Cell Tissue Res (1996) 285:75–82

Cell & Tissue Research



# The effects of soft-water acclimation on gill structure in the rainbow trout *Oncorhynchus mykiss*

**Anna Maria Greco, James C. Fenwick, Steve F. Perry**

Department of Biology, University of Ottawa, 30 Marie Curie, Ottawa, Ontario, Canada K1N 6N5

Received: 14 July 1995 / Accepted: 29 January 1996

**Abstract.** Rainbow trout (*Oncorhynchus mykiss*) were exposed to ion-poor (soft) water to test the hypothesis that naturally induced proliferation of branchial chloride cells causes a thickening of the blood-to-water diffusion barrier. This was achieved by using a combination of scanning and transmission electron-microscopic techniques. Fish were exposed to soft-water conditions ($[Na^+]$= 0.055 mmol l-1, $[Cl^-]\approx 0.029$ mmol $1^{-1}$, $[Ca^{2+}]\approx$ 0.059 mmol $1^{-1}$, and $[K^+]\approx 0.007$ mmol $1^{-1}$) for 1, 2, and 4 weeks. Marked chloride cell proliferation was evident at all sampling times with an approximate doubling of the gill epithelial surface area covered by chloride cells exposed to the water ("chloride cell fractional area"). The increases in chloride cell fractional area resulted from both increased numbers of cells and expanded apical surfaces of exposed individual cells. As a result of chloride cell proliferation, soft-water exposure was associated with a doubling of the lamellar blood-to-water diffusion distance from 3.26±0.08 μm to 6.58±0.43 μm as determined from transmission electron micrographs. These data demonstrated a positive correlation between chloride cell fractional area and blood-to-water diffusion distance. We conclude that, in trout, chloride cell proliferation during soft-water exposure, while presumably benefiting ionic regulation, may impair gas transfer owing to the associated thickening of the blood-to-water diffusion barrier.

**Key words:** Gill – Chloride cell – Gas transfer – Diffusing capacity – *Oncorhynchus mykiss* (Teleostei)

## Introduction

At the teleost gill, the ambient water is separated from the blood by an 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). Pavement cells are the most abundant cell type and cover much of the lamellar and filamental surfaces of the gill. They form an extensive thin surface through which most respiratory gas ($CO_2$ and $O_2$) exchange occurs. The less numerous mucous cells secrete a mucous coat over the apical surface of the epithelium. The physiological role of mucus is unclear, although it is generally believed to impede diffusive ion loss and assist active ion uptake (Handy 1989; Perry and Laurent 1993). The mitochondrion-rich chloride cells are found sparsely distributed on the filament, in the interlamellar regions, and at the bases of lamellae. A role for chloride cells in the freshwater teleost gill in transepithelial $Ca^{2+}$ uptake has been clearly established (Marshall et al. 1992; McCormick et al. 1992), yet its role in NaCl uptake is currently debated. On the basis of correlation, several studies (Laurent et al. 1985; Perry and Laurent 1989; Laurent and Perry 1990; Perry et al. 1992) have implicated the chloride cell in both $Na^+$ and $Cl^-$ uptake. Conversely, other studies (Goss et al. 1992; 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.

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) have shown that various ionoregulatory stressors can induce a significant increase in the relative proportion of chloride cells on all gill epithelial surfaces, especially the lamellar surfaces. These chloride cells are substantially thicker than the pavement cells and 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.

It was recently shown in this laboratory (Bindon et al. 1994a,b) that when the chloride cell density and relative

Funded by operating grants from the Natural Sciences and Engineering Research Council of Canada (J.C.F., S.F.P.).

