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Physiological and Growth Responses of Black Sea Salmon (*Salmo labrax*) to Long-Term Salinity and High Carbon Dioxide Stress

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Abstract

Black Sea salmon (*Salmo labrax*), an anadromous salmonid species of regional importance, is increasingly considered for aquaculture in the Black Sea. This study investigates the physiological and growth responses of Black Sea salmon to seawater transfer, with a particular focus on carbon dioxide (CO_2) stress. The experiment began on 5 July 2022 with 720 fish (76.68±15.34 g) reared under semi-controlled conditions using a freshwater recirculating aquaculture system (RAS). On 12 October 2022, a group of fish was transferred to Black Sea water (18 ppt), and a subgroup was exposed to elevated CO_2 (1000 µatm pCO_2) until the end of the trial on 7 March 2023.

Exposure to carbon dioxide showed negligible or minimal effects on seawater adaptation and growth. In contrast, physiological markers such as gill Na^+/K^+-ATPase (NKA) activity and the expression of *nkaα1a*, *nkaα1b*, and *nkcc1a* genes, along with growth metrics—including specific growth rate (SGR), condition factor (K value), and liver gene expression of i*gf-I, igfbp1b, ghr1,* and *ctsl*—indicated that the fish were not physiologically prepared for seawater transfer in autumn. These findings suggest that the commonly practiced autumn sea transfer in the region may lead to suppressed growth and suboptimal performance.

The results emphasize the importance of aligning seawater transfer with the smoltification window to support fish health and optimize aquaculture outcomes in Black Sea salmon farming.

Introduction

Black Sea salmon (*Salmo labrax*), also referred to as Black Sea trout or brown trout (*Salmo trutta* or *Salmo trutta labrax* in earlier sources), is prized for its delicate and flavourful flesh. It is mainly farmed by small-scale businesses in the Black Sea region (Massa et. al., 2021). These businesses primarily focus on cultivation for tourism and recreational fishing using inland waters. The production of Black Sea salmon reported under the name *Salmo spp.* fluctuated in the last decade reaching a max total production of 2924 tonnes (marine and inland waters) in 2017 (FAO, 2024). However, despite the continued existence of a wild population (Geldiay & Balık, 1996; Innal and Erk'akan, 2006) its production remains hindered by various factors. One of the most outstanding issues that limit the production of Black Sea salmon on larger scale is, indeed, the inability to obtain sufficient supply of eggs. The Black Sea salmon has seen a decline in wild population and is no longer a commercial fishing resource. However, it remains highly valued for recreational fishing.

This species belongs to the Salmonidae family and has common characteristics of most salmonids such as anadromy. Like other anadromous fish, the Black Sea Salmon undertake freshwater migration for spawning, thus playing a significant role in energy transport facilitating ecosystem connectivity between the Black Sea and its rivers (Gende et al., 2002). In their immature stage while residing in freshwater, Black Sea salmon

exhibit scattered black and red spots on their lateral sides. Conversely, upon completing their migration to the sea, matured individuals gradually lose these colours and patterns, acquiring a silvery-white hue (Özel et. al., 2022).

The freshwater-to-seawater migration is a vital aspect of the life cycle of salmonids as well as the transfer of fish to the sea for aquaculture. Facilities established in dams and/or upstream areas of rivers are used to rear fish from hatching to the smolt stage until transferred to seawater. The transfer to seawater could result in various functional impairments in all salmonids, including mortality and growth retardation (Stephen and Rible, 1995; Stien et al., 2013; Vindas et al., 2016; Çakmak et al., 2018). Transfers conducted during the summer months, especially when temperatures are high, exacerbate these issues, sometimes leading producers to lose one-third of their total production. Therefore, the common practice for sea transfer in the region is typically carried out during the autumn months when the waters begin to cool down.

Smoltification -a physiological process in salmonids that develops a coping mechanism against seawater, characterized by a series of morphological, physiological, and behavioural changes- typically emerges upon surpassing a certain developmental threshold - at 10-15 cm in length for most salmonids (McCormick & Saunders, 1987; Kendall et al., 2015). During this process, salmonids undergo hormonal changes in their endocrine system with regards to environmental stimuli (Prunet et al., 1989). Notably, this includes changes in cortisol and thyroid hormones. While these changes in endocrine tissues occur simultaneously, they act independently of each other and get the fish ready for the seawater environment (Hoar, 1988; Björnsson et al., 2011).

Osmoregulation and ion transfer are crucial for salmonid gill function. Specialised cells, which proliferate differently at different life stages, execute the process of osmoregulation. Lamellar chloride cells, found in the gill lamellae, and filament chloride cells, concentrated in the gill filaments, are responsible for the Na+, K+-ATPase (NKA) pump function (Richards et al., 2003; Katoh et al., 2008; McCormick et al., 2009). In the freshwater during the fry stage, the prominent cells are expressed as *nkaα1a* -isoform of the sodium-potassium ATPase enzyme-. In the seawater they are expressed as *nkaα1b* -seawater isoform- and *nkcc1a* -transporter that mediates chloride ions (Cl^-) along with sodium ions (Na^+) and/or potassium ions (K^+)- (Nilsen et al., 2007; Flores & Shrimpton, 2012; McCormick et al., 2013). Gill tissue cell transformation is closely linked to a significant increase in NKA activity, which establishes an osmotic gradient and causes ion efflux. This leads to fish becoming hypoosmotic when exposed to seawater (McCormick, 1995). NKA pump activity directly influences fish survival in seawater, as evidenced by previous research.

