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Effects of short-term acid and aluminum exposure on the parr-smolt transformation in Atlantic salmon (*Salmo salar*): Disruption of seawater tolerance and endocrine status

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ABSTRACT

Episodic acidification resulting in increased acidity and inorganic aluminum (Al_i) is known to interfere with the parr-smolt transformation of Atlantic salmon (*Salmo salar*), and has been implicated as a possible cause of population decline. To determine the extent and mechanism(s) by which short-term acid/Al exposure compromises smolt development, Atlantic salmon smolts were exposed to either control (pH 6.7–6.9) or acid/Al (pH 5.4–6.3, 28–64 $\mu g\,l^{-1}$ Al_i) conditions for 2 and 5 days, and impacts on freshwater (FW) ion regulation, seawater (SW) tolerance, plasma hormone levels and stress response were examined. Gill Al concentrations were elevated in all smolts exposed to acid/Al relative to controls confirming exposure to increased Al_i. There was no effect of acid/Al on plasma ion concentrations in FW however, smolts exposed to acid/Al followed by a 24 h SW challenge exhibited greater plasma Cl^- levels than controls, indicating reduced SW tolerance. Loss of SW tolerance was accompanied by reductions in gill Na^+,K^+-ATPase (NKA) activity and $Na^+,K^+,2Cl^-$ (NKCC) cotransporter protein abundance. Acid/Al exposure resulted in decreased plasma insulin-like growth factor (IGF-I) and 3,3′,5′-triiodo-L-thyronine (T_3) levels, whereas no effect of treatment was seen on plasma cortisol, growth hormone (GH), or thyroxine (T_4) levels. Acid/Al exposure resulted in increased hematocrit and plasma glucose levels in FW, but both returned to control levels after 24 h in SW. The results indicate that smolt development and SW tolerance are compromised by short-term exposure to acid/Al in the absence of detectable impacts on FW ion regulation. Loss of SW tolerance during short-term acid/Al exposure likely results from reductions in gill NKA and NKCC, possibly mediated by decreases in plasma IGF-I and T_3.

1. Introduction

Chronic (year-round) acidification and its associated aluminum (Al) toxicity is a cause of Atlantic salmon (*Salmo salar*) population decline in Norway (Hesthagen, 1989) and Nova Scotia (Watt et al., 1983). Recent studies have suggested that episodic or short-term acidification (single or re-occurring episodes lasting several days) may also affect Atlantic salmon populations in regions of the northeastern United States including Maine where several salmon rivers have been listed as endangered (Magee et al., 2001, 2003; National Academy of Science, 2004). Many rivers and streams in these regions have low concentrations of base cations (Ca^{2+}, Mg^{2+}) and thus poor buffering capacity, making them vulnerable to short-term decreases in pH during precipitation events such as spring snowmelts or fall storms. During episodic acidification, Al leaches from the soil and elevates Al levels of the surrounding watershed. In addition, the solubility of Al increases as a direct result of decreased pH leading to elevated levels of inorganic Al (Al_i), the form of Al that is most toxic to fish (Gensemer and Playle, 1999).

The fish gill, a multifunctional organ involved in ion regulation and respiration, is the major site of acid/Al toxicity (Exley et al., 1991; Gensemer and Playle, 1999). During exposure to acid/Al, Al accumulates both on the surface and within the gill and is often associated with damage to the branchial epithelium (Youson and Neville, 1987; Lacroix et al., 1993; Wilkinson and Campbell, 1993; Teien et al., 2004). Consequently, acid/Al results in the loss of ion regulatory ability due to increased branchial permeability and inhibition of active ion uptake (Booth et al., 1988; McDonald et al., 1991). Increased permeability may be caused by the displacement of Ca^{2+} ions by Al from anionic gill binding sites, resulting in the weakening of intercellular tight junctions (Booth et al., 1988; Freda

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et al., 1991), whereas inhibition of ion uptake may result from damage or alteration of gill chloride cells (Jagoe and Haines, 1997), and decreased gill Na^+,K^+-ATPase (NKA) activity (Staurnes et al., 1993).

Anadromous fishes such as Atlantic salmon may be particularly vulnerable to ion regulatory disturbance resulting from acid/Al exposure, as they face severe ion regulatory challenges when migrating from FW to SW. In the spring, following 2–5 years of residence in FW, Atlantic salmon enter the parr-smolt transformation, a developmental period adaptive for downstream migration and SW residence. This transformation, cued by seasonal changes in photoperiod and temperature, consists of physiological, behavioral, and morphological alterations that work together to enable successful SW entry and survival in the marine environment (Hoar, 1988; McCormick et al., 1998a). Among the physiological changes is an increase in SW tolerance brought about by alterations in osmoregulatory organs (gill, gut, kidney) including the proliferation of gill chloride cells, and the upregulation of gill ion transport proteins such as NKA and NKCC cotransporter (Hoar, 1988; Pelis et al., 2001; Tipsmark et al., 2002).

Several hormones such as cortisol, growth hormone (GH), insulin-like growth factor I (IGF-I), and thyroid hormones increase during the parr-smolt transformation and are thought to play a role in the acquisition of SW tolerance (Hoar, 1988; Björnsson, 1997). In salmonids, cortisol, GH and IGF-I work both independently and in concert to increase SW tolerance by stimulating gill NKA activity and chloride cell proliferation (see review by McCormick, 2001). Recent evidence indicates that cortisol is also involved in the induction of downstream migratory behavior in masu salmon (*Oncorhynchus masou*) smolts (Munakata et al., 2007). Thyroid hormones play a supportive role in the acquisition of SW tolerance through the interaction with both the GH/IGF-I and cortisol axes (McCormick, 2001), and have been also implicated in controlling behaviors such as downstream migration and SW preference (Iwata et al., 1990, 2003; Iwata, 1995; Lerner et al., 2007).

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 smolting can disrupt ion regulatory ability in FW, reduce SW tolerance (Saunders et al., 1983; Staurnes et al., 1993, 1995, 1996; Kroglund and Staurnes, 1999; Magee et al., 2001, 2003; Monette and McCormick, 2008), decrease growth and impair downstream migration and marine survival (Kroglund and Finstad, 2003). The ability of smolts to maintain ion homeostasis in SW appears to be highly sensitive to acid/Al exposure (Staurnes et al., 1993, 1996; Kroglund and Staurnes, 1999), however, the mechanism(s) underlying the increased sensitivity of the smolt hypoosmoregulatory system remain unknown.

