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ADAPTATIONAL RESPONSES OF RAINBOW TROUT TO LOWERED EXTERNAL NaCl CONCENTRATION: CONTRIBUTION OF THE BRANCHIAL CHLORIDE CELL

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Summary

1. Whole-body ionic fluxes and gill chloride cell (CC) morphology were monitored in rainbow trout (*Salmo gairdneri*) exposed acutely or chronically to natural fresh water (NFW; $[Na^+]=0.120\,mmol\,l^{-1}$; $[Cl^-]=0.164\,mmol\,l^{-1}$) or artificially prepared fresh water with reduced [NaCl] (AFW; $[Na^+]=0.017\,mmol\,l^{-1}$; $[Cl^-]=0.014\,mmol\,l^{-1}$).

2. Net fluxes of Na^+ (J_{net}^{Na}) and Cl^- (J_{net}^{Cl}) became extremely negative (indicating net NaCl loss to the environment) upon immediate exposure to AFW exclusively as a result of reduced NaCl influx (J_{in}^{Na} and J_{in}^{Cl}). J_{net}^{Na} and J_{net}^{Cl} were gradually restored to control rates during prolonged (30 days) exposure to AFW.

3. The restoration of J_{net}^{Cl} in AFW was due both to increased J_{in}^{Cl} and to reduced Cl^- efflux (J_{out}^{Cl}), whereas the primary response contributing to the restoration of J_{net}^{Na} was an increase of J_{in}^{Na}.

4. The total apical surface area of branchial CCs exposed to the external environment increased markedly after 24 h in AFW and remained elevated for 1 month as a consequence of enlargement of individual CCs and, to a lesser extent, increased CC density. J_{in}^{Na} and J_{in}^{Cl} were correlated significantly with total CC apical surface area.

5. Plasma cortisol levels rose transiently in fish exposed to AFW. Treatment of NFW-adapted fish with cortisol for 10 days (a protocol known to cause CC proliferation) caused pronounced increases in J_{in}^{Cl} and J_{in}^{Na}, as measured in both NFW and AFW.

6. These results suggest that an important adaptational response of rainbow trout to low environmental [NaCl] is cortisol-mediated enlargement of branchial epithelial CCs which, in turn, enhances the NaCl-transporting capacity of the gill as a result of the proliferation of Na^+ and Cl^- transport sites.

Introduction

Euryhaline fishes are capable of surviving in aquatic habitats of widely varying

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salinities ranging from fresh water (FW) to sea water (SW). Numerous studies have addressed the physiological and biochemical adaptational adjustments associated with euryhalinity (see review by Evans, 1984). These studies have focused on FW to SW transfers and, to a lesser extent, SW to FW transfers. In addition, the extremely variable ionic concentration of FW itself (e.g. see Laurent *et al.* 1985) has prompted interest in the adaptational responses of fish to dilute external media (Olivereau, 1971; Mattheij and Stroband, 1971; Maetz, 1974; Eddy, 1975; Laurent and Dunel, 1980; Laurent *et al.* 1985; Perry and Wood, 1985; McDonald and Rogano, 1986; Avella *et al.* 1987; Leino *et al.* 1987). Typical experiments have usually involved simultaneous reductions in ambient $[Na^+]$, $[Cl^-]$ and $[Ca^{2+}]$; thus the independent effects of these three ions remain poorly defined. It is clear, however, that decreased external [NaCl] alone, or in combination with decreased external $[Ca^{2+}]$, causes diminution of plasma Na^+ and Cl^- levels (Maetz, 1974; McDonald and Rogano, 1986; Avella *et al.* 1987). The traditional explanation for the reduced plasma [NaCl] is the combined effects of (i) lowered rates of branchial Cl^- and Na^+ uptake (J_{in}^{Cl} and J_{in}^{Na}) caused by reduced availability of external Cl^- and Na^+ for the gill ionic uptake mechanisms (McDonald and Rogano, 1986), (ii) increased branchial water influx (e.g. Potts and Fleming, 1970), and (iii) increased branchial efflux of NaCl (Eddy, 1975). The latter two effects are thought to reflect increased ionic and osmotic permeabilities of the gill epithelium caused by the removal of external Ca^{2+}, although McDonald and Rogano (1986) were unable to demonstrate any effects of reduced $[Ca^{2+}]$ (with or without lowered [NaCl]) on branchial ionic effluxes in rainbow trout.

A variety of compensatory physiological adjustments accompany the internal electrolyte imbalances imposed by exposure to dilute media. These include (i) increased urine flow rate (McDonald and Rogano, 1986), (ii) prolactin-induced reductions in gill osmotic permeability (Wendelaar Bonga and van der Meij, 1981), (iii) transient reductions in gill ionic permeability (McDonald and Rogano, 1986) and (iv) persistent increases in the gill ion transport capacity (Maetz, 1974; Perry and Wood, 1985; McDonald and Rogano, 1986; Avella *et al.* 1987). The mechanisms promoting this latter compensatory response are poorly understood. It is evident, however, that proliferation of branchial chloride cells is a common response during exposure of fish to dilute media (Olivereau, 1971; Mattheij and Stroband, 1971; Laurent and Dunel, 1980; Laurent *et al.* 1985; Perry and Wood, 1985; Avella *et al.* 1987) and this response is independent of external Ca^{2+} levels (Laurent *et al.* 1985). The CC is probably an important site of NaCl uptake in FW-adapted fish (Laurent and Hebibi, 1989; P. Laurent and S. F. Perry, in preparation) and therefore CC proliferation in low-[NaCl] water may be an important mechanism contributing to the enhancement of gill NaCl transport capacity as suggested previously (Laurent *et al.* 1985; Avella *et al.* 1987). This hypothesis was tested in the present study by monitoring whole-body ionic fluxes and gill morphology to permit correlation analysis in rainbow trout exposed for varying periods to water deficient in NaCl. We have attempted to implica[illegible] cortisol as a key hormone mediating the compensatory morphological and

physiological adjustments by measuring (i) plasma [cortisol] during exposure to low [NaCl] and (ii) ionic fluxes in low-[NaCl] water after chronic treatment of normal FW trout with cortisol.

Materials and methods

Experimental animals

Rainbow trout (*Salmo gairdneri*) of either sex, but in non-breeding condition [mean mass=213±3.6 g (standard error; S.E.); experimental N=268] were obtained from Thistle Springs Trout Farm (Ashton, Ontario) and transported to the University of Ottawa where they were kept indoors in large (500 l) fibreglass tanks (Living Stream, Toledo, Ohio). All fish were maintained for at least 1 month in dechlorinated City of Ottawa tapwater which hereafter is referred to as natural fresh water (NFW). The chemical composition of NFW is given in Table 1. Fish were fed daily to satiation with commercial floating trout pellets (Purina Trout Chow) except in certain experimental groups (see below). Food always was withheld for 48 h before experimentation. Photoperiod was kept constant at 12 h light: 12 h dark.

Acclimation conditions

After at least 1 month in the laboratory, fish were divided into two groups. One group was maintained in NFW while fish in the other group were exposed acutely or chronically (see below) to continuously flowing artificially prepared low-[NaCl] water, hereafter referred to as artificial fresh water (AFW; the chemical composition of AFW is given in Table 1). AFW was prepared by titrating flowing ($1\,l\,min^{-1}$) deionized water with a stock solution of $NaCl/Ca(NO_3)_2.4H_2O/KOH$

Table 1. *Composition of the acclimation media in which fish were maintained for periods ranging between 1 h and 30 days*

	Natural fresh water (NFW)	Artificial fresh water (AFW)
$[Na^+]$ ($mmol\,l^{-1}$)	0.120±0.001 (510)	0.017±0.001 (108)
$[Cl^-]$ ($mmol\,l^{-1}$)	0.164±0.001 (533)	0.014±0.006 (108)
$[K^+]$ ($mmol\,l^{-1}$)	0.022±0.001 (510)	0.191±0.003 (108)
$[Ca^{2+}]$ ($mmol\,l^{-1}$)	0.473±0.003 (587)	0.417±0.003 (108)
pH	7.72±0.001 (77)	7.36±0.003 (77)
Temperature (°C)	6–12 (77)	8–14 (77)

Values shown are means±1 S.E.; the number of measurements is indicated in parentheses.

using a peristaltic pump to yield nominal NaCl and Ca^{2+} concentrations of 0.01 mmol l^{-1} and 0.45 mmol l^{-1}, respectively. The deionized water was provided by pumping partially deionized water [furnished by two reverse osmosis units (Culligan) connected in parallel] through two mixed-bed ion-exchange columns (Culligan D45P) connected in series. The deionized water was then cooled by passing it through stainless-steel coils immersed in water at ambient temperature. The addition of KOH to the deionized water was necessary to increase the pH to values similar to that of the NFW.

