Aquaculture 519 (2020) 734715

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

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

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ARTICLE INFO

Keywords:
Hypercapnia
CO_2
Salmonids
Recirculating aquaculture systems
Differentially expressed genes

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_2) 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_2 exposure. For this purpose, Atlantic salmon post-smolts (N = 900; 67 ± 8 g) were exposed to six CO_2 treatments (5, 12, 19, 26, 33 and 40 mg/L) for 12-weeks (RAS phase) followed by non-CO_2 exposure for a (< 5 mg/L) period of 6-weeks (seawaterphase). Results from blood analysis of fish exposed to CO_2 for 12 weeks showed that CO_2 lead to significantly higher pH, K^+, HCO_3^- and PCO_2 and lower Na^+ and Cl^- plasma concentrations. Whereas, haematocrit, Ca^+, Mg^{2+}, urea and glucose concentrations were similar among all CO_2 treatments. After 6 weeks in the seawater phase, all the parameters that were previously altered, became similar among all CO_2 treatments. Gill microarray results analysis showed 88 differentially expressed genes, resulting from the CO_2 exposure. At the end of the RAS phase (week 12), fish exposed to high CO_2 (40 mg/L) in comparison to fish exposed to low CO_2 (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_2. 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_2 exposure. Several osmoregulation and acid-base balance parameters as well as gill gene expression levels were altered for as long as CO_2 exposure persisted. Moreover, most of these parameters were linearly related with the environmental CO_2 concentrations (5–40 mg/L range). The data from this study adds to recent findings that CO_2 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_2 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_2) that do not compromise fish physiology, performance, or welfare.

Performance indicators such as survival, feed intake, and growth

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https://doi.org/10.1016/j.aquaculture.2019.734715
Received 31 May 2019; Received in revised form 5 November 2019; Accepted 7 November 2019

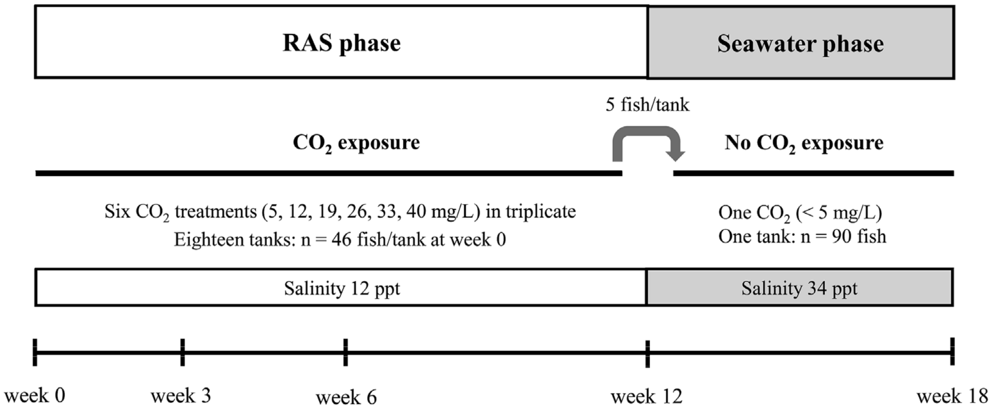

Fig. 1. Experimental design scheme.

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_2 (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_2 (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_2 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_2 exposure as an individual factor (see review by Fivelstad (2013)). Studies focused on the impact of high CO_2 exposure on Atlantic salmon growth, found that growth is impacted by high CO_2 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_2 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_2 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_2 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_2 level for Atlantic salmon production needs to be further investigated, particularly with respect to the physiological and molecular responses of long-term CO_2 exposure.

High CO_2 exposure is known to trigger a series of physiological responses in fish, normally seen as an increase of blood partial pressure of CO_2 (PCO_2) and bicarbonate (HCO_3^-) 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_2 concentrations as low as 1–2 mg/L CO_2. However, it is not uncommon to observed dissolved CO_2 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_2 have found alterations in the concentration of several blood ions, such as Cl^-, Na^+ and HCO_3^- (Fivelstad et al., 1998, 2003b). Although most of these changes were reported for CO_2 concentrations higher than 15 mg/L, it would be expected that Atlantic salmon display compensatory changes in acid-balance at lower concentrations of CO_2.

