Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Preclinical Sciences
and Pathology (PREPAT)

Philosophiae Doctor (PhD)
Thesis 2021:59

Immune responses in Atlantic salmon (*Salmo salar*) following vaccination and Salmonid alphavirus challenge

Immunresponser i atlantisk laks (*Salmo salar*) etter vaksinasjon mot og smitte med Salmonid alphavirus

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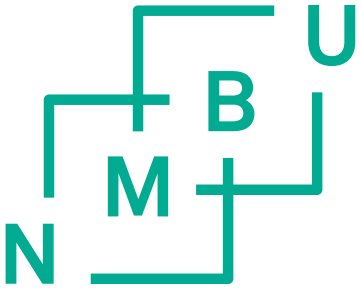

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“We cannot solve our problems with the same thinking we used when we created them.” -
Albert Einstein

CONTENTS

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Fagerstrand, Mai 2021

Anne Flore Bakke

List of papers

Paper I

A time-course study of gene expression and antibody repertoire at early time post vaccination of Atlantic salmon

Authors: Hege Lund, **Anne Flore Bakke**, Ingunn Sommerset, Sergey Afanasyev, Geir Schriwer, Audur Thorisdottir, Preben Boysen, Aleksei Krasnov

Published: Molecular Immunology, vol. 106, p. 99-107, 2019

Paper II

IgM+ and IgT+ B Cell Traffic to the Heart during SAV Infection in Atlantic Salmon

Authors: **Anne Flore Bakke**, Håvard Bjørgen, Erling Olaf Koppang, Petter Frost, Sergey Afanasyev, Preben Boysen, Aleksei Krasnov and Hege Lund.

Published: Vaccines, 2020, *8*(3), 493; https://doi.org/10.3390/vaccines8030493

Paper III

Effect of two constant light regimen on antibody profiles and immune gene expression in Atlantic salmon following vaccination and challenge with Salmonid alphavirus

Authors: **Anne Flore Bakke**, Alexander Rebl, Petter Frost, Sergey Afanasyev, Kristoffer Alnes Røyset, Tina Søfteland, Hege Lund, Preben Boysen, Aleksei Krasnov

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Summary

Norway has perfect conditions for Atlantic salmon (*Salmo salar*) production and is one of the largest producers of salmon for food consumption worldwide, but rearing fish at high densities also means that infectious disease agents can easily transmit between individuals. Vaccines have been an essential contributor to the steady increase in Atlantic salmon production over the last decades by reducing the severity and cases of diseases. To further increase vaccine efficacy and fish welfare, an increased knowledge about immune responses after vaccination and challenge is essential. This PhD work aimed to describe immune responses in Atlantic salmon following vaccination with a multivalent vaccine and challenge with Salmonid alpha virus (SAV).

Our results show that vaccination induces the production of vaccine specific antibodies, and that seroconversion occurs at around 2-4 weeks post vaccination. Additionally, both vaccination and infection with SAV induce the production of non-specific, presumably polyreactive, antibodies. After we compared the serology assays data and gene transcript data from head kidney in paper I, a dissimilarity in the levels of immunoglobulin (Ig) transcripts and soluble Ig protein were evident, and in context with Ig sequencing of the CDR3 region (IgSeq) indicated a traffic of Ig producing B cells from head kidney in response to vaccination. Later results indicated a transfer of B cells from spleen to heart in response to SAV infection, and that this traffic is earlier in vaccinated individuals. Furthermore, results showed a colocalization of B cells and SAV transcribing cells, indicating that B cells traffic to the site of SAV replication to perform their function.

Lastly, the effect of constant light (CL) relative to vaccination were evaluated. Our results showed that the CL exposure highly influenced the gene transcript profile, and the results indicated that all fish experienced an immune suppression shortly after onset of CL. Gene transcript profiles also indicated that the vaccinated groups were more similar in their immune responses against SAV, regardless of the CL treatment prior to vaccination. In summary, our results indicate that vaccinated fish has an advantage against SAV infection and that they are less affected by the duration of CL prior to vaccination.

Sammendrag (Summary in Norwegian)

Norge har perfekte forhold for produksjon av Atlantisk laks (*Salmo salar*) og er en av verdens største produsenter av laks, men oppdrett av fisk ved høy tetthet betyr imidlertid også at smittsomme sykdommer lett sprer seg mellom individer. Det har vært en jevn økning i produksjon av laks de siste tiårene, og vaksinering har vært en viktig bidragsyter til dette. Vaksiner har bidratt ved å redusere alvorlighetsgraden og antall tilfeller av ulike sykdommer. For å ytterligere øke vaksineeffektivitet og fiskevelferden er det viktig med mer kunnskap om immunresponser etter vaksinering og infeksjoner. Dette doktorgradsarbeidet hadde derav som mål å beskrive immunresponser i Atlantisk laks etter vaksinering med en multivalent vaksine og etter smitte med Salmonid alphavirus (SAV).

Våre resultater viste at vaksinasjon fører til produksjon av vaksinespesifikke antistoffer, og at serokonvertering skjer omtrent 2-4 uker etter vaksinasjon. I tillegg fører både vaksinasjon og SAV infeksjon til produksjon av ikke-spesifikke, antagelig polyreaktive, antistoffer. Etter å ha sammenlignet serologianalyse- og gentranskripsjon resultater fra hodenyrene i artikkel I så vi en forskjell i nivåene av immunglobulin (Ig) transkripter og løselig Ig-protein, som i sammenheng med Ig-sekvensering av CDR3-regionen (IgSeq) indikerte en trafikk av Ig-produserende B-celler vekk fra hodenyren i respons på vaksinasjon. Senere viste vi der er en trafikk av B-celler fra milt til hjerte i respons på SAV-infeksjon, og at det foregikk tidligere hos vaksinerte individer. En samlokalisering av Ig produserende B-celler og SAV-transkriberende celler innad i hjerte-vevet indikerte også at B-celler flytter seg til stedet for SAV-replikering for å utføre deres funksjon.

Til slutt ble effekten av konstant lys (CL) i forhold til vaksinasjonstidspunkt evaluert. Resultatene våre viste at CL-eksponeringen i stor grad påvirket gentranskript-profilen til fisken og at alle fisker opplevde en immunsuppresjon kort tid etter CL eksponering. Gentranskripsjon resultater fra de vaksinerte fiskene viste fisk med lignende transkripsjonsprofiler, som indikerer at vaksinert fisk hadde en mer lik immunrespons mot SAV, uavhengig av CL-eksponeringen før vaksinasjon. Kort oppsummert indikerer resultatene at vaksinert fisk har en fordel mot SAV og at de på sikt er mindre påvirket av lengden av konstant lys før vaksinasjon.

1 Introduction

1.1 Background

In Norway, it is common to vaccinate Atlantic salmon prior to sea-water transfer, often with vaccines targeting a combination of different pathogens to safeguard that adequate vaccine efficacy is achieved by the time the fish is transferred to sea. The infectious agents against which the fish are vaccinated is based on the prevalence of diseases in the seawater area to which the fish will be transferred, therefore several mono- and multivalent vaccines covering a combination of different pathogens are currently on the market.

Vaccination against several bacterial pathogens have led to a large decrease in the prevalence of diseases in Norwegian farmed salmon, such as vibriosis and furunculosis, and at the same drastically reduced the use of antibiotics (reviewed by (Håstein, Gudding et al. 2005)). In comparison, vaccination against virus and intracellular bacteria is still a challenge (reviewed by (Dahle and Jørgensen 2019)). Vaccines have been an essential contributor to the steady increase in Atlantic salmon production over the last decades, and vaccine strategies and responses have been extensively explored (Secombes 2008, Wang, Secombes et al. 2013, Munang'andu, Mutoloki et al. 2014, Yamaguchi, Quillet et al. 2019). However, there are still many aspects of the teleost immune system that remain unexplored.

The class of teleost consists of a large and diverse group of species, where the immune system has so far been characterized in only a few species. They are also the first animal phyla to possess both an innate and adaptive immune system which should make them capable of developing an immune response like what we know from mammals, but they also have some clear dissimilarities which makes them a highly interesting group for study of the development of the immune systems (reviewed by (Magnadottir 2010)). Recent studies on fish immune system have also overturned old paradigms and led to the discovery of novel aspects of mammalian immunity, such as the phagocytic abilities of primary B cells (reviewed by (Sunyer 2013)). A better understanding of the salmonid immune responses after vaccination and infection will

be an important contributor to compliment fish welfare strategies for the future and in the fight against pathogens.

1.2 Features of the teleost immune system

Lymphoid structures in teleost

Immune responses are characterized by a well-organized and structured cooperation between several cell types and soluble compounds, which communicate on several different levels and in distinct compartments. Fish represents a diverse and successful group of vertebrates. Many features from the fish immune system are homologous to structures and genes known from the mammalian immune system, but with a varying degree of similar function.

One large difference is arrangement of lymphoid structures. The mammalian immune system is mostly developed within special lymphoid structures known as primary lymphoid organs (bone marrow and thymus) and secondary lymphoid structures (tonsil, lymph nodes, spleen etc.), which are the site of B- and T-cell maturation and the initiation of the immune response, respectively. However, some of these lymphoid structures lack in fish, or other organs may perform similar functions.

Teleost lack both bone marrow and lymph nodes, which in mammals are important lymphoid structures that are essential for development of immune responses, but the anterior part of the fish kidney (the head kidney) is considered to be a functional ortholog to the mammalian bone marrow (Zapata and Amemiya 2000). At present, no known equivalent to mammalian lymph nodes and germinal centers are known in teleost, which is an important site of antibody affinity maturation. A cluster of melanomacrophages, which in fish are normally located in the stroma of the haemopoietic tissue of kidney and spleen, have been suggested to be analogous to lymphoid follicles or germinal centers found within mammalian lymph nodes (reviewed by (Agius and Roberts 2003)). Mucosal surface of a fish is the first line of defense and four mucosal associated lymphoid structures (MALTs) have been described in fish; nose-associated lymphoid tissue (NALT), skin-associated lymphoid tissue (SALT), gill-associated lymphoid tissue (GALT) and gut-associated lymphoid tissue (GALT) (Parra, Reyes-Lopez et al. 2015, Smith, Rise et al. 2019). Despite the lack

of important immunological structures, teleosts still have a functional immune system with many similarities to the mammalian immune system.

Innate immune system – the first line of defense

The first line of defense against invading pathogens is the innate immunes system, which is an evolutionary ancient system present in both invertebrates and vertebrates. The innate immune system is a defense mechanism that occur immediately or shortly after encountering a foreign antigen, but despite an instant response it can often be of short duration. The teleost innate immune system possesses most, if not all, of the elements found in the mammalian innate immune system (reviewed by (Magor and Magor 2001, Magnadóttir 2006, Sunyer 2013), and the innate immune system of fish can be divided into three; mucosal/physical barrier, cellular component and humoral parameters.