*Correspondence to:* S.F. Perry

**Table 1.** Water chemistry variables (mean±1 SEM, *n*=28) for the control (dechlorinated tap water) and soft-water (artificial soft water) conditions used for trout acclimation

| | Dechlorinated tap water | Artificial soft water |
|---|---|---|
| $[Ca^{2+}]$ (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 |

surface area are increased by chronic injections of hormones (growth hormone and/or 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 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 the present study was to conduct a thorough investigation of the effects of naturally induced chloride cell proliferation on the structure of the gill epithelium 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 209–495 g (mean weight=323.1±13.3 g) were purchased from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada) and transferred to the fish-holding facility of The University of Ottawa. Fish were kept indoors in large tanks supplied with flowing, dechlorinated, and aerated City of Ottawa tap water (see Table 1 for water chemistry). The photoperiod was kept constant at 12 h light:12 h 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 500–1 fibreglass tanks (Living Stream, Toledo, Ohio, USA). The first tank (control group, *n*=12) was supplied with running dechlorinated tap water. The second tank (soft-water-acclimated group, *n*=12) was supplied with dechlorinated tap water diluted with running reverse osmosis water (see Table 1 for water chemistry). Total water-flow rates for the tanks were adjusted to 5 l/min. The experimental group was exposed to the soft-water condition by gradually increasing the proportion of reverse osmosis water over a period of three days until the final conditions were met. The third day was recorded as day 1

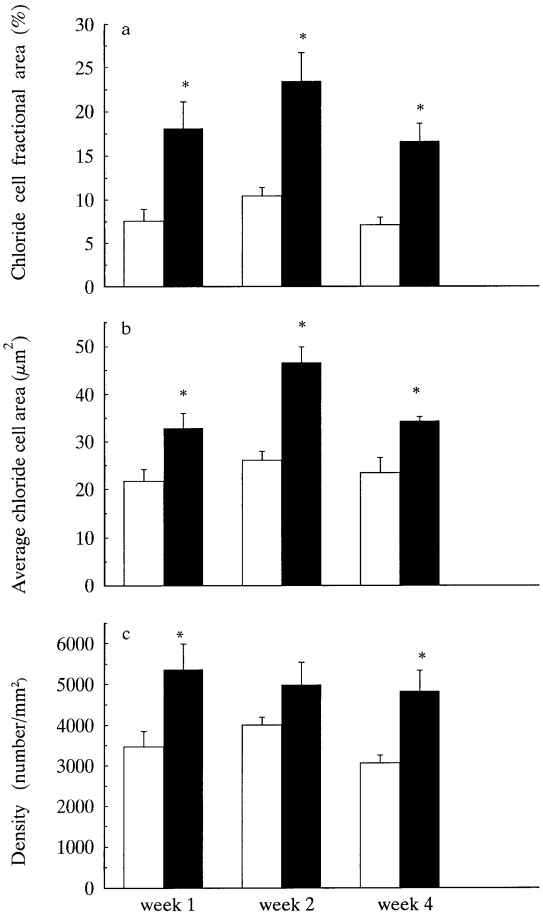


**Fig. 1a–c.** The temporal effects of soft-water acclimation on the surface morphometry of the rainbow trout gill (*open bars* control fish, *solid bars* soft-water fish): chloride cell fractional area (**a**), average chloride cell area (**b**), and chloride cell density (**c**). Values are means±1 SEM (*n*=4). *Asterisks* indicate soft-water values that are significantly different from control values ($P<0.05$)

of soft-water exposure. Soft-water 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 that 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-microscopic study, while the central portion of the second gill arch (right side) was reserved for the transmission electron microscopic study.

