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Water Flow Requirements of Post-smolt Atlantic Salmon (*Salmo salar* L.) Reared in Intensive Seawater Flow-through Systems: A Physiological Perspective †

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Abstract: Environmental challenges related to open sea cage production of Atlantic salmon have sparked interest in developing commercial-scale semi-closed sea systems for post-smolt Atlantic salmon (100–1000 g). Determining the mass-specific water flow required by post-smolts will largely influence the design and dimensioning of such systems. In this experiment, post-smolts were exposed to four levels of specific water flow: 0.2, 0.3, 0.4, and 0.5 L kg fish^{-1} min^{-1}. All treatments involved flow-through seawater with full oxygenation, a salinity of 34‰, and a mean temperature of 9.3 °C. The stocking density was kept stable at 75 kg m^{-3}. Water pH decreased with reduced flow, while partial pressure of carbon dioxide (pCO_2) and total ammonia nitrogen (TAN) in the water increased. The increase in water CO_2 was reflected in the blood with increased pCO_2, HCO_3^-, and decreased Cl^- in the lowest water flow treatment (0.2 L kg fish^{-1} min^{-1}), indicating a typical regulatory response to increased water CO_2 over the eight-week experimental period. No negative effects on osmoregulation, external macroscopic welfare, or performance indicators were observed, suggesting that within the time period of this experiment, post-smolts can compensate for reductions in water flow down to 0.2 L kg fish^{-1} min^{-1}. However, to avoid activating and exhausting potentially energy-costly physiological regulatory mechanisms, it is suggested to keep specific water flow above 0.3 L kg fish^{-1} min^{-1} in large-scale operations with semi-closed sea systems at intermediate temperatures.

Keywords: fish welfare; closed-containment aquaculture systems; water quality; specific water flow

Key Contribution: It is suggested that post-smolt Atlantic salmon should be reared at a water flow of 0.3 L kg fish^{-1} min^{-1} in large-scale operations with semi-closed sea systems at intermediate

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temperatures. This will ensure no negative effects on osmoregulation, external macroscopic welfare, or performance indicators.

1. Introduction

Currently, global aquaculture production of Atlantic salmon post-smolts occurs mainly in sea cages. This part of the production cycle has the potential for the largest environmental impact and is when the industry faces the highest losses [1,2]. Thus, developing new production methods that increase the overall sustainability of salmon aquaculture is of fundamental importance. Extending the time fish spend in closed-containment systems (CCS), where the rearing environment can be controlled and optimized, prior to stocking in open sea cages is expected to produce larger and more resilient post-smolts. This strategy could lower losses, and by shortening the time that fish spend in open sea cages, it will contribute to an overall reduction in environmental impact. Both land-based systems and semi-closed systems in the sea (S-CCS) are currently of interest. However, post-smolt production in CCS and S-CCS is predicted to have higher initial costs compared to open sea cages [3,4]. Investment costs for S-CCS can potentially be offset by increasing stocking density and/or reducing biomass-specific water flow, hereafter referred to as specific water flow (SWF). Nevertheless, economic profitability will depend on whether the resulting rearing conditions have a negative impact on fish physiology, performance, and overall welfare.

Most of the knowledge on water quality requirements for salmon in flow-through systems is based on freshwater studies on earlier life stages, from eggs/fry to smolts [5–8]. Accordingly, it is of great relevance to establish safe limits and guidelines regarding SWF rates for the seawater post-smolt life stage. These water quality requirements will to a large extent influence the design of closed and semi-closed flow-through systems by determining the necessary water inflow volume, retention time, accumulation of feces, carbon dioxide (CO_2), ammonia (NH_3-N), and oxygen supply.

Reduced access to water at any given stocking density will reduce pO_2, pH, and increase pCO_2, total ammonia nitrogen (TAN), in the ambient water and the blood of the fish. Ammonia (NH_3-N) is the toxic metabolic waste product from the catabolism of amino acids and some nucleotides and is mainly excreted through the gills [9,10]. In water, TAN is present in two forms: ionized NH_4^+ and unionized NH_3, and the equilibrium of the two species depends mainly on pH but also on temperature and salinity. NH_3 is considered more toxic since it can easily cross gill membranes [9]. Depending on the energy substrate, fish excrete approximately 10 times more CO_2 than TAN [11,12]. CO_2, produced through the aerobic metabolism of the fish, will accumulate and decrease the water pH, which shifts the TAN equilibrium towards the ionized, less toxic form (NH_4^+). Thus, in intensive flow-through systems without aeration and where oxygen is added to the inflow water, CO_2 has been suggested to be the first limiting production factor [13,14].

Due to the close contact between the gills and the ambient water, ventilation will quickly equilibrate any differences in CO_2 tension between the water and the blood of the fish. The acid-base response to increased ambient CO_2 is a decrease in blood pH, which reduces the oxygen-transporting ability of the blood [15]. However, to compensate for the increased pCO_2 level and reduced pH, fish excrete H^+ and take up HCO_3^- from the surrounding water via the HCO_3/Cl exchanger in the gill epithelium [16], and in general, within 2–7 days, the blood pH is restored [17]. A decreased water exchange rate will not only increase the metabolite load but also the amount of total suspended solids (TSS) and the bacterial load in the system [18]. As decreasing the water flow creates complex water quality issues, it is important to establish safe levels for reduced SWF instead of relying on individual water quality parameters.

From a functional perspective, good fish welfare entails the ability of an animal to adapt to its environment and maintain normal biological function [19,20]. Stressors, like

reduced water quality [21], may require energy-demanding physiological adjustments, known as allostasis, that enable the animal to adapt to a changing environment [19]. Increased blood glucose, osmoregulatory, and hematological changes are examples of physiological alterations that allow the fish to react and compensate for the stressful stimuli [22–24]. However, long-term or repeated stress can lead to an allostatic overload of these adaptive mechanisms with chronic adverse effects on welfare like reduced growth, immune function, and reproductive capacity [25–27]. The rearing environment may not only induce stress responses but is also suggested as a cause of fin and bodily damage in farmed fish, representing a clear welfare issue that must be addressed [28–30].

An earlier study focusing on determining the optimal fish density for post-smolt Atlantic salmon suggested 75 kg m^{-3} as a viable option [30]. However, in that study, a high SWF rate was used in all groups, thus eliminating water quality as a factor for poor performance. Therefore, the aim of this study was to document the potential effects of reduced specific water flow on performance and welfare at the proposed stocking density of 75 kg m^{-3}. Furthermore, open flow respirometry, i.e., the difference in O_2 content between the tank inlet and outlet, flow rate, and biomass were used to estimate oxygen uptake and indirectly the metabolic effect of reduced SWF. It is hypothesized that below the SWF threshold level, higher water CO_2 may result in increased oxygen demand, osmoregulatory and hematological changes, and potentially chronic effects such as poorer growth.

