# MOLECULAR BASIS OF ANTIVIRAL IMMUNE RESPONSES IN TELEOST FISH

by

**©Khalil Eslamloo**

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## ABSTRACT

In addition to the importance of Atlantic cod (*Gadus morhua*) and Atlantic salmon (*Salmo salar*) for fisheries or aquaculture of several countries including Canada, these species are valuable for comparative immunological studies. The Atlantic cod possesses a unique immune system among many teleosts, and the Atlantic salmon may serve as a molecular model for genomics studies in salmonids. Several aspects of the immune response in these species are yet to be determined, and a broader picture of the immune responses of Atlantic cod and Atlantic salmon may enhance the health management of wild or farmed populations of these species. With respect to the importance of macrophages in innate immune responses, the objectives of my thesis were to use a macrophage model and genomics approaches to enhance our understanding of the antiviral immune response of Atlantic cod, and to profile the transcriptome of the dietary fatty acid-dependent and antiviral responses of Atlantic salmon.

In Chapter 2, I used microarray and quantitative reverse transcription-polymerase chain reaction (qPCR) analyses to identify and validate several antiviral biomarker genes encoding receptors, signal transducers, transcription factors and immune effectors in Atlantic cod macrophages. This study revealed that the antiviral immune responses of Atlantic cod macrophages may be activated downstream of RIG-I-like receptor (RLR)- and Toll-like receptor (TLR)-dependent pathways. Chapter 3 fully characterised Atlantic cod *viperin*, one of the putative interferon-stimulated genes (ISGs) identified in Chapter 2. I found Atlantic cod *viperin* to be an evolutionarily conserved gene with an antiviral response that is regulated through endosomal-TLRs-, protein kinase R (PKR), mitogen-

activated protein kinase (MAPK)- and interferon (IFN)-mediated pathways. Using deep sequencing and gene expression analysis, Chapter 4 profiled the immune-related microRNAs (miRNAs) of Atlantic cod macrophages, and discovered both evolutionarily-conserved and teleost-specific miRNAs playing roles in the antiviral immune response of macrophages of this species. Concerning the immune-related functions of omega (n)-3 and n-6 fatty acids, Chapter 5 used an *ex vivo* model to evaluate if varying levels of dietary n-3 and n-6 fatty acids influence the antiviral responses of Atlantic salmon macrophage-like cells (MLCs). Chapter 5 showed that different levels of dietary n-3 and n-6 fatty acids change the fatty acid composition of head kidney leukocytes, and it identified fatty acid-dependent immune-related biomarkers in Atlantic salmon MLCs. Moreover, this chapter identified a large number of biomarker transcripts activated by RLR, TLR, MAPK and IFN pathways during the antiviral response of Atlantic salmon MLCs.

This thesis contributed significantly to the development of a comprehensive picture of the genes, non-coding RNAs and regulating pathways involved in antiviral immune responses of teleosts. In addition, my thesis enhances the general understanding of the immunomodulatory functions of dietary n-3 fatty acids in Atlantic salmon. The novel results generated by this thesis contribute molecular biomarkers that may be used in future immunological studies to aid in the development of aquafeeds and other health management tools for teleosts.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| 2-AP | 2-Aminopurine |
| AA | Amino acid |
| ACL | Atlantic cod larvae cell line |
| AcOH | Glacial acetic acid |
| ACTB | Beta-actin |
| AKT/PKB | Protein kinase B |
| AP1 | Transcription factor AP1 |
| aRNA | Amplified RNA |
| APC | Antigen-presenting cell |
| ASAL | Formalin-killed atypical *A. salmonicida* |
| ATF3 | Cyclic AMP-dependent transcription factor ATF-3 |
| ATF | Activating transcription factor |
| BAD | Bcl-2-associated death promoter |
| BATF3 | Basic leucine zipper transcription factor, ATF-like 3 |
| BLAST | Basic local alignment search tool |
| bp | Base pair |
| BP | Biological process |
| BTY | Bloodthirsty |
| C | Cellular component |
| CARD | Caspase activation and recruitment domains |
| CASP | Caspase |
| CBA | *Carassius auratus* blastulae embryonic |
| CCL13 | CC motif chemokine 13 |
| CCR7 | CC chemokine receptor 7 |
| CD | Cluster of differentiation |
| CD209 | CD209 antigen-like protein |
| cDNA | Complementary DNA |
| CEES | Centre for ecological and evolutionary synthesis |
| CFLAR | CASP8 and FADD-like apoptosis regulator |
| cGRASP | Genomic research on all salmonids project |
| CHQ | Chloroquine |
| CMKLR1 | Chemokine receptor-like 1 |
| CMPK2 | Cytidine monophosphate (UMP-CMP) kinase 2 |
| CREM | cAMP-responsive element modulator-like |
| CSF1R | Macrophage colony-stimulating factor 1, receptor |
| $C_T$ | Threshold cycle |
| CTS | Cathepsin |
| CTSA | Lysosomal protective protein /Cathepsin A |

| | |
|---|---|
| CXCR3 | C-X-C chemokine receptor type 3 |
| CYPA | Cyclophilin a |
| CYTIP | Cytohesin-interacting like protein |
| DC | Dendritic cell |
| DC-SIGN | DC-specific intercellular adhesion molecule-3-grabbing non-integrin |
| DDX58 | DExD/H-box helicase 58 |
| DEG | Differentially expressed genes |
| DENV-2 | Dengue Virus Type-2 |
| DEP | Differentially expressed probes |
| DHA | Docosahexaenoic acid |
| DHX58 | DExH-box helicase 58 |
| DMSO | Dimethyl sulfoxide |
| DPF | Days post-fertilization |
| DPI | Day post-injection |
| dsRNA | Double-stranded RNA |
| DUSP | Dual specificity phosphatase |
| EEF1A | Eukaryotic elongation factor 1 alpha |
| EF1A1 | Elongation factor 1 alpha-1 |
| EIF3/4 | Eukaryotic translation initiation factor 3/4 |
| EPA | Eicosapentaenoic acid |
| ER | Endoplasmic reticulum |
| EST | Expressed sequence tag |
| FABP4 | Fatty acid-binding protein, adipocyte |
| FACS | Fluorescence-activated cell sorting |
| FADD | FAS-associated death domain |
| FADOX | FAD-linked sulfhydryl oxidase ALR-like |
| FCR2 | Fc receptor-like protein 2 |
| FDR | False discovery rate |
| Fig | Figure |
| FSC | Forward scatter |
| g | Grams |
| GAF | Interferon gamma-activated factor |
| GAS | Interferon gamma-activated sequence |
| GCRV | Grass carp reovirus |
| GEO | Gene expression omnibus |
| GIG | GCRV-induced gene |
| GIV | Grouper iridovirus |
| GO | Gene ontology |
| GOI | Gene of interest |

| | |
|---|---|
| GRP94 | Heat shock protein 90, beta, member 1 |
| GSPs | Gene-specific primers |
| h | Hours |
| HCMV | Human cytomegalovirus |
| HCV | Hepatitis C virus |
| HERC | E3 ubiquitin-protein ligase HERC |
| HK | Head kidney |
| hpf | Hours post-fertilization |
| HPI | Hours post-injection |
| HPS | Hours post-stimulation |
| HSC70 | Heat shock cognate 70 kDa |
| HSV-1 | Herpes simplex virus 1 |
| ICAM | Intercellular adhesion molecule |
| IFIH1 | Interferon-induced with helicase C domain 1 |
| IFIT5 | Interferon-induced protein with tetratricopeptide repeats 5 |
| IFN | Interferon |
| IFNA/B/G | Interferon-alpha/beta/gamma |
| IFNAR | Interferon-alpha receptor |
| IFNGR | Interferon-gamma receptor |
| IFNG-rel | Interferon-gamma-related |
| iGTPase | Interferon-inducible GTPase |
| IHNV | Infectious hematopoietic necrosis virus |
| IKK | Inhibitor of nuclear factor kappa-B kinase |
| IL | Interleukin |
| IL1B | Interleukin 1, beta |
| iNOS | Inducible nitric oxide synthase |
| IP44 | Interferon-induced protein 44-like |
| IPN | Infectious pancreatic necrosis |
| IPNV | Infectious pancreatic necrosis virus |
| IRAK | Interleukin 1 receptor-associated kinase |
| IRF | Interferon regulatory factor |
| ISAV | Infectious salmon anemia virus |
| ISG | Interferon-stimulated gene |
| ISGF3 | Interferon-stimulated gene factor 3 |
| ISRE | Interferon-sensitive response element |
| JAK | Janus kinase |
| JAK3 | Tyrosine kinase JAK3 |
| JBARB | Dr. Joe Brown Aquatic Research Building |
| KLF4 | Krueppel-like factor 4 |
| l | Litre |

| | |
|---|---|
| LC | Long chain |
| LCn-3/6 | Long chain n-3/6 |
| LG | Lineage group |
| LGMN | Legumain-like |
| LGP2 | RNA helicase LGP2 |
| LPS | Lipopolysaccharide |
| M | Molar |
| MAPK | Mitogen-activated protein kinase |
| MAP2K | Dual specificity mitogen-activated protein kinase kinase |
| MAP3K | Mitogen-activated protein kinase kinase kinase |
| MAVS | Mitochondrial antiviral-signaling protein |
| MCP | Monocyte chemoattractant protein |
| MCSF | Macrophage colony-stimulating factor |
| MDA5 | Melanoma differentiation-associated protein 5 |
| MF | Molecular function |
| MFP | Minimize false positives |
| mg | Milligram |
| MHCI/II | Major histocompatibility complex I/II |
| MIG1 | Megalocytivirus-induced protein 1 |
| min | Minute |
| MIP2 | Macrophage inflammatory protein 2 |
| MIQE | Minimum information for publication of qPCR experiments |
| miRNA | MicroRNA |
| ml | Millilitre |
| MLCs | Macrophage-like cells |
| mM | Millimolar |
| mRNA | Messenger-RNA |
| MSA | Multiple sequence alignment |
| MTAP | Microtubule aggregate protein homolog isotype |
| MUFA | Monounsaturated fatty acid |
| MUN | Memorial University of Newfoundland |
| MX | Interferon-induced GTP-binding protein Mx |
| MyD88 | Myeloid differentiation primary response gene 88 |
| *n* | Number |
| n-3/n-6 | Omega-3/omega-6 |
| NCBI | National Center for Biotechnology Information |
| ncRNA | Non-coding RNA |
| NEMO | NFKB1 essential modulator or IKKG |
| NFKB1/2 | Nuclear factor (NF)-kappa-B 1/2 |
| NFKBIA | Nuclear factor kappa-B inhibitor alpha |

| | |
|---|---|
| ng | Nanogram |
| NO | Nitric oxide |
| nr | Non-redundant |
| nt | Nucleotide |
| NTC | No-template control |
| NTF2 | Nuclear transport factor 2 |
| OPTN | Optineurin |
| ORF | Open reading frame |
| OSC | Ocean Sciences Centre |
| OsHV-1 | Ostreid herpesvirus |
| *p* | *p*-value |
| P67-PHOX | NADPH oxidase cytosolic protein p67phox |
| PABPC1 | Polyadenylate-binding protein cytoplasmic 1 |
| PAMP | Pathogen-associated molecular pattern |
| PBS | Phosphate-buffered saline |
| PDB | Protein data bank |
| Peli1 | Pellino E3 ubiquitin protein ligase 1 |
| PI | Propidium iodide |
| pI | Isoelectric point |
| PI3K | Phosphoinositide 3-kinase |
| pIC | Polyriboinosinic polyribocytidylic acid |
| PIK3R4 | Phosphoinositide 3-kinase regulatory subunit 4 |
| PKC | Protein kinase C |
| PKR | Interferon-induced, double-stranded RNA-activated protein kinase |
| PLD4 | Phospholipase d4 |
| PPP1CC | Protein phosphatase 1, catalytic subunit, gamma isozyme |
| PrDOS | Protein disorder prediction server |
| pre-miRNA | Precursor miRNA |
| pri-miRNA | Primary miRNA |
| PRR | Pattern recognition receptor |
| PSMB8 | Proteasome subunit beta type-8 |
| PU.1 | Transcription factor PU.1 |
| PUFA | Polyunsaturated fatty acid |
| QC | Quality check |
| qPCR | Quantitative reverse transcription - polymerase chain reaction |
| RAC1 | Ras-related C3 botulinum toxin substrate 1 |
| RACE | Rapid amplification of cDNA ends |
| RAF-1 | Serine/threonine kinase Raf-1 |
| RB | Respiratory burst |
| RCSB | Research collaboratory for structural bioinformatics |

| | |
|---|---|
| redox | Oxidation-reduction |
| RESV | Resveratrol |
| RIG-I | Retinoic acid-inducible gene I |
| RIP1/3 | Receptor-interacting protein 1/3 |
| RLR | RIG-I-like receptor |
| RNF144 | Ring finger protein 144 |
| RNF8 | Ring finger protein 8, E3 ubiquitin protein ligase |
| ROI | Reactive oxygen intermediates |
| RP | Rank products |
| RPL32 | 60S ribosomal protein 32 |
| RPL4A | 60s ribosomal protein l4-a |
| RPLP1 | 60S acidic ribosomal protein P1 |
| RQ | Relative quantity |
| RSAD2 | Radical S-adenosyl methionine SAM domain-containing 2 |
| RTG-2 | Rainbow trout fibroblast-like cell line |
| RTL-W1 | Rainbow trout liver cell line |
| RTS11 | Monocyte/macrophage-like cell line of rainbow trout |
| RUX | Ruxolitinib |
| s | Second |
| S90 | SB202190 |
| SAH | S-adenosylhomocysteine |
| SAM | Significance analysis of microarrays |
| SAV | Salmonid alphavirus |
| SC5D | Lathosterol oxidase |
| SCARB1 | Scavenger receptor class B type I |
| SE | Standard Error |
| SFA | Saturated fatty acid |
| SNTB1 | Beta-1 syntrophin |
| SOCS | Suppressor of cytokine signaling |
| SSC | Side scatter |
| SSH | Suppression subtractive hybridization |
| ssRNA | Single-stranded RNA |
| STAT | Signal transducer and activator of transcription |
| TAB | TAK1-binding protein |
| TAK1 | Transforming growth factor beta (TGFB)-activated kinase 1 or MAP3K7 |
| TANK | TRAF family member-associated NFKB activator |
| TBK | TANK-binding kinase |
| TFBS | Transcription factor binding site |
| TGFB1 | Transforming growth factor beta 1 |

| | |
|---|---|
| Th1/2 | Helper T-cell 1/2 |
| TLR | Toll-like receptor |
| TMOD4 | Tropomodulin-4-like |
| TNF | Tumor necrosis factor |
| TO cells | Atlantic salmon macrophage/dendritic-like TO cells |
| TRAF | TNF receptor-associated factor |
| TRAIP | TRAF-interacting protein |
| TRIF | TIR-domain-containing adapter-inducing interferon-beta |
| TUBB2 | Beta-2 tubulin |
| TUBG1 | Tubulin gamma-1 |
| TYK | Tyrosine kinase |
| U | Unit |
| UBE1L | Ubiquitin-activating enzyme E1-like |
| UTR | Untranslated region |
| VHSV | Viral hemorrhagic septicaemia virus |
| Viperin | Virus inhibitory protein, endoplasmic reticulum ER-associated, IFN-inducible |
| VNN | Viral nervous necrosis |
| ZNFX1 | NFX1-type zinc finger-containing protein 1 |
| µg | Microgram |
| µl | Microlitre |
| µM | Micromolar |

## CO-AUTHORSHIP STATEMENT

I am the first author on all of the manuscripts generated from this thesis. I was primarily responsible for experimental design, implementation of the experiments, sampling, molecular analyses, data analyses and drafting the manuscripts.

Dr. Matthew Rise is a co-author on all of the manuscripts in this thesis. Dr. Rise was involved in the conceptual design of experiments and data interpretation, and he provided guidance in all aspects and stages of the experiments, and reviewed all of the manuscripts.

Xi Xue is a co-author on the manuscripts generated from Chapter 2, 3 and 5. He helped with training, microarray experimental design and data analyses in these chapters.

Dr. Marije Booman is a co-author on the manuscript resulting from Chapter 2, as she helped with experimental design and reviewing of the manuscript.

Nicole Smith is a co-author on the manuscripts resulting from Chapters 2 and 5. She carried out the flow cytometry analyses in these chapters and reviewed the manuscripts.

Sabrina Inkpen is a co-author on the manuscripts resulting from Chapters 3 and 4. She assisted with macrophage isolation, tissue sampling, RNA extraction and qPCR analyses.

Atefeh Ghorbani and Dr. Mani Larijani are co-authors on the manuscript resulting from Chapter 3. They assisted with RNA extraction, protein structure prediction and manuscript preparation.

Dr. Rune Andreassen is a co-author on the manuscript resulting from Chapter 4. Dr. Andreassen took an active role in experimental design, and provided guidance during the miRNA-related experiments, data analyses and manuscript preparation.

Dr. Jennifer Hall is a co-author on the manuscript resulting from Chapter 5. She helped with the qPCR assays in this chapter.

Dr. Albert Caballero-Solares is a co-author on the manuscript resulting from Chapter 5. He helped with experimental design and reviewed the draft of manuscript.

Dr. Christopher Parrish is a co-author on the manuscript resulting from Chapter 5. He provided guidance with the fatty acid analyses, statistical analyses, data interpretation and manuscript preparation.

Dr. Richard Taylor is a co-author on the manuscript resulting from Chapter 5. Dr. Taylor designed and formulated the experimental diets and reviewed the manuscript.

# CHAPTER 1: Introduction

## 1.1. Introduction

World aquaculture production has increased as a result of a plateau in world capture fisheries (i.e., the limitation of naturally supplied aquatic stocks) and a growing human population (FAO, 2016), and the global consumption of fish is predicted to mainly rely on aquaculture products by 2025 (FAO, 2016). The Atlantic cod (*Gadus morhua*) is a crucial species for capture fisheries in several countries (e.g., Canada, Norway and Finland) (Rosenlund and Halldórsson, 2007). However, some wild populations (e.g., in the Northwest Atlantic) of Atlantic cod have been over-exploited (reviewed by Hutchings, 2005; Kjesbu et al., 2014). If problems that negatively affect the profitability of Atlantic cod farming (e.g., infectious diseases and precocious sexual maturation) are solved, then genomics-enabled Atlantic cod aquaculture may be developed to fulfil the demands of human consumption for this species and diversify aquaculture industry (Tørresen et al., 2016 and references therein). The Atlantic cod is susceptible to several diseases caused by viruses [e.g., nodaviruses, infectious pancreatic necrosis virus (IPNV), viral haemorrhagic septicaemia virus (VHSV)] and bacteria (e.g., *Aeromonas salmonicida*, *Vibrio* sp.) (Samuelsen et al., 2006), and the development of molecular tools (e.g., biomarkers) may lead to a better understanding of the health management of this species.

The Atlantic salmon (*Salmo salar*) is one of the most important species in aquaculture, and its global production is still increasing (FAO, 2010, 2016, 2017).

However, Atlantic salmon farming faces several health and nutritional issues. For example, fish oil and fish meal for feed production have become limited (Tacon and Metian, 2008; Tocher, 2015), and thus, the essential polyunsaturated fatty acids (PUFAs) must be replaced by another source. However, the substitution of fish oil with vegetable oils alters dietary PUFAs [e.g., omega-3 (n-3) and n-6 fatty acids], and may influence the immune responses of this species to various pathogens (e.g., bacteria, viruses and parasites) (Kiron, 2012). Farmed and/or wild Atlantic salmon are susceptible to infections caused by several viruses [e.g., infectious salmon anaemia virus (ISAV), IPNV, salmonid alphavirus (SAV)] (Robertsen, 2018), bacteria (e.g., *Vibrio* sp., *Aeromonas salmonicida*, *Piscirickettsia salmonis*) (Toranzo et al., 2005), parasites (e.g., sea lice and protozoans) (Bakke and Harris, 1998; Vollset et al., 2016) and fungi (*Saprolegnia parasitica*) (Songe et al., 2014). Therefore, the need for replacement of dietary fish oil, alongside the susceptibility of Atlantic salmon to several diseases, necessitates evaluating the effects of dietary fatty acids on the immune responses of Atlantic salmon. The current thesis focuses on the antiviral responses of Atlantic cod and salmon.

### 1.2. The fish immune system

The immune system of vertebrates is mainly categorised into the innate and adaptive immune responses. The adaptive immune responses chiefly rely on antigen-specific receptors distributed on the surface of T- and B-lymphocytes (Medzhitov and Janeway Jr, 1998); pathogen-derived antigens bind to these receptors and elicit specific immune responses (Pancer and Cooper, 2006). On the other hand, the innate immune responses are independent of the previous recognition of the pathogen-derived antigens

and are mediated through recognition of pathogen-associated molecular patterns (PAMPs) as well as the activity of phagocytes (e.g., macrophages) (Akira et al., 2006; Medzhitov and Janeway Jr, 1998). While some immune-relevant organs (e.g., thymus and spleen) and cells (e.g., monocytes, lymphocytes and neutrophils) in fishes are homologous to those of mammals, the immune system and responses of fishes have several differences from those of mammalian vertebrates (Rauta et al., 2012; Tort et al., 2003; Watts et al., 2001). For example, fish immune systems lack germinal centres and some immunoglobulins (i.e., IgG, IgE and IgA), and they have a lower antibody affinity compared to those of mammals (Rauta et al., 2012; Tort et al., 2003). The fish innate immune systems play key roles in the defence against pathogens through phagocytosis (e.g., macrophages and dendritic cells), by producing antimicrobial agents (e.g., peptides and lysozyme) and by the production of immune regulators [e.g., cytokines such as IL1B (Interleukin 1 beta) and IL8] (Uribe et al., 2011). Further, fishes are highly dependent upon innate immunity, as it is the basis of their immune responses and the first line of defence of their immune system, and fishes have a limited capacity to mount an adaptive immune response as functional as mammals (Magnadóttir, 2006; Tort et al., 2003; Uribe et al., 2011; Whyte, 2007). With respect to innate immunity, several aspects of the antiviral immune responses of Atlantic cod and Atlantic salmon remain unknown. For example, the global expression of the genes and non-coding RNAs associated with antiviral immune responses of different immune cells (e.g., macrophages) is not fully understood in these species. Therefore, my thesis focuses on the molecular basis of the innate antiviral responses of these species.

### 1.3. Importance of Atlantic cod and Atlantic salmon in immunological research

In addition to the economic importance of Atlantic cod (1.3 million tonnes of fisheries landing per year) and Atlantic salmon (2.4 million tonnes of aquaculture production per year) (FAO, 2017), these species are of paramount importance to fish comparative immunology. The immune system of Atlantic cod has several unique features among all teleosts (Star et al., 2011). For example, this species has high serum IgM concentrations and weak specific antibody responses (Solem and Stenvik, 2006; Star et al., 2011). In addition, the genome of Gadiformes lineage, which includes Atlantic cod, lacks the genes encoding the major histocompatibility complex II (MHC II), CD4, Mx and TLR5 (Toll-like receptor 5) proteins (Malmstrøm et al., 2016; Solbakken et al., 2016; Solbakken et al., 2017). Salmonids (i.e., members of the family Salmonidae), on the other hand, possess pseudotetraploid genomes due to a salmonid-specific whole-genome duplication, which occurred approximately 80 million years ago in the common ancestor of this family (Lien et al., 2016; Quinn et al., 2008). The genomes of salmonids share several features; for example, 99% of the rainbow trout (*Oncorhynchus mykiss*) scaffolds can be mapped onto the chromosome sequences of Atlantic salmon (Lien et al., 2016). The Atlantic salmon genome and transcriptome are well characterised (Davidson et al., 2010; Koop et al., 2008; Lien et al., 2016; Quinn et al., 2008), and ~60% of expressed sequence tags (ESTs) of salmonids belong to Atlantic salmon. Accordingly, Atlantic salmon may serve as a representative salmonid for immunological studies. My thesis concentrates on the expression of transcripts involved in the antiviral responses of Atlantic cod and Atlantic salmon macrophages. Genomic analyses (e.g., microarrays and

RNA sequencing) can profile the transcriptomic responses of cells or tissues to a given immune stimulus (e.g., viruses and viral mimics), thus providing a better understanding of the molecular pathways and regulating factors that are activated (Hyatt et al., 2006; Ozsolak and Milos, 2011). These genomic techniques were used to develop a broader picture of innate antiviral immune responses of Atlantic cod and salmon. The microarray and RNA sequencing approaches employed herein allowed us to identify novel biomarkers for antiviral immune responses of Atlantic cod and Atlantic salmon, and to predict the biological processes and molecular pathways activated by antiviral immune responses of these species.

### 1.4. Available genomics resources for Atlantic cod and Atlantic salmon

There are numerous genomic resources that can facilitate studies of the genes and molecular pathways involved in the immune responses of Atlantic cod and Atlantic salmon. For instance, whole genome sequencing projects have been conducted for both species (Davidson et al., 2010; Johansen et al., 2009; Lien et al., 2016; Star et al., 2011). The sequence of the Atlantic cod genome is available in Ensembl Genome Browser (https://www.ensembl.org/). An updated version of the Atlantic cod genome was recently released (Tørresen et al., 2017), and is available through the Centre for Ecological and Evolutionary Synthesis (CEES: http://cees-genomes.hpc.uio.no/) Genome Browser. There are more than 250,000 (250K) and 490K ESTs for the Atlantic cod and salmon, respectively, in GenBank (https://www.ncbi.nlm.nih.gov/genbank/). There are excellent microarray platforms available for the Atlantic cod [i.e., a 20K Atlantic cod custom-built oligonucleotide (oligo) microarray] and salmon [e.g., Genomic Research on All

Salmonids Project (cGRASP)-designed Agilent 44K salmonid oligonucleotide microarray] (Booman et al., 2011; Jantzen et al., 2011) that may be employed for transcriptomic studies of immune responses.

### 1.5. The innate antiviral immune responses of fishes

Antiviral immune responses of mammals are well characterised (Bonjardim et al., 2009). Although innate immune responses of teleosts and mammals exhibit considerable homologies (Plouffe et al., 2005), several aspects of fish antiviral innate immune responses remain uncharacterised. Innate immune responses may be triggered by the recognition of viral pathogens or PAMPs by pattern recognition receptors (PRRs) (Bonjardim et al., 2009; Kawai and Akira, 2006). Different PAMPs stimulate innate immune responses; for example, synthetic double-stranded RNA (dsRNA) [i.e., polyriboinosinic polyribocytidylic acid (pIC)] and single-stranded RNA (ssRNA) mimic the antiviral responses to dsRNA and ssRNA viruses, respectively, (Kumar et al., 2009a, b). Accordingly, these molecules may be used to assess the antiviral immune responses of different species.

As shown in Fig. 1.1., mammalian TLR3, TLR7 and TLR9 act as endosomal PRRs and recognise dsRNA, ssRNA and CpG-rich DNA viruses, respectively (O'Neill et al., 2013). TLR3 and TLR9 have been structurally characterised in several teleosts [e.g., Atlantic cod, rainbow trout, zebrafish (*Danio rerio*), common carp (*Cyprinus carpio*) and Atlantic salmon] and found to play evolutionarily-conserved roles in teleosts (Palti, 2011; Rebl et al., 2010). In contrast, the function of TLR7 in teleost species remains

undetermined (Palti, 2011). In addition to the recognised orthologues of mammalian TLRs, TLR22 has been identified in various fish and described as a teleost-specific PRR recognising dsRNA (Palti, 2011; Rebl et al., 2010); this suggests that there might be some differences between dsRNA-activated signalling pathways of fishes and mammals.

Stimulation of mammalian TLR3 activates the TRIF (TIR-domain-containing adapter-inducing interferon-B)-dependent pathway via phosphorylation and polyubiquitination of RIP1 (Receptor interacting protein 1) by Peli1 (E3 ubiquitin ligase), and a complex comprised of TRAF6 (TNF receptor-associated factor 6), PKR (Interferon-induced, double-stranded RNA-activated protein kinase), TAK1 (TGFB-activated kinase 1), and TAB2 (TAK1-binding protein) is formed. The activation of this complex results in phosphorylation of TAK1, followed by formation of the IKK (Inhibitor of nuclear factor kappa-B kinase) complex (reviewed by Yu and Levine, 2011). Subsequently, the IKK multiprotein complex [i.e., IKKA, IKKB and NEMO (NFKB1 essential modulator alias IKKG)] phosphorylates NFKBIA (Nuclear factor kappa-B inhibitor alpha), leading to the degradation of NFKBIA, the activation of NFKB1 (Nuclear factor NF-kappa-B) and the expression of inflammatory cytokines (Kawai and Akira, 2007). The NFKB1 of zebrafish was found to be conserved with mammalian vertebrates (Correa et al., 2004). Several factors in the TRIF-dependent pathway have been identified in fish species (reviewed by Langevin et al., 2013; Zhang and Gui, 2012), and some of these factors were found to be pIC-induced in teleosts (e.g., Atlantic cod and Atlantic salmon) (Hori et al., 2012; reviewed by Workenhe et al., 2010; Zenke et al., 2010). Furthermore, downstream of TRIF-dependent signalling pathways, IKKE and

TBK1 (TANK-binding kinase 1) phosphorylate IRFs (interferon regulatory factors; i.e., IRF7 and IRF3), thereby activating IRFs and increasing the production of IFNs (Interferons) and ISGs (IFN-stimulated genes) (Akira et al., 2006; Bonjardim et al., 2009) (see Fig. 1.1.). A similar, but species-dependent, signalling pathway has been demonstrated for IRF7 and IRF3 in bony fishes (reviewed by Zhang and Gui, 2012). While the molecules involved in the TLR-activated TRIF pathway of fish share some characteristics with their mammalian orthologues (Zhang and Gui, 2012), it needs to be validated whether or not this pathway is conserved between higher vertebrates and teleosts.

As described for mammalian species, following binding of TLR7 and TLR9 to their ligands (i.e., ssRNA and CpG-rich DNA from PAMPs or the genome of pathogens, respectively), the activation of the MyD88-dependent (or TRIF-independent) pathway results in the formation of a kinase complex, consisting of IRAK4, IRAK1 and TRAF6, that activates NFKB1. Also, this complex activates IRF7 and may increase the transcription of ISRE (Interferon-sensitive response element)-containing immune effectors (e.g., ISGs and IFNs) (Kawai and Akira, 2006; Koyama et al., 2008; Kumar et al., 2009b). Likewise, teleost (e.g., Atlantic salmon and zebrafish) MyD88 (Myeloid differentiation primary response gene 88) was found to modulate the activation of IRF3/7 and NFKB1 (Iliev et al., 2011; Rebl et al., 2011; Zhang and Gui, 2012) and activate IFN responses.

In addition to endosomal-associated PRRs, PAMPs and viral pathogens may be recognised by RLR (RIG-I-like receptor) family members, initiating the mitochondrial-dependent pathway (Fig. 1.1.). In mammals, ssRNA and dsRNA viruses are recognised by RIG-I [Retinoic acid-inducible gene I, alias DDX58 (DExD/H-box helicase 58)] and MDA5 [Melanoma differentiation-associated protein 5, alias IFIH1 (IFN-induced with helicase C domain 1], respectively; on the other hand, LGP2 [RNA helicase LGP2, alias DHX58 (DExH-box helicase 58)] regulates the activity of other RLR members (Akira et al., 2006; Koyama et al., 2008; Yu and Levine, 2011). Following the stimulation of MAVS (Mitochondrial antiviral-signaling protein) by RLRs, IRF7 and IRF3 are activated through a series of phosphorylation events by TBK1 and IKKE (Akira et al., 2006; Takeuchi and Akira, 2008). RLR elements were identified in several bony fishes, even though RIG-I was found to be lost in the genome of some species (Zhang and Gui, 2012). These previous transcriptomics-based studies showed the up-regulation of the genes (e.g., *lgp2*, *mda5* and *irf7*) in the RLR pathway in Atlantic salmon macrophage/dendritic-like TO cells infected with SAV or in the spleen of pIC-injected Atlantic cod (Booman et al., 2014; Hori et al., 2012; Xu et al., 2015). However, the RLR pathway of some teleosts may differ from that of other vertebrates. For example, it is unknown whether the Atlantic cod genome contains the gene encoding RIG-I (Eslamloo et al., 2016). Different factors in this pathway (i.e., RIG-I, MDA5 and MAVS and IRF3/7) have been reported to be associated with IFN promoter stimulation and ISG production in fishes [e.g., zebrafish and Japanese flounder (*Paralichthys olivaceus*)] (Ohtani et al., 2010; Sun et al., 2011). The IRF3 of goldfish (*Carassius auratus*) was shown to be induced by pIC and IFN, and

to transactivate IFN through binding to ISRE motifs (Sun et al., 2010). Several Atlantic cod *irf*s (e.g., *irf7*) were previously characterised and shown to be induced in the spleen of pIC-injected fish (Inkpen et al., 2015). Teleost *irf3* was found to be an IFNA- and ISAV-induced transcript that chiefly regulates the production of type I IFN in Atlantic salmon TO cells (Bergan et al., 2010). Therefore, it seems that RIG-I and MDA5, as well as their activated pathways, are functionally conserved between teleosts and mammals (Zhang and Gui, 2012). The function of teleost LGP2 is not well understood. While *lgp2* was reported to be an enhancer of the IFN response in Japanese flounder (Ohtani et al., 2010), it was shown to be a repressor of pIC induction as well as RIG-I- and MDA5-mediated activation of IFN promoter in blastulae embryonic cells (CABs) of goldfish (Sun et al., 2011). Further studies are required to have a better understanding of LGP2 functions in antiviral immune responses of fishes.

As in mammalian species, the production of IFNs and ISGs elicits the antiviral immune responses (see Fig. 1.1.). The binding of IFNA or IFNB to IFNAR1/2 initiates the type I IFN pathway. Thereupon, the activation of TYK2 (Tyrosine kinase 2) and JAK1 (Janus kinase 1) leads to the formation of the transcription regulator ISGF3 (Interferon-stimulated gene factor 3), composed of STAT1 (Signal transducer and activator of transcription 1) and STAT2 associated with IRF9, and this complex enhances the transcription of ISGs via binding to ISRE motifs (reviewed by Bonjardim et al., 2009; Sadler and Williams, 2008). Type II IFN (i.e., IFNG) is engaged with homodimeric receptors (IFNGR1/2), and activates JAK1/2. Following the phosphorylation of STAT1 by JAK1/2, IFNG-activated factor (GAF: i.e., STAT1 homodimer) is formed, and

promotes the transcription of IFNG-responsive genes by binding to GAS (IFNG-activated sequence) (Bonjardim et al., 2009; Sadler and Williams, 2008).

Type I and/or II IFNs have been identified and structurally characterised in several teleost species (e.g., Atlantic cod, Atlantic salmon, rainbow trout and zebrafish) (Furnes et al., 2009; Robertsen, 2006; Robertsen et al., 2003). However, considering the fact that some teleosts (e.g., salmonids) have multiple copies of these IFNs, the categorisation of fish IFNs remains controversial. Therefore, they have been named variably (e.g., IFN1-2 or IFNA-B) in different species (Robertsen, 2006; Zou and Secombes, 2011). IFN type I members are sub-grouped into either four (sub-group I) or two cysteine-containing (sub-group II) cytokines. Members of group I are encoded by all vertebrates, whereas genes categorised under group II have only been identified in salmonids and cyprinids, suggesting that they are teleost-specific IFNs (Zou and Secombes, 2011; Zou et al., 2007). In addition to IFNG, the genomes of some teleosts (e.g., zebrafish) encode an IFNG-related molecule (Savan et al., 2009). Despite the sequence divergence found in teleost *ifng* genes, their genomic structure and organisation (i.e., number and size of the exons) are similar to mammalian vertebrate genes (Savan et al., 2009). As in other vertebrates (e.g., human and chicken), the transcription of fish IFNs is thought to be controlled through IRF binding sites (Zou and Secombes, 2011). Moreover, the signal transduction activated by both type I and II IFNs of teleosts seems to be highly comparable to that of mammalian IFNs (Langevin et al., 2013).

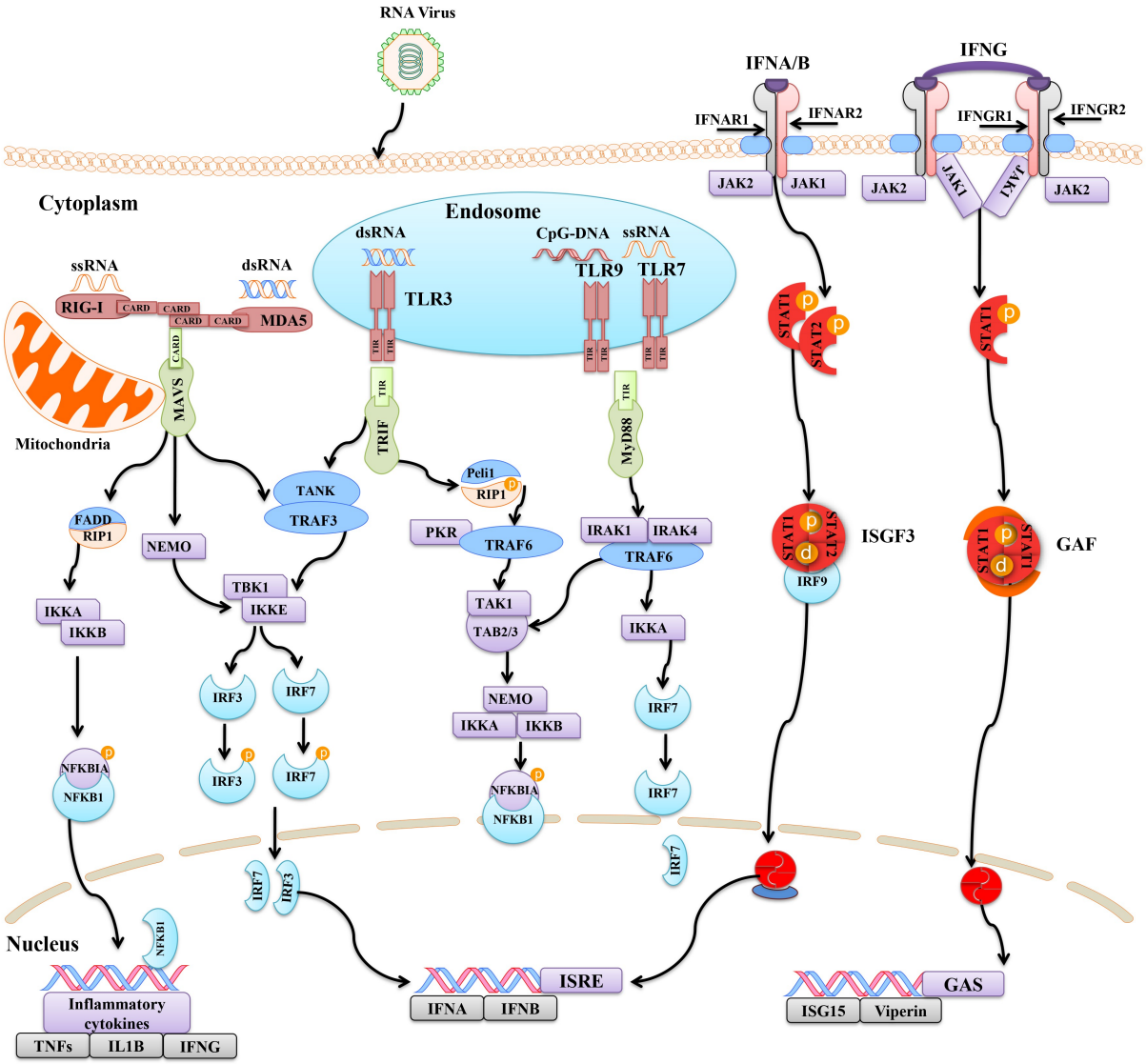


**Figure 1.1. The signalling pathways activated in antiviral immune responses.** This Figure is adapted based upon known mammalian pathways (Bonjardim et al., 2009; Yu and Levine, 2011). MDA5 (melanoma differentiation-associated protein 5), RIG-I (retinoic acid-inducible gene), MAVS (mitochondrial antiviral-signaling protein), FADD (FAS-associated death domain), RIP1 (receptor-interacting protein 1), IKK (NFKBIA kinase), NFKB1 (nuclear factor of kappa light polypeptide gene enhancer in B-cells 1), NFKBIA (NFKB1 inhibitor alpha), TNF (tumour necrosis factor), IL (interleukin), IFN (interferon), NEMO (NFKB1 essential modulator or IKKG), TLR (Toll-like receptor), TRIF (TIR domain-containing adaptor protein inducing IFNB), TRAF (TNF receptor-

associated factor), TANK (TRAF family member-associated NFKB activator), TBK (tank-binding kinase), IRF (IFN regulatory factor), ISRE (IFN-sensitive response element), Peli1 (pellino E3 ubiquitin protein ligase 1), PKR (IFN-induced, double-stranded RNA-activated protein kinase), TAK1 [transforming growth factor beta (TGFB)-activated kinase 1], TAB (TAK1-binding protein), MyD88 (myeloid differentiation primary response gene 88), IRAK (IL1 receptor-associated kinase), IFNGR (IFN-gamma receptor), IFNAR (IFN-alpha receptor), JAK (Janus kinase), STAT1 (signal transducer and activator of transcription 1), GAF (IFNG-activated factor), GAS (IFNG-activated sequence), ISG15 (IFN-stimulated gene 15).

Previously published studies have shown that recombinant teleost type I IFN induces the transcription of ISGs or immune effectors (e.g., *viperin* and *pkr*) in different species (e.g., salmon and rainbow trout) and enhances their resistance against viral infections [e.g., IPNV and Infectious hematopoietic necrosis virus (IHNV)] (Jørgensen et al., 2007; Ooi et al., 2008; Zou and Secombes, 2011). In contrast to mammals, the expression of teleost type I IFN can be regulated by a positive feedback loop via up-regulation of *stat1* (Yu et al., 2010). Type II IFNs of teleosts (e.g., rainbow trout and Atlantic salmon) were shown to activate GAS- and ISRE-containing genes as well as antigen presenting pathways (e.g., MHC) (Zou and Secombes, 2011). In addition, type II IFN of teleosts (e.g., goldfish and Japanese flounder) increased the expression of several ISGs (e.g., *viperin* and *isg15*) and cytokines (e.g., *il1b*) as well as enhanced the phagocytic activity of macrophages (Grayfer and Belosevic, 2009; Grayfer et al., 2010; Jørgensen et al., 2007; Jung et al., 2012). Similar to mammals, both type I and II IFNs in teleosts (e.g., salmon) exhibit negative immunoregulatory functions through activation of the inhibitors of innate immune responses (e.g., Suppressor of cytokine signaling; SOCS) (Skjesol et al., 2014; Zhu et al., 2013), reflecting a conserved negative feedback loop activated by IFNs in mammalian and teleost immune responses.

Using genomics approaches, a large number of putative ISGs (*viperin*, *isg15*, *lgp2*) were identified in Atlantic cod and Atlantic salmon (Hori et al., 2012; Rise et al., 2008; Workenhe et al., 2009; Xu et al., 2015). Several evolutionarily-conserved IFN-induced antiviral agents (e.g., Viperin, ISG15 and TRIM-like proteins) have been identified in teleosts (e.g., Atlantic cod, Atlantic salmon and carp) (Reviewed by Poynter

and DeWitte-Orr, 2016; Workenhe et al., 2010), although many important immune effectors are yet to be structurally and functionally characterised.

### 1.6. Diet-associated immunomodulation

Several nutritional factors (e.g., fatty acids, proteins and vitamins) may influence fish innate immune responses (reviewed by Kiron, 2012). There is a limited supply of forage fish that can be used as lipid and protein sources in aquafeeds (notably for carnivorous species such as Atlantic salmon), as well as increasing demand for aquaculture products as a result of the growing human population (reviewed by Tacon and Metian, 2008; Tocher, 2015). Consequently, the ingredients of carnivorous fish feed must be altered to minimise their marine-based lipid and protein contents. Several plant-based oil sources can be substituted for fish oil in aquafeeds without compromising fish growth performance (Nasopoulou and Zabetakis, 2012; Turchini et al., 2009). However, since the fatty acid content of vegetable oils (e.g., high levels of n-6 fatty acids and/or short chain n-3 fatty acids) differs from that of fish oil (e.g., high level of long chain n-3 fatty acids) (Turchini et al., 2009), this may lead to physiological and immunological changes in different mammalian and fish species (Galli and Calder, 2009; Kiron, 2012). Generally, n-3 and n-6 PUFAs give rise to different eicosanoids with anti- and pro-inflammatory functions, respectively (Calder, 2015; Patterson et al., 2012). For instance, in mammals, n-3 PUFAs [e.g., 20:5(n-3): eicosapentaenoic acid (EPA) and 22:6(n−3): docosahexaenoic acid (DHA)] decrease the expression of cytokines [e.g., TNF (Tumour necrosis factor), IL1B and IL6], adhesion molecules [e.g., ICAM (Intercellular adhesion molecule)] and the activation of NFKB, and increase the expression of the anti-

inflammatory cytokine IL10 (Calder, 2015; Mori and Beilin, 2004). With respect to mammalian macrophages, n-3 PUFAs inhibit the LPS-triggered inflammasome activation more effectively than n-6 PUFAs (Martínez-Micaelo et al., 2016). Moreover, DHA was shown to suppress the translocation of NFKB into the nucleus and inhibit inflammasome activity in mouse macrophages (Williams-Bey et al., 2014). The anti-inflammatory immunoregulation of n-3 PUFAs in LPS-induced mammalian macrophages was found to be dependent on the deacetylation of NFKB subunit p65 (Xue et al., 2012).

The results of studies conducted to determine the impact of dietary vegetable oils on fish immune responses were dependent upon the dietary vegetable oil source and the fish species being studied (reviewed by Kiron, 2012). For example, dietary vegetable oils (i.e., soybean and linseed oils) enhanced antiviral responses (i.e., *mx* expression) in the liver of pIC-injected sea bream (*Sparus aurata*) (Montero et al., 2008). In addition, dietary rapeseed oil changed the head kidney gene expression response in Atlantic salmon intraperitoneally injected with pIC (Caballero-Solares et al., 2017). On the other hand, dietary rapeseed, soybean or camelina oils did not change the immune responses of LPS-treated Atlantic salmon macrophages (Seierstad et al., 2009), LPS-treated Atlantic salmon head kidney leukocytes (Holen et al., 2011), or the spleen of pIC-injected Atlantic cod (Booman et al., 2011). However, the number of studies on dietary PUFA-dependent immune responses in fish is limited, and several aspects of dietary fatty acid-associated immune responses in different fish species need to be investigated. For example, prior to the current research, it remained unknown if various PUFAs or levels of n-3/n-6 fatty acids influence the functions (e.g., antiviral responses) of immune cells (i.e.,

macrophages) of teleost species. Therefore, *in vivo*-, *in vitro* and *ex vivo*-based experiments are needed to test cell-specific immunomodulatory effects of cell membrane PUFA compositions or dietary-derived PUFAs. Considering the economic importance of Atlantic salmon, the present thesis used nutrigenomics and *ex vivo* approaches to examine if various levels of n-3 fatty acids change the function and immune responses of macrophages in this species.

### 1.7. Regulation of immune responses by microRNAs (miRNAs)

Small non-coding RNAs may regulate the gene expression responses to various stimuli in different eukaryotic species (Mattick and Makunin, 2006). The primary molecules of miRNAs (pri-miRNAs) are chiefly transcribed from intergenic (i.e., independent miRNA genes) or intronic regions of DNA, and these pri-miRNAs form hairpin structures consisting of stem-loops with imperfect base-pairing that are then processed by the Drosha complex into precursor miRNAs (pre-miRNAs; ~70 nt hairpins). Following Exportin5-mediated transportation out of the nucleus, Dicer cleaves pre-miRNAs in the cytoplasm. Then, the resulting mature miRNAs (5p or 3p miRs) form an imperfect complementary base-pairing in the 3′-untranslated regions (3′-UTR) and mediate suppressed expression or the degradation of the target genes (reviewed by Chekulaeva and Filipowicz, 2009; Filipowicz et al., 2008). Several miRNAs have been previously found in higher vertebrates (e.g., mammals) to play crucial roles in different immune-related mechanisms such as inflammation, adaptive and innate antiviral immune responses, e.g., IFN- and TLR-dependent pathways (reviewed by Lodish et al., 2008; O'Connell et al., 2012; Sedger, 2013). In addition, miRNAs have been shown to regulate

the development and function of immune cells (e.g., mammalian macrophages) (reviewed by Baltimore et al., 2008). Several miRNAs have been characterised in teleosts (reviewed by Bizuayehu and Babiak, 2014), and some evolutionarily-conserved and teleost-specific miRNAs involved in antibacterial and antiviral responses have been recently identified in different fish species (e.g., rainbow trout and common carp) (reviewed by Andreassen and Høyheim, 2017). Nonetheless, the miRNAs associated with antiviral and cell-specific (e.g., macrophage) responses remain uncharacterised in many teleost species. This thesis was the first report on immune-responsive miRNAs in Atlantic cod.

### 1.8. Fish macrophages and immune responses

Macrophages play several pivotal roles in the innate and adaptive immune responses of vertebrates. For example, mammalian macrophages recognise viral and bacterial pathogens through receptors (e.g., TLRs and RLRs) and initiate innate immune responses. Following activation of pathways downstream of different PRRs, macrophages facilitate pathogen elimination through phagocytosis of pathogens and/or infected cells as well as through the production of inflammatory agents [e.g., IFNG, iNOS (inducible nitric oxide synthase) and lysozyme] (Mosser and Edwards, 2008; Murray and Wynn, 2011). Teleost macrophages have been shown to share functional and molecular features with their mammalian counterparts (reviewed by Hodgkinson et al., 2015; Wiegertjes et al., 2016). As in mammals, teleost macrophages have been described as one of the main phagocytes (along with monocytes and neutrophils) and the most active phagocytic cells (reviewed by Esteban et al., 2015), as fish macrophages show a higher phagocytic activity compared to the neutrophils (Do Vale et al., 2002). Pathogen

(e.g., bacteria) elimination by teleost phagocytes has been reported to be mediated through the production of reactive oxygen intermediates (ROI) and nitric oxide (NO) (reviewed by Grayfer et al., 2014). Mammalian macrophages are categorised into different subgroups according to their receptors, activating factors and functions. M1 cells, classically-activated by pro-inflammatory cytokines or helper T-cell 1 (Th1)-derived cytokines (e.g., IFNG) and different PAMPs [e.g., lipopolysaccharides (LPS), pIC], enhance phagocytosis and the production of antimicrobial agents (e.g., NO), whereas M2 cells, known as regulatory macrophages, are induced by Th2-derived or anti-inflammatory cytokines (e.g., IL4, IL10) (reviewed by Martinez and Gordon, 2014). Type II IFN molecules have been characterised in several teleosts, e.g., Atlantic cod, Atlantic salmon, goldfish and channel catfish (*Ictalurus punctatus*) (reviewed by Zou and Secombes, 2011). Recombinant IFNG was previously shown to facilitate the full development of teleost (e.g., common carp and Atlantic salmon) M1-like macrophages and trigger the STAT1-associated signalling pathway that leads to the enhanced transcription of various cytokines and immunoregulatory factors (e.g., SOCS1/3) (reviewed by Hodgkinson et al., 2015). However, since IFNG-related (IFNG-rel) proteins were found in some teleosts (e.g., rainbow trout and goldfish) (reviewed by Zou and Secombes, 2011), it has been suggested that the cytokine-mediated activation of M1-like macrophages exhibits unique features in teleosts (reviewed by Hodgkinson et al., 2015; Wiegertjes et al., 2016). The homology between teleost (e.g., zebrafish and rainbow trout) and mammalian IL4/13 may suggest the existence of M2 macrophages in fishes, although IL4-induced polarization of fish macrophages has yet to be reported (reviewed

by Hodgkinson et al., 2015; Wiegertjes et al., 2016). Macrophages may also function as antigen-presenting cells (APCs), and thus bridge between innate and adaptive immunity, and teleost APCs display some features similar to their mammalian counterparts (reviewed by Lewis et al., 2014). Although MHC II-expressing macrophage populations have been identified in zebrafish and rainbow trout (Bassity and Clark, 2012; Lewis et al., 2014), several macrophage functions (e.g., interaction with T-cells) associated with antigen presentation of fishes are yet to be determined.

There are several gaps in the current knowledge of the antiviral immune responses and macrophage functions of the Atlantic cod and Atlantic salmon. Some unique features (e.g., the absence of MHC II and expansion of teleost-specific TLRs) of Atlantic cod immune responses (see Section 1.3) are related to macrophage functions (e.g., antigen presentation and PAMP recognition). However, the global gene and non-coding RNA expression responses of these cells to different PAMPs (e.g., dsRNA, ssRNA and LPS) were uncharacterised before the current thesis (i.e., dsRNA) or are yet to be determined (e.g., ssRNA). In addition, the diet-associated immune responses of Atlantic salmon macrophages are poorly understood (see Section 1.6). The current thesis helps to fill some of the identified gaps through developing a better understanding of the genes or non-coding RNAs and molecular pathways underlying the antiviral immune responses of Atlantic cod and Atlantic salmon macrophages.

## 1.9. Overall objectives of thesis

Several aspects of Atlantic cod and Atlantic salmon macrophage function remain undetermined, and my thesis focuses on the antiviral responses of macrophages in these species. Profiling the transcriptomic (i.e., mRNA or miRNA) responses of Atlantic cod and Atlantic salmon macrophages provide a better understanding of the molecular basis of the antiviral responses of these cells through identification of responsive genes and miRNAs as well as characterising putative molecular pathways involved in antiviral immune responses. In addition to the identification of novel biomarkers, the global gene expression analyses reveal signalling pathways activated by antiviral responses that are conserved between the studied species. In the second chapter of this thesis, I used immunogenomics to better understand the genes and molecular pathways involved in the antiviral responses of Atlantic cod macrophages. Microarray and sequencing analyses have been used previously to identify transcripts differentially expressed in response to pIC injection in the spleen of Atlantic cod (Booman et al., 2014; Hori et al., 2012; Rise et al., 2008) or nervous necrosis virus infection in the brain of Atlantic cod (Krasnov et al., 2013). Whereas the microarray analyses used herein evaluated the antiviral responses of Atlantic cod macrophages, and identified biomarkers for immune responses of these cells.

In the third chapter of this thesis, I focused on one pIC-responsive gene (i.e., *viperin*) identified in Chapter 2 and aimed to fully characterise this gene. ISGs play crucial roles in the antiviral immune defence system of teleosts (reviewed by Poynter and DeWitte-Orr, 2016), although several of them (e.g., *viperin*) remain uncharacterised in

Atlantic cod. Viperin is known as a pIC- and virus-responsive ISG that can exhibit antiviral activity in both mammalian vertebrates and teleosts (reviewed by Mattijssen and Pruijn, 2012; Poynter and DeWitte-Orr, 2016). There are several aspects of Atlantic cod *viperin* (e.g., gene expression regulation) that were unknown prior to the current study. Therefore, I used molecular approaches, alongside a macrophage model, to examine if this gene has a conserved structure and expression, and to determine the molecular pathways activating its antiviral response in Atlantic cod.

The roles of miRNAs in antiviral immune responses of several mammalian and teleost species have been determined, but there was no study on the immune-related miRNAs of Atlantic cod. Therefore, the fourth chapter of my thesis used deep sequencing to identify pIC-responsive miRNAs in Atlantic cod macrophages. I hypothesized that both conserved and teleost-specific miRNAs may be involved in the antiviral immune responses of Atlantic cod macrophages.

The EPA+DHA contents of Atlantic salmon feed must be minimised, and the effects of various levels of dietary EPA+DHA on antiviral responses need to be determined. With respect to the importance of macrophages in different immune functions, in the fifth chapter of my thesis, I used an *ex vivo* model as well as cellular and transcriptomic approaches to investigate whether the antiviral responses of Atlantic salmon macrophages change with different levels of dietary EPA+DHA. I expected that various levels of dietary EPA+DHA would influence the fatty acid contents and transcriptome responses of Atlantic salmon macrophages.

The studies presented in this thesis have already improved our understanding of the miRNA and genes involved in the antiviral immune response of Atlantic cod. Further, my thesis provided a broader picture of signalling pathways underlying antiviral immune responses or fatty acid-related immune responses of Atlantic salmon macrophages. The findings of this thesis may be used for development of biomarker genes for assessing antiviral immune responses, and/or for examining fatty acid-dependent immune responses of Atlantic cod and Atlantic salmon.

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# CHAPTER 2

# Transcriptome profiling of the antiviral immune response in Atlantic cod macrophages

## Preface

The research described in Chapter 2 has been published in *Developmental & Comparative Immunology* as: Eslamloo, K., Xue, X., Booman, M., Smith, N.C., Rise, M.L., 2016. Transcriptome profiling of the antiviral immune response in Atlantic cod macrophages. Dev. Comp. Immunol. 63, 187-205; see Co-authorship Statement on page XXIV.

## 2.1. Abstract

A study was conducted to determine the transcriptome response of Atlantic cod (*Gadus morhua*) macrophages to the viral mimic, polyriboinosinic polyribocytidylic acid (pIC), using a 20K Atlantic cod microarray platform and qPCR. This study identified 285 significantly up-regulated and 161 significantly down-regulated probes in cod macrophages 24 h after pIC stimulation. A subset of 26 microarray-identified transcripts was subjected to qPCR validation using samples treated with pIC or phosphate-buffered saline (control) over time (3, 6, 12, 24, 48 h), and 77% of them showed a significant response to pIC. The microarray and qPCR analyses in this study showed that pIC induced the expression of cod macrophage transcripts involved in RLR- and TLR-dependent pathogen recognition (e.g., *tlr3*, *tlr7, mda5* and *lgp2*), as well as signal transducers (e.g., *stat1* and *nfkbia*) and transcription activators (e.g., *irf7* and *irf10*) in the MyD88-independent and dependent signalling pathways. Several immune effectors (e.g., *isg15*s, *viperin*, *herc4*, *mip2* and *ccl13*) were significantly up-regulated in pIC-stimulated cod macrophages. The expression of some transcripts (e.g., *irf7*, *irf10*, *viperin*) was significantly up-regulated by pIC as early as 12 h. All pIC-induced transcripts had peak expression at either 24 h (e.g., *tlr7*, *irf7*, *mip2*) or 48 h (e.g., *tlr3*, *lgp2*, *stat1*). This study suggests possible roles of both vertebrate-conserved (e.g., *tlr3* as an up-regulated gene) and fish-specific (*tlr22g* as a down-regulated gene) receptors in dsRNA recognition, and the importance of conserved and potentially fish-specific interferon stimulated genes in cod macrophages.

## 2.2. Introduction

In addition to the importance of the Atlantic cod (*Gadus morhua*) fishery for several countries (e.g., Canada, Norway and Finland), this species has shown promise as a candidate for northern Atlantic aquaculture (Rosenlund and Halldórsson, 2007). Atlantic cod aquaculture was being developed in several countries (e.g., Canada, Norway and Iceland) as a result of the over-exploitation and dramatic decline of some wild cod populations (Johansen et al., 2009). However, the development of large-scale Atlantic cod aquaculture faced serious issues including viral diseases [e.g., viral nervous necrosis (VNN), caused by nodaviruses] (Samuelsen et al., 2006). Identification of the transcripts and proteins involved in the antiviral immune responses of Atlantic cod [e.g., pattern-recognition receptors (PRRs), transcription activators and immune effectors] can improve the health management of wild and farmed fish, e.g., through the development and application of molecular biomarkers for assessing virus carrier state and response to vaccines or therapeutants. Further, the Atlantic cod possesses a unique immune system among bony fishes [e.g., high serum IgM concentration, weak specific antibody responses, a large number of MHC I (major histocompatibility complex I) and teleost-specific TLR (Toll-like receptor) paralogues; and the absence of transcripts encoding MHC II and CD4 proteins] (Solbakken et al., 2016; Solem and Stenvik, 2006; Star et al., 2011). Several unique features of Atlantic cod immune responses are associated with functions of macrophages that play roles in antigen presentation and pathogen recognition; thus, understanding the genes and mechanisms involved in macrophage immune responses of this species is of evolutionary importance.

Polyriboinosinic polyribocytidylic acid (pIC) is a synthetic double-stranded RNA (dsRNA) viral mimic that can trigger antiviral responses [e.g., the expression of IFNs (interferons) and ISGs (IFN-stimulated genes)] in fish as effectively as pathogenic viruses and more efficiently than other pathogen-associated molecular patterns (PAMPs) such as CpG oligodeoxynucleotides (Strandskog et al., 2008). Nodaviruses are thought to produce dsRNA intermediates in their replication cycle (Wu et al., 1992), and pIC was shown to increase the resistance of grouper (*Epinephelus septemfasciatus*) against VNN challenge (Nishizawa et al., 2011). pIC, mainly recognized by various PRRs in fish (e.g., TLRs), activates the signalling pathways that produce IFNs. Correspondingly, the binding of IFNs to IFNRs (IFN receptors) leads to the transcription of ISGs through the activation of the JAK-STAT signalling pathway (reviewed by Langevin et al., 2013b; Zhang and Gui, 2012).

Microarray analysis of gene expression can provide a profile of the transcriptional responses of cells or tissues to various agents (e.g., bacteria and viruses) (Hyatt et al., 2006). Using a database containing over 150,000 expressed sequence tags (ESTs) identified in various suppression subtractive hybridization (SSH) and normalized cDNA libraries (including several libraries designed to be enriched with virus-responsive transcripts), a 20,000 probe (20K) custom-built oligonucleotide (oligo) Atlantic cod microarray platform was previously designed and quality tested (Booman et al., 2011). This 20K microarray platform has been used to study cod oocyte and larval transcriptomes (Rise et al., 2015; Rise et al., 2014), as well as the effects of diet or elevated temperature on the cod spleen transcriptome responses to immunogenic stimuli

(e.g., pIC) (Booman et al., 2014; Hori et al., 2012; Hori et al., 2013). The current study is the first application of the 20K cod microarray platform to study the macrophage transcriptome.

PRRs that initiate antiviral immune responses, and consequently activate immune signalling pathways, could be tissue- or cell-specific in a species (Langevin et al., 2013a). For example, a tissue-dependent response to viral challenge was reported for teleost-specific *tlr*s [e.g., *tlr22* (*toll-like receptor 22*)] of Atlantic cod (Sundaram et al., 2012). Macrophages play prominent roles in fish immune responses at the cellular and molecular levels through pathogen recognition (e.g., different PAMPs), the regulation of innate and adaptive immune responses, cytokine production [e.g., of TNFs (Tumour necrosis factors) and ILs (Interleukins)], antigen presentation, and phagocytosis (Roca et al., 2007; Whyte, 2007). Viral infections increase IFNs and nitric oxide production, phagocytosis, and respiratory burst activity in fish macrophages (Collet, 2014). Viral infections alter host gene expression. For example, microarray analyses showed a massive transcriptomic response of Atlantic salmon macrophage-like cells to infectious salmon anaemia virus (ISAV) (Schiøtz et al., 2008; Workenhe et al., 2009), and Bakkemo et al. (2011) showed the time-dependent induction of ILs-encoding transcript expression by lipopolysaccharide (LPS) in Atlantic cod macrophages. Using real-time quantitative polymerase chain reaction (qPCR) and a small microarray platform (1056 probes), only 4 pIC-responsive transcripts [i.e., *isg15* (*interferon stimulated gene 15*), *bty* (*bloodthirsty*), *tlr3* (*toll-like receptor 3*) and *enolase 1 α*] were identified in Atlantic cod head kidney cells (Holen et al., 2012). However, a larger microarray platform that provides better coverage of the

transcriptome (as used in the current study) will yield a more complete picture of the antiviral response of head kidney cells or a specific type of Atlantic cod leukocyte (e.g., macrophages). Therefore, the present study aimed to determine the transcriptome profile of the antiviral response of Atlantic cod macrophages, using the 20K Atlantic cod microarray and qPCR.

## 2.3. Materials and methods

### *2.3.1. Animals*

Atlantic cod [1.28 ± 0.08 kg (mean ± SE)], reared in the Dr. Joe Brown Aquatic Research Building (JBARB) of the Ocean Sciences Centre [OSC; Memorial University of Newfoundland (MUN)], were used for this experiment. The fish were kept in a 21 $m^3$ tank and under optimum conditions (5.2-6.4˚C, 95-110% oxygen saturation, and an ambient photoperiod). The fish were fed 3 days per week at a level of 1% body weight, using a commercial diet (Skretting, BC, Canada; crude protein 50%, crude fat 18%, crude fibre 1.5%, calcium 3% and phosphorus 1.4%). All procedures in this experiment were approved by MUN's Institutional Animal Care Committee, based on the guidelines of the Canadian Council on Animal Care.

