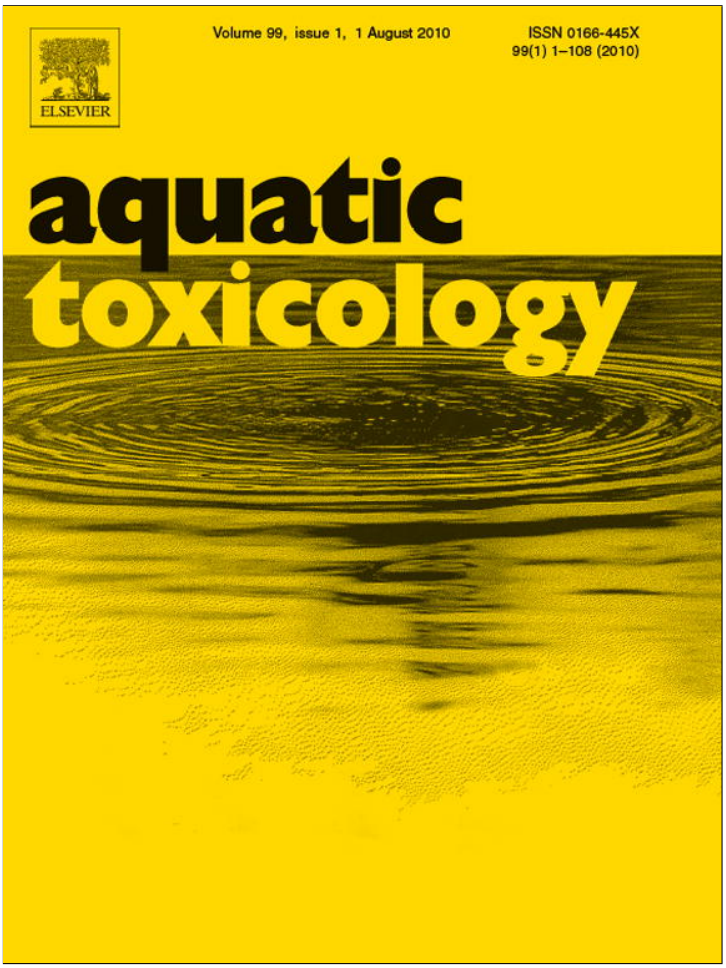

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Physiological, molecular, and cellular mechanisms of impaired seawater tolerance following exposure of Atlantic salmon, *Salmo salar*, smolts to acid and aluminum

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ABSTRACT

We examined the physiological, molecular, and cellular mechanisms of impaired ion regulation in Atlantic salmon, *Salmo salar*, smolts following acute acid and aluminum (Al) exposure. Smolts were exposed to: control (pH 6.5, 3.4 $\mu g\,l^{-1}$ Al), acid and low Al (LAl: pH 5.4, 11 $\mu g\,l^{-1}$ Al), acid and moderate Al (MAl: pH 5.3, 42 $\mu g\,l^{-1}$ Al), and acid and high Al (HAl: pH 5.4, 56 $\mu g\,l^{-1}$ Al) for two and six days. At each time-point, smolts were sampled directly from freshwater treatment tanks and after a 24 h seawater challenge. Exposure to acid/MAl and acid/HAl led to accumulation of gill Al, substantial alterations in gill morphology, reduced gill Na^+/K^+-ATPase (NKA) activity, and impaired ion regulation in both freshwater and seawater. Exposure to acid/MAl for six days also led to a decrease in gill mRNA expression of the apical Cl^- channel (cystic fibrosis transmembrane conductance regulator I), increased apoptosis upon seawater exposure, an increase in the surface expression of mitochondria-rich cells (MRCs) within the filament epithelium of the gill, but reduced abundance of gill NKA-positive MRCs. By contrast, smolts exposed to acid and the lowest Al concentration exhibited minor gill Al accumulation, slight morphological modifications in the gill, and impaired seawater tolerance in the absence of a detectable effect on freshwater ion regulation. These impacts were accompanied by decreased cell proliferation, a slight increase in the surface expression of MRCs within the filament epithelium, but no impact on gill apoptosis or total MRC abundance was observed. However, MRCs in the gills of smolts exposed to acid/LAl exhibited morphological alterations including decreased size, staining intensity, and shape factor. We demonstrate that the seawater tolerance of Atlantic salmon smolts is extremely sensitive to acute exposure to acid and low levels of Al, and that the mechanisms underlying this depend on the time-course and severity of Al exposure. We propose that when smolts are exposed to acid and moderate to high Al concentrations, impaired seawater tolerance results from extensive gill Al accumulation, damage to the epithelium, reduced MRC and transport protein abundance, and a synergistic stimulation of apoptosis in the gill upon seawater exposure. When smolts are exposed to acid and low levels of Al, loss of seawater tolerance appears to be independent of these mechanisms and may result instead from a shift in the phenotype of MRCs present in the gill epithelium.

1. Introduction

Acid rain resulting from industrial emissions of sulfur and nitrogen oxides is a known cause of Atlantic salmon, *Salmo salar*, decline in southern Norway (Hesthagen, 1989) and eastern Canada (Watt, 1987). Recently, acid rain has also been suggested as a cause of Atlantic salmon decline in regions of the northeastern United States including Maine where salmon have been listed as a federally endangered species (National Academy of Science, 2004; McCormick et al., 2009). Acid rain is known to have a variety of adverse affects on aquatic ecosystems including decreased buffering capacity of soils and surface waters, increased soil and water acidity, and increased aluminum (Al) (Driscoll et al., 2001). As a result of their underlying geology, many rivers and streams in New England have poor buffering capacity and are particularly vulnerable to episodic acidification during spring snowmelts and

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fall storms (Driscoll et al., 2001). During precipitation events, Al leaches from the soil elevating water Al concentrations and this, coupled with decreased water pH, leads to increased concentrations of inorganic Al (Al_i), the chemical form of Al most toxic to fish (Driscoll, 1984; Gensemer and Playle, 1999). Inorganic Al accumulates on the surface and within the fish gill, damages the gill epithelium, and ultimately causes impaired ion regulation and respiration (Gensemer and Playle, 1999). These types of disturbances are particularly debilitating to anadromous salmonids that face the physiological challenge of migration from freshwater (FW) to seawater (SW).

After two to five years of stream residence, Atlantic salmon undergo the parr-smolt transformation. This developmental period, cued by changes in photoperiod and temperature, consists of physiological, behavioral, and morphological alterations that are preparatory for SW entry and survival in the marine environment (McCormick et al., 1998). Among the physiological changes is an increase in SW tolerance brought about by alterations in several osmoregulatory organs (gill, gut, and kidney). At the level of the gill, this includes the proliferation of mitochondria-rich cells (MRCs) or chloride cells and the up-regulation of ion transport proteins including Na^+/K^+-ATPase (NKA), $Na^+/K^+/Cl^-$ cotransporter (NKCC), and an apical Cl^- channel (cystic fibrosis transmembrane conductance regulator, CFTR) (Hoar, 1988; Pelis et al., 2001; Singer et al., 2002; Tipsmark et al., 2002).

The changing physiology of the Atlantic salmon smolt makes it one of the most sensitive of the salmon life-stages to acid/Al (Rosseland and Skogheim, 1984; Rosseland et al., 2001; Monette and McCormick, 2008). Exposure to acid/Al during the parr-smolt transformation can disrupt ion regulation (Saunders et al., 1983; Staurnes et al., 1993; Kroglund and Staurnes, 1999; Magee et al., 2003; Monette et al., 2008), increase ventilation frequency (Poleo and Muniz, 1993), decrease growth, and impair downstream migration and marine survival (Staurnes et al., 1996; Kroglund and Finstad, 2003). Previous work in our laboratory has demonstrated that acute exposure to acid/Al disrupts ion regulation of Atlantic salmon smolts during a 24 h SW challenge without affecting ion regulation in FW (Monette et al., 2008). This suggests that the hypoosmoregulatory system of smolts is highly sensitive to acid/Al however the specific mechanisms underlying this heightened sensitivity remain poorly understood.

The present study was conducted to investigate the physiological, molecular, and cellular mechanisms of impaired ion regulation in Atlantic salmon smolts during acute acid/Al exposure. To do this, we exposed smolts to acidic softwater with increasing concentrations of Al for two and six days in the laboratory followed by a 24 h SW challenge at each time-point. We subsequently examined ion regulation, stress physiology, endocrine status, gill ion transporter expression and activity, and gill morphology and cell turnover.

2. Materials and methods

2.1. Fish

Atlantic salmon, *Salmo salar*, were obtained from the Kensington National Fish Hatchery (Kensington, CT, USA), and held at the Conte Anadromous Fish Research Center (Turners Falls, MA, USA). Prior to the start of the study, fish (6 kg biomass $tank^{-1}$) were held in 1.6 m diameter (1000 L) tanks receiving flow-through Connecticut River water (Ca^{2+}, 9.0 mg l^{-1}; Mg^{2+}, 1.5 mg l^{-1}; Na^+, 6.8 mg l^{-1}; K^+, 1.1 mg l^{-1}; Cl^-, 11.0 mg l^{-1}) with a mean residence time of ~4 h. Fish were maintained under natural photoperiod conditions and ambient river temperatures, and fed to satiation twice daily with commercial feed (Zeigler Bros., Garners, PA, USA).

2.2. Experimental design

Laboratory experiments were conducted with one-year-old juvenile Atlantic salmon large enough to be smolts (30–71 g, fork length >14 cm). Smolts were randomly assigned to two replicate tanks (n = 22–24) receiving control (pH 6.5), acid/low Al (LAl: pH 5.2), acid/moderate Al (MAl: pH 5.2 plus nominal addition of 50 μg l^{-1} Al), and acid/HAl (HAl: pH 5.2 plus nominal addition of 85 μg l^{-1} Al). Acid/Al concentrations were chosen to be comparable to those observed in poorly buffered streams in the Northeastern United States. In these streams, when pH is below 6.0, inorganic Al is present at low concentrations due to mobilization from the soil (Driscoll et al., 2001), therefore we did not attempt to have an Al-free treatment condition. Instead, our goal was to examine the impact of acidic water in the presence of varying, environmentally relevant, Al concentrations on Atlantic salmon smolts. All experimental tanks (134 l) received artificial softwater prepared by mixing deionized water (Siemens, Lowell, MA, USA) with dechlorinated city tap water (Turners Falls, MA, USA) (5:1), and target pH and Al concentrations were achieved using 3 N HCl and an $AlCl_3{\cdot}6H_2O$ stock solution (1000 mg l^{-1} Al), respectively. Dilution of tap water resulted in a reduction in ionic strength (including ambient Ca^{2+}) similar in magnitude to that which occurs following episodic rain events in low to moderately buffered streams (Haines et al., 1990). Experimental water was mixed for ≥12 h before entering fish tanks to avoid unstable water conditions, and each tank received continuous flow of 22 l h^{-1}. Temperature was maintained at 10.4–13.3 °C using a re-circulating chiller system. Experimental tanks were aerated continuously maintaining dissolved oxygen at normoxic levels. Measurements of pH were made twice daily from water samples collected at the tank outlet using a bench-top pH meter (Type 145, Corning, Medfield, MA, USA) with a low-ion pH probe (Ross Ultra 8156, Thermo Orion, Beverly, MA, USA). Water samples were also collected at the tank outlet twice daily in acid-washed 50 ml tubes for the measurement of Al, Ca^{2+} and Na^+. Food was withheld for 24 h prior to the start of the study, and fish were starved for the duration of the experiment. Just before the start of the study, seven to ten smolts were sampled directly from their rearing tanks as a reference group (T = 0). Smolts were exposed to the four experimental water chemistries for two and six days. At each time-point, four to five fish per replicate tank were sampled (FW), and simultaneously, four fish per replicate tank were placed into tanks (330 l, maintained at 12 °C) containing 35‰ SW and sampled after 24 h (SW). Seawater was made with Crystal Sea Marine Mix (Marine Enterprises, Inc., Baltimore, MD, USA) and was charcoal-treated and aerated. Smolts taken directly from rearing tanks were also given a SW challenge at the same time as experimental fish as a reference. Plasma Na^+ and Cl^- concentrations after 24 h in 35‰ SW were used as indicators of SW tolerance (Clarke and Blackburn, 1977). Also at each time-point, three smolts per replicate tank were anesthetized with MS-222 (50 mg MS 222 l^{-1}, pH 7.0) and injected intraperitoneally with 5-bromo-2′-deoxyuridine (BrdU; Sigma Aldrich, St. Louis, MO, USA) dissolved in Ringers (140 mM NaCl, 10 mM $NaHCO_3$, 2 mM NaH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 4 mM KCl, pH 7.8) at a dose of 200 μg g^{-1} fish. BrdU is a thymidine analog incorporated into DNA during the synthesis phase of the cell cycle and was used to identify cells undergoing proliferation/replication. After a short recovery, injected smolts were returned to their respective treatment tanks and sampled four hours later.