2. Materials and Methods

2.1. Fish Stock and Rearing Conditions

The fish used in this study were out-of-season smolts of the AquaGen strain produced by Lerøy Vest, Flateråker, in Western Norway. Smolts were produced according to standard rearing protocols; for more details, see [30]. Before the acclimation phase of the experiment, all fish showed normal morphological and physiological signs of smolting, including silvery scales, dark fin margins, a low condition factor, and high gill Na^+ and K^+-ATPase (NKA) activity [31,32].

2.2. Experimental Design

All experimental procedures were approved by the Norwegian Animal Research Authority (reference no. 4692). This study was carried out at the Department of Biological Sciences at the University of Bergen, Norway, between October and December 2012. This was part of a combined experiment, and other aspects of the experiment have previously been published [30]. On 10 October, 2500 smolts (mean ± SEM; fork length = 22.0 cm ± 0.1, weight = 113.6 g ± 1.1) were randomly distributed among eight 1 m^3 square fiberglass tanks (500 L, stocking density 75.0 kg fish m^{-3}) with freshwater (pH adjusted and treated with SiO_2) in a flow-through mode (0.6 L kg $fish^{-1}$ min^{-1}). From the 16th to the 18th of October, the freshwater in each tank was gradually and stepwise replaced with deep flow-through seawater (−105 m; 9.3 °C ± 0.01); i.e., increasing salinity from 0 to 17‰ on 16 October (D1), from 17 to 25‰ on 17 October (D2), and from 25‰ to full strength seawater (34‰) on October 18 (D3).

In seawater, the fish were exposed to a simulated natural photoperiod (60°25′ N). The experimental treatments were established on the 24th of October and included four different levels of specific water flow (SWF): 0.2, 0.3, 0.4, and 0.5 L kg $fish^{-1}$ min^{-1}. Each treatment was conducted in two identical tanks. The chosen SWFs tested are in the range of those previously investigated in other life stages [5–8] and slightly lower to identify a possible threshold level. The fish in all treatments were fed a commercial freshwater dry diet (Optiline 3 mm, Skretting AS, Stavanger, Norway) in 10% excess versus tables (Skretting AS, Stavanger, Norway) with an automatic feeder daily between 09:00–10:00 and 15:00–16.00 throughout the study. A freshwater feed was used to reduce the sinking rate of the pellets, thereby increasing the time they were available to the fish. All tanks were checked twice daily, and dead fish were removed immediately and weighed. Bulk weight measurements of the total biomass in each tank were recorded at the start of the experiment,

in the middle (4 weeks), and at the end (8 weeks). At week 4, the actual biomass gain was recorded and removed to maintain the original treatment density of 75 kg m^{-3} (range 71.2–89.1 kg m^{-3}). At weeks 2 and 6, biomass gain was estimated from sampled fish and removed. The study lasted for 8 weeks and was terminated on December 20.

2.3. Blood Chemistry Sampling and Analysis

All individuals fasted for 24 h prior to sampling. Fish were quickly netted and anesthetized in 200 mg L^{-1} buffered tricaine methanesulphonate (MS222, Sigma-Aldrich, St. Louis, MO, USA). Blood samples from one fish per tank (n = 12 fish) were collected during the gradual acclimation to full-strength seawater (D1–D4) from October 16th to 19th and at the start of the experiment (24 October). Samples from six fish per tank (n = 12 fish) were also collected after 2, 4, 6, and 8 weeks of exposure to different SWF treatments. Blood was sampled with heparinized syringes from the caudal vessels. A sub-sample of blood was immediately analyzed by an ISTAT analyzer (Abbot Norge AS, Billingstad, Norway).

Analytical cassettes (EC8+) were used with the ISTAT analyzer to measure blood levels of glucose, sodium (Na^+), chloride (Cl^-), bicarbonate (HCO_3^-), blood pH, and partial pressure of carbon dioxide (pCO_2). Both blood pCO_2 [33] and plasma pH [17] values were adjusted according to the temperature difference between 37 °C and the temperature of the fish (pH_{blood} equals $pH_{measured}$ + 0.013·ΔT) [17]. Values for HCO_3^- were calculated according to the Henderson-Hasselbach equation [34], in which the solubility of CO_2 and the apparent pK were adjusted according to temperature. When used for diagnostics in fish, deviations between the ISTAT and conventional laboratory values have been found [35–38]. However, it is also the only tool that allows for a large number of parameters of interest to be analyzed simultaneously. This is particularly useful when a more extensive overview of blood chemical changes is needed, and the main objective is to compare relative differences between treatments and not to obtain absolute values for both blood ions and gases. Therefore, identical handling and sampling of fish were prioritized in this study to allow for comparison between treatments.

2.4. Gill Activity Sampling and Analysis

Gill tissue from 12 fish was collected for determination of Na^+, K^+-ATPase (NKA) activity, and NKA α-subunit isoforms during the gradual acclimation to full-strength seawater (D1–D4) and at the start of the experiment (0). Samples from six fish per tank were also collected after 4 and 8 weeks of exposure to different SWF treatments. For NKA activity determination, gill tissue was collected from the second gill arch, immediately immersed in ice-cold SEI buffer, and frozen at −80 °C until subsequent analysis according to the procedure of [31]. Readings were taken at 340 nm for 10 min at 25 °C. Protein in the homogenate was determined by a bicinchoninic acid method [39], and NKA activity is expressed as μmol ADP mg^{-1} protein h^{-1}.

For determination of NKA α-subunit isoforms, gill filaments were preserved in RNA later stabilization solution (Ambion, Foster City, CA, USA) for real-time quantitative PCR analysis. Total RNA was isolated from approximately 50 mg of gill tissue by phenol-chloroform extraction using TRI Reagent® (Sigma, St. Louis, MO, USA) as outlined by [40]. Total RNA concentration and purity were determined by the NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and RNA integrity was evaluated with the Agilent 2100 Bioanalyzer using the RNA 6000 Nano LabChip® kit (Agilent Technologies, Palo Alto, CA, USA). cDNA was reversely transcribed using 2000 ng of total RNA and an oligo (dT) 20 primer in conjunction with the SuperScript III kit (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. Gill NKA α-subunit isoforms (α1a, α1b) and elongation factor 1A (EF α1a) mRNA levels were measured using the CFX-96 Real-Time PCR detection system platform (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Melt-curve analysis verified that the primer sets for each Q-PCR assay generated one single product and no primer-dimer artifacts. Primers and PCR conditions have been published by [41,42]. For each assay, a triplicate, two-fold cDNA dilution series

made from total RNA from different exposure groups was used to determine amplification efficiencies (E). These were calculated as the slope from the plot of log cDNA concentration versus threshold cycle (Ct) values using the following formula: $E = 10^{(-1/\text{slope})}$. This efficiency was used to correct for differences in amplification efficiency when calculating gene expression, according to [43]. Expression is presented as relative to the endogenous reference gene EF α1a [44]. The α1a did not change over time or differ between treatments in the present study.

