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Sensitivity of Na^+/K^+-ATPase isoforms to acid and aluminum explains differential effects on Atlantic salmon osmoregulation in fresh water and seawater

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Abstract: Atlantic salmon (*Salmo salar*) smolts are sensitive to acid rain and associated increases in dissolved inorganic aluminum (Al) resulting in decreased seawater tolerance at this critical life stage. Salmon have two major isoforms of the catalytic alpha subunit of Na^+/K^+-ATPase (NKA), with NKAα1a being the major freshwater (FW) isoform and NKAα1b the major seawater (SW) isoform. Here we evaluate physiological markers of SW preparedness and NKAα1a and NKAα1b isoforms after short-term exposure to acidified water and acidified water with added Al (acid–Al). Atlantic salmon smolts were exposed to low ion FW (Control), low ion acidic water (pH 5.2; acid), and low ion acidic water (pH 5.2) with moderate levels of added inorganic Al (35 $\mu g \cdot L^{-1}$; acid–Al) for 4 days. Acid exposure resulted in loss of salinity tolerance (higher plasma chloride (Cl) after SW exposure) and significantly decreased the levels of gill NKAα1b but not NKAα1a protein abundance. Acid–Al exposure resulted in loss of plasma Cl in FW and higher plasma Cl in SW and decreased NKAα1a and NKAα1b abundance. The loss of salinity tolerance in smolts can be explained by the differential sensitivity of NKA isoforms to acid.

Résumé : Les saumoneaux de saumon atlantique (*Salmo salar*) sont sensibles aux pluies acides et aux augmentations associées d'aluminium (Al) inorganique dissous qui réduisent la tolérance à l'eau de mer à ce stade critique du cycle biologique. Les saumons présentent deux principales isoformes de la sous-unité catalytique alpha de la Na^+/K^+-ATPase (NKA), NKAα1a étant la principale isoforme d'eau douce (ED) et NKAα1b, la principale isoforme d'eau salée (ES). Nous évaluons les marqueurs physiologiques de la préparation à l'eau salée et les isoformes NKAα1a et NKAα1b après une exposition de courte durée à de l'eau acidifiée et de l'eau acidifiée avec de l'aluminium ajouté (acide–Al). Des saumoneaux de saumon atlantique ont été exposés à de l'ED faible en ion (témoin), de l'eau acide faible en ions (pH de 5,2; acide) et de l'eau acide faible en ions (pH de 5,2) avec des teneurs modérées d'Al inorganique ajouté (35 $\mu g \cdot L^{-1}$; acide–Al) pendant quatre jours. L'exposition à l'acide s'est traduite par une baisse de la tolérance à la salinité (teneur plasmatique en Cl plus élevée après l'exposition à l'ES) et une baisse significative des teneurs branchiales en NKAα1b, mais pas de l'abondance de la protéine NKAα1a. L'exposition à l'acide–Al a entraîné une baisse du Cl plasmatique dans l'ED et une hausse du Cl plasmatique dans l'ES et des réductions de l'abondance de NKAα1a et de NKAα1b. La baisse de tolérance à la salinité chez les saumoneaux peut s'expliquer par différentes sensibilités à l'acide des isoformes de NKA. [Traduit par la Rédaction]

Introduction

Environmental acidification has been a long-standing and widespread concern across Europe (Kallend et al. 1983), including Scandinavia (Johannessen et al. 1976), and also in the Northeast USA (Driscoll et al. 2001). The common term "acid rain" is used to refer to wet and dry deposition of all atmospheric substances that cause acidification. These substances arise primarily from man-made sources such as emissions of sulfur dioxide (SO_2) and nitrogen oxides (NO_x) resulting from fossil fuel combustion. The resulting solutions of sulfuric acid and nitric acid are known to cause a variety of adverse effects on terrestrial and aquatic ecosystems (Schofield 1976; Wright and Schindler 1995; Menz and Seip 2004). The poorly buffered soils and impervious rocks of the northeastern United States, combined with the limited acid neutralizing capacity of lakes and streams in the region, increases the mobilization of aluminum (Al) from soils (Driscoll and Schecher 1990). In addition, acidic deposition increases the concentration of soluble Al in forested watersheds and in their associated aquatic ecosystems. Under acidic conditions, Al is converted to its bioavailable (inorganic) forms and becomes more toxic (Cronan and Schofield 1990). The toxicity of inorganic Al to fish is well established (Baker and Schofield 1982; Rosseland et al. 1986; Witters et al. 1990; Kroglund et al. 2007) and is primarily mediated through the gill. Death appears to be due to a combination of ionoregulatory, osmoregulatory, and respiratory dysfunction (Exley et al. 1991). Surface waters are often most acidic in spring after snowmelt and rain events, carrying increased levels of inorganic Al into river and stream systems, which can have negative consequences for downstream-migrating anadromous fishes.

In spring, Atlantic salmon (*Salmo salar*) smolts undergo morphological, behavioral, and physiological changes in preparation for downstream migration and seawater (SW) entry. In anadromous smolts, SW entry is preceded by developmental changes that increase the capacity of the gill to excrete ions. Maintenance of internal osmotic balance in fresh water (FW) and SW is mediated

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in large part by gill ionocytes that contain high levels of Na^+/K^+-ATPase (NKA) (Evans et al. 2005). The NKA enzyme consists of two subunits, α and β, and an associated FXYD-11 protein. The α subunit is responsible for the catalytic and ion regulatory capacity of the enzyme, while the β subunit is involved in protein maturation and membrane anchoring. FXYD-11 is thought to act in a regulatory capacity and is associated with the NKA α subunit (Tipsmark et al. 2010). Transcription studies indicate that there are salinity-dependent isoforms of the α subunit expressed in the gills of tilapia (*Oreochromis mossambicus*) (Tipsmark et al. 2011), rainbow trout (*Oncorhynchus mykiss*) (Richards et al. 2003), and Atlantic salmon (Mackie et al. 2005; Nilsen et al. 2007) and that differential expression of α subunit isoforms in salmonids may be an important mechanism by which anadromous species adjust NKA function in response to changes in salinity (Bystriansky et al. 2006). With the development of isoform-specific homologous antibodies, we were able to further demonstrate that FW (NKAα1a) and SW (NKAα1b) protein isoforms in the gills of Atlantic salmon are normally present in distinct ionocytes (McCormick et al. 2009) and that the increase in the abundance of NKAα1b protein during smolt development is directly linked to the increase in salinity tolerance that occurs at this stage (McCormick et al. 2013).

