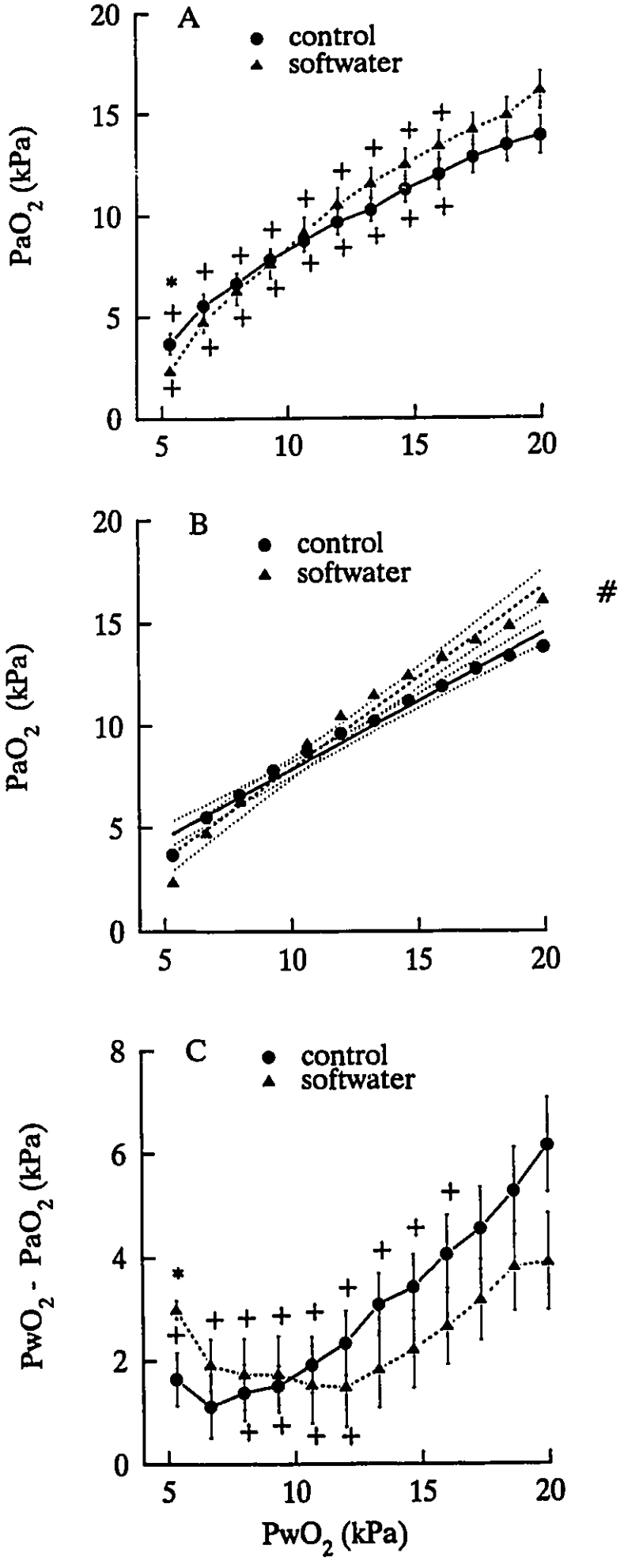
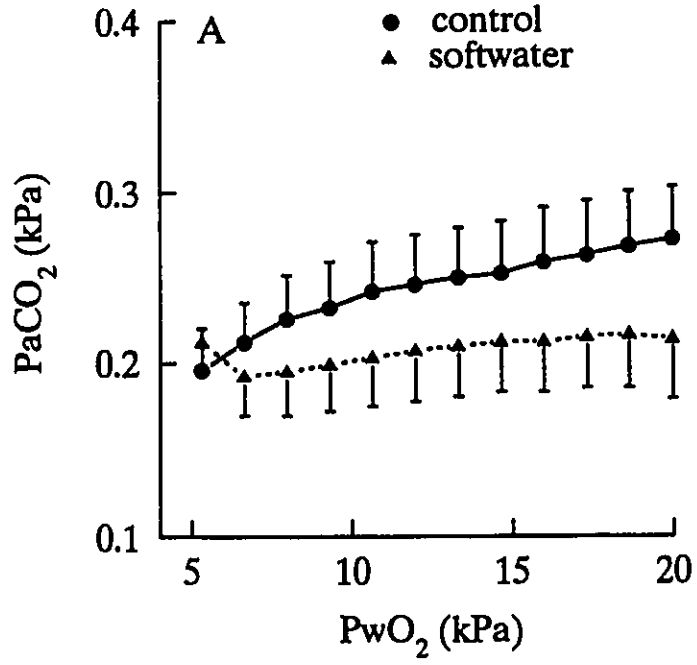

