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Full title: The effects of carbon dioxide on growth performance, welfare, and health of Atlantic salmon post-smolt (*Salmo salar*) in recirculating aquaculture systems

Running head: Carbon dioxide in Atlantic salmon RAS

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Abstract

High carbon dioxide (CO₂) concentrations negatively impact fish, which makes data on its tolerance especially relevant for production systems that can accumulate CO₂ such as recirculating aquaculture system (RAS). The current study evaluates the effect of CO₂ on the growth performance, welfare, and health of Atlantic salmon post-smolts in RAS. This study consisted of two phases. The first was a CO₂ exposure phase, where eighteen tanks were used with six treatments in triplicate: 5, 12, 19, 26, 33 and 40 mg/L of CO₂ during 12 weeks in a 12 ppt salinity RAS (hereafter RAS phase). In the second phase, PIT-tagged fish were transferred to a 34 ppt salinity single flow-through tank at CO₂ < 5 mg/L (hereafter seawater phase) for an additional 6-week experimental period mimicking a seawater phase. Overall, mortality of fish exposed to CO₂ was low and not related to treatments. The mean final body weight was significantly higher in the 5 mg/L treatment compared to CO₂ treatments ≥ 12 mg/L at the end of RAS phase and to CO₂ treatments ≥ 33 mg/L at the end of seawater phase. Moreover, regressions showed that growth significantly decreased linearly with increasing CO₂ in the water. Eye cataracts and visible external damage on skin, operculum, and fins were inexistent and similar among CO₂ treatments. Kidneys showed no signs of mineral deposits in any of the structures of the tissue. However, skin analysis showed that fish exposed to high CO₂ concentrations had a significantly thinner dermis layer (both at the end of RAS and seawater phase) and a significantly thinner epidermis layer and lower mucus cells count (at the end of seawater phase). In conclusion, Atlantic salmon post-smolts cultured in brackish water RAS showed a maximum growth performance at CO₂ concentrations below 12 mg/L. Except skin, no major effects of health and welfare were observed, including cataracts and nephrocalcinosis. Further studies should evaluate the molecular and physiological responses to both short-term and long-term carbon dioxide exposure.

Keywords: salmonids; RAS; closed systems; hypercapnia; CO₂

1. Introduction

Production of Atlantic salmon (*Salmo salar*) has had an impressive cumulative growth of 384 % since 1995 (5 - 8% annual growth), reaching 2 million tonnes per year in 2014 (FAO, 2016). However, production growth has ceased since 2014 and stabilized just above 2M tonnes per year. This stagnation is mainly associated with the reduced availability of licensed sites for sea cage farming, and to the increasing pressure for controlling pathogens and parasites, especially the ectoparasite sea lice (*Lepeophtheirus salmonis*). Alternative production systems to the traditional sea cages are gaining momentum, particularly land-based recirculating aquaculture system (RAS) facilities to produce smolts, post-smolts or all the way to market-size fish (Dalsgaard et al., 2013; Martins et al., 2010; Summerfelt et al., 2016). The majority of facilities being designed or under consideration in Norway at the moment are however to produce post-smolts up to 1 kg (Hagspiel et al., 2018). The distinctive characteristics of RAS (e.g. flexible location, production environment control and biosecurity) along with the converging production costs between sea cage farming and RAS (Liu et al., 2016) are inviting more production of Atlantic salmon in RAS, perhaps to levels as much as 5 - 10 % of the total production by 2020 (DNB Markets, 2017; Iversen et al., 2016). However, others still maintain that RAS production has higher levels of financial uncertainty compared to ocean pens for producing market-size Atlantic salmon (King et al., 2016).

Extensive research efforts are therefore being done to optimize the design and dimensioning of RAS, especially solutions regarding the negative consequences of water re-use, i.e. the accumulation fish metabolites like ammonia and carbon dioxide (CO₂) in the water (Mota et al., 2014; Summerfelt et al., 2015; Terjesen et al., 2013). CO₂ removal is one of the most costly unit processes, since CO₂ levels limit fish density and determines system flow in many cases and therefore energy costs of pumping water through the fish tanks (Colt et al., 2012; Noble et al., 2012; Summerfelt et al., 2000; Summerfelt et al., 2003). To improve CO₂

removal and better manage CO₂ concentration in RAS, it is necessary to generate reliable data on fish tolerance to CO₂. Fish CO₂ tolerance has been vastly studied in the context of increasing CO₂ levels in the sea, for a review see Heuer (2014). However, even the 100-year CO₂ level projections (1 - 2 mg/L) (Ou et al., 2015) are notably lower than the levels typically recorded in commercial aquaculture tanks for salmonids (10 – 20 mg/L) (Fivelstad, 2013; Gorle et al., 2018). Such high CO₂ concentrations observed in tanks producing commercially important species such as Atlantic salmon led to a wide number of studies focusing on the effects of long term exposure to high CO₂ levels (Fivelstad, 2013; Good et al., 2010; Methling et al., 2013; Moran and Støttrup, 2011; Santos et al., 2012; Steinberg et al., 2017; Stiller et al., 2015). These studies showed that high CO₂ concentrations disturb fish acid-balance, induce stress response, increase nephrocalcinosis incidence, energy metabolism, and feed conversion ratio, and reduces body condition factor, feed intake, and growth.

The CO₂ concentrations that trigger negative growth performance in Atlantic salmon smolts and post-smolts have been suggested to range from 15 to 20 mg/L (Fivelstad, 2013; Fivelstad et al., 1998). However, these exposure studies were conducted in flow-through systems, see review by Fivelstad (2013), which are notably distinct from RAS, where water is re-used. A recent study in RAS showed that Atlantic salmon performs similarly when exposed to a CO₂ concentration of 8 or 20 mg/L (Good et al., 2018); however, that study was done in a high-alkalinity (> 200 mg as CaCO₃) freshwater environment. Thus, several comprehensive studies testing a wider range of CO₂ concentrations and different environments and life-stages are needed to better determine the maximum tolerance levels of Atlantic salmon to CO₂.

In recent years, Atlantic salmon post-smolts production in brackish water (12 – 20 ppt) RAS has been increasing. Thus, it is relevant to determine the optimal water quality parameters in these conditions, including CO₂, to ensure optimal fish welfare, health and performance.

Also, how skin integrity copes with a high CO₂ environment is worth investigating, as Atlantic salmon skin epidermis and dermis increases in thickness and mucus cell numbers resulting from transferring fish from freshwater to seawater (Karlsen et al., 2018).

The objective of the present study was to determine the CO₂ concentration in 12 ppt salinity RAS up to which no negative consequences are observed for growth performance, health, or welfare of Atlantic salmon post-smolts. To this end, fish were exposed to six dissolved CO₂ concentrations 5, 12, 19, 26, 33 and 40 mg/L during a 12-week period in RAS. Additionally, fish were subjected to a subsequent 6-week period in flow-through seawater to understand if the effects of earlier CO₂ exposure persist or not, when fish are moved into sea cages to grow to market size.

