# RESEARCH ARTICLE

# Aluminum exposure impacts brain plasticity and behavior in Atlantic salmon (*Salmo salar*)

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## SUMMARY

**Aluminum (Al) toxicity occurs frequently in natural aquatic ecosystems as a result of acid deposition and natural weathering processes. Detrimental effects of Al toxicity on aquatic organisms are well known and can have consequences for survival. Fish exposed to Al in low pH waters will experience physiological and neuroendocrine changes that disrupt homeostasis and alter behavior. To investigate the effects of Al exposure on both the brain and behavior, Atlantic salmon (*Salmo salar*) kept in water treated with Al (pH 5.7, $0.37 \pm 0.04\,\mu mol\,l^{-1}$ Al) for 2 weeks were compared with fish kept in under control conditions (pH 6.7, $<0.04\,\mu mol\,l^{-1}$ Al). Fish exposed to Al and acidic conditions had increased Al accumulation in the gills and decreased gill $Na^+$, $K^+$-ATPase activity, which impaired osmoregulatory capacity and caused physiological stress, indicated by elevated plasma cortisol and glucose levels. Here we show for the first time that exposure to Al in acidic conditions also impaired learning performance in a maze task. Al toxicity also reduced the expression of NeuroD1 transcript levels in the forebrain of exposed fish. As in mammals, these data show that exposure to chronic stress, such as acidified Al, can reduce neural plasticity during behavioral challenges in salmon, and may impair the ability to cope with new environments.**

## INTRODUCTION

Acid deposition causes acidification of many aquatic habitats worldwide; furthermore, it can cause aluminum (Al) toxicity through mobilization of Al from surrounding soil into adjacent waterways (Schindler, 1988). In water, Al can be present in different physico-chemical forms, but it is well established that Al cations are the bioavailable and toxic forms of Al, and that Al associated with organic material such as humic substances are less toxic (Gensemer and Playle, 1999; Teien et al., 2006). Many aquatic organisms are sensitive to such changes in water quality, and the detrimental effects of Al in acidified water have been studied in plants (Lovett et al., 2009), invertebrates (Guerold et al., 2000), amphibians (Brady and Griffiths, 1995) and fish (Kroglund et al., 2008; Poléo et al., 1997). Thus Al toxicity has significant, negative implications for the biodiversity and functioning of many ecosystems (Horne and Dunson, 1995; Lovett et al., 2009). To effectively manage aquatic habitats threatened by acid precipitation and the resulting Al pollution, it is necessary to identify and understand how different species are affected (Dudgeon et al., 2006).

Teleostean fishes, similar to other vertebrates, react to changes in the environment through the stress response – a series of behavioral and physiological adjustments mediated by a number of neuroendocrine pathways (Barton, 2002). In fish, the combined effects of acidified water and Al toxicity produce physiological changes such as disruption of gas and ion transport, altered blood chemistry and hormonal imbalance (Camargo et al., 2009; Neff et al., 2009; Nilsen et al., 2010). Such physiological changes can disrupt behaviors such as foraging and competition (Øverli et al., 2006; Sørensen et al., 2007), and can have consequences for growth and survival (Biro et al., 2007).

Atlantic salmon (*Salmo salar* L.) is an important recreational and commercial species found on both sides of the Atlantic Ocean, but over the past few decades many wild populations have experienced a significant decline (Parrish et al., 1998). Al toxicity in acidified water has been identified as a major contributor to this decrease (Kroglund et al., 2007; McCormick et al., 2009). An increase in Al concentration in water has been linked to an increase in Al accumulation in the gills (Kroglund et al., 2008). Al is known to affect the function of the gills in both parr and smolts, but there appears to be increased sensitivity to this chemical stress in smolts (Monette and McCormick, 2008). This effect is likely due to enhanced stress responsiveness, often seen as elevated plasma glucose and cortisol levels, as the fish adapt to seawater (Monette and McCormick, 2008; Monette et al., 2010). One way in which exposure to Al toxicity can hinder adaptation is through the loss of ion regulatory ability brought about by a decrease in gill $Na^+$,$K^+$-ATPase (NKA) activity, an enzyme required for seawater tolerance (Nilsen et al., 2010). The negative influences of Al on ion secretion during smoltification may therefore contribute to decreased survival in salmon at sea.