The present study was conducted to investigate the effects of a single, short-term, sub-lethal acid/Al pulse, such as might occur after an acid rain event, on the mechanism(s) underlying the increased sensitivity of smolt SW tolerance. We hypothesized that exposure to acid/Al would lead to impaired SW tolerance of smolts as a result of disruption of gill ion transporters and plasma hormones necessary for smolt development. We tested this hypothesis by exposing Atlantic salmon smolts to an acid/Al pulse for 2 and 5 days followed by exposure to SW for 24 h.

2. Materials and methods

2.1. Fish rearing

Atlantic salmon parr were obtained from the White River National Fish Hatchery (Bethel, VT, USA), and raised to smolts at the Conte Anadromous Fish Research Center (Turners Fall, MA, USA). Fish were held in fiberglass tanks receiving flow through (4 l min^{-1}) Connecticut River water (Ca^{2+}, 9.0 mg l^{-1}; Mg^{2+}, 1.5 mg l^{-1}; Na^+, 6.8 mg l^{-1}; K^+, 1.10 mg l^{-1}, Cl^-, 11.0 mg l^{-1}) prior to the initiation of treatment. 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. Laboratory exposure

Laboratory exposures were conducted from May 12 to 16, 2002. Atlantic salmon smolts (28–54 g) were randomly assigned to 2 replicate tanks (20 fish $tank^{-1}$) receiving either control (pH 6.7–6.9) water, or an acid/Al pulse (pH 5.4–6.3, 28–64 μg l^{-1} Al_i). An acid-only treatment was not included in this study, as it has been established that increases in Al_i occur together with decreased pH in rivers experiencing episodic acidification (Lacroix and Townsend, 1987). Experimental tanks (330 l) received artificial soft water prepared by mixing deionized water (Culligan, Keene, NH, USA) with ambient Connecticut River water (4:1), and target pH and Al concentrations were achieved in header tanks using 3 N HCl and an $AlCl_3 \cdot 6H_2O$ stock solution (1000 mg l^{-1} Al), respectively. Dilution of river water resulted in a reduction in ionic strength (including ambient Ca^{2+}, Na^+) similar to that which occurs following episodic rain events in low to moderately buffered streams (Lacroix and Townsend, 1987; Haines et al., 1990). These studies observed 2- to 5-fold decreases in ambient calcium concentrations shortly after periods of increased river discharge in Maine and Nova Scotia. Experimental water was mixed for at least 1 h before flowing into the fish tanks to avoid unstable water conditions. Each tank received a continuous flow of ~4.4 l h^{-1}. Both header and experimental tanks were oxygenated continuously maintaining dissolved oxygen at >10 mg O_2 l^{-1}. Temperature was maintained at 11.4–13.1 °C using a recirculating chiller system. Daily pH measurements were made directly in the tank using a portable pH 105 meter (Corning, Medfield, MA, USA) with a Ross Ultra pH probe (Thermo Orion, Beverly, MA, USA). Daily water samples were taken in acid-washed 50 ml tubes for the measurement of Al and Ca^{2+}. Food was withheld for 24 h prior to the initiation of the study and for the duration of the experiment. Smolts were exposed to one of the two water chemistries for two and five days. At each time-point, five fish replicate $tank^{-1}$ (ten fish $treatment^{-1}$) were sampled, and simultaneously, five fish replicate $tank^{-1}$ (ten fish $treatment^{-1}$) were placed into tanks (330 l, maintained at 12 °C) containing 30 pptSW (charcoal-treated, aerated) and sampled after 24 h. Plasma Na^+ and Cl^- concentrations of smolts after 24 h in 30 pptSW were used as indicators of SW tolerance.

2.3. Tissue collection 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 vessel puncture and centrifuged at 3200*g* for 5 min at 4 °C. Plasma was then removed and stored at −80 °C. Blood for hematocrit measurement was collected in heparinized micro-hematocrit capillary tubes and centrifuged at 13,500*g* for 5 min in a micro-hematocrit centrifuge (Damon/IEC Division, Needham, MA, USA). Plasma Na^+ was measured by flame atomic absorption spectrophotometry (FAAS) (AAnalyst 100, Perkin Elmer, Wellesley, MA, USA). Plasma Cl^- was measured by silver titration using a digital chloridometer (Labconco, Kansas City, MO, USA). 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 as described by McCormick (1993), placed into acid-washed 1.5-ml centrifuge tubes, and stored at −80 °C. Gill biopsies were also 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 determination of NKA activity. The remaining gill tissue was removed for Western immunoblotting, placed in a 1.5-ml centrifuge tube, immediately frozen on dry ice, and stored at −80 °C.

2.4. Water chemistry analysis

Water samples for Al analysis were taken and processed as described by Lacroix and Townsend (1987). 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 (HGA-800/AAnalyst 100, Perkin Elmer, Wellesley, MA, USA) atomic absorption spectrophotometry (GFAAS). Water samples were read in duplicate, and instrument calibration was monitored every ten samples with a reference standard. Acceptable recovery limits of reference standard were 90–110%, and when values were outside this range a re-slope function was applied. Inorganic Al (Al_i) was determined by the cation-exchange column method (Amberlite 120, prepared with Na^+) described by Driscoll (1984). Al present in the column processed samples was called organically bound Al (Al_o). Al_i was then determined by calculating the difference between Al_a and Al_o. Calcium was measured by flame atomic absorption spectrophotometry (AAnalyst 100, Perkin Elmer, Wellesley, MA, USA).

2.5. Gill aluminum analysis

Al accumulation in gill tissue biopsies was analyzed by the method outlined in Teien et al. (2006). Gill biopsies were thawed, dried at 60 °C for 24 h, and weighed to the nearest 0.0001 mg using a Series 30 microbalance (Cahn Instruments, Cerritos, CA, USA). Gill biopsies were then digested by adding 98 μl of 100% trace metal grade

HNO_3 and 2 μl of H_2O_2 to biopsy tubes, and heating at 100 °C until completely 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 (1:10) by the addition of 900 μl of ultrapure water, and Al concentration was analyzed by GFAAS as described above. A background correction was made by subtracting the Al present in digestion blanks. Gill Al was expressed as μg Al g^{-1} gill dry weight.