Smolts are the most sensitive life stage of Atlantic salmon to environmental acidification (Staurnes et al. 1993*a*; Monette and McCormick 2008). Exposure to even mildly elevated Al under acidic conditions for relatively short periods (days) has been shown to alter physiology, resulting in impaired osmoregulatory performance and reduced SW tolerance in salmon smolts (Staurnes et al. 1993*a*; Monette et al. 2008). Monette et al. (2010) demonstrated that smolts exposed to acid plus inorganic Al (Al_i) of ~11 μg·L^{-1} exhibited minor gill Al accumulation, slight morphological modifications in the gill, and impaired SW tolerance in the absence of a detectable effect on FW ion regulation. They proposed that when smolts are exposed to acid and low levels of Al, loss of SW tolerance may result from a shift in the phenotype of ionocytes present in the gill. In this study, we examined how moderate levels of acid and acid plus Al affect the abundance and distribution of NKAα subunit isoforms in Atlantic salmon gills and relate these changes to compromises in osmoregulation in both FW and SW.

Methods

Fish and experimental design

Atlantic salmon were obtained from the Kensington National Fish Hatchery in October 2007 (Kensington, Connecticut, USA) and held at the Conte Anadromous Fish Research Center (Turners Falls, Massachusetts, USA). Fish were reared for 7 months in fiberglass tanks receiving flow-through (4 L·min^{-1}) Connecticut River water at ambient river temperatures (1.5–15.0 °C) with natural photoperiod and fed to satiation twice daily with commercial feed (Zeigler Bros., Garners, Pennsylvania, USA). All experiments were carried out in accordance with USGS institutional guidelines and approved by the USGS, Leetown Science Center Institutional Animal Care and Use Committee (Protocol SP9065).

In May 2008, smolts were transferred to duplicate 1 m diameter experimental tanks (18 fish per tank) and acclimated to low ion water for 24 h. Food was withheld for this 24 h time period and throughout the experiment. Fish were maintained for 4 days in flow-through conditions with the following treatments: low ion water (Control), low ion acidic water (pH 5.2, acid), and low ion acidic water with added aluminum (pH 5.2, 35 μg Al·L^{-1}, acid–Al). Constant temperature (12 ± 1 °C) was achieved with in-tank heat exchangers and chiller systems. Flow of approximately 0.4 L·min^{-1} was maintained throughout the experiment from treatment specific water prepared in 1400 L head tanks. Directional aeration and flow-through conditions created circular flow and mixing throughout the experimental treatments. Low ion conditions were achieved by mixing deionized water (~12 μS, prepared by passing tap water through an ion exchange resin (Siemens, Lowell, Massachusetts, USA) with Connecticut River water (~240 μS) at a ratio of approximately 8:1, resulting in conditions similar to poorly buffered Atlantic salmon streams in New England (~20–45 μS, ~2 mg Ca^{++}·L^{-1}). To acidify tanks, 3 mol·L^{-1} HCl was added to the deionized head tanks until the desired pH was achieved. Aluminum ($AlCl_3 \cdot 6H_2O$ stock solution (1000 mg Al·L^{-1}) was added to deionized and acidified head tanks at a nominal concentration of 40 μg·L^{-1}. All tanks were mixed with aeration for at least 12 h prior to distribution to fish tanks to insure stable water chemistry. After 4 days of treatment,12 fish from each treatment were either sampled or transferred to 35 ppt SW for 24 h. SW-exposed fish were netted and placed directly into 1.2 m diameter, 330 L tanks containing 35 ppt SW (charcoal-treated, filtered, and aerated), maintained at 12 °C, and sampled after 24 h. Sampled smolts were anesthetized with MS-222 (200 mg·L^{-1}, pH 7.0,), weighed to the nearest 0.1 g, and fork and total lengths recorded to the nearest 0.1 cm. Blood was collected from the caudal vasculature in heparinized 1 mL syringes within 6 min of tank disturbance and centrifuged at 3200 × gravity (*g*) for 5 min at 4 °C. Plasma was removed and stored at –80 °C for later analyses. Gill biopsies (four to six primary filaments) for the measurement of Al accumulation were taken and analyzed using established protocols (Monette and McCormick 2008). Gill biopsies were also taken for the measurement of NKA activity and placed into 100 μL SEI buffer (250 mmol·L^{-1} sucrose, 10 mmol·L^{-1} Na_2EDTA, and 50 mmol·L^{-1} imidazole, pH 7.3) and stored at –80 °C for later analysis. Two gill arches were frozen at –80 °C for Western blot analyses. Segments of six to eight filament pairs were trimmed from the gill arch and transferred to 4% paraformaldehyde for 4 h at 4 °C. Following fixation, the segments were rinsed and stored in 70% ethanol at 4 °C for immunohistochemical analysis of NKAα subunit distribution.

Water chemistry analyses

Water samples were taken twice daily from each treatment tank outflow to monitor pH, Al, and Ca^{++}. Measurements of pH in experimental tanks were made using a bench-top pH meter (Type 145, Corning, Medfield, Massachusetts, USA) with a low-ion pH probe (Ross Ultra 8156, Thermo Orion, Beverly, Massachusetts, USA). Calibration was performed before each measurement using low ionic strength pH standards (pH 6.97 and pH 4.10, Pure Water Standards, Thermo Orion). After collection, all Al water samples were acidified with trace metal grade HNO_3 (0.2% final concentration) and stored for later analysis. Al concentration was measured by graphite furnace atomic absorption spectrophotometry (GFAAS; HGA-800/AAnalyst 100, Perkin Elmer, Wellesley, Massachusetts, USA). Total dissolved Al (Al_f) was measured from water samples that were passed through a 0.45 μm nitrocellulose filter and then acidified with trace metal grade HNO_3 (0.2%). Al_i was determined by passing filtered water samples through a strong acid cation-exchange column immediately upon collection (Amberlite 120, prepared with Na^+) as described by Driscoll (1984) and Monette and McCormick (2008). Column-processed samples were immediately acidified (0.2% trace metal grade HNO_3) and analyzed for Al content. Al_i was calculated by subtracting column-processed Al from Al_f. Ca was measured by flame atomic absorption spectrophotometry (AAnalyst 100, Perkin Elmer, Wellesley, Massachusetts, USA). Duplicate water samples were measured and calibration was checked every ten samples against a reference standard.

