Whole-Genome Transcript Expression Profiling Reveals Novel Insights into Transposon Genes and Non-Coding RNAs during Atlantic Salmon Seawater Adaptation

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Simple Summary: This study proposes a novel approach to analyze transcriptome data sets using the Atlantic salmon seawater adaptation process as a model. *Salmon salar* smolts were transferred to seawater under two strategies: (i) fish group exposed to gradual salinity changes (GSC) and (ii) fish group exposed to a salinity shock (SS). mRNA and miRNAs sequencing were performed for gills, intestine, and head kidney tissues. The whole-genome transcript expression profiling revealed specific gene expression patterns among the tissues and treatments. A great abundance of transposable elements was observed in chromosome regions differentially expressed under experimental conditions. Moreover, small RNA expression analysis suggested fewer of miRNAs associated with the smoltification process. However, target analysis of these miRNAs suggests a regulatory role of process such as growth, stress response, and immunity. The findings uncover whole-transcriptome modulation during seawater adaptation of Atlantic salmon, evidencing the interplaying among mRNAs and miRNAs.

Abstract: The growing amount of genome information and transcriptomes data available allows for a better understanding of biological processes. However, analysis of complex transcriptomic experimental designs involving different conditions, tissues, or times is relevant. This study proposes a novel approach to analyze complex data sets combining transcriptomes and miRNAs at the chromosome-level genome. Atlantic salmon smolts were transferred to seawater under two strategies: (i) fish group exposed to gradual salinity changes (GSC) and (ii) fish group exposed to a salinity shock (SS). Gills, intestine, and head kidney samples were used for total RNA extraction, followed by mRNA and small RNA illumina sequencing. Different expression patterns among the tissues and treatments were observed through a whole-genome transcriptomic approach. Chromosome regions highly expressed between experimental conditions included a great abundance of transposable elements. In addition, differential expression analysis showed a greater number of transcripts modulated in response to SS in gills and head kidney. mRNA expression analysis suggested a small number of miRNAs involved in the smoltification process. However, target analysis of these miRNAs showed a regulatory role in growth, stress response, and immunity. This study is the first to evidence the interplaying among mRNAs and miRNAs and the structural relationship at the genome level during Atlantic salmon smoltification.

Keywords: Atlantic salmon; smoltification; genome; mRNAs; miRNAs
1. Introduction

Genomics tools have facilitated the elucidation of the global genomic changes under different conditions. Nowadays, the availability of the Atlantic salmon (Salmo salar) genome [1] allows for the identification of genome regions associated with pivotal biological processes and responses to the aquatic environment. One of the most important biological processes during the salmon lifecycle is the parr-smolt transformation (smoltification), which is primarily influenced by water temperature and photoperiod [1]. This process involves physiological, morphological, endocrinal, and behavioral changes [2,3], which have been extensively studied, owing to their implications in salmon aquaculture [1,4–6].

Previous transcriptomic analyses have elucidated expression changes of genes associated with growth, metabolism, oxygen transport, osmoregulation, protein biosynthesis, and sensory reception during the Atlantic salmon smoltification process [7,8]. Recently, the role of non-coding RNAs (ncRNAs) during Atlantic salmon transition from freshwater (FW) to seawater (SW) has been proposed as a novel regulatory molecular mechanism involved in fish biology. For instance, previous studies have reported 2864 long non-coding RNAs (lncRNAs) differentially modulated in Atlantic salmon gills during the transition from FW to SW [9]. Among them, two putative lncRNAs with the potential to be used as smoltification-timing biomarkers were identified. One was highly regulated in FW, and the other was upregulated in SW. In addition, a putative regulation role of lncRNAs associated with Na⁺/K⁺-ATPase genes, hormone receptors, and thyroid hormone receptors was suggested [9].

Among ncRNAs, microRNAs (miRNAs) are crucial in post-transcriptional regulation, binding to target mRNAs in 3’UTR and repressing translation to proteins [10,11]. Different biological process, such as development, growth, cell division, metabolism, and apoptosis, are regulated by miRNAs [12–15]. For Atlantic salmon, 472 miRNAs have been reported in the miRBase [16]. In addition, a total of 521 miRNAs have been described for Atlantic salmon, with different expression patterns among kidney, head kidney, heart, brain, gills, white muscle, and intestine tissues [17]. Moreover, 71 miRNAs were reported to be differentially modulated in head kidney of Atlantic salmon during the smoltification process [18]. Furthermore, the authors reported a negative-correlation expression pattern of miRNAs with genes associated with hormone biosynthesis, stress management, immune response, and ion transport. In addition, the study reported a cluster of 37 miRNAs highly regulated before the smoltification initiation and another cluster of 17 miRNAs that increased their expression until SW transfer [18]. Despite the evidence suggesting a role of ncRNAs in the regulation of the smoltification process, comprehensive transcriptome analyses linking physiological adaptation to seawater with the complexity of the salmon genome are unexplored. For instance, there is no evidence of how unduplicated and/or duplicated genes involved in the smoltification are modulated and which are the key molecular elements involved. Herein, the molecular interplaying among coding/non-coding genes expressed in different tissues during seawater adaptation is uncovered. The major obstacle to conducting extensive experimental trials is combining the transcriptome time-series with different sequencing approaches (e.g., mRNA vs. small RNA sequencing), and joining the analyzed target-tissues with the experimental conditions tested. The massive amount of transcriptome data is frequently completely unused or at least not straightforwardly used to identify the primary biological processes modulated and their molecular components. Furthermore, evidence that chromatin in the interphase nucleus has nonrandom localization supports the idea that the nuclear architecture is comprised of three-dimensional (3D) genome spaces. This spatial organization plays crucial roles in genome function and cellular processes, such as DNA replication [19,20], transcription [21], DNA-damage repair [22,23], development, and cell differentiation [24]. Chromosomes occupy distinct subnuclear territories, with transcriptionally active loci positioned at their surface [25–30]. Thus, the relationship between gene transcription, gene regulation, and spatial 3D genome structure for relevant biological processes requires further scientific investigation. This study aimed to explore global transcriptome modulation during Atlantic salmon seawater...
adaptation. Herein, a novel approach was developed to analyze time-series differential transcription data from head kidney, gills, and intestine tissues exposed to salinity changes. In parallel, transcriptional dynamics were associated with chromosome regions where differently expressed thresholds between salinity conditions were identified. Notably, transcriptome analyses revealed novel insights into transposon genes and non-coding RNAs involved in smoltification in Atlantic salmon. This study is the first to suggest putative chromosome regions transcriptionally activated in response to salinity stress in anadromous fish.