In addition to physiological adaptations associated with smoltification, the brain undergoes major structural and chemical changes. These arise because of the neuroendocrine and behavioral changes associated with olfactory imprinting that are essential for migration to the ocean and return to natal streams (Ebbesson et al., 1996a; Ebbesson et al., 1996b; Ebbesson et al., 2003; Ebbesson et al., 2007; Ebbesson et al., 2011; Stefansson et al., 2008). The physiological effects of stress also have an impact on behavior, through modification or impairment of signaling pathways that control behaviors such as feeding and aggression (Barton, 2002). During the critical smolting phase, Atlantic salmon have to adapt their behavior to cope with the transition from freshwater to the marine environment, and an additional stress imposed by Al toxicity could impair their ability to adapt to this transition successfully. Because behavioral changes are mediated by chemical pathways in the brain, exposure to a stressor will likely affect the organization or properties of specific neural circuitry that produce behavior (Kolb et al., 2003). Neurogenic differentiation factor (NeuroD) is a member of the family of proneural genes that regulates and controls neural differentiation (Kiefer et al., 2005). Neurogenesis, which includes proliferation, migration, differentiation and survival of neurons, is an integral step in learning and memory that is modulated by stress (Banasr and Duman, 2007). NeuroD protein and mRNA have been used as neural differentiation markers in vertebrates (see Dufton et al., 2005) including fish (Mueller and Wullimann, 2002; Mueller and Wullimann, 2003). Therefore, stress-induced changes in learning could be linked to modifications in neurogenesis through differential expression of NeuroD. As in zebrafish, it is possible that salmon possess multiple NeuroD transcription factors, so for the purposes of this study we investigated changes in the expression of one specific NeuroD transcription factor, NeuroD1 (Liao et al., 1999).

Several behaviors that influence how animals make decisions are underpinned by cognition, a process that spans the way an animal perceives current information, internalizes this and then uses learning and memory processes to determine which behavioral response is most appropriate (Shettleworth, 2010). Learning and memory processes help to support navigation, which is an important behavior for many fish species (Braithwaite and de Perera, 2006; Odling-Smee et al., 2008). As juveniles, Atlantic salmon need to be able to find their way around their river; as smolts, they need to migrate to sea and then, after many months at sea, as adults they return to their natal river or stream to spawn (Braithwaite and de Perera, 2006; Hansen et al., 1993). To be able to complete these different kinds of navigation, salmon need to learn and remember different aspects of their environment. If exposure to Al in acidified water impairs cognitive capacity, this will likely affect the survival of these fish.

The present study was designed to test the effects of chronic exposure to Al in acidified water on Atlantic salmon. Al concentration in the gills was determined to document Al exposure. Plasma glucose and cortisol levels were measured to assess physiological stress. We measured gill NKA activity to assess the physiological effect of the treatment, as Al toxicity is known to reduce NKA activity in salmon smolts (Monette and McCormick, 2008; Nilsen et al., 2010). A simple maze task was used to investigate whether Al exposure affects cognitive ability. We predicted that exposure to an Al-enriched environment would have a negative impact on learning through decreased accuracy in a spatial task. Finally, to investigate potential changes within the brain, we measured neural plasticity through expression of the transcription factor NeuroD in the telencephalon.

## MATERIALS AND METHODS
### Fish and rearing conditions
In February 2010, 250 Atlantic salmon parr were obtained from a wild anadromous strain originating from River Vosso in Norway. They were transported to the Aquatic Laboratory at Bergen High Technology Center and maintained in two $1\,m^2$ indoor tanks with a rearing volume of $500\,l$ supplied with flow-through freshwater at $10\,l\,min^{-1}$, pH adjusted (6.9–7.1) and kept at 8°C. The fish were exposed to a simulated natural photoperiod (60°25′N) and fed a commercial dry diet (T. Skretting A/S, Stavanger, Norway) continuously during the photo-phase.

The experiments were given ethical approval by the Norwegian Animal Research Authority (reference no. 1027636).

### Treatment setup
On 20 March 2010, 104 pre-smolt salmon (mean mass of 22.8±0.3 g and mean fork length of 12.8±0.1 cm; randomly sampled from the original 250) were individually marked using micro PIT tags (Nonatec, Lutronic International, Luxembourg) under anesthesia using $100\,mg\,l^{-1}$ buffered tricaine methanosulphonate (MS-222; Sigma-Aldrich, St Louis, MO, USA). The fish were allowed to recover for 4 weeks before being placed into two different treatments on 19 April: Acid-Al and control (no additional stressor). Both treatment groups consisted of two replicate tanks with 20 or 22 fish in each. Al [$AlCl_3(H_2O)_6$ dissolved in $24\,mmol\,l^{-1}$ HCl] and acid ($1.2\,mol\,l^{-1}$ HCl) were added by peristaltic pumps continuously to one header tank and mixed with control water, creating the Acid-Al water quality. Thus, increased concentration of $AlCl_3$ and decreased pH were the only differences between the treatments. Fish were maintained this way for the entire experiment.