### *2.3.2. Macrophage isolation*

Atlantic cod head kidney macrophages were isolated based on Bakkemo et al. (2011) and Steiro et al. (1998), with some modifications. Briefly, the fish were netted and immediately euthanized with an overdose of MS222 (400 mg $l^{-1}$; Syndel Laboratories,

Vancouver, BC, Canada), and blood was removed from the caudal vein of each fish using a 5 ml syringe with a 25 gauge needle. Each fish was then dissected, and the hematopoietic kidney (i.e., head kidney) was removed and minced through 100 µm nylon cell strainers (Fisherbrand™, Thermo Fisher Scientific, Waltham, MA, USA) in Leibovitz-15+ (L-15+; Gibco, Carlsbad, CA, USA) culture medium supplemented with 2 mM L-glutamine, 4.2 mM $NaHCO_3$, 25 mM HEPES, 1.8 mM glucose, 20 U $ml^{-1}$ heparin (Sigma-Aldrich, St. Louis, MO, USA) and 100 U $ml^{-1}$ penicillin and 100 µg $ml^{-1}$ streptomycin (Gibco). The cell suspension was centrifuged on a discontinuous 25/51% Percoll gradient (GE Healthcare, Uppsala, Sweden) at 300 × g for 40 min at 4˚C, and the macrophage-enriched interface was collected. The resulting macrophage-rich suspension was washed twice in L-15+ by centrifuging at 300 × g for 15 min at 4 ˚C. The cells were diluted in L-15+ medium containing 1% fetal bovine serum (FBS; Gibco) and without heparin in the last washing stage. The dead and viable cells were counted using a hemocytometer and a trypan blue (Sigma-Aldrich) exclusion test, and >96% viability was recorded for isolated cells from all fish. Thereafter, the cells were seeded into 35 mm culture dishes (Corning, Corning, NY, USA) at an equal density of $3 \times 10^7$ cells (in 2 ml L-15+) per dish. The cells were allowed to adhere overnight (16 h) at 10˚C, and then the non-adherent cells were removed by washing the culture dishes 3 times with L-15+. The adherent macrophage-enriched cells are henceforth referred to as macrophages.

### *2.3.3. Cell characterisation*

To determine the effect of pIC on cell death, size and complexity, macrophages were isolated from 3 Atlantic cod and cultured in 6-well plates (Corning, Corning, NY,

USA) as in Section 2.3.2. Samples were plated in duplicate wells. After 24 h in culture, the cells were stimulated with 50 µg $ml^{-1}$ pIC (as determined in Section 2.3.4.) or PBS (control) for 24 and 48 h. Cells were removed from the plate using trypsin-EDTA (0.25%) (Thermo Fisher Scientific, Waltham, MA, USA), washed in 1 ml of fluorescence-activated cell sorting (FACS) buffer (PBS + 1% FBS) for 5 min at 500 × *g* and 4ºC, and stained with 1.0 µg $ml^{-1}$ propidium iodide (PI) (Thermo Fisher Scientific, Waltham, MA, USA) (Kalgraff et al., 2011). Fluorescence was detected from 10,000 cells using a BD FACS Aria II flow cytometer and analysed using BD FACS Diva v7.0 software (BD Biosciences, San Jose, CA, USA). Cell death was determined as PI-positive events, while changes in morphology were determined via a visual assessment of changes in cell size (forward scatter, FSC) and cell complexity (side scatter, SSC). To determine the effect of pIC and sampling time on cell differentiation, cells from the pIC and control groups were stained using Wright-Giemsa stain (Thermo Scientific) at 24 and 48 HPS, according to the manufacturer's instructions. The percentage of macrophage-like cells [i.e., large, adherent cells with cytoplasmic projections (Sørensen et al., 1997)] was determined in three fields of view (magnification 200X) for each sample.

### *2.3.4. Macrophage stimulation*

Six individual Atlantic cod were used for determination of the optimal dose of pIC and the microarray-based identification of antiviral (i.e., pIC-responsive) macrophage transcripts (Fig. 2.1.). For each individual cod, macrophages were isolated and seeded into five 35 mm culture dishes (i.e., a total of 30 dishes from 6 fish). The viral mimic pIC (Sigma-Aldrich) was dissolved in sterile phosphate-buffered saline (PBS) (pH

7.2). Starting 24 h after isolation, the macrophages from each individual were exposed to a range of pIC doses including 0 [PBS (control)], 1, 10, 50, and 100 μg $ml^{-1}$ at 10˚C. A previously published study showed that the highest transcript expression of Atlantic cod *isg15* in response to pIC occurred at 24 h post-stimulation (HPS) (Seppola et al., 2007). Therefore, 24 h after the onset of stimulation (i.e., 48 h after isolation), the media was removed and the macrophages were harvested by adding 800 μl TRIzol (Invitrogen, Burlington, Ontario, Canada) into each culture dish. Samples were kept at -80˚C until RNA extraction. qPCR for known antiviral biomarker transcripts [*isg15*, *viperin* and *stat1* (*signal transducer and activator of transcription 1*), see Sections 2.3.5. and 2.3.8.2.] was used to determine that 50 μg $ml^{-1}$ pIC was the most stimulative dose of pIC (data not shown). Therefore, this treatment group was selected for microarray analysis.

### *2.3.5. RNA extraction and purification*

Total RNA was extracted from TRIzol-lysed samples following the manufacturer's instructions, and RNA pellets were dissolved in 50 μl of DNase/RNase-free water (Gibco). The RNA samples were treated with DNase-I (Qiagen, Mississauga, Ontario, Canada) to degrade residual genomic DNA, and then purified using the RNeasy MinElute Cleanup Kit (Qiagen) according to the manufacturer's instructions. RNA concentration and quality were measured using NanoDrop spectrophotometry (ND-1000), and the RNA integrity was checked by agarose gel electrophoresis (1% agarose gel). The high-purity RNA samples (e.g., with A260/230 and A260/280 ratios > 1.8) with tight 18S and 28S ribosomal RNA bands were used for the gene expression analyses (i.e., microarray and qPCR).

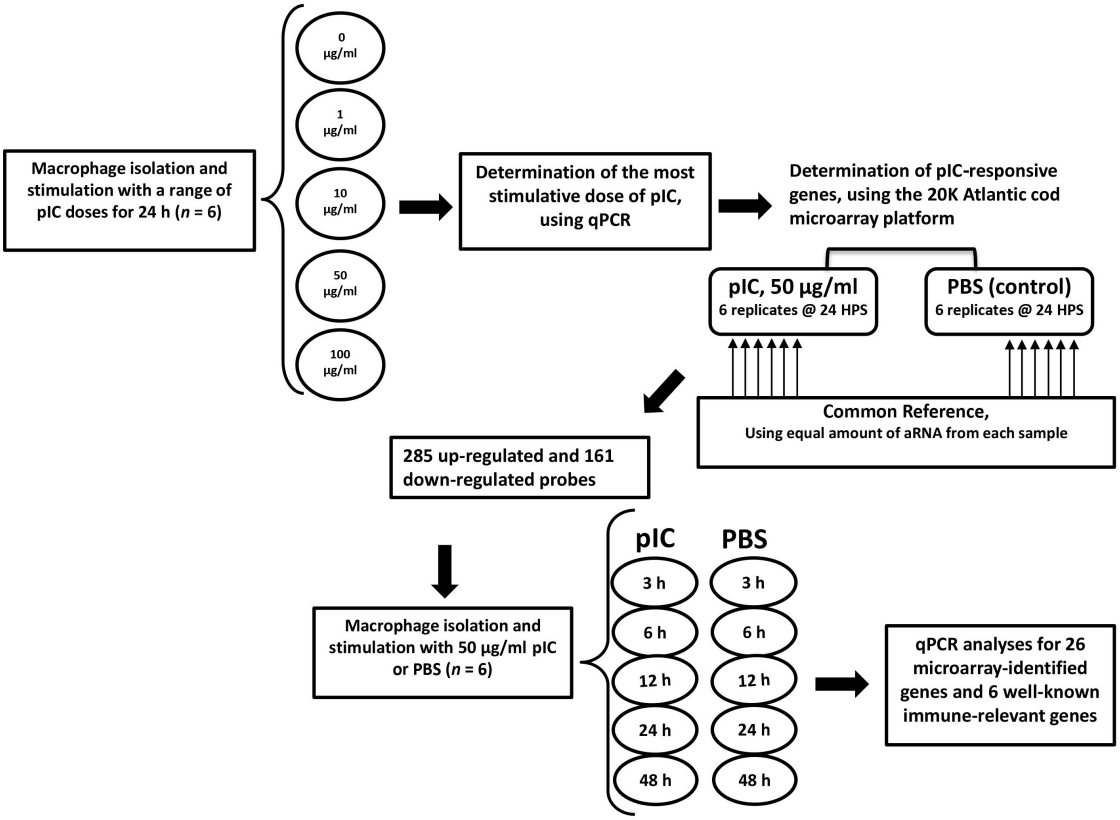


**Figure 2.1. Overview of experimental design.** Macrophages were isolated from 6 Atlantic cod, and the isolated cells from each individual were exposed to different levels of pIC. The pIC-responsive transcripts were determined using the 20K Atlantic cod microarray. qPCR validation was performed using macrophages that were isolated from 6 Atlantic cod and stimulated by pIC over time.

### *2.3.6. Microarray experimental design and hybridization*

This microarray experiment was designed and carried out based on the MIAME guidelines (Brazma et al., 2001). Macrophages exposed to 50 µg $ml^{-1}$ pIC or PBS (control) for 24 h (pIC and PBS treatments for each of 6 individuals) were used to identify the pIC-responsive transcripts in Atlantic cod macrophages (see Fig. 2.1.). The Atlantic cod 20K oligonucleotide microarray platform (Booman et al., 2011) was used for this reference design experiment that included 12 arrays. One microgram of DNase-treated and column-purified RNA was amplified and then labelled using the Amino Allyl MessageAmp™ II aRNA Amplification kit (Ambion, Carlsbad, CA, USA), according to the manufacturer's protocol. The amplified RNA (aRNA) samples were column-purified and their quality and quantity assessed using gel electrophoresis and NanoDrop spectrophotometry, respectively. A common reference was prepared using a pool of all 12 experimental samples (15 µg aRNA from each sample). The individual experimental samples (pIC and control) were labelled with Cy5 (GE Healthcare Life Sciences, Buckinghamshire, UK), whereas the common reference was labelled with Cy3 (GE Healthcare Life Sciences), based on the manufacturer's protocol (in the aRNA Amplification kit). Then, 3 µg of labelled aRNA was fragmented using Ambion Fragmentation Reagents (Ambion). For each array, 3 µg of labelled aRNA of one experimental sample, 3 µg of labelled aRNA of the common reference and 2 µl of LNA blocker (Genisphere, Hatfield, Pennsylvania, USA) were mixed in a formamide-based hybridization buffer (Genisphere) and hybridized to the array overnight (~ 16 h) at 42˚C

(Booman et al., 2011). The detailed protocols for hybridizations and washing are described in Booman et al. (2011).

### *2.3.7. Microarray data acquisition and analyses*

The arrays were scanned at 5 μm resolution using a ScanArray Gx Plus scanner and ScanExpress v4.0 software (Perkin Elmer, Waltham, Massachusetts, USA). Photomultiplier tube (PMT) settings were adjusted for Cy5 and Cy3 to minimize overall signal differences between channels. The raw data were saved as Tiff images, and then Imagene 9.0 (BioDiscovery, El Segundo, California, USA) was used for extracting the signal intensity data. In R and the Bioconductor package marray, the flagged spots were omitted and the resulting data were $\log_2$-transformed and Loess-normalized per subgrid (Booman et al., 2014). Probes with more than 25% missing values in all 12 arrays were removed from the data file, and the missing data were imputed by means of the EM_array method, using the LSimpute package (Bø et al., 2004; Celton et al., 2010). Thereafter, the final data file was composed of 11,797 probes for all arrays (GEO accession number: GSE70808).

Significant differences in transcript expression were found using Significance Analysis of Microarrays (SAM) (Chu et al., 2001; Tusher et al., 2001), as implemented by the Excel Add-in SAM package (Stanford University, CA, USA). A two-class paired comparison analysis with a false discovery rate (FDR) cutoff of 0.05 (i.e., 5%) was performed using SAM to determine the differentially expressed probes between pIC and control groups. The significant transcript list was re-annotated by BLASTx, which aligned the differentially expressed Atlantic cod sequences (i.e., contigs or singletons that

were used for 20K Atlantic cod microarray design) against NCBI's non-redundant (nr) amino acid sequence database (E-value < 1.00E-05), using Blast2GO software (BioBam Bioinformatics S.L., Valencia, Spain) (Conesa et al., 2005; Götz et al., 2008). Blast2GO was used for mapping the significant BLASTx hits to Gene Ontology (GO) terms and creating the GO pie chart of pIC-responsive transcripts (GO Biological Process level 2). GO enrichment analysis was conducted using the Fisher's Exact test (FDR cutoff of 0.05) in the Blast2GO software. A bar chart of enriched GO terms, reduced to the most specific GO terms (Biological Process and Molecular Function), was created. Selected pIC-responsive transcripts that had the same BLASTx hit name were re-assembled using the SeqMan function in the Lasergene 7.20 software package (Madison, WI, USA) to determine if those transcripts represented different putative paralogues. Finally, the median-cantered data of differentially expressed probes were hierarchically clustered using the Pearson correlation and complete linkage clustering function in Genesis software (Rockville, Maryland, USA) as in Booman et al. (2014).

### *2.3.8. qPCR validation*

#### *2.3.8.1. Macrophage isolation and time point sampling*

To validate the microarray results, and to study the pIC response of selected microarray-identified transcripts over time, macrophages were isolated from 6 additional healthy appearing Atlantic cod and seeded in 35 mm culture dishes (10 dishes per fish, for a total of 60 dishes) as previously described in Section 2.3.2. Macrophages isolated from each individual were exposed to either 50 μg $ml^{-1}$ pIC or PBS starting at 24 h post-isolation. The macrophages from each individual were incorporated into both

experimental groups and all time points (i.e., 6 biological replicates for each treatment and time point; see Fig. 2.1.). The stimulated and non-stimulated macrophages were lysed as previously described at 3, 6, 12, 24 and 48 HPS. Each RNA sample was extracted, DNase-treated, column-purified, and subsequently quantity- and quality-checked as described previously.

#### *2.3.8.2. qPCR analysis*

cDNA was synthesized for each sample using 800 ng of RNA, random hexamer primers (250 ng; Invitrogen), 1 µl of dNTPs (10 mM each), 4 µl of 5X first-strand buffer and M-MLV reverse transcriptase (200U; Invitrogen) at 37˚C for 50 min as recommended in the manufacturer's instructions.

This qPCR experiment was conducted according to the MIQE guidelines (Bustin et al., 2009). Viral mimic-responsive transcripts representing various biological functions (e.g., pathogen recognition, signal transduction/transcription control, and immune effectors) and different response patterns (e.g., high fold-change versus low fold-change; up-regulated versus down-regulated transcripts) were selected for qPCR validation with macrophage samples from all 5 time points (Table 2.1.). I also added 6 important immune-related transcripts [*ifng* (*interferon, gamma*), *ifngr1* (*interferon gamma receptor 1*), *p67-phox* (*NADPH oxidase cytosolic protein p67phox*), *bcl-x1*, *il8* (*interleukin 8*) and *ilb1* (*interleukin 1, beta*)] that were not present in the microarray significant transcript list to the qPCR experiment. The candidate normalizer transcripts were selected from the microarray data (i.e., transcripts that were comparably expressed between pIC and PBS control groups) or from previous studies (Booman et al., 2014; Feng and Rise, 2010;

Inkpen et al., 2015). Primers for the transcripts of interest and candidate normalizers were either designed using Primer3web v4.0.0 (http://primer3.wi.mit.edu) or obtained from previous studies (Booman et al., 2014; Feng and Rise, 2010; Hori et al., 2012; Inkpen et al., 2015) (Table 2.1.). The quality and amplification efficiencies of all primer pairs were assessed using two templates (a pool of 3 individuals in the pIC group at 24 HPS for up-regulated transcripts, and a pool of 3 individuals in the PBS control group at 24 HPS for down-regulated transcripts) with technical duplicates of a 5-point [or 4-point for *tlr7* (*toll-like receptor 7*), *znfx1* (*NFX1-type zinc finger-containing protein 1*), *traip* (*TRAF-interacting protein*), *bcl-x1, mig1* (*megalocytivirus-induced protein 1*), *herc4* (*E3 ubiquitin-protein ligase herc4*) and *catalase*], 3-fold serial dilution of cDNA, starting at 10 ng input RNA. Primer sets having 80-100% amplification efficiency (Pfaffl 2001), no amplification in the no-template controls, and a single melting peak (i.e., no evidence of primer dimers in the dissociation curve) were selected for qPCR assays. The absence of genomic DNA contamination in RNA samples was checked with all of the selected primer sets, using no-reverse transcription controls (i.e., samples devoid of reverse transcriptase enzyme during cDNA synthesis) from pooled control and pIC samples of each fish.

To determine the optimal normalizers, the expression of candidate normalizers [*cypa* (*cyclophilin a*), *rplp1* (*60S acidic ribosomal protein P1*), *rpl4a* (*60s ribosomal protein l4-a*), *tubb2* (*beta-2 tubulin*), *hsc70* (*heat shock cognate 70 kDa*), *eif3* (*eukaryotic translation initiation factor 3 subunit*), *eif4* (*eukaryotic translation initiation factor 4*), and *eef1a* (*eukaryotic elongation factor 1 α*)] was assayed in triplicate for one-half of the

samples. Using the geNorm analysis in the qBase software, two functionally distinct normalizers (*eif3* and *rplp1*) that were expressed at a stable level (i.e., with low M-value, a measure of transcript expression stability) across all samples were selected for qPCR assays as in Xue et al. (2015). The qPCR assays of the transcripts of interest were performed in 384 well plates using Power SYBR Green Master Mix (Applied Biosystems, Burlington, Ontario, Canada) and a ViiA7 Real-Time PCR system (Applied Biosystems). The expression of each transcript was measured in triplicate in a total reaction volume of 13 µl consisting of 4 µl of input cDNA (corresponding to 4 ng of input total RNA for each reaction), 6.5 µl of Power SYBR Green Master Mix, 1.04 µl of primers (forward and reverse primers 1.25 µM) and 1.46 µl of nuclease-free water (Gibco). The qPCR program was comprised of one cycle of 50 ˚C for 2 min, one cycle of 95 ˚C for 10 min, and 40 cycles of 95 ˚C for 15 s and 60 ˚C for 1 min, with fluorescence data detected at the end of each cycle. The robustness of assay performance between plates was checked using an inter-plate linker sample [$C_T$ (threshold cycle) value variation < 0.5] as well as no-template controls. ViiA7 software v1.2.2 (Applied Biosystems) was applied to acquire the $C_T$ values, and also to calculate the relative quantity (RQ) values. RQ values were calculated (Pfaffl, 2001) relative to a baseline sample (calibrator, i.e., with the lowest expression) for each transcript of interest, and using the amplification efficiency of each primer pair.

#### *2.3.8.3. qPCR data analyses*

RQ values were used for statistical analyses. The normality of data was initially checked with the Kolmogorov-Smirnov normality test. The gene expression data were analysed using repeated measures two-way ANOVAs, followed by Sidak multiple comparisons *post-hoc* tests in order to identify significant differences between pIC and PBS control groups at each time point, and within a group in multiple time points. Differences were considered statistically significant when $p \leq 0.05$. All data analyses were performed using Prism package v6.0 (GraphPad Software Inc., La Jolla, CA, USA).

## Table 2.1. qPCR primers.

| Transcript name | | Primer sequence 5' to 3' | $R^2$ | Amplification efficiency (%) | Amplicon size (bp) |
|---|---|---|---|---|---|
| **Transcript of interest** | | | | | |
| *toll-like receptor 3* (*tlr3*)[c] | Forward | GCCTGGGTGGTGATTAAGAA | 0.990 | 85.9 | 151 |
| | Reverse | GTCCCCAGTGAAGAGCTGTC | | | |
| *toll-like receptor 7* (*tlr7*)[c] | Forward | AACAAGTAGCGGATGGAAGC | 0.997 | 99.2[e] | 164 |
| | Reverse | ATGTCCAGGGAGACGCTGTA | | | |
| *toll-like receptor 22g* (*tlr22g*)[c] | Forward | GCAGTTCTTCATCACGTAGCC | 0.994 | 97.8 | 200 |
| | Reverse | ACGGAACCAGAGATCACCTT | | | |
| *RNA helicase lgp2* (*lgp2*)[a] | Forward | ACAGAAGCCATCGCAGAAAT | 0.994 | 85.5 | 105 |
| | Reverse | TTTTGCAGCACGAATCAAAC | | | |
| *melanoma differentiation-associated protein 5* (*mda5*)[c] | Forward | GGGAACATGGATCAAGAGGA | 0.996 | 91.7 | 129 |
| | Reverse | GGACACCTCAGACGGACTGT | | | |
| *signal transducer and activator of transcription 1* (*stat1*)[a] | Forward | GCCAATGCCATGTGTTTATG | 0.998 | 90.2 | 100 |
| | Reverse | ACCTGGAGCAGTTCGTCAGT | | | |
| *NF-kappa-B inhibitor alpha* (*nfkbia*)[a] | Forward | GCCAGCAACTGATAAAGCATC | 0.994 | 84.9 | 132 |
| | Reverse | GGTCACAGAGGGAGACAGAAAA | | | |
| *interferon regulatory factor 7* (*irf7*)[b] | Forward | CATGTGCTTTGGGGAGAAGT | 0.988 | 85.3 | 152 |
| | Reverse | TCTGTAGGCTGACGTTGGTG | | | |
| *interferon regulatory factor 10* (*irf10*)[b] | Forward | CCGAGAAGCCCAATAAACTG | 0.997 | 94.9 | 143 |
| | Reverse | ATACTCCTCGCCAAAGCAGA | | | |
| *NFX1-type zinc finger-containing protein 1* (*znfx1*)[a] | Forward | ATGCCACTATCGGTGGACAGA | 0.999 | 81.9[e] | 108 |
| | Reverse | TCAACAGATTATTGCCCTCGG | | | |
| *TRAF-interacting protein* (*traip*)[c] | Forward | AGCCTACTGCACCATTTGCT | 0.990 | 92.2[e] | 108 |
| | Reverse | GCTTGGAACCACTGAAGGAG | | | |
| *interferon gamma receptor 1* (*ifngr1*)[d] | Forward | CAGCGACCATGAAACTCTGA | 0.998 | 95.5 | 137 |
| | Reverse | TGTAGTCGTTGTCGGGACTG | | | |
| *CC chemokine receptor 7* (*ccr7*)[c] | Forward | CAGCACCTGTAGCCCTTACC | 0.989 | 90.9 | 120 |
| | Reverse | GCGCTGTAGCAGAAGGTCAT | | | |
| *bcl-x1*[d,f] | Forward | AGGTGTTCAGGGACAGCATC | 0.997 | 81.9[e] | 157 |
| | Reverse | CAGTGGTCAATGTGGTCGTC | | | |
| *interferon stimulated gene 15-1* (*isg15-1*)[a] | Forward | AGGACCAACAAAGGCTGATG | 0.998 | 91.4 | 110 |
| | Reverse | CAGCCGTCCGTTAAGGTAGA | | | |
| *interferon stimulated gene 15-2* (*isg15-2*)[c] | Forward | GAGCCCAACAACATCCAAGT | 0.998 | 87.8 | 165 |
| | Reverse | GTCCAACTGCTTGCCTTCAT | | | |
| *interferon stimulated gene 15-3* (*isg15-3*)[c] | Forward | CAAGAGTCGCACCTACACCA | 0.992 | 97.8 | 164 |
| | Reverse | CCGCGCTCACATTATAGGAC | | | |
| *interferon, gamma (ifng)*[d] | Forward | TCGCTCTTCATGTTGGTCTG | 0.993 | 89.9 | 121 |
| | Reverse | GGCCTTTCTGTGGATGTTGT | | | |
| *viperin*[a] | Forward | TGTTTCCACACAGCGAAGAC | 0.998 | 88.4 | 108 |
| | Reverse | TCCGCCAGAGAAGTTGATCT | | | |
| *C-C motif chemokine 13* (*ccl13*)[c] | Forward | TGTGCCTCATCCAACTCAGA | 0.996 | 88.1 | 162 |
| | Reverse | AAACCTCTGCATCGTCATCC | | | |
| *interleukin 8* (*il8*)[d] | Forward | ATCGGCTCCCTACTGGTTCT | 0.997 | 80.1 | 125 |
| | Reverse | ATGTGACGACCAATCTGACG | | | |
| *interleukin 1, beta (il1b)*[d] | Forward | AACACGGACGACCTGAAAAG | 0.999 | 83.4 | 126 |
| | Reverse | GCTGATGTACCAACCGGAGT | | | |
| *NADPH oxidase cytosolic protein p67phox (p67-phox)*[d] | Forward | ATAGTAGGCCCCTCGTCCAC | 0.996 | 83.5 | 168 |
| | Reverse | AGCTGTGGTCCAATCCAAAG | | | |
| *macrophage inflammatory protein 2* (*mip2*)[a] | Forward | GTGTTTCCAGCAGATCACTCG | 0.998 | 90.9 | 118 |
| | Reverse | TGTTCCACTTGGTGAGGAGTC | | | |
| *megalocytivirus-induced protein 1* (*mig1*)[c] | Forward | CAGTCCTGGTTTGGTGGTCT | 0.998 | 80.8[e] | 132 |
| | Reverse | GCTTCCTCTGAAACCCAACA | | | |
| *bloodthirsty-1* (*bty-1*)[c] | Forward | GGAGACCAGACCTGCATCAT | 0.994 | 93.5 | 153 |
| | Reverse | ATCCCCTTGACCCCTAAGAA | | | |
| *bloodthirsty-2* (*bty-2*)[c] | Forward | ATAGCAACCTGCTGGGATGT | 0.990 | 82.6 | 171 |
| | Reverse | GATCCGAAGGTCTGGTTCAA | | | |
| *E3 ubiquitin-protein ligase herc4* (*herc4*)[c] | Forward | CGCTCTGTGGATTTGCTACA | 0.989 | 87.8[e] | 116 |
| | Reverse | CCTTCTCTTGAGGGGAAACC | | | |
| *vimentin*[c] | Forward | AGAGCAGGATTTCCGTTCCT | 0.995 | 83.5 | 165 |
| | Reverse | CATGGTTCTGTGTGGACTCG | | | |
| *heat shock protein 90, beta, member 1* (*grp94*)[c] | Forward | GCCTACCAGACAGGCAAAGA | 0.997 | 86.4 | 115 |
| | Reverse | CGCTCGCTACTCTCTTGAGC | | | |

| | | | | | |
|---|---|---|---|---|---|
| *catalase*[c] | Forward | GCGTGCTACCTGGTCAAGAT | 0.997 | 83.3[e] | 145 |
| | Reverse | GACTACGGAAAGCAGGTCCA | | | |
| *lysosomal protective protein* (*ctsa*)[c] | Forward | AGGTCCTCCACAAACCACTG | 0.996 | 85.6 | 125 |
| | Reverse | ACCTACCCAACGATGAAGGA | | | |
| **Normalizers** | | | | | |
| *eukaryotic translation initiation factor 3 subunit* (*eif3*)[c] | Forward | AACTGTCCGTAGTCCGCAAG | 0.995 | 91.1 | 125 |
| | Reverse | CTGCTCAGCGAGAAACAGAA | | | |
| *60S acidic ribosomal protein P1* (*rplp1*)[b] | Forward | TCTGAAGCTAAGGCCCTCAA | 0.995 | 90.4 | 141 |
| | Reverse | ATCGTCGTGGAGGATCAGAG | | | |

[a] The primer sequences for these transcripts were previously published in Hori et al. (2012).
[b] The primer sequences for these transcripts were previously published in Inkpen et al. (2015).
[c] These primers were designed using ESTs or contigs representing the microarray probes.
[d] These transcripts were not present in the microarray significant gene list, and the primers were designed based on nucleotide sequence in NCBI.
[e] The amplification efficiencies of these primers were determined using 4-point serial dilutions of cDNA.
[f] This primer pair was previously published in Feng and Rise (2010).

## 2.4. Results

### *2.4.1. Cell characterisation*

PI staining remained under 4% during the culture period. Cell death increased slightly in both groups at 48 HPS (control, 2.03 ± 0.14% and pIC, 2.35 ± 0.36%) compared to 24 HPS (control, 1.08 ± 0.19% and pIC, 1.68 ± 0.44%). However, cell death was not influenced by pIC stimulation. Based on visual assessment of the flow cytometry dot plots, there was no apparent change in cell size (FSC), or cell complexity (SSC), due to culture time or pIC treatment (Fig. 2.2.). The majority of the adherent cells were identified morphologically as macrophage-like cells; the percentage of macrophage-like cells was not affected by sampling time or pIC at 24 HPS (control, 78.72 ± 2.25% and pIC, 75.86 ± 1.61%) or 48 HPS (control, 76.47 ± 1.24% and pIC, 72.48 ± 0.51%) and sampling time. Fig. 2.3. shows an example of cultured adherent cells.

### *2.4.2. The pIC-responsive transcripts in Atlantic cod macrophages*

A two-class paired comparison of SAM analysis (FDR < 0.05) was used to identify the pIC-responsive transcripts in Atlantic cod macrophages at 24 HPS. SAM found 446 pIC-responsive probes (285 significantly up-regulated and 161 significantly down-regulated probes). After re-annotating the significant transcript list, 92 up-regulated and 99 down-regulated probes remained unknown (see Supplemental Table S2.1. for the complete list of pIC-responsive transcripts). A selection of pIC-responsive transcripts with putative roles in immune responses and/or macrophage functions is represented in

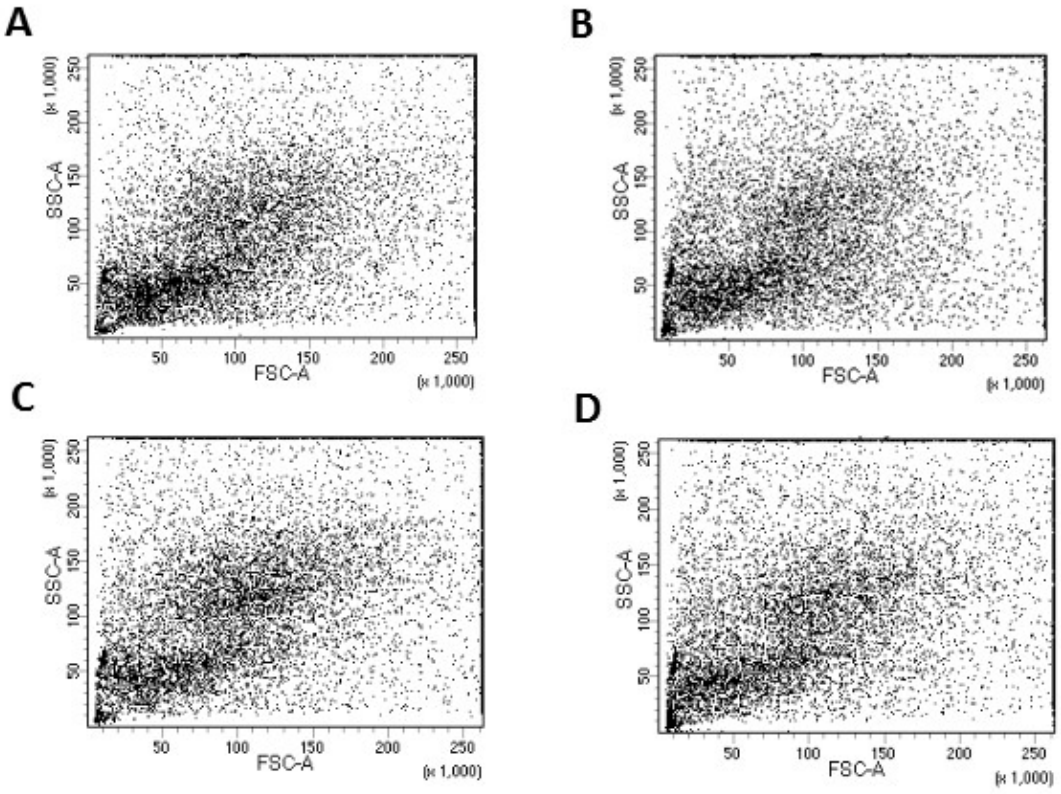


**Figure 2.2. Cell size cell complexity of Atlantic cod macrophage in control and pIC groups.** Representative flow cytometry dot plots ($n$ = 3) of adherent macrophages from Atlantic cod demonstrating no change in cell size (forward scatter, FSC) and cell complexity (side scatter, SSC) in PBS (control) and pIC treated cells over time. A. Control 24 h post-stimulation (HPS); B. pIC 24 HPS; C. Control 48 HPS; D. pIC 48 HPS.

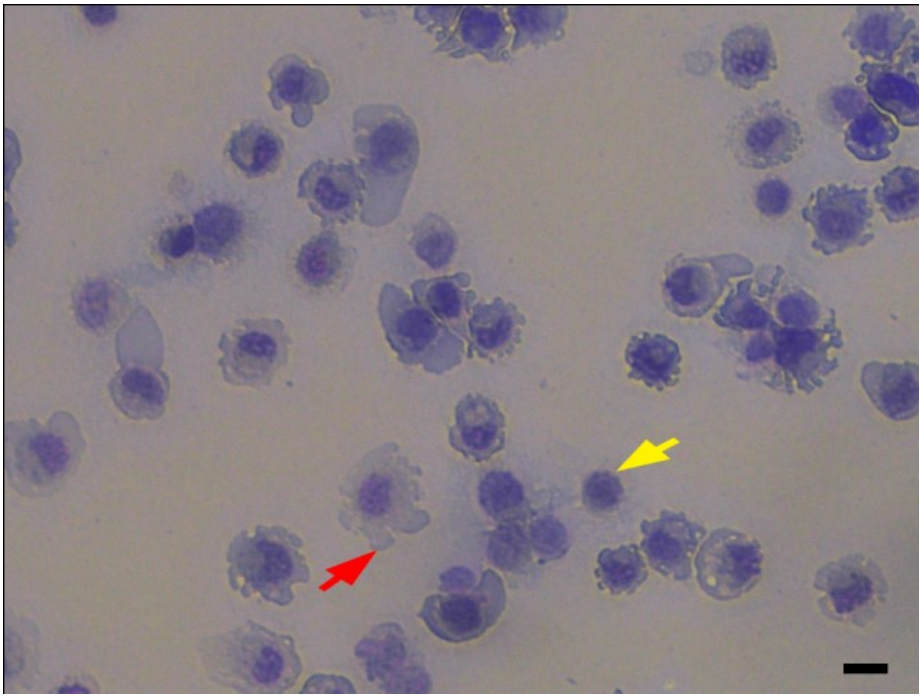

**Figure 2.3. Representative image of adherent Atlantic cod head kidney cells at 48 h post-isolation (24 h post-stimulation).** Magnification: 200X. The red and yellow arrows indicate representative macrophage-like and non-spread cells, respectively. Scale bar = 10 μm.

Table 2.2. Some immune effector transcripts [e.g., *mip2* (*macrophage inflammatory protein 2*), *isg15*, *mig1* and *viperin*) were found to be highly responsive to pIC (more than 6-fold increase in expression). Also, the microarray analysis revealed highly responsive [*lgp2* (*RNA helicase lgp2*), 5.7-fold] and less responsive [*mda5* (*melanoma differentiation-associated protein 5*), *tlr3* and *tlr7,* 1.8 to 2-fold] up-regulated and down-regulated (*tlr22g*, -1.27-fold) transcripts involved in pathogen recognition. Transcripts having roles in signal transduction/transcription control were identified as being up-regulated (e.g., *stat1*, *irf7* (*interferon regulatory factor 7*), *irf10* (*interferon regulatory factor 10*), *nfkbia* (*NF-kappa-B inhibitor alpha*), 1.6 to 3.1-fold) or down-regulated [e.g., *ccr7* (*CC chemokine receptor 7*), -1.47-fold] in response to pIC.

### *2.4.3. GO terms and GO enrichment analyses of pIC-responsive transcripts*

GO terms (Molecular Function, Biological Process, or Cellular Component categories) of pIC-responsive transcripts were mapped using Blast2GO (see Supplemental Table S2.1.). The GO annotation distribution (Biological Process level 2) of up- and down-regulated transcripts is illustrated in Fig. 2.4. The proportion of up-regulated transcripts with GO annotation involved in immune system process and multi-organism process appeared to be higher than that of down-regulated genes. GO enrichment analysis was performed to identify the over- and under-represented GO terms of the pIC-responsive probes compared to the whole array. This analysis identified 315 significantly enriched GO terms (see Supplemental Table S2.2.). After reduction to the

**Table 2.2. Selected[1] immune-related probes differentially expressed between Atlantic cod macrophages in pIC or control groups at 24 HPS.**

| ID | Name [species; GenBank accession number; E-value] [2] | Functional annotation [3] | Fold-change [4] |
|---|---|---|---|
| **38638** | Macrophage inflammatory protein 2 precursor [*Esox lucius*; ACO13449.1; 5.25E-06] [2]; (MIP2 alias CXCL2) | BP: chemotaxis, inflammatory response, immune response, cell chemotaxis; MF: chemokine activity | 15.71 |
| **38607** | Interferon stimulated gene 15-2 [*Gadus morhua*; ACZ02438.1; 4.05E-70] [2]; (ISG15) | NA[5] | 10.41 |
| **44624** | Viperin [*Gadus morhua*; ADG85737.1; 3.33E-137] [5]; (alias RSAD2) | BP: metabolic process; MF: catalytic activity, iron-sulfur cluster binding | 9.85 |
| **43195** | Megalocytivirus-induced protein 1 [*Cynoglossus semilaevis*; AFR33114.1; 9.94E-20][4]; (MIG1) | NA | 8.53 |
| **38611** | Interferon stimulated gene 15-3 [*Gadus morhua*; ACZ02439.1; 1.23E-72]; (ISG15) | NA | 8.48 |
| **38359** | Sacsin [*Chelonia mydas*; EMP28803.1; 3.98E-36] [4] | MF: ATP binding | 6.83 |
| **38617** | Interferon-inducible GTPase_a [*Salmo salar*; ABW94983.1; 8.41E-69]; (iGTPase-a) | BP: metabolic process; MF: GTP binding, hydrolase activity, acting on acid anhydrides | 6.19 |
| **44590** | RIG-I C-terminal domain-containing protein 1 [*Gadus morhua*; ADG85724.1; 5.60E-101]**[11];** LGP2 (alias DHX58) | BP: metabolic process; MF: hydrolase activity, acting on acid anhydrides | 5.72 |
| **44448** | C-C motif chemokine 13-like precursor [*Takifugu rubripes*; NP_001266983.1; 6.15E-14] [2]; (CCL13) | BP: immune response, cell chemotaxis; MF: chemokine activity | 3.93 |
| **43797** | Probable E3 ubiquitin-protein ligase HERC4-like [*Danio rerio*; NP_001139103.1; 7.06E-78] [5] | BP: metabolic process, protein ubiquitination; MF: ubiquitin-protein transferase activity, ligase activity | 3.43 |
| 38618 | Interferon-inducible GTPase_b [*Salmo salar*; ABW94984.1; 1.30E-85]; (iGTPase-b) | BP: metabolic process; MF: GTP binding, hydrolase activity, acting on acid anhydrides | 3.29 |
| **39321** | NFX1-type zinc finger-containing protein 1 [*Dicentrarchus labrax*; CBN80799.1; 4.87E-22] [8]; (ZNFX) | NA | 3.27 |
| **38599** | Interferon regulatory factor 10 [*Miichthys miiuy*; AHB59741.1; 5.19E-32]; (IRF10) | BP: regulation of transcription, DNA-templated; MF: regulatory region DNA binding, sequence-specific DNA binding transcription factor activity | 3.11 |
| **42028** | Secernin-3 [*Danio rerio*; NP_956032.1; 1.41E-95] | BP: proteolysis; MF: dipeptidase activity | 2.90 |
| 38655 | Interferon regulatory factor 7 [*Scophthalmus maximus*; ADQ52413.1; 6.69E-12] [2]; (IRF7) | BP: regulation of transcription, DNA-templated, MF: regulatory region DNA binding, sequence-specific DNA binding transcription factor activit | 2.79 |
| 43099 | Bloodthirsty [*Gadus morhua*; ADM21462.1; 1.04E-42] [13]; (BTY) | MF: zinc ion binding, metal ion binding | 2.61 |
| 38565 | Inducible heat shock protein 70 [*Miichthys miiuy*; CCF23009.1; 1.55E-173]; (HSP70) | BP: response to stress, MF: nucleotide binding, ATP binding | 2.56 |
| 36483 | CXC chemokine [*Scophthalmus maximus*; ACD62783.1; 1.20E-18] | BP: immune response, neutrophil chemotaxis, neutrophil degranulation; MF: chemokine activity | 2.37 |
| **37853** | Interferon-inducible protein Gig2 [*Carassius auratus*; AAP49829.1; 1.04E-36] | BP: metabolic process; MF: NAD+ ADP-ribosyltransferase activity | 2.36 |
| **36407** | CD9 antigen 2 [*Oplegnathus fasciatus*; BAM36395.1; 2.78E-34] | NA | 2.32 |
| **39485** | Helicase MOV-10 [*Salmo salar*; NP_001167174.1; 1.30E-45] [4] | BP: metabolic process; MF: helicase activity | 2.24 |
| 42596 | Toll-like receptor 7 [*Larimichthys crocea*; AGO28200.1; 9.70E-26]; (TLR7) | BP: MyD88-dependent toll-like receptor signaling pathway, immune response, signal transduction, toll-like receptor 7 signaling pathway, defense response to virus; MF: single-stranded RNA binding, transmembrane signaling receptor activity | 2.05 |
| **42754** | TAP1 [*Oncorhynchus mykiss*; ABB52828.1; 2.77E-45] | BP: ATP catabolic process, transport, peptide | 2.03 |

| | | | |
|---|---|---|---|
| | | transport, transmembrane transport; MF: nucleotide binding, ATP binding, peptide transporter activity, ATPase activity, ATPase activity, coupled to transmembrane movement of substances | |
| 40500 | Polymeric immunoglobulin receptor [*Paralichthys olivaceus*; ADK91435.1; 2.94E-44] | NA | 2.03 |
| 36933 | Type 1 double stranded RNA activated protein kinase [*Gadus morhua*; ADG85735.1; 4.09E-69]; (PKR) | BP: protein phosphorylation, phosphorylation; MF: nucleotide binding, protein kinase activity, protein serine/threonine kinase activity, ATP binding, kinase activity | 2.01 |
| **44545** | Interferon regulatory factor 1 [*Gadus morhua*; ADG85733.1; 2.00E-120]$_4$; (IRF1) | MF: regulatory region DNA binding | 1.95 |
| **44919** | Immunoglobulin superfamily member 2, partial [*Chelonia mydas*; EMP37893.1; 3.60E-06] | NA | 1.87 |
| 42622 | TRAF-interacting protein [*Danio rerio*; NP_991170.1; 5.69E-44]; (TRAIP) | MF: zinc ion binding, metal ion binding | 1.83 |
| 44541 | Interferon induced with helicase C domain 1 [*Gadus morhua*; ADG85722.1; 1.65E-162] $_2$; alias Melanoma Differentiation-Associated Protein 5 (MDA5) | BP: metabolic process; MF: helicase activity, hydrolase activity, acting on acid anhydrides | 1.83 |
| 46358 | TNFAIP3-interacting protein 3 [*Myotis davidii*; ELK35575.1; 2.06E-08] | NA | 1.79 |
| 44608 | Toll-like receptor 3 [*Gadus morhua*; ADG85741.1; 2.81E-35] $_3$; (TLR3) | BP: signal transduction | 1.78 |
| **45045** | Ubiquitin carboxyl-terminal hydrolase CYLD, partial [*Ophiophagus hannah*; ETE61821.1; 2.06E-24] | BP: ubiquitin-dependent protein catabolic process; MF: hydrolase activity, ubiquitinyl hydrolase activity | 1.68 |
| 44596 | STAT1 [*Gadus morhua*; ADG85732.1; 8.24E-37] $_4$ | BP: regulation of transcription, DNA-templated, signal transduction; MF: sequence-specific DNA binding transcription factor activity, signal transducer activity | 1.64 |
| **48764** | Interferon-induced protein 44-like protein, partial [*Epinephelus coioides*; AEA39725.1; 1.95E-16] $_2$; (IP44) | NA | 1.64 |
| **38670** | Optineurin [*Salmo salar* ; NP_001133761; 7.65E-30] | NA | 1.63 |
| **39077** | Microtubule aggregate protein homolog isotype-1 [*Paralichthys olivaceus*; BAK52806.1; 1.67E-67]; (MTAP) | NA | 1.62 |
| 44530 | NF-kappa-B inhibitor alpha [*Gadus morhua*; ADG85744.1; 5.08E-44] $_2$; (NFKBIA) | NA | 1.58 |
| 38635 | IL-4 receptor-2 precursor [*Oncorhynchus mykiss*; NP_001233265.1; 2.00E-14] | NA | 1.55 |
| **36797** | Cytotoxic and regulatory T cell protein precursor [*Oncorhynchus mykiss*; NP_001117972.1; 1.87E-52] | NA | 1.53 |
| **38094** | Probable E3 ubiquitin-protein ligase HERC6 [*Bos taurus*; NP_001179573.1; 3.47E-39] | NA | 1.51 |
| 44572 | Mitogen-activated protein kinase 14 [*Gadus morhua*; ADG85751.1; 0]; (MAPK14) | BP: MAPK cascade, protein phosphorylation, phosphorylation; MF: nucleotide binding, protein kinase activity, MAP kinase activity, ATP binding, kinase activity, transferase activity | 1.51 |
| 36400 | Tumor necrosis factor receptor superfamily member 5 precursor [*Salmo salar*; ACI69421.1; 2.66E-43] | NA | 1.41 |
| 40882 | Kinase C eta type [*Salmo salar*; ACN60220.1; 2.86E-136] | BP: phosphorylation, intracellular signal transduction; MF: nucleotide binding, protein kinase activity, protein serine/threonine kinase activity, ATP | 1.41 |

| | | | |
|---|---|---|---|
| | | binding, transferase activity | |
| **43692** | Tumor necrosis factor receptor superfamily member 14 precursor [*Osmerus mordax*; ACO09453.1; 1.57E-37] | NA | 1.4 |
| 40316 | Shugoshin-like 2 (S. pombe) [*Rattus norvegicus*; EDL99015.1; 8.44E-11] | NA | 1.39 |
| 37678 | Krueppel-like factor 4 [*Salmo salar*; ACI34008.1; 6.82E-22]; (KLF4) | MF: nucleic acid binding, metal ion binding | 1.23 |
| 42605 | Toll-like receptor 22g [*Gadus morhua*; AFK76491.1; 5.84E-63]; (TLR22g) | BP: signal transduction | 0.78 (-1.27) |
| **36119** | BCL2/adenovirus E1B interacting protein 3-like a [*Danio rerio*; NP_001012242.1; 4.40E-73] | BP: positive regulation of apoptotic process, negative regulation of programmed cell death, defense response to virus, mitochondrial outer membrane permeabilization | 0.74 (-1.36) |
| 37916 | Immunoglobulin D heavy chain constant region variant b [*Gadus morhua*; AAF72569.1; 2.77E-135] | NA | 0.73 (-1.37) |
| 37913 | Immunoglobulin light chain isotype 2, partial [*Trematomus bernacchii*; ABL60895.1; 5.07E-36] | NA | 0.71 (-1.4) |
| 40298 | CC chemokine CK3 [*Sparus aurata*; ADE58986.1; 1.39E-12] | BP: immune response, cell chemotaxis; MF: chemokine activity | 0.71 (-1.4) |
| 36350 | Catalase [*Oreochromis niloticus*; AEE40963.1; 4.76E-11] | BP: response to oxidative stress, oxidation-reduction process; MF: catalase activity, peroxidase activity, oxidoreductase activity, heme binding | 0.69 (-1.43) |
| 36377 | CC chemokine receptor 7 [*Oncorhynchus mykiss*; NP_001268325.1; 3.78E-55]; (CCR7) | BP: Chemotaxis, inflammatory response, immune response, signal transduction, G-protein coupled receptor signaling pathway, chemokine-mediated signaling pathway; MF: signal transducer activity, G-protein coupled receptor activity, chemokine receptor activity, C-C chemokine receptor activity | 0.68 (-1.47) |
| 44976 | Chymase precursor [*Esox lucius*; ACO13491.1; 2.71E-49] | BP: proteolysis, MF: catalytic activity, serine-type endopeptidase activity | 0.66 (-1.5) |
| 40296 | Lysosomal protective protein [*Tupaia chinensis*; ELW66216.1; 2.08E-47]$_2$; (alias Cathepsin A; CTSA) | BP: Proteolysis; MF: carboxypeptidase activity, serine-type carboxypeptidase activity, peptidase activity, hydrolase activity | 0.64 (-1.57) |
| 37846 | Vimentin [*Oncorhynchus mykiss*; NP_001118201; 9.20E-58] | MF: structural molecule activity | 0.63 (-1.59) |
| 37372 | GRP94a protein [*Oncorhynchus mykiss*; CDG41617.1; 3.94E-61]; (alias HSP90b1) | BP: protein folding, response to stress; MF: ATP binding, unfolded protein binding | 0.58 (-1.73) |

[1] These differentially expressed probes were selected based on their function in immune response (i.e., involved in immune response or known to be an immune-responsive transcript in vertebrates).The probe identifiers (IDs) in bold indicate overlap between differentially expressed probes in the current study and previously published pIC-responsive transcripts in Atlantic cod spleen [(Booman et al. 2014); FO diet, FDR < 0.01, fold change >1.3]. See Supplemental Table S2.1. for the complete list of differentially expressed probes.

[2]Taken from the most significant (i.e., lowest E-value) BLASTx hit (not hypothetical, predicted and unnamed protein) in the Blast2GO annotation. The subscript after the BLASTx hit's name represents the number of differentially expressed probes sharing the same annotation (see Supplemental Table S2.1. for the complete list of differentially expressed probes).

[3] Functional annotation represents the GO (Gene Ontology) terms, including Biological Process (BP) and Molecular Function (MF), for the best BLASTx hit.

[4] Fold-change (pIC/control) for the differentially expressed probes (FDR ≤ 0.05) as outputted by SAM analysis. The numbers in parenthesis represent fold down-regulation calculated as the inverse of fold-change (i.e., 1/fold-change) for the values less than one.

[5] Not available

most specific terms, 64 significantly enriched GO terms (Molecular Function and Biological Process categories) remained and these are depicted in Fig. 2.5. The majority of enriched GO terms were over-represented, and mainly associated with immune response signalling pathways (e.g., negative/positive regulation of MDA5, RIG-I and TLR signalling pathways) and immune effector production (e.g., negative/positive regulation of IFN and IL production). The GO terms 'oxidation-reduction process' and 'oxidoreductase activity' were significantly under-represented among pIC-responsive transcripts (see Supplemental Table S2.1.).

### *2.4.4. Hierarchical clustering analysis of microarray results*

Hierarchical clustering analysis was performed to determine if different groups (pIC and control) were separated based on their transcriptome profiles. All samples were clustered using the median-centred values of 446 differentially expressed probes (Fig. 2.6.). All samples were hierarchically clustered based on a subset of pIC-responsive transcripts that were annotated with GO terms associated with TLR and RLR (RIG-I-like receptor) signalling pathways (Fig. 2.7.). As expected, the samples clustered in two groups according to treatment (pIC or control). However, the level of immune responsiveness of macrophages to pIC differed between individuals.

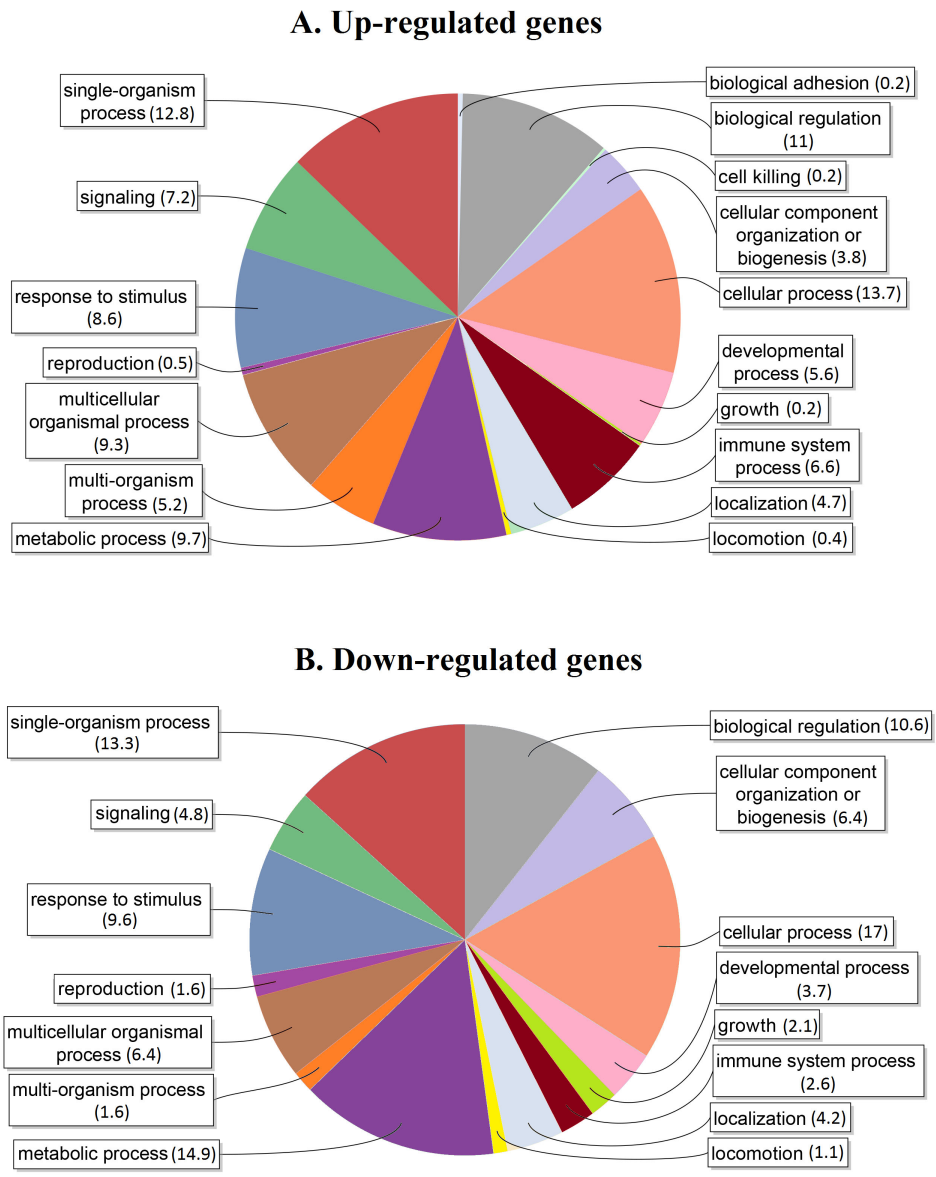


**Figure 2.4. GO term annotation of pIC-responsive transcripts.** The charts illustrate the GO term distribution (Biological Process level 2) of up-regulated (A) and down-regulated (B) transcripts in Atlantic cod macrophages (SAM, FDR < 0.05) exposed to pIC at 24 HPS. The numbers between brackets indicate the percentage of probes with each GO annotation.

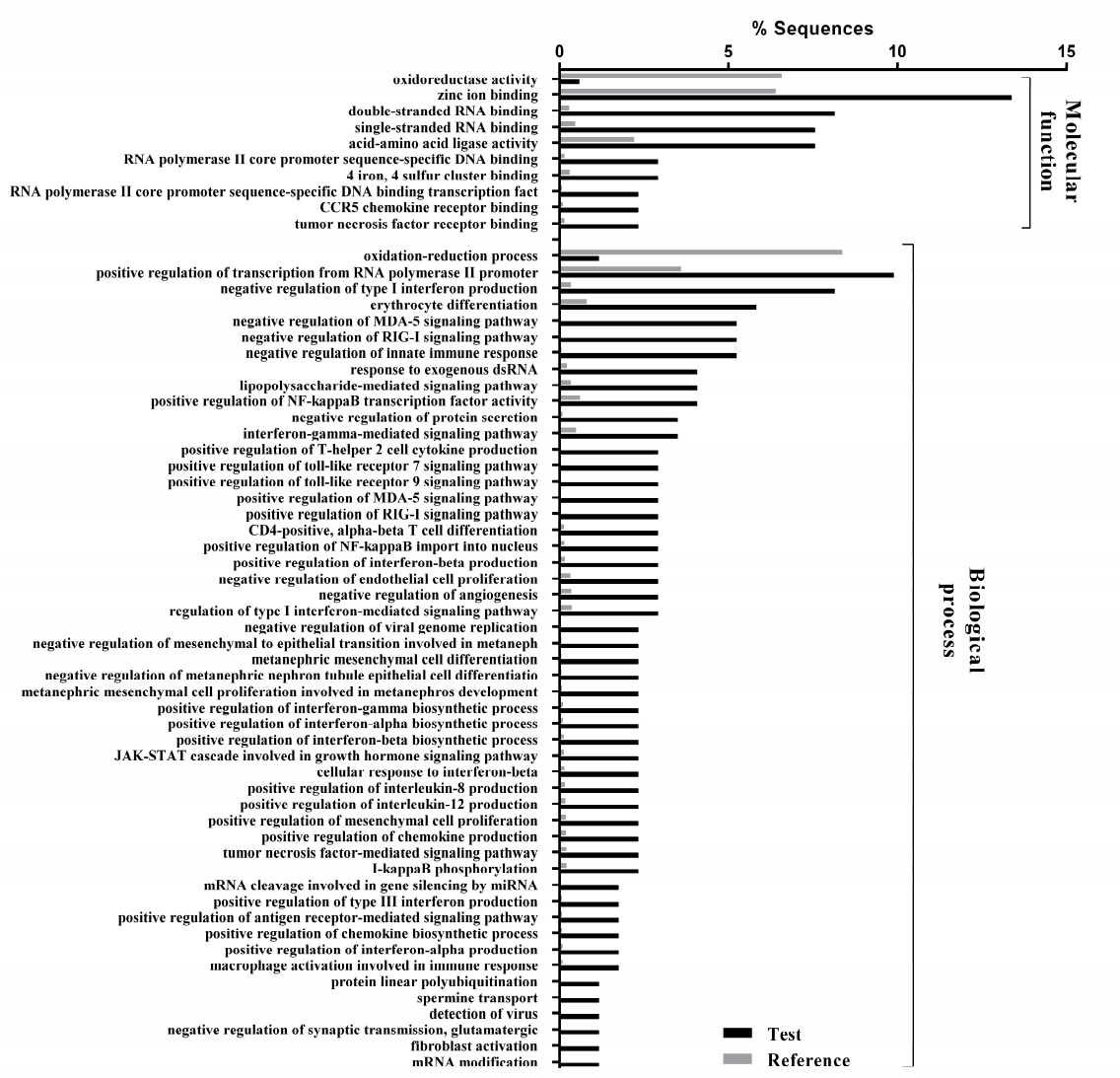


**Figure 2.5. The significantly enriched GO terms, reduced to the most specific terms.** The bar chart depicts the percentage of pIC-responsive probes in Atlantic cod macrophages at 24 HPS (SAM, FDR < 0.05) that were annotated with each GO term (Biological Process and Molecular Function; FDR < 0.05).

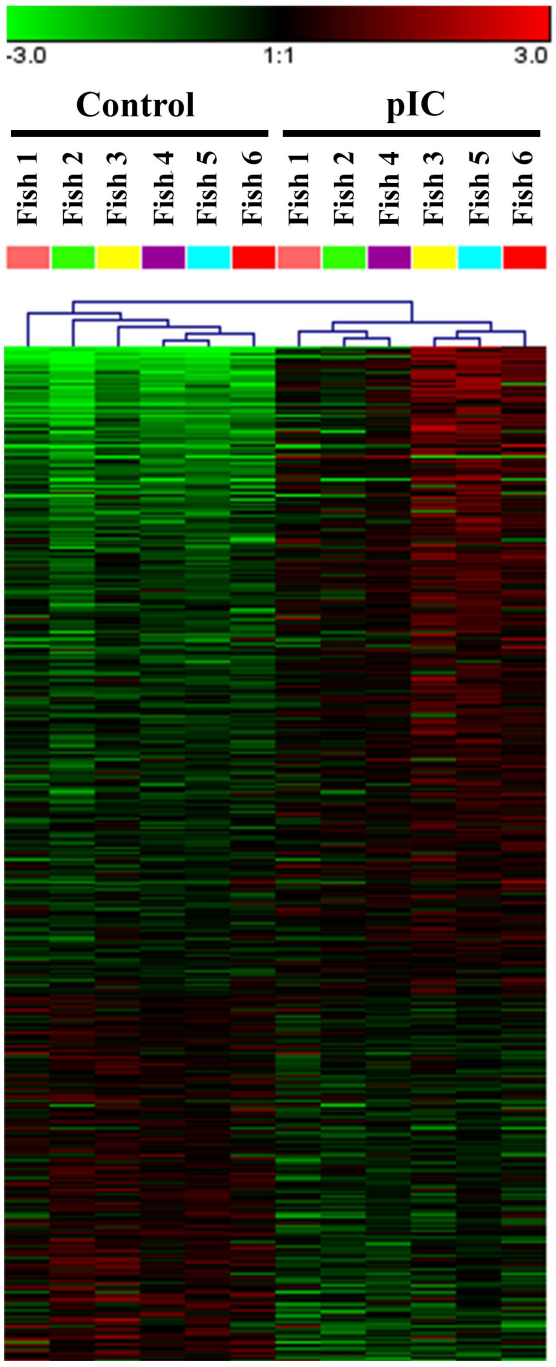


**Figure 2.6. Hierarchical clustering analysis based on all pIC-responsive transcripts.** Samples with the same coloured bar at the top of the figure belong to the same biological replicate.

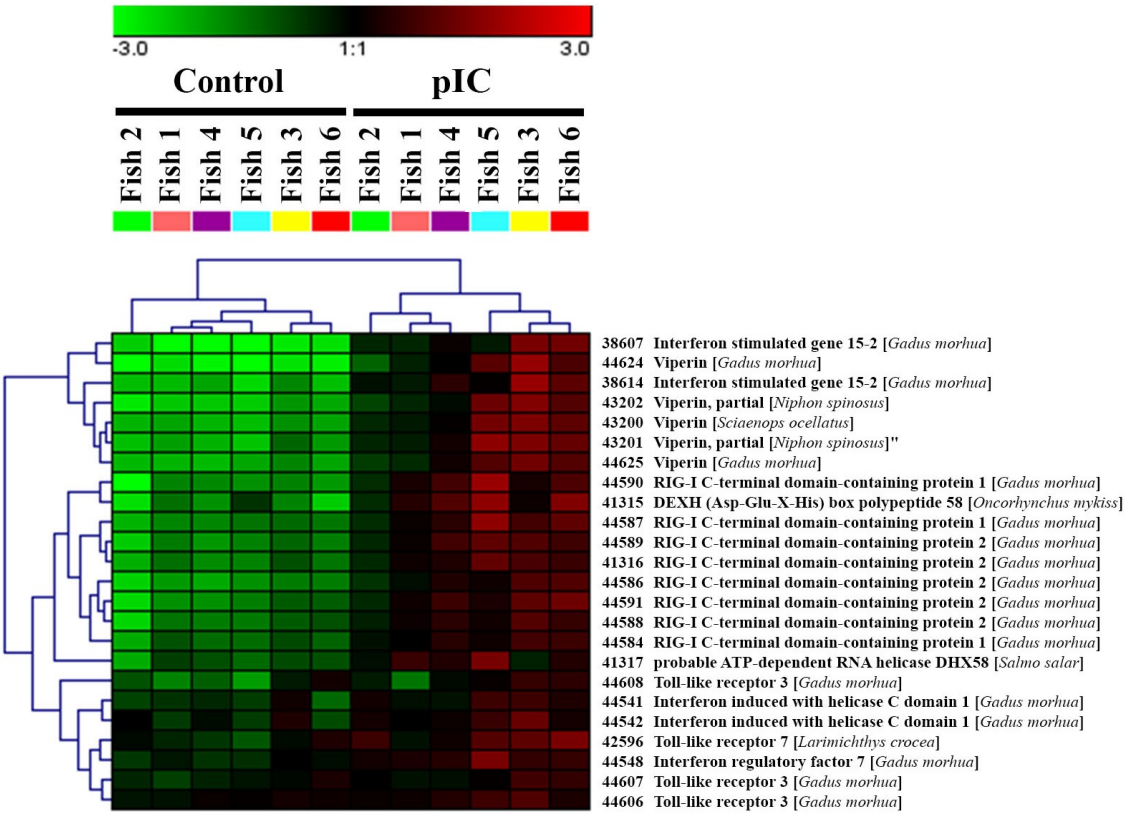


**Figure 2.7. Hierarchical clustering analysis based on a subset of pIC-responsive transcripts involved in pathogen recognition (signaling pathway or regulation of RLR and TLR families).** The transcript names are derived from the most significant BLASTx hit (E-value < 1.00E-05). Samples with the same coloured bar at the top of the figure belong to the same biological replicate.

