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Neuroendocrine indicators of allostatic load reveal the impact of environmental acidification in fish

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ABSTRACT

When mobilized from surrounding soils and binding to gills at moderately low pH, aluminum (Al) cations can adversely affect fish populations. Furthermore, acidification may lead to allostatic overload, a situation in which the costs of coping with chronic stress affects long-term survival and reproductive output and, ultimately, ecosystem health. The brain's serotonergic system plays a key role in neuroendocrine stress responses and allostatic processes. Here, we explored whether sublethal effects of Al in acidified water affects serotonergic neurochemistry and stress coping ability in a unique land-locked salmon population from Lake Bygelandsfjorden, in southern Norway. Fish were exposed to untreated water with pH 6.5 and 74 μg Al l^{-1} or acidified (pH 5.5) water with different aluminum concentrations ([Al]; 74–148 μg l^{-1}) for 5–6 days. Afterward, effects on stress coping ability were investigated by analyzing plasma cortisol levels and telencephalic serotonergic neurochemistry before and after a standardized acute stress test. Before the stress test, positive dose-response relationships existed between [Al], serotonergic turnover rate and plasma cortisol. However, in acutely stressed fish, exposure to the highest [Al] resulted in reduced cortisol values compared with those exposed to lower concentrations, while the positive dose-response relationship between Al concentrations and serotonergic turnover rate persisted in baseline conditions. This suggests that fish exposed to the highest Al concentration were unable to mount a proper cortisol response to further acute stress, demonstrating that neuroendocrine indicators of allostatic load can be used to reveal sublethal effects of water acidification—and potentially, the environmental impacts of other factors.

1. Introduction

Exposure to environmental contaminants can affect the survival of organisms via direct toxicity. However, more often than not, effects are subtler, including chronic or intermittent activation of physiological stress responses, and changes in sexual behavior, prey-capture capability, and disease resilience. Such impacts have been suggested to mediate the effects on long-term organism survival, life history trajectories, and reproductive output, ultimately compromising ecosystem health (Scholz et al., 2012). In stress physiology research, such suboptimal environmental effects have been linked to the concept of allostasis; i.e., the costs of adjusting stress-coping mechanisms to maintain stable biological functions in a changing environment (Korte et al., 2005; McEwen and Wingfield, 2010; Romero et al., 2009). In addition, one or more intense perturbations resulting in more intense chronic stress may impose deficits in the way the brain and other coping systems respond to additional stressors (McEwen, 2000, 2007). Hence, this organismal state, often referred to as allostatic overload, is associated with more direct fitness-decreasing consequences.

The brain's serotonergic (5-HTergic) system a plays a key role in the integration of behavioral and physiological stress responses in vertebrates (Puglisi-Allegra and Andolina, 2015; Winberg and Nilsson,

Abbreviations: ACTH, adrenocorticotropic hormone; ANOVA, analysis of variance; CRF, corticotropic releasing factor; HPA, hypothalamus–pituitary–adrenal axis; HPI, hypothalamus–pituitary–interrenal axis; HPLC, high-performance liquid chromatography; ICP-MS, inductively coupled plasma mass spectrometry; PAHs, polycyclic aromatic hydrocarbons; PCBs, polychlorinated biphenyls; RIA, radioimmunoassay; 5-HT, serotonin; 5-HIAA, 5-hydroxyindoleacetic acid

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1993), and as such is a central mediator of allostatic processes (reviewed by Beauchaine et al., 2011). Specifically, 5-HT modulates the release of glucocorticoids by interacting with the hypothalamic–pituitary–adrenal (HPA) axis, or its homologue in teleost fishes: the hypothalamic–pituitary–interrenal (HPI) axis. Generally, the glucocorticoids make energy available for acute stress-coping responses (i.e., the "fight or flight" reaction), by stimulating glycogenesis and suppressing maintenance functions of the body (Sapolsky et al., 2000). Furthermore, they can affect central stress-coping mechanisms, including changes in brain 5-HT signaling. In the vertebrate brain, 5-HT-mediated signaling is crucial for energy regulation, neural plasticity, behavioral and emotional control, as well as neuroendocrine responses to stress (Andrews et al., 2015; Lanfumey et al., 2008). Sustained serotonergic activation in several animal species has been associated with chronic stress and stress-induced pathologies, such as depression-like states (Graeff et al., 1996; Krishnan and Nestler, 2011; Lanfumey et al., 2008; Lillesaar, 2011). Moreover, an emerging body of literature suggests that chronic stress may lead to a condition whereby normal stress-coping mechanisms become maladaptive (McEwen, 2000, 2007). Consistent with this view, Moltesen et al. (2016) demonstrated that chronic crowding stress induced changes in telencephalic 5-HTergic neurochemistry and the responsiveness of the HPI axis in farmed rainbow trout (*Oncorhynchus mykiss*); that study also emphasized the importance of controlled challenges, to activate the stress axis, when using physiological stress parameters for investigating the impact of suboptimal environmental conditions on fish. Similarly, Vindas et al. (2016) recently showed that a characteristic of chronically stressed salmon in aquaculture was an inability to mount a serotonergic response to further superimposed stressors; i.e., a clear sign of compromised coping ability under allostatic overload. In line with the concept of allostatic load are the many studies showing that prolonged exposure to aquatic environmental contaminants and other chronic stressors will suppress the cortisol response to an acute stressor in teleost fishes (Brodeur et al., 1997; Hontela et al., 1997). However, to our best knowledge, central allostatic neural regulation has not yet been included in studies investigating the effects of environmental contaminants on the HPI-axis capacity or other coping mechanisms in fish.