A standard fixation procedure was used to prepare gill tissue for electron microscopy. Small pieces of tissue were excised and quickly rinsed in ice-cold 0.15 M sodium cacodylate buffer (pH 7.4) to remove excess mucus and blood. They were fixed using 5% glutaraldehyde in 0.15 M sodium cacodylate buffer (292 mOsm) at 4°C for 1 h, rinsed three times in buffer, and then post-fixed at room temperature for 1 h in unbuffered 1% osmium tetroxide. 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 and fixed in glutaraldehyde as described above. The filament pairs were isolated and

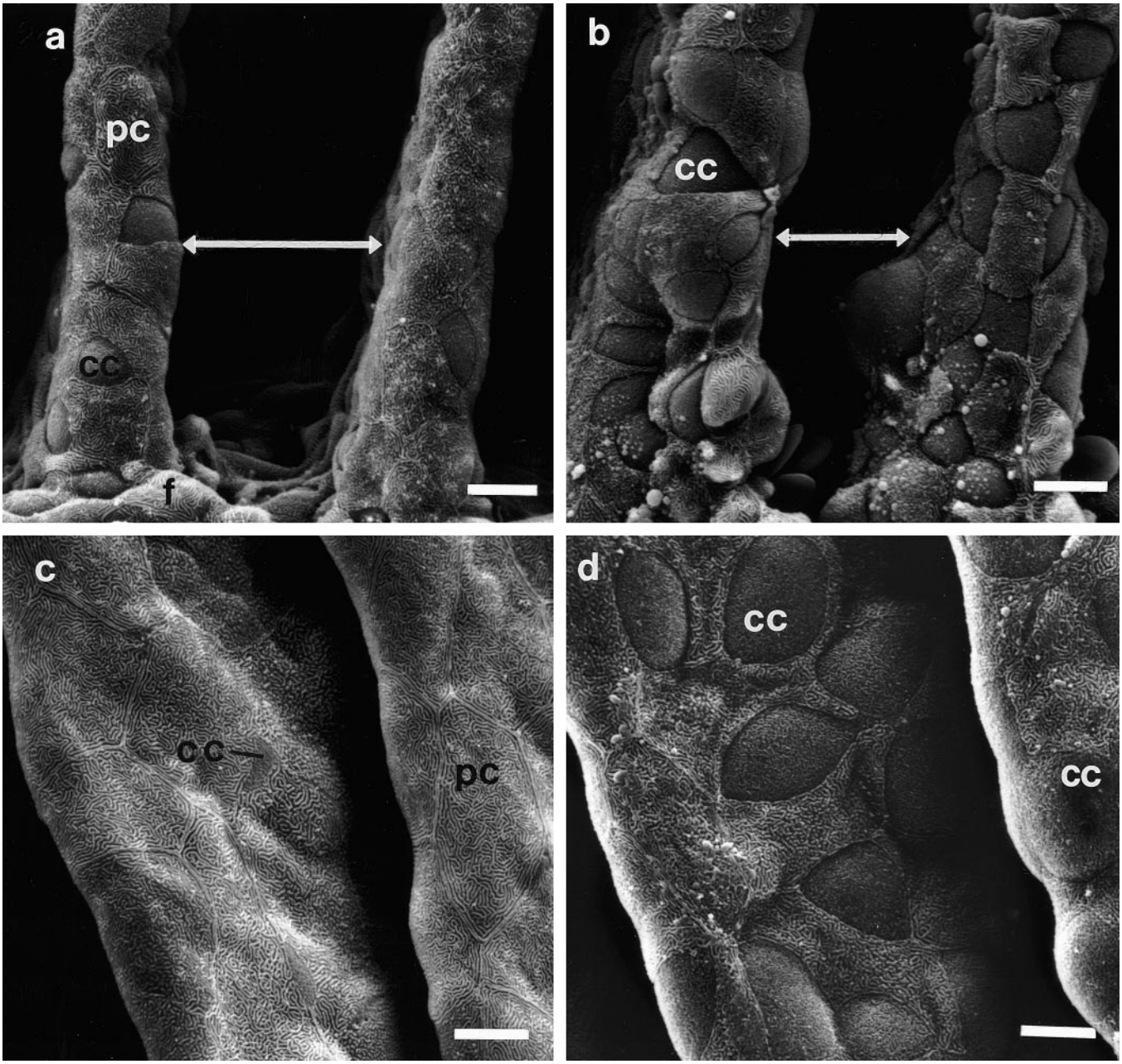


**Fig. 2a–d.** Representative scanning electron micrographs of trout-gill lamellar epithelia under control conditions (**a, c**) and after 2 weeks of soft-water exposure (**b, d**). Observe the obvious proliferation of chloride cells (*cc*) on the lamellae and the thickening of the lamellar bases, thus diminishing the width of water channels (*double-headed arrows*) between lamellae. For clarity, only a few chloride cells and pavement cells (*pc*) are labeled; *f* filament. ×1600 (**a, b**), ×2000 (**c, d**). *Bars*: 10 μm

