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Impacts of short-term acid and aluminum exposure on Atlantic salmon (*Salmo salar*) physiology: A direct comparison of parr and smolts

Michelle Y. Monette [a,b,*], Stephen D. McCormick [a,b]

[a] *Organismic and Evolutionary Biology, University of Massachusetts, 611 North Pleasant Street, Amherst, MA 01003, USA*
[b] *USGS, Leetown Science Center, Conte Anadromous Fish Research Center, One Migratory Way, Turners Falls, MA 01376, USA*

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Abstract

Episodic acidification resulting in increased acidity and inorganic aluminum (Al_i) is known to impact anadromous salmonids and has been identified as a possible cause of Atlantic salmon population decline. Sensitive life-stages such as smolts may be particularly vulnerable to impacts of short-term (days–week) acid/Al exposure, however the extent and mechanism(s) of this remain unknown. To determine if Atlantic salmon smolts are more sensitive than parr to short-term acid/Al, parr and smolts held in the same experimental tanks were exposed to control (pH 6.3–6.6, 11–37 $\mu g\,l^{-1}$ Al_i) and acid/Al (pH 5.0–5.4, 43–68 $\mu g\,l^{-1}$ Al_i) conditions in the lab, and impacts on ion regulation, stress response and gill Al accumulation were examined after 2 and 6 days. Parr and smolts were also held in cages for 2 and 6 days in a reference (Rock River, RR) and an acid/Al-impacted tributary (Ball Mountain Brook, BMB) of the West River in Southern Vermont. In the lab, losses in plasma Cl^- levels occurred in both control parr and smolts as compared to fish sampled prior to the start of the study, however smolts exposed to acid/Al experienced additional losses in plasma Cl^- levels (9–14 mM) after 2 and 6 days, and increases in plasma cortisol (4.3-fold) and glucose (2.9-fold) levels after 6 days, whereas these parameters were not significantly affected by acid/Al in parr. Gill Na^+,K^+-ATPase (NKA) activity was not affected by acid/Al in either life-stage. Both parr and smolts held at BMB (but not RR) exhibited declines in plasma Cl^-, and increases in plasma cortisol and glucose levels; these differences were significantly greater in smolts after 2 days but similar in parr and smolts after 6 days. Gill NKA activity was reduced 45–54% in both life-stages held at BMB for 6 days compared to reference fish at RR. In both studies, exposure to acid/Al resulted in gill Al accumulation in parr and smolts, with parr exhibiting two-fold greater gill Al than smolts after 6 days. Our results indicate that smolts are more sensitive than parr to short-term acid/Al. Increased sensitivity of smolts appears to be independent of a reduction in gill NKA activity and greater gill Al accumulation. Instead, increased sensitivity of smolts is likely a result of both the acquisition of seawater tolerance while still in freshwater and heightened stress responsiveness in preparation for seawater entry and residence.

Keywords: Acid rain; Aluminum; Atlantic salmon; Smolts; Ion regulation; Gill Na^+,K^+-ATPase activity

1. Introduction

Chronic (year-round) acidification and its associated aluminum (Al) toxicity is a known cause of Atlantic salmon population decline in Norway (Hesthagen, 1989) and Nova Scotia (Watt et al., 1983). Recent studies have suggested that episodic acidification (single or re-occurring episodes lasting several days) may also have effects on 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). As a result of their underlying geology, many rivers and streams in these regions have low concentrations of base cations (Ca^{2+}, Mg^{2+}) and consequent poor buffering capacity making them vulnerable to increases in acidity during episodic acidification events such as spring snowmelts and fall storms. During episodic acidification, Al is mobilized from the soil and enters the surrounding water leading to elevated Al concentrations. In addition, the solubility of Al increases as a direct result of decreased pH leading to the increased presence 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

* Corresponding author at: Department of Cellular and Molecular Physiology, Yale School of Medicine, 333 Cedar Street, New Haven, CT 06520, USA. Tel.: +1 203 785 7690.
E-mail address: michelle.monette@yale.edu (M.Y. Monette).

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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 exposure results in the loss of ion regulatory ability due to an increase in branchial permeability and an 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 from anionic gill binding sites by Al, resulting in the weakening of intercellular tight junctions (Booth et al., 1988; Freda et al., 1991), whereas inhibition of ion uptake may result from damage to or alteration of gill chloride cells (Jagoe and Haines, 1997), and decreased gill Na^+,K^+-ATPase (NKA) activity (Staurnes et al., 1993, 1996; Kroglund and Staurnes, 1999; Magee et al., 2003).

Atlantic salmon are among the most sensitive of the salmonid species to acid/Al (Fivelstad and Leivestad, 1984; Rosseland and Skogheim, 1984). After several years of stream residence, Atlantic salmon enter the parr–smolt transformation, a developmental period necessary for seawater (SW) entry and residence (McCormick et al., 1998). This period is marked by the acquisition of SW tolerance (salt secretory capacity) resulting in part from an increase in the number and size of gill chloride cells and gill NKA activity (McCormick et al., 1998). Other physiological changes include silvering, darkening of fin margins, and increased growth and oxygen consumption (Hoar, 1988). Several studies have indicated that Altantic salmon smolts are the most sensitive of the salmon life-stages to ion regulatory disturbance resulting from acid/Al exposure (Rosseland and Skogheim, 1984; Leivestad et al., 1987; Staurnes et al., 1993; Rosseland et al., 2001). However, these studies have made life-stage comparisons during chronic exposures, under severe acid/Al conditions, or during different seasons. Thus, there is a need for direct life-stage comparisons of Atlantic salmon exposed to short-term and moderate acid/Al conditions. In addition, these studies have suggested that increased smolt sensitivity may be due to the acquisition of SW tolerance while still in freshwater, however the specific mechanism(s) underlying this remain unknown.

The present study was conducted to directly compare the impacts of short-term acid/Al on the ion regulatory ability and stress response of Atlantic salmon parr and smolts. We investigated impacts of acid/Al on plasma Cl^-, gill NKA activity, indicators of stress, plasma cortisol and glucose, and gill Al accumulation. Our objectives were to determine if smolts are more sensitive than parr to short-term exposure to moderate acid/Al, and to investigate the mechanism(s) of increased sensitivity. More specifically, we tested the hypotheses that decreased gill NKA activity and/or increased gill Al accumulation underlie increased smolt sensitivity.

2. Materials and methods

2.1. Fish rearing

Atlantic salmon (*Salmo salar*) were obtained from the Kensington National Fish Hatchery (Kensignton, CT), and held at the Conte Anadromous Fish Research Center (Turners Fall, MA). Prior to the initiation of studies, 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}), maintained under natural photoperiod conditions and ambient river temperatures, and fed to satiation twice daily with commercial feed (Zeigler Bros., Garners, PA).

