

Discovery of microRNAs associated with the antiviral immune response of Atlantic cod macrophages

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Abstract:

MicroRNAs (miRNAs) are known to play important immunoregulatory roles in teleosts, although miRNAs involved in the antiviral immune response of Atlantic cod (*Gadus morhua*) were previously uncharacterised. Using deep sequencing and qPCR, the present study was conducted to identify miRNAs responsive to the viral mimic, polyriboinosinic polyribocytidylic acid (pIC) in Atlantic cod macrophages. Macrophage samples isolated from Atlantic cod ($n = 3$) and treated with pIC or phosphate buffered saline (PBS control) for 24 and 72 h were used for miRNA profiling. Following deep sequencing, DESeq2 analyses identified four (miR-731-3p, miR-125b-3-3p, miR-150-3p and miR-462-3p) and two (miR-2188-3p and miR-462-3p) significantly

differentially expressed miRNAs at 24 and 72 h post-stimulation (HPS), respectively. Sequencing-identified miRNAs were subjected to qPCR validation using a larger number of biological replicates ($n = 6$) exposed to pIC or PBS over time (i.e. 12, 24, 48 and 72 HPS). As in sequencing, miR-731-3p, miR-462-3p and miR-2188-3p showed significant upregulation by pIC. The sequencing results were not qPCR-validated for miR-125b-3-3p and miR150-3p as up- and down-regulated miRNAs at 24 HPS, respectively; however, qPCR results showed significant up-regulation in response to pIC stimulation at later time points (i.e. 48 and/or 72 HPS). We also used qPCR to assess the expression of other miRNAs that were previously shown as immune responsive in other vertebrates. qPCR results at 48 and/or 72 HPS revealed that miR-128-3-5p, miR-214-1-5p and miR-451-3p were induced by pIC, whereas miR-30b-3p and miR-199-1-3p expression were repressed in response to pIC.

The present study identified ten pIC-stimulated miRNAs, suggesting them as important in antiviral immune responses of Atlantic cod macrophages. Some pIC-responsive miRNAs identified in this study were predicted to target putative immune-related genes of Atlantic cod (e.g. miR-30b-3p targeting *herc4*), although the regulatory functions of these miRNAs need to be validated by future studies.

Keywords: Poly(I:C), Teleost fish, miRNA, Small RNA sequencing, *Gadus morhua*, Gene expression regulation

Highlights:

- This is the first report on miRNAs involved in antiviral responses of Atlantic cod.
- miR-731-3p and miR-462-3p are co-upregulated by viral mimic in cod macrophages.
- Results support hypothesis on conserved immune roles of miRNA-462/731 in teleosts.
- miR-2188-3p is a late pIC-responsive miRNA in cod macrophages.
- Some identified miRNAs were predicted to target immune-related genes in cod.

1. Introduction

Atlantic cod (*Gadus morhua*) is an economically and ecologically important species in the northern Atlantic (Rosenlund and Halldórsson, 2007). The over-exploitation of some wild Atlantic cod populations has led to increased research on the genomics and aquaculture of this species (Bowman et al., 2011; Johansen et al., 2009; Tørresen et al., 2016). Atlantic cod is susceptible to several viral infections such as viral nervous necrosis (VNN, caused by a member of the genus Betanodavirus) and infectious pancreatic necrosis (IPN, caused by IPNV) (reviewed by Lang et al., 2009; Samuelson et al., 2006). Furthermore, the immune system of Atlantic cod displays several unique features (e.g. the absence of *MHC II* genes in the genome) among teleosts (Star et al., 2011), indicating the evolutionary importance of immunological studies on this fish. A comprehensive understanding of the molecular pathways involved in physiological and immunological responses of Atlantic cod may help to overcome the challenges in health management (e.g. viral diseases) of this species.

Pathogen-associated molecular patterns (PAMPs) can trigger innate immune responses in different species (Bonjardim et al., 2009). Previously, polyriboinosinic polyribocytidylic acid (pIC), a synthetic double-stranded RNA (dsRNA) viral mimic, was utilised in functional genomics studies [using suppression subtractive hybridization (SSH) libraries or DNA microarrays] to characterise the antiviral transcriptome response of cod spleen (Rise et al., 2008) and its association with diet (Booman et al., 2014) and elevated temperature (Hori et al., 2012). In both fishes and mammals, pIC is chiefly recognised via different pattern-recognition receptors (PRRs) [e.g. TLR3 (Toll-like receptor 3) and MDA5 (melanoma differentiation-associated protein 5)] and activates the regulating factors downstream of MyD88 (myeloid differentiation primary response gene 88)-dependent and -independent pathways, resulting in expression of IFNs (interferons) and ISGs

(IFN-stimulated genes) (reviewed by Bonjardim et al., 2009; Langevin et al., 2013; Workenhe et al., 2010; Yu and Levine, 2011). Immune response-mediated gene expression can be regulated through small non-coding RNAs (ncRNAs). MicroRNAs (miRNAs) are processed into 20-24 nt ncRNAs that post-transcriptionally regulate gene expression in different species and play key roles in several biological processes (Chekulaeva and Filipowicz, 2009). The primary miRNA transcripts (pri-miRNAs) are cleaved by Drosha into precursor miRNAs (pre-miRNAs). Thereafter, these are trimmed into the mature or functional miRNAs (5p or 3p miRs) that generally bind to imperfect complementary sequences in the 3'untranslated regions (3'-UTRs) of the target mRNAs, resulting in translational suppression or mRNA degradation (Chekulaeva and Filipowicz, 2009; Winter et al., 2009).

miRNAs are key regulators of antiviral immune responses through various mechanisms (Baltimore et al., 2008; Lodish et al., 2008). With respect to innate immune responses of mammals, pIC stimulation and IFN production elicit miRNA responses via the STAT1 (signal transducer and activator of transcription 1) signalling pathway (reviewed by Sedger, 2013), and IFN-induced miRNAs are suggested to influence the virus-host battle (reviewed by Pedersen et al., 2007; Sedger, 2013). Some mammalian miRNAs (e.g. miR-146 and miR-132) were shown to manage inflammatory responses, following TLR-dependent pathogen recognition and macrophage activation (Pedersen and David, 2008). In addition, several miRNAs (e.g. miR-150) were reported to mediate the differentiation and activation of mammalian immune cells, e.g. macrophages (reviewed by Baltimore et al., 2008). Interestingly, some cellular miRNAs display direct antiviral activities in human (Lecellier et al., 2005). In addition, virus-derived miRNAs were found to negatively regulate antiviral responses and interact with viral replication through targeting host and viral mRNAs (Cullen, 2009).

Small RNA profiling can be applied to improve our understanding of miRNAs involved in antiviral immune responses in teleost fish (Andreassen and Høyheim, 2017). Small RNA deep sequencing previously identified virus-/bacteria-responsive miRNAs in teleosts (Najib et al., 2016; Wang et al., 2016). For example, several differentially expressed miRNAs were identified in olive flounder (*Paralichthys olivaceus*) infected by viral hemorrhagic septicemia virus (VHSV) or megalocytivirus (Najib et al., 2016; Zhang et al., 2014). A total of thirteen miRNAs were associated with viral or bacterial infections in the studied teleost species, indicating that the immune-related responses of these miRNAs are conserved (Andreassen and Høyheim, 2017). While Atlantic cod miRNAs were previously discovered and characterised in early and later life stages (Andreassen et al., 2016; Bizuayehu et al., 2015), there was previously no information on cod miRNAs associated with antiviral immune responses.

Macrophages have crucial functions in innate immune responses of fishes via cytokine expression (e.g. IFNs and ILs), phagocytosis, production of anti-microbial agents [e.g. nitric oxide (NO)], pathogen recognition (e.g. TLRs) and immune regulation (e.g. T-cell activation) (Wiegertjes et al., 2016). Therefore, macrophage studies are of importance to broaden our knowledge of molecular pathways and regulatory mechanisms underlying the antiviral immune responses of fish. The pIC-responsive transcripts (mRNAs) of Atlantic cod macrophages were profiled in a previously published study (Eslamloo et al., 2016). To have a better understanding of antiviral regulators in Atlantic cod, deep sequencing and real-time quantitative polymerase chain reaction (qPCR) were used in the present study to identify the pIC-responsive miRNAs in macrophages of this species.

