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Fooling a freshwater fish: how dietary salt transforms the rainbow trout gill into a seawater gill phenotype

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Summary

Numerous fish species, including rainbow trout (*Oncorhynchus mykiss*), are able to inhabit both freshwater and seawater and routinely migrate between the two environments. One of the most critical adjustments allowing such successful migrations is a remodelling of the gill in which a suite of morphological and molecular changes ensure optimal function in the face of reversing requirements for salt and water balance. The remodelling leads to specific freshwater and seawater gill phenotypes that are readily identified by the orientation and/or quantities of specific ion transporters and the presence or absence of specific cell types. The proximate cues promoting gill phenotypic plasticity are unknown. Here, by assessing the consequences of a salt-enriched diet (in the absence of any changes in external salinity) in the freshwater rainbow trout, we demonstrate that internal salt loading alone, is able to induce various elements of the seawater gill phenotype. Specifically, we show upregulation of three ion transport genes, cystic fibrosis transmembrane conductance regulator (CFTR), $Na^+/K^+/2Cl^-$ co-transporter (NKCC1) and Na^+/K^+-ATPase, which are essential for ionic regulation in seawater, and the appearance of chloride cell-accessory cell complexes, which are normally restricted to fish inhabiting seawater. These data provide compelling evidence that gill remodelling during migration from freshwater to seawater may involve sensing of elevated levels of internal salt.

Key words: chloride cell, euryhalinity, osmoregulation, ionic regulation, gill, CFTR, Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ co-transporter.

Introduction

The fish gill forms an expansive interface separating the external and internal environments. Because of its large surface area and high relative permeability, the gill is the major site of passive movements of salt and water (Evans et al., 2005). The net direction of these passive fluxes is dictated by the existing ionic and osmotic gradients across the gill. Thus, in freshwater (FW), the gradients across the gill promote the continual passive loss of salt and the entry of water. In seawater (SW), the gradients are reversed and hence marine teleost fish are faced with perpetual salt gain and water loss. Despite the opposite directions of net passive salt and water fluxes in the two environments, the hydromineral composition of the body fluids of teleost fish inhabiting FW or SW is remarkably similar. The relative constancy of the internal environments in fish inhabiting FW and SW is achieved by a suite of compensatory physiological processes. In FW fish, salt and water balance is achieved by the excretion of large volumes of dilute urine and the absorption by active transport of Na^+ and Cl^- across the gill. SW fish, on the other hand, osmoregulate by drinking (and absorbing) water, reducing glomerular filtration rate to minimise renal water loss, and by actively extruding NaCl across the gill.

Because the FW fish gill is designed to absorb NaCl from a dilute environment, whereas the SW gill is optimised for NaCl secretion into a hyperionic environment, the successful migration of fish between the two media requires a remodelling of the gill, leading to distinct FW and SW gill phenotypes. In both environments, the gill epithelium is composed of a multitude of cell types (Laurent and Dunel, 1980; Laurent, 1984) including mitochondria-rich cells (MRC), pavement cells, mucous cells and neuroepithelial cells. Although the Na^+ and Cl^- uptake mechanisms also exist in marine teleosts (presumably used for acid–base regulation), net NaCl transport is dominated by well-defined transcellular and paracellular secretory pathways confined to cell types found only in SW. The SW fish gill contains a specific subtype of MRC known as the chloride cell (CC), aptly named because of its proven function in Cl^- secretion (Foskett and Scheffey, 1982). Unlike the MRCs of the FW gill (Perry, 1997), the SW CC is characterised by two distinct features: (1) a smooth concave apical membrane that forms a recessed pit or crypt and (2) its intimate association with an adjacent cell type, termed the accessory cell (AC) (for reviews, see Dunel-Erb and Laurent, 1980; Wilson and Laurent, 2002). Unlike the tight junctions