2.6. Gill NKA activity

Gill NKA activity was measured following the method described by McCormick (1993). Gill biopsies were thawed immediately prior to assay and homogenized in 0.5% SEID (0.1 g sodium deoxycholate in 20 ml SEI) for 10–15 s using a Kontes pellet pestle motor. The homogenate was then centrifuged at 3200*g* for 30 s to remove large debris, 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 mM ouabain, and expressed as μmol ADP mg protein^{-1} h^{-1}.

2.7. SDS–PAGE Western immunoblotting for NKCC

Gill NKCC protein abundance was measured by Western immunoblotting as outlined by Pelis and McCormick (2001). Gill tissue was prepared following partial membrane purification (Zaugg, 1982). Approximately 0.08 g of frozen gill tissue was thawed and placed into 0.8 ml ice-cold SEI with protease inhibitors (Complete mini protease inhibitor cocktail, Roche Applied Sciences, USA) homogenized, and centrifuged for 7 min at 2000*g*. The supernatant was then discarded and the tissue pellet re-suspended in 0.5 ml ice-cold SEI with protease inhibitors and 0.1% deoxycholate. The re-suspended pellet was homogenized, centrifuged for 6 min at 2000*g*, and the supernatant was then assayed for total protein (BCA protein assay, Pierce, Rockford, IL, USA). Samples were then placed in 2× Laemmli buffer and heated for 15 min at 60 °C. Samples were run on a 6% SDS–PAGE gel at 10 μg of protein per lane. Following electrophoresis, proteins were transferred to immobilon PVDF transfer membranes (Millipore, Bedford, MA, USA) at 30 V overnight in 25 mM Tris, 192 mM glycine buffer at pH 8.3. PVDF membranes were blocked in phosphate-buffered saline with 0.05% Triton X-100 (PBST) and 2% nonfat dry milk for 1 h at room temperature, rinsed in PBST, and probed with an NKCC primary antibody (T4; Developmental Studies Hybridoma Bank) diluted 1:1000 in antibody dilution buffer (PBST with 0.01% NaN_3 and 0.1% BSA) for 1 h at room temperature. After rinsing in PBST blots were probed with horseradish peroxidase (HRP) labeled goat anti-mouse IgG, H+L (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) diluted 1:1000 in antibody dilution buffer for 1 h at room temperature. PVDF membranes were developed with the colorimetric substrate diaminobenzidine for approximately 90 s.

Digital photographs were taken of individual gels and band staining intensity was measured using ImageJ version 1.37 (National Institutes of Health, USA). To quantify band staining intensity, individual lanes were selected and ImageJ measured cumulative 8-bit gray scale values and generated a lane profile plot. Peaks of interest were chosen according to positive NKCC bands identified by Pelis et al. (2001). Peaks were then enclosed using a drawing tool, area of each peak was measured, and peak areas were summed for each lane. Gill NKCC protein abundance was expressed as cumulative 8-bit gray scale values.

2.8. Hormone anaylsis

Plasma cortisol was measured by enzyme immunoassay (EIA) as outlined by Carey and McCormick (1998). Plasma GH was measured by radioimmunoassay (RIA) using a specific double-antibody against salmon growth hormone as outlined by Björnsson et al. (1994). Plasma IGF-I was measured by homologous RIA as outlined by Moriyama et al. (1994). Plasma T_4 and T_3 were measured by RIA as outlined by McCormick et al. (1995).

2.9. Statistics

All data are presented as mean ± standard error (SE). For each physiological parameter, potential tank effects were tested by nesting replicate tanks within treatment. Fish from replicate tanks were pooled only if there was no significant tank effect ($P > 0.05$). A three-way analysis of variance (ANOVA) was used to determine the effects of treatment (control, acid/Al), exposure time (2, 5 days) and group (FW, SW) on smolt physiology, unless otherwise noted. In all cases, when significant effects were observed ($P < 0.05$), pairwise comparisons were made using Duncan's post-hoc test. All statistical analyses were performed using Statistica 7.0 (Statsoft, Inc., Tulsa, OK, USA).

3. Results

In control tanks, mean pH ranged from 6.7–6.9 over the course of the study (Fig. 1). Treatment tanks received an acid/Al pulse in which mean pH was initially 5.4, rose to 6.3 after 2 days, and remained between 6.0 and 6.3 for the duration of the study (Fig. 1). Mean Al_{tot} concentrations were 13 ± 0.6 and 86 ± 6.3 μg l^{-1} in control and treatment tanks, respectively (Table 1). Mean Al_i concentration was 43 ± 8.0 μg l^{-1} in treatment tanks (Table 1). Mean Ca^{2+} concentrations were 2.7 ± 0.4 and 2.9 ± 0.4 mg l^{-1} in control and treatment tanks, respectively.

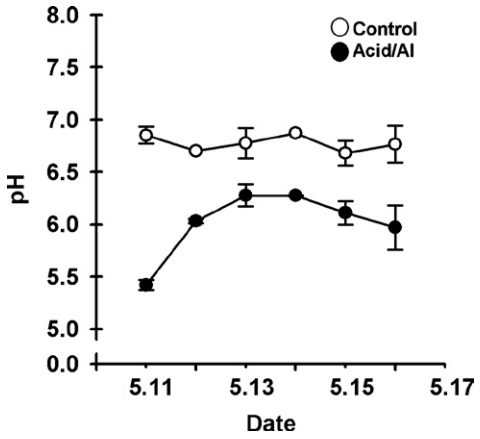

Fig. 1. Time-course pH profile of control (white) and acid/Al (black) tanks over 5 days ($n = 6$). Values are mean ± SE of two replicate tanks.

