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Thresholds for short-term acid and aluminum impacts on Atlantic salmon smolts

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ABSTRACT

Although the negative effects of acid and aluminum (Al) on smolt development have been known for some time, the thresholds for impact of short-term exposure of several days that may occur during episodic acidification have not been systematically examined. In order to determine the levels of acid and Al that impact juvenile Atlantic salmon, smolts and yolk-sac larvae were exposed to three pH levels (6.0, 5.7, and 5.3) and four added Al levels (0, 40, 80 and 175 μg/L total Al) for 48 h. Following this treatment, 10 smolts were sampled in freshwater and another 10 were subjected to a 24 h seawater challenge (35 ppt). Survival of yolk-sac larvae was >96% in all acid and Al treatments. All smolts died within 48 h at pH 5.3, 175 μg L^{-1} Al. There were some mortalities in freshwater at pH 5.3, 80 μg L^{-1} Al and pH 5.7, 175 μg L^{-1} Al, and further mortalities when these fish were transferred to seawater. Mortalities in these groups were associated with decreased plasma chloride in freshwater and higher plasma chloride in seawater, indicating that these smolts had lost seawater tolerance. Gill Na^+/K^+-ATPase (NKA) activity decreased at pH 5.7, 175 μg L^{-1} Al in freshwater, and further decreases were observed at more moderate pH and Al exposures after transfer to seawater. Hematocrit and plasma glucose were the most sensitive physiological responses, increasing at all Al treatments at pH 5.7 and 5.3 in freshwater. There was no detectable increase in gill Al levels at pH 6.0 with added Al, whereas substantial increases in gill Al were observed in all added Al groups at pH 5.7 and 5.3. Our results demonstrate a critical interaction between acid and Al in their effects on smolts, and that episodic acidification events will negatively impact smolt survival in freshwater and after seawater entry.

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1. Introduction

Acid rain is the result of atmospheric deposition of sulfates and nitrates that are primarily generated from coal-fired power plants. It can have a number of negative impacts on aquatic environments, including increased water acidity, decreased buffering capacity of surrounding terrestrial and aquatic environments, and increased mobilization of aluminum (Al) (Driscoll et al., 2001). As pH decreases, Al becomes both more soluble and more toxic, indicating an important interaction in determining their biological effects. When pH drops below 6.0, aluminum becomes charged and more soluble, resulting in increased levels of inorganic aluminum (Al_i), a form that is toxic to aquatic organisms (Gensemer and Playle 1999). The presence and magnitude of these negative impacts are dependent on the surrounding geology and soil; areas with relatively low initial buffering capacity will suffer greater impacts of acidification than areas with greater buffering capacity. In these poorly buffered areas, acid rain can result in chronic acidification in which pH drops below 6.0 for many months at a time. In areas with low to moderate buffering, pH may drop below 6.0 for a few days or more following rain events or snow melt, a phenomenon known as episodic acidification.

Acid rain can have a variety of effects on aquatic ecosystems. Overall productivity, biodiversity and impacts on specific species and life stages can result in both 'bottom up' and 'top down' impacts (Gensemer and Playle, 1999). In fish, the major site of acid and Al toxicity is the gill, with effects on both osmoregulation and respiration (Gensemer and Playle, 1999). Different species and life stages have varying sensitivities to acid and Al impacts. It has been known for some time that smolts are the most sensitive life stage of Atlantic salmon to the impact of acid and Al (Lacroix and Townsend, 1987; Staurnes et al., 1993), and that smolts suffer physiological impacts and mortality at levels of pH and Al that have relatively little impact on other life stages. In addition, under conditions of chronic acid exposure, smolts will fail to develop (or lose) salinity tolerance. In Norway and eastern Nova Scotia where chronic acidification is known to be responsible for extirpations of Atlantic salmon (Clair and Hindar, 2005), it is thought that the major impact of acidification is impaired smolt development causing mortalities once fish enter seawater (Kroglund et al., 2007).

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Most of the research on the impacts of acid and Al on smolt development have been through long-term studies (weeks to months) seeking to understand the impact of chronic acidification. However, a systematic study of the levels of acid and Al that affect smolts under short-term conditions has not been conducted. In eastern Maine where populations of Atlantic salmon have been declining since the 1970's, episodic acidification has been implicated as a contributing factor to their initial and continuing decline (Clegg et al., 2004). Multiple pulses of low pH and elevated Al levels simulating several episodic acidification events resulted in losses of plasma ions in freshwater and mortality after transfer to seawater (Magee et al., 2003). Cage studies conducted in Norway and eastern North America indicate that exposure to low pH and Al conditions can result in mortality within 0.5 to 3 days (McCormick et al., 2009a; Staurnes et al., 1993). Under controlled laboratory conditions compromised salinity tolerance has been detected in smolts exposed to acid and Al conditions for as little as two days (Monette et al., 2008). The present study was designed to determine the thresholds of short-term (2 day) acid and Al exposures on survival and seawater tolerance of Atlantic salmon smolts, and examine a range of physiological parameters that may be influenced by episodic acidification.

2. Material and methods

2.1. Fish and experimental design

Atlantic salmon (*Salmo salar*) parr from the Connecticut River strain were obtained from the Kensington National Fish Hatchery (Kensington, CT, USA), and held at the USGS, Conte Anadromous Fish Research Center (Turners Falls, MA, USA). Prior to the start of the study, 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^{+}, 1.1 mg L^{-1}; Cl^{-}, 11.0 mg L^{-1}, pH 6.8–7.8), maintained under natural photoperiod conditions and ambient river temperatures, and fed to satiation twice daily with commercial feed (Zeigler Bros., Garners, PA, USA). Fertilized eggs from the same Atlantic salmon strain were obtained from White River National Fish Hatchery (Bethel, VT, USA). Eggs were also maintained in Connecticut River water under flow through conditions and a natural photoperiod regime through hatching.