separated at the septum prior to postfixation. Subsequent to dehydration in an ethanol series, 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 pieces were immersed in propylene oxide, and 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 pure Araldite (8 h). The filament pieces were embedded in fresh Araldite in flat molds. They were oriented so that the lamellae would be sectioned transversely when cut. Fourteen sample blocks were made per fish. The Araldite was allowed to harden slowly (24 h at 25°C and 40 h at 60°C).

Five blocks were selected at random per fish. Ultrathin (50–70 nm) transverse sections were cut through the middle regions of the lamellae. Of these, ten sections were picked randomly (ten per block; 50 per fish) and mounted on 200-mesh copper grids. Sections were stained with lead citrate (Reynolds 1963) and saturated uranyl acetate before being examined with a Philips 500 transmission electron microscope. Twenty-five randomly selected lamellar regions were photographed per fish (five micrographs per block; five blocks per fish) to yield micrographs similar to those in Fig. 2. Blood-to-water diffusion distance was determined by randomly placing a circular grid (Weibel and Knight 1964) of equidistant parallel lines over each micrograph (Laurent and Hebibi 1989). A digitizing tablet, connected to a microcomputer run-

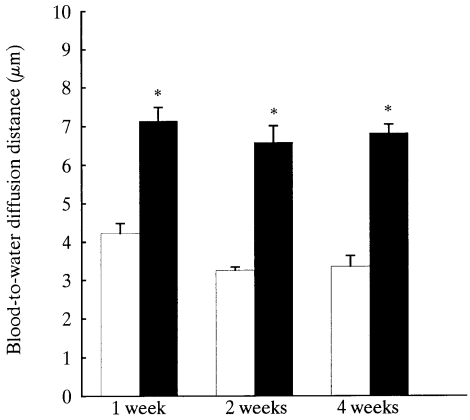


**Fig. 3.** The temporal effects of soft-water acclimation on blood-to-water diffusion distance (*open bars* control fish; *solid bars* soft-water fish). Values are means±1 SEM ($n$=4). *Asterisks* indicate soft-water values that are significantly different from control values ($P$<0.05)

ning Jandel SigmaScan Ver. 3.90, was used for automated measurements. Measurement of the intercept length (lh) of several lines randomly crossing the blood space and the external lamellar surface were taken. This grid was superimposed ten times per micrograph, and approximately 1200 measurements were made per fish. The harmonic mean blood-to-water barrier thickness ($\tau_h$) was calculated with the equation:

$\tau_h = 2/3(l_h)$

### *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 with the scanning electron microscope.

Pairs of filaments, still attached at the septum, were separated from the gill arch after fixation. Each filament pair was dehydrated in ethanol, bathed in 1,1,1,3,3,3-hexylmethyldisilizan (Aldrich, Milwaukee, Wis., USA), and air-dried (Laurent and Hebibi 1989). Pairs of filaments were attached with silver paint to stubs for use in a Philips 500 scanning electron microscope. The tissue was oriented so that the lateral sides of the filaments were parallel to the face of the stub. One photograph from each anterior and posterior filament was taken (total of ten per fish). At a magnification of approximately ×1000, the microscope was focused on the trailing edge of the filament epithelium close to where the lamellae meet the filament and about ten lamellae distal from the septum. This location was chosen as it is the same location from which the transmission electron-microscopic results were derived. The apical chloride cell area was measured by tracing the cell perimeters on micrographs with the digitizing tablet. Chloride cell fractional area (CCFA) and density were determined with the following equations: CCFA=total area of whole and partial chloride cells/picture area×$10^{-6}$ chloride cell density=CCFA/average chloride cell area.

