Welfare effects of environmental hypercapnia quantified by indicators based on morphology and allostatic load in Atlantic salmon (*Salmo salar*)

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ABSTRACT

Water supply is a limited resource in most salmon hatcheries, which is compensated by reduced water flow and oxygenation. However, reduced water exchange can lead to accumulation of CO_2, resulting in environmental hypercapnia, which may have negative impacts on fish welfare. Thus, environmental hypercapnia can be a common welfare problem for salmon in hatcheries, and particularly in recirculating systems (RAS).

In this experiment, Atlantic salmon were exposed to chronic environmental hypercapnia during the last 68 days of the freshwater phase, whereupon effects on physiological stress coping mechanisms and morphological welfare indicators were investigated. Effects on stress coping mechanisms were quantified by measuring changes in brain serotonergic chemistry and plasma cortisol at basal levels and in response to a standardized acute stress test. The results show that exposure to elevated CO_2 saturation in the water compromised stress responsiveness of brainstem serotonergic activity, altered osmotic homeostasis, and suppressed growth indicating that fish experience allostatic overload. However, no effects on morphological welfare indicators were observed. This accentuates the need for physiological measures, including physiological responses to controlled challenges to activate the stress axis, when investigating the welfare status of fish reared in systems with potential high CO_2.

1. Introduction

In general, the public concerns regarding animal welfare seem to focus on avoiding animal suffering (Rushen, 2003). In this context, welfare can be defined as the state of the individual in response to its attempts to cope with its environment (Broom, 1986; 1996). Therefore, the mechanisms for coping with the environment are crucial for minimizing suffering and maintaining a high welfare status. For these reasons, the concept of allostatic load, i.e., the costs of adjusting stress-coping mechanisms to maintain stable biological functions in a changing environment (McEwen and Wingfield, 2010; Romero et al., 2009) has now also been incorporated in the study of animal welfare (Korte et al., 2007).

The brain neurotransmitter serotonin (5-hydroxytryptamine; 5-HT)

plays a key role in stress coping in vertebrates (Puglisi-Allegra and Andolina, 2015; Winberg and Nilsson, 1993) and as such, is a central mediator of allostatic processes (reviewed by Beauchaine et al., 2011). Specifically, the serotonergic system modulates the release of glucocorticoids by interacting with the hypothalamic–pituitary–interrenal (HPI axis in teleost fish) or-adrenal (HPA axis in mammals) axis. Glucocorticoids, in turn, act as adaptive hormones, making energy available for acute stress-coping responses by stimulating gluconeogenesis and suppressing maintenance functions of the body (Sapolsky et al., 2000). Cortisol is the dominant glucocorticoid in teleost fish, and as such, its response to a stressor must be finetuned to ensure proper response. Still, an emerging body of literature suggests that when the stress response is maintained over prolonged periods of time (i.e. chronic stress) it may lead to allostatic overload, a condition whereby normal stress-coping

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mechanisms become maladaptive or even detrimental (McEwen and Wingfield, 2010; McEwen, 2007). Consistent with this view, Moltesen et al. (2016) demonstrated that chronic crowding stress induced changes in central 5-HTergic neurochemistry and the responsiveness of the HPI axis in farmed rainbow trout (*Oncorhynchus mykiss*). Moreover, in the latter study, neuroendocrine responses to a standardized stressor was put forward as indicators of allostatic overload and compromised welfare in fish.

Stocking density, water quality and type of enclosure are key components that have been associated with welfare issues in farmed salmonid fishes. Detrimental conditions are associated with the aforementioned neuroendocrine stress responses, as well as with external body condition, such as fin and body damage (Ellis et al., 2002). This is especially true for semi- and fully-closed containment rearing systems, including recirculating aquaculture systems (RAS). As a result of high fish density and low water exchange rates per fish biomass, carbon dioxide can accumulate in these systems (Summerfelt et al., 2000; Hosfeld et al., 2008). This can lead to environmental hypercapnia, a situation where the rearing water is super-saturated with carbon dioxide. Generally, environmental hypercapnia affects blood-gas exchange which can result in changes in plasma electrolytes, haematocrit and haemoglobin content (Gilmour, 1998; Hayashi et al., 2004). Accordingly, it has been pointed out as one of the largest production-limiting factors in RAS (Khan et al., 2018). Still, studies regarding its effects on plasma cortisol show elusive results, and species-specific differences have been suggested as an underlaying factor for this discrepancy (Petochi et al., 2011). Previous studies in salmonid fishes show mainly acute effects of environmental hypercapnia on cortisol release (Fivelstad et al., 1999). However, it is yet to be evaluated if long term environmental hypercapnia imposes increased allostatic load, which in turn compromises stress coping mechanisms and animal welfare.

In this framework, the aim of the current study was to investigate long-term effects of environmental hypercapnia on the brain serotonergic system, HPI axis reactivity and morphological welfare indicators in farmed Atlantic salmon (*Salmo salar*). In order to accomplish this, salmon were exposed to elevated CO_2 concentrations during the 68 last days of the freshwater phase. This period was chosen since the risk for being exposed to hypercapnia are highest in the end of the freshwater phase when rearing densities are highest. Effects of hypercapnia on the serotonergic system and HPI axis reactivity were assessed by changes in 5-HT neurochemistry and plasma cortisol induced by an acute confinement stress test. In addition, effects on morphological welfare indicators were investigated, along with some supplemental haematological parameters.