2. Materials and methods

2.1. Animals

Atlantic cod [1.64 ± 0.14 kg (mean \pm SE)] reared in a 21 m³ tank and optimal conditions (5.26.4°C, 95-110% oxygen saturation and under an ambient photoperiod) at the Dr. Joe Brown Aquatic Research Building (JBARB) of the Ocean Sciences Centre (OSC) were used for the present study. These fish were fed a commercial diet (Skretting, BC, Canada; crude protein 50%, crude fat 18%, crude fibre 1.5%, calcium 3% and phosphorus 1.4%) 3 days per week (i.e. 1% of body mass for each feeding time). The fish used in this study were euthanized, using an overdose of MS222 (400 mg L⁻¹; Syndel Laboratories, Vancouver, BC, Canada), before dissection and cell isolation. Twelve individuals were used in this study in total (6 fish per experiment; see sections 2.3. and 2.4). All procedures in this study were performed under the approval of Memorial University of Newfoundland's Institutional Animal Care Committee, following the guidelines of the Canadian Council on Animal Care.

2.2. Macrophage isolation

Atlantic cod head kidney macrophages were isolated as described in Eslamloo et al. (2016). Briefly, the head kidney of Atlantic cod was removed by dissection and transferred to L-15+: Leibovitz's L-15 culture medium (Gibco, Carlsbad, CA) supplemented with 2 mM L-glutamine, 4.2 mM NaHCO₃, 25 mM HEPES, 1.8 mM glucose, 20 U ml⁻¹ heparin (Sigma-Aldrich, St. Louis, MO) and 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin (Gibco). The head kidney cells were separated using 100 µm nylon cell strainers (Thermo Fisher Scientific, Waltham, MA) and the resulting cell suspension was centrifuged on a discontinuous 25/51% Percoll gradient (GE Healthcare, Uppsala, Sweden) at 300 × g for 40 min at 4°C. The interface layer, enriched in macrophage-like cells, was collected and washed using L-15+ by centrifuging at 300 × g for 15

min at 4 °C. L-15+ medium in the last washing and the subsequent cell culture contained 1% fetal bovine serum (FBS; Gibco) and no heparin. The cells were added into 35 mm culture dishes (Corning, NY) at an equal density of 3×10^7 cells (in 2 ml L-15+) per dish. After overnight (16 h) cell culture at 10°C, the culture dishes were washed 3 times using L-15+ and the non-adherent cells were discarded. Since the majority of the adherent cells in this stage were identified as macrophage-like cells (Eslamloo et al., 2016), these cells are henceforth referred to as macrophages. Atlantic cod macrophages isolated and cultured by this method were shown to have very high viability during cell culture (Eslamloo et al., 2016).

2.3. Macrophage stimulation and sampling at 24 HPS for sequencing

To determine the pIC-responsive miRNAs in Atlantic cod at 24 h post-stimulation (HPS), macrophages were isolated from six individuals, as described in section 2.2, and cultured into 2 culture dishes (3×10^7 cells per 35 mm culture dish) per fish (in a total of 12 culture dishes). The pIC (Sigma-Aldrich) was diluted in phosphate buffered saline (PBS) (Gibco) at 10 mg ml⁻¹ and used as a stock solution. Starting 24 h after cell isolation, the macrophages were exposed to 50 µg ml⁻¹ pIC or PBS (5 µl of pIC solution or PBS per ml of L-15+) at 10°C (Eslamloo et al., 2016). After 24 h of stimulation, the culture medium of samples was removed; then, culture dishes were sealed with parafilm and kept at -80°C until RNA extraction (see Fig. 1 for experimental design).

2.4. Macrophage stimulation and sampling over time for sequencing and qPCR analyses

An experiment was conducted to determine the time-dependent response of cod macrophages to pIC stimulation. Macrophages were isolated from six different individuals (see section 2.2) and seeded into 8 culture dishes (3×10^7 cells per 35 mm culture dish) per fish (48 culture dishes in total). As in section 2.3, the isolated macrophages were stimulated with 50 µg ml⁻¹ pIC or PBS at 10°C.

Samples were collected at 12, 24, 48 and 72 HPS and kept at -80°C before RNA extraction (see Fig. 1 for experimental design).

2.5. Total RNA extraction

Total RNAs of all samples in both experiments (i.e. macrophages stimulated with pIC and timematched controls) were extracted using the *mirVana* miRNA isolation kit (Ambion, Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The purity and concentration of RNAs were checked using NanoDrop spectrophotometry (ND-1000), and 1% agarose gel electrophoresis was used to assess RNA integrity. All RNA samples showed tight 18S and 28S ribosomal RNA bands and A260/280 ratios greater than 1.8. Also, A260/230 ratios of the majority (i.e. 41 out of 48 samples) of the samples were higher than 1.8; it was determined that lower A260/230 ratios did not influence the qPCR assays performed with the templates (see section 2.7.).

2.6. Deep sequencing and analysis of deep sequencing data

The samples were selected for miRNA sequencing of macrophages in both pIC and PBS groups at 24 and 72 HPS, based on the quality and quantity (ranging from 57-405 ng μl^{-1}) of RNA as well as their immune response; the expression of *isg15-1* and *viperin* (see Eslamloo et al. 2016 for primer sequences and qPCR assay method) was assessed in all of the samples (data not shown). Then, macrophage samples (i.e. time-matched pIC and PBS samples from the same individuals) from 3 fish at 24 HPS (i.e. $n = 3$; 6 samples in total; see section 2.3) and 3 fish at 72 HPS (i.e. $n = 3$; 6 samples in total; see section 2.4) that showed high fold-change response of both *viperin* and *isg15* to pIC were selected for sequencing analyses. The library construction and sequencing analyses in this study were carried out at the Norwegian Genomics Consortium's core facility (Oslo, Norway).

The preparation of the 6 libraries for 24 HPS samples (i.e. 3 control and 3 pIC samples from 3 biological replicates) used the Illumina TruSeq Small RNA Library Preparation Kit (Illumina, San Diego, CA), whereas the preparation of the 6 libraries for 72 HPS samples (i.e. 3 control and 3 pIC samples from 3 biological replicates) used the NEBNext® Multiplex Small RNA Library Prep Set for Illumina (New England Biolabs, Inc. Ipswich, MA). Both preparations of libraries were performed with 1 µg total RNA input, following the manufacturers' instructions.

cDNA products of samples in both sequencing experiments (i.e. 24 and 72 HPS) were loaded onto 6% polyacrylamide gels, and the fractions between ~140–160 bp were selected for miRNA sequencing. Twelve small RNA libraries constructed from the 6 macrophage samples (3 pIC and 3 PBS samples from the same individuals) at each time point (12 samples in total at 24 and 72 HPS) were analysed by next generation sequencing using the Illumina Genome Analyzer Iix sequencing platform as in Andreassen et al. (2013). The quality of sequence reads (i.e. fastq files) was checked by the means of FASTQC software. Following removal of adapter-only sequences, the sequence reads were trimmed (i.e. adapter sequences removed). Then, the trimmed sequences were size-filtered to discard the reads shorter than 18 or longer than 24 nucleotides, using the Cutadapt Python Package (Martin, 2011). The sequence reads (i.e. 18 - 24 nt) were mapped to a reference miRNAome (i.e. all of the known mature miRNAs in Atlantic cod) (Andreassen et al., 2016; Bizuayehu et al., 2015), using Novoalign (<http://www.novocraft.com>), and the mapped sequence reads were counted using a Custom-made script in Python. The reads mapped to the mature reference sequences with edit distance of one or less were considered true mature miRNAs.