2. Materials and Methods

2.1. Smoltification and Seawater Transfer

Atlantic salmon smolts (60 ± 6.2 gr) were obtained from a commercial farm (Hendrix Genetics, Boxmeer, The Netherlands) and then transported to the Marine Biology Station, Universidad de Concepción, Dichato, Chile. Fish were maintained in ultraviolet-treated saltwater by single-pass flow-through tank systems on a 12:12 h light:dark cycle, fed daily with a commercial diet, dissolved oxygen level of 8.5 mg/L and pH = 8.0. After ten days of acclimation, a group of 30 smolts was exposed to a gradual salinity change (GSC) by increasing FW salinity to SW. The gradient was set at three salinity points, changing 10 PSU per week over a month. Meanwhile, another group of 30 smolts was exposed to a salinity shock (SS), directly from FW to 32 PSU. Gills, head kidney, and intestine samples were collected during the experiment trial. Samples were collected at FW, 10, 20, and 32 PSU for the GSC group and after a week of acclimation at 32 PSU for the SS group (Figure S1). Both processes were conducted in triplicate. The samples were fixed in RNAlater® (Thermofisher, Waltham, MA, USA) for subsequent RNA isolation. Furthermore, the salmon condition to SW transfer was evaluated by immune histochemistry analyses performed by the VEHICE company. In addition, RT-qPCR expression analysis of ATPase-α and ATPase-β was determined using comparative ∆∆Ct relative expression analysis. Primers and qPCR conditions were similar, as described by Valenzuela-Muñoz, Váldez, and Gallardo-Escárate [9]. All animal procedures were carried out under the guidelines approved by the Ethics Committee of the University of Concepción. The experimental design for the current study considered the Three Rs (3Rs) guidelines for animal testing.

2.2. High-Throughput Transcriptome Sequencing

Total RNA was isolated from each experimental fish group using Trizol Reagent (Ambion®, Austin, TX, USA), following the manufacturer’s instructions. The isolated RNA was evaluated by TapeStation 2200 (Agilent Technologies Inc., Santa Clara, CA, USA), using the R6K Reagent Kit. Three biological replicates were separately sequenced by tissue and sampling point from each experimental fish group. For each replicate, five individuals were used for the RNA extraction and then pooled for library preparation. RNAs with RIN > 8.0 were used for double-stranded cDNA library construction using the TruSeq RNA Sample Preparation Kit v2 (Illumina®, San Diego, CA, USA). The same RNA samples were used for small RNA library synthesis using the TruSeq Small RNA Library Prep Kit (Illumina®, San Diego, CA, USA). All libraries made for RNA and small RNAs were sequenced by the Hiseq (Illumina®, San Diego, CA, USA) platform in Macrogen Inc. Raw data used for the current study are available in SRA-NCBI (Bioproject # PRJNA761374).

2.3. Whole-Genome Transcript Expression Analysis

Raw data from each experimental group were separately trimmed and mapped to the Atlantic salmon genome (GCF_000233385.1) using CLC Genomics Workbench v21 software (Qiagen Bioinformatics, Hilden, Germany). Threshold values for mRNA and small RNA were calculated from the coverage analysis using the Graph Threshold Areas tool in CLC Genomics Workbench v21 software. Here, an index denoted as chromosome genome
expression (CGE) was formulated to explore the whole-genome transcript expression profiling according to:

\[
CGE = \frac{|X_1 - X_n| \times 100}{(X_1 - X_n)}
\]

where X corresponds to mean coverage of transcripts mapped into a specific chromosome region and compared among experimental conditions (e.g., GSC cv. SS; tissues vs. experimental time-points, mRNAs vs. miRNAs). The transcript coverage values for each dataset were calculated using a threshold of 10,000 to 90,000 reads, where a window size of 10 positions was set to calculate and identify differentially transcribed chromosome regions. The CGE index represents the percentage of transcriptional variation between two or more groups for the same locus. This approach allows for visualization of actively transcribed chromosome regions, identification of differentially expressed loci, exploration of mRNA-miRNAs interactions in term of transcriptional activity, and observation of tissue-specific patterns in fish exposed to several experimental conditions. Finally, threshold values for each dataset and CGE index were visualized in Circos plots [31].

2.4. RNA-Seq Data Analysis

Raw sequencing reads were assembled de novo separately for each tissue using the CLC Genomics Workbench v21 software (CLC Bio, Aarhus, Denmark). Assembly was performed with overlap criteria of 70% and a similarity of 0.9 to exclude paralogous sequence variants (Renaut et al. 2010). The settings used were set as mismatch cost = 2, deletion cost = 3, insertion cost = 3, minimum contig length = 200 base pairs, and trimming quality score = 0.05. After assembly, singletons were retained in the dataset as possible representatives of low-expression transcript fragments. Differential expression analysis was set with a minimum length fraction = 0.6 and a minimum similarity fraction (long reads) = 0.5.

Contig sequences obtained from each tissue were blasted to CGE regions to enrich the number of transcripts evaluated by RNA-Seq analysis (Figure 1). In addition, the sequences were extracted from the Atlantic salmon genome near to the threshold areas in a window of 10 kb for each transcriptome. The expression value was set as transcripts per million (TPM). The distance metric was calculated with the Manhattan method, with a mean expression level in 5–6 rounds of k-means, clustering subtracted. Finally, Generalized Linear Model (GLM) available in the CLC software was used for statistical analyses and to compare gene expression levels in terms of the log₂ fold change \( p = 0.005; \text{FDR-corrected} \). In addition, k-means clustering was performed for transposable element (TE) expression values. The metric distance was calculated with the Manhattan method, where the mean expression level in 5–6 rounds of k-means clustering was subtracted.

2.5. Sequence Annotation and GO Enrichment Analysis

Differentially expressed contigs were annotated through BlastX analysis using a custom protein database constructed from GeneBank and UniProtKB/Swiss-Prot. The cutoff E-value was set at 1E-10. Transcripts were subjected to gene ontology (GO) analysis using the Blast2GO plugins included in the CLC Genomics Workbench v12 software (CLC Bio, Qiagen, Germantown, MA, USA). The results were plotted using the cluster Profiler R package [32]. GO enrichment analysis was conducted to identify the most represented biological processes among protein-coding genes located proximally to the identified lncRNAs. Enrichment of biological processes was identified using Fisher’s exact test tool of Blast2GO among the different experimental groups against the control group (FW samples).
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2.6. miRNA Annotation and Expression Analysis in Response to Salinity Changes

Low-quality reads from illumina sequencing data, reads with a quality score of less than 0.05 on the Phred scale, with a short length, or with three or more ambiguous nucleotides were removed using CLC Genomics Workbench software (Version 21, CLC Bio, Aarhus, Denmark). Furthermore, any cleaned sequences matching metazoan mRNA, rRNA, tRNA, snRNA, snoRNA, repeat sequences, or other ncRNAs were deposited in the NCBI databases (http://www.ncbi.nlm.nih.gov/ (accessed on 27 July 2021)), RFam (http://rfam.janelia.org/ (accessed on 27 July 2021)), or Repbase (http://www.girinst.org/repbase/ (accessed on 27 July 2021)) were discarded. Then, the remaining transcripts were counted to generate a unique small RNA list. These sequences were annotated against pre-miRNA and mature miRNA (5' and 3') sequences listed for *Salmo salar* available in the miRbase (release 22) [16,33]. miRNA expression analysis followed a similar protocol, as previously described by our group [34].