The physical barrier includes the fish skin (e.g. scales and mucus), gills and epithelial layer of the gastrointestinal tract (Magnadóttir 2006). Mucus from fish plays a critical role in neutralizing pathogens, and contains lectins, lysozymes, complement proteins, and antimicrobial peptides (AMPs) (Fast, Sims et al. 2002, Ángeles Esteban 2012). If a pathogen makes it through the physical barriers, it will encounter the cellular and humoral part of the innate immune system. Innate immune responses are initiated through the recognition of conserved molecular or structural part on the pathogen. These conserved molecular or structural part are known as pathogen-associated molecular patterns (PAMPs), and include bacterial lipopolysaccharides (LPS), bacterial DNA, double stranded viral RNA (dsRNA) and bacterial flagellin, among others. PAMPs are recognized through the binding of pathogen recognition receptors (PRR), mainly found on cells of the innate immune system like dendritic cells, macrophages, monocytes, neutrophils, and epithelial cells. Many components of the innate immune signaling system are conserved between mammals and teleosts, with clear orthologous relationship, and PRR is one of them (Aoki, Takano et al. 2008). In mammals, several groups of PRRs are known, e.g. Toll-like receptors (TLRs), retinoic acid inducible gene I (RIG-I)-like receptors (RLRs) and C-type lectins (CLRs). Groups of PRRs that have been identified in fish thus far include TLRs, CLRs and complement nucleotide-binding domains and LRR proteins (NLRs). However, some difference between mammalian and teleost PRRs exists. For example, TLR 4 which in best known for its recognition of

lipopolysaccharide (LPS) a component in many gram-negative and some gram-positive bacteria, has been lost from the genome of most fishes (reviewed by (Palti 2011)) . Additionally, innate immune molecules like lectins, complement and natural killer cell receptors are more diverse in teleosts than in mammals (Sunyer, Zarkadis et al. 1998, Vasta, Nita-Lazar et al. 2011, Yoder and Litman 2011). Another important feature of the innate immune cells is their ability to distinguish between infectious non-self and non-infectious self, which are an important feature which enables homeostasis and not a state of constant inflammation.

Humoral innate immune parameters in fish include the complement system, lysozyme, antimicrobial peptides, and acute phase proteins. The complement system is responsible for elimination of pathogens, promotion of inflammatory responses and clearance of apoptotic cells etc. (Nakao, Tsujikura et al. 2011). Three biochemical pathways activate the mammalian complement system: the classical pathway, the alternative pathway, and the lectin pathway. In mammals, the complement system consists of about 30 distinct proteins and membrane-associated proteins, and almost all of them have teleost homologues (Boshra, Li et al. 2006). However, if all of them have identical function in fish and mammals remains unknown.

Lysozyme is a lytic enzyme that performs bacterial lysis by hydrolyzing glycoside bonds on the peptidoglycan layer of the bacterial wall. The two types of lysozymes described in vertebrates, C- and G-type lysozymes, have also been reported in Atlantic salmon (Myrnes, Seppola et al. 2013). Antimicrobial peptides (AMPs) protect against a variety of pathogens by disrupting actions against bacterial membranes. Fish express all of the major class of AMPs, including defensines, cathelicidins, hepcidins, histone-derived peptides, and a fish-specific class of cecropin family, called piscidins (reviewed by (Masso-Silva and Diamond 2014)). Lastly, as in mammals, fish acute phase protein (APPs) are secreted into the blood stream in response to tissue damage, infection, and inflammation by different immune cells. APPS are proteins which serve as inhibitors or mediators of the developing inflammatory response. Some of the most well-known mammalian APPs such as C-reactive protein (CRP) and serum amyloid proteins (SAP) have been identified in Atlantic salmon (Lee, Bird et al. 2017).

Teleosts inhabit a large diverse repertoire of innate immune molecules indicating that the innate immune system plays an important role in teleost's fight against pathogens

(Sunyer, Zarkadis et al. 1998, Vasta, Nita-Lazar et al. 2011, Yoder and Litman 2011). It is often said that the mammalian immune system is more evolved than the teleost immune system. However, a more correct phrase would perhaps be that the immune systems of different organisms are better adapted to the environment where they live.

Initiation of the adaptive immune system

In contrast to the germline encoded innate immune system, the adaptive immune system is an acquired immune system which develops and adapt during the lifespan of an individual. From mammals we know that the adaptive immune system consists of B and T lymphocytes which carry out the humoral and cell-mediated immune response activities, respectively. Mammalian T cells and B cells are developed in the thymus and bone marrow, respectively. The teleost adaptive immune system also consists of T and B cells. In teleost the head kidney is considered a primary lymphoid organ, an equivalent to the bone marrow, and the site of the B cell development (Zapata and Amemiya 2000). Teleost T cells develop in thymus, like in mammals.

All vertebrates have a special collection of genes in their genome encoding for cell surface proteins essential for the adaptive immune system, some of which codes for the major histocompatibility complex (MHC) proteins. Antigen presentation by MHC molecules is an essential part of the adaptive immune response and they are divided into MHC class I, MHC class II and MHC class III. Class I and II are mainly expressed on cell surfaces, while class III mainly consist of soluble molecules. The presence of fish MHC molecules was first reported in 1990 in carp (Hashimoto, Nakanishi et al. 1990), and both MHC class I and II have now been reported in several fish species and believed to function in the same way as in mammals. However, on exception is the lack of MHC class II genes in Atlantic cod (Star, Nederbragt et al. 2011). Nevertheless, they have a higher number of MHC I genes and a unique composition of TLR families and are not exceptionally susceptible to disease under normal conditions.

MHC Class I molecules are found on almost all nucleated cells, while MHC class II are normally found on professional antigen-presenting cells (APC) such as dendritic cells, mononuclear phagocytes, and B cells. MHC class I and class II present peptides derived from cytosolic and extracellular proteins, respectively. Thus, professional APCs must inhabit the ability to phagocytose extracellular components. In this process,

extracellular proteins are endocytosed, digested in lysosomes, and the peptide in loaded to the MHC molecules before transfer to the cell surface.

MHC class I and II binds to two distinct subsets of T cells known as $CD8^+$ cytotoxic T cells (CTLs) and $CD4^+$ T helper (Th) cells, respectively. Roughly, the elimination of intracellular pathogens or tumorous cells are performed by CTLs and natural killer (NK) cells. In teleost, two types of NK cell homologues have been described; non-specific cytotoxic cells and NK-like cells (reviewed by (Fischer, Koppang et al. 2013), (Evans and Jaso-Friedmann 1992)). Extracellular pathogens are dependent on APC and Th cells to initiate an immune response.

$CD4^+$ and $CD8^+$ T cells in teleost

In mammals, immature thymocytes travel from the bone marrow to the thymus where they undergo thymopoiesis, a process which produces self-restricted and self-tolerant T-cells. T cells will only react to antigens that are bound to an MHC-molecule that was present in the thymus during the development of the T cell. Thus, T cells only bind antigens in the context of self MHC. One of the tasks of $CD4^+$ T helper (Th) cells is to aid B cells in initiating a humoral immune response. Mammalian $CD4^+$ Th cells are divided into different subtypes, Th1, Th2, and Th17 etc., which are characterized based on their production of cytokines and their differentiated functions. Many of the cytokines and transcription factors related to the different Th-pathways are present in the teleost genome. However, there is still no firm evidence that different Th-pathways exist in teleost as in mammals (reviewed by (Castro, Bernard et al. 2011)).

Cellular T cell immunity against intracellular pathogens is mainly mediated by $CD8^+$ CTL cells. CTLs recognizing non-self/pathogenic antigens presented to them on MHC class I receptors initiate different pathways that results in promoting the elimination of the infected cell. CTLs are therefore highly important in the fight against viral pathogens, since promotion of cell death also will reduce the number of viable virus. In mammals the cross-presentation of antigens is a process where certain APC, such as dendritic cells (DC), endocytose extracellular e.g. free viral components and present them through MHC class I to CTLs. The ability of cross-presentation in teleosts is sparsely explored, but initial studies on rainbow trout (*Oncorhynchus mykiss*) skin

showed a DC subpopulation which co-expressed CD8α and MHC class II on the cell surface (Granja, Leal et al. 2015).

B cell subtypes and activation

Like T cells, B cells undergo several steps of positive/negative selection, which leads to mature or naïve B cells equipped with an adapted B cell receptor (BCR). In mammals, naïve B cells are generally divided into three subsets, B-1 B cells (B1a and B1b cells), follicular B cells and marginal zone (MZ) B cells. In mammals, follicular B cells, also known as B-2 cells, is the most common type of B cells. In mice, B-1 and B-2 cells originate mainly from fetal liver and bone marrow, respectively (reviewed by (Hardy and Hayakawa 2001, Hardy, Kincade et al. 2007)). B-2 cells can in response to an infection cooperate with T cells in germinal centers (GC) resulting in a high-affinity antibody response. Clonal expansion, affinity maturation and high-affinity antibody production is mainly a feature of B-2 cells (Figure 1). On the other hand, B-1 cells are known to spontaneously secrete natural antibodies, which are defined as antibodies present prior to encountering cognate antigen (Holodick, Rodríguez-Zhurbenko et al. 2017) (Figure 1). A major component of the natural antibody repertoire is characterized as polyreactive antibodies. These antibodies bind with low affinity to a variety of unrelated antigens, and it has been shown that they can protect against both pathogens and own damaged cells (Baumgarth, Herman et al. 1999, Ochsenbein, Fehr et al. 1999, Gunti and Notkins 2015). A similar non-specific response is well known in teleosts, and is thought to be a fundamental defense mechanism in fish (Magnadóttir 2006), mounted to a much higher degree than commonly seen in mammals (Magnadóttir 2006, Coll 2018).

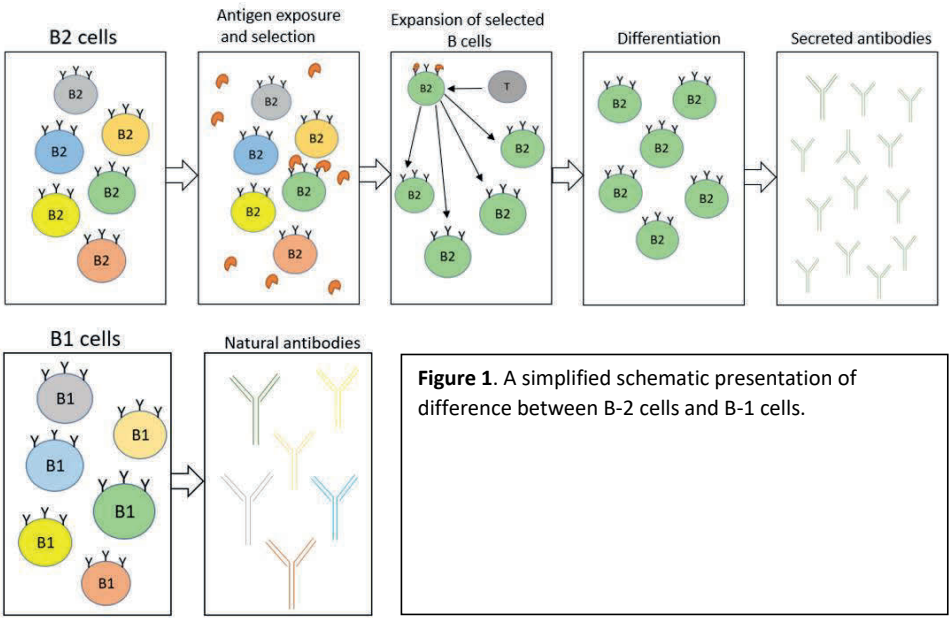

Figure 1. A simplified schematic presentation of difference between B-2 cells and B-1 cells.