Laboratory analyses

Gill Al levels were measured by acid digestion and GFAAS by modification of methods reported in Teien et al. (2006). Gill biopsies were thawed, dried at 60 °C for 24 h in 1.5 mL microcentrifuge tubes, and weighed to the nearest 0.0001 mg using a microbalance (Series 30, Cahn Instruments, Cerritos, California, USA). For acid digestions, 98 μL of 100% trace metal grade HNO_3 and 2 μL of H_2O_2 were added to the dried biopsies and heated at 100 °C until com-

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pletely evaporated (~3 h). The same amounts of HNO_3 and H_2O_2 were again added to biopsy tubes and heated with tube caps on at 60 °C for 1 h. Samples were diluted (9:1) by the addition of 900 μL of trace-metal-free deionized water, and Al concentration was analyzed using GFAAS. A background correction was made for gill biopsy samples by subtracting the Al present in digestion blanks. Gill Al measurements were expressed as μg Al·g^{-1} gill dry mass.

Plasma chloride (Cl) was analyzed by the silver titration method using a Buchler–Cotlove digital chloridometer (LABCONCO, Kansas City, Missouri, USA) and external standards.

Plasma cortisol levels were measured by a validated direct competitive enzyme immunoassay as outlined in Carey and McCormick (1998). Sensitivity as defined by the dose–response curve was 1 to 400 ng·mL^{-1}. The lower detection limit was 0.3 ng·mL^{-1}. Using a pooled plasma sample, the mean intra-assay variation was 5.5% (n = 10), and the mean interassay variation was 8.8% (n = 10).

NKA activity in gill homogenates was determined using a temperature regulated microplate method (McCormick 1993). In this assay, ouabain-sensitive ATPase activity was measured by coupling the production of ADP to NADH using lactic dehydrogenase and pyruvate kinase in the presence and absence of 0.5 mmol ouabain·L^{-1}. Samples (10 μL) were run in duplicate in 96-well microplates at 25 °C and read at a wavelength of 340 nm for 10 min on a THERMOmax microplate reader using SOFTmax software (Molecular Devices, Menlo Park, California, USA). Protein concentration of the homogenate was determined using a bicinchoninic acid protein assay (Pierce, Thermo Fisher Scientific).

NKAα1 isoform abundance and immunohistochemistry (IHC)

NKA isoform-specific antibodies were developed and validated as previously described. Rabbit anti-NKAα1b polyclonal antibodies were developed as previously outlined in McCormick et al. (2009). Chicken anti-NKAα1a IgY polyclonal antibodies were developed as outlined in McCormick et al. (2013). NKAα1a and NKAα1b abundance was quantified by Western immunoblotting and ECL detection on membrane preparations of frozen gill as outlined in McCormick et al. (2013). Gill tissue was homogenized in 10 volumes of PBS (1.9 mmol·L^{-1} NaH_2PO_4, 8.1 mmol·L^{-1} Na_2HPO_4, 138 mmol·L^{-1} NaCl, pH 7.4) containing 30% sucrose (*w*/*v*), 2 mmol·L^{-1} EDTA, 1 mmol·L^{-1} phenylmethylsulfonylflouride, and Complete Mini protease inhibitor tablets (Roche, Indianapolis, Indiana, USA). The tissue homogenate was centrifuged at 5000*g* for 10 min at 4 °C. The supernatant was then centrifuged at 20 000*g* for 10 min at 4 °C. The resulting supernatant was centrifuged at 48 000*g* for 2 h at 4 °C. The final pellet was resuspended in homogenization buffer plus 0.1% Triton X-100 (LabChem, Pittsburg, Pennsylvania, USA). Protein concentration was determined with a bicinchoninic acid protein assay (Pierce, Thermo Fisher Scientific). Samples were then placed in an equal volume of 2× Laemmli buffer, heated for 15 min at 60 °C, and resolved by SDS–PAGE using a 6.75% gel. Protein samples of 10 μg were loaded per lane for the detection of NKAα1a or NKAα1b. Two lanes were reserved on each gel for Precision Plus relative molecular weight markers (Bio-Rad Laboratories, Hercules, California, USA) and a standard consistent tissue preparation reference to control for blot-to-blot variation. Following electrophoresis, proteins were transferred to Immobilon PVDF transfer membrane (Millipore, Bedford, Massachusetts, USA) at 30 V overnight in 25 mmol·L^{-1} Tris, 192 mmol·L^{-1} glycine buffer, pH 8.3. PVDF membranes were blocked with 5% non-fat dry milk in PBST (phosphate-buffered saline with 0.1% Triton X-100) for 1 h at room temperature, rinsed in PBST, and exposed to primary antibody in blocking buffer for 1 h at room temperature(chicken anti-NKAα1a, 0.1 μg·mL^{-1}; rabbit anti-NKAα1b, 0.013 μg·mL^{-1}). After rinsing in PBST, blots were exposed to goat anti-rabbit-HRP or goat anti-chicken HRP (KPL, Maryland, USA) diluted 1:10 000 in blocking buffer for 1 h at room temperature. After rinsing in PBST, blots were incubated for 1 min in a 1:1 mixture of enhanced chemiluminescent (ECL) solution A (396 μmol·L^{-1} coumaric acid, 2.5 μmol·L^{-1} luminol, 100 mmol·L^{-1} Tris, pH 8.5) and ECL solution B (0.018% H_2O_2, 100 mmol·L^{-1} Tris, pH 8.5), then exposed to X-ray film (RPI, Mount Prospect, Illinois, USA). Digital photographs were taken of individual blots and band staining intensity measured using ImageJ (NIH, Bethesda, Maryland, USA). Protein abundance is expressed as a cumulative eight-bit gray scale value. Gray values were standardized to the internal reference lane and corrected for blot-to-blot variation.

Fixed gill tissue for IHC was rinsed in 10 mmol·L^{-1} PBS at room temperature for 30 min and placed in PBS with 30% (*w*/*v*) sucrose for 1 h. Tissue sections were then embedded and frozen in Tissue-Tek OCT embedding medium (Sakura Finetek, Torrance, Callifornia, USA) at –80 °C. Sections (5 μm thick) were cut in a cryostat at –24 °C, lengthwise along the primary filaments. The tissue was placed on Fisherbrand Colorfrost Plus slides (Fisher Scientific, Hampton, New Hampshire, USA), dried, rinsed with PBS, and incubated in 8% normal goat serum in PBS for 30 min at room temperature. Slides were exposed to primary antibody (chicken anti-NKAα1a, 1.9 μg·mL^{-1}; rabbit anti-NKAα1b,1 μg·mL^{-1}) in antibody dilution buffer (0.01% NaN_3, 0.05% Triton X-100, 8% normal goat serum, and 0.02% keyhole limpet hemocyanin in PBS) and incubated overnight at 4 °C. The slides were rinsed several times with PBS and exposed to fluorescently labeled AlexaFlour 488 and 546 secondary antibodies (Invitrogen, Thermo Fisher Scientific Inc., USA) for 2 h at room temperature. After incubation, slides were rinsed several times with PBS, covered by a coverslip, and examined with a Nikon Diaphot-TMD inverted fluorescence microscope with a mercury lamp. From each fish, immunoreactive NKAα1a and NKAα1b cells on the primary filaments and secondary lamellae were counted and expressed per 0.7 mm of primary filament. When present, at least 50 immunoreactive ionocytes from five different tissue sections were analyzed from each of at least five fish per treatment. If fewer than 10 immunoreactive cells were present per section analyzed, all cells present were counted and used for analysis. Immunoreactive cell area (μm^2 per cell) was determined using MetaMorph 4.1.2 (Universal Imaging Corporation, Downingtown, Pennsylvania, USA). A set color threshold defined the NKAα1a, NKAα1b, and co-localized signal, and the nucleus was included in the total cell area.