### *2.4.5. qPCR validation*

To validate the microarray results and to assess the time-dependent expression of pIC-responsive transcripts, Atlantic cod macrophages were exposed to pIC and sampled at different time points. I selected a subset of pIC-responsive transcripts (i.e., 26 microarray-identified and 6 other important immune-responsive transcripts) that were related to various immunological pathways for qPCR validation. All of the microarray-identified transcripts subjected to qPCR were validated as pIC-responsive, except for *traip*, *ccr7*, *grp94* (*heat shock protein 90, beta, member 1*), *catalase* and *vimentin*. No significant change was found between pIC and control groups for any of the assayed transcripts at 3 and 6 HPS.

The qPCR results for transcripts involved in pathogen recognition are shown in Fig. 2.8. The highest expression of *tlr3*, *lgp2* and *mda5* in response to pIC was observed at 48 HPS, while the expression of *tlr7* in the pIC group peaked at 24 HPS. The expression of *tlr22g* transcript decreased in the pIC group at 24 HPS compared with the time-matched controls; there was, however, a significant down-regulation of this transcript in both pIC and control groups at 48 HPS compared to the other time points (Fig. 2.8. C). The maximum induction of *lgp2* in response to pIC was approximately one order of magnitude higher (~ 34-fold) than that of other pathogen recognition-associated transcripts, i.e., *tlr3*, *tlr7* and *mda5* (3.8 to 4.6-fold).

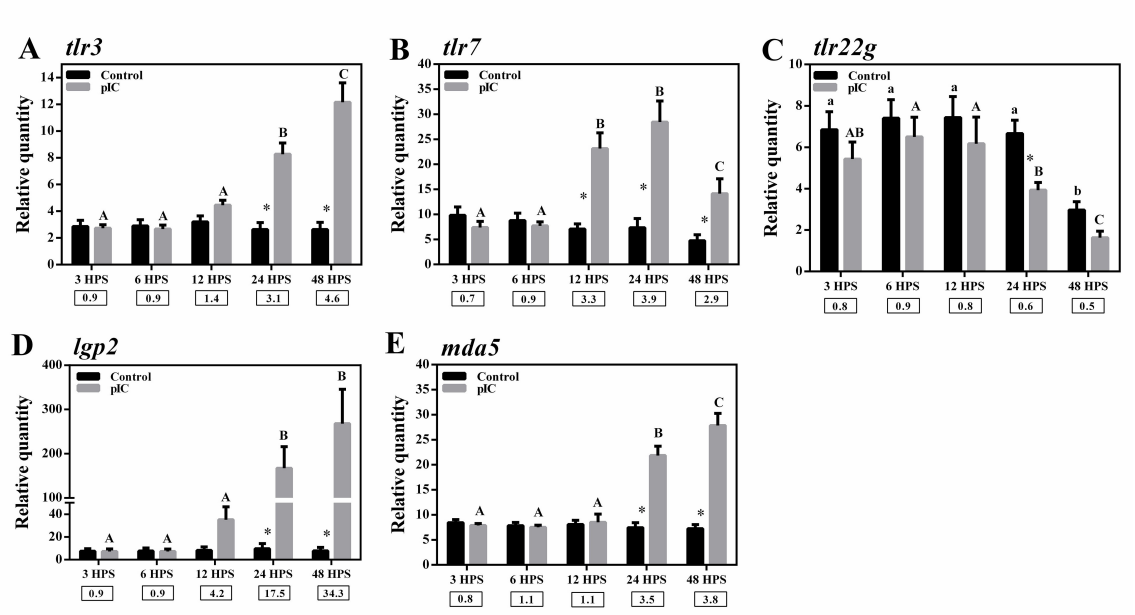


**Figure 2.8. qPCR results for pIC-responsive transcripts involved in pathogen detection.** Data are presented as means ± SE. An asterisk indicates a significant difference between time-matched pIC and control (PBS) groups. Different letters (lower-case for control and upper-case for pIC) represent significant differences within a group over time. The fold-change (pIC/control) values for each time point are shown below the figure panels. Significant differences were observed for all of the represented transcripts [*tlr3*, *tlr7*, *lgp2* and *mda5* ($p < 0.0001$); *tlr22g* (time: $p < 0.0001$ and treatment: $p < 0.05$)].

The qPCR results for pIC-responsive transcripts involved in signal transduction/transcription control are shown in Fig. 2.9. *stat1* and *znfx1* were significantly up-regulated by pIC only at 24 and 48 HPS, compared to both time-matched controls and other sampling times within the pIC group. Both *irf7* and *irf10* were significantly up-regulated at 12, 24 and 48 HPS, with maximum *irf7* induction at 24 HPS (10.6-fold) and maximum *irf10* induction at 48 HPS (12.8-fold); *irf7* transcript expression in pIC-stimulated macrophages at 48 HPS was significantly lower than in pIC-stimulated macrophages at 12 and 24 HPS, although still significantly higher than the time-matched control (Fig. 2.9. C, D). The microarray results were validated for *nfkbia*, a slightly up-regulated pIC-responsive transcript (1.3-fold at 48 HPS). The expression of *traip, ccr7* and *ifngr1* did not change significantly in response to pIC, yet their expression varied in either both groups (i.e., *traip* and *ccr7*) or only the pIC group (*ifngr1*) over time. The expression of *bcl-x1* did not change in Atlantic cod macrophages in response to either sampling time or pIC stimulation (Fig. 2.9. I).

The qPCR results of pIC-responsive transcripts with well-known or putative roles as immune effectors are depicted in Figs. 2.10. and 2.11., respectively. All three paralogues of *isg15* were strongly up-regulated by pIC, and their fold-change increased over time in the pIC group; peaking at 48 HPS. In addition, *isg15-3* was found to be more responsive to pIC (296.2-fold increase at 48 HPS) when compared to 2 other paralogues of *isg15*, but the level of responsiveness of this transcript was highly variable. Out of all *isg15* paralogues, *isg15-2* showed the lowest fold-change (117.5-fold at 48 HPS) and

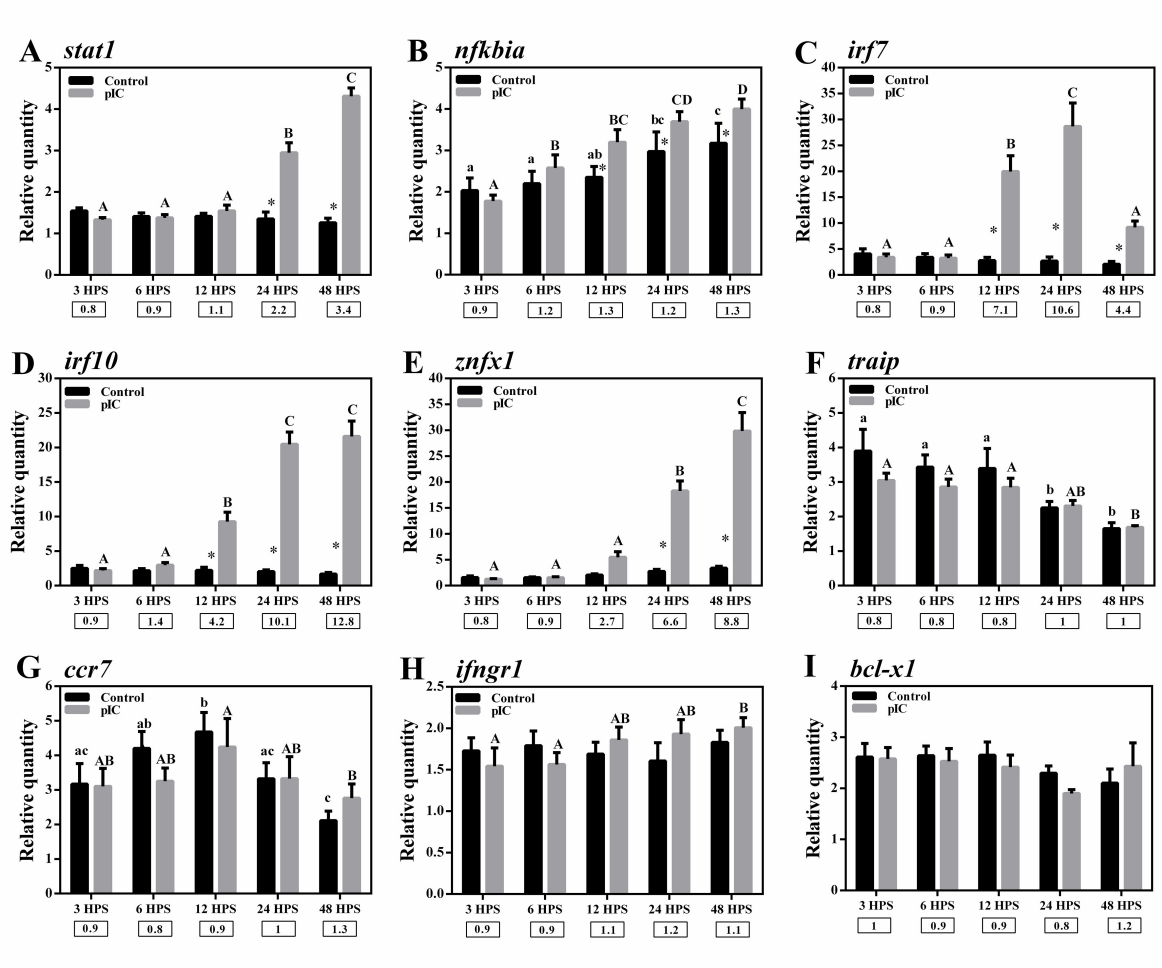


**Figure 2.9. qPCR results for pIC-responsive transcripts involved in signal transduction/transcription control.** Data are presented as means ± SE. An asterisk indicates a significant difference between time-matched pIC and control (PBS) groups. Different letters (lower-case for control and upper-case for pIC) represent the significant differences within a group over time. The fold-change (pIC/control) values for each time-point are shown below the figure panels. Significant differences were observed for all of the represented transcripts [*stat1*, *irf7*, *irf10* and *znfx1* ($p < 0.0001$); *nfkbia* ($p < 0.05$); *traip* (time: $p < 0.0001$); *ccr7* (time: $p < 0.05$); *ifngr1* ( $p < 0.05$)] except for *bcl-x1* ($p = 0.37$).

biological variability in the response to pIC. Likewise, *viperin*, *herc4* and *mig1* were up-regulated by pIC in a time-dependent manner, and the expression of these transcripts significantly increased within the pIC group after the 12 h time point, peaking at 48 HPS (i.e., 79.4- 38.7- and 12.9-fold respectively; Fig. 2.10. E, 2.11. E and 2.11. B). Furthermore, *ifng* and *ccl13* (*CC motif chemokine 13*) showed a strong up-regulation in response to pIC (Fig. 2.10. D, F); but, in contrast to *viperin* or *isg15*s, their expression within the pIC group did not increase significantly after the 24 h stimulation time point. Similar to the microarray results, *mip2* had the highest fold-change in response to pIC (318.2-fold at 24 HPS and 644.7-fold at 48 HPS). Two studied paralogues of *bty* had variable responses to pIC. *Bty-1* was significantly up-regulated in a time-related manner 12-48 h after pIC stimulation, with the highest expression at 48 HPS. However, the expression of *bty-2* was not induced by pIC. The expression of *il8* was not markedly induced by pIC, albeit this transcript was significantly up-regulated within both groups at 24 and 48 HPS in comparison with other sampling times. A similar time-dependent up-regulation was observed for *il1b* in both groups. On the other hand, the expression of *p67-phox* was not affected by pIC or sampling time (Fig. 2.10. I). Similar to the microarray result, *ctsa* (*lysosomal protective protein*) was down-regulated by pIC in Atlantic cod macrophages at 24 and 48 HPS, compared with time-matched controls (Fig. 2.11. I). The microarray results for *vimentin*, *grp94* and *catalase*, as candidate down-regulated transcripts at 24 HPS, were not validated by qPCR assays. While the expression of *vimentin* was not changed by pIC, this transcript was significantly down-regulated in both groups at 48 HPS compared with 3, 6 and 12 HPS (Fig. 2.11. F). While stable

expression was recorded for *grp94* in the pIC group over time, this transcript was up-regulated in the control group at 12 h compared both to other sampling times in the control group and to the time-matched pIC group (Fig. 2.11. G). This suggests that pIC inhibited the up-regulation of *grp94* in Atlantic cod macrophages at 12 HPS. The expression of *catalase* did not vary within the control group over time, but it increased within the pIC group at 48 HPS compared with 3, 6 and 24 HPS (Fig. 2.11. H).

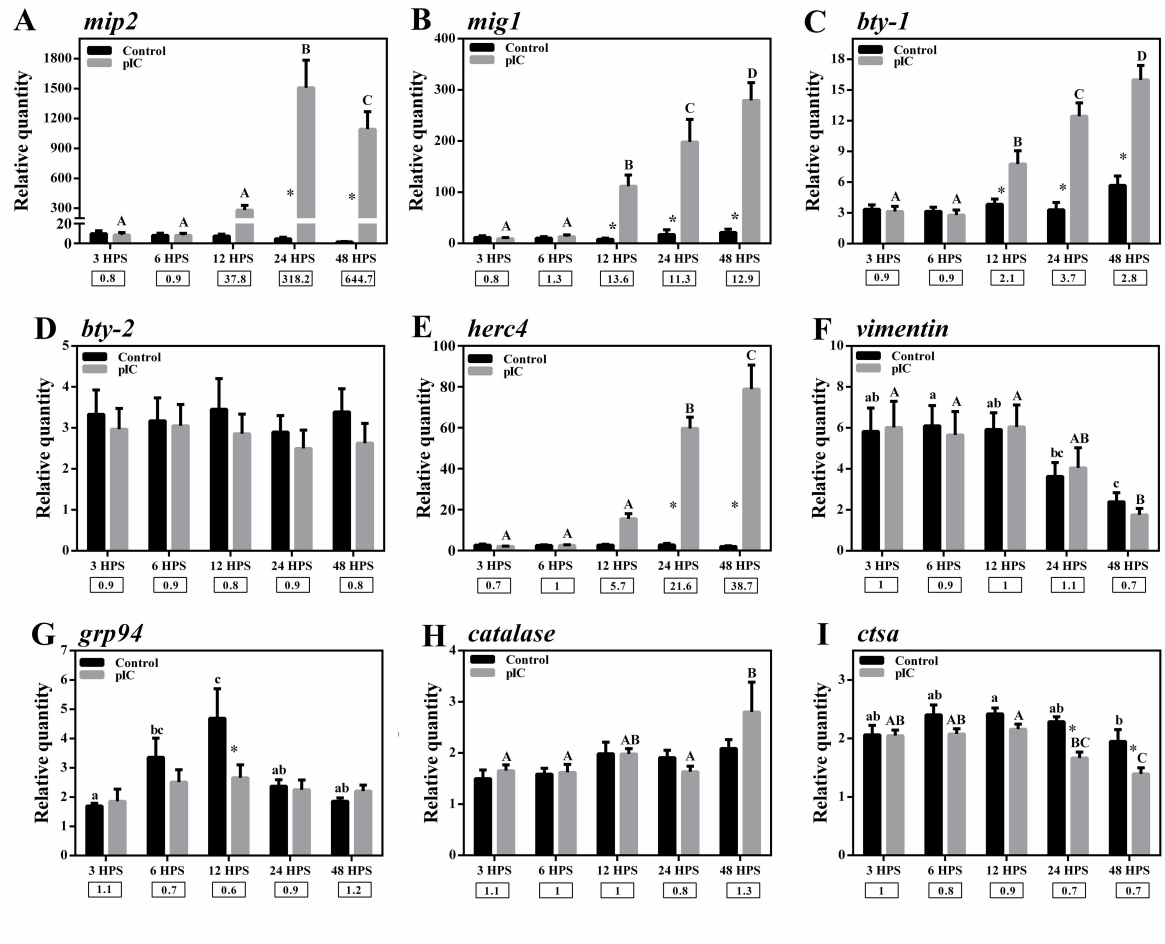


**Figure 2.10. qPCR results for pIC-responsive transcripts encoding well-known immune effectors.** Data are presented as means ± SE. An asterisk indicates a significant difference between time-matched pIC and control (PBS) groups. Different letters (lower-case for control and upper-case for pIC) indicate significant differences within a group over time. The fold-change (pIC/control) values for each time point are shown below the figure panels. Significant differences were observed for all of the represented transcripts [*isg15-1*, *isg15-2*, *viperin* and *ccl13* ($p < 0.0001$); *isg15-3* ($p < 0.05$); *ifng* ($p < 0.01$), *il8* ($p < 0.05$); *il1b* (time: $p < 0.0001$ and treatment: $p < 0.05$)] except for *p67-phox* ($p = 0.46$).

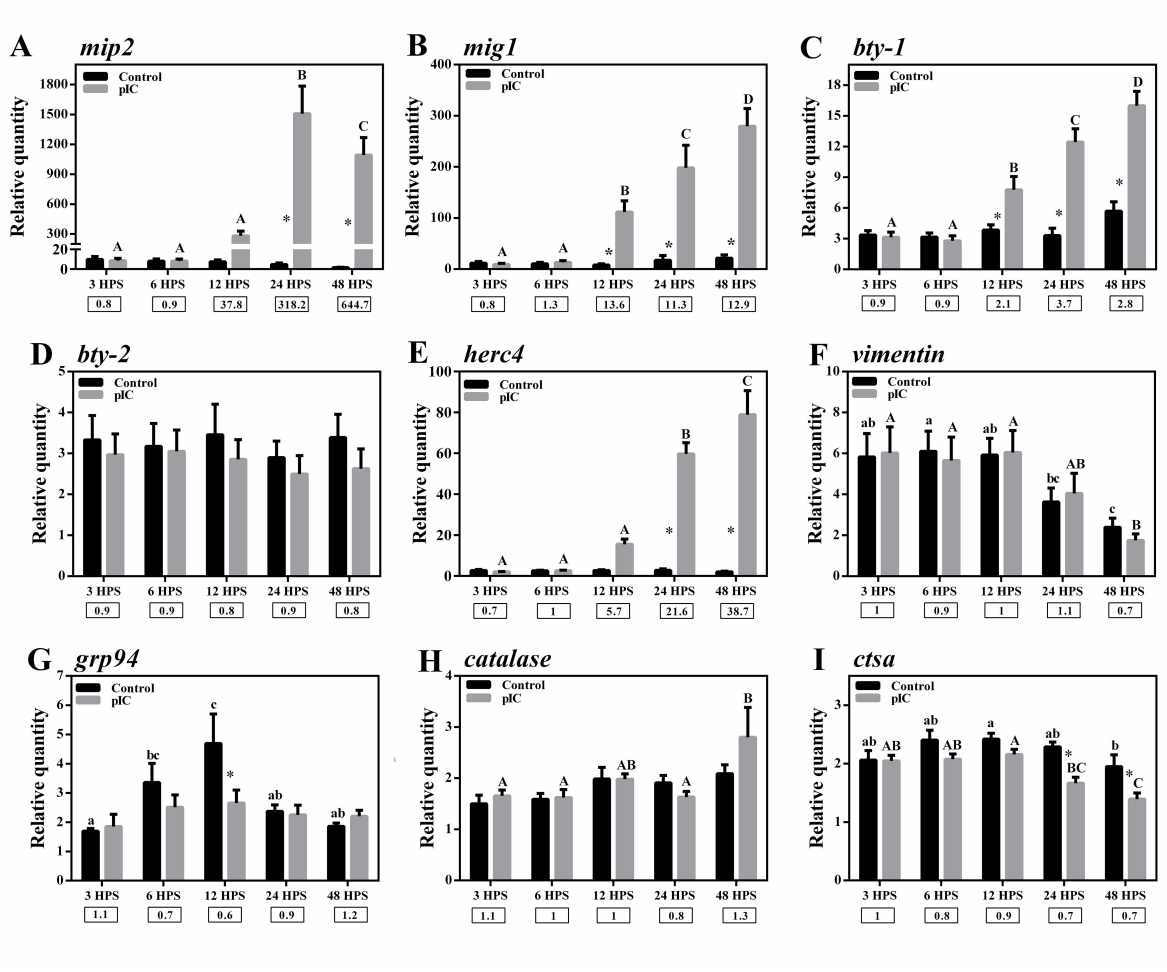


**Figure 2.11. qPCR results for pIC-responsive transcripts encoding putative immune effectors.** Data are presented as means ± SE. An asterisk indicates a significant difference between time-matched pIC and control (PBS) groups. Different letters (lower-case for control and upper-case for pIC) indicate significant differences within a group over time. The fold-change (pIC/control) values for each time point are shown below the figure panels. Significant differences were observed for all of the represented transcripts [*mip2*, *mig1*, *bty-1* and *herc4* ($p < 0.0001$); *vimentin* (time: $p < 0.001$); *grp94* ($p < 0.05$); *catalase* (time: $p < 0.05$); *ctsa* (time: $p < 0.001$ and treatment: $p < 0.0001$)] except for *bty-2* ($p = 0.053$).

## 2.5. Discussion

The present study was conducted using primary cell cultures isolated from Atlantic cod head kidney. While most (~75%) of the cells used in this study showed a macrophage-like morphology (i.e., large, adherent cells with cytoplasmic projections), these cells may include a heterogeneous population of the macrophages, which may consist of M1- and M2-like macrophages (Grayfer et al., 2018). The time-dependent up-regulation of *il1b,* a pro-inflammatory biomarker (Grayfer et al., 2014), in Atlantic cod macrophages may suggest the enhanced inflammatory response of macrophages over time. Cell death was not influenced by time points or pIC stimulation, in agreement with the gene expression results of *bcl-x1* (i.e., an apoptosis-related gene). In addition, cell death (i.e., less than 4% during the experiment) in the present study was comparable to a previous investigation on Atlantic cod macrophages (Steiro et al., 1998). Although pIC (i.e., 30 h exposure) was shown to induce apoptosis in rainbow trout macrophages (RTS11) in a dose-dependent manner (DeWitte-Orr et al., 2005), my results suggest that the dose of the pIC used in this study did not influence cell viability.

The microarray results showed that 446 probes were differentially expressed in pIC-stimulated Atlantic cod macrophages in comparison with control macrophages at 24 HPS. After BLASTx aligning the contig sequences representing pIC-responsive probes against NCBI's nr database, 92 up-regulated and 99 down-regulated probes remained unannotated. The proportion of unannotated pIC-responsive transcripts in down-regulated probes (61.4%) was markedly higher than that of up-regulated probes (34.7%), similar to the results seen in a previously published study on the transcriptome profile of the

antiviral response of Atlantic cod brain (Krasnov et al., 2013). The pIC-responsive transcript list in this study was compared with the spleen transcriptome response of pIC-injected Atlantic cod [Fish oil diet group; FDR < 0.01, fold change > 1.3; (Booman et al., 2014)], which was assessed using the same 20K microarray platform as that used in the current study. One hundred and sixty microarray-identified probes in the current study overlapped with pIC-responsive probes of the Atlantic cod spleen in the control diet group (see Table 2.2. and Supplemental Table S2.1.). The hierarchical clustering analysis revealed that all pIC-stimulated macrophage samples clustered in a separate branch from all control macrophages (Fig 2.6.); this was expected, since the transcripts included in this analysis were identified by SAM as pIC-responsive. The following discussion provides information on the genes and molecular pathways activated by pIC stimulation in Atlantic cod macrophages.

Five transcripts encoding receptors that play key roles in the recognition of dsRNA in Atlantic cod macrophages were identified herein (Fig. 2.8.). The expression of macrophage *tlr3* increased in response to pIC at 24 and 48 HPS compared with time-matched controls, and this was similar to the response of Atlantic cod head kidney cells to pIC (Holen et al., 2012). An earlier induction by pIC (at 12 HPS) in cod macrophages was recorded for *tlr7*, and this transcript was significantly up-regulated by pIC at 24 and 48 HPS (Fig. 2.8. B). TLR3 and TLR7 are evolutionarily conserved PRRs that detect RNA molecules of pathogens in endosomes (Akira et al., 2006; Kumar et al., 2009). TLR3 is responsible for recognition of dsRNA and the initiation of immune responses through the TRIF (TIR domain-containing adaptor protein inducing IFNB)-dependent

pathway in mammals (Akira et al., 2006; Kumar et al., 2009; Yu and Levine, 2011) and fish (Palti, 2011; Workenhe et al., 2010). While TLR7 is known to recognize ssRNA and mediate the production of type I IFN through the MyD88 (myeloid-differentiation primary-response gene 88)-dependent pathway in mammals, its role is unknown in fish (Palti, 2011; Zhang et al., 2014) (see Fig. 2.12. for pathways activated by dsRNA in Atlantic cod). In contrast to other *tlr*s, *tlr22g* was down-regulated by pIC in Atlantic cod macrophages at 24 HPS (Fig. 2.8. C). TLR22 is known as a fish-specific PRR that can recognize dsRNA on cell surfaces (Palti, 2011; Zhang et al., 2014). Sundaram et al. (2012) reported that 12 different paralogues of Atlantic cod *tlr22* showed distinct responses (i.e., were time-, tissue- and paralogue-dependent) to a bath challenge of *V. anguillarum*. In previous studies involving the same 20K cod microarray platform as that used herein, *tlr22g* was not reported as differentially expressed in the spleen of pIC-injected cod (Booman et al., 2014; Hori et al., 2012). Thus, this transcript may have specific roles in pathogen recognition of Atlantic cod head kidney cells.

In agreement with a previous *in vitro* study on pIC stimulation of an Atlantic cod larvae cell line (ACL) (Jensen et al., 2013), *lgp2* and *mda5* were up-regulated at 24 and 48 HPS in the present study. The RLR family (consisting of RIG-I, LGP2 and MDA5) is involved in the detection of cytoplasmic RNA of viruses and the induction of transcription factors [e.g., IRF7/3 and NFKB1 (nuclear factor of kappa light polypeptide gene enhancer in B-cells 1)] through interaction with MAVS/IPS-1 (mitochondrial antiviral-signalling protein) and the phosphorylation of TBK1 (TANK-binding kinase 1) and IKKs (NFKBIA kinase alias IKBK) (Akira et al., 2006; Takeuchi and Akira, 2008).

The current study also revealed the over-representation of GO terms associated with the positive or negative regulation of the MDA5 signalling pathway among pIC-responsive transcripts. RIG-I and MDA5 are responsible for recognition of ssRNA and dsRNA, respectively, and initiation of signalling cascades. Conversely, LGP2, lacking CARDs (caspase activation and recruitment domains), regulates (positively or negatively) or facilitates the activity of RIG-I and MDA5 (Kato et al., 2006; Takeuchi and Akira, 2008; Yu and Levine, 2011). Nonetheless, the function of LGP2 in fish remains controversial (Zhang and Gui, 2012). For example, the over-expression of fish *lgp2* has been shown to promote the induction of IFN and ISGs in Japanese flounder, *Paralichthys olivaceus* (Ohtani et al., 2010) and rainbow trout, *Oncorhynchus mykiss* (Chang et al., 2011), but to negatively regulate pIC-induced activity in goldfish, *Carassius auratus* (Sun et al., 2011b). Previous studies from the Rise lab have reported *lgp2*, but not *mda5*, as a responsive transcript in spleen of pIC-injected cod (Booman et al., 2014; Hori et al., 2012; Rise et al., 2008). The function of *mda5* in pIC recognition and signalling has been described in mammalian macrophages (Semple et al., 2015). Similarly, teleost MDA5 (i.e., in Japanese flounder) was shown to play an important role in the pIC-dependant signalling pathway (Zhou et al., 2014). These findings indicate the importance of *mda5* in antiviral immune responses of cod macrophages.

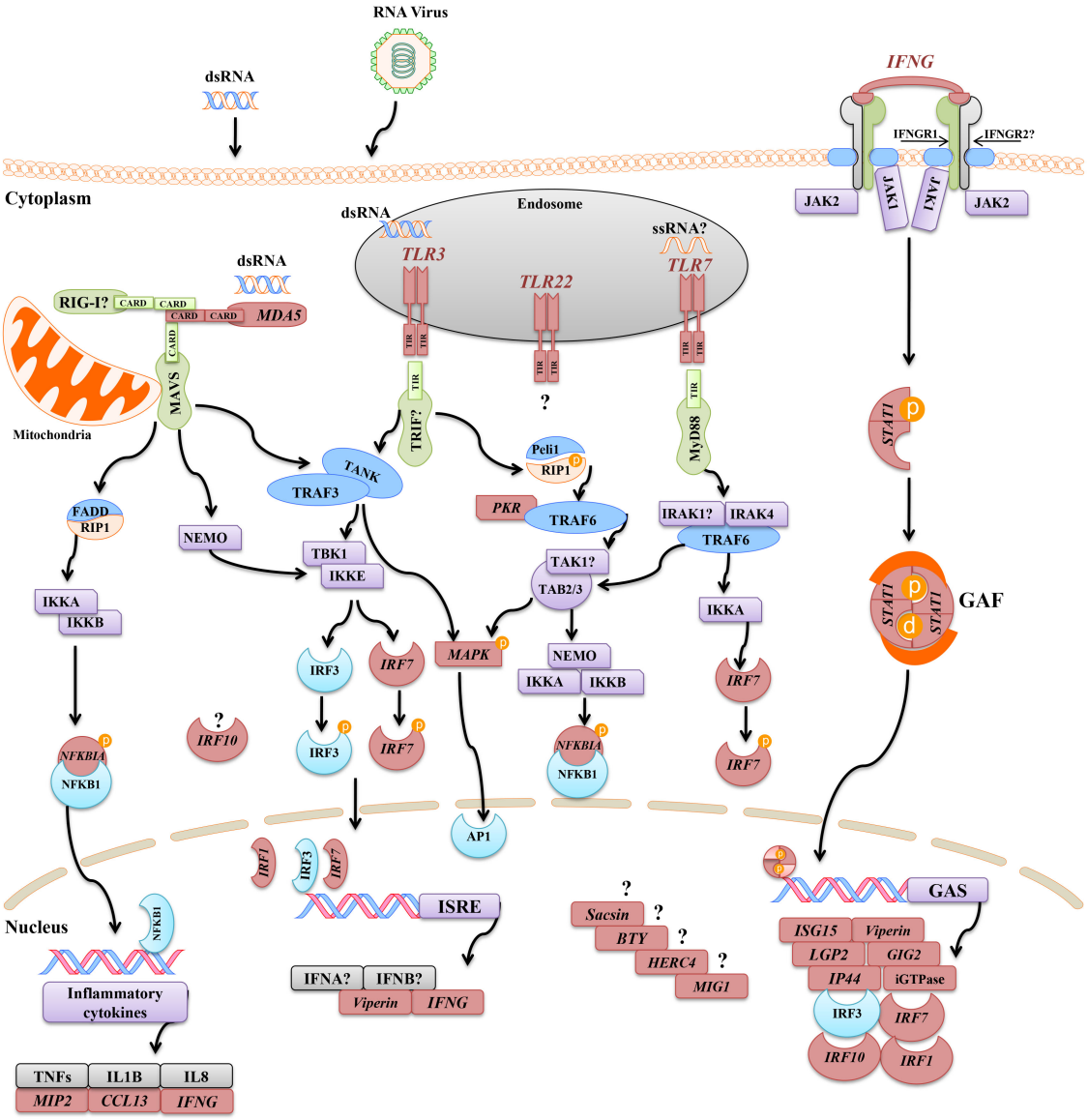


**Figure 2.12. The signalling pathways stimulated by pIC in Atlantic cod macrophages.** This figure was adapted from known mammalian pathways (Akira et al., 2006; Bonjardim et al., 2009; Yu and Levine, 2011). A question mark beside the gene/protein name indicates that it was not found in the Atlantic cod genome using nominal search in the Ensembl annotation of the Atlantic cod genome (http://ensembl.org/Gadus_morhua). A question mark below or above the gene/protein name indicates unknown immune function and signalling activation in mammals. The pIC-responsive genes identified in the present study are shown in italics. MDA5 (melanoma differentiation-associated protein 5), RIG-I (retinoic acid-inducible gene), MAVS (mitochondrial antiviral-signaling protein), FADD (FAS-associated death domain), RIP1 (receptor-interacting protein 1), IKK (NFKBIA kinase), NFKBIA (NF-kappa-B inhibitor alpha), NFKB1

(nuclear factor of kappa light polypeptide gene enhancer in B-cells 1), TNF (tumour necrosis factor), IL (interleukin), MIP2 (macrophage inflammatory protein 2), CCL13 (C-C motif chemokine 13), IFN (interferon), NEMO (NFKB1 essential modulator or IKKG), TLR (Toll-like receptor), TRIF (TIR domain-containing adaptor protein inducing IFNB), TRAF (TNF receptor-associated factor), TANK (TRAF family member-associated NFKB activator), TBK (tank-binding kinase), IRF (IFN regulatory factor), MAPK (mitogen-activated protein kinase), AP1 (transcription factor AP1), ISRE (IFN-sensitive response element), Peli1 (pellino E3 ubiquitin protein ligase 1), PKR (IFN-induced, double-stranded RNA-activated protein kinase), TAK1 [transforming growth factor beta (TGFB)-activated kinase 1], TAB (TAK1-binding protein), MyD88 (myeloid differentiation primary response gene 88), IRAK (IL1 receptor-associated kinase), IFNGR (IFN-gamma receptor), JAK (Janus kinase), STAT1 (signal transducer and activator of transcription 1), GAF (IFNG-activated factor), GAS (IFNG-activated sequence), ISG (IFN-stimulated gene 15), LGP2 (RNA helicase LGP2), GIG2 (IFN-inducible protein gig2), MIG1 (megalocytivirus-induced protein 1), IP44 (IFN-induced protein 44-like protein), iGTPase (IFN-inducible GTPase), BTY (bloodthirsty), HERC4 (E3 ubiquitin-protein ligase herc4).

pIC-responsive transcripts involved in signal transduction and transcription [e.g., *stat1*, *nfkbia*, *pkr* (*dsRNA-dependent protein kinase*), *irf1*, *irf7*, *irf10* and *znfx1*] were up-regulated in macrophages as in previously conducted studies on the spleen transcriptome response of Atlantic cod injected with either pIC or formalin-killed atypical *A. salmonicida* (ASAL) (Booman et al., 2014; Hori et al., 2012; Inkpen et al., 2015; Rise et al., 2008) or the brain transcriptome profiling of nodavirus carrier and/or pIC-stimulated Atlantic cod (Rise et al., 2010). As shown in Fig. 2.12., upon stimulation of TLRs and activation of several proteins (e.g., PKR), the IKK multiprotein complex phosphorylates NFKBIA, leading to the expression of inflammatory cytokines (reviewed by Kawai and Akira, 2007; Yu and Levine, 2011). The microarray analysis in this study showed a 2-fold increase in the expression of *pkr* by pIC. In addition, this study showed the over-representation of GO terms related to the positive regulation of NFKB1 transcription and import into the nucleus in the pIC-responsive transcript list, thus suggesting the possible role of this transcription factor in antiviral immune responses of Atlantic cod macrophages. As proposed by Hori et al. (2012) with regard to the pIC response of cod spleen, the slight but significant up-regulation of *nfkbia* observed in the present study could have occurred as a result of degradation and re-synthesis of NFKBIA in pIC-stimulated Atlantic cod macrophages (see Fig. 2.12.). The up-regulation of z*nfx1* by pIC as seen herein suggests the potential role of this transcript as a mediator of immune-related pathways (e.g., NFKB1) in cod macrophage immune responses. Although z*nfx1* has been reported as a pIC-responsive transcript in fish (Krasnov et al., 2011; Rise et al., 2010), its function in fish remains undetermined.

The microarray analyses of the current study identified *irf1, irf7* and *irf10* as pIC-responsive transcripts in Atlantic cod macrophages. IRFs (e.g., IRF3 and IRF7) bind to interferon-sensitive response elements (ISREs) and increase the production of IFNs and ISGs (Akira et al., 2006; Bonjardim et al., 2009). In previously published studies, the highest expression of *irf7* and *irf10* was found in the spleen of pIC-injected cod at either 6 (Rise et al., 2008) or 24 (Hori et al., 2012) h post-injection. Both *irf7* and *irf10* were significantly up-regulated by pIC at 12 HPS (7.1- and 4.2-fold, respectively), suggesting that these transcripts have important roles in early immune responses of Atlantic cod macrophages. The expression of *irf7* in cod macrophages peaked at 24 HPS (i.e., 10.6-fold up-regulation compared with time-matched control), but its expression was lower in the pIC group at 48 HPS. Goldfish *irf7* has been reported to be highly expressed in monocytes when compared with mature macrophages, suggesting the important role of this transcription factor in early stages of macrophage development (Katzenback et al., 2013). However, a significant down-regulation of Atlantic cod *irf7* at the later time points (e.g., 48 h) was not seen within the control group of the current study. While the *irf10* transcript expression response to pIC remained high at 48 HPS (comparable to that at 24 HPS), the *irf7* transcript expression response to pIC at 48 HPS dropped significantly (Fig. 2.9. C, D). This suggests that *irf10* has a more important role than *irf7* in the later (i.e., at 48 h post-stimulation) response of cod macrophages to pIC.

In this study, *ifng* and *stat1* were up-regulated in the pIC group at 24 and 48 HPS, although the expression of *ifngr1* was not affected by pIC. The production of IFNs and ISGs elicit the antiviral immune response (Fig. 2.12.). Following the binding of IFNG

with IFNGR1/2, IFNG-activated factor (GAF, i.e., a STAT1 homodimer) translocates into the nucleus and facilitates transcription initiation of the IFNG-responsive genes by binding to the IFNG-activated sequence (GAS) (reviewed by Bonjardim et al., 2009; Sadler and Williams, 2008). The GO terms associated with the regulation of IFNG production and the IFNG-dependent response were significantly over-represented in the pIC-responsive transcript list. A previously published study has shown that IFNG stimulates the nuclear localization of STAT1 in goldfish monocytes (Grayfer et al., 2010). Considering the inducibility of cod *stat1* by dsRNA observed in the present and previous studies (Booman et al., 2014; Hori et al., 2012), IFN signalling in Atlantic cod appears to be mainly mediated via IFNG and STAT1.

The engagement of dsRNA with PRRs (e.g., TLRs and RLRs) activates the signalling pathways (e.g., the TRIF-dependent pathway; see Fig. 2.12.), and results in the up-regulation of ISRE-containing immune effectors (e.g., ISGs and IFNs) (Bonjardim et al., 2009; Langevin et al., 2013a). As in previously published studies of the spleen transcriptome response of pIC-injected cod (Booman et al., 2014; Hori et al., 2012; Rise et al., 2008), *isg15*s, *ccl13*, *mip2*, *viperin*, *sacsin*, *mig1*, *gig2*, *herc4*, *bty*, *iGTPase* (*interferon-inducible GTPase*), *ip44* (*interferon-induced protein 44-like*) and *mtap* (*microtubule aggregate protein homolog isotype*) were up-regulated in cod macrophages by pIC. Several well-known antiviral immune effectors conserved among vertebrates (e.g., *isg15*s, *viperin, ccl13*, and *mip2*) were identified in the current study by microarray analysis, and subjected to time-dependent qPCR validation. All three paralogues of Atlantic cod *isg15* showed a strong up-regulation in macrophages after 24 h of pIC

stimulation, peaking at 48 HPS (i.e., > 100-fold up-regulation compared with time-matched controls). Furnes et al. (2009) also reported that the maximum response of all *isg15* paralogues in the head kidney of Atlantic cod occurred 2 days after pIC injection. Mammalian ISG15, as a highly-conserved antiviral protein, hinders several viruses by attaching to the target protein in conjugation with other proteins [e.g., UBE1L (ubiquitin-activating enzyme E1-like) and HERC5], termed ISGylation (Zhang and Zhang, 2011). Additionally, mammalian ISG15 promotes the activation of some virus-responsive proteins such as PKR (Okumura et al., 2013). Likewise, zebrafish ISG15 has been demonstrated to inhibit RNA viruses, and promote the induction of *viperin* and *rig-I* (Langevin et al., 2013b). The present study shows the importance of *isg15* paralogues in the antiviral immune responses of Atlantic cod macrophages.

The microarray-identified transcript list included 5 differentially expressed probes representing *viperin*. Consistent with the expression of *viperin* in ISAV-infected Atlantic salmon macrophage-like cells (Workenhe et al., 2009), the highest expression of *viperin* in pIC-induced Atlantic cod macrophages was recorded at 48 HPS (79-fold up-regulation). Similarly, the induction of *viperin* by pIC, chum salmon reovirus (CSV) and interferon-containing culture medium has been reported in monocyte/macrophage-like cells of rainbow trout at 24 HPS; this suggests that the viral induction of *viperin* in this species occurs via an IFN-dependent pathway (DeWitte-Orr et al., 2007). Virus-induced Viperin is chiefly activated by IRF1 or IRF3 in the RLR-associated pathway (Helbig and Beard, 2014). Similar to mammals, fish *viperin* can be triggered by dsRNA via a RLR-dependent pathway (Wang et al., 2014) and also by type I and II IFN (Aggad et al., 2009;

Sun et al., 2011a). In addition to the inhibitory effects of Viperin on virus replication, it facilitates the induction of IRF7 by interacting with signal mediators [e.g., it recruits IRAK1 and TRAF6 (TNF receptor-associated factor 6) to lipid bodies], and enhances the TLR7- and TLR9-dependent pathogen recognition (Saitoh et al., 2011). The over-representation of GO terms associated with regulation of TLR7 and TLR9 signalling pathways in pIC-responsive transcripts of Atlantic cod macrophages can be explained by the possible role of Viperin in modulation of these PRRs.

In the current study, the GO term "positive regulation of chemokine production" was over-represented in pIC-responsive macrophage transcripts. Chemokines are a superfamily of small cytokines that act as chemoattractants, and regulate leukocyte migration and trafficking (reviewed by Mantovani et al., 2004). A significant increase in *ccl13* expression was observed in pIC-treated Atlantic cod macrophages at 24 and 48 HPS (26.6-fold at 48 HPS). In addition, *mip2*/*cxcl2* showed the strongest response to pIC in Atlantic cod macrophages (a 644.7-fold increase at 48 HPS). Mammalian MIP2 is a crucial chemokine for neutrophils, and is secreted by different cells (e.g., macrophages, epithelial and intestinal cells), and is activated by viruses or LPS via IL1R- and NFKB1-dependent pathways (De Plaen et al., 2006; Di Paolo et al., 2009). CCL13, classified as a MCP (monocyte chemoattractant protein), stimulates the expression of adhesion molecules and pro-inflammatory cytokines (Mendez-Enriquez and García-Zepeda, 2013). *ccl13* and *mip2* have been identified in some fish species such as zebrafish (Baoprasertkul et al., 2005; Peatman and Liu, 2006), but their functions in fish are yet to be determined.

Some pIC-responsive transcripts (e.g., *herc4*, *gig2*, *bty*, and *sacsin*) identified in Atlantic cod macrophages may be categorized as fish-specific immune effectors since they were previously shown to be virus- or pIC-responsive transcripts in Atlantic cod and/or other fish species (Booman et al., 2014; Wang et al., 2013; Workenhe et al., 2009). However, they are not known as virus- or dsRNA-responsive transcripts in higher vertebrates. There was a strong up-regulation of *herc4* in pIC-induced Atlantic cod macrophages (38-fold increase at 48 HPS), as previously seen in ISAV-infected Atlantic salmon macrophage-like cells (Workenhe et al., 2009). However, *herc4* was reported as a weakly down-regulated transcript in the brain of nodavirus carrier Atlantic cod (Rise et al., 2010). Furthermore, *herc6* was identified herein as a pIC-responsive transcript in Atlantic cod macrophages by microarray analysis. HERC4 and HERC6 are members of a family of proteins having HECT and RCC1 domains. Some HERC family members are known to be important proteins in antiviral responses. For example, human HERC5 is the most recently evolved HERC family member and an IFN-induced protein that mediates (i.e., as the main E3 ligase) the process of ISGylation by conjugating to ISG15 and inhibits viral replication (Woods et al., 2014). HERC6 is an E3 ligase for ISG15 in mice, which lack a HERC5 ortholog in their genome (Zhang and Zhang, 2011). Further investigations are needed to determine whether HERC4 or HERC6 functions as an E3 ligase for ISG15 in lower vertebrates such as fishes. The strong induction of Atlantic cod *herc4* by pIC as seen herein suggests that this transcript plays an important role in antiviral immune responses of Atlantic cod.

The time-dependent qPCR results showed that *mig1* was significantly up-regulated by pIC 12 h after pIC stimulation, and its expression peaked at 48 HPS. The highest induction of *mig1* in response to megalocytivirus infection was observed in different tissues (i.e., kidney, spleen and liver) of tongue sole (*Cynoglossus semilaevis*) at 48 h post-injection, although the peak of up-regulation for this gene occurred in virus-treated head kidney lymphocytes of this species at 2 HPS (Wang et al., 2013). Tongue sole *mig1* shares moderate similarity with *gig1* of several teleost species and the VHSV-induced protein of rainbow trout (Wang et al., 2013). Indeed, the pIC-responsive probes identified as *mig1* in this study share significant similarity (e.g., as the second best BLASTx hit) with teleost *gig1* (e.g., goldfish) and the VHSV-induced protein of rainbow trout. This suggests that further studies are needed to completely characterise these pIC-responsive transcripts. Goldfish *gig1* has been recently identified as a novel antiviral fish ISG that is highly responsive to pIC, IFN and UV-inactivated grass carp reovirus (GCRV) through RLR-dependent signalling (i.e., *mda5*, *rig-i*, *mita*, *tbk1* and *irf3*) and *stat1* (Sun et al., 2013; Sun et al., 2014). Similar to the results for *mig1*, the highest fold-induction of *gig1* and *gig2* in pIC-stimulated blastulae embryonic cells of goldfish (CBA) was recorded at 24 and 48 HPS (Jiang et al., 2009; Sun et al., 2014). Moreover, the present microarray analysis showed a 2.36-fold increase in the expression of *gig2* in pIC-induced cod macrophages at 24 HPS. Jiang et al. (2009) reported that goldfish *gig2* is a IFN- and pIC-inducible gene (i.e., harbouring ISREs and GASs in its promoter), and is activated by IRF7. The gene expression results of the present study, along with the over-represented GO terms (e.g., of MDA5 signalling) in pIC-induced cod macrophages,

suggest that this protein family plays an important role in the antiviral responses of Atlantic cod macrophages.

Several pIC-responsive *bty* transcripts were identified by microarray analysis, and further analysis revealed that these pIC-responsive probes of Atlantic cod *bty*s share between 80-94% similarity at the nucleotide level with a fully characterised Atlantic cod *bty* (Genbank: HM140849). The qPCR assays confirmed the microarray results for *bty-1* (probe ID: 40261; see Supplemental Table S2.1.) as this transcript was significantly up-regulated in a time-dependent manner after 12 h of pIC stimulation. The *bty-2* (probe ID: 44336; see Supplemental Table S2.1.) microarray results were not, however, validated by qPCR. My bioinformatics analysis of Atlantic cod *bty*s, using BLAST against the EST and nt databases of NCBI revealed that the Atlantic cod genome encodes a large number of *bty* paralogues (data not shown). qPCR analyses were not performed for other microarray-identified *bty*s (e.g., probe IDs 36195 and 43099) because the qPCR primers failed quality testing. Atlantic cod *bty* has been characterised as a pIC-responsive gene that encodes a tripartite motif (TRIM)-containing protein, and TRIM proteins have crucial functions in numerous biological processes including immune responses (Furnes and Robertsen, 2010). TRIMs are established as IFN-inducible proteins that can act like a PRR, inhibit viruses, and positively or negatively regulate immune signalling through the RIG-I-associated pathway (e.g., the binding to the CARD domain of RIG-I) and transcription factors (e.g., NFKB1 and IRF3) (McNab et al., 2011). Many functions of TRIMs are mediated by the activity of E3 ligases. It has been recently demonstrated that different members of virus-induced TRIMs in rainbow trout possess E3 ubiquitin ligase

activity and affect antiviral signalling by ubiquitination (van der Aa et al., 2012). The microarray results of the present study suggest that different *bty* paralogues play roles in the antiviral immune response of Atlantic cod macrophages.

Similar to previous investigations on pIC-injected cod (Hori et al., 2012; Rise et al., 2010) and ISAV-infected Atlantic salmon macrophage-like cells (Workenhe et al., 2009), the microarray analysis in this study showed that the expression of *sacsin* (6.8-fold) was increased by pIC. This implies that this transcript plays a role in antiviral innate immunity pathways of Atlantic cod. Sacsin is a protein involved in the central nervous system of mammals, although Atlantic salmon *sacsin* has been previously described as a pIC-responsive transcript with expression that is highly correlated with type I IFN (Krasnov et al., 2011). The precise role of *sacsin* in fish antiviral responses is not currently known.

In addition to known fish immune-related transcripts (e.g., *mig1*, *gig2*, *bty* and *tlr22g*), the present study identified several pIC-responsive cod macrophage transcripts that have known functions in mammalian immune responses or macrophage activity but poorly understood functions in fish. These included *cd9*, *mapk14*, *klf4*, *tap1*, and *optineurin*. In mammals, *cd9* is involved in the regulation of cell adhesion, migration and MAPK activation (Peddibhotla et al., 2013). MAPKs can regulate the induction of several transcripts and the inflammatory response in conjunction with nuclear factors (e.g., IRFs and NFKB1) (Arthur and Ley, 2013). In the present study, *mapk14* was up-regulated in Atlantic cod macrophages by pIC (a 1.5-fold increase; Table 2.2.), and it is recognised as a suppressor of TNF in mammalian macrophages (Arthur and Ley, 2013).

Further, *klf4* (*krueppel-like factor 4*), a microarray-identified pIC-responsive transcript in Atlantic cod macrophages (1.2-fold up-regulation; Table 2.2.), is an IFNG-induced gene playing roles in the regulation of signalling activation in mammalian macrophages (Feinberg et al., 2005). In the present study, an up-regulation was recorded for *tap1* (2-fold) and *optineurin* (1.6-fold) by pIC stimulation (Table 2.2.). IFN-induced *tap1* is activated in mammalian macrophages via STAT1 and IRF1, and acts as a pivotal protein in antigen processing of MHC I (Brucet et al., 2004). Mammalian *optineurin* has previously been described as an inhibitor of IFN production in dsRNA-induced cells (Mankouri et al., 2010), but its immune functions in teleosts are yet to be determined.

As identified by the microarray study, Atlantic cod *vimentin* was down-regulated in pIC-exposed macrophages, although this result was not validated by qPCR. Since Vimentin is an intermediate filament protein that can act as a regulator of macrophage differentiation and adhesion (Beneš et al., 2006), the down-regulation of *vimentin* in Atlantic cod macrophages at 48 HPS within both groups could have occurred, due in part, to the reduced requirement for this protein in differentiated and adhered macrophages. The contradiction observed between the microarray and qPCR results (i.e., at 24 HPS) of *vimentin* or other transcripts (i.e., *traip*, *ccr7*, *bty-2*, *catalase* and *grp94*) in this study can be related to several factors as discussed in Booman et al. (2011). For example, it may be explained by misassembled contigs used for microarray design, or variations in the location of qPCR primers and microarray probes, although I generally attempted to place the qPCR primers in the region of the transcript corresponding to informative microarray probes. Moreover, this study used different fish for the microarray study and the time-

dependent qPCR validation (see Section 2.3.8. for details), and individual variation may have led to disagreements between the qPCR and microarray results for some pIC-responsive transcripts.

Heat shock proteins (HSPs) can be involved in immune responses. For instance, they are involved in the activation of macrophages and dendritic cells as well as the production of cytokines and chemokines (van Noort et al., 2012). In disagreement with the transcriptome response of ISAV-infected Atlantic salmon macrophage-like cells (Schiøtz et al., 2008), *hsp70* transcript was found to be up-regulated in pIC-induced Atlantic cod macrophages (Table 2.2.). On the other hand, microarray analysis showed a significant down-regulation of *grp94* (alias *hsp90b1*) in cod macrophages at 24 HPS; this result was not validated by qPCR, where the down-regulation of *grp94* by pIC was only seen at 12 HPS compared with time-matched controls. GRP94 was reported to stimulate the NFKB1 pathway and nitric oxide generation in murine macrophages (Reed et al., 2003), suggesting its importance in macrophage functions.

In this study, GO terms associated with the oxidation-reduction process and oxidoreductase activity were under-represented in the pIC-responsive transcripts list. The oxidation-reduction (redox) molecules, including nitric oxide (NO) and reactive oxygen species (ROS), are mainly produced in macrophages as a result of phagocytosis and cell stimulation, and they have important roles in pathogen (e.g., virus) eradication, apoptosis and signalling pathway activation (Wink et al., 2011). A reduced oxidative response to viral infection has been previously reported in human macrophages (Müller et al., 1990). In addition, a genomics survey of the salmon macrophage-like cell response to ISAV

identified both up- and down-regulated transcripts involved in ROS metabolism (Schiøtz et al., 2008). Although they suggested an inverse correlation between oxidative stress and viral replication, more studies are needed to determine the role of ROS in the antiviral immune responses of fish macrophages.

As identified by microarray and validated by qPCR analysis, *ctsa* was down-regulated in Atlantic cod macrophages by pIC at 24 and 48 HPS. Cathepsins can be involved in cell death by modulation of anti-apoptotic agents (Repnik et al., 2012). However, the expression of *bcl-x1* was not influenced by pIC stimulation in this study. In contrast to this study, *bcl-x1,* an anti-apoptotic biomarker, was shown to have a tissue-specific response to pIC in Atlantic cod (Feng and Rise, 2010). These findings, along with the cell death results reported herein, suggest that pIC stimulation may not influence the apoptosis of Atlantic cod macrophages. A similar result was seen for *p67-phox* (i.e., a NADPH oxidase) in the current study. NADPH oxidases are JAK2/STAT-activated transcripts that play roles in the phagocytosis of fish macrophages (Olavarría et al., 2013). Although there was no change in the expression of Atlantic cod *p67-phox* over time, the time-dependent qPCR results of this investigation may be affected by cell differentiation and macrophage activation. For example, the expression of *li8* and *il1b* were up-regulated in both groups over time. These transcripts can influence macrophage response or activation (Ma et al., 2003). Therefore, further studies are required to fully characterise the macrophage-related functions of these transcripts in Atlantic cod.

## 2.6. Conclusions

The present study showed that Atlantic cod macrophage transcripts involved in MyD88- and TRIF-dependent pathways were induced by pIC, and identified several potentially fish-specific pIC-responsive transcripts (e.g., *tlr22g*, *gig2*, *bty*). Some pIC-responsive transcripts (e.g., *herc4*) identified in this study may have fish-specific immune functions since their orthologues in higher vertebrates are not known as immune responsive genes. Moreover, the time-dependent qPCR assays indicated that the transcriptome response of Atlantic cod macrophages to pIC occurs 12 h after stimulation and peaks at 24 HPS or 48 HPS. Some transcripts (e.g., *il8*, *il1b* and *vimentin*) may play a role in macrophage maturation and activation, as their expression varied over time in the control group. The present study identified a large number of immune-relevant pIC-responsive genes, and this enhances our understanding of the antiviral immune responses of Atlantic cod macrophages. However, I acknowledge that there may be differences between Atlantic cod macrophage gene expression responses to pIC versus their responses to pathogenic viruses. Further studies are required to structurally and functionally characterise the pIC-responsive transcripts of cod macrophages, to profile their transcriptome responses to pathogenic viruses, and to understand the correlation between their activation (e.g., cytokine expression) and antiviral immune responses.

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# CHAPTER 3

## Characterisation and transcript expression analyses of Atlantic cod *viperin*

### Preface

The research described in Chapter 3 has been submitted to *Frontiers in Immunology* as: Eslamloo, K., Ghorbani, A., Xue, X., Inkpen S.M., Larijani, M., Rise, M.L., 2018. Characterisation and transcript expression analyses of Atlantic cod *viperin*; see Co-authorship Statement on page XXIV.

## 3.1. Abstract

Viperin is a key antiviral effector in the immune responses of vertebrates including the Atlantic cod (*Gadus morhua*). Using cloning, sequencing and gene expression analyses, I characterised Atlantic cod *viperin* at the nucleotide and hypothetical amino acid levels, and investigated its transcriptional regulation pathways. Atlantic cod *viperin* cDNA is 1342 bp long, and its predicted protein contains 347 amino acids. Using *in silico* analyses, this study showed that Atlantic cod *viperin* is composed of 5 exons, as in other vertebrate orthologues, and that the radical SAM domain and C-terminal sequences of the predicted Viperin protein are highly conserved among various species. As expected, Atlantic cod Viperin was most closely related to other teleost orthologues. Using computational modelling, this study shows that the Atlantic cod Viperin forms similar overall protein architecture compared to mammalian Viperins. qPCR analyses revealed that *viperin* is a weakly-expressed transcript during the embryonic development of Atlantic cod. In adults, the highest constitutive expression of *viperin* transcript was found in blood compared with 18 other tissues. Using isolated macrophages and synthetic dsRNA (pIC) stimulation, various immune inhibitors were tested to determine the possible pathways regulating the expression of Atlantic cod *viperin*. Atlantic cod *viperin* showed a comparable pIC induction to other well-known antiviral genes (e.g., *interferon gamma* and *interferon-stimulated gene 15-1*) in response to various immune inhibitors. The pIC induction of Atlantic cod *viperin* was significantly inhibited by 2-Aminopurine, Chloroquine, SB202190, and Ruxolitinib. Therefore, endosomal-TLR-mediated pIC recognition and signal transducers (i.e., PKR and p38

MAPK) downstream of the TLR-dependent pathway may activate the gene expression response of Atlantic cod *viperin*. These results also suggest that *viperin* may be transcriptionally regulated during the antiviral response of Atlantic cod by the interferon-activated pathway.

## 3.2. Introduction

Interferon-stimulated genes (ISGs) play crucial roles as immune effectors and regulators in the antiviral immune response of fishes and other vertebrates (Poynter and DeWitte-Orr, 2016; Sadler and Williams, 2008). As in mammals (Akira et al., 2006; Bonjardim et al., 2009; Yu and Levine, 2011), the antiviral response of teleosts is triggered by recognising viruses or "viral mimics" [e.g., synthetic double-stranded RNA (dsRNA): polyriboinosinic polyribocytidylic acid (pIC)] through intracellular TLRs (Toll-like receptors) or RLR (RIG-I-like receptor), and that leads to the activation of the transcription factors and enhances the production of type I and II IFNs (Interferons) (Gitlin et al., 2006; Langevin et al., 2013; Schwarze et al., 2016; Zhang and Gui, 2012). Secreted IFNs initiate the JAK-STAT (Janus kinase - Signal transduction and activator of transcription) signalling pathway that enhances the transcription of ISGs (e.g., *viperin*, *isg15*, and *mx*) containing IFN gamma (IFNG)-activated sequences (GAS) and/or IFN-sensitive response elements (ISRE) in their promoters (Langevin et al., 2013; Poynter and DeWitte-Orr, 2016; Robertsen, 2006).

Viperin [Virus inhibitory protein, endoplasmic reticulum (ER)-associated, IFN-inducible], also known as RSAD2 [Radical S-adenosyl methionine (SAM) domain-containing 2] or VIG1 (Virus-induced gene 1), is an antiviral protein, highly inducible by pIC, lipopolysaccharide (LPS), viruses and bacteria (Fitzgerald, 2011; Severa et al., 2006). The expression of mammalian *viperin* is induced via IFN-dependent and independent pathways, both of which may be activated by the detection of viruses or dsRNA through a member of the RLR family and activation of transcription factors [e.g.,

IRF3 (IFN regulatory factor 3)] (Fitzgerald, 2011; Helbig and Beard, 2014; Mattijssen and Pruijn, 2012). Mammalian Viperin is localised in the ER-derived lipid droplets and inhibits viral replication [e.g., hepatitis C virus (HCV) and influenza] (Fitzgerald, 2011; Helbig and Beard, 2014; Mattijssen and Pruijn, 2012). Viperin expression enhances the TLR-mediated production of type I IFN by forming a signalling complex consisting of IRAK1 (Interleukin-1 receptor-associated kinase) and TRAF6 (Tumor necrosis factor receptor-associated factor 6) on lipid bodies and facilitating the nuclear translocation of IRF7 (Saitoh et al., 2011).

In addition to mammals, the antiviral responsiveness of *viperin* has been observed in teleosts (Workenhe et al., 2010) and an invertebrate species, i.e., the Pacific oyster (*Crassostrea gigas*) (Green et al., 2014; Green et al., 2016). Teleost *viperin* has been characterised and shown to be a pIC and/or LPS-induced gene in various species [i.e., tilapia (*Oreochromis niloticus*), annual fish (*Nothobranchius guentheri*) and red drum (*Sciaenops ocellatus*) (Dang et al., 2010; Lee et al., 2013; Liu et al., 2015; Poynter and DeWitte-Orr, 2016; Workenhe et al., 2010)]. In addition, it has been shown that Atlantic salmon (*Salmo salar*) and crucian carp (*Carassius auratus*) *viperin* gene expression was induced in response to infectious salmon anemia virus (ISAV) and grass carp reovirus (GCRV), respectively (Wang et al., 2014b; Workenhe et al., 2009), and that Viperin exhibited antiviral activity against megalocytivirus in rock bream (*Oplegnathus fasciatus*) (Zhang et al., 2014). As in mammals, *viperin* was shown to be an IFN-induced gene in zebrafish (*Danio rerio*) (Li et al., 2010), and crucian carp *viperin* was suggested to be transcriptionally regulated via the RLR-activated IFN pathway (Wang et al., 2014b).

Although fish Viperins share some characteristics with their mammalian counterparts, the mechanisms involved in Viperin responses to immunogenic stimuli are not well-understood in fishes. Additionally, this gene/protein is not fully characterised in several teleost models.

In addition to its importance in Atlantic fisheries (Rosenlund and Halldórsson, 2007), the Atlantic cod (*Gadus morhua*) exhibits a unique immune system among teleosts (Star et al., 2011). Genomic studies have indicated that the Gadiformes lineage, including Atlantic cod, lacks MHC II (Major histocompatibility complex II), CD4, Mx and TLR5 genes, and shows a unique expansion of genes including MHC I and TLR22 (Malmstrøm et al., 2016; Solbakken et al., 2016; Solbakken et al., 2017). Using transcriptome profiling of antiviral responses, several ISGs including *viperin* have been previously identified in Atlantic cod. *viperin* displayed a strong induction in the brain of nodavirus carrier fish, the spleen and brain of pIC-injected fish, and macrophages stimulated with pIC, but not LPS (Eslamloo et al., 2016; Hori et al., 2012; Rise et al., 2008; Rise et al., 2010; Seppola et al., 2015). However, the full sequence, developmental and tissue expression profiles, and regulating factors of Atlantic cod *viperin* remained unknown. In this study, I fully characterised Atlantic cod *viperin*, at the nucleotide and hypothetical amino acid (AA) levels, and determined its tissue distribution, developmental expression, and the signalling pathways underlying its gene expression regulation during the antiviral response.

## 3.3. Materials and methods

### *3.3.1. Gene cloning, sequencing and sequence assembly*

Gene-specific primers (GSPs) for rapid amplification of cDNA ends (RACE) were designed (see Table 3.1.) using Primer3web v4.0.0 (http://primer3.ut.ee/) and the partial sequence of Atlantic cod *viperin* (obtained from NCBI; GenBank accession: HM046448). A pool of column-purified RNA samples from the spleens of 10 Atlantic cod injected with pIC and sampled at 24 h post-injection (HPI) (5 µg RNA per sample) was used as RNA template for the RACE cDNA synthesis [see Inkpen et al., (2015) and Hori et al., (2012) for experimental design].