2.7. miRNA Target Prediction and Expression Correlation

The computational target prediction algorithm used was RNA22 [35]. The datasets used were the differently modulated transcripts from both groups, GSC fish and SS fish. The RNA22 parameters were set at free energy < −15 kcal/mol and a score > 50. All target genes were annotated by GO analysis, following the protocol described above.

In addition, smoltification-related genes and differently expressed miRNAs were selected for expression-correlation analysis. Pearson’s correlation among TPM values from both datasets, including all samples exposed and not exposed to each salinity condition, were calculated in R software [36]. Plots for correlation analyses were constructed using the Corrplot package [37], considering a correlations with p-value < 0.01 significant.
2.8. RT-qPCR Validation Analysis

Transcription expression profiles of genes associated with the smoltification process and ncRNAs were conducted by RT-qPCR. Briefly, 200 ng/µL of total RNA from three individuals per fish group obtained was used for cDNA synthesis using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific™, USA), following the manufacturer’s instructions. Four genes and their putative miRNAs were validated (Table S4). The comparative ΔΔCt relative expression analysis method was used. Selection of the housekeeping gene for the experiment was based on evaluation of the stability of elongation factor-α (EF-α), β-tubulin, and 18S genes by Normfinder. Here, EF-α was selected for gene normalization. Each RT-qPCR reaction was carried out in a final volume of 10 µL using the commercial PowerUp SYBR Green Master Mix kit (Applied Biosystems®, Waltham, MA, USA). RT-qPCR reactions were performed on the StepOnePlus™ (Applied Biosystems®, Carlsbad, CA, USA) using the following conditions: 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 30 s, ending with 30 s at 72 °C. Statistical analyses were conducted through ANOVA-1 test and Student’s t-test in the GraphPad Prims 8.4.7 package.

miRNA transcription-level validation was achieved by synthetizing cDNA from the same RNA samples using the miScript II RT kit (Qiagen, Germany), with an incubation reaction at 37 °C for 60 min and 5 min at 95 °C. Specific primers were designed for bantam miRNA and were used for amplification by qPCR using the miScript SYBR Green PCR kit (Qiagen, Germany) in a QuanStudio 3 System (Life Technologies, USA). Thermal cycling conditions consisted of an initial denaturation and enzyme activation at 95 °C for 15 min, followed by 40 cycles of 94 °C for 15 s (denaturation), 55 °C for 30 s (annealing), and 70 °C for 45 s (extension). Ssa-mir-455e5p was used as an endogenous control for this reaction [38]. Gene and miRNA expression were quantified using the ΔΔCT comparative method, was previously described (Pfaffl, 2001).

3. Results

3.1. Atlantic Salmon Performance during the Experimental Trial

Immune histochemistry analysis performed in Atlantic salmon exposed to GSC and SS conditions showed chloride cell migration, indicating adequate salmon conditions for SW transfer (Figure S2). Furthermore, this condition was confirmed by RT-qPCR analysis of ATPase-α and ATPase-β subunits (Figure S3). No mortality was recorded in experimental groups.

3.2. Gill-Tissue-Transcription Modulation during Smoltification Process

Whole-genome modulation of Atlantic salmon tissues was evaluated in two groups: Atlantic salmon exposed to gradual salinity change (GSC) and salinity shock (SS). Atlantic salmon gill-tissue whole-genome expression showed low variation in MRNAs between the GSC and SS fish groups (Figure 2A). Moreover, miRNAs had an opposite expression pattern compared with mRNAs, with high threshold values in chromosome areas where mRNAs showed a downregulation. Chromosome expression variation between experimental groups was calculated by CGE index. Despite the low differences in mRNA threshold values between the GCS and SS groups, a group of chromosomes including chr1, chr2, chr4, chr9, chr12, chr13, chr14, chr17, chr18, chr22, and chr25 presented high CGE index values (Figure 2B). In addition, these chromosomes showed high threshold values for miRNAs, with a high CGE index. Furthermore, the synteny analysis of the selected chromosomes exhibits a section with high homology among the highlighting areas (Figure 2B). Notably, chr12 and chr22 showed a large synteny block and high mRNA modulation (red ribbons), and similar patterns were found in chr12 with chr2 in green ribbons. This also draws attention to chr4 and chr13, with high miRNA modulation linking a synteny block.
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Putative differentially expressed chromosome regions were annotated by Blast analysis using a *Salmo salar* protein database. Notably, numerous transposase and transposable elements, *Tcb1*, *HSP70*, and *MCHII* genes were annotated in chromosomes showing high differential expression among experimental fish groups (Table S1). Moreover, RNA-Seq analyses of these selected chromosomes evidenced an interesting expression pattern, where gill samples of FW and fish from the SS group at 32 PSU were grouped in the same cluster, separated from the GSC 32PSU group (Figure 2C). Furthermore, differential expression analysis of transposable elements (TEs) showed that the median of the TEs expressed in fish exposed to GSC was downmodulated (Figure S5).

In addition, contigs annotated as TEs clustered in the Atlantic salmon gill transcriptome. Fish transfer from FW to SW gradually and by salinity shock exhibited different expression (Figure S5). Notably, clusters 1 and 4 were downmodulated after SW transfer at 32 PSU, GSC group. In contrast, cluster 2 showed contigs annotated as upmodulated TEs (Figure S5A). Furthermore, gill samples of Atlantic salmon from FW to 32 PSU by salinity sock showed four clusters, where cluster 1 and 3 showed different expression profiles, up- and down-modulated, respectively. Interestingly, TEs RT-qPCR validation showed a similar expression pattern to RNA-seq analysis in cluster 2 and cluster 3 for GSC and SS, respectively (Figure S5B).

**Figure 2.** Whole-genome transcription of Atlantic salmon gills during smoltification process. (A) Threshold analysis of gills for GSC and SS fish groups. (B) Chromosome regions with high CGE index variation between GSC and SS fish groups. Heatmap in red shows the expression variation between both groups, CGE index. Black line graph indicates genome coverage of threshold areas. In the Circos plot, the ribbons represent the homoeologous regions in salmon genome. (C) RNA-Seq analysis of chromosome regions with high CGE index between experimental groups.
3.3. Intestine Tissue-Transcription Modulation during Smoltification Process

A high regulation of the expression of chr2 to chr9 regions was observed from the whole-genome expression analysis. Moreover, miRNA transcriptional variation was observed in a reduced number of chromosomes, including chr4, chr13, chr15, and chr25 (Figure 3A), suggesting a low regulatory role of miRNAs in the intestine during the smoltification process. Notably, chromosomes with a high CGE index between mRNAs expressed in the GSC and SS fish group showed a high miRNA CGE index (Figure 3B), suggesting local miRNAs regulation, similar to the findings observed in gills. In addition, the synteny analysis among highlighting chromosomes showed an area with a high CGE index and mRNA homology between chr1-chr9 and chr12-chr22. Contrary to was observed in gills, heatmap representations of intestine transcripts group in the same cluster fish at 32 PSU (Figure 3C). In addition, intestine chromosomes with a high CGE index exhibited a great abundance of regulatory elements, such as transposase, transposable elements, and retro-transposable elements (Table S1).

