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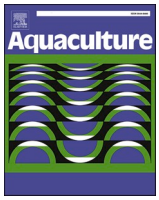

Performance of Atlantic salmon reared under three different regimes of continuous aerobic exercise during the freshwater phase

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ABSTRACT

Freshwater rearing conditions influence the growth and seawater adaptation of Atlantic salmon, and swimming exercise may enhance these adaptive processes. This study examined growth and physiological responses of Atlantic salmon subjected to continuous swimming at three speeds: low (0.5 body length per second, BL/s) and moderate (1.0 and 1.5 BL/s) for 11 weeks in freshwater, followed by transfer to brackish water. Fish trained at 1.0 and 1.5 BL/s demonstrated significantly higher specific growth rates and plasma insulin-like growth factor 1 levels after 5 weeks in brackish water, suggesting that moderate exercise enhances growth. Additionally, the 1.5 BL/s group showed a higher frequency of small-diameter white muscle fibers, suggesting hyperplastic growth. Although the expression of growth-related genes was not affected by swimming speed, moderate exercise groups had significantly lower plasma triglycerides and cholesterol levels, suggesting a shift of energy allocation towards growth. At the end of the freshwater phase, distinct energy allocation strategies were evident: the low-speed swimming group had higher hepatosomatic index and plasma inorganic phosphate levels, whereas the 1.5 BL/s group showed higher muscle adenylate energy charge, indicating enhanced muscle energy status. Fish in moderate swimming groups also had lower cortisol, creatinine (significantly different between 0.5 and 1.5 BL/s), and lactate levels (significantly different between 0.5 and 1.0 BL/s), suggesting an improved stress profile. Swimming exercise did not affect smoltification markers, including NKA activity or plasma sodium and chloride concentrations. Overall, moderate swimming (1.0–1.5 BL/s) improved growth in Atlantic salmon, highlighting potential applications for aquaculture.

1. Introduction

The transition of Atlantic salmon (*Salmo salar*) from freshwater to seawater represents a critical period in the aquaculture production cycle. In 2023, a record number of 16.7 % (62.7 million) of farmed Atlantic salmon died in the seawater phase (Brun, 2024). The high mortality can be attributed to several challenges such as infestations by salmon lice (*Lepeophtheirus salmonis)*, disease and effects of handling and treatment procedures, and poor smolt-quality (Nilsen et al., 2020; Oliveira et al., 2021; Sommerset et al., 2024). To reduce mortality, it is essential to produce salmon that has improved resilience to both diseases and physiological challenges, as well as a capacity for fast growth.

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One method which has shown promising results in producing more robust Atlantic salmon is aerobic swimming exercise. Swimming exercise has been shown to promote growth, increase cardiac growth and performance, aerobic capacity of white muscle, oxygen carrying and extraction capacity, improve skeletal integrity, brain plasticity, decrease the prevalence of male precocity, and reduce aggressive behavior (Balseiro et al., 2018; Castro et al., 2011, 2013a, 2013b; Mes et al., 2020; Nilsen et al., 2019; Solstorm et al., 2016; Timmerhaus et al., 2021; Totland et al., 2011; Totland et al., 1987; Waldrop et al., 2018).

Swimming speed between 0.5 and 1.7 body lengths per second (BL/s) seems to be most effective to improve the growth rate in salmonids (reviewed in: McKenzie et al., 2021). These swimming speeds are well below the critical swimming speed (U_{crit}) of Atlantic salmon, which is defined as the maximum sustained swimming speed before exhaustion (Brett, 1964). For example, Hvas et al. (2017) reported U_{crit} values of 2.3–2.6 BL/s at 8–13 °C for post-smolts (408–491 g), with a peak U_{crit} of 2.7 BL/s at 18 °C. Muscle growth in teleost fish is achieved by a combination of hyperplasia (or recruitment) and hypertrophy of muscle fibers (Koumans et al., 1993). In fast growing species, such as salmonids, muscle growth results mainly from a sustained recruitment of new fibers (Weatherley et al., 1988). However, as the fish size increases, the muscle fiber recruitment contribution decreases while the hypertrophy contribution increases (Stickland, 1988). Swimming exercise has shown to improve growth by hyperplasia in gilthead sea bream (*Sparus aurata*) fingerlings (Moya et al., 2019) and in pacus (*Piaractus mesopotamicus*) (dos Santos et al., 2017). Atlantic salmon post-smolts reared in a semi-closed raceway system in the sea (Preline), which facilitates continuous swimming exercise conditions, exhibited a higher frequency of small muscle fibers, i.e., higher hyperplasia compared to fish reared in an open sea cage after 4 months in seawater (Balseiro et al., 2018). Additionally, studies indicate that sustained swimming exercise induces muscle hypertrophy in fish (Bugeon et al., 2003; Castro et al., 2011; Ibarz et al., 2011; Martin and Johnston, 2005; Palstra et al., 2014; Totland et al., 1987; Walker and Emerson, 1978).

Somatic growth in teleost fishes, as in other vertebrates, is mainly regulated by the growth hormone (Gh)/insulin-like growth factor 1 (Igf1) axis (reviewed in: Fuentes et al., 2013; Johnston et al., 2011; Pérez-Sánchez et al., 2018; Wood et al., 2005)). The Gh, produced in the pituitary gland, modulates the expression of *igf1* (Leung et al., 2008; Pedroso et al., 2009) and *igf2* (Pierce et al., 2010) in fish hepatocytes. Once in circulation, Igf1 inhibits Gh secretion (negative feedback) (Fruchtman et al., 2000; Pérez-Sánchez et al., 1992; Rousseau et al., 1998). On the other hand, Igf1 also stimulates the release of prolactin (Prl) from the pituitary (Fruchtman et al., 2000; Fruchtman et al., 2002). It has been suggested that the plasma concentration of Igf1 is a useful biomarker to assess growth in fish (Beckman, 2011; Kaneko et al., 2015; Picha et al., 2008a). The effects of Igf on peripheral tissues depend on several other factors, including Igf-binding proteins (Igfbps) and Igf receptor (Igf1r) (reviewed in: Ndandala et al., 2022; Reindl and Sheridan, 2012; Shimizu and Dickhoff, 2017)). Igfs are also synthesized in several other tissues, including in muscle, where they exert autocrine/paracrine effects to regulate tissue growth (Eppler et al., 2007; Picha et al., 2008b).

In addition to their role in somatic growth, Igf1, Gh and Prl are key hormones involved in adaptation to changes in salinity (reviewed in: Sakamoto and McCormick, 2006; Seale and Breves, 2022)) in species including tilapia (*Oreochromis mossambicus*), blackchin tilapia (*Sarotherodon melanotheron*), killifish (*Fundulus heteroclitus*), brown trout (*Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (Kajimura et al., 2002; Link et al., 2022; Madsen and Bern, 1992; Mancera and McCormick, 1999; Tipsmark and Madsen, 2009). In salmonids, the parr-to-smolt transformation (smoltification) is a hormone-driven process that enables the fish preparatory adaptation to migrate from freshwater to seawater. Transfer to seawater generally results in reduced levels of Prl and increased levels of Gh and Igf1 (Nilsen et al., 2008; Poppinga et al., 2007; Yada et al., 1991). Synergistically with the Gh/Igf1 axis, cortisol is implicated in increasing gill Na^+-K^+-ATPase activity and promote seawater tolerance (Tipsmark and Madsen, 2009). However, the effect of exercise in the adaptation to seawater remains to be clarified (Esbaugh et al., 2014; Jørgensen and Jobling, 1994).

Freshwater conditions can influence the performance of Atlantic salmon in seawater (Lai et al., 2024). Thus, we aimed to investigate if swimming exercise prior to seawater transfer can have a beneficial impact on fish performance. To achieve this, we subjected fish to 11 weeks of swimming exercise with a regime of either 0.5 BL/s, 1.0 BL/s or 1.5 BL/s (average of the population in the tank) just before transfer to brackish water (26 ‰). These speeds were chosen based on previous studies indicating they fall within the optimal range for promoting growth and physiological benefits in salmonids. A suite of analyses were employed to examine growth of Atlantic salmon and overall performance in response to the different swimming speeds before and after transfer to brackish water.

2. Material and methods

2.1. Experimental design

Atlantic salmon parr from Salmobreed strain were obtained from the land-based RAS facility Lerøy Sjøtroll Kjærelva (Fitjar, Norway) on 1 September 2021, and transported to the lab facilities at High Technology centre (Department of Biological Sciences, UIB, Bergen, Norway). On 3 September 2021 fish (n = 480) were anesthetized with 80 mg/L of $NaHCO_3$-buffered tricaine methanesulfonate (MS-222; MSD Animal Health, Netherlands) before being individually tagged with a Passive Integrated Transponder (Glass tag 2.12 × 12 mm 134.2 kHz ISO FDX B, RFID solutions). The fish PIT-tag was recorded using a PR600 RFID Handheld Reader (Agrident, USA), and fish weight and fork length were measured (body weight of 78.9 ± 18.1 g and fork length 18.6 ± 1.5 cm). Fish were kept in 0.5 m^3 flow-through tanks at 12 °C water temperature and oxygen levels above 80 %, and constant daylight (LD24:0) for one month before being transferred into the experimental tanks. Each tank was equipped with an automated feeder and fish were fed to satiation once a day from 9:00 to 15:00 (6 h) with a commercial feed provided by Lerøy Sjøtroll Kjærelva (3 mm, Ewos Clear Fly 80). All 480 fish (body weight of 98.1 ± 17.6 g and fork length 20.7 ± 1.3 cm) were randomly distributed into 6 circular experimental tanks (80 fish per tank) with a capacity of 460 L (28 September 2021). The water flow in each tank (height: 80 cm; diameter: 104 cm; water depth: 55 cm; with a 20 cm cylinder-shaped separator positioned in the center) was maintained at 0.5 BL/s using a traditional dry-setup pump (CM10, Grundfos, Norway) and monitored with a Flow Watch FW450 (General, USA). The feeding setup, feed, and procedure were as described above. On the 26 October 2021, tanks were allocated into three experimental groups (duplicated tanks per group) with different swimming speeds: 0.5 BL/s, 1.0 BL/s and 1.5 BL/s. Water velocity was based on the average length of the fish in each tank and was adjusted every two weeks based on the specific growth rate of the fish length obtained during samplings. In parallel with the start of swimming exercise, salmon were exposed to an artificial winter signal with short days (LD12:12) for 5 weeks, followed by 6 weeks of exposure to continuous light (LD24:0) to induce smoltification before being transferred to 26 ‰ brackish water (17 January 2022). Due to rapid growth, supplemental oxygen was provided (Pacific Combi, OxyGuard) from 10 December 2021 until the end of the experimental trial (min. 80 % in outlet). During the 5-week growth phase in brackish water (end date: 23 February 2022), the swimming speed was kept at 0.5 BL/s for all groups, and fish were kept under continuous light (LD24:0). **Fig. S1** shows a schematic representation of experimental design and sampling. The temperature and oxygen concentrations were monitored daily in the outlet water of every tank using OxyGuard Commander probes and visualized by an automatic system (VIGO Visual, VIGO5.9). For details on the water temperature and oxygen saturation throughout the experiment please refer to **Fig. S2** and **Table S1**, and for details on

water quality please refer to **Table S2**. No mortality was registered during the trial. A simplified description of the experimental design, until the end of the freshwater phase, is also provided in the study by Keihani et al. (2024).

