



## Molecular and physiological responses to long-term carbon dioxide exposure in Atlantic salmon (*Salmo salar*)



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### ABSTRACT

Optimal water quality is vital for the growth of Atlantic salmon aquaculture production. Recent data showed that Atlantic salmon feed intake and growth reduce linearly with increasing water carbon dioxide (CO<sub>2</sub>) concentrations, suggesting that even relatively low concentrations may impact fish performance. This study evaluated the molecular and physiological responses of Atlantic salmon (*Salmo salar*) to long-term CO<sub>2</sub> exposure. For this purpose, Atlantic salmon post-smolts (N = 900; 67 ± 8 g) were exposed to six CO<sub>2</sub> treatments (5, 12, 19, 26, 33 and 40 mg/L) for 12-weeks (RAS phase) followed by non-CO<sub>2</sub> exposure for a (< 5 mg/L) period of 6-weeks (seawater phase). Results from blood analysis of fish exposed to CO<sub>2</sub> for 12 weeks showed that CO<sub>2</sub> lead to significantly higher pH, K<sup>+</sup>, HCO<sub>3</sub><sup>-</sup> and PCO<sub>2</sub> and lower Na<sup>+</sup> and Cl<sup>-</sup> plasma concentrations. Whereas, haematocrit, Ca<sup>+</sup>, Mg<sup>2+</sup>, urea and glucose concentrations were similar among all CO<sub>2</sub> treatments. After 6 weeks in the seawater phase, all the parameters that were previously altered, became similar among all CO<sub>2</sub> treatments. Gill microarray results analysis showed 88 differentially expressed genes, resulting from the CO<sub>2</sub> exposure. At the end of the RAS phase (week 12), fish exposed to high CO<sub>2</sub> (40 mg/L) in comparison to fish exposed to low CO<sub>2</sub> (5 mg/L), showed 60 down-regulated genes, including genes encoding proteins involved in immune responses, differentiation, and maintenance of tissue structure. There was no evidence for stress and metabolic changes directed to neutralization of disturbance caused with high CO<sub>2</sub>. After 6 weeks in the seawater phase, a switch of expression from down regulated to up-regulated was observed. In conclusion, the present study brings new insights on the molecular and physiological responses of Atlantic salmon post-smolts to long-term CO<sub>2</sub> exposure. Several osmoregulation and acid-base balance parameters as well as gill gene expression levels were altered for as long as CO<sub>2</sub> exposure persisted. Moreover, most of these parameters were linearly related with the environmental CO<sub>2</sub> concentrations (5–40 mg/L range). The data from this study adds to recent findings that CO<sub>2</sub> concentrations below the 15 mg/L threshold still have an impact on Atlantic salmon. This finding may be relevant for a better dimensioning and management of production systems where CO<sub>2</sub> may accumulate in the water such as in recirculating aquaculture systems (RAS).

### 1. Introduction

Fish represents 6.7% of the global population's intake of all protein sources, 50% of which derives from fish aquaculture (FAO, 2018). The pressure to provide such quantities of fish is steering aquaculture towards a higher intensification that is often achieved with larger facilities (Ellis et al., 2016), high fish stocking densities (Calabrese et al., 2017) and reducing water use (Verdegem et al., 2006), all of which are

conditions that can lead to an accumulation of fish metabolites (Martins et al., 2010; Mota et al., 2014; Summerfelt et al., 2015). Fish metabolites can accumulate in production systems like semi-closed containment systems tanks in the sea, or recirculating aquaculture systems (RAS) on land. It is therefore important to know maximum levels of metabolite accumulation such as carbon dioxide (CO<sub>2</sub>) that do not compromise fish physiology, performance, or welfare.

Performance indicators such as survival, feed intake, and growth

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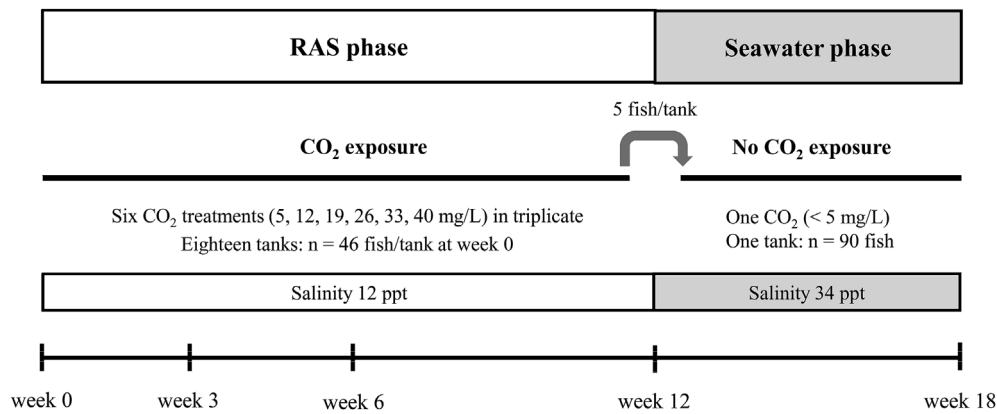


Fig. 1. Experimental design scheme.

have been primarily addressed in studies focusing on important aquaculture species. For instance, turbot (*Scophthalmus maximus*) growth is reduced by 26% when exposed to 26 mg/L CO<sub>2</sub> (Stiller et al., 2015) and Atlantic cod (*Gadus morhua*) condition factor, growth and cataracts prevalence were shown to be impacted at 18 mg/L CO<sub>2</sub> (Moran and Støttrup, 2011; Neves and Brown, 2015). In contrast, rainbow trout (*Oncorhynchus mykiss*) display good growth at both 8 and 24 mg/L CO<sub>2</sub> without impairing fish health (Good et al., 2010). Atlantic salmon (*Salmo salar*), accounts for more than 4% of all finfish production, with an annual production of over 2 million tonnes per year (FAO, 2018). Relative to its aquaculture importance, limited numbers of studies have addressed the impact of CO<sub>2</sub> exposure as an individual factor (see review by Fivelstad (2013)). Studies focused on the impact of high CO<sub>2</sub> exposure on Atlantic salmon growth, found that growth is impacted by high CO<sub>2</sub> exposure (Fivelstad et al., 1998; Martens et al., 2006). However, recent studies have shown that Atlantic salmon growth is reduced linearly with the increase of CO<sub>2</sub> concentration, even at concentrations below 15 mg/L (Fivelstad et al., 2018; Khan et al., 2018; Mota et al., 2019). In general, the impact of CO<sub>2</sub> in Atlantic salmon seems to depend on its life cycle stage (parr, smolt, post smolt), water quality (pH, aluminium, alkalinity) and other production factors, making it difficult to draw an accurate line for an unaffected threshold. For instance, Norwegian authorities (FOR, 2004) suggested a maximum of 15 mg/L. However, in light of recent studies that found that major performance indicators such as growth and feed intake change linearly with increasing water CO<sub>2</sub> concentrations (Fivelstad et al., 2018; Khan et al., 2018; Mota et al., 2019) and that there is a carry-over effect after transfer to seawater (Mota et al., 2019), the acceptable CO<sub>2</sub> level for Atlantic salmon production needs to be further investigated, particularly with respect to the physiological and molecular responses of long-term CO<sub>2</sub> exposure.