B-1 and MZ cells are often referred to as innate-like B cells (ILBs) because of their immune regulatory functions which share properties with innate immune cells. ILBs rapidly respond to Thymus-independent (TI) antigens, and produce low-affinity but broad-reactive antibodies, which contrasts to the high affinity antibodies created by B2 cells in GCs (reviewed by (Zhang 2013)). Mammalian B-1 cell are distinguished from other subsets by their anatomical location, phenotype, self-renewing capacity, phagocytic abilities and their natural antibody production (Hardy and Hayakawa 2001, Berland and Wortis 2002, Gao, Ma et al. 2012). Mammalian B-1 cells can further be divided into B-1a or B-1b dependent on if they are $CD5^+$ or $CD5^-$, respectively (reviewed by (Baumgarth 2016)). B-1 cells are believed to be the major producer of natural IgM antibodies (Baumgarth, Herman et al. 1999).

The definition of natural antibodies (Nabs) includes several criteria; non-specific, broadly cross-reactive, low affinity and germline-like antibodies produced by B-1 cells which were present prior to encountering cognate antigen. However, several studies show an increased B-1 cell-derived IgM that actively contribute to a pathogen-induced immune response (Baumgarth, Chen et al. 2000, Alugupalli, Leong et al. 2004, Cole, Yang et al. 2009, Gil-Cruz, Bobat et al. 2009, Yang, Ghosn et al. 2012, Cunningham,

Flores-Langarica et al. 2014) . The presence of these antigen-induced antibody secreting B-1 cells question the initial definition of natural antibodies, and others have suggested an explicit definition such as the definition by Baumgarth et al. "we suggest the term *natural IgM production* be restricted to the truly antigen-independent elaboration of IgM in the spleen and bone marrow and not be extended to antigen-induced responses by B-1 cells". (Baumgarth, Waffarn et al. 2015). Some years later Holodick et al. writes in a review article "Different *subsets of B cells in different locations are capable of secreting NAbs. Neither a specific isotype nor a specific function can define NAbs. Therefore, the characteristics left to define NAbs include how they are generated (presence or absence of endogenous and/or exogenous antigen) and their structural composition (germline-like or diverse)"* (Holodick, Rodríguez-Zhurbenko et al. 2017). Thus, a consensus definition of IgM antibodies produced by antigen-induced B-1 cells is at present lacking.

In 2006 Li et al. showed the phagocytic activity of rainbow trout B cells *in vitro* and *in vivo* (Li, Barreda et al. 2006). The discovery of phagocytic B cells in fish changed the paradigm that mammalian and other vertebrate B cells did not inhabit phagocytic abilities, which later led to the discovery of phagocytic B cells in the murine peritoneal cavity (Parra, Rieger et al. 2012). The study performed by Parra, Rieger et.al showed that the phagocytic murine B cells were mainly of the B-1 cell subtype and that phagocytosis occurred independent of BCR, leaving the phagocytic receptors unknown (Parra, Rieger et al. 2012). Additionally, these phagocytic B cells were able to effectively present antigen from internalized particles to $CD4^+$ T cells, but any cross-presentation to $CD8^+$ CTL remains unexplored (Parra, Rieger et al. 2012). Based on the phagocytic ability of teleost B-1 cells, phenotypical similarities and the expression of CD5 it has been suggested that most fish B cells are similar to the mammalian B-1 cells (Li, Barreda et al. 2006, Abós, Bird et al. 2018).

The activation of mammalian B cells is performed either in a Thymus-dependent (TD) or Thymus-independent (TI) manner. B-2 cells are activated in response to TD antigens withing lymphoid follicles, which triggers the formation of germinal centers (GC). In cooperation with T follicular helper cells the B-2 cells are then stimulated to divide and differentiate into antibody-secreting cells, resulting in plasma cells (PC) or memory B cells (reviewed by (MacLennan 1994). Alternatively, mammals have a TI

response resulting in a faster antibody response, mainly orchestrated by innate B cells subsets consisting of B-1 cells or marginal zone (MZ) B cells. TI responses are activated by direct recognition of pathogen motifs or BCR stimulation, and do not need the help of T cells (reviewed by (Cerutti, Puga et al. 2011)). In the mammalian paradigm it is believed that memory responses are achieved through TD class-switch responses initiated within GCs, but teleosts do not have lymph nodes or generate cognate GCs (Zapata and Amemiya 2000). However, recent studies question this strict contradiction between TD and TI responses (Alugupalli, Leong et al. 2004, Obukhanych and Nussenzweig 2006, Bortnick, Chernova et al. 2012). These studies indicate that there are multiple routes to B cell memory also in mammals, including variations of TD and TI responses (reviewed by (Good-Jacobson and Tarlinton 2012)), which also might be applicable to teleost. A study performed on rainbow trout (*Oncorhynchus mykiss*) shows that they preferably respond to TI antigens rather than TD antigens, and the results suggest that TD and TI pathways are not as independent in teleosts as it may be in mammals (Granja, Perdiguero et al. 2019).

Structure of immunoglobulins

Immunoglobulins (Ig), also known as antibodies, are proteins found soluble when secreted from cells into all body fluids, or in a membrane-bound form attached to B cells (B cell receptors, BCR). Igs are the main contributors to the humoral immune system, which is also referred to as the antibody-meditated immune system. The binding of an antibody and antigen happens between the paratope of the antibody and an epitope, which is a specific molecular pattern, of an antigen. Once activated, the mammalian B cell starts to proliferate, leading to a clonal expansion, followed by differentiation, and finally leading to Ig-secreting plasma cell or memory cell. Mammalian Ig consist of 5 subtypes, but only three immunoglobulins have so far been identified in teleost: immunoglobulin M (IgM), IgD and IgT, which is determined by their μ, μ_1 and τ-constant gene segment, respectively (Hansen, Landis et al. 2005). In Atlantic salmon, the dominating B cell isotype in kidney, blood and spleen is IgM^+ B cells (Jenberie, Thim et al. 2018), and the circulatory immunoglobulins are mainly of the IgM isotype. Teleost IgM is found as monomer and tetramer, bound to membrane or soluble in serum, respectively (Hordvik 2015). In comparison, mammalian soluble IgM in serum are found as pentamers. A simplified graphic representation of soluble Ig

(sIg) and membrane bound (mIg) of salmon IgT/IgM and human IgM are presented in figure 2.

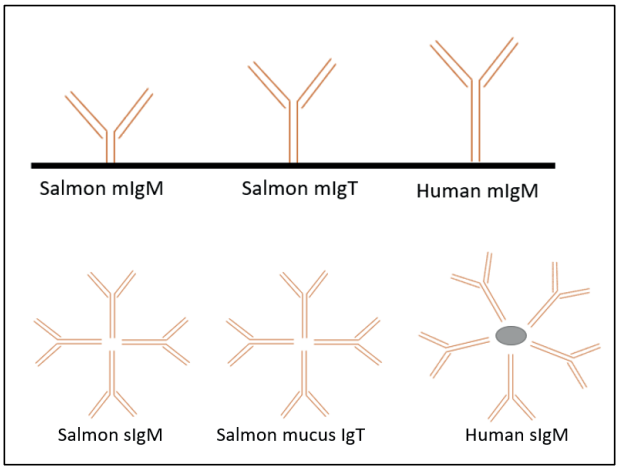

Figure 2. Simplified graphic representation of soluble Ig (sIg) and membrane bound (mIg) salmon IgT/IgM and human IgM (modified from (Tadiso, Lie et al. 2011, Hordvik 2015)).

Antibodies are glycoproteins and their basic structure involves two heavy chains (H) and two light chains (L) held together by disulfide bridges in a y-formation (figure 2). The different antibody regions are named differently; the two arms of the y-formation are known as the fab (fragment antigen-binding)-region, while the stem is known as Fc (fragment crystallizable)-region and the anchoring site for membrane binding. In addition, the outermost part of the fab region is known as the variable region, consisting of one light and one heavy chain. The remaining light and heavy chains then constitute the constant region (figure 3).

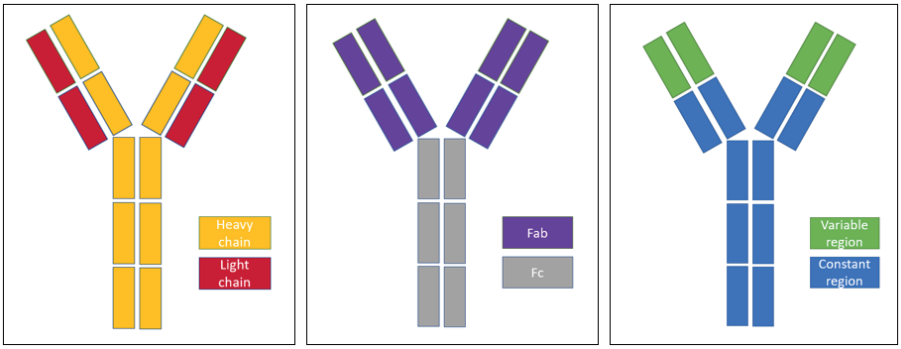

Figure 3. Schematic presentation of terms used for different antibody regions.

In early B cell development, the genes coding for the variable and constant region of an antibody are brought together through a process called somatic recombination. The assembling and diversity of immunoglobulins are then generated by a random rearrangement in individual lymphocytes of different variable (V) region gene segments with diversity (D) and joining (J) gene segment, in a special process known as the V(D)J recombination process. In this process, a novel rearranged exon is generated for each antibody by fusing a V gene segment to downstream D and J segment, and simultaneously removes all excess gene segments. Finally, the V(D)J exon are coupled to a constant region, which determines the antibody isotype. The variable domain of the IgH and IgL is functionally divided into three hypervariable sequences known as complementary-determining regions (CDR). CDR1 and CDR2 are encoded by V gene alone, while CDR3 is encoded by V-J or V-D-J rearrangement junction. Thus, CDR3 is considered the most diverse CDR and is often the target when performing immunoglobulin sequencing in Atlantic salmon (Schroeder and Cavacini 2010, Krasnov, Jorgensen et al. 2017). A total of six CDR makes out the antibody paratope, 3 CDR from each of the IgHV and IgL, respectively.