2. Material and Methods

2.1. Experimental design

This study consisted of two experimental phases (Figure 1). The first was a CO₂ exposure phase, where eighteen tanks were used as experimental units in a mono-factorial design with six treatments (CO₂ concentrations) and three replicates per treatment: 5, 12, 19, 26, 33 and 40 mg/L of CO₂ during 12-weeks in a 12 ppt salinity RAS (hereafter termed RAS phase). In the second phase, PIT-tagged fish were transferred to a 34 ppt salinity single flow-through tank at CO₂ < 5 mg/L (hereafter termed seawater phase) for an additional 6-week experimental period mimicking a seawater phase.

2.2. Experimental fish and feed

All procedures involving fish were approved by the Norwegian Food Safety Authority animal experimentation approval system (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 light (LD 24:00) photoperiod 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. Fish were individually pit-tagged with a smart glass tag (Smartrac, Reichshof-Wehnrath, Germany). Atlantic salmon post-smolts (N=900; 67 ± 8 g) were captured from a main holding tank, ten fish at a time, and then for each catchment of fish, randomly distributed over eighteen experimental tanks (N=50 fish/tank) in Nofima Centre for Recirculation in Aquaculture, Sunndalsøra, Norway (Terjesen et al., 2013). The fish were subsequently allowed to adapt to the rearing and feeding conditions for a 3-week period. After this period, fish body weight and length were measured and 46 fish per tank were stocked back for a 12-week CO₂ exposure period. Mean weight and density at the beginning of the experiment were 71 ± 9 g and 6.4 ± 0.1 kg/m³, respectively. At the end of the 12-week experimental period, five fish per tank (total 90 fish) were randomly selected and transferred to a single flow-through tank (3.3 m³) for an additional 6-week experimental seawater phase, at salinity 34 ppt and where CO₂ was kept below 5 mg/L.

Fish were fed continuously (approx. 23 h day) 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.

2.3 Experimental system and water quality

Eighteen cylindro-conical experimental tanks (V=0.5 m³) were connected to a RAS as previously described (Terjesen et al., 2013). Briefly, 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 (Figure 2). The ten octagonal tanks (3.3 m³) with non-

experimental Atlantic salmon post-smolts aimed to increase fish and feed load in the system to relevant intensity, resulting in an added fish biomass of 1497 ± 265 kg and feed load of 21 ± 3 kg/day (12-week experimental period average). 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. This relatively high-water exchange rate was necessary to control temperature at 13°C . The make-up water (12 ppt salinity) was added into the top of the RAS degasser column.

The different CO_2 concentrations in each fish tank resulted from the specific mix of two water inlets (Figure 2): holding sump 1 ($\text{CO}_2 = 3 \text{ mg/L}$) and holding sump 2 ($\text{CO}_2 = 40 \text{ mg/L}$). Each holding sump ($V = 2.2 \text{ m}^3$ each) had pure oxygen gas added through a diffuser and a submerged pump to assure an adequate mix of the water. The holding sump 2 had CO_2 gas added through a diffuser from a pressurized CO_2 -gas bottle. The CO_2 concentration in the holding sump 2 was continuously monitored (every 1 min.) 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 water quality parameters measured during the two experimental phases are presented in Table 1. Photoperiod was maintained at 24L:00D throughout all experiment. Hydraulic retention time (HRT) for the experimental fish tanks was 45 min. and water flow was checked twice weekly per tank.

2.4 Water sampling and analysis

All water quality parameters were measured and sampled at each fish tank effluent, with the exception of temperature, pH, oxygen, and salinity that were measured in the fish tank. The specific parameters, method of analysis, and frequency are described in Table 2. New factory-calibrated CO₂ sensors (Oxyguard, Denmark and Franatech, Germany, N = 5) were pre-tested over the range of CO₂ concentrations studied (5 – 40 mg/L) and the displayed values were comparable within a 5 mg/L variation at the top range, i.e. 40 mg/L. NH₃-N concentrations were calculated from the total ammonia nitrogen (TAN) concentrations as a function of pH, temperature, and salinity (Johansson and Wedborg, 1980). PCO₂ concentrations were calculated from the CO₂, temperature and salinity values (Table 2 for measurement details), and from the dissociation constants of carbonic acid in seawater (Roy et al., 1993).

2.5 Fish sampling and analysis

Five sampling events took place throughout the experiment: week 0, week 3, week 6, week 12, and week 18. Five fish per tank were sampled in each event; except in week 0 where only two fish per tank were sampled. Fish were anesthetized (0.12 g/L MS-222) prior to handling or euthanized (0.5 g/L MS-222) prior to tissue sampling. Fish mortality was assessed and recorded daily in each fish tank. Individual weight and length were measured in all sampling events, and condition factor and growth were calculated from these measurements.

Condition factor was calculated as:

$$CF = 100 \times W / L^3$$

Where W is individual weight in grams and L is the length in centimeters.

Thermal growth coefficient (TGC) was calculated as:

$$TGC = 1000 \times (W_2^{1/3} - W_1^{1/3}) / (T \times \Delta t)$$

Where T is the water temperature in °C and ΔT is the number of days between time T₁ and T₂.

External welfare indicators were scored at every sampling point by examining fish (N = 5 per replicated tank) for eye cataracts, operculum, skin, and fins condition. Dorsal, pectoral, pelvic and caudal fins were examined and scored from 0 to 5 according to an adapted scale from Hoyle et al. (2007): score 0 was given for lack of damage/erosion and maximum score of 5 was given for most severe damages/erosions. Skin lesions and operculum status was scored from 0 to 2 according to Kolarevic et al. (2013): 0 for lack of damage and 2 for severe damage. Eyes were examined for presence of cataract using a portable slit lamp SL-17 (Kowa, Tokyo, Japan) and were scored between 0 and 4 according to Wall and Bjerkas (1999).

Kidneys were examined for mineral deposits, i.e. nephrocalcinosis. Each kidney was scored by visual examination as 0 (normal kidney), 1 (minor kidney swollen and < 10 nodules), 2 (kidney swollen, grey colour, irregular surface and many but separated nodules), or 3 (kidney swollen, grey colour, irregular surface and interconnected nodules forming continuous areas) adapted from Fivelstad et al. (2003). For histology analysis, a portion of mid kidney tissue (1 cm) was dissected and placed in 10% neutral buffered formalin (CellStor pots, CellPath, Powys, U.K.). The samples were dehydrated and processed according to standard protocols. Paraplast embedded samples were microtome cut (5 μ m) and stained with hematoxylin and eosin (Merck KGaA, Darmstadt, Germany). The stained slides were examined using a standard Nikon Optiphot light microscope (Nikon, Japan). The sections were blind evaluated for pathologies and deviations on tissue morphology.

Skin samples from below the dorsal fin and above the lateral line were dissected and placed in 10% neutral buffered formalin (CellStor pots, CellPath, Powys, U.K.) for histology. Skin samples were dehydrated through graded series of ethanol and embedded in paraffin (Histowax, Histolab Products AB). Sections (5 μ m) were prepared in the anterior-posterior direction using a Microm HM 355S (Thermo Fisher Scientific Inc., MA, USA). Parallel

sections (N=2 per fish) were stained with haematoxylin-eosin (HE, Sigma-Aldrich). Images and measurements were done with Zeiss Axio Observer Z1 equipped with an AxioCam MRc5 camera and AxioVision software (Carl Zeiss Microimaging GmbH, Göttingen, Germany). Overall morphology was examined, number of mucus cells were counted, and epidermis and dermis thickness measured (N=10 measurements per sample) using the AxioVision software (Carl Zeiss Microimaging GmbH). Histology analysis only considered samples from week 12 and week 18 and treatments 5 and 40 mg/L. Skin histology at week 12 additionally analyzed treatment 19 mg/L.