All PCR reactions in the present study were conducted on a Bio-Rad Tetrad 2 Thermal Cycler (Bio-Rad, Hercules, CA). Full-length 5′ and 3′ RACE cDNAs were synthesized using 1 µg total RNA and the SMARTer RACE cDNA Amplification Kit according to the manufacturer's instructions (Clontech, Mountain View, CA). The resulting 5′ and 3′ RACE cDNAs (i.e., 10 µl reaction) were diluted by adding 100 µl of Tricine-EDTA buffer and used for RACE PCR. A touch-down PCR [cycling program: 1 min at 95°C; 5 cycles of (94°C for 30 s, 72°C for 3 min); 5 cycles of (94°C for 30 s, 70°C for 30 s, 72°C for 3 min); 25 cycles of (94°C for 30 s, 68°C for 30 s, 72°C for 3 min); and 1 final extension cycle of 72°C for 10 min] was conducted in 50 µl reactions using the Advantage 2 Polymerase, Advantage 2 PCR buffer, dNTP Mix (Clontech) and GSPs (i.e., *viperin*-GSP; Table 3.1.) as well as Universal Primer Mix provided by the kit (Clontech), following the manufacturer's instructions. Thereafter, 5 µl of the amplified 5′ and 3′

## Table 3.1. Primers used for gene characterisation and expression studies.

| Primer name | | Primer sequence (5' to 3') | Application | Amplification efficiency (%) | Amplicon size (bp) |
|---|---|---|---|---|---|
| **Gene characterisation primers** | | | | | |
| *viperin*-GSP | Forward | AGACGTCTTTCGTCCTGCCTTTGGAT | 3'RACE PCR | N/A | N/A |
| | Reverse | CCCATGTCTGCTTTGCTCCACACATA | 5'RACE PCR | | |
| *viperin*-Nested GSP | Forward | CATCTTGGCCGTTTCCTGTGACAGTT | 3'RACE PCR | N/A | N/A |
| | Reverse | GCATCTTCTGATTGGACTCGGGTACG | 5'RACE PCR | | |
| *viperin*-ORF GSP | Forward | AATTTGAACCATGGTGCCGG | ORF-PCR | N/A | 1060 |
| | Reverse | TATCCATCACCACTCCAGGC | ORF-PCR | | |
| **qPCR primers** | | | | | |
| *viperin* | Forward | TGTTTCCACACAGCGAAGAC | qPCR | 88. 7 | 108 |
| | Reverse | TCCGCCAGAGAAGTTGATCT | qPCR | | |
| *ifng* | Forward | TCGCTCTTCATGTTGGTCTG | qPCR | 99.8 | 121 |
| | Reverse | GGCCTTTCTGTGGATGTTGT | qPCR | | |
| *isg15-1* | Forward | AGGACCAACAAAGGCTGATG | qPCR | 88.8 | 110 |
| | Reverse | CAGCCGTCCGTTAAGGTAGA | qPCR | | |
| *lgp2* | Forward | ACAGAAGCCATCGCAGAAAT | qPCR | 98.1 | 105 |
| | Reverse | TTTTGCAGCACGAATCAAAC | qPCR | | |
| *il1b* | Forward | AACACGGACGACCTGAAAAG | qPCR | 93.1 | 126 |
| | Reverse | GCTGATGTACCAACCGGAGT | qPCR | | |
| *eef1a* | Forward | CAACGTCAAGAACGTCTCCA | qPCR | 88.7 | 197 |
| | Reverse | TGAGCTCGTTGAACTTGCAG | qPCR | | |
| *rpl4a* | Forward | GGTGCCATACAGCTGATCCA | qPCR | 94. 7 | 123 |
| | Reverse | CCAGGCATCACACTGCAGAA | qPCR | | |
| *tubb2* | Forward | AGCCTGGCACTATGGACTCTGT | qPCR | 91. 4 | 129 |
| | Reverse | GCTCGGCTCCCTCTGTGTAG | qPCR | | |
| *eif3* | Forward | AACTGTCCGTAGTCCGCAAG | qPCR | 99.7 | 125 |
| | Reverse | CTGCTCAGCGAGAAACAGAA | qPCR | | |
| *rplp1* | Forward | TCTGAAGCTAAGGCCCTCAA | qPCR | 92. 7 | 141 |
| | Reverse | ATCGTCGTGGAGGATCAGAG | qPCR | | |

GSPs (gene-specific primers), ORF (open reading frame), *viperin* (*virus inhibitory protein, ER-associated, IFN-inducible*), *ifng* (*interferon gamma*), *isg15-1* (*interferon stimulated gene 15-1*), *lgp2* (*RNA helicase lgp2*), *il1b* (*interleukin 1, beta*), *eef1a* (*eukaryotic elongation factor 1 α*), *rpl4a* (*60s ribosomal protein l4-a*), *tubb2* (*beta-2 tubulin*), *eif3* (*eukaryotic translation initiation factor 3*), *rplp1* (*60S acidic ribosomal protein P1*), N/A (not applicable.

RACE products were diluted 50 times using Tricine-EDTA buffer and used for nested PCR. The nested PCR was performed in 50 µl reactions, using Nested GSPs (i.e., *viperin*-Nested GSP; Table 3.1) and Nested Universal Primer provided by the kit (Clontech), following the manufacturer's instructions. The cycling parameters for nested RACE PCR consisted of 1 min at 95°C, followed by 20 cycles of (94°C for 30 s, 68°C for 30 s, 72°C for 3 min), and 1 final extension cycle at 72°C for 10 min.

PCR products were examined on 1.2% agarose gel, and were then extracted using the QIAquick Gel Extraction kit (Qiagen) according to the manufacturer's recommendations. TA cloning of gel-extracted PCR products was performed using pGEM-T-Easy vector (Promega, Madison, WI) at 4°C overnight, using the manufacturer's instructions. Recombinant plasmids were transformed into Subcloning Efficiency DH5α Competent Cells (i.e., chemically-competent cells) (Invitrogen, Burlington, Ontario) following the manufacturer's instructions. The transformed cells were incubated in 300 µl of SOC medium (Invitrogen) for 1 h at 37°C with shaking (~225 rpm), and then cultured on Luria broth (LB)/agar plates containing 100 µg $m1^{-1}$ ampicillin and 40 µl $plate^{-1}$ of 40 mg $ml^{-1}$ X-gal (Sigma, St. Louis, MO) for 16 h at 37°C. Thereafter, colonies were taken using blue/white selection and cultured in LB supplemented with ampicillin (100 µg $ml^{-1}$) at 37°C overnight. Plasmid DNA was extracted using QIAprep Spin Miniprep Kit (Qiagen), following the manufacturer's instructions. The insert sizes of the recombinant plasmids were checked using *Eco*RI (Invitrogen) digestion and agarose gel (1%) electrophoresis. Four colonies from each 5′ and 3′ RACE products were used for sequencing performed at the Genomics and

Proteomics (GaP) facility, Core Research Equipment and Instrument Training (CREAIT) network, Memorial University of Newfoundland. Sequencing was conducted using an ABI 3730 DNA Analyzer with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

Lasergene 7.20 software (DNASTAR, Madison, WI) was used to acquire overlapping sequence from 5′ and 3′ RACE products and assemble the full-length *viperin* cDNA. GSPs amplifying the open reading frame (ORF) were designed (see Table 3.1.) to verify the sequence assembly of full-length *viperin*. The ORF PCR was performed using the TopTaq polymerase kit (Qiagen, Mississauga, Ontario) and cDNA of pIC-stimulated Atlantic cod macrophages (i.e., pIC sub-group of no-inhibitor treatment in the macrophage stimulation experiment; see Section 3.3.7.) in a 50 µl reaction. The PCR reaction was composed of 2 µl of cDNA (representing ~10 ng of input RNA; see Section 3.3.9. for the cDNA synthesis method), 0.5 µM each of forward and reverse GSP, 5 µl of 10X TopTaq PCR buffer, 1.25 U of TopTaq DNA Polymerase, and 200 µM of each dNTP, with the following PCR program: initial denaturation of 3 min at 94 °C, followed by 30 cycles (of 94°C for 30 s, 60°C for 30 s, 72°C for 2 min), and 1 final extension cycle at 72°C for 10 min. The size of the resulting PCR product was determined by gel electrophoresis (1.2% agarose) alongside a 1 kb Plus Ladder (Invitrogen).

### *3.3.2. Sequence characterisation and in silico analyses*

The AA sequence of Atlantic cod Viperin was predicted based upon assembled cDNA sequence using the SeqBuilder software of the Lasergene package (DNASTAR). To map the gene structure and determine intronic regions and genomic location, the cDNA sequence of *viperin* was aligned with the genomic DNA sequence of Atlantic cod obtained from the Ensembl (http://www.ensembl.org) and Centre for Ecological and Evolutionary Synthesis (CEES: http://cees-genomes.hpc.uio.no) Genome Browsers. The AA sequences of Viperin of other fish species, as well as other vertebrate and invertebrate species, were collected from the NCBI GenBank non-redundant (nr) AA database, and used for multiple sequence alignment (MSA) and phylogenetic tree construction. MSA analysis of predicted AA sequences of Atlantic cod Viperin with orthologous sequences in other species was implemented in MEGA6 software using the MUSCLE feature (Edgar, 2004; Tamura et al., 2011). The Radical SAM domain of Atlantic cod Viperin was predicted using the PFAM database (http://pfam.xfam.org/) (Finn et al., 2016). The deduced AA sequences of Viperin homologues were aligned and used to generate a phylogenetic tree using the Neighbour-joining method in MEGA6 software (bootstrapped 10,000 times).

The neighbouring genes of Atlantic cod *viperin* and its conserved synteny with other species were mapped using the Genomicus database (http://www.genomicus.-biologie.ens.fr), powered by the Ensembl database. The neighbouring genes and genomic location of Atlantic cod *viperin* were also confirmed using the CEES Genome Browser (http://cees-genomes.hpc.uio.no).

To gain insight into transcriptional regulation of Atlantic cod *viperin*, putative transcription factor binding sites (TFBSs) were predicted in the proximal promoter region of this gene. The 1000 bp 5′-upstream of the transcription start site of Atlantic cod *viperin* were taken from genomic DNA sequence, available at CEES Genome Browser (http://cees-genomes.hpc.uio.no/). Putative TFBSs were identified using the vertebrates' profile of the TRANSFAC database (http://genexplain.com/transfac/). This prediction was performed using the default parameters (i.e., Minimize False Positives) suggested by the TRANSFAC database, and the predicted TFBSs (core score > 0.8) with putative functions in immune responses were selected and presented herein.

### *3.3.3. Prediction of Viperin protein structure*

The recently-described partial mouse (*Mus musculus*) Viperin crystal structures were used as templates for homology modelling (Fenwick et al., 2017; Roy et al., 2010; Yang et al., 2015; Zhang, 2008). These templates were retrieved from the Research Collaboratory for Structural Bioinformatics - Protein Data Bank (RCSB-PDB) (https://www.rcsb.org/; PDB ID: 5VSL and 5VSM). PyMOL v1.7.6 (http://www.pymol.org/) was used to visualize templates and models. Atlantic cod Viperin was computationally modelled using the default parameters of I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) (Roy et al., 2010; Yang et al., 2015; Zhang, 2008). Twenty models were generated of which four models with the highest C-score (>-1.8) were further analysed. C-score (range -5 to 2), which indicates the confidence of the quality of the predicted model, was estimated by I-TASSER. The majority of Atlantic cod Viperin was homology-modelled based on the mouse partial

Viperin crystal structure with the exception of the first 56 N-terminal AAs and the last 23 C-terminal AAs which were missing from crystal structure and, therefore, were modelled *ab initio* by I-TASSER. The same approach was used to model the full-length mouse Viperin as well as zebrafish, Atlantic salmon, and human (*Homo sapiens*) Viperins. Both [4Fe-4S] cluster and Radical SAM analogue (S-adenosylhomocysteine; SAH) were manually placed in the catalytic pocket of the Atlantic cod Viperin. The surface charge and the isoelectric point of Atlantic cod and full-length mouse Viperin were estimated using the PDB2PQR server (http://nbcr-222.ucsd.edu/pdb2pqr_2.0.0/). Prediction of natively disordered regions of Viperin was conducted using the default parameters of the Protein disorder prediction server (PrDOS) website (http://prdos.hgc.jp/cgi-bin/top.cgi).

### *3.3.4. Animals*

The Atlantic cod used in this experiment were kept in two 21 $m^3$ flow-through tanks (one tank for broodstock fish and one tank for fish used for tissue sampling and macrophage isolation) with optimal conditions (5.2-6.4°C, 95-110% oxygen saturation and an ambient photoperiod) in the JBARB of OSC. The fish [2.29 ± 0.42 kg (mean ± SE)] used for tissue sampling and macrophage isolation were fed 3 days weekly (i.e., at 1% body weight per day) with a commercial diet (Skretting, BC, Canada; crude protein 50%, crude fat 18% and crude fibre 1.5%). The broodstock fish were fed mackerel, herring and squid supplemented with vitamins 2 days per week before and during spawning season. Fish (i.e., for tissue sampling and macrophage isolation experiments) were fasted for 24 h and euthanized with an overdose of MS222 (400 mg $l^{-1}$; Syndel Laboratories, Vancouver, BC) prior to the sampling. All procedures applied in the current

investigation were approved by the MUN's Institutional Animal Care Committee, according to the guidelines of the Canadian Council on Animal Care.

### *3.3.5. Tissue sampling*

To evaluate the constitutive transcript expression of *viperin* in various tissues of adult Atlantic cod, four individuals (i.e., 2 male and 2 females) were used. Following euthanasia and dissection, samples were collected from 19 different tissues (i.e., blood, eye, brain, gill, heart, head kidney, posterior kidney, spleen, liver, gonad, stomach, pyloric caecum, midgut, hindgut, dorsal skin, ventral skin, dorsal muscle, ventral muscle and fin) of each individual; the samples were immediately flash-frozen in liquid nitrogen and kept at -80˚C until RNA extraction.

### *3.3.6. Sampling for developmental series*

Floating fertilized eggs were automatically collected after overnight communal spawning. Then, 1.4 l of fertilized eggs (i.e., 2-cell to 64-cell embryos) [henceforth referred to as zero days post-fertilization (DPF), or Day 0] were distributed into three 50 l conical incubator tanks (350 ml of eggs per tank) with a 25 l $h^{-1}$ flow rate and gentle aeration. The fertilized eggs were incubated and kept in these tanks until the yolk-sac absorption stage (i.e., 20 DPF; before active feeding) at 5.5 to 6.1°C and under an ambient photoperiod. The developmental stage of embryos was determined daily (Hall et al., 2004). The embryos were in blastula/gastrula stages from 1 to 6 DPF (34.4 degree-days). The segmentation period began at 7 DPF (40.2 degree-days), and the golden eye stage was observed at 12 DPF (68.5 degree-days). Hatching started at 15 DPF (86.2

degree-days), and 100% of the embryos were hatched by 18 DPF (103.9 degree-days). To study *viperin* expression during early developmental stages, embryos (~0.5 ml) or larvae (~0.4 ml) were sampled daily [i.e., 0 to 20 DPF] from each incubator tank (i.e., one pooled sample from each replicate tank per day; $n$ = 3), using 500 µm Nitex. The collected samples (~180 embryos or larvae per sample) from each tank were then flash-frozen in liquid nitrogen and kept at -80°C until RNA extraction.

### *3.3.7. Macrophage isolation and stimulation*

Atlantic cod macrophages were isolated from the head kidneys of 5 individuals as described in Eslamloo et al. (2018; 2016). All reagents (e.g., culture medium) and equipment used in this experiment were identical to Eslamloo et al. (2016). Briefly, following fish dissection, the macrophage-like cells isolated from each fish were seeded into 6-well plates (Corning, Corning, NY) at an equal density of $3 \times 10^7$ cells (in 2 ml L-15+) per well (16 wells per fish). The cells were cultured in Leibovitz's L-15 (L-15+; Gibco, Carlsbad, CA) culture medium supplemented with 2 mM L-glutamine, 4.2 mM $NaHCO_3$, 25 mM HEPES, 1.8 mM glucose, 100 U $ml^{-1}$ penicillin, 100 µg $ml^{-1}$ streptomycin (Gibco) and 1% fetal bovine serum (FBS; Gibco) overnight at 10°C. The non-adherent cells were then removed by washing the culture dishes 3 times with L-15+.

The immune inhibitors used in this study were purchased from InvivoGen (San Diego, CA), and the stock solutions were prepared using the manufacturer's instructions. The effective doses of different immune inhibitors used in this experiment were obtained from previously published *in vitro*-based studies in fish (Domínguez et al., 2013; Holen

et al., 2011; Ooi et al., 2006; Zhu et al., 2014), except for Ruxolitinib (RUX) for which the dose was based on an *in vitro* study on mammalian cells (Heine et al., 2013). 2-Aminopurine (2-AP), dissolved in phosphate-buffered saline (PBS) with glacial acetic acid (AcOH, 200:1), was used at 5 mM as an inhibitor of PKR (double-stranded RNA-activated protein kinase) (Ooi et al., 2006). Chloroquine (CHQ), dissolved in water, was used at 80 μM as an inhibitor of endosomal TLR (Zhu et al., 2014). In addition, Dimethyl sulfoxide (DMSO)-dissolved Resveratrol (RESV) and SB202190 (S90) were utilised at 50 μM for inhibiting NFKB (Nuclear factor kappa-B) and p38 MAPK (p38 mitogen-activated protein kinase) pathways, respectively (Domínguez et al., 2013; Holen et al., 2011). To inhibit JAK1/JAK2, DMSO-dissolved RUX was used at 5 μM (Heine et al., 2013). The pIC (Sigma-Aldrich) was dissolved in PBS at 10 mg $ml^{-1}$ and used as a stock solution in the present experiment. Starting 24 h after seeding, Atlantic cod macrophages isolated from each fish were exposed to 5 inhibitors (i.e., 2 wells per inhibitor for each fish). Moreover, macrophages from each individual were subjected to PBS, DMSO (0.57 μl $ml^{-1}$ of L-15+), or AcOH (0.17 μl $ml^{-1}$ of L-15+) as control conditions (i.e., there were a total of 8 experimental groups; 2 wells per condition for each fish). The cells were incubated with inhibitors for 1 h. Afterwards, macrophages from each fish under different inhibitor treatments or controls (2 wells per group) were exposed to either 50 μg $ml^{-1}$ pIC (Eslamloo et al., 2016) or PBS (5 μl of pIC solution or PBS $ml^{-1}$ of L-15+). The culture medium of all groups in this study contained an identical level of PBS (i.e., 38.8 μl $ml^{-1}$ of L-15+). The macrophages from each individual were incorporated into all experimental groups (i.e., 16 conditions in total; 5 biological replicates per group). My

previous study determined the time-dependent pIC responses of Atlantic cod macrophages (Eslamloo et al., 2016), and based on these results, the 24 h post-stimulation time point was selected for assessment of gene expression responses of macrophages in the current study. The samples were collected 24 h after pIC stimulation by removing the media and adding 800 µl of TRIzol (Invitrogen, Burlington, ON) into each culture well plate. TRIzol-lysed samples were kept at -80°C until RNA extraction.

### *3.3.8. RNA extraction and purification*

Total RNA was extracted using TRIzol (Invitrogen) following the manufacturer's instructions. RNase-free (i.e., baked at 220°C for 7 h) ceramic mortars and pestles were used to homogenise the firm tissues (i.e., eye, gill, heart, stomach, pyloric caecum, midgut, hindgut, dorsal skin, ventral skin, dorsal muscle, ventral muscle and fin), whereas other tissue and developmental samples were TRIzol-lysed using RNase-Free Disposable Pellet Pestles (Fisherbrand). Then, the homogenates of tissue and developmental samples were passed through QIAshredder (Qiagen) spin columns and used for RNA extraction following the manufacturer's instructions. The TRIzol-lysed macrophage samples were also processed for RNA extraction according to the manufacturer's recommendations.

Prior to purification, RNA samples (≤ 50 µg) in all experiments were treated with 6.8 Kunitz units of DNaseI (Qiagen) with the manufacturer's buffer (1X final concentration) for 10 min at room temperature to remove residual genomic DNA. RNA purification of adult tissue and developmental series samples (see Sections 3.3.5. and 3.3.6.) was performed using the RNeasy Mini Kit (Qiagen), whereas macrophage

samples were purified using the RNeasy MinElute Cleanup Kit (Qiagen) according to the manufacturer's instructions. RNA concentration and quality were assessed using NanoDrop spectrophotometry (ND-1000), and RNA integrity was assessed by agarose gel electrophoresis (1% agarose). All column-purified RNA samples subjected to gene expression analyses in this study showed acceptable purity (i.e., A260/230 and A260/280 ratios > 1.8) and integrity (i.e., tight 18S and 28S ribosomal RNA bands).

### *3.3.9. qPCR assays*

cDNA synthesis was performed using RNA of each sample (i.e., 1 µg total RNA for macrophage samples or 5 µg total RNA for adult tissue and developmental samples), random primers (250 ng; Invitrogen), 1 µl of dNTPs (10 mM each; Invitrogen) and M-MLV reverse transcriptase (200U; Invitrogen) in the manufacturer's first-strand buffer (1X final concentration) and DTT (10 mM final concentration) at 37°C for 50 min following the manufacturer's instructions.

The qPCR assays in the current study were designed and performed on the basis of the Minimum Information for Publication of qPCR Experiments (MIQE) guidelines (Bustin et al., 2009). The qPCR analyses (including an inter-plate linker and no-template controls) were conducted using a ViiA7 Real-Time PCR system (Applied Biosystems, Burlington, Ontario) in the 384-well format and a qPCR program consisting of one cycle of 50°C for 2 min, one cycle of 95°C for 10 min, and 40 cycles (of 95°C for 15 s and 60°C for 1 min), followed by a dissociation curve analysis (1 cycle at 60–95°C in increments of 0.05°C per second). Fluorescence data detection occurred at the end of

each cycle. qPCR assays (13 µl) were comprised of 6.5 µl of Power SYBR Green Master Mix (Applied Biosystems), 50 nM of each forward and reverse primer (0.52 µl of forward and 0.52 µl of reverse primers per reaction) 1.46 µl of nuclease-free water (Gibco) and 4 µl of cDNA (corresponding to 4 ng of input total RNA for macrophage samples or 10 ng of input total RNA for adult tissue and developmental samples).

The primer sequences in this study were obtained from my previous study (Eslamloo et al., 2016) (see Table 3.1.). Prior to the qPCR assays, primer quality tests were conducted in triplicate using a 5-point, 3-fold, serial dilution of the given cDNA template [starting with cDNA representing 10 ng (macrophage experiment) and 20 ng (adult tissue and developmental experiments) of input total RNA] as well as a no-template control. The cDNA template used for primer quality tests in the macrophage experiment was a pool of 3 individual pIC-stimulated samples, whereas the template used for primer quality tests in the adult tissue and developmental studies was a pool of different adult tissues and developmental samples. Each primer pair selected for qPCR assays showed an absence of amplification in the no-template controls, an amplicon with a single melting peak (i.e., no evidence of primer dimers or non-specific products in the dissociation curve), and amplification efficiency (Pfaffl, 2001) ranging 88 to 100% (Table 3.1.).

To identify suitable normalizer genes for qPCR assays, the expression of candidate normalizers in adult tissue [i.e., *cypa* (*cyclophilin a*), *rplp1* (*60S acidic ribosomal protein P1*), *rpl4a* (*60S ribosomal protein l4-a*), *tubb2* (*beta-2 tubulin*), *hsc70*

(*heat shock cognate 70 kDa*), *eif3* (*eukaryotic translation initiation factor 3*), and *eef1a* (*eukaryotic elongation factor 1 α*)], embryonic and early larval stages (*cypa, eif3, eef1a*, *tubb2* and *beta-actin*) and macrophage stimulation [*ppp1cc* (*protein phosphatase 1, catalytic subunit, gamma isozyme*), *cypa*, *rplp1*, *rpl4a*, *tubb2*, *eif3*, and *eef1a*] experiments was assessed in duplicate using 50% of the samples from each experiment. The expression results were subjected to geNorm analysis using qBase software as in Eslamloo et al. (2016). Normalizers that were expressed similarly (i.e., with low M-value, a measure of transcript expression stability) in the samples were selected for qPCR assays of adult tissue (*eef1a*, *rpl4a* and *rplp1*), embryonic and early larval development (*eif3* and *tubb2*), and macrophage stimulation (*rplp1* and *eef1a*) experiments. In addition to *viperin*, the expression levels of Atlantic cod *ifng, isg15-1* (*interferon stimulated gene 15-1*), *lgp2* (*RNA helicase lgp2*; alias *dhx58*) and *il1b* (*interleukin 1 beta*) were measured in macrophage samples. Since *ifng, isg15-1* and *lgp2* play important roles in antiviral responses (Poynter and DeWitte-Orr, 2016; Workenhe et al., 2010), and they showed a strong pIC induction in Atlantic cod macrophages (Eslamloo et al., 2016), I included them in qPCR assays as positive biomarkers of antiviral responses. On the other hand, since *il1b* is an antibacterial and pro-inflammatory biomarker in fish macrophages (Grayfer et al., 2014), this gene was added to qPCR assays in the macrophage experiment as a negative biomarker for the inhibition of targeted pathways.

The qPCR assays for samples of each experiment were performed in triplicate using 4 ng (macrophage samples) or 10 ng (adult tissue and embryonic and early larval development samples) of input total RNA per reaction. The performance of assays

between qPCR plates used in a given experiment was tested using an inter-plate linker sample [$C_T$ (threshold cycle) value variations were < 0.5] as well as no-template controls. ViiA7 software v1.2.2 (Applied Biosystems) was used to calculate the relative quantity (RQ) values, relative to a calibrator sample (i.e., sample with the lowest normalized expression within each experiment) for each gene of interest, using the $C_T$ values (i.e., gene of interest and normalizers) and the amplification efficiency of each primer pair (see Table 3.1.).

The qPCR results (RQ values) of the tissue and macrophage experiments were statistically analysed using the Prism package v6.0 (GraphPad Software Inc., La Jolla, CA). Kolmogorov-Smirnov tests were performed to check the normality of the data. The transcript expression data in the macrophage experiment were analysed using a repeated measures two-way ANOVA designed for randomized-block experiments, whereas the transcript tissue expression data were analysed by a one-way ANOVA. These analyses were followed by Sidak multiple comparisons *post-hoc* tests to determine the significant differences ($p \leq 0.05$) between adult tissues, as well as between and within the groups in the macrophage experiment. The qPCR results from the developmental study were not subjected to statistical analyses.

## 3.4. Results

### *3.4.1. Characterisation of Atlantic cod viperin sequence*

Assembly of RACE sequencing reads for *viperin*, validated by ORF PCR, generated a 1342 bp cDNA sequence (excluding poly-A tail) (GenBank accession: MH279971; Fig. 3.1.). As predicted using SeqBuilder, the Atlantic cod *viperin* cDNA consisted of a 55 bp 5'-UTR, a 1041 bp (347 AA) ORF and a 246 bp 3'-UTR. Also, three polyadenylation signal (AAUAAA) sequences were found in the 3'-UTR (Fig. 3.1.). As determined by CEES and Ensembl databases, Atlantic cod *viperin* contains 6 exons (i.e., 1: 359 bp, 2: 162 bp, 3: 230 bp, 4: 150 bp, 5: 33 bp, and 6: 408 bp). This gene is located on Linkage Group (LG) 5 of the Atlantic cod genome (CEES, LG05:12624935-12621299). Atlantic cod *viperin* is located downstream of *cmpk2* (*cytidine monophosphate (UMP CMP) kinase 2*) and upstream of *rnf144* (*ring finger protein 144*), and shows synteny similarity to zebrafish and human. Figure 3.2. shows the genomic organisation of the Atlantic cod *viperin* gene and its syntenic comparison with the *viperin* loci of zebrafish and human.

The multiple alignment of putative AA sequences of Atlantic cod Viperin and orthologous sequences from other eukaryotic species is shown in Figure 3.3. This comparison revealed considerable identity (i.e., 61-82%), notably in the radical SAM domain, between the Viperin of Atlantic cod and representatives of different invertebrate phyla [Ciliophora: (i.e., *Tetrahymena thermophila*) and Mollusca: (i.e., *C. gigas*)] or vertebrate classes (e.g., Actinopteri, Amphibia, Reptilia and Mammalia). Also, the radical

SAM domain of Atlantic cod Viperin contained a conserved SAM binding motif (CXXXCXXC) (Viperin AA 85-92; see Fig. 3.3.). However, no discernible conservation in AA sequence was noted in the N-terminus (i.e., first 70 AA) of Viperin putative orthologues. The lowest percentage of similarity (i.e., 61%) was found between Viperin of Atlantic cod and the ciliated protozoan, *T. thermophila* (see Supplemental Table S3.1.), whereas Atlantic cod Viperin showed the highest percentage of similarity to its putative orthologues in teleosts, i.e., rainbow trout (*Oncorhynchus mykiss*) (82%) and Orange-spotted grouper (*Epinephelus coioides*) (79%). The phylogenetic tree of Viperin was constructed using a MSA of putative orthologous sequences from various species (i.e., ciliated protozoan, amphioxus, mollusc, fishes, amphibian, reptiles, bird and mammals) (Fig. 3.4.). As expected, putative orthologous sequences were grouped and sub-grouped based upon the associated phyla and classes. For example, some fish species [e.g., Salmoniformes (*O. mykiss* and *S. salar*) and Cypriniformes (*Cyprinus carpio*, *C. auratus* and *D. rerio*)] within a given order were clustered together (Fig. 3.4.). Moreover, species (*O. mykiss*, *S. salar* and *Esox lucius*) within the Protacanthopterygii superorder were clustered together. The Viperin of the ciliated protozoan, *T. thermophila*, formed a separate branch from the other species in the phylogenetic three. Interestingly, the Viperin of amphioxus (*Branchiostoma japonicum*) and the Pacific oyster (*C. gigas*) were clustered together and separated from other species.

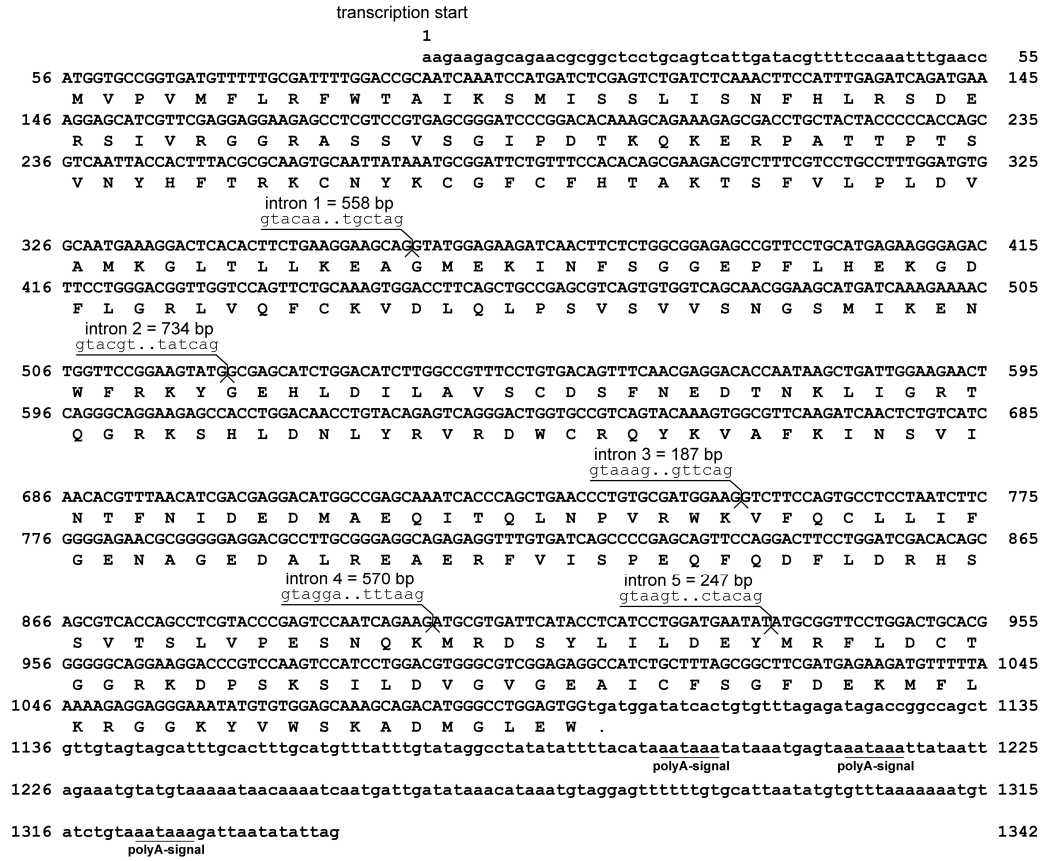


**Figure 3.1. Sequence and the predicted structure of Atlantic cod *viperin*.** Nucleic acid sequence of *viperin* (GenBank accession: MH279971) and inferred amino acid translation. The nucleotide sequences are numbered on both sides, and the inferred amino acids are shown below the coding sequence. The period shows the predicted stop codon. The lower-case letters indicate the non-coding nucleotide sequence, whereas the protein coding sequence of *viperin* is shown in upper-case letters. The intronic sequences were determined using the Ensembl (http://www.ensembl.org) and Centre for Ecological and Evolutionary Synthesis (CEES: http://cees-genomes.hpc.uio.no) Genome Browsers. The polyadenylation signals (polyA-signal: AAUAAA) were found in the 3'UTR.

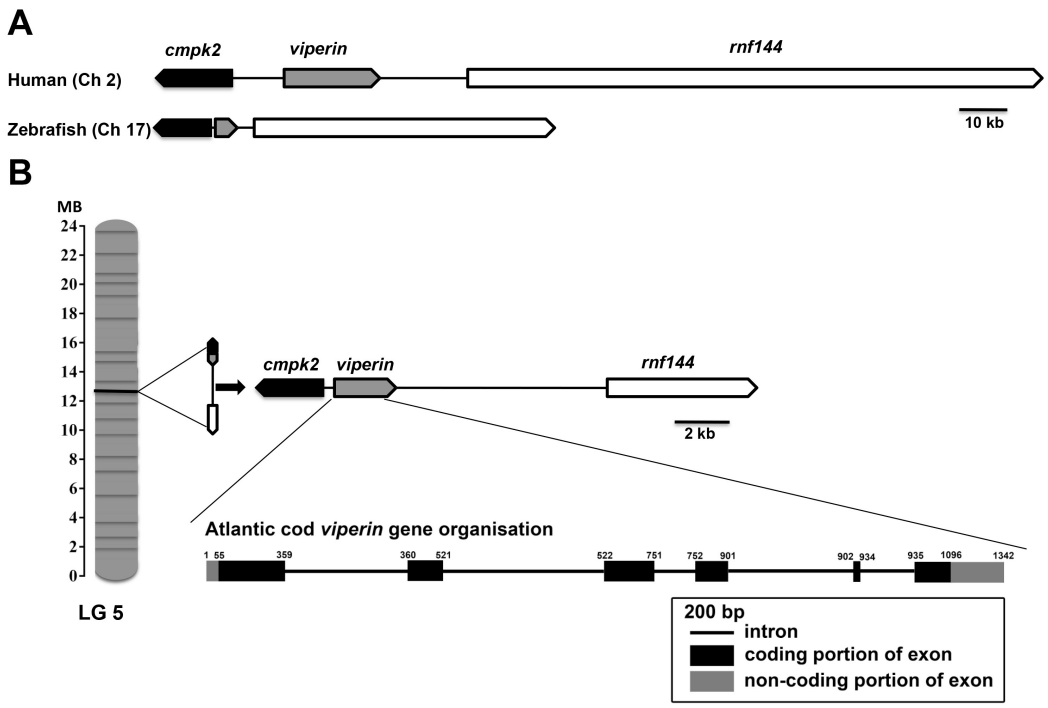


**Figure 3.2. Genome organization and syntenic correspondence of Atlantic cod *viperin*. A:** Syntenic relationship between zebrafish and human *viperin*. **B:** Location of *viperin* and its neighbouring genes in the Atlantic cod genome, and the gene organisation of Atlantic cod introns and exons. Black and grey boxes show coding and non-coding portions of exons, respectively, and the lines connecting the exons represent introns. The synteny was determined using the Genomicus database (http://www.genomicus.-biologie.ens.fr); *viperin* was found to be flanked by *cmpk2* and *rnf144* in the genomes of Atlantic cod, zebrafish and human. Arrows indicate the transcription direction of genes. Linkage Group (LG).

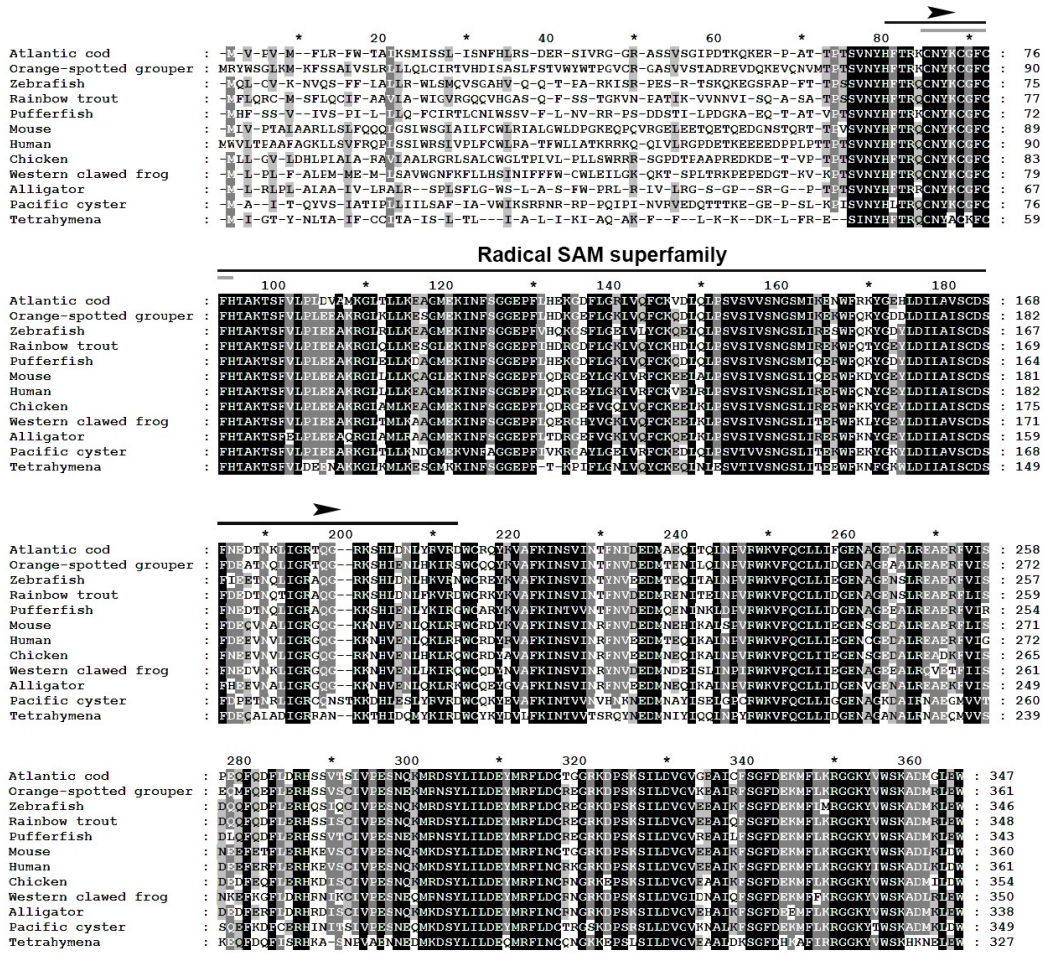


**Figure 3.3. Multiple alignment of Atlantic cod Viperin deduced protein sequence with putative orthologous sequences obtained from the NCBI protein database.** As identified by GeneDoc, black shading with white font shows the 100% conserved residues among the putative orthologous sequences. Dark grey shading with white font and light grey with black font denote 80% and 60% conservation among the residues, respectively. The numbers show the relative positions of the amino acid residues of Viperin putative orthologues. The Radical SAM domain of Viperin was predicted using the PFAM database (http://pfam.xfam.org/), and it includes a conserved SAM binding motif (CXXXCXXC) (i.e., Viperin AA 85-94; marked with a grey line within the Radical SAM domain). Atlantic cod (*Gadus morhua*), orange-spotted grouper (*Epinephelus coioides*), zebrafish (*Danio rerio*), rainbow trout (*Oncorhynchus mykiss*), pufferfish (*Takifugu rubripes*), mouse (*Mus musculus*), human (*Homo sapiens*), chicken (*Gallus gallus*), western clawed frog (*Xenopus tropicalis*), alligator (*Alligator mississippiensis*), Pacific oyster (*Crassostrea gigas*), Tetrahymena (*Tetrahymena thermophila*). See Supplemental Table S3.1. for GenBank accession numbers and the percentage of sequence similarity to Atlantic cod Viperin.

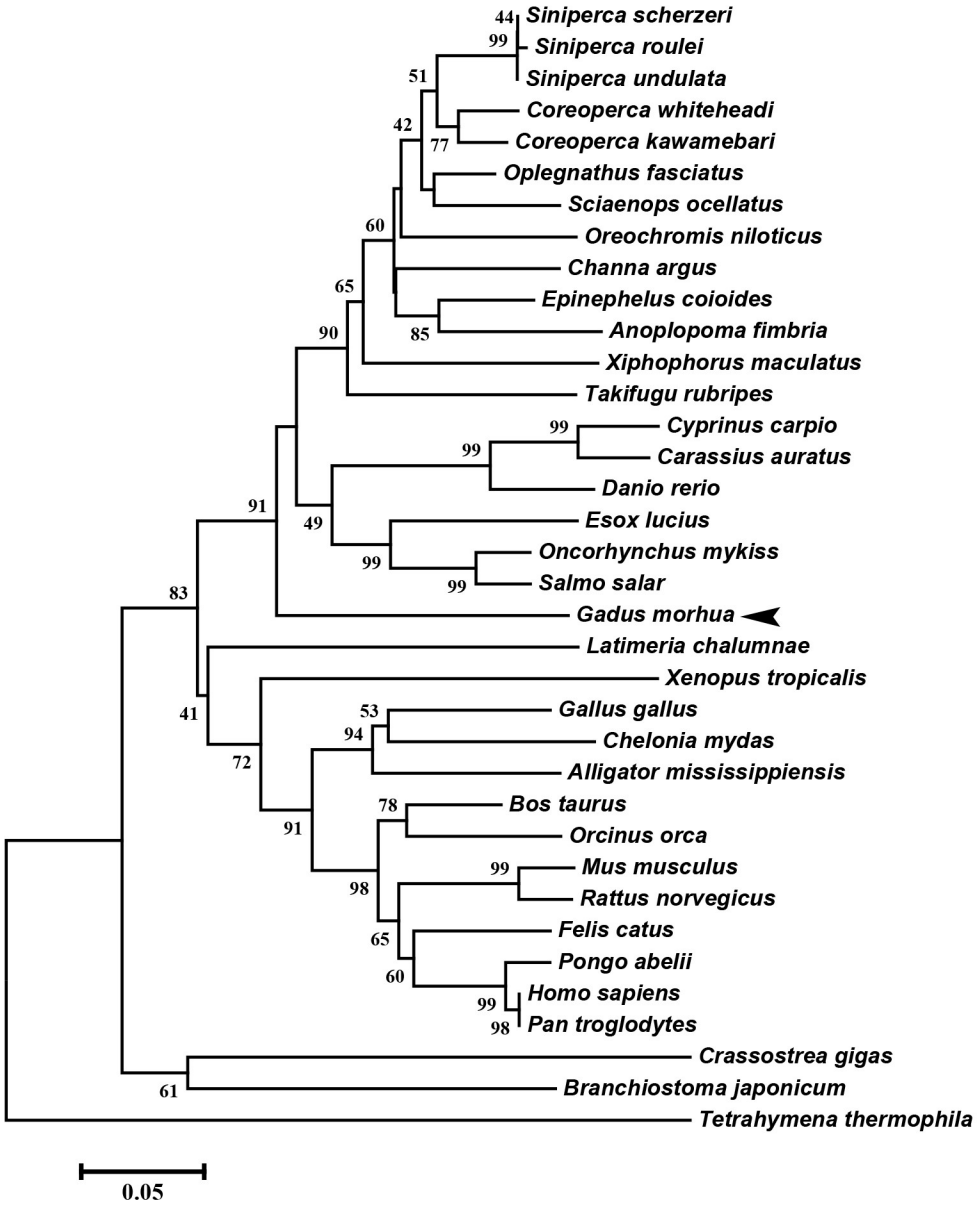


**Figure 3.4. Molecular phylogenetic analysis of Viperin in various species.** Putative Viperin amino acid sequence of Atlantic cod and the Viperin sequences from other species obtained from NCBI protein database were used to infer the evolutionary relationship among Viperin orthologues (See Supplemental Table S3.1. for GenBank accession numbers and the percentage of sequence similarity to Atlantic cod Viperin). The phylogenetic tree was generated using the Neighbour-joining method and bootstrapped 10,000 times using MEGA6 software. The numbers at the branch points represent the bootstrap values. Branch lengths are proportional to calculated evolutionary distances. The scale represents number of substitutions per site. Arrow shows the Atlantic cod Viperin sequence.

*In silico* analysis of the 5′-upstream region was used to predict the immune-related putative TFBSs that may play roles in regulating the expression of Atlantic cod *viperin*. As shown in Fig. 3.5., this analysis predicted IRF3/9, STAT and Activating transcription factor (ATF) motifs located in the proximal promoter of Atlantic cod *viperin*. Moreover, GAS and ISRE, which are the binding motifs for IFNG activation factor (GAF; STAT1 homodimer) and IFN-stimulated gene factor 3 (ISGF3), respectively, were identified in the proximal promoter region of Atlantic cod *viperin*.

### *3.4.2. Structure prediction of Viperin protein*

The recently-described partial mouse Viperin crystal structures (PDB: 5VSL, 5VSM) were used as templates for homology modelling prediction of full-length Atlantic cod and mouse Viperins (Fig. 3.6). Comparison of the predicted structure of Atlantic cod Viperin to the partial crystal structure of mouse Viperin revealed a nearly identical overall architecture, namely a partial $(\beta\alpha)_6$-barrel folding (Fig. 3.6. A). The CXXXCXXC or the radical SAM binding motif within which the cysteine residues ligate three iron atoms of the [4Fe-4S] cluster, and the GEE motif as well as a serine and an arginine which were shown to form hydrogen bonds with SAH, are found to be conserved in Atlantic cod Viperin ($G_{125}G_{126}E_{127}$ and $S_{180}$ and $R_{194}$ in mouse Viperin; $G_{110}G_{111}E_{112}$ and $S_{165}$ and $R_{179}$ in Atlantic cod Viperin; Fig. 3.6. B) (Fenwick et al., 2017). Further, the aromatic residues adjacent to the third cysteine in the CXXXCXXC motif were conserved in the Atlantic cod Viperin ($F_{90}$ and $F_{92}$ in mouse Viperin; $F_{75}$ and $F_{77}$ in Atlantic cod Viperin; Fig. 3.6. B).

To generate a soluble derivative of mouse Viperin for crystallization, the N-terminus region contacting a suggested α-helix (residues 1 to 71) was truncated. The computational modelling also suggests the conservation of this N-terminus α-helix in the Viperin of Atlantic cod, zebrafish, Atlantic salmon and human (Fig. 3.7.). As is the case for the mouse protein (Fenwick et al., 2017), the Viperins of the Atlantic cod as well as the aforementioned other species were predicted to have an intrinsically disordered N-terminal region (Supplemental Figure S3.1.), thus allowing for a lower degree of confidence for prediction of this region.

The majority of AA differences between the Atlantic cod and mouse Viperins are located in the α-helices and loops forming the surface of the protein, while the core of the protein forming the potential catalytic pocket cavity remained remarkably conserved (Identity = 65.1%; homology = 77.7%; Fig. 3.6. C and D). Strikingly, surface charge analysis of cod Viperin revealed that most AA differences between Atlantic cod and mouse Viperin were replacing negative/neutral AAs with more positively-charged residues. This increased the isoelectric point (pI) of the Atlantic cod Viperin compared to its mouse counterpart (mouse Viperin: charge at pH 7 = -5.61, pI = 5.89; Atlantic cod Viperin: charge at pH 7 = +3.78, pI = 8.61; Fig. 3.6. E).

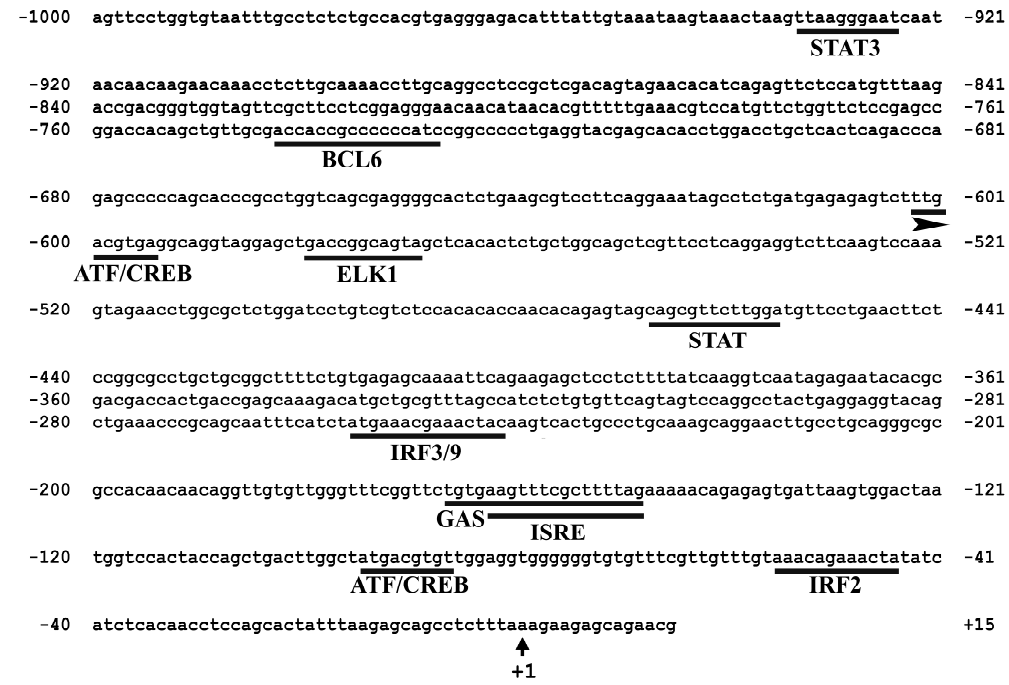


**Figure 3.5. Putative immune-related transcription factor binding sites (TFBSs) predicted in the 5′-upstream region of Atlantic cod *viperin***. The proximal promoter region sequence (i.e., 1000 bp 5′ of the transcription start site) of Atlantic cod viperin was taken from Centre for Ecological and Evolutionary Synthesis Genome Browser (CEES: http://cees-genomes.hpc.uio.no), and TFBSs were predicted using the TRANSFAC database (http://genexplain.com/transfac/). STAT (Signal Transducer and Activator of Transcription), BCL6 (B-cell lymphoma 6), ATF (Activating Transcription Factor), CREB (cAMP Response Element-Binding protein), ELK1 (ELK1, ETS transcription factor), IRF (Interferon Regulatory Factor), GAS (IFNG-Activated Sequence), ISRE (IFN-Sensitive Response Element). +1 indicates the transcription start site of Atlantic cod *viperin*.

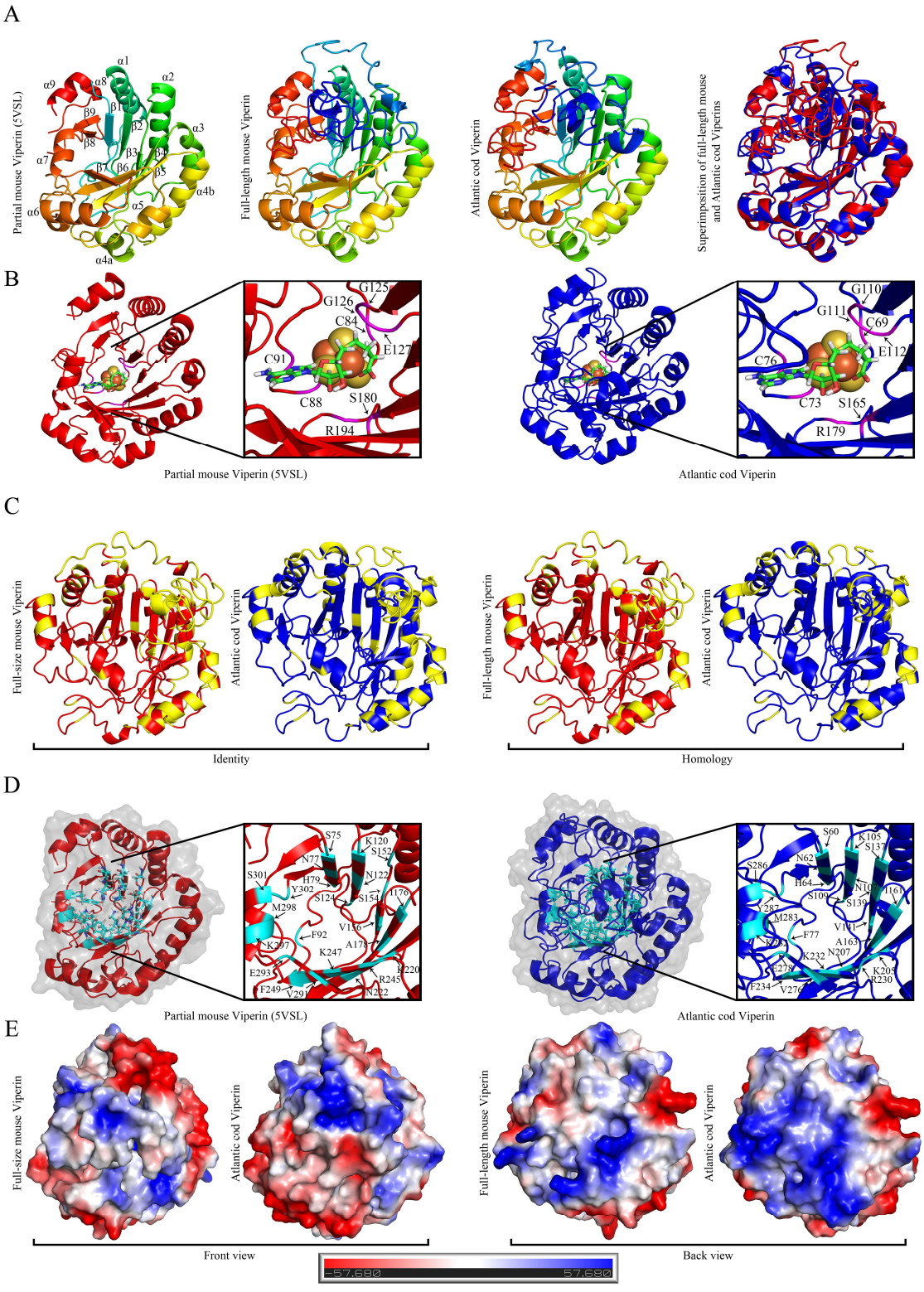
A
Partial mouse Viperin (5VSL)
Full-length mouse Viperin
Atlantic cod Viperin
Superimposition of full-length mouse and Atlantic cod Viperins
B
G125
G126
C84
E127
C91
C88
S180
R194
G110
G111
C69
E112
C76
C73
S165
R179
Partial mouse Viperin (5VSL)
Atlantic cod Viperin
C
Full-size mouse Viperin
Atlantic cod Viperin
Identity
Full-length mouse Viperin
Atlantic cod Viperin
Homology
D
Partial mouse Viperin (5VSL)
Atlantic cod Viperin
E
Full-size mouse Viperin
Atlantic cod Viperin
Front view
Full-length mouse Viperin
Atlantic cod Viperin
Back view
-57.680
57.680

**Figure 3.6. Predicted structure of Atlantic cod and mouse Viperin. A)** From left to right: Representative ribbon structures of crystallized mouse Viperin (PDB ID: 5VSL); full-length mouse Viperin modelled with the additional α-helix missing from the crystal structure; predicated Atlantic cod Viperin structure; and superimposition of modelled full-length mouse (red) and Atlantic cod (blue) Viperins. In first three models, blue to red colour change indicates N to C terminus progression. Loops, β-strands, and α-helices are labelled in mouse crystal structure. **B)** Ribbon model of crystal structure of mouse Viperin (left panel) and predicted structure of Atlantic cod Viperin (right panel) showing the SAH and [4Fe-4S] cluster coordinating residues in magenta. **C)** Comparison between the identity and homology of the mouse and Atlantic cod Viperin. Non-identical/homologous residues are shown in yellow. **D)** Ribbon model of crystal structure of mouse Viperin (left panel) and predicted structure of Atlantic cod Viperin (right panel) showing the amino acid residues forming the catalytic cavity in cyan. **E)** Predicted surface topology of full-length mouse and Atlantic cod Viperins. The positive, neutral, and negative residues are coloured blue, white, and red, respectively.

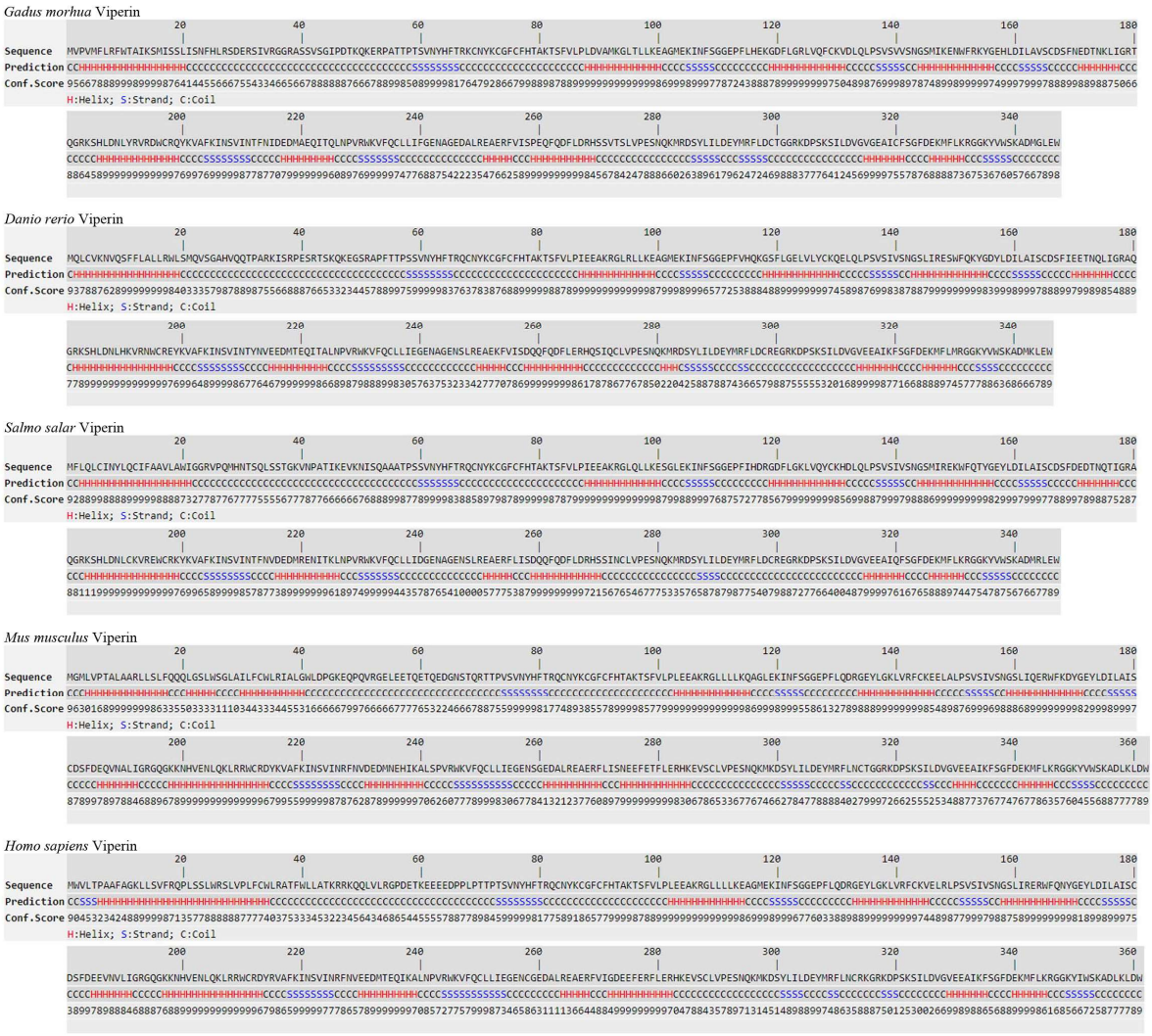


**Figure 3.7. Predicted secondary structure of orthologous Viperins from several species using I-TASSER website.** Confidence score values range from 0 (the lowest confidence) to 9 (the highest confidence). H: Helix, S: Strand, C: Coil.

### *3.4.3. Constitutive expression of Atlantic cod viperin during early and late life stages*

qPCR revealed that *viperin* was a low-expressed gene (i.e., $C_T$ values above 30) during the early developmental stages of Atlantic cod. The expression of the normalizer genes was slightly lower (Fig. 3.8.) during very early Atlantic cod embryonic development (days 0-3), and this appeared to influence the RQ values of *viperin* for these developmental time points. Therefore, these results were not subjected to statistical analyses. However, as illustrated in Fig. 3.8., the expression of Atlantic cod *viperin* was relatively higher from mixed cleavage until the mid-blastula stages (i.e., 0-2 DPF). Thereupon, *viperin* levels dropped to a non-detectable level during gastrula and the early segmentation stages (days 4-7). In other words, Atlantic cod *viperin* was not detected at the onset of zygotic gene expression, suggesting that *viperin* is a maternal transcript. Atlantic cod *viperin* expression increased during the segmentation stage, and then appeared to decrease after hatching.

The constitutive expression of Atlantic cod *viperin* was assessed in 19 different adult tissues. As shown in Figure 3.9., the highest expression of *viperin* transcript was seen in Atlantic cod blood, which was significantly higher than all other tissues. Interestingly, the levels of *viperin* transcript in immune-related tissues, notably head kidney and spleen, were significantly lower than that in blood. Additionally, *viperin* transcript had significantly higher expression in the gill and pyloric caecum compared to the dorsal skin and liver. The transcript expression of *viperin* was relatively, but not significantly, lower in the skin, muscle and fin tissues compared with some digestive tissues (e.g., midgut).