In the humoral immune system, class-switch recombination (CSR) and somatic hypermutation (SHM) are defining features, and the sole reasons for the highly diverse repertoire of antibodies and the production of antibodies with increased affinity. CSR, also known as isotype switching, is a mechanism in mammals which changes the B cells antibody production from one subtype to another. SHM is a mechanism in B cells in which point mutations accumulate in the antibody V regions in response to a

present antigen. In mammals, one known B cell-specific protein is required to induce both CSR and SHM: the enzyme activation-induced cytidine deaminase (AID). Despite the fact that fish inhabit AID there is no evidence that fishes have, or did have, antibody CSR (Wakae, Magor et al. 2006, Patel, Banerjee et al. 2018). In mammals, affinity maturation is aided by follicular T-helper cells and occurs within GC. However, GC lack in teleosts, and for many years they were also believed to lack affinity maturation. Despite that, recent studies have shown the presence of a degree of affinity maturation in Rainbow trout (*Oncorhynchus mykiss*) and that antibodies with increased affinity were more frequently found in a tetrameric form (Cain, Jones et al. 2002, Bromage, Ye et al. 2006, Ye, Bromage et al. 2010).

1.3 Antiviral- and vaccine immune responses in fish

Antiviral immune responses

In the aquatic environment virus outnumber any other microbes and they probably infect all living things (Suttle 2005). Viruses are experts in hiding from the immune system or dampening host defense, and for many viruses the exact natural port of viral entry in fish remains unknown. In farmed Atlantic salmon, viral diseases are a large health threat that may highly influence the fish welfare and have large economical costs for the producer. Vaccines are an important tool in the fight against viral pathogens, and viral vaccines currently on the marked are an important contributor in reducing the number and severity of some viral disease outbreaks such as Salmonid alphavirus (SAV), which is the causative agent for pancreatic disease (PD) (Skjold, Sommerset et al. 2016).

As mentioned earlier, the first line of defense for many viruses is the mucosal barrier covering the fish, and mechanisms such as mucus shedding and reproduction, antiviral peptides and enzymes, mucosal antibodies, all fight pathogens in mucosal layers (reviewed by (Gomez, Sunyer et al. 2013, Salinas 2015). Early teleost innate immune mechanism also includes type I interferon (IFN) production, as in mammals. IFN-1 are induced and secreted upon host cell recognition of viral nucleic acids, and protection of other cells is given by inducing antiviral proteins. Atlantic salmon has an extraordinary repertoire of IFN-1 genes, and has at least six different classes – IFNa, IFNb, IFNc, IFNd,

IFNe and IFNf (Robertsen 2018). IFNs act by binding of IFN receptors resulting in activation of downstream signalling cascades which in turn induces many interferon-stimulated genes (ISGs) which have direct antiviral activities. Some IFNs genes are also included in a group of virus responsive genes (VRG) which are characterized by a their rapid induction, their low tissue specificity and that their expression levels were related to the viral load (Krasnov, Timmerhaus et al. 2011).

Less is also known about specific adaptive immune responses against viral infection in salmonids, but it is expected that the adaptive immune responses differ depending on the viral pathogen encountered. Increased knowledge about viral pathogenesis and adaptive immune responses are highly wanted since this in turn could mean increased viral vaccine efficacy, thus, leading to increased fish welfare for the farmed Atlantic salmon.

Vaccine immune responses

Several types of vaccines against viral and bacterial disease are currently being used worldwide in aquaculture, one of which are vaccines based on inactivated bacteria or virus. The overall aim of vaccination is to reduce the severity of a given disease, which can be done by introducing a weakened (attenuated), killed, or just fractions, from the causative agent. Inactivated vaccines include whole (killed) or fractions of either virus or bacteria, or a combination of such (multivalent inactivated vaccine). Until this date, the majority of licensed vaccines for the aquaculture industry are inactivated whole pathogen vaccines administered with an oil-adjuvant (reviewed by (Biering, Villoing et al. 2005)), commonly given by intraperitoneal (ip.) injection to the abdominal cavity.

In the seven-component vaccine used in this work (Aquavac PD7 vet., MSD AH) an oil-adjuvant is included. The oil emulsion adjuvant increases the immunogenicity by slow release of the antigen in the context of a water (antigenic phase) in oil (paraffin) emulsion. The water in oil emulsion has been shown to generate a long-term immune response with several antigens (reviewed by (Tafalla, Bøgwald et al. 2013)). One aim in vaccine development is to develop a long-term protection or by developing a certain degree of memory. Injection with inactivated pathogens alone are often not enough to initiate a systemic immune response, so an adjuvant is often added to modify or amplify the immune response against the vaccine antigen. Adjuvants were initially

assumed to just aid in the ability to prevent infection and death (efficacy) and the magnitude of an adaptive immune response to the vaccine (potency). However, nowadays it is acknowledged that adjuvants triggers innate immune responses and most immunostimulatory adjuvants are ligands for PRRs (reviewed by (Coffman, Sher et al. 2010)) and that adjuvants can guide the immune response, and different adjuvants for different pathogens are a necessity. Adjuvants are now defined as a group of structurally heterogeneous compounds able to modulate the intrinsic immunogenicity of an antigen (Guy 2007).

Development of a long-term vaccine protection by developing a degree of memory has been debated in fish immunology. The definition of immunological memory was traditionally understood as a long-term acquired protection, but later it was changed to include the capacity to mount a quicker and more efficient immune response upon the second encounter (reviewed by (Pradeu and Du Pasquier 2018)). The definition of immunological memory is widely debated and is still changing. In a viewpoint article from 2016 five leading immunologist share their thoughts on this, but no conclusive or united definition on immunological memory was given (Farber, Netea et al. 2016). Despite that, a consistent feature was that immunological memory is the ability of the immune system to respond to a pathogen more rapidly and effectively upon reencounter, but which cells or product of cells that should be included was not defined. Recent studies show that the traditional concept of immunological memory no longer can be limited to B and T lymphocytes, but should also include various types of "innate" or "innate-like" immune cells such as natural killer cells, macrophages, monocytes, etc., (reviewed (Pradeu and Du Pasquier 2018)). It has been shown that the innate immune system, of both mammals and organisms lacking adaptive immunity, can mount resistance to reinfection. This phenomenon is termed "trained immunity" or "innate immune memory" (reviewed by(Netea, Joosten et al. 2016)). A more rapid immune response after reencountering a pathogen is dependent on the existence of a true secondary response, a feature it has been debated whether teleosts inhabit.

In mammals, the secondary immune response is dominated by IgG antibodies due to class-shift of antibodies, which is an immunological event teleosts do not perform. A distinction between a primary and secondary immune response might therefore not be

as clear in teleosts, as in mammals. A study performed by Cossarini-Dunier et al. showed a peak in antibody levels against the hapten dinitrophenyl keyhole limpet haemocyanin (DNP-KLH) 50 days post immunization of rainbow trout, and that the antibody levels remained elevated for at least 150days (Cossarini-Dunier, Desvaux et al. 1986). The long-lasting elevated antibody levels might be due to a long-lasting primary response. On the other hand, a more rapid secondary responses and significantly higher levels of pathogen specific antibodies after immunization against two bacterial pathogens *Y.ruckeri*(Cossarini-Dunier 1986) and *Aeromonas salmonicida* (Tatner, Adams et al. 1987) has been shown in trout. Additionally, vaccination against infectious pancreatic disease virus prior to challenge showed an increased number of individuals with specific antibodies shortly after challenge in Atlantic salmon (Frost and Ness 1997). Immune responses differ depending on the pathogen encountered.

Taken together, much remains to be understood about pathogen specific immune responses in the Atlantic salmon, and how optimized vaccine responses can be achieved. Hopefully, the work presented in this phd-work can contribute to increased knowledge within this field, which in turn can contribute to increase the animal welfare.

2 Aim of the study

Main objective

Investigate immune responses in Atlantic salmon after vaccination with a multivalent vaccine and following challenge with SAV.

Sub goals

1. Describe the kinetics and levels of specific and non-specific antibodies at various time-points and conditions using multiplex immunoassays and ELISA (**paper I and III**).
2. Explore B cell traffic to target organs following SAV infection using immunoglobulin transcripts, by combining *in situ* imaging and immunoglobulin sequencing (**paper II**).
3. Study the time-resolved immune gene transcription in response to vaccination and SAV infection (**paper I, II and III**).

3 Summary of papers

Paper 1

A time-course study of gene expression and antibody repertoire at early time post vaccination of Atlantic salmon.

Many studies on vaccine responses focus on several weeks post vaccination. In this paper, the aim was to study the early immune responses after vaccination. Salmon parr were vaccinated with aquavac PD7 (MSD Animal Health), and blood plasma and head kidney were sampled at several time-points between 0-35 days post vaccination (dpv). Saline injected control fish were included at all time-points. A combination of microarray analyses, serological assays, and immunoglobulin sequencing (IgSeq) pointed out 14 dpv as an important time-point post vaccination in which the immune system shift from innate to a more adaptive driven immune response. 14dpv was also the time where an increase in vaccine specific antibodies, measured by salmon Ig binding to *Moritella viscosa* sonicate, and A-layer from *Aeromonas salmonicida*, were detected in plasma. In this study, we observed that non-specific antibodies, i.e. those binding to the hapten-carrier complex DNP-KLH (foreign to the fish environment) were present prior to vaccination.

Paper 2

IgM+ and IgT+ B Cell Traffic to the Heart during SAV Infection in Atlantic Salmon.
There is limited knowledge about piscine B cell traffic to sites of vaccination and infection, and their functional roles at these sites. The aim of this paper was to study the immune responses and especially traffic of B cells following a co-habitant SAV infection. *In situ* hybridization (RNAScope) showed increased numbers of immunoglobin (Ig)M$^+$ and IgT$^+$ B cells in the heart in response to SAV challenge, with IgM$^+$ B cells being most abundant. An increase in IgT$^+$ B cells was also evident, indicating a role of IgT$^+$ B cells in nonmucosal tissues and systemic viral infections. The co-localization of SAV transcripts and IgM/IgT transcribing B cells also indicated a traffic of B cells to the site of SAV replication. The co-occurrence of clonotypes,

measured by IgSeq, indicated that B cells migrated from spleen to heart, and that this traffic was initiated earlier in vaccinated fish compared to unvaccinated fish. Transcriptional data indicated that vaccinated fish had an advantage against the ongoing SAV infection showed by signs of cardiac dysfunction in the unvaccinated control group. These results suggest that migration of B cells from lymphatic organs to sites of infection is an important part of the adaptive immune response of Atlantic salmon to SAV, and that vaccinated individuals had an advantage in the fight against SAV.

Paper 3

Effect of two constant light regimen on antibody profiles and immune gene expression in Atlantic salmon following vaccination and challenge with Salmonid alphavirus.