2. Material and methods

2.1. Experimental fish

The experiment was conducted at the Institute of Marine Research, Matre Research Station, Norway. Atlantic salmon (SalmoBreed strain) were hatched and start fed at the Lønningdal Breeding station (Benchmark inc., Norway) and transported as fry (~3 g size) to Matre the 12th of May 2017. The fish were held in three 500 l tanks at 12 °C (>90% O_2 saturation) freshwater until they were individually tagged with PIT-tags (12 mm, FDX-B tag, Biomark Inc. Idaho, USA) the 10th of August. Individual weight and length were recorded at tagging, whereupon 540 fish were distributed into six 500 l tanks ($n = 90$ per tank). Three tanks were designated to each of the two treatment groups; environmental hypercapnia and control, resulting in $n = 270$ per treatment. The tanks were supplied with filtered river water with ambient river temperature and at a flow rate (15 l min^{-1}) which secured oxygen saturation > 85%. The fish were under a continuous photo regime (24:0 D:L) until the 28th of August. Thereafter smoltification was initiated by 6 weeks of 12:12 D: L and reintroduction to a continuous light regime. All fish were vaccinated (Aquavac 6 vet. MSD) on the 12th of October.

2.2. Experiment set-up

Environmental hypercapnia was initiated on the 4th of September (see Production of CO_2 enriched water) and were maintained for 68 days. The *Control* treated group was given a flow rate of 15–30 l min^{-1} which secured oxygen saturation > 85% and prevented hypercapnia for 68 days. The CO_2 concentration was calculated (see next section) from pH measurement in water samples from mid depth within the experimental tanks every 5th day and was maintained <10 mg l^{-1} (mean pH 6.2) for the *Control*. The *Hypercapnia* group was provided with CO_2 enriched water and a stepwise upregulation of CO_2 concentration to simulate the deteriorating effect of growing biomass, where the highest concentration was chosen based on the sub-lethal effects showed by Fivelstad et al. (2003a, 2003b). The CO_2 concentration was periodically increased from 7.5 mg l^{-1} (pH 5.84) between Day 1 and 7, 15.9 mg l^{-1} (pH 5.55) between Day 7 and 16, 20.42 mg l^{-1} (pH 5.40) between Day 16 and 50, and 23.5 mg l^{-1} (pH 5.27) between Day 50 and 68. A summary of the treatments is presented in Fig. 1. The flow rate into each Hypercapnia tank was gradually increased from 3.5 l to 5 l min^{-1} over the 68 days of treatment to secure >85% O_2 saturation. Fish ($n = 3$ per tank; 12 per water treatment) were sampled for baseline stress levels by swiftly netting the fish from the tank and then putting them directly into a lethal bath of metacaine (Finquel vet, 1 g l^{-1}; Western Chemical Inc., Washington DC, USA). In addition, fish for the acute stress test were netted from the tanks and were confined individually for 30 min ($n = 3$ per tank; 12 per water treatment) within a perforated and transparent plastic container (30 × 15 × 10 cm) in a 500 l tank with continuous water flow to prevent hypoxia. After confinement, fish were put in a lethal bath of metacaine. Sampled fish were measured for length (fork length) and weight before blood was sampled from the caudal vein with heparinized syringes. The blood samples were centrifuged for 5 min at 4 °C, 8000 g and plasma was frozen and stored at −20 °C for later analysis of cortisol levels. Following this, the brains were excised and the brain stem (not including the cerebellum) was dissected out. The brain stems were covered in aluminium foil and immediately frozen in dry ice. In addition, a subsample of fish (n = 3 per tank; n = 12 per treatment), were sampled directly from the holding tanks in order to collect blood samples (as described above) for measurements of haematocrit, haemoglobin and plasma Cl$^-$, Na$^+$, and osmolality (mOsm). The sampling procedures was finished within <5 min from netting of each fish. All fish were weighed and measured for length at tagging (experimental Day −25 relative to start of hypercapnia treatment), vaccination (Day 38) and after the hypercapnia treatment period (Day 68), Fig. 1. Mortalities over the entire experimental period consisted of 16 individuals in the control group and 29 individuals in the hypercapnia treated group. This resulted in $n = 254$ and 241 in the control and hypercapnia treated groups, respectively.

