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The effects of long-term 20 mg/L carbon dioxide exposure on the health and performance of Atlantic salmon *Salmo salar* post-smolts in water recirculation aquaculture systems

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ABSTRACT

Previous research and experience has linked elevated dissolved carbon dioxide (CO_2) to reduced growth performance, poor feed conversion, and a variety of health issues in farm-raised fish, including Atlantic salmon *Salmo salar*. Supplemental control measures in water recirculation aquaculture systems (RAS) to reduce CO_2 accumulation, however, such as increased water pumping to decrease tank hydraulic retention time, can represent significant costs for operators. We exposed post-smolt S_0 Atlantic salmon (197 ± 2 g, 423 days post-hatch) to either high (20 ± 1 mg/L) or low (8 ± < 1 mg/L) dissolved CO_2 in six replicated freshwater RAS for 384 days to investigate differences in performance and health as the salmon were grown to harvest size. All RAS were operated at moderate water exchange rates (1.0% of the total recirculating flow), a 24-h photoperiod was provided, fish were fed to satiation, and densities were maintained between 40 and 80 kg/m^3. Over the study period, dissolved oxygen was kept at saturation, mean water temperature was 14.1 ± 0.1 °C, and alkalinity averaged 237 mg/L as $CaCO_3$. At study's end, no significant differences in fish weight (high CO_2 mean weight = 2879 ± 35 g; low CO_2 mean weight = 2896 ± 12 g), feed conversion ratio (1.14 ± 0.12 vs. 1.22 ± 0.13, respectively), or thermal growth coefficient (1.45 ± 0.01 vs. 1.46 ± 0.01, respectively), were observed. No significant differences in survival (high CO_2 mean survival = 99.1 ± 0.4%; low CO_2 mean survival = 98.9 ± 0.3%) or culls due to saprolegniasis (3.5 ± 1% vs. 3.0 ± 1%, respectively) were determined, and no nephrocalcinosis was observed through histopathological evaluation. Blood gas and chemistry evaluation revealed higher pCO_2, bicarbonate, and total CO_2, and lower chloride and glucose, in the high CO_2 cohort. Molecular analyses of gill enzyme regulation showed significantly higher expression of Na^+/K^+ ATPase α1a in high CO_2 fish at 3-weeks post-challenge, indicating physiological adaptation to the higher CO_2 environment without any noticeable long-term impacts on health or performance. Overall, the results of this study suggest that, at 237 mg/L as $CaCO_3$ mean alkalinity, post-smolt Atlantic salmon can be raised in freshwater RAS to harvest size with up to 20 mg/L CO_2 without significantly impacting fish health and performance.

1. Introduction

Increased production and accumulation of dissolved carbon dioxide (CO_2) can become limiting in intensive recirculation aquaculture systems (RAS) when pure oxygen is utilized (Fivelstad, 2013). Hypercapnia and associated acidosis, in response to chronic exposure to elevated dissolved CO_2, are related to reduced feed intake and growth performance of fish (Smart, 1981; Danley et al., 2005; Fivelstad et al., 2007), reduced condition factor (Fivelstad et al., 1998, 2003a,b), and nephrocalcinosis (Landolt, 1975; Fivelstad et al., 1999; Hosfeld et al., 2008). Many of the negative effects of elevated CO_2 can be attributed to impaired oxygen-hemoglobin binding in affected fish (Colt et al., 1991; Wedemeyer, 1996). For Atlantic salmon *Salmo salar*, the impact of elevated CO_2 can be influenced by other water quality parameters, especially dissolved oxygen, alkalinity and dissolved metals, and life-stage (Eshchar et al., 2006; Fivelstad, 2013; Fivelstad et al., 2015, 2017); therefore, the establishment of upper CO_2 limits in salmon culture needs to include consideration of such additional factors. In

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Norway, the safe upper limit of dissolved CO_2 for smolt production has been set at 15 mg/L, based on research in flow-through systems, although it has been suggested that this limit should be reduced in low alkalinity and/or high dissolved metals environments (Fivelstad, 2013). Additionally, there are potentially large bio-economic benefits that can be achieved by using a combination of technologies, e.g., more rapid culture tank hydraulic exchange and application of CO_2 stripping technologies, to prevent CO_2 from accumulating to concentrations that can reduce productivity and fish welfare (Noble et al., 2012). For example, maintaining a CO_2 concentration of 20 mg/L versus 10 mg/L would require pumping approximately 100% more water through the culture tank that in turn substantially increases the size and cost of the RAS water treatment processes and piping (Summerfelt and Vinci, 2004).

Recent interest in raising larger (i.e., 500–1000 g) post-smolts prior to sea cage transfer, as an alternative to the traditional approach of raising S_0 or S_1 smolts for stocking sea cages, has necessitated research to determine optimal culture conditions for these larger, post-smolt salmon in land-based systems. Furthermore, raising Atlantic salmon up to market size entirely in land-based systems, without any utilization of sea cages, has also received significant interest in recent years (Summerfelt and Christianson, 2014; Davidson et al., 2016; Liu et al., 2016). Fivelstad et al. (2015) recently reported specific growth rates for Atlantic salmon post-smolts reared for 3 months at varying CO_2 levels (up to 34 mg/L) in flow-through seawater, and assessed that SGR would not be compromised until CO_2 reached 18.6 mg/L. However, in the same study, nephrocalcinosis was identified at a lower CO_2 concentration of 16 mg/L. Comparable research focused on freshwater systems is currently lacking, as is research examining CO_2 exposure in land-based RAS as Atlantic salmon are raised to harvest size.