In each case, fish were maintained in 500 l opaque fibreglass aquaria; the loading density was approximately 0.02 kg l^{-1}. In addition to the continuous water flow (1 l min^{-1}) to each acclimation aquarium, the water was also aerated vigorously and filtered continuously through cottonwool and charcoal. Fish were fed daily with Purina Trout Chow equivalent to about 1.5 % body mass day^{-1}.

Acute acclimation (less than 4 days)

Fish were transferred from NFW holding aquaria into individual opaque Perspex flux boxes (volume=3 l) supplied with NFW. The water in the flux boxes was aerated and mixed as described by McDonald and Rogano (1986). Fish were allowed to recover for 48 h from the stress associated with capture and handling. Acute exposure to AFW was accomplished by stopping the flow of NFW and pumping AFW from the chronic acclimation tank (see below) to each flux box at a rate of 5 l min^{-1} for 10 min. The flow of AFW was then reduced to about 150 ml min^{-1} for the remainder of the acute exposure period. The flux boxes were flushed for 10 min with AFW at a rate of 5 l min^{-1} at 12 h intervals. The control fish were supplied with NFW from the chronic control acclimation tank in an identical manner. Fish were not fed while in the Perspex boxes. Mortality was zero in the NFW fish and about 4 % in the AFW-exposed fish.

Chronic acclimation (4–30 days)

Appropriate numbers of fish were placed into NFW in each acclimation aquarium. In the control group, NFW flow was continued to the aquarium, whereas in the experimental group the flow of NFW was stopped and the flow of AFW begun (see above). With this gradual process of acclimation, the desired final [NaCl] was achieved within 36 h, mortality was zero, and the fish resumed eating after about 48 h. The control fish were not fed until the AFW-exposed fish resumed eating. Fish were transferred to Perspex flux boxes 48 h before beginning a flux determination.

Cortisol treatment

Two additional groups of fish were kept isolated in NFW in Perspex flux boxes and injected (intramuscularly) once a day with 4 mg kg^{-1} body mass of cortisol (hydrocortisone hemisuccinate sodium salt; Sigma) dissolved in 0.4 ml kg^{-1} of 0.6 % NaCl, for a period of 10 days. Sham-treated fish were injected with 0.6 % NaCl for 10 days. Fish were not fed while in the Perspex boxes.

Experimental protocol

Experiments were performed on intact rainbow trout to determine the effects of acute or chronic acclimation to AFW and cortisol treatment on whole-body net and unidirectional fluxes of Na^+ and Cl^-, gill filamental chloride cell morphology, plasma electrolyte levels and plasma cortisol levels.

In all experiments, ionic fluxes were determined simultaneously on control and experimental fish. Two consecutive 1-h flux determinations (separated by a 15 min rinsing period) were performed. In the first, fluxes were measured in the appropriate acclimation medium (NFW in the control fish, AFW in the experimental fish), whereas in the second, fluxes were always measured in NFW. After completing the second flux, fish were killed by over-anaesthesia with $0.5\,g\,l^{-1}$ ethyl-*m*-aminobenzoate (MS 222; Sigma) neutralized with $1.0\,g\,l^{-1}$ $NaHCO_3$. A blood sample (1.0 ml) was removed by caudal puncture, centrifuged at 13 000 *g* for 1 min, and the plasma removed and stored at −20°C for subsequent analysis of ion concentrations (see below). A portion of gill from the second arch (left side) was excised, washed in water (to remove mucus) and then fixed for 1 h in ice-cold 2.5% glutaraldehyde in $0.15\,mol\,l^{-1}$ sodium cacodylate buffer (pH 7.4). Tissue was rinsed twice in $0.15\,mol\,l^{-1}$ cacodylate buffer and placed in a solution of 2% osmium tetroxide (in cacodylate buffer) for 1 h. Next, the filaments were washed three times in distilled water, immersed for 30 min in 30% followed by 50% ethanol, and then transferred to 70% ethanol. Samples were shipped to Strasbourg in 70% ethanol where the morphological measurements were performed (see below).

To determine the effects of AFW exposure on plasma cortisol levels, a slightly modified protocol was adopted in which fluxes were not measured and acclimation water was not switched to NFW. This was because preliminary experiments demonstrated that the switch from AFW (first flux) to NFW (second flux) often resulted in elevated cortisol levels compared with fish kept in AFW. This was most obvious in the fish exposed to AFW for periods exceeding 1 week.

Whole-body ion fluxes

Whole-body ion fluxes were determined in cortisol/sham-treated and in NFW- and AFW-acclimated fish. In acutely acclimated fish, fluxes were determined at 0, 6, 12, 24 and 48 h, whereas in the chronically acclimated fish, fluxes were determined at 4, 7, 14 and 30 days. Water flow to the flux boxes was stopped for 1 h periods during which the partial pressure of O_2 in the water was maintained by vigorous aeration. Water temperature was kept constant by partially immersing the flux boxes in cooling baths.

Whole-body influxes of Na^+ (J_{in}^{Na}) and Cl^- (J_{in}^{Cl}) were determined on separate groups of fish by monitoring the disappearance of ^{22}Na (as NaCl; Amersham) or ^{36}Cl (as HCl; ICN) from the external environment after the addition of 0.2 μCi (7.4 kBq) to AFW or 2.0 μCi (74 kBq) to NFW. In this way, specific activities of Na^+ and Cl^- were approximately equal in AFW and NFW. A water sample

(10 ml) was removed after a 5 min mixing period and another 1 h later. Activity of ^{22}Na or ^{36}Cl was determined immediately on 5 ml samples while the remainder was stored at 6°C for subsequent ionic analyses (Na^+, Cl^-, Ca^{2+} and K^+). From these measurements, unidirectional fluxes (J_{in} and J_{out}) and net fluxes (J_{net}) were calculated (Maetz, 1956). Backflux correction was not necessary.

Analytical procedures

Water and plasma analysis

Water and diluted (200×) plasma [Na^+], [Ca^{2+}] and [K^+] were determined by flame emission spectrophotometry (Varian model Spectra AA-10). Water and plasma [Cl^-] were determined by amperometric titration (Buchler–Cotlove chloridometer) on undiluted 1 ml and 100 μl samples, respectively. ^{22}Na and ^{36}Cl activities were determined on 5 ml water samples by liquid scintillation counting (LKB 1215 Rackbeta). Water pH was measured using a Fisher Acumet pH meter and gel-filled polymer body electrodes. Water P_{O_2} was monitored using a P_{O_2} electrode (Radiometer E5046) in conjunction with a Radiometer PHM-71 acid–base analyser and BMS3 MK2 blood micro system.

Plasma [cortisol] was measured using a commercial ^{125}I liquid-phase radioimmunoassay (Biomega Inc.) on duplicate 20 μl samples.

Gill histology and morphometric measurements

In Strasbourg, most of the gill arch tissue was removed with a razor blade but a few pairs of filaments were left attached to the septum of the arch. After complete dehydration in absolute ethanol, the filamental tissue was soaked in two successive baths (2 min each) of 1,1,1,3,3,3-hexamethyldisilizan (Aldrich) and air-dried. This method is as acceptable as critical-point drying for scanning electron microscopy (SEM) and causes little shrinkage or deformation of the filamental or lamellar epithelia. The pairs of filaments were glued with silver paint onto specimen stubs of a Stereoscan 100 (Cambridge Ltd) scanning electron microscope and gold-coated. The tissue was oriented in a way that maintained the side of the filaments parallel with the stub plate.