2.2. Sampling

Three samplings were performed during the trial, and 10 fish per tank were collected at each sampling point (see **Fig. S1**). All fish were deprived of feed 24 h prior to sampling. Fish were euthanized with a lethal dose of $NaHCO_3$-buffered MS-222 (200 mg/L). Blood was collected from the caudal vein using 1 mL heparinized syringes with 25 G needles and centrifuged for 3 min at 5000 rpm to separate the plasma. Plasma samples were immediately frozen in dry ice and stored at −80 °C until further use. The fish PIT-tag was read and registered using the APR600 RFID Handheld Reader (Agrident, USA). Body weight and fork length were measured for each individual prior to dissection. The liver and gonads were collected and weighed using a VWR LPC-213i balance (VWR, USA). The first gill arch from the left side was placed in SEI buffer (250 mM sucrose, 10 mM Na_2EDTA, 50 mM imidazole, pH 7.3) and immediately frozen in dry ice and subsequently stored at −80 °C until Na^+-K^+-ATPase activity (NKA) analyses were performed. A piece of white muscle (3–5 mm thick) was cut out from posterior to the dorsal fin on the left side of the fish, and it was fixed in 4 % formaldehyde using BiopSafe containers (BiopSafe ApS, Denmark) at 4 °C for 48 h, for histology analysis. A white muscle sample from the same area was also collected and frozen directly in dry ice and kept at −80 °C for biochemical analysis. For gene expression analysis, a sample of white muscle (from the same area as for histology and biochemical analyses), and the pituitary were collected and placed in RNAlater (Invitrogen, Carlsbad, CA, USA). Samples in RNAlater were kept at 4 °C for 24 h and stored at −80 °C until analysis.

Feed waste from each tank was collected daily in individual zip-lock plastic bags and stored at -20 °C. The wet feed waste was measured using a scale (Entris623i-1S, Sartorius Lab Instruments GmbH & Co. KG, Germany) before being dried in a dehydrator (EXC10ELF, Excalibur, USA) for 42 h at 74 °C and subsequently weighed.

2.3. Plasma analyses

The concentration of monovalent ions (Cl^-, Na^+, K^+) in the plasma samples were determined using the potentiometry method with the Ion-Selective Electrode (ISE) module of the Pentra c400 clinical chemistry analyzer (HORIBA, Japan). The calibration of the ISE module was performed using ABX Pentra Standard 1, ABX Pentra Standard 2, and ABX Pentra Reference solutions. Specific electrodes corresponding to each ion were used for the measurement. The potential difference across the electrode membrane was altered by the ions present in the sample, and it was then compared with the reference potential generated by a known ion concentration (Buck, 1981). The other plasma parameters were measured by colorimetric spectrophotometry determination, using the HORIBA's kits ABX Pentra Glucose HK CP for glucose, ABX Pentra Cholesterol CP for cholesterol, ABX Pentra Triglycerides CP for triglycerides, ABX Pentra Lactic Acid kits for lactate, ABX Pentra Enzymatic Creatinine CP for creatinine, ABX Pentra Urea CP for urea, ABX Pentra LDH IFCC CP for LDH, ABX Pentra Magnesium RTU for Mg^{2+}, ABX Pentra Calcium AS CP for Ca^{2+}, and ABX Pentra Phosphorus CP for inorganic phosphorus (P_i) in the Pentra c400 clinical chemistry analyzer. Each required reagent was calibrated using the ABX Pentra Multical and quality control was performed using ABX Pentra N control, as stated in the manufacturer's protocol.

Plasma cortisol was measured using a custom ELISA in a 96-well plate. All wells except the 'non-specifics' received 100 μL cortisol antibody (East Coast Biologics, North Berwick, ME, USA; P01–92-94 M-P); 1:3000 in 50 mM $NaHCO_3$, 50 mM NaH_2CO_3, pH 9.6) and were incubated overnight at 4 °C. The following day, the plates were washed three times with 200 μL/well wash buffer + Tween (100 mM Tris, 0.9 % NaCl, 0.1 % Tween20). Subsequently, non-specific sites were blocked by the addition of 200 μL blocking buffer (100 mM Tris, 0.9 % NaCl, 0.1 % Tween20, 2 % Normal Calf Serum) to each well. Plates were covered and incubated for one hour at room temperature on a plate shaker (300 rpm). Wells were emptied by decanting, after which 10 μL of standard (4–2048 pg cortisol/10 μL assay buffer containing 100 mM Tris, 0.9 % NaCl, 0.1 % 8-anilino-1-naphthalene-sulfonic acid, 0.1 % Tween20) in triplicate, or 10 μL of undiluted plasma in duplicate was added to designated wells. Non-specifics and B0 received 10 μL assay buffer (both in triplicate). After the addition of standards and samples, 90 μL cortisol-HRP conjugate (1:3000; East Coast Biologics) solution was added to all wells. Plates were incubated overnight at 4 °C. The plates were then washed once with wash buffer with Tween, and twice with wash buffer without Tween. 100 μL 3,3′,5,5'-Tetramethylbenzidine (TMB) substrate at room temperature (Sigma Aldrich, St. Louis, MO, USA) was added to each well. After 30 to 60 min (depending on the time required to develop a blue color) incubation in the dark on a plate shaker (300 rpm), 100 μL of stop solution (1 M sulfuric acid) was added to all wells. Absorbance was measured within half an hour at 450 nm.

2.4. Plasma Igf1

To analyze plasma Igf1, plasma was first extracted with an acid-ethanol solution (87.5 % ethanol and 12.5 % 2 N HCl, *v*/v), as previously described by Shimizu et al. (2000). The Igf1 in the extract was quantified by time-resolved fluoroimmunoassay based on the method described by Small and Peterson (2005) using for a standard the recombinant salmon/trout Igf1 (GroPep, Adelaide, SA, Australia). Time-resolved fluorescence was measured using the luminometer Wallac ARVO SX (PerkinElmer, Waltham, MA, USA).

2.5. White muscle biochemical analyses

The nucleotides ATP, ADP and AMP were analysed in the white muscle of Atlantic salmon. Frozen white muscle tissue samples ($n = 6$ per group), 180–250 mg, were homogenized on ice with 0.6 M Perchloric acid PCA (1:5 *w/v*) using an Ultra Turrax T8 homogenizer. The homogenate was centrifuged at 10000 *g* for 5 min at 4 °C, and then 700 μL of supernatant was collected and pH adjusted to 6.5–7 with 1 M KOH. Samples were then kept on ice for 30 min, before the potassium perchlorate was removed by centrifugation (10,000 x*g* for 5 min at 4 °C). Finally, 1 mL of the supernatant was diluted to a final volume of 1.4 mL with phosphate buffer (0.04 M KH_2PO_4 + 0.06 M K_2HPO_4; pH 7). Samples were stored at -20 °C before analysis.

To analyze ATP and its breakdown products, high-performance liquid chromatography (HPLC) method was used with a Waters Alliance 2695 HPLC system (Waters Associates Inc., Milford, MA,) equipped with a 2487 PDA detector. The separation was achieved using a reverse phase C18 Kinetex column 250 × 4.6 mm, with an internal particle diameter of 5 μm. The mobile phase was composed of solvent A (0.04 M KH2PO4 + 0.06 M K2HPO4 pH 7) and solvent B (methanol). The chromatographic conditions were as follows: flow rate of 1.0 mL/min was used, with a starting solvent composition of 100 % solvent A, which was ramped linearly to 16 % B over 8 min. The composition of solvent B was increased linearly over 0.5 min to 60 % B, which was then held for 5 min. The mobile phase was then brought back to 100 % of solvent A over 5 min and equilibrated for a further 3.5 min for a total run time of 22 min. The column was held at 25 °C, with the samples measured at 254 nm, with 10 μL injected on-column. Standard curves were prepared from ATP, ADP and AMP all from Sigma Aldrich (Dorset, England, UK) in concentrations ranging from 0 to 1.0 mM. ATP and its breakdown products were identified by their relative retention time in Empower 2 software and externally quantified using the standard curves.

2.6. Gene expression analyses

Total RNA was extracted from white muscle and pituitary tissues using TRI Reagent (Sigma-Aldrich, USA) following the manufacturer' instructions. A NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA) was used to measure total RNA concentration and purity. To avoid any remnants of genomic DNA, 10 μg of total RNA was treated with TURBO DNase-free Kit (Ambion Applied Biosystem, USA). After, total RNA concentration (ng/μL) was measured using a Qubit 4 Fluorometer (Thermo Fisher Scientific) with a Qubit RNA BR assay kit (Thermo Fisher Scientific, USA), following manufacturer's instructions (Thermo Fisher Scientific, USA). Purity was assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Thermo Fisher Scientific) and RNA integrity was analysed on 25 % of the samples using a 2100 Bioanalyzer with RNA 6000 Nano Kit (Agilent Technologies, CA, USA. cDNA was reversely transcribed using 2 μg of white muscle and 1 μg of pituitary total RNA with oligo(dt)20 primer and the Superscript III kit (Thermo Fisher), according to the manufacturer's instructions.

qPCR was carried out with a CFX96 Real-Time PCR detection system platform (Bio-Rad Laboratories) using the following conditions: 3 min at 95 °C followed by 35 cycles of 15 s at 95 °C and 1 min at 60 °C. The absence of non-specific products and primer dimers was verified in all qPCR assays by melting curve analysis: 65 °C to 95 °C (increment of 0.5 °C every 2 s). For each assay, a triplicate two-fold cDNA dilution series from pooled samples were used to determine each primer pair amplification efficiencies (Table 1). qPCR was carried out using 6.25 μL of iTaq universal SYBR Green Supermix (Bio-Rad, CA, USA), 0.25 μL of each forward and reverse primers (10 mM), 3.25 μL Ultra-Pure Water (Biochrom, Berlin, Germany) and 2.5 μL of 1:20 diluted cDNA for white muscle (12.5 ng) or 2.5 μL of 1:100 diluted cDNA for pituitary (1.25 ng). All reactions were run in duplicate. Two negative control reactions were included in each plate to detect any possible contamination or genomic DNA contamination. A common pooled cDNA sample was used for the inter-calibration of assays among plates. The relative transcription levels of the genes were normalized following the efficiency corrected method (Pfaffl, 2001) using *rps20* and *β-actin* as the endogenous reference genes (Olsvik et al., 2005).