2.5. External Welfare Analysis

External welfare indicators were scored as described in [45]. In short, external welfare indicators were recorded for 10 fish per tank at the final sampling point. Fin condition was assessed according to [46], where each fin was examined and given a score from 0 (no damage/fin erosion) to 5 (severe damage/fin erosion). In addition, the left and right pectoral and pelvic fins were measured for length (mm). Each fish was also examined for the presence of cataracts, skin lesions, and operculum shortening and scored as described in [47].

2.6. Water Quality and Specific Oxygen Consumption

The tank water flow rates were adjusted by measuring the time it took to fill a 10-liter container with outlet water from each tank and were continually monitored and adjusted weekly throughout the experiment. The water velocity in each tank was kept stable and equal by adjusting the angle of the inlet water pipe. The water temperature was measured daily in the outlet of each tank with a handheld YSI 550A (Xylem Inc., Yellow Springs, OH, USA) and was 9.3 °C ± 0.3 throughout the experiment.

To achieve a good basis for accurate MO_2 estimations in the present trial, an automatic oxygen level regulation and monitoring system was used throughout the whole experiment, and various water quality parameters were closely followed (Table 1). The oxygen level in the header tank and in the outlet water was continuously monitored and logged (every 10 min) from each tank by an Oxyguard Commander System (Oxyguard, Farum, Denmark). Based on actual oxygen level information, the same system controlled the injection of oxygen into the inlet water via the corresponding header tank to always maintain oxygen saturation above 80% in the outlet water. The overall specific oxygen consumption (MO_2; $mgO_2\ kg^{-1}\ min^{-1}$) was based on the difference in O_2 between the header tank and tank outlet logged every 10 min throughout the experiment. The specific oxygen consumption () was calculated from the 1st measurement every hour, every second day in the period October 27–December 19 (n = 28), based on the formula:

$$MO_2 = (C_{inlet} - C_{outlet}) \times Q/B$$

C is the oxygen concentration ($mg\ L^{-1}$), Q is the flow ($L\ min^{-1}$), and B is the fish biomass (kg).

Every second week, pH was measured directly in the outlet of each tank (Seven Easy pH meter, Mettler-Toledo AG, Schwerzenbach, Germany; Table 1), and water samples were collected in sealable airtight glass bottles to monitor CO_2 levels [48] and in acid-washed tubes for TAN measurements. The carbon dioxide concentrations were calculated based on the percentage of carbon dioxide in the total carbonate concentration [49]. Before TAN was analyzed, pH was reduced to below 2 in each sample using sulfuric acid (H_2SO_4). TAN concentrations were analyzed according to 'Norwegian Standard 2005, NS-EN ISO 11732' using a Seal autoanalyzer (Omni Process AB, Solna, Sweden).

Table 1. Water quality at 4 different levels of specific water flow in full strength sea water (34‰) over the 8-week experimental period (n = 2 tanks). O_2 (Oxygen), TAN (total ammonia nitrogen) and CO_2 (Carbon dioxide) levels were measured in outlet of each tank. Un-ionized ammonia (NH_3-N; μg L^{-1}) is calculated from TAN, pH, salinity and temperature. Significant differences between SWF treatments are indicated by different letters (one-way ANOVA, $p < 0.05$). Data given are means ± SEM.

Parameter	0.2 L kg^{-1} min^{-1}	0.3 L kg^{-1} min^{-1}	0.4 L kg^{-1} min^{-1}	0.5 L kg^{-1} min^{-1}
Water flow (L min^{-1})	7.5	11.25	15	18.75
Tank exchange time (min)	66.6	44.4	33.3	26.6
Temperature (°C)	9.3 ± 0.01	9.3 ± 0.01	9.3 ± 0.01	9.3 ± 0.01
O_2 (mg L^{-1})	7.97 ± 0.01 ^{a}	8.10 ± 0.01^{b}	9.82 ± 0.01 ^{d}	8.63 ± 0.01 ^{c}
pH	6.94 ± 0.05 ^{a}	7.20 ± 0.05 ^{b}	7.37 ± 0.04 ^{c}	7.46 ± 0.05 ^{c}
CO_2 (mg L^{-1})	15.74 ± 1.83 ^{a}	8.6 ± 0.88 ^{b}	5.60 ± 0.48 ^{bc}	4.79 ± 0.62 ^{c}
TAN (mg N L^{-1})	0.76 ± 0.11 ^{a}	0.48 ± 0.07 ^{b}	0.35 ± 0.05^{b}	0.36 ± 0.05 ^{b}
NH_3-N (μg L^{-1})	1.0 ^{a}	1.2 ^{ab}	1.3 ^{b}	1.6 ^{c}

2.7. Growth and Condition Factor

To assess the effects of reduced SWF on growth and condition factors, a sub-group of 15 randomly selected fish from each tank were individually tagged (11 October, PIT tags, Trovan Ltd., Melton, North Ferriby, UK). Fork length (L) was measured to the nearest 0.1 cm and weight (W) to the nearest 0.1 g and was recorded during tagging and at the end of the experiment. The growth was calculated as a specific growth rate (SGR), where W_1 and W_2 are weights at days T_1 (start of experiment) and T_2 (after 8 weeks), according to the equation:

$$SGR = (\ln W_2 - \ln W_1) \times 100/(T_2 - T_1).$$

Fulton's condition factor (K) was calculated based on the formula:

$$K = 100W \times L^{-3}$$

2.8. Statistics

Statistical analyses and graphics were made using STATISTICA (version 12). All data sets were tested for normality using the Kolmogorov-Smirnov test. The Hartley F-max test was used to test for homogeneity of variances. A two-way nested ANOVA [50] with tanks nested within specific water flow (SWF) and sample time points was used to compare the effect of treatment time on physiological parameters (n = 12). A two-way nested ANOVA with the tank nested in SWF was also used to assess the effect of SWF on performance parameters (SGR, weight, fork length, and condition factor) of individually tagged fish (n = 27–30). The overall MO_2 (n = 28) after 8 weeks of treatment was tested with a one-way ANOVA, and a two-way ANOVA [50] was used to compare the effect of SWF and time of day on MO_2 (n = 28). The change in MO_2 after feeding was tested with a linear regression, and the parallelism of regression lines was tested with an analysis of covariance (ANCOVA [50]). All welfare score data (n = 20), except fin length, were arcsine square root transformed. Subsequently, the effect of SWF on external welfare was analyzed with a two-way nested ANOVA, with tank nested in SWF. Differences in water quality were also tested with a one-way ANOVA (n = 2 tanks). Significant ANOVAs were followed by a Student-Newman-Keuls multiple comparison post hoc test (SNK). All data given are means ± SEM. A significance level (α) of 0.05 was used unless stated otherwise.