between neighbouring epithelial cells in the FW gill, the junctions between CCs and ACs in SW are leaky (Sardet et al., 1979) and allow for the passive paracellular extrusion of Na^+ (Degnan and Zadunaisky, 1980). Cl^- efflux is a multi-step process beginning with the entry of Na^+ and Cl^- into the CC across the basolateral membrane by a $Na^+/K^+/2Cl^-$ co-transporter (NKCC1) (Degnan et al., 1977; Zadunaisky, 1984). The favourable chemical gradients for Na^+ and K^+ movements are established by the activity of the Na^+ pump (Na^+/K^+-ATPase; NKA). Ultimately, Cl^- moves passively into the SW across the apical membrane though cystic fibrosis transmembrane conductance regulator (CFTR) anion channels (Singer et al., 1998). Thus, the FW and SW gill phenotypes can be readily distinguished on the basis of morphology and the relative expression of mRNAs of at least three ion transport proteins, NKA, NKCC1 and CFTR; all of which increase (Singer et al., 1998; Pelis et al., 2001) or are re-compartmentalised (Marshall et al., 2002) upon transfer from FW to SW.

It was previously demonstrated (Salman and Eddy, 1987) that rainbow trout fed a diet enriched in NaCl exhibited a significant increase in the number of branchial MRCs and an increase in NKA activity. These authors (Salman and Eddy, 1987) remarked on the similarity between the responses elicited by salt ingestion and those occurring when FW fish are transferred to SW. Both processes result in transient increases in internal salt levels. Thus, it is conceivable that the transformation of the FW gill into the SW phenotype during natural migration of fish into SW could involve sensing increasing concentrations of external and internal salt. However, because fish migrating between FW and SW will experience simultaneous changes in both external and internal salt levels, it has not yet been possible to discern the proximate cue(s) promoting gill remodelling. The idea that internal salt sensing may promote physiological responses accompanying migration of fish into SW was proposed (Nearing et al., 2002) after demonstrating that polyvalent cation receptors (CaRs) (Brown et al., 1993) can act as salinity receptors in fish. Given this context, the present study tested the hypothesis that internal salt loading, in the absence of any change in external salinity, was sufficient to evoke a SW gill phenotype. This was accomplished by examining gill morphology and expression levels of NKA, NKCC1 and CFTR in control fish and fish fed a high-salt diet.

Materials and methods

All procedures involving animals were carried out according to institutional guidelines, which are in accordance with those of the Canadian Council on Animal Care (CCAC). Rainbow trout *Oncorhynchus mykiss* (Walbaum) were housed in circular fibreglass tanks containing dechlorinated water (13°C). They were kept in the facility under a 12 h:12 h light:dark photoperiod during which time they were fed daily with commercial trout pellets. After at least 1 month, fish were weighed, divided into two groups and housed in separate tanks. One group was fed daily with the usual diet (1.5% body mass per day) while the other group was fed a diet supplemented with 11% NaCl. To ensure that both groups received similar daily caloric intakes, the salt-fed fish were given 11% more food per day; all food was consumed within 30 min. The salt-fed fish were gradually acclimated to the salt-enriched food over a 10 day period during which time the salt content of the food was increased by 1% per day. Thus, the tenth day was considered to be the first day of the treatment period.

After 4 weeks of feeding, fish were killed by a sharp blow to the head and gill tissue was removed and processed for immunocytochemistry, transmission electron microscopy, western blot analysis, NKA activity and real-time reverse transcription PCR (real rime RT–PCR).

Immunocytochemistry

Tissues were fixed in 4% paraformaldehyde, cryoprotected in sucrose and sectioned as described previously (Georgalis et al., 2006). Sections were incubated for 2 h at room temperature with one of two primary antibodies: α5 (1:100), a mouse monoclonal antibody against the α_1 sub-unit of chicken Na^+-K^+-ATPase or T4 1:10, a mouse monoclonal antibody against the carboxyl terminus of human colon NKCC (Lytle et al., 1995). Both antibodies have been used successfully for immunocytochemistry in a wide variety of vertebrate species including fish (Pelis et al., 2001). For negative controls, sections were incubated with buffer lacking primary antibodies. Immunofluorescence was detected after incubating the sections with a 1:400 dilution of Alexa Fluor 546-coupled to goat anti-mouse IgG (Fisher, Ottawa, ON, Canada).