High CO_2 exposure has also been shown to trigger a series of molecular responses in fish. For example, genes linked to high CO_2 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_2 (Dennis et al., 2015). To the best of our knowledge, only a few genes have been analysed for Atlantic salmon in a CO_2 context, namely the H^+ -ATPase, Na^+/K^+ ATPases (alpha 1a and 1b subunits) and heat shock protein (HSP70) (Good et al., 2018), and only the expression of Na^+/K^+ ATPase alpha 1a was increased as a result of a high CO_2 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_2 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_2 treatments (5, 12, 19, 26, 33 and 40 mg/L) for 12-weeks (RAS phase) followed by non-CO_2 exposure period of 6-weeks (seawater phase). The objective of this exposure was to determine the CO_2 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_2 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_2 exposure phase where Atlantic salmon were exposed for 12 weeks, with 6 treatment groups (5, 12, 19, 26, 33 and 40 mg/L CO_2) 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_2 were transferred to a single flow-through system at $CO_2 < 5$ 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 (SalmoBreed, 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-Wehnrath, Germany) and randomly distributed over eighteen cylindro-conical experimental tanks ($V = 0.5\,m^3$) 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_2 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 ($3.3\,m^3$) for an additional 6-week experimental seawater phase, at salinity 34 ppt and where CO_2 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 bioreactor and a degasser column, two holding sump units, and ten octagonal fish biomass tanks. The total RAS water volume was 79 m^3, 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_2 concentrations in each fish tank was achieved by mixing inlets from holding sump 1 (CO_2 = 3 mg/L) and holding sump 2 (CO_2 = 40 mg/L). The holding sump 2 had CO_2 gas added through a diffusor from a pressurized CO_2-gas bottle, and the concentration was continuously monitored through a CO_2 sensor (OxyGuard, Denmark) connected to an analogue unit (Pacific, OxyGuard, Denmark). Due to the acidifying action of the CO_2 in holding sump 2, it was necessary to control the pH. To stabilize the pH at 6.9, a solution with $NaHCO_3$ (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, Kremsmunster, 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_2). Plasma PCO_2 and HCO_3^- were calculated from TCO_2, blood pH and water temperature using the Henderson-Hasselbalch equation:

$$PCO_2 = TCO_2 / (\alpha \times 10^{\,pH - pK1} + 1)$$

And

$$HCO_3^- = TCO_2 - (\alpha \times PCO_2)$$

where PCO_2 is partial pressure of CO_2 in mm Hg, TCO_2 is total CO_2 in plasma in mmol/L, α is solubility constant of CO_2 in mmol/L/mm Hg, pH is blood pH and pK_1 is the first dissociation constant of CO_2. Carbon dioxide solubility and pK_1 were obtained from Boutilier et al. (1984).

Sodium (Na^+), potassium (K^+), magnesium (Mg^{2+}), calcium (Ca^{2+}), chloride (Cl^-) 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_2 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_2 on blood pH, serum Cl^-, serum Na^+, plasma HCO_3^-, serum K^+ and plasma PCO_2 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_2 was analysed using a linear regression as the $PCO_{2\,plasma}$ depends on $PCO_{2\,water}$, whereas the

Table 1
Blood parameters of Atlantic salmon exposed to six different CO_2 concentrations for 12 weeks (RAS phase).