2.3. Tissue sampling and analysis

Fish were anesthetized with MS-222 (100 mg MS 222 l^{-1}, pH 7.0), weighed to the nearest 0.1 g, and fork and total lengths recorded to the nearest 0.1 cm. All fish were sampled within

6 min of tank disturbance. Blood was collected in heparinized 1 ml syringes via caudal puncture and centrifuged at 3200 × *g* for 5 min at 4 °C. Plasma was removed and stored at −80 °C for later analyses. Plasma Na^+ was measured by flame atomic absorption spectrophotometry (AAnalyst 100, PerkinElmer, Wellesley, MA, USA). Plasma Cl^- was measured by silver titration using a digital chloridometer (Labconco, Kansas City, MO, USA). Plasma cortisol was measured by direct enzyme immunoassay (Carey and McCormick, 1998). Plasma prolactin was measured by radioimmunoassay (Hirano et al., 1985). Plasma glucose was measured by enzymatic coupling with hexokinase and glucose 6-phosphate dehydrogenase (Stein, 1963). Gill biopsies (4–6 primary filaments) for the measurement of Al accumulation were taken and analyzed as previously described (Monette and McCormick, 2008). Gill biopsies were also taken for the measurement of NKA activity, placed into 100 μl SEI (250 mM sucrose, 10 mM Na_2EDTA and 50 mM imidazole, pH 7.3) and stored at −80 °C for later analysis (McCormick, 1993). The second right gill arch of each fish was removed, placed into an autoclaved 1.5 ml centrifuge tube and stored at −80 °C for RNA extraction. For immunocytochemistry, the first right gill arch of each fish was removed, cut into pieces (3–5 mm), and fixed overnight in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4) at 4 °C. Fixative was replaced with 70% ethanol the next day. For scanning electron microscopy (SEM), the second left gill arch of each fish was removed and fixed in 2% PFA-2% glutaraldehyde in 0.1 M PB for 24 h, post-fixed in 1% osmium tetroxide in the same buffer for 1 h, and stored in 70% ethanol.

2.4. Water chemistry analyses

Water samples for the measurement of total Al (Al_{tot}) and inorganic Al (Al_i) were collected, processed, and analyzed as previously described by Monette and McCormick (2008). In brief, total Al (Al_{tot}) was analyzed from unfiltered water samples, whereas dissolved Al (Al_a) was analyzed from filtered (0.45 μm, nitrocellulose) water samples. Water samples were acidified (0.2%) with trace metal grade HNO_3 immediately upon collection and Al concentration was measured using graphite furnace atomic absorption spectrophotometry (HGA-800/AAnalyst 100, Perkin Elmer, Wellesley, MA, USA). Inorganic Al (Al_i) was determined by the cation-exchange column method (Amberlite 120, prepared with Na^+) (Driscoll, 1984). Al retained in the samples processed through the column was termed organically bound Al (Al_o). Al_i was determined by calculating the difference between Al_a and Al_o. Ca^{2+} and Na^{2+} were measured by flame atomic absorption spectrophotometry. Water chemistry values are reported in Table 1.

2.5. Gill NKA activity

Gill NKA activity was measured following the method described by McCormick (1993). Gill biopsies were thawed immediately prior to assay, 25 μl of 0.5% SEID (0.1 g sodium deoxycholate in 20 ml SEI) was added to each microcentrifuge tube with tissue and homogenized for 10–15 s using a Kontes pellet pestle motor. The homogenate was then centrifuged at 3200 × *g* for 30 s and the supernatant assayed both for NKA activity and total protein (BCA protein assay, Pierce, Rockford, IL, USA). This kinetic assay was run at 25 °C for 10 min in a temperature-controlled plate reader (Thermomax, Molecular Devices, Menlo Park, CA, USA) and read at a wavelength of 340 nm. Gill NKA activity was calculated as the difference in the production of ADP in the absence and presence of 0.5 mmol l^{-1} ouabain, and expressed as μmol ADP mg $protein^{-1}$ h^{-1}.

2.6. Real-time quantitative PCR

Total RNA was extracted from gill samples (approximately 20 mg tissue) using Isogen buffer (Nippon Gene, Kanda, Tokyo, Japan) according to the manufacturer's instructions, and RNA concentrations were determined using a microplate spectrophotometer (SpectraMax 190, Nihon Molecular Devices, Kanda, Tokyo, Japan). Total RNA was then treated with RNase-free DNase I (Takara, Shiga, Japan). Reverse transcription was carried out using SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Real-time PCR was performed with an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with TaqMan probe (Applied Biosystems, Foster City, CA, USA). The cDNA fragments as the standards for CFTR I, CFTR II, caspase-3B and β-actin were amplified with primers designed based on the Gene Bank accession numbers AF155237, AF161070, DQ008069, and AF012125, respectively (Table 2). After denaturation at 95 °C for 10 min, amplification was carried out by 50 cycles of 95 °C for 15 s and 60 °C for 1 min. In each assay standard cDNAs, positive control sample (cDNA from pooled gill tissue), and samples were run in triplicate. All data were normalized to mRNA levels of β-actin.

2.7. Antibodies

A mouse monoclonal antibody against a conserved sequence of the α-subunit of NKA (α5; Developmental Studies Hybridoma Bank) was diluted 1:1000 and used for immunocytological detection of NKA. A rabbit monoclonal antibody against active human caspase-3 (BD PharMingen, San Diego, CA, USA) was diluted 1:1000 and used for immunocytochemical detection of caspase-3. This antibody recognizes the active form of caspase-3 which consists of a heterodimer of 17 and 12 kD subunits, derived from a 32 kD proenzyme. As a positive antibody control, gill biopsies were incubated in culture media containing 14 μM camptothecin (apoptosis inducer; Sigma Aldrich, St. Louis, MO, USA) for 24 h at 15 °C. Gill biopsies incubated with camptothecin exhibited very high levels of caspase-3 immunoreactivity, whereas immunoreactivity was less in biopsies incubated without camptothecin (M.Y. Monette and S.D. McCormick, unpublished). A mouse monoclonal antibody

Table 1
Nominal acid/Al exposures and measured water chemistry parameters in exposure tanks over the time-course of six days. Values are mean ± SE.

Treatment	Nominal pH/Al	pH (n = 23–36)	Al_{tot} (μg l^{-1}) (n = 10–29)	Al_i (μg l^{-1}) (n = 5–20)	Ca^{2+} (mg l^{-1}) (n = 9–26)	Na^{2+} (mg l^{-1}) (n = 9–28)
Control	6.5/0	6.5 ± 0.02 (6.4–6.9)	6.3 ± 0.5 (4.0–15)	3.4 ± 0.5 (1–11)	2.1 ± 0.08 (1.2–2.6)	2.0 ± 0.13 (1.3–3.7)
Acid/LAl	5.2/0	5.3 ± 0.02 (5.1–5.4)	11 ± 2.3 (4.8–32)	11 ± 3.4 (4.5–24)	2.5 ± 0.04 (2.3–2.7)	2.3 ± 0.19 (1.5–3.8)
Acid/MAl	5.2/50	5.4 ± 0.03 (4.9–5.7)	43 ± 1.7 (37–60)	42 ± 3.5 (36–55)	2.0 ± 0.10 (1.4–2.7)	2.4 ± 0.08 (2.0–2.9)
Acid/HAl	5.2/85	5.3 ± 0.06 (5.0–5.5)	71 ± 3.3 (58–90)	56 ± 5.7 (42–68)	2.6 ± 0.04 (2.4–2.7)	2.5 ± 0.27 (1.6–3.8)

Range is given in parentheses below.

Table 2
Design of primers and fluorogenic probes for cloning of standard cDNAs.

Gene	Forward primer	Reverse primer	Probe
CFTR I	5′-GTTGAAGAGGCAAGGAGATAGGTT-3′	5′-GACTAGAGGCACTTGGATGAGTCA-3′	5′-CAAGGAATCGTGGAAAGATGCGCTG-3′
CFTR II	5′-TGGGAGGAGGGATCTTGGA-3′	5′-CCTTACATCCTATCTCCCCATCTC-3′	5′-TTGTCCTCCAGTGTGGTTGGG-3′
Caspase-3B	5′-AGCCAGTCCGCCTCCTTT-3′	5′-TGTGCCATAGAAGAACCCCTCT-3′	5′-TGTGTGATGCTGAGCCATGGA-3′
β-Actin	5′-TTCAACACCCCTGCCATGTA-3′	5′-ACGGCCAGAGGCGTATAGG-3′	5′-TGGCCATCCAGGCCGTGTTGT-3′

recognizing BrdU (Roche Applied Sciences, Indianapolis, IN, USA) was diluted 1:1000 and used for immunocytochemical detection of proliferating cells. Alexa-Fluor 546 goat anti-mouse and Alexa-Fluor 488 goat ant-rabbit (Molecular Probes, Eugene, OR, USA) were used as secondary antibodies where appropriate. Antibody control experiments (no antibodies, secondary antibodies without primary, fish not injected with BrdU) showed no specific staining and low background (M.Y. Monette and S.D. McCormick, unpublished). Double staining of the same sections was performed for NKA and caspase-3.

2.8. Immunocytochemistry

Fixed tissue was rinsed in 10 mM phosphate buffered saline (PBS), placed in PBS with 30% (w/v) sucrose for 1 h and frozen in embedding medium (Tissue Tek, VWR International, West Chester, PA, USA). 5 μm tissue sections, both parallel (sagittal) and perpendicular (cross) to the long axis of the filament, were cut in a cryostat at −24 °C. Sections were placed on poly-L-lysine-coated slides, dried at room temperature for 10 min, rinsed in PBS, and incubated in 2% normal goat serum in PBS for 0.5 h at room temperature. For BrdU immunocytochemistry only, slides were incubated in 2N HCl in PBS for 0.5 h at room temperature (to denature DNA and make BrdU available for antibody detection) prior to incubation with normal goat serum. Slides were incubated with primary antibody in antibody dilution buffer (0.01% NaN_3, 0.1% bovine serum albumin, 2% normal goat serum and 0.02% keyhole limpet hemocyanin in PBS) overnight at 4 °C. After incubation, slides were rinsed 3× with PBS, exposed to secondary antibody for 2 h at room temperature, and again rinsed 3× with PBS. Sections were mounted in PBS, covered by a cover slip, and examined with a Nikon inverted fluorescent microscope with a mercury lamp. All images were taken with a Nikon digital camera set to a two second shutter speed.