Epithelial surfaces on the trailing edge of the filaments at the point of separation from the septum and close to the base of lamellae were located and focused on the video screen. Anterior and posterior filaments were photographed. To obtain a good compromise between the size of the area on screen and the definition of the scanning image, photographs were taken at 1000× magnification which yielded a field area of 8360 μm^2. At least four non-contiguous fields were photographed from each filament for subsequent morphometric measurements. 48 fields were measured per fish.

The apical surface area of individual chloride cells was determined by outlining the perimeter of chloride cells on a digitizer tablet (Summagraphics) assisted by a microcomputer (CBM 8096SK) and a morphometry program. The error in the measurements is less than 1 % as long as the cursor displacement speed is kept

constant (Cornelisse and van den Berg, 1984). From these measurements, the mean apical surface area per unit of filamental surface was calculated. The number of chloride cells per unit of epithelial surface area (chloride cell density) was determined using low-magnification SEM photographs.

Data analysis

The statistical significance of the differences in means within and between groups was determined using two-way ANOVA followed by Student–Newman–Keuls test. 5 % was accepted as the fiducial limit of significance. The significance of Spearman correlation coefficients was determined by ANOVA using a statistical software program (Statgraphics).

Results

Effects of AFW exposure on whole-body ion fluxes

Upon immediate exposure to AFW, fish experienced pronounced losses of both Cl^- and Na^+ to the environment, as indicated by the reversal of J_{net}^{Cl} (Fig. 1A) and J_{net}^{Na} (Fig. 2A) from positive values of about 30–50 μmol kg^{-1} h^{-1} to markedly negative values. The initial reductions of J_{net} were due entirely to lowered rates of J_{in}^{Cl} (Fig. 1A) and J_{in}^{Na} (Fig. 2A); J_{out} of either ion was unaffected in acutely exposed fish. J_{net}^{Cl} and J_{net}^{Na} gradually increased (i.e. became less negative) as the duration of acclimation increased. After 1 month of acclimation to AFW, the net fluxes determined in AFW were not significantly different from the net fluxes in control (NFW-acclimated) fish (Figs 1A, 2A).

The gradual restoration of J_{net}^{Cl} was due to the combined effects of increased J_{in}^{Cl} and decreased J_{out}^{Cl} (Fig. 1A). The compensatory changes in unidirectional Cl^- fluxes became apparent after 4 days of acclimation to AFW (J_{in}^{Cl} was elevated transiently at 24 h). The progressive increases in J_{in}^{Cl} in AFW, though highly significant, were not sufficient to restore J_{in}^{Cl} to control rates.

Unlike the adjustment of J_{net}^{Cl}, the gradual increase in J_{net}^{Na} was due exclusively to an elevation of J_{in}^{Na} (except for a transitory decrease in J_{out}^{Na} at 2 weeks) that was apparent after only 6 h of AFW-exposure (Fig. 2A). The changes in J_{in}^{Na} were more pronounced than the changes in J_{in}^{Cl} but still insufficient to raise J_{in}^{Na} to control values, even after an exposure of 1 month.

The time-dependent increases in J_{net} and J_{in} associated with AFW exposure (Figs 1, 2) were much greater when determined in NFW. Pronounced increases in J_{net}^{Cl} and J_{net}^{Na} were observed after 48 h and 24 h, respectively, and persisted throughout the remainder of the AFW acclimation period. The stimulation of J_{net} was due to increased rates of J_{in} and, to a lesser extent, reduced J_{out} (J_{net}^{Cl} only). It is noteworthy that the increases in J_{in}^{Na} measured in AFW occurred more rapidly (significantly increased after 6 h) than when measured in NFW (significant *sustained* increase after 4 days).

Gill morphological adjustments

The morphometric changes in the filamental chloride cell population that accompanied the acclimation of trout to AFW are illustrated in Figs 3–6. The

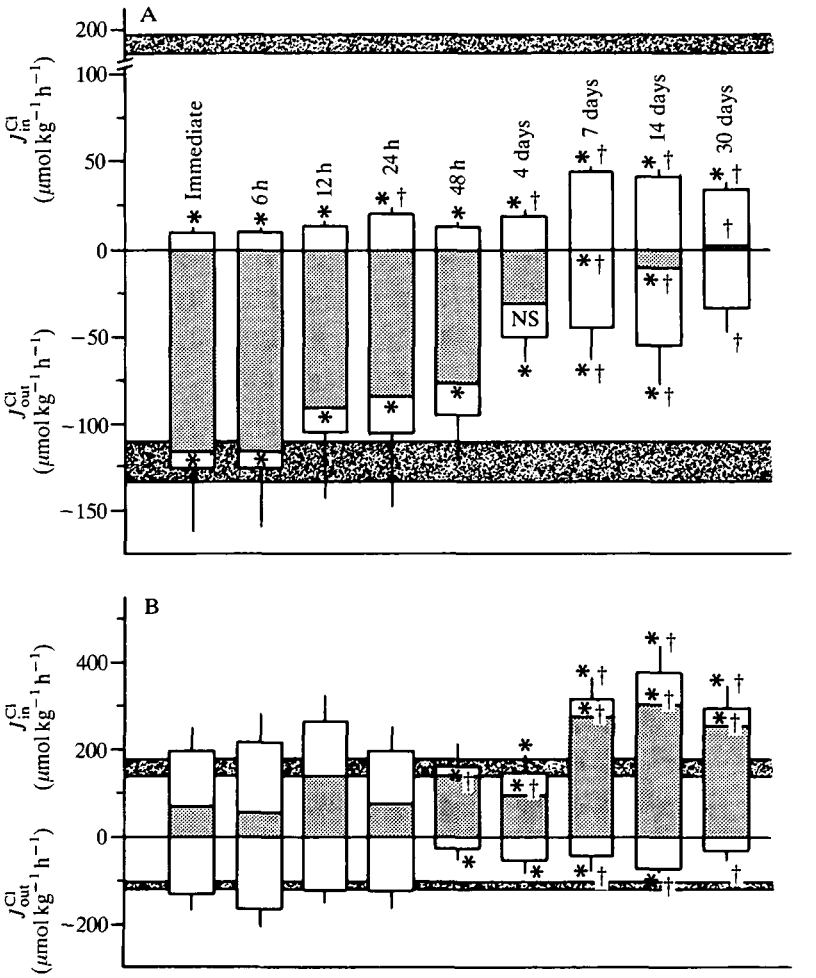


Fig. 1. The effect of acclimation to artificial fresh water (AFW) (N=6 different fish at each acclimation time) on whole-body chloride fluxes in rainbow trout (*Salmo gairdneri*) determined during two successive 1 h flux periods. Open bars indicate unidirectional Cl^- fluxes (±1 S.E.) and the shaded areas, net Cl^- fluxes. The first flux determination (A) was measured in the appropriate acclimation medium (natural fresh water, NFW, in the control fish; AFW in the experimental fish) whereas the second flux (B) was measured in NFW after a 15 min rinsing period. For clarity, the unidirectional flux values in the control fish (NFW-acclimated; N=6 different fish at each acclimation time) have been averaged, since there were only minor changes over time, and are represented by the horizontal stippled bars (±1 S.E.). * Significantly different from the control fish at corresponding time; † significantly different from the immediate exposure value; NS, non-significant. Note the difference in scale of the vertical axes of A and B.