A
control
softwater
$PaO_2$ (kPa)
B
control
softwater
#
$PaO_2$ (kPa)
C
control
softwater
$PwO_2 - PaO_2$ (kPa)
$PwO_2$ (kPa)

**FIGURE 3.6:** A) Effect of environmental hypoxia on arterial carbon dioxide partial pressure ($PaCO_2$) in control (solid line; n = 7), and softwater acclimated rainbow trout (dotted 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 $PaCO_2$ and $PwO_2$ in control fish (slope = 0.0047 ± 0.0011 kPa $PaCO_2$ $kPa^{-1}$ $PwO_2$) and softwater exposed rainbow trout (slope = 0.002 ± 0.001 kPa $PaCO_2$ $kPa^{-1}$ $PwO_2$). # indicates a significant difference ($p < 0.06$) in the rate of change of $PaCO_2$ between the two groups. 95 % confidence limits are shown as dotted lines.


A
control
softwater
0.4
0.3
0.2
0.1
PaCO2 (kPa)
5
10
15
20
PwO2 (kPa)

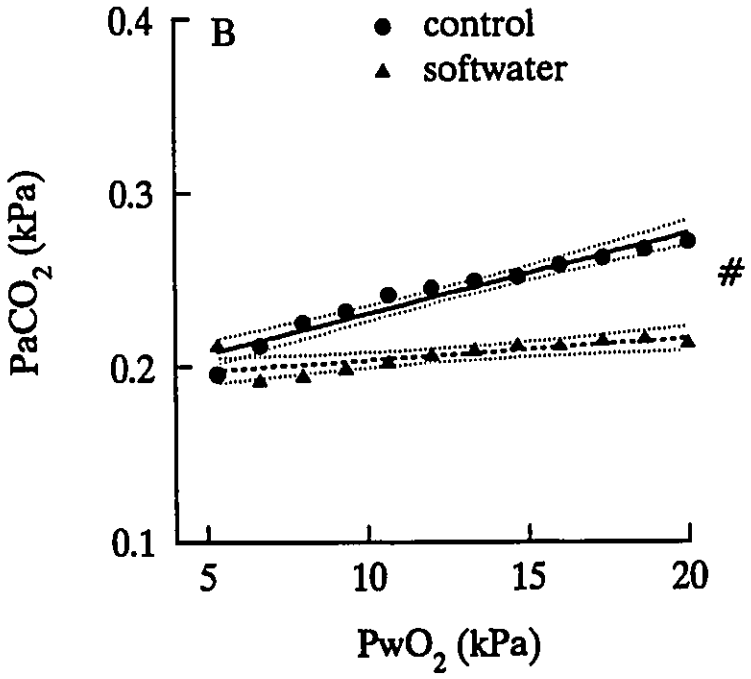
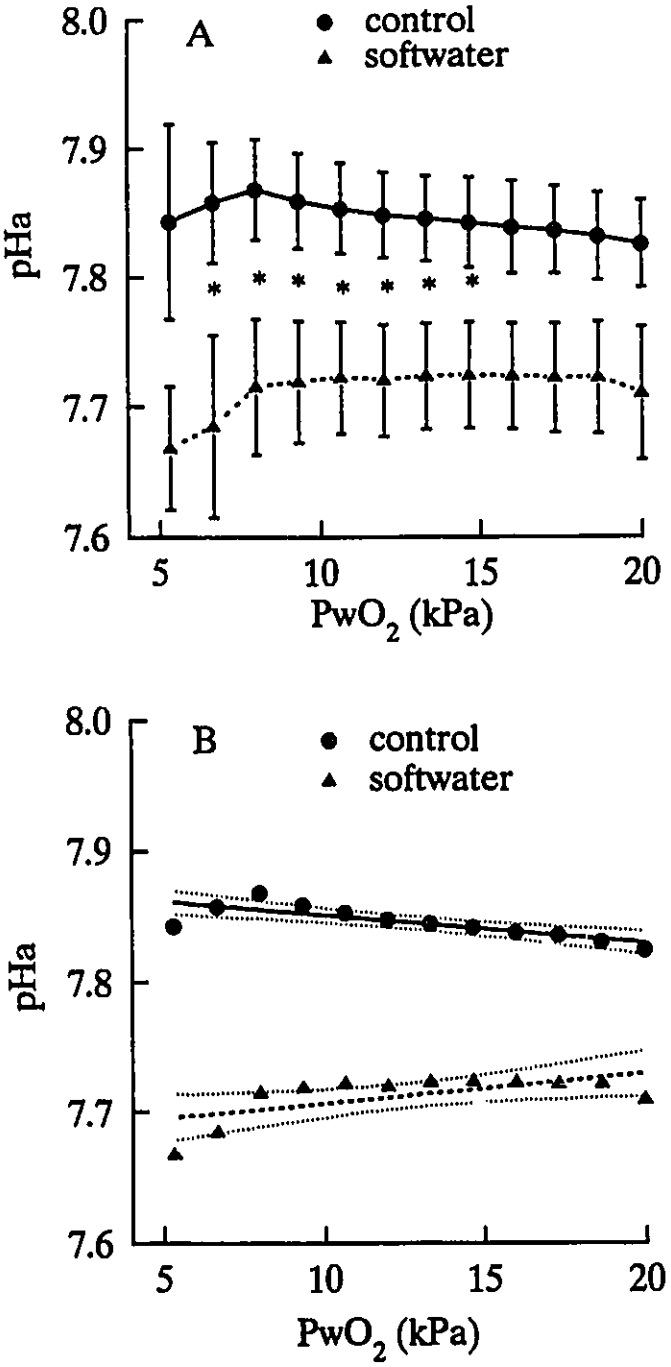