2.6. Statistics

Statistical analysis was performed with IBM SPSS Statistics V25 (IBM, Corp., USA). The effect of CO₂ on fish performance parameters was analyzed by linear regressions. Growth performance parameters and water quality comparisons among treatments were performed using one-way ANOVA followed by post-hoc Tukey HSD test (equal variances assumed) or Games-Howell test (unequal variances). Skin parameter differences between treatments at week 12 (5, 19 and 40 mg/L) were compared using an ANOVA followed by post-hoc Tukey HSD test and at week 18 (5 and 40 mg/L) were tested using a *t* test. Homogeneity of variances was tested using Levene's test. A significant level (α) of 0.05 was used for all analyses. Data are presented as mean \pm standard deviation (SD).

3. Results

3.1. Water Quality

Water quality parameters measured during the two experimental periods (RAS and seawater phase) are summarized in Table 1. Differences in water quality among CO₂ treatments at the

end of RAS phase were related to experimental treatments: higher CO₂ treatments had significantly higher dissolved CO₂ (in mg/L and mm Hg), and significantly lower pH and unionized ammonia nitrogen (NH₃-N). The exception was dissolved O₂ that was significantly lower in the treatment 5 mg/L compared to the treatment 40 mg/L, but all values were above 85% of air saturation at 92 ± 0 and 95 ± 1 %, respectively. All other averaged water quality parameters at the end of RAS phase did not significantly differ among treatments.

3.2. Fish performance

Overall mortality of fish exposed to CO₂ was low, with one dead fish in total throughout the experimental period (Table 3). Average fish tank density during the RAS phase was 6 kg/m³ and 21 kg/m³, at week 6 and 12 respectively, whereas during the Seawater phase density was 6 kg/m³ and 9 kg/m³, at week 12 and 18 respectively. Growth curves show that the fish body weight was similar among CO₂ treatments up to about mid-way through the RAS phase (week 0, 3 and 6; P -value > 0.05) while and from there on fish body weight significantly differed towards the end of RAS phase (week 12; P -value < 0.001) and the subsequent seawater phase (week 18; P -value = 0.003) (Figure 3). Additionally, pairwise comparisons among the treatments showed that the mean final body weight (FBW) was significantly higher in the 5 mg/L treatment compared to CO₂ treatments ≥ 12 mg/L at the end of RAS phase (week 12; P -value < 0.001). Moreover, fish in the CO₂ treatment 5 mg/L presented a significantly higher FBW when compared to CO₂ treatments ≥ 33 mg/L at the end of seawater phase (week 18; P -value = 0.003) (Table 3). Figure 4 shows the relationship between CO₂ in the water and fish growth as thermal growth coefficient (TGC), where TGC significantly decreases linearly with increasing CO₂ in the water at the end of RAS phase and at the end of seawater phase. Comparisons among treatments show that TGC was significantly higher in the 5 mg/L treatment compared to CO₂ treatments ≥ 12 mg/L during RAS phase (week 12; P -

value<0.001), whereas no differences among groups were observed during the seawater phase (week 18; P -value=0.095).

3.3. Fish health and welfare

External welfare observations on eye cataracts and damage on skin, operculum, and fins were similar among CO₂ treatments, with low overall scores for each of the five samplings (week 0, 3, 6, 12 and 18): <0.1 (0 – 4 index) cataracts, < 0.5 (0 – 2 index) for skin and operculum and, < 1 for fins (0 – 5 index). The visual kidney scores for nephrocalcinosis at each of the five samplings were similar among treatments, with only a few fish (5 out of 396 experimental fish analyzed) showing minor calcium deposits (score 1). Histology of kidney samples analyzed (N=24) on the two extreme groups, i.e. only 5 mg/L and 40 mg/L showed normal morphology in all analyzed samples (end of RAS phase and seawater phase). Specifically, there were no signs of mineral deposits in any of the structures of the kidney tissue.

Histology results of skin, including dermis, epidermis, scales, and mucus cells are shown in Figure 5. At the end of RAS phase, the dermis layer was significantly thinner (P -value=0.047) in fish from treatment 19 mg/L (23 % thinner) and 40 mg/L (22 %) compared to fish from the 5 mg/L treatment, but epidermis and scale thickness were similar between these three CO₂ treatments (Figure 6a). Mucus cells number did not differ between any of these three treatments. At the end of seawater phase, both dermis (41 % thinner; P -value=0.009) and epidermis (15 %; P -value=0.007) were significantly thinner in fish from the 40 mg/L treatment compared to the 5 mg/L treatment, but scale thickness was similar between these two CO₂ treatments (Figure 6b). The number of mucus cells were significantly lower (P -value=0.010) between the 5 mg/L treatment (22.4 ± 3.6 cells/100 μ m) compared to the 40 mg/L treatment (11.4 ± 1.4 cells/100 μ m).

4. Discussion

The current study shows that growth in Atlantic salmon post-smolts is negatively linear-related to CO₂ exposure, indicating that the growth penalty of CO₂ starts at lower concentrations than previously reported (<12 mg/L). Results suggest that exposing Atlantic salmon post-smolt to an increase in CO₂ of 10 mg/L would correspondingly decrease their TGC by approximately 0.2 units. As the highest TGC averaged 2.2 during this study, an approximate 10% of growth reduction was observed for every 10 mg/L increase in CO₂, over the range of CO₂ concentrations studied (5 – 40 mg/L). Moreover, CO₂ exposure effects observed during RAS phase were carried over to seawater phase. Furthermore, fish exposed to high CO₂ concentrations (40 mg/L) presented a thinner skin dermis. However, in contrast, fish survival, eye cataracts, nephrocalcinosis and several external welfare indicators of Atlantic salmon post-smolts were not affected by the 12-week exposure to CO₂ concentrations up to 40 mg/L in RAS.

The water quality during the experiment was, with the exception of CO₂, within the recommended levels for Atlantic salmon post-smolts (Thorarensen and Farrell, 2011). This was also the case for NH₃-N. Despite significantly higher NH₃-N concentrations for the control group (1.5 µg/L) compared to the other CO₂ treatments, the levels were far below the threshold value (> 14 µg/L) previously shown to negatively impact Atlantic salmon (Kolarevic et al., 2012; Kolarevic et al., 2013). Dissolved oxygen also differed among the treatments, which was likely due to a higher feed intake from the fish exposed to lower CO₂ concentrations (data not shown). Nevertheless, the oxygen concentrations were above the optimal 85 % saturation for all the treatments (Thorarensen and Farrell, 2011). Water pH was another quality parameter that differed among treatments; increasing CO₂ levels led to a decrease in pH levels due to the acidic effect of CO₂. The interaction between these both variables on fish performance was shown previously (Fivelstad, 2013; Fivelstad et al., 1998;

Steinberg et al., 2017). However, in our study, the decrease of pH due to the CO₂ supply was partly counteracted by adding a sodium bicarbonate solution, which stabilized the pH levels (> 6.7) at levels previously shown not impact Atlantic salmon growth performance (Fivelstad et al., 2004) so that the effect observed in the current study could be only caused by the different CO₂ levels.