The resulting data were analysed and the differentially expressed miRNA in pIC-stimulated cod macrophages at 24 and 72 h were identified using DESeq2 package in R (Love et al., 2014). In

DESeq2, the size factor, dispersion and the normalized counts for each sample were estimated. Thereafter, the \log_2 Fold Change was calculated for all the miRNAs in each experiment (i.e. 24 and 72 HPS), and the significant miRNA lists were implemented by DESeq2 (adjusted p value < 0.1). IsomiRs are mature miRNAs that have non-template changes of one nucleotide, mainly at the 3' end of the reference mature miRNA (Nielsen et al., 2012). The presence of isomiR variants (non-template sequences) among the differentially expressed miRNAs was checked through merging reads from the datasets of small RNA sequencing, followed by collapsing the file into unique reads using FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). The file was imported into Sequencher software (Gene Codes, Ann Arbor, MI), and the reads aligned to each of the mature miRNA sequence of the differentially expressed miRNAs. Variant reads showed the length variants of the mature miRNAs were not considered as isomiRs. The identified isomiRs were used for target gene prediction analysis (see section 2.9.).

2.7. qPCR analysis of miRNA expression

The miRNA sequencing results of both experiments (i.e. 24 and 72 HPS) were subjected to qPCR validation using samples from all individuals (i.e. 6 control and 6 pIC samples from 6 biological replicates) in the time-dependent pIC stimulation experiment (section 2.4) at all four sampling points (48 samples in total; Fig. 1). cDNAs were synthesized using 400 ng of total RNA and the miScript II RT Kit (Qiagen, Hilden, Germany) in 20 μ l reactions as recommended by the manufacturer's instructions. The cDNAs were diluted by adding 200 μ l of RNase-free water (Qiagen) for use in the qPCR assays. The sequences of mature miRNAs of interest were used as forward specific primers for assays (Table 1). The primers were provided as desalted and in de-ionized water (100 μ M) by the manufacturer (Sigma-Aldrich). A universal primer, provided by the miScript SYBR Green PCR Kit (Qiagen), was used as a reverse primer in qPCR assays. Two

miRNAs (miR-25-3p and miR-210-5p), suggested as the most suitable normalizers for miRNA expression in Atlantic cod (Andreassen et al., 2016), were used as normalizers in the current study. These normalizers were expressed stably in our qPCR study (i.e. geometric mean of normalizers' C_T less than 0.3 cycles different for time-matched pIC and PBS groups). The A260/230 ratio of some samples (i.e. 7 out of 48 samples) was lower than 1.8; however, this did not affect the qPCR assays, as there was no correlation between the expression of normalizers and A260/230 ratio of the samples (data not shown). The quality (i.e. acceptable amplification efficiency, no amplification in the no-template controls and absence of primer-dimer formation) of all primer pairs (including one specific and one universal primer, see below) in this study were checked using duplicates of a 4-5 point (see Table 1), 3-fold serial dilution of a cDNA template (i.e. a pool of cDNA from 3 individuals in both PBS and pIC groups at 24 and 72 HPS from the time-dependent experiment). qPCR assays for normalizers and miRNAs of interest were performed in duplicate using 12.5 μ l of 2X QuantiTect SYBR Green PCR Master Mix, 2.5 μ l of 10X miScript Universal Primer, 2.5 μ l specific forward primer (10 μ M), 5 μ l RNase-free water (Qiagen), and 2.5 μ l of diluted cDNA template representing 4.5 ng of input total RNA. All qPCR assays were conducted in an AriaMx Real-time PCR System (Agilent Technologies, Santa Clara, CA) using 96-well plates. The PCR program consisted of one cycle of 95 °C for 15 min, and 40 cycles of 94°C for 15 s, 55°C for 30 s and 70°C for 30 s, followed by a final melting point analysis. The performance of qPCR analyses between plates was determined using an inter-plate linker sample and no-template controls. Agilent AriaMx software v1.0 (Agilent Technologies) was applied to obtain C_T (or C_q) values. In Excel, the relative quantity (RQ) values of each miRNA of interest were then calculated (Pfaffl, 2001) using a calibrator, i.e. the individual that showed the

lowest expression (i.e. highest normalized C_T value: $RQ = 1$) of a given miRNA of interest compared to the other samples.

After checking the normality of data using the Kolmogorov-Smirnov normality test, RQ data of each miRNA of interest were analysed by means of a repeated measures two-way ANOVA test. Thereafter, statistically significant differences ($p \leq 0.05$) between time-matched pIC and PBS groups and between different sampling points within each group were identified by Sidak multiple comparisons *post hoc* tests. The qPCR data in this study were analysed using the Prism package v6.0 (GraphPad Software Inc., La Jolla, CA).

2.8. In silico sequence analysis of upstream genome sequence of the pIC-responsive miRNAs

The 1000 bp 5'-flanking regions of the immune-responsive miRNAs in this study were obtained from the Atlantic cod genomic DNA sequence in Ensembl (<http://useast.ensembl.org/index.html>) and Centre for Ecological and Evolutionary Synthesis (CEES: <http://cees-genomes.hpc.uio.no/>) Genome Browsers. The clustered miRNA pairs (i.e. miRNA-462/731, miRNA-30b/e and miRNA-451/144) were analysed by use of the 5'-flanking region of the 5' gene in each pair, while miR-214-1 and miR-125b were analysed as individual miRNAs since there was more than 1000 bp distance between the locations of these miRNAs and other miRNAs in their cluster (Andreassen et al., 2016). Putative transcription factor binding sites (TFBSs) were predicted by the TRANSFAC database (<http://genexplain.com/transfac/>), using the vertebrates profile and the default parameters (i.e. Minimize False Positives) as suggested by the database. The identified putative TFBSs that may play roles in immune responses and showed a core score > 0.8 were selected and presented herein. Moreover, the 1000 bp 5'-flanking regions of ten non-pICresponsive miRNAs (i.e. exhibiting ~1-fold change in sequencing results at 72 HPS) were analysed as described to check the frequency of predicted TFBSs in a set of non-immune related miRNAs.

2.9. Target gene prediction for pIC-responsive miRNAs

The pIC-responsive transcripts (i.e. up- or down-regulated) with immune-related functions previously identified in Atlantic cod macrophages were selected for target gene prediction analysis. Fifty transcripts used for target gene prediction, as well as information on their function and regulation, are listed in Supplemental Table S2 [also see Table 2 in Eslamloo et al., (2016)]. The 3'UTRs of these transcripts [i.e. the contigs used for microarray probe design] were determined using the SeqBuilder software of the Lasergene package (DNASTAR, Madison, WI) and by BLASTx aligning the contigs against NCBI's non-redundant (nr) amino acid sequence database.

The target prediction analysis was performed by RNAhybrid software (Rehmsmeier et al., 2004), using the 10 sequences of the pIC-responsive miRNAs and the partial (for 41 transcripts) or complete (for 9 transcripts) 3'UTR sequences from the pIC-responsive transcripts in Atlantic cod macrophages [see Supplemental Table S2 and Eslamloo et al., (2016)]. In addition, one variant identified as an isomiR of miR-2188-3p was detected and included in the *in silico* predictions.

The RNAhybrid prediction was conducted using the following parameters: no G:U in seed (resulting in the identification of targets with perfect "seed" match), helix constraint 2-8 and max loops 9. Also, to obtain the prediction with high stability, minimum free energy was adjusted to 15 for the RNAhybrid.

3. Results

3.1. Deep sequencing

We used two sets of experimental samples to discover pIC-responsive miRNAs by deep sequencing. Table 2 provides an overview of the read numbers obtained from the deep sequencing

on all the samples used in the present study. The deep sequencing of 24 HPS samples was conducted using TruSeq library preparation. The total number of reads obtained from sequencing for 24 HPS samples ranged from 4.2 to 6.7 million reads. After trimming and filtering, the reads were mapped to the reference and more than 70% of reads from the 24 HPS were mapped into the reference Atlantic cod miRNA datasets (i.e. approximately 1 million reads in each sample).

The preparation of libraries for 72 HPS samples was performed using the NEBNext kit, and the total number of reads for these samples varied between 11.2 to 36.2 million. Following trimming and filtering, the total reads for each sample were used for mapping. Although the number of reads mapped into Atlantic cod miRNAs was more than one million in most of the 72 HPS samples, the percentages of mapped miRNAs were comparatively lower than those of the 24 HPS samples. The sequencing results of all samples are available in the SRA database of NCBI (see accession numbers in Table 2).