### Water sampling and speciation of Al
It was expected that some of the Al added as Al cations to the water was transformed and thus would be present in less bioavailable particulate and colloidal forms (Teien et al., 2006). To obtain information about the final distribution of Al species and the Al exposure, on 28 April both the total concentration of Al and the Al speciation in the Acid-Al exposure and the control water were determined. The water in the tanks was fractionated with respect to size (molecular mass) and charge using *in situ* $0.45\,\mu m$ membrane filtration and ultra filtration (Amicon H1P10-20 hollow fibre operating at 69–103 kPa, nominal molecular mass cut-off 10 kDa; Danvers, MA, USA) and cation chromatography (Amberlite) (Teien et al., 2004). Fractionated water was acidified and Al was determined using inductively coupled plasma atomic emission spectrometry (ICP-AES). Thus, the concentration of different Al species was derived and defined according to the methods applied: Al tot, total dissolved Al in unfiltered samples; particulate Al, total concentration of Al associated with particles, i.e. larger than $0.45\,\mu m$; colloidal or high molecular mass (HMM) Al, total concentration of HMM Al species and Al associated with colloids, i.e. molecular mass ranging from 10 kDa to $0.45\,\mu m$; low molecular mass (LMM) Al, total concentration of LMM Al species, i.e. ultrafiltered (nominal molecular mass less than 10 kDa); and LMM $Al_{cation}$, cationic LMM Al species, i.e. ultrafiltered (nominal molecular mass less than 10 kDa), retained by Amberlite (Na form).

The water quality in the tanks was controlled by monitoring pH, temperature and conductivity. In addition, major cations ($Ca^{2+}$, $Na^+$, $Si^+$, $Mg^{2+}$) and anions ($SO_4^{2-}$, $Cl^-$, $F^-$, $NO_3^-$) were analyzed in the collected water samples (Teien et al., 2004).

Table 1. Water quality in control and Acid-Al ($N$=3) treatments

| | Temperature (°C) | Conductivity (mS m$^{-1}$) | pH pH | TOC (µmol l$^{-1}$) | Cl$^-$ (µmol l$^{-1}$) | NO$_3^-$ (µmol l$^{-1}$) | SO$_4^{2-}$ (µmol l$^{-1}$) | Ca (µmol l$^{-1}$) | K (µmol l$^{-1}$) | Mg (µmol l$^{-1}$) | Na (µmol l$^{-1}$) | Si (µmol l$^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 7.9±0 | 3.5±0 | 6.7 | 174.8±16 | 155[a] | 1.61[a] | 30.18[a] | 49.9±5.0 | 56.27±7.7 | 16.5±0 | 160.9±0 | 71.2±10 |
| Acid-Al | 8.1±0 | 3.8±0 | 5.7 | 174.8±16 | 206±0.0 | 1.61±0 | 29.15±0 | 47.4±0 | 46.04±0 | 16.5±0 | 160.9±0 | 78.3±0 |

Data are means ± s.d. TOC, total organic carbon.
[a]$N$=1.

The control water quality was characterized with a pH of 6.75 and was relatively low in ionic strength (3.5 mS m$^{-1}$ in conductivity and 49.9 µmol l$^{-1}$ of Ca; Table 1). In the control water the Al concentration was 1.46 µmol l$^{-1}$, where 70% was present as colloidal and not bioavailable Al species (Table 2). The concentration of Al cations was less than 0.04 µmol l$^{-1}$ in the control water. Due to the addition of AlCl$_3$ and acid, the pH decreased to 5.7 and the concentration of Al increased from 1.46 to 3.29 µmol l$^{-1}$ in the Acid-Al exposure. This is similar to the concentration of the Al in several rivers in Norway and thus is highly relevant, with both the colloidal Al fraction being less bioavailable (Teien et al., 2005) and the LMM Al fraction being increased in the Acid-Al water. The concentration of LMM Al cations in the Acid-Al water was 0.38±0.09 µmol l$^{-1}$ (mean ± s.d.).

### Sampling
Two weeks after the start of the treatment and 1 day prior to the behavior trials, 10 fish from each treatment group were quickly dip-netted and anesthetized in 200 mg l$^{-1}$ MS-222. Individual fish were weighed (wet mass in g) and measured (fork length in cm), and then blood was collected from the caudal vessels with heparinized tuberculin syringes. Blood was subsequently centrifuged (1500$g$, 10 min, 4°C), and plasma aliquots were frozen on dry ice. One drop of blood was used for glucose measurement (see below). One gill arch was placed in a pre-weighed scintillation vial for gill Al measurements. The day after behavior trials, another 10 fish from each treatment group were sampled following the same procedure as above. Brains were rapidly dissected and immediately placed in RNAlater (Ambion, Austin, TX, USA) and stored at −80°C for subsequent quantification of telencephalic NeuroD1 mRNA expression. One gill arch was placed in ice-cold SEI buffer (250 mmol l$^{-1}$ sucrose, 10 mmol l$^{-1}$ EDTA, 50 mmol l$^{-1}$ imidazole, pH 7.3) and frozen for determination of NKA activity. Telencephalic NeuroD1 mRNA expression and NKA activity samples were taken after behavioral testing to determine how physiology and neural plasticity after the maze challenge were affected by Acid-Al. All samples were stored at −80°C until assayed.

### Gill aluminum
The concentration of Al in gills ($N$=10) was determined by the method of Teien et al. (Teien et al., 2006), and refers to Al precipitated on the gill surface, Al incorporated in the gill cells and Al associated with mucus on gills.