In Norway, salmon are commonly vaccinated towards end of the freshwater period to safeguard that adequate vaccine efficacy is achieved by the time the fish is transferred to sea. However, in addition to vaccination, fish are often subjected to several manipulations that can affect the immune system and resistance to pathogens later in life. In the present study, we investigated how the responses to vaccination and viral infection varied depending of time of constant light (CL) onset relative to vaccination. Changes in CL is used by the aquaculture industry to initiate smoltification, but endocrine changes in this period have been associated with immune suppression. Transcriptional differences were most prominent in the heart, and results from heart samples indicated that the fish experienced an immune suppression shortly after onset of CL. A comparison between one group exposed to CL 2 weeks prior to vaccination (2-PRI) and one group that was put on constant light at the time of vaccination (0-PRI), was performed. The latter group had lower levels of circulating vaccine specific antibodies at 6wpw, which coincided with the ongoing immune suppression in the 0-PRI group shown by gene expression data. However, results indicated that vaccination eliminates any transcriptional differences after challenge caused by the CL exposure relative to the time of vaccination.

4 General discussion

The kinetics of specific antibodies after vaccination and SAV challenge

Fish vaccines have been developed based on a trial-and-error approach which includes pathogen identification, cultivation and vaccine formulation that contains whole cell preparation and oils (reviewed by (Tafalla, Bøgwald et al. 2013)). This strategy has worked quite efficiently for inactivated extracellular bacterial pathogens such as *A.salmonicida* and *M.viscosa* (reviewed by (Håstein, Gudding et al. 2005)). In contrast, the production of effective vaccines against intracellular pathogens (such as SAV) has not been an easy task (reviewed by (Sommerset, Krossøy et al. 2005, Tafalla, Bøgwald et al. 2013, Dhar, Manna et al. 2014), which might be seen in the context with that several aspects of the teleost immune responses remains unknown or unexplored.

In this PhD work, we evaluated the levels of specific antibodies after vaccination with a multivalent vaccine (Aquavac PD7 (MSD Animal health)) which includes inactivated *A.salmonicida,* Salmonid Alphavirus (SAV) and *M.viscosa*. In paper I, antibody levels against two of the vaccine components, A-layer (from *A.salmonicida*) and *M.viscosa* sonicate, were measured. Results indicated a seroconversion at about 14 days post vaccination, and by 21 days post vaccination (dpv) the presence of vaccine specific antibodies was evident in all vaccinated individuals. Our results coincide with an earlier study that showed increased levels of antibodies binding to whole washed cells of *A.salmonicida* at 3 wpv in salmon (Erdal and Reitan 1992). However, the authors also included A-layer isolated from *A.salmonicida* but only a weak to moderate antibody activity against A-layer was detected (Erdal and Reitan 1992).

In paper I, the number of Ig transcripts in head kidney samples were measured by RT-PCR and microarray. Results showed a peak in Ig at 14dpv, which overlapped with the first sign of seroconversion. However, a decrease in Ig transcripts from 14dpv towards 35dpv was evident, but this did not coincide with the serology data which all showed an increase in antibody levels. Despite that 14dpv was the time point with the highest amount of Ig transcripts, this was also the time point with the lowest variation in the CDR3 region shown by immunoglobulin sequencing (IgSeq). Less variance at 14dpv indicates that the transcribed Ig measured by RT-PCR and microarray was transcribed

by a limited set of B cell clonotypes. After 14dpv, the CDR3 variance slowly increased while the number of Ig transcripts decreased. In contrast, the levels of Ig in serum measured by serological assays remained relatively stable towards the end of the trail (35dpv). Based on these results, we hypothesized that the Ig transcribing B cells present in head kidney at 14dpv had migrated out of the head kidney to continue performing their function elsewhere.

In paper III, antibody levels against recombinant E2-protein (from SAV), in addition to antibody levels against A-layer (from *A.salmonicida*) and *M.viscosa* sonicate, were measured after vaccination and SAV challenge in two groups with different exposure to constant light (CL) prior to vaccination. One group was put on CL 2 weeks prior to vaccination (2-PRI) and one group was put on CL stimulation at the time of vaccination (0-PRI). To evaluate the smoltification status, a commercially available method termed SmoltTimer® were performed. SmoltTimer® includes the quantification of freshwater ATPase-genes and a SmoltTimer®-value is calculated based on PatoGens proprietary technology. Despite that the SmoltTimer® values showed two groups at different stages in the smoltification process, no large differences in total, specific or non-specific, antibody levels between groups were seen at 0wpv except a slightly higher level of E2-binding antibodies in the 2-PRI group. At 6wpv the 2-PRI group had significantly higher levels of vaccine specific antibodies than the 0-PRI group against all three vaccine-relevant antigens. In conclusion, serology assay results indicate that the group which was exposed to CL 2 weeks prior to vaccination had an advantage, resulting in faster or more potent antibody production after vaccination. Lastly, low levels of antibodies binding to vaccine antigens were evident after challenge also in the 2-PRI control group, shown by increased levels of antibodies binding to A-layer and *M.viscosa* sonicate. That unvaccinated control fish produce antibodies binding to vaccine antigens can be explained by an increased amount of polyreactive antibodies that are able to bind several antigens. To some extent, the increase of non-specific antibodies (DNP-KLH reactive) from before to after challenge was more prominent in the control groups, which may suggest that some control fish developed tentatively polyreactive antibodies which also could bind to the vaccine antigens.

Despite having identical names, the mammalian and teleost IgM differ in structure and can be found as a pentamer and a tetramer in serum, respectively. In response to an

antigenic stimulation, mammalian IgM producing B cells can perform two developmental stages known as CSR and affinity maturation. These mechanisms enable the B cells to change the production of antibodies from one isotype to another, resulting in the substitution of low affinity IgM antibodies with high affinity IgG antibodies. The site for affinity maturation in salmonids remains unknown and hence categorizing fish antibodies into mammalian categories, such as "natural" or "adaptive" antibodies, may not be applicable. It is not unlikely that fish IgM has similar functions as mammalian IgM. However, being the most dominating Ig subtype in fish they most likely have additionally functions not covered by mammalian IgM.

Non-specific antibodies constitute a major part of humoral immune responses in Atlantic salmon.

The presence of serum antibodies that bind to nonself antigens or an unspecific/non-specific antibody response in teleosts are well known (Gonzalez, Charlemagne et al. 1988, Gonzalez, Matsiota et al. 1989, Magnadóttir, Gudmundsdóttir et al. 1995, Kachamakova, Irnazarow et al. 2006). In the present PhD work, we addressed the issue with non-specific antibody binding by measuring antibodies binding to 2,4-Dinitrophenyl hapten conjugated to keyhole limpet hemocyanin (DNP-KLH). The presence of antibodies binding to model antigens, such as DNP-KLH, in fish has been reported (Gonzalez, Charlemagne et al. 1988, Sinyakov, Dror et al. 2002, Magnadottir, Gudmundsdottir et al. 2009). Similarly, 2,4,6-trinitrophenyl (TNP) has been used to demonstrated the presence of natural antibodies in teleost and other fish species (Gonzalez, Charlemagne et al. 1988). DNP is a synthetic molecule, not common either on land or in the aquatic environment, and only differs from TNP in that the latter has one extra nitrophenyl-group. Studies performed on mice of different strain, age and sex show that the titer of antibody binding to DNP can be used as an index for polyreactive antibodies (Gunti and Notkins 2015). In mammals, a major part of natural antibodies is polyreactive antibodies, which can bind with low affinity to a several unrelated antigens (Gunti and Notkins 2015). Based on these previous data, we postulated that fish antibodies binding to DNP-KLH would have similar properties as in mammals. In a technical sense, we measure the polyreactivity of the serum and not

the levels of polyreactive antibodies, but we assume that an increase in anti-DNP-KLH antibodies can be correlated with an increase in polyreactive antibodies.

Results from paper I showed that vaccination with a multivalent vaccine caused an increase in non-specific antibodies, in addition to vaccine specific antibodies, in serum. Results from paper III confirmed this, and a similar increase in non-specific antibodies after vaccination was evident. Additionally, results from paper III showed that an increase in non-specific antibodies was evident in unvaccinated fish in response to SAV infection. In conclusion, our results indicated that both vaccination and a viral challenge induce the production of non-specific antibodies, but their function remained uncertain.

Antibodies reacting to targets not included in an antigenic challenge, such as a vaccine, have often been noted and may consist of different overlapping entities and are described under different names depending on context, such as "non-specific, heterologous, polyreactive or natural antibodies". The traditional definition of mammalian natural antibodies is antibodies that are present prior to encountering cognate antigen, and they are known to provide a first line of defense against invaders which gives time for a specific response to be mounted (Holodick, Rodríguez-Zhurbenko et al. 2017). A major component of these natural antibodies is polyreactive antibodies of the IgM class, which are known to consist of low specificity but high avidity antibodies (Zhou, Tzioufas et al. 2007, Gunti and Notkins 2015). Mammalian polyreactive antibodies mainly consist of IgM antibodies, seen in the context with IgM being the most prominent Ig class in fish (Mashoof and Criscitiello 2016), further supported our assumption that the antibodies binding to DNP-KLH were of a polyreactive nature.

To further elaborate on the nature of these non-specific, presumably polyreactive antibodies, we performed a study where we tested fish serum samples against several avian infectious agents (unpublished results). As it is not likely that the fish has been in contact with DNP-KLH, it is not likely that a group of fish have been in contact with several avian infectious agents. A binding of antibodies in fish serum samples to several avian infectious agent would further strengthen our assumption that fish serum inhabits a high degree of polyreactivity, which presumably is caused by polyreactive antibodies. The fish serum samples used were the same as used in paper

III. Samples were from before vaccination (TP0), 9 weeks post vaccination (wpv) and 6 weeks post SAV challenge (wpc) and tested against a poultry serological kit (Charles river (Supplemental information 2019)) including antigens from 14 infectious agent (Table 1 and figure 4) (unpublished results). Results is presented in figure 4 showed that both vaccination and SAV challenge resulted in an increase of non-specific, presumably polyreactive, antibody fraction. Altogether, our results indicate that there is a high proportion of polyreactive antibodies in fish serum samples.

Table 1. Overview of infectious agents from the poultry kit, and corresponding symbol used in figure 1.