2.2. Laboratory exposure

Laboratory exposures were conducted from May 12–18, 2005. Atlantic salmon parr (9.2–12.8 cm) and smolts (14.7–16.7 cm) were randomly assigned to two replicate tanks receiving control (pH 6.5, 0 μg l^{-1} Al) or acid/Al (pH 5.2, 50 μg l^{-1} Al) conditions. An acid only treatment was not included in this study, as it has been established that increases in inorganic Al occur together with decreased pH in rivers experiencing episodic acidification (Lacroix and Townsend, 1987). Each experimental tank contained 10 parr and 10 smolts. Food was withheld for 24 h prior to the initiation of the study, and fish were starved for the duration of the experiment. Parr and smolts were exposed to the two experimental water chemistries for 2 and 6 days, and five fish per tank were sampled at each time-point. Prior to the start of the experiment, eight parr and eight smolts were sampled directly from their rearing tanks as a reference group ($T = 0$). Experimental tanks (186 l) received artificial soft water prepared by mixing deionized water (Siemens, Lowell, MA) 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 2–5-fold decreases in ambient calcium concentrations shortly after periods of increased river discharge in Maine and Nova Scotia. Experimental water was mixed for >1 h before entering fish tanks to avoid unstable water conditions, and each tank received continuous flow of 14 l h^{-1}. Temperature was maintained at 10.3–12.4 °C using a re-circulating chiller system. Both header and experimental tanks were oxygenated continuously with airstones maintaining dissolved oxygen at >10 mg O_2 l^{-1}. pH measurements were made twice daily from water samples collected at the tank outlet using a bench top pH meter 145 (Corning, Medfield, MA) with a Ross Ultra pH probe (Thermo Orion, Beverly, MA). 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^+.

2.3. Field exposure

Cage studies were conducted from May 17–23, 2005. Atlantic salmon parr (10.3–14.0 cm) and smolts (14.3–18.2 cm) were transported to two tributaries of the West River in Southern

Vermont. Prior to transport, nine parr and seven smolts were sampled directly from their rearing tanks as reference groups ($T=0$). Two replicate cages each containing eight parr and eight smolts were placed into two tributaries of the West River; the Rock River (RR), a reference stream, and Ball Mountain Brook (BMB), an acid/Al impacted-stream. Cages were 76 cm × 46 cm × 31 cm and constructed of 3 cm wooden supports with 1 cm plastic mesh on the outside. Cages were placed behind large boulders or inside scour pools to ensure that they had adequate flow but were protected from both high flow and reduced water levels. During the time-course of the study temperature ranged from 8.7 to 11.0 °C in RR and 8.1 to 11.2 °C in BMB. Parr and smolts (four fish/cage) were sampled after 2 and 6 days. pH was measured directly in the stream after 0, 2, 3 and 6 days using a portable pH 105 meter (Corning, Medfield, MA) with a Ross Ultra probe (Thermo Orion, Beverly, MA, and water samples were taken at the same time as described above.

2.4. Sampling protocol and tissue collection

All fish 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. Blood was collected in heparinized 1 ml syringes from the caudal vessels and centrifuged at 3200 × *g* for 5 min. Plasma was then removed and stored at −80 °C. 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 taken for the measurement of NKA activity, placed into 100 μl SEI (250 mM sucrose, 10 mM Na_2EDTA and 50 mM imidazole, pH 7.3) and stored at −80 °C.

2.5. 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, PerkinElmer, Wellesley, MA) atomic absorption spectrophotometry (GFAAS). Water samples were read in duplicate and instrument calibration was monitored every 10 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 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. Ca^{2+} and Na^{2+} were measured by flame atomic absorption spectrophotometry (AAnalyst 100, PerkinElmer, Wellesley, MA).

2.6. Plasma anaylsis

Plasma Cl^- was measured by silver titration using a digital chloridometer (Labconco, Kansas City, MO). Plasma cortisol was measured by enzyme immunoassay (EIA) as outlined by Carey and McCormick (1998). Plasma glucose was measured by enzymatic coupling with hexokinase and glucose 6-phosphate dehydrogenase (Stein, 1963).

2.7. Gill aluminum analysis

Al accumulation in a gill tissue biopsy 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). 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 (9:1) by the addition of 900 μl of ultrapure water, and Al concentration was analyzed by GFAAS as described above. A background correction was made for gill biopsy samples by subtracting the Al present in digestion blanks. Gill Al was expressed as μg Al g^{-1} gill dry weight.

2.8. Gill NKA activity

Gill NKA activity was measured following the method described by McCormick (1993). Gill biopsies were thawed immediately prior to assay, and 25 μl of 0.5% SEID (0.1 g sodium deoxycholate in 20 ml SEI) added to the microcentrifuge tube with tissue and homogenized for 10–15 s using a Kontes pellet pestle motor. The homogenate was then centrifuged at 3200 × *g* for 30 s, and the supernatant assayed both for NKA activity and total protein (BCA protein assay, Pierce, Rockford, IL). This kinetic assay was run at 25 °C for 10 min in a temperature-controlled plate reader (Thermomax, Molecular Devices, Menlo Park, CA) 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}.

2.9. Statistics

All data are presented as mean ± standard error (S.E.). For each physiological parameter, potential tank/cage effects were tested by nesting replicate tanks and cages within treatment and stream, respectively. Fish from replicate tanks/cages were pooled only if there was no significant tank/cage effect ($P>0.05$). A one-way ANOVA on ranks was used to test differences between parr and smolts sampled at the start of each study ($T=0$). For the laboratory study, a three-way ANOVA on ranks was used to determine the effect of treatment (control, acid/Al), exposure time (2 and 6 days), and life-stage (parr, smolt) on physiology. For the field study, a three-way ANOVA on ranks was used to determine the effect of stream (RR, BMB), exposure

time (2 and 6 days), and life-stage (parr, smolt) on physiology. For both studies, a one-way ANOVA on ranks was used to test differences between fish sampled at the start of each study ($T=0$) and control and RR fish sampled after 2 and 6 days. 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

3.1. Laboratory exposure

Over the course of the study, pH ranged from 6.29 to 6.56 and 4.99 to 5.42 in control and treatment tanks, respectively (Table 1). Mean Al_{tot} concentrations were 33 ± 5 and $72 \pm 3\ \mu g\ l^{-1}$ and mean Al_i concentrations were 20 ± 5 and $53 \pm 4\ \mu g\ l^{-1}$ in control and treatment tanks, respectively (Table 1). Ca^{2+} and Na^+ concentrations ranged from 0.9 to 1.8 and 1.7 to 2.8 mg l^{-1}, respectively, and were similar in all tanks (Table 1).