We sought to investigate “typical” (i.e., 8–10 mg/L) and elevated (20 mg/L) dissolved CO_2 and its effects on Atlantic salmon post-smolt performance and health while raised in freshwater RAS up to a harvest size of approximately 3 kg. Specifically, we examined growth performance, survival, feed conversion, thermal growth coefficient, blood chemistry, histopathology, and gill enzyme gene expression as post-smolt Atlantic salmon were raised from approximately 300 g to 3000 g in replicated freshwater RAS at normal and elevated CO_2 levels over the course of 1 year. The overall objective of this study was to determine whether long-term exposure to 20 mg/L would significantly impact post-smolt Atlantic salmon health and performance. The absence of negative effects under these conditions would inform the RAS-based post-smolt and growout salmon industry that potentially costly efforts to reduce CO_2, such as increasing water recirculation flow to reduce culture tank hydraulic retention time, might be unnecessary.

2. Materials and methods

2.1. Water recirculation aquaculture systems

This study utilized six replicated 9.5 m^3 freshwater RAS, which have previously been described in detail (Davidson et al., 2011; Good et al., 2011a; Davidson et al., 2013). Briefly, each RAS consisted of a 5.3 m^3 circular dual-drain tank, radial flow separator, 60-μm drum filter, fluidized sand biofilter, and a degassing column overtopping a low head oxygenator (LHO) (Fig. 1). The overall water recirculation flow was 380 L/min, with makeup water flushing rate set at 1.0% of the total recirculation flow. The makeup water used for RAS flushing was obtained from an on-site spring source. System hydraulic retention time (HRT) was 1.54 days; culture tank HRT was 15 min.

2.2. Atlantic salmon

Fertilized, eyed Atlantic salmon eggs (St. John River strain) were obtained from a domestic producer, hatched on-site, and raised in a flow-through system at 12 °C under constant photoperiod (i.e. 24 h light, zero hours dark, or LD24:0) until 40 g, at which point photoperiod was reduced to provide a 6-week LD12:12 “winter”, followed by a return to LD24:0, in order to induce smoltification. Post-smolts were then maintained in a partial water reuse system (80% exchange) until transfer to the six study RAS. Approximately 890 Atlantic salmon were randomly selected from this initial group for stocking each RAS, at 197.2 ± 1.9 g (± SE) in mean weight.

2.3. Experimental conditions

Throughout the study period, dissolved oxygen was maintained at saturation, mean water temperature was 14.1 ± 0.1 °C, and alkalinity averaged 237 mg/L as $CaCO_3$ in all RAS. Initial stocking density was approximately 33 kg/m^3; as the study progressed, salmon were maintained at densities between 40 kg/m^3 to 80 kg/m^3, with fish randomly culled periodically to maintain equal densities within this range among all rearing units. A constant LD24:0 photoperiod was provided. All salmon populations were fed using automated feeders (T-drum 2000CE, Arvo-Tec, Finland), which administered feed every second hour. Feeding rates were based on standardized feeding charts, but also on observations of feeding activity and wasted feed, such that fish were fed to satiation. A slow-sinking trout feed (Zeigler Brothers, Inc., Gardners, PA, USA) with a 48:24 protein-to-fat ratio was initially used; however, approximately three months into the study, feed was switched to a commercial salmon diet (EWOS, Vancouver, BC, Canada). This mid-study change in diet was based solely on feed availability at the time and was applied simultaneously to all RAS within each treatment to eliminate the possibility of confounding. Mean feed loadings of 0.34 ± 0.01 kg/day per m^3/day make-up water were maintained for the high and low CO_2 treatments, with no significant differences in feed loadings between RAS or treatment groups.

Rotational water velocity was initially set to provide swimming exercise of 1–2 body-lengths per second (BL/s); however, as fish grew in length over the study period, additional rotational velocity was required to provide exercise within this swimming speed range, while maintaining a tank hydraulic retention time of 15 min. Therefore, a small submersible pump (Model R100, BJM Pumps LLC, Old Saybrook, CT, USA) was installed in the sidewall box of each tank, when salmon were 647 days post-hatch in age, to jet water through a vertical in-tank spray bar, and as such maintained swimming speed between 1 and 2 BL/s for the remainder of the study.

Following stocking, the salmon were acclimated to the RAS environment for approximately one month, after which three RAS were randomly selected to receive additional CO_2 to attain the ‘high’ treatment concentration of 20 ± 1 mg/L. At this time, the salmon were 423 days post-hatch in age and approximately 300 g mean weight. Elevation of dissolved CO_2 concentration was achieved by co-transferring pure CO_2 feed gas with O_2 feed gas within the LHOs of the high CO_2 RAS. The remaining three RAS received no additional CO_2, such that levels in these systems were at normal, ‘low’ levels (8 ± < 1 mg/L).

2.4. Data collection

2.4.1. Water quality

Water samples were collected weekly from the RAS side drains and were assessed on-site for the following parameters: alkalinity, CO_2, nitrite nitrogen, nitrate nitrogen, true color, total ammonia nitrogen (TAN), total suspended solids (TSS), and UV transmittance. On-site water sampling and testing methodologies have been described previously (e.g. Davidson et al., 2011); all water quality parameters were measured according to methods detailed in APHA (2005) and HACH (2003). In particular, dissolved CO_2 was measured following Hach Method 8223-Burret titration (HACH, 2003). Dissolved oxygen, pH, and temperature were monitored continuously with a SC100 Universal Controller (Hach Company, Loveland, CO, USA) utilizing probes, at