Infectious agents	Symbol	Infectious agents	Symbol
Avian encephalomyelitis virus	✳	Avian hepatitis E virus	●
Avian influenza virus	⊗	Infectious bursal disease virus	×
Avian leukosis virus subgroup A	○	Infectious bronchitis virus	△
Avian leukosis virus subgroup B	□	Infectious laryngotracheitis virus	⊠
Avian leukosis virus subgroup J	◇	Fowl pox virus	⬢
Avian nephritis virus	■	Newcastle disease virus	⬡
Avian adenovirus	◆	Avian paramyxovirus type 2	▲

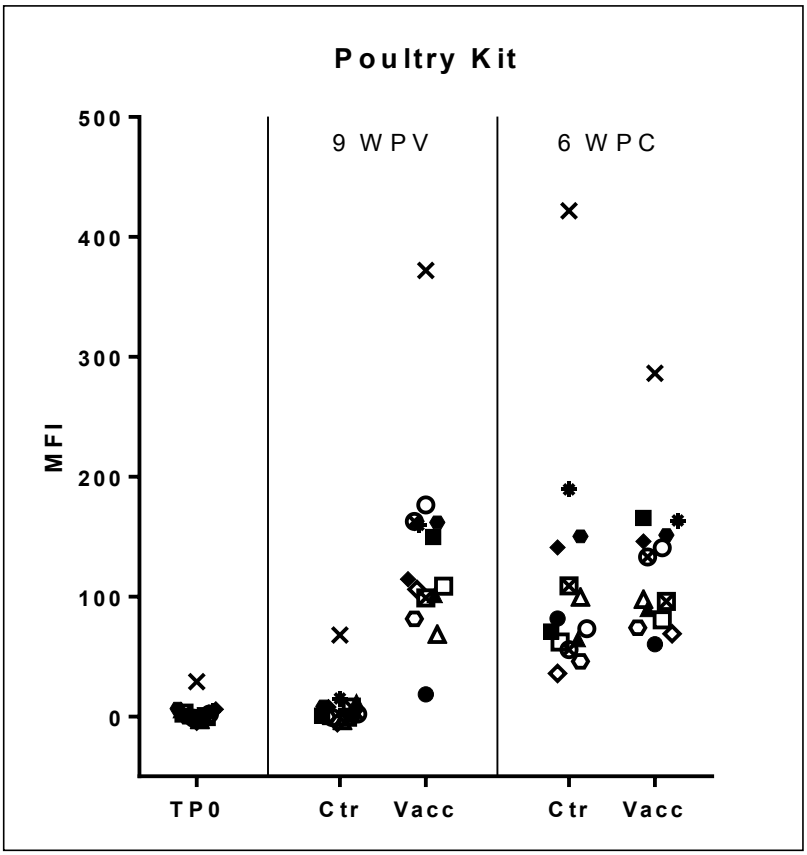

Figure 4. Results from poultry kit. Each symbol marks the average Median Fluorescent Intensity (MFI) of n=7 against one antigen. A total of 14 infectious agents were tested.

An arising question was if the polyreactivity was a property of all circulating salmon antibodies or restricted to a certain compartment. To evaluate this, we calculated the ratios of total and model antigen-binding antibodies, expecting to see a stable ratio if polyreactivity was a universal feature. Data from paper III, including some additional time-points (not published), were used to assess the ratio between total Ig and the antigens used in the multiplex immunoassay for the 0-PRI group. We found that the ratio fluctuated over time in both vaccinated and saline injected fish, indicating that the antibodies binding to DNP-KLH constituted only a proportion of the total amount of Ig (figure 5A) (and unpublished results). Ratios of vaccine-specific antibodies over total Ig fluctuated markedly following vaccination, and for SAV, also after challenge (Figure 5B-D), in a pattern distinct from DNP-KLH, illustrating that these represented an at least partly different fraction of the antibody pool (Figure 5B-D).

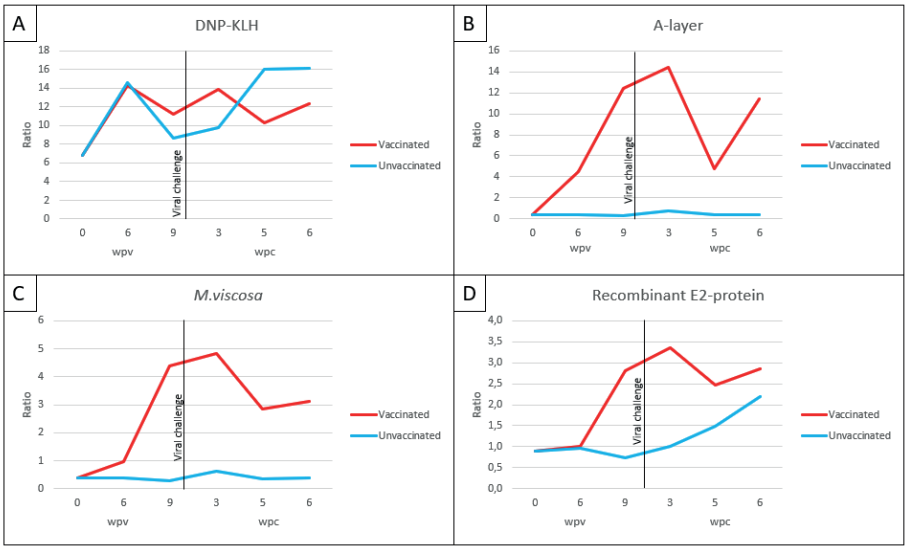

Figure 5. Ratio between total amount of Ig and antibodies measured against 4 antigens, as indicated, in the multiplex immunoassay in n=8 individual fish displayed as a mean at each sampling time-point; **A**: DNP-KLH, **B**: A-layer, **C**: *M.viscosa*, **D**: Recombinant E2-protein.

Results from paper I and III showed that antibodies of a polyreactive nature were present already before vaccination (paper I - figure 6C and paper III - figure 3D). A background level of circulating polyreactive antibodies would presumably be beneficial for the fish even in the absence of an infection, as a possible first line of defence. A study performed in mice showed that natural IgM is essential for immediate protection against pathogens by indicating that natural IgM had a protective role in early trapping of viral and bacterial pathogens in the spleen (Ochsenbein, Fehr et al. 1999).

In conclusion, our results indicate that polyreactive antibodies are present prior to vaccination, their increased production is induced by vaccination and by SAV challenge, and that polyreactive antibodies constitute only a proportion of the total amount of Ig. Despite some structural differences between mammalian and teleost IgM, it is likely that polyreactive IgM antibodies found in Atlantic salmon has a role in the protection against pathogens, as seen in mammals and *in vitro* in rainbow trout (Gonzalez, Matsiota et al. 1989).

Vaccination affects B cell traffic in Atlantic salmon after SAV challenge

Considerable research and review articles on salmonid B cells, site of hematopoiesis, different B cell subsets, etc., have already been published (Zwollo, Cole et al. 2005, Wakae, Magor et al. 2006, Fillatreau, Six et al. 2013, Parra, Takizawa et al. 2013, Parra, Korytar et al. 2016, Zhang, Wang et al. 2017, Jenberie, Thim et al. 2018, Magadan, Jouneau et al. 2018, Peñaranda, Jensen et al. 2019, Perdiguero, Martín-Martín et al. 2019). However, traffic of B cells in response to an infection is a less explored field. In paper I, high-throughput immunoglobulin sequencing (IgSeq)(Krasnov, Jorgensen et al. 2017) was used to study Ig clonotypes in head kidney which had indicated an egress of B cells from head kidney in response to vaccination. In paper II, IgSeq and *in situ* hybridization of IgM and IgT transcripts was combined to further elaborate on the traffic of B cells in response to SAV infection. The distribution and the semi-quantitative amount of B cells in the two main target organs for SAV, pancreas and heart, was then studied by *in situ* hybridization. After SAV infection, a degeneration of pancreatic tissue was evident, which is a typical symptom of pancreas disease (PD) (Taksdal, Olsen et al. 2007). The degeneration of tissue most likely affected the RNA integrity in the present study, and few Ig transcribing cells were seen in the pancreatic tissue samples. Heart samples was therefore the main target organ in our study.

Our results showed that prior to infection, IgM transcribing cells was the most common B cell type in the heart. After infection, the amount of both IgM and IgT transcribing B cells steadily increased in the heart. Ig transcribing B cells were mainly distributed in the atrium and the *stratum spongiosum* of the heart ventricle. Additionally, both Ig transcribing B cells colocalized with SAV transcripts in the *stratum spongiosum* of the heart ventricle, showing that Ig transcribing B cell traffic to the site of virus replication. The increase of IgT transcribing B cells in the heart was also confirmed by RT-PCR analysis of IgT transcript in the heart of both vaccinated and unvaccinated fish, which is shown in figure 6 (unpublished results, same IgT primer sequence as used in paper I). IgT have mainly been linked to mucosal immunity in teleosts (Zhang, Salinas et al. 2010). However, previous reports of IgT^+ cells in the heart (Al-Jubury, LaPatra et al. 2016) and IgT transcripts in the head kidney (Abos, Estensoro et al. 2018, Nuñez-Ortiz, Moore et al. 2018) and spleen (Castro, Jouneau et

al. 2013) of salmonid fish have indicated a role for IgT+ B cells also in nonmucosal tissues and in systemic viral infections in salmonids, and our results further support this.

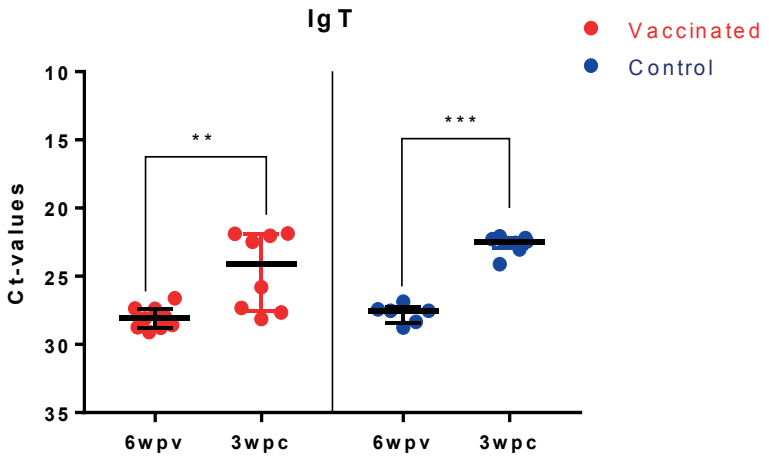

Figure 6. IgT transcripts in heart analyzed by RT-PCR at 6 weeks post vaccination (wpv) and 3 weeks post SAV challenge (wpv) in vaccinated and control fish. Statistical analysis was performed by the GraphPad Prism 7 software, using the non-parametric Mann-Whitney test, and significantly differences are indicated by *. P values <0.05 were considered significant.

By measuring the co-occurrence of clonotypes IgSeq results were presented as HS100 (leader clonotypes in the heart that are also found in the spleen) and SH100 (spleen clonotype leaders also found in the heart). The HS100 was twice as high in vaccinated fish as in saline injected control fish at 21dpc indicating that vaccinated fish had an earlier or more rapid traffic of IgM transcribing B cell from spleen to the heart. Simultaneously, SH100 indicated that a large fraction of the clonotypes that had migrated in vaccinated fish were recently expanded clonotypes. Similar increase in HS100 and SH100 was seen in saline injected fish, but at a later time-point (42dpc). The increase in saline injected fish at 42dpc overlapped with the presence of the peak levels of IgM and IgT transcribing B cells, as shown by *in situ* hybridization. This could indicate that the B cells migrating to the heart, and which co-localized with SAV transcripts, were recently expanded clonotypes from the spleen.

Vaccination and SAV challenge trigger major changes in gene transcription.