Data analyses

Values are reported as mean ± standard error (SE). All statistical analyses were conducted using Statistica (StatSoft Inc., USA). From each measured parameter that met ANVOA assumptions, significance of treatment effect was analyzed by one-way ANOVA followed by post hoc Student–Newman–Keuls test ($p < 0.05$). When data did not meet the required assumptions of ANOVA, nonparametric indices (Mann–Whitney *U* test) and Kruskal–Wallis one-way ANOVA were used to determine significance of treatment effect ($p < 0.05$).

Results

Water chemistry

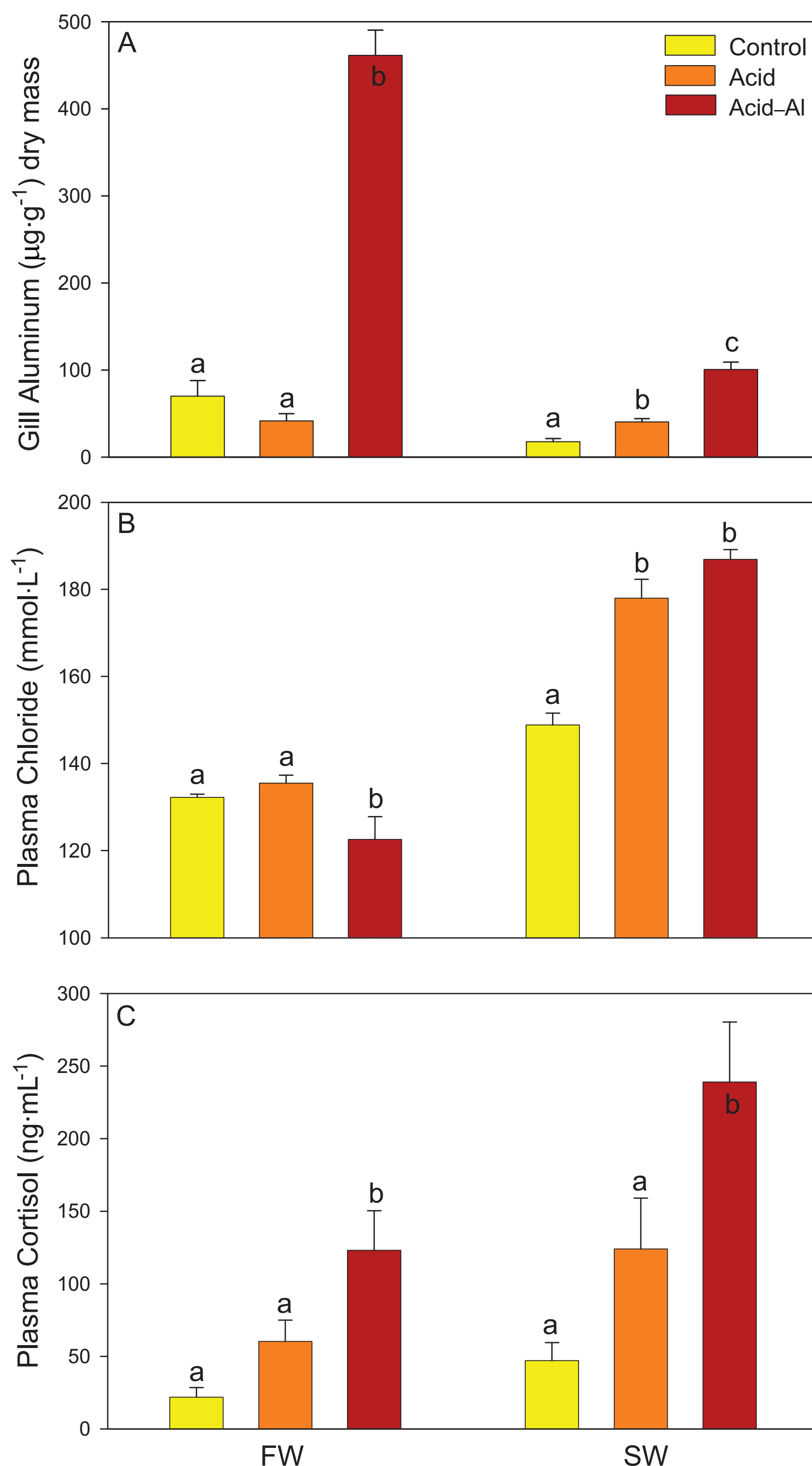
Water pH values were within targeted ranges for control and acidified tanks; Control pH treatment tank means were 6.6 ± 0.05, acid tank means were 5.2 ± 0.07, and acid–Al tank pH means were 5.3 ± 0.06.

Al_f measured 3.0 ± 0.4 μg·L^{-1} in Control tanks. Acidified tanks measured 6.5 ± 1.4 μg·L^{-1}. Al_f for acid–Al averaged 25.0 ± 2.7 μg·L^{-1}. Al_i measured 2.1 ± 0.6 μg·L^{-1} in control treatment tanks. Acidified tanks measured 5.5 ± 1.1 μg·L^{-1}, and Al_i for acid–Al treated water measured 21.2 ± 2.9 μg·L^{-1}.

Physiological responses

FW gill Al levels were not significantly different in Control (70.8 ± 19.3 μg·g^{-1}) and acid treatment groups (41.5 ± 8.4 μg·g^{-1}). Gill Al was

Fig. 1. Gill aluminum (A), plasma chloride (B), and plasma cortisol (C) following 5 days of treatment in fresh water (FW) and after a 24 h seawater (SW) challenge (yellow: Control, orange: acid only, and red: acid–Al). Values are mean + standard error (n = 12). Letter change from "a" indicates significant difference from Control (b) or acid and Control (c) ($p < 0.05$, Student–Newman–Keuls test). In FW, one-way ANOVA indicated significant effect of treatment on gill Al ($p < 0.001$), plasma chloride ($p = 0.023$), and plasma cortisol ($p = 0.002$). In SW, one-way ANOVA indicated significant effect of treatment on gill Al ($p < 0.001$), plasma chloride ($p < 0.001$), and plasma cortisol ($p < 0.001$). [Colour online.]