Figure 3. Whole-genome transcription in Atlantic salmon intestine during smoltification process. (A) Threshold analysis of gills for GSC and SS fish groups. (B) Chromosome regions with high CGE index variation between GSC and SS fish groups. Heatmap in red shows the expression variation between both groups, CGE index. Black line graph indicates genome coverage of threshold areas. In the Circos plot, the ribbons represent the homoeologous regions in salmon genome. (C) RNA-Seq analysis of chromosomes regions with high CGE index between experimental groups.
3.4. Head-Kidney Transcriptome Modulation during Smoltification Process

From chromosome analysis using read sequences obtained for head kidney tissue from Atlantic salmon exposed to GSC and SS, high modulated areas were observed in all Atlantic salmon genomes (Figure 4A). Additionally, head-kidney miRNA analysis of whole Atlantic salmon genome suggested an important regulatory function during the smoltification process in this tissue. Chromosomes chr2, chr3, chr4, chr9, chr13, chr15, chr17, chr20, chr24, and chr25 had high CGE indexes for mRNA and miRNA between head kidney tissue of fish exposed to GSC and SS (Figure 4B). Interestingly, while the synteny analysis of gill and intestine tissue showed homology among mRNAs areas, a synteny block in head kidney tissue was observed in regions with a high miRNA CGE index, such as chr13-chr15, chr20-chr24, chr3-chr6, and chr13-chr14 (Figure 4B). Interestingly, among the genes annotated in these chromosomes, hemoglobin, HSP70, TLR8, and a large number of transposase and transposable elements were found (Table S1). Notably, TEs expressed in head kidney were upregulated in both experimental conditions, GSC and SS (Figure S5).

Figure 4. Whole-genome transcription in Atlantic salmon head kidney during smoltification process. (A) Threshold analysis of gills for GSC and SS fish groups. (B) Chromosome regions with high CGE index variation between GSC and SS fish groups. Heatmap in red shows the expression variation between both groups, CGE index. Black line graph indicates genome coverage of threshold areas. In the Circos plot, the ribbons represent the homoeologous regions in salmon genome. (C) RNA-Seq analysis of chromosome regions with high CGE index between experimental groups.
3.5. Differential Expression Analysis of Atlantic Salmon during Seawater Transfer

Nucleotide sequences near 10kb of each coverage threshold area were extracted from the Atlantic salmon genome. Later, sequences were blasted to the contigs obtained from the de novo assembly performed for each tissue. These sequences were used as a reference for RNA-Seq analysis using the filtered data of each tissue. Gill tissue of fish exposed to GSC and SS at 32 PSU showed similar expression patterns. On the other hand, samples obtained from the control group (FW) and fish exposed to 10 and 20 PSU were grouped in the same cluster (Figure S4A). From the differential expression analysis between gills of fish exposed to GSC and SS compared with the control group (FW), a larger number of transcripts differently modulated in response to the SS, with 2528 transcripts, compared to 646 transcripts differently expressed in the GSC group, suggesting a signification effect of SS in mRNA expression modulation (Figure 5A, Table S2). From GO analysis, the differentially expressed transcripts were annotated as biological processes, such as response to a steroid hormone, positive regulation of gene expression, muscle cell migration, localization of cell, insulin secretion, defense response, eye morphogenesis, and cell motility, with a large number of transcripts in the GSC group (Figure 5A).

![Figure 5](image-url)

**Figure 5.** Differential expression analysis and GO enrichment of Atlantic salmon exposed to GSC and SS. (A) DEGs and GO enrichment of gills tissue. (B) DEGs and GO enrichment of intestine tissue. (C) DEGs and GO enrichment of head kidney tissue.
Similar expression patterns were found between intestine fish samples from GSC and SS under the 32 PSU condition. In the control group (FW), fish exposed to 10 and 20 PSU were clustered in the same group (Figure S4B). A total of 3528 transcripts were highly modulated in the intestine of the GSC group, while in SS fish group, 1483 DE transcripts in intestine tissue were obtained (Figure 5B, Table S2). GO enrichment analysis of differently modulated transcripts in intestine tissue showed BP processes associated with response to hormones, organic substance metabolic processes, metabolic processes, ion transmembrane transport, cellular processes, and some processes associate with immune response, with a greater number of transcripts in the GSC group (Figure 5B).

Transcriptome expression analyses showed a similar expression profile between head-kidney samples of fish at 32 PSU from GSC and SS groups. Notably, the head-kidney samples obtained from the GSC fish group at 10 PSU showed high expression levels of transcripts downregulated under the other evaluated conditions (Figure S4C). Interestingly, differential expression analysis showed a large number of transcripts modulated in the SS group, with 2480, compared with to 681 transcripts modulated in response to the GSS condition (Figure 5C, Table S2). GO analysis showed that the most relevant modulated BP were in response to oxidative stress, response to growth factor, positive regulation of transcription, and positive regulation of response to extracellular stimulus, among others (Figure 5C).

3.6. miRNA Regulation in Atlantic Salmon during Smoltification

The small RNAs obtained from Illumina sequencing for each tissue were annotated using the *Salmo salar* miRNA database published in miRBase release 22.1 [16,33]. A total of 478 miRNAs were annotated, like those reported in the miRBase for Atlantic salmon. In gills, two clusters of transcriptional profiles were identified, the first one grouping the control group (FW) with fish exposed to 10 PSU, and a second cluster grouping samples obtained from fish exposed to GSC at 20, 32 PSU and the SS group at 32 PSU (Figure 6A). Unlike gills, the transcriptional patterns of miRNAs of SS and GSC were grouped by salinity (32 PSU) in the same cluster. Furthermore, in intestine and head kidney tissue, fish exposed to GSC (32 PSU) evidenced an upregulation compared to fish from the SS group. In contrast, GSC fish at 20 and 10 PSU were fish sampled in freshwater (Figure 6B,C).

![Figure 6](image-url). miRNA expression profile during gradual salinity changes and salinity shock in gills, intestine, and head kidney of Atlantic salmon.
Notably, clustering analysis showed different miRNAs with expression profile changes related to the FW condition between GSC and SS groups. Interestingly, miRNAs with expression changes between FW and GSC showed an upregulation in gill samples after transferring to 32 PSU (Figure S6A). Furthermore, these miRNAs expressed in response to GSC, ssa-miR-143, ssa-miR-21b, and ssa-miR-10d, were validated by RT-qPCR. Expression evaluation showed an up-modulation during salinity changes from FW to 32 PSU, similar to the in silico analysis (Figure S6B). Furthermore, four cluster exhibited significant expression changes in fish groups exposed to SS (Figure S6A). In addition, RT-qPCR analysis in gill samples obtained at each sample point (Figure S1) showed similar expression patterns of ssa-miR-181 and ssa-miR-30d from cluster 1, and ssa-miR-10b from cluster 2 (Figure S6B).

