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Variation in salinity tolerance, gill Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ cotransporter and mitochondria-rich cell distribution in three salmonids *Salvelinus namaycush*, *Salvelinus fontinalis* and *Salmo salar*

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Summary

We compared seawater tolerance, gill Na^+/K^+-ATPase and $Na^+/K^+/2Cl^-$ cotransporter (NKCC) abundance, and mitochondria-rich cell (MRC) morphology of three salmonids, lake trout *Salvelinus namaycush*, brook trout *Salvelinus fontinalis* and Atlantic salmon *Salmo salar*. They were transferred directly from 0 p.p.t. (parts per thousand; freshwater) to 30 p.p.t. seawater, or transferred gradually from 0 to 10, 20 and 30 p.p.t. at 1-week intervals and kept in 30 p.p.t. for 3 weeks. The survival rates of lake trout, brook trout and Atlantic salmon were 80%, 50% and 100% following direct transfer, and 80%, 100% and 100% during gradual transfer, respectively. Plasma Na^+, K^+ and Cl^- concentrations in surviving lake trout increased rapidly and remained at high levels in 30 p.p.t. of both direct and gradual transfer, whereas those in brook trout showed a transient increase following direct transfer but did not change significantly during gradual transfer. Only minor changes in plasma ions were observed in Atlantic salmon smolts in both direct and gradual transfer. These results suggest that lake trout retains some degree of euryhalinity and that brook trout possesses intermediate euryhalinity between lake trout and Atlantic salmon smolts. Gill Na^+/K^+-ATPase activity of lake trout and brook trout increased in seawater, whereas that of Atlantic salmon smolts was already upregulated in freshwater and remained high after seawater exposure. NKCC abundance was upregulated in parallel with gill Na^+/K^+-ATPase activity in each species. Immunocytochemistry with anti-Na^+/K^+-ATPase α-subunit and anti-NKCC revealed that the two ion transporters were colocalized on the basolateral membrane of gill MRCs. Immunopositive MRCs were distributed on both primary filaments and secondary lamellae in all three species kept in freshwater; following transfer to seawater this pattern did not change in lake trout and brook trout but lamellar MRCs disappeared in Atlantic salmon. Previous studies on several teleost species have suggested that filament and lamellar MRCs would be involved in seawater and freshwater acclimation, respectively. However, our results in lake trout and brook trout suggest that lamellar MRCs could be also functional during seawater acclimation.

Key words: mitochondria-rich cell, chloride cell, salinity tolerance, Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ cotransporter, salmonid, lake trout, brook trout, Atlantic salmon.

Introduction

Euryhaline teleost fish, such as killifish *Fundulus heteroclitus* and Mozambique tilapia *Oreochromis mossambicus*, have been used as model animals for the study of osmo- and iono-regulation in teleost fish. Numerous studies on these euryhaline species demonstrate that mitochondria-rich cells (MRCs, often referred to as chloride cells or ionocytes) in the skin and gill epithelia are responsible for the secretion of excess ions in seawater, and possibly for ion uptake in freshwater (for a review, see Evans et al., 2005). The mechanism of active NaCl secretion by MRCs in seawater consists primarily of the cooperative action of three major ion transporters: basolaterally located Na^+/K^+-ATPase and $Na^+/K^+/2Cl^-$ cotransporter (NKCC), and an apical Cl^- channel. Recent immunocytochemical studies clearly demonstrated that all three proteins are colocalized to MRCs in seawater-acclimated fish, indicating the cells are involved in active ion secretion (McCormick et al., 2003; Hiroi et al., 2005).

Anadromous salmonids are another group of model species that have been widely used in studies of osmo- and ionoregulation in fish. In most anadromous salmonids, seawater tolerance and gill Na^+/K^+-ATPase activity increase during parr–smolt transformation coincident with seaward migration (McCormick and Saunders, 1987). In Atlantic

salmon *Salmo salar* and brown trout *Salmo trutta*, protein abundance of Na^+/K^+-ATPase and NKCC increase during seawater acclimation and smolting, and these ion transporters were colocalized to gill MRCs (Pelis et al., 2001; Tipsmark et al., 2002). In Atlantic salmon, brown trout and chum salmon *Oncorhynchus keta*, gill MRCs were distributed on both the primary filaments and secondary lamellae in freshwater, whereas lamellar MRCs disappeared and filament MRCs enlarged following seawater exposure, suggesting that lamellar MRCs are responsible for ion uptake in freshwater, and filament MRCs are for ion secretion in seawater, respectively (Pisam et al., 1988; Uchida et al., 1996; Seidelin et al., 2000; Pelis et al., 2001). However, studies on MRCs and ion transporters have mostly been limited to species of the genera *Salmo* and *Oncorhynchus*.

The genus *Salvelinus* is regarded as a primitive salmonid group relative to *Salmo* and *Oncorhynchus*, and displays a more restricted pattern of seaward migration (Hoar, 1976). For example, lake trout *Salvelinus namaycush* is a non-anadromous species largely restricted to cold freshwater lakes, and brook trout *Salvelinus fontinalis* largely non-anadromous, but anadromous populations are present in the northern part of their distribution (McCormick and Naiman, 1984a; McCormick and Naiman, 1984b; McCormick et al., 1985). Therefore, it is expected that *Salvelinus* could exhibit a lower seawater tolerance than *Salmo* and *Oncorhynchus*, and possess primitive mechanism of ion regulation. In the present study, we selected three salmonid species, lake trout, brook trout and Atlantic salmon, which were expected to exhibit varying degrees of salinity tolerance, and examined changes in plasma ion and cortisol levels, gill Na^+/K^+-ATPase activity, and protein abundance and localization of Na^+/K^+-ATPase and NKCC in the gills during transfer from freshwater to seawater.