In Black Sea aquaculture size-based decision process is commonly followed for seawater transfer. This often leads to a growth stunt phenomenon. At this point, a debate regarding the primary factors controlling smoltification such as temperature (McCormick et. al., 2002) light (Morro et. al., 2019), salinity (Pino-Martinez et. al., 2024) and their relationship to growth stunt smolts is ongoing. The smolt status of the cultured Black Sea salmon has not been thoroughly evaluated but speculated based on its growth performance in the sea (after harvesting). Its condition during the autumn months before the transfer takes place remains unknown.

Ocean acidification (OA), stemming from rising atmospheric carbon dioxide (CO_2) concentrations, poses a considerable global environmental concern. As CO_2 dissolves in seawater, it lowers the pH of marine ecosystems. CO_2 levels are expected a rise to approximately 1000 µatm leading to an estimated reduction of approx. 0.3 pH units by the year 2100 (Meinshausen et al., 2011). This trajectory, outlined by the Intergovernmental Panel on Climate Change (IPCC – Scenario RCP 8.5) in 2019 and onwards, highlights the pressing need for proactive measures to mitigate the adverse effects of CO_2 (Bopp et al., 2013; Pörtner et al., 2014; McNeil & Sasse, 2016). The consequences of OA extend beyond mere pH reduction, disrupting marine environment and prompting intensive research into its ecological and physiological impacts (Orr et al., 2005). Salmonids -due to their complex life cycles involving migration between freshwater and seawater habitats- may face particular challenges when it comes to adapting to OA conditions (Thorstad et al., 2012). This serious issue highlights the urgent need for further research into its physiological impacts.

The objective of this research is to fill knowledge gaps on the transfer of Black Sea salmon to seawater and to assess the long-term effects of carbon dioxide stress on the fish. Through a comprehensive analysis of physiological indicators and growth, this paper aims to elucidate the effects of seawater exposure on smolt development. It also intends to assess the potential implications of these findings for the management of Black Sea aquaculture.

Material and Methods

Experimental Setup

The fish was bred in early December 2020, hatched in February 2021 and moved to 500L fiber glass rearing tanks in late February 2021. The experiment was designed in two phases. On 5 July 2022 (Phase I), 720 Black Sea salmon, 1 year old, with an average weight of 76.68±15.34 g and length of 18.56±1.22 cm were randomly distributed among 9 tanks supported with a temperature-controlled freshwater recirculating aquaculture system (RAS) (water retention time: 45-60 min, temperature set: 13.5±0.5°C, TAN: <1 mg/l -

balanced with water exchange-, O_2: 9.27±0.26, pH: 7.08±0.25, light support: 12L:12D, initial stocking density: 12.25 kg/m^3). Salinity and Carbon dioxide (CO_2) treatment was established in triplicates in October 2022 (Phase II) supported with filtered brackish water (18 ppt) pumped out of Black Sea: one group kept in Fresh Water (Group-F) until the end of experiment, one transferred to Sea Water (Group-S), and one transferred to Sea Water supplied with CO_2 (Group-C) (Figure 1). Sea water temperature followed the pattern of the mixed water inlet (adjusted daily by rationing water coming from different depths averaging 13.56±1.89°C), and the freshwater RAS temperature was mirrored the changes in the sea water temperature during second phase (gradually cooled down to 12°C by February). Dissolved oxygen was maintained above 80% on the outlet water. Fish were fed three times a day a total of 3% of body weight (recalculated every month) with extruded commercial feed (containing 45% protein, 20% fat, 6% ash and 2% cellulose). The dose of carbon dioxide is controlled using a pH-stat system set to maintain pH 7.8 (1000 µatm pCO_2) with a 0.05 hysteresis. The survival rate for groups S, C and F were 98.8%, 97.2% and 96.1% respectively. The experiment is ended on 7 March 2023.

Samplings

Initially, samples were planned to be taken every two weeks. However, after the third sampling point, the intervals were extended due to the similarity in results. Gene expression analyses were not performed for the samples collected at the fourth sampling point, as NKA activity results showed minimal variation. During Phase I, (from early-August to mid-October 2022 until the seawater transfer of the fish) a total of 180 fish were sampled (n=4/tank/sampling) and 5 samplings were performed. A final sampling of 36 fish took place at the end of the Phase II on 7 March 2023 resulting a total of 6 samplings. These fish were used to assess the growth, smolt and health status during the experiment. Fish were euthanized with an overdose of Benzocaine (Saltan et. al., 2023). Total length and body weight were measured. The second gill arch from left side and liver were collected in RNALater for gene expression analysis. The second gill arch from right side was kept in SEI Buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) to analyse NKA activity. Gill samples in SEI buffer were immediately stored at −80°C until analysis and RNALater samples were kept overnight at 4°C before being moved to −80°C for storage.

Growth Parameters

The condition factor (K) was determined using the formula K = 100*W/L^3, where W represents the body weight in grams, and L as the total length in centimetres. Specific growth rate (SGR) per day was calculated based on the collected data using the formula SGR (%) = (ln (W_{final}) − ln ($W_{initial}$) × 100) divided by the time past (t in days), where W_{final} denotes the final body weight and $W_{initial}$ indicates the initial body weight, both measured in grams.