2.7. Histology analysis of white muscle

After 48 h of formaldehyde fixation, white muscle tissue samples were washed in 50 % ethanol for 30 min before being stored in 70 % ethanol at 4 °C until processing. Muscle tissue samples ($n = 36$) were processed by an external laboratory (Pharmaq Analytiq, Norway). Samples were fixed in buffered formalin (4 % formalin, 0.08 M sodium phosphate, pH 7.0), processed using a Thermo Scientific Excelsior tissue processor (Thermo Fisher Scientific, UK) and embedded in paraffin (Histowax, 56–58 °C) using Tissue-Tek TEC 5 (Sakura Finetek Europe B. V., The Netherlands). The embedded tissues were sectioned at a thickness of 1.5–2.0 μm using a Leica RM 2255 Microtome. Tissue sections were mounted on glass slides and stained with haematoxylin-eosin (HE). The stained slides were scanned using an Aperio ScanScope AT Turbo slide scanner and read with Aperio ImageScope (Leica Biosystems, USA).

Muscle sections were further examined using QuPath software v0.4.3 (Bankhead et al., 2017). A circular region with a diameter of 1000 μm was randomly selected from the white skeletal muscle in each sample. Within this predefined area, muscle fibers were assessed based on their maximum diameter, as well as the total number of fibers. The circular region was divided into four quadrants, and fibers in contact with the border were only considered in two opposite quadrants of the four.

2.8. Calculations

The specific growth rate (% day^{-1}) between two sampling points was calculated as:

$$SGR\,(\%) = \frac{Ln(W_2) - Ln(W_1)}{T_2 - T_1} \times 100$$

The condition factor was measured in g/cm^3 following the equation (Froese, 2006):

$$CF = \left(\frac{W}{L^3}\right) \times 100$$

The feed consumption (C) in the tank was calculated as described by Helland et al. (1996) using the equation:

$$C = \frac{(Feed\,fed \times \%of\ feed\ dry\ matter) - (Dry\ feed\ waste/Recovery)}{\%of\ feed\ dry\ matter}$$

Table 1
Sequence of the specific primers used for qPCR mRNA expression analysis.

Gene	GenBank acc no.	Sequence (5′ → 3′)	Amplicon (bp)	E (%)	R^2
[1]*igf1*	[a] NM_001123623.1	F: GATGTCTTCAAGAGTGCGATGTG R: CGCCGAAGTCAGGGTTAGG	84	90	0.996
[2]*igf2*	NM_001123647.1	F: ATTGCGCTGGCACTTACTCT R: CACTCCTCCACGATACCACG	176	89	0.972
[3]*igf1ra1*	XM_014124120.2	F: TGCACAACTCCATCTTCACC R: GGGGCTCTCCTTCTGTCCTA	132	97	0.999
[2]*igf1ra2*	XM_014149105.2	F: TAATGGGACGGACGGAGAATC R: GTGAAGGGCTGTAGGTTGGG	99	89	0.985
[2]*prl*	XM_014192393.1	F: TCAAGAAGACTCTCACTTTCCAC R: TGAGTGCTTGCTCCTTGTC	107	99	0.997
[4]*gh1*	NM_001123676.1	F: GGTTTCCCAGATACAGATTAG R: GCTCAGAGTAATAGTCAATATAG	197	87	0.997
[4]*gh2*	XM_014204437.1	F: GGGTGAAATGGGAACTTGTAGAG R: CCATCTGTGGACATACCAAAAGC	52	97	0.995
[5]*β-actin*	NM_001123525.1	F: CCAAAGCCAACAGGGAGAAG R: AGGGACAACACTGCCTGGAT	91	98/93	0.994/0.997
[5]*rps20*	NM_001140843.1	F: GCAGACCTTATCCGTGGAGCTA R: TGGTGATGCGCAGAGTCTTG	85	98	0.996/0.997

[1] Pierce et al. (2004).
[2] Present work.
[3] Hevrøy et al. (2013).
[4] Pino Martinez et al., (2021).
[5] Olsvik et al. (2005).
[a] Two paralogs were found but it was not possible to design paralog-specific primers, thus a primer that amplifies both was used.

The feed conversion ratio (FCR) as:

$$FCR = \frac{C}{Final\ biomass - (Initial\ biomass + Removed\ biomass)}$$

The feed intake (FI) as:

$$FI = \frac{C}{Biomass} \times 100$$

The feed conversion efficiency (FCE) as:

$$FCE = \frac{Final\ biomass - (Initial\ biomass + Removed\ biomass)}{C} \times 100$$

The hepatosomatic index (HSI) (Chellappa et al., 1995) and the gonado-somatic index (GSI) were calculated based on the liver and gonad weight (W_o) and the fish weight (W) using the equation:

$$Organ\ index\,(\%) = \frac{W_o}{W} \times 100$$

The Adenylate Energy Charge (AEC) was determined by the equation (Atkinson and Walton, 1967).

$$AEC = \frac{[ATP] + 0.5[ADP]}{[ATP] + [ADP] + [AMP]}$$

2.9. Statistical analysis

Data exploration was conducted to identify outliers and evaluate the data distribution. A generalized linear mixed model (GLMM) with gamma distribution (log-link function) was used to model the response variables (R) as a function of categorical variables swimming speed (V) and sampling time point (S). Tank (t) was added as random intercept, $b_t \sim N(0, \sigma_i^2)$, to account for possible tank effect:

$$R_{V,S,t} = \beta_0 + \beta_1 V + \beta_2 S + \beta_3 (V \times S) + b_t + \varepsilon_i$$

For cortisol a GLMM with tweedie distribution (log-link function) was used to account for zeros (values were assumed to be zeros when these were below the detection level).

For the GSI a binomial distribution with logit-link function was applied to determine the probability of maturing males in response to treatment and sampling point. Onset of maturation was set at GSI > 0.06 % as defined by Pino Martinez et al. (2023).

Post-hoc tests with Tukey approximation were used to analyze pairwise differences and contrasts between groups (swimming speed and sampling point).

Feeding related analyses (FI, FCR and FE) were performed using a generalized additive model (GAM) with gamma distribution (log-link function). The explanatory variables used were swimming speed (V) and phase (P, indicating either freshwater phase or grow-out phase in brackish water) as categorical variables, and days (D) as continuous effects with thin-plate smooth splines by V restricted to 5 knots.

$$R_{V,D,P,t} = \beta_0(t) + \beta_1 V + s(D|V) + \beta_3 P + u_t + \varepsilon_i$$

For the analysis of white muscle fiber diameter, fiber measurements were categorized into bins with intervals of 20 μm, from 0 to 300 μm. To capture and aggregate larger diameters, an additional bin was defined for measurements greater than 300 μm. The categorization facilitated a clear analysis of frequency distributions across diameter classes in relation to the experimental factors. The frequency of white muscle fiber diameter was analysed using a GLM with beta distribution (logit-link function). The explanatory variables were swimming exercise (V), sampling (S), and bin diameter interval (B), each treated as categorical predictors.

$$R_{V,S,B} = \beta_0 + \beta_1 V + \beta_2 S + \beta_3 B + \beta_4 (V \times S) + \beta_5 (V \times B) + \beta_6 (S \times B) + \beta_7 (V \times S \times B) + \varepsilon_i$$

Data exploration and statistical analyses were performed in RStudio R.4.3.2 with the packages *glmmTMB* (Brooks et al., 2017), *mgcv* (Wood, 2017), *DHARMa* (Hartig, 2021) which was used to validate the model fit, (simulated) residual distribution and to evaluate residual distributions, and *emmeans* (Lenth et al., 2023) for *post-hoc* testing. Statistical significance was set at $p < 0.05$. All data are presented as mean ± 95 % confidence interval (CI).

3. Results

3.1. Fish biometry

At the start of the acclimation period, fish from all treatments had similar mean body weights (0.5 BL/s = 99.1 ± 18.1 g; 1.0 BL/s = 98.6 ± 17.2 g; 1.5 BL/s = 96.5 ± 17.5 g), lengths (0.5 BL/s = 20.4 ± 1.33 cm; 1.0 BL/s = 20.4 ± 1.29 cm; 1.5 BL/s = 20.3 ± 1.31 cm), and condition factors K (0.5 BL/s = 1.15 ± 0.06; 1.0 BL/s = 1.16 ± 0.07; 1.5 BL/s = 1.15 ± 0.06). Using GLMM, we evaluated the effects of swimming speed and sampling time on several biometry parameters: SGR, K, HSI and probability of males maturing. The effect of tank, accounting for potential tank-specific cluster effects in all statistical models, showed that the variation attributed to different tanks was not relevant (**Fig. S3**).

The SGR of the fish was significantly affected by sampling time and its interaction with swimming speed (**Table S3**). After transfer to brackish water, at 17 weeks, the SGR in the 0.5 BL/s was significantly lower compared to the 1.0 BL/s group ($p < 0.01$) and the 1.5 BL/s group ($p < 0.05$). No significant differences in SGR were observed between swimming speeds during the freshwater phase (Fig. 1A, **Table S4**). Additionally, the SGR at 17 weeks (grow-out phase in brackish water) was significantly lower compared to the SGR during freshwater phase ($p < 0.0001$) across all swimming speeds. Similar results were obtained when analyzing SGR by phase (Fig. 1B, **Tables S5** and **S6**).

Both the condition factor K and HSI were only significantly affected by sampling time (**Table S3**). K significantly increased over time and was higher in the grow-out phase (17 weeks) compared to the first sampling (5 weeks) across all groups ($p < 0.05$) and compared to 11 weeks for the 1.5 BL/s group (Fig. 1C, **Table S4**).

For HSI, at the end of the freshwater phase (11 weeks), the group trained at 0.5 BL/s was significantly higher compared to the moderate swimming groups ($p < 0.05$) (Fig. 1D, **Table S4**). A significant increase in HSI was observed between 5 weeks and 17 weeks for 1.0 BL/s ($p < 0.01$) and 1.5 BL/s ($p < 0.05$); and for the 1.0 BL/s between 11 weeks and 17 weeks ($p < 0.001$). Since HSI is determined by both liver and body weight, the strong correlation between them ($r^2 = 0.93$ for 0.5 and 1.5 BL/s groups, and $r^2 = 0.91$ for the 1.0 BL/s group, with all p-values <0.001) was nearly identical for all groups (**Fig. S4**), indicating that swimming affects the compound variable HSI without altering the relationship between body and liver weight.

The proportions of males and females sampled differed between sampling points but were similar between swimming speeds (**Fig. S5A**). At 5 weeks, nearly all males were classified as immature, regardless of swimming speed (**Fig. S5B**). The proportion of maturing males increased with time and was higher at 11 and 17 weeks. When evaluating which variables affected probability of male maturing, only sampling time had a significant effect (**Table S3**). The probability of maturing males was significantly higher at 11 and 17 weeks compared to the first sampling (5 weeks) for the 0.5 BL/s group (**Fig. S5C**, **Table S4**). No female maturation was observed during the trial (data not shown).