Materials and methods

Animals and experimental protocols

A direct transfer experiment from freshwater (0 p.p.t.; parts per thousand) to seawater (30 p.p.t.) and a gradual transfer experiment from 0 p.p.t. to 10, 20 and 30 p.p.t. were conducted in May and June 2001, with 1-year old lake trout (*Salvelinus namaycush* Walbaum), brook trout (*Salvelinus fontinalis* Mitchill) and Atlantic salmon (*Salmo salar* Linnaeus). Lake trout were obtained from the Salisbury State Fish Hatchery (Salisbury, VT, USA) and brook trout were a non-anadromous form obtained from the Sunderland State Fish Hatchery (Sunderland, MA, USA). Atlantic salmon were obtained as parr from the White River National Fish Hatchery (Bethel, VT, USA) and were smolt during the transfer experiments. Total length of all three species used in these experiments ranged from 15 to 20 cm. All fish were acclimatized to laboratory conditions with flow through Connecticut river water for at least 2 weeks prior to experiments. Dechlorinated tapwater was used during all experiments and seawater was produced by dissolving Instant Ocean (Marine Enterprises International, Baltimore, MD, USA) in dechlorinated tapwater. For the direct transfer experiment, 10 individuals of each species were initially sampled, then 20 fish were transferred by dip net from the freshwater tank to another tank filled with recirculating seawater (30 p.p.t.). Ten individuals were sampled at 24 h and all surviving fish (5–10) were sampled 7 days after transfer. The gradual transfer experiment was conducted by increasing the salinity of the tank in a stepwise fashion, from 0 p.p.t. to 10 p.p.t., 20 p.p.t. and 30 p.p.t. at 1-week intervals, and kept at 30 p.p.t. for 3 weeks. Salinities were raised by removing water from the tank and adding an appropriate amount of artificial seawater (40 p.p.t.). Eight individuals were sampled five times, just before raising the salinity (at 0 p.p.t., 10 p.p.t. and 20 p.p.t.) and after 1 and 3 weeks in 30 p.p.t. About 50 individuals of each species were used for the gradual transfer experiment. The water temperature was maintained at 10.2–10.7°C and the fishes were fed commercial Atlantic salmon diet (Zeigler Bros., Gardners, PA, USA) daily throughout the experiment period, except for the days of transfer and days on which sampling occurred. Mortalities were monitored daily and dead fish were excluded from all analyses. Plasma Na^+, K^+ and Cl^- concentrations and cortisol levels, and gill Na^+/K^+-ATPase activity were measured in all surviving individuals at each sampling time. Western blotting and immunocytochemistry were conducted on the gill tissue of fish kept in 0 p.p.t. and those transferred gradually and kept in 30 p.p.t. for 3 weeks.

Plasma ion and cortisol measurement

The fish were anesthetized with tricaine methane sulphonate (100 mg l^{-1}, neutralized and buffered with $NaHCO_3$, pH 7.0) and blood was drawn from the caudal vessels into heparin-treated syringes. Blood was centrifuged at 5000 ***g*** for 5 min at 4°C, and aliquots of plasma were stored at –80°C. Plasma Na^+, K^+ and Cl^- concentrations were measured using an electrolyte analyzer (AVL Scientific, Roswell, GA, USA). Plasma cortisol levels were measured using a direct enzyme immunoassay as outlined elsewhere (Carey and McCormick, 1999).

Gill Na^+/K^+-ATPase activity measurement

Gill Na^+/K^+-ATPase activity was measured according to the microassay protocol of McCormick (McCormick, 1993). Approximately 4–6 primary filaments from just above the septum were severed, with fine point scissors, from the anesthetized fish, immersed in 100 μl of ice-cold SEI buffer (150 mmol l^{-1} sucrose, 10 mmol l^{-1} EDTA, 50 mmol l^{-1} imidazole, pH 7.3) and frozen at –80°C. The filaments were thawed, homogenized in SEI buffer containing 0.1% deoxycholic acid and centrifuged at 5000 ***g*** for 30 s to remove large debris. 10 μl samples were run in two sets of duplicates, one set containing the assay mixture and the other assay mixture plus 0.5 mmol l^{-1} ouabain. The resulting ouabain-sensitive ATPase activity measurement is expressed as μmol ADP mg^{-1} protein h^{-1}. Protein concentrations were determined using the Bicinchoninic Acid (BCA) Protein Assay (Pierce, Rockford, Il, USA). Both assays were run on a THERMOmax microplate reader using SOFTmax software (Molecular Devices, Menlo Park, CA, USA).

Antibodies

A rabbit polyclonal antiserum directed against a synthetic peptide corresponding to part of the highly conserved region of the Na^+/K^+-ATPase α-subunit (NAK121) (Ura et al., 1996; Katoh et al., 2000) was used as the primary antibody for western blotting (diluted 1:5000) and immunocytochemistry (1:1000). The amino acid sequence of the synthetic peptide was Cys-Val-Thr-Gly-Val-Glu-Glu-Gly-Arg-Leu-Ile-Phe-Asp-Asn-Leu-Lys-Lys-Ser. A mouse monoclonal antibody directed against 310 amino acids at the carboxyl terminus of human colonic NKCC1 (T4; developed by Dr Christian Lytle and Dr Bliss Forbush III, and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health & Human Development and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA, USA) was used at a concentration of 0.15 μg ml^{-1} for western blotting, and 0.3 μg ml^{-1} for immunocytochemistry. These primary antibodies were diluted in PBS containing 0.05% Triton X-100, 0.02% keyhole limpet hemocyanin, 0.1% bovine serum albumin, 10% normal goat serum and 0.01% sodium azide. Negative control experiments (without primary antibody) showed no specific staining in both western blotting and immunocytochemistry.

SDS-PAGE and western blotting

Gill filaments were cut away from the gill arch and homogenized in 10 vol of ice-cold 10 mmol l^{-1} phosphate-buffered saline (PBS, pH 7.2), containing 30% sucrose and a Complete Mini protease inhibitor cocktail tablet (Roche, Indianapolis, IN, USA). The homogenate was centrifuged at 5000 ***g*** for 10 min at 4°C and at 20 000 ***g*** for 10 min at 4°C to sediment mitochondria and cellular debris. The supernatant was centrifuged at 48 000 ***g*** for 1 h at 4°C. The pellet was resuspended in the homogenization buffer, and total protein was determined using the BCA protein assay. Proteins were placed in Laemmli sample buffer (50 mmol l^{-1} Tris–HCl pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate, 6% 2-mercaptoethanol, 0.05% Bromophenyl Blue), heated to 60°C for 15 min, and separated using 7.5% and 6% polyacrylamide gels for Na^+/K^+-ATPase and NKCC, respectively, at 10 μg of total protein per lane. Proteins were then transferred to an Immobilon-P transfer membrane (Millipore, Bedford, MA, USA). The membranes were preincubated in 2% skim milk/PBST (0.05% Triton X-100 in PBS) for 1 h at room temperature, and incubated with anti-Na^+/K^+-ATPase or anti-NKCC for 1 h at room temperature. The membranes were washed three times in PBST followed by a 1-h incubation at room temperature in peroxidase-labeled affinity purified goat antibodies to rabbit IgG (for anti-Na^+/K^+-ATPase) or mouse IgG (for NKCC) (diluted 1:1000, KPL, Gaithersburg, MD, USA) in PBST. Following five washes in PBST, immunoreactivity was visualized with PBS containing 0.05% diaminobenzidine tetrahydrochloride, 0.01% $CoCl_2$ and 0.01% H_2O_2 for 30–90 s. Digital images of the membranes were obtained using a flatbed scanner and the intensity of the bands was determined by densitometry with Gel Plotting Macros on the public domain NIH image program (version 1.63, http://rsb.info.nih.gov/nih-image/).