Plasma Cl^- levels of parr and smolts sampled prior to the start of the study ($T=0$) were 136 ± 2.1 and 137 ± 1.7 mM, respectively (Fig. 1A). Plasma Cl^- levels of control parr and smolts were significantly lower (9–13 mM) than $T=0$ fish after both time-points ($P<0.01$, one-way ANOVA; Fig. 1A). Plasma Cl^- levels of parr were not affected by acid/Al, whereas plasma Cl^- levels of treated smolts were significantly lower (9–14 mM) than control smolts after 2 and 6 days, indicating disturbance of ion regulatory ability in this group (Fig. 1A). Plasma Cl^- levels of treated smolts were significantly lower (13 mM) than treated parr after 2 days (Fig. 1A).

Gill NKA activity of $T=0$ parr and smolts was 3.1 ± 0.2 and 7.2 ± 0.6 μmol ADP mg protein $^{-1}$ h^{-1}, respectively (Fig. 1B). Gill NKA activity of control parr did not differ from $T=0$ parr after either time-point ($P>0.65$; one-way ANOVA), whereas gill NKA activity of control smolts was significantly lower (34%) than $T=0$ smolts after 6 days (Fig. 1B). Gill NKA activity of both control parr and smolts did not differ from acid/Al treated fish throughout the study (Fig. 1B). Gill NKA activity of smolts was significantly greater (38%-2.3-fold) than parr in all groups (Fig. 1B). An elevation in gill NKA activity is typically used as an indicator of smolt development in Atlantic salmon (McCormick, 1993), therefore the observed life-stage differences in NKA activity confirm the status of parr and smolts used in the laboratory.

Plasma cortisol levels of $T=0$ parr and smolts were 1.1 ± 0.3 and 26 ± 8.0 ng ml^{-1}, respectively (Fig. 2A). Plasma cortisol levels of control parr were significantly greater (5–14-fold) than $T=0$ parr after both time-points ($P<0.01$, one-way ANOVA), whereas plasma cortisol levels of control smolts did not differ from $T=0$ smolts after either time-point ($P>0.20$, one-way ANOVA; Fig. 2A). Plasma cortisol levels of parr were not affected by acid/Al, whereas plasma cortisol levels of treated smolts were significantly greater (4.3-fold) than control smolts after 6 days (Fig. 2A). Plasma cortisol levels of both control and treated smolts were significantly greater (9–12-fold) than parr after 2 days, but were not significantly elevated in either group after 6 days (Fig. 2A).

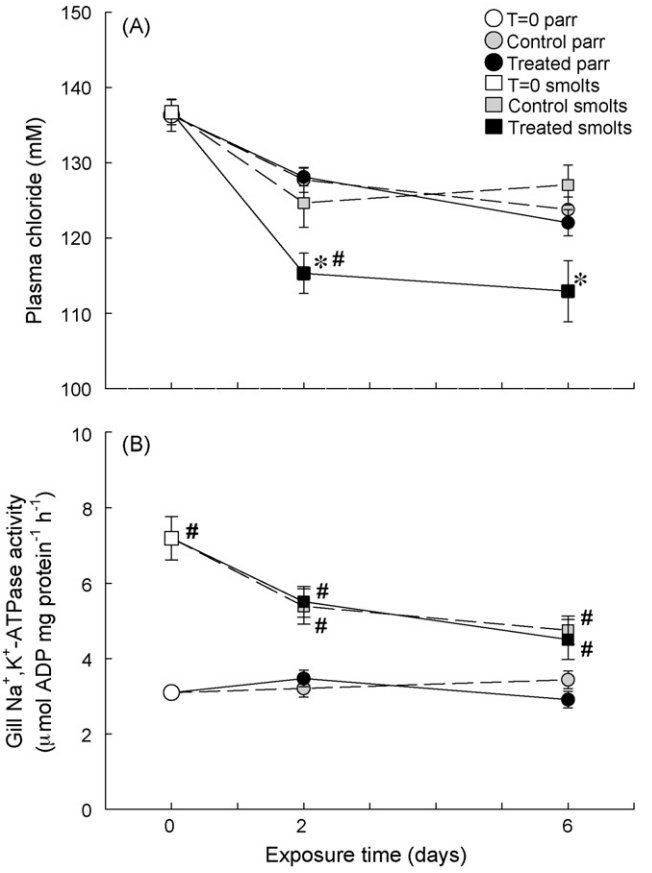

Fig. 1. Impacts of short-term laboratory exposure to acid/Al on the ion regulatory ability of Atlantic salmon parr and smolts. Plasma Cl^- (A) and gill NKA activity (B) levels of control and treated parr and smolts after 2 and 6 days. Values are mean ± S.E. ($n=7$–10). An * indicates a significant difference between control and treatment within exposure time and life-stage (Duncan's; $P<0.05$). An # indicates a significant difference between parr and smolt within a treatment and an exposure time (Duncan's; $P<0.05$). Values at day 0 represent parr and smolts sampled prior to the start of the study ($T=0$). Three-way ANOVA for plasma Cl^- levels determined significant effects of treatment ($P=0.004$) and life-stage ($P=0.01$), and significant treatment/life-stage ($P=0.02$) and timing/life-stage ($P=0.04$) interactions. Three-way ANOVA for gill NKA activity determined a significant life-stage effect ($P<0.001$).

Plasma glucose levels of $T=0$ parr and smolts were 3.5 ± 0.3 and 5.3 ± 0.8 mM, respectively (Fig. 2B). Plasma glucose levels of both control parr and smolts did not differ from $T=0$ fish throughout the study ($P>0.10$, one-way ANOVA; Fig. 2B). Plasma glucose levels of parr were not affected by acid/Al, whereas plasma glucose levels of treated smolts were significantly greater (2.9-fold) than control smolts after 6 days (Fig. 2B). Plasma glucose levels of treated smolts were also greater than control smolts after 2 days, but this difference was not statistically significant ($P=0.11$, Duncan's post-hoc test; Fig. 2B). Plasma glucose levels of smolts were significantly greater (51%-4.1-fold) than parr in all groups throughout the study (Fig. 2B).