Survival rates among Atlantic salmon post-smolts exposed up to 40 mg/L CO₂ levels during 12 weeks were close to 100 %, and remained so through all 6 weeks during the following seawater phase. Thus, it seems that Atlantic salmon post-smolts are able to withstand high levels of CO₂ without major mortalities, as similar survival rates were found in other long-term exposure CO₂ studies up to 15 - 20 mg/L with Atlantic salmon (Fivelstad, 2013).

Fish body weight and growth rates are key indicators to assess fish culture conditions. In flow-through systems it was shown that CO₂ concentrations up to 15 to 20 mg/L reduce Atlantic salmon growth rates, see review by Fivelstad (2013). However, a study performed in freshwater RAS exposing Atlantic salmon up to harvest weight in fresh water to two CO₂ concentrations of 8 and 20 mg/L showed no difference in growth performance between these two treatments (Good et al., 2018). Similarly, in the current study, where six CO₂ treatments were used, the results of growth were consistent with Good et al. (2018) in the same range, 12 and 19 mg/L. However, the present study went beyond a 2-treatment design and tested six CO₂ treatments and a wider range of CO₂ concentrations (5 – 40 mg/L). This allowed a linear regression analysis that showed a significantly negative relationship between fish growth and CO₂ concentration, which was not visible only when two concentrations were compared. Likewise, a negative linear relationship between fish growth and the concentration of CO₂ that fish are exposed to was recently shown for a seawater flow-through system (Fivelstad et al., 2018). However, in contrast to our study, Fivelstad et al. (2018) divided the 12-week

experimental period into four periods. In the first three periods polynomial or non-relationships between fish growth and the concentration of CO₂ were observed, and only in the last period was this negative linear relationship shown. Another study with pikeperch (*Sander lucioperca*), also observed a linear decrease in final body weight with increasing CO₂ levels (Steinberg et al., 2017). To our best knowledge, our study showed for the first time that there is a linear decrease of growth when CO₂ concentration is increased in Atlantic salmon post-smolts raised in brackish water RAS.

A seawater phase grow-out in cages is commonly used after the land phase to grow Atlantic salmon post-smolt into market size fish (4-5 kg). The fish performance during the sea phase depends on several factors, particularly the previous culture conditions during the land phase, which nowadays occurs increasingly in RAS. In the current study, fish were grown for an extra 6 weeks in a system mimicking a seawater phase (34 ppt salinity, flow through, <5 mg/L CO₂). We observed that the fish that were previously exposed to CO₂ concentrations equal to or higher than 33 mg/L presented a lower body weight, whereas all other CO₂ treatments were able to recover the previous weight deficit, when compared to fish exposed to 5 mg/L. Noteworthy, a linear regression (P -value=0.004) for these 6 weeks growth shows that the effects of CO₂ exposure previously observed in the RAS phase persist into seawater phase. This result shows that it is important to consider CO₂ exposure in the earliest phases, even when part of the grow out phase is conducted in an environment low in CO₂ such as a seawater phase.

External welfare indicators were generally optimal throughout the experiment for fish in all CO₂ treatments without major signs of external injuries. Score values of the eye cataracts and damage on skin, operculum, and fins were similar among CO₂ treatments and comparable to other studies on Atlantic salmon (Kolarevic et al., 2013). Cataracts have been reported to increase in fish raised in RAS (Davidson et al., 2017; Neves and Brown, 2015)

and at high CO₂ levels (Moran et al., 2012), but in our study no significant cataracts were observed in fish raised in RAS even when fish were exposed to high CO₂ concentrations. Other factors have been linked to cataract formation, such as rapid changes in environmental parameters, e.g. water temperature, salinity and nutrition (Bjerkås and Sveier, 2004). The absence of eye cataracts in our trial, where water quality parameters were maintained stable during the 12 week RAS phase, highlights the advantage of the environmental control attained in RAS.

Nephrocalcinosis, a kidney lesion that comprises renal degeneration with mineral deposits and nodule formations, is a pathology commonly associated to high CO₂ concentration exposure. For instance, a previous study showed that 88% of the Atlantic salmon smolts exposed to 16 mg/L of CO₂ for 54 days in fresh water presented nephrocalcinosis (Fivelstad et al., 2003). In a second study, 34% of Atlantic salmon post-smolts exposed to 21–30 mg/L CO₂ for 12 weeks in seawater also presented nephrocalcinosis (Fivelstad et al., 2018). In contrast, nephrocalcinosis was not found in another study where Atlantic salmon post-smolts were exposed up to 44 mg/L CO₂ for 43 days in seawater, (Fivelstad et al., 1998) neither in a study where Atlantic salmon were exposed up to 20 mg/L of CO₂ for 384 days in freshwater (Good et al., 2018) nor was it found in another study where rainbow trout were exposed to 24 mg/L CO₂ for 6 months (Good, et al., 2010). In the current study, the incidence of nephrocalcinosis was minor, with 5 fish out of the 396 fish analysed showing minor signs even when exposed to artificially high concentrations of CO₂ (up to 40 mg/L) for 12 weeks. The results from the present study show that environmental factors other than elevated CO₂ must have an important role in the development of nephrocalcinosis. Diet composition, namely high concentrations of calcium coupled with phosphorus, may also cause nephrocalcinosis (Richardson et al., 1985; Smart et al., 1979). Water chemistry, in particular the source of alkalinity, may cause nephrocalcinosis, as a change of calcium

carbonate for sodium bicarbonate seems to reduce the prevalence of nephrocalcinosis in Nile tilapia (*Oreochromis niloticus*) grown in RAS (Chen et al., 2001). This topic should be further studied for Atlantic salmon, since nephrocalcinosis incidence is recurrent in commercial conditions and poses important economic losses.

Skin is the first defense barrier to the external environment and plays an important role in fish protection and preservation from suboptimal water quality. In the current study, skin histology showed that fish exposed to 5 mg/L CO₂ at the end of RAS phase had normal dermis, whereas fish from 19 and 40 mg/L showed thinner dermis. The epidermis and mucus cells, of the first treatment showed a good organization of cells lining the basement membrane and flat cells at the outer part of the epidermis. In contrast, the other two treatments resulted in a more uneven epidermis and thus could lead to a more permeable barrier and ion selectivity disturbances. These changes in the skin layer morphology and thickness could be due to a higher CO₂ concentration exposure, as a previous study have shown that suboptimal water quality can change skin properties (Sveen et al., 2016).