Results were analyzed statistically with unpaired two-sample $t$-tests between sample means. The fiducial limit was 5%.

### *Water analysis*

Water [$Na^+$], [$Ca^{2+}$], and [$K^+$] were determined by flame-emission spectrophotometry using a Varian Spectra AA 250 Plus. [$Cl^-$] was determined by a mercuric thiocyanate spectrophotometric assay (Zall et al. 1956).

## Results

### *Epithelial surface structure*

Three surface morphometric parameters were measured. Chloride cell fractional area (Fig. 1a), average chloride cell area (Fig. 1b), and chloride cell density (Fig. 1c) were assessed in control and soft-water-acclimated trout over a 4-week period. CCFA was significantly elevated by approximately 100% in the soft-water-acclimated fish at all sampling times (1, 2, and 4 weeks). This increase in CCFA was caused by the combined effects of an increase in average chloride cell area and density, except at 2 weeks when chloride cell density was not significantly different from that of controls.

Representative scanning electron micrographs of control and soft-water-acclimated trout are shown in Fig. 2. These micrographs convey the general morphological appearance of the control and soft-water-acclimated fish gill epithelia. Proliferation of chloride cells was observable over the entire surface of the gill epithelium in soft-water-acclimated fish. The proliferation of chloride cells led to a pronounced thickening of the lamellae and, in turn, this decreased the width of the interlamellar water channels in the soft-water-exposed trout (Fig. 2a,b).

### *Blood-to-water diffusion distance*

The thickness of the lamellar diffusion barrier increased significantly in trout acclimated to soft water when compared to controls at all time periods (Fig. 3). Representative transmission electron micrographs illustrating differences in the blood-to-water diffusion distances are shown for control and soft-water fish in Figs. 4 and 5 (magnified view), respectively. Clearly the proliferation of the voluminous chloride cells (which are found, but sparsely on the lamellae of control fish) was the cause of the doubling of the diffusion barrier in the soft-water trout.

The experimental protocol used in this study in which gill CCFA 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 ($P$<0.05) with CCFA (Fig. 6).

