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 to both brain and behavior, Atlantic salmon (*Salmo salar*) kept in water treated with Al (pH 5.7, 0.37±0.04 µmol l^{-1} of Al) for 2 weeks were compared to fish kept in a control condition (pH 6.7, <0.04 µmol l^{-1} of Al). Fish exposed to Al and acidic conditions had increased Al accumulation in the gills and decreased gill Na^+, K^+-ATPase activity, which impaired osmoreguatory 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 coping ability to new environments.

Keywords: parr-smolt transformation; telencephalon; salmonid; spatial learning; chronic mild stress; physiology

1. 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 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 produces 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 like 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 their natal streams (Ebbesson *et al.*, 1996a,b, 2003, 2007, 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 fresh water to the marine environment, and an additional stress imposed by Al toxicity could impair their ability to adapt to this transition successfully. Since 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, 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 Dumas, 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, 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 current 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.

2. Materials and Methods

2.1 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 $1m^2$ indoor tanks with a rearing volume of 500 L supplied with flow-through fresh water at 10 l/min, pH

adjusted (6.9-7.1) and kept at 8ºC. The fish were exposed to a simulated natural photoperiod (60º25'N) and fed a commercial dry diet (T. Skretting A/S, Stavanger, Norway) continuously during the photo-phase.

2.2 Treatment Set-up

On March 20th 2010, 104 pre-smolt salmon (mean weight 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 buffered tricaine methanosulphonate (MS222; Sigma, St. Louis, MO, USA). The fish were allowed to recover for 4 weeks before being placed into 2 different treatments on April 19th; Acid-Al and control (no additional stressor). Both treatment groups consisted of 2 replicate tanks with 20 or 22 fish in each. Al ($AlCl_3(H_2O)_6$ dissolved in 24 mM HCl) and acid (1.2 M 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.

2.3 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 April 28th 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 µm membrane filtration and ultra filtration (Amicon H1P10-20 hollow fibre operating at 10-15 psi, nominal molecular mass cut-off 10 kDa) and cation chromatography (Amberlite, Teien *et al.*, 2004). Fractionated water was acidified and Al 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 µm.
- Colloidal or HMM Al = total concentration of high molecular mass Al-species and Al associated with colloids, *i.e.* molecular mass ranging from 10 kDa-0.45 µm.
- LMM Al = total concentration of low molecular mass Al-species, *i.e.* ultrafiltered (nominal molecular mass less than 10 kDa).
- LMM Al_{cation} = cationic low molecular mass 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).

The control water quality was characterized with a pH of 6.75 and was relatively low in ionic strength (3.5 mS/m in conductivity and 49.9 µmol 1^{-1} of Ca, Table 1). In the control water the Al concentration was 1.46 µmol 1^{-1}, where 70% was present as colloidal not bioavailable Al-species (Table 2). The concentration of Al cations was less than 0.04 µmol 1^{-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 1^{-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 1^{-1} (mean ± S.D.).

2.4 Sampling

Two weeks after the start of the treatment and one day prior to the behavior trials, 10 fish from each treatment group were quickly dip-netted and anaesthetized in 200 mg/l MS222. Individual fish were weighed (wet weight 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 *x* **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 Inc., 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 mM sucrose, 10 mM EDTA, 50 mM 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.

2.4.1 Gill Aluminum

The concentration of Al in gills (n = 10) was determined by the method of 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.

2.4.2 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). Analytical cassettes of the type EC8+ were used for analyses of plasma glucose (mmol 1^{-1}).

2.4.3 Plasma Cortisol

Cortisol (n = 10) was measured using a RIA in a 96-well plate. All wells except the 'non-specifics' received 100 µl cortisol antibody (Cortisol Antibody[xm210] monoclonal and IgG purified (Abcam); 1:2000 in 50 mM $NaHCO_3$, 50 mM NaH_2CO_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 wash buffer (100 mM Tris, 0.9% NaCl, 0.02% NaN_3). Subsequently, non-specific sites were blocked by the addition of 100 µl blocking buffer (100 mM Tris, 0.9% NaCl, 0.02% NaN_3, 0.25% Normal Calf Serum) to each well. Plates were covered and incubated for one hour at 37˚C. Subsequently, 10 µl of standard (4–2048 pg cortisol/10 µl assay buffer containing 100 mM 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, 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, USA) and were covered. Beta-emission was quantified by a 3 min count per well using a Microbeta Plus (Wallac/PerkinElmer, 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).

2.4.4 Gill NKA activity

Gill NKA activity (n = 10) was determined by the method of McCormick (1993). Briefly, this kinetic assay utilizes 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 done 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 µmol ADP h^{-1} per mg protein.