3. Results

3.1. Blood Chemistry

Measurements conducted prior to the establishment of the experimental SWF treatments showed that seawater transfer and acclimation did not affect blood pCO_2 and HCO_3^- levels ($p < 0.05$; results not shown). Both blood pCO_2 (Figure 1A) and HCO_3^- (Figure 1B) levels were significantly influenced by SWF and the duration of treatment

($p < 0.001$). The most prominent increase in blood pCO_2 level was in the lowest SWF treatment (0.2 L kg fish^{-1} min^{-1}) which had higher levels compared to other treatments from week 2 till the end of the experiment ($p < 0.01$). Blood HCO_3^- level followed a similar development as blood pCO_2, with an initial rise in blood HCO_3^- in all treatments in the first two weeks of the experiment ($p < 0.001$; Figure 1B), followed by a period of stabilization. Similarly to the blood pCO_2, the HCO_3^- level was also significantly higher in the 0.2 L kg fish^{-1}·min^{-1} treatment from week two and on ($p < 0.01$). Furthermore, the 0.3 L kg fish^{-1} min^{-1} treatment had elevated HCO_3^- levels compared to the two higher SWF treatments (0.4 and 0.5 L kg fish^{-1} min^{-1}; $p < 0.05$).

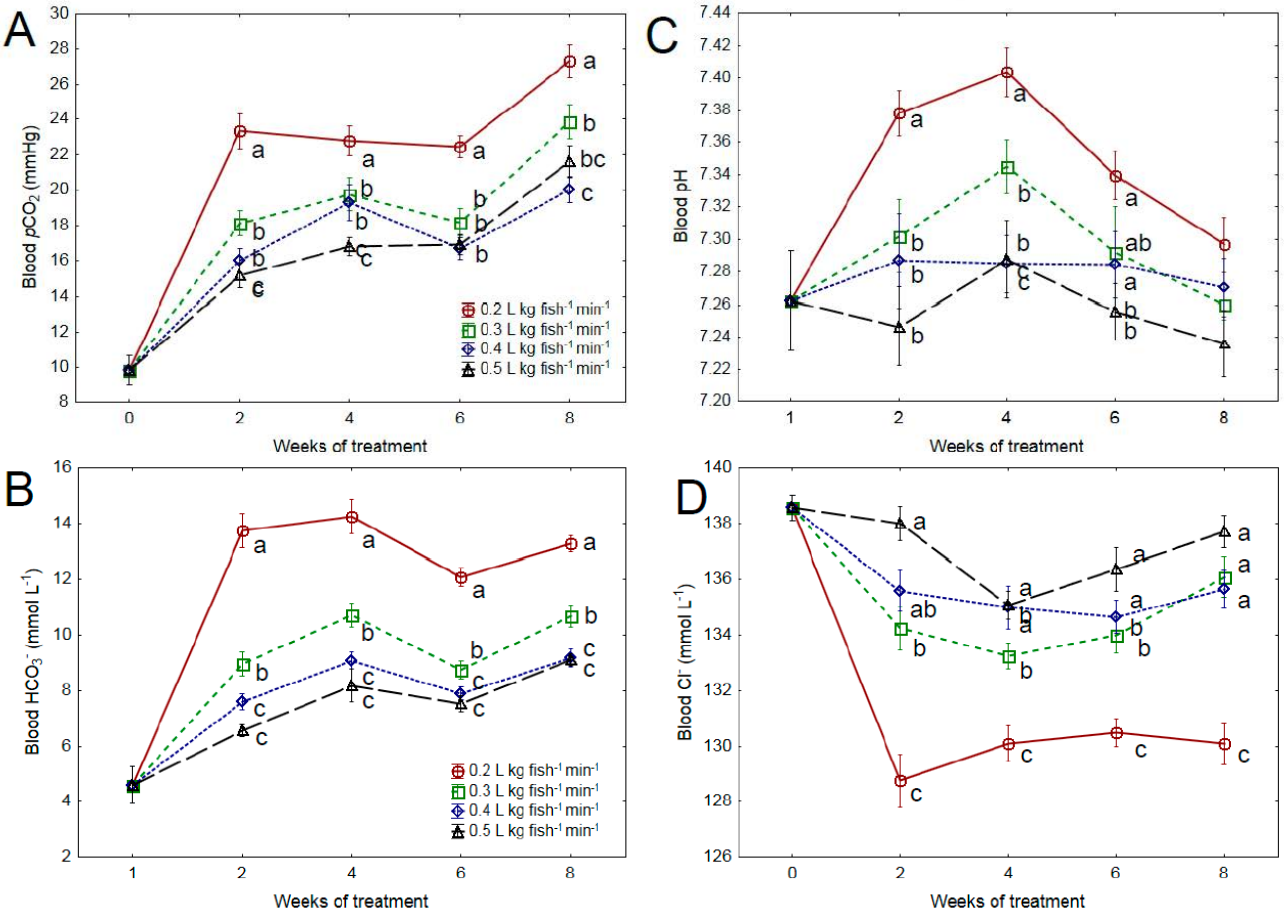

Figure 1. (**A**) Blood pCO_2 (mmHg), (**B**) blood bicarbonate (HCO_3^-, mmol L^{-1}), (**C**) blood pH levels, and (**D**) blood chloride (Cl^-, mmol L^{-1}) in Atlantic salmon post-smolts reared at four different levels of specific water flow (SWF: 0.2, 0.3, 0.4, and 0.5 L kg fish^{-1} min^{-1}). Significant differences between SWF treatments at each time point are indicated by different letters (two-way nested ANOVA, $p < 0.05$). $n = 12$, and all data given are means ± SEM.

Blood pH was significantly influenced by time and reduced SWF (Figure 1C, $p < 0.001$). In the lowest SWF treatment (0.2 L kg fish^{-1} min^{-1}), blood pH was significantly elevated compared to the highest SWF (0.5 L kg fish^{-1} min^{-1}) for the first 6 weeks of the experiment ($p < 0.001$).

Prior to the establishment of SWF treatments, a significant increase in blood chloride levels (Cl^-) was observed when increasing the salinity from 25 to 34 ppt (D3; $p < 0.05$). Cl^- levels remained at the increased level until the start of the experiment (results not shown). Plasma Cl^- levels were inversely related to the observed changes in blood pCO_2 and HCO_3^- in all treatments and decreased in a dose-response manner to reduced specific water flow (Figure 1D, $p < 0.001$). In the 0.2 L kg fish^{-1} min^{-1} treatment, Cl^- levels were significantly lower than in other treatments throughout the experiment ($p < 0.01$). There

was also a reduction in blood Cl^- concentration in the 0.3 L kg fish^{-1} min^{-1} treatment compared to the 0.5 L kg fish^{-1} min^{-1} treatment at weeks 2 and 6.

Prior to the establishment of SWF treatments, there was a significant increase in blood sodium (Na^+) when increasing the salinity from 0 ppt to 17 ppt on D1. Na^+ levels stabilized at this level until the start of the experiment (results not shown). Neither exposure time nor SWF influenced blood sodium levels (Na^+, $p > 0.05$, Figure 2).