Gill Al levels were $13 \pm 3\ \mu g\ g^{-1}$ for both $T=0$ parr and smolts (Fig. 3). Gill Al levels of all control fish remained between

Table 1
Measured water chemistry parameters in the laboratory study examining the impacts of acid/Al on the physiology of Atlantic salmon parr and smolts

Exposure	pH	Al_{tot} ($\mu g\,l^{-1}$)	Al_i ($\mu g\,l^{-1}$)	Ca^{2+} ($mg\,l^{-1}$)	Na^{+} ($mg\,l^{-1}$)
Control	6.40 ± 0.03 (20) (6.29–6.56)	33 ± 5 (6) (18–49)	20 ± 5 (5) (11–37)	1.4 ± 0.1 (12) (0.9–1.8)	2.2 ± 0.1 (12) (1.7–2.6)
Acid/Al	5.23 ± 0.04 (26) (4.99–5.42)	72 ± 3 (6) (61–83)	53 ± 4 (6) (43–68)	1.3 ± 0.1 (12) (1.0–1.8)	2.2 ± 0.1 (12) (1.7–2.8)

Values are mean ± S.E. of all measurements made throughout the 6-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.

9 ± 2 and 26 ± 10 $\mu g\,g^{-1}$ throughout the study and did not differ from $T = 0$ fish ($P > 0.25$, one-way ANOVA; Fig. 3). Gill Al of treated parr was significantly greater (6.5–19-fold) than control parr after 2 and 6 days (Fig. 3). Gill Al of treated parr increased by 69% between days 2 and 6 ($P < 0.05$; Fig. 3). Gill Al of treated smolts was significantly greater (12–15-fold) than control smolts after 2 and 6 days (Fig. 3). Gill Al of treated parr was significantly greater (two-fold) than treated smolts after 6 days.

3.2. Field exposure

Over the course of the study, pH ranged from 7.44 to 7.55 at the reference site (RR) and from 5.59 to 5.85 at the acid/Al-impacted site (BMB) (Table 2). Mean Al_{tot} concentrations were 36 ± 12 and 186 ± 9 $\mu g\,l^{-1}$ and mean Al_i concentrations were 7 ± 4 and 53 ± 7 $\mu g\,l^{-1}$ at RR and BMB, respectively (Table 2). Mean Ca^{2+} concentrations were 2.5 ± 0.1 and 0.7 ± 0.1 $mg\,l^{-1}$, and mean Na^{+} concentrations were 5.9 ± 0.3 and 8.8 ± 0.4 $mg\,l^{-1}$ at RR and BMB, respectively (Table 2).

Plasma Cl^{-} levels were 136 ± 0.5 and 136 ± 0.8 mM for $T = 0$ parr and smolts, respectively (Fig. 4A). Plasma Cl^{-} levels of RR parr and smolts did not differ from $T = 0$ fish after either time-point ($P > 0.05$, one-way ANOVA; Fig. 4A). Parr and smolts held at BMB experienced losses in plasma Cl^{-} levels compared to RR fish, but impacts were greater in smolts after 2 days. Plasma Cl^{-} levels of BMB parr were significantly lower (9–30 mM) than RR parr after 2 and 6 days (Fig. 4A). Plasma

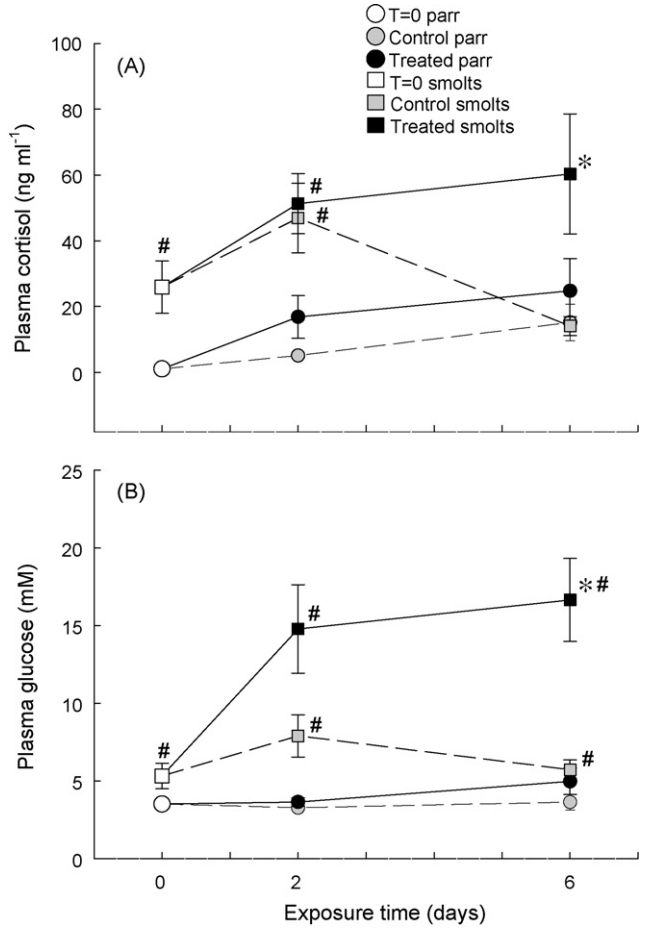

Fig. 2. Impacts of short-term laboratory exposure to acid/Al on the stress response of Atlantic salmon parr and smolts. Plasma cortisol (A) and plasma glucose (B) levels, of control and treated parr and smolts after 2 and 6 days. Values are mean ± S.E. ($n = 7$–10). An * indicates a significant difference between control and treatment within exposure time and life-stage (Duncan's; $P < 0.05$). An # indicates a significant difference between parr and smolt within a treatment and an exposure time (Duncan's; $P < 0.05$). Values at day 0 represent parr and smolts sampled prior to the start of the study ($T = 0$). Three-way ANOVA for plasma cortisol levels determined significant effects of treatment ($P = 0.002$) and lifestage ($P < 0.001$), and a significant timing/life-stage interaction ($P = 0.002$). Three-way ANOVA for plasma glucose levels determined significant effects of treatment ($P < 0.001$) and life-stage ($P < 0.001$).

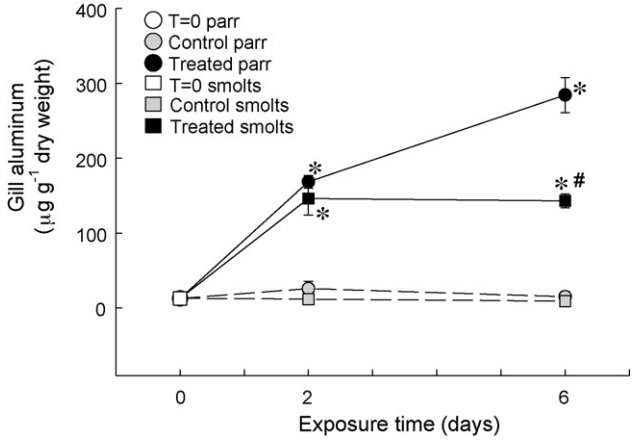

Fig. 3. Impacts of short-term laboratory exposure to acid/Al on gill Al accumulation of Atlantic salmon parr and smolts. Gill Al levels of control and treated parr and smolts after 2 and 6 days. Values are mean ± S.E. ($n = 8$–10). An * indicates a significant difference between control and treatment within exposure time and life-stage (Duncan's; $P < 0.05$). An # indicates a significant difference between parr and smolt within a treatment and an exposure time (Duncan's; $P < 0.05$). Values at day 0 represent parr and smolts sampled prior to the start of the study ($T = 0$). Three-way ANOVA for gill Al determined significant effects of treatment ($P < 0.001$) and life-stage ($P < 0.001$), and a significant treatment/time ($P = 0.002$) interaction.