Immunocytochemistry

The fresh second gill arches were cut into small pieces (3–5 mm), fixed in 4% paraformaldehyde in 0.1 mol l^{-1} phosphate buffer (pH 7.4) for 6 h at 4°C, and preserved in 70% ethanol. The gill tissue was rinsed in PBS, placed in PBS containing 30% sucrose for 30 min and then frozen in Tissue-Tek OCT Compound (Sakura Finetek, Torrance, CA, USA). Sections (7 μm) were cut in a cryostat at –24°C, parallel to the long axis of primary filaments, and placed on poly-L-lysine-coated slides. Slides were preincubated with 2% normal goat serum in PBS for 30 min at room temperature, and then incubated simultaneously with anti-Na^+/K^+-ATPase and anti-NKCC overnight at 4°C. Following three washes with PBS, the slides were exposed to Alexa Fluor 488-labeled anti-rabbit and Alexa Fluor 546-labeled anti-mouse secondary antibodies (diluted 1:500; Molecular Probes, Eugene, OR, USA) for 2 h at room temperature. The sections were incubated with 1 μmol l^{-1} 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) for 1 min, rinsed five times in PBS and mounted using SlowFade-Light antifade reagent (Molecular Probes) under coverslips. The sections were examined under a Nikon inverted fluorescence microscope using FITC (for Alexa Fluor 488), Rhodamine (for Alexa Fluor 546) and DAPI filter sets, and images were recorded with a cooled CCD camera (Penguin 150CL, Pixera, Los Gatos, CA, USA). From each fish, immunoreactive MRCs on the primary filaments and secondary lamellae were separately counted from sagittal sections of the trailing edge of gill filaments (1600 μm of primary filament/sagittal section, containing at least 150 MRCs on the primary filament) and expressed per millimeter of primary filament. The individual cell size (Na^+/K^+-ATPase-immunopositive area) was measured using NIH image.

Statistics

The temporal changes in plasma ions and cortisol concentrations and gill Na^+/K^+-ATPase activity were analyzed by a one-way analysis of variance (ANOVA) and the Tukey–Kramer *post-hoc* test. Prior to analysis, data displaying heterogeneity of variances were log-transformed by $X'=\log_{10}(X+1)$ (Zar, 1999). Significant differences in band intensity of western blotting, and number and size of MRCs between fish kept at 0 p.p.t. and those transferred gradually and kept in 30 p.p.t. for 3 weeks were analyzed by the nonparametric Wilcoxon rank-sum test. All analyses were conducted using JMP 5.0.1 (SAS Institute, Cary, NC, USA) and $P<0.05$ was used to reject the null hypothesis.

Results

Direct transfer experiment

During the direct transfer experiment from 0 p.p.t. to 30 p.p.t., there were no mortalities in the three species for the

first 24 h. However, 20% of the remaining lake trout and 50% of the remaining brook trout died during the following 6 days, while no mortalities were observed in Atlantic salmon. All surviving individuals of each species ate well at each feeding, and there were no observable differences in feeding or other behaviors.

In lake trout, plasma Na^+, K^+ and Cl^- concentrations increased significantly 24 h after transfer to 30 p.p.t., and remained high after 7 days (Fig. 1A). The three ions in brook trout also increased significantly at 24 h, but decreased at 7 days, in the five fish that survived (Fig. 1B). In Atlantic salmon, plasma Na^+ and Cl^- increased significantly but slightly less than in the other two species, and no significant change was observed in plasma K^+ (Fig. 1C).

Plasma cortisol concentration in lake trout increased following transfer and reached 643± 123 ng ml^{-1} at 7 days (Fig. 1D). In brook trout and Atlantic salmon, plasma cortisol increased rapidly at 24 h and decreased to 42±33 and 27±8 ng ml^{-1} respectively at 7 days (Fig. 1E,F).

Initial gill Na^+/K^+-ATPase activity in lake trout and brook trout was relatively low (below 2 μmol ADP mg^{-1} protein h^{-1}; Fig. 1G,H). Gill Na^+/K^+-ATPase activity increased significantly at 7 days in lake trout (1.5±0.1 at day 0; 4.0±0.4 at day 7), and not significantly (P=0.092, the Tukey–Kramer test) but slightly increased in brook trout (2.0±0.2 at day 0; 3.0±0.2 at day 7). In Atlantic salmon, a relatively high level of gill Na^+/K^+-ATPase activity (approximately 10 μmol ADP mg^{-1} protein h^{-1}) was observed in 0 p.p.t. and remained high following transfer to 30 p.p.t. (Fig. 1I).