Parameters	CO_2 treatment (mg/L)						Regression	
	5	12	19	26	33	40	R^2	*P*-value
Haematocrit (% PCV)^1	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^1	7.25–7.31^a	7.32–7.41^a	7.45–7.54^b	7.47–7.53^b	7.53–7.562^b	7.52–7.56^b	0.787	< **0.001**
Na^+ (mmol/L)^2	158.1 ± 0.1^a	157.5 ± 0.4^{ab}	155.7 ± 1.8^{ab}	155.3 ± 1.9^{ab}	155.2 ± 0.5^{ab}	154.7 ± 1.1^b	0.559	< **0.001**
K^+ (mmol/L)^2	2.7 ± 0.2^a	2.7 ± 0.1^a	3.4 ± 0.2^{a,b}	3.3 ± 0.4^{a,b}	3.5 ± 0.2^{a,b}	4.2 ± 0.6^b	0.671	< **0.001**
Ca^{2+} (mmol/L)^2	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^{2+} (mmol/L)^2	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^- (mmol/L)^2	128.6 ± 2.8^a	125.1 ± 3.3^{a,b}	119.5 ± 2.8^{b,c}	119.7 ± 2.1^{b,c}	114.6 ± 1.7^{c,d}	111.1 ± 2.8^d	0.854	< **0.001**
HCO_3^-(mmol/L)^3	11.4 ± 1.0^a	15.8 ± 0.6^b	21.0 ± 0.5^c	24.1 ± 2.5^{c,d}	26.5 ± 1.1^{d,e}	29.4 ± 1.4^e	0.948	< **0.001**
PCO_2 (mmHg)^3	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)^2	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)^1	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^1, serum^2, or calculated^3.
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_3^- and serum Cl^- was analysed using a Pearson correlation as these two variables are independent from each other. The PCO_{2water} data set is the measured CO_2 concentration in each tank instead of the fixed CO_2 treatment concentration. Gill microarray data analysis was carried out with Nofima's bioinformatics package STARS (Krasnov et al., 2011) as described in (Pellizzari 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 log2-ER were calculated and normalised with the locally weighted non-linear regression (lowess). The exposure groups were compared, i.e. shown comparations were made between the treatments (5 mg/L and 40 mg/L) at week 12 and week 18, using the low CO_2 exposure treatment (5 mg/L) as baseline. Differentially expressed genes (DEG) were selected by criteria of significant log2-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_2 for 12 weeks shows that CO_2 leads to a significantly higher pH, K^+, HCO_3^- and PCO_2 and lower Na^+ and Cl^- concentrations (Table 1). Haematocrit, Ca^+, Mg^{2+} urea and glucose concentrations were unaffected by CO_2 treatments ($P > 0.05$). Pairwise comparisons among treatments further show that the lowest observed adverse effect level for HCO_3^- was 12 mg CO_2/L, pH and Cl^- was 19 mg CO_2/L, and, Na^+ and K^+ was 40 mg CO_2/L. Fig. 2 shows the effect of CO_2 on these 5 parameters together with PCO_2 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_2, were maintained throughout the CO_2 exposure. After 6 weeks in the seawater phase, where CO_2 was kept below < 5 mg/L, all these six parameters (pH, K^+, HCO_3^-, PCO_2, Na^+ and Cl^-) that were previously altered, became similar among all CO_2 treatments and were within the 15% variation compared to week 0, except for K^+ that varied by 50%. The strong relationship between serum Cl^- and plasma HCO_3^- is further illustrated in Fig. 3A ($P < 0.001$). A linear regression shows the relationship between plasma and water partial pressures of CO_2 (Fig. 3B, $P < 0.001$).

3.2. Gill microarray

At the end of the RAS phase (week 12), fish exposed to high CO_2 (40 mg/L) in comparison to fish exposed to low CO_2 (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_2 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_2-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_2 (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_2 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_2.

Microarray did not find significant changes in metabolism. However, a panel of genes that were down-regulated in salmon exposed to high CO_2 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_2.

4. Discussion

The current study shows that several osmoregulation and acid-base balance parameters have positive (pH, K^+, HCO_3^- and PCO_2) or negative (Na^+, Cl^-) linear relationships with environmental CO_2 concentrations. The current study also shows, that the physiological compensatory regulation is maintained as long as CO_2 exposure persists, returning to control levels when CO_2 exposure is ended. Changes in the Atlantic salmon gill microarray expression showed that long-term high CO_2 exposure lead to relatively small transcriptome changes, since a