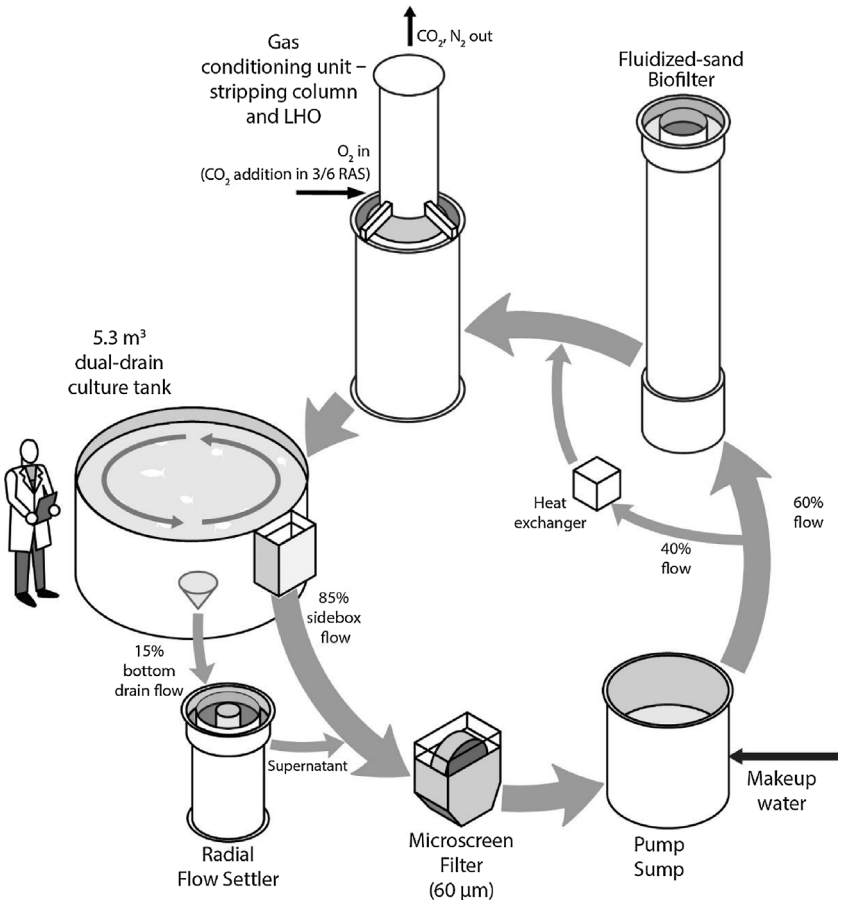

Fig. 1. Process flow diagram of experimental RAS utilized (N = 6), with n = 3 treated with additional CO_2 to maintain the high CO_2 treatment of 20 mg/L (drawing by K. Sharrer, The Conservation Fund Freshwater Institute).

each RAS, including LDO (dissolved oxygen) and Digital Differential pH Sensor probes (Hach Company). Unionized ammonia was determined based on TAN, pH, and temperature data using tables provided by Timmons et al. (2002).

2.4.2. Fish performance

Growth performance was assessed through length and weight sampling events carried out every second month; sample sizes ranged from approximately 60–120 salmon per RAS, and were calculated with the following formula:

$$n = (Z * (\text{stdev.}_{\text{grams}}/\text{accepted error}_{\text{grams}}))^2$$

where Z = 1.65 (relative to a 90% confidence interval), and the accepted error was 5 g. Performance data were used to generate growth curves, and to calculate thermal growth coefficients (in conjunction with daily water temperature data) using the following formula (Iwama and Tautz, 1981):

$$[FBW^{1/3} - IBW^{1/3}]/\Sigma\,[T \times D] \times 100$$

where FBW = final body weight; IBW = initial body weight; T = water temperature; and D = number of days. Feed conversion ratios (FCR) were calculated for each RAS, for both the entire study period and for individual 2-month intervals, following the formula:

FCR = cumulative feed delivered/biomass gain

Finally, all mortalities were noted daily to assess mean survival in each treatment group. Culls due to clinical saprolegniasis, a common disease associated with ubiquitous freshwater oomycete species of the *Saprolegnia* genus, were also recorded for each RAS and treatment group.

2.4.3. Fish health and physiology

Histopathology was assessed over four separate sampling events. Specifically, at treatment commencement (day 423) and at approximately 1-month (day 465) and 2-month (day 465) time points, anterior and posterior kidney were sampled from 5 fish per RAS for examination for nephrocalcinosis, and at study's end (day 807) samples of gill, heart, spleen, liver, and anterior and posterior kidney were collected from 5 fish per RAS for overall tissue health assessment. All sampled fish were euthanized prior to tissue collection with 200 mg/L tricaine methanesulfonate. All samples were preserved in histological grade 10% formalin solution for at least one week prior to shipment to an independent veterinary pathologist, who was blinded to the treatment group origins of all sampled tissues. Tissues were routinely processed and stained with H&E (haemotoxylin and eosin) stain; additional sections were stained with GMS (Gomori's methenamine silver) reagents for the detection of fungal elements. A 0-to-6 point grading scale was developed to quantify the severity of each lesion type observed, with 0 representing normal tissue and 6 representing very severe lesions.

At study's end, 5 fish per RAS were captured at random, euthanized, then quickly bled via caudal venipuncture using a 21-gauge 1.5-inch needle and 5 mL syringe. Whole blood samples were analyzed using an i-Stat 1 portable analyzer (Abbott Laboratories, Abbott Park, IL) with CG4 + and CHEM8 + cartridges, measuring pH, pCO_2, pO_2, HCO_3, total CO_2, O_2 saturation, and lactate, and sodium, potassium, chloride, calcium, glucose, creatinine, hematocrit, and hemoglobin levels, respectively.

Gill samples for gene expression analyses were collected pre-study and at 1-week and 3-weeks, and the expressions of i) proton ATPase (H

Table 1
Water quality parameters (mean ± SE) in the high and low CO_2 treatment groups averaged over the 384-day study period (n = 3).