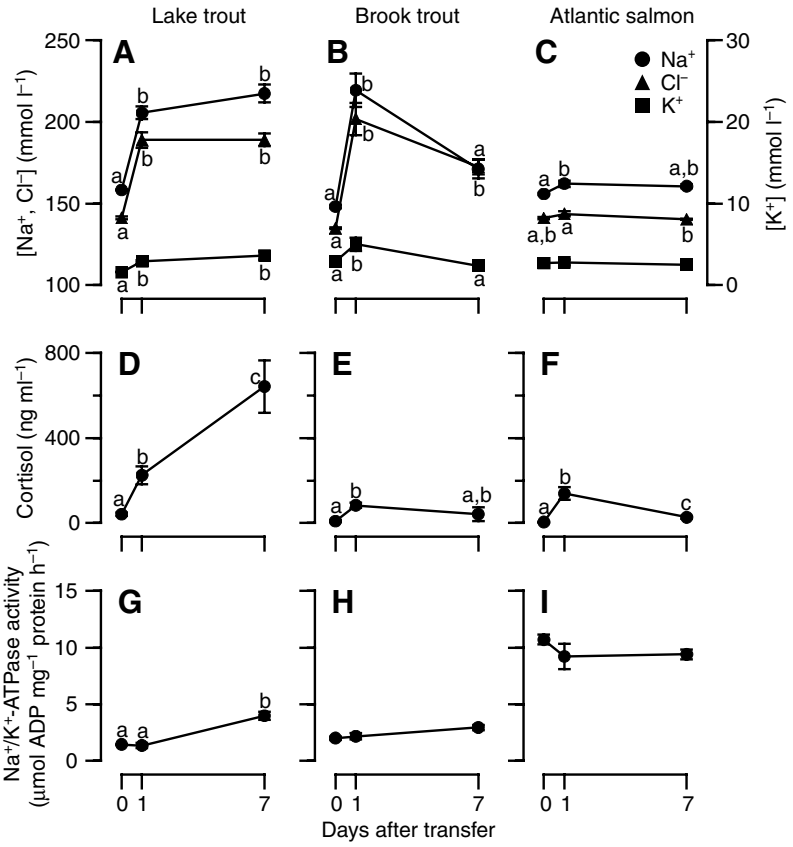

Fig. 1. Changes in plasma Na^+, Cl^-, K^+ (A–C) and cortisol (D–F) concentrations, and gill Na^+/K^+-ATPase activity (G–I) in lake trout (A,D,G), brook trout (B,E,H) and Atlantic salmon (C,F,I) following direct transfer from 0 p.p.t. to 30 p.p.t. Each value represents the mean ± s.e.m., N=10 (except for 7 days in A,D,G: N=8; in B,E,H: N=5). Symbols in A–C: circles, Na^+; triangles, Cl^-; squares, K^+. Values with different lowercase letters are significantly different (e.g. a significant difference is observed between 'a' and 'b', but not between 'a' and 'a,b', or between 'b' and 'a,b', P<0.05, Tukey–Kramer test).

Gradual transfer experiment

No mortalities were observed in brook trout and Atlantic salmon throughout the gradual transfer experiment, but 20% of lake trout died following transfer to 30 p.p.t. All surviving individuals of each species ate well at each feeding, and there were no observable differences in feeding or other behaviors.

Plasma Na^+ and Cl^- concentrations in lake trout increased gradually in accordance with external salinities, and remained high in 30 p.p.t., whereas K^+ did not show a significant change (Fig. 2A). No significant changes were observed in the three ions in brook trout and Atlantic salmon, excepting Cl^- of Atlantic salmon, which showed slight but significant increases following transfer (Fig. 2B,C).

Plasma cortisol concentration slightly decreased in 10 p.p.t. but increased in 30 p.p.t. in lake trout (Fig. 2D). No significant changes in plasma cortisol were observed in brook trout (Fig. 2E). Plasma cortisol in Atlantic salmon fluctuated and a significant increase was observed between day 0 and 35 (Fig. 2F).

Gill Na^+/K^+-ATPase activity in lake trout and brook trout increased in accordance with external salinities, whereas the activity in Atlantic salmon was already high in 0 p.p.t. (approximately 10 μmol ADP mg^{-1} protein h^{-1}) and remained high following transfer (Fig. 2G–I).

Western blotting

The anti-Na^+/K^+-ATPase revealed a single band with a molecular mass of 100 kDa, which is near the predicted molecular mass of the Na^+/K^+-ATPase α-subunit, but the bands from fish gradually acclimated to 30 p.p.t. shifted upward approximately 4 kDa from those of fish kept in 0 p.p.t., in all three species examined (Fig. 3A). Significant differences were not found in the band intensity of 0 p.p.t.- and 30 p.p.t.-acclimated fish (Fig. 4A). The anti-NKCC antibody revealed three broadly stained bands with molecular masses centered at 120, 150 and 250 kDa in samples from Atlantic salmon kept in 0 p.p.t. and those acclimated to 30 p.p.t. (Fig. 3B). Three

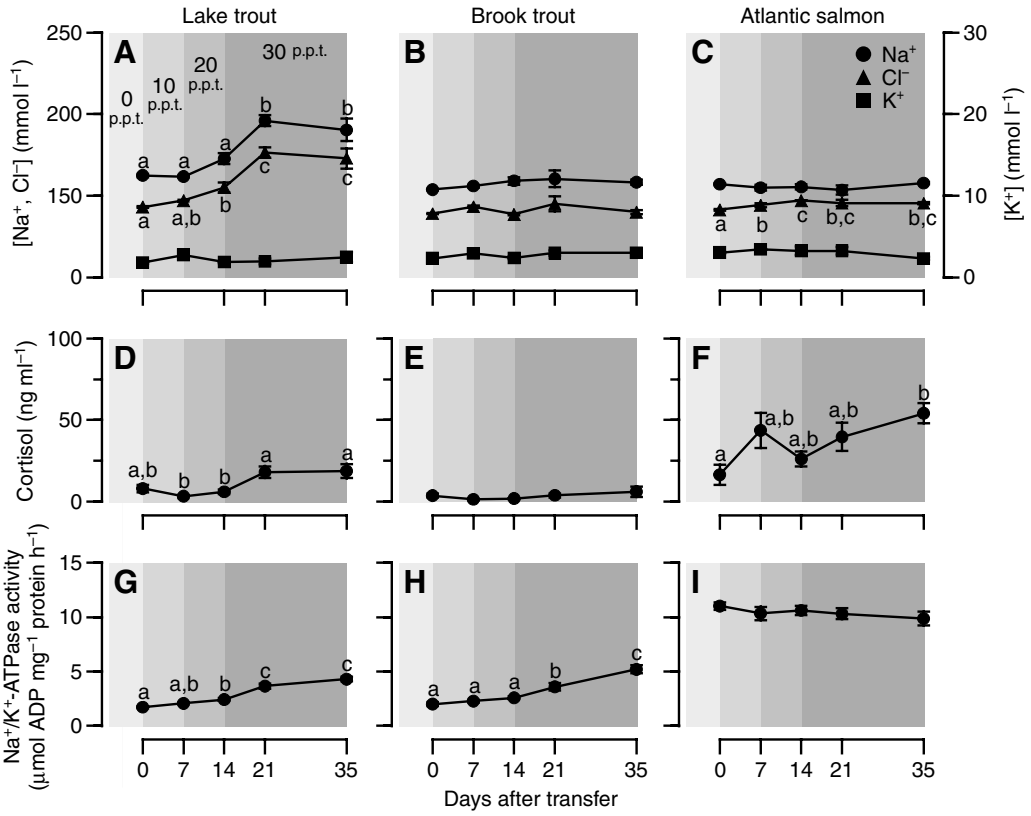

Fig. 2. Changes in plasma Na^+, Cl^-, K^+ (A–C) and cortisol (D–F) concentrations and gill Na^+/K^+-ATPase activity (G–I) in lake trout (A,D,G), brook trout (B,E,H) and Atlantic salmon (C,F,I) following gradual transfer from 0 p.p.t. to 10, 20 and 30 p.p.t. Salinities: before day 0, 0 p.p.t.; day 0–7, 10 p.p.t.; day 7–14, 20 p.p.t.; day 14–35, 30 p.p.t. Symbols in A–C: circles, Na^+; triangles, Cl^-; squares, K^+. Each value represents the mean ± s.e.m., N=8. Groups with different lowercase letters are significantly different (P<0.05, Tukey–Kramer test).