### Plasma glucose
Plasma glucose (mmol l$^{-1}$) levels ($N$=10) were determined immediately after blood collection using a portable i-STAT clinical analyser (Harrenstien et al., 2005). Values were corrected for the temperature difference between ambient water temperature and the temperature-adjusted (37°C) values displayed by the instrument in accordance with the i-STAT procedure (Eliason et al., 2007) and ISTAT analyzer (Abbot Norge AS, Fornebu, Norway). Analytical cassettes of the type EC8+ were used for analyses of plasma glucose (mmol l$^{-1}$).

### Plasma cortisol
Cortisol ($N$=10) was measured using a radioimmunoassay in a 96-well plate. All wells except the 'non-specifics' received 100 µl cortisol antibody [Cortisol Antibody[xm210] monoclonal and IgG purified (Abcam, Cambridge, MA, USA); 1:2000 in 50 mmol l$^{-1}$ NaHCO$_3$, 50 mmol l$^{-1}$ NaH$_2$CO$_3$, 0.02% NaN$_3$, pH 9.6] and were incubated overnight at 4°C. The following day, the plates were washed three times with 200 µl well$^{-1}$ wash buffer (100 mmol l$^{-1}$ Tris, 0.9% NaCl, 0.02% NaN$_3$). Subsequently, non-specific sites were blocked by the addition of 100 µl blocking buffer (100 mmol l$^{-1}$ Tris, 0.9% NaCl, 0.02% NaN$_3$, 0.25% normal calf serum) to each well. Plates were covered and incubated for 1 h at 37°C. Subsequently, 10 µl of standard (4–2048 pg cortisol 10 µl$^{-1}$ assay buffer containing 100 mmol l$^{-1}$ Tris, 0.9% NaCl, 0.1% 8-anilino-1-naphthalenesulfonic acid, 0.02% NaN$_3$) or 10 µl of undiluted plasma was added to designated wells. Non-specifics and B$_0$ received 10 µl assay buffer. After the addition of standards and samples, 90 µl (333 Bq) of $^3$H-hydrocortisone (PerkinElmer, Waltham, MA, USA, 1:10,000 in assay buffer) solution was added to all wells. Plates were incubated overnight at 4°C. The plates were then washed three times with wash buffer. After the final wash step, all wells received 200 µl of Optiphase hisafe-3 scintillation liquid (PerkinElmer) and were covered. Beta-emission was quantified by a 3 min count per well using a Microbeta Plus (Wallac/PerkinElmer, Waltham, MA, USA). The cortisol assay had inter- and intra-assay variations of 12.5 and 3.5%, respectively. The cortisol antibody had the following cross-reactivities: cortisol 100%; 11-deoxycortisol 0.9%; prednisolone 5.6%; corticosterone 0.6%; 11-deoxycorticosterone, progesterone, 17-hydroxyprogesterone, testosterone, estradiol and estriol all <0.01% (Gorissen et al., 2012).

### Gill NKA activity
Gill NKA activity ($N$=10) was determined by the method of McCormick (McCormick, 1993). Briefly, this kinetic assay utilizes

Table 2. Water concentrations of different Al fractions in control and Acid-Al ($N$=3–6) treatments

| | Al total (µmol l$^{-1}$) | Particulate Al (µmol l$^{-1}$) | Colloidal Al (µmol l$^{-1}$) | LMM Al (µmol l$^{-1}$) | LMM Al cations (µmol l$^{-1}$) |
|---|---|---|---|---|---|
| Control | 1.46±0.01 | 0.03±0.02 | 1.03±0.06 | 0.40±0.05 | <0.04 |
| Acid-Al | 3.29±0.10 | 0.03±0.08 | 1.96±0.17 | 1.33±0.13 | 0.38±0.09 |

Data are means ± s.d. LMM, low molecular mass.

the hydrolysis of ATP, which is enzymatically coupled to the conversion of NADH to $NAD^+$ by pyruvate kinase and lactic dehydrogenase with or without the addition of ouabain, the specific inhibitor of NKA. Readings were taken at 340 nm for 10 min at 25°C. Protein in homogenate was determined by a bicinchoninic acid method (Smith et al., 1985). The ouabain-sensitive, $K^+$-dependent NKA-specific activity is expressed in $\mu mol\,ADP\,h^{-1}\,mg^{-1}$ protein.

### RNA isolation, cDNA synthesis and real-time quantitative PCR

The whole brain ($N$=10) was thawed in RNAlater Ice (Ambion) following the manufacturer's instructions, and the telencephalon was isolated under a dissecting microscope by cutting away the olfactory bulbs and then cutting vertically between the telencephalon and the hypothalamus. Total RNA was then directly isolated from the telencephalon by phenol-chloroform extraction using TRI Reagent (Sigma-Aldrich) as outlined by Chomczynski (Chomczynski, 1993). Total RNA concentration and purity was determined by the NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the RNA integrity was evaluated with the Agilent 2100 Bioanalyzer using the RNA 6000 Nano LabChip kit (Agilent Technologies, Palo Alto, CA, USA). Total RNA was treated with the TURBO DNA-free kit (Ambion, Austin, TX, USA) and cDNA reversely transcribed using 2 μg total RNA and Oligo $d(T_{15})$ in conjunction with the SuperScript III kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions.