B
control
softwater
0.4
0.3
0.2
0.1
PaCO2 (kPa)
#
5
10
15
20
PwO2 (kPa)

**FIGURE 3.7:** 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 $PwO_2$ in the control fish (slope = -0.0026 ± 0.0025 pH units $kPa^{-1}$ $PwO_2$) and softwater acclimated fish (slope = 0.0021 ± 0.0035 pH units $kPa^{-1}$ $PwO_2$). 95 % confidence limits are shown as dotted lines.


A
control
softwater
8.0
7.9
7.8
7.7
7.6
pHa
5
10
15
20
PwO2 (kPa)
B
control
softwater
8.0
7.9
7.8
7.7
7.6
pHa
5
10
15
20
PwO2 (kPa)

# DISCUSSION

*Gill morphology and ionic regulation in softwater acclimated fish*

As in previous studies (Laurent *et al.* 1985; Perry and Wood, 1985; Avella *et al.* 1987; Leino *et al.* 1987; Spry and Wood, 1988; Perry and Laurent, 1989; Laurent and Hebibi, 1989; Laurent *et al.* 1994), acclimation of fish to softwater caused a pronounced proliferation of chloride cells on both the filamental and lamellar epithelia of the gill. This response is thought to promote ion uptake from the ion poor softwater (e.g. Avella *et al.* 1987; Perry and Laurent, 1989).

In the present study, trout were maintained for two weeks in softwater. After this period, plasma $Cl^-$ levels were significantly depressed and, although not significant, there was a trend toward reduced plasma levels of $Ca^{2+}$ and $Na^+$ (Table 3.2). 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. But it is not unreasonable to suggest 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 softwater and must markedly influence the gill blood-to-water diffusion barrier at such times. In Chapter 2 of this thesis, using fish of the same stock and acclimation conditions as in the present study, a two-fold increase in the lamellar diffusion distance was reported (from $3.26 \pm 0.08$ to $6.58 \pm 0.43$ μm) based on detailed analysis of transmission electron micrographs. Using a similar analytical procedure, Bindon *et al.* (1994a) observed a comparable thickening of the

lamellar diffusion barrier associated with the chloride cell proliferation accompanying chronic treatment of trout with cortisol and/or growth hormone. Such exhaustive quantitative analyses were beyond the scope of this present chapter but based on 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.* 1994a and results obtained in Chapter 1), it is reasonable to assume that the thickness of the diffusion barrier was increased markedly in the present experiments.