Table 2
Measured water chemistry parameters of 2 tributaries of the West River during the 6-day cage study

Stream	pH	Al_{tot} ($\mu g\,l^{-1}$)	Al_i ($\mu g\,l^{-1}$)	Ca^{2+} ($mg\,l^{-1}$)	Na^{+} ($mg\,l^{-1}$)
RR	7.47 ± 0.03 (4) (7.44–7.55)	36 ± 12 (4) (16–69)	7 ± 4 (3) (2–15)	2.5 ± 0.1 (4) (2.1–2.7)	5.9 ± 0.3 (4) (4.9–6.4)
BMB	5.75 ± 0.06 (4) (5.59–5.85)	186 ± 9 (4) (164–207)	53 ± 7 (3) (42–66)	0.7 ± 0.1 (4) (0.7–0.8)	8.8 ± 0.4 (4) (8.1–9.8)

Values are mean ± S.E. of all measurements made throughout the 6-day study. Number of measurements made for each parameter is given in parentheses to the right. Range is given in parentheses below.

Cl^- levels of BMB smolts were significantly lower (20–43 mM) than RR smolts after 2 and 6 days (Fig. 4A). Plasma Cl^- levels of BMB smolts were significantly lower (32 mM) than BMB parr after 2 days (Fig. 4A).

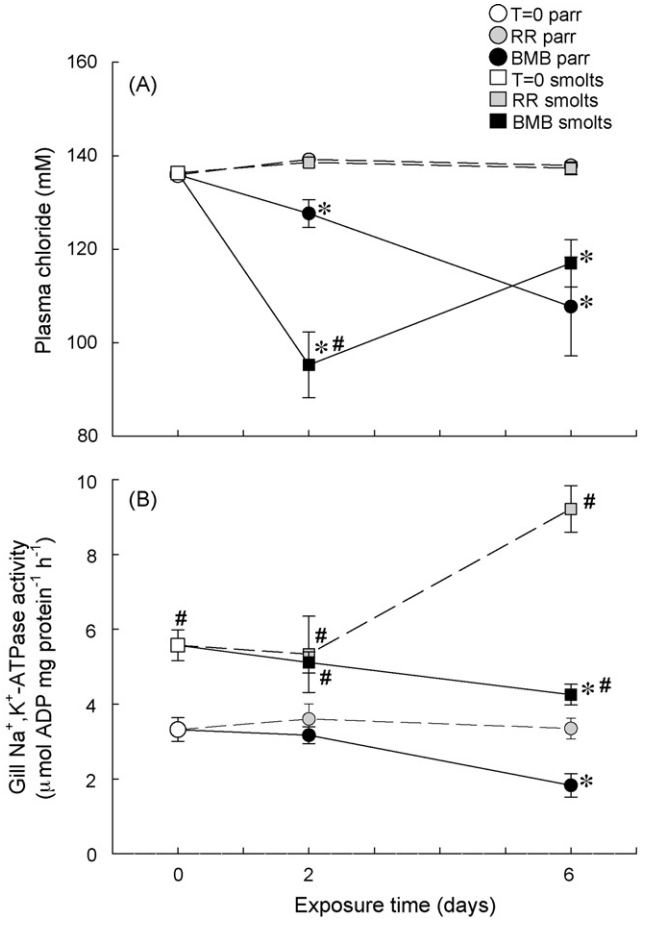

Fig. 4. Impacts of short-term field exposure to acid/Al on the ion regulatory ability of Atlantic salmon parr and smolts. Plasma Cl^- (A) and gill NKA activity (B) levels of parr and smolts held at a reference site (RR) and an acid/Al-impacted site (BMB) for 2 and 6 days. Values are mean ± S.E. (n = 4–16). An * indicates a significant difference between stream within an exposure time and life-stage (Duncan's; $P < 0.05$). An # indicates a significant difference between parr and smolt within a stream and an exposure time (Duncan's; $P < 0.05$). Values at day 0 represent parr and smolts sampled prior to the start of the study ($T = 0$). A significant cage effect was found for RR smolts at both time-points of exposure. For this group only, an outlier cage (one out of four replicate cages) was removed from the analysis, as it differed by ≥6 standard deviations. Three-way ANOVA for plasma Cl^- levels determined a significant stream effect ($P < 0.001$). Three-way ANOVA for gill NKA activity determined significant effects of stream ($P < 0.001$) and life-stage ($P < 0.001$), and significant stream/time ($P < 0.001$) and life-stage/time ($P = 0.008$) interactions.

Gill NKA activity of $T = 0$ parr and smolts was 3.3 ± 0.3 and 5.6 ± 0.4 μmol ADP mg protein $^{-1}$ h^{-1}, respectively (Fig. 4B). Gill NKA activity of RR parr was not significantly different from $T = 0$ parr after either time-point ($P > 0.55$, one-way ANOVA), whereas gill NKA activity of RR smolts was significantly greater (65%) than $T = 0$ smolts after 6 days (Fig. 4B). Gill NKA activity of parr was not affected by stream after 2 days, whereas after 6 days, gill NKA of BMB parr was significantly lower (45%) than RR parr (Fig. 4B). Gill NKA activity of smolts was not affected by stream after 2 days, whereas after 6 days, gill NKA activity of BMB smolts was significantly lower (54%) than RR smolts (Fig. 4B). Gill NKA activity of smolts was significantly greater (47%-2.8-fold) than parr in all groups throughout the study (Fig. 4B). As in the laboratory, the observed life-stage differences in NKA activity confirm the status of parr and smolts used in the field.

Plasma cortisol levels of parr and smolts were 8.1 ± 3.6 and 25 ± 4.6 ng ml^{-1}, respectively (Fig. 5A). Plasma cortisol levels of RR parr were significantly greater (6.9-fold) than $T = 0$ parr after 2 days, whereas plasma cortisol levels of RR smolts were not significantly different from $T = 0$ smolts after either time-point ($P = 0.19$, one-way ANOVA; Fig. 5A). Plasma cortisol levels of BMB parr were not affected by stream after 2 days, but were significantly greater (9.6-fold) than RR parr after 6 days (Fig. 5A). Plasma cortisol levels of BMB smolts were significantly greater (3.5–15-fold) than RR smolts after 2 and 6 days (Fig. 5A). Plasma cortisol levels were not significantly different between parr and smolts in any group throughout the study.