Real-time quantitative PCR was conducted with gene-specific primers in conjunction with SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) using the MJ Research Chromo 4 System Platform (Bio-Rad Laboratories, Hercules, CA, USA). The forward and reverse primers for salmon brain NeuroD1 (GenBank accession no. BT058820) were: CAATGGACAG-CTCCCACATCT (forward) and CCAGCGCACTTCCGTATGA (reverse). For the assays, the thermal cycling protocols contained 5 μl cDNA (200 ng RNA), $200\,nmol\,l^{-1}$ of each primer and 12.5 μl SYBR Green Master Mix in a total volume of 25 μl. The thermal cycling protocol consisted of 10 min at 95°C followed by 45 cycles at 95°C for 15 s and 60°C for 1 min. Melt-curve analysis verified that the primer sets for each Q-PCR assay generated one single product and no primer–dimer artifacts. For each assay, triplicate twofold cDNA dilution series made from different exposure groups were used to determine amplification efficiencies ($E$), calculated as the slope from the plot of log cDNA concentration *versus* threshold cycle ($C_t$) values using the following formula: $E=10^{-1/slope}$. This efficiency was used to correct for differences in amplification efficiency when calculating gene expression according to Pfaffl (Pfaffl, 2004). Expression is presented as relative to the endogenous reference gene ribosomal protein L 23 (RPL 23), which did not vary between treatments in the present study. The RPL 23 forward primer was ATGCTGCCAGCATTTGAAGCAATCCT and the 23 RPL reverse primer was CTTTACATCATCTCTGTCAAGGGCA-TCAA.

### Maze trial procedure

After 15 days in the treatment tanks, behavioral trials started (control, $N$=40; Acid-Al, $N$=42). Learning ability was assessed using a maze that was placed inside the test tanks (similar to the holding tanks) that either contained Acid-Al treated water or control freshwater at a depth of 50 cm. Fish were tested in the same water as their home tanks: fish exposed to Acid-Al were always run in the Acid-Al conditions for the maze, and control fish were tested in control water conditions. The maze was raised up on a platform

creating a water depth of 15 cm inside the maze, providing a place of shelter and shade beneath the maze. The maze consisted of a central arena that opened out to four arms that each led to a doorway (Fig. 1). There were three 'false' exits and one 'true' exit leading out into the open area in the test tank. The 'true' exit was marked with a small black circular piece of plastic (radius 1.5 cm) attached to the wall next to the exit.

Fish were tested individually. Each day, fish were netted out of their holding tank, scanned using a hand-held PIT-tag reader for identification and then placed in a transparent start cylinder (radius 8 cm) in the center of the maze. After 10 s the cylinder was lifted remotely and the fish was free to explore the maze. Trials ended after 5 min, or when the fish escaped successfully. Once fish had left the maze they were allowed to swim freely around the exterior of the maze to help provide a potential social stimulus for fish still to be tested (although the sides of the central arena were opaque, the doorways at each of the false exits were transparent). This methodology is similar to that of Sovrano et al. (Sovrano et al., 2002), where the motivation for fish to leave the maze was social reinstatement. As the maze was brightly illuminated, the main motivator to leave the maze was probably the aversion to bright light, promoting escape into the main tank where the fish could find shade and shelter underneath the maze apparatus. Testing of fish within each tank was randomized; no fish was selected as the first fish more than once across the different test trials. Fish were returned to their holding tank after all fish in that tank had been screened in the maze. No fish attempted to re-enter the maze. The maze was taken out of the tank after all the fish from a tank had been tested. This made it easier to net and remove the fish. During this stage, we also flushed the tank with clean water before the start of the next block of trials.

Fish were tested once per day in two blocks of trials: block 1, trials 1–3, followed by a 2 day break, and then block 2, trials 4–6. All trials were filmed using a camera positioned over the top of the test tanks. The videos were screened after the maze trials were
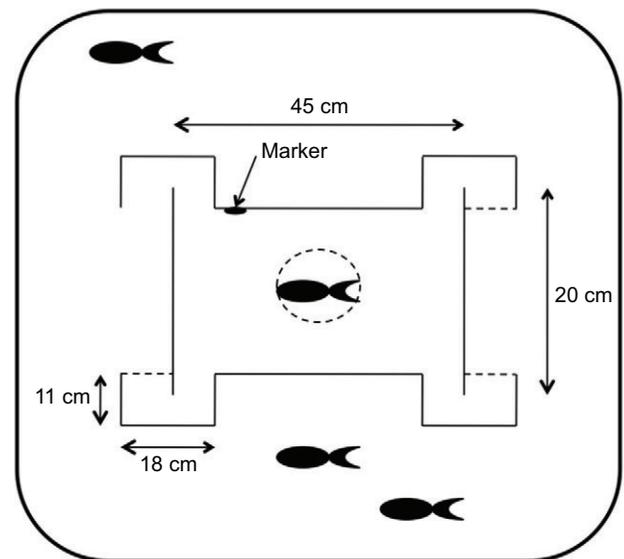


Fig. 1. Plan view (not drawn to scale) of the maze with test fish inside the isolation tube. The maze was raised up on a platform, creating a water depth of 15 cm inside the maze. Each exit (arm) was enclosed but the central arena was open. Solid lines indicate opaque walls that were used to minimize external cues; dashed lines are transparent walls.