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significantly elevated in acid–Al treated fish (461.3 ± 29.0 µg·g^{-1}) as compared with Control and acid treatment groups. After 24 h in SW, gill Al of acid treated fish (40.4 ± 3.7 µg·g^{-1}) was elevated over Control (17.6 ± 3.6 µg·g^{-1}), and the acid–Al group showed a significant elevation over both Control and acid treated fish (100.8 ± 8.3 µg·g^{-1}; Fig. 1A).

In FW, plasma Cl of Control (132 ± 0.7 mmol·L^{-1}) and acid treated fish (136 ± 1.8 mmol·L^{-1}) were not significantly different from each

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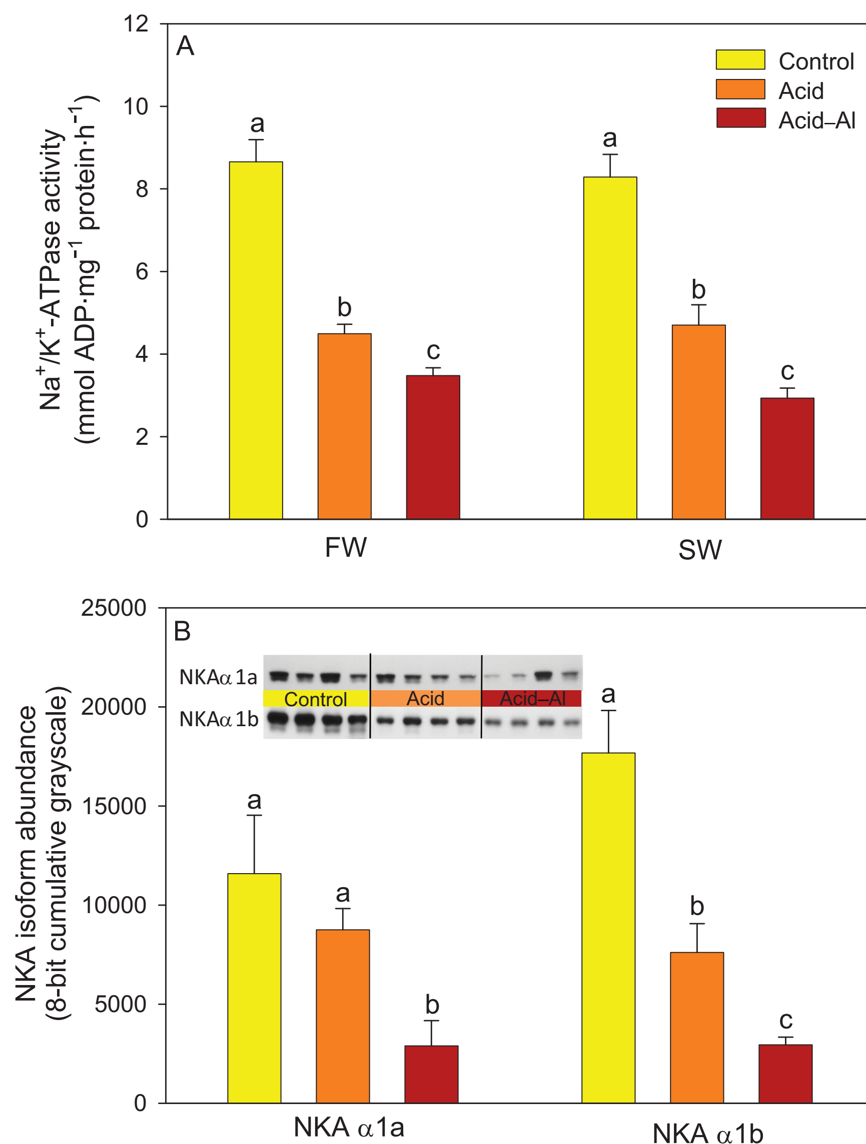
Fig. 2. Gill Na^+/K^+-ATPase (NKA) activity (A) following 5 days of treatment in FW and after a 24 h SW challenge (yellow: Control, orange: acid only, and red: acid–Al). Values are mean + standard error (n = 12). Letter change from "a" indicates significant difference from Control (b) or acid and Control (c) (p < 0.05, Student–Newman–Keuls test). One-way ANOVA indicated significant effect of treatment on gill NKA activity in FW (p < 0.001) and following 24 h SW challenge (p < 0.001). Gill NKA isoform abundance (B) as determined by Western blotting (inset) was measured in FW after treatment. Values are mean + standard error (n = 7). One-way ANOVA indicated significant effect of treatment on NKAα1a abundance (p = 0.017) and NKAα1b abundance (p < 0.001). [Colour online.]



other, but plasma Cl of acid–Al treated fish was significantly lower (123 ± 5.2 mmol·L^{-1}). After a 24 h SW challenge, Control fish plasma Cl levels (149 ± 2.7 mmol·L^{-1}) were significantly lower than that of both acid (178 ± 4.4 mmol·L^{-1}) and acid–Al (187 ± 2.2 mmol·L^{-1}) treated fish (Fig. 1B).