The mobilization of aluminum (Al) cations from surrounding soils into surface waters via atmospheric acid deposition can negatively impact the biodiversity and functioning of aquatic ecosystems (Horne and Dunson, 1995; Lovett et al., 2009). Generally, in teleost fishes, Al toxicity in moderately acidic water (pH 5–6) is caused by Al's precipitation and polymerization, resulting in Al deposition on the gill surface. This then induces mucus production and cell swelling, which affects overall membrane permeability, resulting in increased ion effluxes, reduced ion uptake, and gas exchange. The last in particular leads to decreased arterial O_2 tension and raised arterial CO_2 tension (for references, see the review by Gensemer and Playle, 1999). Atlantic salmon (*Salmo salar* L.) seems especially sensitive to water acidification, given that several studies show direct lethal effects of Al cations and low pH in this species (see references in Kroglund et al., 2001), with other work demonstrating sublethal/late effects of acidification on its learning (Grassie et al., 2013) and susceptibility to ectoparasites (Finstad et al., 2012), all of which indicates acidification may impose an allostatic load in this species. A unique landlocked Atlantic salmon population, "Bleke", lives in Lake Bygelandsfjorden in the watershed of the Otra River in southern Norway, where it faced near extinction due to a combination of water acidification and hydropower development (Wright et al., 2017). During the last two decades, this population's size has increased under a restoration program that included the lake's liming and rebuilt spawning areas and fish passages. Yet, the water quality requirements for the Bleka remain largely unknown. The documented sensitivity and effects thereof renders this particular teleost population ideal for testing the tentative hypothesis that long-term exposure to sublethal environmental toxicants impairs allostatic responsiveness of aquatic organisms.

This study's aim was two-fold: Firstly, to explore whether sublethal effects of Al in acidified water are reflected in neuroendocrine changes that are indicative of allostatic load. Secondly, to investigate whether such changes could be applied to gauge the water quality requirements of this land-locked Atlantic salmon population. To address both objectives, fish from the Bleka population were exposed to acidified water with different concentrations of Al for five to six days. Their plasma cortisol levels and telencephalic 5-HT neurochemistry were then determined, both before (baseline) and after a standardized acute stress test, and Al deposition on their gills investigated to quantify the physiological impact of greater Al concentrations.

2. Material and methods

2.1. Experimental animals

Six-month-old (0+ years) Atlantic salmon, originating from the land-locked population in Otra, hatched and reared at the Syrtveit hatchery and weighing 8.3 ± 2.2 g (mean ± standard deviation), were used in the experiment. Before experimentation, all fish individuals were kept in water from the Otra River, with its pH regulated to 6.5, and at a natural water temperature. Due to seasonally low water temperatures (ranging from 2 to 4 °C), the fish were fed at a minimal rate (approximately 0.1% of body weight day^{-1}) before experimentation, they went unfed during the experiment. Moreover, fish were kept in constant dim light by covering half of the rearing tanks, following the standard rearing conditions for Otra River's land-locked Atlantic salmon, before and during their experimentation.

2.2. Experiment protocol

The water pH was set to 5.5 and controlled by Pi regulation of a peristaltic pump, which added H_2SO_4 to the inlet of a 1.6-m-long mixing tube with a pH sensor (Hamilton Polylite +) positioned at the outlet. The inflow to the mixing tube consisted of regular water from the hatchery facility (pH 6.5) at a flow rate of 30 l min^{-1}. The acidified water was collected in a tank with overflow, whereupon it was delivered to four 50 l mixing tanks. Inflow to the mixing tanks was 6 l min^{-1} plus any minor overflow. Aluminum at concentrations of 0.72, 0.36, 0.18, and 0 g l^{-1} were delivered into each mixing tank via peristaltic pumps at a flow rate of 3 ml min^{-1}. Each mixing tank had two outlets linked to two 50-l exposure tanks, which resulted in nominal Al concentrations of 75, 35, 17,5 and 0 µg l^{-1} in each respective pair of exposure tanks.