3.2. Identification of differentially expressed miRNAs by DESeq2 analysis

DESeq2 analysis (adjusted p value < 0.1) was applied to identify miRNAs differentially expressed between the pIC and the time-matched control groups at 24 and 72 HPS. Table 3 shows the results of DESeq2 analyses at 24 and 72 HPS. DESeq2 analysis on pIC-exposed Atlantic cod macrophages at 24 HPS identified four differentially expressed miRNAs (Table 3). There was an up-regulation in expression of miR-462-3p, miR-125b-3-3p and miR-731-3p in pIC-stimulated samples at 24 HPS. On the other hand, miR-150-3p was found as a downregulated miRNA by pIC at 24 HPS (adjusted p value 0.09). DESeq2 analysis of 72 HPS samples showed two miRNAs significantly induced by pIC stimulation in Atlantic cod macrophages. Similar to the 24 HPS sequencing results, the expression of miR-462-3p was increased in pIC-stimulated Atlantic

cod macrophages at 72 HPS. In addition, the expression of miR-2188-3p in cod macrophages was found to be up-regulated by pIC stimulation at 72 HPS (Table 3).

3.3. qPCR analyses of DESeq2-identified pIC-responsive miRNAs

Table 1 shows the sequence and quality control results (e.g. amplification efficiencies) of the primers used for qPCR assays in this study. Atlantic cod macrophages stimulated by pIC ($n = 6$) and controls ($n = 6$) sampled at four time points (12, 24, 48 and 72 HPS) were used for the qPCR assays (see section 2.4). Five miRNAs identified as differentially expressed by DESeq2 were subjected to qPCR analyses to validate sequencing results using additional biological replicates and to evaluate the time-dependent pIC responses. The qPCR results of pIC- and PBS-exposed groups are illustrated in Figure 2.

The expression of miR-731-3p was significantly up-regulated by pIC stimulation at 24 HPS (1.5fold increase), and it continued to increase at 48 and 72 HPS (2.1- and 2.5-fold, respectively) (Fig. 2A). Similarly, the expression of miR-462-3p increased in cod macrophages over time in response to pIC stimulation, showing a significant difference at 24 HPS (2.6-fold increase; Fig. 2B), with the highest fold-change recorded at 72 HPS (4.1-fold increase). As in the results of DESeq2 analysis at 24 and 72 HPS, miR-462-3p of Atlantic cod macrophages showed the strongest response to pIC among all qPCR-assayed miRNAs.

DESeq2 results identified miR-150-3p as down-regulated by pIC at 24 HPS. This finding was not in agreement with the qPCR assay. The expression of miR-150-3p was not significantly changed by pIC stimulation at 12 and 24 HPS; nonetheless, it was significantly up-regulated by pIC at 48 and 72 HPS (1.7- and 1.9-fold, respectively; Fig. 2C). The increased expression of miR-125b-3-3p found by qPCR at 24 and 48 HPS (1.2- and 1.3-fold, respectively) was not significant. However, this miRNA showed an up-regulation that was significant at 72 HPS (2.2fold; Fig. 2D). qPCR

analyses also validated the DESeq2 results for miR-2188-3p, which showed significantly increased expression in pIC-stimulated cod macrophages at 48 and 72 HPS (1.4- and 1.6-fold, respectively; Fig. 2E). However, the expression of miR-2188-3p was significantly down-regulated within the control group at 72 HPS compared to the 12 and 24 HPS. This indicates that pIC stimulation of Atlantic cod macrophages prevented the time-dependent down-regulation of miR-2188-3p that was observed in the control group at later time points.

3.4. qPCR assays of other selected miRNAs qPCR was also applied to assess the expression of six other miRNAs (i.e. miR-30b-3p, miR-1283-5p, miR-214-1-5p, miR-451-3p, miR-144-3p and miR-199-1-3p) previously found as immunerelated miRNAs in other vertebrates. These miRNAs also showed non-significant changes in expression in the DESeq2 analysis (data not shown). Two of the selected miRNAs were miR30b-3p and miR-128-3-5p, which are known to be IFN-responsive and to have important functions in immune responses of mammals (reviewed by Lodish et al., 2008; Pedersen et al., 2007). In addition, the mature miRNAs from miRNA-214-1/199-1 and miRNA-451/144 gene clusters (Andreassen et al., 2016) were selected for qPCR. These miRNAs are also clustered in mouse (Juan et al., 2009; Rasmussen et al., 2010), and were previously shown to be immuneresponsive in other teleost species [i.e. Japanese flounder (*Paralichthys olivaceus*) and Nile tilapia (*Oreochromis niloticus*)] (Wang et al., 2016; Zhang et al., 2014).

Figure 3 illustrates the results of these six miRNAs at all time points. There was a downregulation in miR-30b-3p of cod macrophages in response to pIC stimulation at all the time points, although a significant difference was only seen at 48 HPS (0.5-fold decrease; Fig. 3A). On the other hand, miR-128-3-5p of Atlantic cod macrophages was significantly up-regulated in response to pIC at 48 and 72 HPS (1.7- and 2-fold increase respectively; Fig. 3B). The qPCR results of miR-30b-3p

and miR-128-3-5p showed a similar direction (i.e. up- or down-regulation) to DESeq2 results at 72 HPS (data not shown).

A significant increase in response to pIC was also revealed for miR-214-1-5p at 48 and 72 HPS compared to the time-matched controls (3.4- and 2.9-fold increase respectively; Fig. 3C).

Further, miR-451-3p showed an up-regulation in pIC-stimulated Atlantic cod macrophages at 72 HPS compared to the time-matched controls (1.7-fold increase; Fig. 3E). A similar fold-change trend, although not significant, was also observed for these miRNAs (miR-214-1-5p and miR451-3p) in DESeq2 results obtained at 72 HPS (data not shown). The expression of miR-199-13p was down-regulated by pIC at 48 HPS (0.7-fold; Fig. 3D). The expression of miR-144-3p (Fig. 3F) was not influenced by pIC.

3.5. In silico analysis of promoter of pIC-responsive miRNAs

We used 1000 bp 5'-upstream regions of the miRNA genes or clustered miRNA genes found as immune-responsive in this study to identify putative immune-related TFBSs. As shown in Supplemental Table S1, *in silico* analyses predicted several TFBSs in the 5'-upstream region of all the miRNAs studied herein. Some immune-related putative TFBSs including IRFs (Interferon regulatory factors), PU.1 (Transcription factor PU.1), ATFs (Activating transcription factors) and STAT (Signal transducer and activator of transcription) motifs were predicted upstream of miRNAs sequences. The binding motif for STAT1, known also as IFNG-activated sequence (GAS), was identified in the proximal promoter region of the miRNA-462/731 cluster, suggesting that the transcriptional regulation of this cluster may be mediated by IFNs (Supplemental Figure S1). However, we also found some immune-related potential TFBSs in the 5'-upstream regions of ten non-pIC-responsive miRNAs (i.e. miRNAs with ~1-fold change in response to pIC); indeed, there was no correlation between the frequency of predicted

immunorelated TFBSs and immune responsiveness of miRNAs (data not shown). For example, the proximal promoter regions of 2 and 3 non-pIC-responsive miRNAs (out of 10) showed putative binding sites for STAT1 and IRF3, respectively (data not shown). This suggests that *in silico* based prediction of TFBSs may be influenced by randomness due to short sequences of the putative binding motifs. Therefore, these results should be regarded with caution, and the true regulatory effects of predicted TFBSs herein must be experimentally validated in the future.

3.6. Target gene prediction for pIC-responsive miRNAs

The immune-relevant pIC-responsive transcripts in Atlantic cod macrophages [see Supplemental Table S2 and Eslamloo et al., (2016)] were included in the *in silico* analysis to predict whether any of these may be targeted by the pIC-responsive miRNAs. Table 4 shows the results of these *in silico* predictions. In total, 3'UTR sequences of 7 transcripts showed target sites for 5 different miRNAs. The predicted targets were microarray-identified transcripts up-regulated in Atlantic cod macrophages by pIC at 24 HPS [see Table 2 in Eslamloo et al., (2016)]. *cytotoxic and regulatory T-cell protein precursor* and *interferon stimulated gene 15-3* had target site matches with miR-451-3p and miR-128-3-5p, respectively. On the other hand, miR-199-1-3p was predicted to target both *bloodthirsty-1* and *bloodthirsty-3* transcripts. In addition, miR-2188-3p and its isomiR variant were identified to target the 3'UTR of *CXC chemokine*. The pIC-induced *herc4* (*probable E3 ubiquitin-protein ligase HERC4-like*) was identified as a putative target for miR-30b-3p that was down-regulated (significant suppression at 48 HPS) by pIC in the present study, suggesting the possible role of miR-30b-3p in the regulation of *herc4*.