At the end of seawater phase, for the 5 mg/L treatment, the skin (dermis, epidermis, scales and mucous cells) appeared normal, whereas for the 40 mg/L treatment more connective tissue and connective tissue with a looser structure under the scales was observed and severe morphological deviations in the epidermis, including thin layer and condensed cells. The cause for these deviations in the skin in this treatment is uncertain. They could result from the combination several factors, including the adaptation from a high CO₂ environment (RAS phase) into a low CO₂ environment (seawater phase) and from the transfer of an isosmotic environment (RAS phase; 12 ppt salinity) to a hyperosmotic environment (seawater phase; 34 ppt salinity). Changes in the skin epidermal and dermal thickness were shown after Atlantic salmon seawater transfer (Karlsen et al., 2018). Furthermore, these authors demonstrated that a thinner dermis and epidermis may be associated with a reduction

in the immune activity in the skin. This may suggest that a sub-optimal rearing environment, such as high CO₂ concentrations or a sudden salinity change can have implications on skin defence mechanisms against infectious agents. However, the causative agent for thinner and less organized dermis observed in our study is unknown and thus the mechanisms behind this fish response should be addressed in future studies.

The current study brings a new insight on the growth of Atlantic salmon post-smolts in brackish water RAS and shows that maximum growth performance is obtained in CO₂ concentrations below 12 mg/L. Skin health seems to be compromised in fish exposed to very high CO₂ concentrations (40 mg/L). In contrast, with the exception of skin, no major effects of health and welfare were observed for the indicators here analyzed up to an exposure to 40 mg/L, including cataracts and nephrocalcinosis. The data from this study is relevant for the design, dimensioning, and management of RAS. Further studies should evaluate the molecular and physiological responses to both short-term and long-term carbon dioxide exposure to understand the fish adaptation mechanism to these conditions.

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Table 1. Summary of water quality measured at the fish tank effluent for RAS and seawater phase.

Parameters	RAS phase						ANOVA	Seawater
	5 mg/L	12 mg/L	19 mg/L	26 mg/L	33 mg/L	40 mg/L	<i>P-value</i>	phase
CO ₂ (mg/L)	5.3 ± 0.1 ^a	13.2 ± 0.1 ^b	19.8 ± 0.8 ^c	26.3 ± 0.6 ^d	32.0 ± 0.7 ^e	38.5 ± 0.7 ^f	< 0.001	2.2
PCO ₂ (mm Hg) ¹	2.0 ± 0.0 ^a	4.9 ± 0.1 ^b	7.4 ± 0.3 ^c	9.8 ± 0.2 ^d	12.0 ± 0.2 ^e	14.4 ± 0.2 ^f	< 0.001	0.8
O ₂ (%) saturation) ²	92 ± 0 ^a	93 ± 0 ^{a,b}	93 ± 2 ^{a,b}	93 ± 0 ^{a,b}	94 ± 1 ^{a,b}	95 ± 1 ^b	0.018	91
Temperature (°C) ²	12.8 ± 0.1	12.7 ± 0.0	12.7 ± 0.0	12.8 ± 0.0	12.7 ± 0.0	12.7 ± 0.0	0.647	8.4
Salinity (ppt) ²	11.9 ± 0.1	11.9 ± 0.1	11.9 ± 0.1	11.8 ± 0.0	11.9 ± 0.1	11.9 ± 0.0	0.786	33.9
pH ¹	7.66 ± 0.02 ^a	7.15 ± 0.00 ^b	6.99 ± 0.03 ^c	6.87 ± 0.02 ^d	6.81 ± 0.01 ^e	6.74 ± 0.01 ^f	< 0.001	7.8
Turbidity (ntu)	8.6 ± 3.2	7.5 ± 3.0	8.0 ± 2.9	7.0 ± 3.6	7.2 ± 2.9	7.6 ± 2.6	0.995	0.16
TSS (mg/L)	9.1 ± 3.9	11.1 ± 4.5	9.6 ± 2.3	9.8 ± 3.1	9.9 ± 3.9	9.2 ± 1.8	0.984	-
Alkalinity (CaCO ₃ mg/L)	116 ± 13	120 ± 11	138 ± 20	151 ± 33	148 ± 25	165 ± 25	0.096	122
TAN (mg/L)	0.12 ± 0.02	0.13 ± 0.02	0.18 ± 0.01	0.13 ± 0.02	0.10 ± 0.04	0.12 ± 0.06	0.213	-
NH ₃ -N (µg/L) ¹	1.5 ± 0.5	0.5 ± 0.5	0.3 ± 0.3	0.2 ± 0.2	0.2 ± 0.2	0.1 ± 0.1	< 0.001	-

	0.3 ^a	0.1 ^b	0.2 ^b	0.00 ^b	0.1 ^b	0.0 ^b		
NO ₂ -N (mg/L)	0.03 ±	0.04 ±	0.04 ±	0.03 ±	0.03 ±	0.02 ±	0.864	0.02
	0.02	0.02	0.01	0.01	0.00	0.00		
NO ₃ -N (mg/L)	17.2 ±	16.7 ±	17.2 ±	17.1 ±	16.4 ±	16.5 ±	0.996	0
	11.9	12.0	11.1	10.8	12.1	11.8		

¹ Calculated values.

² Parameters measured inside fish tanks.

Superscript alphabets indicate significant differences, post-hoc Tukey HSD test, $P < 0.05$.

Values are given as treatment mean ± SD (n=3, except seawater phase where n=1). See Table 2 for specific parameter measurement frequency.

PCO₂; partial pressure of CO₂ in the water; TSS – total suspended solids, TAN – total ammonia nitrogen.

Table 2. Water quality parameters, method of sampling, and analysis and frequency.

Parameter	Method of analysis and sampling	Frequency
Dissolved oxygen	DO Handy Polaris, OxyGuard, Denmark	Daily
Dissolved CO ₂ (holding sump 2)	Dissolved CO ₂ sensor, OxyGuard Pacific, OxyGuard, Denmark	Daily
Dissolved CO ₂ (fish tanks)	Dissolved CO ₂ sensor, Franatech, Germany	Twice weekly
pH	pH-electrode Sentix 980, WTW, Germany	Twice weekly
Temperature	DO Handy Polaris, OxyGuard, Denmark	Twice weekly
Conductivity	IDS conductivity, TetraCon 925, WTW, Germany	Twice weekly
Total ammonia nitrogen (TAN)	Method HI 93700, C203 2008 photometer, Hanna Instruments, Quebec, Canada (U.S.EPA, 1983)	Monthly
Nitrite nitrogen (NO ₂ -N)	Method HI 93707, C203 2008 photometer, Hanna Instruments, Quebec, Canada (U.S.EPA, 1983)	Monthly
Nitrate nitrogen (NO ₃ -N)	Method 350.1, Automated analyzer, Flow Solution IV, OI Analytical, College Station, TX, USA	Monthly
Total alkalinity	Method 8203, Digital titrator, Hach, USA.	Monthly
Total suspended solids (TSS)	Method 2540 D (TSS dried at 103 -105 °C) (APHA, 2005)	Monthly
Turbidity	Turbiquant 1500 IR, Merck, Darmstadt, Germany (APHA, 1999)	Monthly

Table 3. Summary of performance parameters of Atlantic salmon post-smolt exposed to six dissolved CO₂ concentrations during RAS and seawater phase.