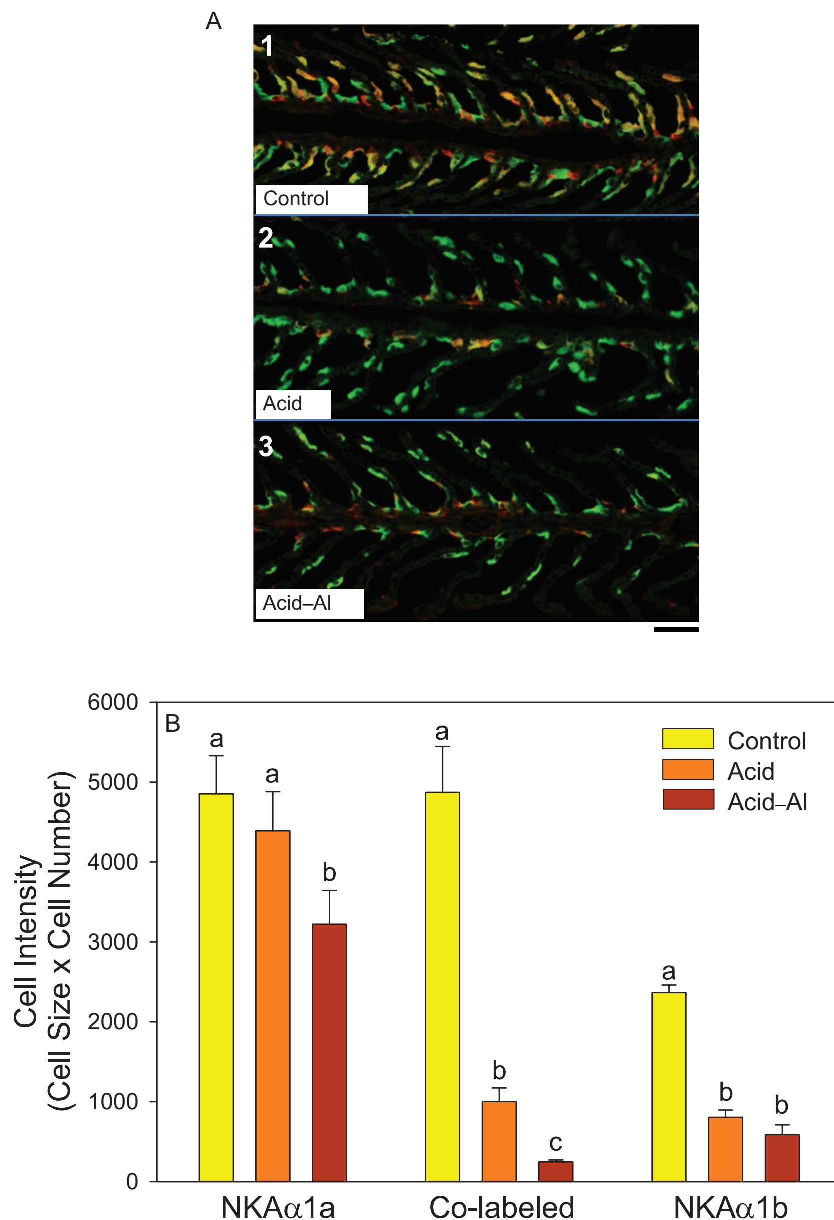
FW plasma cortisol was not significantly different between Control (21.9 ± 6.6 ng·mL^{-1}) and acid treated fish (60.2 ± 14.6 ng·mL^{-1}). Cortisol was significantly elevated in acid–Al treated fish (123.0 ± 27.3 ng·mL^{-1}). Following a 24 h SW challenge, plasma cortisol in Control (47.1 ± 12.4 ng·mL^{-1}) and acid (123.9 ± 35.0 ng·mL^{-1}) treated fish were lower than that of acid–Al (239.1 ± 41.3 ng·mL^{-1}) treated fish. Although plasma cortisol levels of acid treated fish were elevated over Control levels in both FW and SW, these trends were not found to be statistically significant (Fig. 1C).

Measured FW gill NKA activity (μmoles ADP·mg protein^{-1}·h^{-1}) was significantly higher in Control (8.5 ± 0.55) than in acid (4.5 ± 0.23) treated fish. There was a further significant reduction in NKA activity in acid–Al (3.5 ± 0.19) treated fish. The same trend was observed following a 24 h SW challenge (Control 8.3 ± 0.55, acid 5.0 ± 0.45, acid–Al 2.9 ± 0.24). Although both acid and acid–Al treated fish showed a reduction in gill NKA activity, the reduction was significantly greater in acid–Al treated fish as compared with acid alone in both FW and SW (Fig. 2A).

Gill NKA isoform abundance

Analysis of NKA isoform abundance showed a trend towards reduced gill FW NKAα1a isoform abundance as measured by Western blotting but was not significantly lower in acid treated fish (8754 ± 1440) as compared with Control (11584 ± 2938). Gill NKAα1a abundance was significantly decreased in acid–Al treated fish (3064 ± 1158). NKAα1b isoform abundance was significantly decreased below Control (1764 ± 2897) in acid treated fish (7611 ± 1580). A further significant reduction in NKAα1b abundance was

Fig. 3. Representative immunohistochemical sections (A) and summary data of NKA isoform-specific ionocytes following acid and Al exposure in FW (B). Ionocytes stained positively for NKAα1a (green), co-labeled NKAα1a and NKAα1b (yellow-orange), and NKAα1b (red) in Control (1), acid (2) and acid–Al (3) treated fish. Scale bar = 70 μm. (B) Immunohistochemical ionocyte integrated cell intensity, defined as mean cell size × mean cell number. Bar shows combined lamellar and filamental ionocyte intensity for all treatment groups and NKAα1a (yellow), co-labeled (orange), and NKAα1b (red) cell expression (n = 6). Letter change from "a" indicates significant difference from Control (b) or acid only and Control (c) ($p < 0.05$, Student–Newman–Keuls test). One-way ANOVA indicated significant effect of treatment on NKAα1a cell intensity in acid–Al chloride cells only ($p = 0.02$). There was a significant effect of treatment on co-labeled cell intensity of acid treated fish and a further significant reduction in acid–Al treated fish ($p < 0.001$ and $p < 0.001$). There was also a significant effect of treatment on NKAα1b integrated cell intensity in cells of acid and acid–Al treated fish ($p < 0.001$) relative to Control. [Colour online.]



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seen in acid–Al (2947 ± 498) as compared with acid treated fish (Fig. 2B).

Gill NKA IHC

FW gill NKA isoform IHC (Fig. 3A) showed the effects of acid and acid–Al treatment on gill chloride cell number and size and is represented in Table 1. There was no significant reduction in the number of NKAα1a positive cells on gill filament or lamellae as compared with Control in either acid or acid–Al treated FW fish. There was a trend for reduced cell area of lamellar NKAα1a immunoreactive cells in acid and acid–Al treatment groups as compared with Control, but it was not found to be significant. There was a significant reduction of total cell area of NKAα1a positive filamental cells in acid treated fish as compared to Control as well as in acid–Al treated fish. Co-labeled cells (NKAα1a and NKA α1b immunoreactive cells) were significantly reduced in number in both the lamellae and filament of acid treated fish as compared with Control fish. On the filament there was a significant further reduction in the number of immunopositive cells in acid–Al as compared with acid. Co-labeled cells in both lamellae and filament of acid and acid–Al treated fish were significantly reduced in size as com-

Table 1. Effect of acid and acid–Al treatment on Na^+/K^+-ATPase (NKA) immunoreactive cell number (No., counted from 700 μm of primary filament–lamellae per sagittal section) and size (cross-sectional area, μm^2) in Atlantic salmon smolts.