There were no mortalities in any group over the course of the study. One fish in one of the acid/Al treatment tanks exhibited abnormal swimming behavior and was removed from the study after 3 days. Gill Al of control smolts in FW was 23 ± 7.0 and 9 ± 1.1 μg g^{-1} after 2 and 5 days, respectively (Fig. 2). Gill Al of treated smolts in FW was 87 ± 9.7 and 84 ± 24 μg g^{-1} after 2 and 5 days, respectively (Fig. 2). Gill Al of treated smolts was significantly greater (3.8- to 9.0-fold) than controls after both time-points (Fig. 2).

Plasma Na^+ levels were not significantly affected by acid/Al, however, transfer to SW resulted in significant increases (12–20 mM) in plasma Na^+ levels in all groups (Fig. 3A). Acid/Al had no significant effect on plasma Cl^- levels in FW at either time-point, however, after 2 days of treatment and a subsequent SW challenge, plasma Cl^- levels of treated smolts were significantly greater (8 mM) than controls indicating a reduction in SW tolerance in this group (Fig. 3B). Plasma Cl^- levels of smolts exposed to 5 days of acid/Al and a subsequent SW challenge also tended to be greater than controls (Fig. 3B). Transfer to SW resulted in significant increases in plasma Cl^- levels in most groups however, increases were greater in treated smolts (Fig. 3B). After 2 days, transfer to SW had no significant effect on plasma Cl^- levels in control smolts, but resulted in a 13 mM increase in treated smolts. After 5 days, transfer to SW resulted in a 13 and 23 mM increase in plasma Cl^- levels in control and treated smolts, respectively.

Exposure to acid/Al reduced gill NKA activity (Fig. 3C). After 2 days, gill NKA activity of treated smolts was 38% and 41% lower than controls before and after a SW challenge, respectively, however, these differences were not statistically significant ($P = 0.11$

Table 1
Nominal Al exposures and measured water chemistry parameters in control and acid/Al tanks over the time-course of 5 days

Exposure condition	Nominal Al_{tot} (μg l^{-1})	Al_{tot} (μg l^{-1})	Al_i (mg l^{-1}) (μg l^{-1})	Ca^{2+} (mg l^{-1})
Control	0	13 ± 0.6 (7) (11–16)	Nd	2.7 ± 0.38 (10) (0.9–4.1)
Acid/Al	100	86 ± 6.3 (8) (56–109)	43 ± 8 (5) (28–64)	2.9 ± 0.36 (10) (0.9–4.0)

Values are mean ± SE of all measurements made throughout the 5-day study in both replicate tanks. Number of measurements made for each parameter is given in parentheses to the right. Range is given in parentheses below. Nd = not determined.

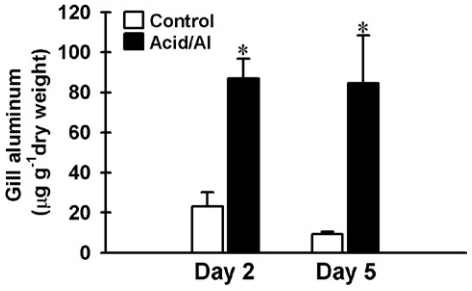

Fig. 2. Effects of short-term acid/Al exposure on gill Al accumulation of Atlantic salmon smolts. Gill Al of control (white bars) and treated (black bars) smolts after 2 and 5 days (FW only). Values are mean ± SE (n = 8–10). An (*) indicates a significant difference from control within a time-point (Duncan's; P < 0.05). Two-way ANOVA for gill Al levels determined significant effects of treatment (P < 0.001) and time (P = 0.03).

and P = 0.05; Fig. 3C). Gill NKA activity of treated smolts was significantly lower (36%) than controls after 5 days in FW (Fig. 3C). Gill NKA activity of smolts exposed to 5 days of treatment remained depressed after a 24 h SW challenge, however, controls also exhibited depressed gill NKA activity (Fig. 3C).

The T4 antibody to the NKCC cotransporter recognized three major bands centered at ~285, 160 and 120 kDa (Fig. 4A). This agrees well with the molecular weights of NKCC for Atlantic salmon previously reported by Pelis et al. (2001). According to these authors, the band at 285 kDa likely represents the glycosylated form of NKCC, while the bands at 160 and 120 kDa represent degradation products. Remaining bands present in the blot may represent NKCC associated with other peptides or NKCC with different degrees of glycosylation (Tipsmark et al., 2002). Quantification of Western blots (cumulative 8-bit gray scale from all three bands) revealed that acid/Al resulted in reductions in NKCC protein abundance (Fig. 4B). After 2 days, gill NKCC of treated smolts tended to be lower in both FW and SW compared to controls. Gill NKCC of treated smolts was significantly lower (27%) than controls after 5 days of acid/Al exposure and a subsequent SW challenge (Fig. 4B). After 5 days, transfer to SW resulted in a significant increase (2-fold) in gill NKCC in both control and treated smolts (Fig. 4B).

Plasma GH levels ranged from 14 to 37 ng ml^{-1} and from 9 to 35 ng ml^{-1} in control and treated smolts, respectively. Acid/Al had no significant effect on plasma GH levels, however, plasma GH levels of smolts exposed to 2 days of acid/Al were slightly lower than controls in FW (Fig. 5A). Transfer to SW resulted in significant increases (92% to 3.6-fold) in plasma GH levels in all groups (Fig. 5A).