From each fish, immunopositive NKA, caspase-3, and BrdU cells were counted within cross-sections of the filament and sagittal sections of lamellar epithelia. Mean numbers of NKA-, caspase-3-, and BrdU-positive cells for each experimental group were obtained using means calculated from each fish. Cell or staining area (μm^2 $cell^{-1}$), staining intensity (mean gray value/pixel), and shape factor were obtained from NKA-positive cells using Image J version 1.37 (NIH, Bethesda, MD, USA). Shape factor is defined as $4\pi(A/P^2)$ (where A is the area and P is the perimeter of the immunoreactive region), with values of 1.0 indicating a circular shape, and values of 0 an elongate shape. A single threshold level for each image was used to quantify immunoreactive cells. Background staining intensity was obtained by averaging intensity in two non-cellular regions of each image and subtracting that from each staining intensity value obtained for that image. At least 100 immunoreactive MRCs were analyzed from five different tissues sections from four individuals per experimental group and more than 4800 MRCs were measured overall.

2.9. Scanning electron microscopy

Five fish from each treatment were used in SEM analysis. The second gill arch from the left side of each fish was excised, and the central region of each arch (~1 mm), bearing up to 15 filaments in both anterior and posterior rows, was used for SEM analysis. Small pieces containing two to five individual filaments were cut free from the central part of each arch by a razor blade, completely dehydrated in ethanol, critical-point dried with liquid CO_2, mounted on carbon tape, sputter-coated with gold-palladium, and examined with a scanning electron microscope (Hitachi S 2700, Tokyo, Japan) at an accelerated voltage 20 kV. The gross anatomy of the gills and surface ultrastructure of the afferent (trailing) edge of the filament below respiratory lamellae but not including the interlamellar areas were examined. Photographs at 2000× magnification of two randomly chosen rectangle areas (2400 μm^2) from three different filaments per fish and five fish per experimental group (a total of 30 areas per group) were used for quantification of mitochondria-rich cell (MRC) and mucous cell (MC) density. Cell types were identified according to surface morphology as previously reported for salmonids (Perry, 1997). MRC and MC density was quantified by counting the number of apical apexes of MRCs and pores of MCs.

2.10. Statistics

All data are presented as mean ± standard error of the mean (SEM). For each physiological parameter, potential tank effects were tested by nesting replicate tanks within treatment. Significant tank effects ($P < 0.05$) were not observed for any analyses, therefore fish from replicate tanks were pooled. A two-way analysis of variance (ANOVA) on ranks was used to determine the effect of treatment (control, LAl, MAl, HAl) and salinity (FW, SW) on physiological parameters unless otherwise noted. Data at days two and six were treated separately. For immunocytochemistry data, a three-way ANOVA on ranks was used to determine the effect of treatment (control, LAl, MAl), salinity (FW, SW), and cell location (filament, lamellae) on cell number and morphology. In all cases, when significant effects were observed ($P < 0.05$), comparisons to control were made using Duncan's post hoc test. All statistical analyses were performed using Statistica 7.0 (Statsoft, Inc., Tulsa, OK, USA).

3. Results

3.1. Mortality and gill aluminum concentrations

There were no mortalities in the control, acid/LAl, or acid/MAl treatments. In the acid/HAl treatment, 34% of smolts died by day two in FW, and 50% of smolts transferred to SW at this time-point died within 24 h. All remaining smolts in the acid/HAl treatment were dead by the end of day four therefore this treatment is not represented at day six.

To confirm exposure of smolts to elevated levels of waterborne Al_i, we measured Al concentration in a gill tissue biopsy. After two days of treatment, gill biopsies exhibited a dose-dependent increase in Al concentration and were 4-fold to 120-fold greater than controls in FW (Fig. 1A). Following SW challenge, gill Al concentrations remained elevated relative to controls in all acid/Al treatments, but a significant proportion (40–95%) was lost from the gill demonstrating the rapid (≤24 h) depuration of Al from the epithelium after exposure to salt water (Fig. 1A). After six days, gill Al concentrations were 4-fold greater than controls in the acid/LAl

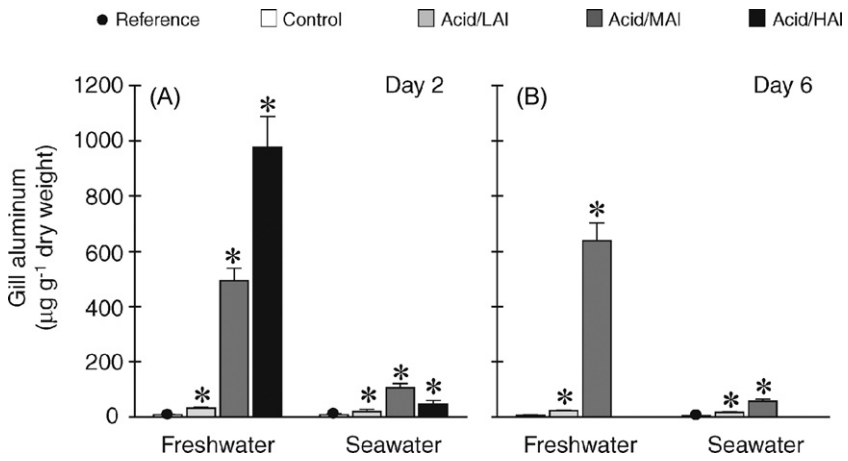

Fig. 1. Gill Al accumulation of Atlantic salmon smolts exposed to control, acid/LAl, acid/MAl, and acid/HAl for two and six days in FW followed by a SW challenge at each time-point. Values are mean ± SE (n = 4–18). An * indicates a significant difference ($P < 0.05$) from control within salinity (FW, SW). The black dot at day two in FW represents reference fish sampled just prior to the start of the study, whereas those at days two and six in SW represent reference fish sampled after a SW challenge. Two-way ANOVA for gill Al accumulation determined effects of treatment and salinity ($P < 0.001$) after both time-points.

treatment and 120-fold greater in the acid/MAl treatment in FW (Fig. 1B). Following SW challenge, gill Al concentrations in the acid/MAl treatment remained elevated relative to controls but were 91% lower than their respective FW value (Fig. 1B). Gill Al concentrations in the acid/LAl treatment also remained elevated relative to controls in SW but did not differ from their respective FW value (Fig. 1B).

3.2. Plasma ion concentrations and gill NKA activity

After two days, smolts in the acid/MAl and acid/HAl treatments exhibited decreases (7–10 mM) in plasma Na^+ concentrations in FW and increases (7–20 mM) in SW, whereas exposure to acid/LAl had no effect (Fig. 2A). After six days, exposure of smolts to acid/LAl had no effect on plasma Na^+ concentrations in FW but led to a 24 mM increase relative to control values when fish were given a SW challenge, demonstrating impaired SW tolerance (Fig. 2B). Smolts exposed to acid/MAl for six days exhibited decreases (16 mM) in plasma Na^+ concentrations in FW and increases (12 mM) in SW, indicating impaired ion regulation in both media (Fig. 2B).

After two days, smolts in the acid/HAl treatment exhibited decreases (11 mM) in plasma Cl^- concentrations in FW and increases (24 mM) in SW (Fig. 2C). Exposure to acid/MAl had no effect on plasma Cl^- concentrations in FW but led to a 12 mM increase relative to controls when fish were given a SW challenge (Fig. 2C). Exposure of smolts to acid/LAl resulted in a slight but significant increase (3 mM) in plasma Cl^- levels in FW but had no effect on Cl^- levels in SW (Fig. 2C). Similar to plasma Na^+ concentrations, six-day exposure to acid/LAl had no effect on plasma Cl^- concentrations in FW but led to a 25 mM increase relative to control values when fish were given a SW challenge (Fig. 2D). Smolts exposed to acid/MAl for six days exhibited decreases (17 mM) in plasma Cl^- in FW and increases (20 mM) in SW again indicating impaired ion regulation in both media (Fig. 2D).

Reference fish had high levels of gill NKA activity (8.5–11.0 μmol ADP mg protein^{-1} h^{-1}) confirming the smolt status of fish used in this study and activity levels remained high in controls throughout the study (Fig. 2E and F). After two days, exposure to acid/LAl and acid/MAl had no effect on gill NKA activity levels whereas exposure to acid/HAl led to a 38% reduction in enzyme activity in FW relative to controls and a 47% reduction in SW (Fig. 2E). After six days, exposure to acid/LAl had no effect on gill NKA activity of smolts despite the observed loss of SW tolerance in this treatment, whereas exposure to acid/MAl led to a 50% reduction in enzyme activity in FW and a 35% reduction in SW (Fig. 2F).

3.3. Real-time quantitative PCR

Expression of CFTR I mRNA in the gills of smolts was two orders of magnitude greater than expression of CFTR II mRNA (Fig. 3A–D). Acid/Al exposure had no effect on gill CFTR I mRNA expression after two days (Fig. 3A). After six days, exposure to acid/LAl had no effect on gill CFTR I mRNA expression, whereas exposure to acid/MAl led to a 5-fold reduction in expression relative to controls (Fig. 3B). One-way ANOVA revealed a significant treatment effect on gill mRNA levels of CFTR II and the cysteine protease, caspase-3B, after two and six days, respectively, however there were no significant post hoc comparisons (Fig. 3C and F).

3.4. Plasma glucose and plasma hormones

Exposure to acid/LAl had no effect on plasma glucose levels under any condition (Fig. 4A and B). After two days, smolts exposed to acid/MAl and acid/HAl had plasma glucose levels that were 2- and 3-fold greater than controls in FW, respectively. Following SW challenge, plasma glucose levels returned to control levels in the acid/MAl group but remained elevated in the acid/HAl group (Fig. 4A). After six days, plasma glucose levels of smolts in the acid/MAl treatment were 4-fold greater than controls, but this effect disappeared after 24 h in SW (Fig. 4B).

After two days plasma cortisol levels of smolts remained unaffected by treatment with acid/LAl and acid/MAl, whereas in the acid/HAl treatment, cortisol levels were 6.5-fold greater than controls in FW (Fig. 4C). Plasma cortisol levels of smolts in the acid/HAl treatment returned to control levels after 24 h in SW (Fig. 4C). After six days, exposure to acid/LAl had no effect on plasma cortisol levels, whereas there was a strong but highly variable tendency for levels to be greater than controls in the acid/MAl treatment in both FW and SW (Fig. 4D).

Acid/Al treatment had no effect on plasma prolactin levels of smolts (Fig. 4E and F).