Treat	No. or size	NKAα1a		Co-labeled		NKAα1b	
		Lamellar	Filamental	Lamellar	Filamental	Lamellar	Filamental
Control	Cell no.	26.4±3.1	28.2±2.8	24.0±5.2	21.6±2.5	10.0±0.7	28.6±2.2
Acid	Cell no.	30.2±3.1	25.7±1.4	2.7±0.8*	9.9±1.3*	0.9±0.2*	14.9±1.8*
Acid–Al	Cell no.	23.7±2.9	23.6±2.6	0.4±0.6**	2.9±0.5**	0.8±0.4*	12.1±2.0*
Control	Size	82.7±4.5	95.2±2.9	99.9±3.6	113.7±6.2	60.0±5.9	62.5±3.4
Acid	Size	74.3±5.6	81.3±4.8*	72.4±4.9*	80.8±4.7*	49.6±6.7*	51.5±3.5*
Acid–Al	Size	66.0±2.2	69.7±4.3*	60.7±6.0*	68.4±3.0*	51.1±7.2*	45.0±1.9*

Note: A single asterisk (*) indicates a significant effect of treatment using one-way ANOVA and Neuman–Keuls post hoc analysis ($p < 0.05$). A double asterisk (**) indicates significant difference from both Control and acid treated fish.

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pared with Control. NKAα1b cell numbers on both lamellae and filament were significantly lower in acid and acid–Al than in Control. In addition, NKAα1b positive cell size was also significantly reduced below that of Control in acid and acid–Al.

The product of cell size and cell number is represented as integrated cell intensity (Fig. 3B). There was no significant reduction in integrated cell intensity of NKAα1a cells on gills of acid treated fish (4389 ± 490) as compared with Control (4852 ± 477). There was a significant reduction in integrated cell intensity in NKAα1a cells in acid–Al treated fish (3222 ± 421) as compared with Control. Co-labeled cell intensity was significantly reduced in acid and further reduced in acid–Al (acid 1002 ± 169; acid–Al 246 ± 25) as compared with Control (4871 ± 574). The further reduction in intensity of co-labeled cells in acid–Al as compared with acid was significant. NKAα1b integrated cell intensity was significantly reduced in both acid and acid–Al (acid 805 ± 90; acid–Al 589 ± 120) relative to Control (2366 ± 92).

Discussion

We sought to examine how moderate levels of acid and acid plus Al impacts NKA α subunit isoform protein in Atlantic salmon gills and relate these changes to compromises in osmoregulation in both FW and SW. The present study is the first to demonstrate the relatively rapid impact (4 days) of acid and acid–Al treatment on abundance and distribution of the FW and SW NKA protein isoforms in Atlantic salmon during smolting. We show a differential sensitivity of gill NKA isoforms in salmon smolts exposed to acid and acid–Al conditions. Western blotting demonstrated that FW gill NKAα1a isoform protein abundance was not significantly affected by acid but was significantly reduced by acid–Al. IHC-measured integrated cell intensity of detected proteins showed that NKAα1a immunopositive cells were unchanged by acid but significantly reduced by acid–Al treatment. The SW type isoform, NKAα1b, protein abundance was significantly reduced after acid treatment and was even further reduced by acid–Al conditions. NKAα1b IHC showed a significant decrease in integrated cell intensity in both acid and acid–Al treatments relative to control. This differential sensitivity of the FW and SW NKA isoforms explains the sensitivity of osmoregulatory physiology of salmon smolts, in which both acid and acid–Al affected SW osmoregulation, whereas only acid–Al conditions significantly affected ion regulation in FW.

In FW, loss of plasma Cl indicates a reduced ability to maintain plasma ions. Previous studies have shown that acidic conditions can increase efflux of ions and reduce the capacity for ion uptake in salmonids (Booth et al. 1988; Gensemer and Playle 1999). Here, FW plasma Cl was not affected by acid alone, whereas acid–Al did have a significant impact. These results match the relative impact of acid versus acid–Al treatment on the NKAα1a isoform abundance and integrated cell intensity. In contrast, both acid and acid–Al conditions reduced the ability of smolts to maintain ion balance after a 24 h SW challenge. This indicates that salinity tolerance is more sensitive to prior acid–Al exposure than is osmoregulation in FW and is consistent with previous results (Monette et al. 2010). SW challenge tests have previously demonstrated that acidic water exposure impairs salinity tolerance during smolting (Farmer et al. 1989; Staurnes et al. 1993*a*; Rosseland and Staurnes 1994; Bjerknes et al. 2003) and that even short-term exposure of Atlantic salmon released into acidic waters impairs osmoregulation, salinity tolerance, and marine survival (Staurnes et al. 1996*a*; Kroglund et al. 2007). Earlier studies examining short-term acid and Al exposure have shown an effect on osmoregulatory proteins, with reduced NKA activity and reduction of Na^+, K^+, $2Cl^-$ (NKCC) cotransporter protein abundance (Monette et al. 2008). These studies showed that smolt development and SW tolerance are compromised by short-term exposure to acid and Al in the absence of detectable impacts on FW ion regulation and proposed the loss of SW tolerance during short-term acid and Al exposure likely results from reductions in gill NKA and NKCC. Our results further demonstrate that the loss of salinity tolerance is due specifically to the loss of the SW isoform NKAα1b and associated ionocytes.

Our findings on the impact of acid and Al exposure on NKA isoform protein abundance and ionocyte abundance are generally consistent with previous studies on transcriptional responses of NKA isoforms (Nilsen et al. 2010). These authors found that with treatments of either pH 5.9, 15 $\mu g\ Al_i \cdot L^{-1}$ or pH 5.6, 30 $\mu g\ Al_i \cdot L^{-1}$ for 3–9 days resulted in reduced gill mRNA levels of NKAα1b and reduced NKA activity in Atlantic salmon smolts. Reduced levels of gill NKAα1a mRNA were found in their more severe treatment but not in their moderate treatment. Interestingly, these authors also found that the transcriptional response to SW was also altered by prior severe acid treatment, with NKAα1a mRNA increasing more than controls after SW exposure and NKAα1b increasing less, both responses indicative of reduced capacity for salt secretion. Although the severity and duration of treatment was different between Nilsen et al. (2010) and the present study, both studies indicate that NKAα1b is more sensitive than NKAα1a to acid exposure during smolt development.