4. Discussion

In the present study, differential expression analyses (DESeq2) of small RNA sequencing results were used to discover miRNAs potentially involved in the antiviral immune response of Atlantic cod macrophages. The library preparations of 24 HPS and 72 HPS samples were performed using different kits. The variations observed between two time points in number of reads mapped to miRNAs may have contributed to the inter-experiment variations in the total and miRNA mapped reads, but it did not influence the time-matched comparisons between pIC and control groups at 24 HPS and 72 HPS. However, the lower number of reads mapped to miRNAs in the pIC-treated samples compared to time-matched controls at 72 HPS may have affected the sensitivity of detection of differentially expressed miRNAs at this time point (Campbell et al., 2015). Some significant pIC-responsive miRNAs (e.g. miR-731-3p, miR-128-3-5p, miR-214-15p) detected by qPCR at 72 HPS were not identified as differentially expressed by DESeq2 analysis at this time point. This may be attributed to the lower percentage of mapped-miRNAs reads in pIC samples at 72 HPS and a larger number (i.e. 6 replicates in qPCR vs. 3 replicates in sequencing) of samples subjected to the qPCR analysis. The differences between pIC and control samples observed only within the second experiment (72 HPS) may be caused by various factors such as the pIC-dependent molecular and cellular responses. Nonetheless, our previous studies showed that pIC does not influence cell death of Atlantic cod macrophages at 24 and 48 HPS (Eslamloo et al., 2016) or 72 HPS (data not shown), and cell death remains under 3% in both stimulation groups at all sampling points.

In the present study, DESeq2 analyses identified five differentially expressed miRNAs at the two time points (24 HPS and 72 HPS). qPCR results showed a time-dependent up-regulation by pIC for miR-731-3p and miR-462-3p, starting at 24 HPS with a fold-change peak recorded at 72 HPS.

In Atlantic cod (Andreassen et al., 2016), these miRNAs were found to be clustered in the genome and potentially co-transcribed as in other teleost species, i.e. rainbow trout (*Oncorhynchus mykiss*) (Schyth et al., 2015), Atlantic salmon (Andreassen and Høyheim, 2017) and zebrafish (*Danio rerio*) (Thatcher et al., 2008). These two teleost-specific miRNAs have been reported as virus- and bacteria-responsive in several teleost species (Andreassen and Høyheim, 2017). Similar to the findings of the current study, miR-731 and miR-462 were shown to be up-regulated in virus-challenged teleosts e.g. in Japanese flounder challenged with megalocytivirus infection (Zhang et al., 2014), rainbow trout challenged with VHSV, RTL-W1 (i.e. rainbow trout liver cell line) stimulated with pIC for 48 h (Schyth et al., 2015) and Atlantic salmon challenged with salmonid alphavirus (SAV) (Andreassen et al., 2017). In addition, these miRNAs were up-regulated in the muscle of VHSV-vaccinated rainbow trout in a timedependent manner, i.e. induction at 1 day post-injection (DPI) and peaking at 21 DPI (Bela-ong et al., 2015).

We identified a putative GAS binding site in the proximal promoter of the Atlantic cod miRNA462/731 cluster along with other immune-relevant putative TFBSs (e.g. IRFs), although the activation of the predicted motifs herein need to be validated by future studies. Upon induction of IFNG-dependent pathway, STAT1 homodimer, termed as IFNG-activated factor (GAF), binds to GAS and increases the expression of IFNG-activated transcripts (Bonjardim et al., 2009). Similar motifs were identified in the promoter region of the miRNA-462/731 cluster in rainbow trout (Schyth et al., 2015) and Atlantic salmon (Andreassen et al., 2017), and the potential conservation among species suggests that these motifs may be associated with immune responsiveness of the miRNA-462/731 cluster. These miRNAs also showed a time-dependent up-regulation in response to injection of IFNG-expressing plasmids (Bela-ong et al., 2015). In our previously published study (Eslamloo et al., 2016), the induction of IFNG pathway and its

associated genes (e.g. activation of *stat1*) was seen in pIC-treated cod macrophages at 24 HPS, which is similar to the time-dependent up-regulation of miRNA-462/731 cluster herein. Therefore, this induced miRNA cluster may have an IFN-dependent expression in Atlantic cod macrophages. The megalocytivirus-induced miR-731-5p of Japanese flounder was revealed to enhance the early-stage viral replication and inhibit virus-induced apoptosis of the splenocytes, and to suppress IRF7 expression by targeting its transcript (Zhang et al., 2016). In our previous study (Eslamloo et al., 2016), *irf7* expression significantly decreased by sampling time in pIC-stimulated cod macrophages at 48 HPS compared to those at 24 HPS. However, we used *in silico* analysis for searching target sites with perfect seed matches, but did not identify any target motif for miR-462/miR-731 (5p or 3p) in the 3' UTR of Atlantic cod *irf7*, which was previously characterised (Inkpen et al., 2015). Taken together, it seems that these miRNAs co-transcribed from the miRNA-462/731 cluster have some conserved immune-related functions in teleost species. However, they may target different immune-relevant transcripts (e.g. viral- vs. bacterial-responsive transcripts) and play various roles in different teleosts (Andreassen et al., 2017; Xu et al., 2015; Zhang et al., 2016).

As identified by deep sequencing at 72 HPS and confirmed by qPCR assay, the expression of miR-2188-3p was up-regulated in response to pIC stimulation at 48 and 72 HPS. As found herein, miR-2188-5p of olive flounder (*Paralichthys olivaceus*) and Atlantic salmon was responsive to VHSV and SAV infections, respectively (Andreassen et al., 2017; Najib et al., 2016). However, the immunoregulatory function of this miRNA remains undetermined.

In the present study, individual-dependent variations between the experiments may have affected the qPCR results of miR-125b-3-3p (see first paragraph of discussion). Nonetheless, qPCR showed an up-regulation for miR-125b-3-3p in pIC-stimulated cod macrophages at 72 HPS, which was similar to its fold-change trend seen in 72 HPS sequencing. Likewise, the expression of Japanese

flounder miR-125b increased 6 days post-megalocytivirus infection (Zhang et al., 2016). In higher vertebrates, miR-125b increased the pIC-triggered expression of *ifna/b* in airway epithelial cells of human (Zhang et al., 2012). Collectively our findings, along with previous results from different species, suggest that miR-125b may play some evolutionarily conserved immune-related functions.

In contrast to our sequencing results at 24 HPS, qPCR showed no significant difference between pIC and control groups in expression of miR-150-3p at 24 HPS, and this miRNA was upregulated in response to pIC stimulation at 48 and 72 HPS. We used the cod macrophage stimulated with pIC over time (i.e. the second experiment) for qPCR validation of sequencing at both 24 and 72 HPS. Hence, the differences between qPCR and sequencing results may arise from individual-dependent variations of pIC responses in the first and second experiments. Mammalian miR-150 is a well-characterised regulator of lymphocyte development, notably Bcell maturation (Baltimore et al., 2008). miR-150 was a hypoxia-induced miRNA in zebrafish larvae (Huang et al., 2015), but its function in teleost antiviral responses is yet to be determined.

In addition to the miRNAs discovered by DESeq2 analysis, five miRNAs showed significant responses to pIC by qPCR (Fig. 3). The majority of these qPCR-assayed miRNAs showed the same fold-change direction (i.e. up- or down-regulation) to deep sequencing results at 72 HPS (data not shown). Atlantic cod miR-30b-3p and miR-128-3-5p were significantly down- and upregulated, respectively, in pIC-treated macrophages at later time points. Moreover, miR-30b-3p was predicted to target *herc4*, which was an induced transcript by pIC in Atlantic cod macrophages at 24 and 48 HPS (Eslamloo et al., 2016). With respect to inverse correlation of expression of miRNAs and their target transcripts (Huntzinger and Izaurralde, 2011), the opposite regulation of miR-30b-3p and its putative target suggests the potential influence of this miRNA on transcript level of *herc4*. Mammalian miR-30 and miR-128 are IFNB-inducible miRNAs, suggesting their

potential contribution in IFNB-dependent antiviral activity (reviewed by Lodish et al., 2008; Pedersen et al., 2007). The immunomodulatory features of miR-30 and miR-128 remain undescribed in fish species.