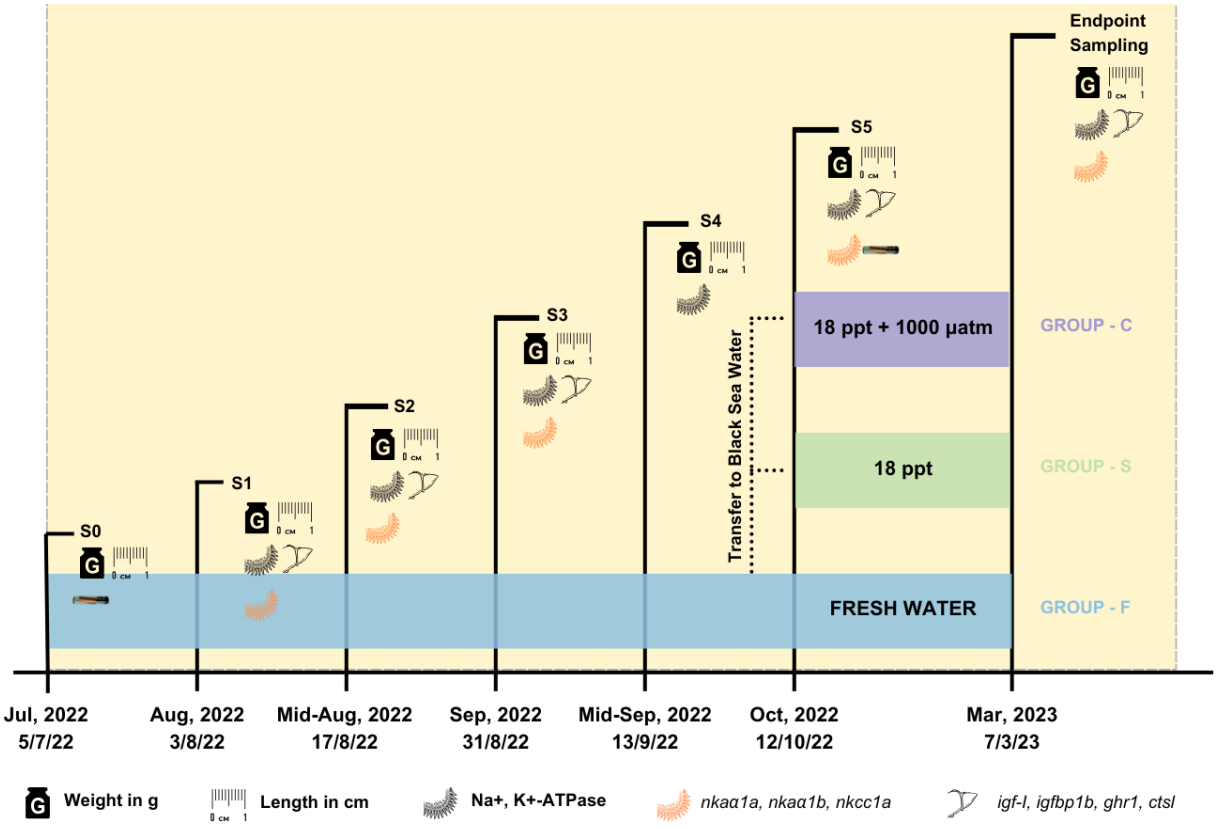

Figure 1. The initial measurements and grouping took place in July 2022, first sampling in August 2022 and the last sampling in March 2023. A group of fish transferred to Black Sea water, with and without additional carbon dioxide, in October 2022.

NKA Activity Assay

Na^+, K^+-ATPase activity (NKA) was assessed following the protocol outlined in McCormick (1993). This technique involves linking ATP hydrolysis to the generation of NAD^+ via pyruvate kinase and lactate dehydrogenase enzymes. The assay is conducted with and without the presence of ouabain, a potent NKA inhibitor. Discrepancies in ATP hydrolysis in the presence and absence of ouabain were assayed at 25 °C - 340 nm over a 10-minute period at 10-second intervals using a Multiskan GO microplate reader (Thermo) and SkanIt RE 7.0.2 software. Protein concentration in the homogenate was determined using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Massachusetts, USA) in triplicate. The final NKA values were calculated as the ouabain-sensitive fraction of the ATP hydrolysis and were expressed as µmol ADP × mg $protein^{-1}$ × $hour^{-1}$.

RT-qPCR Assay

Real-time quantitative PCR (RT-qPCR) was used to quantify the expression levels of *nkaα1a*, *nkaα1b* and *nkcc1a* genes in the gills, as well as *igf-I, igfbp1b, ghr1* and *ctsl* genes in liver samples and presented as fold change value. Total RNA isolation was performed from 20-25 mg of sample tissue using the PureLink RNA Mini Kit (Invitrogen) according to the manufacturer's protocol. The concentration of isolated RNA samples at 260-280 nm was determined using the NanoDrop™ 8000 Spectrophotometer (Thermo Fisher), and RNA quantities for cDNA synthesis were calculated using the equation 1/RNA concentration (µg). Subsequently, cDNA synthesis was carried out using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's instructions. The cDNA samples were stored at -20°C until real-time PCR application. For RT-PCR, previously published primers (Nilsen et al., 2007) and Nucleogene qPCR Sybr Green Master Mix (2x) were used according to the manufacturer's instructions. The amplification protocol included an initial denaturation step at 95°C for 15 min, followed by 45 cycles of denaturation at 95°C for 10 sec, annealing at 58-60°C for 1 min, and extension at 72°C for 10 sec, followed by a melting curve analysis stage (10 sec at 95°C, 5 sec from 65 to 95°C with a 0.5°C increment, and 5 sec at 95°C). PCR reactions were prepared with 25 µl SYBR Green Master Mix (Nucleogene), 0.40 µM primer, and 5 µl cDNA for each sample. Each plate included a negative control and a pool sample for calibration between plates for each target gene. The relative transcription levels of the genes were normalized using the reference gene *ef1α* (Olsvik et al., 2005) as it exhibited no significant changes over time and treatment following the Pfaffl et al. (2004) methodology. The primers used in this study are shown in Table 1. The amplification efficiency (E) was calculated by synthesizing cDNA from total RNA isolated from different groups using a 5-fold dilution series, and each series was performed in triplicate. The E value was calculated as the slope of the log RNA concentration against the threshold cycle using the formula E=10(-1/slope), and it was utilized to correct differences in amplification efficiencies between samples (Pfaffl et al., 2004).