The experiment was performed over two consecutive parts. The first investigated how the different Al treatments affected the neuroendocrine stress response, and the second considered the effect of Al on gill physiology. The water temperature was 2–4 °C during the entire experiment. Twenty fish were put into each experimental tank 4 days before Al exposure, to diminish any effects of transfer stress; during this period, fish were exposed to regular water from the hatchery (pH 6.5). After this period, the fish were exposed to pH 5.5 and the four Al concentrations in two replicates. In addition, 20 fish in each of one-of-two exposure tanks were kept in the typical hatchery water, at pH 6.5, to serve as the unexposed controls. Water samples to analyze Al concentrations were taken at the end of each study.

To facilitate the sampling in the first study, the fish in one of the tanks in each duplicate were sampled after 5 days and the fish from the other paired tank sampled after 6 days of exposure. Baseline stress levels were sampled by swiftly netting three fish from each exposure tank and directly anesthetizing each in MS 222 at a concentration of 0.5 g l^{-1}. In addition, four individuals from each exposure tank were subjected to confinement stress, by keeping each individual fish in a 1.5-m × 0.5-m × 0.2-m (length × width × depth) aquarium, with the water surface just above the dorsal fin, for 30 min. This acute stress test principle followed previous studies' methodology for detecting both

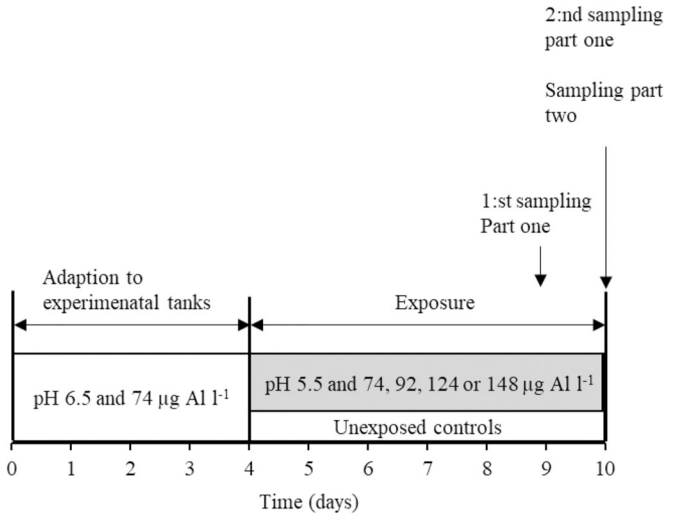

Fig. 1. The experiment spanned two consecutive parts (i.e., part 1 and 2). The first investigated how the different treatments affected the neuroendocrine stress response; the second investigated the effect of Al on gill physiology. In both, the fish were allowed to adapt to experimental tanks in water from the Otra River (total [Al] 74 µg l^{-1} and pH 6.5). After a four-day acclimation period, fish were exposed to water from Otra acidified with H_2SO_4 to pH 5.5 added with different levels of $AlCl_3$ resulting in the total aluminum concentrations of 74, 92, 124, and 148 µg Al l^{-1} in the treatment groups. Control fish were kept in Otra River water having a total [Al] 74 µg l^{-1} and pH 6.5. In study 1, the fish were sampled after 5 and 6 days of exposure (on day 9 and 10) and in study 2, they were sampled after 6 days of exposure (day 10) to the treatments.

heritable and environmentally induced differences in the stress-coping ability of teleost fishes (e.g., Basic et al., 2013; Johansen et al., 2012; Vindas et al., 2016; Øverli et al., 2004). Fish were confinement-stressed in the same water that they had previously been exposed to. After their confinement, fish were netted and anesthetized in MS 222 (0.5 g^{-1}). This resulted in samples sizes of $n = 6$ for the baseline and $n = 8$ for the confinement-stressed condition in each of the five treatments (four Al concentration groups and the control group). Blood (approximately 0.1 ml) was collected from the caudal vasculature of anesthetized fish using a syringe pre-treated with heparin, whereupon the fish were humanely killed by decapitation and the telencephalon quickly dissected out from each of them. Then the blood samples were rapidly transferred to Eppendorf tubes and centrifuged at 1500*g* for 10 min at 4 °C. Following centrifugation, blood plasma samples were frozen on dry ice and stored at −80 °C. Dissected telencephalons were wrapped individually in aluminum foil, frozen on dry ice, and stored at −80 °C.

In the second study, five fish from each tank were sampled and killed by a blow to the head, whereupon their gills were dissected and removed and frozen on dry ice. The experimental procedures in study one and two are summarized in Fig. 1. The experiment was conducted in accordance with the Guidelines of the European Union Council (86/609/EU) and Norwegian legislation for the use of laboratory animals. The experimental protocol was approved by the ethics committee of the Norwegian food safety authority (permit number 14193).