Atlantic cod miR-451-3p was significantly up-regulated in pIC-exposed macrophages at 72 HPS, although the expression of miR-144-3p, located in the same miRNA gene cluster, did not significantly change by pIC over time. Similarly, small RNA sequencing identified miR-451 to be a late virus-inducible miRNA in Japanese flounder (Zhang et al., 2014). As in zebrafish (Pase et al., 2009) and mouse (Rasmussen et al., 2010), miRNA-451 and miRNA-144 are clustered together in the genome of Atlantic cod (Andreassen et al., 2016). Mammalian miRNA-451/144 cluster was reported to play a crucial role in the late erythroblast maturation, although the influence of miR-451 on the target gene was stronger than that of miR-144 (Rasmussen et al.,

2010). Zebrafish miR-451 expression increased the maturation of erythroid cells (Pase et al., 2009).

In the current study, miR-214-1-5p of Atlantic cod was significantly induced by pIC at 48 and 72 HPS. Contrary to this expression pattern, miR-199-1-3p was only down-regulated by pIC at 48 HPS. As reported in mammals (Juan et al., 2009), Atlantic cod miR-214-1 and miR-199-1 are located in the same gene cluster, but they are not closely located (i.e. more than 3000 bp distance) together in this cluster (Andreassen et al., 2016). Some immune-related functions of miR-214, e.g. inhibition of tumour cells (Qiang et al., 2011), were previously explained in the higher vertebrates. The expression of miR-214 was up-regulated in *Streptococcus agalactiae* challenged Nile tilapia at 48 and 72 hours post-infection (Wang et al., 2016). Further studies are needed to determine the immunomodulatory functions of miR-451 and miR-214 in Atlantic cod.

Despite being closely located in the same gene clusters, miR-144-3p and miR-451-3p did not display a co-expression pattern in Atlantic cod macrophages. The post-transcriptional regulation

of miRNAs was demonstrated (reviewed by Cullen, 2004) to provoke tissue- and developmental stage-specific expression of mammalian miRNAs through Drosha-mediated processing of premiRNA (Obernosterer et al., 2006; Thomson et al., 2006). Correspondingly, differential expression of the miRNAs located in the same gene clusters seen herein may be attributed to post-transcriptional regulation.

In silico analysis predicted target transcripts for pIC-responsive miRNAs identified herein (Table 4). The miRNAs showed no reverse regulation with their putative targets, except for miR-199-13p and miR-30b-3p at 48 HPS. miRNAs have been well-documented to cause both translational repression and/or transcriptional degradation (Chekulaeva and Filipowicz, 2009; Huntzinger and Izaurralde, 2011). We acknowledge that there may be some false positives among the predicted targets. However, while some pIC-responsive miRNA in the present study showed the same fold-change direction with their putative targets, these miRNAs may influence their putative targets at the translational level. Furthermore, the same fold-change direction of a true miRNA target pair may be explained by contribution of the miRNAs to a fine tuning of the immunestimulated transcripts. In this regulating pathway, the expression of miRNAs increases along with the transcripts (e.g. through an immune-activated transcription factor) to ensure a balanced immune response, leading to protecting the host and minimizing the immune response damages by suppressing the inflammatory responses (Andreassen and Høyheim, 2017). An improved version of the Atlantic cod genome has been recently published (Tørresen et al., 2017). However, the majority of Atlantic cod transcripts are not yet fully-characterised and the 3' UTRs for a large number of transcripts remained unsequenced; therefore, *in silico* target predictions against the currently available Atlantic cod transcriptome do not provide comprehensive information on putative targets of the pIC-responsive miRNAs. Further studies at the gene and protein expression

levels are needed to determine the correlation between pIC-responsive miRNA in cod macrophages and their putative targets.

In conclusion, the present study identified 10 pIC-responsive miRNAs in Atlantic cod macrophages. Three of these, miR-731-3p, miR-462-3p and miR-2188-3p are teleost-specific miRNAs up-regulated by viral mimic stimulation. This suggests that the miRNA-462/731 cluster and miR-2188 may have similar functions in antiviral immune responses in Atlantic cod and other teleosts. The ten identified miRNAs in the present study may be involved in gene expression regulation of immune responses. In future research, the miRNAs identified in the current study could be functionally characterised using *in vitro* analyses (e.g. gene silencing or overexpression in cell assays). Thus, while the present study was the first report on the immuneresponsive miRNAs in Atlantic cod, further studies need to be conducted in order to understand the interaction between cod miRNAs and antiviral responses of Atlantic cod.