similar bands were observed in samples from lake trout acclimated to 30 p.p.t. and only the upper band was visible in samples form brook trout acclimated to 30 p.p.t., whereas immunopositive bands were almost invisible in samples from the lake trout and brook trout kept in 0 p.p.t. (Fig. 3B). Significant differences were observed in the band intensity of 0 p.p.t.- and 30 p.p.t.-acclimated lake trout and brook trout, and not observed in samples from Atlantic salmon (Fig. 4B).

Immunocytochemistry

In all three species kept in 0 p.p.t., Na^+/K^+-ATPase immunoreactivity was localized to large cuboidal cells on the primary filaments and low-cuboidal cells on the secondary lamellae (green color in Fig. 5A,E,I), and NKCC immunoreactivity was colocalized to these cells (red color in Fig. 5B,F,J). The size, shape and location indicated that these cells were MRCs. The immunoreactivity of Na^+/K^+-ATPase and NKCC was detectable throughout the cell except for the nucleus. The colocalization of Na^+/K^+-ATPase and NKCC within MRCs was also observed in fishes acclimated to 30 p.p.t., but the distribution pattern of MRCs in Atlantic salmon was different from that of lake trout and brook trout: MRCs were still distributed on the primary filaments and secondary lamellae in lake trout and brook trout (Fig. 5C,D,G,H), but MRCs were found primarily on the primary filaments and were rare on the secondary lamellae in Atlantic salmon (Fig. 5K,L). The number of Na^+/K^+-ATPase-immunoreactive MRCs in the filaments and lamellae were not significantly different between the 0 p.p.t.- and 30 p.p.t.-acclimated groups, except for lamellar MRCs in Atlantic salmon (Fig. 6A,B). The size of Na^+/K^+-ATPase-immunoreactive MRCs in the filaments and lamellae increased significantly in individuals acclimated to 30 p.p.t. relative to those in 0 p.p.t., except for lamellar MRCs in Atlantic salmon (Fig. 6C,D).

Discussion

Plasma ion concentrations showed different responses in each of the three salmonid species following direct and gradual transfer experiments from freshwater to seawater. In lake trout, plasma ion concentrations increased rapidly and remained at high levels in 30 p.p.t. following both direct and gradual transfer, whereas those in brook trout showed a transient increase following direct transfer but did not change significantly during gradual transfer. Only slight increases were observed in Atlantic salmon smolts in both direct and gradual transfer. These plasma ion responses to seawater indicate varying degrees of salinity tolerance in the three species. Lake trout had a survival rate of 80% following both direct and gradual transfer to 30 p.p.t. but were not able to maintain plasma ion concentrations within a narrow range. Their relatively high survival rates in seawater may indicate that they are resistant to, but perhaps incapable of long-term survival in seawater. Although 50% of brook trout died following direct transfer to seawater, they all survived gradual acclimation and their plasma ion responses suggest that brook trout possess intermediate euryhalinity between lake trout and Atlantic

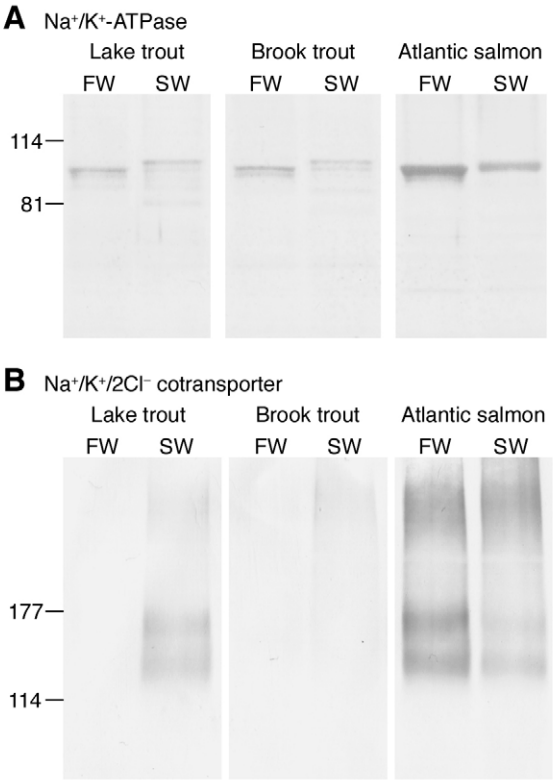

Fig. 3. Representative western blots of Na^+/K^+-ATPase (A) and $Na^+/K^+/2Cl^-$ cotransporter (B) from the gills of lake trout, brook trout and Atlantic salmon kept in 0 p.p.t. (FW) and those transferred gradually and kept in 30 p.p.t. for 3 weeks (SW). Molecular mass standards are indicated on the left.

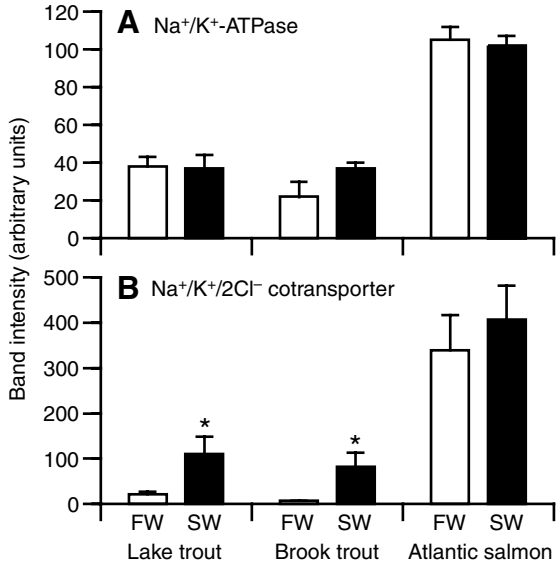

Fig. 4. Band intensities of western blots of Na^+/K^+-ATPase (A) and $Na^+/K^+/2Cl^-$ cotransporter (B) from the gills of lake trout, brook trout and Atlantic salmon kept in 0 p.p.t. (FW) and those transferred gradually and kept in 30 p.p.t. for 3 weeks (SW). Each value represents the mean ± s.e.m., N=4. Asterisks indicate significant differences between FW and SW values (P<0.05, Wilcoxon rank-sum test).