2.3. Analysis of water and gill Al levels

Gill tissue was freeze-dried, weighed, and then digested in concentrated, trace metal-grade nitric acid (HNO_3) overnight at 50 °C. Samples were then diluted to 10% HNO_3 and trace elements measured on an Agilent 7700 Q-ICP-MS. For quality control, we concurrently ran certified reference materials: DORM-4 (fish protein); and DOLT5 (dogfish liver), both from the National Research Council of Canada, and IAEA-436 (tuna Fish flesh homogenate) from the International Atomic Energy Agency. Results are expressed as µg Al per g of gill dry weight. Water samples were analyzed using ICP-MS.

The nominal concentrations of 0, 17.5, 35, and 70 added µg Al l^{-1} corresponded to total aluminum concentrations of 74, 89, 118, and 141 µg Al l^{-1} in the first part and 73, 94, 132, and 152 Al l^{-1} in the second part of the study, respectively. Since the discrepancies between the round fell within the specified accuracy of the method (i.e., 20%) specified by the accredited laboratory of NIVA (https://www.niva.no/en/services/laboratory-services), total Al treatment concentrations are thus reported here as the averaged values of first and second part of the study: namely, as 74, 92, 124, and 148 µg Al l^{-1}.

2.4. Analysis of 5-HT brain neurochemistry and plasma cortisol

The frozen telencephalon samples were homogenized in 4% (w/v) ice-cold perchloric acid (PCA) containing 0.2% EDTA and 94.2 ng ml^{-1} of 3,4-dihydroxybenzyl amine hydrobromide deoxyepinephrine (the internal standard), by using an MSE 100 W ultrasonic disintegrator. Prior to analysis, each sample was thawed on ice, and centrifuged at 17000 rpm for 5 min. Then its supernatant was removed, from which 5-HT and its principal catabolite, 5-hydroxtrindolaceticacid (5-HIAA), were quantified using high-performance liquid chromatography (HPLC) with electrochemical detection. The HPLC system consisted of a solvent-delivery system (Shimadzu, LC-10AD), an auto injector (Famos, Spark), a reverse phase column (4.6 mm × 100 mm, Hichrom, C18, 3.5 µm) and an ESA Coulochem II detector (ESA, Bedford, MA, USA) with two electrodes, at −40 and +320 mV. A conditioning electrode (ESA 5020), with a potential of +400 mV, was employed before the analytical electrodes, to oxidize any possible contaminants present. The mobile phase consisted of 86.25 mM l^{-1} of sodium phosphate, 1.4 mM l^{-1} of sodium octyl sulfate and 12.26 µM l^{-1} of EDTA in deionized (resistance 18.2 MW) water containing 7% acetonitrile brought to a pH of 3.1 with phosphoric acid. Samples were quantified by comparison with standard solutions of known concentrations and corrected for recovery of the internal standard using the HPLC software (CSW, DataApex Ltd, Czech Republic).

Plasma cortisol was analyzed using a radioimmunoassay (RIA). Plasma samples were diluted in 1:2 ratio in a RIA buffer (containing 0.05% NaN_3), followed by heating for 1 h at 80 °C to denature the proteins. After cooling for 1 h, samples were centrifuged at 1500 rpm for 20 min at 4 °C, after which the supernatant containing the cortisol was collected and stored at 4 °C. Samples were assayed in duplicate, with every tube containing 100 µl of a given plasma sample, 200 µl of anti-cortisol antibody (ab1949; Abcam), and 50 µl of hydrocortisone (1, 2, 6, 7-3 H (N); PerkinElmer). The plasma concentration of cortisol was measured by a specific RIA following the procedure described by Mayer et al. (1990), which included a comprehensive validation of the steroid RIA for Atlantic salmon plasma, in addition to a comparison between the above heating method with the extraction, followed by thin-layer chromatographic separation of the steroids. Since no significant difference was found between the two methods, the heat treatment method was chosen. The intra- and inter-assay CV values for the cortisol assay were 6.3% and 12.1%, respectively.

2.5. Statistics

All values are presented as means ± standard error of mean. A residual analysis of treatment groups was performed and the baseline cortisol values were log-transformed to obtain a normal distribution. The effects of acidification and increased Al concentration on plasma cortisol, 5-HT, 5-HIAA, and 5-HIAA/5-HT were investigated separately using one-way analysis of variance (ANOVAs) in the baseline and confinement-stressed fish. Likewise, the effects of acidification and increased Al concentration on the gill-deposited Al were investigated by a one-way ANOVA. Significant differences between treatment groups were investigated by the HSD post hoc test. All statistical analyses were