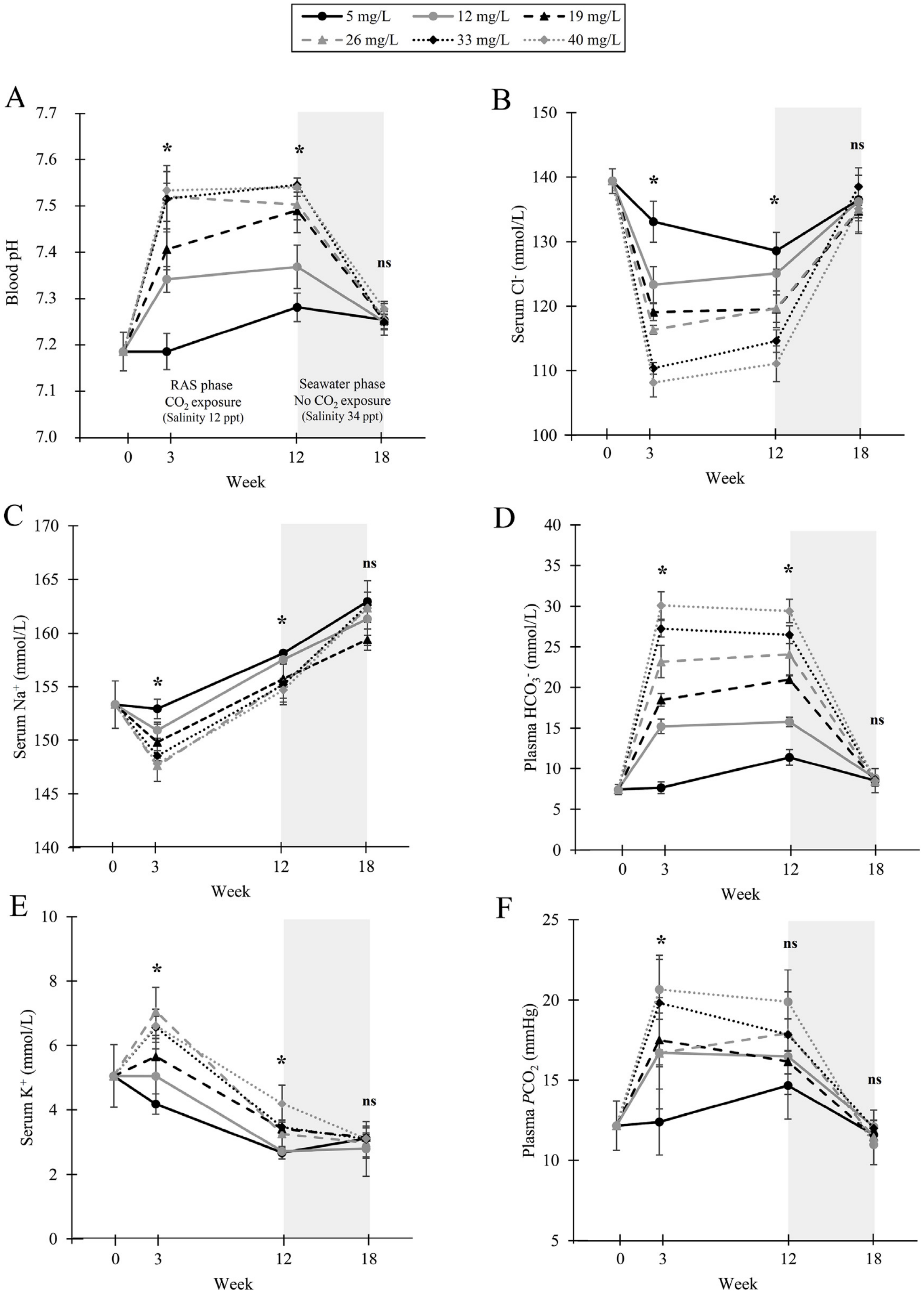

Fig. 2. The effect of CO_2 on blood pH (A), serum Cl^- (B), serum Na^+ (C), plasma HCO_3^- (D), serum K^+ (E) and plasma PCO_2 (F) concentration (in mmol/L) during an 18-week experimental period. Two periods shown: RAS phase (white area) with CO_2 exposure and, seawater phase (grey area) without CO_2 exposure. * indicates significant differences among at least two CO_2 treatments. ns – non-significant differences.

total of only 88 genes were differentially expressed. Nonetheless, the transcriptome changes suggested that a high CO_2 exposure lead to a down-regulation of several genes followed by a hyper compensation after this CO_2 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_2: respiratory compensation through an increased ventilation, and metabolic compensation (Perry and Gilmour, 2006). In the latter, H^+ and HCO_3^-, resulting from the hydration of CO_2 in the plasma, are exchanged with the environment to regulate internal pH levels. These effluxes are generally accompanied by influxes of Na^+ and Cl^-,