### *3.4.4. Pathway inhibition and viperin induction*

Different inhibitors were used to gain a better understanding of the signalling pathways activating Atlantic cod *viperin* during the antiviral response. As shown in Figure 3.10. A, 2-AP, an inhibitor of the PKR-dependent pathway, significantly repressed the pIC induction of Atlantic cod *viperin* compared to the AcOH-matched control. However, there was no significant difference between group-matched PBS and AcOH controls, and this shows that *viperin* suppression in the 2-AP group was not influenced by the AcOH in which 2-AP was dissolved. CHQ also inhibited *viperin* transcript expression in the pIC-stimulated Atlantic cod macrophages compared to the DMSO-matched vehicle control, indicating that TLR-activated pathways play an important role in the Atlantic cod *viperin* antiviral response. The expression of *viperin* transcript in pIC-treated macrophages was likewise significantly reduced in the S90 group compared to its pIC-treated DMSO vehicle control, suggesting that inhibition of the MAPK pathway strongly affects Atlantic cod *viperin* induction. Finally, the present results revealed that Atlantic cod *viperin* may be a JAK1/JAK2-activated gene downstream of the IFN pathway, as there was a decrease in expression in the RUX-exposed pIC group compared to the pIC-treated DMSO vehicle control. On the other hand, there was no significant difference between the RESV (i.e., NFKB inhibitor) group and its DMSO-matched control. In addition, with respect to the role of *ifng* in innate immune responses and *viperin* induction, the expression levels of *ifng* as well as two important IFN-induced genes, i.e., *isg15-1* and *lgp2*, were assessed in the macrophage samples (Fig. 3.10. B-D). The expression profiles of *isg15-1* and *lgp2* (Fig. 3.10. C-D) in response to various immune

inhibitors were similar to *viperin*. Although there was a significant increase in the level of *isg15-1* and *lgp2* in the pIC sub-group of RESV treatment compared to its PBS control, the pIC induction of these genes in the RESV-treated samples was significantly attenuated compared to the pIC-stimulated DMSO vehicle control (Fig. 3.10. C-D). Further, Atlantic cod *ifng* showed a largely comparable expression pattern to *viperin*, and its pIC induction was significantly suppressed by 2-AP, CHQ, S90 and RUX. However, in contrast to *viperin* and similar to *isg15-1* and *lgp2*, the pIC induction of *ifng* was significantly decreased by RESV (i.e., 5.1-fold pIC induction) compared to the DMSO control group (13.5-fold pIC induction). Unlike *isg15-1* and *lgp2*, though, there was no significant difference between the PBS control and pIC sub-groups of RESV treatment for *ifng*, indicating the suppressed induction of this gene by RESV. These findings suggest that, while the expression of all genes studied herein is regulated through PKR, TLR, MAPK and IFN pathways, only *ifng* shows a significant NFKB-dependent transcriptional activation. None of the inhibitors used in this study influenced *viperin*, *ifng*, *isg15-1* or *lgp2* expression in non-stimulated macrophages, and the inhibitors only suppressed the expression of these antiviral genes in the pIC group compared with pIC-stimulated controls (PBS, DMSO or AcOH). This suggests that the constitutive expression of these antiviral biomarkers in Atlantic cod macrophages is regulated independent of the inhibited pathways. The expression of *il1b* was measured as an antibacterial and pro-inflammatory biomarker to examine the specificity of the immune inhibitors used in the current study. The transcription of *il1b* was not induced by pIC stimulation in any of the treatments (Fig. 3.10. E). The expression of *il1b* remained

unchanged in the RESV group compared to the DMSO-matched control. Unlike the antiviral biomarkers, the constitutive expression of *il1b* was suppressed by 2-AP and S90 (Fig. 3.10. E), suggesting that PKR and MAPK pathways may have roles in regulation of *il1b* basal expression in Atlantic cod macrophages. Interestingly, *il1b* expression increased in the RUX-exposed group compared to the DMSO-matched control. Also, the expression of *il1b* was higher in PBS group of CHQ treatment compared to its PBS-matched control group. These results show that the expression profile of *il1b* is different from those of the antiviral biomarkers subjected to qPCR assays, suggesting that the inhibitors in the present study influenced their specific targets in immune pathways. In the present study, there were no significant differences between DMSO, PBS and AcOH control groups within pIC or PBS treatment, showing that the DMSO vehicle and AcOH used herein did not change the basal or pIC-induced expression of Atlantic cod *viperin, ifng*, *isg15-1*, *lgp2* and *il1b*. Figure 3.11. summarises the pathway characterisation results of the current study and illustrates inhibitors, the target molecules, and their effects on the antiviral immune responses of Atlantic cod macrophages.

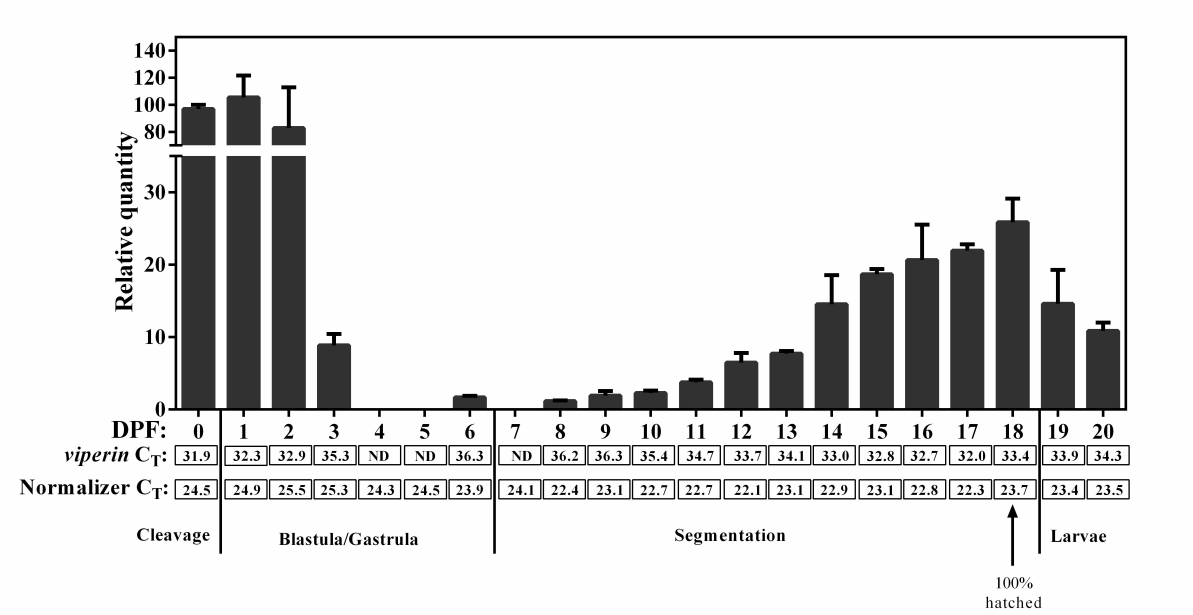


**Figure 3.8. qPCR results for constitutive expression of Atlantic cod *viperin* during embryonic and early larval development**. Relative quantity (RQ) data are presented as means ± SE. The numbers below each bar show the sampling time [i.e., day post-fertilization; (DPF)], and 0 DPF were samples taken less than 12 h after fertilization (see Section 3.3.6.). Cleavage, Blastula/Gastrula, Segmentation and Larvae describe the developmental stage at which samples were collected (Hall et al., 2004). $C_T$ (Cycle threshold) values for *viperin* below each bar represent the average $C_T$ of 3 pooled samples at the given time point, and ND are "not detectable" samples. Normalizer $C_T$ values are the average of the geometric mean value of 2 normalizers in 3 pooled samples (See Materials and Methods for details). These results were not analysed statistically since the $C_T$ values of the normalizers in the first 4 sampling time points were slightly higher compared with the other sampling time points.

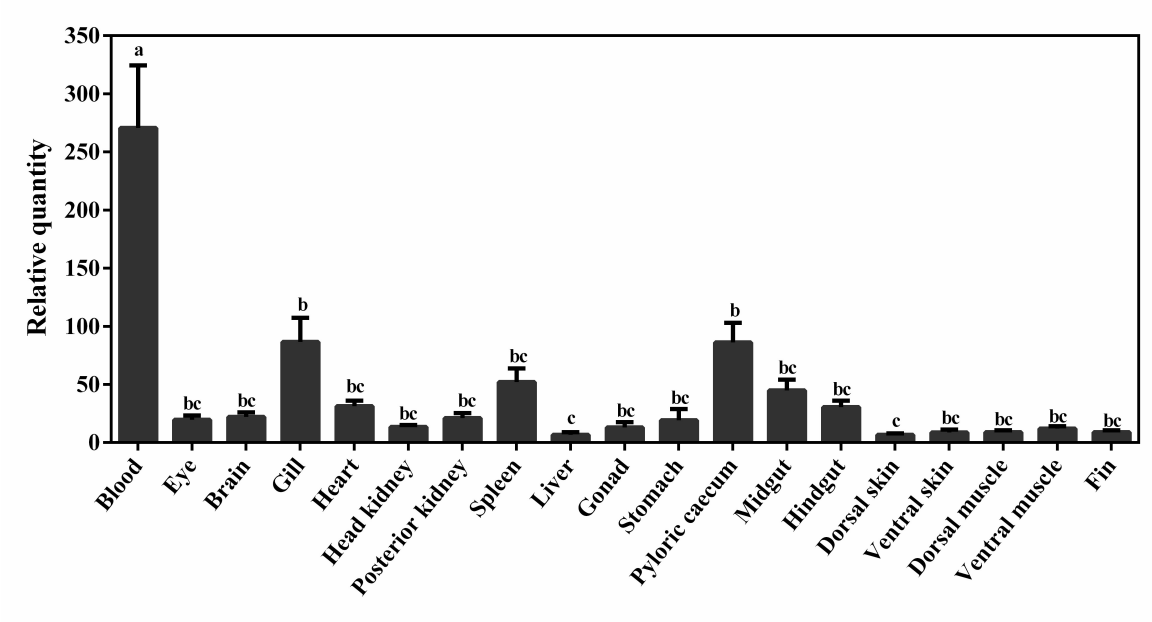


**Figure 3.9. qPCR results for constitutive expression of *viperin* in different tissues of adult Atlantic cod.** Relative quantity (RQ) data are presented as means ± SE ($n$ = 4). Different letters represent significant differences ($p < 0.05$) in *viperin* transcript expression between various tissues.

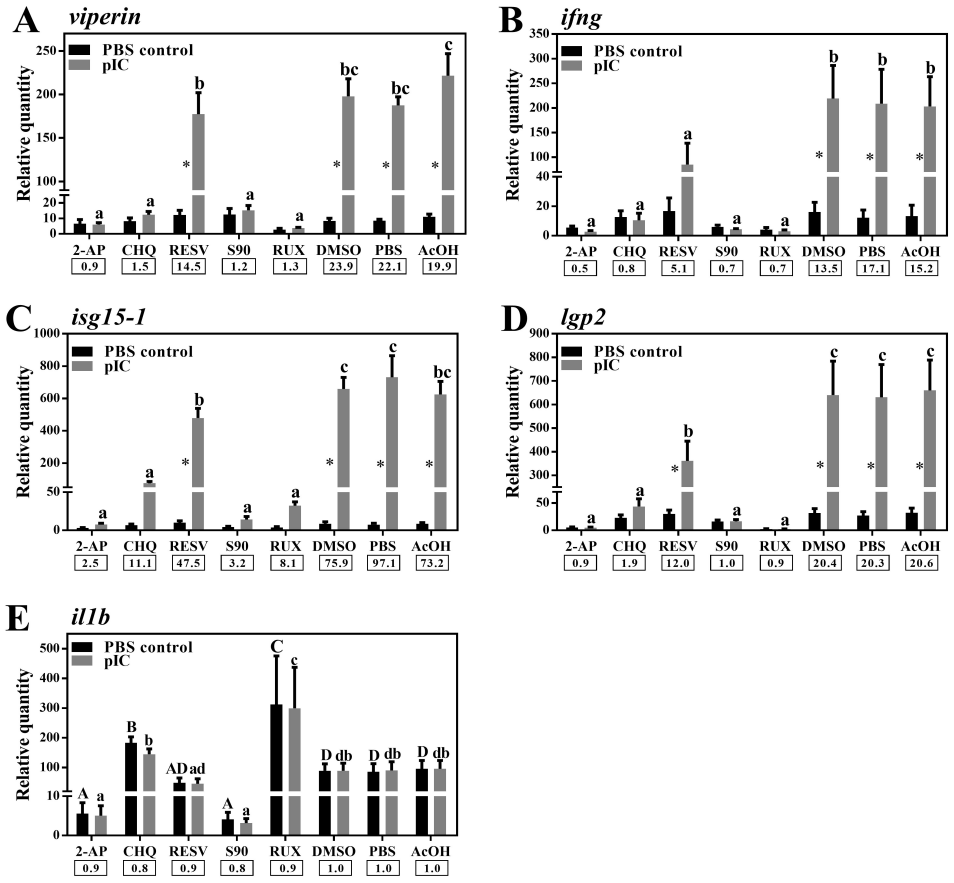


**Figure 3.10. qPCR results showing the effects of different inhibitors on pIC induction of Atlantic cod *viperin* (A), *ifng* (B), *isg15-1* (C), *lgp2* (D) and *il1b* (E).** Atlantic cod macrophages were exposed to different inhibitors [i.e., 2-Aminopurine (2-AP), Chloroquine (CHQ), Resveratrol (RESV), SB202190 (S90) and Ruxolitinib (RUX) groups] or control groups [i.e., Dimethyl sulfoxide (DMSO)-, phosphate-buffered saline (PBS) and glacial acetic acid (AcOH) controls] for 1 h, and then subjected to pIC or PBS for 24 h. The DMSO group is considered to be a vehicle control for the RESV, S90 and RUX treatments, whereas PBS and AcOH groups are considered to be control groups for the 2-AP and CHQ treatments, respectively. RQ data are presented as means ± SE. An asterisk shows a significant difference ($p < 0.05$) between treatment-matched pIC and control (PBS) groups. Different letters (upper-case for PBS and lower-case for pIC) represent significant differences between PBS- or pIC-stimulated inhibitor and control treatment groups. The pIC induction fold-change (pIC/control) within each treatment (i.e., inhibitors or control groups) is shown below the bars.

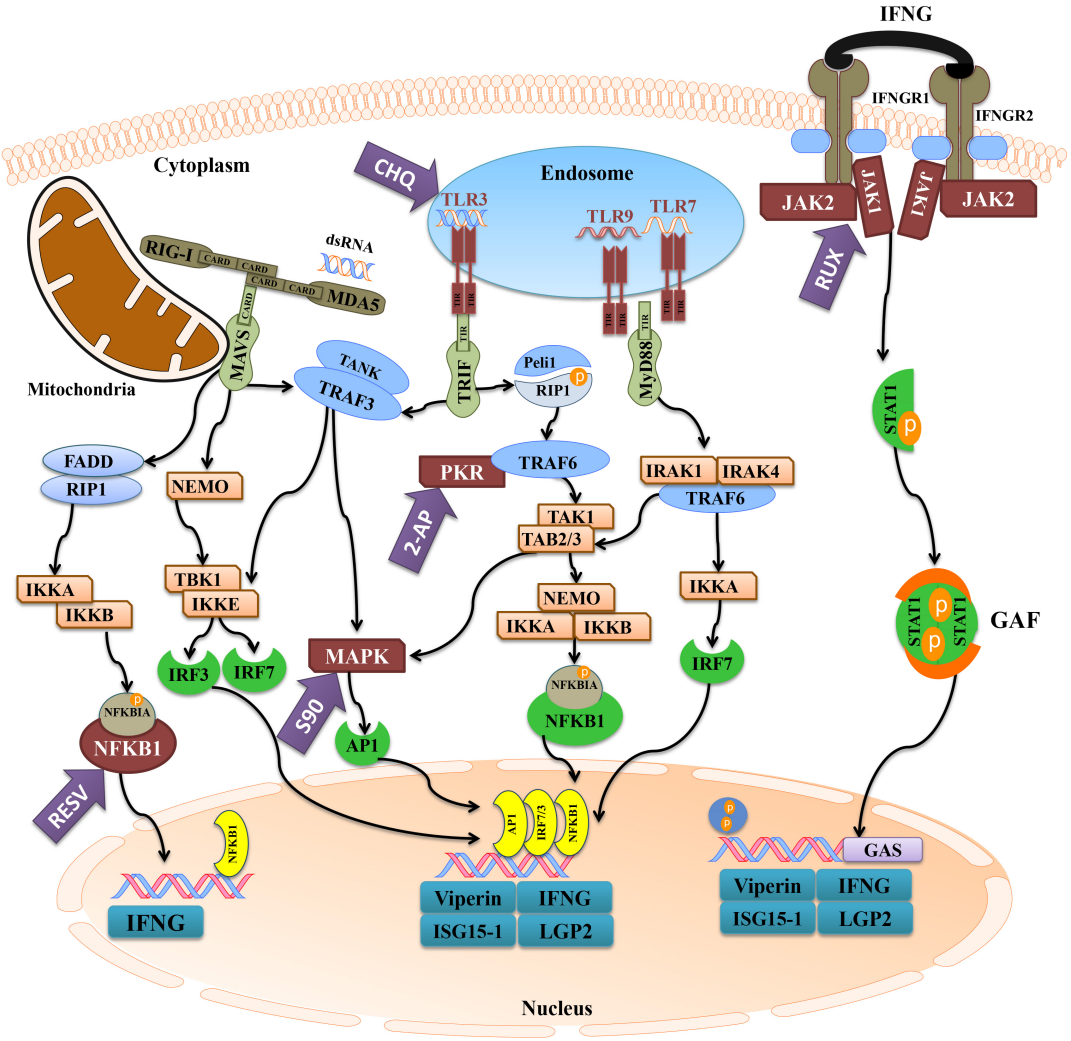


**Figure 3.11. The activation of antiviral pathways in Atlantic cod.** This figure was adapted from known mammalian pathways (Akira et al., 2006; Bonjardim et al., 2009; Yu and Levine, 2011), and illustrates the effects of immune inhibitors used herein on the antiviral immune response of Atlantic cod *viperin*, *ifng*, *isg15-1* and *lgp2*. The purple arrows represent the immune inhibitors used in this study, and the targets of inhibitors are shown in maroon. The turquoise boxes downstream of each pathway indicate the studied pIC-responsive genes that were influenced by the inhibitors. 2-AP (2-Aminopurine), CHQ (Chloroquine), RESV (Resveratrol), S90 (SB202190), RUX (Ruxolitinib), MDA5 (melanoma differentiation-associated protein 5), RIG-I (retinoic acid-inducible gene), MAVS (mitochondrial antiviral-signalling protein), FADD (FAS-associated death domain), RIP1 (receptor-interacting protein 1), IKK (NFKBIA kinase), NFKBIA (NF-kappa-B inhibitor alpha), NFKB1 (nuclear factor kappa-B 1), IFN (interferon), NEMO

(NFKB1 essential modulator or IKKG), TLR (Toll-like receptor), TRIF (TIR domain-containing adaptor protein inducing IFNB), TRAF (TNF receptor-associated factor), TANK (TRAF family member-associated NFKB activator), TBK (tank-binding kinase), IRF (IFN regulatory factor), MAPK (mitogen-activated protein kinase), AP1 (transcription factor AP1), Peli1 (pellino E3 ubiquitin protein ligase 1), PKR (IFN-induced, double-stranded RNA-activated protein kinase), TAK1 [transforming growth factor beta (TGFB)-activated kinase 1], TAB (TAK1-binding protein), MyD88 (myeloid differentiation primary response gene 88), IRAK (interleukin-1 receptor-associated kinase), IFNGR (IFN-gamma receptor), JAK (Janus kinase), STAT1 (signal transducer and activator of transcription 1), GAF (IFNG-activated factor), GAS (IFNG-activated sequence), ISG15-1 (IFN-stimulated gene 15-1), LGP2 (RNA helicase LGP2).

### 3.5. Discussion

The sequencing results showed that the Atlantic cod *viperin* transcript is 1342-bp long and consists of 6 exons. Exon 5 of Atlantic cod *viperin* is the shortest, whereas exons 1 and 6 are relatively longer compared to the other exons. The transcript size of Atlantic cod *viperin* is comparable with other fish species such as red drum (Dang et al., 2010), mandarin fish (*Siniperca chuatsi*) (Sun and Nie, 2004), Chinese perch (*Siniperca chuatsi*) and Ara (*Niphon spinosus*) (Chen et al., 2010). As in Atlantic cod, *viperin* transcripts of red drum (Dang et al., 2010) and human (Chin and Cresswell, 2001) were reported to include short 5'-UTRs. Similar to Atlantic cod, *viperin* genes of various vertebrates, e.g., mandarin fish (Chen et al., 2010; Sun and Nie, 2004), chicken (*Gallus gallus*) (Goossens et al., 2015) and mouse (*Mus musculus*) (Qiu et al., 2009), include 6 exons. The *viperin* exons in these species show a comparable size distribution to exons of Atlantic cod *viperin*, suggesting an evolutionarily-conserved exon/intron organisation for *viperin* in vertebrates. The present study found *viperin* to be flanked by *rnf144* and *cmpk2* in the genome of Atlantic cod, which showed a conserved synteny to human and zebrafish. Previously published studies showed the same gene synteny for *viperin* of various fish (i.e., Elephant shark and tilapia), avians [i.e., chicken and zebra finch (*Taeniopygia guttata*)] and mammals [i.e., mouse and chimpanzee (*Pan troglodytes*)] (Goossens et al., 2015; Lei et al., 2015). Taken together, it seems that the genomic arrangement of *viperin* and its flanking genes, notably the opposite orientation of *viperin* and *cmpk2,* is conserved in vertebrate evolution.

As expected, the phylogenetic tree showed that the relatedness of Viperin putative orthologues appears to agree with taxonomic classification. Previous studies using MSAs and molecular phylogenetic analyses of Viperin in different species obtained comparable results to the current study (Dang et al., 2010; Lei et al., 2015; Milic et al., 2015; Sun and Nie, 2004; Zhang et al., 2014; Zhong et al., 2015). MSA analyses revealed high levels of diversity in N-terminal AA sequences of putative orthologous Viperins among the representative species of different phyla and classes; in contrast, the radical SAM domain and the C-terminus are highly conserved.

This is the first study to predict the protein structure of a teleost Viperin using the crystal structure of a mammalian Viperin, and it suggests that the overall structure of Viperin of Atlantic cod and mouse is similar. Specifically, the AA residues involved in coordinating the radical SAM domain, as well as the [4Fe-4S] cluster of Atlantic cod, were shown to be highly conserved compared to its mammalian orthologue. As in duck (*Anas platyrhynchos*) (Zhong et al., 2015) and red drum (Dang et al., 2010), a conserved SAM binding motif (CXXXCXXC) was observed (i.e., Viperin AA 85-92) in Atlantic cod Viperin. The conserved aromatic residues adjacent to the third cysteine in the CXXXCXXC motif were suggested to modulate the oxidation-reduction midpoint of the [4Fe-4S] cluster (Sofia et al., 2001). The conservation of these residues in Atlantic cod Viperin seen herein suggests that the [4Fe-4S] cluster is likely used for the same function in both species. Moreover, computational modelling revealed a catalytic cavity that is conserved between the Atlantic cod Viperin and its mammalian orthologue. Consistent with Viperin of human, duck and crucian carp (Hinson and Cresswell, 2009b; Wang et

al., 2014a; Zhong et al., 2015), computational modelling analyses predicted the formation of α-helix in the N-terminal region of Atlantic cod Viperin, though the N-terminus of all Viperins modelled herein are predicted to be a disordered region. Taken together, these results indicate that Atlantic cod Viperin exhibit the overall conserved structure observed/predicted in other Viperin orthologues, highly suggestive of a comparable functional role.

The radical SAM Superfamily domain is found in hundreds of proteins that play a wide range of roles (Frey et al., 2008). Viperin has been well-documented to exhibit antiviral activities against human viruses [e.g., Zika, HCV and human cytomegalovirus (HCMV)] (Chin and Cresswell, 2001; Helbig et al., 2011; Hoek et al., 2017; Jiang et al., 2008). The radical SAM domain of Viperin is a key factor determining the antiviral roles of this protein (Jiang et al., 2008; Shaveta et al., 2010). The antiviral properties of mammalian Viperin chiefly rely on the interaction of its C-terminus with viruses [HCV and Dengue Virus Type-2 (DENV-2)] (Helbig et al., 2013; Wang et al., 2012). Similar to the mammalian Viperin, the overexpression of this protein by intramuscular injection of Viperin plasmid enhanced the resistance of rock bream against megalocytivirus (Zhang et al., 2014). Accordingly, the conserved antiviral activity of mammalian and teleost Viperin may be attributed to the conserved SAM domains (e.g., the near-identical structures of SAM domains of Atlantic cod and mouse reported herein). While N-terminal residues of mammalian Viperin were not necessary for antiviral activity of this protein, they may modulate Viperin antiviral activity (Jiang et al., 2008). The N-terminal amphipathic α-helix anchors Viperin into the ER membrane, and is needed for protein

localisation in lipid droplets (Hinson and Cresswell, 2009a, b). Correspondingly, the mammalian Viperin N-terminus assists the inhibition of lipid droplet-dependent viral replication (reviewed by Mattijssen and Pruijn, 2012). ER localisation of Viperin was previously shown in rock bream (Zhang et al., 2014) and crucian carp (Wang et al., 2014a). The evolution of fish Viperin involved the positive selection of N-terminal residues (Padhi, 2013). Therefore, the positively-selected N-terminus and the conserved C-terminus of Viperin may reflect species-dependent and ancestral functions, respectively, of this protein in antiviral responses (Padhi, 2013). The present protein structure findings suggest that the ability of teleost Viperin to bind to ER-associated lipid droplets, most likely, remained conserved, despite a large diversity between N-terminal residues of Viperin orthologues. Further studies are needed to test the relationship between diversity in the N-terminal amphipathic α-helix and lipid binding and antiviral functions of teleost Viperin.

Atlantic cod *viperin* was weakly expressed during embryonic development. However, the expression of *viperin* was higher between the mixed cleavage and mid-blastula stages (i.e., day 0-2) compared with subsequent embryonic stages (e.g., gastrula, early segmentation). This shows the existence of *viperin* transcript prior to the onset of zygotic gene expression. This embryonic expression profile of *viperin,* in combination with its presence in adult fish gonads, suggests that *viperin* is a maternal transcript in Atlantic cod. Maternal molecules (e.g., transcripts and proteins) play an important role in defence responses of fish during early life stages (Magnadóttir et al., 2005; Swain and Nayak, 2009). Some maternal (i.e., pre-mid-blastula expression) transcripts (e.g., *irf*7,

*ifngr1* and *cathelicidin*) involved in innate immune responses were previously identified in Atlantic cod (Rise et al., 2012; Rise et al., 2014; Seppola et al., 2009). There was a considerable decrease in level of Atlantic cod *viperin* transcript after the mid-blastula stage (i.e., in gastrula to early segmentation stages, 4-8 dpf), followed by an increase in expression between 13 and 20 dpf. The transition from maternal to zygotic gene expression occurs at mid-blastula stage (i.e., the maternal-embryo transition) (Lubzens et al., 2017; Rise et al., 2012; Traverso et al., 2012). Therefore, with respect to the expression and degradation patterns of maternal transcripts (Traverso et al., 2012), the non-detectable levels of Atlantic cod *viperin* immediately following the mid-blastula stage may be attributed to the degradation of maternal transcripts. It remains unknown if Viperin has any function in oogenesis or early embryogenesis. Nonetheless, viral hemorrhagic septicemia virus (VHSV)-induced *viperin* transcript was reported in eyed eggs and hatching fry of rainbow trout (Castro et al., 2015). Additionally, levels of *viperin* transcript increased in 48-h post-fertilization (hpf) larvae of zebrafish and 24-hpf D-veliger larvae of oyster infected with herpes simplex virus 1 (HSV-1) and Ostreid herpesvirus (OsHV-1), respectively (Ge et al., 2015; Green et al., 2016). A steady increase in Atlantic cod *viperin* transcript expression was observed from early segmentation until hatch, and a slight decrease in *viperin* expression was seen after hatch. Therefore, if *viperin* functions are conserved in teleost fish larvae, then its increasing constitutive expression in later stages of Atlantic cod embryonic development may provide information on the ontogeny of antiviral defence in this species. Further investigations are needed to determine whether *viperin* is a virus-responsive transcript in

Atlantic cod larvae. However, similar to the *viperin* results seen herein, some immune-relevant transcripts (*cxc chemokine*, *interleukin 8*, *atf3*, and *gaduscidin-1*) of Atlantic cod were reported to increase during hatching, and it was suggested that this induction may be involved in preparing cod embryos at the defensome level to combat environmental pathogens that may be encountered post-hatch (Rise et al., 2012).

This study showed that the constitutive expression of Atlantic cod *viperin* varied among different tissues. Atlantic cod *viperin* was strongly expressed in blood, and interestingly, *viperin* levels in immune-related tissues (i.e., head kidney and spleen) were significantly lower than that in blood. Likewise, the expression of *viperin* in the blood of red drum (Dang et al., 2010), large yellow croaker (*Larimichthys crocea*) (Zhang et al., 2018) and duck (Zhong et al., 2015) was higher than other tissues, including the immune-related and hematopoietic tissues (i.e., the kidney of red drum and the bursa of Fabricius of the duck). The head kidney is the hematopoietic site in teleost species (Geven and Klaren, 2017), and it contains a large number of differentiating cells (e.g., myeloid progenitor cells). The tissue-dependent expression of *viperin* in vertebrates may be associated with the differentiation stage of immune cells, as its expression is lower in hematopoietic tissues than in the blood. The expression of Atlantic cod *viperin* transcript in an intestinal tissue (i.e., pyloric caecum) was higher than some tissues (e.g., fin, skin and muscle). The intestinal expression of *viperin* was previously reported in different species [Rock bream, amphioxus (*Branchiostoma japonicum*) and duck] (Lei et al., 2015; Zhang et al., 2014; Zhong et al., 2015). As in other vertebrates (Peterson and Artis, 2014), the teleost intestine contains various immune cells (e.g., granulocytes and

macrophages) (Rombout et al., 2011), and *viperin* expression in the digestive system of Atlantic cod may be attributed to the mucosal immunity in this species. The results suggest that some aspects of cell- and tissue-dependent expression of *viperin* may be conserved among vertebrates. However, the function of Viperin in uninfected cells and tissues is still unknown (Mattijssen and Pruijn, 2012), and further studies are needed to determine if the constitutive expression of *viperin* is related to its immune or potential non-immune roles.

The present study examined if different pathway inhibitors may change the pIC response of Atlantic cod *viperin* and other well-known antiviral genes (i.e., *ifng, isg15-1* and *lgp2*). In addition to these genes, the expression of *il1b* was assessed, as a pro-inflammatory and antibacterial biomarker, to check if the inhibitors used in this study have gene-specific effects. Previous studies have documented the induction of Atlantic cod *viperin* in pIC-exposed macrophages or larval cell line (ACL cells), as well as in the spleen of pIC-injected fish (Eslamloo et al., 2016; Hori et al., 2012; Jensen et al., 2013; Rise et al., 2008). Similarly, the pIC-induced expression of *viperin* has been confirmed by several *in vivo*- or *in vitro*-based studies in Pacific oyster (Green et al., 2014), crucian carp (Wang et al., 2014b), red drum (Dang et al., 2010), tilapia (Lee et al., 2013), large yellow croaker (Zhang et al., 2018), annual fish (Liu et al., 2015), amphioxus (Lei et al., 2015), duck (Zhong et al., 2015) and mice (Severa et al., 2006). Previous studies failed to find induction of Atlantic cod *viperin* in macrophages or ACL cells stimulated with different LPSs (Seppola et al., 2015), as well as in spleens of Atlantic cod injected with different LPSs or formalin-killed atypical *Aeromonas salmonicida* (Hori et al., 2013;

Seppola et al., 2015). I also found similar results in LPS-treated Atlantic cod macrophages (unpublished data). The current study did not examine the effects of inhibition of immune-related pathways on the antibacterial response of Atlantic cod *viperin*. However, antibacterial induction of vertebrate *viperin* was reported in LPS-stimulated tilapia (Lee et al., 2013) and chicken (Goossens et al., 2015) as well as mouse macrophages (Severa et al., 2006) and dendritic cells (Boudinot et al., 2000). Further, LPS injection slightly up-regulated *viperin* expression in the spleen of orange-spotted grouper, but *viperin* induction was stronger in response to pIC or viral [i.e., grouper iridovirus (GIV)] stimulation (Wu et al., 2012). Thus, it seems that, while the transcriptional regulation of vertebrate *viperin* by the antiviral response is conserved among vertebrates, *viperin* may have species-dependent antibacterial responses.

Figure 3.11. depicts the inhibitors, the targeted factors in antiviral immune responses and the summary of pathway characterisation results in this study. My research showed that 2-AP, CHQ, S90 and RUX significantly suppressed the pIC-triggered expression of *viperin* in Atlantic cod macrophages, and similar results were seen for *ifng, isg15-1* and *lgp2* (Fig. 3.11.). In agreement with the present study, 2-AP was previously reported to block pIC induction of *viperin* in a monocyte/macrophage-like cell line (RTS11) of rainbow trout (DeWitte-Orr et al., 2007) and pIC inducibility of *mx* promoter in Japanese flounder (*Paralichthys olivaceus*) embryo cells (Ooi et al., 2006). 2-AP is a known inhibitor of IFN-induced PKR autophosphorylation (Hu and Conway, 1993). However, Sugiyama et al. (2012) showed that the inhibitory effects of 2-AP on IFNB transcription may occur independent of PKR, through inhibiting Akt and consequently

nuclear translocation of activated IRF3. The inhibitory mechanisms of 2-AP in fish species are yet to be determined, and 2-AP-associated repression of *viperin* and other studied genes in Atlantic cod may be caused by PKR- or IRF3-dependent mechanisms. In contrast to antiviral biomarker genes, 2-AP suppressed the basal expression of *il1b* in Atlantic cod macrophages. In agreement with this result, 2-AP-dependent inhibition of *il1b* expression was reported in human (Youssef et al., 2009). Taken together, it seems that PKR-regulated expression of *il1b* is conserved, and 2-AP suppresses PKR-derived immune responses of Atlantic cod in a gene-specific manner.

To determine TLR-dependent responses of Atlantic cod *viperin*, this study used CHQ, which blocks the pIC response and TLR signalling by hindering endosomal acidification, thereby impairing PAMP recognition by intracellular TLRs (e.g., TLR3) (de Bouteiller et al., 2005). CHQ can also suppress autophagy by hindering lysosomal acidification, and it can be used as a drug with diverse functions (e.g., in malaria and cancer treatments) (Maycotte et al., 2012). However, immunosuppressive and antiviral activities of CHQ are associated with its roles in endosomal pH modulation and blocking nucleic acid binding to TLRs (Rutz et al., 2004; Savarino et al., 2003). Therefore, the CHQ-mediated immunosuppression seen herein may be attributed to its effects on antiviral responses initiated by endosomal TLRs. CHQ was previously shown to inhibit the antiviral activity of rainbow trout macrophages (Jørgensen et al., 2001), to decrease pIC induction of *il1b* in gilthead seabream (*Sparus aurata*) macrophages (Muñoz et al., 2014) and to block R848 (i.e., TLR7 ligand) response [e.g., *myd88* (*myeloid differentiation primary response 88*) and *il6*] in peripheral blood leukocytes of Japanese

flounder (Zhou and Sun, 2015). The expression of *il1b* was not influenced by pIC stimulation in the current investigation, but this gene previously showed a slight up-regulation (i.e., 1.4-fold increase at 24 h post-stimulation) in response to pIC in Atlantic cod macrophages (Eslamloo et al., 2016). Slight differences between *il1b* results of previous and present studies may be due to biological variability of immune responses among individuals. However, CHQ enhanced the expression of *il1b* in the control group, and this gene expression profile (i.e., diverged response of antibacterial and antiviral biomarkers) reflects the specific effects of CHQ on the antiviral response of Atlantic cod macrophages. Collectively, it seems that CHQ influences intracellular TLRs of teleosts, and that activation of Atlantic cod *viperin*, *ifng*, *isg15-1* and *lgp2* is highly dependent upon the endosomal recognition of pIC.

S90 is known to inhibit the activity of p38 MAPK (Nemoto et al., 1998). S90 was found to be a strong inhibitor of LPS-induced inflammation relevant genes (e.g., *il1b* and *tnfa*) in head kidney leukocytes of Atlantic salmon (Holen et al., 2011). Generally, p38 MAPK is a well-established key regulator of inflammatory responses (Schieven, 2005). Therefore, the suppressed constitutive expression of *il1b* in the current study may be explained by p38 MAPK-mediated regulation of inflammatory cytokines, as in a previous study involving murine macrophages (Baldassare et al., 1999). Nonetheless, with respect to S90-related inhibition of the virus-responsive IFNs, RIG-I-dependent p38 was suggested to be a pivotal factor in antiviral responses of mammalian dendritic cells (Mikkelsen et al., 2009). In Atlantic salmon, transcriptome profiling of antiviral responses of macrophage-like cells identified several pIC-responsive MAPKs (Eslamloo

et al., 2017). While the association of MAPK activation and antiviral responses of teleosts is not yet fully understood, the current findings suggest an indirect or direct role of the p38 pathway in induction of Atlantic cod *ifng* and the other putative IFN-induced genes (i.e., *viperin*, *isg15-1* and *lgp2*).

RUX blocks the activation of JAK1/JAK2 following the engagement of induced (e.g., pIC and LPS) type I and II IFNs with the IFN receptors (Mascarenhas and Hoffman, 2012; Pattison et al., 2013). As in Atlantic cod *viperin*, RUX significantly suppressed the expression of *ifng*, *isg15-1* and *lgp2* in the present study, suggesting the activation of these genes downstream of JAK1/JAK2-dependent pathway. There is no report on RUX-based inhibition of IFN-dependent responses of fish species, although RUX has been reported to significantly reduce the production of IFNG in mice (Heine et al., 2013). In mammalian macrophages, RUX suppressed LPS-induced expression of IFN-regulated genes (Febvre-James et al., 2018) and the IFN-mediated response of genes containing STAT-binding sites in their promoters (Pattison et al., 2013). Similar to crucian carp *viperin* (Wang et al., 2014b), the proximal promoter region of Atlantic cod *viperin* contains putative binding sites for GAS and ISRE, suggesting the IFN- or STAT-dependent regulation of this gene. Although the *viperin* putative TFBSs (i.e., GAS and ISRE sites) identified herein are compatible with the JAK1/JAK2-dependent Atlantic cod *viperin* transcript expression, these *in silico* results need to be experimentally validated by future studies. ISRE-regulated activation, as well as IFN receptor (IFNR)- and IRF-dependent induction of *viperin* have been described for mammalian macrophages (Severa et al., 2006), and chicken *viperin* transcript was found to be IFN-responsive (Goossens et

al., 2015). Likewise, stimulation of Atlantic salmon TO cells with recombinant IFN (Sun et al., 2011) or overexpression of IFN in zebrafish embryos (Aggad et al., 2009) up-regulated the expression of teleost *viperin*. Also, induction of teleost *viperin* through activating factors downstream of MDA5 and IFN pathways was reported in crucian carp (Wang et al., 2014b). In the present study, I observed a comparable gene expression profile between *viperin* and *ifng* in response to different inhibitors (except for RESV). Moreover, two putative IFN-induced genes (i.e., *isg15-1* and *lgp2*) studied herein showed expression profiles that were similar to that of *viperin*, suggesting that these genes may share signalling pathways (e.g., IFN-related pathway) activating their pIC response. Conversely, *il1b* in this study showed a different expression pattern and its expression increased in the RUX group compared to the DMSO control group. Likewise, RUX-mediated JAK inhibition increased the expression of pro-inflammatory cytokines (e.g., *il6*) in mouse macrophages (Pattison et al., 2012). The qPCR results of the current study showed that RUX-mediated immune inhibition variably influenced the response of genes with different putative functions (e.g., antiviral *vs.* antibacterial roles) and regulatory pathways. RQ values of all samples from all treatments (i.e., pIC and PBS samples of inhibitor and control groups) and Pearson correlation coefficient tests were used to examine correlations between expression of *ifng* and other assessed genes. There were significant correlations ($p < 0.0001$) between the expression of Atlantic cod *ifng* and other antiviral genes [i.e., *viperin* (R: 0.71), *isg15-1* (R: 0.70) and *lgp2* (R: 0.94)], but no correlation was seen between *ifng* and *il1b* expression (R: 0.04; $p = 0.74$). This suggests that the IFN pathway plays important roles in transcriptional regulation of Atlantic cod

*viperin*, *isg15-1* and *lgp2*. Additionally, the repressed antiviral response of Atlantic cod *viperin*, *isg15-1* and *lgp2* by other immune inhibitors (e.g., 2-AP and CHQ) may also be attributed to their influence on IFNG secretion, as *ifng* expression was significantly influenced by these inhibitors. Taken together, these results, alongside previous studies, suggest that IFN-dependent regulation of *viperin* may be conserved among vertebrates. However, a previous study suggested that the pIC induction of rainbow trout *viperin* may be independent of protein synthesis (DeWitte-Orr et al., 2007). Therefore, future studies using protein synthesis inhibitors and recombinant IFNs are needed to confirm IFN inducibility of Atlantic cod *viperin*.

RESV can inhibit NFKB through preventing NFKBIA (Nuclear factor kappa-B inhibitor alpha) phosphorylation (Adhami et al., 2003), and it was shown to down-regulate the immune responses of head kidney leucocytes in turbot (*Scophthalmus maximus*). However, RESV did not significantly change the expression of Atlantic cod *viperin* transcript in response to pIC herein. Conversely, the pIC response of Atlantic cod *ifng* was suppressed by RESV. Also, there was a decrease in pIC induction of *isg15-1* and *lgp2* in the RESV group compared to the pIC-stimulated DMSO vehicle control group. In agreement with these results, RESV suppressed virus-induced IFNG expression in mice (Zang et al., 2011). These results suggest that the TLR- or RLR-activated NFKB may enhance the expression of Atlantic cod *ifng*, and may influence the intensity of the pIC response of *isg15-1* and *lgp2*. Although the current findings suggest NFKB-independent stimulation for Atlantic cod *viperin*, further studies using a wider range of RESV doses

and multiple sampling points are needed to evaluate the time- and dose-dependent effects of RESV on Atlantic cod *viperin* expression.

### 3.6. Conclusions

The present study showed that Atlantic cod *viperin* is an evolutionarily conserved gene that has similar gene organisation and flanking genes to its putative orthologues in other vertebrates. Atlantic cod Viperin exhibits a close phylogenetic relationship with Viperin of other teleosts. A highly-conserved protein structure reported herein suggests a functional role for the Atlantic cod Viperin comparable with that of other Viperin orthologues. Atlantic cod *viperin* transcript showed a tissue-specific constitutive expression and was most strongly expressed in the blood. The inhibitory effects of 2-AP, CHQ and S90 on pIC induction of *viperin* transcript in Atlantic cod macrophages revealed that the expression of this gene may be dependent upon PKR, intracellular TLRs and MAPK, and/or possibly the factors (e.g., IRFs) activated downstream of these pathways. Moreover, the RUX-associated suppression of Atlantic cod *viperin* was in agreement with the GAS and ISRE motifs predicted in the proximal promoter of this gene. These data, alongside a significant correlation seen between *viperin* and *ifng* expression in response to different immune inhibitors, suggest the IFN-mediated regulation of Atlantic cod *viperin*.

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# CHAPTER 4

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

## Preface

The research described in Chapter 4 has been published in *Molecular Immunology* as: Eslamloo, K., Inkpen, S.M., Rise, M.L., Andreassen, R., 2018. Discovery of microRNAs associated with the antiviral immune response of Atlantic cod macrophages. Mol. Immunol. 93, 152-161; see Co-authorship Statement on page XXIV.

**4.1. 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) 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) that were exposed to pIC or PBS over time (i.e., for 12, 24, 48 and 72 HPS). As in sequencing, miR-731-3p, miR-462-3p and miR-2188-3p were significantly up-regulated by pIC. Although the sequencing results were not qPCR-validated for miR-125b-3-3p and miR-150-3p, the qPCR did show significant up-regulation of these transcripts in response to pIC stimulation at later time points (i.e., at 48 and/or 72 HPS). Also, qPCR was used 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, and this data strongly suggests

that miRNAs are important in the 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.

## 4.2. Introduction

The 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). The 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; Samuelsen et al., 2006). Furthermore, the immune system of the Atlantic cod displays several unique features (e.g., the absence of *MHC II* genes in the genome) among teleosts (Star et al., 2011), suggesting that the immunological studies on this fish are of evolutionary importance. In addition, 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 the 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 bind to imperfect complementary sequences in the 3′-untranslated regions (3′-UTRs) of target mRNAs, and result in translational suppression or mRNA degradation (Chekulaeva and Filipowicz, 2009; Winter et al., 2009).

miRNAs are key regulators of antiviral immune responses, and act through various mechanisms (Baltimore et al., 2008; Lodish et al., 2008). With respect to the 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 thought 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 humans (Lecellier et al., 2005). Finally, virus-derived miRNAs have been reported to negatively regulate antiviral responses and interact with viral replication through the targeting of host and viral mRNAs (Cullen, 2009), suggesting the importance of miRNAs in virus-host interactions.

Small RNA profiling can be applied to improve our understanding of the involvement of miRNAs in the antiviral immune responses of teleost fish (Andreassen and Høyheim, 2017). Small RNA deep sequencing has 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 [e.g., Atlantic salmon (*Salmo salar*) and zebrafish (*Danio rerio*)], indicating that the immune-related responses of these miRNAs are conserved (Andreassen and Høyheim, 2017). While the early and later life stage Atlantic cod miRNAs have been discovered and characterised (Andreassen et al., 2016; Bizuayehu et al., 2015), there is currently no information on cod miRNAs associated with the antiviral immune response.

Macrophages have crucial functions in the 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 important if we are to broaden our knowledge of molecular pathways and regulatory mechanisms underlying the antiviral immune responses of fish. pIC-responsive Atlantic cod macrophage transcript (mRNAs) expression was 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 pIC-responsive macrophage miRNAs in this species.

## 4.3. Materials and methods

### *4.3.1. Animals*

Atlantic cod [1.64 ± 0.14 kg (mean ± SE)], reared in a 21 $m^3$ tank and optimal conditions (5.2-6.4˚C, 95-110% oxygen saturation and an ambient photoperiod) at the JBARB of 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 at each feeding). 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 4.3.3. and 4.3.4.). All procedures in this study were performed under the approval of Memorial University of Newfoundland's Institutional Animal Care Committee, and followed the guidelines of the Canadian Council on Animal Care.

### *4.3.2. 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 $NaHCO_3$, 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 to 35 mm culture dishes (Corning, NY) at an equal density of $3 \times 10^7$ 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).

### *4.3.3. Macrophage stimulation and sampling at 24 HPS for sequencing*

To identify pIC-responsive miRNAs in Atlantic cod at 24 h post-stimulation (HPS), macrophages were isolated from six individuals, as described in Section 4.3.2., and placed in 2 culture dishes ($3 \times 10^7$ cells per 35 mm culture dish) per fish (for a total of 12 culture dishes). Starting 24 h after cell isolation, the macrophages were exposed to 50 µg $ml^{-1}$ pIC [in phosphate buffered saline (PBS)] 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 was removed, then the culture dishes were sealed with parafilm and kept at -80˚C until RNA extraction (see Fig. 4.1. for experimental design).

### *4.3.4. Time-dependent pIC response of macrophages and sampling for sequencing and qPCR analyses*

Macrophages were isolated from six different individuals (see Section 4.3.2.) and seeded into 8 culture dishes ($3 \times 10^7$ cells per 35 mm culture dish) per fish (48 culture dishes in total). As in Section 4.3.3., the isolated macrophages were stimulated with 50 µg $ml^{-1}$ pIC or PBS at 10˚C. Samples for qPCR and sequencing were collected at 12, 24, 48 and 72 HPS and kept at -80˚C before RNA extraction (see Fig. 4.1. for experimental design).

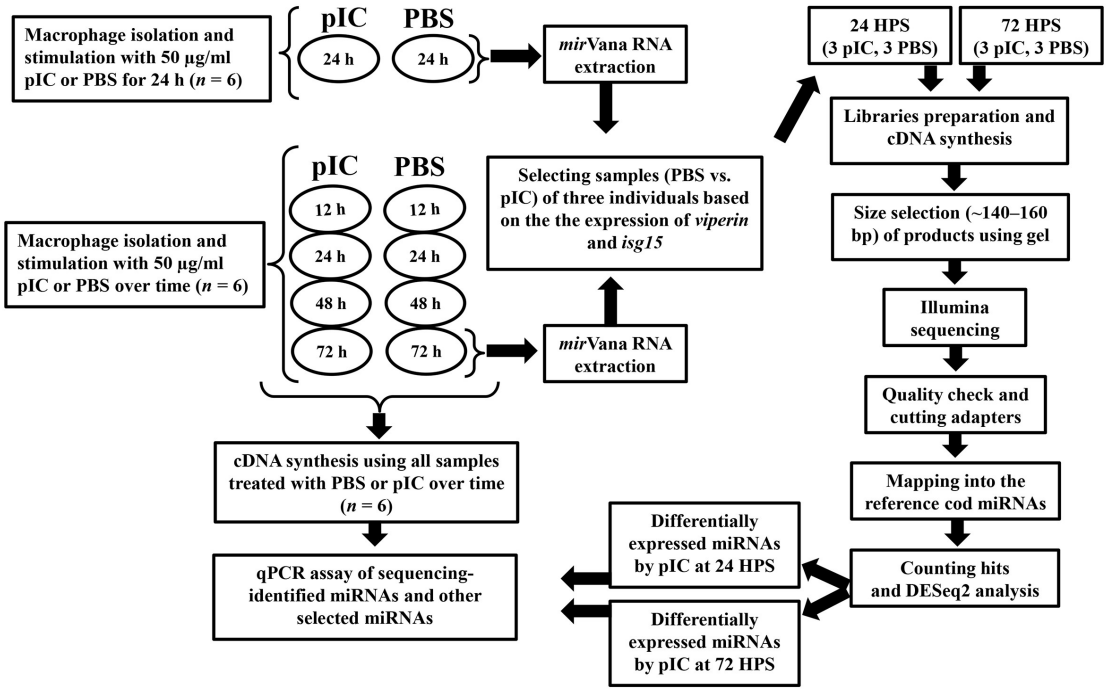


**Figure 4.1. Overview of experimental design.** Macrophages were isolated from six Atlantic cod in each experiment (12 individual in total), and were stimulated with pIC. The control and pIC samples from 3 individuals in each experiment were used for deep sequencing, and the differentially expressed miRNAs were identified using DESeq2 analyses. qPCR analyses were performed using all of the samples (6 fish) in the second experiment.

### *4.3.5. Total RNA extraction*

The total RNA of all samples in both experiments (i.e., macrophages stimulated with pIC and time-matched 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 RNA 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. However, it was determined that lower A260/230 ratios did not influence the qPCR assays performed with the templates (see Section 4.3.7.).

### *4.3.6. Deep sequencing and analysis of the deep sequencing data*

The samples were selected for macrophage miRNA sequencing in both pIC and PBS groups at 24 and 72 HPS, based on the quality and quantity (ranging from 57-405 ng $\mu l^{-1}$) of RNA as well as their immune response (i.e., the expression of *isg15-1* and *viperin*) [see Eslamloo et al. (2016) for primer sequences and qPCR assay method]. 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 4.3.3.) and 3 fish at 72 HPS (i.e., $n = 3$; 6 samples in total; see Section 4.3.4.) that showed a high fold-change response in 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 of total RNA input, following the manufacturers' instructions.

cDNA products of samples from both sequencing experiments (i.e., at 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. Reads mapped to the mature reference sequences with an 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., at 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 (Neilsen 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 then 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 that 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 4.3.9.).

### *4.3.7. qPCR analysis of miRNA expression*

The miRNA sequencing results of both experiments (i.e., at 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 4.3.4.) at all four sampling points (48 samples in total; Fig. 4.1.). qPCR was used to examine the expression of six other miRNAs (i.e., miR-30b-3p, miR-128-3-5p, miR-214-1-5p, miR-451-3p, miR-144-3p and miR-199-1-3p) that have previously been identified as immune-related miRNAs in other vertebrates. Two of the selected miRNAs were miR-30b-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 immune-responsive in other teleost species [i.e., Japanese flounder (*Paralichthys olivaceus*) and Nile tilapia (*Oreochromis niloticus*)] (Wang et al., 2016; Zhang et al., 2014), suggesting that these miRNAs may have a conserved regulation in immune responses of teleosts.

cDNAs were synthesized using 400 ng of total RNA and the miScript II RT Kit (Qiagen, Hilden, Germany) in 20 µl reactions as recommended by the manufacturer's instructions. The cDNAs were diluted by adding 200 µl 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 4.1.). The primers were provided desalted, and

in de-ionized water (100 µM), by the manufacturer (Sigma-Aldrich). A universal primer, provided by the miScript SYBR Green PCR Kit (Qiagen), was used as a reverse primer in the qPCR assays. Two miRNAs (miR-25-3p and miR-210-5p), identified as the most suitable normalizers for miRNA expression in Atlantic cod (Andreassen et al., 2016), were used as normalizers in this study. These normalizers were expressed stably in qPCR assays of the present study (i.e., the geometric mean of normalizers' $C_T$ was 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 the 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 4.1.), 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 µl of 2X QuantiTect SYBR Green PCR Master Mix, 2.5 µl of 10X miScript Universal Primer, 2.5 µl of specific forward primer (10 µM), 5 µl of RNase-free water (Qiagen), and 2.5 µl 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 variability in 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 Cq) 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. 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).

**Table 4.1. Primers used for qPCR analyses.**

| | Primer sequence 5' to 3' [1] | $R^2$ | Amplification efficiency (%) [2] |
|---|---|---|---|
| **Sequencing-identified miRNAs** | | | |
| miR-731-3p | ACCGGGAATCTCGTGTCAGCTA | 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 | TAGCAATGGTAAGGGTTCC | 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 primers. 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 miR-30b-3p were slightly wider than that of 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 this parameter was determined for the other miRNAs using 5-point serial dilutions.

### *4.3.8. 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 the 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 using the 5'-flanking region of the 5' miRNA in each pair, while miR-214-1 and miR-125b were analysed as individual miRNAs since there was more than a 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-pIC-responsive miRNAs (i.e., exhibiting no fold-change in sequencing results at 72 HPS) were analysed as described above to check the frequency of predicted TFBSs in a set of non-immune related miRNAs.

### *4.3.9. 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 S4.2. [also see Table 2. in Eslamloo et al., (2016); Chapter 2, Table 2.2. ]. 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.

Target prediction analysis was performed using 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. To obtain a prediction with high stability, minimum free energy was adjusted to -15 for the RNAhybrid.

## 4.4. Results

### *4.4.1. Deep sequencing*

This study used two sets of experimental samples to identify pIC-responsive miRNAs by deep sequencing. Table 4.2. 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 of the 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 the reads from the 24 HPS were mapped into the reference Atlantic cod miRNA datasets (i.e., approximately 1 million reads for each sample).

The preparation of libraries for the 72 HPS samples was performed using the NEBNext kit, and the total number of reads for these samples varied between 11.2 and 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 for 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 4.2.).

**Table 4.2. An overview of the samples used for deep sequencing and the results of the sequencing.**

| Sample ID[1] | Sample characteristics | Library prep [2] | Total number of reads [3] | Trimmed and filtered reads [4] | Reads mapped to miRNAs [5] | Accession number [6] |
|---|---|---|---|---|---|---|
| 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 | 1,656,324 | 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 |
| Fish3b-72-P | pIC, 72 HPS | NEBNext | 28,057,048 | 7,108,218 | 1,143,310 | SRR3884866 |

[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 4.3.3.), and "b" shows individuals from experiment 2 (Section 4.3.4.).
[2] Library preparation methods, i.e., NEBNext or TruSeq; all samples from the same time point were sequenced using the same library preparation method.
[3] Total number of reads in raw fastq file for each sample.
[4] Total number of reads after cutting adapters and filtering reads by size (18-24 bp).
[5] Reads mapped to the reference (i.e., all known mature miRNAs of Atlantic cod).
[6] Accession number of sequencing results for each sample submitted to the NCBI SRA database (http://www.ncbi.nlm.nih.gov/sra).

### *4.4.2. 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 4.3. 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 4.3.). 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 down-regulated 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 4.3.).

### *4.4.3. qPCR analyses of DEseq2-identified pIC-responsive miRNAs*

Table 4.1. 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 4.3.4.). 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 for the pIC- and PBS-exposed (control)

**Table 4.3. 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] | *p* value [5] | *p* adj [6] |
|---|---|---|---|---|---|---|
| **Identified at 24 HPS** | | | | | | |
| 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 |
| **Identified at 72 HPS** | | | | | | |
| 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 the samples in the given time point.
[2] $Log_2$-transformed fold-change (pIC/control) as implemented by DESeq2.
[3] Log-fold Standard Error.
[4] Wald statistic (i.e., pIC vs. control).
[5] Wald test *p* value.
[6] Adjusted *p* values.

groups are shown in Figure 4.2. The expression of miR-731-3p was significantly up-regulated by pIC stimulation at 24 HPS (1.5-fold increase), and it continued to increase at 48 and 72 HPS (2.1- and 2.5-fold, respectively) (Fig. 4.2. A). Similarly, the expression of miR-462-3p increased in cod macrophages over time in response to pIC stimulation. The difference was significant at 24 HPS (2.6-fold increase; Fig. 4.2. B), 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 showed the strongest response to pIC among all qPCR-assayed miRNAs.

The DESeq2 results identified miR-150-3p as down-regulated by pIC at 24 HPS (Table 4.3.). 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, this miRNA was significantly up-regulated by pIC at 48 and 72 HPS (1.7- and 1.9-fold, respectively; Fig. 4.2. C). The DESeq2 results for miR-125b-3-3p were not validated by qPCR analyses. However, this miRNA was up-regulated at 72 HPS (by 2.2-fold; Fig. 4.2. D). qPCR analyses 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. 4.2. E). However, this was not an effect of pIC, but due to the fact that 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 suggests 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.

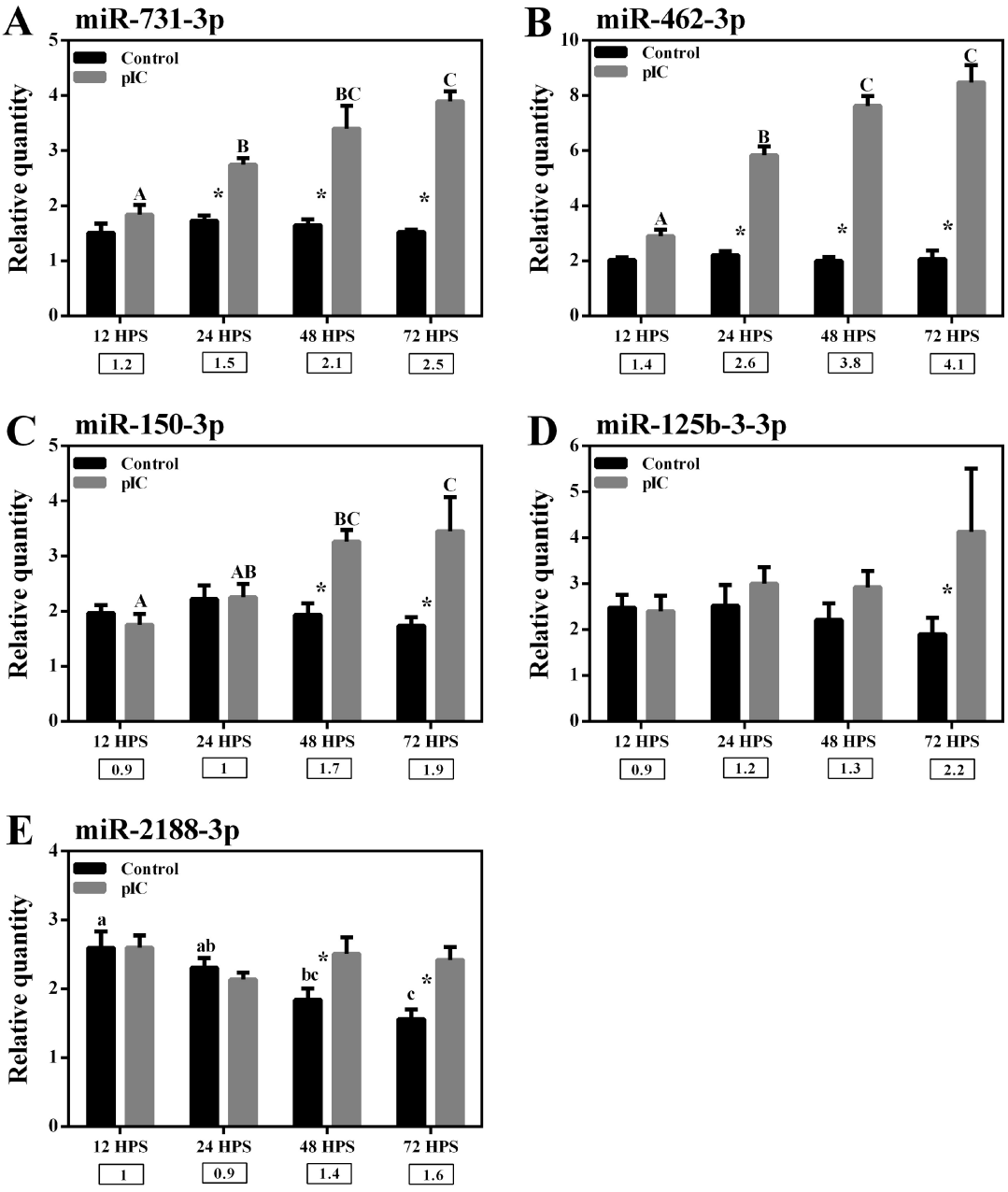


**Figure 4.2. qPCR results for pIC-responsive miRNAs identified by sequencing.** Data are presented as mean ± SE. An asterisk indicates a significant difference ($p < 0.05$) between time-matched pIC and control (PBS) groups. Different letters (lower-case for control and upper-case for pIC) represent significant differences within a group over time. The fold-change (pIC/control) for each time point is shown below the figures.

### *4.4.4. qPCR results for other selected miRNAs*

Figure 4.3. shows the results of six miRNAs known to play an immune role in other vertebrates. miR-30b-3p expression decreased in response to pIC stimulation at all the time points; however, a significant difference was only identified at 48 HPS (a 0.5-fold decrease; Fig. 4.3. A). On the other hand, miR-128-3-5p was significantly up-regulated in response to pIC at 48 and 72 HPS (1.7- and 2-fold increases respectively; Fig. 4.3. B). 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. 4.3. C). Further, miR-451-3p showed an up-regulation (by 1.7-fold) in pIC-stimulated Atlantic cod macrophages at 72 HPS compared to the time-matched controls (Fig. 4.3. E). While the expression of miR-199-1-3p was down-regulated by pIC at 48 HPS (0.7-fold; Fig. 4.3. D), the expression of miR-144-3p (Fig. 4.3. F) was not influenced by pIC.

### *4.4.5. In silico analysis of the promoter region of pIC-responsive miRNAs*

In this study, 1000 bp 5′-upstream regions of the immune-responsive miRNA genes or clustered miRNA genes were used to identify putative immune-related TFBSs. As shown in Supplemental Table S4.1., *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

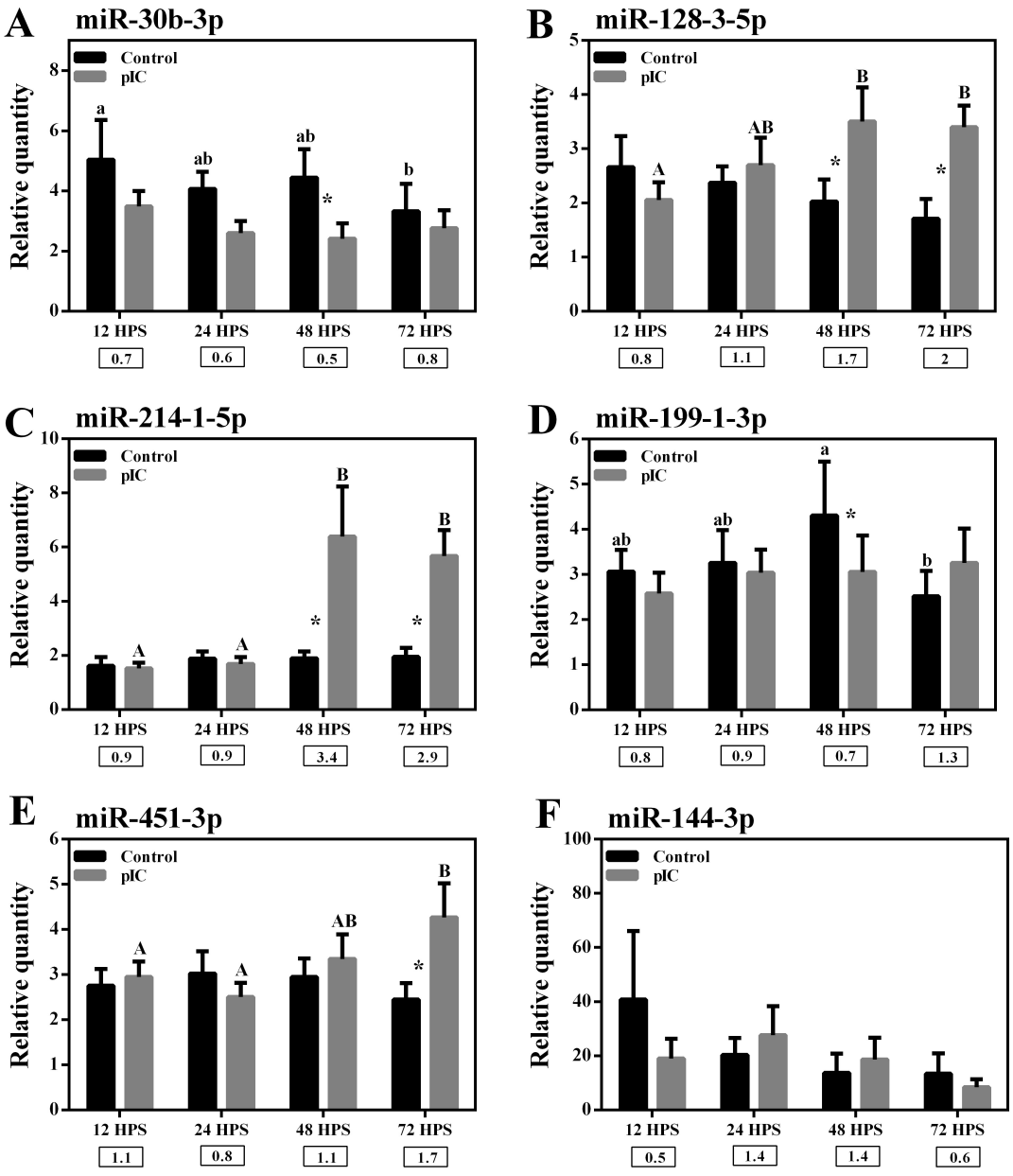


**Figure 4.3. qPCR results for other selected miRNAs.** Data are presented as means ± SE. An asterisk indicates a significant difference ($p < 0.05$) between time-matched pIC and control (PBS) groups. Different letters (lower-case for control and upper-case for pIC) represent significant differences within a group over time. The fold-change (pIC/control) for each time point is shown below the figures.

for STAT1, also known 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 (Fig. 4.4.). However, *in silico* analysis also found some immune-related potential TFBSs in the 5′-upstream regions of ten non-pIC-responsive miRNAs (i.e., miRNAs with no-fold change in response to pIC). Indeed, there was no correlation between the frequency of predicted immune-related TFBSs and the 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.