All three papers presented in this PhD study included gene transcript data analyzed by at least one of the following methods; RT-PCR, microarray or the multigene expression assay on the BioMark HD platform. Evaluation of gene expression using transcriptional data are based on comparison of the number of transcripts in one group against another group: E.g. vaccinated group against saline injected group, or before vaccination against after challenge within the same group. An upregulated gene then means a gene with an increased number of transcripts in one of the groups, or from one time-point to another, and *vice versa* for downregulated genes. How data is presented is to some degree dependent on the method used, but often Ct-values, fold change of Ct-values or number of differentially expressed genes (DEG) are presented. A comparison between several time-points and treatment groups can also be performed by normalizing the data between all groups, and further study the up- or down-regulation of genes compared to the average (Fundel, Haag et al. 2008). It would be tempting to try to compare gene transcription across the studies presented in this PhD, but several issues like different time points and normalization methods complicate that. A direct comparison of genes across these studies was therefore not attempted. However, some key points from each paper should be mentioned.

In paper I, the number of DEG were calculated, and like the results from the serological assays and Ig transcripts, 14dpv was found to be the time-point with highest number of DEG. The DEG included genes linked to B cells, T cells, acute phase proteins, chemokines etc., whereas most DEG was genes linked to innate immune responses and inflammatory responses. Gene transcripts linked to innate immune responses were characterized by a constant or prolonged change in vaccinated fish, while genes associated with B cells were either up-regulated only at 1dpv or not later than 3dpv. However, a group of B cell related genes that can take part in B cell differentiation including *rag1*, *rag2* and *cd5* were up-regulated from 14dpv. All microarray results were presented as vaccinated (n=5) to saline-injected (n=4) individuals (paper I, figure 3 & 9). These results indicated that at 14dpv the immune system shifted to a more adaptive driven immune response, based on the increased expression of adaptive immunity genes.

In paper II, microarray analysis was performed on heart samples from vaccinated and unvaccinated (saline injected) fish. Prior to challenge, only minor differences between vaccinated and unvaccinated fish were found, and therefore not discussed in detail. On the other hand, larger transcriptional differences were evident after challenge and most prominent in unvaccinated fish. The transcriptome profile was similar to that reported after SAV infection by others (Johansen, Thim et al. 2015, Hillestad, Makvandi-Nejad et al. 2020, Robinson, Krasnov et al. 2020). The transcription data indicated that vaccinated fish had an advantage against the ongoing SAV infection, as shown by signs of cardiac dysfunction in the unvaccinated control group, including down-regulated genes involved in muscle contraction and energy metabolism which is typical for PD (Johansen, Thim et al. 2015, Lund, Røsæg et al. 2016). Furthermore, several B cell related genes were upregulated at 21 dpc in unvaccinated fish compared to vaccinated fish. Two of these B cell related genes were *BTK tyrosine protein kinase* and *pi3k regulatory subunit 6*, which in mammals are known to be indispensable for B lymphocyte development (Satterthwaite, Li et al. 1998) and lymphocyte proliferation (Fruman 2004), respectively. Increased number of transcripts related to B cell development indicates that the B cells in unvaccinated fish might be at an earlier developmental stage than B cells from vaccinated fish.

Lastly, in paper III the effect of constant light (CL) relative to vaccination and SAV challenge was examined using a newly developed multigene transcription assay for the BioMark HD platform (Fluidigm). In the life cycle of a salmonid, CL in itself is known to stimulate and triggers profound transcriptional changes through the smoltification process (Seear, Carmichael et al. 2010) and the smoltification process has previously been associated with immune suppression (Johansson, Timmerhaus et al. 2016). However, the aim of this paper was not to study the effect of CL on smoltification, but rather to see if the duration of CL prior to vaccination affected immune responses triggered by the vaccine, and later by SAV challenge. The duration of CL prior to vaccination was planned so that one group was vaccinated during the smoltification (2-PRI) and the other just prior to initiation of smoltification (0-PRI). The SmoltTimer values showed us that we had successfully managed to vaccinate the two groups at different stages of the smoltification process.

Our results showed that the fish exposed to CL prior to vaccination (2-PRI) were immune suppressed at the time of vaccination, which was in coherence with a previous report showing immune suppression during smoltification (Johansson, Timmerhaus et al. 2016). Despite being vaccinated while undergoing the smoltification process and having an immune suppression, the 2-PRI group had the highest levels of vaccine specific antibodies at 6wpv. In relation to gene expression, the opposite was observed in the 0-PRI group, which was immune supressed at 6 wpv. After challenge, transcriptional changes typical for virus-infected Atlantic salmon were evident (Johansen, Thim et al. 2015). However, any transcriptional differences between light treatment groups before challenge had disappeared after challenge for the vaccinated groups. Based on gene transcript profiles, results indicate that the vaccinated groups were more similar in their immune responses against SAV, regardless of the CL treatment prior to vaccination. In contrast, the control groups had large transcriptional differences after challenge. The transcriptional profile of the 0-PRI control group resembled more that of vaccinated fish, than the 2-PRI control group. The 2-PRI control group had an overall lower number of immune gene transcripts at 6wpc. In conclusion, gene transcription data indicated the following sequence of gene transcription events: immune suppression began no later than two weeks after onset of CL, lasted no less than six weeks, but the immune system had recovered by eight weeks after onset of CL.

We have here showed the use of transcriptional data (transcripts) to substantiate and be seen in the context of serological assay (protein). Gene transcription analysis is indispensable when performing descriptive and hypothesis-free analysis, as those performed in this PhD work, and may lay the foundation for hypothesises that later can be verified through falsifiable trials.

5 Methodological considerations

Limitation in experimental design

The broad aim of this PhD work was to describe immune responses taking place after vaccination and challenge, and since all animal experiments should be planned with the 3Rs (Reduce, Replace and Refine) in mind (Russell and Burch 1959) – this PhD work was conducted based on samples received from already planned vaccination and/or challenge trials by partners on the IMCOM project. Samples used were solely from vaccination trials using a multivalent vaccine (Aquavac PD7) and from SAV challenge trial.

A negative aspect of using samples from pre-planned trials might be the lack of relevant controls for what you wish to study. When describing an immunological event in fish in response to a manipulation, a relevant control would be an identical group of unmanipulated fish. Thus, when studying immune responses after vaccination and after challenge, relevant controls would be non-injected and non-infected fish, respectively. We only had non-injected fish, not non-infected fish. In paper III the experimental design gets even more complicated with the comparison of two groups with different CL exposure, which additionally experienced two separate SAV infection dynamics. The dynamic of a shedder-cohabitant viral challenge will not be identical in two different tanks. Therefore, a direct comparison of the disease pathogenesis was not possible. If the challenge trial had been conducted in one, or preferably several tanks consisting of a randomized mix of the two different CL groups, it would be possible to compare disease pathogenesis. Despite the lack of some control groups, our descriptive findings make biological sense and provide informative data for further studies.

Bio-Plex multiplex immunoassays

The multiplex bead-based immunoassay is a time and labour-saving method which allows us to measure antibody levels to several antigens simultaneously in a small sample volume. The method is based on Luminex technology which is known for its wide dynamic range and high sensitivity. Due to its wide dynamic range it is especially

suitable for studying the kinetics of antibodies over time, since all samples often can be analysed within the same dilution. A quantification of antibody titers can be determined by performing end-point titrations for each sample, which enables the possibility of comparing antibody titers against different antigen. However, this is a very time-consuming process and not a necessity when studying the kinetics of antibodies and was therefore not performed in the present work.

The multiplex bead-based immunoassay can be used with pre-coated magnetic beads from commercial kits, but the supplier also enables the user to coat beads on their own with any desirable proteins. However, the latter entails more responsibility for the user with regards to validations. A validation process for several of the vaccine-relevant antigens as well as for the DNP-KLH model antigen have been performed in our lab (unpublished). In this PhD work, non-specific antibody binding was addressed by showing binding to the hapten-carrier complex DNP-KLH. DNP alone is a small molecule and a carrier molecule is needed for the model antigen to be used in immunological assays. The carrier molecule chosen for our studies was KLH. Other carrier- molecules (ovalbumin, LPS and BSA) had previously been tested in the lab. With the exception of LPS, the carrier molecules showed little to no antibody binding capacity by themselves (Krasnov, Sommerset et al. 2020).

In paper III, an increase of challenge specific antibodies (rE2-protein) was evident in the control groups after SAV challenge. Another study performed in our lab showed that the levels of antibodies binding to rE2-protein also increased after an experimental infection with Piscine orthoreovirus (PRV) in SAV negative fish (Teige, Aksnes et al. 2020). This indicated a degree of nonspecific binding of fish serum to the recombinantly produced protein. In paper III the background levels of antibodies binding to rE2-protein before vaccination/challenge was higher compared to the two other vaccine-relevant antigens in the assay, which might be explained by a degree of nonspecific binding as shown by Teige et.al. Despite that, a marked increase in SAV specific antibodies shown by binding to rE2 was evident after challenge in both vaccinated and saline injected fish.

Another study, performed in a controlled experimental environment, used rE2 protein in an ELISA when evaluating the vaccine efficacy of DNA vaccines targeting SAV. They found that there was correlation between elevated serum antibody levels against

recombinant E2 and protection against virus infection (Chang, Gu et al. 2017). In a study performed by Veenstra et al. they found that specific antibodies develop in response to a SAV1 infection in Rainbow trout, but not after vaccination with inactivated SAV (oil-adjuvanted emulsion), which indicated that different protective mechanisms were triggered by infection and vaccination, respectively (Veenstra, Hodneland et al. 2020). Thus, it is likely to believe that vaccination and natural SAV infection can initiate different protective mechanism also in Atlantic salmon. If the antibodies we measured prior to SAV challenge using rE2 are strictly SAV specific or if they inhabit a degree of polyreactivity remains unclear, but they have been shown to participate in the fight against SAV (Chang, Gu et al. 2017). Despite the lack of a validation process for the recombinant E2 protein, these results indicate that in a controlled experimental setting the recombinant protein is suitable for detection SAV specific antibodies, but caution should be taken if used on serum samples from field experiments where more unknown factors are likely to affect the readout.

Currently, the use of serological assays for field samples in fish is limited (reviewed by (Jaramillo, Peeler et al. 2017)). In the aquaculture industry, health surveillance screening is based on herd diagnostics performed by lethal sampling of a fixed number of fish, followed by a combination of histological examination and screening for pathogens by PCR. In comparison, other production animals like poultry flocks can be weekly tested using serology assays to evaluate the flock health by individually screening for pathogen specific antibodies. A similar screening of pathogen specific antibodies on fish serum samples would greatly reduce the number of sacrificed fish since non-lethal sampling would be possible and would also be an economical benefit for the producer. Furthermore, serology screening for pathogen specific antibodies are not as time limited as the use of blood for detection of virus. The pathogenic virus will most likely only be detectable in blood at peak viremia, if at all. However, much work remains before serology screening for pathogen specific antibodies can be a standard procedure in the field but should be the aim. Serology pathogen screening will increase fish welfare by reducing the number of individuals sacrificed for health and disease monitoring.