Parameters	High CO_2	Low CO_2
TAN (mg/L)	0.16 ± 0.01	0.16 ± 0.02
Unionized Ammonia (mg/L)*	0.001 ± < 0.001	0.003 ± < 0.001
Nitrite Nitrogen (mg/L)	0.075 ± 0.013	0.080 ± 0.016
Nitrate Nitrogen (mg/L)	12 ± < 1	12 ± < 1
Alkalinity (mg/L)	233 ± 5	241 ± 2
CO_2 (mg/L)*	20 ± < 1	8 ± < 1
True Color (Pt-Co units)	11 ± < 1	12 ± < 1
UV Transmittance (%)	89 ± < 1	88 ± 1
TSS (mg/L)	0.9 ± < 0.1	1.1 ± < 0.1
pH*	7.41 ± 0.01	7.86 ± 0.03
DO (mg/L)	10.1 ± < 0.0	10.1 ± < 0.0

* Significant ($p < .05$) differences between high and low CO_2 systems.

ATPase), ii) Na^+/K^+ ATPase α1b, iii) Na^+/K^+ ATPase B α1a, and iv) heat shock protein 70 (HSP70), were assessed at these time points. Gill H ATPase is typically utilized for acid excretion (Evans et al., 2005), while Na+/K+ ATPase is involved in osmoregulation; expression of isoform α1b increases with seawater acclimation, while the α1a isoform expression decreases during this transition (McCormick et al., 2009). Gill HSP70 expression is typically increased in response to a wide range of environmental stressors (Basu et al., 2002). To assess gene expression, small sections of the middle of the left second gill arch were collected from 5 fish per RAS at each sampling point. Samples were preserved in RNA*later*™ (Invitrogen) prior to analyses to assess the expression of the aforementioned genes using primers and methods described previously in detail (Kolarevic et al., 2012a,b, 2014). Briefly, gills were homogenized using the Precellyser 24 (Bertin Technologies, France) and Trizol (Invitrogen) and RNA extracted using the Micro to Midi Kit® (Invitrogen). All samples were DNase treated (DNase1, Invitrogen). The quality of the RNA was assessed spectrophotometrically (NanoDrop Technologies, DE, USA) and 0.5 µg RNA was reverse transcribed to cDNA using oligo(dT) primer and random hexamers and the Taqman Gold RT-PCR kit (Applied Biosystems). The cDNA synthesis was performed with 10 min primer incubation at 25 °C, 1 h RT step at 48 °C and 5 min RT inactivation at 95 °C. Real-time qPCR was conducted using the Light cycler 480 and SYBR Green chemistry (Roche, Switzerland) at the following thermal cycling conditions: 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s, 60 ± 1 °C for 15 s and 72 °C for 15 s. Further, specificity was assessed by the melting curves, determined post PCR (95 °C for 15 s, 60 °C for 1 min and 97 °C continuous). For relative quantification, duplicate C_T values were averaged, and values differing > $0.5C_T$ were removed and re-analyzed. Relative expression was calculated according to Pfaffl et al. (2002), with normalization against 18S rRNA (Singer et al., 2002; Jørgensen et al., 2007); Forward: *GCCCTATCAACTTTCGATGGTAC* and Reverse: *TTTGGATGTGGTAGCCGTTTCTC*), which was found to be stably transcribed in control and test samples according to the BestKeeper software (Pfaffl et al., 2004). The efficiency of the PCR reactions was calculated for all primer pairs by six times 1:5 dilution series of a cDNA mix of all used samples. Efficiencies were between 1.8 and 2.0 as estimated using the LightCycler® 480 Software (version 1.5.0.39).

2.5. Statistical analyses

Water quality and whole blood data were analyzed using two-sample *t*-tests of mean data per RAS (i.e., n = 3) to determine individual outcome variables associated with CO_2 treatment; data for individual water quality parameters were averaged per RAS over the entire study period, while whole blood data were derived from the discreet sampling point at study's end. For growth performance, TGC, and FCR data, *t*-tests were performed for data collected at each sampling point or interval, to identify differences between treatment groups for these outcomes at or during specific timepoints or intervals. Ordered logit regression was carried out for the scored histopathology data, for each sampling point and tissue lesion type. The above analyses were carried out using STATA software (StataCorp, College Station, TX). For the gill gene expression data, transcription ratios were analyzed using the Relative Expression Software Tool (REST) and tested for significance by the Pair Wise Fixed Reallocation Randomization Test© (Pfaffl et al., 2002).

Means and standard errors (SE) reported for gill gene expression data analyses were based on replication of individual fish within treatments (n = 15) (Jørgensen et al., 2007; Kolarevic et al., 2012a,b); all other means reported in this study were based on n = 3 replication (i.e., 3 RAS per treatment), and all reported error values are standard errors.

3. Results

Elevated CO_2 affected water quality parameters in a predictable manner (Table 1); significantly ($p < .05$) higher CO_2 resulted in significantly lower pH and unionized ammonia. No differences were observed in the remaining water quality parameters measured, e.g., O_2, NO_2-N, NO_3-N, TAN, true color, UV transmittance, and TSS.

Comparably high survival was observed within each cohort over the study period: 99.1% ± 0.4 and 98.9% ± 0.3 survival in the high and low CO_2 groups, respectively. Culls due to saprolegniasis were also comparable: 3.5% ± 1 and 3.0% ± 1 for the high and low CO_2 treatments, respectively, over the entire study period. Growth performance (Fig. 2) was virtually identical between treatment groups. Final mean fish weights for the high and low CO_2 conditions were 2879 ± 35 and 2896 ± 12 g, respectively. Low CO_2 fish had significantly higher mean weight at day 689 post-hatch, after water rotational velocities were increased at day 647 post-hatch; however, no previous or subsequent differences in mean weight were observed between treatment groups. Furthermore, thermal growth coefficients (Fig. 3) were comparable between high and low CO_2 groups in this period, as well as the rest of the study, except for a significantly higher mean TGC in the low CO_2 group during the period immediately following the initiation of the high and low CO_2 treatments (i.e., 453–507 days post-hatch). For all subsequent time periods, however, TGC was not significantly different between the two treatments. Mean FCRs for each treatment group were comparable ($p > .05$) over the entire study period: 1.02 ± 0.03 and 1.03 ± 0.02 for the high and low CO_2 groups, respectively.