### *4.4.6. Predicted target genes 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 4.4. 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)]. As predicted, *cytotoxic and regulatory T-cell protein precursor* and *interferon stimulated gene 15-3* had target site matches with

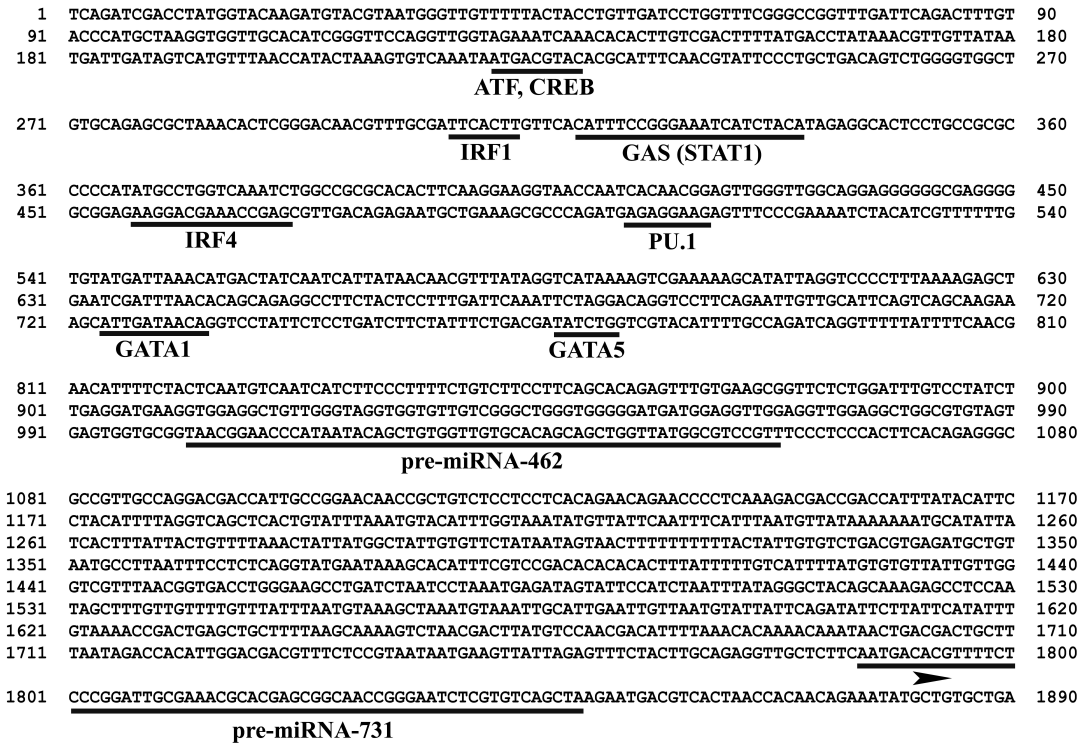


**Figure 4.4. The transcription factor binding sites predicted in the 5′-upstream region of the miR-462/miR731 cluster of Atlantic cod.** CREB (cAMP response element-binding protein), ATF (Activating transcription factor), IRF (Interferon regulatory factor), GAS (IFNG-activated sequence), STAT1 (Signal transducer and activator of transcription 1), PU.1 (Transcription factor PU.1), GATA (GATA binding protein).

**Table 4.4. Predicted target genes for pIC-responsive miRNAs in Atlantic cod macrophages.**

| Predicted target gene [1] | Probe ID [2] | Regulation [3] | miRNAs |
|---|---|---|---|
| *interferon stimulated gene 15-3* | 38611 | Up-regulated gene | miR-128-3-5p |
| *CXC chemokine* | 36483 | Up-regulated gene | miR-2188-3p[4] |
| *probable E3 ubiquitin-protein ligase herc4-like* | 43797 | Up-regulated gene | miR-30b-3p |
| *cytotoxic and regulatory T-cell protein precursor* | 36797 | Up-regulated gene | miR-451-3p |
| *optineurin* | 38670 | Up-regulated gene | miR-128-3-5p |
| *bloodthirsty-1* | 40261 | Up-regulated gene | miR-199-1-3p |
| *bloodthirsty-3* | 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 a given microarray probe and the contig used for designing the probe [see Eslamloo et al., (2016)].

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

[4] 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 S4.2.

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. miR-2188-3p and its isomiR variant were identified to target the 3'UTR of *CXC chemokine*. Finally, 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, this suggests that miR-30b-3p potentially plays a role in the regulation of *herc4*.

### 4.5. 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 the 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-214-1-5p) 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 have been due to various factors, such as the pIC-dependent molecular and cellular responses. Nonetheless, 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 that cell death remains under 3% in both stimulation groups at all sampling points.

In the present study, the 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 peak fold-change 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 (Thatcher et al., 2008). These two teleost-specific miRNAs have been reported as virus- and bacteria-responsive in several 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), in rainbow trout challenged with VHSV, RTL-W1 (i.e., a rainbow trout liver cell line) stimulated with pIC for 48 h (Schyth et al., 2015) and in 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 time-dependent manner (Bela-ong et al., 2015), i.e., induction at 1 day post-injection (DPI) and peak expression occurring at 21 DPI.

*In silico* analysis identified a putative GAS binding site in the proximal promoter of the Atlantic cod miRNA-462/731 cluster along with other immune-relevant putative TFBSs (e.g., IRFs), although the activity of the predicted motifs herein needs to be validated by future studies. Upon induction of the IFNG-dependent pathway, the STAT1 homodimer, termed 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 showed a time-dependent up-regulation in response to the injection of IFNG-expressing plasmids (Bela-ong et al., 2015). In a previously published study (Eslamloo et al., 2016), the induction of IFNG 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. The TFBS results found herein, alongside my previous study, suggest that miRNA-462/731 cluster may have an IFN-dependent expression in Atlantic cod macrophages. The megalocytivirus-induced miR-731-5p of Japanese flounder enhanced early-stage viral replication, inhibited virus-induced apoptosis of splenocytes, and suppressed IRF7 expression by targeting its transcript (Zhang et al., 2016). As reported in

a previous study (Eslamloo et al., 2016), *irf7* expression decreased significantly with sampling time in pIC-stimulated cod macrophages (i.e., from 24 HPS to 48 HPS). However, the *in silico* analysis in this study 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 by 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. This is consistent with the data for miR-2188-5p of olive flounder and miR-2188-3p Atlantic salmon, which were 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 for miR-125b-3-3p (see the first paragraph of discussion). Nonetheless, qPCR showed an up-regulation for miR-125b-3-3p in pIC-stimulated cod macrophages at 72 HPS. In agreement with this result, the expression of Japanese flounder miR-125b increased 6 days post-megalocytivirus infection (Zhang et al., 2016), and miR-125b increased the pIC-triggered expression of *ifna*/*b* in airway epithelial cells of human (Zhang et al., 2012). Collectively, the findings of the present

study, along with previous results from different species, suggest that miR-125b may have some evolutionarily-conserved immune-related functions.

In contrast to the sequencing results of the current study at 24 HPS, qPCR analysis showed no significant difference between the pIC and control groups with regard to the expression of miR-150-3p at 24 HPS, and this miRNA was up-regulated in response to pIC stimulation at 48 and 72 HPS (Table 4.3. and Fig. 4.2.). Atlantic cod macrophages stimulated with pIC over time (i.e., the second experiment) were used for qPCR validation of sequencing at both 24 and 72 HPS. Hence, the differences between the qPCR and sequencing results may arise from individual-dependent variations in the pIC responses in the first and second experiments. Mammalian miR-150 is a well-characterised regulator of lymphocyte development, notably B-cell maturation (Baltimore et al., 2008), and teleost miR-150 was a hypoxia-induced miRNA in zebrafish larvae (Huang et al., 2015). The immune-related functions of teleost miR-150 are yet to be determined.

In addition to the miRNAs discovered by DESeq2 analysis, five miRNAs showed significant responses to pIC by qPCR (Fig. 4.3.). Atlantic cod miR-30b-3p and miR-128-3-5p were significantly down- and up-regulated, respectively, in pIC-treated macrophages at later time points. Moreover, miR-30b-3p is predicted to target *herc4*, which is a pIC-induced transcript in Atlantic cod macrophages at 24 and 48 HPS (Eslamloo et al., 2016). With respect to the inverse correlation between the expression of miRNAs and their target transcripts (Huntzinger and Izaurralde, 2011), the opposite

regulation of miR-30b-3p and its putative target suggests that this miRNA affects transcript levels 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).

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, was not significantly affected. These results support the previous small RNA sequencing data showing that miR-451 was a late virus-inducible miRNA in Japanese flounder (Zhang et al., 2014). These findings suggest that miR-451 may play an immunoregulatory role in the late antiviral responses of these species. 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 late erythroblast maturation, although the influence of miR-451 on the target gene was stronger than that of miR-144 (Rasmussen et al., 2010). Further, 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 macrophages 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 located close to each other (i.e., they are more than 3000 bp apart) 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 documented in the higher vertebrates. The expression of miR-214 was up-regulated in *Streptococcus agalactiae*-challenged Nile tilapia at 48 and 72 h 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 cluster, miR-144-3p and miR-451-3p were not co-expressed 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 pre-miRNA (Obernosterer et al., 2006; Thomson et al., 2006). Therefore, the differential expression of miRNAs located in the same gene cluster seen herein may be attributed to post-transcriptional regulation.

*In silico* analysis performed in my thesis predicted target transcripts for pIC-responsive miRNAs (Table 4.4.). The miRNAs showed no reverse regulation with their putative targets (i.e., at the transcript level), except for miR-199-1-3p 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). I acknowledge that there may be some false positives among the predicted targets. However, while some pIC-responsive miRNAs 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 immune-stimulated transcripts. In this model, the expression of miRNAs increases along with the transcripts (e.g., through an immune-activated transcription factor) to ensure a balanced immune response that leads to the protection of the host and minimized immune-mediated damages to cells by suppressing the inflammatory responses (Andreassen and Høyheim, 2017). An improved version of the Atlantic cod genome has recently been 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.

### 4.6. Conclusions

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 immune-responsive 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.

## 4.7. References


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# CHAPTER 5

# Transcriptome profiling of antiviral immune and dietary fatty acid dependent responses of Atlantic salmon macrophage-like cells

## Preface

The research described in Chapter 5 has been published in *BMC Genomics* as: Eslamloo, K., Xue, X., Hall, J.R., Smith, N.C., Caballero-Solares, A., Parrish, C.C., Taylor, R.G., Rise, M.L., 2017. Transcriptome profiling of antiviral immune and dietary fatty acid dependent responses of Atlantic salmon macrophage-like cells. BMC Genomics, 18(1), p.706; see Co-authorship Statement on page XXIV.

## 5.1. Abstract

Due to the limited availability and high cost of fish oil, and the global expansion of aquaculture production, the use of fish oil in aquafeeds must be reduced without compromising fish health. Therefore, the present study was conducted to determine whether different levels of vegetable and fish oils alter the antiviral response of salmon macrophage-like cells (MLCs). Atlantic salmon (*Salmo salar*) were fed diets containing 7.4% (FO7) or 5.1% (FO5) fish oil. Vegetable oil (i.e., rapeseed oil) was used to replace fish oil in experimental diets. These diets were designed to be relatively low in EPA+DHA (i.e., FO7: 1.41% and FO5: 1%), but near the requirement level, and resulted in comparable growth. After a 16-week feeding trial, MLCs isolated from fish in these dietary groups were stimulated by a viral mimic (dsRNA: pIC), and sampled at 6 h (for qPCR analyses) and at 24 h (for both microarray and qPCR analyses). In addition, head kidney leukocytes were isolated to determine the fatty acid composition of the cells.

The fatty acid composition of head kidney leukocytes varied between the two dietary groups (e.g., 20:5n-3 was higher in the FO7 group). Following microarray assays using the 44K salmonid platform, Rank Products (RP) analysis showed that there were 14 and 54 differentially expressed probes (DEP) (PFP<0.05) between the two diets in the control and pIC groups (FO5 vs. FO7), respectively. Nonetheless, Significance Analysis of Microarrays (SAM, FDR<0.05) identified only one (i.e., *transmembrane protein 115-like*) DEP between the pIC groups of the two diets. This study, however, identified a large number (i.e., 890 DEP in FO7 and 1128 DEP in FO5 that overlapped between SAM and RP) of pIC-responsive transcripts, and several of them were involved in TLR-/RLR-

dependent and cytokine-mediated pathways. Although only 2 out of 9 RP-selected diet-responsive transcripts were validated by qPCR as differentially expressed, all of the 35 selected pIC-responsive transcripts were qPCR-validated.

*Fatty acid-binding protein adipocyte* (*fabp4*) and *proteasome subunit beta type-8* (*psmb8*) were significantly up- and down-regulated, respectively, in the MLCs of fish fed the FO5 diet, suggesting that they are important diet-responsive, immune-related, biomarkers for future studies. Although the different levels of dietary fish and vegetable oil involved in this study affected the expression of some transcripts, the MLC immune-related pathways and functions activated by pIC were comparable in both groups. Moreover, the qPCR data reveal transcripts that responded early to pIC (e.g., *lgp2*, *map3k8*, *socs1*, *dusp5* and *cflar*) and others that were time-responsive (e.g., *scarb1-a*, *csf1r*, *traf5a*, *cd80* and *ctsf*) in salmon MLCs. The present study provides a comprehensive picture of the putative molecular pathways (e.g., RLR-, TLR-, MAPK- and IFN-associated pathways) activated by the antiviral response of salmon MLCs.

## 5.2. Introduction

The nutritional modulation of fish innate immune responses by different diets (e.g., proteins and amino acids, lipids and fatty acids, carbohydrates, vitamins and minerals) has been well-documented (Kiron, 2012; Trichet, 2010). Fatty acids, notably polyunsaturated fatty acids (PUFAs), may suppress or stimulate innate immune responses (e.g., antigen presentation or phagocytosis) of various immune cells (e.g., T-cells, B-cells, natural killer cells and macrophages) (Galli and Calder, 2009; Pablo and Cienfuegos, 2000). For example, dietary omega (n)-3, n-6 or n-9 fatty acids can alter (i.e., increase or decrease) the production of ILs (Interleukins) and TNFs (Tumour necrosis factor) as well as the activity (e.g., phagocytosis) and proliferation of leukocytes (e.g., T-cells and macrophages) (Calder, 2008; Galli and Calder, 2009; Martínez-Micaelo et al., 2016; Pablo and Cienfuegos, 2000). Indeed, the inhibitory roles and anti-inflammatory functions of n-3 fatty acids [e.g., eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3)] are mediated by the suppression of cytokines (e.g., IL1B and IL6) and by activating anti-inflammatory factors (Calder, 2015). Importantly, several studies have reported the EPA- and DHA-dependent suppression of pathogen-associated molecular pattern (PAMP)-induced responses via NFKB (Nuclear factor kappa-B) signalling in mammalian macrophages (Williams-Bey et al., 2014; Xue et al., 2012). On the other hand, n-6-derived eicosanoids play pro-inflammatory roles in the immune responses (Patterson et al., 2012). Hence, optimal levels of n-3/n-6 fatty acids contribute to a robust and effective immune response.

Similar to other vertebrates, fishes must acquire essential polyunsaturated fatty acids (e.g., linoleic acid, linolenic acid, EPA and DHA) from the diet (Kiron, 2012). Aquaculture production has been steadily growing (Shepherd and Jackson, 2013). However, over-fishing and the dramatic collapse of many marine fish stocks have led to a limited supply of the marine ingredients that provide the required long chain n-3 fatty acids in aquafeeds (Tacon and Metian, 2008; Tocher, 2015). Hence, there are concerted efforts to replace fish oils with vegetable oils in fish diets. Unfortunately, diets containing high levels of vegetable oils may have low levels of some essential n-3 fatty acids (e.g., EPA and DHA) and an unbalanced n-6/n-3 ratio. In mammals, different ratios of dietary n-6/n-3 have been shown to change the fatty acid composition of the plasma, as well as immune function and macrophage activation (e.g., IL6 and TNF expression) (Turek et al., 1998; Watkins et al., 2000), and it can be attributed to their influence on eicosanoid biosynthesis. Further, the consumption of vegetable oil-rich diets can cause variations in fish immunological responses and resistance to pathogens in a species- and lipid source-dependent manner (Fracalossi and Lovell, 1994; Kiron, 2012). For example, sea bream (*Sparus aurata*) fed soybean and linseed oil containing diets had reduced leukocyte phagocytic activity and increased expression of the *mx* gene (i.e., myxovirus resistance, interferon-inducible gene) in response to viral mimic stimulation (Montero et al., 2008). Higher levels of vegetable oil in the diet have been reported to up-regulate the expression of *tlr3* (*toll-like receptor 3*) and *tlr7* in the head kidney of polyriboinosinic polyribocytidylic acid (pIC)-injected Atlantic salmon (*Salmo salar*) (Caballero-Solares et al., 2017). On the other hand, Booman et al. (2014) reported that camelina oil-containing

diets (i.e., replacement of 40 or 80% of fish oil with camelina oil) did not change the antiviral immune response of Atlantic cod (*Gadus morhua*) at the transcriptome level. It seems that immunomodulatory effects of dietary vegetable oils are dependent on studied species, vegetable oil sources and levels of oils in test diets, and further investigations are required to have a better understanding of the influence of dietary vegetable oils on immune responses of teleosts.

The production of Atlantic salmon, one of the most economically important aquaculture finfish species, is increasing worldwide (FAO, 2010). The largest proportion of fish oil used in the global aquaculture industry is consumed by farmed Atlantic salmon (Tacon and Metian, 2008; Tocher, 2015), but this usage (e.g., forage fish equivalents needed to produce a unit of salmon) has been declining over the last two decades (Tacon and Metian, 2008). Still, there is a need to further decrease the level of fish oil in salmon aquafeeds, and to determine if the immune function (e.g., antiviral response) of salmon is influenced by lower EPA+DHA intake. Previous studies have demonstrated that the replacement of fish oil with soybean or rapeseed oils does not change the susceptibility of Atlantic salmon to bacterial (*Aeromonas salmonicida*) infection, the phagocytic activity of macrophages, or cytokine (TNF and IL1B) expression of lipopolysaccharide (LPS)-stimulated head kidney leukocytes (HKLs) in this species (Gjøen et al., 2004; Seierstad et al., 2009). Since the Atlantic salmon is susceptible to several viral pathogens [e.g., infectious salmon anaemia virus (ISAV)], it is of paramount importance to determine if replacement of fish oil with vegetable oil in the diet will alter the salmon antiviral response. To address this issue, two diets (FO7: 7.4% fish oil and FO5: 5.1% fish oil)

which were relatively low in EPA+DHA [1.4% and 1% of the diet; 4.74% and 3.57% of the fatty acids, respectively] were fed to salmon for 16 weeks. These diets contained lower EPA+DHA levels as compared to a previous study (Seierstad et al., 2009), and were close to the EPA+DHA requirement level (4.4% of fatty acids) of Atlantic salmon (Rosenlund et al., 2016). Rapeseed oil contains n-3 fatty acids (~7%) and high levels of monounsaturated fatty acids (~63% MUFA) that increase its resistance to oxidation and provide the required energy for fish (Bell et al., 2001; Miller et al., 2008; Turchini and Mailer, 2011). Therefore, this was used as the vegetable oil source as it is one of the most suitable candidates for fish oil substitution in Atlantic salmon feed.

Macrophages play key roles in the innate immune responses of fish through pathogen recognition, cytokine production and phagocytosis (Hodgkinson et al., 2015), and their functions can be greatly affected by dietary fatty acids (Calder, 2008). Microarray analyses may be used to assess global gene expression changes associated with immunological responses (Hyatt et al., 2006), and yield a comprehensive picture of molecular pathways activated by an immune stimulus. Microarray analyses were previously employed to profile the transcriptome responses of salmon macrophage-like cells to ISAV infection (Schiøtz et al., 2008; Workenhe et al., 2009). The present study characterises the transcriptome and functional response of Atlantic salmon macrophage-like cells to a viral mimic, and the immunomodulatory effect of low dietary EPA+DHA on these cells, using microarrays, real-time quantitative polymerase chain reaction (qPCR), fatty acid analysis and cellular assays [i.e., phagocytosis and respiratory burst (RB)].

## 5.3. Materials and methods

### *5.3.1. Fish and experimental diets*

Two diets (5 mm pellets) with different levels of fish oil (i.e., FO7: 7.4% and FO5: 5.1% of the diet), and therefore different levels of DHA and EPA (i.e., FO7: 1.41% and FO5: 1.00% of the diet), were formulated and produced by EWOS [EWOS Innovation (now Cargill Innovation Center), Dirdal, Norway] for use in this study (Table 5.1.). These diets were designed to provide an optimal growth for Atlantic salmon. This is because the data of Ruyter et al. (2000) showed that the salmon growth is maximised around 1.25% EPA+DHA as a proportion of the diet, which is near the middle of the formulated range of the diets used herein. When measured, the proportion of EPA+DHA in diet FO5 was 3.57% of total fatty acids, and in diet FO7 it was 4.74%, which represents an increase of a third. Growth data in two long-term trials by Rosenlund et al. (2016) suggest Atlantic salmon require dietary EPA+DHA at 2.7 to 4.4% of total fatty acids. Fatty acid proportions of the diets used in the present study are on both sides of the 4.4% value. The low EPA+DHA diets used in the current experiment were associated with comparable growth performance of salmon and may be regarded as practical diets for salmon farming.

Atlantic salmon smolts were transported from a local farm and held at the JBARB (Dr. Joe Brown Aquatic Research Building, Ocean Sciences Centre, MUN) in a 3,000 l tank for four months, using a flow-through seawater system. Two weeks prior to the beginning of the experiment, fish were PIT (passive integrated transponder) tagged and

**Table 5.1. The composition of experimental diets.**

| Ingredients | omega-3LC1.4 (FO7) % | omega-3LC1 (FO5) % |
|---|---|---|
| Fish meal | 5 | 5 |
| Animal byproduct | 21.6 | 21.4 |
| Vegetable protein | 33.3 | 33.8 |
| Fish oil | 7.4 | 5.1 |
| Vegetable oil | 19.9 | 22.1 |
| Binder | 10.4 | 10.4 |
| Premix | 2.4 | 2.4 |
| EPA+DHA content | 1.41 | 1 |
| **Fatty acids %[1]** | | |
| 14:0 | 1.96 ± 0.011 | 1.42 ± 0.011 |
| 16:0 | 7.83 ± 0.034 | 7.34 ± 0.030 |
| 16:1n-7 | 1.92 ± 0.006 | 1.52 ± 0.006 |
| 18:0 | 2.30 ± 0.021 | 4.38 ± 0.059 |
| 18:1n-7 | 2.40 ± 0.059 | 2.57 ± 0.015 |
| 18:1n-9 | 41.25 ± 0.067 | 43.41 ± 0.147 |
| 18:2n-6 | 15.74 ± 0.036 | 16.56 ± 0.038 |
| 18:3n-3 | 6.34 ± 0.024 | 6.69 ± 0.016 |
| 20:1n-9 | 4.29 ± 0.014 | 3.41 ± 0.021 |
| 20:5n-3 | 2.47 ± 0.012 | 1.82 ± 0.015 |
| 22:1n-11(13) | 4.57 ± 0.021 | 2.88 ± 0.322 |
| 22:1n-9 | 0.73 ± 0.005 | 1.00 ± 0.288 |
| 22:6n-3 | 2.27 ± 0.018 | 1.75 ± 0.035 |

[1] Data (means ± SE) expressed as a percentage of identified FAME (fatty acid methyl esters) on an as-fed basis. Only fatty acids present at ≥ 1.00% of total are shown.

then randomly distributed into eight 500 l tanks (40 fish per tank, 4 replicate tanks per dietary group). The fish [initial weight ($n$ = 160; mean ± SE): FO7, 178.64 ± 2.2 g; FO5, 179.28 ± 2.39 g] were fed to satiation for 16 weeks using the experimental diets twice a day; water temperature was ~12°C and photoperiod was 12-h light: 12-h dark. Fish fork length and weight were measured at the beginning and the end of the 14-week feeding trial, and the fish were held for additional 2 weeks under the experimental conditions before cell isolation and sampling. Water quality parameters (e.g., temperature and oxygen saturation) were checked daily during the feeding trial. Fish were feed deprived for 24 h before any handling or sampling.

All procedures (Protocol number: 14-71-MR) in the current study were approved by MUN's Institutional Animal Care Committee, according to the guidelines of the Canadian Council on Animal Care.

### *5.3.2. Macrophage-like cell isolation*

Atlantic salmon anterior (head) kidney cells were isolated as in previous studies on salmon macrophages (Bridle et al., 2005; Fast et al., 2008; Rise et al., 2004) with some modifications. Briefly, Atlantic salmon were euthanized with an overdose of MS222 (400 mg $l^{-1}$; Syndel Laboratories, Vancouver, BC, Canada). After dissection, the head kidney was removed and transferred into Leibovitz-15+ (L-15+; Gibco, Carlsbad, CA, USA) medium supplemented with 2% fetal bovine serum (FBS; Gibco), 10 U $ml^{-1}$ of heparin (Sigma-Aldrich, St. Louis, MO, USA) and 100 U $ml^{-1}$ of penicillin and 100 µg $ml^{-1}$ of streptomycin (Gibco). Head kidney samples were then minced using 100 µm

nylon cell strainers (Thermo Fisher Scientific, Waltham, MA, USA), and the resulting cell suspension was washed and pelleted by centrifugation at 400 × g for 5 min at 4˚C. The cell suspension was centrifuged on a discontinuous 34/51% Percoll gradient (GE Healthcare, Uppsala, Sweden) at 400 × g for 30 min at 4˚C, and the interface enriched in monocyte/macrophage-like cells was collected. The cells were washed twice (400 × g for 5 min at 4˚C) and suspended in L-15+ with 2% FBS and without heparin.

The cells were counted using a hemocytometer and then seeded into 6-well plates (Corning™, Corning, NY, USA) at a density of $3 \times 10^7$ viable cells (in 2 ml L-15+) per well. The cell viability was above 96% as determined by a trypan blue (Sigma-Aldrich) exclusion method. The cells were cultured overnight (~16 h) at 15˚C, and the non-adherent cells were removed by washing the plates 3 times with L-15+. The cells were then cultured in L-15+ containing 5% FBS at 15˚C. Monocyte/macrophage-like cells are henceforth referred to as macrophage-like cells (MLCs). Since the cultured cells in this chapter included a mixture of monocytes and macrophages and showed a low phagocytic activity (see Section 5.4.2), they were described as MLCs.

### *5.3.3. Sampling and stimulation of MLCs in dietary groups for gene expression analyses*

Two fish per replicate tank in each dietary group were used for cell isolation, pIC stimulation and gene expression analyses (i.e., 8 biological replicates per group). MLCs of each fish were isolated as previously described in Section 5.3.2., and the cells were seeded in 6-well plates (i.e., $3 \times 10^7$ cells per well). A stock solution of pIC [Sigma-

Aldrich; 10 mg $ml^{-1}$ in phosphate-buffered saline (PBS)] was prepared. Starting 24 h after seeding, MLCs isolated from each fish were exposed to PBS (control) or 10 µg $ml^{-1}$ pIC (i.e., 1 µl of the stock solution per ml of L-15+) at 15˚C. Samples from each individual were lysed by pipetting using 800 µl of TRIzol (Invitrogen, Burlington, Ontario, Canada) at 6 ($n$ = 6) and 24 ($n$ = 8) h post-stimulation (HPS). Since the number of cells isolated from 2 individuals (i.e., 2 out of 8 fish) in each dietary group was not enough for seeding 4 culture wells, the pIC- and PBS-treated cells from these individuals were only sampled at 24 HPS. The collected samples were kept at -80˚C until RNA extraction and analyses. An overview of the experimental design is illustrated in Fig. 5.1.

Based upon a pilot study described in the Section 5.3.11., 24 HPS was used as the main time point for microarray and qPCR analyses, and 6 HPS samples were collected to assess the early pIC response of a subset of microarray-identified transcripts selected for qPCR validation.

### *5.3.4. Sampling of MLCs for cellular activity analyses*

In addition, MLCs were isolated from 11 fish fed the FO7 diet and 9 fish fed the FO5 diet (from 4 tanks in FO7 and 3 tanks in FO5; one week after cell isolation in Section 5.3.3.). One of the FO5 replicate tanks was excluded from sampling since fish in that tank were exposed to hypoxia stress after the first sampling (i.e., gene expression sampling; see Section 5.3.2.). The isolated cells were seeded in 6-well plates at an equal density of $10^7$ viable cells per well.

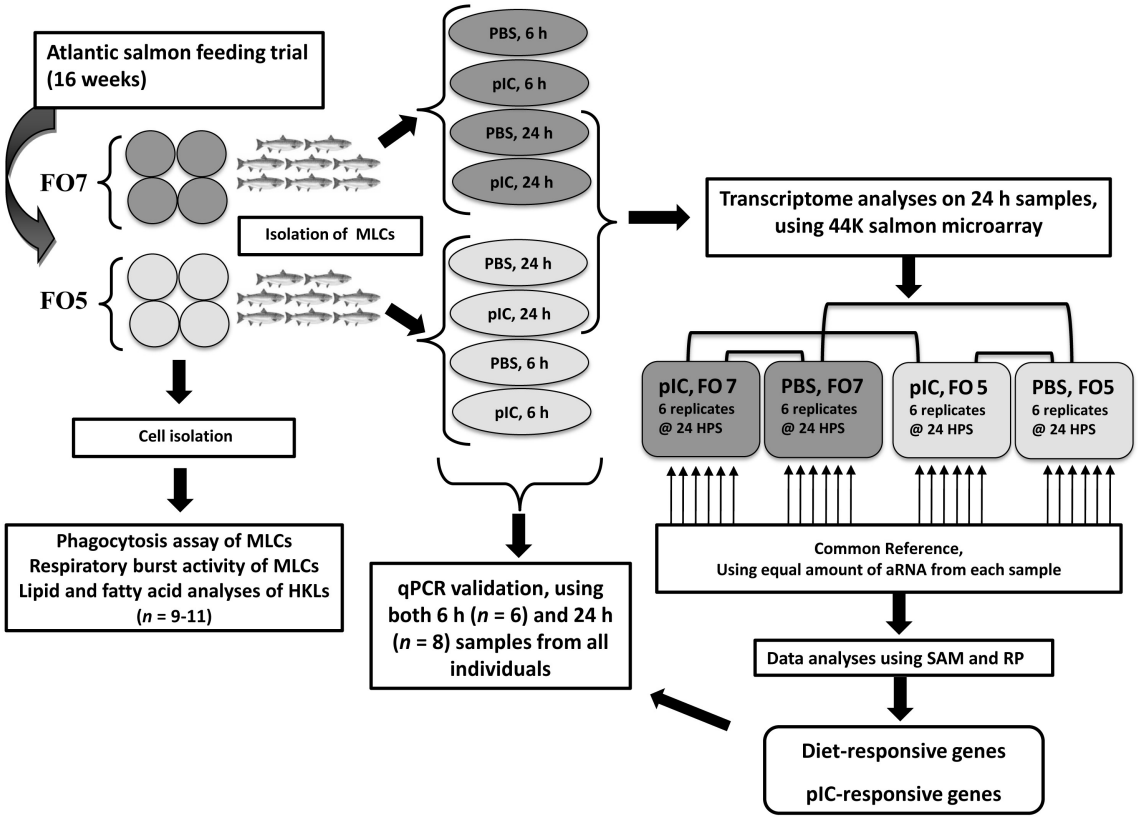



**Figure 5.1. Overview of experimental design.** Following a 16-week feeding trial, macrophage-like cells (MLCs) were isolated from 8 Atlantic salmon in each dietary group, and the isolated cells from each individual were exposed to PBS or 10 µg $ml^{-1}$ pIC for 6 h or 24 h. The diet- and pIC-responsive transcripts were determined by the means of microarray analyses on 24 h time point samples. Subsets of microarray-identified transcripts were subjected to qPCR assays, using all samples at both time points. Phagocytosis and respiratory burst assays were conducted on MLCs, whereas the lipid and fatty acid analyses were performed on head kidney leucocytes (HKLs).

#### *5.3.4.1. Phagocytosis assay*

Starting 24 h after seeding, MLCs were washed once in culture medium, and 1 µm Fluoresbrite YG (yellow-green) microspheres (Polysciences, Warrington, PA) were added at a ratio of 1:30 (cell: microsphere) (Øverland et al., 2010). Twenty-four hours after microsphere exposure and culturing at 15˚C, MLCs were rinsed with culture medium and de-adhered using 500 µl of trypsin-EDTA (0.25%; Thermo Fisher Scientific, Waltham, MA). Thereafter, the trypsinized MLCs were diluted in 5 ml of culture medium, centrifuged (5 min at 500 × g) at 4˚C and re-suspended in 500 µl of fluorescence-activated cell sorting (FACS) buffer (PBS + 1% FBS). Then, propidium iodide (PI) was added to the samples to determine the dead cells. Fluorescence was detected and analysed from 10,000 cells using a BD FACS Aria II flow cytometer and BD FACS Diva v7.0 software (BD Biosciences, San Jose, CA). The percentage of cells that phagocytized beads, as well as the number of beads phagocytized per cell, were determined as FITC positive events. Cell death was assessed as PI-positive events, and the dead cells were excluded from analyses.

#### *5.3.4.2. Respiratory burst (RB) assay*

MLCs were rinsed once with culture medium and then incubated in 500 µl of respiratory burst assay buffer (L-15 media + 1% BSA + 1 mM $CaCl_2$) for 15 min. One microlitre of dihydrorhodamine 123 (DHR) (5 mg $ml^{-1}$) was diluted in 1 ml of PBS and used as a stock solution; then, 50 µl of the solution was added to the cells for 15 min. DHR is a non-fluorescent dye that becomes fluorescent rhodamine under reactive oxygen

conditions. Following DHR addition, 200 μM of phorbol myristate acetate (PMA), or PBS as a negative control, was added to MLCs for 45 min to stimulate reactive oxygen species (ROS) production (Kalgraff et al., 2011). Afterward, MLCs were removed from the plates using trypsin-EDTA, and re-suspended in FACS buffer (PBS + 1% FBS) as described in Section 5.3.4.1. Fluorescence detection and analyses were performed using 10,000 cells, a BD FACS Aria II flow cytometer and BD FACS Diva v7.0 software (BD Biosciences). The negative control cells were used to set the baseline for non-ROS producing cells. The percentage of MLCs that produced ROS was determined as cells with rhodamine fluorescence levels greater than the negative control, and PI positive cells were excluded from analyses.

### *5.3.5. Sampling for fatty acid analyses*

HKLs were sampled from 4 replicate FO7 tanks (11 individuals) and 3 replicate FO5 tanks (10 individuals), as explained in Section 5.3.2. After Percoll gradient centrifugation (see Section 5.3.2.), the interface was taken and pelleted by centrifugation at 400 × g for 5 min at 4˚C. The pelleted cells were re-suspended in PBS, and washed twice in a lipid-cleaned glass tube by centrifugation at 400 × g for 5 min at 4˚C. The resulting HKLs, enriched in monocyte/macrophage-like cells, were covered with 3 ml of chloroform (HPLC-grade), and the headspace of each tube was filled with nitrogen. Thereafter, the tubes were capped tightly, sealed using Teflon tape and stored at -20˚C until lipid extraction.

### *5.3.6. Lipid and fatty acid analyses*

Lipids were extracted from samples based on Parrish (1999). Lipid class composition of the samples was determined using an Iatroscan Mark VI TLC–FID (Mitsubishi Kagaku Iatron, Inc., Tokyo, Japan) (Parrish, 1987). The fatty acid profile of the samples was measured after fatty acid methyl ester (FAME) derivatization as previously described by Hixson et al. (2013). Also, similar reagents and equipment to Hixson et al. (2013) were used for lipid and fatty acid analyses.

The lipid class and fatty acid data were analysed using SPSS v16.0.0 (Armonk, North Castle, NY). Firstly, the normality of the data were assessed using the Kolmogorov-Smirnov normality test. Differences between lipid classes and fatty acid contents of salmon HKLs in two dietary groups were then analysed using an unpaired t-test ($p \leq 0.05$).

### *5.3.7. RNA extraction and purification*

Total RNA was extracted from the TRIzol-lysed samples following the manufacturer's instructions. To degrade any residual genomic DNA, total RNA samples were treated with 6.8 Kunitz units of DNase I (RNase-Free DNase Set, Qiagen, Mississauga, Ontario, Canada) with the manufacturer's buffer (1X final concentration) at room temperature for 10 min. DNase-treated RNA samples were column-purified using the RNeasy MinElute Cleanup Kit (Qiagen) following the manufacturer's instructions. RNA integrity was verified by 1% agarose gel electrophoresis, and RNA purity was

assessed by A260/280 and A260/230 NanoDrop UV spectrophotometry. Column-purified RNA samples had A260/280 and A260/230 ratios above 1.8.

### *5.3.8. Microarray experimental design and hybridization*

MLCs, isolated from 6 individuals (i.e., samples from three replicate tanks) in each dietary group, and stimulated with pIC or PBS for 24 h, were subjected to microarray analyses [i.e., 12 samples from each dietary group (6 pIC and 6 PBS), 24 samples in total; see Fig. 5.1]. The microarray experiment was designed and performed according to the MIAME guidelines (Brazma et al., 2001). These analyses were carried out using the consortium for Genomic Research on All Salmonids Project (cGRASP)-designed Agilent 44K salmonid oligonucleotide microarray (Jantzen et al., 2011) as described in Xue et al. (2015). Briefly, anti-sense amplified RNA (aRNA) was *in vitro* transcribed from 800 ng of each individual sample RNA (DNase-treated and column-purified) using the Amino Allyl MessageAmp™ II aRNA Amplification Kit (Ambion, Carlsbad, CA, USA) following the manufacturer's instructions. The quality and quantity of the aRNAs were checked by agarose gel electrophoresis and NanoDrop spectrophotometry. Amplified RNA from all 24 samples (i.e., 10 µg from each sample) was pooled and used as a common reference in this experiment. Twenty micrograms of aRNA (i.e., experimental samples or common reference) were precipitated, using standard ethanol precipitation methodology, and re-suspended in coupling buffer. Thereafter, the experimental samples were labelled with Cy5 (GE Healthcare Life Sciences, Buckinghamshire, UK), and the common reference was labelled with Cy3 (GE Healthcare Life Sciences), following the manufacturer's instructions. The efficiency of

labelling and aRNA concentration were assessed using spectrophotometry (i.e., microarray feature in NanoDrop). The labeled aRNA (i.e., 825 ng) from each experimental sample was mixed with an equal amount of labelled aRNA from the common reference, and the resulting pool was fragmented following the manufacturer's instructions (Agilent, Mississauga, ON). Each labelled aRNA pool (i.e., an individual sample and common reference) was co-hybridized to a 44K microarray at 65°C for 17 h with rotation (10 rpm) using an Agilent hybridization oven.

### *5.3.9. Microarray data acquisition and analysis*

The microarray slides were scanned at 5 μm resolution with 90% of laser power using a ScanArray Gx Plus scanner and ScanExpress v4.0 software (Perkin Elmer, Waltham, Massachusetts, USA), and the Cy3 and Cy5 channel photomultiplier tube (PMT) settings were adjusted to balance the fluorescence signal. The raw data were saved as TIFF images, and the signal intensity data were extracted using Imagene 9.0 (BioDiscovery, El Segundo, California, USA). Using R and the Bioconductor package marray, the low-quality or flagged spots on the microarray were discarded from datasets, followed by $\log_2$-transformation and Loess-normalization of data (Booman et al., 2014). Thereafter, the probes with absent values in more than 25% of all 24 arrays were omitted from the dataset, and the missing values were imputed using the EM_array method and the LSimpute package (Bø et al., 2004; Booman et al., 2014; Celton et al., 2010). The final dataset that was used for statistical analyses consisted of 12,983 probes for all arrays (GEO accession number: GSE93773).

The differentially expressed probes (DEP) between different treatments were determined using Significance Analysis of Microarrays (SAM) (Tusher et al., 2001) and Rank Products (RP) (Brown et al., 2016; Hong et al., 2006). The Excel add-in SAM package (Stanford University, CA) and two-class comparison analysis with a false discovery rate (FDR) cutoff of 0.05 were used to identify the diet-responsive transcripts between groups (i.e., FO7, PBS vs. FO5, PBS and FO7, pIC vs. FO5, pIC) and pIC-responsive transcripts within groups (e.g., FO7, PBS vs. FO7, pIC). The diet- and pIC-responsive transcripts were also found using RP analysis at a percentage of false-positives (PFP) threshold of 0.05, as implemented by the Bioconductor package of R. The resulting significant transcript lists were re-annotated using the contigs or singletons (Jantzen et al., 2011) that were used for designing the given informative 60mer oligonucleotide probes on the array. The BLASTx searches of NCBI's non-redundant (nr) amino acid sequence database (E-value < 1e-05) were carried out using Blast2GO software (BioBam Bioinformatics S.L., Valencia, Spain) (Conesa et al., 2005; Götz et al., 2008).

SAM and RP apply distinct approaches to detect DEP in microarray experiments (Hong et al., 2006; Tusher et al., 2001), and the overlap of transcripts identified by both techniques represent very high-trust gene lists (i.e., few false positives) as demonstrated by Brown et al. (2016). Therefore, the microarray-identified pIC-responsive probes that overlapped between the SAM and RP analyses in each group were subjected to further functional analyses [i.e., gene ontology (GO) analysis and the Fisher's exact test]. The resulting BLASTx hits were mapped to GO terms of pIC-responsive transcripts in each

dietary group (GO Biological Process level 2). GO enrichment analysis was performed (Fisher's exact test, FDR cutoff of 0.05) using Blast2GO software. The Ancestor Chart feature of QuickGO (http://www.ebi.ac.uk/QuickGO) was used to categorise and select a subset of enriched GO terms related to immunity. The Pearson correlation and complete linkage clustering function in the Genesis software (Rockville, Maryland, USA) (Sturn et al., 2002) were employed for the hierarchical clustering of median-centred data of DEP as described in Booman et al. (2014).

### *5.3.10. qPCR validation*

Transcript levels of a subset of genes identified as differentially expressed in the microarray analyses were validated using qPCR. These genes included a subset of diet-responsive up- or down-regulated transcripts identified by RP analysis. Additionally, pIC-responsive transcripts (e.g., up- and down-regulated) that are involved in different molecular functions (e.g., pathogen recognition, signal transduction, transcription factors and immune effectors) and immune pathways [e.g., IFN (Interferon) and TLR] were selected for qPCR validation (Table 5.2.). These transcripts were mainly selected from pIC-responsive transcripts in both dietary groups, and that overlapped between the SAM and RP analyses. This study assessed the expression of two transcripts (*tlr3* and *tlr7*) that play important roles in dsRNA signalling pathways but were absent from the microarray platform. In addition, *mx-b* was included in the qPCR analyses since this transcript previously showed a differential expression in response to dietary rapeseed oil in head kidney of pIC-stimulated salmon (Caballero-Solares et al., 2017). Transcript levels of

these genes of interest (GOIs) were measured in all of the samples (i.e., both PBS- and pIC-treated) from each dietary group collected at both 6 ($n$ = 6) and 24 ($n$ = 8) HPS.

First-strand cDNA templates for qPCR were synthesized in 20 µl reactions from 800 ng of DNaseI-treated, column-purified total RNA using random primers (250 ng; Invitrogen) and M-MLV reverse transcriptase (200 U; Invitrogen) with the manufacturer's first-strand buffer (1X final concentration) and DTT (10 mM final concentration) at 37°C for 50 min.

All PCR amplifications were performed in 13 µl reactions using 1X Power SYBR Green PCR Master Mix (Applied Biosystems, Burlington, Ontario, Canada), 50 nM of both the forward and reverse primers, and the indicated cDNA quantity (see below). Amplifications were performed using the ViiA 7 Real-Time PCR system (384-well format) (Applied Biosystems); the real-time analysis program consisted of 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min, with fluorescence detection at the end of each 60°C step.

The qPCR assays used in the current study were designed and performed following MIQE guidelines (Bustin et al., 2009). Primers used in this study were designed using Primer3web v4.0.0 (http://primer3.wi.mit.edu) (Table 5.2.). The performance and amplification efficiencies of all primer pairs were tested prior to use in the experimental studies. Briefly, for diet-responsive transcripts, they were assessed using a cDNA template generated from a pool of 8 individuals from pIC- and PBS-stimulated samples at 24 HPS from both dietary groups; for pIC-responsive up-regulated transcripts,

they were assessed using a cDNA template generated from a pool of 6 individuals from pIC-stimulated samples at 24 HPS from both dietary groups; for pIC-responsive down-regulated transcripts, they were assessed using a cDNA template generated from a pool of 6 individuals from PBS-stimulated samples at 24 HPS from both dietary groups. The standard curves for all primer pairs (i.e., GOIs and candidate normalizers) were generated using a 5-point, 3-fold serial dilution of the given cDNA template (starting with cDNA representing 10 ng of input total RNA) as well as a no-template control. The primer quality tests were performed in triplicate. Only primer pairs generating an amplicon with a single melting peak, no primer-dimer present in the no-template control and an amplification efficiency (Pfaffl, 2001) between 80-110% were used for qPCR analyses (Table 5.2.).

Transcript levels of the GOIs were normalized to transcript levels of two endogenous control genes. To select these endogenous controls, qPCR primers pairs were designed for seven candidate normalizers, [i.e., *actb* (*beta-actin*), *rpl32* (*60S ribosomal protein 32*), *ef1a1* (*elongation factor 1 alpha-1*), *pabpc1* (*polyadenylate-binding protein cytoplasmic 1*), *eif3d* (*eukaryotic translation initiation factor 3 subunit D*), *tubg1* (*tubulin gamma-1*) and *ntf2* (*nuclear transport factor 2*)], and quality tested as described above. Thereafter, the fluorescence threshold cycle ($C_T$) values of 50% of the experimental samples (including PBS- and pIC-treated samples at both 6 and 24 HPS from both dietary groups) were measured in duplicate for each of these transcripts using cDNA representing 3.2 ng of input total RNA, and then analysed using geNorm in the qBase software (Vandesompele et al., 2002). Two transcripts, *eif3d* and *rpl32*, were expressed

comparably (i.e., with lowest M-values; a measure of transcript expression stability) in all samples tested, and thus, were selected as the normalizers for the experimental qPCR assays.

When primer quality testing and normalizer selection were completed, qPCR analyses of transcript (mRNA) expression levels of the GOIs were performed. In all cases, cDNA representing 3.2 ng of input RNA was used as template in the PCR reactions. On each plate, for every sample, the GOIs and endogenous controls were tested in triplicate, and a plate linker sample (i.e., a sample that was run on all plates in a given study) and a no-template control were included. The relative quantity (RQ) of each transcript was determined using the ViiA 7 Software Relative Quantification Study Application (Version 1.2.3) (Applied Biosystems), with normalization to both *eif3d* and *rpl32* transcript levels, and with amplification efficiencies incorporated. For each GOI, the sample with the lowest normalized expression (mRNA) level was set as the calibrator sample (i.e., assigned an RQ value = 1).

RQ values of each transcript of interest were subjected to statistical analyses. Prior to analyses, the normality of the data were checked using the Kolmogorov-Smirnov normality test. A two-way ANOVA (i.e., dietary groups and sampling times as factors) was then used to analyse qPCR results between dietary groups (e.g., FO7, PBS vs. FO5, PBS), whereas the significant differences within each dietary group (between pIC and PBS) were assessed using a repeated measures two-way ANOVA (i.e., stimulation groups and sampling times as factors). These analyses were followed by Sidak multiple

comparison *post-hoc* tests to determine significant differences ($p \leq 0.05$) in the time- and treatment-matched results between dietary groups as well as the significant differences in time-matched pIC or PBS groups within each dietary group and within pIC and PBS groups at different time points. All data analyses of qPCR results in the current study were conducted in the Prism package v6.0 (GraphPad Software Inc., La Jolla, CA, USA).

### *5.3.11. Determination of time-dependent response of salmon MLCs to pIC*

Prior to the diet-related experiment and to determine the time-dependent response to pIC, salmon MLCs were isolated from 4 individuals, weighing 1.78 ± 0.09 kg, as described in Section 5.3.2. The resulting cells were seeded into 35 mm (i.e., similar size to one well of a 6-well plate) culture dishes (Corning™) at an equal density of $3 \times 10^7$ viable cells per dish. MLCs from each individual were incorporated into all groups and sampling points. After 24 h of culture, MLCs were treated with PBS or 10 μg $ml^{-1}$ pIC (Sigma-Aldrich) [stimulative dose of pIC for salmon MLCs (Berg et al., 2009)]; then, the samples were collected at 3, 6, 12, 24 and 48 HPS by removing the medium and adding 800 μl of TRIzol (Invitrogen). RNAs were extracted as described previously. The expression of selected biomarker genes [i.e., *gig1*, *mx*, *viperin* and *lgp2* (*RNA helicase lgp2*)] involved in the antiviral immune response was assessed by qPCR (see Section 5.3.10.).

Expression levels of all of the assayed antiviral biomarker transcripts were significantly up-regulated by pIC at 12 HPS, peaked at 24 HPS and were significantly lower within the pIC group at 48 HPS compared to 24 HPS (data not shown). Since the

peak of pIC response in salmon macrophages occurred at 24 HPS, this time point was chosen for the global gene expression analyses of pIC-stimulated MLCs in the diet-related experiment. Additionally, there were non-significant increases in expression of *gig1*, *mx* and *viperin* and a significant up-regulation of *lgp2* in response to pIC at 6 HPS (data not shown); therefore, since the early pIC response in salmon MLCs occurred at 6 HPS, this time point was included in the qPCR studies.

## Table 5.2. Primers used in qPCR studies.

| Gene name | GenBank accession number | | Primer sequence 5' to 3' | $R^2$ | Amplification efficiency (%) | Amplicon size (bp) |
|---|---|---|---|---|---|---|
| **Diet-responsive transcripts** | | | | | | |
| *Fc receptor-like protein 2* (*fcr2*) | DY734226 | F: | TCTTCATGCACCGTGTCACT | 0.993 | 91.2 | 148 |
| | | R | CTCCCTCAGTCACAGGAAGG | | | |
| *fatty acid-binding protein, adipocyte* (*fabp4*) | NM_001141203 | F: | GACTTGGGACGGCAAGACTA | 0.992 | 97.9 | 128 |
| | | R: | CAGCAGACTGGAATCACACC | | | |
| *FAD-linked sulfhydryl oxidase ALR-like* (*fadox*) | GE791133 | F: | TTAGGGCGTAACACCTGGTC | 0.998 | 102.3 | 144 |
| | | R: | CAGGTCCTCAGCACACTCCT | | | |
| *legumain-like* (*lgmn*) | EG917238 | F: | AGTCCGCTCCTCAAACTCAA | 0.997 | 100.8 | 184 |
| | | R: | TGCTGTACCTGGTTTTGCTG | | | |
| *lathosterol oxidase* (*sc5d*) | NM_001140116 | F: | TGGCGACAACACTCACAGAT | 0.992 | 103.9 | 172 |
| | | R: | TGAGCAAGGCACTCAACTCA | | | |
| *MHC-I* | AF504022 | F: | CATGAAGATGTGGAGCATGG | 0.982 | 101.0 | 131 |
| | | R: | AGACCCGTGACTTGAACCAC | | | |
| *proteasome subunit beta type-8* (*psmb8*) | BT058447 | F: | TACGCCTATGGTGTGATGGA | 0.993 | 100.2 | 189 |
| | | R: | GTACAGGTCGCCAACATCCT | | | |
| *tropomodulin-4-like* (*tmod4*) | DW569648 | F: | GCTACCACTTCACCCAGCAG | 0.994 | 98.1 | 153 |
| | | R: | CAAGGGGCTGAGTTGATTGT | | | |
| *phospholipase d4* (*pld4*) | GE792176 | F: | GCAGTCCAACAGGCTTACTGT | 0.998 | 102.2 | 159 |
| | | R: | ATCTCGCTGACTTGGGTCAT | | | |
| **pIC-responsive transcripts** | | | | | | |
| **Receptors** | | | | | | |
| *RNA helicase lgp2* (*lgp2*)[a] | BT045378 | F: | TCCAAGACCCGTAAAAGCAC | 0.999 | 89.1 | 189 |
| | | R: | GGTGGAGATCAGGAGGTTGA | | | |
| *C-X-C chemokine receptor type 3* (*cxcr3*) | NM_001140493 | F: | GGTGTTGGTGCTGGTCTTTT | 0.998 | 90.9 | 150 |
| | | R: | GCGAACGTAACCACCAGACT | | | |
| *CD209 antigen-like protein e* (*cd209e*) | NM_001141449 | F: | ATGTACTGGATCGGCCTGAG | 0.998 | 93.6 | 109 |
| | | R: | AACCACAATCCCCTTGTCTG | | | |
| *toll-like receptor 3* (*tlr3*)[a] | BK008646 | F: | AATATGGCGCTGGTGAAGAG | 0.997 | 92.5 | 135 |
| | | R: | CGCAAAGGTGAACACTGAGA | | | |
| *toll-like receptor 7* (*tlr7*)[a] | HF970585 | F: | CACCAACACAGAGCTGGAGA | 0.994 | 98.3 | 184 |
| | | R: | GCCTTGGAAAACTTGCTGAG | | | |
| *scavenger receptor class B type I-like* (*scarb1-a*) | NM_001204894 | F: | AGGGGATAATGGAGGGGATT | 0.995 | 92.1 | 153 |
| | | R: | ACACTGGGGAGTTGGATCTG | | | |
| *scavenger receptor class B type I* (*scarb1-b*) | NM_001123612 | F: | TTCCCAACCCCACTTCTACA | 0.996 | 90.3 | 128 |
| | | R: | ACACATTCAGAGGCACACCA | | | |
| *macrophage colony stimulating factor 1, receptor 1* (*csf1r*) | CB515019 | F: | GGTTGAGGAGTTGGAGCTGT | 0.998 | 91.2 | 174 |
| | | R: | TTGATGATGTCGGAGCTGTC | | | |
| *chemokine receptor-like 1* (*cmklr1*) | BT044928 | F: | ATGTTCAGCAGCGTCTTCCT | 0.999 | 80.2[b] | 150 |
| | | R: | TACAGTCAGGGCAGCAGAGA | | | |
| *CD209 antigen-like protein d (cd209d)* | BT048497 | F: | AGAGAGGAGCAGACCTGGTG | 0.993 | 88.7 | 187 |
| | | R: | GCACCATTATCAGGCTGGTT | | | |
| **Signal transduction** | | | | | | |
| *mitogen-activated protein kinase kinase kinase 8* (*map3k8*) | NM_001173785 | F: | GGTGAACGTGTGACTGATGC | 0.996 | 99.7 | 144 |
| | | R: | GGCAGCTACAGAAACCACCT | | | |
| *suppressor of cytokine signaling 1* (*socs1*) | EG924375 | F: | CTGTAGGATGGTCGCTCACA | 0.999 | 94.4 | 133 |
| | | R: | ACACTGTTTGGATGGGTGCT | | | |
| *suppressor of cytokine signaling 3* (*socs3*) | GE794538 | F: | ATGGTAGCCCACAGCAAGTT | 0.997 | 89.2 | 179 |
| | | R: | GCCTCCTTGCCATTGATAGA | | | |
| *dual specificity phosphatase 5* (*dusp5*) | BT049175 | F: | CCGTCGCCTCAAGAAGATTA | 0.995 | 91.3 | 131 |
| | | R: | GGACAACAACCGAGTTCAGG | | | |
| *TNF receptor-associated factor 5-like a* (*traf5a*) | DY720479 | F: | AGAGGGGCCGTAAAGAACTG | 0.997 | 98.2[b] | 140 |
| | | R: | GCATGGACGTGCTGACACTA | | | |
| *tyrosine kinase JAK3* (*jak3*) | DY728848 | F: | TTCCCTCTCATCTTGGATGC | 0.998 | 98.1 | 121 |
| | | R: | ACTCTGCCCTCCCAGAATTT | | | |
| *cytohesin-interacting like* (*cytip*) | DY700802 | F: | CGTCTTTGTGTGATGTGACG | 0.997 | 104.5 | 151 |
| | | R: | GTCAAAGGGAGAATGCTTGC | | | |
| *inhibitor of nuclear factor kappa-B kinase subunit alpha* (*ikka*) | GE780687 | F: | AAAGACTGCACCCGACAAAC | 0.992 | 93.8 | 111 |
| | | R: | TTGTGTCACCAAGGATGCTG | | | |
| *mitogen-activated protein kinase 13* (*mapk13*) | NM_001141648 | F: | ACAGGCGATTGACCTTCTTG | 0.999 | 95.7 | 133 |
| | | R: | ATATGGCTGTGGCTCAGGAC | | | |
| *cd80* | EG933501 | F: | CAGTGACACAGATCCCAGCA | 0.996 | 97.3 | 107 |
| | | R: | GTTGTTAGTTTGCGCCTGTG | | | |
| *dual specificity phosphatase 6* (*dusp6*) | NM_001165367 | F: | AGAAAAGAGGGGAAGCGAAG | 0.994 | 90.7 | 167 |
| | | R: | AGCCTTTCGTCGTGAAGCTA | | | |
| *dual specificity phosphatase 22-a* (*dusp22a*) | NM_001140429 | F: | CTGCGAGAGACATGGGAAAT | 0.990 | 105.8 | 120 |
| | | R: | CAGACAGGATGTGGGTGATG | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Transcription factors** | | | | | | |
| *cAMP-responsive element modulator-like* (*crem*) | CB508094 | F:<br>R: | GCTCTCTATGCAAGCCCTAGTC<br>AGACAAAATGCCCCCAGAG | 0.992 | 98.4 | 110 |
| *interferon regulatory factor 7* (*irf7*) | BT045216 | F:<br>R: | CCAGTGCCACCAGTCCTAAT<br>GGTGATCTCCAAGTCCCAGA | 0.999 | 94.5 | 105 |
| *cyclic AMP-dependent transcription factor ATF-3* (*atf3*) | BT059485 | F:<br>R: | CCAACTTCACTCCCCTGGTA<br>GGCTTCGGACTGTCTTTCTG | 0.993 | 91.9 | 128 |
| *basic leucine zipper transcription factor, ATF-like 3* (*batf3*) | NM_001141610 | F:<br>R: | GAGCCTCTCTGTCCCATCAG<br>TGCATGGTATGGTGGCTATC | 0.995 | 95.7 | 102 |
| *signal transducer and activator of transcription 1* (*stat1*) | DW551983 | F:<br>R: | GGTCCACACAAATCAACGTG<br>CTTTGCAGGGCCTTCTTCTT | 0.998 | 97.1 | 154 |
| **Immune effectors** | | | | | | |
| *ring finger protein 8, E3 ubiquitin protein ligase* (*rnf8*) | NM_001173788 | F:<br>R: | CACGAGTCGGGAAAGATTTG<br>TATCATTAGGGGGCAGCTTG | 0.996 | 100.3 [b] | 116 |
| *CASP8 and FADD-like apoptosis regulator* (*cflar*) | EG868690 | F:<br>R: | AGCTGGAGGAGAAGGACCAT<br>ATCTGGCTCCTGGATTCCTT | 0.998 | 98.1 | 153 |
| *interferon-induced GTP-binding protein Mx* (*mx-b*) [a] | NM_001139918 | F:<br>R: | ACGCACCACTCTGGAGAAAT<br>CTTCCATTTCCCGAACTCTG | 0.995 | 93.2 | 184 |
| *optineurin* (*optn*) | NM_001140289 | F:<br>R: | GAGCCAGCTAGAGCAGAGGA<br>CCCACCTCCTTAGTGACCAG | 0.997 | 91.8 | 144 |
| *E3 ubiquitin-protein ligase herc3* (*herc3*) | DY693327 | F:<br>R: | GGTGACTGCTGGTCCTCATT<br>GGGTCACGAGTGCGTAGAAT | 0.999 | 92.8 | 113 |
| *E3 ubiquitin-protein ligase herc6* (*herc6*) | EG915319 | F:<br>R: | CACCGGCTAACAGTGATGTG<br>GCACCCAGTTGTCTGAAGGT | 0.998 | 92.6 | 128 |
| *interferon, gamma (ifng)* | AJ841811 | F:<br>R: | CCGTACACCGATTGAGGACT<br>GCGGCATTACTCCATCCTAA | 0.998 | 94.9 | 133 |
| *viperin* | BT047610 | F:<br>R: | ACCATTTTACCCGACAGTGC<br>TCCCCAAGAAATCACCTCTG | 0.995 | 103.2 | 183 |
| *beta-1 syntrophin* (*sntb1*) | BT072489 | F:<br>R: | CACTCATGCCCAAAGCCTAT<br>ATAAGCCATGTTCGCCTCTG | 0.997 | 95.3 | 194 |
| *cathepsin-L1-like* (*ctsl1*) | DW575971 | F:<br>R: | TAGTTGTGGCTGCTGCTCTG<br>TAGGACTTGATGCCCTGGTC | 0.997 | 86.8 | 199 |
| *cathepsin-f* (*ctsf*) | NM_001140206 | F:<br>R: | TGCTGTGTTTTAGGCAGAGG<br>CAGGGGAACATAACGGACAC | 0.995 | 98.7 | 122 |
| **Normalizers** | | | | | | |
| *60S ribosomal protein 32 (rpl32)* | BT043656 | F:<br>R: | AGGCGGTTTAAGGGTCAGAT<br>TCGAGCTCCTTGATGTTGTG | 0.997 | 101.7 | 119 |
| *eukaryotic translation initiation factor 3 subunit D* (*eif3d*) | GE777139 | F:<br>R: | CTCCTCCTCCTCGTCCTCTT<br>GACCCCAACAAGCAAGTGAT | 0.995 | 101.4 | 105 |

F= Forward primer; R= Reverse primer

[a] These transcripts were not present in the microarray significant gene list.

[b] The amplification efficiencies of these primers were determined using 4-point serial dilutions of cDNA.

The diet-responsive transcripts were selected from RP-identified gene lists. The pIC-responsive transcripts were selected from microarray-identified transcripts overlapping between SAM and RP in both dietary groups (783 DEP; see Fig. 5.3.), except for *cd209d* (RP-identified in the FO5 group), and *stat1* and *irf7* (SAM-identified in both groups).

## 5.4. Results

### *5.4.1. Fish growth*

Fish growth was not significantly different between the FO7 and FO5 groups after 14 weeks of the feeding trial [final weight (means ± SE): FO7, 340.6 ± 5.97 g ($n$ = 138); FO5, 339.7 ± 6.21 g ($n$ = 140)]. The growth performance data of the diets used herein were published in Beheshti-Foroutani et al. (2018).

### *5.4.2. Phagocytosis and RB*

In this study, the phagocytosis (18.3% phagocytic cells in the FO7 and 17.6 phagocytic cells in the FO5) of the salmon MLCs was not significantly influenced by diet (Fig. 5.2.). In addition, the percentage of MLCs exhibiting RB (10.9% in the FO7 and 7.9% in the FO5) did not significantly change between the dietary groups.

### *5.4.3. Lipid and fatty acid analyses*

There were some differences in the composition of lipid classes in HKLs isolated from salmon fed experimental diets (Table 5.3.). The proportion of HKL free fatty acids was significantly higher in the FO5 group (1.99 ± 0.44%) than in the FO7 group (0.58 ± 0.16%) (Table 5.3.). There was a significant increase in sterols in HKLs isolated from salmon fed the FO5 diet as compared to those fed the FO7 diet (Table 5.3.). However, HKLs of salmon in the FO5 group had a lower proportion of phospholipids as compared to the FO7 group. Phospholipids were found to be the most dominant lipid class in the salmon HKLs. No significant differences were detected for other lipid classes (e.g.,

hydrocarbons and triacylglycerols) of HKLs isolated from salmon fed the FO5 and FO7 diets (Table 5.3.).