3.5. Immunocytochemistry

Many cells within the filament and lamellar epithelia were positively stained for NKA in FW and SW (Figs. 5 and 6A–C). Positively stained cells within the filament epithelium appeared large and

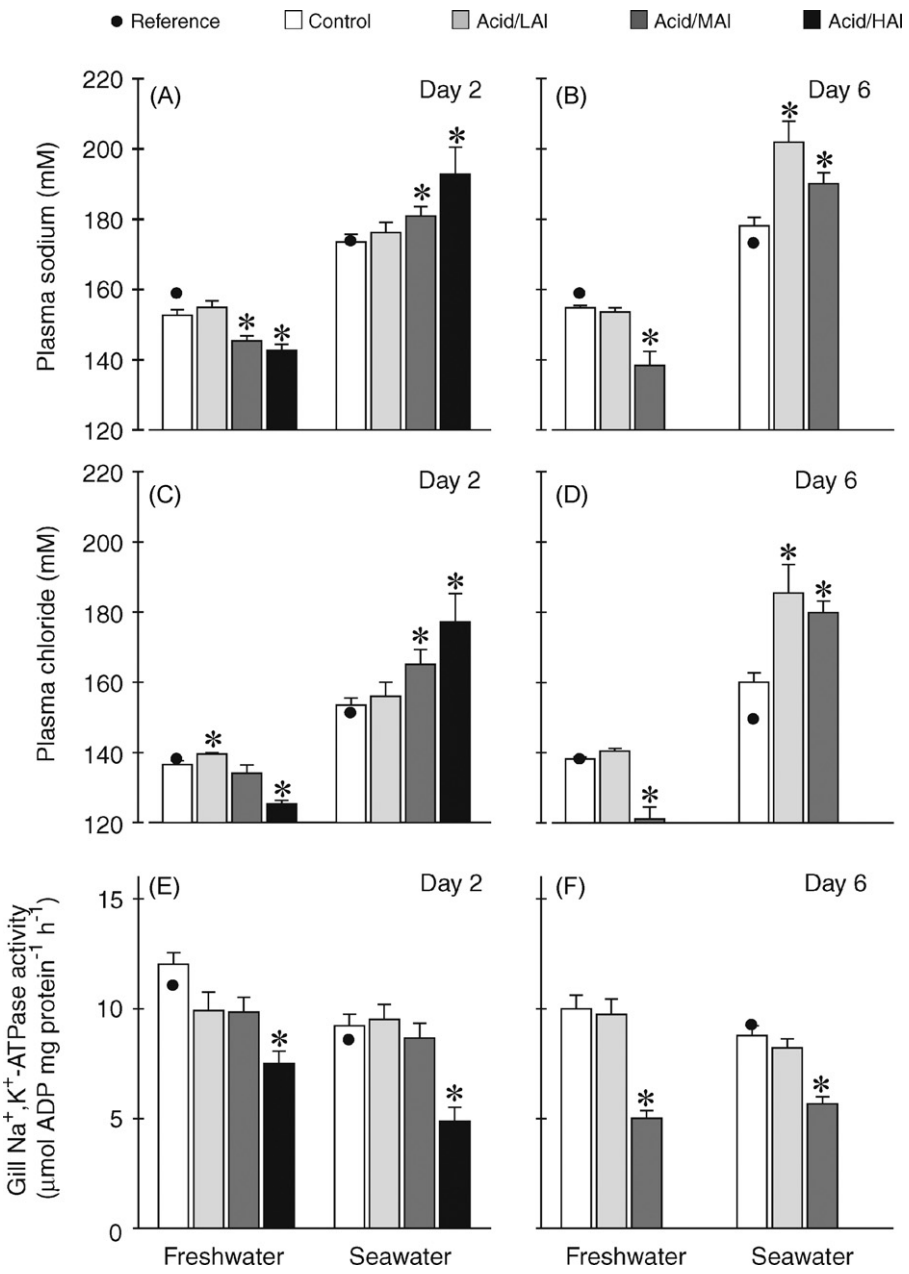

Fig. 2. Plasma Na^+, plasma Cl^- and gill NKA activity of smolts exposed to control, acid/LAl, acid/MAl, and acid/HAl for two and six days in FW followed by a SW challenge at each time-point. Values are mean ± SE (n = 4–18). An * indicates a significant difference (P < 0.05) from control within salinity (FW, SW). The black dot at day two in FW represents reference fish sampled just prior to the start of the study, whereas those at days two and six in SW represent reference fish sampled after a SW challenge. Two-way ANOVA for plasma Na^+ and Cl^- determined a treatment effect (P < 0.02) after six days, a salinity effect (P < 0.001) after both time-points, and a treatment/salinity interaction (P < 0.001) after both time-points. Two-way ANOVA for gill NKA activity determined a treatment effect (P < 0.001) after both time-points, and a salinity effect (P = 0.004) after two days.

columnar, whereas those in the lamellar epithelium were slightly smaller and more circular (Table 3). The size, shape, and location of NKA-positive cells indicated that they were mitochondria-rich cells (MRCs) or chloride cells (Figs. 5 and 6A–C). No other cell type exhibited positive NKA staining above background levels. NKA immunoreactivity was seen throughout the cell except for the nucleus, consistent with a basolateral/tubular distribution. Exposure to acid/LAl for six days had no significant effect on the number of total MRCs in the gills of smolts (Fig. 7). However, in this treatment MRCs appeared smaller than those in control fish in the filament epithelium, and less bright and more elongate (significantly lower shape factor) in the lamellar epithelium in FW (Table 3). Smolts in the acid/MAl treatment had 46% fewer MRCs than controls in the lamellar epithelium in FW and 67% fewer in SW (Fig. 7B). MRC area, staining intensity, and shape factor in the acid/MAl group did not appear to be altered by treatment (Table 3), however many MRCs within the filament epithelium were small and had very low levels of NKA immunoreactivity (Fig. 8A).

Cells immunoreactive for caspase-3 were found within the filament and lamellar epithelia in FW and SW, but at a much lower frequency than NKA-positive cells (Figs. 5 and 6D–F). Exposure to acid/LAl and acid/MAl for six days had no effect on the number of caspase-3-positive cells in the gills of smolts in FW (Fig. 7C and D). By contrast, the number of caspase-3-positive cells on the primary filament of smolts exposed to acid/MAl for six days followed by a

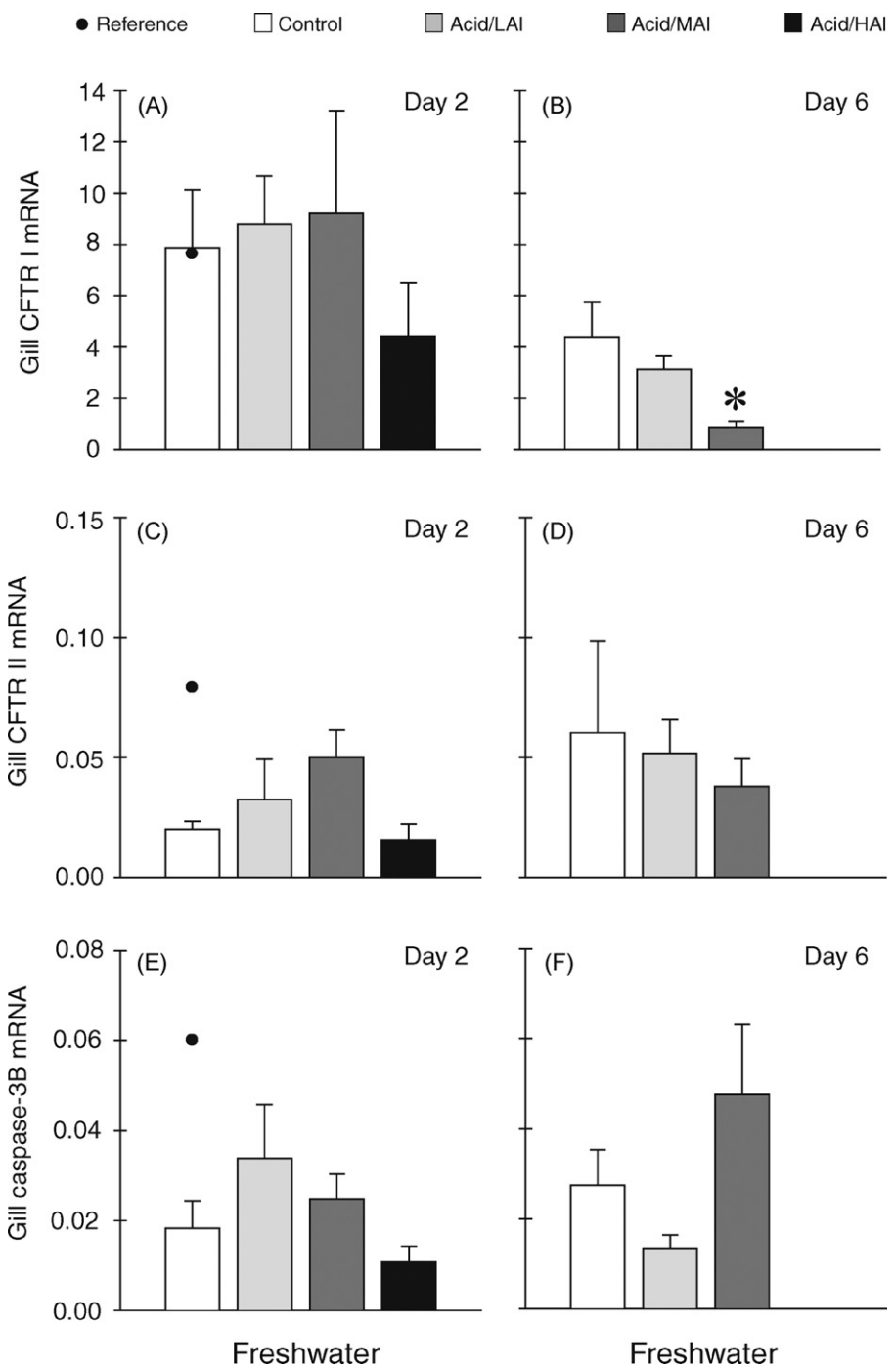

Fig. 3. CFTR I, CFTR II, and caspase-3B mRNA abundance in the gills of Atlantic salmon smolts exposed to control, acid/LAl, acid/MAl, and acid/HAl for two and six days in FW. Values are mean ± SE (n = 6–8). Data are expressed as a ratio to β-actin mRNA levels. An * indicates a significant difference ($P < 0.05$) from control. The black dot at day two in FW represents reference fish sampled just prior to the start of the study. One-way ANOVA of CFTR I mRNA abundance determined a treatment effect ($P < 0.001$) after six days. One-way ANOVA of CFTR II mRNA abundance determined a treatment effect ($P = 0.03$) after two days. One-way ANOVA for gill caspase-3B mRNA abundance determined a treatment effect ($P = 0.01$) after six days.

SW challenge was 3-fold greater than controls (Fig. 7C). The number of caspase-3-positive cells on the secondary lamellae of smolts in this treatment also tended to be greater than controls (Fig. 7D). The location and size of caspase-3-positive cells often indicated that they could be MRCs, however double staining of the same section revealed that the majority of caspase-3-positive cells did not exhibit NKA immunostaining (Fig. 8B). Transfer to SW resulted in increases in the number of caspase-3-positive cells within the gill in most groups, with this effect appearing to increase in a dose-dependent manner with acid/Al exposure (Fig. 7C and D).