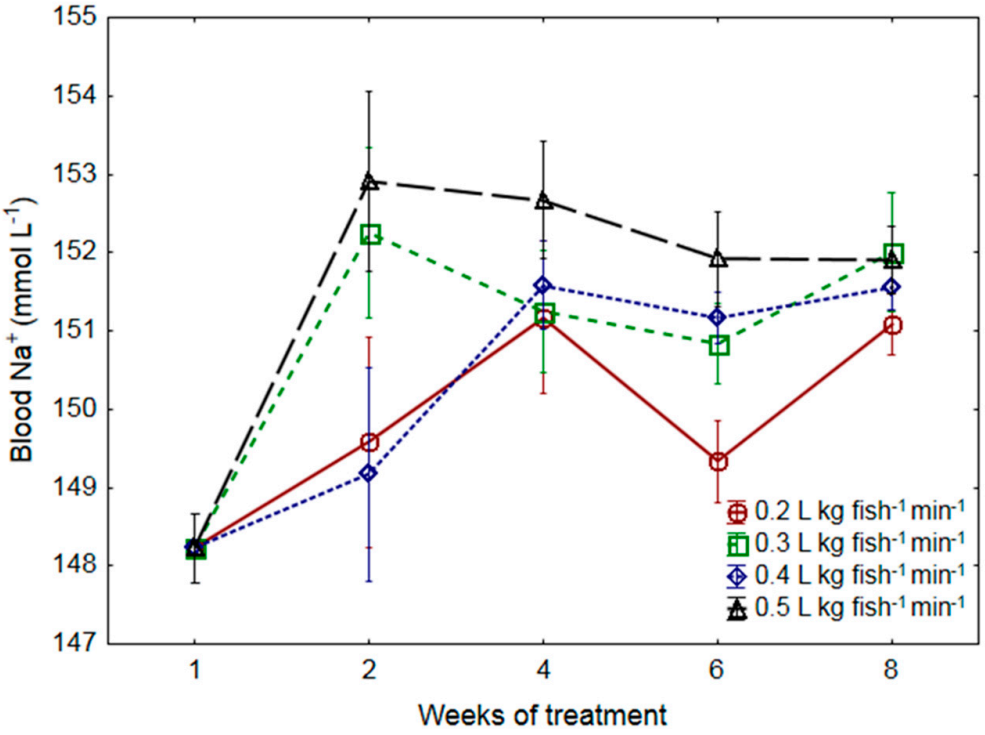

Figure 2. Blood sodium (Na^+; mmol L^{-1}) levels in Atlantic salmon post-smolts raised at four different levels of specific water flow (SWF: 0.2, 0.3, 0.4, and 0.5 L kg fish^{-1} min^{-1}). Significant differences between different levels of SWF at each time point are indicated by different letters (two-way nested ANOVA, $p < 0.05$). $n = 12$, and all data given are means ± SEM.

No significant differences in blood glucose between treatments were observed at any of the sampling points (results not shown).

3.2. Gill NKA α1a and α1b mRNA Levels and Gill NKA

A gradual increase in gill NKA was observed during the seawater acclimation period, reaching peak levels of 11.2, 12.2, 12.3, and 15.1 µmol ADP mg protein^{-1} h^{-1} in the 0.2, 0.3, 0.4, and 0.5 L kg fish^{-1} min^{-1} treatments, respectively, at week 2 (Figure 3). During the gradual 4-day transition to seawater, D1 to D4, a significant decline in gill NKA α1a (freshwater isoform) mRNA levels was recorded, which reached transcript levels close to 0 after 2 weeks in seawater (Figure 4, $p < 0.001$). There were no significant differences among groups during this period. In the same period, the seawater isoform NKA α1b mRNA showed the opposite pattern, with levels increasing to reach peak levels at the start of the experiment. Following the peak, gill NKA α1b mRNA levels declined to significantly lower levels by the end of the experiment (Figure 4, $p < 0.05$). No differences among treatments were registered.

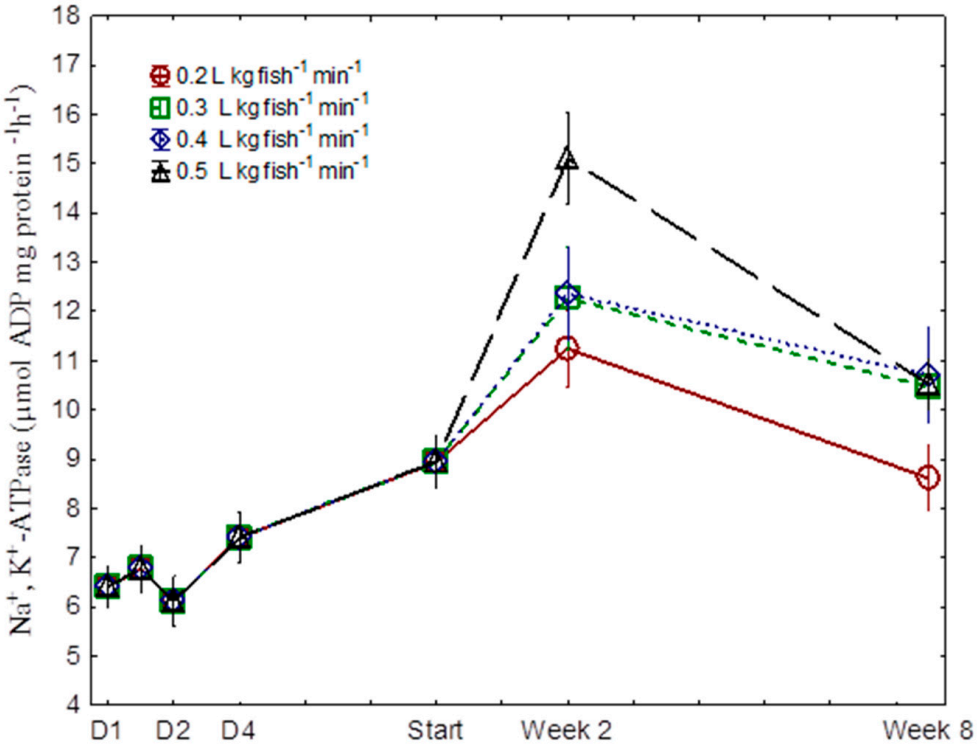

Figure 3. Gill Na+, K+-ATPase (NKA) activity in Atlantic salmon post-smolts reared at four different levels of specific water flow (SWF: 0.2, 0.3, 0.4, and 0.5 L kg fish^{-1} min^{-1}). D1–D4 refers to the four days of gradual acclimation to full-strength sea water. Significant differences between different levels of SWF at each time point are indicated by different letters (two-way nested ANOVA, $p < 0.05$). $n = 12$, and the data given are means ± SEM.