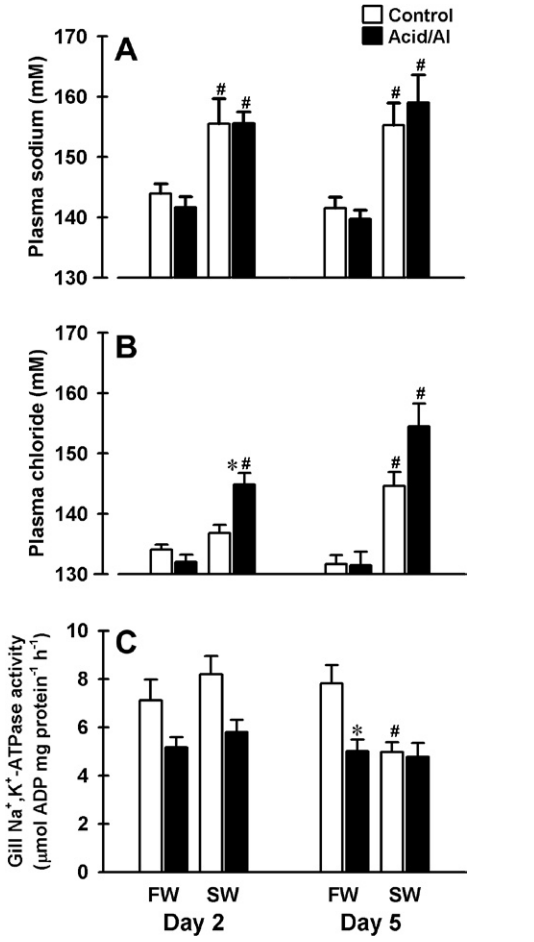

Fig. 3. Effects of short-term acid/Al exposure on the FW ion regulatory ability and SW tolerance of Atlantic salmon smolts. Plasma Na^+ (A), plasma Cl^- (B), and gill NKA activity (C) levels of control (white bars) and treated (black bars) smolts after 2 and 5 days (FW) and a subsequent 24 h SW challenge (SW). Values are mean ± SE (n = 9–10). An (*) indicates a significant difference from control within a group (FW, SW) and a time-point (Duncan's; P < 0.05). A (#) indicates a significant difference between FW and SW within a treatment and a time-point (Duncan's; P < 0.05). Three-way ANOVA for plasma Na^+ levels determined a significant group effect (P < 0.001). Three-way ANOVA for plasma Cl^- levels determined a significant group effect (P < 0.001), and significant treatment/group (P = 0.01) and group/time (P = 0.02) interactions. Three-way ANOVA for gill NKA activity determined a significant treatment effect (P < 0.001) and a significant group/time interaction (P = 0.02).

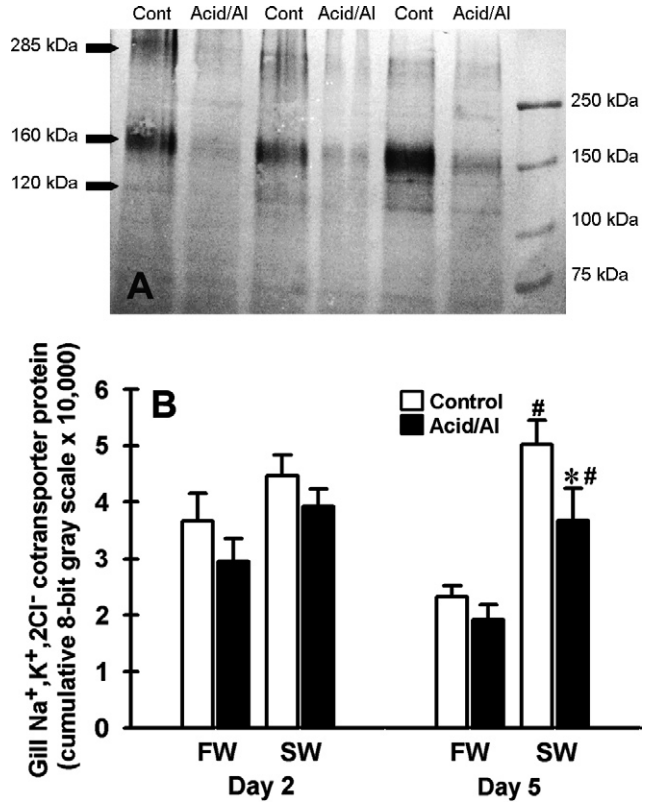

Fig. 4. Effects of short-term acid/Al exposure on gill NKCC protein abundance of Atlantic salmon smolts. Representative western blot of gill NKCC protein in control and treated smolts after 5 days of treatment and a subsequent 24 h SW challenge (SW) (A). The Western blot was probed with the T4 monoclonal primary antibody and three immunoreactive bands centered at ~285, 160 and 120 kDa were obtained. Gill NKCC protein abundance (cumulative 8-bit gray scale × 10,000) of control (white bars) and treated (black bars) smolts after 2 and 5 days (FW) and a subsequent 24 h SW challenge (SW) (B). Values are mean ± SE (n = 7–9). An (*) indicates a significant difference from control within a group (FW, SW) and a time-point (Duncan's; P < 0.05). An (#) indicates a significant difference between FW and SW within a treatment and a time-point (Duncan's; P < 0.05). Three-way ANOVA for gill NKCC protein abundance determined significant effects of treatment (P = 0.02), group (P < 0.001), time (P = 0.04), and a significant group/time interaction (P = 0.04).