2.3. Production of CO_2 enriched water

Environmental hypercapnia was induced in a two-step process involving a stock solution and a mixing tank. Pressurized CO_2 gas (AGA, Germany) was dissolved into the stock solution tank through a fine diffusor. Thereafter stock solution was added to a volume of crude water in the mixing tank to make a final CO_2/water solution with a constant CO_2 level. Adequate mixing of the water in both tanks were secured by submerged aquarium circulation pumps. The gas pressure from the CO_2 cylinder was reduced until the gas cloud emerging from the diffusor failed to reach the water surface, to ensure that the CO_2 bubbles from the diffusor were totally assimilated in water. The stock solution tank contained 150 l and the water volume was kept constant by input of new crude water through a flotation valve as the solution was consumed. The amount of CO_2 added to the stock solution tank was regulated by a Liquiline CM 442 Universal four-wire multichannel transmitter (Endress and Hauser, Germany) with input from a submerged Memosense Liquiliner pH sensor (Endress and Hauser,
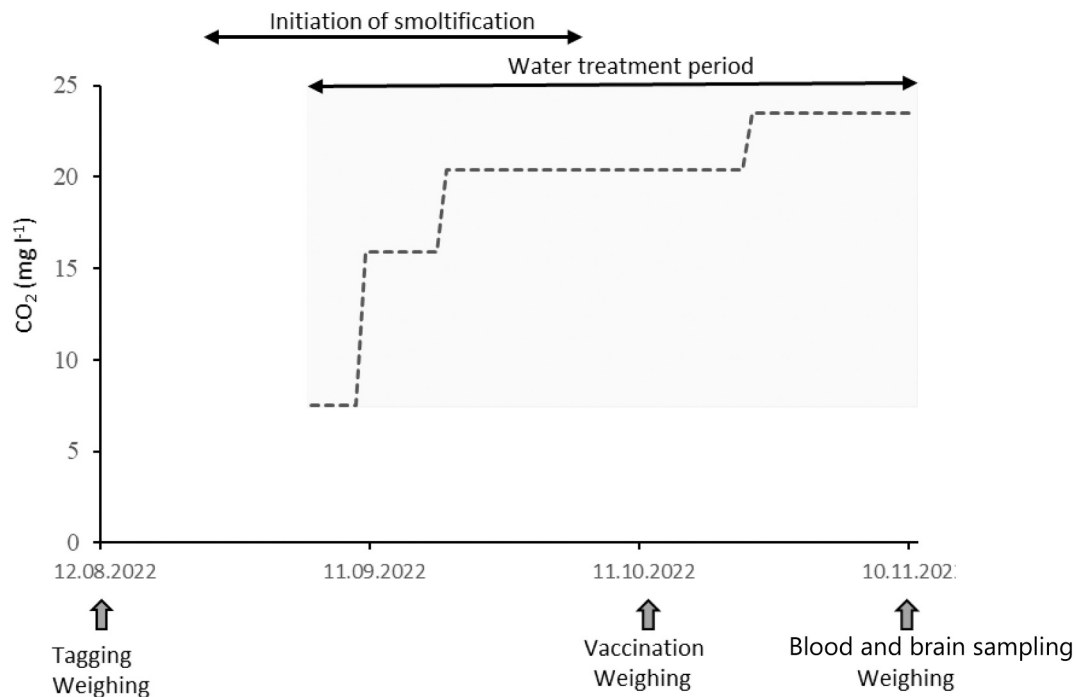
Fig. 1. Diagram illustrating activities throughout the experimental period. Weight and length measures were conducted at tagging, vaccination, and finally after smoltification at the end of the CO_2 water treatment period. A light cycle of 12:12 DL was applied for 42 days to induce smoltification, otherwise fish were kept under continuous light (24:0 DL). During the water treatment period the hypercapnia treated group of fish was exposed to increasing levels of CO_2 while the control treated group was kept in normal holding water without addition of CO_2.

Germany). The controllers limit switch (on/off) operated a magnet valve that opened for CO_2 gas when pH exceeded the pre-set value of pH = 4.1. The stock solution was pumped with a dose pump (IWAKI EWN-R C36, Japan) into a mixing tank (tank 2) similar to the stock solution tank, also supplied with a flotation valve for new water to keep the volume constant. The pH setpoint in the stock solution was chosen to balance the pump capacity and to maintain the target pH in the mixing tank. The CO_2 content in the mixing tank was controlled by a second identical pH sensor giving feedback to the dose-pump via a controller in the transmitter. The pump received a 4–20 mA signal from the controller allowing the pump to give a modulated response to keep the pH constant according to a setpoint value. The water solution from the mixing tank was continuously pumped (IWAKI MD-30RZ, Japan) to the experimental tanks where the fish were kept. The CO_2 concentrations (mgl^{-1}) in the exposure tanks was calculated by values of total alkalinity, pH and temperature using the CO2sys.exe program, (Pierrot et al., 2006). Total alkalinity was determined by end-point titration (TIM840, NB pH/EP/IP Routine Potentiometric Titrator, Radiometer Analytical Denmark) and adjusted by Gran-calculation to correct values. Total alkalinity was determined on weekly basis to adjust for changes in crude water alkalinity. Titrant was 0,05 M HCl, and the 100 ml samples were pre-dosed with 3 ml titrant to seed up the analysis. The pH set value in the transmitter was adjusted on demand after each total alkalinity determination to obtain the correct CO_2 content for exposure water.

2.4. Morphological welfare indicators

At the final sampling after the period with environmental hypercapnia, welfare indicators were scored during weighing and length measurements, using the scoring system of morphological welfare indicators in Noble et al. (2018). In short, the scoring levels range from 0 to 3, where 0 indicate no deviation from normal, 1 minor deviation, 2 clear deviation and 3 severe deviation. Morphological indicators consisted on evaluating eye injuries, scale loss, hemorrhage, wounds, jaw and vertebral deformities, emaciation, fin status and opercular damages.

The latter was scored by the following where 0 referred to a non-damaged operculum which covers the gills, 1 to that the operculum partly covered the hindermost gill, 2 to that the operculum leaved the hindmost gill uncovered and 3 to that the hindmost gill was uncovered on both sides of the head.

2.5. Analysis of 5-HT brain stem neurochemistry

Frozen brain stem samples were homogenized in 4% (w/v) ice-cold perchloric acid (PCA), containing 0.2% EDTA and an internal standard (94.2 ng ml^{-1} of 3,4-dihydroxybenzyl amine hydrobromide deoxyepinephrine), by using an MSE 100 W ultrasonic disintegrator. The brain tissue/4% PCA solution ratio was ≈ 10%. Prior to analysis, each sample was thawed on ice, and centrifuged at 17,000 rpm at 4 °C for 5 min. Subsequently, the supernatant was removed. 5-HT and its principal catabolite, 5-Hydroxyindolacetic acid (5-HIAA) in the supernatant were quantified using high-performance liquid chromatography (HPLC) with electrochemical detection. Generally, the ratio between monoamine catabolite and parent monoamine have been related to release and production and thus have been used as proxy for monoaminergic activity (Shannon et al., 1986). In the present study serotonergic activity was quantified by the [5-HIAA]/[5-HT] ratio. The HPLC system consisted of a solvent-delivery system (Shimadzu, LC-10 CE), an auto injector (Famos, Spark), a reverse phase column (4 × 150 mm, C18, ReproSil-Pur 120 C18 5 μm) and an ESA Coulochem II detector (ESA, Bedford, MA, USA) with two electrodes, at −40 mV 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 of sodium phosphate, 1.4 mM of sodium octyl sulfate and 12.26 μM 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).