Morphometric analysis

Six control and six salt-fed fish were assessed. For each fish, six gill sections derived from the second arch were examined using epifluorescence microscopy. Photos from 'randomly' selected areas of the mid regions of gill filaments (10–28 per section) were taken at 20× magnification. Digital images were then analysed using web-based imaging software (Scion, Frederick, MD, USA) to determine numbers and surface areas of NKA-immunopositive MRCs.

Transmission electron microscopy

Pairs of gill filaments were excised, immersion fixed in 1.5% paraformaldehyde/1.5% glutaraldehyde/0.1 mol l^{-1} cacodylate buffer pH 7.3, and processed for embedding in Spurr's resin (EMS, USA). Sections were stained with lead citrate and uranyl acetate and viewed with a Phillips CM10 transmission electron microscope (TEM). Sections from 12 fish (six controls and six salt-fed) were examined.

Western blotting

Proteins were prepared and quantified using standard procedures (e.g. Shahsavarani et al., 2006). Samples (50 μg) were size fractionated by reducing SDS-PAGE using 7%

separating and 5% stacking polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad, Mississanga, ON, Canada). After transfer, each membrane was blocked for 1 h in TBS-T (1× PBS, 0.1% Tween 20), 5% milk powder and then incubated overnight (4°C) with α5 (1:400) primary antibody. The membranes were then incubated for 1 h at room temperature with anti-mouse Ig, horseradish peroxidase (1:5000). The specific bands were detected by enhanced chemiluminescence (ECL; Pierce; SuperSignal West Pico Chemiluminescent Substrate, Rockford, IL, USA). For negative controls, blots were incubated with buffer lacking primary antibodies.

Blots were stripped and probed with the T4 antibody (1:50; overnight at 4°C). To assess for equal loading of proteins, blots were re-stripped and probed with an anti-β-tubulin antibody (1:1000, Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) for 1 h at 37°C.

The density of the antigenic bands was determined by scanning the films, and then analyzing the digital images using commercial software (Quantity One v4.1.1, Bio-Rad). The results are presented as the ratio of NKA or NKCC1 to tubulin band density.

Na^+/K^+-ATPase activity

Tissue from the second gill arch was added to SEI buffer (150 mmol l^{-1} sucrose, 10 mmol l^{-1} EDTA, 50 mmol l^{-1} imidazole), placed in liquid N_2 and stored at –80°C. NKA activity was determined in samples homogenized in SEI buffer containing 0.5% sodium deoxycholate using the microplate method of McCormick (McCormick, 1993).

Real-time RT–PCR

Total RNA was extracted from 30 mg of tissue using Invitrogen TRIzol Reagent and re-suspended in 30 μl of nuclease-free water. Reverse transcription was performed using Stratascript Reverse Transcriptase Kit (Stratagene). Complementary DNA was synthesized as per kit instructions with the following changes: final reaction volume was adjusted to 12.5 μl, and 0.5 μg of total RNA was used with 0.15 μg of random hexamer primers.

Real-time RT–PCR was performed using a MX 4000 Multiplex Quantitative PCR System (Stratagene) using a Brilliant SYBR Green QPCR Master Mix (Stratagene) as per the instructions of the manufacturer with the following modifications: the total reaction volume was reduced to 25 μl; 0.5 μl of cDNA template was used; and primer concentrations were 0.150 nmol l^{-1} for each primer. The following primers were used:

CFTR FWD 5′-GAGATGTGGTCTTGTCCCTTTCTTT-G-3′
CFTR REV 5′-GGCCACCATGAAAAACTAAAGAG-TAC-3′
NKA FWD 5′-YCATTTCCAGCTCCCTGAT-3′
NKA REV 5′-KAGGCCAACGAAGCACAGA-3′
NKCC1 FWD 5′-CGAGACCAAGGCACTCTACA-3′
NKCC1 REV 5′-ATGTCTCGCTCCTTCCAGTC-3′
Actin FWD 5′-CCAACAGATGTGGATCAGCAA-3′
Actin REV 5′-GGTGGCACAGAGCTGAAGTGGTA-3′
18s FWD 5′-TCTCGATTCTGTGGGTGGT-3′
18s REV 5′-CTCAATCTCGTGTGGCTGA-3′