salmon. Little plasma ion changes and no mortalities in Atlantic salmon indicate excellent euryhalinity of salmon smolts. Plasma cortisol levels were elevated significantly at 1 day after direct transfer in all three species, and an extremely high value was observed after 7 days in lake trout. Since cortisol has been viewed as a seawater-adapting hormone (McCormick, 1995; McCormick, 2001), these elevated cortisol levels may be responsible for the seawater acclimation process. However, the sustained high plasma cortisol levels observed in lake trout could indicate unusually high stress levels, perhaps caused by high plasma ion concentrations following direct transfer. Gradual acclimation of lake trout resulted in slightly lower plasma ions and substantially lower plasma cortisol.

Based on the primarily lacustrine distribution of lake trout, we expected their salinity tolerance to be quite poor. Although they were not able to acclimate to long-term exposure to high salinity, lake trout maintained low plasma ions in 10 and 20 p.p.t., and most could withstand short-term exposure to 30 p.p.t. We are not aware of any previously published studies that have examined the salinity tolerance of lake trout. Anadromy is rare or non-existent in lake trout (Rounsefell, 1958), but Martin and Olver (Martin and Olver, 1982) review several studies that found lake trout in brackish waters (generally less than 10 p.p.t.) of the Arctic region, that may be the result of opportunistic feeding. In addition, lake trout have been introduced into the brackish waters of the Baltic Sea. The presence of salinity tolerance may also be the result of selection pressures related to colonization of new regions during periods of changing glaciation (Wilson and Hebert, 1998).

Although lake trout demonstrated a larger than expected tolerance for salinity, plasma ions and cortisol were much higher than in brook trout or Atlantic salmon after exposure to 30 p.p.t., indicating a limited capacity for long-term ion regulation in seawater. In response to seawater, lake trout were able to upregulate the Na^+/K^+-ATPase activity and abundance, NKCC abundance and MRC size and number to levels similar to that seen in brook trout. This indicates that the limited capacity of seawater acclimation in lake trout is unlikely to be related to limitations in the gill ion transporters measured in the present study. We suggest that other mechanisms of ion regulation are involved in this difference, and these might include other transporters in the gill (e.g. the apical Cl^- channel), gill permeability, or fluid and ion transport by the gut or kidney.

Na^+/K^+-ATPase plays a central role in a currently accepted model for ion secretion by MRCs. Na^+/K^+-ATPase located on the basolateral membrane of MRCs creates low intracellular Na^+ and contributes to a highly negative charge within the cell. The Na^+ gradient is used to transport Na^+, K^+ and Cl^- into the cell through basolateral NKCC. Cl^- then leaves the cell down on an electrical gradient through an apical Cl^- channel, which is homologous to human cystic fibrosis transmembrane conductance regulator (CFTR) (Silva et al., 1977; Marshall,