Statistical Analysis

Statistical analyses were conducted using R version 4.4.0 with RStudio desktop version 2024.04.0+735 software packages. One-way ANOVA or t-tests were applied to detect statistical differences between groups. To meet the assumptions of normal distribution and homogeneity of variance, data were transformed to a logarithmic base. Samples from the first sampling point (S1), representing the biological baseline, were considered as the reference group. Significant differences ($P<0.05$) between groups were determined using Tukey HSD post-hoc tests.

Table 1. Primer sequences used to determine expression levels of relevant genes

Sample	Gene	Primer Sequence (5' > 3')	Reference
Gills	*nkaα1a*	F: CCAGGATCACTCAATGTCACTCT R: CAAAGGCAAATGGGTTTAATATCAT	Nilsen et.al., 2007
	nkaα1b	F: GCTACATCTCAACCAACAACATTACAC R: TGCAGCTGAGTGCACCAT	Nilsen et.al., 2007
	nkcc1a	F: GATGATCTGCGGCCATGTTC R: CTGGTCATTGGACAGTTCTTTG	Nilsen et.al., 2007
Liver	*igf-I*	F: TGCGGAGAGAGAGGCTTTTA R: AGCACTCGTCCACAATACCA	Rolland et.al., 2015
	igfbp1b	F: AGTTCACCAACTTCTACCTACC R: GACGACTCACACTGCTTGGC	Gabillard et.al., 2006
	ghr1	F: CGTCCTCATCCTTCCAGTTTTA R: GTTCTGTGAGGTTCTGGAAAAC	Gabillard et.al., 2006
	ctsl	F: CAACTACCTGCAGGCACCTA R: ACATGATCCCTGGTCCTTGAC	Rolland et.al., 2015
Housekeeping	*ef1α*	F: CCCCTCCAGGATGTCTACAAA R: CACACGGCCCACGGGTACT	Genge et.al., 2013

Results

Phase I

Samples were collected between August 2022 and March 2023. NKA activity and the related gene expressions were analysed in the gill samples. The expressions of *nkaα1a*, *nkaα1b*, and *nkcc1a* gene regions were measured using RT-PCR (Figure 2A). The freshwater marker *nkaα1a* transcription showed no significant changes in summer and autumn, while a downward regulation was observed in spring ($P<0.1$). The transcription of *nkaα1b* did not show a significant change throughout the experimental period ($P>0.05$). The most noteworthy upward change was observed for the seawater marker *nkcc1a* transcription in spring samples ($P<0.001$). NKA pump activity during this period remained consistent in summer and autumn, staying below 2 µmol ADP/mg protein per hour. It then peaked in March 2023 reached above 6 µmol ADP/mg protein per hour (Table 2).

The mRNA expressions of the *igf-I*, *igfbp1b*, *ghr1*, and *ctsl* gene regions, which are linked to growth performance, were measured using RT-PCR in liver samples collected from August 2022 to March 2023 (Figure 2B). The expression of *igf-I* in the liver remained mostly stable from August to March ($P>0.05$), with a slight downregulation noted in the October samples ($P<0.1$). The transcription of *igfbp1b* significantly decreased in the March samples ($P<0.01$) but showed no notable changes during the other periods. No significant differences were observed in the expression of *ghr1* and *ctsl* genes throughout the same timeframe ($P>0.05$). The specific growth rate (SGR) remained relatively stable at 0.95±0.02% body weight gained per day during summer and autumn but declined to 0.47±0.04% in spring (Table **2**). The condition factor (K) also showed a steady trend, averaging 1.18±0.012. The most significant variation in K was observed in July, at the start of the experiment, with an average of 1.13±0.01 ($P<0.05$).

Phase II

Following the seawater transfer conducted in October 2022, physiological and growth-related parameters were evaluated at the end of the exposure period to assess the effects of seawater conditions and carbon dioxide exposure.

Gene Expression

Na^+/K^+-ATPase (NKA) activity and associated gene expression were assessed in gill tissue among three groups: fish kept in freshwater RAS (March-F), fish transferred to Black Sea water (March-S), and fish transferred to Black Sea water with additional CO_2 exposure (March-C). No significant differences were found in the expression of *nkaα1a* among the groups (Figure 3A). However, *nkaα1b* expression was significantly upregulated in seawater-exposed groups ($P<0.05$), and *nkcc1a* expression showed a significant increase across all groups ($P<0.001$). Correspondingly, NKA activity in all groups increased during this period, exceeding 6 µmol ADP/mg protein per hour (Table 2).