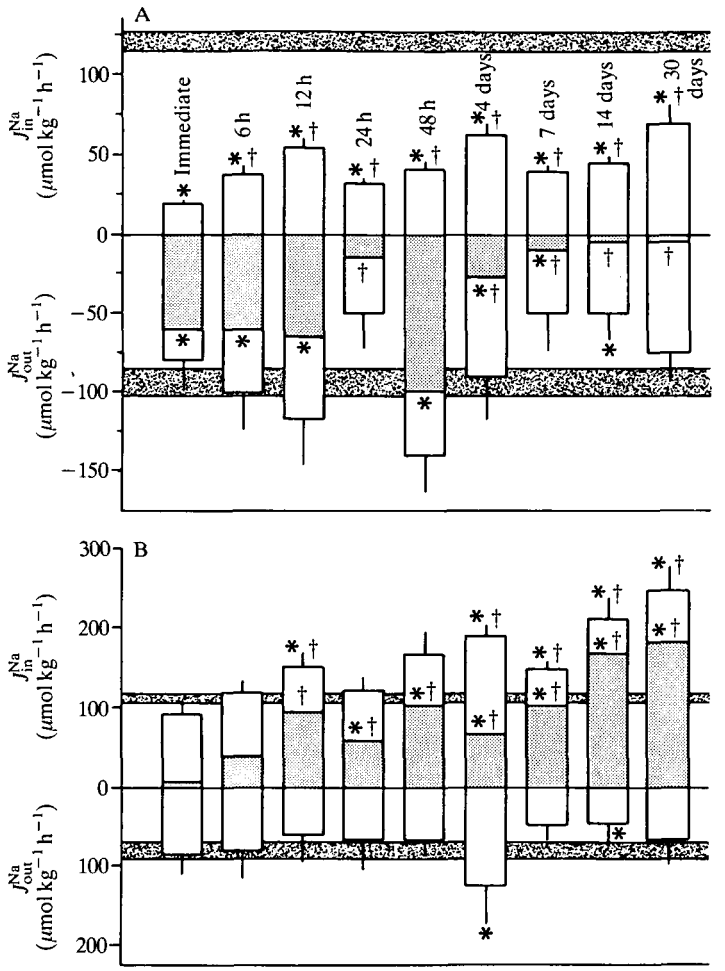


Fig. 2. The effect of acclimation to AFW on whole-body sodium fluxes in rainbow trout (*Salmo gairdneri*). All other details as in Fig. 1.

total filamental surface area (Fig. 3A) occupied by chloride cells with exposed apical surfaces increased 1.5- to 2.5-fold after 24 h in AFW when compared with the NFW-acclimated fish (compare Figs 4 and 5). Occasionally, the NFW-acclimated fish also displayed increased total chloride cell apical surface area during the acclimation period (e.g. at 24 h and 4 weeks; Fig. 3). The increased apical surface area of chloride cells during acclimation was due almost exclusively to increased apical surface areas of individual chloride cells (Fig. 3B) rather than to increased numbers of cells per unit surface area (Fig. 3C). Although the numbers of chloride cells did increase during AFW exposure (Figs 3C, 5), we feel this result may be a statistical artefact related to the significantly reduced chloride cell density in the immediately exposed group (Fig. 3C). Further, except at 48 h,

the AFW-exposed fish did not display elevated cell numbers in comparison with the NFW-acclimated group (Fig. 3C).

Scanning electron micrographs of gill tissue from trout displaying high rates of J_{in}^{Cl} and J_{in}^{Na} measured in NFW are shown in Fig. 6A (J_{in}^{Cl}=623.1 μmol kg^{-1} h^{-1}) and 6B (J_{in}^{Na}=273.5 μmol kg^{-1} h^{-1}), respectively. The enlargement of chloride cells and protuberance of mucus cells is very evident. In Fig. 6A the expansion of chloride cells and proliferation of mucus cells have nearly obliterated the once plentiful pavement cell population. The fish from which these gill tissues were

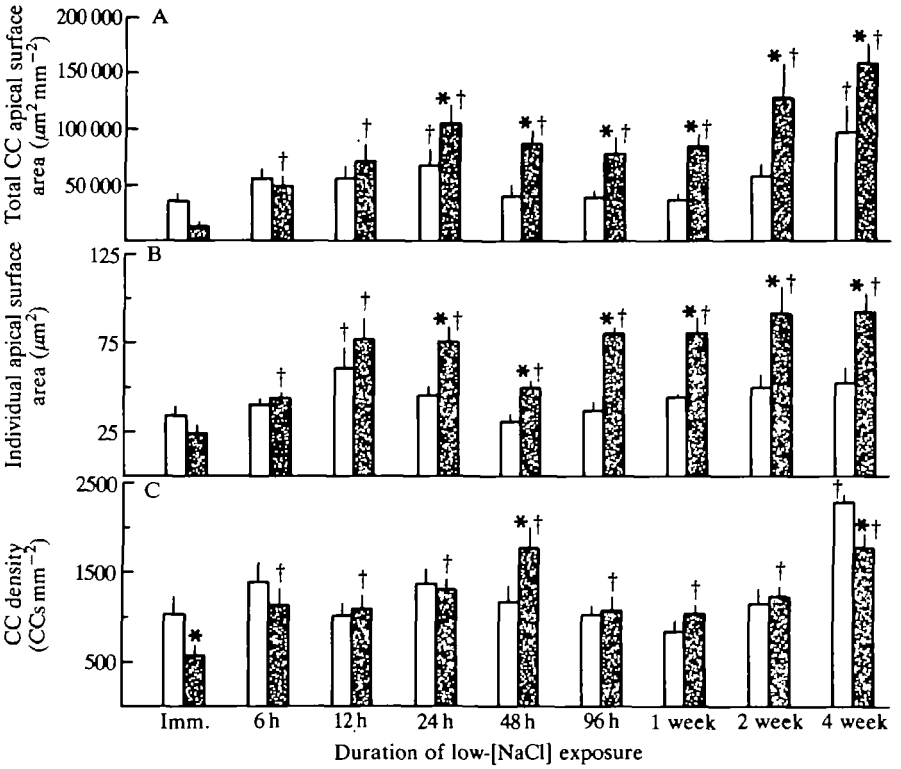
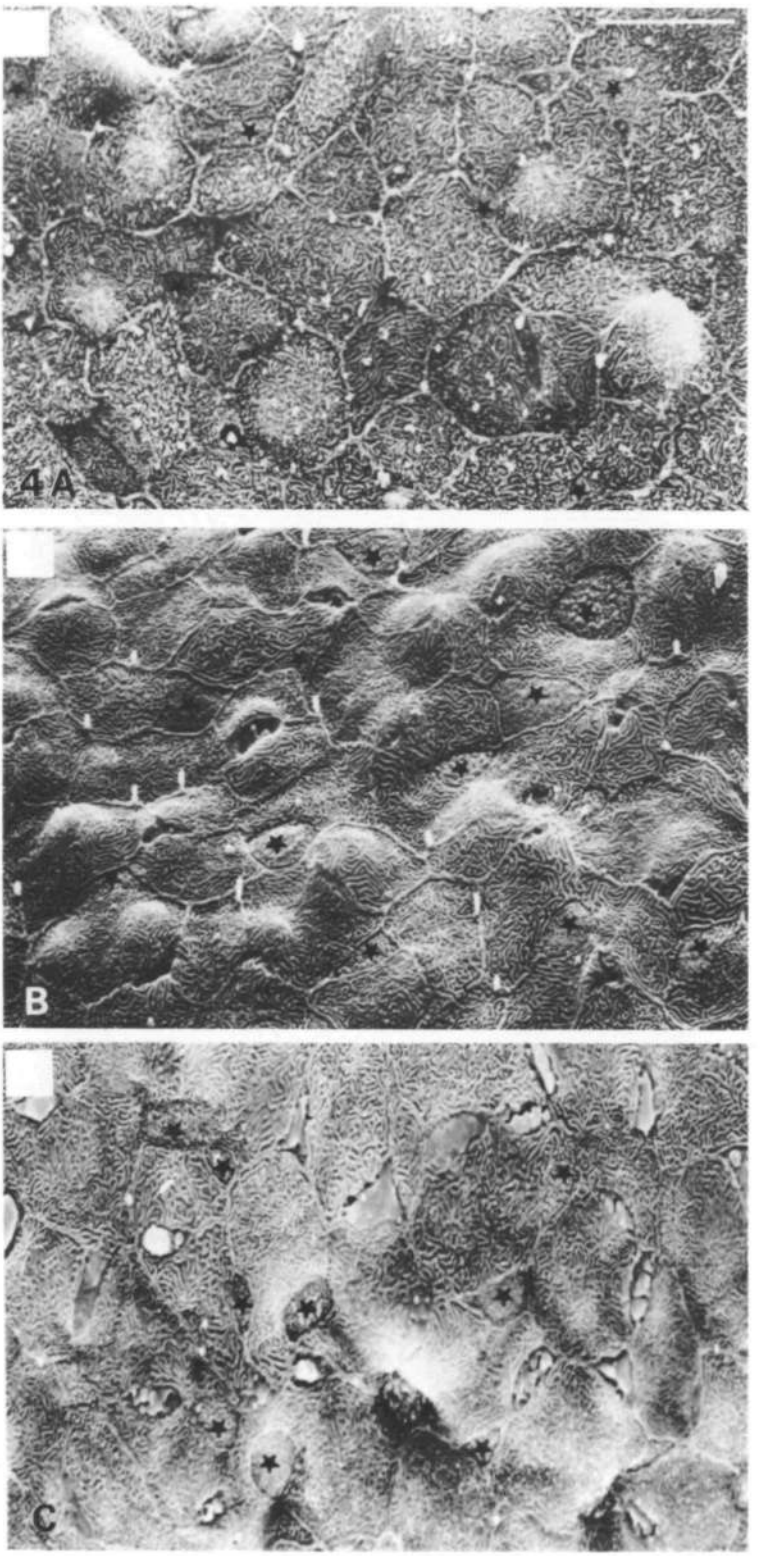