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

The whole brain (n = 10) was thawed in RNAlater Ice following manufacturer 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 telencephalon by phenol-chloroform extraction using TRI Reagent® (Sigma, St. Louis, MO, USA) as outlined by 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 TURBO DNA-free™ kit (Ambion, Austin, TX, USA) and cDNA reversely transcribed using 2µg total RNA and Oligo $d(T_{15})$ in

conjunction with the SuperScript III kit (Invitrogen, Carlsbad, CA, USA) following the manufactures instructions.

Real-time quantitative PCR were conducted with gene specific primers in conjunction SYBR Green Master Mix (ABI; Applied Biosystems, Foster City, CA, USA) using the MJ Research Chromo 4 System Platform (Bio-Rad Laboratories Inc., CA, USA). The forward and reverse primers for salmon brain NeuroD1 (GenBank accession. number. BT058820) were: CAATGGACAGCTCCCACATCT (forward) and CCAGCGCACTTCCGTATGA (reverse). For the assays, the thermal cycling protocols contained 5 µl cDNA (200 ng RNA), 200 nM of each primer and 12.5 µl SYBR Green Master Mix in at 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 seconds 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 two-fold 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 (Ct) values using the following formula: $E = 10^{(-1/\text{slope})}$. This efficiency was used to correct for differences in amplification efficiency when calculating gene expression according to 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 CTTTACATCATCTCTGTCAAGGGCATCAA.

2.5 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 seconds the cylinder was lifted remotely and the fish was free to explore the maze. Trials ended after 5 minutes, 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.* (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 2 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 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.

2.6 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. Weight and fork length, neuroD1 mRNA expression and NKA activity were compared between Acid-Al and control groups in unpaired Student's *t*-tests. Gill Al levels were Log_{10}-transformed before these data were compared in a Student's *t*-test. Reciprocal transformation was used for the plasma glucose levels before these data were compared

in a Student's *t*-test. Plasma cortisol levels were compared between Acid-Al and control groups in a Mann-Whitney *U* test. For the behavioral data, there was no differences 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 in 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 arises 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 the standard error of the mean (S.E.M.). For all other data variation around the mean is represented as the standard deviation (S.D.). All analyses were conducted in StatView (version 5.0.1) and significance was tested at $\alpha < 0.05$.

3. Results

3.1 Weight and Length

There was no significant difference in weight (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 two weeks of treatment.

3.2 Physiology

3.2.1 Gill Al

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

3.2.2 Plasma Glucose

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

3.2.3 Plasma Cortisol

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

3.2.4 NKA Activity

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

3.3 NeuroD1 mRNA

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

3.4 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 (Fig. 3; Acid-Al = 3.6 ± 0.3, control = 2.9 ± 0.2, ANOVA: $F_{1,67} = 4.23$, $P = 0.04$). There was no effect of treatment across block 1 trials (ANOVA: $F_{1,68} = 0.03$, $P = 0.86$).

4. 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 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 and cortisol increased. Together, these

388 data suggest that exposure to Al toxicity in acidified water has a negative impact on both
389 the brain and learning behavior in salmon. Such an effect is likely to have a negative
390 influence on the ability of the fish to cope with the transition from freshwater to the
391 marine environment, a time when the fish need to perform critical behaviors such as
392 predator avoidance, social interactions and navigation (McCormick *et al.*, 1998;
393 Ebbesson and Braithwaite, 2012). Furthermore, it is possible that the reduced forebrain
394 neural plasticity and cognitive deficit at the critical smolt stage also affect imprinting, by
395 altering the olfactory-telencephalic plasticity associated with smoltification (Ebbesson *et*
396 *al.*, 1996a, 2003; Folgueira *et al.*, 2004). Memories of the natal stream formed during
397 imprinting are later used to return as adults (Hasler and Scholz 1983; Yamamoto *et al.*
398 2010), and thus impaired imprinting could have a profound impact on return success.
399 Such behavioral processes are likely to involve the area of the brain involved in spatial
400 learning and memory, namely the dorsolateral (Dl) area of the telencephalon (Braithwaite
401 and Ebbesson, 2012).