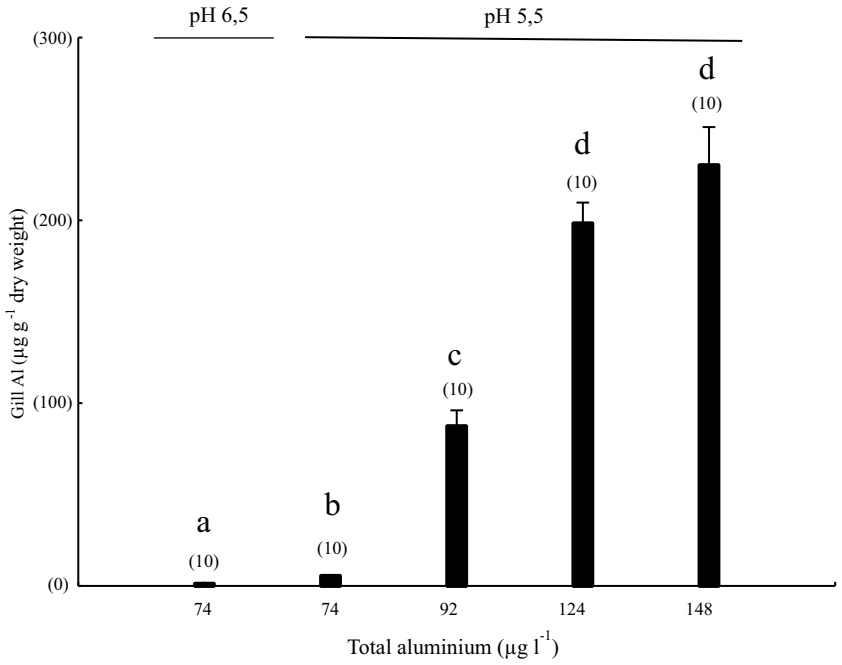

Fig. 2. Aluminum concentrations in the gills of Atlantic salmon (*Salmo salar*) originating from a landlocked population in the Otra River. Fish were exposed to the Otra River's water (total [Al] 74 µg l^{-1} and pH 6.5), or this water acidified with H_2SO_4 to pH 5.5. Different levels of $AlCl_3$ were added to the acidified water (pH = 5.5), generating total aluminum concentrations of 74, 92, 124, and 148 µg Al l^{-1} in the treatment groups. Differing letters indicate significant differences between groups ($P < 0.05$); $n = 6$ in each group.

performed in Statistica v13 (Tibco software).

3. Results

3.1. Gill Al

There was a significant dose-response of gill Al to the increased Al concentrations in the tank water (ANOVA; $F_{(4, 45)} = 89$, $P < 0.001$; Fig. 2). Values for fish exposed to 92, 124 and 148 µg Al l^{-1} were significantly higher than for those exposed to non-acidified (pH 6.5) and acidified (pH 5.5) control water ($P < 0.001$). Moreover, gill Al in fish exposed to 148 and 124 µg Al l^{-1} were significantly higher than in fish exposed to 92 µg Al l^{-1} ($P < 0.05$). There were no significant differences in gill Al between fish exposed to non-acidified and acidified control water ($P < 0.99$), or between fish exposed to 124 and 148 µg Al l^{-1} ($P < 0.28$).

3.2. Neuroendocrine parameters: baseline conditions

The Al treatment in combination with reduced pH affected the baseline levels of plasma cortisol (ANOVA; $F_{(4, 25)} = 3.9$, $P < 0.05$; Fig. 3A). Generally, exposure to Al in combination with reduced pH affected plasma cortisol in a dose dependent manner. This was reflected in that exposure to the highest Al concentration resulted in significantly elevated plasma cortisol levels compared fish exposed to 96 and 124 µg Al l^{-1} ($P < 0.01$ and 0.05 respectively). Furthermore, plasma cortisol was significantly elevated in fish exposed to 74, 124 and 148 µg Al l^{-1} compared to fish exposed to non-acidified control water ($P < 0.01$, 0.05 and 0.001 respectively). However, fish exposed to 96 µg Al l^{-1} did not differ significantly from fish exposed to non-acidified control water ($P < 0.07$). Moreover, in fish exposed to reduced pH, there were no significant difference between fish exposed to 74 and 148 µg Al l^{-1} ($P < 0.11$).

The Al treatments also affected the baseline 5-HIAA/5-HT ratios (ANOVA; $F_{(4, 23)} = 6.2$, $P < 0.01$) in a dose-dependent manner (Fig. 3B). This led to significantly elevated values in fish exposed to 148 µg Al l^{-1} compared with fish exposed to low pH in combination 74, 92 and 124 µg Al l^{-1}, as well with fish exposed to the non-acidified control water ($P < 0.01$, $P < 0.01$, $P < 0.05$, and $P < 0.01$, respectively).