Table 3. Summary of physiological parameters in control and Acid-Al fish

| Physiological parameter | Control | Acid-Al | $P$ |
|---|---|---|---|
| Gill Al ($\mu mol\,g^{-1}$ dry mass) | 0.11±0.01 | 2.00±0.15 | <0.01 |
| Gill NKA activity ($\mu mol\,ADP\,h^{-1}\,mg^{-1}$ protein) | 14.6±1.6 | 9.3±2.4 | <0.01 |
| Plasma glucose ($mmol\,l^{-1}$) | 5.3±0.5 | 7.7±2.8 | 0.01 |
| Plasma cortisol ($ng\,ml^{-1}$) | 14.5 | 43.0 | <0.01 |

All values are reported as means ± s.d. excluding the plasma cortisol levels, which are median values.

completed and the total number of arms visited during each trial was recorded using Etholog v2.2.5 (Ottoni, 2000). All videos were analyzed with the observer blind to whether a fish was a control or Acid-Al treated fish.

### Statistical analysis

Data were tested for equality of variance and were transformed when there was heterogeneity of variance across the groups being compared. If data did not meet the assumptions of normality and homoscedasticity, non-parametric tests were applied. Mass and fork length, neuroD1 mRNA expression and NKA activity were compared between Acid-Al and control groups using unpaired Student's $t$-tests. Gill Al levels were $log_{10}$-transformed before these data were compared using a Student's $t$-test. Reciprocal transformation was used for the plasma glucose levels before these data were compared using a Student's $t$-test. Plasma cortisol levels were compared between Acid-Al and control groups using a Mann–Whitney $U$-test. For the behavioral data, there was no difference in the performance of fish in the maze from replicate tanks ($F_{1,455}=3.33$, $P=0.07$), thus tanks were pooled for behavioral analysis. The number of arms entered was square-root transformed before being compared using a repeated-measures ANOVA with treatment as a factor. The data were compared across two blocks of three trials, the first block covering trials 1–3 and the second block trials 4–6. Fish were excluded if they never learned to leave the maze and just froze during a trial without visiting any exits (excluded: 11 control, 0 Acid-Al). Freezing is not an uncommon response for salmon, and we believe that the zero value for the Acid-Al fish arose because these fish were more active. The increased activity levels exhibited by the Acid-Al fish may be due to increased levels of stress, resulting in all the Acid-Al fish visiting at least one exit. For the behavior data, variation around the mean is represented as s.e.m. For all other data, variation around the mean is represented as the s.d. All analyses were conducted in StatView (version 5.0.1) and significance was tested at $\alpha=0.05$.

### RESULTS
#### Mass and length

There was no significant difference in mass (Acid-Al=29.6±4.3 g, control=29.0±3.5 g; $t$-test: $t_{1,18}=0.36$, $P=0.72$) or fork length (Acid-Al=14.4±0.8 cm, control=14.2±0.6 cm; $t$-test: $t_{1,18}=0.88$, $P=0.39$) of fish after 2 weeks of treatment.

#### Physiology
##### Gill aluminum

Gill Al levels were higher in the Acid-Al exposed fish compared with the control fish ($t$-test: $t_{1,17}=23.04$, $P<0.01$; Table 3).

##### Plasma glucose

Fish from the Acid-Al treatment were hyperglycemic, whereas those from the control treatment were not ($t$-test: $t_{1,18}=3.83$, $P=0.01$; Table 3).

##### Plasma cortisol

Fish from the Acid-Al treatment had higher plasma cortisol levels than control fish (Mann–Whitney: $U=11.0$, $P<0.01$; Table 3).

##### NKA activity

Fish from the Acid-Al treatment had significantly lower NKA activity than control fish ($t$-test: $t_{1,18}=5.82$, $P<0.01$; Table 3).

##### NeuroD1 mRNA

Fish from the Acid-Al treatment had significantly lower neuroD1 mRNA levels (relative to RPL 23) than control fish (Acid-Al=4.6±1.4, control=6.9±2.3; $t$-test: $t_{1,18}=2.67$, $P=0.02$; Fig. 2).