In liver samples, growth-related gene expression was analyzed for the same groups. In the March-F group, *igfbp1b* was significantly downregulated ($P<0.05$), while *igf-I*, *ghr1*, and *ctsl* remained unchanged. In the March-C group, *igf-I* was significantly downregulated, and *ctsl* was significantly upregulated ($P<0.01$) (Figure 3B). No significant differences in gene expression were observed in the March-S group.

To assess the effects of carbon dioxide exposure, gene expression data from the March-C and March-S groups were compared. Although trends of up- or downregulation were observed in several gene regions, none of these differences were statistically significant (Figure 4). The respective p-values were as follows: *nkaα1a* ($P=0.28$), *nkaα1b* ($P=0.61$), *nkcc1a* ($P=0.29$), *igf-I* ($P=0.079$), *igfbp1b* ($P=0.25$), *ghr1* ($P=0.50$), and *ctsl* ($P=0.37$).

Growth Performance

Specific growth rate (SGR) decreased to 0.45±0.02%, 0.50±0.02%, and 0.53±0.02% body weight gain per day for the March-F, March-S, and March-C groups, respectively (Table 2). The condition factor (K value) differed significantly between the freshwater and seawater groups ($P < 0.01$), but no significant difference was observed between the two seawater treatments. The mean K values were 1.22±0.01 (March-F), 1.38±0.02 (March-S), and 1.36±0.01 (March-C).

Discussion

Salmonids typically migrate to seawater during either spring or autumn (Hayes et al., 2008; Satterthwaite et al., 2009; Winter et al., 2016). The migration of natural Black Sea population takes place either in the spring (1+ year with 18.4±0.7 cm in length) or in the autumn (2+ year with 24.3±1.3 cm in length) (Aksungur et.al., 2011). However, in Black Sea aquaculture, sea transfer occurs in autumn, particularly in October. In this study, seawater adaptation in cultured Black Sea salmon was monitored before autumn till spring by assessing osmoregulation through molecular analysis of gill gene transcription and NKA pump activity with the help of previously identified markers of freshwater (*nkaα1a*) and (*nkaα1b* and *nkcc1a*) of seawater adaptation (Nilsen et al., 2007; Flores & Shrimpton, 2012; McCormick et al., 2013). The freshwater marker *nkaα1a* remained stable until started to be downgraded (90% confidence) in spring, along with an increase in the seawater marker *nkcc1a* ($P<0.001$) in March. Until seawater transfer is completed, nkaα1b did not show significant changes. This is confirming its role in adaptation to post-seawater