Plasma glucose levels of $T = 0$ parr and smolts were 4.2 ± 0.4 and 5.2 ± 0.4 mM, respectively (Fig. 5B). Plasma glucose levels of RR parr and smolts were not significantly different from $T = 0$ fish after either time-point ($P > 0.01$, one-way ANOVA; Fig. 5B). Plasma glucose levels of BMB parr were significantly greater (2.7–7.0-fold) than RR parr after 2 and 6 days (Fig. 5B). Plasma glucose levels of BMB smolts were significantly greater (3.7–4.4-fold) than RR smolts after 2 and 6 days (Fig. 5B). Plasma glucose levels of RR smolts were significantly greater (46–54%) than RR parr throughout the study (Fig. 5B).

Gill Al levels of $T = 0$ parr and smolts were 8.7 ± 3.2 and 13 ± 3.1 μg g^{-1}, respectively (Fig. 6). Gill Al levels of all RR fish were between 52 ± 10 and 92 ± 16 μg g^{-1} and were significantly greater (4.2–11-fold) than $T = 0$ fish after both time-points (Fig. 6). Gill Al of BMB parr was significantly greater (8.7–16-fold) than RR parr after 2 and 6 days (Fig. 6). Gill Al of BMB smolts was significantly greater (7.2–11-fold) than RR smolts after 2 and 6 days (Fig. 6). Gill Al of BMB parr was significantly greater (2.3-fold) than BMB smolts after 6 days (Fig. 6).

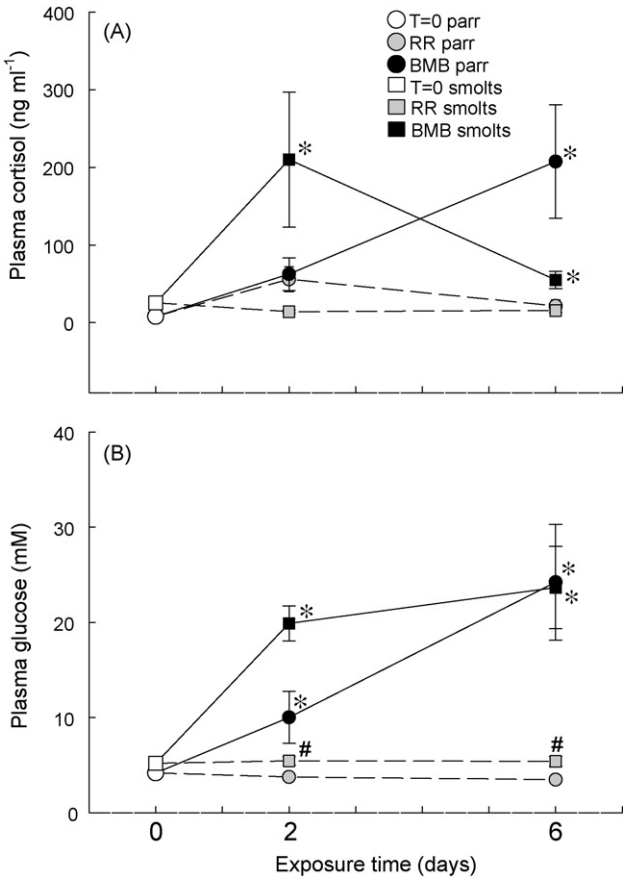

Fig. 5. Impacts of short-term field exposure to acid/Al on the stress response of Atlantic salmon parr and smolts. Plasma cortisol (A) and plasma glucose (B) levels of parr and smolts held at a reference site (RR) and an acid/Al-impacted site (BMB) for 2 and 6 days. Values are mean ± S.E. (n = 4–16). An * indicates a significant difference between stream within an exposure time and life-stage (Duncan's; $P < 0.05$). An # indicates a significant difference between parr and smolts within a stream and an exposure time (Duncan's; $P < 0.05$). Values at day 0 represent parr and smolts sampled prior to the start of the study ($T = 0$). A significant cage effect was found for RR smolts at both time-points of exposure. For this group only, an outlier cage (one out of four replicate cages) was removed from the analysis, as it differed by ≥6 standard deviations. Three-way ANOVA for plasma cortisol levels determined a significant stream effect ($P < 0.001$). Three-way ANOVA for plasma glucose levels determined significant effects of stream ($P < 0.001$) and life-stage ($P < 0.001$), and a significant stream/lifestage interaction ($P < 0.05$).

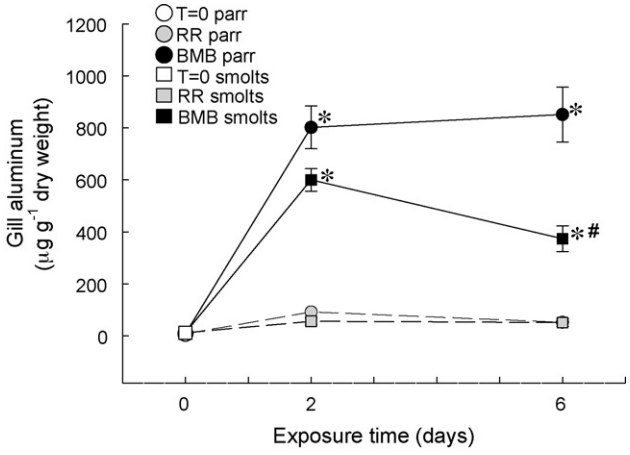

Fig. 6. Impacts of short-term field exposure to acid/Al on gill Al accumulation of Atlantic salmon parr and smolts. Gill Al levels of parr and smolts held at a reference site (RR) and an acid/Al-impacted site (BMB) for 2 and 6 days. Values are mean ± S.E. (n = 4–16). An * indicates a significant difference between stream within an exposure time and life-stage (Duncan's; $P < 0.05$). An # indicates a significant difference between parr and smolt within a stream and an exposure time (Duncan's; $P < 0.05$). Values at day 0 represent parr and smolts sampled prior to the start of the study ($T = 0$). A significant cage effect was found for RR smolts at both time-points of exposure. For this group only, an outlier cage (one out of four replicate cages) was removed from the analysis, as it differed by ≥6 standard deviations. Three-way ANOVA for gill Al determined significant effects of stream ($P < 0.001$) and life-stage ($P = 0.002$).