402 We predicted that fish exposed to a prolonged period of elevated Al would be less
403 accurate in the maze task than control fish. While there was an overall decrease in the
404 number of mistakes made as the experiment progressed for fish from both groups, salmon
405 from the Acid-Al treatment made more mistakes than control fish. It is possible that
406 poorer learning in Acid-Al fish is linked to the observed decrease in neural plasticity, in
407 line with a lower level of NeuroD1 mRNA expression in the telencephalon. The
408 relationship between NeuroD1 gene expression and neurogenesis during early
409 development has been identified in fish (Korzh *et al.* 1998; Mueller and Wullimann,
410 2003), and it is well established that neurogenesis in the telencephalon is known to occur

411 throughout adult life in fish (Lema *et al.* 2005; Zupanc, 2008; von Krogh *et al.* 2010).
412 The telencephalon is one of the most sensitive regions to stress induced changes in the
413 fish brain (Sørensen *et al.*, 2011). A possible explanation for the reduced NeuroD1
414 expression in the telencephalon of Acid-Al fish was the increased levels of stress, shown
415 by the higher levels of plasma glucose and cortisol in this group of fish before testing in
416 the maze. The functional consequences of stress are dependent on the magnitude and
417 duration of the stressor but chronic stress is known to have detrimental effects on learning
418 and memory (Conrad, 2010). Therefore, decreased neural plasticity suggested by a
419 decrease in NeuroD1 mRNA expression may have mediated some of the observed
420 behavioral changes.

421 Both cortisol and plasma glucose levels have been used as indicators of physiological
422 stress in fish (Barton, 2002; Barton and Iwama, 1991; Begg and Pankhurst, 2004;
423 Wendelaar Bonga, 1997). When cortisol is released in response to a stressful event it
424 mobilizes fuels such as glucose to bring the fish back to homeostasis (Gregory and
425 Wood, 1999). In the current study Acid-Al fish had higher levels of both plasma glucose
426 and cortisol than control fish, suggesting of a higher stress level due to activation of the
427 endocrine hypothalamic-pituitary-interrenal (HPI) axis. The effects of acidification on
428 Atlantic salmon have been studied and similarly, it was found that low pH was strongly
429 correlated with negative physiological effects (Liebich *et al.*, 2011). Thus in the current
430 study, fish exposed to Al toxicity in acidic water will have experienced high allostatic
431 load through physiological changes associated with the HPI axis, which may have led to
432 the observed changes in learning behavior. In addition, exposure to Al toxicity lowered
433 NKA activity in the gill. This is similar to the results of other studies in salmon that found

434 negative impacts on ion-regulatory development during smoltification (Nilsen *et al.*,
435 2010). While Al-impairment of ion regulatory development is detrimental for a smolt and
436 has been implicated in low rates of returning adults (Kroglund and Finstad, 2003;
437 Monette *et al.*, 2008), recent studies have shown that ion regulatory capacity can partially
438 recover following episodic acidification and Al exposure (Kroglund *et al.*, 2012). In the
439 current study, the mechanisms through which Al exposure affected gill condition are
440 unknown; Al toxicity may have acted directly on NKA activity, however, it may have
441 acted indirectly by stimulating the HPI axis and inducing changes in NKA activity.

442 Our data showing impaired learning in Acid-Al treated fish adds a new consideration
443 in terms of how Al toxicity in acidified water affects salmon. The effect appears to extend
444 beyond physiological changes to also altering learning behavior. It is known that smolts
445 have a heightened sensitivity to Al in acidified water compared with parr (Monette and
446 McCormick 2008). In parr, many of the smolt-related parameters, such as neural
447 plasticity, hormones, and NKA activity are at baseline levels thus making it difficult to
448 detect impacts on them. We chose to test the fish during a significant developmental
449 period in their life cycle because the toxicity of Al in fresh water is enhanced, potentially
450 leading to detrimental effects later on in life. Certainly, we found effects of Al exposure
451 in terms of increased levels of glucose and cortisol. Taken on its own, however, it is not
452 clear whether an increase in allostatic load, brought about by altered stress physiology, is
453 causing distress or eustress in the fish (Korte *et al*., 2007, 2009). With the addition of our
454 data on behavioral impairment and decreased neural plasticity, a decrease in cognitive
455 ability suggests that the impact of Al toxicity is playing a negative role, causing distress
456 in the exposed fish. These kinds of negative effect 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, 2003, 2007, 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 Al toxicity effects on neural and behavioral consequences 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.

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765

Figure Legends

Fig. 1. Plan view (not drawn to scale) of the maze with test fish inside 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.

Fig. 2. Relative NeuroD1 mRNA expression in the telencephalon for control and Acid-Al fish (mean ± S.D.). * Denotes significant difference.

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.). * Denotes significant difference.