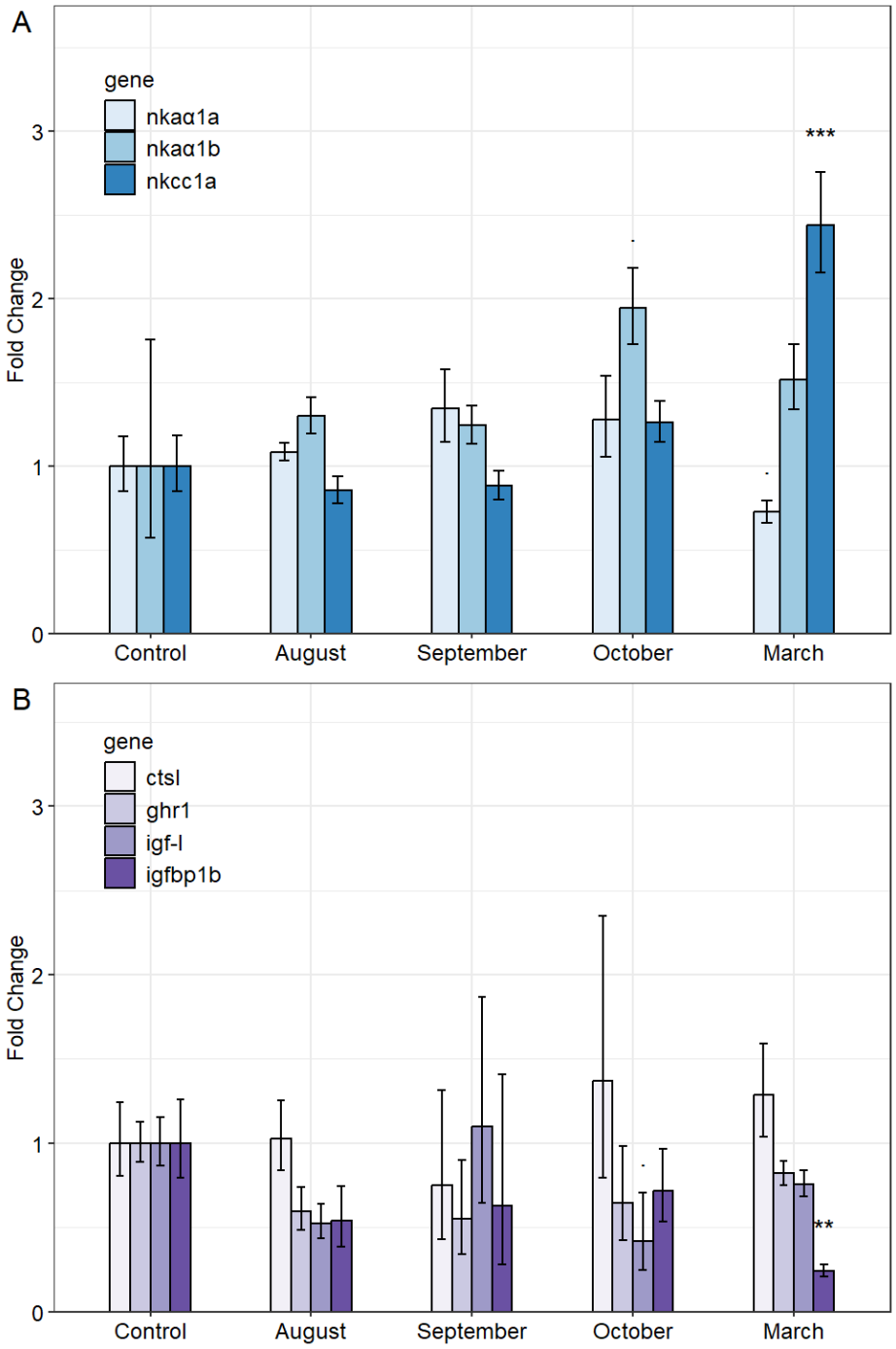

Figure 2. Temporal representation of Black Sea salmon reared in freshwater RAS showing average fold change in A) gill *nkaα1a*, *nkaα1b*, and *nkcc1a* mRNA expression and B) liver *ctsl*, *ghr1*, *igfbp1b*, and *igf-I* mRNA expression (n=6, Control group represents the biological baseline (S1), all points presented as average fold change±SE, ***: P<0.001, **: P<0.01, .: P<0.1).

transfer for Black Sea salmon. NKA pump activity also stagnated until peaking in spring. Results of molecular markers such as *nkaα1b* and *nkcc1a*, alongside stagnated NKA activity, explains why seawater transfer during autumn fall outside the optimal transfer window. The findings also demonstrate that the characteristic changes that could help them to do well in seawater environment predominantly occurred in spring. Until then, fish physiologically compelled to prefer freshwater which does not align with the typical transfer time in Black Sea aquaculture.

The study provides important insights into the impact of untimely seawater transfers on growth performance. To assess these potential issues in our study, gene expression analyses of key growth-related regions (*igf-I*, *igfbp1b*, *ghr1*, and *ctsl*) were conducted on liver samples using RT-PCR. This analysis was compared with a group of fish kept in freshwater RAS over a period from August 2022 to March 2023, covering the autumn sea transfer typically performed by regional farmers. The expression of *igf-I*, a gene known to enhance NKA pump activity and promote growth in both in vivo and in

Table 2. Condition Factor (K), Specific Growth Rate (SGR-% body weight gained per day), and NKA pump activity (µmol ADP/mg protein h) for Black Sea salmon used in the trial from August 2022 to March 2023. All values are presented as means±SE. Data in brackets represent the data from the sampled fish. Different letters within the same phase and column indicate significant differences.

		K		SGR		NKA pump
Sampling Point	**Phase I**	**All**	**Sampled**	**All**	**Sampled**	**activity**
S1	August (Day 0)	1.13±0.01^{b}	(1.12±0.03^{b})	-	-	0.80±0.12
S2	Mid-August (Day 14)	-	(1.18±0.02^{ab})	-	(0.96±0.06^{a})	0.94±0.09
S3	September (Day 28)	1.19±0.01^{a}	(1.14±0.02^{b})	0.90±0.02^{a}	(0.93±0.06^{a})	1.42±0.24
S4	Mid-September (Day 41)	-	(1.24±0.04^{a})	-	(0.95±0.04^{a})	1.21±0.22
S5	October (Day 70)	1.23±0.00^{a}	(1.22±0.01^{ab})	0.90±0.02^{a}	(0.95±0.03^{a})	1.74±0.18
	Phase II					
S6	March – F (Day 146)	1.22±0.01^{b}	(1.23±0.02^{a})	0.45±0.02^{b}	(0.47±0.04^{a})	6.57±2.01
S6	March – S (Day 146)	1.38±0.02^{a}	(1.31±0.02^{a})	0.50±0.02^{ab}	(0.56±0.07^{a})	11.40±1.50
S6	March – C (Day 146)	1.36±0.01^{a}	(1.29±0.03^{a})	0.53±0.02^{a}	(0.46±0.09^{a})	6.22±1.52