## 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 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 to the

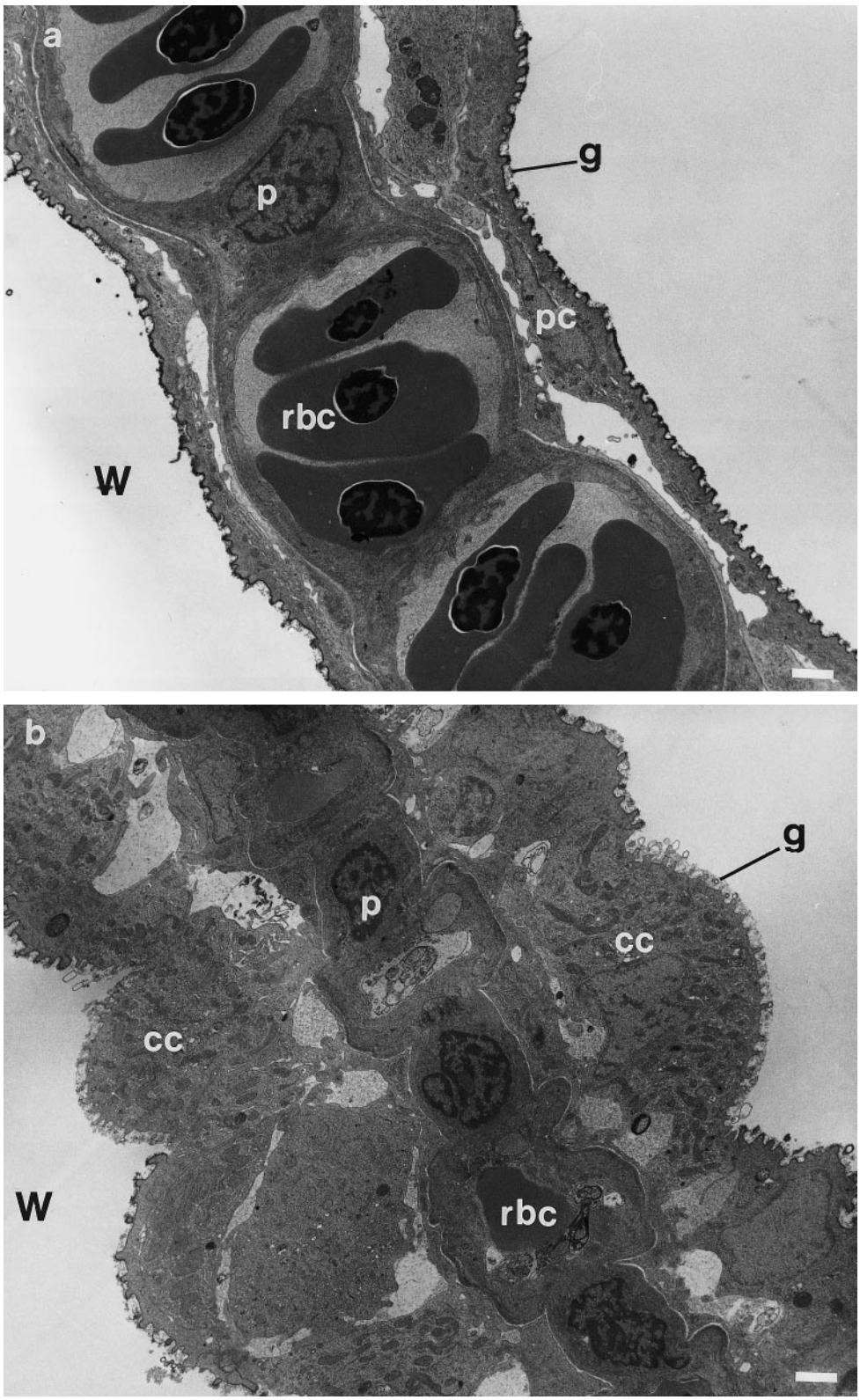


**Fig. 4a, b.** Representative transmission electron micrographs showing transverse sections of trout gill lamellae under control conditions (**a**) and after 2 weeks of exposure to soft water (**b**). Note the significant thickening of the gill epithelium after exposure to soft water. The apparent difference in size of the blood channels in the two micrographs is solely a result of the plane of sectioning. *cc*, Chloride cell, *g*, glycocalyx, *p*, pillar cell, *pc*, pavement cell, *rbc*, red blood cell, *W*, water channel. ×5000. *Bars*: 1 μm

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 dilute environments. Owing to the dual role of the teleost gill in gas exchange and ionic regulation, such a strategy could conceivably affect gas transfer negatively. Thus, the goal of this study was to investigate the effects of exposure to an ion-deficient medium on