Differential expression analysis was performed between GSC and SS salmon groups using sampled tissues in FW as a control. Differences in the number and class of miRNAs among tissues were found. For instance, gill samples showed a large number of miRNAs differently expressed in response to SS, including SSA-miR-122-5p, ssa-miR-122-3p, and ssa-miR-122-2-3p upregulated in GSC group. Moreover, according to prediction target analysis, SSA-miR-122-5p has as target gene the sodium/potassium-transporting ATPase subunit beta-3-like and thyroid hormone receptor interactor 11, with delta G values of −17.3 and −16.2, respectively, While SSA-miR-365-5p is the most downregulated miRNA in this tissue (Figure 7).

A larger number of miRNAs in the intestine were differently expressed in samples obtained from fish exposed to GSC. However, no significant differences in the expression change values were observed (Figure 7). However, upregulated SSA-miR-155-3p miRNA showed a putative binding site to myosin-11-like, probable cation-transporting ATPase, and collagen Type XI Alpha2 in the GSC group, with delta G values of −14.5, −13.5, and −14.7, respectively (Table S3). Moreover, ssa-miR-20a-1-3p was upregulated, and among its target genes, the membrane heat shock 70 kDa protein and toll-like receptor 13 were identified, with delta G values of −12.54 and −18.2, respectively (Table S3).

The head kidney showed a similar number of differently expressed miRNAs between CGS and SS fish groups. Nevertheless, the miRNAs differently modulated between groups were different. For instance, SSA-miR-499a-5p, ssa-miR-192a-3p, ssa-miR-192b-3p, and ssa-miR-200b-5p were overexpressed in the GSC group. Target gene analysis determined that cathelicidin antimicrobial peptide has a putative binding site to ssa-miR-192a-3p, with a delta G value of −15.2, while ssa-miR-122-5p and ssa-miR-122-2-3p were upregulated in response to SS, evidencing a putative binding site to immunoglobulin tau heavy chain (delta G −15.2) (Table S3).

Interestingly, some miRNAs are expressed in two tissues, suggesting a role in salinity changes. For instance, ssa-miR-499a-5p miRNAs was overexpressed in gills and head kidney tissues in response to GSC. Another example is ssa-miR-196b-3p, which is overexpressed in the intestine and head kidney. Regarding the response to SS, the miRNAs ssa-miR-27d-5p is upmodulated in the gills and intestine. Others interesting miRNAs are ssa-miR-144-3p and ssa-miR-301a-5p, downregulated in gills and head kidney from fish exposed to SS (Figure 7). Furthermore, target analysis of commonly expressed miRNAs evidenced a putative role in the modulation of collagen genes in the case of ssa-miR-499a-5p and ssa-miR-27d-5p. Additionally, it was a gene associated with cell differentiation was observed as G-protein-signaling modulator 2-like, with a target site to ssa-miR-144-3b. Finally, among the SSMiR-301a-5p target genes was identified clathrin heavy chain 1-like. (Table S3).
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Figure 7. miRNA differential expression analysis of Atlantic salmon tissues under both conditions, GSC and SS. Tables show the fold-change values of miRNA for each tissue; red: upregulated, blue: downregulated.

3.7. GO Enrichment of miRNA Target Genes

The differentially expressed mRNAs in each tissue of fish groups exposed to GSC and SS were evaluated as target genes of differently modulated miRNAs. Notably, GO enrichment analysis of putative mRNAs suggests that gradual salinity changes trigger a greater number of regulatory responses than the salinity shock in the evaluated tissues. This is reflected in BP number, which seems to be modulated by differential transcribed miRNAs in the GSC fish group (Table 1). Response to stimulus, cell communication, response to stress, and immune system process were found among the Biological Process (BPs) putatively modulated by miRNAs in the three tissues (Table 1). The fish group exposed to SS exhibits a reduced number of BPs putatively modulated by differentially expressed miRNAs (Table 1). Furthermore, among the tissues, different target processes were identified. For instance, among the most representative processes in gills were pattern-recognition receptor-signaling pathways and response to ATP. On the other hand, regulation of mononuclear cell proliferation and antigens processing a presentation by MHC class II were observed in the intestine. While, the BPs found in head kidney were glycosylation and transposition (Table 1).


**Table 1.** GO enrichment analysis of differently modulated putative miRNA target genes in Atlantic salmon exposed to gradual salinity changes (GSC) and salinity shock (SS).

| Biological Process | GSC Atlantic Salmon N° GO Term | GSC Atlantic Salmon GO Term | SS Atlantic Salmon N° GO Term | SS Atlantic Salmon GO Term |
|--------------------|--------------------------------|-----------------------------|-------------------------------|-----------------------------|
| Cellular process   | 269                            | Pattern recognition receptor signaling pathway | 126                          | Regulation of Pattern recognition receptor signaling pathway |
| Metabolic process  | 259                            | Regulation of mononuclear cell proliferation | 8                            | Regulation of lymphocyte proliferation |
| Response to stimulus | 243                         | Antigen processing and presentation of peptide | 8                            | Regulation of lymphocyte proliferation |
| Macromolecule metabolic process | 241                      | Macromolecule glycosylation | 42                           | Nucleobase-containing compound metabolic process |
| Biological regulation | 239                        | Regulation of mononuclear cell proliferation | 8                            | Regulation of lymphocyte proliferation |
| Regulation of biological process | 234               | Antigen processing and presentation of peptide antigen | 7                           | Regulation of lymphocyte proliferation |
| Cellular biosynthetic process | 233              | Antigen processing and presentation of peptide antigen | 7                           | Regulation of lymphocyte proliferation |
| Response to stimulus | 243                         | Antigen processing and presentation of peptide antigen | 7                           | Regulation of lymphocyte proliferation |
| Organic cyclic compound metabolic process | 229 | Microtubule polymerization | 3                            | Sperm-egg recognition |
| Cellular response to stimulus | 232            | Transposition, DNA-mediated | 55                           | Regulation of platelet activation |
| Gene expression | 266                            | Transposition, DNA-mediated | 55                           | Regulation of platelet activation |
| Regulation of gene expression | 202                | DNA integration | 82                           | DNA integration |