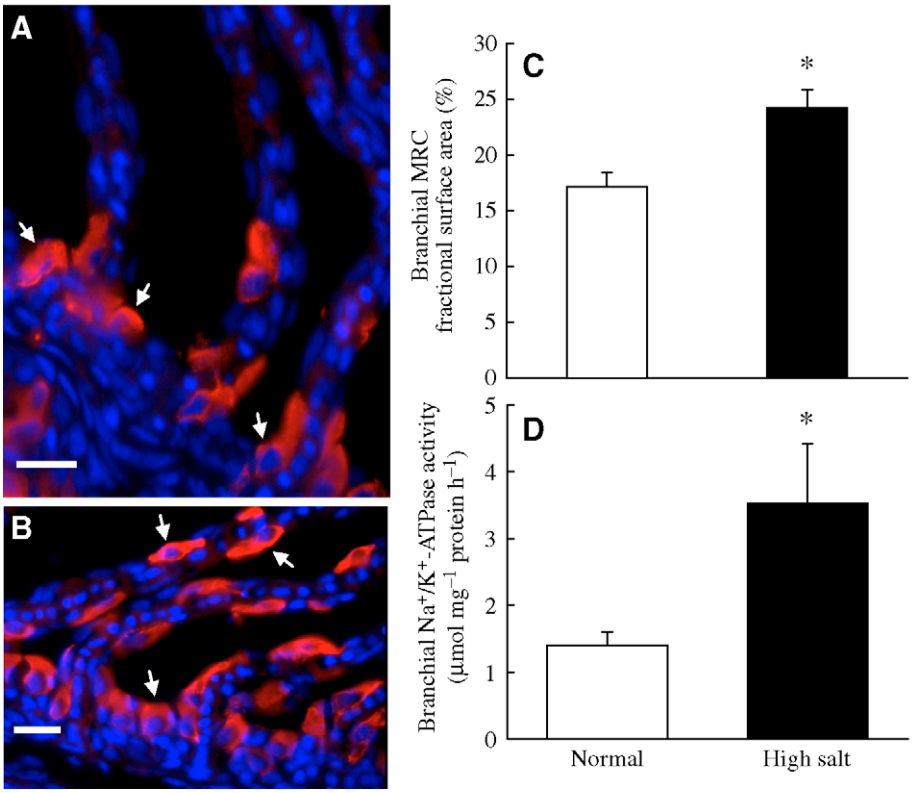

Fig. 1. Elevated dietary salt intake increases the number of mitochondria-rich cells and Na^+/K^+-ATPase activity in trout gill. (A,B) Representative images depicting Na^+/K^+-ATPase (NKA) immunofluorescence (red, arrows) in gill sections from (A) control and (B) salt-fed fish; nuclei are stained blue. (C) Morphometric analysis (N=6 for each group) demonstrated a significant increase (P=0.006) in the fractional surface area of mitochondria-rich (NKA immunopositive) cells in the salt-fed (filled bars) fish. (D) Branchial NKA activity (N=10 for each group) was increased threefold (P=0.011) by salt feeding (filled bars). Scale bars, 10 μm. Asterisks in C and D indicate statistically significant difference from control values.

PCR products initially were purified and sequenced to ensure that primers were indeed amplifying the target genes. At the end of each run, a dissociation curve was established to determine the purity of the amplicons in each reaction. Control samples (diluted RNA samples) were examined at random to test for the presence of genomic DNA contamination.

Data presentation and statistical analysis

Data are presented as means ± 1 standard error of the mean (s.e.m.). Statistical analysis was performed using Sigma Stat (version 3.0, SPSS Inc. Chicago, IL, USA). Except for real-time RT–PCR results, the data were assessed for statistical significance using an unpaired Student's *t*-test. If tests for normality failed, the data were analysed using a Mann–Whitney rank sum test. The effects of dietary salt on expression of mRNAs in the gill as determined by real-time RT–PCR were assessed using one-sample Student's *t*-tests. In all cases, significance was set at $P<0.05$.