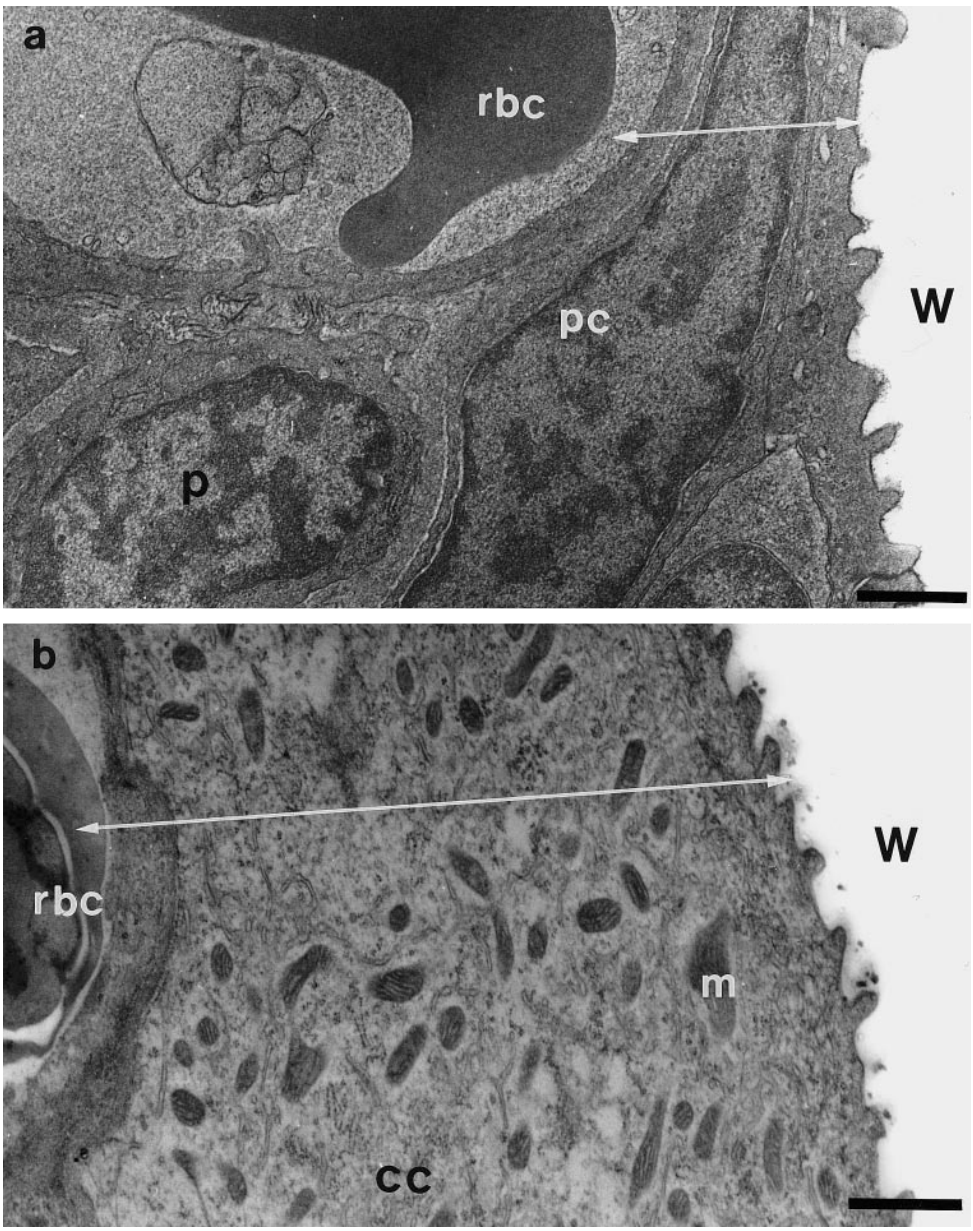


**Fig. 5a, b.** Representative transmission electron micrographs of lamellar transverse sections in control (**a**) and soft water-acclimated (**b**) trout. The *double-headed arrows* represent possible diffusional paths of $O_2$ and $CO_2$ across the blood-to-water barrier. The path in the soft-water trout is greater than that in the control animal, owing to the presence of thicker chloride cells (*cc*), in the former, and thinner pavement cells (*pc*), in the latter. *m*, Mitochondrion, *p*, pillar cell, *rbc*, red blood cell, *W*, water channel. ×16 500. *Bars*: 1 μm

chloride cell structure with particular emphasis on the relationship between chloride cells and the blood-to-water diffusion distance.