In addition, the Al treatments affected the 5-HIAA concentrations significantly (ANOVA; $F_{(4, 23)} = 3.9$, $P < 0.05$). Specifically, baseline concentrations in fish exposed to 148 µg Al l^{-1} were elevated compared to fish exposed to 92 µg Al l^{-1} and acidified control water ($P < 0.05$ in both cases; Table 1). But no significant differences in baseline 5-HIAA concentrations were detected between fish exposed to baseline non-

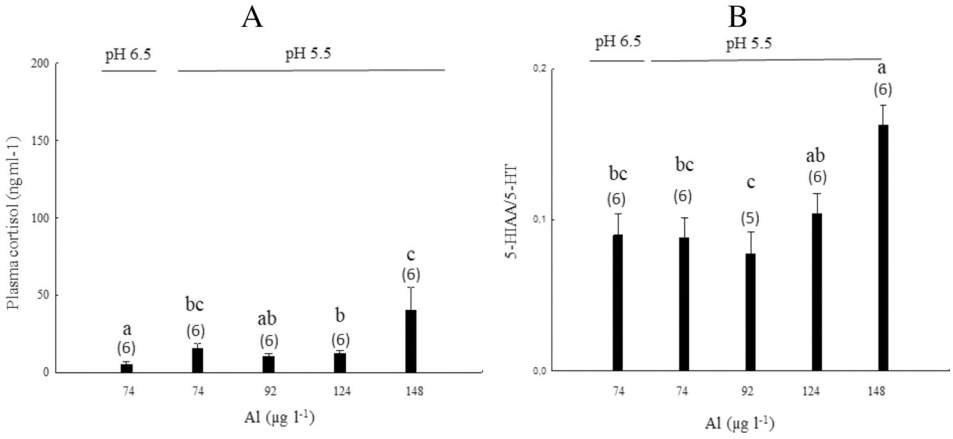

Fig. 3. Plasma levels of cortisol (A) or serotonergic turnover (5-hydroxyindoleacetic acid (5-HIAA)/serotonin (5-HT)) (B) under baseline conditions in Atlantic salmon (*Salmo salar*) originating from a landlocked population in the Otra River. Fish were exposed to the Otra River's water (total [Al] 74 µg l^{-1} and pH 6.5), or this water acidified with H_2SO_4 to pH 5.5. Different levels of $AlCl_3$ were added to the acidified water (pH = 5.5), generating total aluminum concentrations of 74, 92, 124, and 148 µg Al l^{-1} in the treatment groups. Numbers within parentheses correspond to sample sizes of each treatment group; differing letters indicate significant differences between groups ($P < 0.05$).

Table 1
Concentrations of 5-hydroxyindoleacetic acid (5-HIAA) and serotonin (5-HT) under baseline conditions or after 0.5 h of confinement stress in Atlantic salmon (*Salmo salar*) originating from a landlocked population in the Otra River. Fish were exposed to Otra River's water (total [Al] 74 μg l^{-1} and pH 6.5) or this water acidified with H_2SO_4 to pH 5.5. Different levels of $AlCl_3$ were added to the acidified water to generate total aluminum concentrations of 74, 92, 124, and 148 μg Al l^{-1} in the treatment groups.

	Treatment (μg Al l^{-1}, pH)				
	74, 6.5	74, 5.5	92, 5.5	124, 5.5	148, 5.5
Baseline					
5-HIAA	38 ± 7.7(6)^{ab}	33 ± 7.7(6)^{a}	36 ± 8.4(6)^{ab}	47 ± 7.7(6)^{ab}	69 ± 7.7(6)^{ab}
5-HT	509 ± 70(6)^{a}	410 ± 64(6)^{a}	460 ± 70(5)^{a}	460 ± 64(5)^{a}	440 ± 22(5)^{a}
Confinement stress					
5-HIAA	57 ± 7.2(7)^{a}	50 ± 6.8(8)^{a}	59 ± 7.8(8)^{a}	59 ± 6.8(8)^{a}	75 ± 6.8(8)^{a}
5-HT	480 ± 51(7)^{a}	413 ± 47(8)^{a}	450 ± 55(8)^{a}	420 ± 48(8)^{a}	450 ± 48(8)^{a}

Concentrations are ng g^{-1} brain tissue.
Values are mean ± S.E. (N).
Differing letters indicate significant differences between treatments under baseline conditions, or after the 0.5-h confinement stress conditions imposed on the fish.

acidified control water and fish exposed to 92, 124, and 148 μg Al l^{-1}, and acidified control water (respectively $P < 0.88$ and $P < 0.99$, $P < 0.24$, and $P < 0.77$). Moreover, the fish exposed to 92 and 124 μg Al l^{-1} had similar concentrations of 5-HIAA, but the Al treatments did not affect the 5-HT concentration of fish in a significant way (ANOVA; $F_{(4, 23)} = 0.29$, $P = 0.88$; Table 1).

3.3. Neuroendocrine parameters: acute stress test

The Al treatments also affected the plasma levels of cortisol in acute confinement-stressed fish (ANOVA; $F_{(4, 35)} = 4.7$, $P < 0.01$; Fig. 4A), but in a curvilinear manner. Plasma cortisol was significantly higher in fish exposed to either 92 or 124 μg Al l^{-1} than the non-acidified control group ($P < 0.05$ in both cases); however, plasma cortisol in fish exposed 148 μg Al l^{-1} did not differ from fish in the non-acidified control water ($P < 0.99$), and was indeed lower compared to fish exposed to low pH in combination with 92 and 124, μg Al l^{-1} ($P < 0.05$ in both cases).