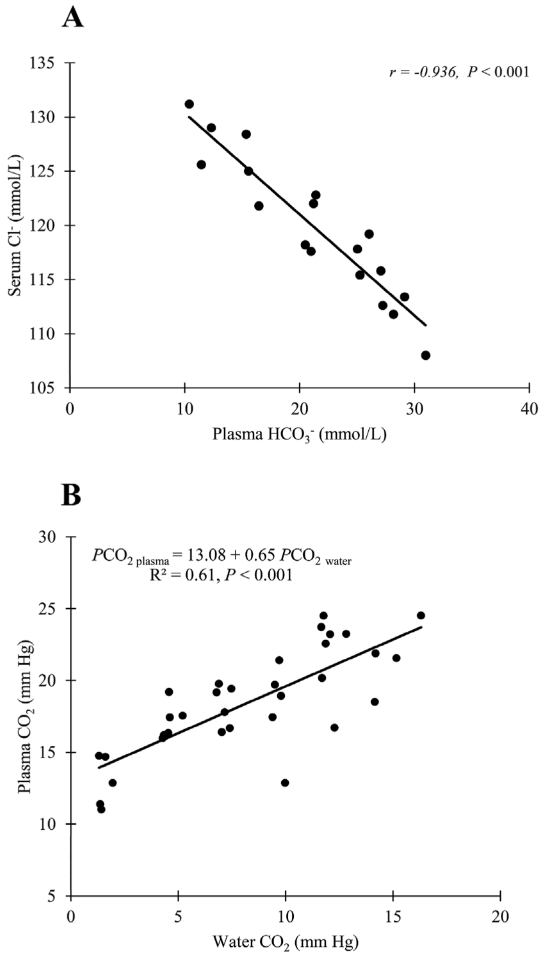

Fig. 3. (A) Correlation between plasma HCO$_3^-$ and serum Cl$^-$ concentrations (in mmol/L) at the end of a 12-week CO$_2$ exposure (RAS phase). Mean tank values presented (n = 18). (B) Linear regression between plasma and water partial pressures of CO$_2$ (in mm Hg) at week 3 and 6 (RAS phase). Mean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

[a] Genes represented on the microarray platform.
[b] Yates corrected chi square.

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 CO$_2$ at the end of a 12-week CO$_2$ exposure (RAS phase – R) and at the end of a 6-week follow-up without CO$_2$ exposure (seawater phase – S). Differentially expressed genes are indicated with bold.

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

[a] 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 CO2 at the end of a 12-week CO2 exposure (RAS phase – R) and at the end of a 6-week follow-up without CO2 exposure (seawater phase – S). Differentially expressed genes are indicated with bold.

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

[a] For genes with several variants, mean values are presented.

thought to be gill Na$^+$/H$^+$ and Cl$^-$/HCO$_3^-$ exchanges (Claiborne et al., 1997). In the present study, a linear decrease of Cl$^-$ and Na$^+$ with CO$_2$ concentration was found, likely resulting from the above-mentioned compensatory mechanisms. Moreover, compensatory Cl$^-$/HCO$_3^-$ exchange were clearly observed in the present study through the correlation between serum Cl$^-$ and plasma HCO$_3^-$ in Fig. 3B. A decrease in plasma 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 Cl$^-$ were found below 15 mg/L CO$_2$ fish exposure. Few studies have measured plasma Na$^+$ in the context of Atlantic salmon aquaculture CO$_2$ exposure studies, and those that have, only found effects at very high CO$_2$ exposures > 26 mg/L (Fivelstad et al., 1998). This contrasts with the current study, where we show a linear regression between CO$_2$ concentration and plasma Na$^+$ in the range of 5–40 mg/L, lower levels than previously reported.

Partial pressure of CO$_2$ (PCO$_2$) remained significantly elevated as a result of high CO$_2$ exposure at weeks 3 and 6, as shown in Fig. 3A. Linear analysis yielded the following relationship: $[PCO_{2\ \text{plasma}}] = 13.08 + 0.65\ [PCO_{2\ \text{water}}]$ in mmHG. This relationship continued while the CO$_2$ exposure period lasted. This has been previously