Fig. 3. The effect of acclimation to AFW on branchial morphological parameters including (A) the total apical surface area of filamental chloride cells (CCs), (B) the apical surface area of individual CCs and (C) the number of CCs per unit of filamental epithelium (CC density). The control fish (NFW-acclimated; N=12 different fish at each acclimation time) are represented by the clear bars; the experimental fish (AFW-acclimated; N=12 different fish at each acclimation time) by the stippled bars. Values shown are means±1 S.E. All other details as in Fig. 1. Imm, immediate.

Fig. 4. Representative scanning electron micrographs of gill filamental tissue showing the distribution and appearance of pavement cells (unlabelled) and chloride cells (labelled with stars) in control (NFW-acclimated) rainbow trout after (A) immediate exposure, (B) 48 h of exposure and (C) 2 weeks of exposure to NFW. Note the relative constancy of the filamental CC population as a function of time in the control fish. The horizontal bar in A represents 20 μm (same magnification for A, B and C).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B
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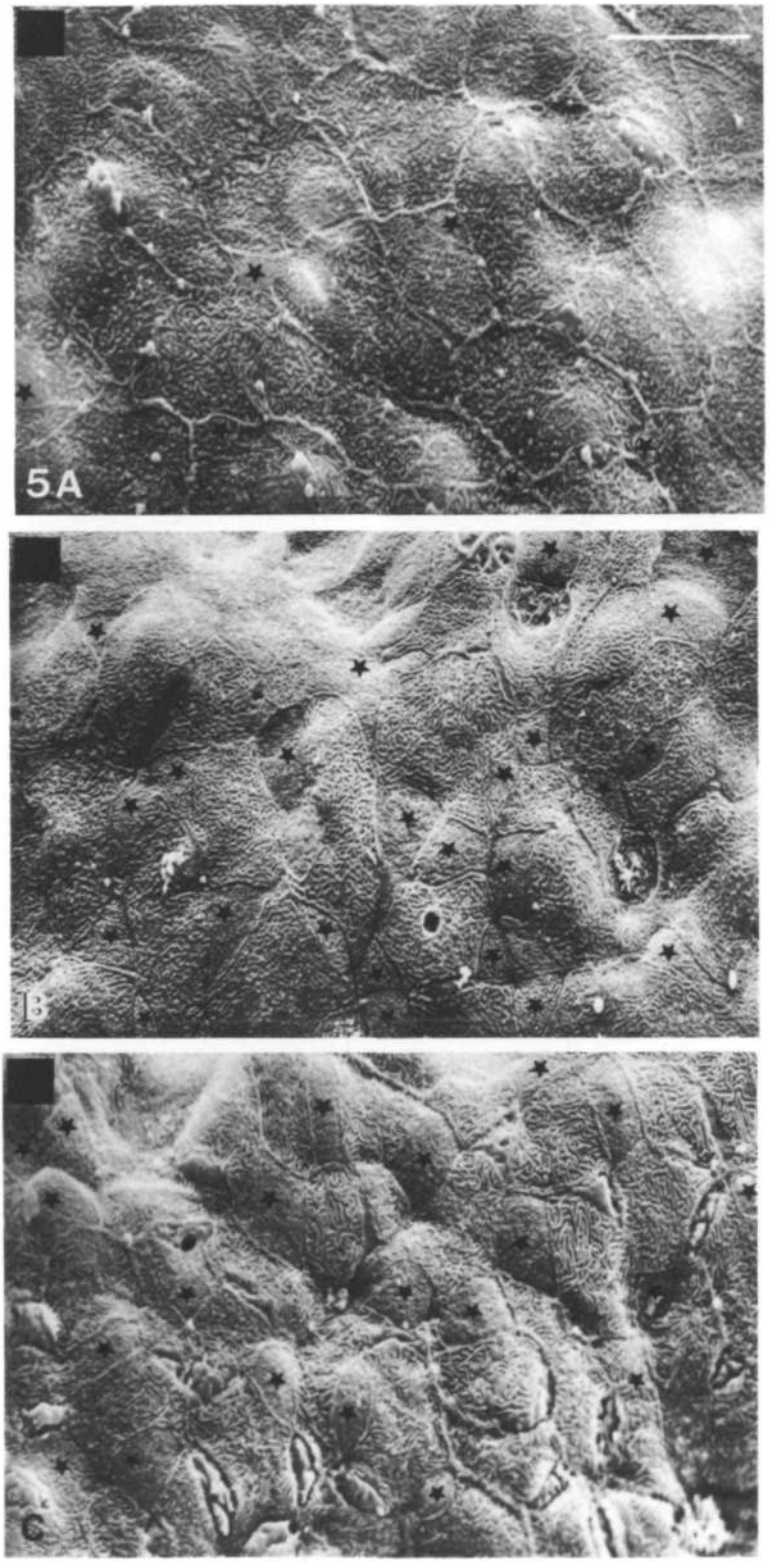
5A
B
C

Fig. 5. Representative scanning electron micrographs of gill filamental tissue showing the distribution and appearance of pavement cells (unlabelled) and chloride cells (labelled with stars) in experimental (AFW-acclimated) rainbow trout after (A) immediate exposure, (B) 48 h of exposure and (C) 2 weeks of exposure to AFW. Note the approximate doubling in the size of the individual CCs, the increased CC density and the fivefold increase in total CC apical surface area in the fish exposed for 48 h and 2 weeks. The horizontal bar in A represents 20 μm (same magnification for A, B and C).

taken not only displayed much higher than average measured rates of ion uptake but also two of the largest total chloride cell apical surface areas [151 272 $\mu m^2 mm^{-2}$, Fig. 6A; 222 761 $\mu m^2 mm^{-2}$, Fig. 6B]. The apparent relationship between total chloride cell apical surface area and rates of ion uptake was the basis of a subsequent detailed quantitative correlation analysis.

Correlation between chloride cell surface area and ion uptake

The experimental protocol utilized in this study, in which gill morphometric measurements were conducted on tissue from every fish in which J_{in}^{Na} or J_{in}^{Cl} was measured, permitted correlation of ion uptake rates during AFW acclimation with various morphometric parameters. Both J_{in}^{Cl} and J_{in}^{Na} determined in AFW or NFW were correlated significantly with total filamental chloride cell apical surface area (Fig. 7). Ion uptake rates were also significantly correlated with apical surface area of individual chloride cells (data not shown). No significant correlations existed between rates of ion uptake and chloride cell numbers (data not shown).

Plasma [cortisol] and effects of cortisol on ion uptake

Plasma cortisol levels were measured at selected times during the 30 day acclimation to AFW. Between 12 h and 48 h in AFW there were significant three- to fourfold increases in plasma [cortisol]. Cortisol levels returned to control values by 1 week and were not significantly different after 1 month (Table 2).

Cortisol-treated fish displayed elevated J_{in}^{Cl} and J_{in}^{Na} determined both in NFW and in AFW (Table 3). Plasma cortisol levels were not measured in these experiments but a previous study using a similar protocol (Perry and Wood, 1985) showed that [cortisol] increased to approximately 700 ng ml^{-1}. Repeated injections of cortisol cause large oscillations in plasma cortisol levels, so the very high [cortisol] reported by Perry and Wood (1985) may represent the peak of such an oscillation. Nevertheless, this protocol is known to cause an increase of total apical surface area of the chloride cells (Perry and Wood, 1985; P. Laurent and S. F. Perry, in preparation).