High CO<sub>2</sub> exposure is known to trigger a series of physiological responses in fish, normally seen as an increase of blood partial pressure of CO<sub>2</sub> (PCO<sub>2</sub>) and bicarbonate (HCO<sub>3</sub><sup>-</sup>) during pH compensation for acid-base balance (Heuer and Grosell, 2014). Other effects such as the reduction of oxygen uptake capacity, anti-predatory behavior and growth (Ou et al., 2015), or the compromise of olfactory system and central brain function (Porteus et al., 2018) were observed at CO<sub>2</sub> concentrations as low as 1–2 mg/L CO<sub>2</sub>. However, it is not uncommon to observed dissolved CO<sub>2</sub> concentration between 10 and 20 mg/L in commercial aquaculture systems (Gorle et al., 2018). Ion transport, osmoregulation and acid-base balance studies on Atlantic salmon exposed to CO<sub>2</sub> have found alterations in the concentration of several blood ions, such as Cl<sup>-</sup>, Na<sup>+</sup> and HCO<sub>3</sub><sup>-</sup> (Fivelstad et al., 1998, 2003b). Although most of these changes were reported for CO<sub>2</sub> concentrations higher than 15 mg/L, it would be expected that Atlantic salmon display compensatory changes in acid-balance at lower concentrations of CO<sub>2</sub>.

High CO<sub>2</sub> exposure has also been shown to trigger a series of

molecular responses in fish. For example, genes linked to high CO<sub>2</sub> exposure (*c-fos*), hypoxia (*hif1-α*) and glucocorticoid receptor (*gr-2*) were up-regulated in bluegill (*Lepomis macrochirus*) gills, heart and erythrocytes after 1 h exposure to 30 mg/L CO<sub>2</sub> (Dennis et al., 2015). To the best of our knowledge, only a few genes have been analysed for Atlantic salmon in a CO<sub>2</sub> context, namely the H<sup>+</sup>-ATPase, Na<sup>+</sup>/K<sup>+</sup> ATPases (alpha 1a and 1b subunits) and heat shock protein (HSP70) (Good et al., 2018), and only the expression of Na<sup>+</sup>/K<sup>+</sup> ATPase alpha 1a was increased as a result of a high CO<sub>2</sub> exposure (20 mg/L). The use of microarrays allows for the simultaneous examination of the expression of thousands of genes and can find differentially expressed genes, which are up- or down-regulated. The use of this tool in CO<sub>2</sub> exposure studies can provide a better overview of the response parameters in Atlantic salmon. The current study is a deeper investigation of an 18-week research trial reported earlier (Mota et al., 2019), and was conducted at the Nofima Centre for Recirculation in Aquaculture, Sunndalsøra, Norway. This study focused on the effects of carbon dioxide on growth performance, welfare, and health of Atlantic salmon. In contrast, the present study focuses on the molecular and physiological responses to long-term carbon dioxide exposure in Atlantic salmon. Atlantic salmon post-smolts were exposed to six CO<sub>2</sub> treatments (5, 12, 19, 26, 33 and 40 mg/L) for 12-weeks (RAS phase) followed by non-CO<sub>2</sub> exposure period of 6-weeks (seawater phase). The objective of this exposure was to determine the CO<sub>2</sub> concentration in which no effects are observed in ion transport, osmoregulation and acid-base balance of Atlantic salmon post-smolts (*Salmo salar*). Moreover, the effect of CO<sub>2</sub> on transcriptome expression of gills was assessed on a 15 thousand oligonucleotide DNA gene microarray.

## 2. Material and methods

### 2.1. Experimental design

The current study consisted of two experimental phases (Fig. 1). The first was a CO<sub>2</sub> exposure phase where Atlantic salmon were exposed for 12 weeks, with 6 treatment groups (5, 12, 19, 26, 33 and 40 mg/L CO<sub>2</sub>) using 3 replicate tanks per treatment. This experimental phase was done in a recirculating aquaculture system (RAS) (hereafter termed RAS phase). In the second phase, a fraction of the fish previously exposed to CO<sub>2</sub> were transferred to a single flow-through system at CO<sub>2</sub> < 5 mg/L (hereafter termed seawater phase) for an additional 6-week experimental period. The experimental fish and rearing conditions were described in more detail in Mota et al. (2019).

### 2.2. Fish and rearing conditions

Fish handling and testing conditions were approved by the Norwegian Food Safety Authority (FOTS) with the reference ID 9165.

Atlantic salmon eyed eggs (*Salmo* *Breid*, Os, Norway) were hatched and raised in a flow-through system (Nofima Research Station for Sustainable Aquaculture, Sunndalsøra, Norway) at 9 °C under continuous photoperiod (LD 24:00) until 44 g, at which point they received a 6-week winter stimulus (LD 12:12) followed by a return to LD24:00 to induce smoltification. Atlantic salmon post-smolts (N = 900; 67 ± 8 g) were individually pit-tagged with a smart glass tag (Smartrac, Reichshof-Wehrath, Germany) and randomly distributed over eighteen cylindro-conical experimental tanks (V = 0.5 m<sup>3</sup>) connected to a RAS (N = 50 fish/tank) in Nofima Centre for Recirculation in Aquaculture, Sunndalsøra, Norway. The fish were subsequently allowed to adapt to the rearing and feeding conditions for a 3-week period in a 12 ppt salinity RAS, followed by a 12-week CO<sub>2</sub> exposure period (RAS phase). At the end of the 12-week experimental period, five fish per tank (total 90 fish) were randomly selected and all transferred to a single flow-through tank (3.3 m<sup>3</sup>) for an additional 6-week experimental seawater phase, at salinity 34 ppt and where CO<sub>2</sub> level at the fish tank outlet averaged 2.2 mg/L.

Fish were fed continuously over 23 h with an automatic belt feeder over satiation (120–140%) using a commercial diet (3–4 mm, Nutra Olympic, Skretting, Norway). Satiation percentage was adjusted according to the feed spill observed.

The RAS consisted of a microscreen belt filter, a moving bed bio-reactor and a degasser column, two holding sump units, and ten octagonal fish biomass tanks. The total RAS water volume was 79 m<sup>3</sup>, water exchange rate was approx. 1180 L/kg feed (39% water system volume/day), and system hydraulic retention time was approx. 2.8 days.