2.6. Analysis of plasma cortisol

Cortisol in plasma was quantified using validated ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) analysis as described in Aerts et al. (2015).

In brief, after defrosting, the volume of plasma was standardized at 1 mL and pipetted into a 12 ml tube. Subsequently, 3990 µl of water (Type I) and 10 µl of a cortisol-d$_4$ of 0.5 µg/l was added as internal standard. When the available plasma volume was <1 ml, the volumes of water and internal standard were adjusted accordingly. The mixed solution was vortex-mixed for 30 s to homogenize, ultra-purified using solid phase extraction and analyzed on an Acquity UPLC BEH C18 (1.7 µm; 2.1 mm × 100 mm) column by means of UPLC-MS/MS (Xevo TQS, Waters, Milford, USA). Since in future research matrix-matched calibration curves are not feasible, calibration curves were made in H2O/MeOH (80:20, *v*/v). Subsequently, the stock factor is 100 and results were corrected. Data analysis was performed using Targetlynx software from Waters. Results were reported as the value (ng/mL or µg/l) ± the expanded measurement uncertainty (U) (ng/ml or µg/l) with a coverage factor (k) of 2 (95% confidentiality interval).

2.7. Analysis of blood parameters

Shortly after blood sampling, small subsamples were centrifuged in capillary tubes (StatSpin MP Centrifuge) to measure the haematocrit in duplication as the fraction of red blood cells of the total blood volume. Simultaneously, haemoglobin was measured with an assay kit using 10 µl of blood (MAK115, Sigma-Aldrich). The mean corpuscular haemoglobin concentration (MCHC) was then calculated as haemoglobin divided by the haematocrit percentage. The remaining blood was centrifuged at 5000 *g* for 5 min in Eppendorf tubes. The plasma supernatant was then transferred to new Eppendorf tubes and stored at −80 °C for later analyses.

The plasma osmolality was quantified with freeze point determination in 20 µl subsamples using a Fiske 210 Micro-Sample Osmometer (Advanced Instruments), and the concentration of plasma Na$^+$ and Cl$^-$ were measured using ion selective electrodes in a Cobas 9180 electrolyte analyzer (Roche Diagnostics).

2.8. Statistics

Values are presented as mean ± standard error of the mean (S.E.M.) unless specified otherwise. Effects of hypercapnia on blood parameters (haematocrit, haemoglobin and plasma Cl-, Na+, and osmolality) were analyzed by student's *t*-test. Effects of hypercapnia on brainstem serotonergic activity weres analyzed with a two-way analysis of variance (Two-way ANOVA), with stress conditions and water treatment as independent variables. Serotonergic activity was log-transformed to obtain normal distribution and homoscedasticity. The distribution of the cortisol data did not allow analysis with a two-way ANOVA. Instead, effects on plasma cortisol were analyzed by a one-way Kruskal Wallis ANOVA with multiple comparisons and control-baseline, control-acute stress, hypercapnia-baseline and hypercapnia-acute stress as independent variables. Effects on growth and condition factors were investigated with a repeated measures ANOVA, with time as dependent variable and water treatment as an independent variable. Nested design ANOVAs were not considered since there were no significant tank effect within treatments (growth $P < 0.24$ and condition factor $P < 0.10$). Differences between treatment groups were investigated by conducting Tukey HSD post hoc tests. Finally, since there were no significant tank effects on opercular damages (Kruskal-Wallis ANOVA; $H_{(5, 460)} = 8.6$, $P < 0.12$), the effect of hypercapnia on opercular damages were analyzed with a non-parametric Mann-Whitney *U* test with water treatments as independent variables. All statistical analyses were performed in Statistica v13 (Tibco software).

3. Results

3.1. Fish growth

There were significant interaction effects between water treatment and time on fish weights (two-way repeated ANOVA; $F_{(3,490)} = 50$, $P < 0.001$). Fish exposed to environmental hypercapnia showed significant lesser weight than control treated fish in measures done during the water treatment period (vaccination and smoltification, Fig. 2 A; $P < 0.001$). This weight difference was not present at the start of the experiment 30 days before the water treatment period ($P < 0.99$). For complete ANOVA values see Fig. 2 A.

There were also significant interaction effects between water treatment and time on condition factor ($F_{(3, 490)} = 15$, $P < 0.001$). Fish exposed to environmental hypercapnia showed significantly lower condition factor than control treated fish during the water treatment period (vaccination and smoltification, Fig. 4 B; $P < 0.001$). This water treatment induced difference was not present before water treatment period ($P < 1.0$). Complete ANOVA values are presented in Fig. 2 B.

3.2. Blood parameters

The haemoglobin concentration was significantly elevated in the hypercapnia group ($t_{(22)} = 2.5$; $P < 0.05$), while the haematocrit appeared to be slightly elevated compared to controls, however, this difference was not significant ($t_{(22)} = 1.6$; $P = 0.125$) (Fig. 3 A,B). The resultant MCHC was therefore unaffected by treatment ($t_{(22)} = 0.21$; $P = 0.214$) (Fig. 3 C). Acclimation to hypercapnia altered the ion-regulatory homeostasis of Atlantic salmon smolts by significantly reducing plasma osmolality, Na$^+$, and Cl$^-$ compared to control conditions ($t_{(22)} = -2,6$; $P < 0.05$, $t_{(22)} = -4.9$; $P < 0.001$, and $t_{(22)} = -9.0$; $P < 0.001$, respectively) (Fig. 3 D,E,F).