Laboratory experiments were conducted with one-year-old juvenile Atlantic salmon (26–59 g, fork length ≥ 14 cm) that all displayed silvering, darkened fin margins and reduced condition factor characteristic of smolts. All exposures were conducted between April 26 and May 15 which is the normal peak of smolt development under the rearing conditions described above. In addition, 20 Atlantic salmon yolk-sac larvae were placed in screened chambers within each of the smolt treatment tanks for assessment of survival. Smolts were randomly assigned to treatment tanks (n = 20) at pH 6.0, 5.7 and 5.3. Each pH group received either no added Al, or the nominal addition of 40, 80, or 175 μg L^{-1}. Several groups were replicated (see Table 1), though limitation in tank numbers prevented us from replicating all groups. Acid/Al concentrations were chosen to be comparable to those observed in poorly buffered Atlantic salmon streams in New England. Experimental tanks were 1 m in diameter, contained 134 L volume and from a header tank received artificial soft water prepared by mixing deionized water (prepared by passing tap water through an ion exchange resin; Siemens, Lowell, MA, USA) with Connecticut River (Turners Falls, MA, USA) in a 5:1 ratio. Target pH and Al concentrations were achieved in header tanks (1400 L) 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+}) similar in magnitude to that which occurs following episodic rain events in low to moderately buffered streams (Haines et al., 1990). Target levels for both calcium and sodium were maintained at 1.5–2.0 mg L^{-1} throughout the study. Experimental water was mixed for at least 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 12.5 °C +/− 1 °C using a re-circulating chiller system. Both header and experimental tanks were aerated, continuously maintaining dissolved oxygen at >90% saturation. 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 smolts were starved for the duration of the experiment. Just before the start of the study, ten smolts were sampled directly from their rearing tanks (T_0). Smolts were exposed to acid and Al treatments for 48 h after which 10 fish per tank were sampled directly from the exposure tank and the remaining 10 fish per tank were subjected to a seawater challenge. Seawater challenge fish were placed directly into 1.2 m diameter, 330 L tanks containing 35‰ SW (charcoal-treated, aerated) maintained at 12 °C and sampled after 24 h. Plasma Cl^{-} concentrations after 24 h in 35‰ SW were used as indicators of SW tolerance (Clarke and Blackburn, 1977).

Table 1
pH, total dissolved aluminum (Al_{tot}) and inorganic aluminum (Al_i) for treatment groups. pH values are the means of at least five values and aluminum the means of two values taken over the two day exposure period. Treatment groups with more than one row were those that were replicated. nd = not detectable (<5 μg L^{-1}).

Nominal pH	Added Al	pH	Al_{tot} (μg L^{-1})	Al_i (μg L^{-1})
6.0	0	6.06	25	nd
	0	6.03	17	nd
	40	5.95	52	23
	80	6.00	108	52
	175	6.05	169	51
5.7	0	5.73	30	nd
	0	5.67	25	nd
	0	5.73	16	nd
	40	5.72	52	32
	80	5.68	106	67
	80	5.80	87	37
	175	5.76	157	103
5.3	0	5.30	32	10
	0	5.26	44	nd
	40	5.39	64	41
	80	5.43	110	88
	175	5.22	184	140

2.2. Laboratory analyses

Smolts were anesthetized with MS-222 (100 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. All fish were sampled within six minutes of tank disturbance. Blood was collected from the caudal vasculature in heparinized 1 mL syringes and centrifuged at 3200 gravity *(g)* for 5 min at 4 °C. Plasma was removed and stored at −80 °C for later analyses. One hematocrit tube of blood was collected from each sample and centrifuged in an IEC MB microhematocrit centrifuge (Thermo Scientific, Waltham, MA, USA) for 5 min and hematocrit was determined. Gill biopsies (4–6 primary filaments) for the measurement of Al accumulation were taken and analyzed as previously described (Monette and McCormick, 2008b). Gill biopsies were also taken for the measurement of Na^{+}/K^{+}-ATPase activity and 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.

Na^{+}/K^{+}-ATPase activity was determined with a kinetic assay run in 96-well microplates at 25 °C and read at a wavelength of 340 nm for

10 min as described in (McCormick, 1993). Gill tissue was homogenized in 125 μL of SEID (SEI buffer and 0.1% deoxycholic acid) and centrifuged at 5000×*g* for 30 s. Two sets of duplicate 10 μL samples were run, one set containing assay mixture and the other assay mixture and 0.5 mM ouabain. The resulting ouabain-sensitive ATPase activity is expressed as μmoles ADP/mg protein/h. Protein concentrations are determined using BCA (bicinchoninic acid) Protein Assay (Pierce, Rockford, Il, USA). Both assays were run on a THERMOmax microplate reader using SOFTmax software (Molecular Devices, Menlo Park, CA, USA).

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 average intra-assay variation was 5.5% (n = 10) and the average inter-assay variation was 8.8% (n = 10).

Plasma chloride was analyzed by the silver titration method using a Buchler-Cotlove digital chloridometer (LABCONCO, Kansas City, MO, USA) and external standards. Plasma glucose was measured by the hexokinase and glucose-6-phosphate dehydrogenase enzymatic method (Stein, 1963).