3.2. Feed intake

A GAM was used to analyze the combined impact of swimming speed, day of the experiment (as a continuous time variable), and water phase (freshwater vs. brackish water) on fish FI, FCR and FCE (**Fig. S6**, **Table S7**). For FI, the model explained 86.9 % of the deviance (adjusted R-squared = 0.884). Fish in the 1.0 BL/s and 1.5 BL/s groups had significantly ($p < 0.05$) higher FI compared to the 0.5 BL/s group. Fish in

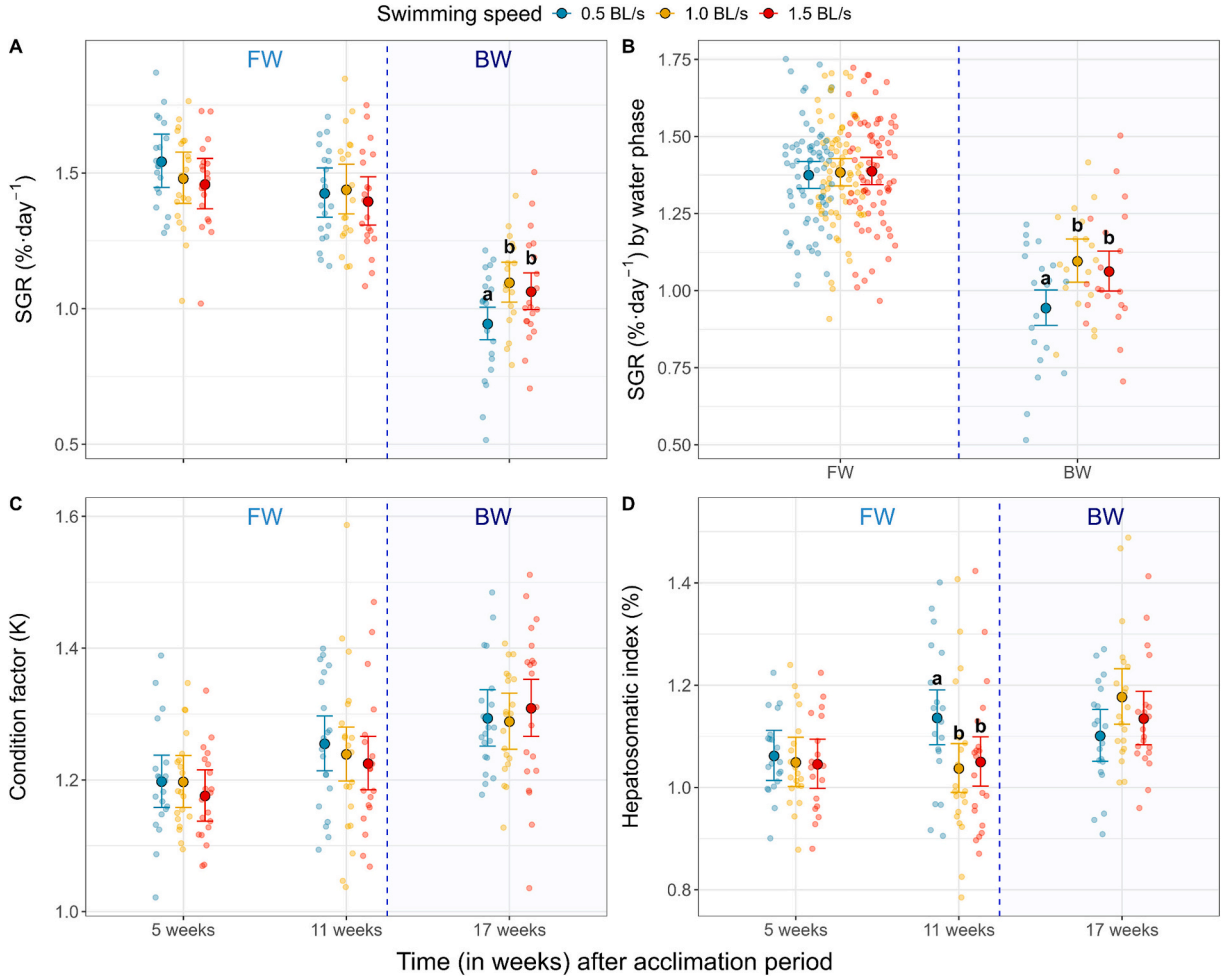

Fig. 1. Growth performance of Atlantic salmon reared under three swimming-speeds. **(A)** Specific growth rate (SGR) expressed as %/day; **(B)** SGR (%/day) by water phase (freshwater vs. brackish water); (**C**) Condition factor (K); and **(D)** Hepatosomatic index (HSI), expressed in %. The plots display the model predicted means (large dots) and 95 % CIs (error bars). Raw data points are represented by smaller dots (horizontally jittered for visualization purposes). The freshwater phase (FW) and brackish water phase (BW) are indicated. The SGR at 17 weeks, i.e., in brackish water, was calculated based on the final weight of the fish and the weight at the end of the freshwater phase. Only significant differences ($p < 0.05$) between swimming speed groups at a given sampling time point (or by phase for panel (**B**)) are indicated by different letters. For details regarding statistical significance, including significant differences between sampling points at each swimming speed, please refer to **Tables S4** and **S6**. The number of individuals included in each analysis is presented in **Table S24**.

the freshwater phase exhibited a significantly higher FI compared to the brackish phase ($p < 0.0001$). There was a significant nonlinear relationship between day and swimming speed within each swimming speed ($p < 2e\text{-}16$), and there was a significant random effect of tank ($p < 2e\text{-}16$). For FCR and FCE, the model explained 59.3 % of the deviance (adjusted R-squared = 0.591). No significant differences in FCR and FCE were found between swimming speeds. However, the phase had a significant effect on both parameters, with the freshwater phase showing significantly higher FCR and FCE than the brackish water phase ($p < 0.0001$). There was a significant nonlinear relationship between day and swimming speed within each swimming speed ($p < 2e\text{-}16$), and the random effect of tank was also significant ($p < 2e\text{-}16$).

3.3. Plasma metabolites and ions

A GLMM was used to analyze the impact of swimming speed, sampling time, and interaction between those two variables on several plasma metabolites (Fig. 2A-G). Swimming speed significantly affected cholesterol and had a marginally significant effect on glucose and creatinine levels (**Table S8**). The sampling time had a significant effect on cortisol, lactate, glucose, triglycerides and urea plasma levels (**Table S8**). The interaction between swimming speed and sampling time significantly affected cortisol, triglycerides and had a marginal significant effect on lactate (**Table S8**). Post-hoc tests revealed that both triglycerides and cholesterol at 17 weeks were significantly higher in the 0.5 BL/s group compared to the 1.0 BL/S ($p < 0.01$ and $p < 0.05$, respectively), and triglycerides also presented significant lower levels in the 1.5 BL/s group ($p < 0.01$) (**Table S9**). At 11 weeks, the 0.5 BL/s group had significantly higher levels of cortisol and creatinine compared to 1.5 BL/s group ($p < 0.05$), and significantly higher levels of lactate compared to the 1.0 BL/s group ($p < 0.05$) (**Table S9**). For information regarding the effect of sampling time within each swimming speed, please refer to **Table S9**. Additionally, the effect tank was not relevant to explain the variability in plasma metabolites levels (**Fig. S7**).

Two plasma enzymes, lactate dehydrogenase (LDH) and creatine kinase, were analysed as function of swimming speed, sampling time, and interaction between those two variables on several plasma metabolites (Fig. 2H and I). None of the variables analysed influenced LDH, and creatine kinase was only affected by sampling time (**Table S8**). Creatinine kinase plasma levels were significantly higher at 11 weeks compared to 5 weeks for the 0.5 and 1.5 BL/s ($p < 0.05$). There was a significant reduction in creatinine kinase levels between 11 and 17 weeks ($p < 0.05$) for the 1.5 BL/s group (**Table S9**).

Analyses of 6 plasma ions (Cl^-, Na^+, K^+, Mg^{2+}, Ca^{2+}, and P_i)

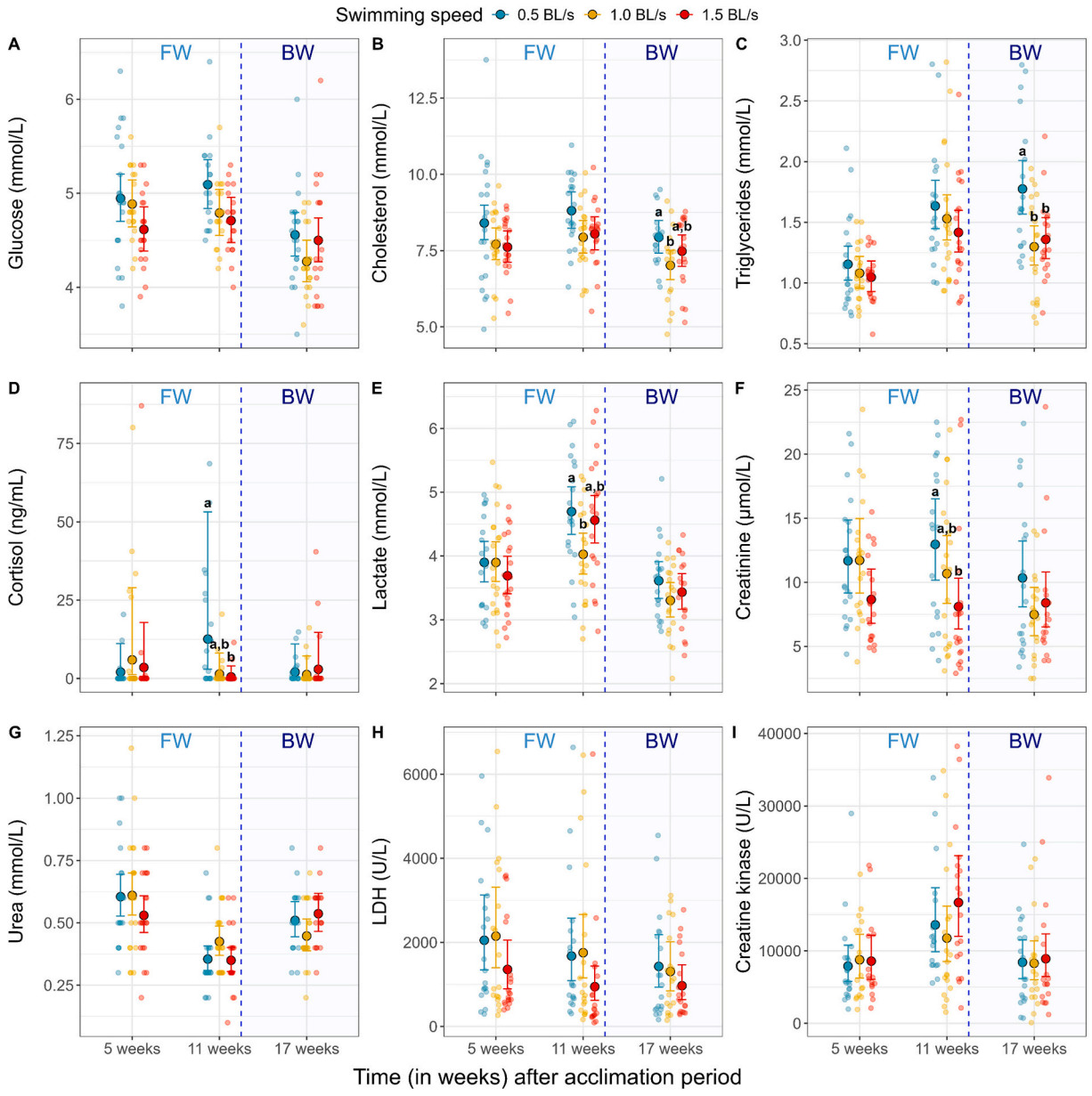

Fig. 2. Plasma metabolites of Atlantic salmon reared under three swimming-speed regimes: **(A)** glucose, **(B)** cholesterol, **(C)** triglycerides, **(D)** cortisol, **(E)** lactate, **(F)** creatinine, and **(G)** urea; and enzymes **(H)** lactate dehydrogenase (LDH), and **(I)** creatine kinase. The plots display the model predicted means (large dots) and 95 % CIs (error bars). Raw data points are represented by smaller dots (horizontally jittered for visualization purposes). The freshwater phase (FW) and brackish water phase (BW) are indicated. Only significant differences ($p < 0.05$) between swimming speed groups at a given sampling time point are indicated by different letters. For details regarding statistical significance, including significant differences between sampling points at each swimming speed, please refer to **Table S9**. The number of individuals included in each analysis is presented in **Table S24**.