4. Discussion

To maintain ion homeostasis in freshwater, fish must combat the passive influx of water and efflux of plasma ions, which they accomplish by excreting a dilute urine and by taking up ions across the gill (Evans et al., 2005). Consequently, when the integrity or function of the gill is disturbed, loss of plasma Cl^- is observed. Laboratory exposure to short-term acid/Al impaired ion regulatory ability in smolts as indicated by reduced plasma Cl^- levels, but had no detectable impact on parr. In the field where conditions were potentially more severe (physical transfer to streams, lower ambient calcium concentrations), both life-stages held in an acid/Al-impacted stream (BMB) exhibited ion regulatory disturbance, but the pattern of ion loss differed between parr and smolts. Smolts held at BMB experienced large and rapid declines in plasma Cl^- levels after 2 days, with levels dropping to near the lethal threshold (<95–100 mM) reported for Atlantic salmon smolts (Staurnes et al., 1993). This was followed by partial recovery of plasma Cl^- levels after 6 days, suggesting that if smolts survive the initial toxic effects of acid/Al they may be able to recover ion homeostasis. However, acclimation to acid/Al would likely come as a cost to multiple aspects of physiology and behavior as has been shown in other salmonids (Wilson et al., 1994a, b, 1996). In contrast to smolts, parr held at BMB experienced only minor declines in plasma Cl^- levels after 2 days, clearly indicating lower sensitivity relative to smolts under these conditions. Parr at BMB continued to lose plasma Cl^- throughout the 6-day study, although levels did not approach the lethal threshold (<60 mM) reported for this life-stage (Lacroix and Townsend, 1987). The greater susceptibility of smolts to ion perturbations caused by acid/Al exposure is consistent with previous studies that have used either long-term or indirect approaches (Rosseland and Skogheim, 1984; Leivestad et al., 1987; Staurnes et al., 1993; Rosseland et al., 2001). The difference in short-term sensitivity demonstrated here has important implications, as the magnitude of initial ion losses may be closely related to fish survival during acid/Al exposure (Booth et al., 1988).

This study reports the effects of a single dose of acid and Al on Atlantic salmon parr and smolts. Based on previously published research and unpublished research from our own laboratory, it is likely that exposure to lower pH or higher Al would have resulted in more severe physiological consequences. Booth et al. (1988) exposed adult brook trout to three different pH levels (pH 5.2, 4.8, 4.4) with increasing total Al concentrations (0, 111, 333 $\mu g\,l^{-1}$) for up to 11 days and found that both mortality

and net ion losses increased with decreasing pH and increasing Al, with almost 100% mortality at the highest Al concentration at each pH level. We have found that Atlantic salmon smolts exposed to pH 5.2 with increasing Al_i concentrations (10, 41, 88, 140 $\mu g\,l^{-1}$) for 2 days suffer large losses in plasma ions when Al_i is 88 $\mu g\,l^{-1}$ ($Al_t = 107\ \mu g\,l^{-1}$) and 100% mortality when Al_i is 140 $\mu g\,l^{-1}$ ($Al_t = 179\ \mu g\,l^{-1}$) (Monette and McCormick, unpublished). However, in the same study, mortality and plasma ion losses of smolts exposed to 92 $\mu g\,l^{-1}$ of Al_t are decreased when pH is 5.6 and no impacts are observed when pH is >6.0. Together these results clearly demonstrate that the magnitude of physiological response (i.e. loss of ion regulatory ability) depends on the interaction of pH and Al levels.

In this study, plasma cortisol and glucose concentrations were measured as indicators of the stress response. Previous studies have shown that both parameters are affected by acid/Al exposure in salmonids (Brown et al., 1990; Waring et al., 1996; Kroglund et al., 2001). In our laboratory study, acid/Al-treated smolts experienced significant increases in plasma cortisol and glucose levels after 6 days, whereas acid/Al had no statistically significant effect in parr. In the field, acid/Al exposure caused elevations in plasma cortisol and glucose levels in both life-stages, but the time-course of impact differed between life-stages. Smolts held at BMB experienced large and rapid increases in both plasma cortisol and glucose levels, whereas increases in parr occurred more slowly. These observed life-stage differences reflect the patterns of plasma Cl^- losses in parr and smolts. Together, these results demonstrate that during short-term acid/Al exposure the stress response of smolts is more rapid than that of parr, suggesting a heightened sensitivity of the hypothalamic-pituitary-interrenal (HPI) axis in smolts. Smolts have been shown to have a heightened stress response during an acute handling stress, and it is thought that this may be important to survival during downstream migration and SW entry (Barton et al., 1985; Carey and McCormick, 1998). However, in this study, increased stress sensitivity of smolts may also be related to greater ion regulatory disturbance and/or other aspects of acid/Al exposure. There may be several advantages to a heightened HPI response including rapid mobilization of energy stores for damage repair and/or acclimation processes and increased respiratory capacity. However, negative consequences include decreased energy resources for other energetic demands such as downstream migration and predator avoidance, as well as negative long-term effects on growth and immunity.

We sought to examine the mechanisms(s) of increased smolt sensitivity by testing the hypothesis that a greater loss in gill NKA activity underlies increased sensitivity. Na^+,K^+-ATPase, an enzyme located in the basolateral membrane of the gill epithelium, plays a major role in teleost ion regulation in both FW and SW (Evans et al., 2005). In Atlantic salmon, gill NKA activity increases during the parr–smolt transformation and is directly related to the ability to maintain plasma ion homeostasis in SW (McCormick et al., 1998). In the present study, gill NKA activity levels of parr and smolts held at BMB for 6 days were lower than reference fish held at RR. In these fish declines in plasma Cl^- levels are likely due, in part, to an inhibition of ion uptake via reductions in gill NKA activity. Negative impacts on gill NKA activity are consistent with previous studies examining effects of long-term (weeks–months) acid/Al exposure on Atlantic salmon smolts (Staurnes et al., 1993; Magee et al., 2003). Decreased gill NKA activity may be attributed to increased chloride cell death via apoptosis and necrosis (Verbost et al., 1995), to the direct inhibition of enzyme activity by Al ions (Silva and Goncalves, 2003), or to the increased appearance of immature gill chloride cells with low levels of NKA protein (Wendelaar Bonga et al., 1990). Interestingly, gill NKA activity of smolts held at RR was significantly greater than $T = 0$ smolts after 6 days which may reflect the seasonal rise in gill NKA activity that occurs in smolts during the spring. This was not observed in smolts held at BMB, indicating that exposure to acid/Al may inhibit this aspect of smolt development.