The majority of proliferating (BrdU-positive nuclei) cells in the gills of smolts were located within the filament epithelium near the central venous sinus with very few cells staining within the lamellar epithelium (Fig. 5G–I). Positively stained cells within the filament epithelium were generally much smaller than MRCs and the location and size of BrdU-positive cells were consistent with nuclear staining of newly differentiating cells (Fig. 5G–I). The number of BrdU-positive cells of smolts exposed to acid/LAl for six days was 37% lower than controls within the filament epithelium and 3-fold lower within the lamellar epithelium (Fig. 7E). The number of

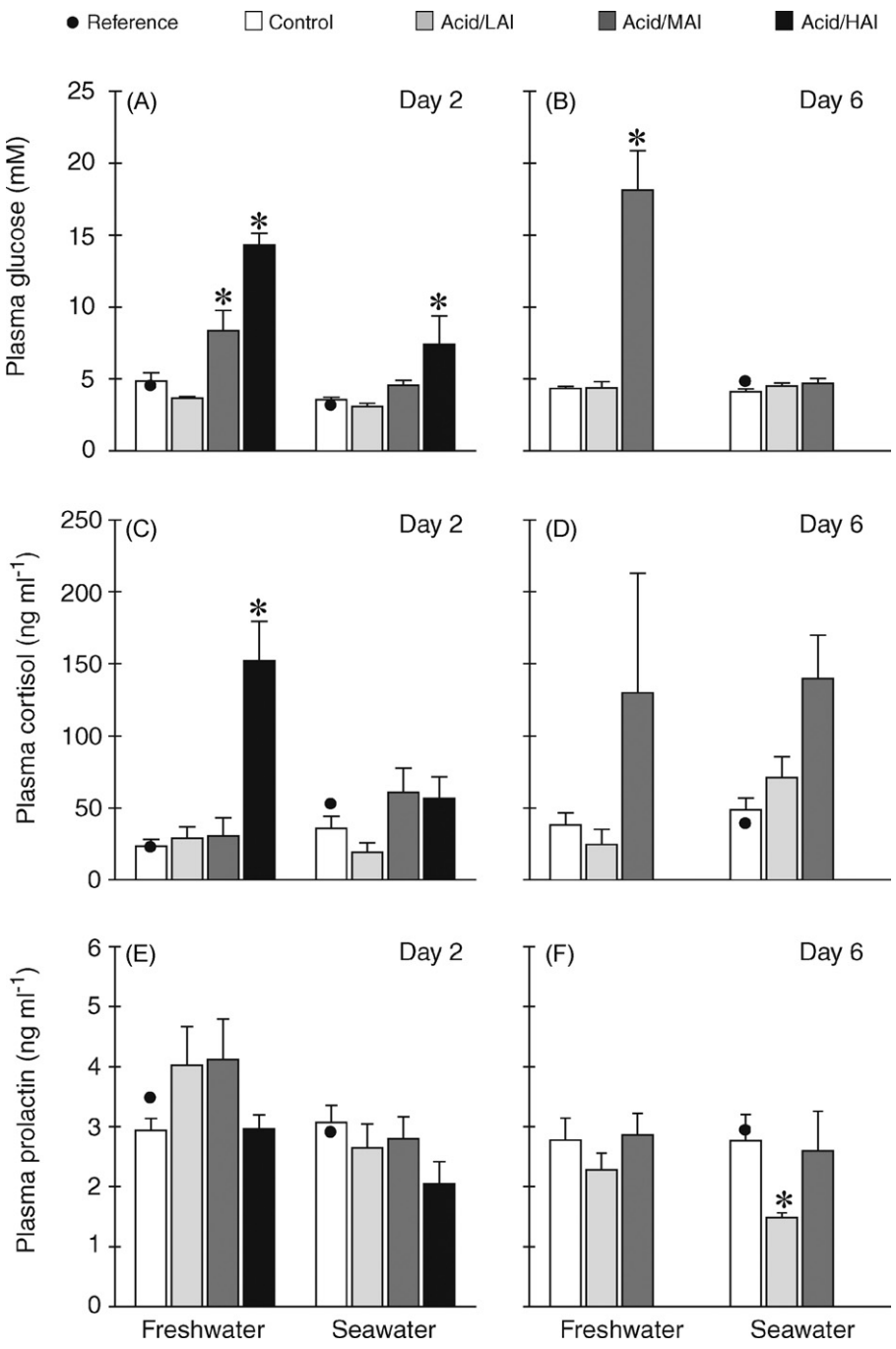

Fig. 4. Plasma glucose, cortisol and prolactin concentrations of smolts exposed to control, acid/LAl, acid/MAl, and acid/HAl for two and six days in FW followed by a SW challenge at each time-point. Values are mean ± SE (n = 4–18). An * indicates a significant difference (P < 0.05) from control within salinity (FW, SW). The black dot at day two in FW represents reference fish sampled just prior to the of the study, whereas those at days two and six in SW represent reference fish sampled after a SW challenge. Two-way ANOVA for plasma glucose determined a treatment (P < 0.001) and salinity effect (P < 0.03) after both time-points. Two-way ANOVA for plasma cortisol determined a treatment effect (P < 0.05) after both time-points, a salinity effect (P = 0.003) after six days, and a treatment/salinity interaction (P = 0.04) after two days. Two-way ANOVA for plasma prolactin determined a salinity effect (P < 0.02) after both time-points.

BrdU-positive cells in the acid/MAl treatment tended to be greater than controls within the filament epithelium and was 42% lower than controls within the lamellar epithelium (Fig. 7E and F).

3.6. Scanning electron microscopy

In control smolts, long gill filaments bore well-shaped lamellae separated by large interlamellar spaces (Fig. 9A). The outermost layer of filament epithelium was composed mainly of pavement cells (PVCs) with a surface ornamented by a system of highly branched microridges (Fig. 9B). Less abundant MRCs and mucous cells (MCs) were spread between PVCs. Mitochondria-rich cells (MRCs) were located mostly in the area below respiratory lamellae and in the interlamellar spaces. Flat apical apexes of MRCs had trapezoid, triangular, polygonal or oval shape and bore thin and short microvilli (Fig. 9B). Mucous cells were located only in the filament epithelium, mostly in close proximity to MRCs and only small granules of secretion were visible within pores of MCs (Fig. 9B).

Acid/LAl treatment affected both the macro- and microstructure of the gills. The most distinctive response was the thickening

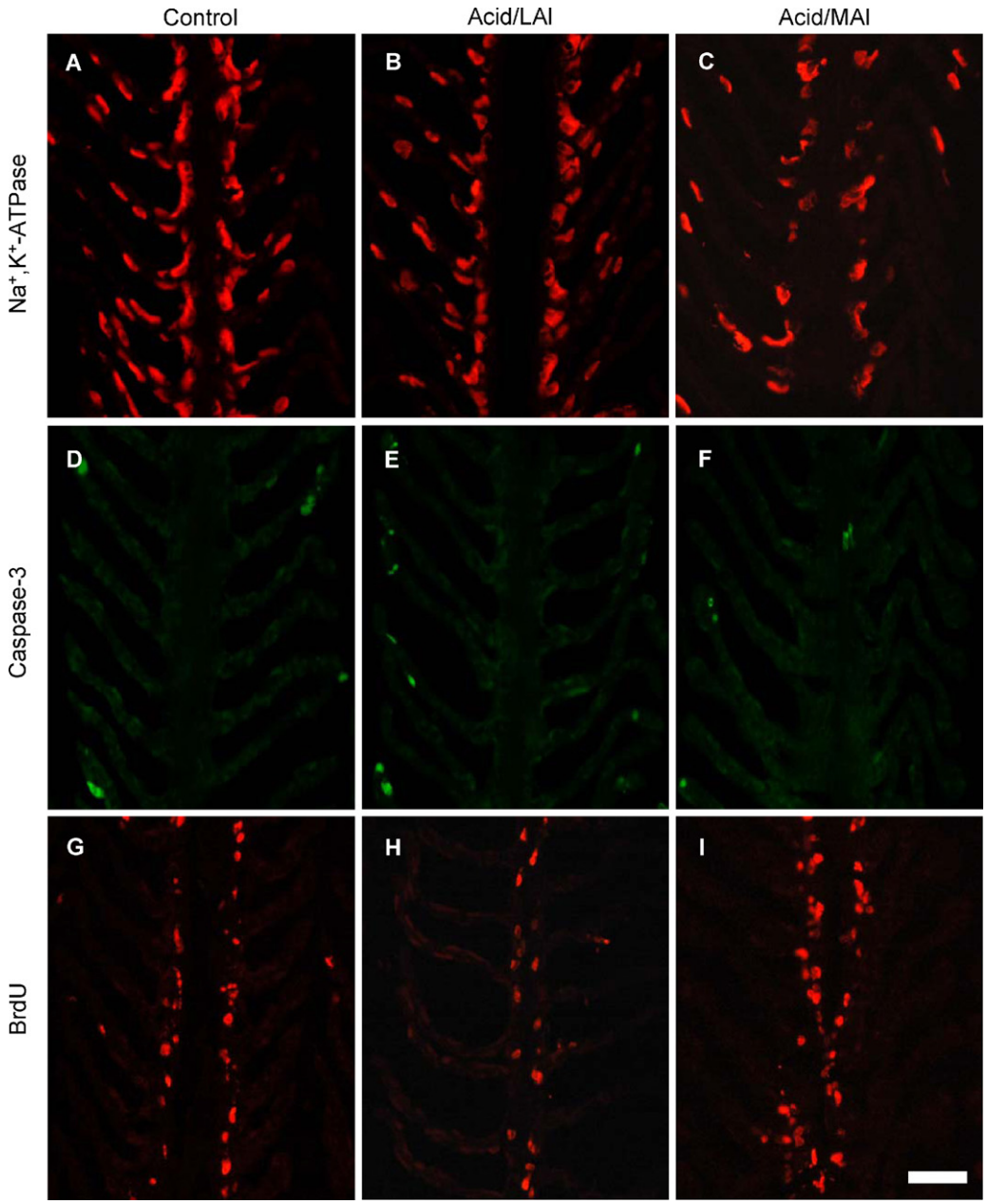

Fig. 5. NKA (A–C), caspase-3 (D–F), and BrdU (G–I) immunoreactivity in the gills of Atlantic salmon smolts exposed to control, acid/LAl, and acid/MAl for six days in FW. Scale bar, 40 μm.

of respiratory lamellae especially in the distal parts of filaments (Fig. 9C). Interlamellar spaces became irregular, and groups of 3–4 lamellae separated with a very narrow interlamellar space were common (see asterisks, Fig. 9C). In acid/LAl exposed smolts, MRC density in the outermost layer of the filament epithelium was 10% greater than controls (Table 4). Apical apexes of MRCs in this group became more convex, and microvilli appeared less regular and stubby (Fig. 9D). The density of MCs in acid/LAl exposed smolts increased 41% relative to controls (Table 4). In this group, large globes of secretion were observed on the surface of the filament epithelium, and small patches of mucus were seen on the surface of some filaments (Fig. 9E).

The effect of acid/MAl treatment on gill morphology was much more severe than that in acid/LAl group. Gills lost their regular structure due to partial or complete fusion of respiratory lamellae in the distal and middle parts of filaments and to reduction of interlamellar spaces along filaments (see arrows, Fig. 9F). In the acid/MAl exposed smolts, MRC density increased 20% over controls, while MC density increased 56% (Table 4). Apical apexes of MRCs in this group were enlarged, convex, with knob-like or almost "vestigial" microvillae (Fig. 9G) and hyperplasia of MCs was coupled with their functional hyperactivity (Fig. 9H). Copious amounts of mucus covered the filament epithelium and masked surfaces of MRCs and PVCs (Fig. 9G and H). Deposition of mucus was also seen in the narrow spaces between lamellae (Fig. 9F). These same severe morphological alterations were observed in smolts exposed to acid/HAl for two days (M.Y. Monette and S.D. McCormick, unpublished).