revealed that swimming speed influenced K^+, and sampling time affected Na^+, K^+, Mg^{2+}, P_i (Fig. 3, **Table S10**). The interaction between swimming speed and sampling time significantly affected K^+, and a marginal effect on P_i (**Table S10**). None of the variables analysed influenced plasma Cl^- and Ca^{2+} levels (**Table S10**). Post-hoc test analyses showed that at 11 weeks K^+ was significantly lower in the 1.0 BL/s compared to the 0.5 BL/s group ($p < 0.05$) (Fig. 3C, **Table S11**). P_i was significantly lower in the 1.0 BL/s compared to the 0.5 BL/s group ($p < 0.01$) (Fig. 3F, **Table S11**). The plasma Na^+ levels were significantly higher at 17 weeks compared to 5 weeks across swimming speed groups (Fig. 3B, **Table S11**). On the other hand, Mg^{2+} was significantly lower at 17 weeks compared to 5 and 11 weeks across swimming speeds (Fig. 3D, **Table S11**). The effect of tank was not relevant (**Fig. S8**).

3.4. Gill NKA activity

Using GLMM, we evaluated the effects of swimming speed and sampling time on the gill NKA activity (**Fig. S9**). Sampling time had a significant effect on the gill NKA activity levels (**Table S12**), which increased significantly over time (**Fig. S9**, **Table S13**). Swimming speed and the interaction between swimming speed and sampling time had no effect on the NKA activity levels (**Table S12**). The effect of tank, accounting for potential tank-specific cluster effect in the statistical model, showed that the variation attributed to different tanks was minimal (**Fig. S10**).

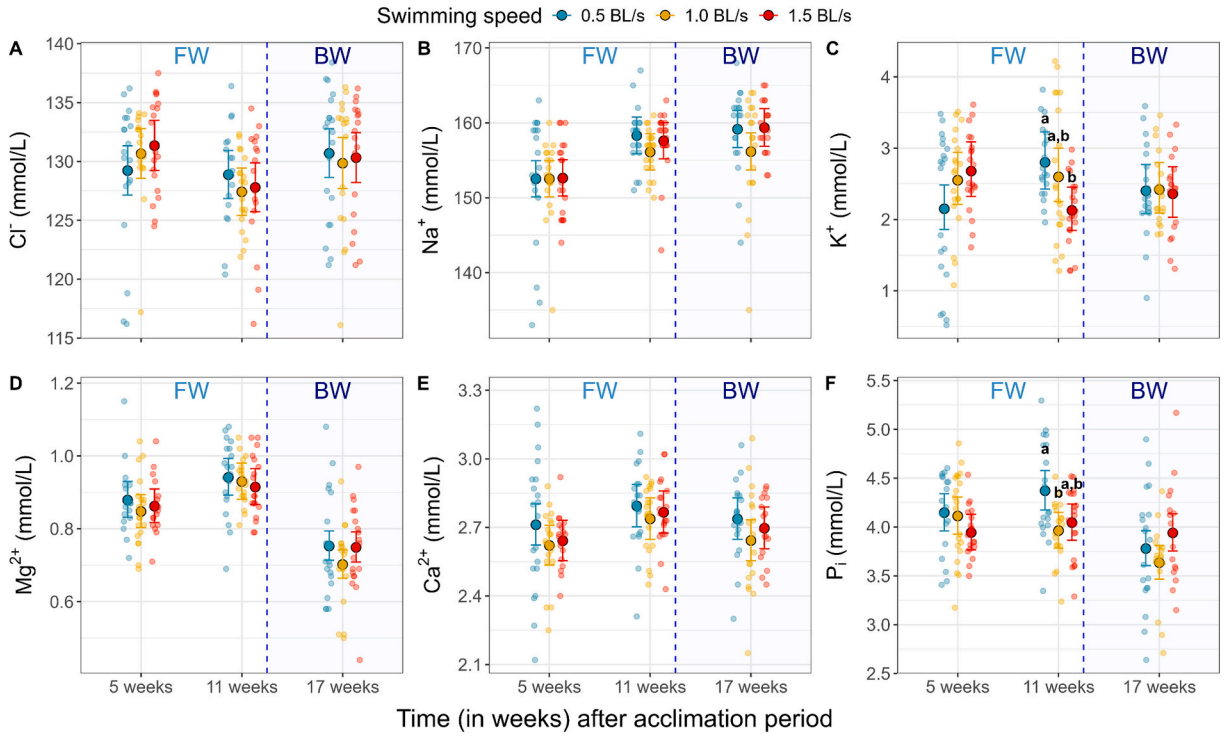

Fig. 3. Plasma ion levels of Atlantic salmon reared under three swimming-speeds: **(A)** chloride (Cl^{-}), **(B)** sodium (Na^{+}), **(C)** potassium (K^{+}), **(D)** magnesium (Mg^{2+}), **(E)** calcium (Ca^{2+}), and **(F)** inorganic phosphorus (P_i). The plots display the model predicted means (large dots) and 95 % CIs (error bars). Raw data points are represented by smaller dots (horizontally jittered for visualization purposes). The freshwater phase (FW) and brackish water phase (BW) are indicated. Only significant differences ($p < 0.05$) between swimming speed groups at a given sampling time point are indicated by different letters. For details regarding statistical significance, including significant differences between sampling points at each swimming speed, please refer to **Table S11**. The number of individuals included in each analysis is presented in **Table S24**.

3.5. AEC and ATP and its breakdown products

A GLMM was used to analyze the impact of swimming speed, sampling time, and interaction between those two variables on the AEC, ATP, ADP and AMP (Fig. 4, **Fig. S11**). The effect of tank, accounting for potential tank-specific cluster effect in all statistical models, revealed negligible variation among tanks for AEC, ATP, and AMP, but moderate tank-related variability for ADP (**Fig. S12**). The AEC was significantly

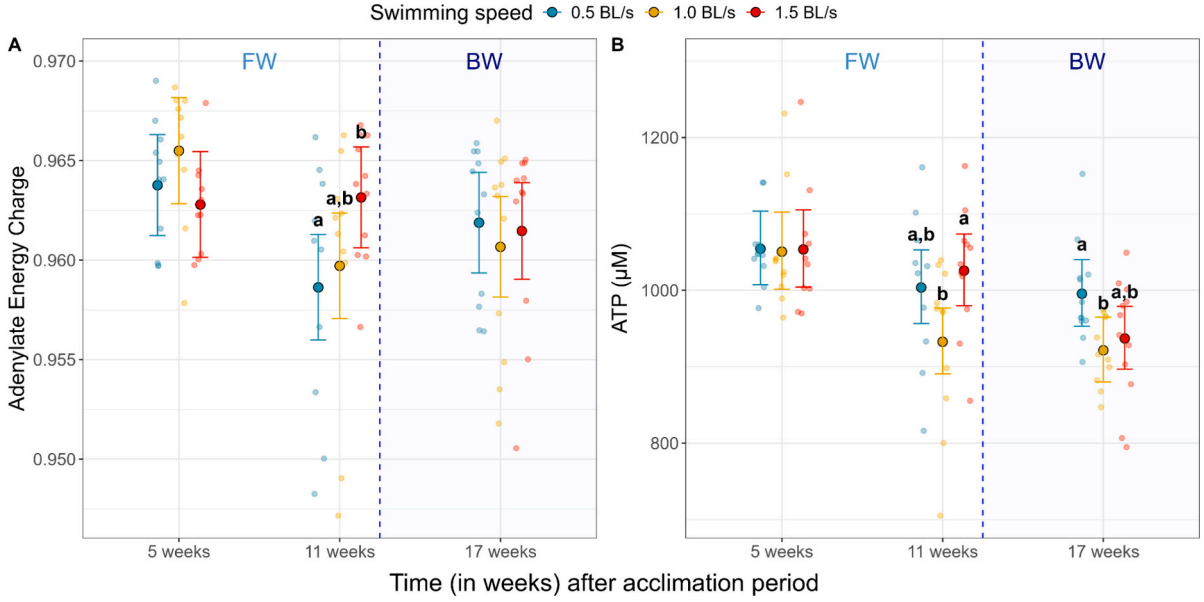

Fig. 4. Adenylate energy change (AEC) and ATP levels in the white muscle of Atlantic salmon reared under three swimming-speed regimes: **(A)** AEC and **(B)** ATP. The plots display the model predicted means (large dots) and 95 % CIs (error bars). Raw data points are represented by smaller dots (horizontally jittered for visualization purposes). The freshwater phase (FW) and brackish water phase (BW) are indicated. Significant differences ($p < 0.05$) between swimming speed groups at a given sampling time point are indicated by different letters. For details regarding statistical significance, including significant differences between sampling points at each swimming speed, please refer to **Table S15**. The number of individuals included in each analysis is presented in **Table S24**.

affected by sampling time and the interaction between swimming speed and sampling time (**Table S14**), whereas we only found a significant effect of the interaction for ADP (**Table S14**). The GLMM analyses showed that none of the fixed variables analysed had a significant effect on the ATP and AMP levels in the muscle (**Table S14**). However, post-hoc tests revealed that at the end of the freshwater phase (11 weeks), AEC was significantly higher ($p < 0.05$) in the 1.5 BL/s compared to the 0.5 BL/s (Fig. 4A, **Table S15**), whereas significantly lower ($p < 0.05$) levels of ADP were registered for the 1.0 BL/s compared to the 0.5BL/s group (**Fig. S11A**, **Table S15**). For ATP, post-hoc tests revealed that the 1.0 BL/s had significantly lower levels ($p < 0.05$) compared to the 1.5 BL/s at 11 weeks and to the 0.5 BL/s group at 17 weeks (Fig. 4B, **Table S15**).