The different CO<sub>2</sub> concentrations in each fish tank was achieved by mixing inlets from holding sump 1 (CO<sub>2</sub> = 3 mg/L) and holding sump 2 (CO<sub>2</sub> = 40 mg/L). The holding sump 2 had CO<sub>2</sub> gas added through a diffuser from a pressurized CO<sub>2</sub>-gas bottle, and the concentration was continuously monitored through a CO<sub>2</sub> sensor (OxyGuard, Denmark) connected to an analogue unit (Pacific, OxyGuard, Denmark). Due to the acidifying action of the CO<sub>2</sub> in holding sump 2, it was necessary to control the pH. To stabilize the pH at 6.9, a solution with NaHCO<sub>3</sub> (50–75 g/L) was added via an electromagnetic metering pump (Iwaki Norge, Oslo, Norway) controlled by an automatic pH control system (Walchem, MA, USA).

Water quality in fish tanks was maintained within the recommendations for Atlantic salmon post-smolts (Thorarensen and Farrell, 2011). The average (± SD) water quality parameters were: RAS phase, oxygen (93 ± 1% saturation), temperature (12.7 ± 0.0 °C), salinity (11.9 ± 0.1 ppt) and, pH (6.6–8.2) and; seawater phase, oxygen (91 ± 1% saturation), temperature (8.4 ± 0.1 °C), salinity (33.9 ± 0.3 ppt) and pH (7.8–7.9). Photoperiod was maintained at constant light (24 h) throughout both experimental phases.

### 2.3. Blood parameter analyses

At weeks 0, 3, 6, 12 and 18, five fish per tank, except at week 0 (only 3 fish per tank), were euthanized (0.12 g/L MS-222) and blood samples were collected from caudal vessels using two different Vacuette® vacuum tubes (Greiner Bio-One, Kremsmünster, Austria) one containing heparin (for plasma) and the other one containing a clot activator (for serum).

Blood pH and glucose were determined from the blood collected in vacuum tubes containing heparin within 5 min of sampling using an I-STAT Portable Clinical Analyser with EC8+ cartridges (Abbott Laboratories, Chicago, USA). The obtained pH value was temperature-corrected to match experimental temperature according to (Roth and Rotabakk, 2012):

$$\text{pH corrected} = \text{pH measured} - 0.015 \times (T - 37)$$

where T is the water temperature (°C) from where the fish were sampled.

Hematocrit was obtained by filling two microcapillary tubes from the same heparinized vacuum tubes and centrifuged at 12 000 rpm for 3 min. A scale was used to determine the % of packed cell volume (PCV).

The remaining blood from heparinized vacuum tubes together with the blood containing a clot activator were centrifuged at 3200 rpm for 10 min. The plasma and the serum were transferred to Eppendorf tubes. Serum was flash-frozen in liquid nitrogen and stored at –80 °C until assayed.

The plasma was immediately analysed using a carbon dioxide analyser (Ciba Corning 965, Essex, UK) for plasma total carbon dioxide (TCO<sub>2</sub>). Plasma PCO<sub>2</sub> and HCO<sub>3</sub><sup>–</sup> were calculated from TCO<sub>2</sub>, blood pH and water temperature using the Henderson-Hasselbalch equation:

$$\text{PCO}_2 = \text{TCO}_2 / (\alpha \times 10^{\text{pH} - \text{pK}_1} + 1)$$

And

$$\text{HCO}_3^- = \text{TCO}_2 - (\alpha \times \text{PCO}_2)$$

where PCO<sub>2</sub> is partial pressure of CO<sub>2</sub> in mm Hg, TCO<sub>2</sub> is total CO<sub>2</sub> in plasma in mmol/L, α is solubility constant of CO<sub>2</sub> in mmol/L/mm Hg, pH is blood pH and pK<sub>1</sub> is the first dissociation constant of CO<sub>2</sub>. Carbon dioxide solubility and pK<sub>1</sub> were obtained from Boutillier et al. (1984).

Sodium (Na<sup>+</sup>), potassium (K<sup>+</sup>), magnesium (Mg<sup>2+</sup>), calcium (Ca<sup>2+</sup>), chloride (Cl<sup>–</sup>) and urea were determined from the Eppendorf's serum using an automated clinical chemistry system (Pentra C400, Horiba, CA, USA). For this clinical automated system analysis, serum, i.e. plasma without the clotting factors of blood (fibrinogens), was used instead of plasma, due to its capacity to provide more consistent ion measurements.

### 2.4. Gill microarray analyses

At weeks 12 and 18, gill samples were dissected from euthanized fish (0.12 g/L MS-222) from only the 5 and the 40 mg/L treatments (n = 6 fish/treatment/week, 2 fish per tank, in total 24 samples). The 2nd arch gill from the right side was immediately flash-frozen in liquid nitrogen and stored at –80 °C until assayed. Microarray analyses were performed on individual samples using Nofima's Atlantic salmon oligonucleotide microarray SIQ-6 (GPL16555) containing 60-mer probes to transcripts of 15 k genes. Microarrays were fabricated by Agilent Technologies; all reagents and equipment were purchased from the same source. Total RNA (200 ng per reaction) was labelled with Cy3 using a Low Input Quick Amp Labeling Kit and fragmented with a Gene Expression Hybridization Kit. Hybridization was performed for 17 h in an oven at 65 °C at a rotation speed of 10 rpm. Arrays were washed for 1 min with the Gene Expression Wash Buffer I at room temperature, and 1 min with the Gene Expression Wash Buffer II at 37 °C and scanned.

### 2.5. Statistics

Statistical analyses were performed with IBM SPSS Statistics V25 (IBM, Corp., USA). ANOVAs homogeneity of variances was assessed using Levene's test and normality using Shapiro-Wilk test. Linear regressions and correlation assumptions were visually examined through predicted probability (P–P) plots for normality and scatterplots of the residuals for homoscedasticity. A significant level (α) of 0.05 was used for all analyses. Data are presented as mean ± standard deviation (SD). The effect of CO<sub>2</sub> on fish blood parameters at the end of RAS phase (12 week, Table 1) was analysed using linear regressions followed by a post-hoc Tukey HSD test. The effect of CO<sub>2</sub> on blood pH, serum Cl<sup>–</sup>, serum Na<sup>+</sup>, plasma HCO<sub>3</sub><sup>–</sup>, serum K<sup>+</sup> and plasma PCO<sub>2</sub> concentrations was further assessed at weeks 0, 3 and 18 using one-way ANOVA followed by a post-hoc Tukey HSD test. The relationship between water and plasma partial pressures of CO<sub>2</sub> was analysed using a linear regression as the PCO<sub>2plasma</sub> depends on PCO<sub>2water</sub>, whereas the

**Table 1**  
Blood parameters of Atlantic salmon exposed to six different CO<sub>2</sub> concentrations for 12 weeks (RAS phase).