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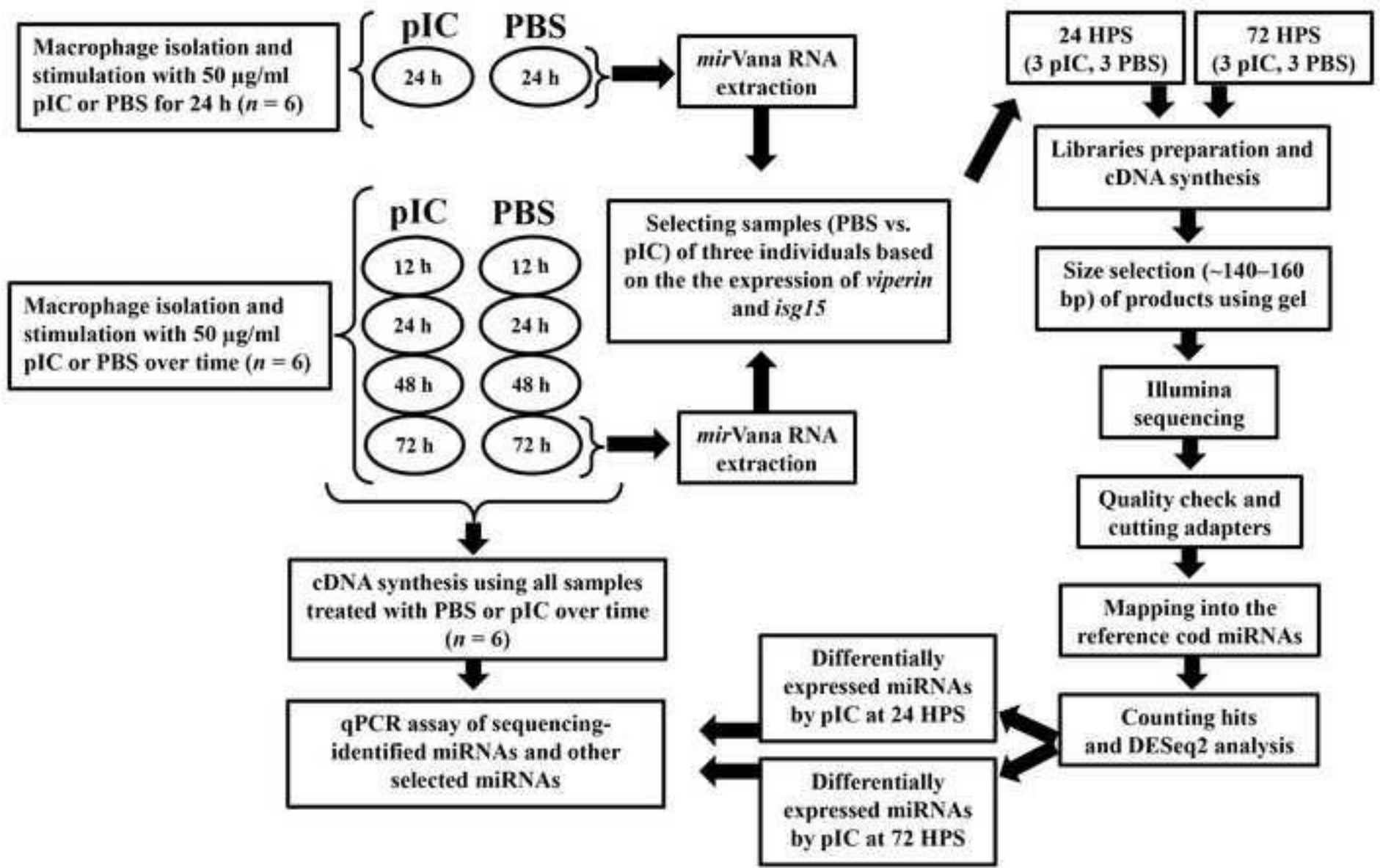
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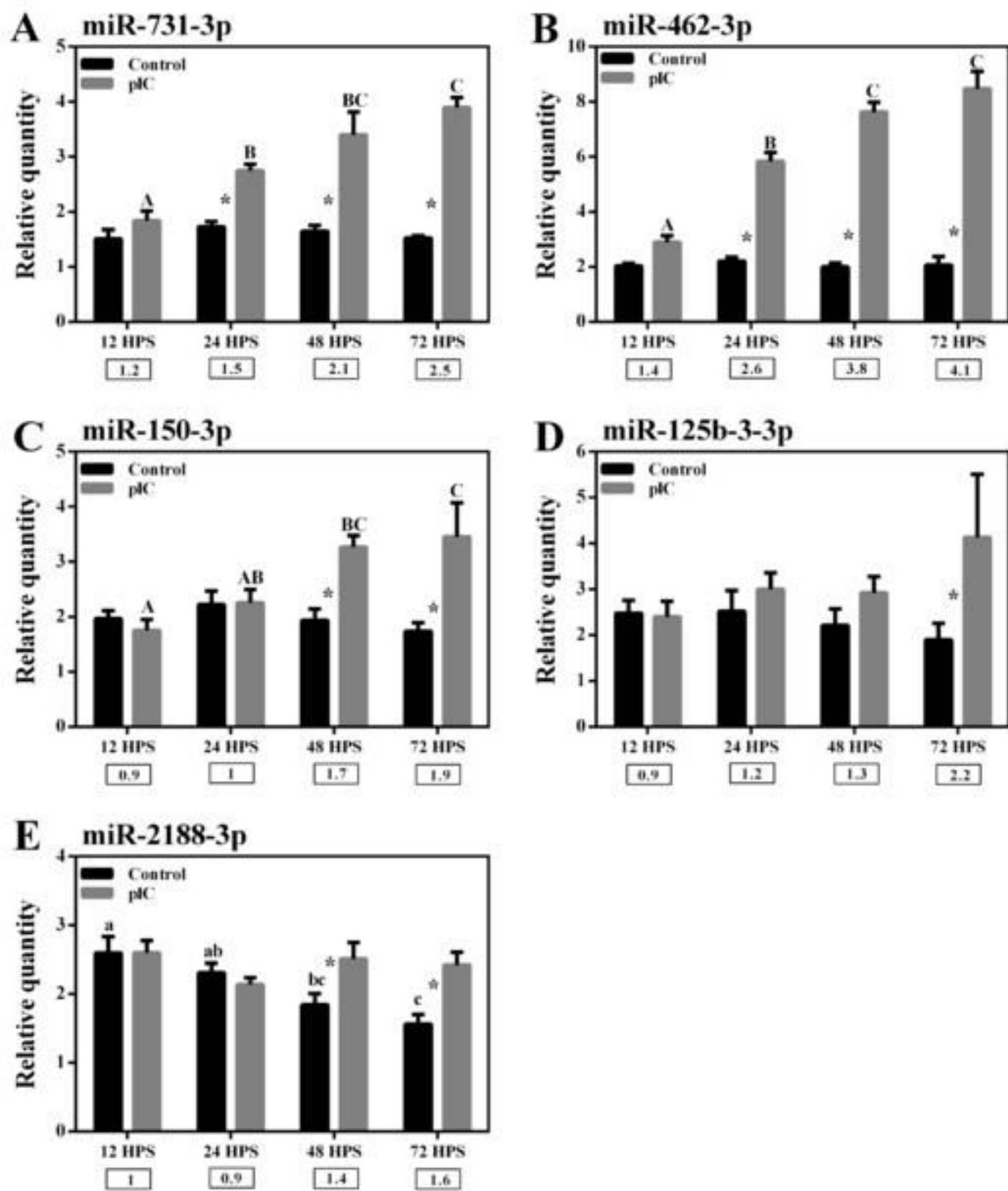
Fig. 1. Overview of experimental design. Macrophages were isolated from six Atlantic cod in each experiment (12 individual in total), and were stimulated with pIC. The control and pIC samples from 3 individuals in each experiment were used for deep sequencing, and the differentially expressed miRNAs were identified using DESeq2 analyses. qPCR analyses were performed using all of the samples (6 fish) in the second experiment.

Fig. 2. qPCR results for pIC-responsive miRNAs identified by sequencing. Data are presented as mean \pm SE. An asterisk indicates significant difference ($p < 0.05$) between timematched pIC and control (PBS) groups. Different letters (lower-case for control and upper-case for pIC) represent significant differences within a group over time. The fold-change (pIC/control) for each time point is shown below the figures.

Fig. 3. qPCR results for other selected miRNAs. Data are presented as mean \pm SE. An asterisk indicates significant difference ($p < 0.05$) between time-matched pIC and control (PBS) groups. Different letters (lower-case for control and upper-case for pIC) represent significant differences within a group over time. The fold-change (pIC/control) for each time point is shown below the figures.

Figure 1
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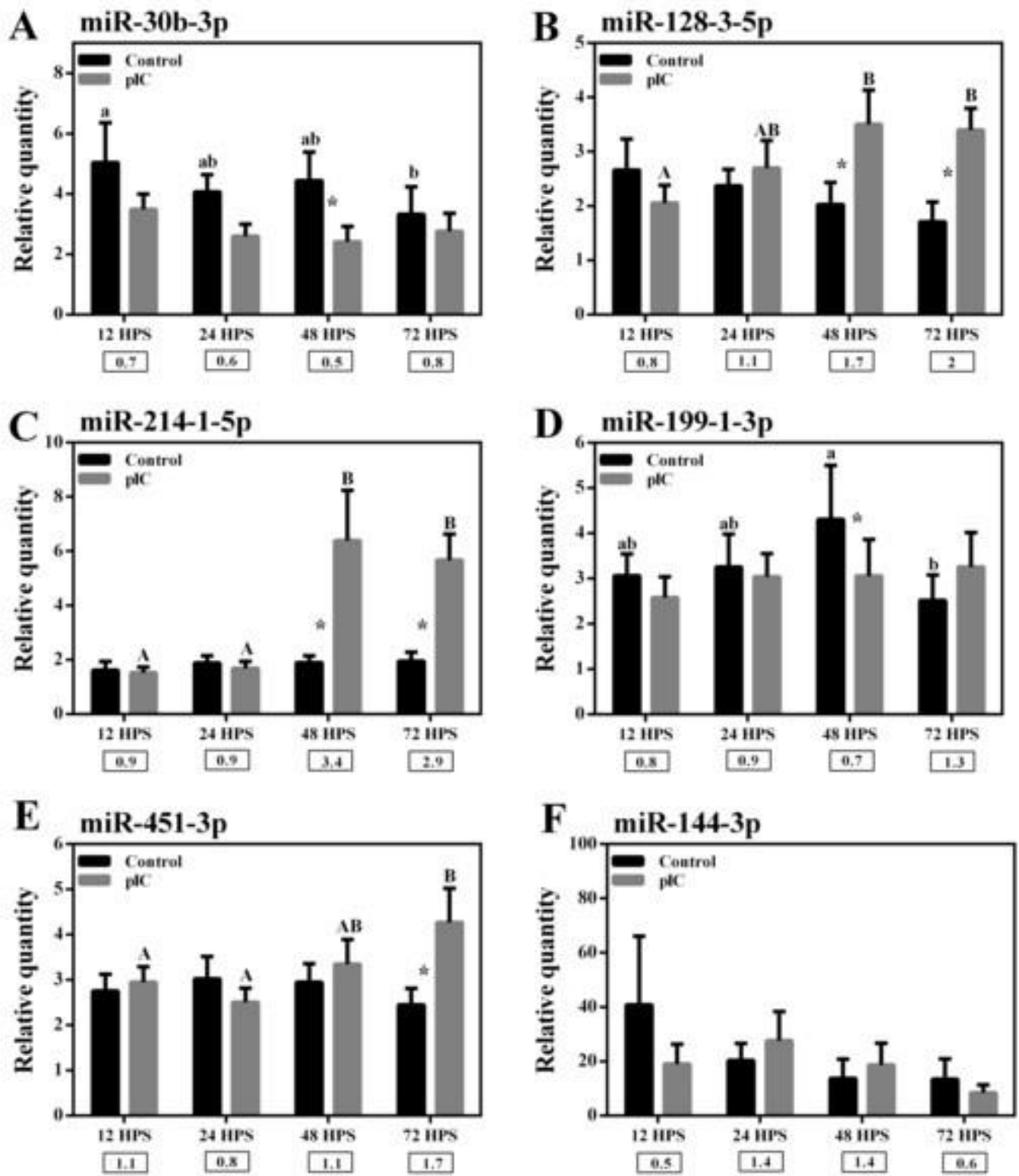