In both the lab and the field, we observed declines in plasma Cl^- levels, despite no detectable impact on gill NKA activity. Also, when negative impacts on gill NKA activity were observed (field study, 6 days), the magnitude of activity loss was similar for parr and smolts. This suggests that under the conditions present in this study, impaired ion regulatory ability and thus increased smolt sensitivity may not be explained by reductions in gill NKA activity (i.e. ion uptake) alone. Instead, it is likely that ion losses are due to the stimulation of passive ion efflux resulting from increases in paracellular permeability as has been found for other salmonids (Booth et al., 1988; Freda et al., 1991). However, we cannot rule out the possibility that there were significant impacts of acid/Al on the *in vivo* activity of gill NKA as the assay employed in this study is a measure of total NKA protein present in the gill epithelium, and is not a measure of how much of the enzyme is working *in vivo*. Also, recent work has indicated that the alpha 1a and 1b isoforms of NKA are differentially regulated in the gill during acclimation to seawater in salmonids (Bystriansky et al., 2006). Thus, it is possible that acid/Al has significantly altered NKA isoform expression and subsequent ion transporting capacity without affecting total NKA protein in the gill.

We hypothesized that another mechanism underlying increased smolt sensitivity during acid/Al exposure may be greater gill Al accumulation. We observed elevated gill Al in parr and smolts during both laboratory and field exposures to acid/Al. Previous studies have found that elevations in gill Al occur in salmonids during both short- and long-term acid/Al exposure (Neville, 1985; Lacroix and Townsend, 1987; Kroglund et al., 2001; Teien et al., 2004, 2006; Winter et al., 2005). It is also known that gill Al is directly related to water Al_i concentration (Booth et al., 1988; Kroglund et al., 2001; Teien et al., 2006). Interestingly, in both the lab and the field, gill Al accumulation was two-fold greater in parr compared to smolts after 6 days. This is consistent with previous findings that smaller fish accumulate more gill copper and may be due to the greater surface area to volume ratio present in smaller fish (Kamunde et al., 2001; Taylor et al., 2002). Alternatively, smolts may have a lower capacity for gill Al accumulation or are better able to eliminate Al from the gill (i.e. sloughing of mucus-bound Al). Finally, exposure to acid/Al has been shown to increase degeneration of chloride cells in the gills of fish (Wendelaar Bonga et al., 1990; Verbost et al., 1995; Jagoe and Haines, 1997). Since gill

chloride cells can accumulate Al (Youson and Neville, 1987), increased chloride cell death may represent a mechanism to eliminate Al from the gill. Given the greater chloride cell abundance in the gill epithelium of smolts, increased chloride cell death may explain lower gill Al levels of smolts. Regardless of the mechanism, smolts exhibited impaired ion regulatory ability at lower concentrations of gill Al than parr, indicating a lower threshold of gill Al to cause damage and/or elicit a physiological response. It is likely that this is a result of the reorganization of the gill to increase salt secretory capacity that occurs during the parr–smolt transformation. Together, these results provide further evidence for the increased sensitivity of smolts however greater gill Al accumulation does not appear to play a role in increased sensitivity.

The present study clearly demonstrates that smolts are more sensitive than parr to impacts of short-term acid/Al however this appears to be independent of gill NKA activity and gill Al accumulation. Instead, it may be speculated that increased smolt sensitivity results from morphological changes in the gill epithelium during smolting, including an increase in the number and size of chloride cells (McCormick et al., 1998), as well as ultrastructural changes in chloride cell associations with other cells in the gill (Pisam et al., 1988; Mizuno et al., 2000). In particular, Pisam et al. (1988) demonstrated that accessory cells linked to apical portions of chloride cells by shallow junctions were present in Atlantic salmon smolts but not in parr. This change in chloride cell morphology is thought to play a role in the paracellular pathway of Na^+ excretion in teleosts, and may be necessary for SW adaptation. It is likely that this ultrastructural change renders smolt gills more permeable and therefore more vulnerable to rapid ion efflux during episodic acid/Al exposure. Increased smolt sensitivity may also occur from changes in the presence of other ion transporters/channels in the gill epithelium (i.e. Na^+, K^+,$2Cl^-$ cotransporter and apical Cl^- channel) in preparation for SW entry and residence.

In the laboratory study, plasma Cl^- levels of control parr and smolts were significantly lower than fish sampled prior to the start of the study ($T = 0$) indicating that there was an effect on plasma Cl^- independent of acid/Al treatment. This effect may be due to fish handling and/or transfer to smaller experimental tanks, as previous work has shown reductions in plasma Cl^- levels (15 mM) after an acute handling and confinement stress in Atlantic salmon (Carey and McCormick, 1998), and this response is well known to be part of the general stress response in fish (Wendelaar Bonga, 1997). Loss of plasma Cl^- may also be part of a physiological response to an acute reduction in ambient ion concentrations (including calcium, sodium and chloride) that was part of our experimental design. Hard water acclimated rainbow trout gills exhibit a greater increase in permeability than soft water acclimated gills during exposure to Al *in vitro* (Gundersen and Curtis, 1995). Increased membrane permeability might then allow for greater metal accumulation and this has been shown by Taylor et al. (2002) who found that rainbow trout previously acclimated to hard water exhibited greater gill copper accumulation than soft water acclimated fish. These effects are most likely due to changes in chloride cell size and density shown to occur during soft-water acclimation in salmonids (Greco et al., 1996; Uchida et al., 2002). Acute reductions in ionic strength in this study may thus have exacerbated observed impacts on physiological indices and gill Al concentrations. However, reductions in ambient ion concentrations are known to occur during increased discharge events in several rivers in both Maine and Nova Scotia, where acid/Al impacts are believed to be present (Lacroix and Townsend, 1987; Haines et al., 1990). Furthermore, we have found that prior acclimation to low ion water for 10 days does not prevent loss of ions or elevations in plasma cortisol and glucose levels in response to similar levels of acid/Al as used in the present study (Monette and McCormick, unpublished).

In conclusion, we have demonstrated by direct comparison that smolts are more sensitive than parr to impacts of short-term exposure to low pH and moderate Al_i in soft water. This is indicated by greater and more rapid losses in plasma Cl^- levels, heightened stress responsiveness, and a lower level of gill Al resulting in impaired ion regulation. We also provide evidence that under the conditions present in this study, increased smolt sensitivity appears to be independent of a reduction in gill NKA activity and greater gill Al accumulation. We suggest that smolts are more vulnerable to rapid ion losses as a result of the reorganization of the gill that has occurred during the parr–smolt transformation in preparation for seawater entry and residence. The heightened sensitivity of the smolt life-stage has substantial implications for salmon populations in regions affected by acid precipitation, as this critical developmental period occurs in the spring when episodic acidification due to seasonal rainfall and snowmelt may be greatest. Furthermore, compromised ion regulatory ability of smolts may have significant impacts on downstream migration and marine survival, which could in turn have population level effects.

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