Gill Al accumulation was analyzed by modification of the protocol described by 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). For acid digestions, 98 μL of 100% trace metal grade HNO_3 and 2 μL of H_2O_2 were added to tubes with biopsies and heated 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 (9:1) by the addition of 900 μL of deionized water, and Al concentration was analyzed using graphite furnace (HGA-800/AAnalyst 100, Perkin Elmer, Wellesley, MA, USA) atomic absorption spectrophotometry (GFAAS). Water samples were read in replicates of two, and calibration was checked every ten samples with a reference standard. 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 weight. In separate validation experiments, the values from biopsies were not different from those detected in whole gill arches (Monette, unpublished results).

Water samples were taken twice daily for each trial. 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% trace metal grade HNO_3) and Al concentration was measured using GFAAS as described above.

Labile or inorganic Al (Al_i) was determined by passing a water sample, via peristaltic pump, through a strong acid cation-exchange column immediately upon collection (Amberlite 120, prepared with Na^+) as described by Driscoll (1984). Column processed samples were then acidified (0.2% trace metal grade HNO_3) immediately, and analyzed for Al as described above. This Al fraction was called organically bound Al. Inorganic Al was determined by calculating the difference between dissolved and organic Al fractions. Calcium and sodium were measured by flame atomic absorption spectrophotometry (AAnalyst 100, Perkin Elmer, Wellesley, MA, USA).

2.3. Data analyses

Our original design was a complete 3×4 (pH×added Al), but the loss of all fish in the pH 5.3, 175 added Al group precluded an analysis of all remaining groups by two-way analysis of variance (ANOVA). We therefore used a two-way ANOVA for analysis of the effect of three pH treatments and added Al of 0, 40 and 80 on individual fish. If a significant effect of pH, added Al or an interaction was found, we used a one-way ANOVA followed by Dunnett's test to compare all groups to the reference group (pH 6.0, no added Al). We compared the ability of pH and Al_i to explain physiological responses using factorial general linear regression models of pH, Al_i or the two together on physiological parameters in Atlantic salmon smolts. Measured values of pH and Al_i in each of seventeen tanks were regressed on mean values of physiological parameters measured from all surviving fish in those tanks. There was no significant correlation between pH and Al_i ($p = 0.26$). All statistics were run using the Statistica (StatSoft Inc., Tulsa, OK, USA) software package.

3. Results

3.1. Water chemistry

As expected, there was a detectable amount of Al in the water sample with no added Al due to its natural presence in river water (Table 1). Increased added Al resulted in higher levels of total Al that were near the target levels and independent of pH. It was clear, however, that levels of inorganic Al were not independent of pH. At pH 6.0, increased added Al up to 80 μg L^{-1} resulted in slight increases in Al_i, but further increases in total Al did not increase Al_i. At any given level of added Al, Al_i increased with decreasing pH.

3.2. Impacts on smolts in freshwater

There were no mortalities in the pH 6.0 groups over the 2 d freshwater exposure period (Fig. 1). All fish died in the most severe acid Al treatment of pH 5.3, 175 μg L^{-1} added Al. There was 20% mortality in the pH 5.3, 80 Al group, 5% in the pH 5.7, 175 Al group, and no mortalities in the remaining pH 5.7 and 5.3 groups. There were no mortalities of yolk-sac larvae over the 2 day exposure period in any group.

Gill Al levels at time 0, in all pH 6.0 groups, and the pH 5.7 and 5.3 with no added Al were all low and similar, with mean values below 40 μg g^{-1} (Fig. 1). All of the added Al groups at pH 5.7 and 5.3 had significantly elevated values of between 250 and 700 μg g^{-1}. There was a general trend for increasing gill Al with increasing added Al at pH 5.7 and 5.3, although the levels of gill Al were lower at 175 than at 80 Al at pH 5.7. There were significant effects of pH, Al, and a significant interaction on gill Al (two-way ANOVA, $p<0.0001$).

Plasma chloride in freshwater did not differ among the time 0 and pH 6.0 groups (Fig. 2). There was a general trend for plasma chloride to decrease with decreasing pH with 0 added Al, but these differences were not statistically significantly. Significant losses of plasma chloride were seen in the pH 5.7, 80 and 175 Al groups, and the pH 5.3, 80 Al group. There was a significant effect of pH, Al, and a significant interaction on plasma chloride (two-way ANOVA, $p<0.0001$).

Gill Na^+/K^+-ATPase activity in freshwater did not differ among the time 0 and pH 6.0 groups (Fig. 3), though there was substantial variation both within and among groups. There was a general trend for gill Na^+/K^+-ATPase activity to decrease with increasing added Al in the pH 5.7 and 5.3 groups, but the only significant difference was in the pH 5.7, 175 Al group. There was a significant effect of pH ($p = 0.008$), but not Al nor a significant interaction on gill Na^+/K^+-ATPase activity (two-way ANOVA, $p>0.14$).

Hematocrit in freshwater did not differ among the time 0 and pH 6.0 groups (Fig. 4). There was a general trend for hematocrit to increase with decreasing pH with 0 added Al, with a significant increase at pH 5.3. There were significant increases in hematocrit at all added Al groups (40, 80 and 175) at pH 5.7 and 5.3, with hematocrit values increasing with increased added Al in each group. There was a significant effect of pH, Al, and a significant interaction on hematocrit (two-way ANOVA, $p<0.0001$).