Parameters	CO <sub>2</sub> treatment (mg/L)						Regression	
	5	12	19	26	33	40	R <sup>2</sup>	P-value
Haematocrit (% PCV) <sup>1</sup>	43.5 ± 0.8	44.8 ± 1.2	43.8 ± 0.6	42.6 ± 3.6	42.8 ± 0.6	43.3 ± 1.5	0.070	0.306
pH <sup>1</sup>	7.25–7.31 <sup>a</sup>	7.32–7.41 <sup>a</sup>	7.45–7.54 <sup>b</sup>	7.47–7.53 <sup>b</sup>	7.53–7.56 <sup>b</sup>	7.52–7.56 <sup>b</sup>	0.787	< 0.001
Na <sup>+</sup> (mmol/L) <sup>2</sup>	158.1 ± 0.1 <sup>a</sup>	157.5 ± 0.4 <sup>ab</sup>	155.7 ± 1.8 <sup>ab</sup>	155.3 ± 1.9 <sup>ab</sup>	155.2 ± 0.5 <sup>ab</sup>	154.7 ± 1.1 <sup>b</sup>	0.559	< 0.001
K <sup>+</sup> (mmol/L) <sup>2</sup>	2.7 ± 0.2 <sup>a</sup>	2.7 ± 0.1 <sup>a</sup>	3.4 ± 0.2 <sup>ab</sup>	3.3 ± 0.4 <sup>ab</sup>	3.5 ± 0.2 <sup>ab</sup>	4.2 ± 0.6 <sup>b</sup>	0.671	< 0.001
Ca <sup>2+</sup> (mmol/L) <sup>2</sup>	2.7 ± 0.1	2.8 ± 0.1	2.8 ± 0.1	2.9 ± 0.2	2.9 ± 0.1	2.8 ± 0.1	0.136	0.132
Mg <sup>2+</sup> (mmol/L) <sup>2</sup>	0.8 ± 0.1	0.8 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	0.023	0.551
Cl <sup>-</sup> (mmol/L) <sup>2</sup>	128.6 ± 2.8 <sup>a</sup>	125.1 ± 3.3 <sup>ab</sup>	119.5 ± 2.8 <sup>bc</sup>	119.7 ± 2.1 <sup>bc</sup>	114.6 ± 1.7 <sup>cd</sup>	111.1 ± 2.8 <sup>d</sup>	0.854	< 0.001
HCO <sub>3</sub> <sup>-</sup> (mmol/L) <sup>3</sup>	11.4 ± 1.0 <sup>a</sup>	15.8 ± 0.6 <sup>b</sup>	21.0 ± 0.5 <sup>c</sup>	24.1 ± 2.5 <sup>cd</sup>	26.5 ± 1.1 <sup>d,e</sup>	29.4 ± 1.4 <sup>e</sup>	0.948	< 0.001
PCO <sub>2</sub> (mmHg) <sup>3</sup>	14.7 ± 2.1	16.5 ± 2.4	16.2 ± 1.5	17.9 ± 2.6	17.8 ± 1.0	19.9 ± 2.0	0.457	0.002
Urea (mmol/L) <sup>2</sup>	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	0.6 ± 0.1	0.007	0.736
Glucose (mmol/L) <sup>1</sup>	4.7 ± 0.4	4.9 ± 0.1	4.8 ± 0.1	4.9 ± 0.0	4.7 ± 0.3	4.8 ± 0.2	0.000	0.977

Parameters measure from blood<sup>1</sup>, serum<sup>2</sup>, or calculated<sup>3</sup>.

Superscript alphabets (post-hoc Tukey HSD test) and values in bold (linear regression analysis) indicate significant differences,  $P < 0.05$ .

Values are given as treatment mean ± SD (n = 3, 15 fish per treatment).

relationship between plasma HCO<sub>3</sub><sup>-</sup> and serum Cl<sup>-</sup> was analysed using a Pearson correlation as these two variables are independent from each other. The PCO<sub>2,water</sub> data set is the measured CO<sub>2</sub> concentration in each tank instead of the fixed CO<sub>2</sub> treatment concentration. Gill microarray data analysis was carried out with Nofima's bioinformatics package STARS (Krasnov et al., 2011) as described in (Pelizzari et al., 2013). Briefly, the mean intensities of all microarrays were equalized. Expression ratios (ER) were calculated by dividing the individual values for each feature to the mean value of the feature in all samples. The log<sub>2</sub>-ER were calculated and normalised with the locally weighted non-linear regression (lowess). The exposure groups were compared, i.e. shown comparisons were made between the treatments (5 mg/L and 40 mg/L) at week 12 and week 18, using the low CO<sub>2</sub> exposure treatment (5 mg/L) as baseline. Differentially expressed genes (DEG) were selected by criteria of significant log<sub>2</sub>-ER > |0.8| (1.74-fold),  $p < 0.05$ . STARS software annotates genes with GO, KEGG and custom vocabulary, which supplements public databases. Enrichment analysis compared the numbers of genes per functional category and pathway among DEG and on the microarray platform. Over-presentation of terms linked to not less than five DEG was assessed with Yates' corrected chi-square test.

### 3. Results

#### 3.1. Blood parameters

The linear regression from fish exposed to CO<sub>2</sub> for 12 weeks shows that CO<sub>2</sub> leads to a significantly higher pH, K<sup>+</sup>, HCO<sub>3</sub><sup>-</sup> and PCO<sub>2</sub> and lower Na<sup>+</sup> and Cl<sup>-</sup> concentrations (Table 1). Haematocrit, Ca<sup>2+</sup>, Mg<sup>2+</sup> urea and glucose concentrations were unaffected by CO<sub>2</sub> treatments ( $P > 0.05$ ). Pairwise comparisons among treatments further show that the lowest observed adverse effect level for HCO<sub>3</sub><sup>-</sup> was 12 mg CO<sub>2</sub>/L, pH and Cl<sup>-</sup> was 19 mg CO<sub>2</sub>/L, and, Na<sup>+</sup> and K<sup>+</sup> was 40 mg CO<sub>2</sub>/L. Fig. 2 shows the effect of CO<sub>2</sub> on these 5 parameters together with PCO<sub>2</sub> throughout the RAS and the seawater phase. Here it is illustrated that these physiological alterations started as early as 3 weeks after the exposures and, except for PCO<sub>2</sub>, were maintained throughout the CO<sub>2</sub> exposure. After 6 weeks in the seawater phase, where CO<sub>2</sub> was kept below < 5 mg/L, all these six parameters (pH, K<sup>+</sup>, HCO<sub>3</sub><sup>-</sup>, PCO<sub>2</sub>, Na<sup>+</sup> and Cl<sup>-</sup>) that were previously altered, became similar among all CO<sub>2</sub> treatments and were within the 15% variation compared to week 0, except for K<sup>+</sup> that varied by 50%. The strong relationship between serum Cl<sup>-</sup> and plasma HCO<sub>3</sub><sup>-</sup> is further illustrated in Fig. 3A ( $P < 0.001$ ). A linear regression shows the relationship between plasma and water partial pressures of CO<sub>2</sub> (Fig. 3B,  $P < 0.001$ ).