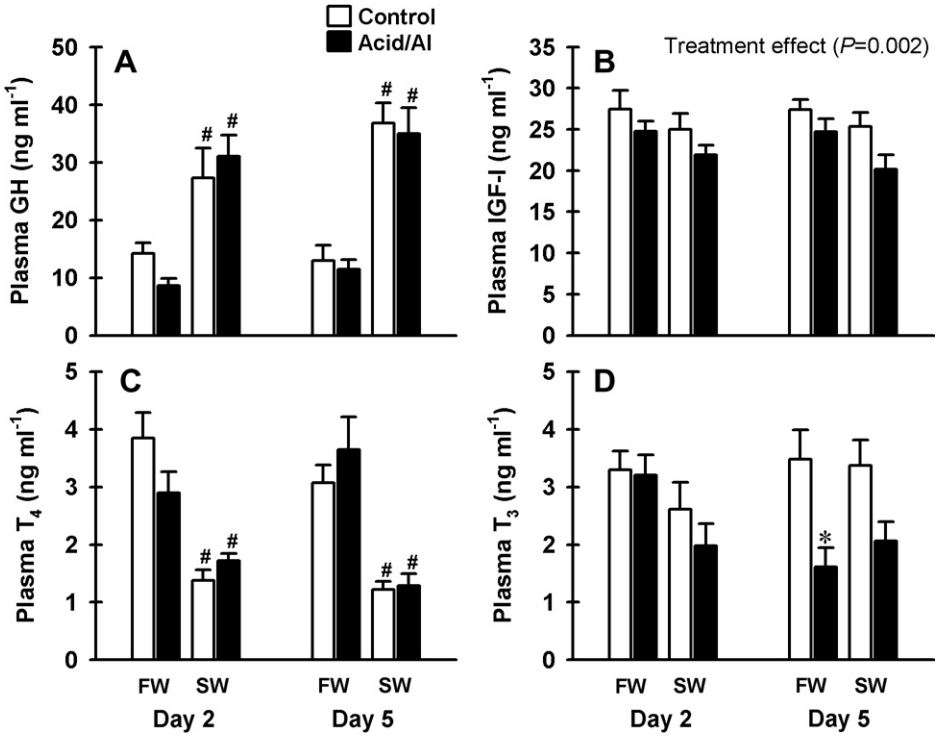

Fig. 5. Effects of short-term acid/Al exposure on plasma GH (A), IGF-I (B), T_4 (C) and T_3 (D) levels of control (white bars) and treated (black bars) smolts after 2 and 5 days (FW) and a subsequent 24 h SW challenge (SW). Values are mean ± SE (n = 8–10). An (*) indicates a significant difference between treatment and control within a group (FW, SW) and a time-point (Duncan's; P < 0.05). An (#) indicates a significant difference between FW and SW within a treatment and a time-point (Duncan's; P < 0.05). Three-way ANOVA for plasma GH levels determined a significant group effect (P < 0.001). Three-way ANOVA for plasma IGF-I levels determined significant effects of treatment (P = 0.002) and group (P = 0.049). Three-way ANOVA for plasma T_4 levels determined a significant group effect (P < 0.001). Three-way ANOVA for plasma T_3 levels determined a significant treatment effect (P = 0.002) and a significant group/time interaction (P = 0.04).

Plasma IGF-I levels ranged from 25 to 28 ng ml^{-1} and from 20 to 25 ng ml^{-1} in control and treated smolts, respectively. When the entire data set was analyzed, three-way ANOVA determined that exposure to acid/Al had a significant treatment effect on plasma IGF-I levels (P = 0.002). Despite no significant post-hoc comparisons, plasma IGF-I levels were reduced in all acid/Al exposed groups relative to their respective controls (Fig. 5B). The greatest reduction in plasma IGF-I was seen in smolts exposed to acid/Al for 5 days and subsequently transferred to SW for 24 h (P = 0.058 Fig. 5B).

Plasma T_4 levels ranged from 1.2 to 3.8 ng ml^{-1} and from 1.3 to 3.6 ng ml^{-1} in control and treated smolts, respectively. Acid/Al had no significant effect on plasma T_4 levels (Fig. 5C). Transfer to SW resulted in significant decreases (41% to 2.8-fold) in plasma T_4 levels in all groups (Fig. 5C).

Plasma T_3 levels ranged from 2.6 to 3.5 ng ml^{-1} and from 1.6 to 3.2 ng ml^{-1} in control and treated smolts, respectively. Exposure to acid/Al significantly reduced circulating levels of T_3 (Fig. 5D). After 5 days, plasma T_3 levels of treated smolts were significantly lower (2.2-fold) than controls in FW (Fig. 5D). Plasma T_3 levels of treated smolts also tended to be lower than controls after 24 h in SW (Fig. 5D).

After 2 days, hematocrit of treated smolts was significantly greater (17%) than controls in FW, but returned to control levels after a 24 h SW challenge (Fig. 5B). Acid/Al had no effect on hematocrit after 5 days (Fig. 6A). Transfer to SW resulted in significant decreases (14–23%) in hematocrit in all but one group (Fig. 6A).

Plasma cortisol levels ranged from 11 to 22 ng ml^{-1} and from 8 to 22 ng ml^{-1} in control and treated smolts, respectively. Acid/Al had no significant effect on plasma cortisol levels, however, transfer to SW tended to increase plasma cortisol levels in all groups (Fig. 6B).

Acid/Al exposure had a significant effect on plasma glucose levels (Fig. 6C). Plasma glucose levels of treated smolts were significantly greater (39% to 2.3-fold) than controls after both 2 and 5 days in FW (Fig. 6C). After both time-points of exposure, plasma glucose levels of treated smolts returned to control levels after 24 h in SW (Fig. 6C).

4. Discussion

The present study demonstrates that exposure to a single, short-term pulse of acid/Al compromises the development of SW tolerance in smolts as indicated by a loss in the ability to regulate plasma Cl^- levels during a 24 h SW challenge. Loss of SW tolerance is likely due in part to negative impacts on gill ion transport proteins, NKA and NKCC cotransporter. Previous studies, examining the effects of acid/Al exposure, have demonstrated disruption of SW tolerance in smolts, however, these studies also reported losses of plasma ions in FW and/or mortality (Staurnes et al., 1995, 1996; Kroglund and Staurnes, 1999; Magee et al., 2001, 2003). In this study, the disruption of SW tolerance by acid/Al exposure was observed in the absence of detectable effects on FW ion regulation, emphasizing the subtle impacts that short-term, sub-lethal exposure to acid/Al can have on the smolt hypoosmoregulatory system. This has important implications for the survival of Atlantic salmon smolts in nature, for impaired SW tolerance is associated with reduced growth performance and impaired predator avoidance (Jarvi, 1989; Handeland et al., 1996, 2004). It also supports the idea that during smolt development, environmental impacts during FW residence can have a great impact on survival in the marine environment.