4. Discussion

In this study, we examined the impacts of acute acid/Al exposure on ion regulation of Atlantic salmon smolts by employing an integrated physiological, molecular, and cellular approach. As a continuation of our previous work, we were particularly interested

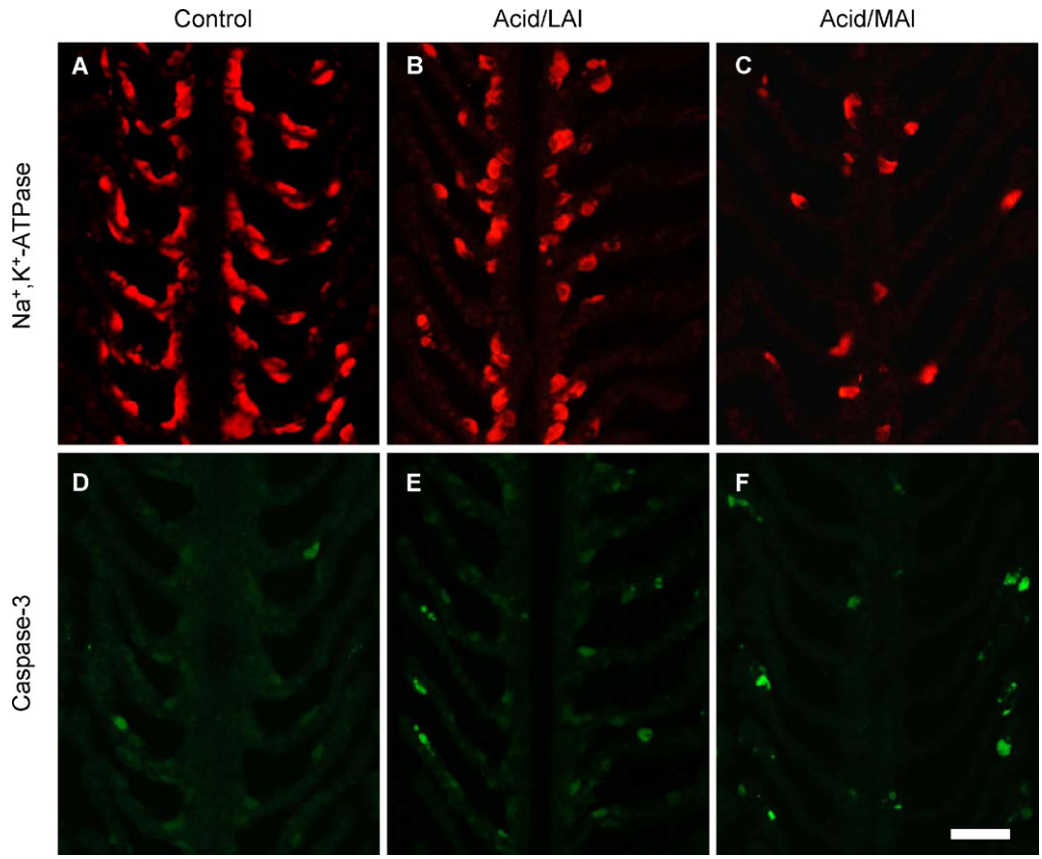

Fig. 6. NKA (A–C) and caspase-3 (D–F) immunoreactivity in the gills of Atlantic salmon smolts after exposure to control, acid/LAl, and acid/MAl for six days in FW followed by a SW challenge. Scale bar, 40 μm.

in examining mechanisms of impaired SW tolerance including impacts on CFTR and MRC dynamics in the gill. We determined that there are two mechanisms underlying the loss of SW tolerance of smolts during acid/Al exposure that depend on the time-course and severity of Al exposure.

Table 3
Cell area, staining intensity, and shape factor of NKA-immunoreactive cells in the gills of Atlantic salmon smolts exposed to control, acid/LAl, and acid/MAl for six days (FW) followed by a SW challenge (SW). Values are mean ± SE (n = 4–6).

	Freshwater		Seawater	
	Filament	Lamellae	Filament	Lamellae
Cell area (μm²)				
Control	114 ± 7.0	102 ± 4.5	90.6 ± 9.5	81.9 ± 9.5
Acid/LAl	86.4 ± 5.3*	85.0 ± 4.9	98.0 ± 14	84.2 ± 9.4
Acid/MAl	109 ± 4.5	102 ± 8.6	86.2 ± 12	85.6 ± 10
Staining intensity				
Control	33.7 ± 0.6	36.0 ± 0.7	31.1 ± 0.9	33.5 ± 1.6
Acid/LAl	31.2 ± 1.1	31.9 ± 1.0*	34.3 ± 2.0	35.6 ± 1.5
Acid/MAl	34.3 ± 0.8	35.9 ± 1.5	34.2 ± 1.0	35.9 ± 1.4
Shape factor				
Control	0.48 ± 0.01	0.57 ± 0.03	0.51 ± 0.02	0.56 ± 0.02
Acid/LAl	0.50 ± 0.03	0.51 ± 0.02*	0.55 ± 0.03	0.62 ± 0.04
Acid/MAl	0.45 ± 0.01	0.55 ± 0.01	0.51 ± 0.01	0.60 ± 0.02

Approximately 200 cells were counted from five sagittal sections per individual, and at least four individuals were measured per treatment.

* Indicates a significant difference (P < 0.05) from control within salinity (FW, SW) and location (filament, lamellae). Three-way ANOVA for cell area determined a salinity effect (P = 0.006) and a treatment/salinity interaction (P = 0.045). Three-way ANOVA for staining intensity determined an effect of cell location (P = 0.018) and a treatment/salinity interaction (P = 0.002). Three-way ANOVA for shape factor determined an effect of salinity (P < 0.001), cell location (P < 0.001), and a treatment/salinity interaction (P = 0.026).

4.1. Exposure to acid and moderate or high levels of Al

Exposure of smolts to acid/MAl and acid/HAl led to impaired ion regulation accompanied by large reductions in gill NKA activity, confirming our previous findings that acute acid/Al exposure leads to negative impacts on gill ion transport proteins (Monette et al., 2008). However, as NKA is known to play a role in both FW and SW ion regulation (Evans et al., 2005), we wanted to investigate acid/Al impacts on a gill ion transport protein that is thought to be primarily involved in salt secretion. To do this, we examined the effects of acid/Al exposure on mRNA levels of CFTR I and II in the gills of smolts. Currently available CFTR antibodies do not recognize CFTR protein in the gills of Atlantic salmon therefore we were limited to measuring gene transcription. Similar to other salt secretory epithelia, CFTR provides the major Cl^- exit pathway in the teleost gill. In euryhaline teleosts, CFTR appears in the apical membrane of MRCs upon SW exposure, and is typically absent or found at low levels when fish are in FW (Marshall et al., 2002; Katoh and Kaneko, 2003; McCormick et al., 2003; Scott et al., 2004; Hiroi et al., 2005,

Table 4
Density of mitochondria-rich (MRC) and mucous (MC) cells in the unit** of gill epithelium in Atlantic salmon smolts exposed to control, acid/LAl and acid/MAl for six days in (FW). Values are mean ± SE (n = 30).

Treatment	MRC	MC
Control	5.9 ± 0.19	4.1 ± 0.19
Acid/LAl	6.5 ± 0.22*	5.8 ± 0.19*
Acid/MAl	7.1 ± 0.19*	6.4 ± 0.25*

** Unit is the rectangular area of 2400 μm² on the afferent (trailing) edge of filaments located below respiratory lamellae.

* Indicates a significant difference (P < 0.05) from control. One-way ANOVA determined a treatment effect on both MRC and MC density (P < 0.001).

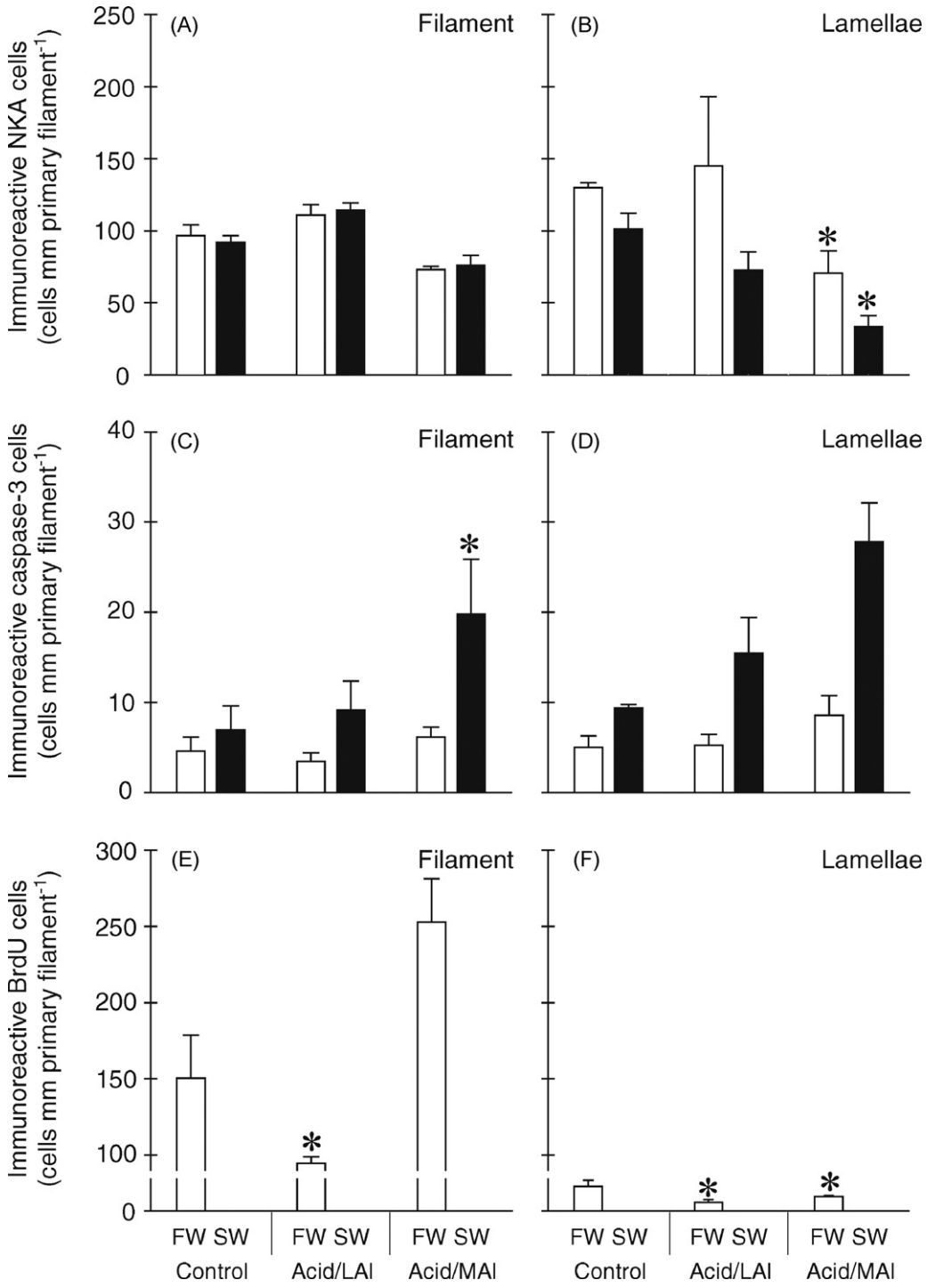

Fig. 7. Quantification of gill NKA-, caspase-3-, and BrdU-immunoreactive cell number in Atlantic salmon smolts exposed to control, acid/LAl and acid/MAl for six days in FW followed by a SW challenge. Cells within the filament epithelium were counted from 20 cross-sections per individual. Cells within the lamellar epithelium were counted from five sagittal sections per individual. Values are mean ± SE (n = 4–6). An * indicates a significant difference (P < 0.05) from control within salinity (FW, SW) and location (filament, lamellae). Three-way ANOVA for gill NKA-immunoreactive cell number determined effects of treatment (P < 0.001) and salinity (P = 0.007), and treatment/location (P = 0.004) and salinity/location (P = 0.006) interactions. Three-way ANOVA for gill caspase 3-immunoreactive cell number determined effects of treatment (P = 0.003), salinity (P < 0.001), and location (P = 0.02). Two-way ANOVA for gill BrdU-immunoreactive cell number determined effects of treatment (P < 0.001) and location (P < 0.001), and their interaction (P = 0.02).