3.3. Stress responsiveness: plasma cortisol and brain stem serotonergic activity

The Kruskal Wallis ANOVA indicated significant differences in plasma cortisol between the four experimental groups; control baseline, hypercapnia basline, control exposed to an acute stress test and hypercapnia exposed to an acute stress test ($H_{(3,36)} = 29.5$, $P < 0.001$). More specific, the acute stress test resulted in a significant elevation of plasma cortisol compared to baseline in control ($P < 0.001$) and hypercapnia treated ($P < 0.001$) fish, Fig. 4. However, there were no significant differences between stress tested control and hypercapnia treated fish ($P < 0.16$). Moreover, there were no significant diffrences between control and hypercapnia treated fish in basline cortisol ($P < 0.11$).

5-HT activty showed significant interaction effects between hypercapnia and the acute stress test (two-way ANOVA: $F_{(1,32)} = 6.8$, $p < 0.05$). Baseline values were significantly higher in fish exposed to environmental hypercapnia compared to control treated fish ($P < 0.001$). Moreover, control treated fish reacted with an increase of 5-HT activity in response to the acute stress test ($P < 0.001$). In contrast to this, fish exposed to hypercapnia did not show a significant change in 5-HT activity to acute stress ($P < 0.54$). For complete two way ANOVA values see Fig. 5.

3.4. Morphological welfare indicators

Except for opercular damages, we observed very few external deviations (one fish with vertebral deformities and two fish showing wounds). Still, there were no significant differences in opercular damages between treatment groups (Mann-Whitney U = 26,293, $P < 0.82$; Fig. 6).
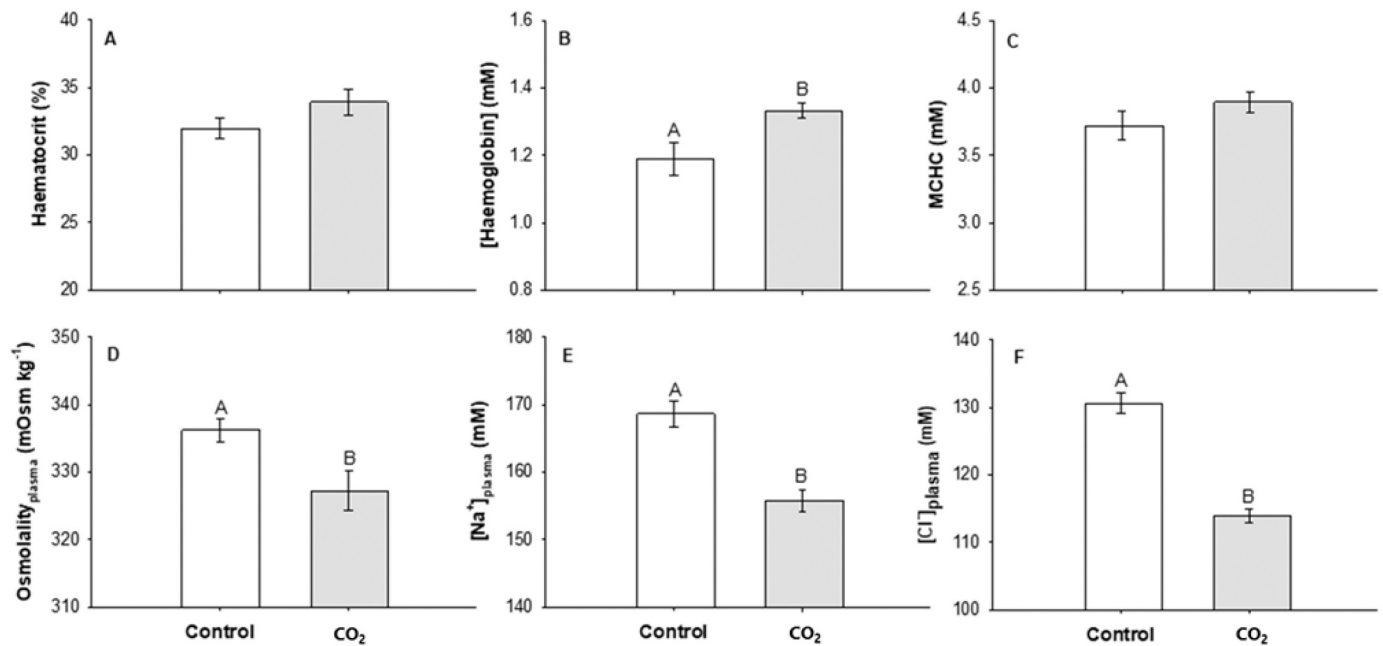
Fig. 2. Weight (A) and condition factor (B) trajectories of Atlantic salmon exposed to environmental hypercapnia (CO_2) or control groups during the 68 last days of the freshwater phase. There were no measurements done at start of the CO_2 water treatment period and estimated trajectories are indicated by dotted lines. * indicate significant ($P < 0.05$) differences within the same date. $n = 254$ and 241 for control and hypercapnia treated groups, respectively.
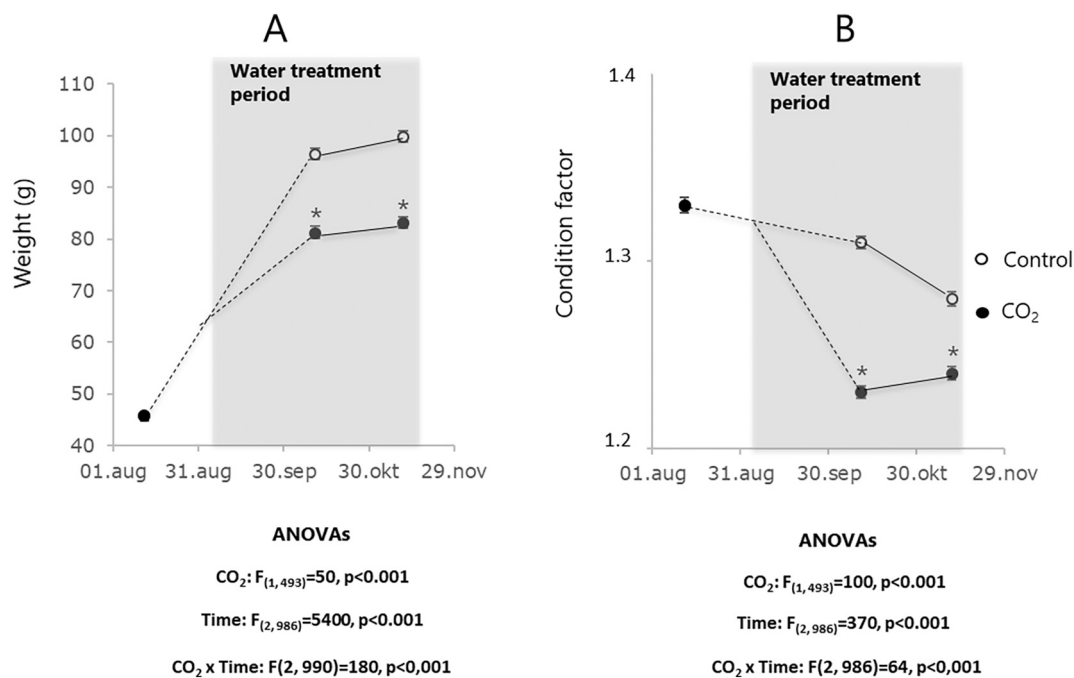