Parameters	CO ₂ treatment (mg/L)						Regression	
	5	12	19	26	33	40	R ²	P-value
Overall mortality (count)	0	0	0	0	1	0	-	-
RAS phase (week 0 - 12)								
Initial body weight (g)	72 ± 1	72 ± 1	70 ± 1	70 ± 2	70 ± 2	72 ± 1	0.152	0.548
Final body weight (g)	270 ± 11	249 ± 9	234 ± 7	220 ± 4	213 ± 7	206 ± 6	0.883	< 0.001
Condition factor (week 12)	1.27 ± 0.04	1.24 ± 0.03	1.18 ± 0.03	1.17 ± 0.07	1.16 ± 0.02	1.21 ± 0.03	0.252	0.034
Seawater phase (week 13 – 18)								
Final body weight (g)	402 ± 20 ^{a,b}	411 ± 48 ^a	342 ± 42 ^{a,b,c}	304 ± 46 ^{b,c}	289 ± 18 ^c	293 ± 34 ^c	0.632	< 0.001
Condition factor (week 18)	1.19 ± 0.03 ^{a,b}	1.23 ± 0.03 ^a	1.18 ± 0.02 ^{a,b}	1.15 ± 0.05 ^{a,b}	1.15 ± 0.03 ^{a,b}	1.13 ± 0.04 ^b	0.424	0.003

Superscript alphabets indicate significant differences, post-hoc Tukey HSD test, $P < 0.05$.

Values are given as mean ± SD (n= 3).

Figure captions

Figure 1. Experimental design.

Figure 2. Scheme of the recirculating aquaculture system used.

Figure 3. Growth curve for fish exposed to six CO₂ concentrations (5, 12, 19, 26, 33 and 40 mg/l) during an 18-week experimental period. Two periods shown: RAS phase (white area) with CO₂ exposure and seawater phase (grey area) without CO₂ exposure. *Indicates significant differences among CO₂ treatments. NS – non-significant differences.

Figure 4. Linear regression models between measured CO₂ in the water and thermal growth coefficient (TGC) during RAS phase (week 0 - 12) and Seawater phase (week 13 – 18). Note: Water CO₂ during seawater phase was < 5 mg/L for all fish; fish were individually pit-tag and are grouped based on the RAS phase tanks and CO₂ treatments.

Figure 5. Skin histology of fish exposed to 5 and 40 mg/L CO₂ at the end of RAS phase (week 12) and seawater phase (week 18). e = epidermis, d = dermis, s = scales, at = adipose tissue, ct = connective tissue. Arrows point at mucus cells. Scale bar = 100 µm. Vertical lines show measurement points.

Figure 6. Dermis, epidermis and scale layer thickness of fish exposed to 5, 19 and 40 mg/L of CO₂ at the end of RAS phase (week 12) and fish exposed to 5 and 40 mg/L at end of seawater phase (week 18). *Indicates significantly differences between CO₂ treatments. NS – non-significant differences.