*Respiratory gas transfer during normoxia*

A striking feature in the softwater acclimated fish was the approximate 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 hyperventilating. In theory, hyperventilation in the absence of other adjustments will increase the trans-branchial diffusion gradients for $O_2$ and $CO_2$ (Wood and Perry, 1985) and thereby serve to elevate $PaO_2$ and lower $PaCO_2$. Thus, hyperventilation can be viewed as a compensatory mechanism to counteract the potential loss of gas transfer efficiency associated with a thickened diffusion barrier. On the other hand, 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.* (1994b)

observed a marked elevation of $PaCO_2$ with no change in $PaO_2$ 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 on $O_2$ transfer under normoxic conditions (Daxboeck *et al.* 1982; Malte and Weber, 1985), it is perhaps surprising that $PaO_2$ did not increase with increasing ventilation. 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 $PaO_2$.

In softwater acclimated fish, $O_2$ uptake and $CO_2$ excretion were not significantly different than in the control fish. The apparent (not statistically significant) increase in $O_2$ uptake in softwater 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, 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 nevertheless will 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 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 chloride cells.

In contrast to the results of the present study, Bindon *et al.* (1994b) 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 likely explains the increased $PaCO_2$ in the hormone-treated fish (Bindon *et al.* 1994b), 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 softwater (see Table 3.1) also may have contributed to the maintenance of $CO_2$ excretion independent of ventilation adjustments. Adjacent to the gill epithelium is a micro-environment or boundary layer, the chemistry of which can vary markedly from 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 $PCO_2$ of the boundary layer contributing to the maintenance of a favorable $PCO_2$ gradient across the gill. The reduced concentration of $HCO_3^-$ in the softwater (see Table 3.1) 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. Based on 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 $PO_2$. Furthermore, the hyperventilatory response known to accompany hypoxia (e.g. see review by Randall, 1982) lowers $PaCO_2$. The extent of the reduction in $PaCO_2$ 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 more natural condition to further investigate 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 rate of change of $PaO_2$ during progressive hypoxia in the softwater fish provided evidence of impaired $O_2$ uptake and suggested a loss of $O_2$ transfer efficiency in these fish during hypoxia. This view was reinforced by graphically relating the water-arterial blood $PO_2$ difference ($PwO_2$ - $PaO_2$) to $PwO_2$ (Figure 3.3). Generally, during hypoxia the efficiency of gas transfer increases owing to a variety of factors including hyperventilation and lamellar recruitment. Consequently the difference between $PwO_2$ and $PaO_2$ is diminished. In the present study, the apparent $O_2$ uptake efficiency as estimated by $PwO_2$ - $PaO_2$ 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 efficiency continued to increase throughout most of the hypoxic period, whereas in the softwater fish, an obvious inflection was observed at 12 kPa $PwO_2$ at which point the apparent efficiency of $O_2$ uptake began to decrease ($PwO_2$-$PaO_2$ 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 $PaO_2$ during hypoxia is their blunted hyperventilatory response (Figures 3.1 and 3.2). Although ventilation volume was not measured directly, the absence of any changes in breathing frequency, coupled with an attenuated ventilatory amplitude response, likely indicates that ventilation volumes during hypoxia were reduced in the softwater fish. The lack of a change in breathing frequency may have simply

reflected the fact that breathing frequencies already were 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, $PaCO_2$ remained constant during hypoxia in the softwater acclimated fish. It seems likely that the efficiency 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 during hypoxia at the lowest $PwO_2$ values (5.3 kPa). However, there are several problems associated with closed system respirometry when used at such severe levels of hypoxia. In particular, a reduction in $PwO_2$ 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.* 1994b) or a thickening of the diffusion barrier (Bindon *et al.* 1994a) associated with chloride cell proliferation, Laurent and Hebibi (1989) reported a reduction in the blood-to-water diffusion distance in trout exposed to softwater. 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.