2008). In salmonids, CFTR I mRNA is up-regulated in the gill during the parr-smolt transformation in preparation for salt secretion upon SW entry, and this occurs while fish are still in FW (Nilsen et al., 2007). The up-regulation of Cl^- secretory mechanisms while still in FW may be disadvantageous for smolts, therefore it is possible that CFTR becomes active and/or is inserted into the apical membrane only upon SW exposure. In the present study, smolts in the acid/MAl group exhibited large reductions in gill CFTR I mRNA expression by day six. Interestingly, we did not observe a significant effect of acid/Al on gill CFTR II mRNA levels. This may be due to a limited ability to detect differences due to low levels of CFTR II mRNA in the gill or alternatively, CFTR I and II genes may be dif-

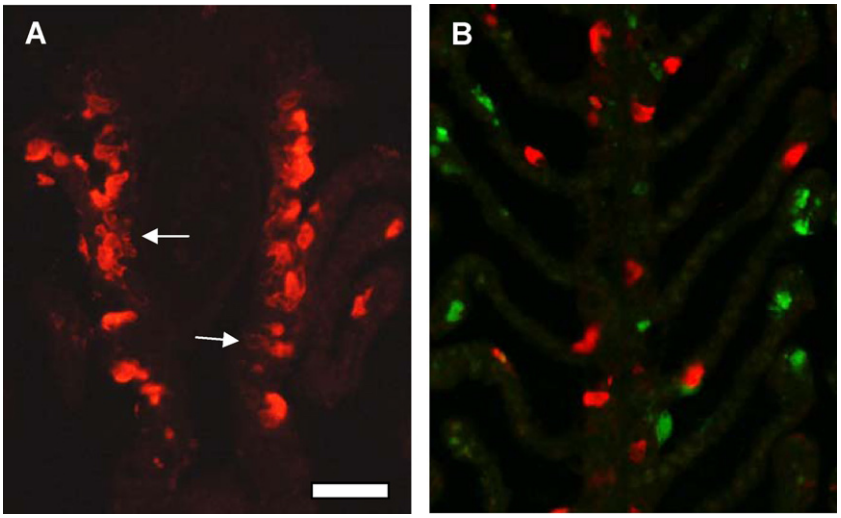

Fig. 8. NKA immunoreactivity in a filament cross-section of a smolt exposed to acid/MAl for six days followed by a SW challenge (A). Arrows point to MRCs exhibiting low levels of NKA immunoreactivity. NKA (red) and caspase-3 (green) immunoreactivity in the gills of a smolt exposed to acid/MAl for six days followed by a SW challenge (B). Double staining was performed on the same section and the images merged to examine colocalization of NKA and caspase-3 immunoreactivity (red + green = yellow). Scale bar, 40 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

ferentially regulated or have different physiological roles (Singer et al., 2002; Nilsen et al., 2007). Together, these results provide the first demonstration of a negative impact of acid/Al on CFTR in the gill. Reductions in gill CFTR I mRNA indicate that acid/Al exposure has led to the disruption of the preparatory up-regulation in gene expression typically seen during the parr-smolt transformation and this is likely to be directly linked to impaired SW tolerance. However, in this study we have demonstrated an impact of acid/Al on gill CFTR mRNA, thus future studies are needed to determine effects on CFTR protein abundance and function.

A second objective of this study was to identify the cellular mechanism underlying the loss of NKA and CFTR in the gills of smolts, and we did this by examining MRC abundance after six days of exposure to acid/MAl using both immunocytochemistry and SEM analyses. Previous studies have examined the effect of acid/Al exposure on gill MRC abundance in Atlantic salmon in FW (Smith and Haines, 1995; Jagoe and Haines, 1997), but examination of changes after SW exposure have not been conducted. To better understand the mechanisms underlying the loss of SW tolerance following acute acid/Al exposure, we examined MRC abundance and morphology in FW and after 24 h in SW. By employing both immunocytochemical and SEM techniques we were able to examine the impacts of acid/Al on both the total number of MRCs (NKA-positive) in the gill, as well as the number of surface exposed and thus presumably "active" MRCs in the filament epithelium (SEM analysis). After exposure to acid/MAl, smolts exhibited a large reduction in the number of total gill MRCs (Fig. 7B), accompanied by an increase in the surface expression of MRCs in the filament epithelium (Table 4). The loss of gill MRCs and their individual ion uptake capacity (possibly linked to loss of NKA activity) can explain impaired ion regulation in FW and after SW exposure. We suggest that increased surface expression of MRCs in the filament epithelium represents an attempt to increase the number of active MRCs in the gill in order to compensate for impaired ion regulation.

To investigate the loss of MRCs, we examined apoptosis in the gills of smolts by measuring gill mRNA and protein expression of the cysteine protease, caspase-3B. Caspases are responsible for many of the irreversible cellular changes that occur during apoptosis. Two caspase isoforms 3A and 3B, have recently been identified in Atlantic salmon (Takle et al., 2006). Despite a large reduction in the number of total MRCs in the gills of smolts upon exposure to acid/MAl for six days, we did not observe an effect on caspase-3B transcription. Interestingly, when smolts were exposed to acid/MAl for six days followed by a SW challenge, loss of MRCs within the gill was accompanied by an increase in the number of cells positive for caspase-3 protein as compared to controls (Fig. 7C and D). In fact, most groups exhibited an increase in the number of cells positive for caspase-3 protein in the gill after transfer to SW and this seemed to occur in a dose-dependent manner with Al exposure. This result supports previous evidence for a role of apoptosis in SW acclimation (Wendelaar Bonga and van der Meij, 1989; Takahashi et al., 2006). We also suggest there is a synergistic effect of acid/Al and SW exposure on the stimulation of apoptosis in the gill of smolts which may explain the additional and rapid ($\leq$24 h) loss of total MRCs within the lamellar epithelium upon transfer to SW. We conclude that the synergistic effect of acid/Al and SW exposure on the stimulation of apoptosis in the gill is one mechanism underlying the extreme sensitivity of the smolt hypoosmoregulatory system and suggest that this is likely to occur with other environmental contaminants.

To examine whether we could detect apoptotic MRCs in the gills of smolts, we used double-labeling with NKA and caspase-3 antibodies. Double-labeling of the same gill section revealed that the majority of caspase-3-positive cells were not co-localized with NKA (Fig. 8B). In mammals, inhibition of NKA plays a fundamental role in apoptosis and may actually trigger cell death (Yu, 2003); thus, the disappearance of NKA protein from MRCs may precede caspase-3 expression. The intracellular mechanism underlying Al-induced apoptosis in mammals involves the direct targeting of mitochondria (Savory et al., 2003). As a result of their high density of mitochondria, MRCs may be more susceptible to Al-induced apoptosis than other cell types in the gill. During the parr-smolt transformation, salmon experience an increase in the number and size of MRCs in the gill (McCormick et al., 1998), and it is therefore possible that increased abundance of MRCs is one explanation for the high sensitivity of smolts to acid/Al exposure compared to other salmon life-stages.

We observed a tendency for increased cell proliferation (i.e. BrdU-positive cells) within the filament epithelium after exposure to acid/MAl for six days (Fig. 7E). Increased cell proliferation may represent the synthesis of new MRCs from undifferentiated stem cells to counteract the large declines in total MRCs within the gill and/or as part of a toxicant-induced damage response. One mechanism thought to mediate MRC proliferation in the gill is an increase