Fig. 4B shows that Al treatments affected 5-HIAA/5-HT ratio (ANOVA; $F_{(4, 31)} = 4,2$, $P < 0.01$) in a dose-dependent manner in the acute confinement-stressed fish. Exposure to 148 μg Al l^{-1} led to significantly elevated values in fish compared with those exposed to non-acidified and acidified control water ($P < 0.05$ in both cases), and tended to be higher in fish exposed to 148 μg Al l^{-1} than those exposed to 92 μg Al l^{-1} ($P < 0.07$). However, the Al treatments neither affected the 5-HT (ANOVA; $F_{(4, 32)} = 0.28$, $P < 0.89$) nor 5-HIAA ($F_{(4, 32)} = 1.9$, $P < 0.13$) concentrations in telencephalon of the fish (Table 1).

4. Discussion

In this study, we demonstrated a positive dose dependency between brain 5-HT turnover rate and Al concentrations in moderately acidified water. This pattern was observed during baseline conditions and after 30 min of imposed confinement stress in the experimental fish. Although this dose dependency was reflected in baseline cortisol values, it did not follow the same general pattern in the confinement-stressed fish. In contrast to baseline cortisol, exposure to the highest Al concentrations resulted in decreased cortisol values compared with fish exposed lower Al concentrations under confinement-stressed conditions.

Generally, 5-HT controls the HPI/A axis by stimulating the release of corticotropic releasing factor (CRF) from the hypothalamus, resulting in the secretion of adrenocorticotropic hormone (ACTH) from the pituitary. This then stimulates production and secretion of cortisol from the internal cells in the head kidney in teleost fishes and the adrenal cortex in mammals. Several studies have suggested extrahypothalamic regulation of the HPI/A axis activity (McEwen, 2007; Moltesen et al., 2016). For example, in mammals, 5-HT in the limbic system in the telencephalon plays a key role in controlling the HPA axis by discriminating among perturbations that may potentially threaten the allostatic regulation. Such extrahypothalamic HPI/A axis control is supported by several studies in teleost fishes, which showed associations between telencephalic—including limbic—5-HT signaling and HPI axis activity (Höglund et al., 2000; Höglund et al., 2001; Silva et al., 2015; Øverli et al., 2004). Moreover, in a recent study, Moltesen et al. (2016) demonstrated that chronic crowding stress resulted in reduced HPA axis activation which was reflected in the telencephalic 5-HT turnover rate, thus suggesting that 5-HT in this brain region is involved in allostatic

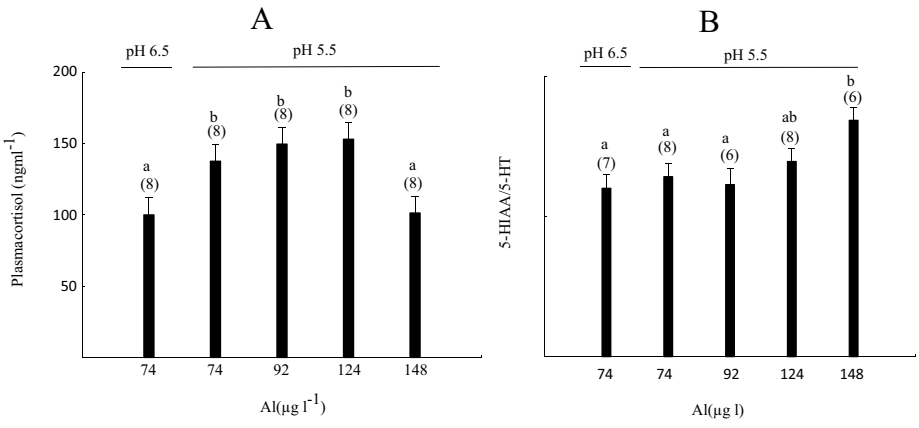

Fig. 4. Plasma levels of cortisol (A) or serotonergic turnover (5-hydroxyindoleacetic acid (5-HIAA)/serotonin (5-HT)) (B) after 0.5 h of confinement stress in Atlantic salmon (*Salmo salar*) originating from a landlocked population in the Otra River. Fish were exposed to water from the Otra River (total [Al] 74 μg l^{-1} and pH 6.5), or this water acidified with H_2SO_4 to pH 5.5. Different levels of $AlCl_3$ were added to the acidified water (pH 5.5), generating total aluminum concentrations of 74, 92, 124, and 148 μg Al l^{-1} in the treatment groups. Numbers within parentheses correspond to sample sizes of each treatment group; differing letters indicate significant differences between groups ($P < 0.05$).