| Biological Process | Intestine Tissue Biological Process N° GO Term | Intestine Tissue Biological Process GO Term | Intestine Tissue Biological Process N° GO Term | Intestine Tissue Biological Process GO Term |
|--------------------|-----------------------------------------------|---------------------------------------------|-----------------------------------------------|---------------------------------------------|
| Cellular process   | 269                            | Regulation of mononuclear cell proliferation | 8                            | Regulation of mononuclear cell proliferation |
| Metabolic process  | 259                            | Regulation of lymphocyte proliferation | 8                            | Regulation of lymphocyte proliferation |
| Organic substance metabolic process | 255 | Macromolecule glycosylation | 42                           | Nucleobase-containing compound metabolic process |
| Cellular metabolic process | 255 | Macromolecule glycosylation | 42                           | Nucleobase-containing compound metabolic process |
| Primary metabolic process | 158          | Protein glycosylation | 42                           | Nucleobase-containing compound metabolic process |
| Nitrogen compound metabolic process | 154 | Pyrimidine nucleotide-sugar transmembrane transport | 19                          | Pyrimidine nucleotide-sugar transmembrane transport |
| Cellular biosynthetic process | 153 | Nucleotide-sugar transmembrane transport | 19                          | Pyrimidine nucleotide-sugar transmembrane transport |
| Biosynthetic process | 153 | Nucleotide-sugar transmembrane transport | 19                          | Pyrimidine nucleotide-sugar transmembrane transport |
| Organic substance biosynthetic process | 152 | ARF protein signal transduction | 10                          | Regulation of ARF protein signal transduction |
| Macromolecule metabolic process | 151 | DNA integration | 82                           | DNA integration |
| Biological regulation | 149                        | DNA integration | 82                           | DNA integration |
| Organonitrogen compound metabolic process | 149 | DNA integration | 82                           | DNA integration |
| Response to stimulus | 147                        | DNA integration | 82                           | DNA integration |
| Cellular macromolecule metabolic process | 147 | DNA integration | 82                           | DNA integration |
| Regulation of biological process | 146                   | DNA integration | 82                           | DNA integration |
| Localization | 144                                  | DNA integration | 82                           | DNA integration |
| Cellular nitrogen compound metabolic process | 143                 | DNA integration | 82                           | DNA integration |
| Regulation of cellular process | 143               | DNA integration | 82                           | DNA integration |
| Organic cyclic compound metabolic process | 143            | DNA integration | 82                           | DNA integration |
| Transport | 142                                 | DNA integration | 82                           | DNA integration |
| Establishment of localization | 142                   | DNA integration | 82                           | DNA integration |
| Immune system process | 124                        | DNA integration | 82                           | DNA integration |
| Immune response | 119                                 | DNA integration | 82                           | DNA integration |
| Defense response | 108                                 | DNA integration | 82                           | DNA integration |
| Response to biotic stimulus | 103                     | DNA integration | 82                           | DNA integration |
In particular, the evaluation of expression changes of miRNAs and their putative targets by tissue suggested a putative regulatory role of ssa-miR-205-5p in the regulation serine/threonine kinase or the regulation of the HSP70 gene by ssa-miR-19c-3-5p in gills exposed to GSC (Table 2, Figure S6). On the other hand, in the case of SS fish gills, a putative regulatory role of ssa-miR-19d-5p and ssa-miR-222b-5p with ATPase inhibitor can be mentioned and transposase, respectively (Table 2). Moreover, a putative regulation of myosin and annexin A2-like genes in the intestine tissue of GSC fish was observed by SSA-miR-92a-5p and SSA-miR-15a-3p, respectively. In addition, in SS fish group was observed a negative correlation expression between free fatty acid receptor and low-density lipoprotein receptor genes, with miRNAs 30a-3p and 125b-5p, respectively (Table 2). In the case of head kidney tissue of GSC fish, ssa-miR-128-1-5p showed a negative correlation expression with the SPATA5 gene, and laminin gene exhibited a binding site to ssa-miR-194a-3p in fish exposed to GSC (Table 2).

Finally, expression-correlation analysis was conducted among the differently expressed miRNAs and smoltification-related genes and TEs to evidence the investigated role observed during smoltification. Expression-correlation analysis exhibited a negative correlation value between ssa-miRNA-204-5p with the TE Tcb1 and the sodium/potassium-transporting ATPase subunit alpha and beta. Additionally, TEs showed negative correlation values with miRNAs ssa-miRNA-19c-3-5p, ssa-miRNA-138-5p, and ssa-miRNA-10a-2-3p (Figure 8). Interestingly, positive correlation values were found in some isoforms of sodium/potassium-transporting ATPase subunit alpha and beta and the miRNAs belonging to the families ssa-miRNA-206, ssa-miRNA-214, and ssa-miRNA-219. This result could be associated with specific regulatory roles between miRNAs and transcript isoforms.

![Figure 8](image_url)