Table 1. qPCR primers

Table 4

Sequencing-identified miRNAs	Primer sequence 5' to 3' ¹	R ²	Amplification efficiency (%) ²
miR-731-3p	ACCGGGAATCTCGTGTTCAGCTA	0.997	83.5
miR-462-3p	GCTGGTTATGGCGTCCGTT	0.997	85.1
miR-150-3p	CGCTGGGCAGGCTTTGGGGGGCG	0.994	82.6
miR-125b-3-3p	ACGGGTTGGGTTCTTGGGAGCT	0.894	80.7
miR-2188-3p	GCTGTGTGGGGTCAGACCTATC	0.991	83.5
Other selected miRNAs			
miR-30b-3p	CTGGGAGAGGGGTGTTTATGCT	0.999	81.2
miR-128-3-5p	GGGGGCCGTTACACTGTCAGAGA	0.953	80.1
miR-214-1-5p	TGCCTGTCTACACTTGCTGTGC	0.985	81.7
miR-451-3p	TAGCAATGGTAAGGGTTC	0.988	88.1
miR-144-3p	CTACAGTATAGATGATGTACT	0.992	102.1
miR-199-1-3p	ACAGTAGTCTGCACATTGGTT	0.994	96.4
Normalizers			
miR-25-3p	CATTGCACTTGTCTCGGTCTGA	0.999	92.1
miR-210-5p	AGCCACTGACTAACGCACATTG	0.999	88.7

¹ Mature miRNA sequences were used as forward specific primers, whereas a universal primer was used as a reverse primer (see Section 2.7). All primers showed no amplification in the no-template controls and generated an amplicon with a single melting peak. However, the melt curves of miR-125b-3-3p and miR30b-3p were slightly wider than those of the other assayed miRNAs.

² The amplification efficiencies of miR-150-3p, miR-125b-3-3p, miR-2188-3p and miR-214-1-5p were determined using 4-point serial dilutions of cDNA, whereas 5-point serial dilutions were used for the other miRNAs.

Table 2. Overview of the samples used for deep sequencing and the sequencing results

Sample ID ¹	Sample	Library	Total	Trimmed Reads reads ²³	miRNAs ³	Accession ⁴
Fish1a-24-C	Control, 24 HPS	TruSeq	5,143,154	1,401,169	1,016,914	SRR3884814
Fish1a-24-P	pIC, 24 HPS	TruSeq	6,695,993	1,354,795	966,023	SRR3884815
Fish2a-24-C	Control, 24 HPS	TruSeq	6,511,402	1,318,259	1,021,423	SRR3884824
Fish2a-24-P	pIC, 24 HPS	TruSeq	6,066,301	1, ⁴ 56,324	1,274,926	SRR3884828
Fish3a-24-C	Control, 24 HPS	TruSeq	4,213,889	1,193,053	970,455	SRR3884830
Fish3a-24-P	pIC, 24 HPS	TruSeq	5,450,651	1,646,542	1,200,154	SRR3884832
Fish1b-72-C	Control, 72 HPS	NEBNext	13,645,051	5,078,178	3,947,280	SRR3884833
Fish1b-72-P	pIC, 72 HPS	NEBNext	24,117,460	5,283,583	1,935,385	SRR3884836
Fish2b-72-C	Control, 72 HPS	NEBNext	13,674,604	6,821,307	5,675,852	SRR3884834
Fish2b-72-P	pIC, 72 HPS	NEBNext	36,256,043	8,322,376	622,611	SRR3884837
Fish3b-72-C	Control, 72 HPS	NEBNext	11,205,514	6,350,751	5,363,422	SRR3884835
<u>Fish3b-72-P</u>	<u>pIC, 72 HPS</u>	<u>NEBNext</u>	<u>28,057,048</u>	<u>7,108,218</u>	<u>1,143,310</u>	<u>SRR3884866</u>

¹ Sample ID: Similar fish numbers (e.g. Fish1a) indicate pIC and PBS-matched samples isolated from the same individuals. The “a” represents individuals from experiment 1 (section 2.3), and “b” shows individuals from experiment 2 (section 2.4).

² Total number of reads after cutting adapters and filtering reads by size (18-25 bp).

³ Reads mapped to the reference (i.e. all known mature miRNAs of Atlantic cod).

⁴ Accession number of sequencing results for each samples submitted to the NCBI SRA database (<http://www.ncbi.nlm.nih.gov/sra>).

Table 3. The responsive miRNAs identified in pIC-stimulated Atlantic cod macrophages at 24 and 72 HPS

	Base mean ¹	Log ₂ fold-change ²	lfcSE ³	Stat ⁴	<i>p</i> value ⁵	<i>p</i> adj ⁶
<u>Identified at 24 HPS</u>						
miR-731-3p	408.17	0.89	0.16	5.389	7.05E-08	1.64E-05
miR-125b-3 -3p	42.29	1.31	0.32	4.090	4.30E-05	0.005
miR-150-3p	36.35	-0.97	0.29	-3.244	0.001	0.091
miR-462-3p	33.32	1.32	0.42	3.144	0.001	0.096
<u>Identified at 72 HPS</u>						
miR-2188-3p	749.63	0.90	0.20	4.388	1.140E-05	0.004
miR-462-3p	36.28	1.33	0.33	4.019	5.835E-05	0.010

¹ The mean of normalized read counts for all of the samples in the given time point.

² Log₂-transformed fold-change (pIC/control) as implemented by DESeq2.

³ Log₂-fold change Standard Error

⁴ Wald statistic (i.e. pIC vs. control)

⁵ Wald test *p* value

⁶ Adjusted *p* values

Table 4. Predicted target genes for pIC-responsive miRNAs in Atlantic cod macrophages

Predicted target gene ¹	Probe ID ²	Regulation ¹	miRNAs
<i>interferon stimulated gene 15-3</i>	38611	Up-regulated gene	miR-128-3-5p
<i>CXC chemokine</i>	36483	Up-regulated gene	miR-2188-3p ²
<i>probable E3 ubiquitin-protein ligase herc4-like</i>	43797	Up-regulated gene	miR-30b-3p
<i>cytotoxic and regulatory T-cell protein precursor</i>	36797	Up-regulated gene	miR-451-3p
<i>optineurin</i>	38670	Up-regulated gene	miR-128-3-5p
<i>bloodthirsty-1</i>	40261	Up-regulated gene	miR-199-1-3p
<i>bloodthirsty-3</i>	43099	Up-regulated gene	miR-199-1-3p

¹ Gene names presented are based on BLASTx hit annotation results in Eslamloo et al. (2016).

² This indicates the identifier (ID) associated with a given microarray probe and the contig used for designing the probe (see Eslamloo et al., 2016).

¹ The expression response of each gene in pIC-stimulated Atlantic cod macrophages as found in Eslamloo et al. (2016).

² This miRNA was identified to have an isomiR variant (sequence 5' - 3': GCTGTGTGGGGTCGGACCTATC), and both miR-2188-3p and its isomiR were predicted to target *CXC chemokine*.

The full list of the genes subjected to the target prediction analyses and their putative functions are presented in Supplemental Table S2.