Plasma ions

Plasma [Na^+] and [Cl^-] were significantly depressed after 4 days in AFW (Table 4). The pattern of change was essentially identical for both ions. The greatest reduction (38.5 mmol l^{-1} for Na^+; 42.8 mmol l^{-1} for Cl^-) was observed after 2 weeks of acclimation. Even after 1 month of acclimation, levels of both ions

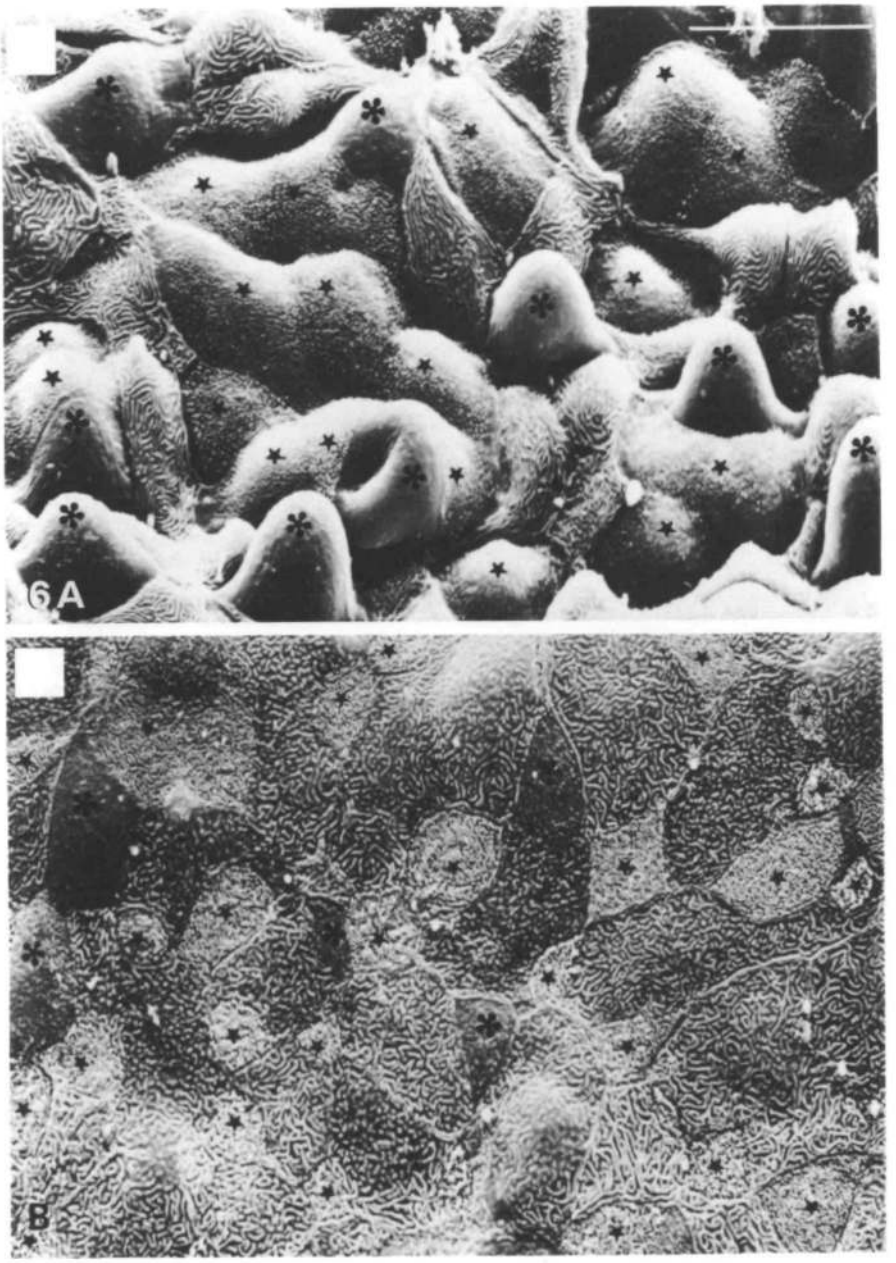

Fig. 6. Representative scanning electron micrographs of gill filamental tissue from fish displaying high rates of (A) chloride uptake (J_{in}^{Cl}=623.1 μmol kg^{-1} h^{-1}; this fish was acclimated to AFW for 2 weeks) and (B) sodium uptake (J_{in}^{Na}=273.5 μmol kg^{-1} h^{-1}; this fish was acclimated to AFW for 1 month) measured in NFW. Note the extensive increase in the proportion of the filamental surface area occupied by CCs (labelled with stars) and the appearance of numerous mucus cells (labelled with asterisks). The horizontal bar in A represents 20 μm (same magnification for A and B).

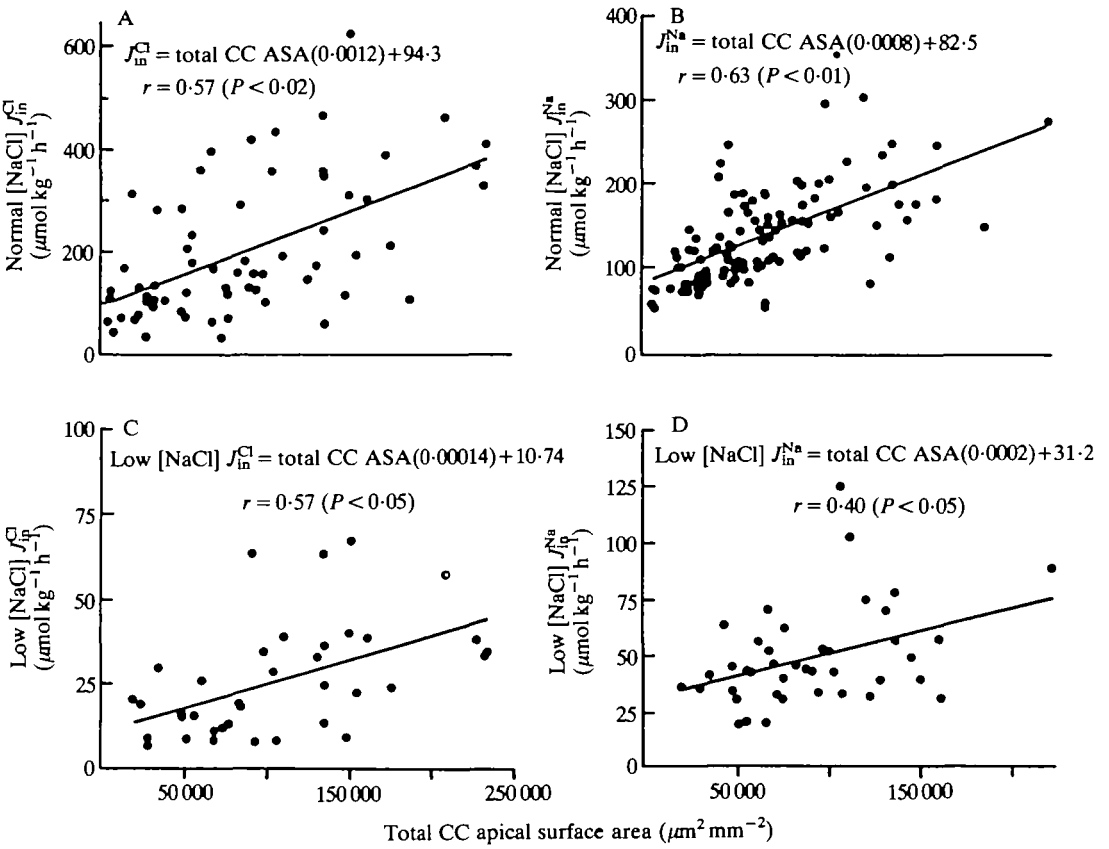

Fig. 7. The relationship between filamental total chloride cell (CC) apical surface area (ASA) and ion uptake (J_{in}^{Cl} and J_{in}^{Na}) in fish acclimated to NFW or AFW for 1 h to 30 days. Fluxes were determined either in (A, B) NFW (normal [NaCl] J_{in}^{Cl}; J_{in}^{Na}) or (C, D) AFW (low [NaCl] J_{in}^{Cl}; J_{in}^{Na}).