Despite observed impacts of acid/Al on plasma Cl^- levels of smolts after 24 h in SW, plasma Na^+ levels were not affected by

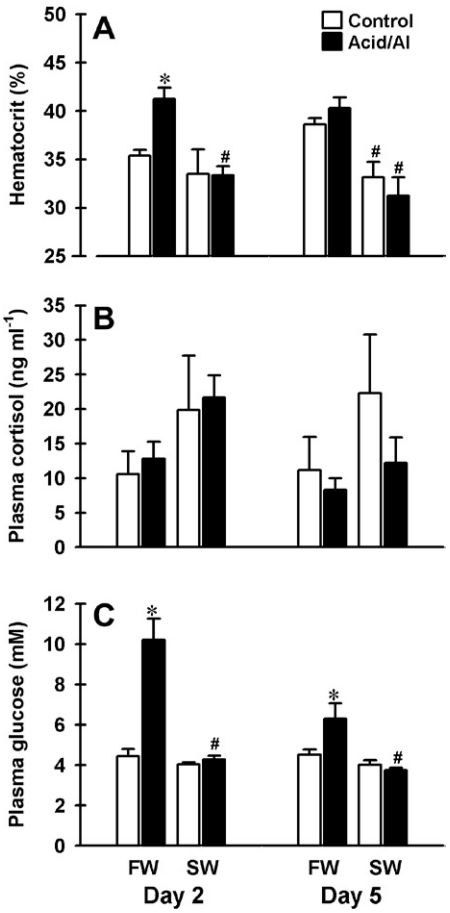

Fig. 6. Effects of short-term acid/Al exposure on the stress response of Atlantic salmon smolts. Hematocrit (A), plasma cortisol (B), and glucose levels (C) of control (white bars) and treated (black bars) smolts after 2 and 5 days (FW) and a subsequent 24 h SW challenge (SW). Values are mean ± SE (n = 8–10). An (*) indicates a significant difference from control within a group (FW, SW) and a time-point (Duncan's; P < 0.05). An (#) indicates a significant difference between FW and SW within a treatment and a time-point (Duncan's; P < 0.05). Three-way ANOVA for hematocrit determined a significant group effect (P < 0.001) and a significant treatment/group interaction (P = 0.002). Three-way ANOVA for plasma cortisol levels determined a significant group effect (P = 0.03). Three-way ANOVA for plasma glucose levels determined significant effects of treatment (P < 0.001) and group (P < 0.001), and significant treatment/group (P < 0.001) and treatment/time interactions (P = 0.01).

treatment. This has been previously observed in Atlantic salmon smolts and may be due to a greater increase in gill anion permeability relative to cation permeability under low Ca^{2+}, acidic conditions (Staurnes et al., 1993). Evidence also suggests that other metals such as copper, zinc and mercury can directly inhibit Cl^- secretion in fish (Crespo and Karnaky, 1983; Weber et al., 2006). Thus, it is possible that the mechanism of Cl^- secretion (active Cl^- secretion involving several ion transporters) is more sensitive to the toxic actions of acid/Al than the mechanism of Na^+ secretion which involves a paracellular transport pathway.

Short-term acid/Al exposure led to substantial reductions in gill NKA activity confirming previous findings in Atlantic salmon smolts (Staurnes et al., 1996; Magee et al., 2003). Greater plasma Cl^- levels of treated smolts after 24 h in SW are likely directly related to observed losses in gill NKA activity, as many studies have found a direct link between gill NKA activity and SW tolerance in smolts (see review by Folmar and Dickhoff, 1980). Interestingly, in the present study, FW ion regulation was not affected by reductions in gill NKA activity, suggesting that the ability to maintain ion homeostasis in SW is more sensitive to decreases in gill NKA activity. One possible cause of decreased gill NKA activity is the direct inhibition of enzyme activity by Al. In mammals, dietary Al directly inhibits NKA activity in the brain (Silva and Goncalves, 2003; Silva et al., 2005), and in fish, metals such as copper and zinc inhibit gill NKA activity *in vitro* (Crespo and Karnaky, 1983; Li et al., 1998).

Exposure to short-term acid/Al resulted in an overall reduction in gill NKCC protein abundance. This is the first study to report impacts of acid/Al on NKCC in any fish. In teleosts, basolaterally located NKA and NKCC work in concert with an apical Cl^- channel (cystic fibrosis transmembrane conductance regulator; CFTR) to drive Cl^- secretion (Evans et al., 2005). Gill NKCC protein abundance increases during the parr-smolt transformation and this has been linked to the development of SW tolerance (Pelis et al., 1999, 2001; Tipsmark et al., 2002). Therefore, it is likely that a reduction in gill NKCC protein abundance is contributing to the observed loss of SW tolerance during acid/Al exposure. Interestingly, the greatest impact of acid/Al on NKCC was seen after 5 days of exposure and subsequent transfer to SW (Fig. 4B). This suggests that acid/Al may also interfere with the upregulation of gill NKCC protein typically seen in Atlantic salmon after transfer to SW (Pelis et al., 2001; Tipsmark et al., 2002).

Reductions in both gill NKA activity and NKCC protein abundance suggest that exposure to short-term acid/Al may be altering the distribution of gill chloride cells, the location of these proteins in the gill epithelium. Impacts on gill NKA and NKCC may occur as a result of increased chloride cell death, as increased necrosis and apoptosis of gill chloride cells occurs during acid/Al exposure in other teleosts (Wendelaar Bonga et al., 1990; Verbost et al., 1995). Reductions in NKA activity and NKCC protein may also occur as a result of alterations in the type of chloride cells present in the gill epithelium. For instance, Wendelaar Bonga et al. (1990) found an overall increase in the number of chloride cells in the gills of tilapia exposed to low pH but the majority of these cells were immature or degenerating cells with poorly developed basolateral tubular systems. The authors suggested that these cells likely had low to no NKA activity and subsequent poor ion transporting capacity. It is possible that the appearance of immature chloride cells represents the upregulation of FW (ion uptake) chloride cells, and that this is a mechanism of acclimation to acid/Al.

This is the first study to examine the impacts of acid/Al on the GH–IGF-I system in salmonids, and we have demonstrated that acid/Al exposure decreases circulating levels of IGF-I in smolts. The role of the GH–IGF-I system in smolt development, specifically the acquisition of SW tolerance, is well established (McCormick, 2001). In Atlantic salmon, plasma levels of GH and IGF-I increase throughout the months of smolting in preparation for SW entry (Agustsson et al., 2001; McCormick et al., 2002). Also, treatment with exogenous GH and IGF-I increases SW tolerance of smolts (McCormick, 1996), which is thought to be mediated by an increase in gill NKA activity and number and size of gill chloride cells (Sakamoto et al., 1993). Similar to the present study, environmental impacts resulting in reductions in circulating IGF-I levels have been associated with impaired tolerance for SW (McCormick et al., 2005; Lerner et al., 2007).