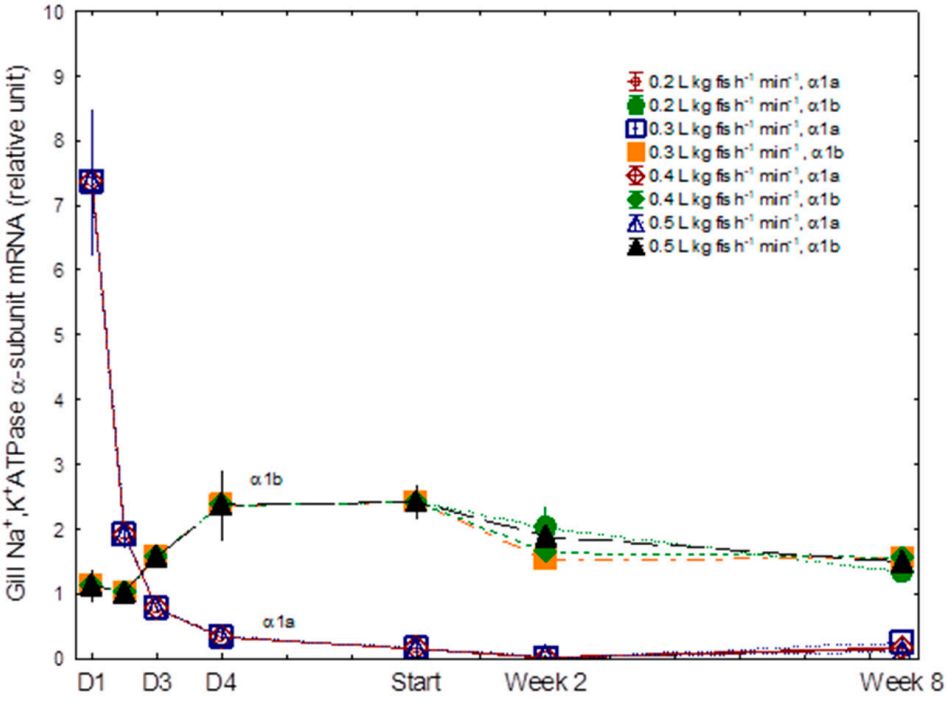

Figure 4. Gill NKA α1a and α1b relative mRNA expression in Atlantic salmon post-smolts reared at four different levels of specific water flow (0.2, 0.3, 0.4, and 0.5 L kg fish^{-1} min^{-1}). See Figure 5 for other details.

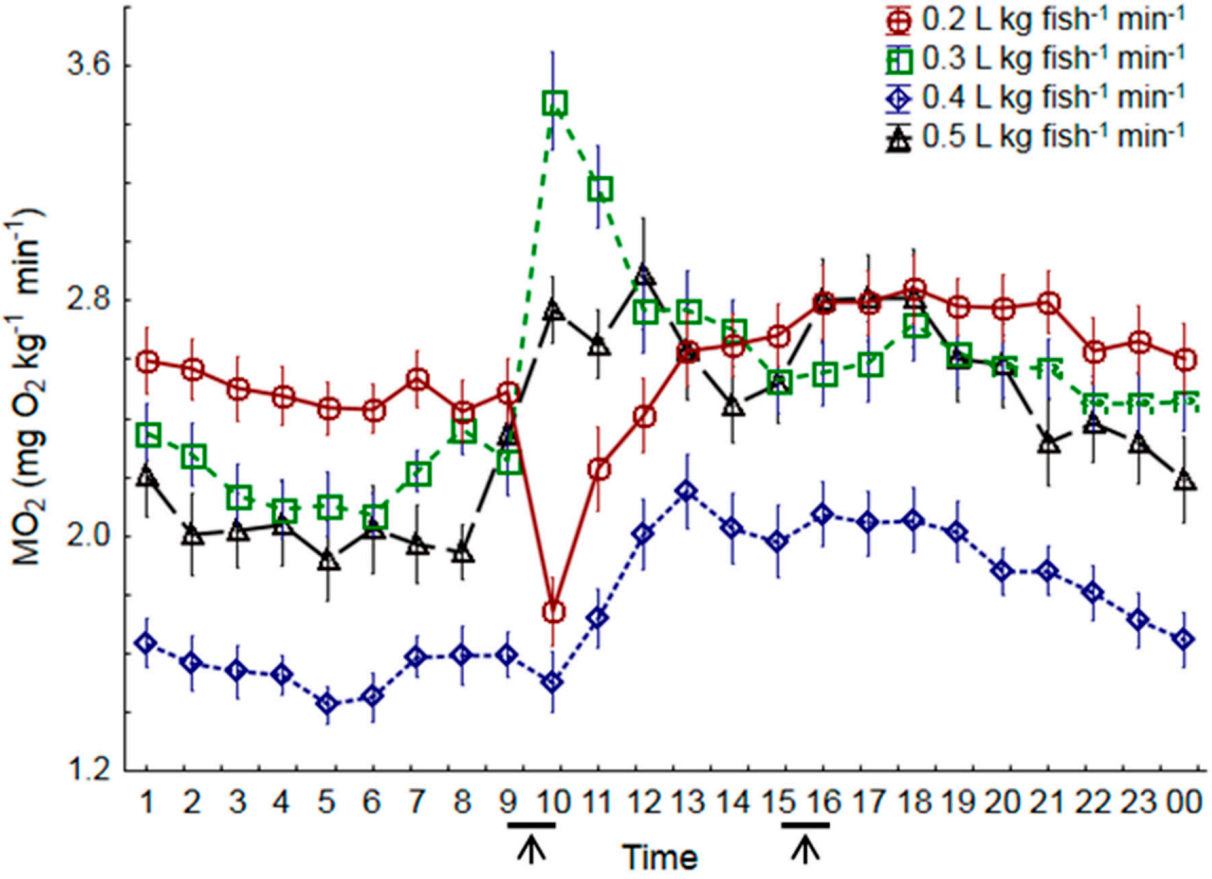

Figure 5. Diurnal specific oxygen consumption (MO_2) in Atlantic salmon post-smolts reared at four different levels of specific water flow (0.2, 0.3, 0.4, and 0.5 L kg fish^{-1} min^{-1}) for 8 weeks. ↑ indicates feeding time. n = 28 days, and all data are given as means ± SEM.

3.3. External Welfare Indicators

Fin damage such as erosion, splitting, malformations, and fin ray damage were the most commonly observed pathologies. However, these were not affected by SWF, nor were the lengths of the pelvic and pectoral fins ($p > 0.05$, lengths not shown). Overall, reduced SWF did not affect any of the external welfare indicators (fin, skin, cataracts, and operculum shortening) studied ($p > 0.05$, Table 2).