Figure 1

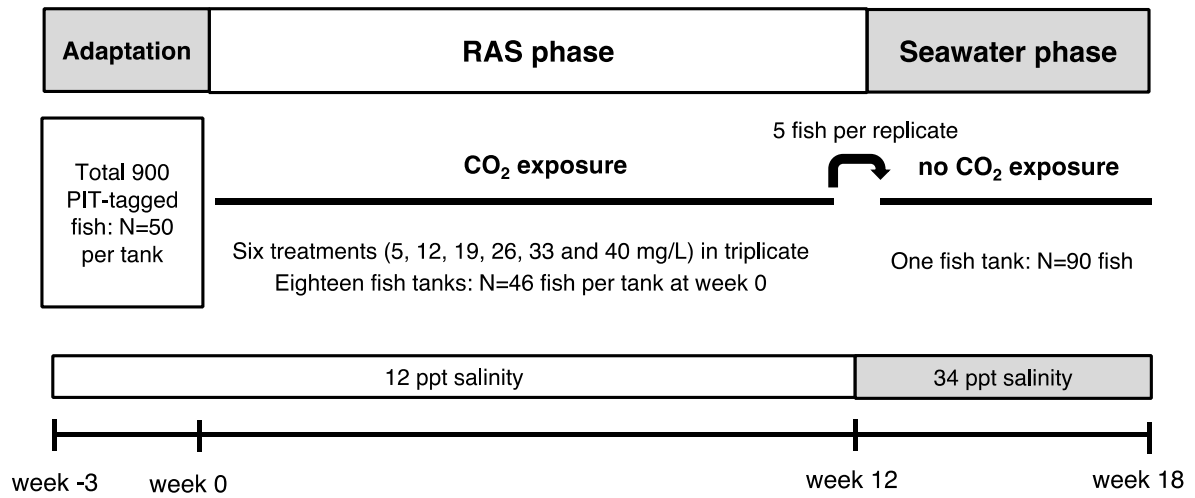


Figure 2

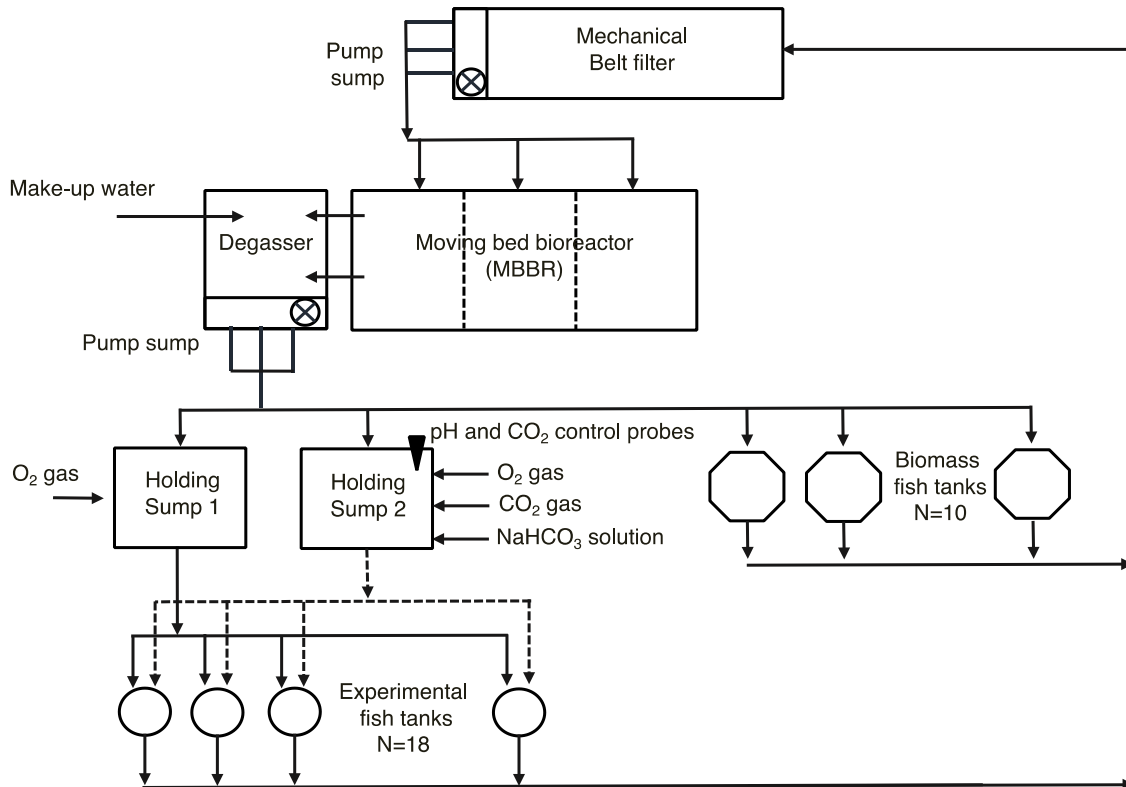


Figure 3

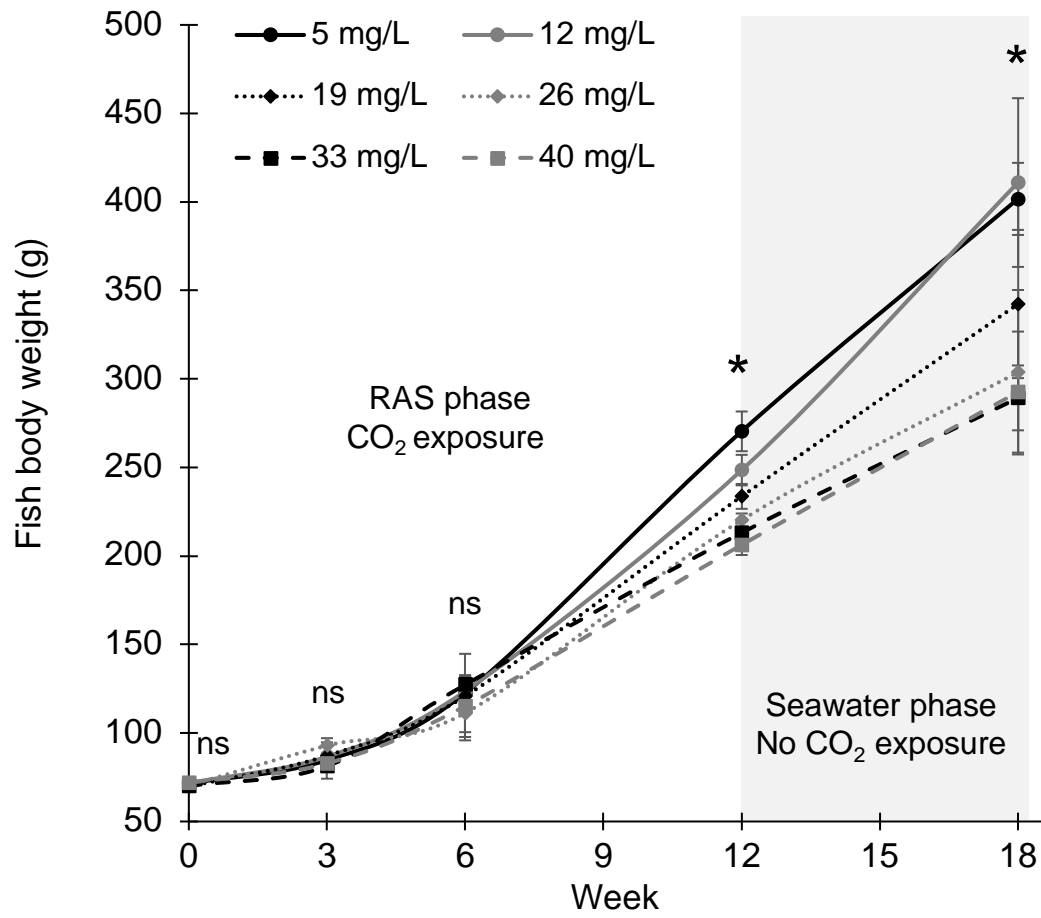


Figure 4

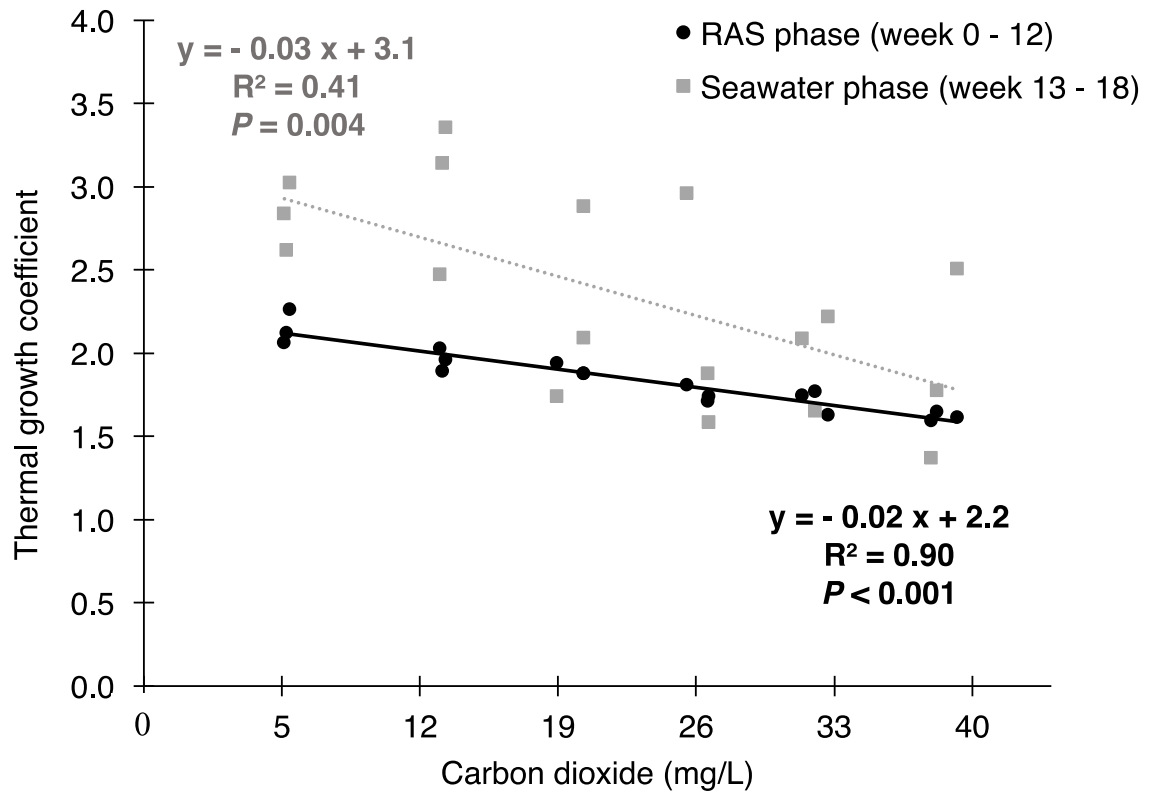
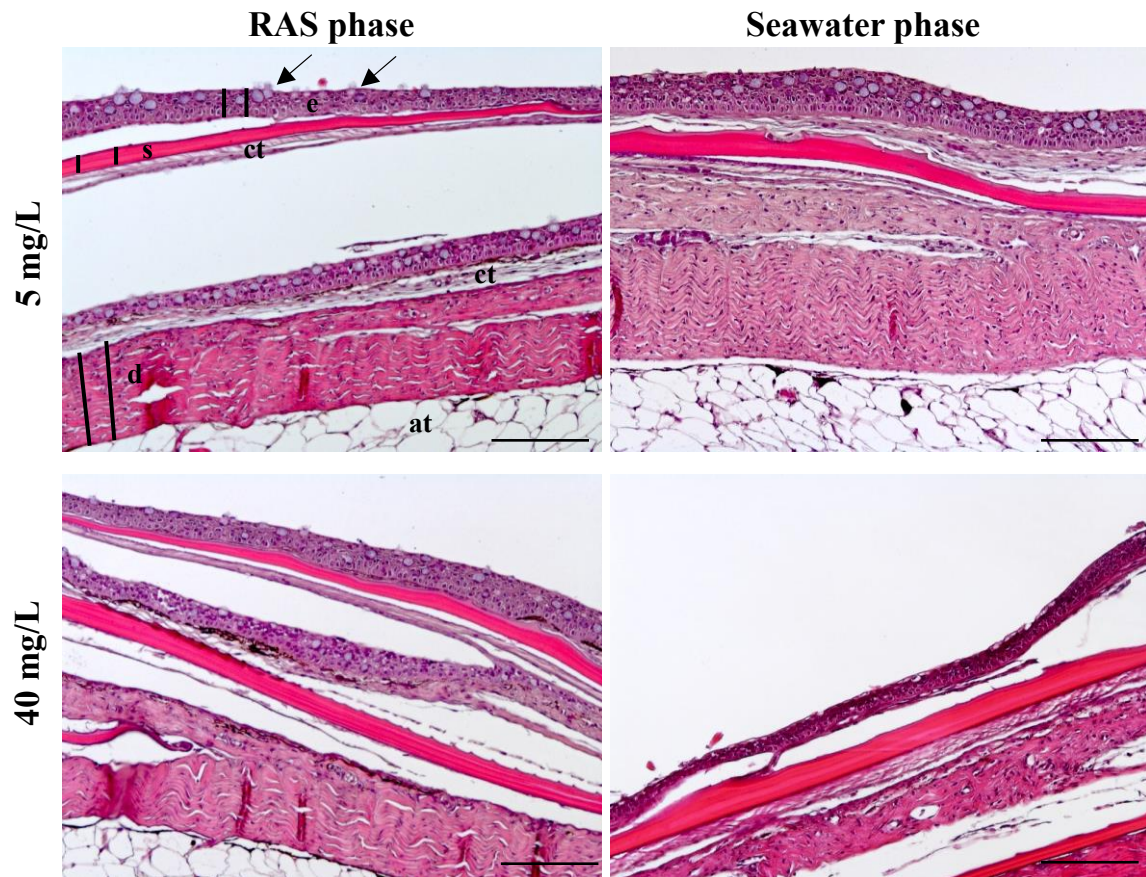
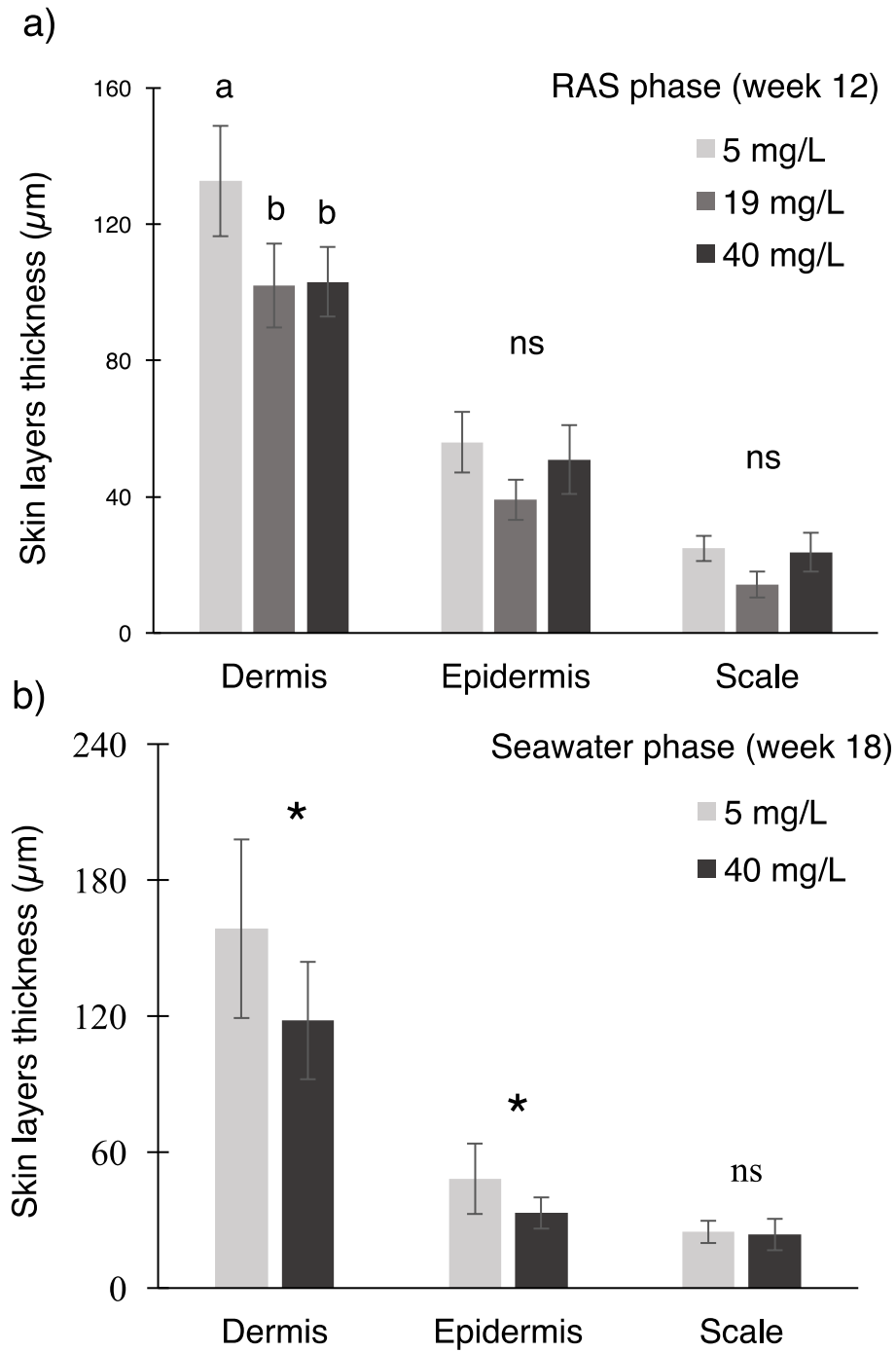


Figure 5



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Figure 6



Highlights

- Atlantic salmon post-smolts were exposed to six CO₂ concentrations (5 – 40 mg/L) for 12 weeks in 12 ppt salinity RAS
- Fish showed no mortality, cataracts, nephrocalcinosis or signs of external injuries.
- Skin dermis layer was significantly thinner in fish exposed to 40 mg/L of CO₂.
- Body weight and growth were significantly lower at CO₂ concentrations ≥ 12 mg/L.
- Effects of CO₂ exposure during the RAS phase were carried over during an additional 6-week experimental period to mimic a seawater phase.