Whole blood gas and chemistry analyses revealed several significant differences between fish exposed to the two treatments (Table 2). Specifically, pCO_2, bicarbonate, and total CO_2 were significantly higher in high CO_2 fish, while chloride and glucose were lower in fish exposed to 20 mg/L CO_2. Histopathology did not reveal evidence of nephrocalcinosis in kidney tissue at any sampling point. Renal tubulointerstitial inflammation was noted in several kidneys – specifically, degeneration and subsequent sloughing of renal tubular cells was observed, with progressive peritubular inflammation and development of chronic granulomatous foci; further staining with GMS reagents did not detect the presence of fungal elements, and no other infectious agents were observable. This lesion type, however, was noted at low prevalence and was not significantly associated with either treatment group. No other abnormal lesions were observed in any tissues collected throughout the study period.

Expression of selected genes in gill tissues demonstrated several statistically significant differences between CO_2 treatment groups and/or 1-week and 3-week post-CO_2 treatment initiation sampling points (Fig. 4). The expression of the Na^+/K^+ ATPase α1a subunit was not significantly different between treatment groups at 1-week; however, a significant reduction in ATPase α1a expression was observed in the low CO_2 group between 1- and 3-weeks, while the expression of this gene in the high CO_2 group remained significantly elevated, compared to the

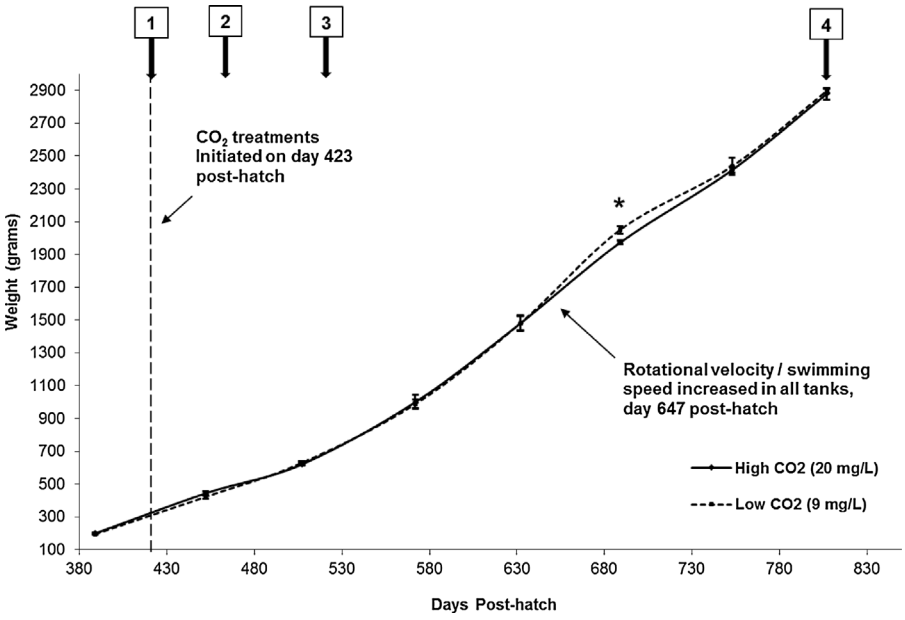

Fig. 2. Growth curves for post-smolt Atlantic salmon weight between 423 and 807 days post-hatch, exposed to either high (20 ± 1 mg/L) or low (8 ± 1 mg/L) dissolved carbon dioxide (n = 94–118 fish per RAS, depending on sampling point). Data points represent treatment means (n = 3 RAS per treatment) with standard error bars. Asterisks represent significant ($p < .05$) differences in mean weight between treatment groups at specific time points. Numbers at top indicate timing of histopathology sampling events, 1–4.

low CO_2 group, at the 3-week point. The expression of the Na^+/K^+ ATPase α1b subunit was not significantly different between treatment groups at 1- and 3-weeks post-study initiation; however, expression in both treatment groups was significantly lower at 3-weeks compared to 1-week post initiation. No significant differences were noted between treatment groups and/or sampling times for H ATPase and HSP70.

4. Discussion

The major finding of this study was that Atlantic salmon post-smolts raised to harvest size in freshwater RAS did not differ in growth performance and overall feed conversion when exposed to long-term concentrations of either 20 mg/L CO_2 or 8 mg/L, while maintaining dissolved oxygen at 100% saturation and alkalinity at 237 mg/L as $CaCO_3$ (approximately 4 mM). These results are similar to those of Fivelstad et al. (2015), who modeled that post-smolt specific growth rate (SGR) in flow-through seawater would not be significantly impacted until a threshold of 18.6 mg/L CO_2 was reached. Similarly, Fivelstad et al. (2017) determined that SGR was only minimally impacted between 15 and 20 mg/L CO_2 for Atlantic salmon parr in freshwater at 15 °C and low alkalinity (≤24 mg/L). Aside from these two studies, however, it is often difficult to compare our findings with many previously published experiments due to differences in important and potentially confounding water quality parameters, such as temperature, salinity, alkalinity, and pH, as well as fish life-stage. Fivelstad (2013) provides an overview of previously published work in this area and summarizes the different environmental conditions under which these studies were performed. For example, Hosfeld et al. (2008) found significant performance reduction in salmon exposed to 17–18 mg/L CO_2 (i.e., comparable to the present study); however, this study examined smolts (as opposed to post-smolts) in low alkalinity water (0.09 mM) at 7–8 °C and 5.9 pH. The fish performance impact demonstrated by Hosfeld et al. (2008) could therefore have been associated with CO_2 in relation to the smolt life-stage (i.e., fish already under relatively high physiological stress), or the low alkalinity environment that likely led to the acidic pH with the addition of CO_2 (and, in turn, potentially facilitated the effects of toxic aluminum). The present study provides further insight into the effects of CO_2 on the health and