Table 2. External welfare analysis of post-smolt Atlantic salmon after 8 weeks exposure to four different levels of specific water flow (SFW: 0.2, 0.3, 0.4 and 0.5 L kg fish^{-1} min^{-1}). Each indicator was given a score, from 0 (no damage) to 2 (severe damage) for operculum, cataract, and skin lesions. Fins are scored from 0 (no damage) to 5 (severe damage). No Significant differences between SWF treatments were detected (two-way nested ANOVA, $p > 0.05$). n = 20 and data given are means ± SEM.

Parameter	0.2 L kg^{-1} min^{-1}	0.3 L kg^{-1} min^{-1}	0.4 L kg^{-1} min^{-1}	0.5 L kg^{-1} min^{-1}
Cataract (0–2)	0.1 ± 0.1	0.2 ± 0.1	0 ± 0	1 ± 0.1
Skin (0−2)	0.1 ± 0.1	0.4 ± 0.1	0.1 ± 0.1	0.4 ± 0.1
Operculum (0–2)	0 ± 0	0.1± 0.1	0.1 ± 0.1	0.2 ± 0.1
Fin (0−5)				
Dorsal	2.0 ± 0.4	2.35 ± 0.3	2.8 ± 0.3	2.3 ± 0.4
Caudal	1.3 ± 0.1	1.5 ± 0.2	1.4 ± 0.2	1.4 ± 0.1
AnalPectoral	0.3 ± 0.2	0.5 ± 0.1	1.2 ± 1.3	0.5 ± 0.2
Pelvic	2.4 ± 0.1	2.3 ± 0.2	2.0 ± 0.1	2.2 ± 0.1
	1.4 ± 0.1	1.7 ± 0.1	1.8 ± 0.2	1.7 ± 0.1

3.4. Oxygen Consumption

The overall highest mean oxygen consumption was observed in the two treatments with the lowest SWF, with 2.6 mg O_2 kg^{-1} min^{-1} being consumed both in the 0.2 and 0.3 L kg $fish^{-1}$ min^{-1} treatments (Table 3, $p < 0.05$). The overall MO_2 was 2.4 mg O_2 kg^{-1} min^{-1} in the 0.5 L kg $fish^{-1}$ min^{-1} treatment, a significant reduction compared to the 0.2 and 0.3 L kg $fish^{-1}$ min^{-1} treatments ($p < 0.05$). In the 0.4 L kg $fish^{-1}$ min^{-1} treatment, the MO_2 was 1.8 mg O_2 kg^{-1} min^{-1}, which was significantly lower than all other treatments ($p < 0.001$). A significant increase in oxygen consumption was observed during the period 08:00–13:00 in the 0.3, 0.4, and 0.5 L kg $fish^{-1}$ min^{-1} treatments (Figure 5, $p < 0.05$). During 13:00–15:00, the oxygen consumption in the same treatments started to decline, and 10–12 h later (approximately 02:00), values returned to the baseline MO_2 for the treatment ($p < 0.05$). Such a pattern was not observed in the lowest SWF treatment (0.2 L kg $fish^{-1}$ min^{-1}); instead, here the MO_2 significantly decreased following the commencement of feeding (09:00–10:00) ($p < 0.05$), resulting in non-parallel regression lines ($p < 0.05$, ANCOVA) for this feeding time.

Table 3. Specific growth rate (SGR; % bodyweight day^{-1}), fork length (L), Weight (W), condition factor (K) and specific oxygen consumption (MO_2; mg kg^{-1} min^{-1}) of Atlantic salmon post-smolts reared at four different levels of specific water flow (SWF: 0.2, 0.3, 0.4 and 0.5 L kg $fish^{-1}$ min^{-1}). All data were collected after an experimental period of 8 weeks (20 December). Data given are means ± SEM. Significant differences between SWF treatments are indicated by different letters ($p < 0.05$, two-way nested ANOVA).

Parameter	0.2 L kg^{-1} min^{-1}	0.3 L kg^{-1} min^{-1}	0.4 L kg^{-1} min^{-1}	0.5 L kg^{-1} min^{-1}
Density range (kg m^{-3}, $n = 2$)	75.2–82.1	75.1–82.1	75.8–83.6	73.1–83.1
Mortality (count, $n = 2$)	1	1	4	9
SGR (% day^{-1}, $n = 27$–30)	0.75 ± 0.03	0.78 ± 0.03	0.77 ± 0.03	0.77 ± 0.03
W start (g, $n = 30$)	121.1 ± 2.4 [a]	113.8 ± 1.5 [b]	110.7 ± 2.3 [b]	108.7 ± 1.7 [b]
W end (g, $n = 27$–30)	207.4 ± 7.1	195.0 ± 5.0	190.7 ± 5.6	187.2 ± 4.8
L end (cm, $n = 27$–30)	26.7 ± 0.3	26.4 ± 0.2	26.1± 0.3	25.9 ± 0.2
K end ($n = 27$–30)	1.07 ± 0.01	1.05 ± 0.01	1.06 ± 0.01	1.07 ± 0.01
MO_2 (mg kg^{-1} min^{-1}, $n = 28$)	2.56 ± 0.02 [a]	2.55 ± 0.03 [a]	1.75 ± 0.02 [c]	2.38 ± 0.03 [b]

3.5. Growth and Condition Factors

The fish density range was similar in all treatments (Table 3). The mortality was low in the experiment and not related to the experimental treatment ($p > 0.25$). Fish in the 0.2 L kg $fish^{-1}$ min^{-1} had a significantly higher average start weight of 121.4 g compared to 113.8, 110.7, and 108.7 g in the 0.3, 0.4, and 0.5 L kg $fish^{-1}$ min^{-1}, respectively ($p < 0.05$, Table 3). Fish in the 0.2 L kg $fish^{-1}$ min^{-1} also had a higher average fork length than other treatments and a higher condition factor than fish in the 0.3 and 0.5 L kg $fish^{-1}$ min^{-1} at the start of the experiment ($p < 0.05$, results not shown). At the end of the experiment, there was no longer a significant difference in weights between treatments ($p > 0.05$, Table 3). Furthermore, no difference in average length or condition factor was registered at the end of the experiment. Furthermore, reduced SWF did not affect the specific growth rate during the experimental period ($p > 0.45$, Table 3).