Fig. 3. Blood haematocrit (A), haemoglobin (B), as well as the mean corpuscular haemoglobin concentration (MCHC; calculated as the ratio of haemoglobin to the haematocrit; C) osmolality (D) sodium (Na^+; E) and chloride (Cl^-; F) concentration, parameters in Atlantic salmon smolts after exposure to environmental hypercapnia (CO_2) or control water during the last 68 days of the freshwater phase. Values with different letters indicate significant differences ($P < 0.05$). $n = 12$ in each group.

4. Discussion

This study demonstrates that exposure to environmental hypercapnia during the 68 last days of the freshwater phase is associated with altered osmoregulatory homeostasis, and suppressed brain 5-HTergic stress reactivity, together with suppressed growth and a reduced condition factor in Atlantic salmon smolts.

There is comprehensive knowledge regarding various long-term effects of hypercapnia in fish due to a substantial body of research on the effects of increased atmospheric carbon dioxide levels and acidification of the sea (for references see reviews by Heuer and Grosell, 2014; Ellis et al., 2017; Esbaugh, 2018). However, studies on the stress physiological impact of environmental hypercapnia in freshwater fish kept at CO_2 levels relevant for RAS have mainly been focusing on acute effects (1–8
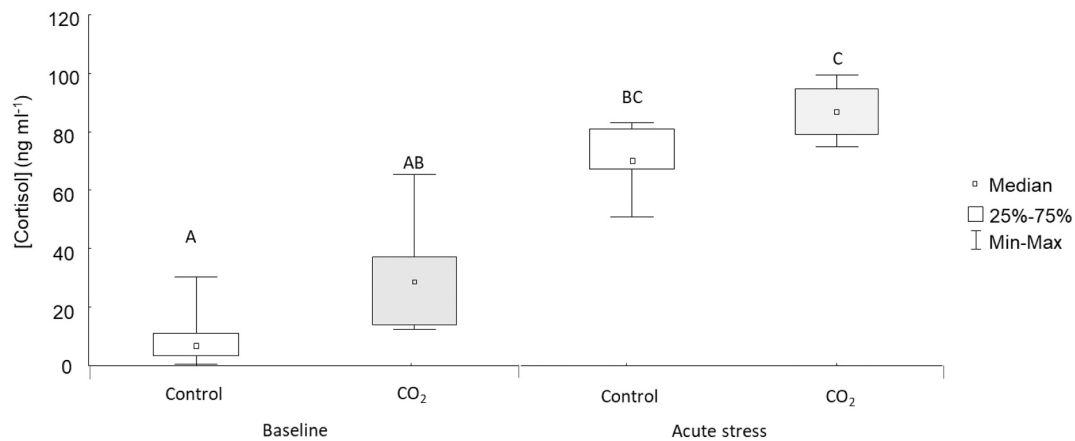
Fig. 4. Baseline and post-acute stress plasma cortisol in Atlantic salmon smolts after exposure to environmental hypercapnia (CO_2) or control water during the last 68 days of the freshwater phase. Values with different letters indicate significant differences ($P < 0.05$), $n = 9$ in each group.