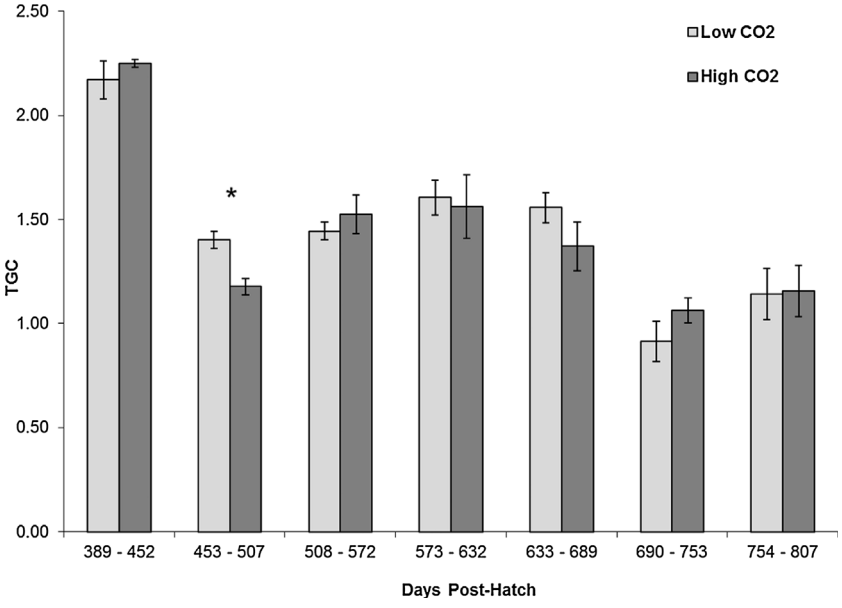

Fig. 3. Thermal growth coefficients (TGC) for post-smolt Atlantic salmon exposed to high (20 mg/L) or low (8 ± 1 mg/L) dissolved carbon dioxide, based on mean water temperatures (continuous measurement) during intervals between fish weight assessments (n = 94–118 fish per RAS, depending on sampling point). Data points represent treatment TGC means (n = 3 RAS per treatment) with standard error bars. Asterisks represent significant ($p < .05$) differences in mean weight between treatment groups at specific time points.

Table 2
Means and standard errors of measured whole blood gas and chemistry parameters in Atlantic salmon (2888 g ± 17; 807 d post-hatch) exposed for 384 days to high (20 ± 1 mg/L) and low (8 ± 1 mg/L) dissolved carbon dioxide (n = 3).

Parameter	CO_2	Mean	SE	*p*-value
Sodium (mmol/L)	High	148	± < 1	.051
	Low	152	± 1	
Potassium (mmol/L)	High	3.50	± 0.10	.174
	Low	3.30	± 0.07	
Chloride (mmol/L)	High	127	± < 1	.004
	Low	132	± 1	
Calcium (mmol/L)	High	1.52	± 0.02	.077
	Low	1.57	± 0.01	
Glucose (mg/dL)	High	66.0	± 0.3	.003
	Low	71.6	± 1.6	
Hematocrit (%PCV)	High	33.9	± 1.5	.082
	Low	39.7	± 2.1	
Hemoglobin (g/dL)	High	11.5	± 0.5	.083
	Low	13.5	± 0.7	
pH	High	7.11	± 0.02	.059
	Low	7.06	± 0.01	
pCO_2 (mmHg)	High	61.6	± 1.5	.010
	Low	48.7	± 2.4	
Bicarbonate (mmol/L)	High	19.7	± 0.3	< .001
	Low	13.7	± 0.3	
Total CO_2 (mmol/L)	High	21.5	± 0.3	< .001
	Low	15.2	± 0.5	
pO_2 (mmHg)	High	10.3	± 0.5	.489
	Low	11.1	± 0.9	
O_2 saturation (%)	High	7.08	± 0.46	.645
	Low	7.70	± 1.15	
Lactate (mmol/L)	High	2.68	± 0.06	.162
	Low	3.03	± 0.19	

performance of Atlantic salmon; however, as with all published studies in this area, care must be taken through consideration of other concurrent and possibly confounding subject and/or environmental variables.

To our knowledge, this is the first study examining post-smolt Atlantic salmon performance in freshwater RAS up to harvest size under elevated CO_2 conditions. The influence of high alkalinity in this study, however, needs to be considered, as CO_2 toxicity has been suggested to increase in low alkalinity environments (Summerfelt et al., 2000; Fivelstad, 2013), although to our knowledge no side-by-side experimental groups to test the effect of alkalinity have been studied in the same trial for salmon. It should be noted that carbonate alkalinity levels in the present study were quite high even in comparison to full-strength seawater, i.e. ~4 mM study conditions compared to approximately 2.3 mM in seawater. Unlike in flow-through systems, alkalinity can be efficiently supplemented in RAS with the addition of sodium bicarbonate, calcium hydroxide, or sodium hydroxide (Loyless and Malone, 1997), which is necessary to maintain alkalinity lost to nitrification in RAS. Alkalinity provides an overall buffering effect through the neutralization of acid (Stumm and Morgan, 1996), and hence the addition of CO_2 to high alkalinity water would not produce the corresponding drop in pH (and the negative physiological consequences in fish) that would otherwise be observed in low alkalinity water. Given that freshwater supplies of Norwegian salmon farms typically have low alkalinity (0.06–0.09 mM) (Kristensen et al., 2009; Fivelstad, 2013), the overall lack of effect of elevated CO_2 in the present study must be considered with caution, as upper tolerance limits for CO_2 in other salmon producing areas of the world, such as Norway, could be considerably lower.