Plasma cortisol in freshwater did not significantly differ among the time 0 and pH 6.0 groups (Fig. 5), although there was substantial variability both within and among groups. Plasma cortisol was

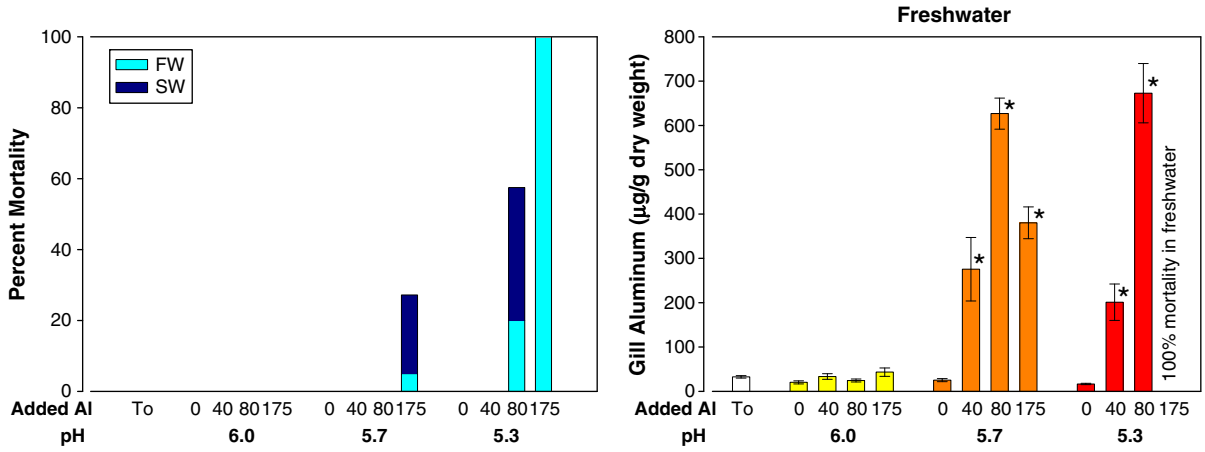

Fig. 1. Mortality and gill Al in Atlantic salmon smolts held for 2 d in freshwater under varying pH and added Al conditions. Values are mean ± standard error. Asterisk indicates significant difference from the reference group (pH 6.0, 0 µg L^{-1} added Al; $p<0.05$, Dunnett's test).

significantly higher than the reference group at 80 Al at both pH 5.7 and 5.3. Plasma glucose also did not differ among the time 0 and pH 6.0 groups (Fig. 5). There was a trend for plasma glucose in freshwater to increase with decreasing pH with no added Al, but there was no statistically significant difference among the 0 added Al groups. Plasma glucose increased with increasing added Al at pH 5.7 and 5.3. There was a significant effect of pH, Al and a significant interaction on plasma glucose (two-way ANOVA, $p<0.0001$).

Results of factorial general linear regression modeling of the effects of pH and Al_i and the two factors together on physiological parameters are shown in Table 2. In all cases there was no significant effect of pH alone on physiological responses, whereas Al_i has significant explanatory power in most cases. Although pH had relatively little explanatory power on its own, there was a substantial increase in total explanatory power (up to 0.31 increase in r^2) when pH was included with Al_i.

3.3. Impacts on smolts after seawater challenge

There were mortalities after 24 h in seawater in the pH 5.7, 175 Al and pH 5.3, 80 Al, but no mortalities in any of the other groups (Fig. 1). Plasma chloride of the reference group after 24 h in seawater was 144 mM, only slightly higher than when the fish were sampled directly from freshwater, indicative of a high level of salinity tolerance characteristic of smolts. There was no significant difference in plasma chloride after seawater challenge with increasing added Al at pH 6.0 (Fig. 2). At pH 5.7 and 5.3 plasma chloride after seawater challenge generally increased with increasing added Al, with significant differences at pH 5.7 and 175 Al and pH 5.3 and 80 Al. There was a significant effect of pH ($p=0.011$), Al ($p=0.020$) but no significant interaction ($p=0.22$) on plasma chloride after seawater challenge (two-way ANOVA).

Gill Na^+/K^+-ATPase activity was not significantly different among the pH 6.0 groups (Fig. 3), though there was substantial variation both within and among groups. There was a general trend for gill Na^+/K^+-ATPase activity to decrease with increasing added Al in the pH 5.7 and 5.3 groups, but the only significant difference was in the pH 5.3, 80 Al group. Gill Na^+/K^+-ATPase activity was generally lower after 24 h in seawater than in fish sampled directly from freshwater, and this was especially apparent with decreasing pH and increasing added Al. There was a significant effect of pH ($p=0.0003$), Al ($p=0.0066$) and a significant interaction ($p=0.0019$) on gill Na^+/K^+-ATPase activity after seawater challenge (two-way ANOVA).