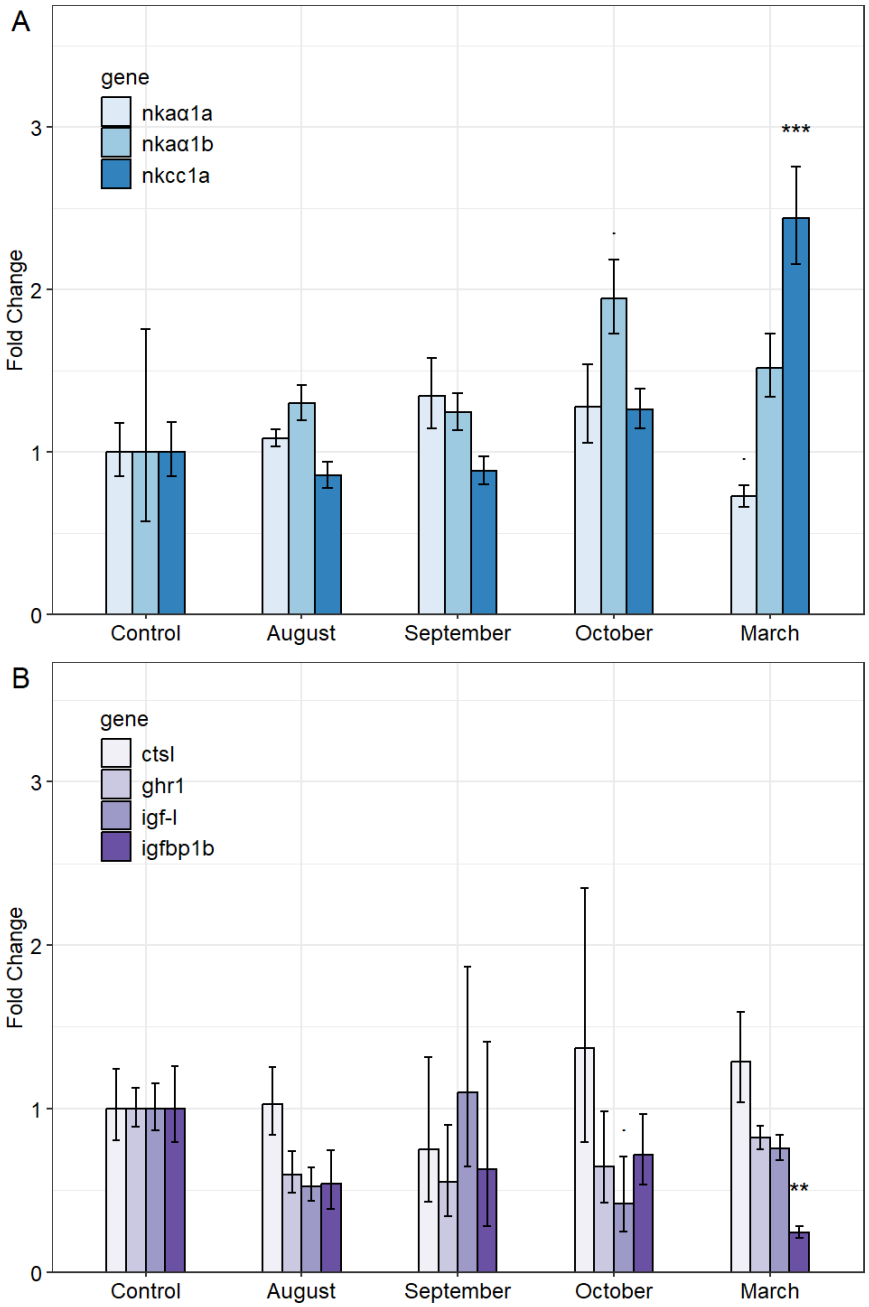

Figure 3. End of experiment representation of average fold change in (A) gill nkaα1a, nkaα1b, and nkcc1a mRNA, and (B) liver ctsl, ghr1, igfbp1b, and igf-I mRNA in March 2023. (n=12; March-F: group of fish kept in freshwater RAS, March-S: group of fish transferred to seawater, March-C: group of fish transferred to seawater and exposed to CO2; Control group represents the biological baseline (S1); significance: '***' 0.001 '**' 0.01 '*' 0.05).

vitro studies (McCormick et al., 1991; Madsen & Bern, 1993), was generally higher in all groups except those exposed to CO_2 in seawater, indicating a potential hormonal imbalance in these fish (Figure 3B). Meanwhile, *igfbp-1*, a protein that inhibits the growth effects of *igf-I* and *igf-II* (Rajaram et al., 1997; Shimizu et al., 2011a; Shimizu et al., 2011b), showed a significant decrease with the onset of smoltification in spring for the freshwater group. This decrease suggests that *igfbp-1* plays a key role in suppressing growth before smoltification. Before the seawater transfer in phase 2, SGR was significantly higher. The expression patterns of the endocrine markers related to smoltification and growth in the March freshwater samples aligned with previous studies (Shimomura et al., 2012; Kaneko et al., 2015; Suzuki et al., 2020), indicating the potential for substantial growth in the following subsequent period. These findings emphasize that moving fish to seawater and force to adapt seawater conditions not only limits the growth benefits associated with seawater transfer but also risks economic losses by extending production timelines. It appears that the advantage of seawater has not been fully realised, resulting in fish achieving similar growth rates to those kept in freshwater. The transcription of *ctsl*, which is involved in tissue and cell differentiation during smoltification (Lysenko et al., 2017), peculiarly did not show significant changes in this experiment for the Group F and S. This lack of change may indicate that the process of tissue differentiation in the Black Sea salmon was delayed or not fully initiated during the autumn sea transfer, which further underscores the importance of aligning transfers with smoltification timing. To ensure successful seawater transfer in autumn, external factors like light and temperature—which are known to trigger smoltification—must be considered. This aligns with previous research emphasizing the influence of temperature and salinity in regulating smoltification (Morro et al., 2019; Pino-Martinez et al., 2024). These adjustments could synchronize smoltification with production cycles, enabling more efficient and sustainable aquaculture practices.