Based on mammalian studies, evidence suggests that the majority of circulating IGF-I is derived from the liver and works through endocrine mechanisms (Sjogren et al., 1999, 2002). Therefore, it is possible that the reduced plasma IGF-I levels observed in this study resulted from decreased hepatic IGF-I production. However, recent evidence indicates that local production of IGF-I acting in an autocrine/paracrine manner is also important for normal growth and development (Yakar et al., 1999). In salmonids, branchial expression of IGF-I mRNA increases during SW acclimation (Sakamoto and Hirano, 1993) and the parr-smolt transformation (Sakamoto et al., 1995), suggesting a role of local IGF-I production in osmoregulation. Thus, declines in branchial IGF-I production may have also

played a role in reduced plasma IGF-I given that the fish gill is the major organ of acid/Al toxicity. However, it is not clear if branchial IGF-I production contributes to circulating levels of this hormone in fish. Together, these data suggest that loss of SW tolerance in smolts during acid/Al exposure may be mediated, in part, by negative impacts on the GH–IGF-I system.

Exposure of smolts to acid/Al for 5 days resulted in a significant reduction in circulating T_3, but not T_4, levels. Previous data on the effects of acid/Al on the thyroid axis in salmonids are inconsistent and appear to be dependent on species, developmental stage, and time-course of exposure (Brown et al., 1984, 1986, 1990a, 1990b; Waring et al., 1996; Waring and Brown, 1997). Acid/Al exposure lowers hepatic T_4 5′-monodeiondinase activity in rainbow trout, by decreasing both the affinity of the enzyme for its T_4 substrate, and the amount of functional enzyme present in the liver (Brown et al., 1990b). Thus, it is possible that lower plasma T_3 levels during acid/Al exposure in the present study are the result of decreased hepatic T_4 to T_3 conversion. Such disruption of thyroid endocrinology may not only be of consequence for physiological smolt development, but also for downstream migratory and SW preference behaviors (Iwata, 1995; Lerner et al., 2007), adding to the detrimental effects of acid/Al exposure on salmon populations.

The present study clearly demonstrates negative impacts of acid/Al on the GH–IGF-I and thyroid systems in smolts; however, the mechanisms of acid/Al action remain to be determined. Reductions in circulating IGF-I and T_3 may occur in response to the down-regulation of salt secretory mechanisms (including NKA activity and NKCC cotransporter protein abundance) during acid/Al exposure in FW. This is likely to occur as smolts switch to upregulating ion uptake mechanisms in order to acclimate to acid/Al conditions. It is also possible that effects on these hormones are the result of specific toxic actions of acid and/or Al. In mammals, ingestion of Al results in decreased plasma IGF-I levels and increased levels of IGF-I binding proteins. However, these effects are at least partially explained by a reduction in food intake (Capdevielle and Scanes, 1995). In fish, others metals such as cadmium and mercury decrease IGF-I mRNA expression in scale bone cells, demonstrating an effect on non-hepatic IGF-I production (Suzuki et al., 2004). Acid/Al impacts on the GH–IGF-I system may also be due to a general stress response, as both long-term and acute stressors can alter plasma GH and IGF-I levels in salmonids (Pickering et al., 1991; Farbridge and Leatherland, 1992; McCormick et al., 1998b).

In the present study, increased hematocrit during acid/Al exposure may result from both increased spleen production of red blood cells and osmotic disturbance leading to red blood cell swelling (Wendelaar Bonga, 1997). The observed hyperglycemia is likely a component of the general stress response mediated by the stimulatory action of catecholamines on hepatic glucose release. This is supported by findings that plasma epinephrine and norepinephrine levels increase 10-fold in rainbow trout exposed to elevated Al (Witters et al., 1991). Interestingly, the effects of acid/Al on both hematocrit and plasma glucose do not persist after a 24 h exposure to SW. Decreased hematocrit in treated smolts is likely due to reduced plasma volume and/or red blood cell shrinking resulting from the osmotic challenge of SW transfer, while return of plasma glucose to control levels may be a result of increased energetic demand from osmo/iono-regulatory mechanisms.

Despite observed elevations in plasma glucose levels of treated smolts in FW, elevations in plasma cortisol levels were not seen in these groups, however, we cannot rule out that plasma cortisol has played a role in the hyperglycemia response. It is possible that cortisol was transiently elevated upon initial exposure to acid/Al, but returned to normal levels prior to the first sampling (2 days). This is supported by Carey and McCormick (1998) who demonstrated that plasma cortisol levels of Atlantic salmon smolts increase after an acute handling stress, but return to normal levels within 8 h. Alternatively longer exposures may be necessary for the observation of a cortisol response. In rainbow trout, elevation of plasma cortisol levels were observed only after 7–8 days of exposure to acid/Al (Brown et al., 1984, 1990b).

In all groups, transfer to SW increased plasma cortisol and GH levels, and decreased plasma T_4 levels supporting the roles and interactions of all three hormone systems in SW acclimation (Björnsson et al., 2002). Similar transitory changes resulting from SW transfer have been reported for these hormones in Atlantic salmon (Björnsson, 1997; Björnsson et al., 1998) and coho salmon (Young et al., 1995). However, it is not clear whether transitory responses in these hormones following SW transfer are involved in hypoosmoregulation and/or reflect temporary changes in hormone secretion and clearance rates (Björnsson, 1997; Björnsson et al., 2002).

In conclusion, the present study demonstrates that exposure to a single, short-term, sub-lethal pulse of acid/Al compromises smolt development by disrupting SW tolerance in the absence of detectable effects on FW ion regulation. In addition, loss of SW tolerance can be explained by decreases in gill NKA activity, NKCC protein abundance, and plasma levels of IGF-I, and T_3. We propose that in response to short-term acid/Al, smolts attempt to upregulate ion uptake mechanisms while still in FW as part of a damage/repair and/or acclimation process, and that this comes as a direct cost to smolt development, specifically the acquisition of SW tolerance. This has many implications for natural salmon populations, as smolts with compromised SW tolerance may experience decreased survival upon seawater entry due to direct mortality and increased susceptibility to predation (Jarvi, 1989; Handeland et al., 1996). Understanding these impacts in natural systems experiencing episodic acidification is critical, as sub-lethal acid/Al exposure of smolts may play a role in decreased ocean survival and reduced populations of Atlantic salmon.

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