The reason for increased sensitivity of NKAα1b ionocytes to acid and Al exposure is still unclear. Decreased gill NKA activity has been attributed to increased chloride cell death via apoptosis and necrosis (Verbost et al. 1995), direct inhibition of enzyme activity by Al ions (Silva and Goncalves 2003), or the increased appearance of immature gill chloride cells with low levels of NKA protein (Wendelaar Bonga et al. 1990). Here, lower levels of NKAα1b ionocytes after only a few days of acid–Al exposure is in line with the findings of Verbost et al. (1995), where gill apoptosis and necrosis increased significantly in just 24 h of acid and Al conditions, suggesting that increased cell death may be involved. However, Monette et al. (2010) did not see co-localization of apoptotic marker Caspase 3B with NKA positive cells in FW acid–Al exposed gills. A more detailed examination of apoptosis and necrosis in response to acid–Al exposure effects on NKA isoforms is necessary to deter-

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mine what role, if any, these processes play in reducing SW tolerance. Here, it is not immediately obvious why these NKAα1b cells should be more sensitive, especially since they are thought to be inactive until stimulated by exposure to SW (McCormick et al. 2013). Perhaps some inherent characteristic of the NKAα1b ionocyte, even when relatively inactive, makes it more sensitive to acid and Al.

Plasma cortisol levels are known to rise in the spring during the parr–smolt transformation (Specker 1982; Barton et al. 1985; Young 1986). Cortisol has been shown to increase hyperosmoregulatory abilities by acting directly to increase gill NKA activity (McCormick and Bern 1989; Bisbal and Specker 1991) and cause differentiation of ionocytes (Foskett et al. 1983; McCormick 1990). Atlantic salmon show a rise in basal cortisol levels during smolting, but also exhibit a heightened stress response at this life stage (Carey and McCormick 1998). During exposure to copper, cortisol has been shown to protect against necrosis but cause increased apoptosis of ionocytes (Bury et al. 1998). It is possible that both types of ionocytes are susceptible to increased turnover after acid exposure, but that the importance of NKAα1a to ion uptake necessitates signaling that maintains their numbers to ensure FW survival. It will therefore be of interest to examine the necrosis, apoptosis, and differentiation of both NKAα1a and NKAα1b and of transitioning ionocytes in smolt gills after acid and Al exposure.

The presence of co-labeled cells (NKAα1a and NKAα1b immunopositive cells) may also play a role in this observed acid sensitivity. In comparison with NKAα1b immunopositive cells, co-labeled cells were most dramatically reduced in number relative to control and between treatments. There was a 41% loss in NKAα1b cells in acid treatment relative to Control and a further loss of 19% from acid to acid–Al for a loss of 66% of NKAα1b immunopositive cells in acid–Al relative to Control. The total number of co-labeled cells was reduced by 72% with acid treatment relative to Control, and the acid–Al treated group was reduced by a further 73% as compared with acid for a total loss of 92% in acid–Al treated fish as compared with Control. The presence of co-labeled cells in smolt gills has been demonstrated previously (McCormick et al. 2013). These cells are present on both the lamellae and filament of juvenile Atlantic salmon as part of the peak SW preparatory stages of smolting. McCormick et al. (2013) proposed a model in which NKAα1b and co-labeled cells of FW smolts have no external exposure and are inactive prior to exposure to SW, then are rapidly activated in SW. They also propose that most NKAα1a cells are deactivated and either transform to NKAα1b cells or undergo apoptosis. If co-labeled cells are transitioning either to SW type cells or undergoing apoptosis, exposure to an environmental stressor such as acid or acid–Al may increase the likelihood that these cells are targeted for apoptosis. This supports the findings of Monette et al. (2010) where they proposed that smolts exposed to moderate acid–Al may experience increased apoptosis and that loss of SW tolerance appears to be independent of the mechanisms at play in high acid–Al conditions and may result instead from a shift in the phenotype of mitochondria-rich cells present in the gill epithelium (Monette et al. 2010).

Many studies have demonstrated a reduction in NKA activity in response to acid and acid–Al exposure in Atlantic salmon (Staurnes et al. 1993*a*, 1993*b*, 1996*b*; Magee et al. 2003; Kroglund et al. 2007; Monette et al. 2008; Nilsen et al. 2010). In this study, NKA activity in FW was reduced in smolts treated with acid and further reduced in acid–Al treated fish. The same pattern was observed after 24 h in SW, with a decrease in NKA activity with increasing severity of treatment. Since the activity is presumably a mixture of both isoforms, the moderate loss of gill NKA activity in the acid treatment likely reflects the decrease of the NKAα1b isoform, while the more severe loss of gill NKA activity in the acid–Al treatment reflects the decrease of both isoforms.

The observation that the SW-type NKA cells were more dramatically affected by acid than FW-type cells may help explain the observed increased sensitivity of the smolt life stage as compared with parr (Monette and McCormick 2008). The distribution and abundance of the FW and SW NKA isoforms in Atlantic salmon parr has been shown by McCormick et al. (2013). Parr show a low number of SW type chloride cells relative to smolts even during the smolt season, with NKAα1a as the primary NKA isoform. The increased sensitivity of NKAα1b to the effects of acid and acid–Al, and the low number of these NKAα1b type chloride cells in parr may impart to them some increased tolerance in comparison with smolts.

Increased inorganic Al usually accompanies decreased pH in acid-impacted streams (Baldigo et al. 2007). High levels of dissolved organic carbon (DOC) generally reduce the effects of Al on fish (Witters et al. 1990) where DOC can bind to and therefore remove the more harmful inorganic Al from surface waters. However, DOC may also increase acidity in the form of humic acids and transport additional complexed Al from soils into surface waters (Gensemer and Playle 1999; Laudon et al. 2005). Findings from Liebich et al. (2011) suggest that physiological effects on *S. salar* smolts are strongly correlated with stream pH and that in rivers and streams with low DOC concentrations, the threshold for physiological effects and mortality probably occurs at a higher pH and shorter exposure period than in rivers with higher DOC. Although the acid alone conditions used in this lab may not completely reflect natural conditions of some high DOC streams that occur in eastern North America, we were able to isolate the effects of acid alone and remove the impacts of acid precipitation leaching Al from soils. Under the conditions created in this experiment, we were able to detect the subtle effect that acidic water without Al has on NKAα1b isoform abundance and distribution in the relatively rapid time frame similar to that seen under spring snowmelt and precipitation events typical in northeastern North America. Since significant loss of salinity tolerance occurred even in the absence of increased gill Aluminum, we must also note that gill Al as a biomarker may have only limited utility in rivers where acid conditions do not result in strongly elevated inorganic Al.

In conclusion, we have shown that the FW and SW NKA isoforms are differentially impacted by acid and acid–Al. The greater sensitivity of the NKAα1b isoform to prior acid–Al is likely responsible for the reduced salinity tolerance of Atlantic salmon smolts that occurs after such exposures. Our results further support the importance of increased gill NKAα1b isoform abundance as a critical component of salinity tolerance and that measurement of both NKAα1a and NKAα1b isoforms and their associated ionocytes are important to understanding alterations in osmoregulatory capacity of salmon.

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