Significant differences in several blood parameters were observed in this study. Firstly, it should be mentioned that the reliability of the i-Stat system for determining fish blood parameters, in relation to more established laboratory methods, has been called into question, largely due to system measurements and calculations being carried out using algorithms derived from human blood at 37 °C (DiMaggio et al., 2010; Gallagher et al., 2010; Harter et al., 2014). While these and other studies have demonstrated significant discrepancies between i-Stat versus laboratory results when analyzing fish blood, the usefulness of the i-Stat in experimental studies, in which the effects of two or more treatments are compared (and hence precision, versus accuracy, becomes the predominant concern), is often overlooked. Although a formal assessment of i-Stat precision was not carried out, in the authors' experience the i-Stat has demonstrated its precision in repeated measurements from the same individuals, and has been very useful, for example, in determining differences in salmonid whole blood parameters between treatment groups in experiments comparing the effects of water ozonation, nitrate-nitrogen, or exposure to RAS versus flow-through environments (Good et al., 2011a,b; Davidson et al., 2014). In the present experiment, blood chemistry findings were mostly in agreement with those published in previous studies, specifically that Atlantic salmon in hypercapnic conditions demonstrate a reduction in plasma chloride and elevation in plasma pH and bicarbonate (Fivelstad et al., 2003a; Hosfeld et al., 2008; Fivelstad, 2013; Fivelstad et al., 2017). Reduced chloride under these conditions is likely the result of increased gill bicarbonate/chloride exchange as fish take up bicarbonate at the expense of chloride in order to buffer the initial acidosis related to elevation in blood pCO_2 (Fivelstad et al., 2003a, 2015, 2017). Reduced sodium was observed by Hosfeld et al. (2008) in association with elevated CO_2; however, the reduction in sodium noted in this study did not quite reach the level of statistical significance (i.e., p = .051). The opposite was observed for blood glucose: Hosfeld et al. (2008) reported no association between glucose and elevated CO_2, whereas in the present study elevated CO_2 was associated with significantly decreased blood glucose. The etiology of the observed lower blood glucose in the high CO_2 treatment is unknown, although we hypothesize that in the absence of elevated CO_2 salmon were more susceptible to capture stress, leading to relatively higher catecholamine release and a consequent increase in blood glucose.

Fivelstad et al. (2003a) found that increasing CO_2 (up to 24 mg/L) in freshwater prior to smolt transfer to seawater increased kidney calcium content in conjunction with increased prevalence of nephrocalcinosis, as well as an increase in whole body calcium content following 4 weeks in seawater. Whole blood calcium was not significantly different between CO_2 treatment groups in the present study, and no nephrocalcinosis was observed. Again, comparisons between the present study and previous research are often difficult to make in light of differences, among other things, in life stage, water temperature, alkalinity, and pH conditions under which the studies were carried out. In the case of Fivelstad et al. (2003a), research was carried out under conditions of significantly lower temperature (7–9 °C), alkalinity (0.6 mM), and pH (6.3–6.6) in salmon undergoing smoltification, and it is conceivable that any of these variables, or combination thereof, could have facilitated nephrocalcinosis progression. Nephrocalcinosis has been commonly observed in association with elevated CO_2 (Harrison, 1979; Hosfeld et al., 2008), yet this pathology was not noted in the present study. Lesions associated with this condition – specifically, granuloma formation in the renal tubules (Harrison and Richards, 1979) – were observed on histological examination of kidneys collected throughout the present study, although at low prevalence and not associated with either CO_2 treatment group. Additional staining (e.g. Von Kossa's stain) to observe mineralization within the granulomas was not carried out in the present experiment, however, and further research in this area should include such staining. Other studies examining nephrocalcinosis in Atlantic salmon have provided conflicting results; for example, Fivelstad et al. (2007) reported an absence of nephrocalcinosis in parr exposed to > 30 mg/L CO_2 for 47 days in