processes in teleost fishes. Other studies have shown the effects of allostatic load further downstream of the stress axis. For example, Madaro et al. (2015) found that repeated unpredictable stress resulted in a lowered cortisol response to a novel acute stressor and induced changes in the expression of genes related to the CRF and glucocorticoid signaling at both the hypothalamic and the pituitary level. Furthermore, earlier work revealed reduced HPI axis reactivity in fish exposed to diverse environmental pollutants, including effluents from bleached craft mills (Hontela et al., 1997; Jardine et al., 1996), heavy metals, PAHs, and PCBs (Hontela et al., 1992), which was associated with changes at the interrenal level. In our study, exposure to the highest concentration of Al resulted in a suppressed cortisol response to acute confinement stress compared with fish exposed to lower Al concentrations. However, this suppression of HPI axis reactivity in fish exposed to the highest Al concentration was not reflected in the telencephalic 5-HT turnover rate. On the contrary, it showed a positive relationship to the water's Al concentrations, both during baseline conditions and in response confinement stress. That the dampened HPI axis reactivity in the highest Al concentrations was not reflected in the changes in telencephalic 5-HT turnover rate agrees with findings of Madaro et al. (2015), thus suggesting that the underlying mechanistic factors operate further downstream in the stress axis. However, such impairment between telencephalic HPI axis reactivity and telencephalic 5-HT turnover rate was not present in fish exposed to chronic crowding stress in work by Moltesen et al. (2016); this suggests the factors involved in dampening the HPI axis reactivity could occur at different levels of the stress axis.

Given that the HPI/A axis is under a multilevel negative feedback control, it may be hypothesized that different types of chronic/repeated stress affect the HPI axis on diverse levels. Madaro et al. (2015), for instance, did not find any effects of chronic uncontrollable stress on the interrenal function, while Brodeur et al. (1997) showed that heavy metals were capable of inducing a lower cortisol response to an ACTH challenge as well as cell differences at the intrarenal level. In mammals, the 5-HT in the limbic system is involved in control of several responses related to social, emotional, and motivational stimuli. This makes it is tempting to suggest that the dampening effects of chronic crowding stress on telencephalic 5-HT and HPI reactivity in the Moltesen et al. (2016) study were related to social/emotional stress and feedback mechanism in limbic structures, while exposure to Al is more closely related to an uncontrollable stress, thereby having effects at the hypothalamic and pituitary level (Madaro et al., 2015). Further research, which should include other neurotransmitter systems and more specific subregions of the brain, is needed to confirm this context dependency in the central mechanism underlying chronic stress and allostatic processes in fish.

In addition to its glucocorticoid function, because cortisol acts as the main mineralocorticoid in teleost fish, it is essential for restoring their hydromineral homeostasis (Wendelaar Bonga, 1997). Accordingly, increased plasma cortisol levels have been suggested to be a response in such fish to the ion imbalance that ensues following their exposure to acidified waters. Our results show that Al exposure in moderate acidic water affects the fish response to confinement stress. This suggests that chronically elevated cortisol levels, associated with its mineralocorticoid functions, can affect the acute response to stressors related to the fight or flight glucocorticoid function of cortisol. In line with this reasoning, Wendelaar Bonga (1997) pointed out that many environmental pollutants evoke an integrated stress response in fish in addition to their toxic effects at the cell and tissue levels in teleost fish. Thus, when coupled to our results, this implies that sublethal effects of acidification may be unraveled by investigating the neuroendocrine response to a controlled challenge.

The results from this study revealed dose-response between Al concentrations (74–148 μg total Al l^{-1}) in the tank water and gill Al agree well with the model proposed by Gensemer and Playle (1999), which predicts 100–200 μg total Al l^{-1} μg l^{-1} in soft water should correspond with Al accumulation on the gill surface. In our study, the highest tank Al concentration resulted in an accumulation of 230 ± 11 (mean ± S.E.) μg g^{-1} Al dry gill weight. The fact that we did not observe any mortality at this concentration is in line with other studies on anadromous Atlantic salmon parr, where lethal effects of episodic exposure (< 10 days) only appear when gill Al exceeds 400 μg g^{-1} dry weight (for those references, see Kroglund et al., 2007). In a recent study, increased mortality together with an Al accumulation of 173 μg g^{-1} dry weight was observed in the land-locked "Bleke" salmon when reared in cages in water with slightly gas oversaturated water (Barlaup, 2018). This finding prompted the authors to suggest that the additive stress of gas oversaturation might be underlying factor for the increased mortality the found. Our results suggest that neuroendocrine indications of allostatic load appear at a gill Al around 200 μg g^{-1} dry weight, and so do lend some support to a poorer ability to cope with additive stressors, such as gas oversaturation. In addition to anthropogenic stressors, including gas oversaturation in waters downstream of power plants, water acidification and other aquatic pollutants, fish experience a number of natural stressors in their environment. So, in further studies that combine such stressors, such as those of gas oversaturation and acidification of water, applying neuroendocrine measures of allostatic load can potentially reveal their effects on stress resilience and other fitness related traits in threatened fish populations.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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