#### Behavior

For both the control and Acid-Al fish, the average number of arms visited decreased across trials, suggesting that performance improved as the fish became more experienced with the maze (ANOVA: $F_{5,407}=3.57$, $P<0.01$). Examining the behavior of the fish across the two blocks (block 1: trials 1–3 and block 2: trials 4–6) revealed a significant effect of treatment across block 2 trials, with Acid-Al fish making more mistakes on average than control fish (Acid-Al=3.6±0.3, control=2.9±0.2, ANOVA: $F_{1,67}=4.23$, $P=0.04$; Fig. 3). There was no effect of treatment across block 1 trials (ANOVA: $F_{1,68}=0.03$, $P=0.86$).

### DISCUSSION

Two weeks of exposure to Al in acidified water caused Al accumulation on the gills and impaired the spatial learning ability of smolting Atlantic salmon. The Acid-Al-exposed fish made more mistakes, indicating that acquisition of the task was inhibited, and had reduced neural plasticity indicators in their forebrain. Exposure to Al imposed a physiological stress, as levels of plasma glucose
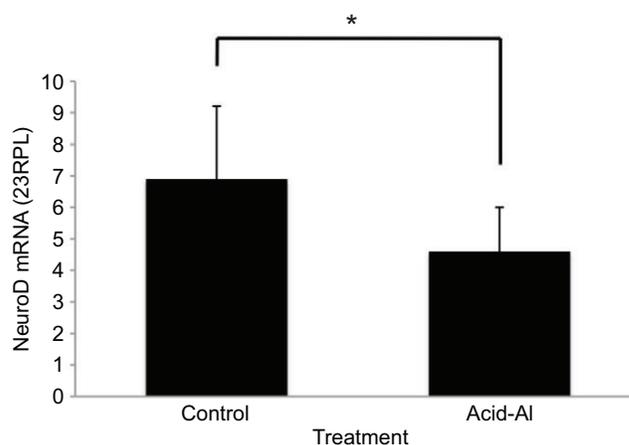


Fig. 2. Relative NeuroD1 mRNA expression in the telencephalon for control and Acid-Al fish (mean ± s.d.). *Significant difference between treatments ($P<0.05$).
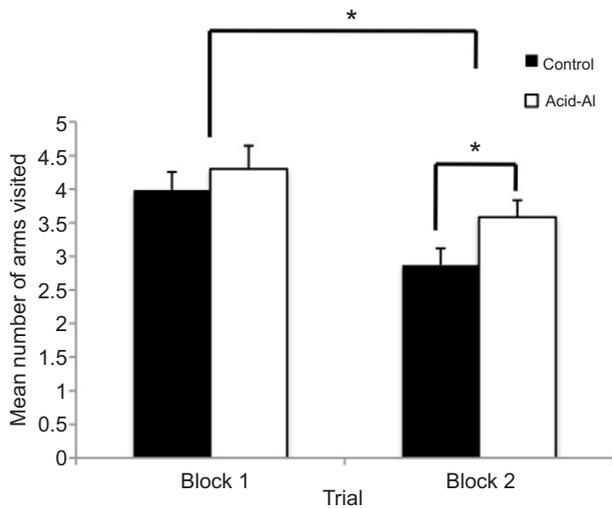
Fig. 3. The average number of maze arms visited for control and Acid-Al fish in block 1 and block 2 of the experiment (mean ± s.e.m.). *Significant difference between blocks or treatments ($P$<0.05).

and cortisol increased. Together, these data suggest that exposure to Al toxicity in acidified water has a negative impact on both the brain and learning behavior in salmon. Such an effect is likely to have a negative influence on the ability of the fish to cope with the transition from freshwater to the marine environment, a time when the fish need to perform critical behaviors such as predator avoidance, social interactions and navigation (McCormick et al., 1998; Ebbesson and Braithwaite, 2012). Furthermore, it is possible that the reduced forebrain neural plasticity and cognitive deficit at the critical smolt stage also affect imprinting, by altering the olfactory-telencephalic plasticity associated with smoltification (Ebbesson et al., 1996a; Ebbesson et al., 2003; Folgueira et al., 2004). Memories of the natal stream formed during imprinting are later used to return as adults (Hasler and Scholz, 1983; Yamamoto et al., 2010), and thus impaired imprinting could have a profound impact on return success. Such behavioral processes are likely to involve the area of the brain involved in spatial learning and memory, namely the dorsolateral area of the telencephalon (Ebbesson and Braithwaite, 2012).

We predicted that fish exposed to a prolonged period of elevated Al would be less accurate in the maze task than control fish. While there was an overall decrease in the number of mistakes made as the experiment progressed for fish from both groups, salmon from the Acid-Al treatment made more mistakes than control fish. It is possible that poorer learning in Acid-Al fish is linked to the observed decrease in neural plasticity, in line with a lower level of NeuroD1 mRNA expression in the telencephalon. The relationship between NeuroD1 gene expression and neurogenesis during early development has been identified in fish (Korzh et al., 1998; Mueller and Wullimann, 2003), and it is well established that neurogenesis in the telencephalon is known to occur throughout adult life in fish (Lema et al., 2005; Zupanc, 2008; von Krogh et al., 2010). The telencephalon is one of the most sensitive regions to stress-induced changes in the fish brain (Sørensen et al., 2011). A possible explanation for the reduced NeuroD1 expression in the telencephalon of Acid-Al fish was the increased levels of stress, shown by the higher levels of plasma glucose and cortisol in this group of fish before testing in the maze. The functional consequences of stress are dependent on the magnitude and duration of the stressor, but

chronic stress is known to have detrimental effects on learning and memory (Conrad, 2010). Therefore, decreased neural plasticity suggested by a decrease in NeuroD1 mRNA expression may have mediated some of the observed behavioral changes.