4. Discussion

4.1. Water Quality and Blood Chemistry

As SWF is reduced, less new water dilutes the metabolic waste from the fish in the culture tank. The effect of this increased intensity was clear on the water quality in this study. Both TAN and CO_2 increased as conditions intensified, and with a rise in CO_2 levels, the pH declined accordingly. The mean CO_2 level in the water was three times higher in the lowest SWF treatment compared to the highest SWF. In this experiment, the first blood samples were taken after 2 weeks, and blood pH had been restored in the 0.3, 0.4,

and 0.5 L kg fish^{-1} min^{-1} treatments but was significantly increased in the lowest SWF treatment (0.2 L kg fish^{-1} min^{-1}), likely due to the elevated pCO_2 and increased level of blood bicarbonate. Furthermore, to maintain electroneutrality in the blood, the branchial chloride influx rate is reduced, subsequently decreasing plasma chloride levels [51]. This compensatory response was sustained in the 0.2 L kg fish^{-1} min^{-1} treatment throughout the whole experiment. Long-term reductions in Cl^- in response to increased water CO_2 have earlier been observed in post-smolts exposed to 26 mg L^{-1} of CO_2 for 43 days [52] and in parr exposed to 17–18 mg L^{-1} of CO_2 for 42 days in freshwater [53]. As no effects on growth were observed in this study, which is considered a chronic stress response, the scope for the physiological compensatory responses (described above) was likely not exceeded in the lowest SWF. However, these responses are energy-intensive, as shown by increased oxygen consumption. It can be speculated that if the experiment had lasted longer, a redistribution of energy from maintenance functions such as growth and immune functions may have been observed in the lowest SWF (0.2 L kg fish^{-1} min^{-1}) treatment.

4.2. Osmoregulation

The sharp decrease in NKA α1a isoform expression and the increase in NKA α1b with increased salinity support earlier findings demonstrating that α1b is the seawater adaptive isoform and α1a is the freshwater isoform [32,41,54,55]. There was no difference in gene expression of either of the NKA isoforms or differences in enzyme activity between treatments, suggesting that within the time span of this study, reducing SWF down to 0.2 L kg fish^{-1} min^{-1} does not impact osmoregulation in post-smolts in seawater.

4.3. External Welfare Indicators

Cataracts, fin, skin, and opercular damage represent injuries to live tissue and have been associated with common rearing practices in farmed salmonids [20,29,45]. Damaged epithelia can lead to osmotic disturbances, represent invasion routes for pathogens, and therefore increase the risk for disease [56,57]. In the present study, reduced flow did not have a detectable effect on any of the macroscopic external welfare indicators studied; to our knowledge, this has not earlier been addressed in relation to reduced specific water flow. The present results are in line with [30], which showed that post-smolts in stocking densities up to 75 kg m^{-3} had no negative effect on the same external welfare indicators, and [47], which concluded that sub-optimal water quality conditions did not affect external welfare in Atlantic salmon. In a skin health analysis that was performed as part of the present study, it was found that a SWF of 0.3 L kg fish^{-1} min^{-1} and below activated transcription of genes associated with immune responses and mucus production in the skin [58]. Hence, a skin health analysis is suggested as a useful tool for detecting early responses to environmental changes.

4.4. Oxygen Consumption

In any aquaculture facility, the balance between oxygen consumption (MO_2) and oxygen supply is critical; however, it has been difficult to estimate MO_2 rates in commercial operations due to the large number of effectors and their unpredictability (reviewed in [3]). The overall MO_2 was lowest in the 0.4 L kg fish^{-1} min^{-1} treatment and not in the highest SWF treatment, 0.5 L kg fish^{-1} min^{-1} (control), as might be expected; this is likely due to the large number of factors affecting MO_2. Although measures were taken to maintain equal water velocities in each tank, it cannot be excluded that a slightly higher water velocity in the 0.5 L kg fish^{-1} min^{-1}, due to a higher SWF rate, caused the increased MO_2 in this treatment.

The diurnal variation in MO_2 with an increase following feeding is known as specific dynamic action (SDA) and accounts for all metabolic expenses associated with digestion, absorption, and storage of nutrients [59]. In the present experiment, the SDA effect was most apparent after the first meal in the 0.3–0.5 L kg fish^{-1} min^{-1}, whereas no clear SDA effect was observed in the lowest SWF treatment over a 24-h period. Instead, at

the lowest SWF (0.2 L kg fish^{-1} min^{-1}), the overall oxygen demand over the 24-h period was higher, indicating that energy was allocated to physiological compensatory processes instead. Similarly, [60] found a reduced SDA effect in sea bass, *Dicentrarchus labrax*, and turbot, *Scophthalmus maximus*, exposed to hypoxic conditions; for sea bass, this effect was explained by a reduced feed intake. Reduced feed intake was not observed in the turbot, indicating that behavioral adaptations like reduced swimming activity may be important in reallocating energy during unfavorable water quality conditions. In line with this, the decreased MO_2 observed after feeding in the 0.2 L kg fish^{-1} min^{-1} treatment in the present trial may indicate decreased activity. In support of this, Ref. [61] found that chronic but mild CO_2 exposure increased feed intake and decreased swimming activity in sea bass; similarly, no effects on growth were observed in that study. Overall, the diurnal fluctuations and the effect of SWF on oxygen demand need to be taken into consideration when designing the oxygen supply system for large-scale semi-closed sea systems.

4.5. Growth

No effects on any of the growth indicators measured were detected in the present study. This is consistent with [62], who did not detect any effects on growth or FCR after 145 days of exposure to 0.2 L kg fish^{-1} min^{-1} in 8 °C seawater. Assuming that CO_2 is the first limiting factor in our experiment, our results are also in agreement with [52], which saw only slight effects on growth in post-smolts after 43 days of CO_2 exposure up to 26 mg L^{-1} in 15–16 °C seawater.

4.6. Application at the Industrial Scale

This experiment investigated four rearing intensities at an intermediate (9.3 °C) temperature. A higher experimental temperature would have increased the fish metabolism and intensified the rearing conditions accordingly, and vice versa for lower temperatures. Thus, the lower SWF limit suggested in this study could be too low for higher temperatures and too conservative for temperatures lower than 9.3 °C [63]. However, according to available reports from commercial S-CCS production [64–66], a temperature of 9.3 ± 2 °C appears to be highly relevant for most of the production period in the reported S-CCS cases. The SWF limit will also depend on post-smolt size since smaller fish have a higher mass excretion rate [10]. According to recent post-smolt growth models [67], it takes approximately 4–7 months to produce post-smolts from 100 g up to 1 kg. Therefore, results from the present study need to be verified in longer-term, large-scale studies covering this size range for post-smolt Atlantic salmon. Although the SWF guidelines in this paper should be applied with consideration to the prevailing environmental and biological factors, this paper reveals information that is highly requested by salmon farmers considering or producing post-smolts in S-CCS.

5. Conclusions

The present study shows that post-smolt Atlantic salmon kept at an intermediate temperature (9.3 °C) in flow-through systems can elicit physiological responses to compensate for reduced specific water flow down to 0.2 L kg fish^{-1} min^{-1}. However, the responses observed have an energetic cost, as revealed by increased oxygen consumption. Hence, based on the present results, it is suggested that without any in-tank water treatment, specific water flow should be maintained above 0.3 L kg fish^{-1} min^{-1} at a post-smolt size of 100–200 g because physiological regulatory responses are energy-costly. Future studies should concentrate on the longer-term effects and the allostatic costs of physiological regulatory responses to decreased water flow/increased CO_2.

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