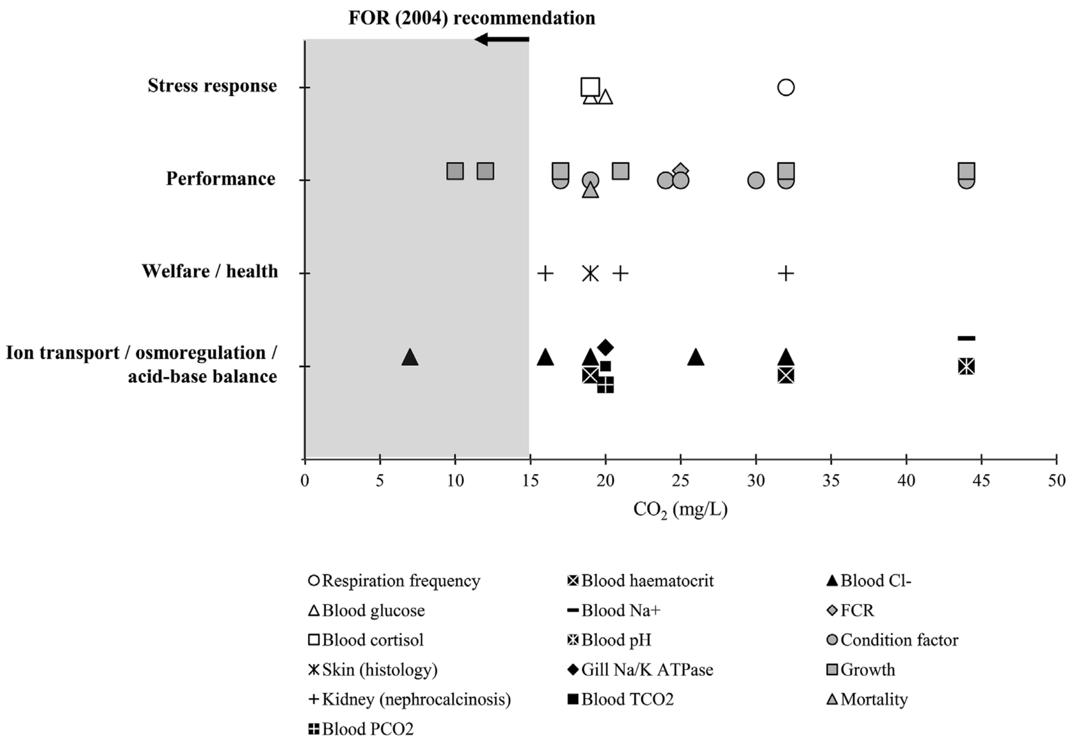

Fig. 4. Overview of the lowest effect reported from a CO_2 exposure experiment in Atlantic salmon (parr, smolt, post-smolt and adult) grouped in four categories of effects (stress response, performance, welfare/health and, ion transport/osmoregulation/acid-base balance). Detailed infromation presented in the online Supplemental Table 1.

shown for Atlantic salmon exposed to 20 mg/L CO_2 (Good et al., 2018) and high PCO_2 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_2 treatments compared to the 5 mg/L treatment, throughout the study. Eevated pH levels in fish exposed to high environmental CO_2 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_2 an initial drop in of blood pH is followed by an increase of plasma HCO_3^- 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_2 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_2, 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_2 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_2-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_2 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_2 treatment (20 mg/L) had a nearly significant ($P = 0.059$) higher plasma pH compared to the low CO2 treatment (10 mg/L). More detailed studies should investigate the precise mechanisms behind this long-term elevated pH mechanism during CO_2 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_2 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_2 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_2 exposure on the Atlantic salmon skin layer morphology and thickness. Specifically, fish exposed to high CO_2 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_2 exposure in combination with low pH and aluminum water (Fivelstad et al., 2003a). Nevertheless, studies focusing solely on CO_2 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_2, particularly given that a large part of genes showed a compensatory up-regulation after a 6-week non-CO_2 exposure (seawater phase). The results discussed here compare a low CO_2 concentration (5 mg/L) and a very high CO_2 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_2 has an impact on gill tissue global gene expression.

The concentration of CO_2 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_2 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_2 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_2 concentrations. Moreover, from the same experiment as is reported here, we earlier showed that growth was negatively linear-related to CO_2 exposure, where an increase in CO_2 of 10 mg/L would correspond to an approximate 10% growth reduction in the range studied (average TGC: 2.2, range CO_2: 5–40 mg/L) (Mota et al., 2019). Two other studies on Atlantic salmon showed a similar relationship between growth and CO_2 exposure with a linear growth reduction with an increase in CO_2 exposure (Fivelstad et al., 2018; Khan et al., 2018) and FCR increase with increasing CO_2 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_2 safety threshold. The combination of evidence of physiological impacts from this study, and growth performance impacts from (Mota et al., 2019) of CO_2 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_2 exposure. Several osmoregulation and acid-base balance parameters were altered and these physiological alterations are maintained as long as CO_2 exposure persists. Molecular responses measured in Atlantic salmon gills exposed to CO_2 experienced an increase of down-regulated genes with various functions, which changed to up-regulation when the CO_2 exposure ended. The data from this study adds to recent findings that CO_2 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_2 may accumulate in the water.

Acknowledgments

This project is a part of CtrlAQUA SFI, Center for research-based innovation funded by the Research Council of Norway (project number 237856/O30) and the partners of the center. The authors would like to thank Tonje Vikan, Marianne Hansen and Gerrit Timmerhaus at Nofima for the laboratorial analysis and Cassandra Roberts for proofreading the manuscript.

Appendix A. Supplementary data

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

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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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