HKLs isolated from salmon in both dietary groups showed a comparable profile for many fatty acids (Table 5.3.). However, significant differences were found for some fatty acids. For example, linoleic acid (18:2n-6) and dihomo-gamma-linolenic acid (20:3n-6) in HKLs isolated from fish in the FO5 group were higher than those of the FO7 group. In contrast, EPA (20:5n-3) of salmon HKLs was lower in the FO5 group as compared to the FO7 group (Table 5.3.). The sum of long-chain n-6 fatty acids (LCn-6) and the LCn-6/LCn-3 ratio of HKLs were higher in the FO5 group as compared to the FO7 group.

### *5.4.4. Microarray results*

#### *5.4.4.1. Diet-responsive transcripts in salmon MLCs*

To identify diet- and pIC-responsive transcripts in salmon MLCs, the expression data were analysed using both SAM and RP. Only one DEP was found by SAM when comparing pIC-stimulated MLCs from fish fed the two diets (FO5/FO7) (i.e., *transmembrane protein 115-like*; 1.87-fold up-regulated in FO5). RP identified 14 and 54 DEP (PFP < 0.05) between the two diets in the PBS and pIC groups, respectively (Fig. 5.3.), and 12 of these DEP were found in both lists and showed a similar response (i.e., up- or down-regulation response to a given diet). Supplemental Table S5.1. shows the diet-responsive probes in the pIC and PBS groups. Transcripts involved in lipid metabolism (e.g., *fatty acid-binding protein, adipocyte*; *fabp4*) as well as immune

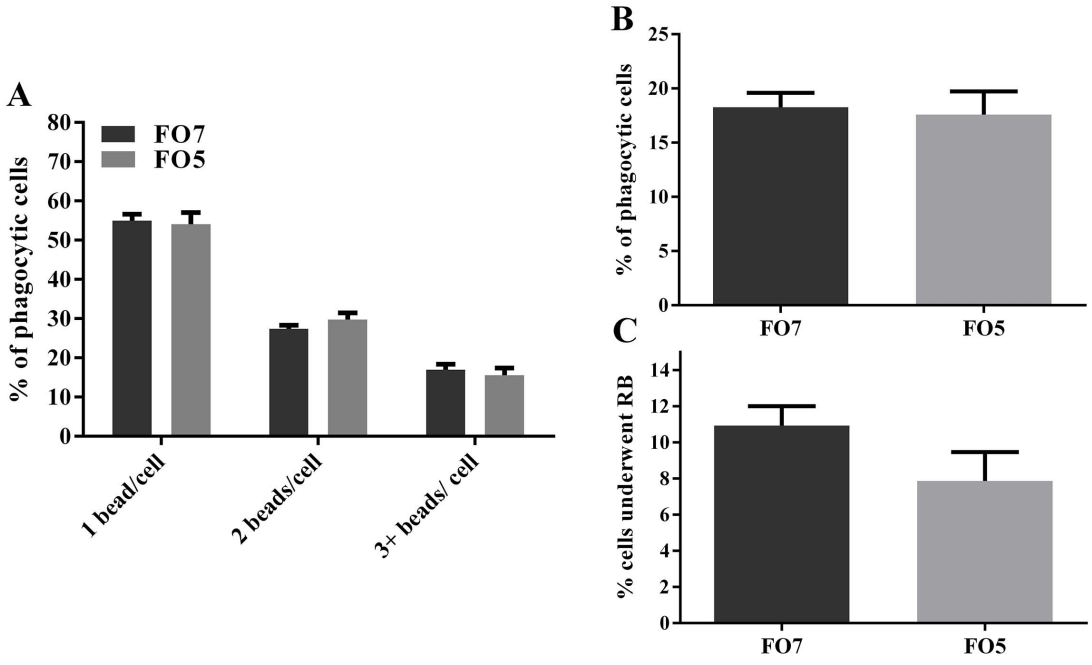



**Figure 5.2. Cellular functions of macrophage-like cells (MLCs) isolated from salmon in the FO7 and FO5 dietary groups.** Data are presented as means ± SE. No significant differences ($p > 0.05$) were found between the groups using an unpaired t-test. **A:** phagocytosis of MLCs based on the number of the beads ingested by phagocytic cells, **B:** The percentage of phagocytic salmon MLCs in the dietary groups, **C:** The percentage of salmon MLCs in each dietary group that exhibited respiratory burst (RB).

**Table 5.3. The lipid class and fatty acid composition of salmon head kidney leukocytes (HKLs) in different dietary groups.**

| Lipid class % | FO7 | FO5 | *p* value |
|---|---|---|---|
| Hydrocarbons | 0.76 ± 0.104 | 0.43 ± 0.177 | 0.110 |
| Triacylglycerols | 0.83 ± 0.182 | 1.94 ± 0.641 | 0.124 |
| Free fatty acids | 0.58 ± 0.162 | 1.99 ± 0.442 | **0.012** |
| Sterols | 11.38 ± 0.318 | 12.51 ± 0.371 | **0.032** |
| AMPL [1] | 5.71 ± 0.787 | 6.06 ± 0.909 | 0.777 |
| Phospholipids | 80.74 ± 0.816 | 76.80 ± 1.695 | **0.044** |
| **Fatty acids % [2]** | | | |
| 14:0 | 1.17 ± 0.027 | 0.97 ± 0.030 | **< 0.0001** |
| 15:0 | 0.24 ± 0.003 | 0.22 ± 0.005 | **0.001** |
| 16:0 | 18.28 ± 0.189 | 17.96 ± 0.245 | 0.314 |
| 16:1n-7 | 0.77 ± 0.032 | 0.73 ± 0.050 | 0.473 |
| 17:0 | 0.21 ± 0.004 | 0.21 ± 0.005 | 0.200 |
| 16:4n-1 | 2.19 ± 0.289 | 2.08 ± 0.356 | 0.814 |
| 18:0 | 6.36 ± 0.110 | 6.45 ± 0.149 | 0.621 |
| 18:1n-9 | 17.08 ± 0.337 | 17.81 ± 0.349 | 0.151 |
| 18:1n-7 | 3.15 ± 0.031 | 3.16 ± 0.043 | 0.840 |
| 18:2n-6 | 4.58 ± 0.084 | 4.93 ± 0.142 | **0.041** |
| 18:3n-6 | 0.20 ± 0.005 | 0.27 ± 0.011 | **< 0.0001** |
| 18:3n-3 | 0.76 ± 0.025 | 0.79 ± 0.038 | 0.568 |
| 18:4n-3 | 0.26 ± 0.008 | 0.30 ± 0.023 | 0.073 |
| 20:1n-9 | 1.14 ± 0.037 | 0.98 ± 0.036 | **0.005** |
| 20:2n-6 | 0.63 ± 0.020 | 0.63 ± 0.030 | 0.922 |
| 20:3n-6 | 1.57 ± 0.046 | 1.88 ± 0.078 | **0.003** |
| 20:4n-6 | 5.23 ± 0.152 | 5.67 ± 0.190 | 0.080 |
| 20:4n-3 | 0.56 ± 0.017 | 0.55 ± 0.018 | 0.739 |
| 20:5n-3 | 6.37 ± 0.150 | 5.77 ± 0.131 | **0.008** |
| 22:1n-11(13) | 0.24 ± 0.024 | 0.32 ± 0.062 | 0.232 |
| 22:1n-9 | 0.27 ± 0.051 | 0.46 ± 0.112 | 0.131 |
| 22:5n-3 | 0.74 ± 0.027 | 0.69 ± 0.023 | 0.245 |
| 22:6n-3 | 23.74 ± 0.448 | 23.00 ± 0.473 | 0.271 |
| 24:1 | 0.72 ± 0.024 | 0.74 ± 0.029 | 0.450 |
| Bacterial | 1.14 ± 0.047 | 1.05 ± 0.049 | 0.196 |
| Σ SFA [3] | 26.41 ± 0.298 | 25.94 ± 0.399 | 0.346 |
| Σ MUFA [4] | 24.48 ± 0.397 | 25.25 ± 0.482 | 0.229 |
| Σ PUFA [5] | 48.53 ± 0.594 | 48.28 ± 0.624 | 0.774 |
| Σ LC n-3 [6] | 31.58 ± 0.551 | 30.16 ± 0.531 | 0.080 |
| Σ LC n-6 | 7.68 ± 0.171 | 8.47 ± 0.226 | **0.012** |
| LCn-6/ LCn-3 | 0.24 ± 0.005 | 0.28 ± 0.007 | **0.001** |
| P/S | 1.84 ± 0.040 | 1.87 ± 0.047 | 0.662 |
| Σ n-3 | 33.43 ± 0.583 | 32.13 ± 0.449 | 0.098 |
| DHA/EPA ratio | 3.74 ± 0.087 | 4.00 ± 0.102 | 0.068 |

Values are means ± SE. Bold ***p* values** indicate a significant ($p < 0.05$) difference between groups

[1] Acetone mobile polar lipids

[2] Data are expressed as a percentage of identified FAME (fatty acid methyl ester), for fatty acids that were present at ≥ 0.2% of the total fatty acids.

[3] Saturated fatty acid

[4] Monounsaturated fatty acid

[5] Polyunsaturated fatty acid

[6] Long chain n-3

Other fatty acids present at < 0.2%: 15:0, *i*16:0, 16:1n-5, *i*17:0, *ai*17:0, 16:2n-4, 17:1, 18:2n-4, 18:3n-4, 20:0, 20:3n-3, 22:0, 22:5n-6

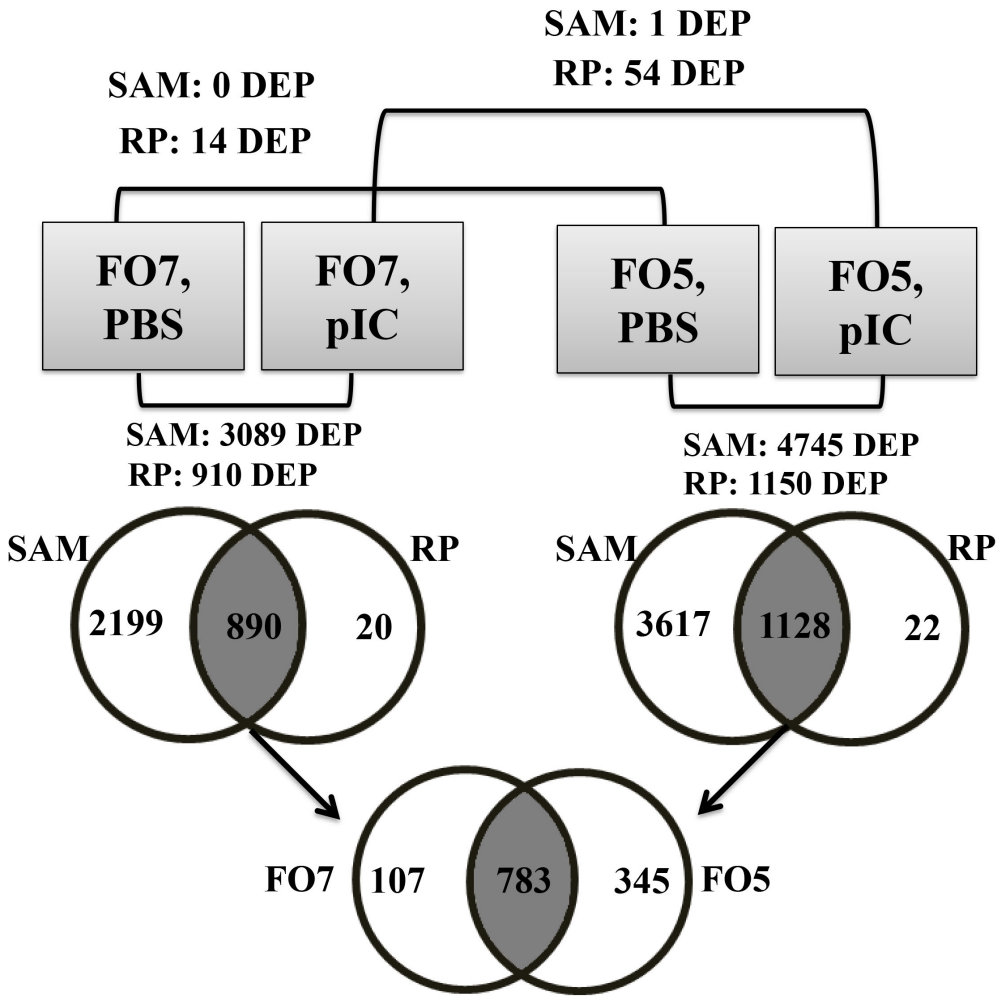



**Figure 5.3. Overview of microarray results.** The diet- and pIC-responsive probes identified by SAM (FDR < 0.05) and RP (PFP < 0.05) analyses. The differentially expressed probes (DEP) by diet and pIC are shown in Supplemental Table S5.1. and S5.2., respectively.

responses (e.g., *Fc receptor-like protein 2* and *MHC-I*) were identified as diet-responsive by RP. A subset of 9 diet-responsive transcripts was subjected to qPCR validation.

#### *5.4.4.2. pIC-responsive transcripts in salmon MLCs*

Supplemental Table S5.2. presents the pIC-responsive probes within FO5 or FO7 groups. SAM found that pIC stimulation resulted in 3089 DEP (FDR < 0.05) within the FO7 group (pIC vs. PBS), whereas RP identified 910 DEP (PFP < 0.05) within this group (3109 DEP in total). Further, SAM found 4745 DEP (FDR < 0.05) following pIC stimulation within the FO5 group (pIC vs. PBS), while RP only identified 1150 DEP (PFP < 0.05) in this group (4767 DEP in total). Venn diagrams show that 890 and 1128 DEP overlapped between the SAM and RP significant pIC-responsive gene lists for the FO7 and FO5 groups, respectively. Between these pIC-responsive probes, 107 and 345 of them were only identified as SAM- and RP-overlapped in the FO7 and FO5 groups, respectively. Figure 5.3. illustrates the microarray results and overlapping pIC-responsive probes between experimental groups.

#### *5.4.4.3. Hierarchical clustering analyses of microarray results*

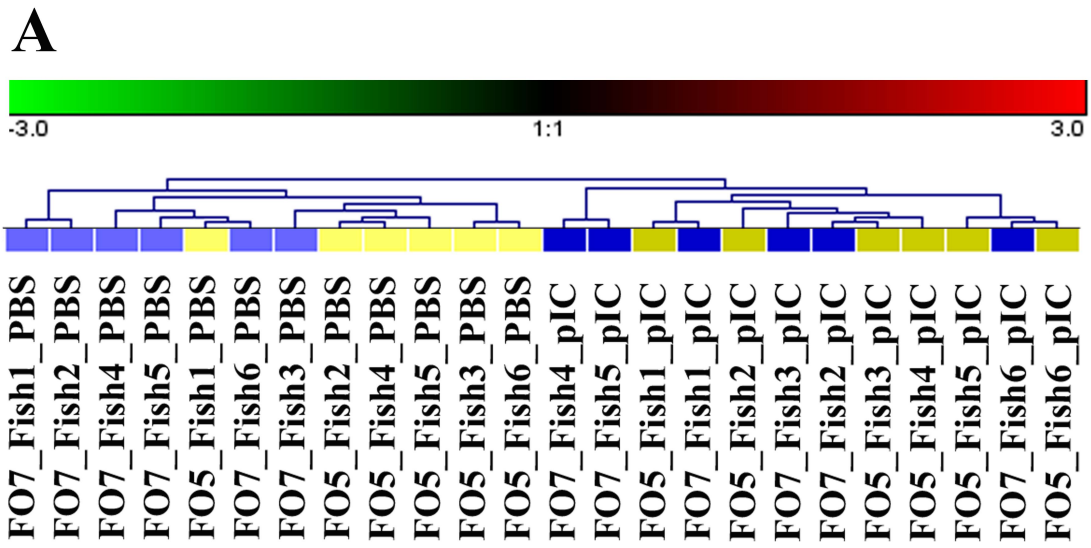
Hierarchical clustering analyses were performed to determine if samples isolated from a dietary group shared a similar transcriptome profile. The microarray data were clustered using the whole microarray dataset (Fig. 5.4. A), the pIC-responsive probes (i.e., 1235 DEP) identified by both SAM and RP (Fig. 5.4. B) and a subset of pIC-responsive transcripts involved in response to cytokine (i.e., transcripts associated with cellular response to cytokine stimulus and/or response to cytokine GO terms; Fig. 5.4. C).

Samples from the pIC or PBS treatments of both dietary groups showed similar transcriptome profiles (i.e., whole microarray dataset), as they were only clustered together according to the stimulation groups (Fig. 5.4. A). The majority of the samples in the PBS treatment of each diet (i.e., 5 samples in FO5 and 4 samples in FO7) grouped closely together (Fig. 5.4. A), and this indicates similar constitutive global gene expression of MLCs isolated from a given diet. However, this diet-related clustering was not found in pIC-stimulated samples (Fig. 5.4. A). Using a subset of the pIC-responsive probes (Fig. 5.4. B), the samples were separated into two clusters based upon their stimulation group (i.e., pIC and PBS), and no grouping was detected based on the dietary treatment. Similar results were observed for the clustering of samples using a subset of 53 pIC-responsive probes with putative roles in response to cytokines [i.e., transcripts associated with cellular response to cytokine stimulus (GO:0071345) and/or response to cytokine (GO:0034097) GO terms] (Fig. 5.4. C).

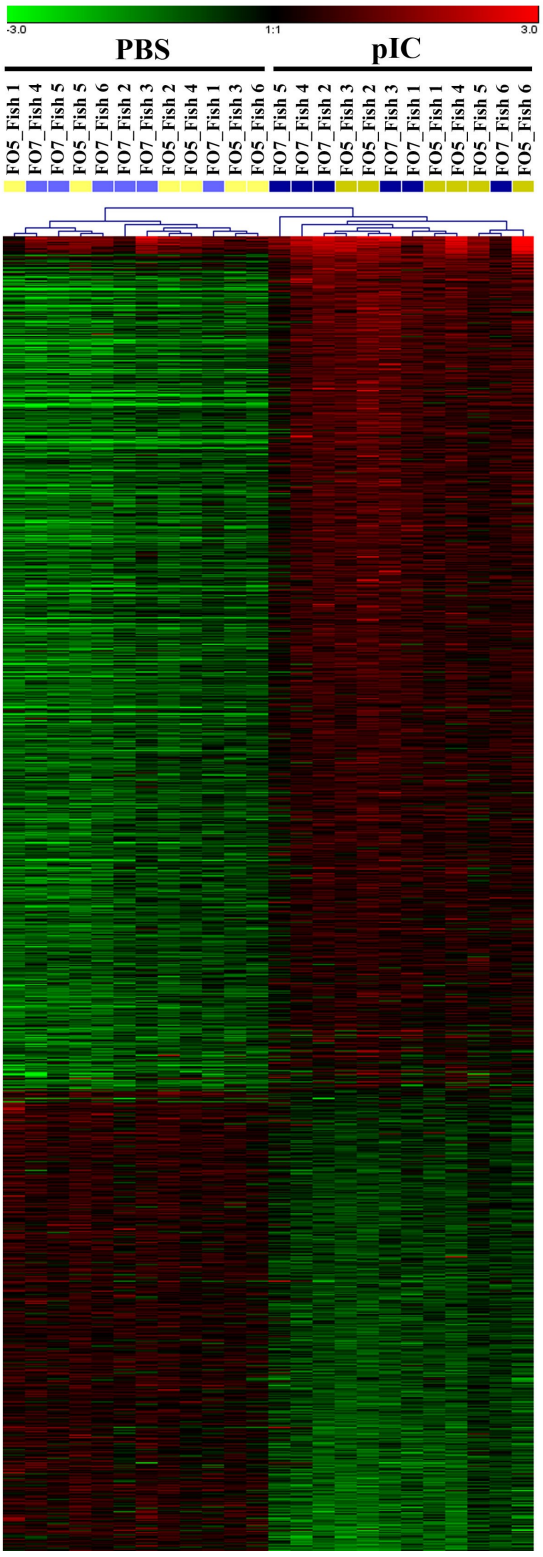
#### *5.4.4.4. GO terms and GO enrichment analyses of pIC-responsive transcripts in dietary groups*

GO terms (i.e., Molecular Function, Biological Process, or Cellular Component categories) for DEP from the pIC treatment in each dietary group were obtained (see Supplemental Table S5.2.), and GO annotation distributions (Biological Process level 2) of pIC-responsive transcripts overlapping between SAM and RP analyses in each dietary group were created (Fig. 5.5.). The proportions of pIC-responsive transcripts associated with different GO annotation (e.g., signalling and immune system process) in the FO7 group were highly comparable to those of the FO5 group.

**Figure 5.4. Hierarchical clustering analyses. A:** Clustering of samples using the whole microarray dataset; **B:** Clustering of samples based on of pIC-responsive transcripts overlapping between SAM- and RP-identified lists. **C:** Clustering of samples based on a subset of pIC-responsive transcripts involved in cytokine-mediated pathways [i.e., associated with GO terms "cellular response to cytokine" (GO:0071345) and/or "response to cytokine" (GO:0034097)]; the transcript names are derived from the significant BLASTx hits (E-value < 1e-05) as implemented by Blast2GO. Coloured blocks at the top of the figures indicate the dietary and stimulation groups: light blue, PBS FO7; dark blue, pIC FO7; light yellow, PBS FO5; dark yellow, pIC FO5.



**B**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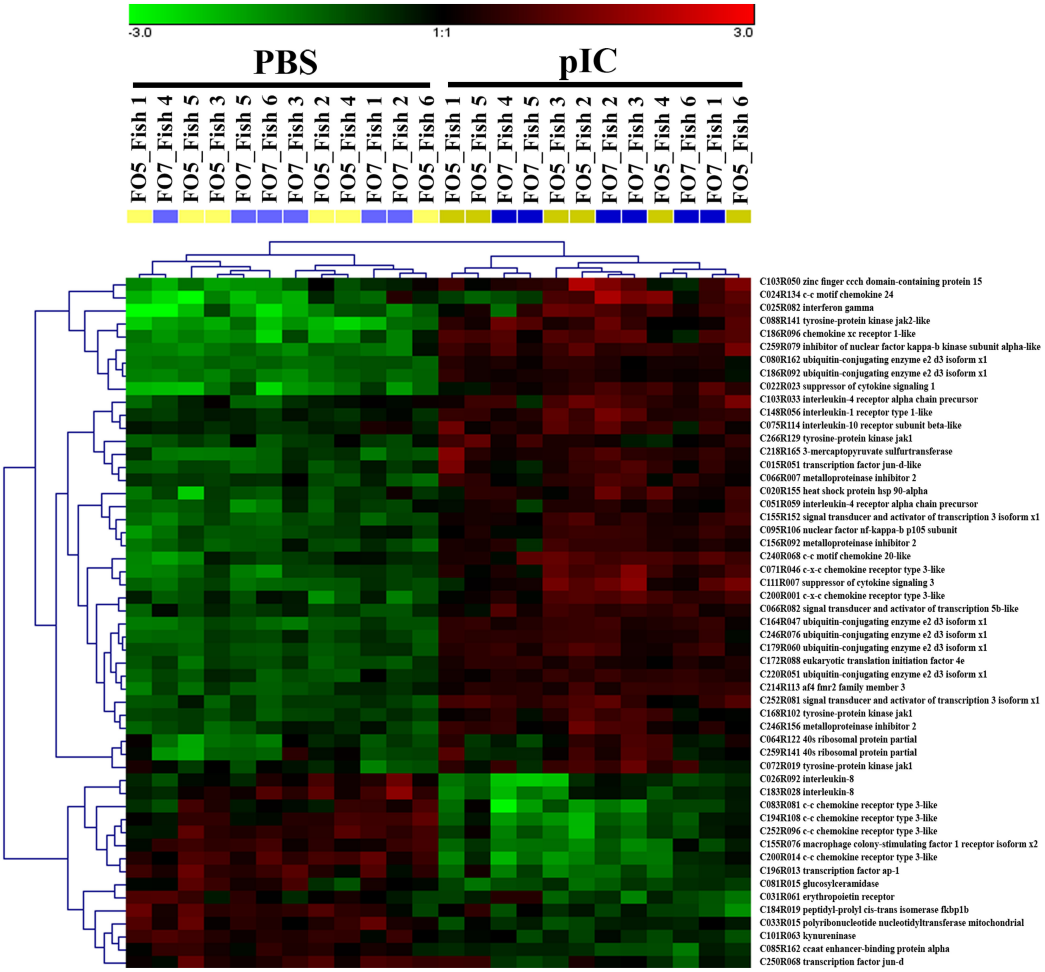
C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FO5_Fish 6
C103R050 zinc finger ccch domain-containing protein 15
C024R134 c-c motif chemokine 24
C025R082 interferon gamma
C088R141 tyrosine-protein kinase jak2-like
C186R096 chemokine xc receptor 1-like
C259R079 inhibitor of nuclear factor kappa-b kinase subunit alpha-like
C080R162 ubiquitin-conjugating enzyme e2 d3 isoform x1
C186R092 ubiquitin-conjugating enzyme e2 d3 isoform x1
C022R023 suppressor of cytokine signaling 1
C103R033 interleukin-4 receptor alpha chain precursor
C148R056 interleukin-1 receptor type 1-like
C075R114 interleukin-10 receptor subunit beta-like
C266R129 tyrosine-protein kinase jak1
C218R165 3-mercaptopyruvate sulfurtransferase
C015R051 transcription factor jun-d-like
C066R007 metalloproteinase inhibitor 2
C020R155 heat shock protein hsp 90-alpha
C051R059 interleukin-4 receptor alpha chain precursor
C155R152 signal transducer and activator of transcription 3 isoform x1
C095R106 nuclear factor nf-kappa-b p105 subunit
C156R092 metalloproteinase inhibitor 2
C240R068 c-c motif chemokine 20-like
C071R046 c-x-c chemokine receptor type 3-like
C111R007 suppressor of cytokine signaling 3
C200R001 c-x-c chemokine receptor type 3-like
C066R082 signal transducer and activator of transcription 5b-like
C164R047 ubiquitin-conjugating enzyme e2 d3 isoform x1
C246R076 ubiquitin-conjugating enzyme e2 d3 isoform x1
C179R060 ubiquitin-conjugating enzyme e2 d3 isoform x1
C172R088 eukaryotic translation initiation factor 4e
C220R051 ubiquitin-conjugating enzyme e2 d3 isoform x1
C214R113 af4 fmr2 family member 3
C252R081 signal transducer and activator of transcription 3 isoform x1
C168R102 tyrosine-protein kinase jak1
C246R156 metalloproteinase inhibitor 2
C064R122 40s ribosomal protein partial
C259R141 40s ribosomal protein partial
C072R019 tyrosine-protein kinase jak1
C026R092 interleukin-8
C183R028 interleukin-8
C083R081 c-c chemokine receptor type 3-like
C194R108 c-c chemokine receptor type 3-like
C252R096 c-c chemokine receptor type 3-like
C155R076 macrophage colony-stimulating factor 1 receptor isoform x2
C200R014 c-c chemokine receptor type 3-like
C196R013 transcription factor ap-1
C081R015 glucosylceramidase
C031R061 erythropoietin receptor
C184R019 peptidyl-prolyl cis-trans isomerase fkbp1b
C033R015 polyribonucleotide nucleotidyltransferase mitochondrial
C101R063 kynureninase
C085R162 ccaat enhancer-binding protein alpha
C250R068 transcription factor jun-d

The Fisher's exact test (FDR < 0.05) was used to determine the over- and under-represented GO terms of the pIC-responsive transcripts (i.e., overlapped between SAM and RP) in each dietary group compared to the whole array. This analysis showed 110 and 117 significantly enriched GO terms by pIC stimulation in the FO7 and FO5 groups, respectively (see Supplemental Table S5.3.). Moreover, 88 enriched GO terms by pIC treatment overlapped in both lists and they shared a similar trend (i.e., over- or under-represented GO terms) between two dietary groups (see Supplemental Table S5.3. and Table 5.4.). Some GO terms (e.g., intracellular organelle and cytoskeleton) in the Cellular Component category were significantly under-represented in both groups (Supplemental Table S5.3.). A subset of enriched GO terms that were associated with immune responses is presented in Table 5.4. GO terms involved in immune responses [e.g., cytokine receptor activity, chemokine receptor activity, response to cytokine, chemokine-mediated signalling pathway and MyD88 (myeloid differentiation primary-response gene 88)-independent Toll-like receptor signalling pathway] were significantly over-represented in pIC-responsive gene lists of both dietary groups (Table 5.4.). Nonetheless, GO terms associated with Toll-like receptor 3 signalling pathway and negative regulation of type I interferon production were only significantly over-represented in the pIC-responsive transcript list of the FO7 group (see Table 5.4.), and this may be influenced by the lower number of pIC-responsive probes identified in FO7 compared to that in FO5.

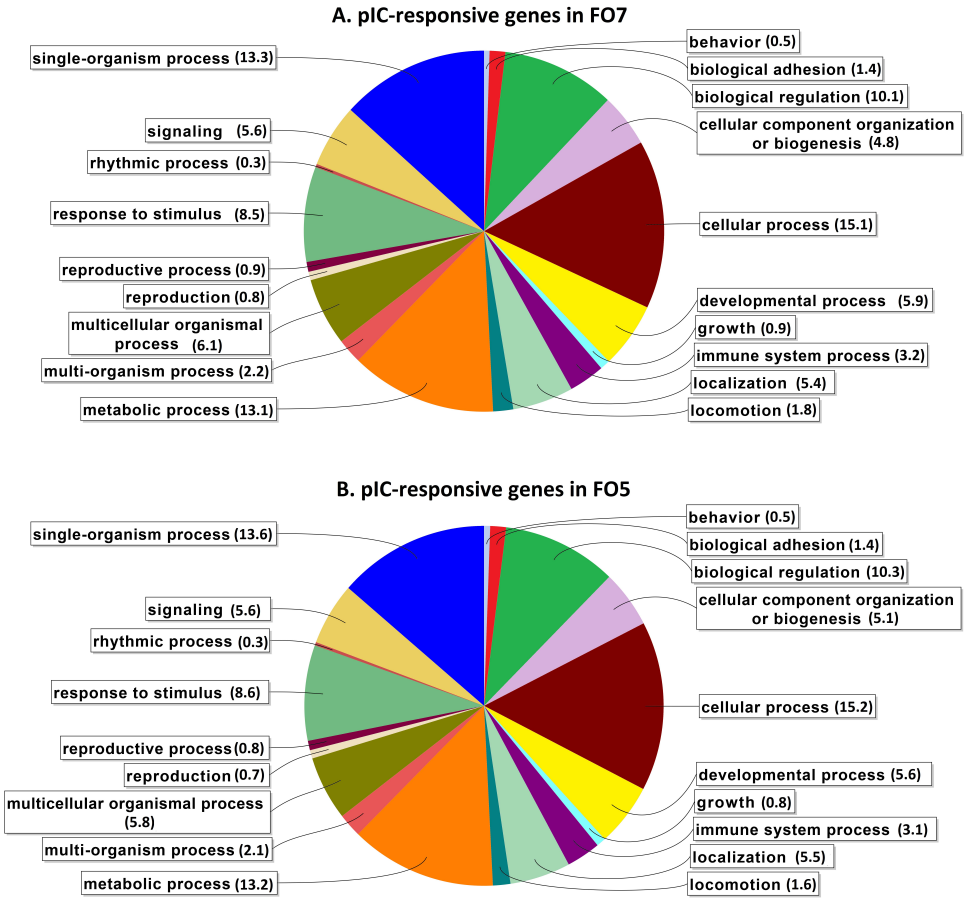


**Figure 5.5. GO term annotation of pIC-stimulated transcripts in the two dietary groups.** The charts illustrate the distribution of GO terms (Biological Process level 2) for pIC-responsive transcripts (i.e., overlapped between SAM- and RP-identified lists) of MLCs from salmon fed the FO7 **(A)** and FO5 **(B)** diets. The numbers between the parentheses represent the percentage of probes with each GO annotation.

**Table 5.4. An immune-related subset of enriched GO terms in pIC-responsive transcripts (overlap between SAM and RP analyses) within each dietary group.**

| GO ID | GO Term[1] | Category[2] | Number of probes with GO[3] | | Over/ Under |
|---|---|---|---|---|---|
| | | | Test FO7 | Test FO5 | |
| GO:0004950 | chemokine receptor activity | F | **8** | **8** | OVER |
| GO:0004896 | cytokine receptor activity | F | **11** | **14** | OVER |
| GO:0071345 | cellular response to cytokine stimulus | P | **37** | **43** | OVER |
| GO:0070098 | chemokine-mediated signaling pathway | P | **9** | **9** | OVER |
| GO:0034097 | response to cytokine | P | **40** | **50** | OVER |
| GO:0045647 | negative regulation of erythrocyte differentiation | P | **5** | **5** | OVER |
| GO:0030219 | megakaryocyte differentiation | P | **9** | **9** | OVER |
| GO:0035666 | TRIF-dependent toll-like receptor signaling pathway | P | **8** | **8** | OVER |
| GO:0002756 | MyD88-independent toll-like receptor signaling pathway | P | **8** | **8** | OVER |
| GO:0006954 | inflammatory response | P | **25** | **29** | OVER |
| GO:0034138 | toll-like receptor 3 signaling pathway | P | **8** | 8 | OVER |
| GO:0002250 | adaptive immune response | P | **15** | 16 | OVER |
| GO:0019221 | cytokine-mediated signaling pathway | P | **24** | 27 | OVER |
| GO:0032480 | negative regulation of type I interferon production | P | **6** | 6 | OVER |

[1] This subset of enriched GO terms associated with immune responses was selected using the Ancestor Chart feature of the QuickGO website (http://www.ebi.ac.uk/QuickGO). The full list of enriched GO terms is presented in Supplemental Table S5.3.

[2] **F:** Molecular Function and **P:** Biological Process

[3] Numbers of probes annotated with each GO term in the pIC-responsive gene list that was common to SAM and RP of each dietary group. Bold numbers indicate a significant over-representation (Fisher's exact test, FDR < 0.05) in the pIC-responsive gene list of the given dietary treatment, compared to the whole 44K salmon microarray. Total number of probes annotated with at least 1 GO term was 666 and 865 for the FO7 and FO5 groups, respectively.

### *5.4.5. qPCR validation*

#### *5.4.5.1. Diet-responsive transcripts*

A subset of 9 diet-responsive transcripts identified by RP analysis was subjected to qPCR validation (Fig. 5.6.). Table 5.5. represents the comparison between microarray and qPCR results for these 9 transcripts. All of the qPCR-assayed diet-responsive transcripts, except for *MHC-I*, showed similar directions of changes (up- or down-regulation) to the microarray results (Table 5.5.). However, the microarray results were only significantly validated for 2 (i.e., significant differential expression) of the studied transcripts. The expression of *fabp4* was significantly higher in both the PBS and pIC treatments (a 5.2- and 4.3-fold increase, respectively) of the FO5 group at 24 HPS, compared to those of the FO7 group (Fig. 5.6. B). On the contrary, *psmb8* (*proteasome subunit beta type-8*) expression was strongly suppressed by the FO5 diet in PBS- and pIC-stimulated salmon MLCs at 24 HPS, and the level of this transcript was very low or undetectable by the qPCR assays in the majority of the samples in the FO5 group (Fig. 5.6. G). The RP result for *lgmn* (*legumain-like*) was not confirmed at 24 HPS, but an up-regulation similar to the microarray was observed at 6 HPS in the pIC-treated MLCs of salmon fed the FO5 diet (a 1.83-fold increase) compared to those fed the FO7 diet (Fig. 5.6. D and Table 5.5.). Also, *sc5d* (*lathosterol oxidase*) and *pld4* (*phospholipase d4*) expression did not vary between the dietary treatments, but these transcripts were shown by both microarray and qPCR to be down-regulated (in at least one of the dietary groups) by pIC stimulation at 24 HPS (Fig. 5.6. E and I; Supplemental Table S5.2.).

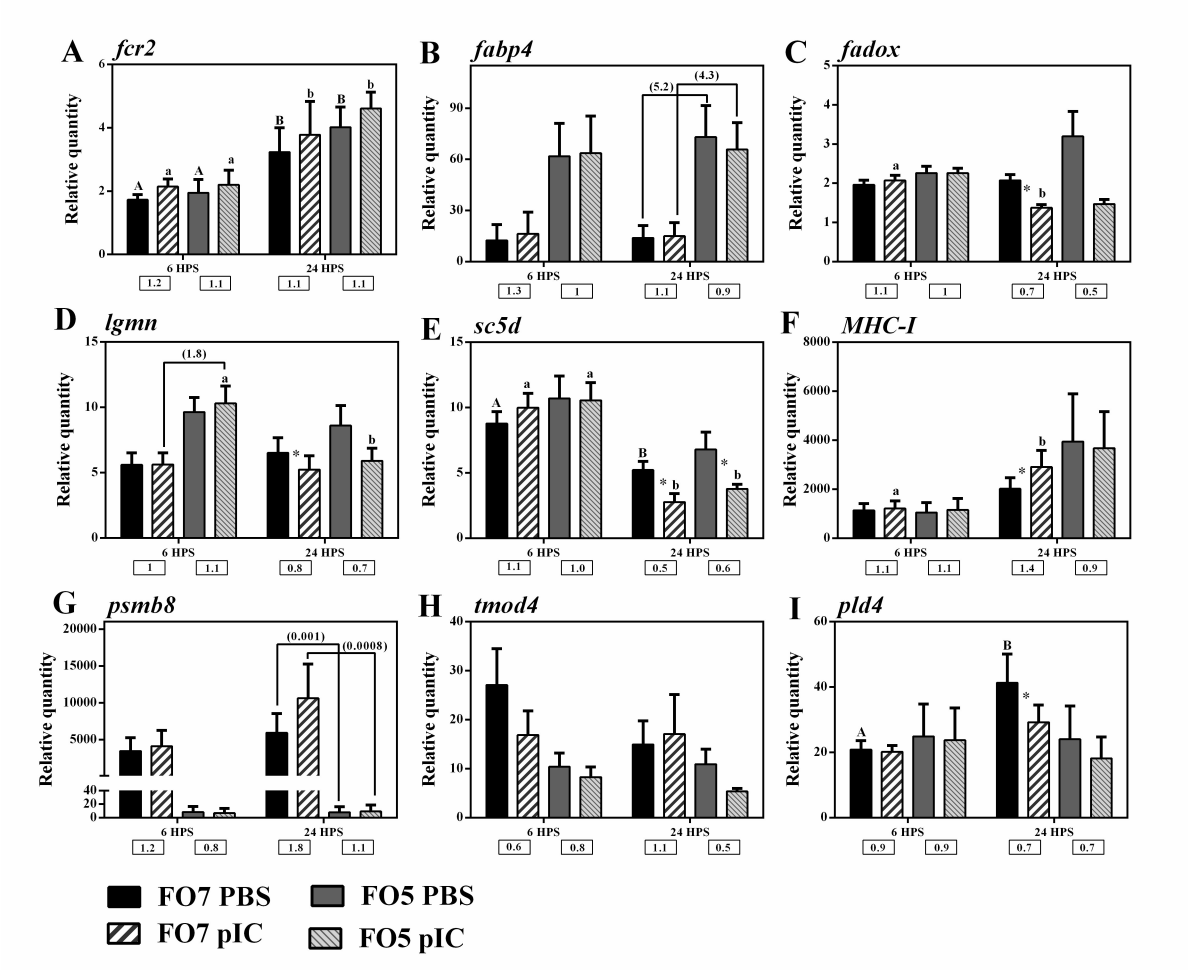


**Figure 5.6. qPCR results for transcripts identified by microarray analyses as diet-responsive.** Data are presented as means ± SE. Fold changes on the line connecting the stimulation-matched treatments of two diets show the significant differences between the PBS or pIC treatments of dietary groups ($p < 0.05$). An asterisk represents a significant difference between time-matched pIC and PBS groups in each dietary treatment ($p < 0.05$). Different letters (upper-case for PBS and lower-case for pIC) indicate the significant differences within PBS or pIC groups in a dietary treatment over time ($p < 0.05$). The fold-change (pIC/control) values are shown below the figure panels.

**Table 5.5. Comparison between the microarray and qPCR results of a subset of 9 diet-responsive transcripts identified by Rank Product (RP) analyses.**

| Microarray Probe ID | Name | FO5/FO7, Microarray fold-change PBS@ 24 HPS [1] | FO5/FO7, Microarray fold-change pIC @ 24 HPS [1] | FO5/FO7, qPCR fold-change PBS @ 6 HPS | FO5/FO7, qPCR fold-change pIC @ 6 HPS | FO5/FO7, qPCR fold-change PBS @ 24 HPS | FO5/FO7, qPCR fold-change pIC @ 24 HPS | qPCR, *p* value PBS [2] | qPCR, *p* value pIC [2] |
|---|---|---|---|---|---|---|---|---|---|
| C148R063 | *Fc receptor-like protein 2* (*fcr2*) | 3.63 | 3.72 | 1.13 | 1.03 | 1.24 | 1.21 | 0.42 | 0.53 |
| C108R146 | *fatty acid-binding protein, adipocyte (fabp4)* | 2.72 | 2.98 | 4.99 | 3.91 | 5.29* | 4.39* | 0.0012 | 0.003 |
| C126R012 | *FAD-linked sulfhydryl oxidase ALR-like (fadox)* | 2.21 | - | 1.15 | 1.09 | 1.54 | 1.070 | 0.082 | 0.22 |
| C001R074 | *lathosterol oxidase (sc5d)* | - | 1.96 | 1.22 | 1.06 | 1.30 | 1.36 | 0.15 | 0.36 |
| C146R053 | *legumain-like (lgmn)* | - | 1.83 | 1.72 | 1.83* | 1.32 | 1.13 | 0.024 [3] | 0.022 |
| C096R058 | *tropomodulin-4-like (tmod4)* | - | 0.43 | 0.38 | 0.49 | 0.73 | 0.31 | 0.039 [3] | 0.064 |
| C153R016 | *phospholipase d4 (pld4)* | 0.43 | 0.47 | 1.19 | 1.17 | 0.58 | 0.62 | 0.46 | 0.56 |
| C027R162 | *MHC class I antigen* | 0.29 | 0.32 | 0.92 | 0.95 | 1.95 | 1.26 | 0.44 | 0.72 |
| C164R003 | *proteasome subunit beta type-8 (psmb8)* | 0.28 | 0.23 | 0.0024 | 0.0016 | 0.0013* | 0.00088* | 0.011 | 0.016 |

[1] Fold changes between PBS- or pIC-matched groups of FO5 and FO7 at the same time (FO5/FO7). A dash (-) represents no differential expression between groups for a given comparison in the microarray analyses.
[2] The *p* values for qPCR results as implemented by two-way ANOVA between PBS- and pIC-matched groups of dietary treatments.
[3] Significant *p* values were observed for the PBS-matched groups of dietary treatments, but no significant difference was found by Sidak multiple comparisons *post-hoc* test
* A significant difference ($p < 0.05$) between the stimulation-matched groups (pIC and PBS) of dietary treatments in qPCR assay.

#### *5.4.5.2. pIC-responsive transcripts*

The qPCR results for pIC-responsive transcripts are presented based on their functions (e.g., receptors and transcription factors) in immune pathways (Figs. 5.7.-5.10.). These pIC-responsive transcripts were selected for qPCR validation from transcripts identified by both SAM and RP in both dietary groups (783 DEP; see Fig. 5.3.), except for *cd209d* (RP-identified in the FO5 group), and *stat1* and *irf7* (SAM-identified in both diet groups). This study included representative transcripts associated with different immune pathways (e.g., IFN, TLR and MAPK) and with different responses (e.g., suppressed or induced) to confirm the microarray results. In addition, some microarray-identified transcripts were subjected to qPCR validation, as they were known to have immune- or macrophage-related functions in higher vertebrates but were not well-characterised in fish species (see Discussion for details). The microarray results were qPCR-validated for all of the pIC-responsive transcripts (for at least one of the dietary groups). However, no significant differences were found between the pIC responses of the two dietary groups, except for *dusp22a* (*dual specificity phosphatase 22-a*) at 6 HPS (Fig. 5.8. L). Thus, the description of the expression results for pIC-responsive transcripts (see below) are for both dietary groups unless otherwise noted.

qPCR analyses measured the expression of 10 transcripts (i.e., 8 microarray-identified transcripts as well as *tlr3* and *tlr7* that were absent in the microarray platform) that play roles as PRRs (pattern recognition receptors) or other receptors (Fig. 5.7.). The expression of *lgp2* and *cxcr3* (*C-X-C chemokine receptor type 3*) was up-regulated in salmon MLCs in response to pIC at both 6 and 24 HPS, with the expression at 24 HPS

being significantly higher (Fig. 5.7. A, B). The up-regulation (i.e., more than 2-fold) of *cd209e* (*cd209 antigen-like protein e*) and *tlr7* (*toll-like receptor 7*) in pIC-stimulated salmon MLCs was only observed at 24 HPS (Fig. 5.7. C, E). While no pIC induction was recorded for *tlr3* in salmon MLCs, this transcript showed a time-dependent up-regulation within the PBS groups of both diets at 24 HPS compared to 6 HPS (Fig. 5.7. D). No early pIC response was seen for microarray-identified down-regulated transcripts with putative roles as receptors [i.e., *scarb1-a* (*scavenger receptor class B type I-like*), *scarb1-b*, *csf1r* (*macrophage colony stimulating factor 1, receptor 1*), *cmklr1* (*chemokine receptor-like 1*) and *cd209d*], and significant down-regulation was only found at 24 HPS for them (Fig. 5.7.). There was a time-dependent up-regulation for *scarb1-a*, *scarb1-b* and *csf1r* in salmon MLCs within the PBS groups at 24 HPS compared to the earlier time point. In other words, pIC stimulation suppressed the time-dependent response of these transcripts (Fig. 5.7. F-H). Two different paralogues (i.e., 90% similarity at the nucleotide level) of salmon *scarb1* responded similarly to pIC. Nonetheless, the down-regulation of the *scarb1-a* (0.1-fold) in response to pIC was stronger than that of *scarb1-b* (0.4-fold), as seen in the microarray results [*scarb1-a* (probe ID: C089R130), 0.39-fold and *scarb1-b* (probe ID: C118R093), 0.47-fold].

Twelve pIC-responsive transcripts involved in signal transduction were subjected to qPCR validation (Fig. 5.8.). The expression of *map3k8* (*mitogen-activated protein kinase kinase kinase 8*), *socs1* (*suppressor of cytokine signaling 1*), *socs3* and *dusp5* (*dual specificity phosphatase 5*) in salmon MLCs was significantly induced by pIC at

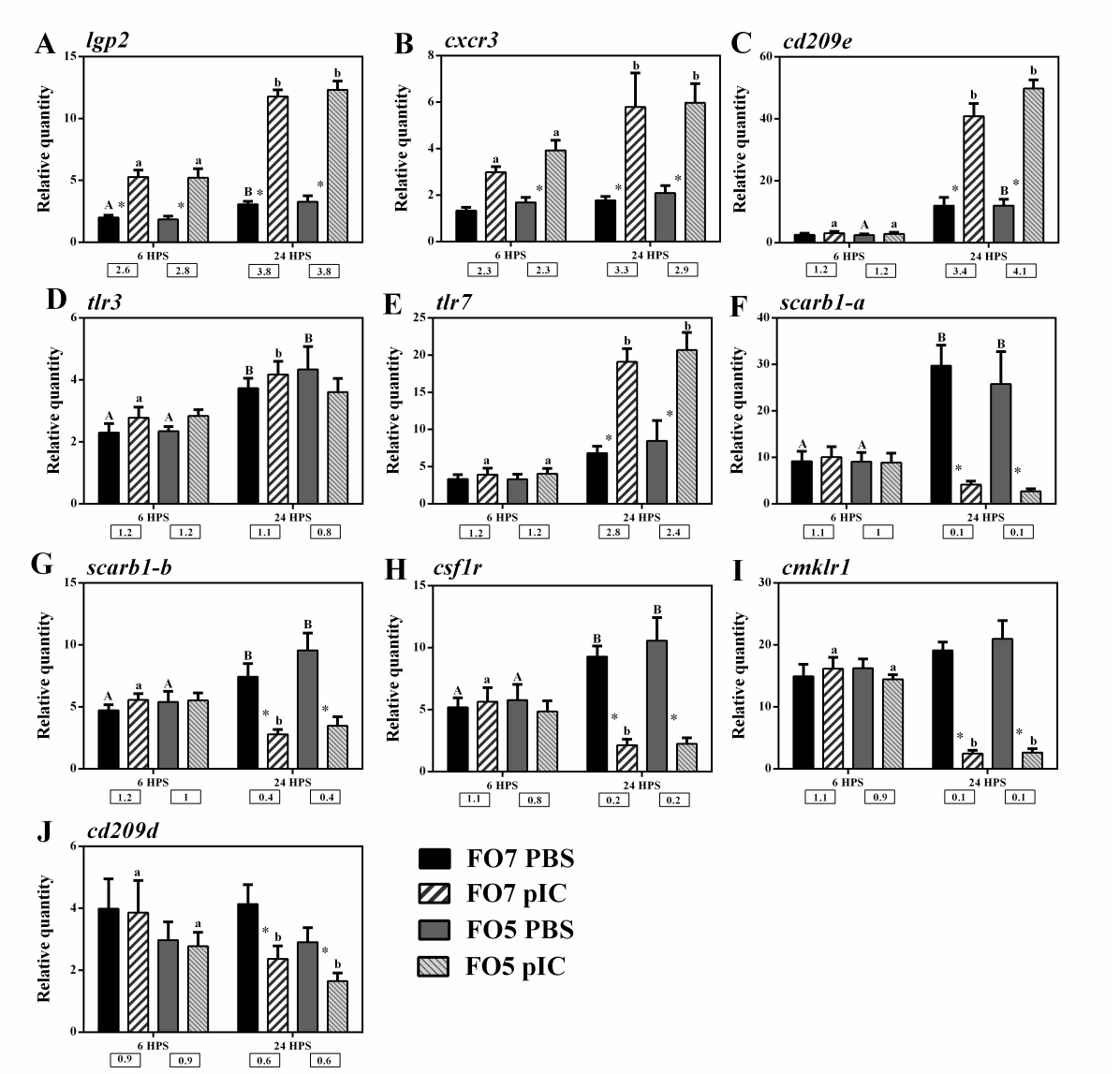


**Figure 5.7. qPCR results for pIC-responsive transcripts that play roles as PRRs or other receptors.** Data are presented as means ± SE. An asterisk represents a significant difference between time-matched pIC and PBS groups in each dietary treatment ($p < 0.05$). Different letters (upper-case for PBS and lower-case for pIC) indicate the significant differences within PBS or pIC group of a dietary treatment over time ($p < 0.05$). The fold-change (pIC/control) values are shown below the figure panels.

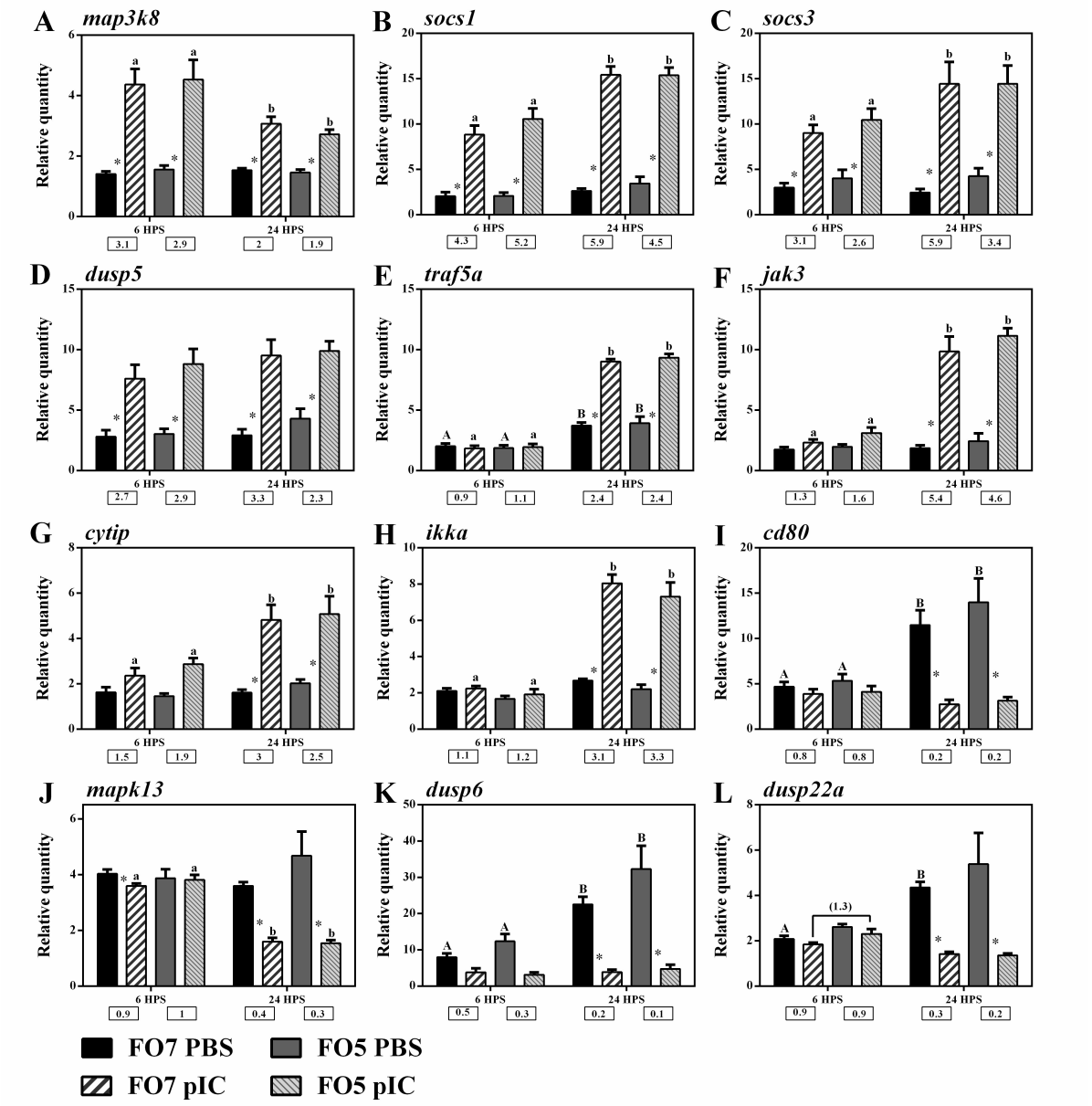


**Figure 5.8. qPCR results for pIC-responsive transcripts involved in signal transduction.** Data are presented as means ± SE. Fold changes on the line connecting the stimulation-matched treatments of two diets show the significant differences between PBS or pIC of dietary groups ($p < 0.05$). An asterisk indicates a significant difference between time-matched pIC and PBS groups in each dietary treatment ($p < 0.05$). Different letters (upper-case for PBS and lower-case for pIC) indicate the significant differences within PBS or pIC group of a dietary treatment over time ($p < 0.05$). The fold-change (pIC/control) values are shown below the figure panels.

both sampling points (Fig. 5.8. A-D). Although the *dusp5* response to pIC was unaffected by time, there was a decrease in *map3k8* expression, as well as an increase in transcript levels of *socs1* and *socs3*, within the pIC groups of both dietary treatments at 24 HPS compared to the earlier time point. The transcription of *traf5a* (*TNF receptor-associated factor 5-like a*), *jak3* (*tyrosine kinase JAK3*), *cytip* (*cytohesin-interacting like*) and *ikka* (*inhibitor of nuclear factor kappa-B kinase subunit alpha*) increased more than 2.4-fold in pIC-stimulated MLCs at 24 HPS (Fig. 5.8. E-H). Despite the time-dependent induction of *traf5a* in both PBS and pIC groups at 24 HPS, the up-regulation of this transcript was strengthened by pIC stimulation. The expression of *cd80*, *mapk13* (*mitogen-activated protein kinase 13*), *dusp6* and *dusp22a* was significantly reduced in pIC-stimulated salmon MLCs at 24 HPS (Fig. 5.8. I-L).

The present study measured the relative quantity of 5 pIC-responsive transcription factor encoding transcripts in salmon MLCs by qPCR (Fig. 5.9.). Salmon *crem* (*cAMP-responsive element modulator-like*) was significantly induced by pIC at both time points (1.7- and 6.1-fold increase at 6 and 24 HPS, respectively), and its expression was significantly suppressed by sampling time within the PBS group at 24 HPS compared to 6 HPS (Fig. 5.9. A). Moreover, *stat1* (*signal transducer and activator of transcription 1*) and *irf7* (*interferon regulatory factor 7*) showed a time-dependent up-regulation within the PBS and pIC groups (i.e., except for pIC group of FO5 in *irf7*) at 24 HPS. However, the pIC induction (at least 1.4-fold) of *stat1* and *irf7* was only significant in the FO7 group (Fig. 5.9. E, B). The other studied transcription factors [i.e., *atf3* (*cyclic AMP-dependent transcription factor ATF-3*) and *batf3* (*basic leucine zipper transcription*

*factor, ATF-like 3)*] were positively regulated in pIC-stimulated MLCs at 24 HPS (Fig. 5.9. C-D). The expression of *atf3* was significantly repressed within the PBS group of FO7 diet at 24 HPS compared to 6 HPS.

The microarray results for 10 pIC-responsive transcripts that play putative roles as immune effectors were confirmed by qPCR analyses (Fig. 5.10.). The expression of the *rnf8* (*ring finger protein 8, E3 ubiquitin protein ligase*) and *cflar* (*CASP8 and FADD-like apoptosis regulator*) in salmon MLCs was significantly induced by pIC at 6 HPS (1.7- and 2.4-fold increase) and peaked at 24 HPS (2.4- and 3.4-fold increase; Fig. 5.10. A-B). Similar results were seen for *mx-b*, *optn* (*optineurin*) and *herc3* (*E3 ubiquitin-protein ligase herc3*), but the significant differences between PBS and pIC treatments at the early time point were only observed for the FO7 group (Fig. 5.10. C-E). Salmon *mx-b* (i.e., included in the qPCR study, but not identified by microarray) was also a time-responsive transcript (i.e., its expression increased within the PBS group over time). The expression of *herc6*, *ifng* (*interferon, gamma*), *viperin*, *sntb1* (*beta-1 syntrophin*) and *ctsl1* (*cathepsin-L1-like*) did not vary between the PBS- and pIC-treated groups at 6 HPS (Fig. 5.10. F-J), but they were up-regulated in response to pIC at 24 HPS (between 3.4- to 7.8-fold increase). Salmon *ctsf* (*cathepsin-f*) expression significantly increased within the PBS group at the latter time point, although it was significantly down-regulated by pIC at 24 HPS, compared to the time-matched PBS group and the pIC group at 6 HPS (Fig. 5.10. K).

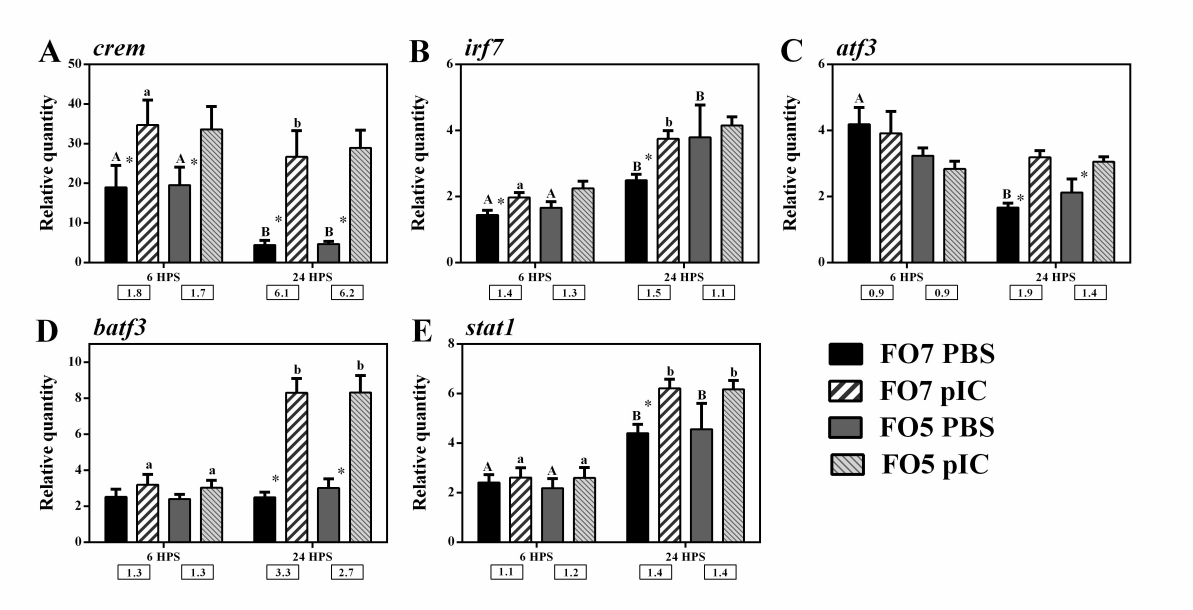


**Figure 5.9. qPCR results for pIC-responsive transcripts that play roles as transcription factors.** Data are presented as means ± SE. An asterisk represents a significant difference between time-matched pIC and PBS groups in each dietary treatment ($p < 0.05$). Different letters (upper-case for PBS and lower-case for pIC) indicate the significant differences within PBS or pIC group of a dietary treatment over time ($p < 0.05$). The fold-change (pIC/control) values are shown below the figure panels.

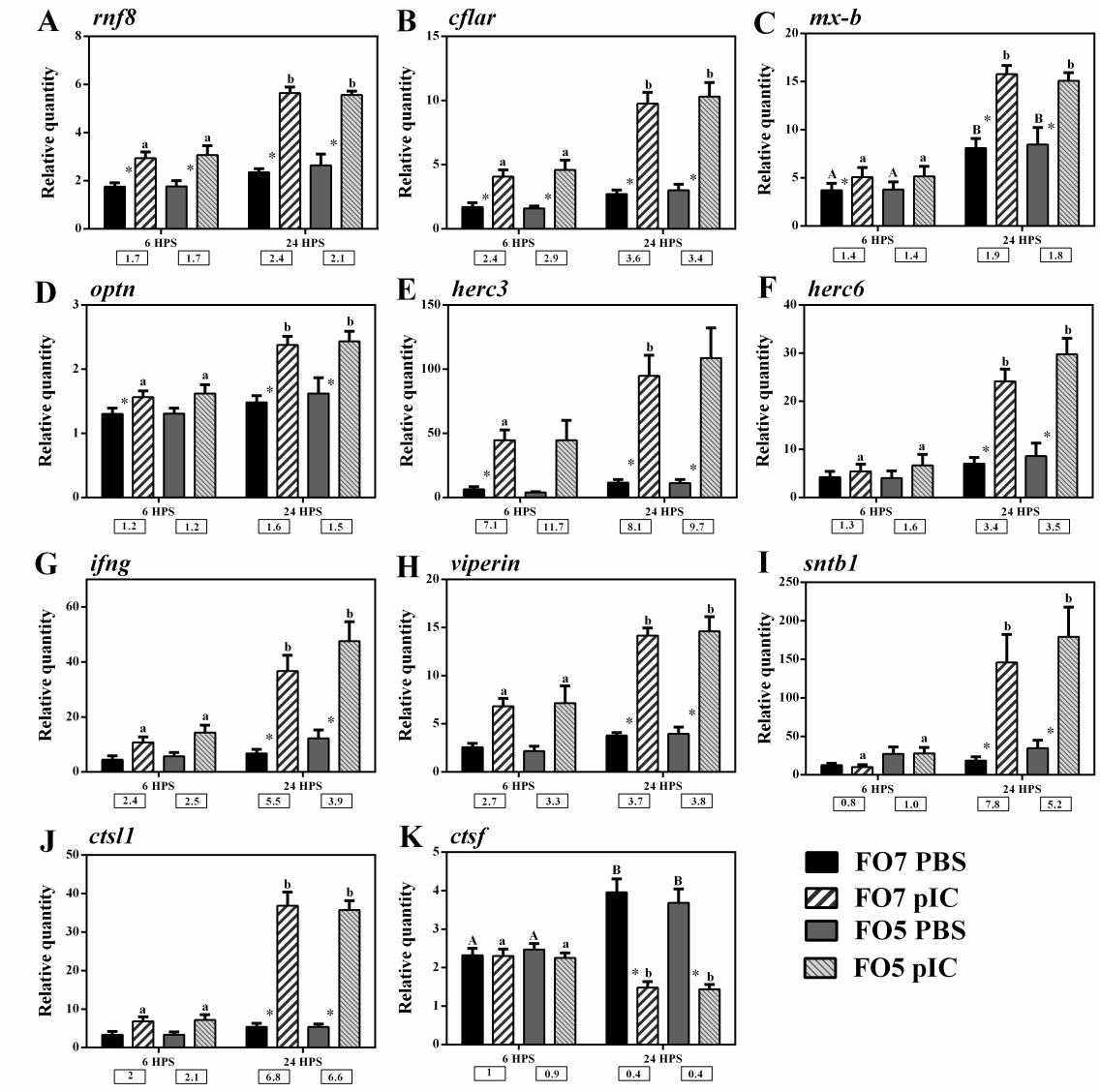


**Figure 5.10. qPCR results for pIC-responsive transcripts that play roles as immune effectors.** Data are presented as means ± SE. An asterisk represents a significant difference between time-matched pIC and PBS groups in each dietary treatment ($p < 0.05$). Different letters (upper-case for PBS and lower-case for pIC) indicate the significant differences within PBS or pIC group of a dietary treatment over time ($p < 0.05$). The fold-change (pIC/control) values are shown below the figure panels.