Hematocrit after seawater challenge was not significantly different among the pH 6.0 groups, nor was there a trend for an effect of low pH exposure as there was in freshwater (Fig. 4). The only significant difference in hematocrit after exposure to seawater was in the pH 5.3, 80 Al group. There was a significant effect of pH ($p=0.020$), Al

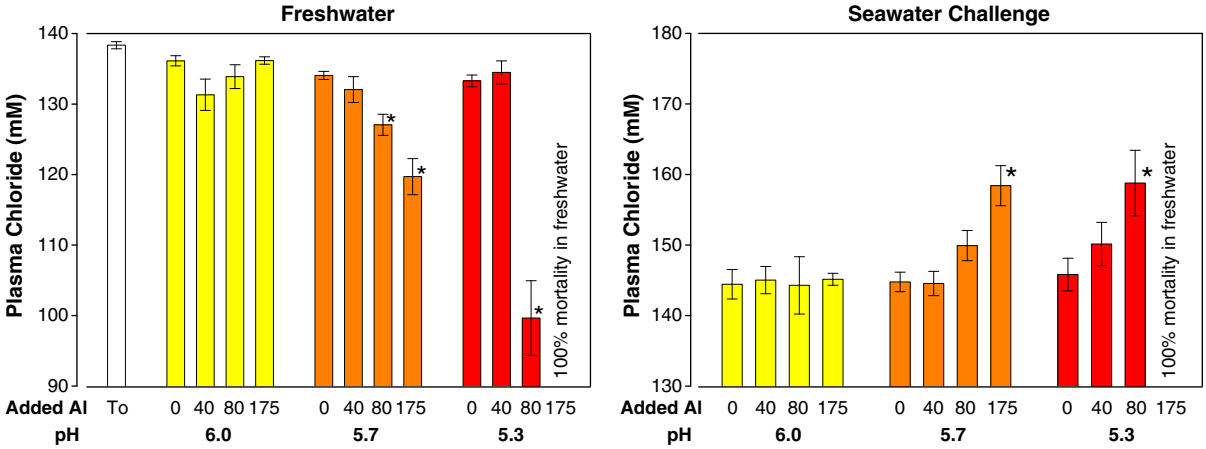

Fig. 2. Plasma chloride (mM) in Atlantic salmon smolts held for 2 d in freshwater (left panel) under varying pH and added Al conditions and after a 24 h seawater challenge (right panel). Values are mean ± standard error. Asterisk indicates significant difference from the reference group (pH 6.0, 0 µg L^{-1} added Al; $p<0.05$, Dunnett's test).

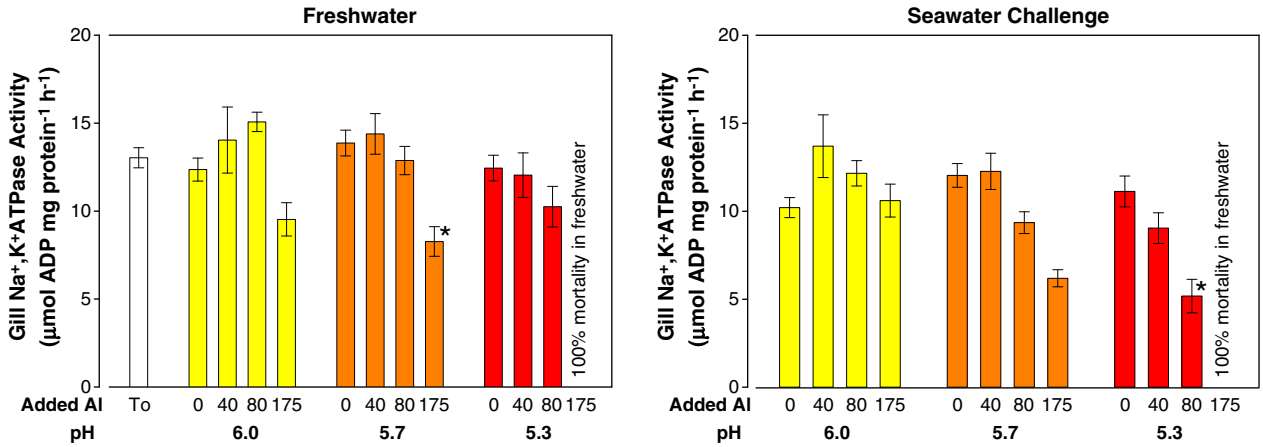

Fig. 3. Gill Na^+/K^+-ATPase activity (µmol ADP mg protein h^{-1}) in Atlantic salmon smolts held for 2 d in freshwater (left panel) under varying pH and added Al conditions and after a 24 h seawater challenge (right panel). Values are mean ± standard error. Asterisk indicates significant difference from the reference group (pH 6.0, 0 µg L^{-1} Al; $p<0.05$, Dunnett's test).

($p=0.0005$) and a significant interaction ($p=0.00015$) on plasma chloride (two-way ANOVA).

4. Discussion

The results of the present study indicate that substantial physiological impacts and even mortality can occur after short-term (2 d) exposure to acid and Al conditions. Our results are consistent with other studies that have found relatively rapid impacts (Monette and McCormick, 2008a; Staurnes et al., 1996) on Atlantic salmon smolts. In addition, we demonstrate the critical interaction of acid and Al, and provide information on the thresholds for episodic exposure to pH and inorganic Al that result in mortality, loss of salinity tolerance and physiological impacts.