**Figure 8.** Correlation analysis of expression pairs among smoltification-related genes, TEs, and differential expressed miRNAs. TPM values. Corrplot analyses were conducted on differentially expressed smoltification-related genes, TEs, and differential expressed miRNAs (fold change > 141 and p-value < 0.01) in the combination of all the data, including exposure to the three tissue and GSC and SS conditions. Only Pearson’s correlation values that were significant (p-value > 0.01) are shown in the plot. Red pies correspond to significant negative correlations, and blue pies correspond to significant positive correlations. The completeness of the pies corresponds to the correlation level, where pies closer to circular shape correspond to values more proximal to 1 in Pearson’s calculation.
| Tissue | miRNA             | Fold Change GSC | Fold Change SS | De novo Assembly | Fold Change GSC | Fold Change SS | Delta G | Description                                      |
|--------|-------------------|-----------------|----------------|------------------|-----------------|----------------|---------|-------------------------------------------------|
| Gills  | ssa-miR-204-5p    | 4,02            | 0              | contig_19100      | 2,43            | 0              | −3,60   | PREDICTED: non-syndromic hearing impairment protein 5-like isoform X2 [Salmo salar] |
|        | ssa-miR-30a-3-3p  | 0               | 2,28           | contig_58533      | −3,12           | 0              | −3,50   | PREDICTED: kynureninase-like [Salmo salar]        |
|        | ssa-miR-92b-3p    | 0               | 1,22           | contig_2303       | 2,07            | 0              | −1,92   | PREDICTED: ATPase inhibitor, mitochondrial precursor [Salmo salar] |
|        | ssa-miR-214-3p    | 0               | 1,35           | contig_3322       | 2,75            | 0              | −1,82   | PREDICTED: fibrinogen beta-like isoform X1 [Salmo salar] |
|        | ssa-miR-19c-5p    | −2,06           | 0              | contig_51580      | 3,29            | 0              | −3,00   | PREDICTED: protein-tyrosine kinase 6-like [Salmo salar] |
|        | ssa-miR-92a-5p    | 3,72            | 0              | contig_4409       | −4,12           | 0              | −3,00   | PREDICTED: low-density lipoprotein receptor-related protein 2-like [Salmo salar] |
|        | ssa-miR-30a-3-3p  | 0               | 2,28           | contig_3114       | −1,94           | 0              | −1,94   | PREDICTED: free fatty acid receptor 3-like [Salmo salar] |
| Intestine | ssa-miR-123b-5p  | 0               | 1,26           | contig_4543       | −1,97           | 0              | −1,97   | PREDICTED: myosin heavy chain, fast skeletal muscle-like [Salmo salar] |
|        | ssa-miR-30a-3-3p  | 0               | 2,28           | contig_44524      | −2,17           | 0              | −1,97   | PREDICTED: protein C3-like [Salmo salar]         |
|        | ssa-miR-92a-5p    | 3,72            | 0              | contig_51499      | −3,15           | 0              | −3,15   | PREDICTED: myosin heavy chain, fast skeletal muscle-like [Salmo salar] |
|        | ssa-miR-15a-3p    | 1,72            | 0              | contig_9149       | −2,1            | 0              | −2,1    | PREDICTED: myosin heavy chain, fast skeletal muscle-like [Salmo salar] |
|        | ssa-miR-140-3p    | −1,53           | 0              | contig_25304      | 4,52            | 0              | 3,12    | PREDICTED: myosin heavy chain, fast skeletal muscle-like [Salmo salar] |
|        | ssa-miR-456-3p    | 3,35            | 0              | contig_24874      | −1,73           | 0              | −1,73   | PREDICTED: myosin heavy chain, fast skeletal muscle-like [Salmo salar] |
|        | ssa-miR-585-3p    | 0               | 5,51           | contig_66659      | 0               | −1,37         | −1,37   | PREDICTED: myosin heavy chain, fast skeletal muscle-like [Salmo salar] |
|        | ssa-miR-74-5p     | 4                | 0              | contig_28976      | −5,05           | 0              | −5,05   | PREDICTED: myosin heavy chain, fast skeletal muscle-like [Salmo salar] |
| Head kidney | ssa-miR-8157-3p  | −1,57           | 0              | contig_122554     | 6,77            | 0              | 5,20    | PREDICTED: transcription factor E2F7-like [Salmo salar] |
|        | ssa-miR-121-1-5p  | −1,57           | 0              | contig_72217      | 6,3             | 0              | 4,83    | PREDICTED: transcription factor E2F7-like [Salmo salar] |
|        | ssa-miR-214-3p    | 0               | 1,8            | contig_21752      | −1,47           | 0              | −1,47   | PREDICTED: transcription factor E2F7-like [Salmo salar] |
|        | ssa-miR-8157-3p   | −2,18           | 0              | contig_46746      | 1,67            | 0              | −0,67   | PREDICTED: transcription factor E2F7-like [Salmo salar] |
|        | ssa-miR-205a-5p   | 0               | 1,99           | contig_45675      | −1,03           | 0              | −1,03   | PREDICTED: transcription factor E2F7-like [Salmo salar] |
|        | ssa-miR-214-3p    | 0               | 1,8            | contig_95191      | 0               | −1,84         | −1,84   | PREDICTED: transcription factor E2F7-like [Salmo salar] |
|        | ssa-miR-194a-3p   | 7,34            | 0              | contig_28663      | −3,31           | 0              | −3,31   | PREDICTED: transcription factor E2F7-like [Salmo salar] |
|        | ssa-miR-462a-3p   | −1,86           | 0              | contig_22955      | 2,08            | 0              | 3,96    | PREDICTED: transcription factor E2F7-like [Salmo salar] |
|        | ssa-miR-122-5p    | 0               | 3,15           | contig_68369      | −1,89           | 0              | −1,89   | PREDICTED: transcription factor E2F7-like [Salmo salar] |
|        | ssa-miR-125a-5p   | 0               | 2,32           | contig_31191      | −1,03           | 0              | −1,03   | PREDICTED: transcription factor E2F7-like [Salmo salar] |
|        | ssa-miR-449a-5p   | 24,47           | 0              | contig_44769      | −2,24           | 0              | −2,24   | PREDICTED: transcription factor E2F7-like [Salmo salar] |
|        | ssa-miR-192b-5p   | 13,39           | 0              | contig_65873      | −2,18           | 0              | −2,18   | PREDICTED: transcription factor E2F7-like [Salmo salar] |
|        | ssa-miR-212a-5p   | −1,83           | 0              | contig_49183      | 2,56            | 0              | 4,40    | PREDICTED: transcription factor E2F7-like [Salmo salar] |

Table 2: Expression modulation of miRNAs and their putative target genes in Atlantic salmon exposed to GSC and SS.
4. Discussion

This study proposes a novel approach to evaluate transcriptome data, where time-series of gene-expression profiling from different tissues and experimental conditions are evaluated. Here, the whole-genome expression-profiling of mRNAs and miRNAs of Atlantic salmon during the smoltification process were explored. Furthermore, the methodology to determine the chromosome gene expression (CGE index) was described. CGE index indicates the coverage differences among two conditions along the Atlantic salmon genome, reflecting differences in the number of mapped reads in specific genomic regions. The study included three different Atlantic salmon tissues with osmoregulation function — gills, intestine, and head kidney. Fish were exposed to gradual salinity changes and salinity shock.

Interestingly, synteny analysis shows high homology among chromosomes with a high CGE index. Furthermore, these chromosomes showed a large number of mobile genetic elements as transposable elements (TE) from Tc1 family and transposase genes. The transposable elements are highly represented in the Atlantic salmon genome. For instance, only the transposable elements of the Tc1-mariner family class represent the 12.89% of the genome [1]. Moreover, this group of transposable elements was associated with signal transduction, regulation of transcription, and defense response [39]. Additionally, it has been reported that transposon expression in rainbow trout is triggered by an external stimulus, such as stress, toxicity, or bacterial antigens [40]. In addition, the growth hormone gene highly associated with the smoltification process, a transposon insertion in a specific isoform of growth hormone gene (gh2) promoter of Atlantic and chinook salmon, has been described. Furthermore, the authors identified a second Tc1 transposon only in the gh2 gene of Atlantic salmon, associated with speciation events [41]. Notably, a previous study performed for our group reported a high presence of TE located near IncRNAs with a putative role in Atlantic salmon seawater adaptation [9]. We suggest a strong regulatory event associated with TEs during Atlantic salmon smoltification. Moreover, this study reports upregulation of TEs in head kidney tissue of Atlantic salmon exposed to GSC and SS. In contrast, gill tissue showed upregulation of TEs in fish exposed to SS, suggesting a putative role of TEs in salmon seawater adaptation. However, additional functional studies are needed to demonstrate the role of TEs during the smoltification process.

Differential expression patterns during parr-smolts transition have been reported by cDNA microarray analysis, highlighting up-modulation of genes associated with growth, metabolism, oxygen transport, and osmoregulation [7]. Additionally, upregulation of genes related to transcription, oxygen transport, electron transport, and protein biosynthesis has been reported [8]. Furthermore, an RNA-Seq study of Atlantic salmon gills showed expression variation during gradual salinity changes, showing higher modulation of processes associated with stress response, cell division and proliferation, tissue development, and collagen catabolic process [9]. Additionally, significant enrichment of genes related to immune response, response to stress, and growth have been described in Atlantic salmon smolt head kidney tissue following seawater transfer [18]. In addition, the authors reported an immune response downregulation after a week in seawater [42]. Moreover, a downregulation of the immune system has been suggested by Shwe, Østbye, Krasnov, Ramberg, and Andreassen [18], associated with the pathogen susceptibility reported for Atlantic salmon during the first period in seawater [42].

Interestingly, a remarkable difference in the number of transcripts differently expressed (DE) among fish exposed to GSC and SS was observed in the present study. It is possible to suggest that salinity shock triggers a higher transcription response than GSC, where the number of DE transcripts was low. GO enrichment analysis of evaluated tissues exhibit BPs associated with protein metabolic process, localization and cell motility, biosynthesis, metabolism, immune response, response to oxidative stress, and response to growth factor. Interestingly, eye morphogenesis was also identified among GO terms identified in gills tissue. In vertebrates, the eyes have a relevant osmoregulatory role [43]. In Coilia nasus, expression variation of eye transcriptomes was observed between hyperosmotic
and hypoosmotic conditions. Among the differentially regulated genes were annotated
genes associated with immune response, metabolism, and transport [43].