Both cortisol and plasma glucose levels have been used as indicators of physiological stress in fish (Barton, 2002; Barton and Iwama, 1991; Begg and Pankhurst, 2004; Wendelaar Bonga, 1997). When cortisol is released in response to a stressful event, it mobilizes fuels such as glucose to bring the fish back to homeostasis (Gregory and Wood, 1999). In the present study, Acid-Al fish had higher levels of both plasma glucose and cortisol than control fish, suggesting a higher stress level due to activation of the endocrine hypothalamic-pituitary-interrenal (HPI) axis. The effects of acidification on Atlantic salmon have been studied and, similarly, it was found that low pH was strongly correlated with negative physiological effects (Liebich et al., 2011). Thus in the present study, fish exposed to Al toxicity in acidic water will have experienced high allostatic load through physiological changes associated with the HPI axis, which may have led to the observed changes in learning behavior. In addition, exposure to Al toxicity lowered NKA activity in the gill. This is similar to the results of another study in salmon that found negative impacts on ion-regulatory development during smoltification (Nilsen et al., 2010). While Al impairment of ion regulatory development is detrimental for a smolt and has been implicated in low rates of returning adults (Kroglund and Finstad, 2003; Monette et al., 2008), a recent study has shown that ion regulatory capacity can partially recover following episodic acidification and Al exposure (Kroglund et al., 2012). In the present study, the mechanisms through which Al exposure affected gill condition are unknown; Al toxicity may have acted directly on NKA activity, or it may have acted indirectly by stimulating the HPI axis and inducing changes in NKA activity.

Our data showing impaired learning in Acid-Al treated fish add a new consideration in terms of how Al toxicity in acidified water affects salmon. The effect appears to extend beyond physiological changes to also altering learning behavior. It is known that smolts have a heightened sensitivity to Al in acidified water compared with parr (Monette and McCormick, 2008). In parr, many of the smolt-related parameters, such as neural plasticity, hormones and NKA activity, are at baseline levels, thus making it difficult to detect impacts on them. We chose to test the fish during a significant developmental period in their life cycle because the toxicity of Al in freshwater is enhanced, potentially leading to detrimental effects later on in life. Certainly, we found effects of Al exposure in terms of increased levels of glucose and cortisol. Taken on its own, however, it is not clear whether an increase in allostatic load, brought about by altered stress physiology, is causing distress or eustress in the fish (Korte et al., 2007; Korte et al., 2009). With the addition of our data on behavioral impairment and decreased neural plasticity, a decrease in cognitive ability suggests that the impact of Al toxicity is playing a negative role, causing distress in the exposed fish. These kinds of negative effects may have consequences for the ability of smolts to imprint on their natal streams (Yamamoto et al., 2010); it has been established that the parr–smolt transformation is a critical period for neural development, including cell differentiation and proliferation (Ebbesson et al., 1996b; Ebbesson et al., 2003; Ebbesson et al., 2007; Ebbesson et al., 2011). Olfactory imprinted memory is formed during smolting and the loss of neural plasticity could therefore impair the ability of Atlantic salmon to return to their natal stream (Dittman et al., 1996).

The negative impact of Al toxicity on cognitive ability during such a critical period has implications for how we can make

management decisions for fish that are at a high risk of exposure. Further studies investigating the neural and behavioral consequences of Al toxicity are needed to determine the breadth of these impacts. Taking the various negative effects together, salmon exposed to Al in acidic conditions appear to be forced into adapting to the adverse environment and this increases the allostatic load to a critical level. Avoiding such effects during the smolting stage could prove to be an important goal for salmon management.

### AUTHOR CONTRIBUTIONS

L.O.E.E., V.A.B. and C.G. participated in the experimental design and all experiments. All authors participated in drafting the manuscript. L.O.E.E., J.N., V.T. and V.A.B. were involved in maze design and behavioural studies. L.O.E.E., C.G., T.O.N., S.O.H., S.O.S. and V.T. carried out sampling. H.-C.T. conducted water chemistry analysis. L.O.E.E., T.O.N. and V.T. participated in gene expression analyses. G.F. and M.G. analysed cortisol and S.O.H. analysed glucose. L.O.E.E., C.G. and V.A.B. conceived the study and drafted the final manuscript. All authors participated in editing the final manuscript.

### COMPETING INTERESTS

No competing interests declared.

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