Acute stress: $F_{(1, 32)}=20$, $p<0.001$

CO_2: $F_{(1, 32)}=58$, $p<0.001$

Acute stress x CO_2: $F_{(1, 32)}=6.8$, $p<0.05$

Fig. 5. Baseline or post-acute stress brain stem serotonergic activity, quantified as the ratio between the serotoninergic metabolite 5-Hydroxyindoleacetic acid (5-HIAA) and serotonin (5-HT), in Atlantic salmon (*Salmo salar*) smolts after exposure to environmental hypercapnia (CO_2) or control water during the last 68 days of the freshwater phase. Values with different letters indicate significant differences ($P < 0.05$), n = 9 in each group.
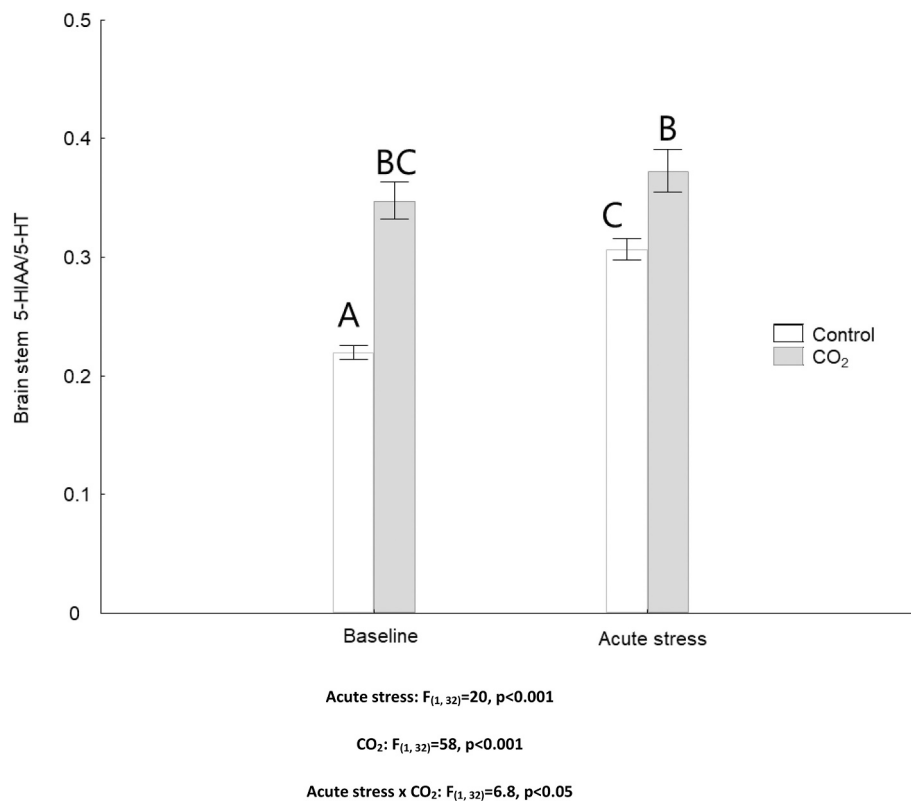
days of exposure; Fivelstad et al., 1999; Evans et al., 2005, Perry and Gilmour, 2006). The primary consequence of acute hypercapnia exposure is a respiratory-acidosis that the fish will attempt to correct for via changes in ion-balance, driven by branchial ion-exchange with the environment. This is typically characterized by a gradual upregulation of plasma bicarbonate together with a downregulation of plasma Cl^-, where after blood pH becomes compensated while pCO_2 remains elevated (Damsgaard et al., 2015; Hvas et al., 2016; Brauner et al., 2019). The effects of chronic hypercapnia have previously been assessed in seawater-adapted Atlantic salmon post-smolts where the fish showed typical compensatory acid-base responses (Gharbi et al., 2019). However, our study was performed in freshwater that has a much reduced

buffer capacity and a reversed osmotic gradient relative to seawater. As such, the sensitivity to hypercapnia in Atlantic salmon would presumably be greater in freshwater owing to a lower availability of counter exchange-ions and a greater potential reduction in water pH levels. Our data shows that the major plasma ions along with plasma osmolality were downregulated in fish after 68 days of exposure to environmental hypercapnia in freshwater. While we did not measure the full acid-base status of the fish, the reduction in plasma Cl^- is in accordance with CO_2 acclimated fish following acid-base compensation (e.g., Brauner et al., 2019). However, the causes for a reduction in plasma osmolality that also was partly driven by a reduction in Na^+ are unknown to us, but this could suggest a general struggle to maintain an adequate ion-balance
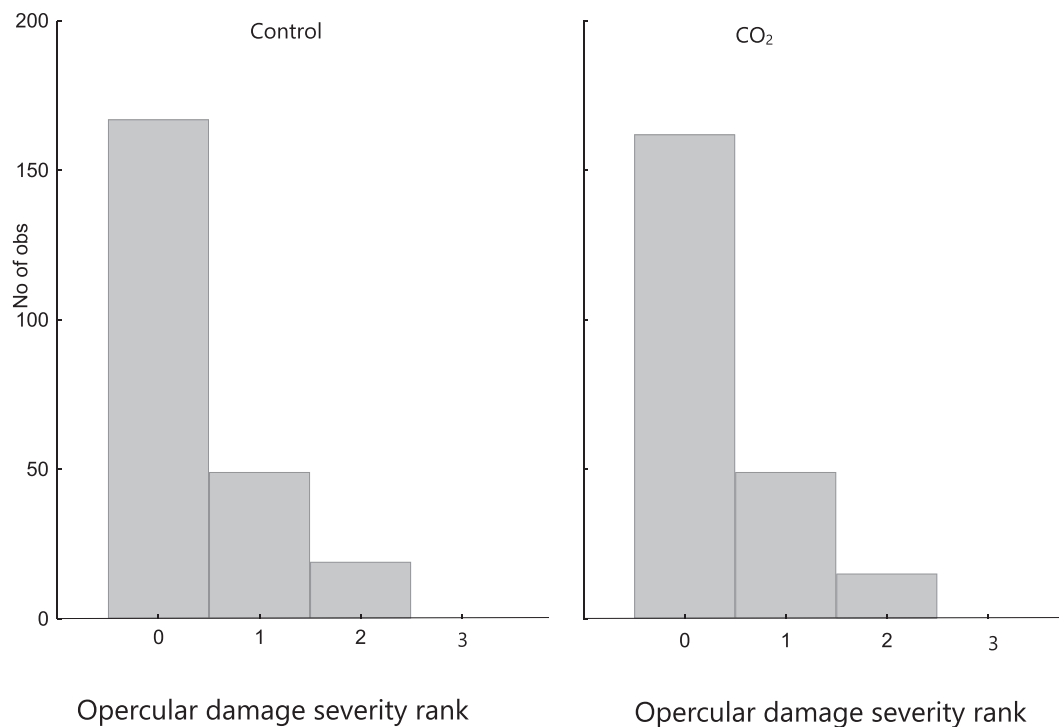
Fig. 6. Histograms of opercula damages in Atlantic salmon smolts after being exposed to environmental hypercapnia (CO_2) or control water during the last 68 days of the freshwater phase. Damage severity was ranked from 0 to 3, where 0 referred to a non-damaged operculum which covers the gills, 1 to that the operculum partly covered the hindermost gill, 2 to that the operculum left the hindmost gill uncovered and 3 to that the hindmost gill was uncovered on both sides of the head.