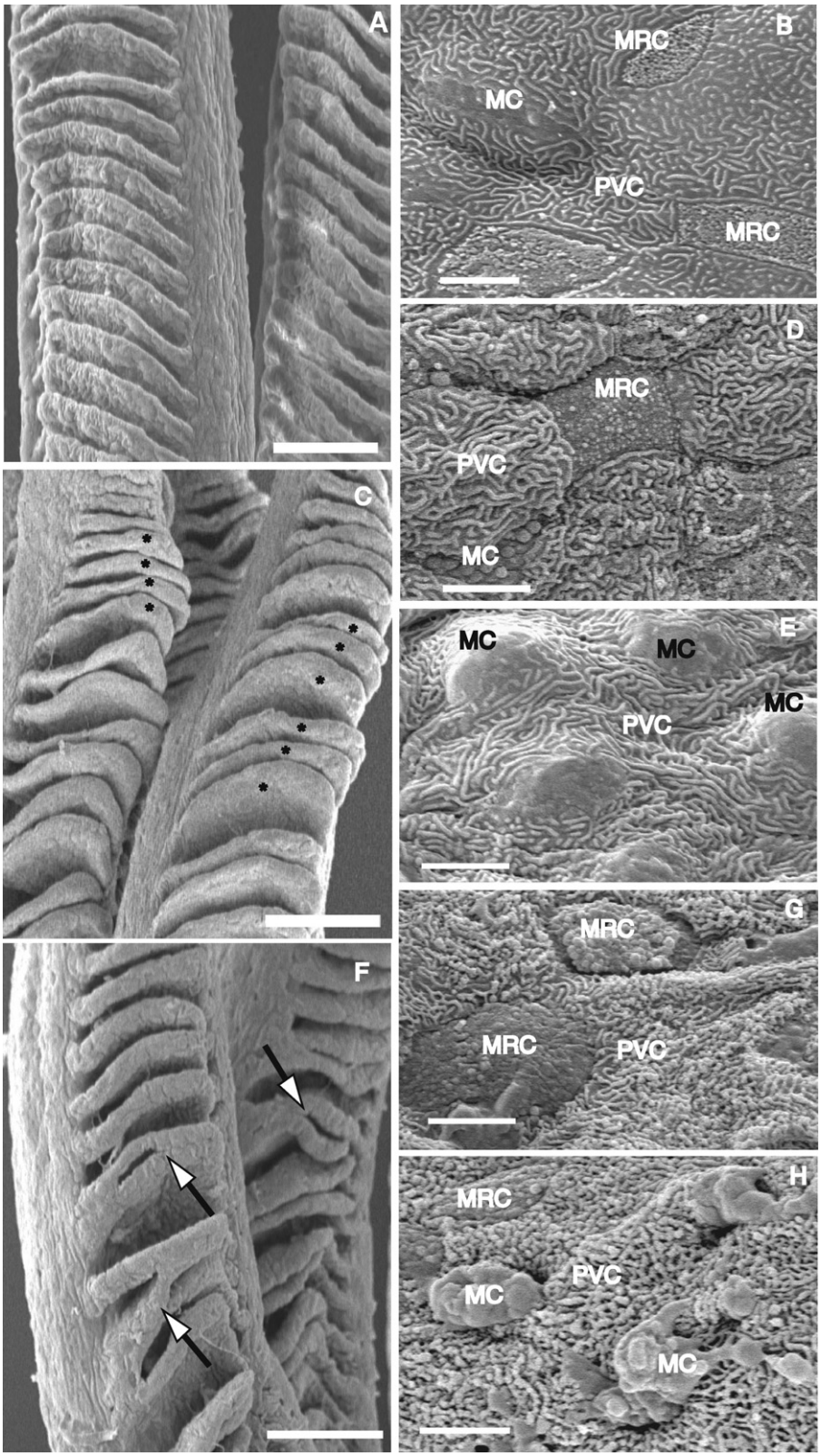

Fig. 9. Scanning electron micrographs of gill filaments of Atlantic salmon smolts exposed to control (A and B), acid/LAl (C–E), and acid/MAl (F–H) for six days in FW. (A) Part of gill filaments bearing thin respiratory lamellae separated by wide interlamellar spaces. (B) Filament epithelium. Note complex surface pattern of pavement cell, flat apical crypts of MRCs equipped with short microvilli, and small secretory granules visible within the pore of the mucous cell. (C) Part of gill filaments bearing thick respiratory lamellae. Note very close disposition of lamellae (asterisks) separated by very narrow interlamellar spaces. (D) Filament epithelium. Note convex MRC apical crypt with stubby microvilli, surface structure of pavement cells and small patches of mucus on pavement cells. (E) Filament epithelium. Mucous cell filled with huge globes of secretion. (F) Part of gill filament showing fusion of respiratory lamellae (arrows). (G) Filament epithelium. Mitochondria-rich cells with convex and almost smooth apical crypts (left, bottom) and MRCs with crypt bearing knob-like microvilli. Note mucus deposition on the surface of MRCs and PVCs. (H) Filament epithelium. Releasing of huge mucus globes on the epithelial surface. Deposition of copious mucus on epithelial surface. MC – mucus cell; MRC – mitochondria-rich cell; PVC – pavement cell. Scale bars: A, C and F – 100 μm; B, D, E, G and H – 5 μm.

in circulating levels of cortisol (Laurent and Perry, 1990). Cortisol is often referred to as a SW-adapting hormone in fish and during the parr-smolt transformation circulating levels of cortisol increase and are involved in the up-regulation of salt secretory mechanisms (McCormick, 2001). However there is also a large body of evidence supporting a role for cortisol in ion uptake (McCormick, 2001). We observed elevations in plasma cortisol after exposure to acid/HAl for two days in FW and a tendency for an increase in the acid/MAl group after six days (Fig. 4B and C). In addition to being part of a general stress response, elevated plasma cortisol levels may be part of a compensatory response to stimulate cell proliferation in the gill in order to rebuild ion regulatory machinery. Interestingly, despite the known role of prolactin in promoting ion uptake mechanisms in fish (Manzon, 2002), we did not observe a significant effect of acid/Al on circulating prolactin levels in spite of impacts on plasma ions in FW. Previous work has demonstrated that water acidification stimulates prolactin secretion in tilapia, *Oreochromis mossambicus* (Wendelaar Bonga et al., 1984), whereas exposure to acid/Al leads to a reduction in plasma prolactin in brown trout, *Salmo trutta* (Waring et al., 1996) suggesting that the regulation of plasma prolactin during exposure to acid/Al may be variable and species specific.

It is clear that in the presence of moderate to high Al levels, smolts exhibit a large accumulation of Al in the gill accompanied by dramatic changes in gill morphology including swelling and fusion of lamellae and increased mucus production. This type of morphological damage has been demonstrated after exposure to acid/Al in several salmonid species (Tietge et al., 1988; Mueller et al., 1991; Matey and Komov, 1992; Ledy et al., 2003) and is a "classic" response to environmental contaminants (Mallatt, 1985). In these groups, elevated levels of the stress response indicators plasma cortisol and glucose were also observed. The combination of extensive gill Al accumulation, damage to the epithelium, and the induction of a stress response may indicate that in these groups Al is the major toxicant. Although not examined in this study, it is likely that gill Al accumulation and subsequent damage to the epithelium is also affecting other major functions of the gill including respiration as has been found in other salmonids (Wood and McDonald, 1987). Future studies are needed to examine the time-course of both Al depuration from the gill and morphological repair after acute acid/Al exposure and specifically how these processes differ when fish are in FW and SW.

4.2. Exposure of smolts to acid and low levels of aluminum

Under conditions of acid and low Al, plasma ion concentrations remained unaffected by acid/Al treatment in FW, but exhibited large increases in SW by day six, indicating that SW tolerance was substantially compromised. These plasma ion responses demonstrate that the SW tolerance of smolts is more sensitive to acid/Al impacts than FW ion regulation, in agreement with previous studies (Saunders et al., 1983; Kroglund and Finstad, 2003; Monette et al., 2008; Kroglund et al., 2008). Interestingly, after two days of treatment in FW, we observed a slight but significant increase in plasma Cl^- concentration in the absence of an impact on plasma Na^+ concentrations (Fig. 2A and C). The uncoupling of plasma Na^+ and Cl^- during exposure of smolts to acid/Al has been previously reported by our lab (Monette et al., 2008) as well as others (Staurnes et al., 1993) and may indicate that acid/Al is differentially affecting ion regulatory processes of the gill. Alternatively, acid/Al exposure may have impacted other gill functions that are coupled to Na^+ and Cl^- homeostatic mechanisms such as acid/base and ammonia exchange; however, the examination of this was beyond the scope of the present study.

In contrast to the acid/MAl and acid/HAl treatments, impaired SW tolerance in the acid/LAl treatment occurred in the absence of alterations in gill NKA activity, CFTR I mRNA expression, and the number of total MRCs in the gill. However, MRCs in the gills of smolts exposed to acid/LAl for six days differed in morphology from controls when fish were in FW, with cells appearing smaller within the filament epithelium and less bright and more elongate within the lamellar epithelium (Table 3). Several studies have identified morphological differences in MRCs in the gills of fish acclimated to FW and SW, with cells in SW typically being larger and staining brighter for NKA (Pisam et al., 1988; Uchida et al., 1996; Pelis et al., 2001; McCormick et al., 2003; Hiroi et al., 2005; Inokuchi et al., 2008). This has led to the speculation that at least two phenotypes of MRCs exist; FW-type (ion uptake) and SW-type (salt secretory), and several studies have now begun to directly link morphological differences in MRCs with their molecular ion transporting properties (i.e. ion transporter expression) (Inokuchi et al., 2009). Further, by following *in vivo* sequential changes of individual MRCs in the yolk-sac membrane of tilapia, *O. mossambicus*, during SW acclimation, Hiroi et al. (1999) observed that FW-type MRCs are transformed into SW-type MRCs demonstrating the functional plasticity of individual MRCs. Thus, we suggest that morphological alterations in the MRCs of smolts represent a shift in cell phenotype as part of a compensatory response to acid/Al exposure. We hypothesize this shift would be from SW-type to FW-type cells (smaller, less bright), therefore reducing the salt secretory capacity of the gill and thus SW tolerance without impacting FW ion regulation; however, we were not able to directly test this hypothesis as there are not yet clearly defined markers for these two functional MRC types in the gills of Atlantic salmon. Alternatively, morphological alterations may indicate incipient MRC necrosis and/or apoptosis; however, this is less likely given the lack of severe morphological damage to the gill in this treatment group.

SEM analysis revealed a slight increase in the surface expression of MRCs in the filament epithelium of smolts exposed to acid/LAl for six days in FW; however, unlike the acid/MAl group this occurred in the absence of plasma ion losses in FW. It is possible that under low Al conditions, an increase in the number of surface exposed and thus "active" MRCs is sufficient to prevent ion losses in FW, whereas when Al concentrations are moderate or high loss of total MRCs is so extensive that this is not sufficient and therefore plasma ion losses in FW are observed. Futhermore, smolts in the acid/LAl group exhibited decreased cell proliferation (BrdU-positive cells) within the gill suggesting a down-regulation of cell turnover in the gill. Together, these observations support the conclusion that smolts in this group have not responded to acid/Al by an up-regulation of new MRC synthesis from undifferentiated stem cells, but rather a shift in the phenotype of pre-existing MRCs in the gill. We hypothesize that this shift in phenotype and increased surface expression of MRCs in FW has resulted in the loss of SW tolerance under acid/LAl conditions.

Smolts exposed to acid/LAl exhibited low levels of gill Al accumulation and slight morphological alterations including thickening of respiratory lamellae and enhanced mucus production. Interestingly, a significant stress response (elevated plasma cortisol and glucose) was not observed in this group despite impaired ion regulation in SW. This suggests that induction of a stress response in smolts may be related to severe gill damage from Al and/or ion losses in FW as such impacts were not observed in this group but were observed at higher Al concentrations. In addition, lack of a stress response in this group provides further support for the conclusion that loss of SW tolerance has occurred via two different mechanisms that depend on the severity of Al exposure. As was previously mentioned we did not attempt to examine individual pH and Al effects in this study, as our goal was to simulate environmentally relevant water chemistry, and previous studies have extensively described individual pH and Al effects in other salmonid species (see review by Gensemer and Playle, 1999). In the present

study, we detected a slight but significant elevation in gill Al accumulation in the low Al group as compared to controls, despite the fact that the mean Al_i exposure for these two groups differed by less than 8 μg/l. These results suggest that even very low Al_i concentrations (possibly below the detection limit of the Al assays employed in many studies) may result in physiological impacts. Thus it is possible that physiological affects that others have attributed to acid alone may in fact be the result of acid in the presence of very low Al_i concentrations.

5. Conclusions

We have demonstrated that the SW tolerance of Atlantic salmon smolts is extremely sensitive to the combined exposure of acid and Al and can be impaired in the absence of detectable impacts on FW ion regulation. By employing an integrated physiological, molecular, and cellular approach we have identified two mechanisms underlying the loss of SW tolerance which depend on the time-course and severity of Al exposure. Exposure of smolts to low pH and moderate or high levels of Al leads to gill Al accumulation, morphological damage to the epithelium, and decreases in total MRCs, NKA activity, and CFTR I transcription. In this situation, we conclude that loss of SW tolerance of smolts has resulted from substantial Al accumulation in the gill leading to the loss of ion transport proteins typically up-regulated during the parr-smolt transformation in preparation for SW entry. In addition, there appears to be a synergistic effect of acid/Al and SW exposure on the stimulation of apoptosis in the gill which we suggest is contributing to the extreme sensitivity of the smolt hypoosmoregulatory system. In this situation, increased surface expression of MRCs may represent an attempt to combat ion losses in FW, whereas increased cell proliferation may represent the up-regulation of MRC replacement possibly mediated by elevated levels of circulating cortisol. By contrast, in the presence of low pH and low levels of Al, loss of SW tolerance is independent of these mechanisms and may result instead from a shift in the phenotype of pre-existing MRCs in the gill. We hypothesize this shift would be from SW-type to FW-type cells, therefore reducing the salt secretory capacity of the gill. Together, these results have substantial implications for salmon populations in regions affected by episodic acidification. Although no direct mortality was observed in the acid/LAl treatment after exposure to SW for 24 h, smolts with impaired SW tolerance may have decreased preference for SW (Lerner et al., 2007), delayed migration, and increased susceptibility to predation (Jarvi, 1989; Handeland et al., 1996). This is likely to increase mortality during downstream migration, SW entry, and marine residence ultimately leading to population level effects.

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