Table 2. *The effect of exposure to artificial fresh water (AFW) on plasma cortisol levels in rainbow trout*

	[Cortisol] (ng ml^{-1})	
Time	NFW	AFW
12 h (6)	23.7±7.8	78.7±13.3*
24 h (6)	30.1±6.3	104.2±21.1*
48 h (5)	27.4±8.4	97.8±19.8*
1 week (5)	32.5±6.0	50.0±16.1
30 days (6)	20.9±4.8	30.4±8.2

Values shown are means±1 S.E.; numbers of fish are indicated in parentheses.
* Significantly different from value at corresponding time in natural fresh water (NFW).

Table 3. *The effects of chronic (10 day) cortisol treatment on whole-body chloride uptake (J_{in}^{Cl}) and sodium uptake (J_{in}^{Na}) in rainbow trout during successive 1 h flux periods, first in natural fresh water (NFW) and then in artificial fresh water (AFW; low external [NaCl])*

	Cortisol-treated		Sham-treated	
	NFW	AFW	NFW	AFW
J_{in}^{Na} (μmol kg^{-1} h^{-1})	220.0±37.8* (6)	36.3±3.4*	122.4±17.3 (6)	18.6±3.2
J_{in}^{Cl} (μmol kg^{-1} h^{-1})	233.7±29.9* (6)	29.0±2.4*	138.4±21.5 (6)	15.0±2.3

Values shown are means±1 S.E.; numbers of fish are indicated in parentheses.
* Significantly different from sham-treated fish.

remained depressed compared with the control (NFW) fish. No changes in plasma $[K^+]$ or $[Ca^{2+}]$ were observed (data not shown).

Discussion

Methodology

The objective of the present study was to examine the adaptational responses of rainbow trout to lowered external [NaCl] alone, rather than to 'soft water' which usually refers to water deficient in Na^+, Cl^- and Ca^{2+} (e.g. McDonald and Rogano, 1986). It is clear, however, that we were only partially successful in preparing low-[NaCl] water (AFW) otherwise identical to City of Ottawa tapwater

Table 4. *The time-dependent effects of exposure to artificial fresh water (AFW) on sodium and chloride concentrations in rainbow trout plasma*

	$[Na^+]$ (mmol l^{-1})		$[Cl^-]$ (mmol l^{-1})	
Time	NFW (12)	AFW (12)	NFW (6)	AFW (6)
Immediate	127.3±2.4	128.4±1.7	120.4±2.4	121.3±2.9
6 h	128.8±2.2	129.2±2.5	118.4±1.8	117.5±1.7
12 h	127.2±4.3	126.4±4.7	118.6±3.6	114.1±3.7
24 h	128.4±3.7	132.9±3.9	122.0±2.9	121.5±3.4
48 h	134.3±5.3	134.1±4.4	124.0±4.8	120.0±5.1
4 days	139.7±3.6	131.2±2.2*	135.4±2.9	120.5±2.5*
1 week	136.7±3.3	126.7±4.9*	136.0±2.5	121.4±1.1*
2 weeks	136.1±1.6	97.6±2.3*	124.6±4.0	81.8±1.9*
30 days	133.3±1.5	105.9±2.2*	132.6±1.8	89.2±2.8*

Values shown are means±1 S.E.; numbers of fish are indicated in parentheses.
* Significantly different from value at corresponding time in natural fresh water (NFW).

(NFW; see Table 1). KOH was added to AFW to raise pH near to NFW values and consequently $[K^+]$ in AFW was about ninefold higher than in NFW. The elevation of $[K^+]$ in AFW to a final concentration of $0.19\,mmol\,l^{-1}$ (Table 1) was not reflected by increased plasma $[K^+]$ in the AFW-acclimated fish. The use of KOH to adjust pH of dilute acclimation media is a common laboratory procedure (e.g. Graham *et al.* 1982). In these instances, the maintenance of circumneutral pH is considered more important than constant external $[K^+]$. The concentration of $[Ca^{2+}]$ in AFW was 12% less than in NFW ($0.42\,mmol\,l^{-1}$ compared to $0.47\,mmol\,l^{-1}$) but still above the boundary separating 'hard' and 'soft' waters ($0.4\,mmol\,l^{-1}$; Marier *et al.* 1979). Thus, it is likely that the physiological disturbances and adaptational responses associated with acclimation to AFW were due solely to reduced ambient [NaCl].

Whole-body, rather than branchial, ion fluxes were measured in this study to avoid implanting urinary bladder cathethers into 240 rainbow trout in various states of acclimation. Renal effluxes of Na^+ and Cl^- in resting trout in NFW are about 10–15% of branchial effluxes (Perry *et al.* 1987*a*,*b*) and largely unaffected by exposure to ion-poor water despite significant increases in urine flow rate (McDonald and Rogano, 1986). For this reason, together with the relative ionic impermeability of fish skin, whole-body ion fluxes essentially indicate branchial fluxes. Thus, throughout this paper we have attributed changes in the rates of whole-body ion fluxes to changes in gill ion movements.

At each acclimation time (immediate to 30 days), fluxes were measured first in acclimation medium and then in NFW. The rationale for this protocol was to increase the ability to detect adaptational flux adjustments (we considered it feasible that changes in ion-transporting capacity would be more obvious in NFW than AFW). This methodology did not permit us to distinguish quantitatively between adjustments in the affinity of the ion transporters from adjustments in maximal transporting capacity. This is normally accomplished by measuring ion uptake (J_{in}) over a wide range of external [NaCl] until saturation of the ion-transporting mechanisms is achieved. Changes in transport capacity are thought to arise from altered numbers of ion transport sites, while changes in affinity are presumably caused by alteration(s) of existing ion carriers. It is important to emphasize that adjustments of ion uptake measured both in AFW and in NFW can potentially reflect changes in affinity and/or altered numbers of ion transport sites.

Ion flux adjustments in AFW

The approximately 10-fold reduction in external [NaCl] caused pronounced reductions in J_{in}^{Cl} and J_{in}^{Na} in a manner predicted by the Michaelis–Menton relationship between J_{in} and external NaCl concentration (e.g. K_m for J_{in}^{Cl} in trout=$0.25\,mmol\,l^{-1}$ Cl^-; Kerstetter and Kirschner, 1972). The reductions of J_{in}^{Na} and J_{in}^{Cl} were the sole causes of the NaCl losses occurring upon exposure to AFW. The classical view of Na^+ and Cl^- uptake in freshwater fishes (e.g. Maetz, 1971) is that absorption from the external medium is accomplished by electroneutral exchange with the internal counter ions NH_4^+/H^+ and HCO_3^-/OH^-, respect-

ively, or alternatively with internal Na^+ and Cl^-. The latter process is termed exchange diffusion. Several variations of this scheme have been proposed (e.g. Kirschner, 1988), although in each the idea of inward movement of Na^+ and Cl^- across the apical membrane of gill epithelial cells coupled to outward movement of similarly charged ions with 1:1 stoichiometry is conserved. Although it is likely that exchange diffusion does occur in the fish gill, the absence of any reduction in J_{out}^{Na} or J_{out}^{Cl} during the pronounced reductions in J_{in}^{Na} and J_{in}^{Cl} indicates that exchange diffusion is not an obligatory component of Na^+ or Cl^- efflux. This seems most certain for J_{out}^{Na} because of the absence of any potential counter ion in AFW, but less certain for J_{out}^{Cl} because of the possibility of Cl^- efflux coupled to uptake of nitrate substituting for chloride [$Ca(NO_3)_2 \cdot 4H_2O$ was used in preparing AFW]. The constancy of NaCl efflux during the initial 4 days of acclimation to AFW is in contrast to the results of the study of McDonald and Rogano (1986) in which J_{out}^{Cl} and J_{out}^{Na} were reduced during the initial 3 days of exposure to 'soft water'. The reductions of Na^+ and Cl^- efflux reported by McDonald and Rogano (1986) are surprising since ambient [Ca^{2+}] was reduced in that study and similar reductions of external [Ca^{2+}] have been shown to increase the branchial permeability to NaCl in previous studies (Eddy, 1975; McDonald *et al.* 1983).