Results

After 4 weeks of consuming a salt-enriched diet, fish demonstrated an increase in the numbers of NKA immunopositive MRCs and a concomitant increase in the fractional surface area of branchial MRCs (Fig. 1C; $P=0.006$). Increased numbers of MRCs were observed on both the filament and lamellar epithelia (Fig. 1A,B); there was no difference in the average size of MRCs between the two groups. Branchial NKA activity was increased 2.5 times ($P=0.011$) in the salt-fed fish (Fig. 1D), a result that was consistent with elevated levels of NKA protein (Fig. 2A,B; $P=0.029$) and mRNA (Fig. 2C). In control fish, NKCC1 was undetectable either by immunofluorescence (Fig. 3A) or by western blotting (Fig. 2A,B). However, the fish being fed a salt-enriched diet displayed obvious branchial expression of NKCC1 that was co-localized with NKA and thus appeared to be uniquely confined to MRCs (Fig. 3B). Moreover, western blots clearly revealed the presence of NKCC1 protein (immunoreactive band at 160 kDa) in three of the four salt-fed fish that were examined (Fig. 2A,B). The levels of NKCC1 and CFTR mRNA were increased 6- and 15-fold, respectively, compared to control fish (Fig. 3C).

A unique feature of the SW trout gill epithelium is the presence of concave apical crypts formed by the close association of the apical membranes of adjacent ACs and chloride cells. This morphology was observed only in the salt-fed fish (Fig. 4), but there were no obvious interdigitations of the AC into the MRC as would be expected of a true SW phenotype. Nor was there any obvious decrease in the depth or strand numbers of the AC-MRC tight junctional complexes. Thus, increased paracellular leakiness, a hallmark of the SW gill, may not have been induced by a salt-enriched diet. In the control fish, the surface of the apical membrane of the MRCs varied from smooth (as in the salt-fed fish) to being highly ornamented with microplicae (Fig. 4C) and tended to be slightly convex or flat. Although similar examples of FW MRC morphology were observed in the SW specimens, apical crypts and ACs were never observed in the FW sections.

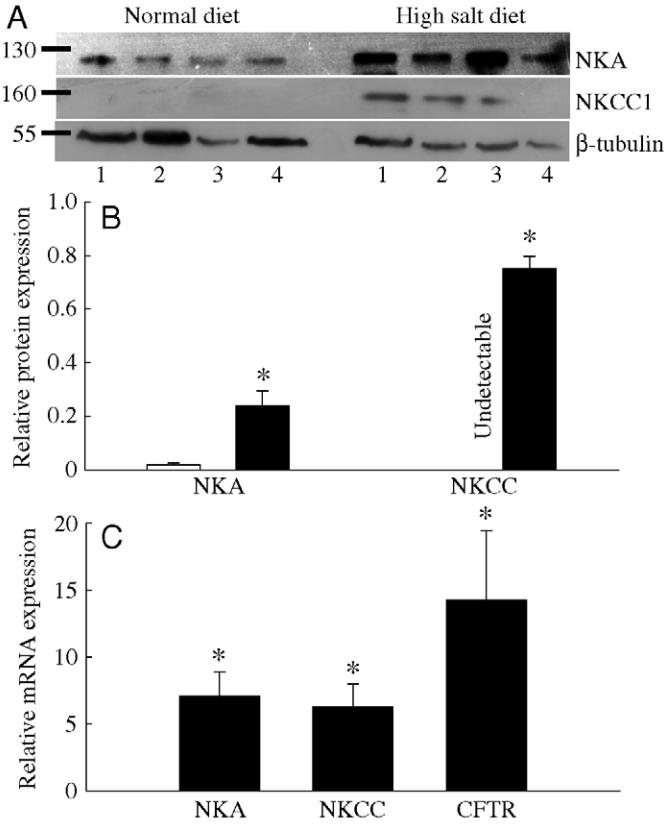

Fig. 2. Elevated dietary salt intake increases the expression of genes involved in ion secretion in seawater. (A,B) The results of western blot analysis revealed a marked increase in Na^+/K^+-ATPase (NKA; band at 115 kDa) and $Na^+/K^+/2Cl^-$ co-transporter (NKCC1; band at 160 kDa) protein in fish fed a high salt diet (filled bars; $N=4$ for each group). Blots were cropped for presentation purposes. (C) The levels of NKA, NKCC1 and cystic fibrosis membrane transmembrane conductance regulator (CFTR) mRNAs in salt-fed fish ($N=14$) were increased 5- to 15-fold relative to control fish ($N=9$). Asterisks in B and C indicate statistically significant difference from control values ($P<0.05$).