The chloride cell fractional area in control and soft-water-acclimated fish was assessed by scanning electron microscopy. Such a technique allows the observation of surface morphological structures. Thus the exposed cells, the sites believed to function in ion uptake, are readily distinguished. The filament epithelium is flat, and because of this property, it can easily be oriented parallel to the specimen stub and photographed without distortion. For this reason, the present study of chloride cell structure was restricted to the filament. Even though gas transfer occurs predominantly over the lamellae, and not over the filament, the method is justified, nonetheless, as Laurent and Perry (1990) reported that increases in CCFA on the filament were mirrored by similar increases on the lamellae.

Exposure of fish to ion-poor water elicits a variety of compensatory physiological adjustments. Wendelaar Bonga and van der Meij (1981) found that soft-water 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 (reviewed by Hirano 1986). McDonald and Rogano (1986) reported reductions in gill ionic permeability upon exposure to soft water. Specifically, there was a significant reduction of both $Na^+$ and $Cl^-$ efflux in rainbow trout during the first three days of exposure to soft water.

Positive correlations between chloride cell surface area and the rates of $Na^+$ and $Cl^-$ uptake have been report-

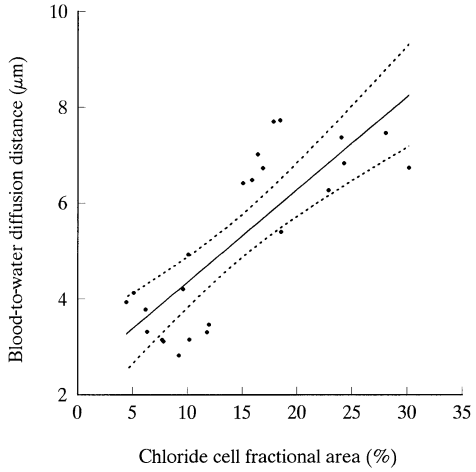


**Fig. 6.** Correlation between chloride cell fractional area and the blood-to-water diffusion distance ($n$=24). Values are individual data points obtained from control and soft-water fish at all sampling times [$r$ (correlation coefficient)=0.81; $y$=0.19×+2.42]. *Broken lines* indicate 95% confidence interval

ed (Perry and Laurent 1989; Laurent and Perry 1990; Perry et al. 1992). As the apical surface areas of chloride cells increase, 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 of exposure of rainbow trout to soft water, there was a stimulation of $Na^+$ and $Cl^-$ transport which was accompanied by a fourfold increase in plasma cortisol levels during the first 12–48 h. 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 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 (Laurent et al. 1994; Morgan et al. 1994). Further, Goss et al. (1992) found that $Cl^-$ uptake was positively correlated to CCFA, but $Na^+$ 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 pavement cell. Regardless, the proliferation of branchial chloride cells is an important physiological response of the teleost to ion-poor water.

### *Chloride cell structure and gas transfer*

In this study, chloride cell proliferation induced by soft-water acclimation caused a marked increase in the blood-to-water diffusion distance and also, by consequence, a narrowing of interlamellar water channels. 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 interlamellar water-channel areas would be expected to restrict the flow of ventilatory water near the lamellae and, 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 soft water, Thomas et al. (1988) noted that these fish experienced a lower $PaO_2$ than controls at the same water PO2. A loss of resistance to hypoxia in these soft-water-acclimated rainbow trout could correspond to the decrease in the diffusing capacity observed in this present study. Thus, the hypothesis of Thomas et al. (1988) that fish experience reduced gas transfer as a result of chloride cell proliferation is supported both by the results of this study and those of Bindon et al. (1994a,b). 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 soft-water exposure. Given the protruding nature of lamellar chloride cells and the positive correlations between chloride cell surface area and blood-to-water diffusion distance reported here and elsewhere (Bindon et al. 1994a), 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 hemoglobin $O_2$-binding affinity. Further studies on physiological compensatory adjustments are warranted to directly address the impact of this soft-water-acclimation-induced chloride cell proliferation on gas exchange in the rainbow trout.

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