In the present study, all smolts died at pH 5.3, 175 Al, and in the slightly less severe conditions of pH 5.3, 80 Al and pH 5.7, 175 Al there was 5–20% mortality and significant loss of plasma ions. Reduction of plasma chloride to 90–100 mM was associated with mortality in this and other studies on Atlantic salmon smolts (Magee et al., 2003; McCormick et al., 2009a; Monette and McCormick, 2008a; Staurnes et al., 1993), and may represent a lower limit for survival. We found that yolk-sac larvae under the same acid and Al conditions as smolts experienced no mortality. The heightened sensitivity of smolts to acid and Al exposure has been observed in previous studies (Lacroix and Townsend, 1987; Monette and McCormick, 2008a; Rosseland et al., 2001), and likely relates to the osmoregulatory changes associated with the development of seawater tolerance that occurs during smolt development. The mechanism of this increased sensitivity in smolts, however, has not been determined. Loss of plasma ions appears to be the major toxic action of Al in fish, and may involve both increased ion efflux through disruption of passive barriers and loss of influx due to interference with active transport mechanisms (Gensemer and Playle, 1999). Parr actually accumulate more gill Al than smolts under the same acid and Al exposure (Monette and McCormick, 2008a), indicating that the altered uptake kinetics is not likely to be the source of greater smolt sensitivity. It has been observed that both ion efflux and influx increase during smolting (Primmett et al., 1988), which appears to result in greater ion losses in smolts in response to a variety of external stressors (McCormick et al., 2009b).

Based on mortality and elevated plasma chloride after seawater challenge, exposure of smolts to low pH and elevated Al for as little as 2 d results in loss of salinity tolerance. These results are consistent with previous studies that have also found loss of salinity tolerance after short term (12–48 h) exposure to acid and Al conditions (McCormick et al., 2009a; Staurnes et al., 1996). Our results demonstrate statistically significant loss of salinity tolerance when pH is 5.7 or less and inorganic Al is >80 µg L^{-1}. These values represent the threshold for 2 d exposure, and smolts will undoubtedly show increased sensitivity with increasing time. When exposed to pH 5.3 and 11 or 43 µg L^{-1} Al_i, Atlantic salmon did not show loss of salinity

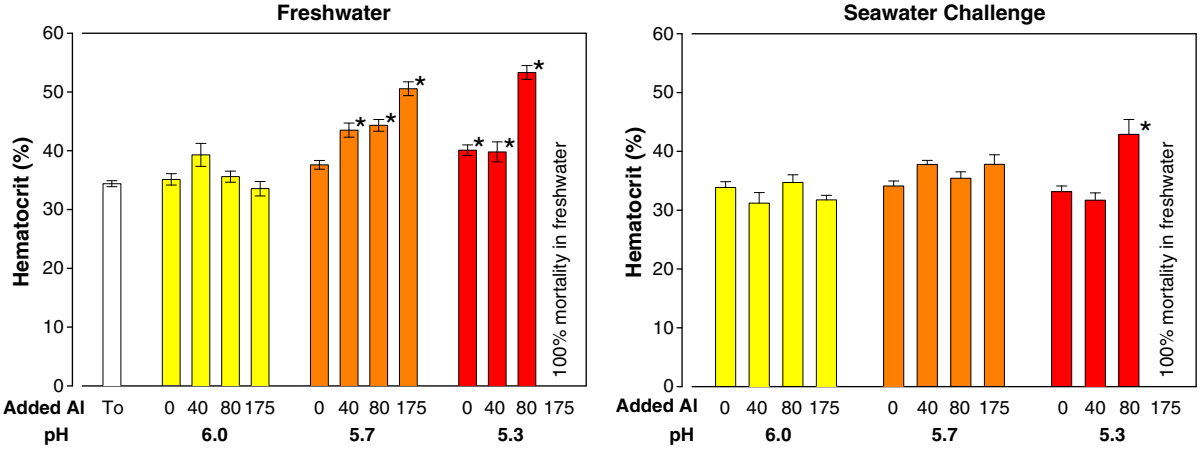

Fig. 4. Hematocrit (% volume red blood cells) in Atlantic salmon smolts held for 2 d in freshwater (left panel) under varying pH and added Al conditions and after a 24 h seawater challenge (right panel). Values are mean ± standard error. Asterisk indicates significant difference from the reference group (pH 6.0, 0 µg L^{-} Al; $p<0.05$, Dunnett's test).

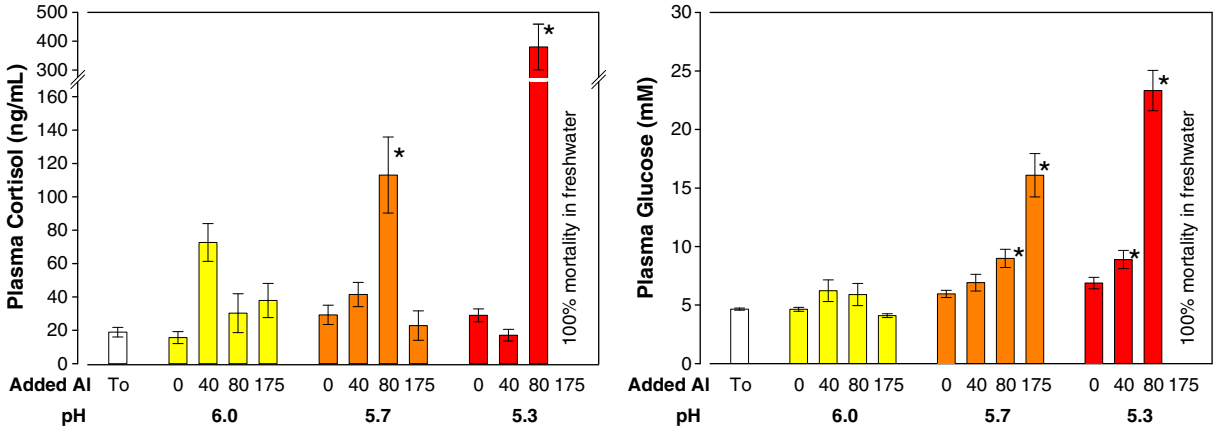

Fig. 5. Plasma cortisol (ng mL^{-1}) (left panel) and glucose (mM) (right panel) in Atlantic salmon smolts held for 2 d in freshwater under varying pH and added Al conditions. Values are mean ± standard error. Asterisk indicates significant difference from the reference group (pH 6.0, 0 μg L^{-1} added Al; $p<0.05$, Dunnett's test).