Discussion

Two MRC sub-types are present in the gills of SW fish (Laurent, 1984). One cell type, termed the chloride cell is believed to arise from the transformation of the FW α-MRC sub-type (Pisam and Rambourg, 1991) upon transfer to SW. In the present study, we have demonstrated that dietary salt loading, in the absence of any change in external salinity, can elicit a remodelling of the gill, resulting in the appearance of MRCs resembling the SW chloride cell. A feature of the SW gill that was observed only in the salt-fed fish was the presence of a second MRC cell type, the so-called accessory cell (Laurent, 1984), which is located adjacent to chloride cells. Although the occurrence of accessory cells in FW rainbow

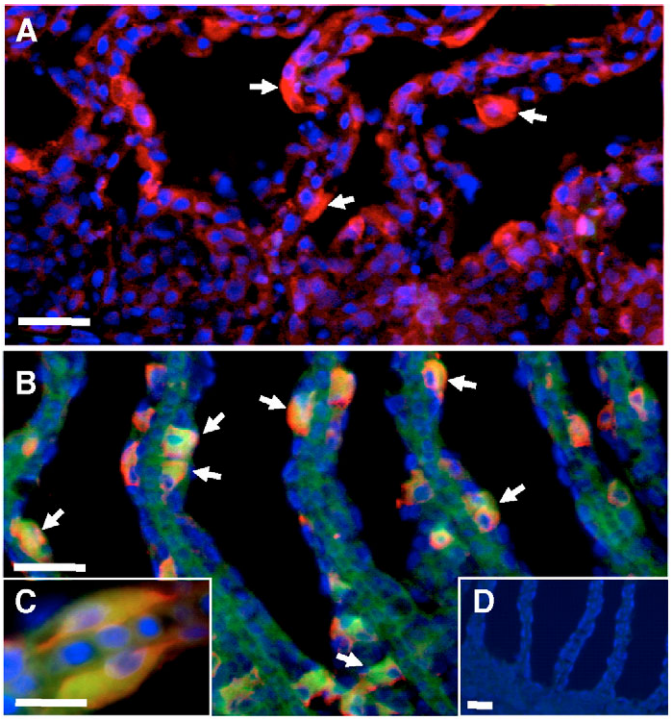

Fig. 3. Elevated dietary salt intake increases the expression of NKCC1 in branchial mitochondria-rich cells. Localisation of Na^+/K^+-ATPase (NKA; red), NKCC1 (green) and nuclei (blue) in gill sections from (A) control or (B,C) salt-fed fish. Areas of co-localisation of NKA with NKCC1 appear as yellow/orange. D is a representative image depicting a gill section from which both primary antibodies were omitted. Arrows in A indicate cells exhibiting NKA immunofluorescence, and in B co-localisation of NKA and NKCC1. Scale bars, 10 μm.