A compensatory reduction in net NaCl loss was apparent after about 4 days in AFW. The adaptational flux adjustments serving to restore J_{net}^{Na} and J_{net}^{Cl} gradually to normal values, however, were markedly different for each ion, although certain responses were shared.

Restoration of J_{net}^{Cl}

The gradual restoration of J_{net}^{Cl} in AFW-acclimated fish was due to increased J_{in}^{Cl} as well as reduced J_{out}^{Cl}. Both these adaptational responses were apparent after 4 days of acclimation. The persistent reduction of J_{out}^{Cl} indicates a diminution of branchial Cl^- permeability in response to the lowered [NaCl]. Such a compensatory response to acclimation to dilute media has not been reported previously, although McDonald and Rogano (1986) demonstrated a reduction of both J_{out}^{Cl} and J_{out}^{Na} in the first 3 days of a 9 day exposure of trout to 'soft water'. It is difficult to speculate on the mechanism(s) causing reduced Cl^- permeability because the transepithelial pathway for branchial Cl^- (or Na^+) efflux in freshwater fish is poorly defined. A general reduction in branchial ion permeability is unlikely because Na^+ efflux was unaffected (see below). The delay in the onset of J_{out}^{Cl} reduction in the present study suggests the involvement of a slow-acting hormone. Prolactin is a possible candidate since its secretion is known to increase in dilute media (Wendelaar Bonga and van der Meij, 1981). Furthermore, prolactin decreases gill osmotic permeability and induces electrolyte retention (see review by Hirano, 1986). Prolactin is believed to decrease gill water permeability by affecting the conductance of epithelial paracellular pathways; thus, it is conceivable that Cl^- efflux is paracellular. The prolactin-induced proliferation of mucus cells (Wendelaar Bonga and Meis, 1981; see also Fig. 6) and subsequent mucus secretion may also limit Cl^- efflux. Regardless of the mechanism, the specificity of

the efflux modulation suggests differential routes for Na^+ and Cl^- efflux in the gill of freshwater fish.

The other component contributing to the gradual increase of J_{net}^{Cl} during acclimation to AFW was sustained stimulation of J_{in}^{Cl} after 4 days. We did not determine J_{max} in the present study thus we are unable to provide definitive evidence that the Cl^--transporting capacity (J_{max}) of the gill increased. However, the pronounced elevation of J_{in}^{Cl} in AFW-acclimated fish, measured in NFW, above the control rates as well as published values for J_{max} (Kerstetter and Kirschner, 1972) does suggest an augmentation of maximal transporting capacity. The delayed stimulation of J_{in}^{Cl} is not consistent with affinity-modulation but implies a gradual proliferation of Cl^- transport sites (see below).

Restoration of J_{net}^{Na}

The primary response correcting the loss of Na^+ in AFW was stimulation of J_{in}^{Na}. The initial compensatory response occurred within 6 h and for this reason probably involved an increase of the affinity rather than the capacity for Na^+ transport. Unlike the stimulation of J_{in}^{Cl}, there was obvious dissociation (except at 12 h) between the time course of the augmentation of uptake in AFW *versus* augmentation in NFW. This also supports our view that a sustained increase in ion transport capacity did not occur until 4 days of AFW acclimation had passed. The cause of the increased Na^+ transport affinity after 6 h in AFW is unknown, although mobilization of circulating catecholamines could be involved. Girard and Payan (1980) showed that elevated epinephrine levels increased J_{in}^{Na} in a perfused trout head preparation. This response, however, is not reproducible *in vivo* (McDonald and Rogano, 1986; Vermette and Perry, 1987). It is unlikely that elevated [cortisol] is involved because acute (12 h) intra-arterial infusion of cortisol does not affect J_{in}^{Na} (P. Laurent and S. F. Perry, in preparation). An additional possibility is that increased mucus secretion may create a zone of elevated $[Na^+]$ immediately adjacent to the gill because of the ability of mucus to bind cations. It is puzzling that the rapid increase in the affinity of the Na^+ uptake mechanism, which was obvious during the AFW flux, was not apparent during the NFW flux (Fig. 2). A possible explanation is that the factor(s) causing the acute affinity increase for Na^+ is activated only in AFW and is inactivated immediately upon reimmersion in NFW.

Although the mechanism causing the acute increase in J_{in}^{Na} is unclear, we suggest that after 4 days of exposure to AFW the persistent stimulation of both Na^+ and Cl^- transport was due to cortisol-mediated proliferation of ion uptake sites. This, in turn, resulted from increased apical surface area of branchial chloride cells.

Relationship between chloride cell morphology and ion uptake in AFW-exposed fish

Evidence is accruing that the branchial chloride cell is an important site of Na^+ and Cl^- uptake in FW fish (Laurent *et al.* 1985; Gardaire *et al.* 1985; Avella *et al.* 1987; Laurent and Hebibi, 1989; P. Laurent and S. F. Perry, in preparation). This

is not surprising considering the high density of mitochondria within CCs that are required to sustain ATP production to fuel active ion uptake. Most recently, it was demonstrated *in vitro* that CCs do indeed have higher rates of oxidative metabolism in comparison with other gill epithelial cell types (Perry and Walsh, 1989). The experiments that have been performed to identify the CC as a site of Na^+ and Cl^- uptake (e.g. P. Laurent and S. F. Perry, in preparation) have provided no data on the involvement of the other epithelial cell types (e.g. pavement cells) in ion absorption. Chloride cells exist on both lamellar and filamental epithelia (e.g. Laurent *et al.* 1985), although the density of CCs on the filament is considerably greater. However, when the density of filamental CCs is low, very few, if any, CCs are observed on the lamellae (P. Laurent and S. F. Perry, in preparation). There have been attempts to separate the function of filamental and lamellar CCs using perfused trout head preparations. This approach has taken advantage of the fact that the filamental and lamellar epithelia are presumed to be perfused by separate and distinct circulations, thereby permitting assessment of the independent contribution of each epithelium to ion uptake (e.g. Perry and Wood, 1985; Gardaire *et al.* 1985). It is clear, however, that these two epithelia are not necessarily structurally or functionally independent, since there are large extracellular spaces connecting the filament and lamellar surfaces (Laurent and Dunel, 1980). Thus, the conclusion (based on perfused head experiments) that lamellar epithelial cells are responsible for approximately 80 % of Na^+ uptake while the other 20 % occurs *via* filamental CCs (Gardaire *et al.* 1985) may oversimplify the actual partitioning of uptake. It seems likely that, regardless of epithelial location, CCs will transport Na^+ and Cl^- into the circulation provided that perfusion is adequate. In the present study we have determined the morphometry of filamental CCs because the quantification is much simpler than using lamellar epithelia.

The gradual increase in CC apical surface area during AFW acclimation and significant correlations between J_{in} and CC apical surface area, taken together with the results of previous studies showing the CC as an important site of ion uptake, indicate that enlargement of CC apical surface area is an important adaptational response to AFW exposure. This increase in surface area presumably allows greater access to ion-transporting sites on the chloride cell. The enlargement of individual CCs and increase in numbers of transport sites may not occur simultaneously. It would appear that incorporation of new ion-transport sites occurs more slowly, because increases in CC surface area were obvious after 24 h whereas sustained stimulation of ion uptake was only apparent after 4 days of AFW acclimation.

The changes in CC morphology during acclimation of fish to AFW were probably due to the transient elevation of plasma cortisol levels. Experimental treatment of fish with cortisol causes pronounced hypertrophy and proliferation of CCs (P. Laurent and S. F. Perry, in preparation), which is associated with stimulation of J_{in}^{Cl} and J_{in}^{Na} both in AFW and in NFW. These results demonstrate that low [NaCl] in the absence of any appreciable change in $[Ca^{2+}]$ can promote

cortisol mobilization and subsequent CC hypertrophy, which supports the contention that the CC is a common site of Ca^{2+} (Perry and Flik, 1988), Na^+ and Cl^- absorption. Despite these adaptational responses, plasma [NaCl] remained depressed after 30 days of exposure to AFW, indicating that acclimation was incomplete or that trout are incapable of re-establishing 'normal' plasma [NaCl] in severely NaCl-deficient water.

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