#### 3.2. Gill microarray

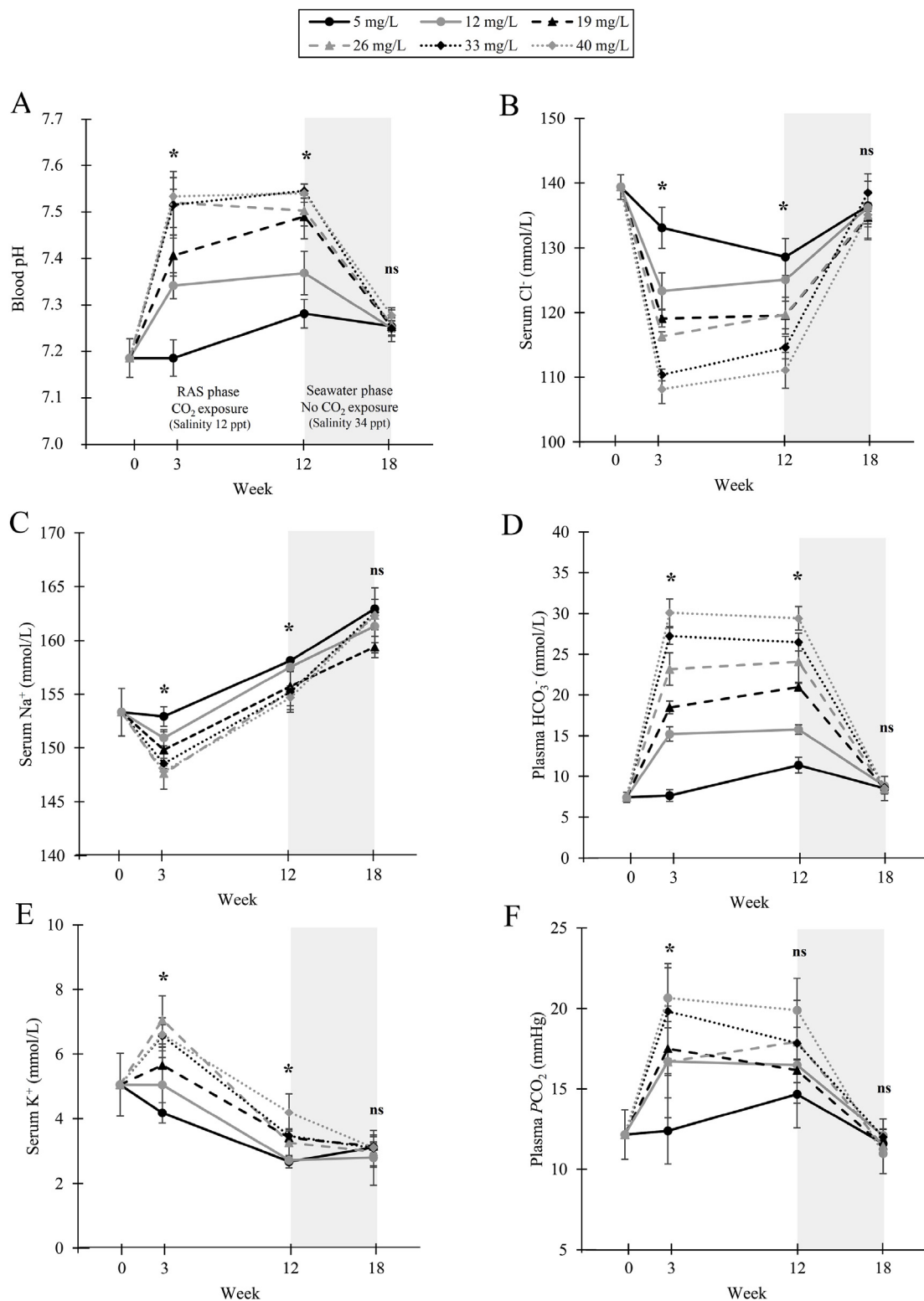
At the end of the RAS phase (week 12), fish exposed to high CO<sub>2</sub> (40 mg/L) in comparison to fish exposed to low CO<sub>2</sub> (5 mg/L), showed 71 DEG of which 60 were down-regulated. At week 18, when the fish had been kept in a flow-through tank with low CO<sub>2</sub> concentration for 6 weeks, the number of DEG had become lower (44) and 38 genes were now up-regulated including 27 genes that earlier were suppressed during the CO<sub>2</sub>-exposure. Enrichment analysis is a simple explorative tool that shows trends in transcriptome changes. Usually it requires a larger number of DEG. However, in this study several GO terms were significantly over-represented and most of them were associated with immune responses (Table 2). At week 12, 22 of 27 DEG with known or predicted immune functions were down-regulated in salmon exposed to high CO<sub>2</sub> (Table 3). Changes were observed in innate immunity without a visible effect on acquired immunity. The most affected functional groups were lectins, chemokines, complement and antiviral proteins represented respectively with seven, six, three and five DEG.

It is worth mentioning the up-regulation of the *matrix metalloproteinase 9* in CO<sub>2</sub> exposed fish. This gene encoding matrix degrading enzyme is characterised by having strong responses to stress and inflammation in Atlantic salmon (Sveen et al., 2018). At week 18, only two immune genes were differentially expressed, both were up-regulated in fish previously exposed to high CO<sub>2</sub>.

Microarray did not find significant changes in metabolism. However, a panel of genes that were down-regulated in salmon exposed to high CO<sub>2</sub> at week 12 encode proteins that may be important for the structure of gill tissue. Most of the DEG presented in Table 4 have unknown roles in Atlantic salmon, but mammalian homologs of several genes are associated with the development of various tissues including blood vessels and epidermis. *Claudin*, *otoancorin* and *nephronectin* are important for contacts between cells and extracellular matrix. Several down-regulated genes control secretion or encode mucosal proteins. At week 18 expression of these genes was either equal or higher in salmon exposed to 40 mg/L CO<sub>2</sub>.