3.6. White muscle histology

A GLMM was used to analyze the impact of swimming speed, sampling time (limited to 11 and 17 weeks for this analysis), fiber diameter (categorized in 20 μm bins), and the interaction between these variables on the frequency of white muscle fibers (Fig. 5, **Table S16**). Post-hoc tests revealed differences between swimming speeds in the brackish water phase (**Table S17**). There was a significantly higher ($p < 0.05$) frequency of muscle fibers within the 40–59 μm diameter range for the 1.5 BL/s compared to the other two swimming groups. Conversely, a higher frequency of fibers with diameters within the 120- 139 μm was found for the 0.5 BL/s group compared to the 1.5 BL/s (Fig. 5, **Table S17**).

3.7. Plasma Igf1

A GLMM was used to analyze the impact of swimming speed, sampling time, and interaction between those two variables on plasma Igf1 (Fig. 6). The effect of tank, accounting for potential tank-specific cluster effect in the statistical model, showed that the variation attributed to different tanks was very small (**Fig. S13**). The plasma Igf1 was significantly affected by sampling time and its interaction with swimming speed (**Table S18**). After transfer to brackish water, at 17 weeks, the Igf1 in the 0.5 BL/s was significantly lower compared to the 1.0 BL/s group ($p < 0.05$) and the 1.5 BL/s group ($p < 0.01$). No significant differences in Igf1 were observed between swimming speeds during the freshwater phase (Fig. 6, **Table S19**).

3.8. Gene expression analysis in the white muscle

A GLMM was used to evaluate the expression of *igf1*, *igf2*, *igf1ra1*, and *igf1ra2* in the white muscle of Atlantic salmon in response to swimming speed, sampling time, and the interaction between these two variables (Fig. 7). The effect of tank, accounting for potential tank-specific cluster effect in the statistical models, showed that the variation attributed to different tanks was minimal (**Fig. S14**). The expression of *igf1* was significantly affected by the fixed variables swimming speed, sampling time and the interaction between those (**Table S20**), whereas the expression of the other three genes in white muscle was not significantly impacted (**Table S20**). Post-hoc tests revealed that *igf1* mRNA expression at 5 weeks was significantly higher ($p < 0.01$) in the 1.0 BL/s group compared to the 0.5 and 1.5 BL/s. The *igf1* and *igf2* expression significantly increased between 5 and 11 weeks for 1.5 BL/s group ($p < 0.05$), and *igf1* also increased for the 0.5 BL/s group ($p < 0.01$) (**Table S21**). On the other hand, the mRNA expression of the *igf receptors* (*igf1ra1* and *igf1ra2*) significantly decreased ($p < 0.05$) between 11 and 17 weeks for the 1.0 BL/s group (**Table S21**).

3.9. Gene expression analysis in the pituitary

Using GLMM, we evaluated the effects of swimming speed, sampling time, and the interaction between those two variables on the expression of *gh1*, *gh2*, and *prl* in Atlantic salmon pituitary (Fig. 8). The effect of tank, accounting for potential tank-specific cluster effect in the statistical models, showed that the variation attributed to different tanks was minimal to negligible (**Fig. S15**). The expression of *gh1* and *gh2* was not significantly affected by any of the fixed variables while *prl* expression was significantly affected by sampling time (**Table S22**). In the sampling point after transfer to brackish water, at 17 weeks, *prl* mRNA levels were significantly higher than at 5 and 11 weeks across all the swimming speed groups (**Table S23**).

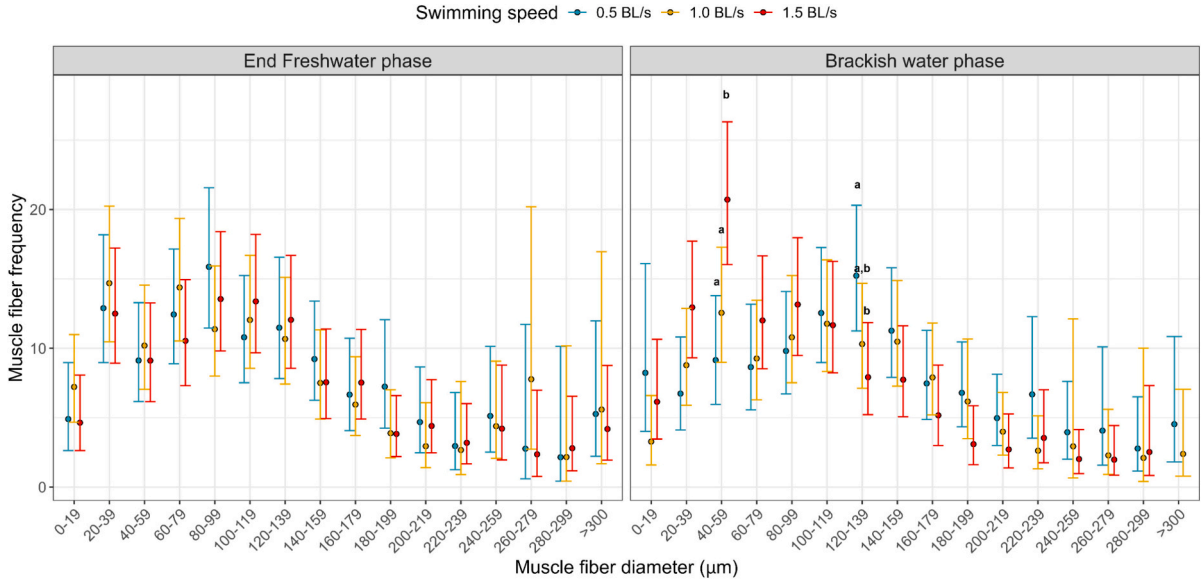

Fig. 5. Frequency of white muscle fibers in 20 μm interval groups of Atlantic salmon reared under three swimming-speed regimes. The plots display the model predicted mean values (large dots) along with 95 % CI. Analyses were performed at the end of freshwater phase (after 11 weeks of swimming exercise), and at the brackish water phase (end of the experiment). Significant differences ($p < 0.05$) between swimming speed groups within a given muscle fiber diameter group are indicated by different letters (please refer to **Table S17**). The number of individuals included in the analysis is presented in **Table S24**.

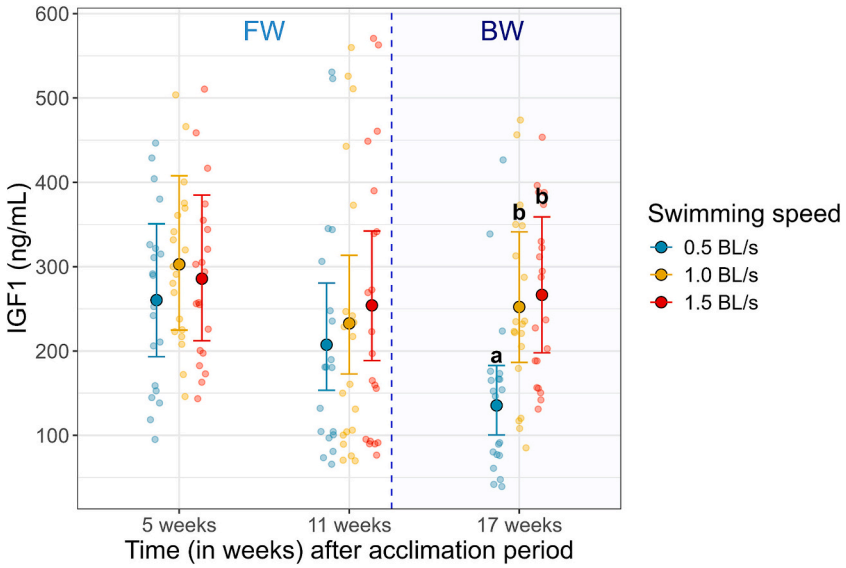

Fig. 6. Plasma levels of insulin-like growth factor 1 (Igf1) of Atlantic salmon reared under three swimming-speed regimes. The plot displays the model predicted means (large dots) and 95 % CIs (error bars). Raw data points are represented by smaller dots (horizontally jittered for visualization purposes). The freshwater phase (FW) and brackish water phase (BW) are indicated. Only significant differences ($p < 0.05$) between swimming speed groups at a given sampling time point are indicated by different letters. For details regarding statistical significance, including significant differences between sampling points at each swimming speed, please refer to **Table S19**. The number of individuals included in the analysis is presented in **Table S24**.

4. Discussion

Increasing evidence suggests that swimming exercise is linked to improved growth performance and health benefits in Atlantic salmon (Balseiro et al., 2018; Castro et al., 2011; Castro et al., 2013b; Mes et al., 2020; Nilsen et al., 2019; Timmerhaus et al., 2021; Totland et al., 1987). In agreement with this hypothesis, our study found physiological and biochemical responses to varying swimming speeds and showed that moderate-intensity exercise (between 1.0 and 1.5 BL/s) during the freshwater phase optimizes growth performance following transfer to brackish water while supporting energy homeostasis in Atlantic salmon.

4.1. Swimming exercise enhanced growth performance

Continuous swimming exercise during the freshwater phase did not result in significant differences in the SGR between the three swimming speeds during this phase. However, after 5 weeks of being transferred into brackish water, Atlantic salmon that had been subjected to moderate swimming speeds (1.0 BL/s and 1.5 BL/s) exhibited a significantly higher SGR compared to the 0.5 BL/s group. Thus, the observed enhanced growth in the brackish water phase following swimming exercise in the freshwater phase suggests a potential link between exercise-induced effects and subsequent growth performance. This aligns with previous studies reporting that swimming exercise at moderate speeds enhances growth (Castro et al., 2011; Jørgensen and Jobling, 1993; Totland et al., 2011), and underscores the importance of freshwater rearing conditions for growth and seawater/brackish adaptation in Atlantic salmon.

As previously mentioned, muscle growth in Atlantic salmon results from a combination of hypertrophy (increase in fiber size) and hyperplasia (increase in fiber number). As fish grow, the frequency of larger muscle fibers tends to increase, while that of smaller fibers decreases (Stickland, 1988; Weatherley et al., 1988). In the current study, the 1.5 BL/s group showed a significantly higher frequency of small fibers with diameter between 40 and 59 μm, along with non-significant trends towards higher frequencies of fibers in the 20–39 and 60–79 μm diameter ranges during the brackish water phase. These results suggest that higher hyperplasia in the white muscle is linked to previous moderately high swimming speed intensities, i.e., 1.5 BL/s. Swimming exercise has been shown to induce hyperplasia in the muscle of Atlantic salmon (Balseiro et al., 2018), and other teleost species (dos Santos et al., 2017; Moya et al., 2019; Rasmussen et al., 2011). Given that muscle growth is a gradual and dynamic process, it is likely that the observed recruited muscle fibers will increase in volume during the brackish water phase, resulting in higher hypertrophy for the 1.5 BL/s group, as has been observed in previous studies for fish under swimming exercise (Bugeon et al., 2003; Ibarz et al., 2011; Palstra et al., 2014; Totland et al., 1987). However, this hypothesis remains to be confirmed, as the final sampling in this study was conducted only after 5 weeks in brackish water, and growth in fish is continuous.