In the second phase of the experiment, a group of fish reared in Black Sea water was subjected to elevated carbon dioxide levels. A pH-controlled solenoid valve system was employed to reduce the water pH from approximately 8.3 to 7.8, thereby increasing the partial pressure of carbon dioxide from ~350 µatm to ~1000 µatm. The seawater used in this study, sourced from the Black Sea, had a measured total alkalinity of 164±1.44 mg $CaCO_3$/L, indicating a relatively robust buffering capacity facilitated the stabilisation of pH levels during CO_2 dosing. This CO_2-exposed group was subsequently compared with a control group, which was transferred to Black Sea water without any additional CO_2 treatment. No statistically significant variations in gene expression were observed between the two groups (Figure 4). Furthermore, growth performance indicators such as specific growth rate (SGR) and condition factor (K value) also exhibited no significant deviations. These findings align with the findings reported by McCormick and Regish (2018), who similarly observed limited growth differences under CO_2 exposure.

Compared to the initial sampling point (Figure 3), both groups demonstrated increased expression of

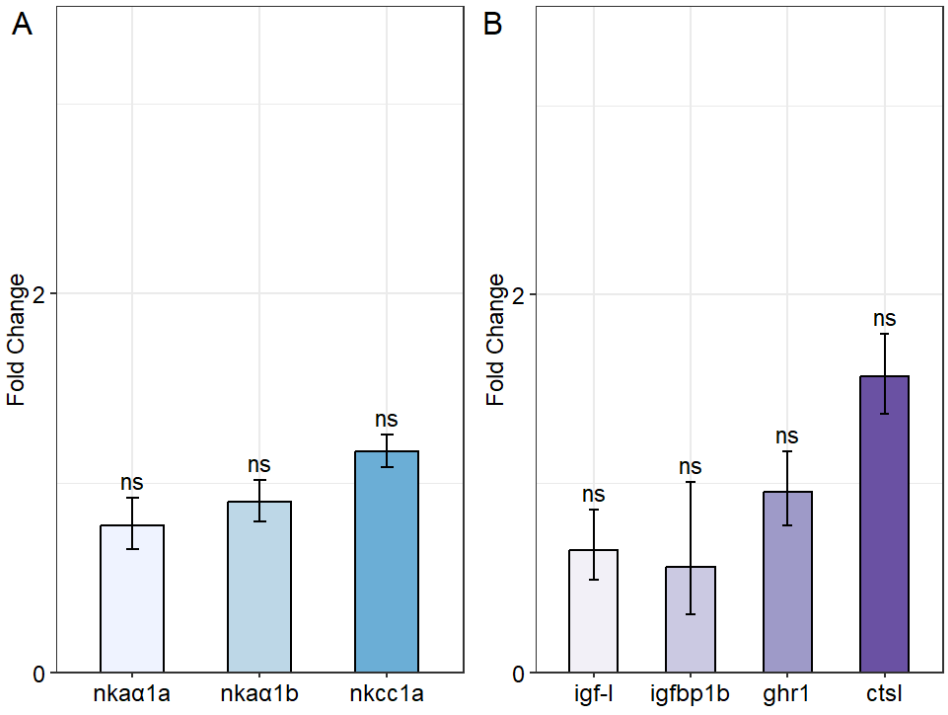

Figure 4. End of experiment representation of Average Fold Change in gill (nkaα1a, nkaα1b, nkcc1a) and liver (ctsl, ghr1, igfbp1b, igf-I) mRNA of fish moved to seawater and exposed to CO2. (n=12, ns=not significant, standard error is given as error bars).

nkaα1b and *nkcc1a*, indicating successful adaptation to seawater. Simultaneously, a decrease in *igf-I* expression was observed, potentially indicating a trend towards growth inhibition following seawater transfer. Although *ctsl* expression was upregulated in the CO_2-exposed group, indicating potential cellular or tissue-level responses, these alterations did not manifest in measurable growth differences. Overall, while Black Sea salmon demonstrated slower growth after seawater transfer, they still exhibited superior growth compared to fish that remained in freshwater. These findings indicate that CO_2 exposure may have an impact on molecular processes. Nevertheless, the observed alterations may not be solely attributable to CO_2 but rather result from its combination with sea transfer. Consequently, these results do not provide definitive evidence of direct hormonal disruption.

Conclusion

The key finding from this experiment is that smoltification in cultured Black Sea salmon occurs in the spring. This presents a challenge for fish farmers in the Black Sea region, as their seawater production cycle typically begins in autumn. The effects of carbon dioxide exposure were minimal and negligible. However, the observed changes in *ctsl* transcription, which reflect cellular and tissue differentiation, suggest some level of cellular adaptation. Additionally, the smoltification process took place in the group of fish kept in freshwater suppressed growth more significantly than carbon dioxide exposure did. The findings underscore the critical interplay between smoltification timing, growth performance, and aquaculture management, laying the foundation for future studies to refine and implement effective strategies for sustainable salmon farming in the Black Sea.

Ethical Statement

This study was carried out in accordance with the HADMEK guidelines. Experimental work was ethically reviewed, approved, and registered (January 28, 2022 - No.325.04.02-11) by the local representative of Animal Experiments Ethics Committee (SUMAE). All samplings and methods were performed as required by the Regulation on the Working Procedures and Principles of Animal Experimentation Ethics Committees dated February 15, 2014.

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Author Contribution

First author: Writing – Original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Project administration, Funding acquisition, Conceptualization. Second author: Writing – review & editing, Supervision, Project administration, Conceptualization. Third author: Writing – review & editing, Methodology, Investigation, Formal analysis. Fourth author: Writing – review & editing, Methodology, Investigation, Formal analysis.

Conflict of 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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