### 4. Discussion

The current study shows that several osmoregulation and acid-base balance parameters have positive (pH, K<sup>+</sup>, HCO<sub>3</sub><sup>-</sup> and PCO<sub>2</sub>) or negative (Na<sup>+</sup>, Cl<sup>-</sup>) linear relationships with environmental CO<sub>2</sub> concentrations. The current study also shows, that the physiological compensatory regulation is maintained as long as CO<sub>2</sub> exposure persists, returning to control levels when CO<sub>2</sub> exposure is ended. Changes in the Atlantic salmon gill microarray expression showed that long-term high CO<sub>2</sub> exposure lead to relatively small transcriptome changes, since a

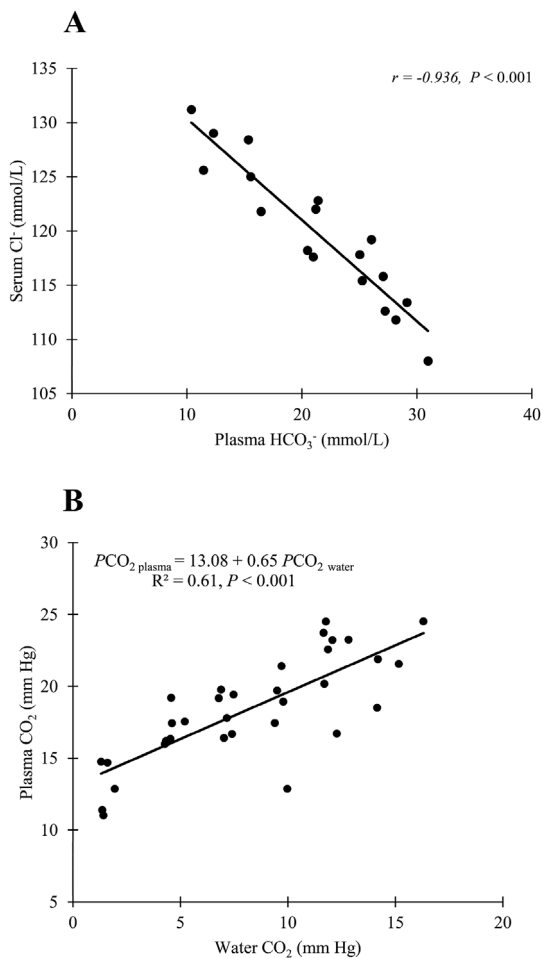


**Fig. 2.** The effect of CO<sub>2</sub> on blood pH (A), serum Cl<sup>-</sup> (B), serum Na<sup>+</sup> (C), plasma HCO<sub>3</sub><sup>-</sup> (D), serum K<sup>+</sup> (E) and plasma PCO<sub>2</sub> (F) concentration (in mmol/L) during an 18-week experimental period. Two periods shown: RAS phase (white area) with CO<sub>2</sub> exposure and, seawater phase (grey area) without CO<sub>2</sub> exposure. \* indicates significant differences among at least two CO<sub>2</sub> treatments. ns – non-significant differences.

total of only 88 genes were differentially expressed. Nonetheless, the transcriptome changes suggested that a high CO<sub>2</sub> exposure lead to a down-regulation of several genes followed by a hyper compensation after this CO<sub>2</sub> exposure was ended.

Fish gills are a major osmoregulatory organ, thought to account for 90% of acid-base compensation fluxes (Claiborne et al., 2002). Fish

have two mechanisms to cope with high environmental CO<sub>2</sub>: respiratory compensation through an increased ventilation, and metabolic compensation (Perry and Gilmour, 2006). In the latter, H<sup>+</sup> and HCO<sub>3</sub><sup>-</sup>, resulting from the hydration of CO<sub>2</sub> in the plasma, are exchanged with the environment to regulate internal pH levels. These effluxes are generally accompanied by influxes of Na<sup>+</sup> and Cl<sup>-</sup>,



**Fig. 3.** (A) Correlation between plasma  $\text{HCO}_3^-$  and serum  $\text{Cl}^-$  concentrations (in mmol/L) at the end of a 12-week  $\text{CO}_2$  exposure (RAS phase). Mean tank values presented ( $n = 18$ ). (B) Linear regression between plasma and water partial pressures of  $\text{CO}_2$  (in mm Hg) at week 3 and 6 (RAS phase). Mean tank values presented ( $n = 33$ , 3 tank values missing).

**Table 2**

Enrichment of Gene Ontology categories in the list of differentially expressed genes (DEG).

GO category	DEG	All <sup>a</sup>	<i>P</i> -value <sup>b</sup>
Carbohydrate binding (lectins)	5	186	0.001
Chemokine activity	5	57	< 0.001
Defense response to virus	6	172	< 0.001
Immune response	11	587	< 0.001
Inflammatory response	7	430	0.01

<sup>a</sup> Genes represented on the microarray platform.

<sup>b</sup> Yates corrected chi square.

thought to be gill  $\text{Na}^+/\text{H}^+$  and  $\text{Cl}^-/\text{HCO}_3^-$  exchanges (Claiborne et al., 1997). In the present study, a linear decrease of  $\text{Cl}^-$  and  $\text{Na}^+$  with  $\text{CO}_2$  concentration was found, likely resulting from the above-mentioned compensatory mechanisms. Moreover, compensatory  $\text{Cl}^-/\text{HCO}_3^-$  exchange were clearly observed in the present study through the correlation between serum  $\text{Cl}^-$  and plasma  $\text{HCO}_3^-$  in Fig. 3B. A decrease in plasma  $\text{Cl}^-$  was previously reported in other Atlantic salmon studies (Fig. 4) but, with the exception of one other study (Fivelstad et al., 2018), no effects in plasma  $\text{Cl}^-$  were found below 15 mg/L  $\text{CO}_2$  fish exposure. Few studies have measured plasma  $\text{Na}^+$  in the context of Atlantic salmon aquaculture  $\text{CO}_2$  exposure studies, and those that have, only found effects at very high  $\text{CO}_2$  exposures > 26

**Table 3**

Expression of genes encoding proteins with known or predicted immune functions in Atlantic salmon gills. Data are ratios of means in groups exposed to 40 mg/L and 5 mg/L  $\text{CO}_2$  at the end of a 12-week  $\text{CO}_2$  exposure (RAS phase – R) and at the end of a 6-week follow-up without  $\text{CO}_2$  exposure (seawater phase – S). Differentially expressed genes are indicated with bold.

Gene	R40-R5	S40-S5	Function
Gig2 family (3 genes) <sup>a</sup>	-2.1	1.3	Antiviral
ISG15	-2.5	1.9	Antiviral
Ubiquitin protein ligase E3A	-1.6	<b>1.8</b>	Antiviral
CC chemokine with stalk CK2	-1.9	1.0	Chemokine activity
C-C motif chemokine 8	-1.9	1.3	Chemokine activity
C-X-C chemokine 2	<b>2.5</b>	1.8	Chemokine activity
C-X-C chemokine 9	<b>2.5</b>	1.7	Chemokine activity
Small inducible cytokine A13 (2 genes) <sup>a</sup>	-1.9	1.3	Chemokine activity
C-type lectin 4E	-1.8	1.3	Carbohydrate binding
C-type lectin M4	<b>3.0</b>	<b>2.4</b>	Carbohydrate binding
Fish-egg lectin	-2.8	1.3	Carbohydrate binding
Leukolectin (2 genes) <sup>a</sup>	-2.6	1.2	Carbohydrate binding
Rhamnose binding lectin	-2.9	-1.3	Carbohydrate binding
Complement component C7	<b>2.0</b>	1.7	Complement cascade
Complement component C8	<b>2.0</b>	1.3	Complement cascade
Complement component C9	-1.8	-1.2	Complement cascade
TAP2b	-1.7	1.0	Antigen presentation
Matrix metalloproteinase-9	<b>2.5</b>	-1.1	Immune response
TNF receptor member 11B	-2.1	1.0	Immune response

<sup>a</sup> For genes with several variants, mean values are presented.