Previously, our group characterized long, non-coding RNAs of Atlantic salmon gills
during the smoltification process and reported on the relevance of non-coding RNAs
in regulation of this biological process [9]. Interestingly, the study observed that highly
regulated IncRNAs were located near genes associated with the process as growth, cell
death, catalytic activity, and apoptotic process [9]. The role of miRNAs during smoltification
and the early seawater phase was described by Shwe, Østbye, Krasnov, Ramberg, and
Andreassen [18] in head kidney of Atlantic salmon. The authors reported 71 DE miRNAs
during their evaluation time. Among the relevant miRNAs identified by the authors,
the miRNA from family mir-192 has been associated with hypoxia [44]. In the present
study, the mir-192 family was overexpressed in response to GSC in head kidney and
down modulated in gills of the SS fish group. Another miRNA family associated with
hypoxia [44], and downregulated in the intestine in our study was mir-181. Notably, in
the present study, SSA-miR-499a-5p was up-regulated in gills and head kidney tissues of
Atlantic salmon exposed to GSC. Moreover, from the target analysis, it is possible to suggest
that miRNA has a role in fish growth, with gene collagen as its target. In contrast, miRNA
was downregulated in head kidney of the SS fish group. Similar results were reported
by Shwe, Østbye, Krasnov, Ramberg, and Andreassen [18], who observed a decrease in
ssa-miR-499a-5p expression in Atlantic salmon head kidney during transfer from FW to SW.

Finally, GO enrichment analysis of putative target genes of differently modulated
miRNAs from the GSC group showed a large number of transcripts associated with cellular
process, response to stimulus, cell communication, response to stress, and immune system
process in gills, intestine, and head kidney tissues. A similar process was reported to be
associated with target genes of miRNAs differently expressed in Atlantic salmon head
kidney during seawater adaptation [18]. In contrast, in this study, a small number of target
genes was annotated in response to salinity shock. For instance, biological process as a
response to A4TP, antigens processing a presentation by MHC class, and glycosylation
were identified in this study. These results suggest bounded Atlantic salmon miRNA
modulation in response to salinity shock, compared with gradual salinity change, where
Atlantic salmon miRNAs display regulation of a large number of biological processes.
Functional analysis will be conducted to demonstrate the regulatory role of the highlight
miRNAs identified in this study.

5. Conclusions

The CGE index proposed in this study explains differences in expression profiling
among fish exposed to gradual salinity change and salinity shock during seawater transfer.
Furthermore, a great abundance of transposable elements was identified among the chro-
mosomes, with significant expression differences between groups. This is the first study
showing the harmony among mRNA and miRNA transcription profiles at the genome
level of Atlantic salmon during the smoltification process. The proposed transcriptome
analysis revealed significant differences among tissues, time, and experimental conditions.
In addition, the relevance of miRNA function in the regulation different biological process
is suggested, such as growth, stress response, catabolism, and immune response.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/
10.3390/biology11010001/s1. Figure S1. Experimental design diagram showing the GSC and SS
conditions for Atlantic salmon smolts (n = 30 fish/tank). Sample points (S) are indicated with
red arrow. T1-T4 indicate the sample points. For GSC Atlantic salmon group T1, T2, and T3
correspond a week after salmons were at 10, 20, and 32 PSU, respectively. For the SS group, the
T1 and T2 are 2 and 3 weeks before salinity shock, and T3 is a week after salinity shock (32 PSU).
Figure S2. Histochemistry analyses showing the localization of chloride cells in the gill filament
epithelium. (A) FW gills sample; (B) GSC 10 PSU; (C) GSC 20 PSU; (D) GSC 32 PSU; (E) GSC 32 PSU.
(A) Na+/K+-ATPase positive cells located in the middle region of the superficial interlamellar space
and near the lamellar vascular axis. B-C Na+/K+-ATPase positive cell deeper in the epithelium.
Na+/K+-ATPase positive cells are indicated with black arrows. Magnification 60 ×. Figure S3. RT-qPCR analysis of ATPase-α and ATPase-β subunits in Atlantic salmon gills exposed to GSC and SS. Significant differences between experimental conditions are indicate with asterisk \((p < 0.05)\). Figure S4. Heatmap representation of Atlantic salmon transcriptome for gills, intestine and head kidney tissues exposed GSC and SS conditions. Red and blue colors represent the gene expression levels from high to low transcription values. Figure S5. Cluster gene expression analysis of transposable elements (TEs) during SW transfer in Atlantic salmon gills. (A) Expression values of TE transcripts in Atlantic salmon gradually transferred from FW to SW (GSC) and from FW to salinity shock (SS), respectively. The four gene clusters were detected by K-means algorithm using TPM values. (B) RT-qPCR validation for transposable elements Tcb1 and Tcb2 for GSC and SS, respectively. For GSC Atlantic salmon group T1, T2, and T3 correspond a week after salmons were at 10, 20, and 32 PSU, respectively. For the SS group, the T1 and T2 are 2 and 3 weeks before salinity shock, and T3 is a week after salinity shock (32 PSU). Elongation factor was used as endogenous control for normalize data. Figure S6. Cluster gene expression analysis of miRNAs during SW transfer in Atlantic salmon gills. (A) Expression values of TE transcripts in Atlantic salmon gradually transferred from FW to SW (GSC) and from FW to salinity shock (SS). The four gene clusters were detected by K-means algorithm using TPM values. (B) RT-qPCR validation for ssa-miR-143, ssa-miR-21b, ssa-miR-10d expressed in response to GSC, and ssa-miR-181, ssa-miR-10b and ssa-miR-30d expressed in gills samples exposed to SS. For GSC Atlantic salmon group T1, T2, and T3 correspond a week after salmons were at 10, 20, and 32 PSU, respectively. For the SS group, the T1 and T2 are 2 and 3 weeks before salinity shock, and T3 is a week after salinity shock (32 PSU). Ssa-mir-455-5p was used as endogenous control for normalize data. Figure S7. RT-qPCR validation of candidate miRNAs and their putative target gene. Fold-changes (Log2) of gene expression were calculated using the FW condition as control group. Elongation factor and Ssa-mir-455-5p were used as endogenous control for mRNAs and miRNAs, respectively. Table S1. chr clusters. Table S2. DEG transcripts annotation. Table S3. miRNA prediction. Table S4. Primer list.

Author Contributions: V.V.-M. and C.G.-E. designed the experiment; B.P.B. performed RNA extraction and RT-qPCR analysis. H.E.-S. software analysis. V.V.-M. coordinated the experimental trials. V.V.-M., C.G.-E., B.P.B., G.N.-A., D.V.-M. and J.A.V. participated in writing of the manuscript. All authors have read and agreed to the published version of the manuscript.

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