## 5.5. Discussion

### *5.5.1. Effects of experimental diets on the cellular functions and fatty acid composition of MLCs*

Neither phagocytosis nor the RB of salmon MLCs varied between diets, and the percentage of phagocytic cells in the current study was comparable to the results of a study on Atlantic salmon macrophages (Smith et al., 2018). Similarly, the phagocytosis of rainbow trout (*Oncorhynchus mykiss*) HKLs did not change with different levels of dietary plant-based n-3 and n-6 (Kiron et al., 2011), and the RB and pro-inflammatory cytokine expression of HKLs remained unchanged in salmon fed different levels of fish and vegetable oil (Seierstad et al., 2009). However, in both present and previous (Seierstad et al., 2009) studies, the lowest level of RB was seen in a diet with the highest level of vegetable oil, and further investigations are needed to have a better understanding of modulation of teleost RB by dietary vegetable oil. Some changes were observed in the lipid and fatty acid content of salmon HKLs fed different diets. For example, linoleic acid (18:2n-6) and free fatty acids increased, but EPA (20:5n-3) decreased in HKLs isolated from salmon fed the FO5 diet as compared to those fed the FO7 diet (Table 5.3.). In the present study, the proportions of sterols and phospholipids were significantly higher and lower, respectively, in MLCs of the FO5 group as compared to the FO7 group. Thus, the higher levels of dietary vegetable oil may influence the membrane structure of salmon MLCs. As in the current study, Seierstad et al. (2009) showed that the sum of n-6 fatty acids increased, and EPA+DHA levels decreased in HKLs when salmon were fed with a vegetable oil diet. However, the proportions of EPA+DHA in the experimental diets of

the present study were lower than those used in Seierstad et al. (2009) (i.e., at least 3.4% EPA+DHA in diet). The current study showed that feeding a diet containing 1% EPA+DHA for 16 weeks did not influence cellular functions (i.e., phagocytosis and RB). However, unbalanced levels of n-3 or n-6 can alter the immune or inflammatory responses of mammalian macrophages (Calder, 2008; Williams-Bey et al., 2014; Xue et al., 2012). Hence, some diet-associated variations in MLC gene expression, discussed in the following paragraphs, may have arisen from the differences in the fatty acid content of the cells between dietary treatments.

### *5.5.2. Impact of experimental diets on transcript expression of salmon MLCs*

Hierarchical clustering analyses using the whole microarray dataset showed that most of the PBS control samples from the same dietary treatment (especially FO5) grouped closely together. The comparable constitutive global gene expression of the PBS samples from the FO5 or FO7 dietary group may be explained by the effects of diets on the lipid and fatty acid content of the cells. The RP analyses identified 54 and 14 DEP between the pIC and PBS groups of the two dietary treatments, respectively (Supplemental Table S5.1.). Nine candidate diet-responsive transcripts identified by microarray analyses were subjected to qPCR validation, and the majority of them showed similar down- or up-regulation trends compared with the microarray results (Table 5.5.). The expression of *psmb8* and *fabp4* was significantly different between the PBS- and pIC-matched groups of FO5 and FO7 (Fig. 5.6.). The expression of *psmb8* was strongly suppressed in the MLCs by lowering the level of fish oil in the diet. PSMB8 (alias LMP7) is an IFN- and TNF-induced immunoproteasome subunit involved in peptide

processing of MHC-I pathway of antigen presenting cells (APCs) (Groettrup et al., 2010). A previous study reported a significant up-regulation of *psmb8* and *MHC-I* in salmon HKLs after 3 days of stimulation with ISAV or pIC (Jørgensen et al., 2006). Lungfish (*Protopterus dolloi*) *psmb8* was found to be an IFN- and pIC-induced gene (Tacchi et al., 2013). In the present study, *MHC-I* expression increased significantly (by 1.4-fold) in pIC-stimulated MLCs within the FO7 group at 24 HPS, and a non-significant up-regulation (i.e., 1.8-fold) was seen for *psmb8* in this group. However, these transcripts were not induced by pIC in the FO5 group. In addition to immunoregulatory functions, immunoproteasomes are thought to eliminate oxidant-damaged proteins, resulting in cell protection against oxidative stress induced by immune responses (Krüger and Kloetzel, 2012; Warnatsch et al., 2013). Further studies are needed to determine the correlation between dietary EPA/DHA and immune-derived oxidative stress with regard to *psmb8* expression.

The qPCR assays in the present study did not validate the microarray results for *lgmn* (i.e., up-regulated in pIC group of FO5 at 24 HPS). However, the qPCR results showed that the expression of this transcript significantly increased in the pIC treatment of the FO5 group at 6 HPS compared to that of the FO7 group. *lgmn* is associated with macrophage activity and differentiation in mammals (Edgington-Mitchell et al., 2016; Solberg et al., 2015), and it has been shown to be more highly expressed in mature macrophages compared with monocytes in goldfish (Barreda et al., 2004). If *lgmn* function is conserved in mammalian and teleost macrophages, then my results suggest that dietary fish oil (i.e., EPA/DHA) may influence salmon macrophage function.

As identified by microarray analyses and validated by qPCR, *fabp4* was significantly up-regulated in both the PBS and pIC groups of the FO5 diet as compared to those of the FO7 diet. FABP family proteins are lipid chaperones that regulate the transfer of specific lipids to different compartments of cells, thereby influencing cell signalling, lipid storage, membrane synthesis and lipid-mediated transcriptional control (Furuhashi and Hotamisligil, 2008). In mammals, different members of the FABP family are expressed in a tissue-specific manner, and *fabp4* is known to be transcribed in some immune-related cells such as macrophages and dendritic cells (Furuhashi and Hotamisligil, 2008). Mammalian FABP4, which plays a role in cell lipid transport in differentiated adipocytes and macrophages, was suggested to be a modulator of energy homeostasis (Syamsunarno et al., 2013). Further, *fabp4*-deficient macrophages of mice developed impaired cholesterol trafficking, suppressed IKK (Inhibitor of nuclear factor kappa-B kinase) signalling and, consequently, had decreased production of inflammatory cytokines (Makowski et al., 2005). Human macrophages showed the higher expression of *fabp4* in response to PUFA oxidation via the mediation of Akt (Protein kinase B)- and ERK (Extracellular signal-regulated kinase)-dependent signalling pathways (Lázaro et al., 2013). Importantly, FABP4 was reported to increase the expression of inflammatory genes in human macrophages and to be involved in the development of atherosclerosis (Furuhashi et al., 2016). There is no information on *fabp4* role in the activity and lipid metabolism of fish macrophages. However, as in mammalian *fabp2* (Furuhashi and Hotamisligil, 2008), the highest expression of Atlantic salmon *fabp2* was found in the intestine (Venold et al., 2013). There was also a decrease in *fabp2* expression by

intestinal inflammation caused by dietary soybean meal (Venold et al., 2013). Collectively, these studies suggest a possible correlation between dietary-induced immune responses of Atlantic salmon and the expression of *fabp*s. In addition, it seems that *fabp4* may be a key gene in Atlantic salmon macrophage function, as in higher vertebrates. The significant up-regulation of salmon *fabp4* in response to the higher level of dietary n-6 fatty acids in the present study suggests that its expression may be PUFA-dependent. The microarray analyses did not reveal a significant difference in inflammatory biomarkers between the dietary groups, although the larger number of microarray-identified pIC-responsive probes in the FO5 group compared to the FO7 group may be affected by the inflammation- or immune-related roles of *fabp4*. In the present study, the 4-fold up-regulation of *fabp4* occurred in response to a relatively small decrease in EPA+DHA content of the diet. Therefore, a larger difference in dietary EPA+DHA, or a longer feeding trial, in future studies may increase the fatty acid-associated responses of *fabp4*. Further studies are required to characterise *fabp4* in Atlantic salmon and to determine the fatty acid metabolism- and immune-related functions of this gene in Atlantic salmon macrophages.

The present study used an *ex vivo* approach to determine dietary fatty acid-dependent transcriptomic responses in Atlantic salmon MLCs. The choice of an *ex vivo* model allowed us to evaluate the impact of dietary DHA+EPA on the cell type of interest (i.e., macrophages) and to assess the antiviral response and cellular function of each individual fish fed a given experimental diet. However, it is noteworthy that the antiviral immune response of fish assessed by *in vivo* studies may vary from these *ex vivo*

experiments, due to different contributing factors such as paracrine signalling. Moreover, the cell isolation and culture procedures used in the present study could influence the fatty acid composition of cell membranes, and potentially modulate the diet-associated responses of MLCs to immune stimuli. Still, since MLCs from both diet groups in this study were subjected to the same conditions for cell culture and immune stimulation, the observed changes in fatty acid composition and gene expression occurred in response to the experimental diets. This suggests that the results of the current *ex vivo* study reflect the impact of dietary PUFAs on the antiviral immune response of salmon MLCs. The current findings show that 1% and 1.4% EPA+DHA diets for Atlantic salmon have different effects on the expression of some macrophage transcripts (i.e., *psmb8*, *fabp4* and *lgmn*) with putative roles in inflammation and/or macrophage function. This suggests that they are important immune-related diet-associated biomarkers. Moreover, these differentially regulated transcripts, alongside the fatty acid composition results, suggest that a relatively small change in EPA+DHA intake may alter membrane lipids and gene expression in the immune cells of salmon.

### *5.5.3. Global transcript expression of pIC-stimulated salmon MLCs*

SAM identified 3089 DEP responsive to pIC (i.e., 890 DEP overlapping with RP) within the FO7 group and 4745 DEP responsive to pIC (i.e., 1128 DEP overlapping with RP) within the FO5 group (Fig. 5.3.). With respect to the DEP overlapping between SAM and RP, 612 and 705 DEP were up-regulated by pIC within the FO7 and FO5 groups, whereas 278 and 423 DEP were down-regulated by pIC within the FO7 and FO5 groups, respectively. The number of microarray-identified, pIC-responsive probes in the current

study was higher than previously published studies on ISAV-infected salmon MLCs (Schiøtz et al., 2008; Workenhe et al., 2009). These differences may be influenced by the microarray platforms (i.e., a 44K in the current study vs. 16K or 1.8K in previous studies) used in the different studies. Furthermore, the inter-study variation may arise from the difference in cell types (e.g., primary cell culture vs. cell line) and stimulating agents (e.g., viral mimic vs. viral pathogen) used in the present study compared to these previous studies. As in the present study, the RNA-seq analyses, performed by Xu et al. (2015), showed a massive gene expression response [i.e., 3149 differentially expressed genes (DEG)] in IFN-treated Atlantic salmon macrophage/dendritic-like TO cells. Furthermore, the ratios of up-regulated to down-regulated transcripts by pIC in the microarray results of the present study are similar to those of pIC-stimulated cod macrophages (Eslamloo et al., 2016) and IFN-exposed salmon TO cells (Xu et al., 2015). The higher number of DEP responsive to pIC in the FO5 group compared with the FO7 group could be influenced by biological variability in basal transcript expression and/or the pIC response. There was a strong response to pIC for all of the microarray-studied samples, as samples belonging to the same stimulation group (i.e., PBS or pIC) clustered together.

#### *5.5.3.1. pIC-responsive transcripts with putative roles as PRRs or other receptors*

As identified by microarray analyses and validated by qPCR, pIC stimulation changed the expression of several transcripts encoding PRRs and other receptors in the salmon MLCs. The expression of *tlr9* (identified by microarray) and *tlr7* (studied by qPCR), known as the endosomal PRRs activating the MyD88-dependent pathway, was up-regulated by pIC in salmon MLCs. Mammalian TLR7 and TLR9 are responsible for

recognising ssRNA viruses and CpG-rich bacterial DNA/dsDNA viruses, respectively (Fig. 5.11.) (O'Neill et al., 2013; Sato et al., 2006), yet their functions are poorly understood in fishes (Palti, 2011; Zhang et al., 2014). Atlantic salmon TLR9 was shown to bind with synthetic oligonucleotides but in a CpG-independent manner, and this indicates the evolutionarily conserved feature of TLR9 binding to DNA (Iliev et al., 2013b). TLR3 is the main PRR detecting dsRNA in mammals and fishes (O'Neill et al., 2013; Zhang et al., 2014), even though the expression of its encoding transcript was not affected by pIC in salmon MLCs in the current study. This expression pattern was similar to *tlr3* in the spleen of pIC-injected Atlantic cod (Hori et al., 2012), but it does not agree with the results for Salmonid alphavirus (SAV)-infected TO cells (Xu et al., 2015). Nevertheless, the GO terms associated with TRIF (TIR-domain-containing adaptor protein inducing IFNB)-dependent TLR and TLR3 signalling pathways were over-represented in the pIC-responsive transcript list of the present study. This reveals that the TRIF-dependent pathway downstream of TLR3 was activated by pIC (see Fig. 5.11. for pIC-activated signalling pathways).

The up-regulation of RLR (RIG-I-like receptors) family members (i.e., *rig-i* alias *ddx58*, *lgp2* alias *dhx58*, and *mda5* alias *ifih1*) was observed in pIC-treated salmon MLCs (Supplementary Table S5.2.), similar to that reported in TO cells 48 h post-exposure to SAV (Xu et al., 2015) and RTG-2 cells (i.e., rainbow trout fibroblast-like cell line) 24 h after pIC stimulation (Chang et al., 2011). MDA5 and RIG-I are involved in mitochondrial-dependent recognition of dsRNA and ssRNA viruses in the cytoplasm

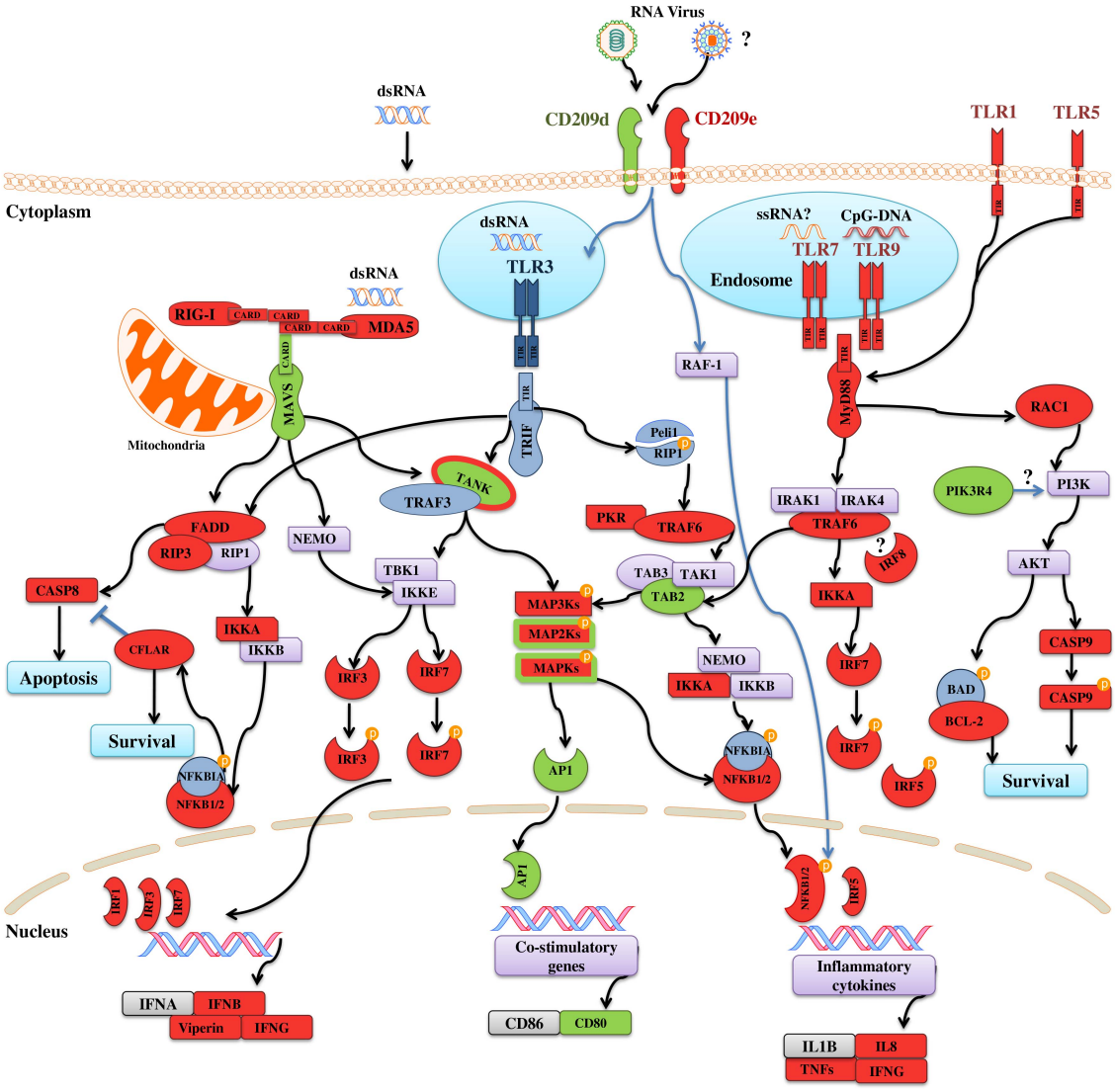


**Figure 5.11. The activated PRRs and signalling pathways by pIC in Atlantic salmon MLCs.** This figure was adapted from known mammalian pathways (Akira et al., 2006; Arthur and Ley, 2013; Bonjardim et al., 2009; Mocarski et al., 2012; Repnik et al., 2012; Sprokholt et al., 2016; Takeuchi et al., 2008; Yu and Levine, 2011). A question mark below or above the gene/protein name indicates that its regulating mechanism is unknown in mammals. The transcripts up- and down-regulated by pIC in the present study are drawn in red and green, respectively. The genes/proteins drawn in both red and green represent both the up- and down-regulation of different probes associated with them. The microarray result for *irf7* was only qPCR-validated for the FO7 group. MDA5 (melanoma differentiation-associated protein 5), RIG-I (retinoic acid-inducible gene),

LGP2 (RNA helicase LGP2), MAVS (mitochondrial antiviral-signaling protein), FADD (FAS-associated death domain), RIP1/3 (receptor-interacting protein 1/3), IKK (inhibitor of nuclear factor kappa-B kinase), NFKB1/2 (nuclear factor NF-kappa-B 1/2), NFKBIA (NF-kappa-B inhibitor alpha), CASP (caspase), CFLAR (casp8 and fadd-like apoptosis regulator), TNF (tumour necrosis factor), IL (interleukin), IFN (interferon), NEMO (NFKB1 essential modulator or IKKG), TLR (toll-like receptor), TRIF (TIR domain-containing adaptor protein inducing IFNB), TRAF (TNF receptor-associated factor), TANK (TRAF family member-associated NFKB activator), TBK (tank-binding kinase), IRF (IFN regulatory factor), MAP3K (mitogen-activated protein kinase kinase kinase), MAP2K (dual specificity mitogen-activated protein kinase kinase), MAPKs (mitogen-activated protein kinase), AP1 (transcription factor AP1), Peli1 (pellino E3 ubiquitin protein ligase 1), PKR (IFN-induced, double-stranded RNA-activated protein kinase), TAK1 [transforming growth factor beta (TGFB)-activated kinase 1 or MAP3K7], TAB (TAK1-binding protein), RAF-1 (serine/threonine kinase Raf-1), MyD88 (myeloid differentiation primary response gene 88), IRAK (IL1 receptor-associated kinase), RAC1 (ras-related C3 botulinum toxin substrate 1), PI3K (phosphoinositide 3-kinase), PIK3R4 (phosphoinositide 3-kinase regulatory subunit 4), AKT/PKB (protein kinase B), BAD (Bcl-2-associated death promoter). Orange circles show phosphorylation.

(Fig. 5.11.), whereas LGP2 is a positive or negative regulator of other RLRs (Akira et al., 2006; Takeuchi and Akira, 2008; Yu and Levine, 2011). Fish MDA5 and RIG-I have been suggested to exhibit evolutionarily conserved functions, but the molecular function of LGP2 in fishes is not fully understood (Zhang and Gui, 2012). The up-regulation of *lgp2* by pIC at 6 HPS observed herein shows the importance of this transcript in the early antiviral responses of salmon MLCs.

The current study identified some pIC-responsive transcripts that can facilitate the entrance of pathogens into cells. In this study, *cd209e* expression increased over time within both the pIC and PBS groups, and it was up-regulated (more than 3-fold) in response to pIC at 24 HPS. Conversely, *cd209d* was down-regulated by pIC at 24 HPS. CD209 acts as a PRR and facilitates the entry of pathogens into the endosomes, resulting in activation of MHC-I-dependent antigen presentation. Additionally, it modulates the TLR-dependent signalling pathway and promotes the DNA affinity of NFKB (Sprokholt et al., 2016; van Kooyk and Geijtenbeek, 2003). Zebrafish *cd209* was shown to be associated with several APCs and an important gene for adaptive immunity (Lin et al., 2009). The distinct regulation of salmon *cd209e* and *cd209d* by pIC stimulation seen in the present study suggests that these transcripts have distinct functions in the immune responses of salmon MLCs.

qPCR analyses showed a time-dependent up-regulation for *csf1r* and both paralogues of *scarb1* within the PBS groups, and significant suppression by pIC at 24 HPS. Mammalian SCARB1 is a high-density lipoprotein (HDL) receptor that changes the

cholesterol content of cell plasma membranes via mediating lipid transfer, but it can also be employed as a co-receptor for viral internalisation into host cells (Canton et al., 2013; Shen et al., 2014). Similar to the findings of the present study, zebrafish CD36 (a family member of SCARB) was down-regulated in response to bacterial infection (Fink et al., 2015). CSF1R is an important biomarker for teleost fish macrophage maturation (Hanington et al., 2009). Therefore, the present results suggest a suppressed macrophage maturation in the pIC group over time. Also, the down-regulation of salmon *csf1r* in pIC-stimulated MLCs may be attributed to SOCS1 (i.e., a pIC-induced transcript in this study; Fig. 5.8. B), as described for other fish species (Hanington et al., 2009). A soluble isoform of teleost CSF1R was found to be a regulator of inflammatory cytokines (Rieger et al., 2015). The different isoforms of CSF1R in salmon macrophages are yet to be structurally and functionally characterised.

The present investigation identified several pIC-responsive chemokine receptors in salmon MLCs (Supplemental Table S5.2.). Further, there was an up-regulation of salmon *cxcr3* in pIC-stimulated MLCs in the present study, and teleost *cxcr3* (e.g., in common carp, *Cyprinus carpio*) was previously reported to be a MCSF (Macrophage colony-stimulating factor)- and IFNG-induced transcript involved in macrophage trafficking and macrophage-mediated responses (Torraca et al., 2015; Wang et al., 2008). Contrary to the results for *cxcr3* in the present study, pIC strongly repressed *cmklr1* in salmon MLCs at 24 HPS (Fig. 5.7. I). Mammalian CMKLR1 mediates macrophage adhesion and migration as well as inflammatory responses (Yoshimura and Oppenheim, 2011), but its role in fish macrophages remains undescribed. In the current investigation,

the transcript expression results (i.e., positive or negative regulation), along with over-representation of GO terms associated with chemokine receptor activity and chemokine-mediated signalling pathway (see Table 5.4.), reveal the importance of different chemokine receptors in antiviral immune responses of salmon MLCs.

#### *5.5.3.2. pIC-responsive transcripts involved in signal transduction and transcriptional regulation*

The current study identified a large number of pIC-responsive transcripts involved in signal transduction and transcription control. The qPCR analyses showed both early and late up-regulation responses to pIC for several transcriptional regulators (i.e., *map3k8*, *socs1*, *socs3*, *dusp5*, *crem* and *irf7*), whereas other studied signal transductors and transcription factors (i.e., *traf5a*, *jak3*, *cytip*, *ikka*, *atf3*, *batf3* and *stat1*) were only up-regulated by pIC at 24 HPS (Figs. 5.8. and 5.9.). The qPCR assays showed that expression of *cd80*, *mapk13*, *dusp6* and *dusp22a* was suppressed by pIC stimulation at 24 HPS. As illustrated in Fig. 5.11., the pIC stimulation of salmon MLCs activated the MAVS (Mitochondrial antiviral-signalling protein)-, TRIF- and MyD88-dependent signalling pathways downstream of RLRs and TLRs. Opposite to the induction of *rig-i* and *mda5*, the expression of *mavs*, which plays a role as their adaptor, was down-regulated by pIC in salmon MLCs. The RLR pathway and MAVS activity appear to be conserved between fish and mammalian species (Poynter et al., 2015). Human *mavs* was down-regulated in pIC-stimulated glial cells, and its knockdown was associated with suppression of inflammatory cytokines (Wan et al., 2016). Accordingly, the inhibition of *mavs* in the present study may be related to the immunoregulatory functions of this gene.

In addition to this pathway, the microarray results (Supplemental Table S5.2.) showed an up-regulation for transcripts encoding signalling adaptors (e.g., TRAF6) and kinases [e.g., PKR (IFN-induced, double-stranded RNA-activated protein kinase)] that are known to trigger a series of events activating transcription factors. IKKs phosphorylate NFKB inhibitor, resulting in translocation of NFKBs into the nucleus and the production of cytokines and inflammatory proteins (reviewed by Kawai and Akira, 2007). The qPCR results for *ikka* expression (i.e., 3-fold up-regulation at 24 HPS), along with the identification of *nfkb1/2* as pIC-responsive transcripts by microarray analyses, indicate that NFKB-related transcription responses are important in the antiviral mechanisms of salmon MLCs. In agreement with a previous study on pIC-induced cod macrophages (Eslamloo et al., 2016), members of the IRF family (e.g., *irf1*, *irf3* and *irf7*) were slightly up-regulated (1.4- to 1.7-fold) by pIC in salmon MLCs. IRF7 and IRF3, known as the main family members involved in virus-associated responses, boost the transcription of *ifn*s and IFN-sensitive response element (ISRE)-containing genes (see Fig. 5.11. and 5.12.) following phosphorylation by IKKA or IKKE (Bonjardim et al., 2009; Tamura et al., 2008). Additionally, other microarray-identified IRFs (e.g., *irf8*) in this study were previously shown to promote IFN induction in mammals by interacting with adaptors in the MyD88-dependent pathway (reviewed by Tamura et al., 2008) (see Fig. 5.11.), and it suggests the importance of IRFs in antiviral immune responses of Atlantic salmon MLCs.

MAPK-dependent induction by the TLR pathway can play crucial roles in the innate immune and inflammatory responses (Arthur and Ley, 2013). The current microarray analyses identified multiple pIC-regulated transcripts at different levels of the

MAPK cascade (see Fig. 5.11.), suggesting that this pathway is activated in the innate antiviral immune responses of salmon MLCs. As validated by qPCR, *map3k8* was up-regulated by pIC at both the early and the late time points. Mammalian MAP3K8 regulates antiviral responses via IRF3 phosphorylation (Schmid and Sachs, 2014). MAPK13 (alias p38 delta), a kinase involved in inflammatory responses, stimulates important transcription factors such as AP-1 in mammalian macrophages (Yang et al., 2014). Therefore, the co-down-regulation of *mapk13* and *ap-1* by pIC (Fig. 5.11.), seen herein, suggests that *mapk13* function may be conserved in fish and mammalian macrophages. The activation of MAPKs is also managed by the DUSPs via negative feedback loops (Schmid and Sachs, 2014), and the present study revealed the negative (e.g., *dusp22a* and *dusp6*) or positive (e.g., *dusp5*) regulation of different members of the DUSP family in pIC-stimulated MLCs. In agreement with the current findings, a previous microarray analysis identified *dusp5* as a CpG- and LPS-induced transcript in mononuclear phagocytes of Atlantic salmon (Iliev et al., 2013a). Mammalian *dusp5* is an LPS- and MCSF-induced gene that can restrict macrophage differentiation (Schmid and Sachs, 2014). Similar to Atlantic salmon MLCs, pIC repressed the expression of *dusp6* in mammalian (Tan et al., 2013) and rainbow trout macrophages (Iliev et al., 2006), suggesting that *dusp6*'s role in inactivating the MAPKs of macrophages may be conserved between fish and mammals. DUSP22 was shown to be a negative regulator for STAT3 in cancer cells (Lin et al., 2015), but its role in antiviral responses is undetermined. While it appears that MAPKs and their regulating factors are crucial parts

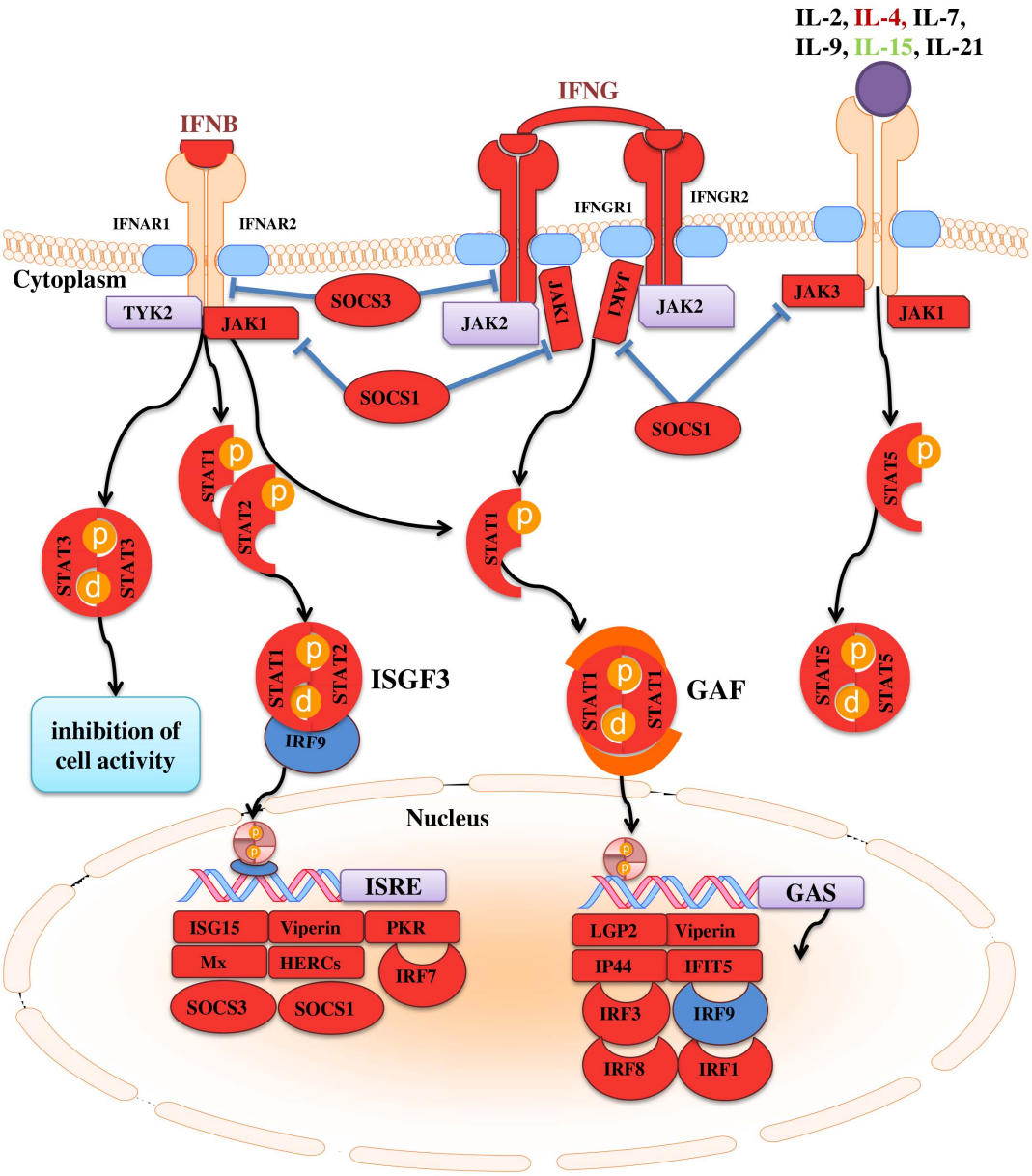


**Figure 5.12. The cytokine-mediated pathways activated by pIC in Atlantic salmon MLCs.** This figure was adapted from known mammalian pathways (Bonjardim et al., 2009; Kubo et al., 2003; O'Shea et al., 2004; Xie, 2013; Yu and Levine, 2011). The transcripts up- and down-regulated by pIC in the present study are drawn in red and green, respectively. The microarray results of *stat1* and *irf7* were only qPCR-validated for the FO7 group. IFNB/G (interferon beta/gamma), IFNAR (IFN alpha receptor), IL

(interleukin), IFNGR (IFN-gamma receptor), TYK2 (tyrosine kinase 2), JAK (Janus kinase), SOCS (suppressor of cytokine signaling), STAT (signal transducer and activator of transcription), IRF (IFN regulatory factor), ISGF3 (IFN-stimulated gene factor 3), ISRE (IFN-sensitive response element), GAF (IFNG-activated factor), GAS (IFNG-activated sequence), ISG15 (IFN-stimulated gene 15), LGP2 (RNA helicase LGP2), IP44 (IFN-induced protein 44-like), HERC (E3 ubiquitin-protein ligase HERC), IFIT5 (interferon-induced protein with tetratricopeptide repeats 5). Orange circles show phosphorylation.

of the antiviral responses of salmon MLCs, the precise functions of genes in this pathway are yet to be determined for fishes.

The current microarray-identified and qPCR-validated transcripts (e.g., up-regulation of *ifng*, *jak3* and *traf5a*), as well as the GO enrichment results (e.g., over-representation of cytokine/chemokine-mediated signalling pathway), reveal the IFN-induced responses in salmon MLCs by pIC stimulation. As depicted in Fig. 5.12., IFNG and IFNB elicit antiviral immune responses by up-regulating IFN-induced genes (Bonjardim et al., 2009; Ivashkiv and Donlin, 2014). In addition to *ifng* and *ifnb,* in the current study, pIC stimulation of salmon MLCs led to the co-up-regulation of *ifngr1*/*2* as well as kinases (i.e., *jak1*) and transcription factors (i.e., *stat1*, *stat2* and *stat3*) in the IFN pathway (Fig. 5.12.). Similar trends were reported for salmon *jak1*, *stat1* and *stat2* in IFN- and SAV-infected TO cells (Xu et al., 2015). Correspondingly, the pIC-activated IFN pathway in salmon MLCs is assumed to be conserved with higher vertebrates. Mammalian JAK3 and TRAF5 are well-established as kinases associated with receptors of several cytokines (e.g., IL2) (O'Shea et al., 2004) and as a signalling adaptor in cytokine-activated pathways (e.g., IL17) (Xie, 2013), respectively. The pIC-associated induction of *traf5a* and *jak3*, alongside the other microarray-identified pIC-responsive cytokines in this study (e.g., *il4*, *il12* and *stat5*; see Supplemental Table S5.2.), suggests the importance of cytokine-activated pathways in the antiviral responses of salmon MLCs. Additionally, qPCR analyses showed an up-regulation in immune-regulating factors (i.e., *socs1*/*3*, *crem*, *atf3* and *batf3*; Fig. 5.8. and 5.9.). Mammalian SOCS1 and SOCS3 bind to chemokine receptors and JAKs, thereby inactivating JAK/STAT

signalling (Kubo et al., 2003). These genes were shown to be LPS- and CpG-DNA-inducible in mammalian macrophages and a contributing factor in PAMP-induced hypersensitivity (Kubo et al., 2003). The induction of *socs1* and *socs3* by pIC in the current study, together with similar results previously reported for SAV-exposed TO cells (Xu et al., 2015), suggest that *socs1/3* of Atlantic salmon macrophages may display a conserved function with their orthologues in higher vertebrates (see Fig. 5.12.). Multiple alternatively spliced CREMs bind to promoters of cytokine genes, provoking gene repression or activation through methylation-dependent mechanisms (reviewed by Rauen et al., 2013). The regulatory role of BATF3 is chiefly linked to the development of APCs (Murphy et al., 2013). ATF3 was found to control IFN signalling and to repress PAMP-stimulated cytokine responses in mammalian macrophages (Khuu et al., 2007; Labzin et al., 2015). Consistent with the present study, Feng and Rise (Feng and Rise, 2011) characterised *atf3* as an evolutionarily-conserved and pIC-inducible transcript in Atlantic cod. Although the findings of the current study showed the involvement of cAMP-dependent factors such as *atf3* in the antiviral responses of salmon MLCs, the precise functions of these factors in teleost macrophages are yet to be investigated.

There was a down-regulation in *cd80* in salmon MLCs in response to pIC, but it was up-regulated in trout leukocytes following LPS stimulation (Zhang et al., 2009). CD80 is a co-factor on the surface of APCs that regulates T-cell proliferation through engagement with CD28 (Chen and Flies, 2013). Although CD80 is not a well-characterised protein in fishes, it has been shown to be functionally and structurally conserved in rainbow trout (Zhang et al., 2009). The results of the current study suggest

that the transcriptional regulation of salmon *cd80* may be similar to that of higher vertebrates since there was a co-down-regulation of *cd80* and *ap-1* by pIC in this study (Fig. 5.11.; Supplemental Table S5.2.). In contrast to *cd80*, the expression of *cytip* (alias *pscdbp*) was increased by pIC in salmon MLCs (Fig. 5.8. G). Mammalian CYTIP regulates T cell-APC adhesion in lymphocytes (Hofer et al., 2006). It appears that pIC stimulation changes the expression of the genes involved in the antigen presenting function of salmon macrophages.

#### *5.5.3.3. pIC-responsive transcripts with putative functions as immune effectors*

Figure 5.10. represents a subset of immune effectors activated through PRR- or IFN-mediated pathways in salmon MLCs. Mammalian RNF8 is responsible for ubiquitination of H2A in response to DNA damage (Chaurushiya et al., 2012). Interestingly, some viruses target RNF8 via phosphorylation-based degradation and enhance viral replication/transcription (Chaurushiya et al., 2012), indicating that RNF8 is important in the virus-host battle. The transcript expression results, along with the over-representation of histone H2A ubiquitination processes in the pIC gene list seen herein, may reflect the activation of the DNA repair pathway in salmon during the antiviral response. Additionally, there was a significant induction of *herc4* (2-fold increase; only microarray-identified), *herc3* and *herc6* in pIC-exposed salmon MLCs. These are members of a protein family containing HECT and RCC1 domains. Likewise, *herc4* expression increased in pIC-stimulated cod macrophages (Eslamloo et al., 2016) and ISAV-exposed salmon MLCs (Workenhe et al., 2009). Different HERCs (i.e., HERC5 in human or HERC6 in mice) in higher vertebrates were reported to be IFN-responsive and

E3 ligase proteins that play roles in the ISGylation process via interaction with ISG15 (Woods et al., 2014; Zhang and Zhang, 2011), thereby inhibiting viral replication. Despite the species-dependent E3 ligase activity of different HERCs, it remains unknown whether the members of this family in fish mediate ISGylation. As in the present study, there was an up-regulation of *mx* and *viperin* in rainbow trout monocyte/macrophage cells exposed to Chum salmon reovirus (CSV) (DeWitte-Orr et al., 2007), ISAV-stimulated salmon MLCs (Workenhe et al., 2009) and IFN-induced salmon TO cells (Sun et al., 2011). Similar to its mammalian orthologue, the induction of fish *viperin* occurs through the dsRNA-stimulated RLR pathway (Wang et al., 2014). Mammalian Viperin restricts viral replication via an unknown molecular mechanism (Helbig et al., 2011). Furthermore, mammalian Mx exhibits antiviral functions against several RNA viruses (e.g., interference with viral genome replication) (Haller et al., 2007), and a previously published study confirmed the inhibition of infectious pancreatic necrosis virus (IPNV) replication with salmon Mx (Larsen et al., 2004). Collectively, the above data suggest that pIC activates the PRR- and IFN-dependent antiviral agents in salmon MLCs.

This research showed that *sntb1* is induced (by more than 5-fold increase) in pIC-treated salmon MLCs at 24 HPS. SNTB1 modulates mammalian macrophage lipid efflux (Tamehiro et al., 2015), but its function in the antiviral responses is not well-understood. The qPCR assays showed a slight up-regulation of *optn* in pIC-exposed salmon MLCs (1.5-fold); this was, however, a lower fold-change than that seen in the microarray results. Mammalian OPTN is a virus- and pIC-induced protein that can inhibit virus-induced IFNB production (Mankouri et al., 2010). This study revealed the activation of

several apoptosis-related factors in pIC-stimulated salmon MLCs. Nonetheless, this induction was seen for both pro-apoptotic [e.g., *casp8* (*caspase 8*) and *casp9*] and anti-apoptotic (e.g., *bcl2*) agents (Fig. 5.11.; Supplement Table S5.2.). The expression of *ctsl1* increased more than 6-fold in pIC-triggered salmon MLCs at 24 HPS, whereas there was a down-regulation (a 0.4-fold decrease) of *ctsf* in salmon MLCs by pIC. Similarly, *ctsa* expression was lowered by pIC in cod macrophages (Eslamloo et al., 2016). CTSs can facilitate cell death by means of degradation of the anti-apoptotic proteins or activation of granule-mediated apoptosis (reviewed by Repnik et al., 2012). Moreover, CTSF influences the MHC-II pathway in macrophages via processing of Ii (Invariant chain) (Shi et al., 2000). The suppression of *ctsf* by pIC in the present study may be due to the involvement of this gene in a different molecular pathway (e.g., MHC-II). The expression of *cflar* was positively regulated by pIC at both early (by more than 2-fold) and late (by more than 3-fold) time points. CFLAR (alias cFLIP) controls cell apoptosis in mammals by inhibiting the CASP8-mediated pathway (Mocarski et al., 2012), but its function is not well-understood in fishes. More studies are needed to determine the PAMP-mediated regulation of apoptosis pathways in salmon MLCs.

## 5.6. Conclusions

I used various cellular and molecular approaches to determine the effects of different levels of dietary EPA+DHA on the antiviral immune responses of salmon MLCs. Although the fatty acid composition of the diets did not influence the cellular

functions of salmon MLCs, they changed the lipid class and n-3 and n-6 proportions of HKLs. The lower level of EPA+DHA (i.e., 1% vs. 1.4%) in the diet influenced the expression of some genes (i.e., *fabp4*, *psmb8* and *lgmn*) in salmon MLCs. The up-regulation of *fabp4* and *lgmn*, with putative inflammatory- or macrophage-related functions, in the higher vegetable oil diet group in this study suggests immunomodulatory effects of dietary n-6 fatty acid level on salmon macrophages. This study suggests *fabp4* and *psmb8* are important diet-responsive immune-related biomarkers for future studies. However, the results of the current *ex vivo*-based study do not necessarily reflect the dietary fatty acid-associated responses in Atlantic salmon at various ages. Thus, further *in vivo* and *ex vivo*-based investigations using a wider range of levels of dietary EPA+DHA, as well as various life stages, are suggested to broaden the current knowledge of immunomodulatory effects of dietary n-3 and n-6 fatty acids in salmon. The pIC-stimulated transcripts identified by microarray and validated by qPCR provide a better understanding of the molecular pathways activated by the antiviral response in salmon MLCs. These results showed that different TLR- and RLR-dependent signalling pathways (e.g., IRFs, NFKB, and STATs) are stimulated by pIC. Further, the present results indicate the importance of MAPKs and their associated regulators in signal transduction of PRR- and cytokine-mediated pathways in salmon. The present study also identified several IFN-induced immune effectors (e.g., *viperin* and *herc6*), which may play roles in the inhibition of viral replication. Since the current study utilised a viral mimic rather than a live pathogen, further studies are required to evaluate the effects of dietary EPA+DHA on salmon MLC responses to viral infections. Moreover, the pIC-

responsive genes identified in the present investigation should be functionally characterised to have a more comprehensive picture of their mechanistic roles in antiviral responses of salmon.

## 5.7. References


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# CHAPTER 6: Summary

## 6.1. Summary of the results

In the second chapter of this thesis, a version of which has been published as Eslamloo et al., (2016), I conducted research that aimed at developing a better understanding of the antiviral immune response of Atlantic cod macrophages. This study was conducted using a heterogeneous population of Atlantic cod macrophages, which may include different macrophage subsets (e.g., M1-and M2-like macrophages) (Grayfer et al., 2018). A molecular biomarker of macrophage maturation (*macrophage colony stimulating factor receptor*) was not assessed in this study, as its primers failed the quality control assays. However, in this study, the time-dependent up-regulation of pro-inflammatory biomarkers [e.g., *il1b* (*interleukin 1 beta*)] may suggest the activation of pro-inflammatory macrophages over time. Unfortunately, different populations of Atlantic cod macrophages are yet to be characterised. This study identified several polyriboinosinic polyribocytidylic acid (pIC)-responsive transcripts in Atlantic cod macrophages, and revealed that IFN (Interferon)- and cytokine-mediated signals downstream of the TLR (Toll-like receptor) /RLR (RIG-I-like receptor)-dependent pathways were activated by pIC. The Atlantic cod genome includes several paralogues for both evolutionarily conserved (e.g., *tlr7* and *tlr8*) and teleost-specific (e.g., *tlr22* and *tlr25*) *tlr*s (Solbakken et al., 2016). The down-regulation of *tlr22g* in Atlantic cod macrophages stimulated with pIC reflects the importance of this paralogue in macrophage pathogen recognition. Furthermore, this study detected transcripts with putative roles at various levels in immune pathways (e.g., receptors, transcription factors

and immune effectors), and showed that the genes encoding immune effectors responded more strongly to pIC compared to genes encoding receptors or transcription factors. The number of pIC-stimulated genes identified herein was lower than *in vivo* studies of the transcriptomic response of the spleen of Atlantic cod to pIC injection (Booman et al., 2014; Hori et al., 2012). However, the proportions of up- to down-regulated transcripts in my study and a previous study on antiviral response of Atlantic cod brain (Krasnov et al., 2013) were comparable. Unfortunately, 43% of the identified pIC-responsive transcripts in my study remained unknown (i.e., had no significant BLASTx hit) due to the fact that the majority of Atlantic cod transcripts are not fully characterised. However, the 255 known transcripts found herein provided a more complete picture of mechanisms involved in the antiviral responses of Atlantic cod macrophages, and these transcripts represent a valuable resource for future investigations of macrophage functions and the development of antiviral biomarkers in this species.

In the third chapter of my thesis, I fully characterised Atlantic cod *viperin* [i.e., an antiviral factor identified in Chapter 2 (Eslamloo et al., 2016) of this thesis as well as previously published studies (Hori et al., 2012; Rise et al., 2008)]. This study enhanced our knowledge of antiviral immune responses of Atlantic cod macrophages, acquired in Chapter 2. This research revealed that Atlantic cod *viperin* has a conserved nucleotide sequence and predicted protein structure, suggesting that this protein may play evolutionarily conserved roles in Atlantic cod. In addition, *viperin* was suggested as a maternal and weakly-expressed transcript during embryonic and early larval stages in Atlantic cod, and this indicates that this transcript may play roles (immune or non-

immune) in very early development of Atlantic cod. Among 19 different tissues subjected to qPCR, the strongest expression of Atlantic cod *viperin* was recorded in the blood, which is similar to other vertebrate species (Dang et al., 2010; Zhong et al., 2015). However, more studies are required to determine the functions of *viperin* in non-infected cells/tissues. In this chapter, Atlantic cod macrophages and various inhibitors were also used to develop an understanding of the signalling pathways involved in regulation of this gene. My results suggested that endosomal-TLR-mediated pIC recognition, PKR (Interferon-induced, double-stranded RNA-activated protein kinase), MAPK (Mitogen-activated protein kinase) and IFN pathways may be involved in transcriptional activation of Atlantic cod *viperin*. In addition, Atlantic cod *viperin* showed a comparable expression profile to the other antiviral biomarker genes (e.g., *interferon-stimulated gene 15*) and a distinct expression profile compared to an antibacterial and pro-inflammatory biomarker (*il1b*). This suggests that Atlantic cod *viperin* shares regulating pathways with other antiviral genes assessed herein and that the pathway inhibitors influenced their specific targets. The promoter region of Atlantic cod *viperin* contains putative binding sites for IFN-activated transcription factors [i.e., Interferon gamma-activated sequence (GAS) and Interferon-sensitive response element (ISRE)]. Based upon this research, it appears that the antiviral response of Atlantic cod *viperin* may be dependent upon the activated factors downstream of the IFN pathway. However, IFN-dependent induction of Atlantic cod *viperin* should be experimentally validated in future studies. In addition to *viperin* findings, the immune inhibition results of this chapter deepen our knowledge of the pathway activated by antiviral immune responses of Atlantic cod. I identified several

pIC-responsive and putative IFN-induced genes and predicted their regulating pathways in the second chapter. Pathway characterisation results of Chapter 3 verified that the immune factors (i.e., PKR, TLRs, MAPK and IFN), the transcripts of which were identified to be pIC-responsive in Chapter 2, play crucial roles in the pathogen recognition and signal activation of antiviral responses in Atlantic cod macrophages.

I aimed to broaden our understanding of pIC-responsive genes in Atlantic cod macrophages that was developed by the research in Chapters 2 and 3, to investigate the regulation of antiviral immune responses in Atlantic cod macrophages by non-coding RNAs. Therefore, in the fourth chapter of my thesis, a version of which has been published as Eslamloo et al., (2018), the microRNAs (miRNAs) involved in the antiviral immune response of Atlantic cod macrophages were profiled. This was the first report on immune-responsive miRNAs in this species, and it identified ten teleost-specific (e.g., miR-731-3p and miR-462-3p) or evolutionarily-conserved (i.e., immune-responsive in fishes and mammals; e.g., miR-125b-3-3p and miR-150-3p) miRNAs involved in the antiviral immune response of Atlantic cod. As in other teleost species (Andreassen and Høyheim, 2017; Bela-ong et al., 2015), my results revealed that miR-731 and miR-462 are clustered in the genome of Atlantic cod and co-expressed in response to pIC stimulation. Although the promoter region of the miR-731/miR-462 cluster contained putative binding sites (e.g., GAS) for some important immune-related transcription factors, the association of these predicted transcription factor binding sites (TFBSs) with the expression of this miRNA cluster must be experimentally validated in the future. The qPCR assays in this chapter showed that the strongest antiviral response of miRNA

occurred between 48-72 h post-stimulation (HPS). In addition, some identified Atlantic cod miRNAs (e.g., miR-214-1 and miR-199-1) were located in the same gene cluster, but showed a different expression pattern in response to pIC. This suggests that these miRNAs may be subjected to a post-transcriptional regulation as in other vertebrates (Thomson et al., 2006). Finally, some putative mRNA targets were identified for pIC-responsive miRNAs of the Atlantic cod. The target identification approaches linked the results of this chapter to the pIC-responsive genes identified by Chapter 2, and provided a preliminary knowledge of miRNA-mRNA interactions of antiviral responses in Atlantic cod macrophages. The majority of the predicted target genes showed a similar fold-change direction to the miRNAs, suggesting a putative gene expression regulation at the translational level by miRNAs (Chekulaeva and Filipowicz, 2009). However, *herc4* (*probable E3 ubiquitin-protein ligase herc4-like*), identified as pIC-induced in Chapter 2, was predicted to be targeted by miR-30b-3p, and their inverse pIC response suggests that miR-30b-3p may regulate the expression of *herc4* through mRNA degradation (Huntzinger and Izaurralde, 2011). The improved characterisation of the Atlantic cod transcriptome in the future may enhance the target gene prediction analyses for pIC-responsive miRNAs in this species.

The fifth chapter of my thesis, a version of which has been published as Eslamloo et al., (2017), used pIC stimulation and an *ex vivo* model to determine if two diets with different levels of dietary n-3 fatty acids alter the antiviral immune response of Atlantic salmon macrophage-like cells (MLCs). The eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA) contents of the practical diets were designed to be higher

(FO7: 7.4% fish oil, 1.4% EPA+DHA) and lower (FO5: 5.1% fish oil, 1% EPA+DHA) than the EPA+DHA level (i.e., 1.25%) that is needed for the optimal growth of Atlantic salmon. The results revealed that a 30% decrease in dietary EPA+DHA influences the lipid classes and fatty acid composition of n-3 and n-6 proportions of Atlantic salmon head kidney leukocytes (HKLs). As identified by microarray and validated by qPCR, different levels of n-3 fatty acids in the diet changed the expression of some transcripts [i.e., *fabp4* (*fatty acid-binding protein adipocyte*) and *psmb8* (*proteasome subunit beta type-8*)] in Atlantic salmon MLCs. Since these transcripts are known to play roles in immune or inflammatory responses (Groettrup et al., 2010; Makowski et al., 2005), I believe that they are important fatty acid-dependent immune-related biomarker genes for Atlantic salmon. With respect to the immunomodulatory functions of the n-3 and n-6 fatty acids (Kiron, 2012; Trichet, 2010), the transcriptional response of the Atlantic salmon MLCs may be associated with the fatty acid content of these cells. Given the diet-dependent transcriptional changes in Atlantic salmon MLCs that were observed, I speculate that a larger difference in n-3 fatty acid content of Atlantic salmon diets may have a larger effect on fatty acid composition of the membrane of the immune cells, and therefore, trigger a stronger immune-related gene expression response. As the pIC-responsive transcriptome profiles of MLCs in both dietary groups were comparable overall, the present investigation suggests that diets containing 5% fish oil (i.e., 1% EPA+DHA) may be used for Atlantic salmon farming without a large effect on immune-related global gene expression. In addition to the evaluation of fatty acid-dependent responses, this chapter profiled the transcriptome responses of Atlantic salmon MLCs to

pIC. Microarray and qPCR analyses identified several transcripts with putative roles as receptors, transcription factors and immune effectors involved in RLR-, TLR-, MAPK- and IFN-associated pathways. Transcripts encoding pathogen recognition receptors (e.g., *tlr7* and *lgp2*) were up-regulated by pIC, but *csf1r* (*macrophage colony stimulating factor 1, receptor*), a biomarker for macrophage maturation (Hanington et al., 2009), was suppressed by pIC stimulation in both dietary groups. This research revealed that pIC either suppresses or induces a large number of transcripts involved in MAPK pathway; this result suggests that this pathway is important in the antiviral immune responses of Atlantic salmon MLCs. Finally, this chapter showed that different members (e.g., *cd209e* and *cd209d*) of a gene family may respond differently to pIC stimulation in Atlantic salmon MLCs. The results of this chapter paint a broader picture of transcripts and pathways playing roles in the antiviral immune response of Atlantic salmon MLCs.

### 6.2. Comparison of antiviral responses in Atlantic cod and Atlantic salmon

In the second and fifth chapters of my thesis, the pIC-responsive transcripts of Atlantic cod macrophages and Atlantic salmon MLCs were identified. Both transcriptome analyses were conducted on pIC-stimulated cells at 24 h post-stimulation. In the Atlantic cod macrophages, 446 (i.e., 285 up-regulated and 161 down-regulated) pIC-stimulated differentially expressed probes (DEP) were identified using Significance Analysis of Microarrays (SAM) analysis and a 20K microarray platform. Using a 44K salmonid platform, SAM and Rank Products (RP) identified 3089 and 910 DEP, respectively, in pIC-stimulated Atlantic salmon MLCs of the FO7 group (i.e., 890 overlapping DEP; 612 up-regulated and 278 down-regulated). The number of DEP by

pIC was higher in the Atlantic salmon, but the percentages of down-regulated transcripts (~33%) in both species were comparable. Although the salmon microarray platform contained a larger number of features compared to the Atlantic cod microarray, the number of probes passing thresholds and subjected to the differential expression analyses was relatively close (i.e., 12,983 probes in Atlantic salmon vs. 11,797 probes in Atlantic cod) in both studies. The larger number of pIC-identified transcripts in Atlantic salmon may be attributed to its pseudotetraploid genome (Lien et al., 2016; Quinn et al., 2008) and regulation of various paralogues of a gene by pIC. In both species, pIC induced the transcription of pattern recognition receptors (PRRs) in endosomal- (e.g., *tlr7*) and mitochondrial-dependent (e.g., *mda5*, *melanoma differentiation-associated protein 5*) pathways. Furthermore, in both species, pIC influenced the expression of genes with putative functions in signal transduction and transcription activation downstream of TRIF (TIR-domain-containing adapter-inducing interferon-beta)- and MyD88 (Myeloid differentiation primary response gene 88)-dependent pathways. The pIC induction of both types of *ifns* was seen in Atlantic salmon, whereas only type II *ifn* was found to be stimulated by pIC in Atlantic cod. The results of these chapters showed that pIC alters the expression of several putative IFN-induced transcripts (e.g., *viperin* and *lgp2*) in Atlantic salmon MLCs and Atlantic cod macrophages. The qPCR assays revealed that the pIC response of Atlantic salmon MLCs and Atlantic cod macrophages starts at 6 and 12 HPS, respectively. In addition, these microarray and qPCR studies identified several pIC-induced putative immune effectors. However, pIC stimulation caused a stronger fold-change response of immune effectors in Atlantic cod macrophages (e.g., 20- and 45-fold

induction for *ifng* and *viperin,* respectively) in comparison with Atlantic salmon MLCs (e.g., 5.5- and 3.7-fold induction for *ifng* and *viperin,* respectively). Overall, the results of these two chapters suggest that the macrophages of these two species share some similar pathways (e.g., TLR-, TRIF-, IFN-dependent pathways) that are activated by pIC stimulation.

### 6.3. Perspectives and future research

Although multiple *in vivo* or *in vitro*-based studies have used immunogenomics to develop a better understanding of antiviral immune responses in Atlantic cod (Eslamloo et al., 2016; Holen et al., 2012; Hori et al., 2012; Hori et al., 2013; Krasnov et al., 2013; Rise et al., 2008; Rise et al., 2010), some aspects of the antiviral immune responses of this species remain unknown. For example, this thesis was the first study that used a 20K probe microarray to profile the antiviral transcriptome response of Atlantic cod macrophages, but the antiviral responses of various immune-related cells (e.g., dendritic cells, T- and B-lymphocytes) are yet to be examined. The 20K Atlantic cod microarray used in the current study is a valuable and trusted genomics resource generated by the Atlantic Cod Genomics and Broodstock Development Project. However, since the Atlantic cod genome and transcriptome are now characterised, the future immunogenomics studies in this species may involve the use of RNAseq. Chapter 2 used pIC to evaluate the antiviral responses of Atlantic cod macrophages. However, with respect to pathogen-host interactions (Zhang and Gui, 2015), pIC-triggered immune responses may be different than exposure to a pathogenic virus, and further studies need to determine the transcriptome profile of Atlantic cod macrophages in responses to

different viruses (e.g., nodaviruses). Several transcripts identified herein remain unknown (Eslamloo et al., 2016), and a larger number of fully-characterised Atlantic cod transcripts may enhance our understanding of the results of the second chapter in the future. The antiviral immune responses of fishes are regulated through several molecules such as type I and II IFNs (Zhang and Gui, 2012), and the recombinant proteins of these immune regulators identified herein (e.g., IFNG) may be used in future *in vitro* studies to develop a better knowledge of their immune function in Atlantic cod macrophages. Also, several immunology tools are yet to be developed for Atlantic cod. For example, the establishment of immune-related cell lines (e.g., macrophages) for Atlantic cod may facilitate the immunological studies with this species. Finally, considering the lack of important elements in the antigen presentation system (e.g., Major histocompatibility complex II, MHC-II) of Atlantic cod (Star et al., 2011) and the importance of macrophages as antigen-presenting cells (APCs) (Jakubzick et al., 2017), Atlantic cod macrophages may be employed in future studies to determine the antigen presentation and T-cell activation pathways of this species.

My third chapter is focused upon *viperin* as an important ISG identified in Chapter 2. Although this study characterised this gene, and identified the putative activation pathway of Atlantic cod *viperin*, the IFN inducibility and the putative TFBSs of Atlantic cod *viperin* must be experimentally (i.e., luciferase assay) validated in future studies. This study was conducted at the transcript level; however, Atlantic cod Viperin antiviral response at the protein level may be different and must be determined by future studies. Mammalian Viperin functions (e.g., viral inhibition) have been associated with

its localisation in the endoplasmic reticulum (ER)-derived lipid droplets (Fitzgerald, 2011). Development of a monoclonal antibody for Atlantic cod Viperin in the future may facilitate studies (e.g., immunoblotting and immunohistochemistry) and determine if this protein in Atlantic cod has conserved functions. Furthermore, gene silencing and overexpression assays, alongside viral challenge experiments, should be used in studies to test if Atlantic cod *viperin* exhibits antiviral activity. While this chapter focused on the characterisation and expression analyses of an important Atlantic cod antiviral gene, many other immune-responsive genes that are not well-characterised in cod, but have well-studied immune functions in other vertebrates, were identified. The future characterisation of these genes will be important in determining whether they have conserved structures and functions.

Immune-responsive miRNAs were previously identified in several teleost species (Andreassen and Høyheim, 2017), and my fourth chapter is the first report on miRNAs involved in antiviral immune responses of Atlantic cod. Nonetheless, several aspects of miRNA-associated immune responses in this species are yet to be discovered. Teleost-specific miR-451-3p and miR-731-3p have been functionally studied in some species (Bela-ong et al., 2015; Zhang et al., 2016), and the gene regulatory roles of these miRNAs can be tested in future *in vitro* assays for Atlantic cod. As well, host-virus miRNA interactions have been documented for mammalian species (Ghosh et al., 2008), and further investigations should evaluate if the miRNAs discovered herein interfere with viral replication in Atlantic cod.

The fifth chapter of this thesis shows that different levels of dietary EPA+DHA led to changes in the gene expression response of Atlantic salmon MLCs, and identified some biomarker genes involved in fatty acid-related immunomodulation in this species (Eslamloo et al., 2017). The diets used in this study had comparable effects on the pIC response of Atlantic salmon MLCs. However, a larger difference in dietary EPA+DHA, or longer feeding trials, may result in stronger long chain-polyunsaturated fatty acid (LC-PUFA)-dependent gene expression responses in Atlantic salmon. The diets used here were designed based upon the minimum EPA+DHA requirement of Atlantic salmon, and further study on other species may develop a more comprehensive understanding of LC-PUFA-related immune responses. The *ex vivo* and viral mimic (pIC) stimulation approaches used herein may generate different results in comparison with *in vivo* studies and viral challenges. Therefore, future studies should evaluate whether different levels of dietary EPA+DHA can influence the antiviral response to pIC injection or viral infection. Two EPA+DHA-dependent immune-related transcripts (*fabp4* and *psmb8*), identified herein, can be used for future gene overexpression/suppression analyses to determine their functions in fatty acid-dependent immune and inflammatory responses. The links between the fatty acid composition of the cell membrane and the immune responses of mammals were previously determined through *in vitro* studies (Calder, 2008). Given that fatty acid-related immune responses may be species-dependent, future studies in Atlantic salmon should use *in vitro*-based experiments to manipulate the fatty acid composition of teleosts cells (e.g., macrophage cell line) and determine ratios of fatty acids (e.g., n-3 to n-6 fatty acids) in the teleost cell membrane that are optimal for antiviral or antibacterial

immune responses. Furthermore, since fatty acids alter pathogen recognition, further studies may be conducted using various immune inhibitors to evaluate which pathogen recognition pathways are influenced by fatty acids. The microarray results of Chapter 5 identified a large number of pIC-stimulated genes, several of which contain multiple paralogues in Atlantic salmon. The characterisation of these genes, and paralogue-specific studies, may broaden our current knowledge of the structure, regulation and functions of these genes. Finally, comparative immunological studies in the future may determine impact of gene duplication on immune responses in different fish species.

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