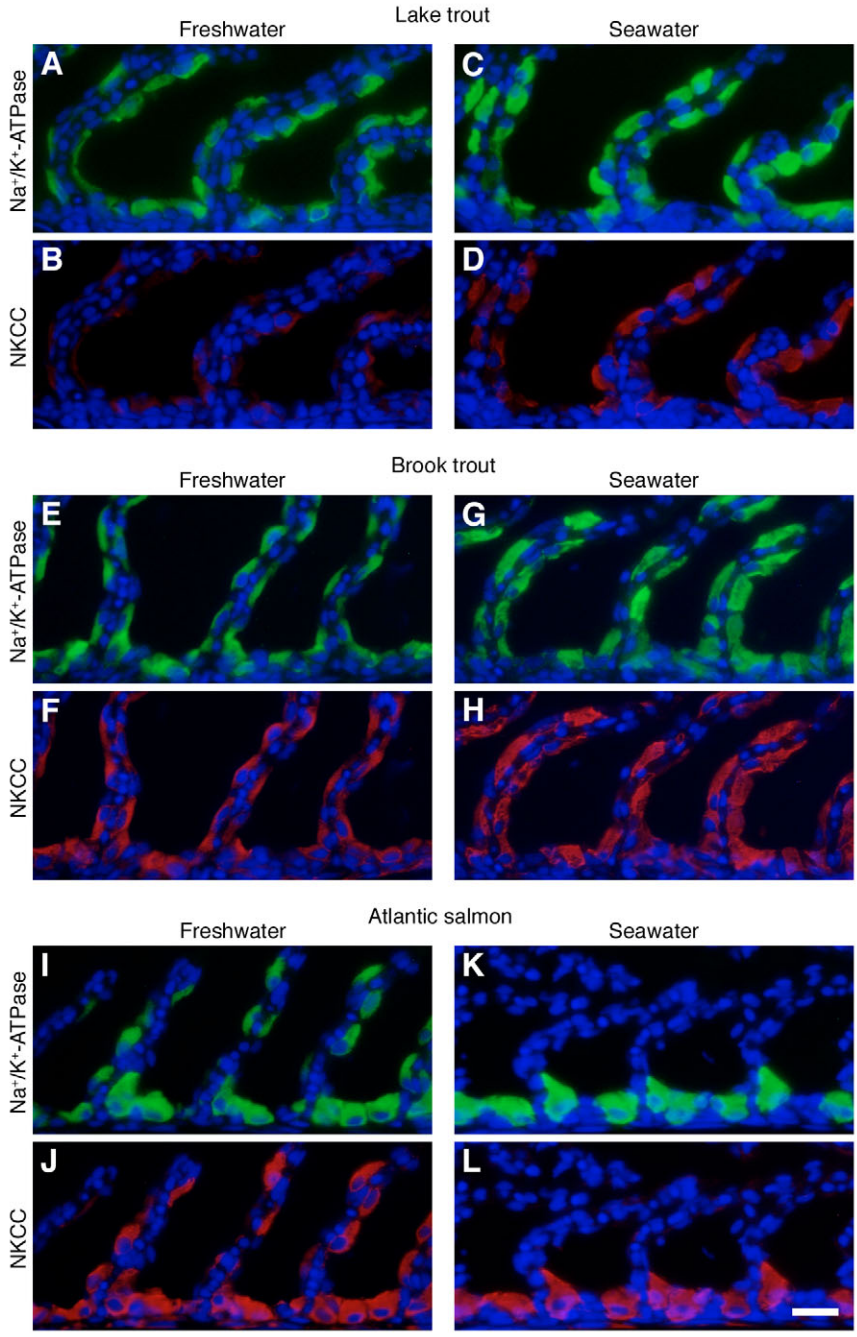

Fig. 5. Na^+/K^+-ATPase (green in A,C,E,G,I,K) and $Na^+/K^+/2Cl^-$ cotransporter (red in B,D,F,H,J,L) immunoreactivity in the gills of lake trout (A–D), brook trout (E–H) and Atlantic salmon (I–L) kept in 0 p.p.t. (freshwater; A,B,E,F,I,J) and those transferred gradually and kept in 30 p.p.t. (seawater) for 3 weeks (C,D,G,H,K,L). The nuclei were counterstained with DAPI (blue). The primary filaments are horizontal, and about three secondary lamellae are seen perpendicular to each filament. Scale bar, 20 μm.

1995; Singer et al., 1998; Marshall et al., 2002). In the present study, gill Na^+/K^+-ATPase activity of lake trout and brook trout increased following direct and gradual transfer to seawater, indicating that Na^+/K^+-ATPase was upregulated in order to hypo-osmoregulate. By contrast, Na^+/K^+-ATPase activity of Atlantic salmon was relatively high in both freshwater and seawater, and the high activity indicates that Na^+/K^+-ATPase was already upregulated in freshwater as a preparative adaptation for seawater entry, and accounts for the excellent euryhalinity of salmon smolts compared to lake trout and brook trout. In all three species, western blotting with anti-Na^+/K^+-ATPase revealed no significant differences in the immunopositive band intensity between freshwater- and seawater-acclimated fish, which were not in accordance with the results of Na^+/K^+-ATPase activity or the immunohistochemical data. This difference may arise from several sources, including a more activated form of Na^+/K^+-ATPase in seawater and/or measurement of inactive forms of Na^+/K^+-ATPase by western blotting. The molecular mass of the immunopositive band from seawater-acclimated fish shifted slightly upward to that of fish kept in freshwater in all three species examined. Recently, five α-subunit isoforms of Na^+/K^+-ATPase (α1a, α1b, α1c, α2 and α3) were identified from rainbow trout (*Oncorhynchus mykiss*), and mRNA level of the α1a isoform decreased whereas α1b level increased following seawater exposure (Richards et al., 2003). This reciprocal mRNA expression of the two isoforms was also confirmed in Atlantic salmon and Arctic char (*Salvelinus alpinus*), indicating that the α1a and α1b isoforms play different roles in freshwater and seawater acclimation in salmonids (Bystriansky et al., 2006). Since the amino acid sequence of the synthetic peptide used to produce our anti-Na^+/K^+-ATPase antibody was conserved in the deduced amino acid sequences of all five α-subunit isoforms in rainbow trout identified by Richards et al. (Richards et al., 2003), the antibody is likely to be immunoreactive with all α-subunit isoforms. Because the calculated molecular masses of the deduced amino acid sequence of rainbow trout α1a and α1b isoforms are similar (112.9 and 112.7 kDa, respectively), it seems unlikely that the slight difference in molecular mass of immunopositive bands between freshwater- and seawater-acclimated fish reflects different pattern of protein abundance of the α1a and α1b isoforms. The slight difference might be due to different degrees of phosphorylation and/or glycosylation. It will be important to measure protein abundance and cellular localization of these isoforms to determine their physiological function in freshwater and seawater.

Western blotting with anti-NKCC revealed three

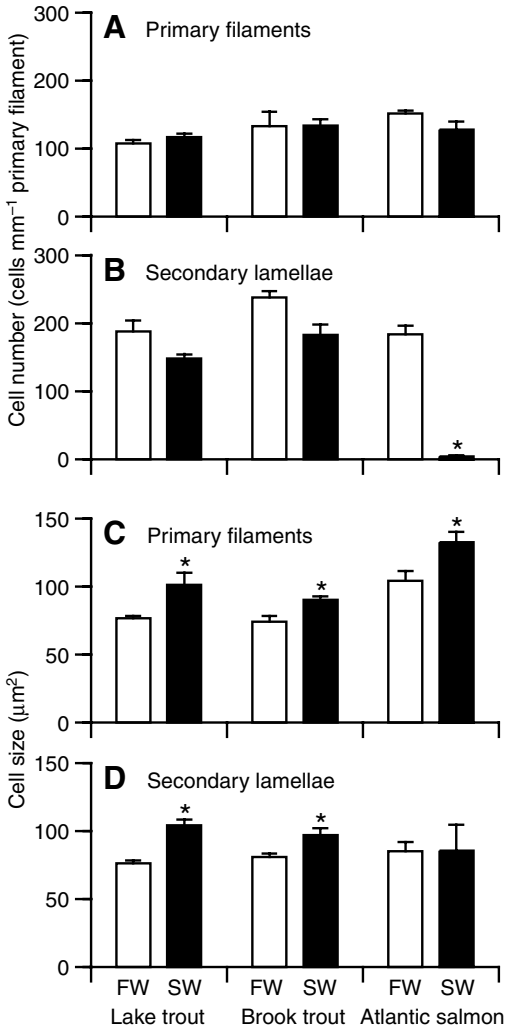

Fig. 6. Quantification of Na^+/K^+-ATPase-immunoreactive mitochondrion-rich cell numbers (A,B) and size (C,D) in the gills of lake trout, brook trout and Atlantic salmon kept in 0 p.p.t. (FW) and those transferred gradually and kept in 30 p.p.t. for 3 weeks (SW). Mitochondria-rich cells on the primary filaments (A,C) and secondary lamellae (B,D) were counted and analyzed separately. Each value represents the mean ± s.e.m., N=4. Asterisks indicate significant differences between FW and SW (P<0.05, Wilcoxon rank-sum test).