# CHAPTER 4

# GENERAL DISCUSSION

The purpose of this thesis research was to test the hypothesis *that a naturally induced chloride cell proliferation would impede gas transfer at the level of the gill.* The prediction was that softwater acclimation would result in a proliferation of chloride cells, which because of their bulging morphology, would increase the blood-to-water diffusion distance. The diffusing capacity of the lamellae would be reduced and gas exchange impaired.

The results presented in Chapters 2 and 3 have demonstrated some of the morphological and physiological effects that softwater acclimation has on rainbow trout, and some of the possible compromises which could occur during periods of ionoregulatory challenge are shown. Owing to the dual role of the fish gill in gas exchange and ionic regulation, and the fact that gas exchange is inversely proportional to the thickness of the gill surface (Chapter 1), any strategy which involves increasing the thickness of the diffusion barrier would, in the absence of compensatory adjustments, negatively affect gas transfer.

## MORPHOLOGICAL STUDIES

The goal was to investigate the effects of softwater exposure on chloride cell morphology, in particular the relationship existing between chloride cells and the blood-to-water diffusion barrier thickness. Evidence was collected (Chapter 2) to support the first part of the hypothesis of this thesis, *that softwater acclimation would cause an increase in chloride cell prevalence, resulting in an increased blood-to-water diffusion distance.* The scannning electron microscopy confirmed a chloride cell proliferation in the softwater fish as the chloride cell fractional area (CCFA) was significantly greater in these fish compared

to control fish. The CCFA increased as a result of both an increase in number and of exposed apical surface area of individual chloride cells. Apparently, fish use the chloride cell proliferation response as an adaptive strategy to optimize ion uptake from dilute environments (softwater).

An intensive transmission electron microscopy study showed that the proliferation of chloride cells doubled the blood-to-water diffusion barrier thickness of the lamellae compared to control fish, and by consequence, a narrowing of the interlamellar water channels was obvious. Ventilatory interlamellar water flow would also be reduced.

**PHYSIOLOGICAL STUDIES**

The primary focus of this thesis was to investigate the effects that pronounced morphological changes of branchial chloride cells have on respiratory parameters. The most obvious feature in the normoxic softwater acclimated fish was the approximate 36 % increase in ventilation frequency as compared to controls. Ventilation frequency did not change during the exposure to hypoxia. This hyperventilation was used as a compensatory mechanism to counteract the potential decrease of gas transfer efficiency in the face of an increased diffusion barrier thickness.

During hypoxia, both $O_2$ and $CO_2$ transfer was impaired in the softwater acclimated fish. A loss of $O_2$ transfer efficiency in these fish during hypoxia was evident compared to the control fish which, unlike the softwater fish whose uptake efficiency decreased quickly, increased their $O_2$ uptake efficiency throughout most of the hypoxic period. At high $PwO_2$, softwater plasma oxygen levels ($PaO_2$) were maintained but the ability to compensate for the increased barrier thickness was lost during hypoxia.

$PaCO_2$ in the softwater fish remained constant during hypoxia. It is possible that the efficiency of $CO_2$ transfer was already maximal during normoxia (due to high ventilation frequency) and that further increases in transfer efficiency were prevented by the thickened barrier.

The blood acid-base status of the softwater fish was characterized by a metabolic acidosis, possibly arising from the increased numbers of $Cl^-/HCO_3^-$ exchangers present on the chloride cell proliferated gills. Such a response would increase base excretion ($HCO3^-$) in exchange for $Cl^-$ uptake and contribute to a base deficit (acidosis).

**FUTURE RESEARCH**

The goal of this thesis was to determine the relationship between a naturally-induced chloride cell proliferation and respiratory function. Indeed, the hypothesis of this thesis is supported. Gas exchange is compromised at the expense of enhanced ion uptake mechanisms which cause an increase in the blood-to-water diffusion distance. More study should be undertaken to reveal any other possible physiological functions that are compromised by softwater acclimation, for example, does the resulting chloride cell proliferation render fish more susceptible to environmental perturbations such as acid-base disturbances? Also, an investigation into the effects of long-term softwater exposure on physiological functions such as growth would be of interest.

Additional morphological examinations of the gill tissue itself focusing in on gap junctions and their involvement in ion movement during softwater acclimation and the determination of Krogh's constant of diffusion for the different components of the gill epithelium might also be endeavored.

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