**Table 4**

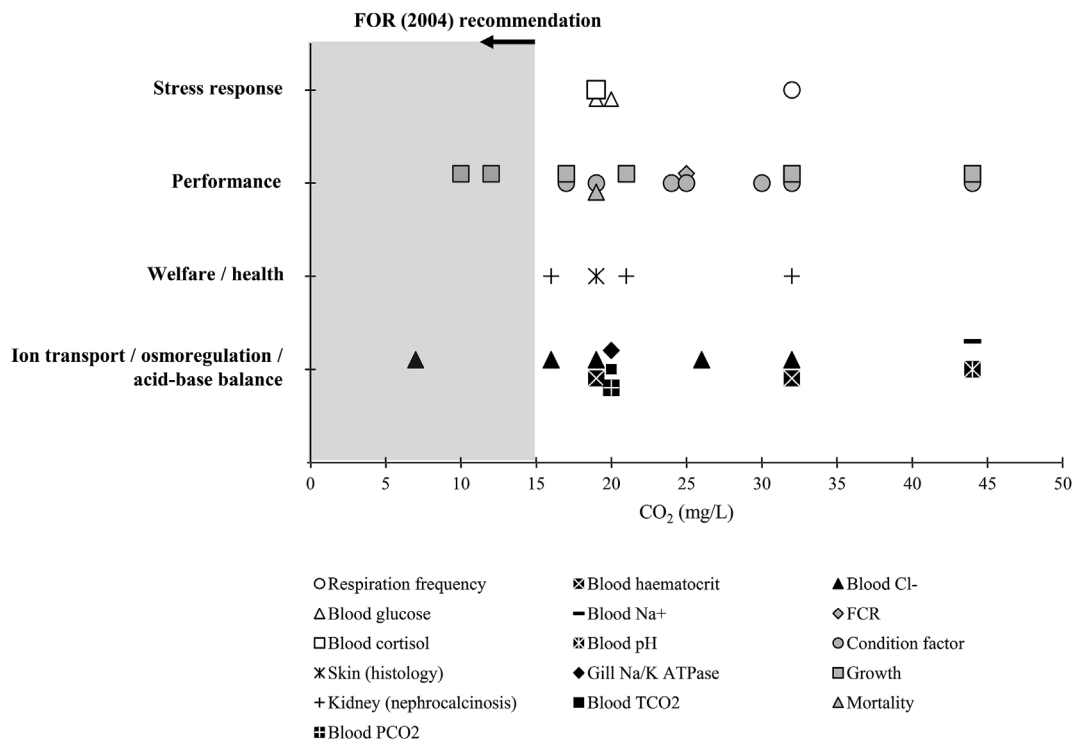
Expression of genes encoding proteins involved in tissue development and maintenance in Atlantic salmon gills. Data are ratios of means in groups exposed to 40 mg/L and 5 mg/L  $\text{CO}_2$  at the end of a 12-week  $\text{CO}_2$  exposure (RAS phase – R) and at the end of a 6-week follow-up without  $\text{CO}_2$  exposure (seawater phase – S). Differentially expressed genes are indicated with bold.

Gene	R40-R5	S40-S5	Function
Claudin-like protein ZF4A22	-2.5	<b>3.1</b>	Cell adhesion molecules
Otoancorin	-1.9	1.1	Cell-matrix adhesion
Nephronectin variant 2	-2.9	<b>3.7</b>	ECM organization
Fibulin-1	<b>2.0</b>	1.1	ECM organization
Angiogenin-1/RNase ZF3	-10.1	-1.1	Angiogenesis
Extracellular matrix protein 1	-3.6	1.2	Angiogenesis
EGF-like domain	-2.9	<b>4.0</b>	Angiogenesis
G-protein coupled receptor 183	-1.5	<b>1.8</b>	Angiogenesis
Growth factor independent 1.1	-1.9	<b>2.2</b>	Definitive hemopoiesis
Fatty aldehyde dehydrogenase	-2.2	<b>3.1</b>	Epidermis development
Ankyrin repeat and SAM domain	-2.2	<b>2.4</b>	Heart development
Lim homeobox protein 3	-2.0	<b>2.3</b>	Neuron differentiation
Homeobox protein HoxC8ba	-2.2	<b>2.9</b>	Pattern specification
Zymogen granule membrane 16 (2 genes) <sup>a</sup>	-4.4	-1.2	Secretion
GMP Giant mucus protein	-1.2	<b>2.6</b>	Secretion
Glucocorticoid receptor	-2.7	<b>3.4</b>	Sodium reabsorption

<sup>a</sup> For genes with several variants, mean values are presented.

mg/L (Fivelstad et al., 1998). This contrasts with the current study, where we show a linear regression between  $\text{CO}_2$  concentration and plasma  $\text{Na}^+$  in the range of 5–40 mg/L, lower levels than previously reported.

Partial pressure of  $\text{CO}_2$  ( $\text{PCO}_2$ ) remained significantly elevated as a result of high  $\text{CO}_2$  exposure at weeks 3 and 6, as shown in Fig. 3A. Linear analysis yielded the following relationship:  $[\text{PCO}_2 \text{ plasma}] = 13.08 + 0.65 [\text{PCO}_2 \text{ water}]$  in mmHG. This relationship continued while the  $\text{CO}_2$  exposure period lasted. This has been previously



**Fig. 4.** Overview of the lowest effect reported from a CO<sub>2</sub> exposure experiment in Atlantic salmon (parr, smolt, post-smolt and adult) grouped in four categories of effects (stress response, performance, welfare/health and, ion transport/osmoregulation/acid-base balance). Detailed information presented in the online Supplemental Table 1.

shown for Atlantic salmon exposed to 20 mg/L CO<sub>2</sub> (Good et al., 2018) and high PCO<sub>2</sub> levels were shown to led to altered blood pH.