Fig. 1

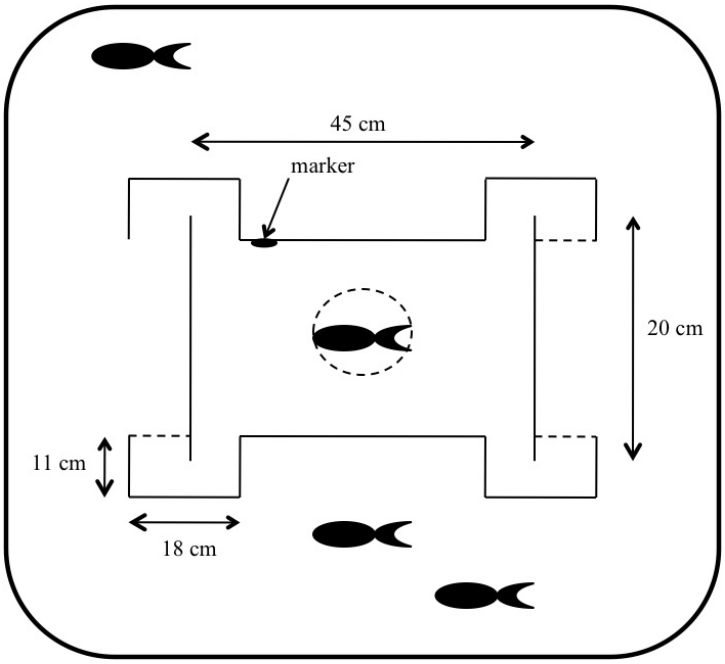

Fig. 2

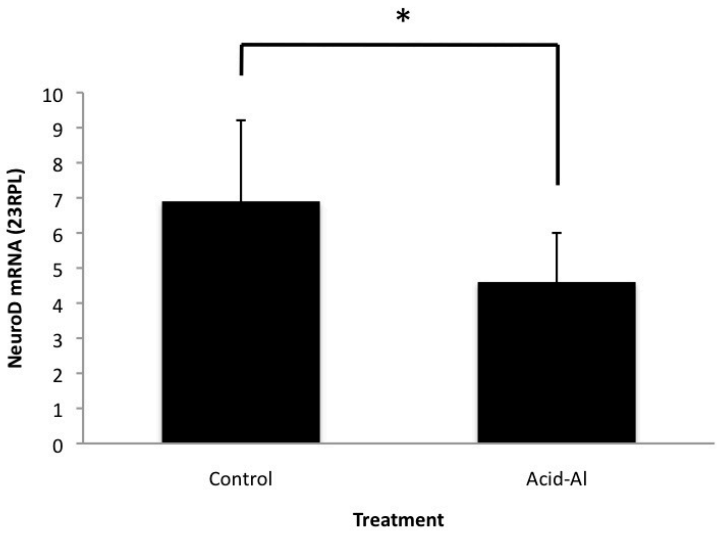

Fig. 3

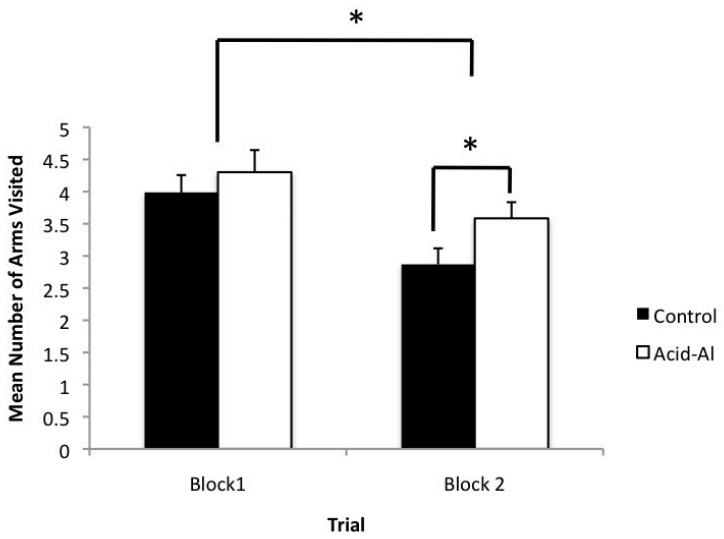

Table 1. Water quality in control and Acid-Al (N=3), mean ± S.D.

	Temp.	Cond.	pH	TOC	Cl^-	NO_{3-}	SO_4^{2-}	Ca	K	Mg	Na	Si
	°C	mS/m		µmol l^{-1}	µmol l^{-1}	µmol l^{-1}	µmol l^{-1}	µmol l^{-1}	µmol l^{-1}	µmol l^{-1}	µmol l^{-1}	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

[a]*N=1*

Table 2. Water concentrations of different Al-fractions in control and Acid-Al, (N= 3-6), mean ± S.D.

	Al tot 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

Table 3. Summary of physiological parameters in control and Acid-Al fish. All values are reported as mean ± S.D. excluding the plasma cortisol levels which are median values.

Physiological Parameters	Control	Acid-Al	p-value
Gill Al (μmol g^{-1} dry weight)	0.11 ± 0.01	2.00 ± 0.15	<0.01
Gill NKA activity (μmol ADP h^{-1} per mg 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