tolerance after 2 days, but the same doses after 6 days resulted in significant loss of salinity tolerance (Monette et al., submitted for publication). Based on this observation and the strongly elevated gill Al observed at all added Al levels at pH 5.3 and 5.7, we predict that when pH is below 6.0 and inorganic Al above 30 μg L^{-1} for more than 2 days, Atlantic salmon smolts will lose salinity tolerance. These thresholds for impact are similar to those observed for Atlantic salmon smolts in Norway exposed to a wide variety of acid and Al conditions for up to 14 days (Kroglund et al., 2008). The absence of pH effects at 5.3 or 5.7 with no added aluminum are consistent with previous studies that found no long-term effect of exposure to acid only (pH 5.4–5.9 with Ali 'removed' through addition of citrate) on salinity tolerance of Atlantic salmon smolts (Fivelstad et al. 2004).

In the present study gill Na^+/K^+-ATPase activity was not a strong predictor of changes in osmoregulatory status. For instance, there were no significant changes at pH 5.3, 80 μg L^{-1} added Al even though plasma chloride levels in freshwater and after seawater challenge were significantly compromised under these conditions. This lack of correspondence is somewhat surprising given the strong relationship between gill Na^+/K^+-ATPase activity and salinity tolerance for smolting in general, and in particular for previous studies that have examined the effect of long-term exposure to acid and Al on smolt development and salinity tolerance (Kroglund et al., 2007; Saunders et al., 1983; Staurnes et al., 1993). One possible explanation is that the relatively high variability in gill Na^+/K^+-ATPase activity among individuals (relative to other physiological variables) makes it more difficult to detect changes, especially in the short-term when the magnitude of change is small relative to long-term exposures. Recent molecular evidence suggests that there are multiple isoforms of Na^+/K^+-ATPase expressed in the gill, including two forms of the catalytic alpha subunit that are up-regulated in freshwater and seawater, respectively (Madsen et al., 2009; Nilsen et al., 2007; Richards et al., 2003). We have recently found that the abundance of the Na^+/K^+-ATPase α1a is greater in freshwater and that the abundance of Na^+/K^+-ATPase α1b is greater in seawater (McCormick et al., submitted). Furthermore, the abundance of both subunits is greater in smolts than in parr (McCormick, unpublished results). It is likely that the current measurement of Na^+/K^+-ATPase activity measures both of these isoforms. If these isoforms are differentially affected by acid and Al exposure (a possibility we are currently examining), then activity measurements in freshwater may be limited in their ability to detect physiologically relevant impacts. It is of interest to note that the levels of gill Na^+/K^+-ATPase activity went down in all groups after seawater exposure, but decreased to a greater degree in fish that had previously been exposed to acid and Al. Thus, there was a greater effect of prior acid and Al exposure and greater correspondence with salinity tolerance of gill Na^+/K^+-ATPase activity after 24 h in seawater compared to that in freshwater.

In addition to the possibility of acid and Al having direct impacts on Na^+/K^+-ATPase activity, there may be other pathways for their effects on salinity tolerance. Other active transporters or passive mechanisms of ion transport may also be affected. Salt secretion in teleosts is dependent on the $Na^+/K^+/Cl^-$ cotransporter, which decreases when smolts are exposed to moderate acid and Al (Monette et al., 2008). Al can interfere with calcium binding sites in the fish gill that are integral to transepithelial permeability (Gensemer and Playle, 1999). Chloride cells (also known as mitochondria-rich cells) are responsible for salt secretion and increase size and abundance during smolt development (Pisam et al., 1988). Al, under acidic conditions, has been shown to preferentially accumulate in chloride cells (Youson and Neville, 1987) and alter their abundance (Jagoe and Haines, 1997). Monette et al. (submitted for publication) found evidence that under severe acid and Al conditions loss of salinity tolerance is associated with a decrease in the abundance of chloride cells, whereas under more moderate conditions loss of salinity tolerance was associated with a shift to smaller chloride cells. The shift to smaller chloride cells probably represents an attempt to compensate for compromised plasma ion levels, but may come at the cost of reduced numbers of chloride cells capable of salt secretion and thus an overall loss of seawater tolerance.

During freshwater exposure, hematocrit showed a very sensitive response to the acid and Al treatments in the present study, increasing with all added Al at pH 5.7 and 5.3 (Fig. 4). Even pH 5.3 with no added Al resulted in a significant increase in hematocrit. In this case we cannot rule out the possibility that this effect is also due to increased inorganic Al, since 10 μg L^{-1} Al_i was detected in this group (Table 1). Increased hematocrit likely represents evidence of acid/Al effects on the respiratory function of the gill (Gensemer and Playle, 1999), as the fish try to counteract reduced oxygen exchange by up-regulating the carrying capacity of the blood. Such reductions in respiratory function may be the result of morphological damage to the gill (Jagoe and Haines, 1997), or increased mucus production which can reduce respiratory efficiency (Ledy et al., 2003). It is of interest to note the rapid recovery of hematocrit after 24 h in seawater, where only the pH 5.3, 80 Al showed a lasting effect of prior acid/Al exposure. This indicates that there may have been more long term damage to the gill in this treatment group.