In the present experiment blood pH remained significantly elevated in the 19–40 mg/L CO<sub>2</sub> treatments compared to the 5 mg/L treatment, throughout the study. Elevated pH levels in fish exposed to high environmental CO<sub>2</sub> exposure have been reported previously by Fivelstad et al. (1998), but contrasts with the observation from the study by Good et al. (2018) where fish exposed to 8 and 20 mg/L showed no differences in pH levels. Typically, during short-term exposure to high CO<sub>2</sub> an initial drop in of blood pH is followed by an increase of plasma HCO<sub>3</sub><sup>-</sup> to regulate the acid-base balance, resulting in a return of pH to initial levels (Pörtner et al., 2004). For instance, this was observed by Cameron and Randall (1972), when an increase of CO<sub>2</sub> exposure led to a linear reduction of blood pH in rainbow trout. In another study, on Pacific hagfish, (*Eptatretus stoutii*) exposed to very high environmental CO<sub>2</sub>, the authors observed a blood pH drop from 8.0 to below 7.0 in the first day, and in the subsequent days an increase of pH levels was observed, rising to 7.6 after 4 days, though notably still lower compared than the control treatment (Baker et al., 2015). In contrast, blood pH was previously found to increase as a result of high CO<sub>2</sub> exposure in rainbow trout. (Eddy et al., 1977). The same authors reported that normal blood pH levels were observed after 12–24 h exposure end. In the present study we found a higher pH level in high CO<sub>2</sub>-exposed fish compared to the lowest exposure group, a situation which continued until the termination of the experiment. These results could be due to the duration of the CO<sub>2</sub> exposure, or to a different mechanism in post-smolt salmon in a 12 ppt salinity RAS environment, compared to earlier studies. To note that in the Good et al. (2018) study the high CO<sub>2</sub> treatment (20 mg/L) had a nearly significant ( $P = 0.059$ ) higher plasma pH compared to the low CO<sub>2</sub> treatment (10 mg/L). More detailed studies should investigate the precise mechanisms behind this long-term elevated pH mechanism during CO<sub>2</sub> exposure in Atlantic salmon.

Fish barriers tissues such as gut, skin and gill are the first affected by changes in rearing environment. High environmental CO<sub>2</sub> was shown to impact gene expression in bluegill and silver carp (Dennis et al., 2015).

In the present study, transcriptome analyses did not reveal changes in ion metabolism. Apparently, compensation of disturbances did not require stable stimulation of genes involved in maintenance of osmotic balance. There was also no evidence for responses to stress and hypoxia – only one stress marker (*matrix metalloproteinase 9*) was up-regulated in salmon exposed to high CO<sub>2</sub> at week 12. Still, the effect of treatment was manifested with down-regulation of dozens of functionally related genes. Immune genes are a highly labile part of salmon transcriptome, their down-regulation may indicate competition for resources. For example, massive suppression is observed during smoltification and adaptation to seawater (Johansson et al., 2016). In this study, changes were much smaller by scale and compensation was achieved shortly after the end of exposure. Down-regulation of a small group of genes involved in development and maintenance of tissue was in concordance with previously shown effects of high CO<sub>2</sub> exposure on the Atlantic salmon skin layer morphology and thickness. Specifically, fish exposed to high CO<sub>2</sub> had a thinner dermis and uneven epidermis (Mota et al., 2019). Gills are directly exposed to the surrounding environment, and hypertrophy and hyperplasia of epithelial cells and adhesion of lamellae have been observed as a result of CO<sub>2</sub> exposure in combination with low pH and aluminum water (Fivelstad et al., 2003a). Nevertheless, studies focusing solely on CO<sub>2</sub> effects did not find any histopathological changes in the gills of Atlantic salmon (Fivelstad et al., 2007, 2015). Similarly, to immunity, the number of DEG mentioned above was not sufficient to warren firm conclusions on potential functional consequences of exposure to CO<sub>2</sub>, particularly given that a large part of genes showed a compensatory up-regulation after a 6-week non-CO<sub>2</sub> exposure (seawater phase). The results discussed here compare a low CO<sub>2</sub> concentration (5 mg/L) and a very high CO<sub>2</sub> concentration (40 mg/L), which is not common, but can nevertheless occur during commercial production of Atlantic salmon. To our knowledge, we show here for the first time that exposure to CO<sub>2</sub> has an impact on gill tissue global gene expression.

The concentration of CO<sub>2</sub> that has been previously recommended as safe for Atlantic salmon is 15 mg/L (FOR, 2004); thus implying that

there is a threshold here, below which there are no major impacts of CO<sub>2</sub> on fish welfare, health and performance. Several studies on Atlantic salmon support this recommendation, since very few parameters measured were found altered below this threshold as Fig. 4 shows. However, these results could be due to a lack of tests below the 15 mg/L threshold. Studies in other fish species in the context of ocean acidification have shown significant impacts of CO<sub>2</sub> at concentrations as low as 1–2 mg/L (Ou et al., 2015; Porteus et al., 2018). In the present study, several osmoregulation and acid-base balance parameters were shown to have positive or negative linear relationships with environmental CO<sub>2</sub> concentrations. Moreover, from the same experiment as is reported here, we earlier showed that growth was negatively linear-related to CO<sub>2</sub> exposure, where an increase in CO<sub>2</sub> of 10 mg/L would correspond to an approximate 10% growth reduction in the range studied (average TGC: 2.2, range CO<sub>2</sub>: 5–40 mg/L) (Mota et al., 2019). Two other studies on Atlantic salmon showed a similar relationship between growth and CO<sub>2</sub> exposure with a linear growth reduction with an increase in CO<sub>2</sub> exposure (Fivelstad et al., 2018; Khan et al., 2018) and FCR increase with increasing CO<sub>2</sub> exposure (Khan et al., 2018). Other authors studying Atlantic salmon (Khan et al., 2018) and Atlantic cod (*Gadus morhua*) (Moran and Støttrup, 2011), have previously suggested the need of revising the CO<sub>2</sub> safety threshold. The combination of evidence of physiological impacts from this study, and growth performance impacts from (Mota et al., 2019) of CO<sub>2</sub> exposure in Atlantic salmon, advocates for a revision of the existing threshold.

The present study brings new insights on the molecular and physiological responses of Atlantic salmon post-smolts to long-term CO<sub>2</sub> exposure. Several osmoregulation and acid-base balance parameters were altered and these physiological alterations are maintained as long as CO<sub>2</sub> exposure persists. Molecular responses measured in Atlantic salmon gills exposed to CO<sub>2</sub> experienced an increase of down-regulated genes with various functions, which changed to up-regulation when the CO<sub>2</sub> exposure ended. The data from this study adds to recent findings that CO<sub>2</sub> concentrations below the 15 mg/L threshold still have an impact on Atlantic salmon, and this finding may be relevant for a better design and dimensioning of production systems where CO<sub>2</sub> may accumulate in the water.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aquaculture.2019.734715>.

## Author contributions

Experimental design: VCM, TON, JK, BFT.  
Data collection and analysis: VCM, JG, MG, JK, TON, AK, BFT.  
Manuscript draft: VCM.

Final manuscript review and approval: VCM, JG, MG, TON, JG, AK, BFT.

## Declaration of competing interestCOI

The authors have no conflict of interest to declare.

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