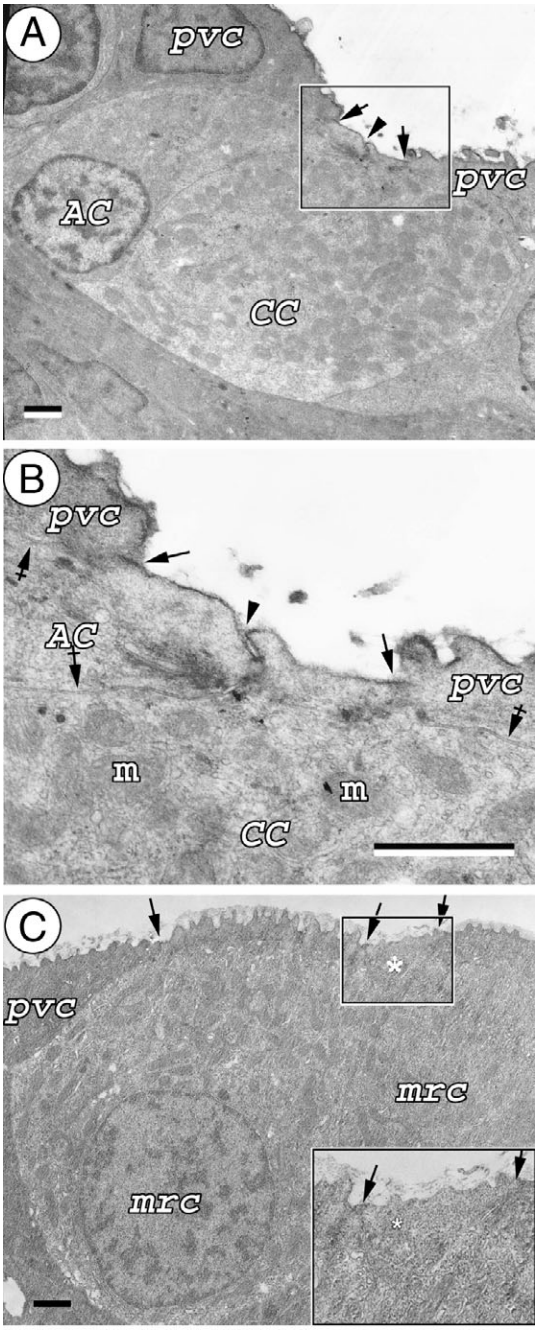

Fig. 4. Elevated dietary salt intake results in the appearance of seawater-like chloride cells. Representative transmission electron micrographs of gill mitochondria-rich cells (MRC) from (A,B) high-salt-fed or (C) control trout. The MRCs can be identified by their abundance of mitochondria (m) and tubular elements. In the high-salt-fed trout, a large mitochondria-rich chloride cell (CC) is flanked by a smaller accessory cell (AC). The apical surfaces of these two adjacent cells form a shallow crypt; this area has been enlarged 2× in B. (C) In the control trout, two large MRCs are shown with microplicae elaborating their apical membranes. Pavement cells (PVC) with less pronounced microplicae are seen between (asterisk) and to the left. This area has been enlarged 2× in the inset. Arrows and arrowheads indicate the MRC–PVC and CC–AC tight junctions, respectively. The lateral membranes of MRCs are indicated by crossed arrows. Scale bars, 1 μm.

trout gill was reported previously (Pisam et al., 1989), it is only in SW fish where the neighbouring apical membranes of the accessory cell and chloride cell share a common apical crypt. Thus, the appearance of such structures in the salt-fed fish is indicative of the SW phenotype.

The chloride cells in SW fish specifically function in net Cl^- secretion, a process that requires the participation of two ion transport proteins, NKCC1, expressed on the basolateral membrane, and CFTR, expressed on the apical membrane. Although these proteins may also exist in FW MRCs (Marshall et al., 2002; Hiroi et al., 2005), their intracellular localisation is markedly different than in SW; specifically, NKCC1 is targeted to the basolateral membrane whereas CFTR is inserted into the apical membrane. Indeed, the diffuse staining pattern of NKCC1 in chloride cells and its co-localization with NKA suggest that this co-transporter, as in SW, is found on the basolateral membranes. Perhaps the more important finding, however, was that the expression of both genes was increased by dietary salt loading, a response that is typical of the transfer of FW fish to SW (Evans et al., 2005). Thus, it is likely that internal salt sensing is an important cue promoting gill remodelling during migration of fish into SW. However, at

least one element of the SW phenotype was not elicited by salt loading, namely the disappearance of MRCs from the lamellae. Additionally, whether or not leaky junctions appeared between accessory cells and adjacent chloride cells will require further evaluation using higher resolution electron microscopy. Nevertheless, it would appear that one or more additional cues including increasing external salinity is required to complete the transformation of the FW gill into the full SW phenotype.

It is possible that salt feeding may pre-adapt FW fish for life in SW. Indeed, it was previously reported that feeding a salt-enriched diet to Chinook salmon *Oncorhynchus tshawytscha* (Zaugg et al., 1983), rainbow trout (Salman and Eddy, 1990) or brook charr *Salvelinus fontinalis* (Pellertier and Besner, 1992) significantly improved their survivability upon transfer to SW. Based on the results of the present study, we suggest that the mechanism underlying the increased survivability is salt-induced structural and molecular re-modelling of the gill into a partial SW phenotype.

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