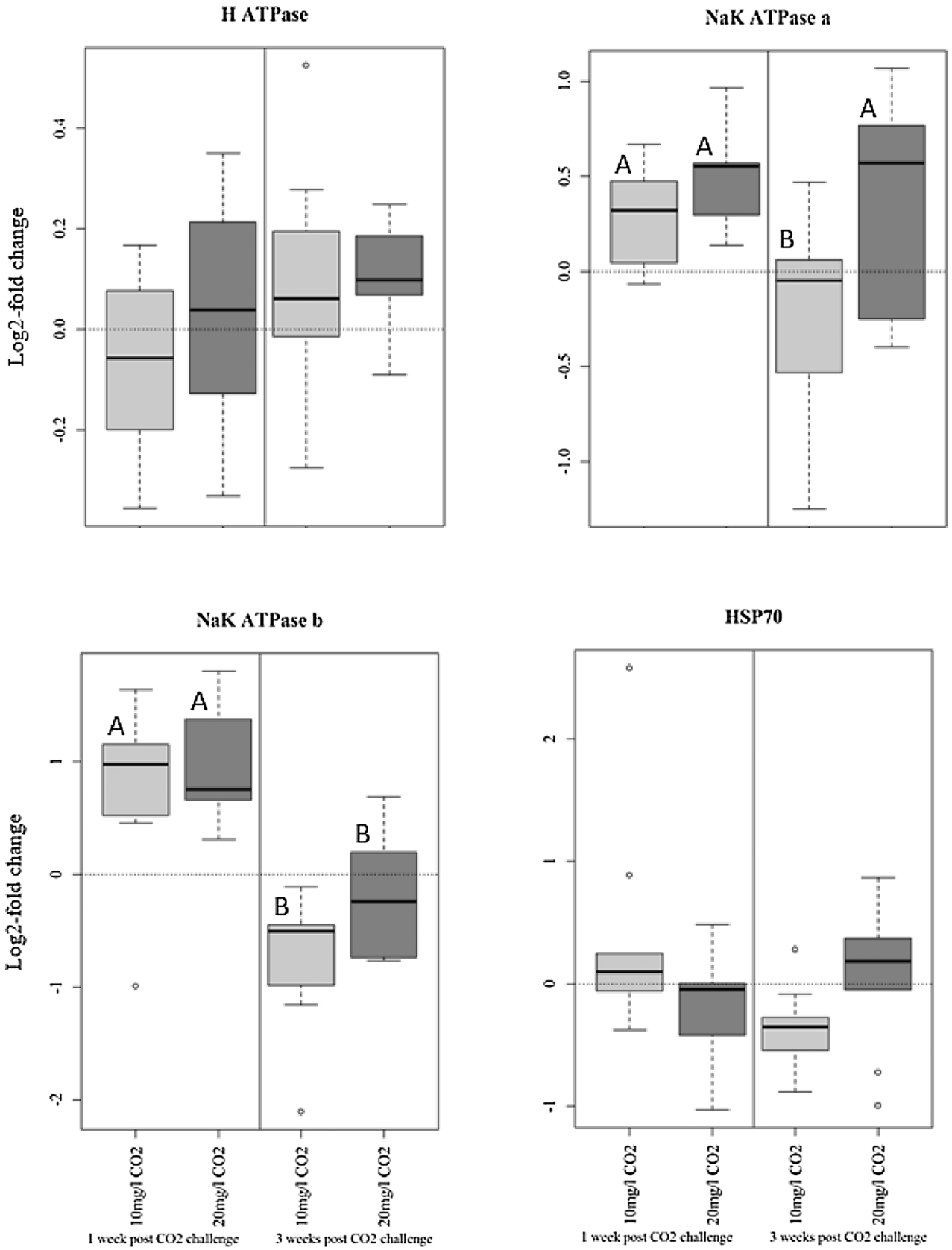

Fig. 4. Expression of selected genes in gill of fish kept either in high or low CO_2 groups, assessed at 1-week and 3-weeks following treatment initiation. Error bars represent standard errors of the mean. For NaK ATPase α1a and α1b, significant differences ($p < .05$) in gene expression between treatment groups and/or sampling points are represented by different letters over boxes.

freshwater, while Hosfeld et al. (2008) reported increased nephrocalcinosis in smolt exposed to up to 24 mg/L CO_2 in freshwater prior to sea transfer. In addition, in a study on post-smolts in flow-through full-strength sea water, nephrocalcinosis was found at concentrations above 16 mg/L CO_2 (Fivelstad et al., 2017). Clearly, more studies should be undertaken to unravel the mechanisms behind nephrocalcinosis and its relation to CO_2 in salmon; at present, the precise etiology of this pathological process remains unknown (Fivelstad et al., 2017).

Evidence of physiological adaptation to elevated CO_2 is apparent in the TGC, gill gene expression, and growth performance data. Specifically, significantly lower TGC was observed in the 20 mg/L CO_2 group immediately following treatment initiation, and this is evidenced by a slightly steeper growth curve in the low CO_2 group during this time interval; however, TGC was not significantly different between treatment groups for the remainder of the experiment, indicating that this was a transient change likely in response to the initial challenge of the higher CO_2 environment. When exposed to elevated CO_2 environments, fish correct the initial resultant acidosis through increased bicarbonate uptake over a period of 2–7 days (Heisler, 1986), and it is therefore likely that this process, along with other physiological adaptations, temporarily diverts energy away from somatic growth during early stages of elevated CO_2 exposure. Although not significantly so, TGC was lower in the 633–689 days post-hatch interval in the 20 mg/L CO_2 group, corresponding with lower mean weight in the same group around this time, and this was likely a response to the increase in water rotational velocity (and hence swimming speed) initiated at day 647 post-hatch. Finally, a significant decline in gill Na^+/K^+ ATPase α1a expression was noted at 3 weeks' post-treatment initiation in the low CO_2 group, whereas expression remained at comparable levels to measurements at 1-week post-initiation in the 20 mg/L CO_2 group. Continued expression of Na^+/K^+ ATPase α1a at 3 weeks' post-treatment could indicate acclimation to higher CO_2 levels during this early timeframe; however, further research is required to fully understand why expression of this particular isoform appears related to higher CO_2 conditions during the first month post-exposure. It is noteworthy that Fivelstad et al. (2017) also found that the effects of CO_2 in flow-through sea water was higher earlier in the trial than towards the end, including periods with no significant effects of CO_2 on growth rate.

Finally, certain findings of this study should be view with some caution, as n = 3 replication often does not provide a high level of statistical power, and hence truly significant differences between certain parameters might have been obscured. For example, a significant difference in whole blood sodium between treatment groups was not determined (p = .051); however, power analysis using the treatment means, common standard deviation, and number of replicates per treatment indicated a power of 0.44, which is below the often 'desired' power level of 0.80. Further research utilizing additional replication would be useful to investigate in more detail the effects of elevated dissolved CO_2 on a similar range of outcomes in Atlantic salmon.

5. Conclusions

Post-smolt Atlantic salmon performed equally in 20 mg/L and 8 mg/L CO_2 RAS, up to a harvest size of approximately 3 kg. While evidence of physiological acclimation to the elevated CO_2 conditions was apparent, this did not affect overall mean weight, TGC, FCR or nephrocalcinosis. This study, therefore, provides evidence that the upper limit of chronic exposure to CO_2 in high alkalinity freshwater RAS is likely higher than 20 mg/L, and that efforts (and associated costs) to reduce CO_2 concentrations to below 20 mg/L are potentially unnecessary. Further research is warranted to examine post-smolt performance and health in lower alkalinity systems (i.e., < 50 mg/L as $CaCO_3$) in response to elevated dissolved CO_2, as well as studies to pinpoint safe upper CO_2 limits for post-smolts in a range of production settings with variable temperature and salinity.

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