The condition factor (K) is commonly used in aquaculture to provide a general indication of the fish welfare and overall health, reflecting the balance between weight (stored energy) and length in fish. All swimming speed groups had a condition factor above 1 during freshwater and brackish water phases, suggesting that the fish is in good health with adequate fat reserves (Stien et al., 2013). Notably, swimming exercise did not impact the condition factor of the fish. On the other hand, feed intake in Atlantic salmon under moderate swimming speeds was significantly higher compared to the 0.5 BL/s group. The higher consumption of feed could be explained by the fish's need for additional energy to maintain sustained swimming without compromising growth. This is also in agreement with previous findings that demonstrated that daily feed intake increased with increasing current speeds (Jørgensen and Jobling, 1993, 1994). Although FCR and FCE were not significantly different between swimming speed groups, it was affected by the phase, i.e., between freshwater and brackish water, basically showing that fish in the freshwater phase used in general more efficiently their feed resources.

Growth can also be affected by poor water quality or early maturation. In the present study, water quality was within optimal ranges for farmed salmon and therefore should not have affected growth performance nor fish welfare. While sustained swimming exercise can delay testicular development in male European seabass (Graziano et al., 2018) and in Atlantic salmon (Waldrop et al., 2018), we did not observe any significant differences in the male maturation probability between swimming speeds in the present study.

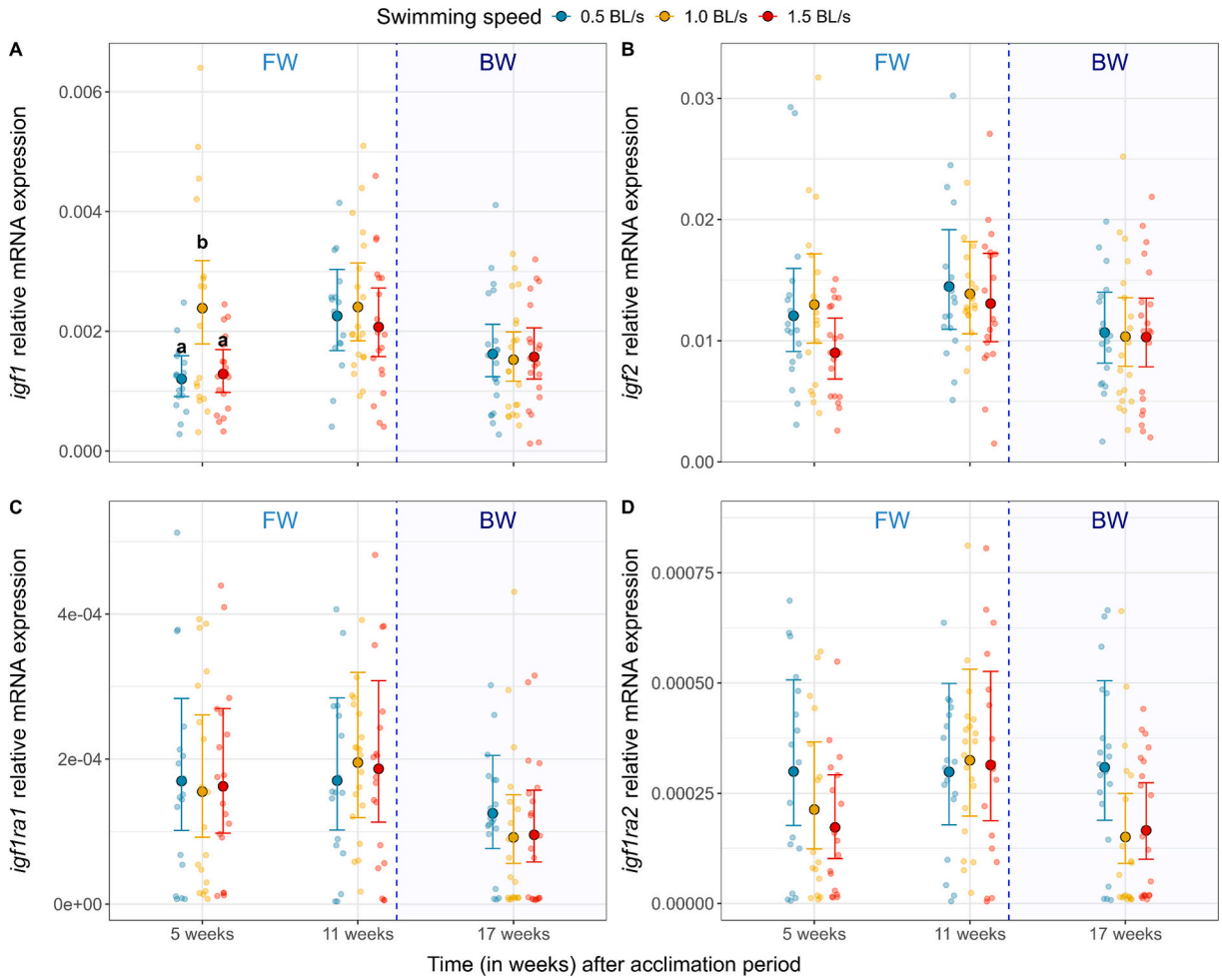

Fig. 7. Expression levels of insulin-like growth factor 1 (*igf1*) and receptor *igfra* genes in the white muscle of Atlantic salmon reared under three swimming-speeds: **(A)** *igf1*, **(B)** *igf2*, **(C)** *igf1ra1*, and **(D)** *igf1ra2*. The plots display the model predicted means (large dots) and 95 % CIs (error bars). Raw data points are represented by smaller dots (horizontally jittered for visualization purposes). The freshwater phase (FW) and brackish water phase (BW) are indicated. Only significant differences ($p < 0.05$) between swimming speed groups at a given sampling time point are indicated by different letters. For details regarding statistical significance, including significant differences between sampling points at each swimming speed, please refer to **Table S21**. The number of individuals included in the analysis is presented in **Table S24**.

4.2. Gh/Igf axis effect on growth

The expression levels of the genes *igf1, igf2*, and their receptors *igf1ra1* and *igf1ra2* in white muscle are crucial for the regulation of growth, muscle development, and metabolism in teleost fishes (Reindl and Sheridan, 2012; Reinecke et al., 2005; Wood et al., 2005). In particular, the *igf1* is the dominant growth factor in mature muscle, driving growth in response to feeding, hormonal signals, and environmental factors. In the present study, the upregulation of *igf1* mRNA in the 1.0 BL/s group at 5 weeks of swimming exercise suggests that the Igf1 pathway plays a role in driving enhanced growth. Similarly, plasma Igf1 levels were higher in the 1.0 and 1.5 BL/s groups during the brackish water phase, indicating that the Igf axis is involved in mediating the increased growth rate observed in these fish. However, in contrast to our findings, Palstra et al. (2020) reported that in seabream, there were no significant differences in plasma Igf1 levels. This difference highlights the complexity of the factors influencing growth in teleosts. Nevertheless, our results also support the hypothesis that moderate swimming speeds influence growth-related pathways. Previous studies have shown that plasma Igf1 levels increase in teleosts exposed to moderate exercise (Blasco et al., 2015; Sánchez-Gurmaches et al., 2013). Igf1 has been identified as a key biomarker for assessing growth in fish species (Beckman, 2011; Kaneko et al., 2015; Picha et al., 2008a). Thus, the increased growth rates and muscle fiber hyperplasia observed in the present study may be linked to elevated plasma Igf1 levels. On the other hand, we did not observe any effects of swimming speed on the mRNA expression of *igf2* or the receptors *igf1ra1* and *igf1ra2*, consistent with findings reported for seabream (Palstra et al., 2020). This may suggest that *igf2* in white muscle is not a key regulator of growth promoted by exercise. However, it would be of interest to also analyze Igf2 plasma levels to verify this hypothesis. Additionally, it should be acknowledged that Igfbps were not analysed in this study, despite their significant role in modulating the bioavailability and activity of Igfs (Clemmons, 2018; Shimizu and Dickhoff, 2017).

Gh is a major regulator of somatic growth in Atlantic salmon (Björnsson, 1997). However, in this study *gh1* and *gh2* mRNA expression in the pituitary was not affected by swimming speed. There was no influence of swimming speed on the expression of *prl* mRNA in the pituitary either. It is known that Igf1 can enhance *prl* and inhibit *gh* expression in teleosts (Fruchtman et al., 2001; Mohammed-Geba et al., 2016). However, in the present study, we did not observe any relationship between plasma Igf1 and *prl* or *gh* mRNA expression. One possible explanation for these results is the involvement of these hormones in the smoltification process. Studies have demonstrated that following seawater transfer, *prl* mRNA expression decreases, while the *gh* mRNA expression increases in Atlantic salmon (Ágústsson et al.,

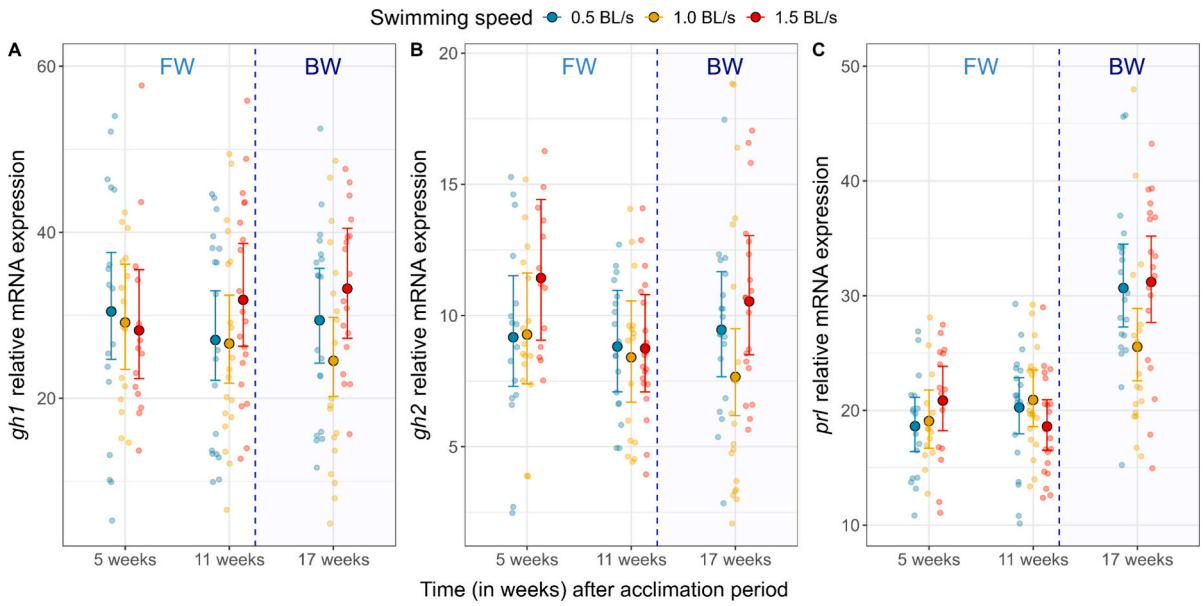

Fig. 8. Expression levels of **(A)** *gh1*, **(B)** *gh2*, and **(C)** *prl* in the pituitary of Atlantic salmon reared under three swimming-speeds. The plots display the model predicted means (large dots) and 95 % CIs (error bars). Raw data points are represented by smaller dots (horizontally jittered for visualization purposes). The freshwater phase (FW) and brackish water phase (BW) are indicated. Only significant differences ($p < 0.05$) between swimming speed groups at a given sampling time point are indicated by different letters. For details regarding statistical significance, including significant differences between sampling points at each swimming speed, please refer to **Table S23**. The number of individuals included in the analysis is presented in **Table S24**.