immunopositive bands, which were consistent with previous studies on Atlantic salmon and brown trout (Pelis et al., 2001; Tipsmark et al., 2002). The band intensity increased in lake trout and brook trout during seawater acclimation and was already high in freshwater in Atlantic salmon. These changes in NKCC protein abundance were in parallel with Na^+/K^+-ATPase activity, and suggest that NKCC was also upregulated during seawater acclimation of lake trout and brook trout, and already upregulated in salmon smolts in freshwater in preparation for seawater entry. NKCC is also likely to play a crucial role during seawater acclimation of salmonids concomitantly with Na^+/K^+-ATPase. In mammals, the $Na^+/K^+/2Cl^-$ cotransporter occurs in two major isoforms: a secretory isoform termed NKCC1 and an absorptive isoform termed NKCC2 (Xu et al., 1994; Payne and Forbush, 1994). NKCC1 is especially prominent in the basolateral membrane of chloride secretory epithelial cells. NKCC2 is found primarily in the apical membrane of epithelial cells in the thick ascending limb of the loop of Henle. The anti-NKCC antibody used in the present study (T4) has been shown to recognize both secretory and absorptive isoforms in a diverse variety of animal tissues (Lytle et al., 1995). The upregulation of NKCC in seawater and the basolateral distribution determined by immunocytochemistry (addressed in the next paragraph) suggest that this is the secretory isoform.

In all three species, Na^+/K^+-ATPase and NKCC immunoreactivity was colocalized in gill MRCs, being present throughout the cell except for the nucleus. It has been demonstrated that the basolateral membrane of MRCs is continuous with the tubular system, which extends throughout the whole cytoplasm of MRCs (Pisam and Rambourg, 1991), and Na^+/K^+-ATPase was present on both the basolateral membrane and the tubular system (Karnaky et al., 1976; Wilson et al., 2000). Therefore, the staining pattern of Na^+/K^+-ATPase and NKCC throughout MRCs is likely to represent a basolateral/tubular distribution, which is consistent with the currently accepted model for ion secretion by MRCs cited above.

The colocalization of Na^+/K^+-ATPase and NKCC was observed not only in seawater-acclimated fish but also in freshwater-acclimated fish. The currently proposed models for ion uptake mechanisms by MRCs include Na^+/K^+-ATPase but not NKCC (Evans et al., 2005). There are several possible explanations for the presence of NKCC in the gill of freshwater salmonids. NKCC may be present to provide as minimal level of salt secretory capability in the event of encountering salt water or a high salt item in the diet (Perry et al., 2006). Alternatively, the presence of NKCC immunoreactivity in MRCs in freshwater may indicate that this transporter has some direct physiological function in ion uptake, or is involved in cell volume or acid/base regulation. Wilson et al. (Wilson et al., 2000) hypothesized that NKCC may be involved in ammonia excretion by the mudskipper *Periophthalmodon schlosseri* gills.

A noticeable finding in the present study is variation in distributional pattern of MRCs between lake trout/brook trout and Atlantic salmon: Na^+/K^+-ATPase- and NKCC-immunopositive MRCs were distributed on both primary filaments and secondary lamellae in all three species kept in freshwater; following transfer to seawater, the pattern did not change in lake trout and brook trout but lamellar MRCs disappeared in Atlantic salmon. The disappearance of lamellar MRCs and enlargement of filament MRCs in seawater have not only been reported in salmonids (Atlantic salmon, brown trout and chum salmon), but also in other teleost species such as Japanese eel *Anguilla japonica*, American shad *Alosa sapidissima* and Japanese sea bass *Lateolabrax japonicus*, and these observations have led to the hypothesis that lamellar MRCs are involved in ion uptake in freshwater and filament

MRCs are responsible for ion secretion in seawater (Pisam et al., 1988; Uchida et al., 1996; Sasai et al., 1998; Hirai et al., 1999; Seidelin et al., 2000; Zydlewski and McCormick, 2001; Pelis et al., 2001). However, lamellar MRCs were still found in lake trout and brook trout acclimated to seawater. These lamellar MRCs were immunopositive for both Na^+/K^+-ATPase and NKCC and increased their size significantly in seawater along with filament MRCs. Therefore, it seems likely that both lamellar and filament MRCs are involved in ion secretion in seawater-acclimated lake trout and brook trout. Demonstrating apical localization of CFTR in addition to basolateral localization of Na^+/K^+-ATPase and NKCC in MRCs is expected to provide more definite evidence that these cells are involved in active ion secretion. We have recently confirmed that a large number of MRCs were still on the lamellae after seawater exposure of Hawaiian goby *Stenogobius hawaiiensis*, and that the lamellar MRCs had similar immunoreactivity of Na^+/K^+-ATPase, NKCC and CFTR to that of MRCs on the filaments (McCormick et al., 2003). These results therefore do not provide evidence for differential function of MRCs on the filaments and lamellae. Two isoforms of CFTR (sCFTR-I and sCFTR-II) were identified from Atlantic salmon and gill mRNA levels of the former were significantly elevated and those of the latter were transiently elevated following seawater exposure (Chen et al., 2001; Singer et al., 2002; Singer et al., 2003). However, immunocytochemical detection of CFTR in MRCs of salmonid has proven difficult, yet this will be essential for a better understanding of the function of MRCs. The present study does not provide an explanation as to why the disappearance of lamellar MRCs in seawater does not occur in lake trout and brook trout but in Atlantic salmon. However, the genus *Salvelinus*, which includes lake trout and brook trout, is regarded as a primitive salmonid group and therefore the disappearance of lamellar MRCs in salmon may be an advanced adaptive mechanism for hypo-osmoregulation in seawater. The absence of MRCs on the secondary lamellae may also increase the efficiency of respiration of Atlantic salmon in seawater.

A large number of MRCs were observed in both freshwater- and seawater-acclimated fish of all three species, but only in Atlantic salmon did MRCs disappear from the secondary lamellae after seawater transfer. Although it has been considered that MRCs are involved in ion uptake in freshwater, the ion uptake mechanism by MRCs is controversial and less understood than their ion secretory mechanism (Hirose et al., 2003; Evans et al., 2005). For instance, two models have been proposed for Na^+ uptake mechanism in freshwater. An original model involves an apically located amiloride-sensitive electroneutral Na^+/H^+ exchanger (NHE), and an alternative model incorporating an amiloride-sensitive epithelial sodium channel (ENaC) and vacuolar-type H^+-ATPase is currently more accepted. However, fish ENaC has yet to be identified by molecular cloning or database searches of fish genomes, whereas NHE has been cloned and shown to be present in the apical membrane of MRCs in Japanese dace *Tribolodon hakonensis* (Hirata et al., 2003). The contrasting behavior of MRCs on the secondary lamellae of trout and salmon may provide a useful comparative model for examining ion uptake mechanisms in salmonids.

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