following several weeks of chronic hypercapnia exposure. Still, it is important to address that the CO_2 concentration was step-wise increased in our study (Fig. 1) and it cannot be excluded that the fish could have adapted to the highest CO_2 level if this had been maintained over a longer period of time. Moreover, it is important to note that hypercapnia is associated with lowering pH, an effect which is dependent on the buffer capacity of the water. In fresh water, toxic effects of environmental acidification are largely dependent on metal ions binding to the gills, and thereby affecting membrane permeability (for references, see the review by Gensemer and Playle, 1999). Following this, Fivelstad et al. (2003a, 2003b) showed increased levels of Al on the gills together with lowered plasma Cl^- in salmon parr exposed to environmental hypercapnia in soft water (freshwater with low alkalinity). Accordingly, some of the changes in blood chemistry in the present study could be related to changes in gill membrane permeability induced by acidification of the water.

Generally, allostatic overload can be defined as a physiological state when unpredictable/uncontrollable chronic or repeated stress impose deficits in biological coping mechanisms (McEwen, 2000; McEwen, 2007; Schreck, 2000; Vindas et al., 2016). Moreover, 5-HT-mediated signalling is crucial for energy regulation and neuroendocrine responses to stress (Andrews et al., 2015; Lanfumey et al., 2008), and as such, is central in stress coping and allostatic processes (reviewed by Beauchaine et al., 2011). In accordance with this, sustained serotonergic activation has been associated with chronic stress in several animal species (Graeff et al., 1996; Lanfumey et al., 2008; Lillesaar, 2011). Similar functions of the 5-HT system have been demonstrated in fish. For example, Vindas et al. (2016) showed sustained elevated brainstem 5-HT activity in chronically stressed moribund fish, and that these fish did not respond with an additional increase in 5-HT activity when exposed to an acute stressor, while their cortisol response to the acute stressor remained intact. Interestingly, our results show a similar pattern; environmental hypercapnia increased baseline brainstem 5-HT activity, reaching similar values as acutely stressed fish where incapable of responding to the acute stressor with an increased 5-HT activity,

while the cortisol response to the acute stressor persisted. Recently, it has been suggested that changes in the relation between HPI axis reactivity and central 5-HT activity is an indicator of increased allostatic load in fish (Höglund et al., 2020; Höglund et al., 2021). Following this view, the current results clearly show that environmental hypercapnia affects stress coping mechanisms and impose increased allostatic load in farmed Atlantic salmon.

In accordance with our results, long-term exposure to environmental hypercapnia has been shown to result in growth reduction in rainbow trout (*Oncorhynchus mykiss*; Smart et al., 1979), and Atlantic salmon (Fivelstad et al., 1999; Fivelstad et al., 2003a, 2003b; Hosfeld et al., 2008; Gharbi et al., 2019). Similar to the aforementioned studies, environmental hypercapnia resulted in decreased condition factor in this experiment. The reduced growth and condition factor in fish exposed to high CO_2 levels has been suggested to be related to stress induced energy expenditure and decreased feed intake (Hosfeld et al., 2008). Apart from the effects on neuroendocrine stress coping mechanisms, allostatic load has been associated with the energy expenditure needed for stabilize the body's functions (Schreck, 2010). Thus, the suppressed growth in fish exposed to environmental hypercapnia in the present study lends further support to that high CO_2 levels in the water imposes allostatic load on farmed fish. Still, it is important to note that we were not able to investigate to which extent decreased feed intake contributed to poorer growth in the present study.

In addition to these CO_2 induced effects, our results show an overall decrease in condition factor over the FW period, which is in line with a general decrease in condition factor associated with smoltification (for references see; Björnsson et al., 1989). Moreover, there were reduction in growth after vaccination in the present study. This is coherent to earlier studies showing suppressed growth up to one month after vaccination (Berg et al., 2006; Sørum and Damsgård, 2004).

Damage to the exterior of the fish, such as damages on the skin and fins, are often used as operational welfare indicators. However, the results of the current study show that these types of welfare indicators are not always reflected in stress coping mechanisms. Considering that

stress coping is central in animal welfare (Korte et al., 2007), this emphasize the need for controlled challenges to activate the stress axis when investigating the welfare status of fish reared in systems with potentially high environmental CO_2.

In conclusion, we here show that chronic exposure to hypercapnia leads to increased baseline brainstem 5-HT activity together with changes in baseline ion balance and a compromised stress responsiveness of the brainstem 5-HT activity, as well as suppressed growth. Taken together, these results suggest that environmental hypercapnia imposed an allostatic overload on fish. Considering that CO_2 levels in RAS can reach critical levels due to dynamic processes such as fish biomass, bacterial aerobic metabolism and degassing (Skov, 2019), our results accentuates the importance of obtaining physiological measures of stress coping mechanisms and environmental tolerance limits on longer time scales when evaluating fish welfare in these types of rearing systems. Thus, to elucidate the relevance of farm welfare indicators (OWIs), such as feed intake and damages to the exterior of the fish, it is important to relate these indicators to physiological functions. Furthermore, in view of the growing realization that conditions in the early rearing phase may determine later performance, robustness, and animal welfare in aquaculture (e.g. Kristensen et al., 2012; Frisk et al., 2020), our results accentuate the need to incorporate hypercapnia as a possible factor in the developmental programming of fishes.

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.

Data availability

Data will be made available on request.

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