Levels of plasma cortisol and glucose observed for the time 0 and reference group were typical for smolting Atlantic salmon, indicating

Table 2
Results of factorial general linear regression of pH and Al_i on physiological parameters in Atlantic salmon smolts. Measured values of pH and Al_i in each of seventeen tanks were regressed on mean values of physiological parameters measured from all surviving fish in those tanks. Bold and asterisk indicate significant r^2 values ($p<0.05$).

	pH	Al_i	pH and Al_i
Freshwater			
Gill Al	0.04	**0.50***	**0.63***
Plasma Cl	0.09	**0.48***	**0.76***
Gill NKA activity	0.01	0.23	0.24
Hematocrit	0.17	**0.46***	**0.69***
Plasma cortisol	0.06	0.24	**0.58***
Plasma glucose	0.16	**0.56***	**0.87***
Seawater challenge			
Plasma Cl	0.05	**0.42***	**0.54***
Gill NKA activity	0.04	**0.42***	**0.59***
Hematocrit	0.02	0.22	0.36

that the basic experimental conditions were not stressful. Plasma glucose showed a clear graded response, with increasing levels as pH became lower and added Al was higher (Fig. 5). Plasma cortisol was significantly elevated at 80 Al at both pH 5.7 and 5.3. Surprisingly there was no detectable increased in plasma cortisol at the pH 5.7 and 175 Al, a treatment that resulted in high plasma glucose and loss of plasma chloride in freshwater. Increased plasma cortisol can occur within minutes and be sustained for hours or days (Mommsen et al., 1999). Since the present study examined only a single time-point after exposure, it is possible that plasma cortisol had increased in the pH 5.7, 175 Al group, and subsequently declined. In spite of this anomaly, the present results are in agreement with previous studies that have found activation of the primary and secondary stress response following exposure to acid and Al (Brown et al., 1990; Monette et al., 2008; Nagae et al., 2001).

Cortisol has been shown to be involved in ion uptake in freshwater teleosts, including proliferation of chloride cells (Laurent and Perry, 1990). A single injection of cortisol increased the capacity of the medaka (*Oryzias latipes*) to maintain plasma ions after exposure to low pH (Yada and Ito, 1999). The observed increase in cortisol in response to acid and Al exposure may be part of an adaptive response to restore plasma ions. However, the sustained elevation of cortisol may have negative long-term consequences (Wendelaar Bonga, 1997).

It was clear that the levels of Al_i in the present study were influenced by pH, with increasing levels of Al_i corresponding to decreasing pH. This is consistent with the known solubility of Al which is low at pH 7.0 and increases with decreasing pH. With the exception of mixing zones, it is generally accepted that Al has little biological action when pH is between 6.0 and 8.0 due to its insolubility, but increases in toxicity with decreasing pH (Gensemer and Playle, 1999). The results of the present study are consistent with this view, in that toxicity, gill Al accumulation and physiological impacts, only occurred when pH was below 6.0, and there was a strong relationship between Al_i and most of the observed responses (Table 2). A strong relationship between Al_i, gill Al and physiological impacts on Atlantic salmon smolts have been observed in laboratory and field studies and for a wide range of exposures times (Kroglund et al., 2007; Kroglund and Finstad, 2003; McCormick et al., 2009a; Monette and McCormick, 2008a). We observed an increase in gill Al with increasing Ali, though this effect was more pronounced with decreasing pH, further underscoring the interaction between acid and aluminum.

The acid and aluminum conditions imposed in these laboratory studies are environmentally relevant, corresponding to those that have been observed in Atlantic salmon rivers in Norway and eastern North America (Haines et al. 1990, Kroglund et al. 2008, McCormick et al., 2009a, 2009b). Episodic acidification is variable in space and time, but can last from a few days to several weeks, depending on the flow conditions and buffering capacity of receiving soil and water (Haines et al. 1990). The impact of acid and aluminum on Atlantic salmon smolts in nature will depend on several factors, including the severity and length of exposure, and the possibility of recovery if fish have the opportunity to migrate through waters without reduced pH and elevated inorganic aluminum. If severe enough, acid and aluminum exposure can be lethal to smolts. If non-lethal, the recovery from acid and aluminum exposure has been shown to take days to weeks, and is dependent on the water quality of the recovery water (Kroglund et al. 2001). In short rivers with poor buffering throughout their length (such as those in Eastern Maine), there will be little chance for recovery. In longer river systems the chance for recovery will likely depend on the location of exposure, the downstream water quality, and the time prior to seawater entry. Delays in seawater entry that might be imposed by effects of acid and aluminum on either freshwater or estuarine migration are likely to increase mortality through predation.

Our results demonstrate that exposure periods of 2 d to moderate acid and Al conditions can result in mortalities in freshwater. Additional mortalities and elevated plasma ions after seawater exposure also indicate that seawater tolerance can be lost after exposure to moderate pH and Al conditions in as little as 2 d. The loss of salinity tolerance will have direct effects on the capacity of fish to survive in the marine environment (Kroglund et al., 2007). These results have important implications for the impacts of episodic acidification on Atlantic salmon. Atlantic salmon rivers that experience pH of 5.7 or less and elevated inorganic Al ($\geq 30\ \mu g\ L^{-1}$) for 2 d or more in spring are likely to experience mortality in freshwater, or loss of salinity tolerance and reduced survival in the marine environment, thereby threatening the long term population viability of Atlantic salmon populations.

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