2003). Therefore, the effects of Igf1 on *gh* and *prl* expression may have been masked by the transfer into brackish water and the changes in environmental conditions during smoltification. The interaction between the smoltification process and exercise-induced hormonal regulation may have obscured any clear exercise-specific effects on *gh* and *prl* mRNA expression regulation.

4.3. Energy dynamics

Hepatosomatic index (HSI) is an indirect biomarker of fish energy reserves and growth rate (Chellappa et al., 1995). In our study, the observed differences in HSI between swimming speed groups were likely driven by changes in liver weight, as we found a strong correlation between liver weight and body weight. At the end of the freshwater phase, which corresponded to 11 weeks of swimming exercise, Atlantic salmon exposed to moderate swimming speeds had a significantly lower HSI compared to fish in the low swimming speed group (0.5 BL/s). This result contrasts with previous findings in Atlantic salmon, where moderate exercise was associated with a higher HSI, although this was observed after a longer training period of about 5 months in seawater, rather than 1.5 months in freshwater (Nilsen et al., 2019). Our findings suggest that there were distinct differences in energy allocation strategies among the swimming speed groups, with the moderate exercise groups appearing to direct more energy towards swimming activity and muscle growth. Moreover, following the transition to brackish water, where all fish were reared at the same swimming speed (0.5 BL/s) for 5 weeks, differences in HSI were no longer significant, suggesting a shift in energy allocation.

The relationship between the HSI and the AEC in white muscle likely reflects broader energy allocation strategies within the organism. While HSI gives insight into the liver energy storage, white muscle AEC measures the immediate energy status of muscle tissue (Atkinson, 1977; Haya et al., 1983). Normal AEC values range from 0.7 to 0.95, indicating a balance between ATP production and utilization (De la Fuente et al., 2014). In this trial, the AEC values were high implying that the white muscle tissue was in a high energy state, possibly because of exercise. The 1.5 BL/s group exhibited significantly higher AEC than the 0.5 BL/s group at the end of the freshwater phase, in contrast to the HSI results. These results suggest that higher moderate swimming speeds (1.5 BL/s) enhances energy capability in muscle (as indicated by higher AEC), while 1.0 BL/s swimming speed may optimize energy usage through efficient ATP turnover (lower ADP). It is plausible that Atlantic salmon in the 0.5 BL/s group prioritize storing energy in the liver for long-term use, leading to lower AEC values in muscle. This pattern of energy allocation could be driven by metabolic priorities that favor long-term energy storage over immediate availability for muscular activity.

Further insights into the energy metabolism of these fish are provided by the significant differences in inorganic phosphate (Pi) and plasma triglyceride levels among the swimming speed groups. Pi is crucial for ATP production and plays a role in the distribution of energy for cellular metabolism (Wagner, 2023). Lower Pi levels in the 1.0 and 1.5 BL/s groups suggest more efficient energy utilization during exercise, possibly indicating better oxidative phosphorylation efficiency. Similarly, plasma triglycerides, which are essential for providing energy to cells, were significantly higher in the 0.5 BL/s group during the brackish water phase, compared to the moderate speed groups. This suggests that fish in the lower-speed group may be relying more heavily on stored lipids for energy.

4.4. Swimming exercise and stress-resilience

Cortisol is a well-known stress hormone in fish, with elevated levels often indicating a physiological response to environmental or metabolic stress (Barton and Iwama, 1991; Bonga, 1997). Thus, differences in cortisol levels between swimming speed groups, particularly the higher levels in the 0.5 BL/s group compared to the moderate intensity groups at the end of freshwater phase (after 11 weeks of swimming exercise), suggest that fish under moderate exercise may be under less stress. It has been shown that fish under swimming exercise exhibit social schooling behavior and therefore less aggressive interactions and reduced hierarchies, resulting in more food available for subordinate fish (Adams et al., 1995; Balseiro et al., 2018; Brännäs, 2009; Solstorm et al., 2016). This finding aligns with studies showing that regular exercise can reduce stress hormone levels, improving fish welfare and adaptation (Castro et al., 2011). In fact, Keihani et al. (2024), subjected the fish in the present study to a crowding stress event and found that fish with

moderate swimming speed were better equipped to handle additional stress events. Nonetheless, it should be noted that cortisol levels reported here remained within the acceptable range for unstressed Atlantic salmon (Lai et al., 2021; Madaro et al., 2023).

At the end of the freshwater phase, after 11 weeks of continuous swimming exercise, plasma creatinine and potassium were lower in fish subjected to moderate swimming speeds (but only significant for the 1.5 BL/s group). These results are possibly linked to the stress levels of the fish, in line with the plasma cortisol levels. Increased plasma potassium levels in Atlantic salmon in response to stress has previously been demonstrated (Solstorm et al., 2015). The lower levels of creatinine in the moderate swimming speed groups could indicate utilization of energy stores during swimming exercise. On the other hand, lactate levels were significantly lower in the 1.0 BL/s group. Elevated plasma lactate levels in the 0.5 BL/s combined with the higher cortisol levels may reflect a stress response. Overall, the 1.0 BL/s group appeared to handle stress better while maintaining aerobic metabolism. Interestingly, these statistically significant differences observed between swimming groups at 11 weeks disappeared after 5 weeks in brackish water, where all groups underwent low-intensity swimming speed at 0.5 BL/s. This suggests that continuous exercise may be necessary for fish to maintain benefits related to stress resilience.

4.5. Smoltification

The enzyme activity of the gill NKA is often used as a biomarker for smoltification. As expected during smoltification and subsequent salinity change, NKA activity levels increased after the winter signal and then again in the brackish water phase. In brackish water, the higher salinity means higher levels of monovalent ions (Na^+, Cl^- and K^+) as well as divalent ions (Ca^{2+} and Mg^{2+}) in the environment. The increase in salinity caused a significant increase in plasma Na^+, consistent with expected osmoregulatory adjustments to brackish water (Al-Jandal and Wilson, 2011; Arnesen et al., 1998). In contrast, no significant changes were observed for Cl^-, suggesting that this ion was effectively regulated in response to the elevated salinity challenge. The plasma Mg^{2+} levels were unexpectedly lower in the brackish water phase, which might suggest an active regulation to maintain osmotic balance for this ion, although further research is needed to help clarify this observation.

In this study, no significant differences in NKA activity were observed between fish subjected to various swimming speeds. Indeed, Jørgensen and Jobling (1993) observed that swimming exercise did not affect Atlantic salmon osmoregulatory capacity (plasma osmolality and Cl^-). In a more recent study, Esbaugh et al. (2014) found that although plasma Na^+ and Cl^- concentrations were not affected by exercise, increased swimming speed led to up-regulation of expression of the seawater osmoregulatory related genes in the gills. Similarly, in the present study, plasma levels of Cl^-, Na^+ and the divalent ions Mg^{2+} and Ca^{2+} were not affected by swimming speed. Altogether, these data suggest that exercise does not affect Atlantic salmon smoltification directly.

The condition factor, K, characteristically decreases during smoltification, because of the increase in length relative to body mass in this period (Handeland and Stefansson, 2002; McCormick et al., 2013). However, in the present study, an increase in K is observed in the brackish water phase. This result might be attributed to the swimming exercise in the freshwater phase; however, further research is needed to test this hypothesis.

5. Conclusions

This study demonstrates that swimming exercise at moderate intensities (1.0 and 1.5 BL/s) can enhance growth rates and promote muscle development in Atlantic salmon following transfer to brackish water. Although no significant growth differences were observed during the freshwater phase, the results indicate that prior exposure to moderate swimming speeds lead to improved growth performance in the subsequent brackish water phase. Furthermore, the observed changes in the recruitment of small size fibers in the white muscle, Igf1 plasma levels, and energy charge dynamics underscores the importance of aerobic exercise in stimulating growth through both hyperplasia and energy regulation.

The relationship between liver energy reserves (HSI) and muscle energy status (AEC) suggests that different swimming speeds influence energy allocation strategies, with lower-intensity speed prioritizing liver storage and higher-intensity speed supporting muscle energy balance. However, the long-term effects of swimming exercise beyond the initial brackish water phase remain to be explored.

In conclusion, consistent with the findings of Timmerhaus et al. (2021), our results show that moderate swimming speeds (between 1.0 and 1.5 BL/s) offer an optimal balance between growth enhancement and energy efficiency, providing a valuable strategy for aquaculture practices aimed at maximizing growth and welfare in Atlantic salmon.

CRediT authorship contribution statement

Ana S. Gomes: Writing – original draft, Visualization, Supervision, Investigation, Formal analysis, Conceptualization. **Pablo Balseiro:** Writing – review & editing, Supervision, Investigation, Conceptualization. **Mari D. Iversen:** Writing – review & editing, Investigation, Formal analysis. **Fabian Zimmermann:** Writing – review & editing, Visualization, Formal analysis. **Munetaka Shimizu:** Writing – review & editing, Investigation. **Ayaka Izutsu:** Writing – review & editing, Investigation. **Amaya Albalat:** Writing – review & editing, Investigation. **Cindy Pedrosa:** Writing – review & editing, Investigation. **Richard Broughton:** Writing – review & editing, Investigation. **Sara Calabrese:** Writing – review & editing, Investigation. **Marnix Gorissen:** Writing – review & editing, Investigation. **Jan Zethof:** Writing – review & editing, Investigation. **Ivar Rønnestad:** Writing – review & editing, Conceptualization. **Simon MacKenzie:** Writing – review & editing, Conceptualization. **Harald Sveier:** Writing – review & editing, Conceptualization. **Julia F. Buhaug:** Writing – review & editing, Resources. **Sigurd O. Handeland:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

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Ethics statement

The research and samplings were conducted in accordance with the Norwegian Animal Research Authority regulations and approved by the Norwegian Food Safety Authority under the permit number 27401.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2025.742797.

Data availability

Data will be made available on request.

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