Gene transcript assays

For the last decade, there has been a rapid technological development within the field of transcriptomics, and new labour- and time saving methods has entered the market. One such method is the multigene expression assay on the BioMark HD platform (Fluidigm, München, Germany), recently published for assessment of the immune competence (ImCom) of farmed Atlantic salmon (Krasnov, Afanasyev et al. 2020). The first set of samples tested on the ImCom assay are presented in paper III. The BioMark system can process 9216 reactions in a single run (System 2021) and is both a labor- and time saving method compared to conventional RT-qPCR. In such high-throughput methods only a limited sample volume can be used, which may represent a problem with insufficient number of copies of the target in the reaction. This problem is often solved by a preamplification step of the cDNA or DNA. On the other hand, this preamplification step is perhaps the least studied part of a qPCR workflow and might introduce additional bias if not performed correctly. However, studies have shown that the variability of the pre-amplification is lower than the variability caused by the reverse transcription step (Korenková, Scott et al. 2015).

New multiplex methods produce large amount of data simultaneously. This presents a new challenge: data analysis. Within the field of fish immunology, we are currently looking at gene transcripts from a bird perspective, observing which genes are going up and down in response to a manipulation or prior to the manipulation. Since the number of transcripts alone is not very informative, the analysis of transcriptional data is based on comparison of two or more groups. Comparisons should always be performed based on the biological aspect one wish to highlight. Perhaps the data can be used in a similar systematic review like the one written by Caruffo, M. et.al (2020), which gave insight to gene responses to infection in teleosts using microarray data (Caruffo, Mandakovic et al. 2020). All gene transcript data produced in this PhD work, independent of method, can later be an important contributor in data analysis across many different studies. Hopefully, that will increase our understanding of immunological responses in Atlantic salmon.

Methods used to study B cell traffic

Within the field of fish immunology, antibodies targeting specific protein and especially surface receptors on immune cell structures is still in short supply. However, new methods like *in situ* hybridization (ISH) using the RNAScope® technology enables the possibility to detect target RNA in intact cells. RNA ISH techniques were previously known to be technically challenging and with an insufficient sensitivity. However, RNAscope has a unique probe design strategy which simultaneously gives signal amplification and background suppression, and achieves single-molecule visualization at the same time as preserving tissue morphology (Wang, Flanagan et al. 2012). ISH using RNAscope has in the last years been frequently used on Atlantic salmon tissue (Bjoergen, Hordvik et al. 2019, Bjørgen, Hellberg et al. 2019, Bjørgen, Løken et al. 2019, Loken, Bjorgen et al. 2019, Malik, Bjorgen et al. 2019).

During the last decade sequencing methods have become much more available and applicable. Novel methods like immunoglobulin sequencing (IgSeq) has broadened the repertoire of methods available to e.g. study B cell traffic. In comparison, non-sequencing methods often target cell surface receptors on the B cell, making it possible to divide the total amount of B cells present into subtypes of B cells. However, only immunoglobulin sequencing opens the possibility to study both the amount of a clonotype (by calculating the cumulative frequency) and the traffic from one organ to another (by looking for co-occurrence of clonotypes). On the other hand, IgSeq produces large amount of data and data analysis is perhaps the most crucial analytic step. In paper III the following assumptions were made so we could indicate any biological function of the IgSeq data; We assumed that identical Ig sequences are unlikely to occur several places by chance and therefore represent the same clone, that B-cell expansion occurs in the spleen (a major secondary lymphoid organ) and then move toward the heart (target organ for SAV), and that the proliferating B-cells targeting the viral infections would primarily be found among the leaders. In this case we do not know if the assumptions made is the actual case, hence representing a potential bias.

6 Future perspectives

The field of fish immunology is still a relatively new field of research, and much work remains before we fully understand relatively basic immunological responses. To increase the understanding of the immune system of Atlantic salmon and the aspect of non-specific (presumably polyreactive) antibodies, it would be very interesting to further study teleost B cells. Many questions are still to be asked.

> Do teleost B cells consist of different subtypes as in mammals? If so, can these subtypes be linked to "natural" polyreactive antibodies and/or antigen-induced polyreactive antibodies?
>
> Do strictly antigen-specific antibodies develop in response to vaccination?
>
> Do all fish antibodies have a certain degree of polyreactivity?
>
> What is the function of IgT in non-mucosal tissue?
>
> Does an equivalent of germinal centres exist in teleost, or is B cell proliferation conducted peripherally at the site of infection?
>
> Affinity maturation: how does it work and to what degree is it performed?

Serological assays, as the multiplex immunoassay presented in this PhD work, enables the testing of several antigens simultaneously on relatively small sample amounts. Additionally, serological assays for fish opens the possibility of non-lethal sampling, giving us the opportunity to study changes in antibody levels over time, but also affinity. A recent study showed the use of chaotropic reagents in a bead-based multiplex immunoassay to assess the antibody avidity (functional affinity) to *Plasmodium falciparum* merozoite antigens (Taylor, Bobbili et al. 2020). A multiplex immunoassay approach and non-lethal sampling over time can be used to assess the antibody affinity maturation against several antigens simultaneously in Atlantic salmon. Additionally, the development of anti-salmonid IgT antibodies would enable implementation of this antibody class in the multiplex immune assay.

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Appendix: Enclosed papers I-III

Paper I

I

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A time-course study of gene expression and antibody repertoire at early time post vaccination of Atlantic salmon

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ABSTRACT

The majority of studies of vaccine responses in Atlantic salmon have focused on several weeks after vaccination, and employed a limited number of marker genes. In this study, novel techniques were used to examine a broad panel of expressed genes and antibody repertoire of Atlantic salmon following vaccination. Salmon parr were vaccinated with a multivalent oil-based vaccine, and blood plasma and head kidney were sampled at several time-points between 0–35 days post vaccination. Saline-injected fish were used as control at all time-points. Microarray analyses showed increased expression of immune genes from the first day to the end of study in the head kidney of vaccinated fish. Genes up-regulated in the late phase included several leukocyte markers and components of the oxidative burst complex. A suite of genes that can take part in B cells differentiation were up-regulated from day 14, at which time secretory IgM transcripts also peaked. This coincided with marked increased plasma titres of non-vaccine specific antibodies binding to a hapten-carrier antigen DNP-KLH, while antibodies to bacterial components of the vaccine, *Moritella viscosa* and *Aeromonas salmonicida,* first showed significantly elevated antibody levels at day 21, and at a markedly lower magnitude than the non-vaccine specific titres. Sequencing of the variable region of IgM heavy chain (CDR3) revealed higher cumulative frequencies of unique clonotypes in vaccinated salmon starting from day 14 when specific antibodies were first detected. Reduced sequence variance of CDR3 suggested expansion of recently emerged clonotypes. Overall, the results presented here follow a broad panel of gene expression, immunoglobulin sequencing and plasma antibody titres in the first few weeks after vaccination of Atlantic salmon, pointing to a potentially important contribution of non-vaccine specific antibody responses early in the vaccine response.

1. Introduction

Vaccines play an essential role in commercial aquaculture (Sommerset et al., 2005; Biering et al., 2005; Hastein et al., 2005; Brudeseth et al., 2013). The formulation and improvement of vaccines require both empirical approaches and profound understanding of the immune processes that occur after vaccination. Processes taking place in the lymphatic organs of vaccinated fish, and local innate responses at the injection site have been extensively explored using microscopy and expression of marker genes with emphasis on the early events (Munang'andu et al., 2014; Secombes, 2008; Wang and Secombes, 2013; Ye et al., 2011, 2013; Yamaguchi et al., 2018; Haugland et al., 2005). However, adaptive immune responses have mainly been studied several weeks after vaccination, when antigen-specific antibodies and T cells can be detected. Moreover, the majority of published studies of vaccinated salmonid fish have employed limited panels of marker genes, while the use of broader transcriptomic analyses has been infrequent. The profiles of early gene expression, such as those involved in B cell development, are therefore less known.

In recent years, novel analytic techniques have revolutionized the studies of antibodies and the antibody repertoire. Deep sequencing of the variable region of immunoglobulins (Ig-seq) enables a

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comprehensive description of antibody repertoires (Krasnov et al., 2017; Castro et al., 2013). IgM is the key effector of systemic B cell responses in fish, and sequencing of the variable region of IgM heavy chain transcripts including the CDR3 (complementarity determining region 3) enables the examination of repertoires of antibody specificity. Particular CDR3 sequences are referred to as clonotypes, and these are classified as unique when found in one individual, or shared when found in many (or all) individuals (Krasnov et al., 2017; Magadan et al., 2015). Complementing these molecular techniques, new developments in protein level detection such as the xMAP multiplex immunoassay technology enables the detection of systemic antibodies to multiple antigens (Houser, 2012). With this technology, specific antibodies against several vaccine antigens can be analysed simultaneously from the same sample (Morgan et al., 2004). In contrast to a specific antibody response to antigens exposed via vaccine or infection, a non-specific antibody activity can be found in normal serum of most vertebrates (Panda and Ding, 2015), including several fish species (reviewed by (Magnadottir, 2006)), but has commonly been dismissed as background noise in serological assays. Non-specific antibodies (NSAB) are believed to be polyreactive, binding with medium to low affinity to various self-antigens as well as some foreign antigens to which the host has never been exposed. A useful method to estimate the titres of non-specific antibodies is the serological binding to model antigens consisting of hapten-carrier complexes, and the presence of antibodies to model antigens in fish has been reported (Gonzalez et al., 1988; Magnadottir et al., 2009; Sinyakov et al., 2002). However, their functional importance is not yet well-understood (Sinyakov et al., 2002; Magnadottir et al., 1999; Coll, 2018).

Here, we report a time-course study of early immune responses in Atlantic salmon (*Salmo salar*), covering a period from 1 to 35 days post vaccination (dpv). Transcriptome profiling in the head kidney, the primary lymphatic organ, with genome-wide Atlantic salmon DNA microarrays explored processes in both arms of immunity and outlined a number of genes with possible roles in differentiation and maturation of B cells after vaccination. Starting from day 14, an increase in non-vaccine specific antibody titres against a hapten-carrier antigen was observed in vaccinated fish, which coincided with a marked differential expression of multiple genes. Titres of non-vaccine specific antibodies were markedly higher than titres against specific vaccine antigens. An increase in the cumulative frequencies of unique clonotypes in vaccinated fish was accompanied by a decrease in the variance of the nucleotide sequences of CDR3 in vaccinated fish, indicating an expansion of B cells producing specific antibodies.

2. Material and methods

2.1. Fish

The experiment was performed at Veso Vikan Research Facility (Namsos, Norway). Atlantic salmon with a mean weight of 40.5 g (+/-11.1 g) were assigned by random netting to two different treatment groups (vaccinated and saline-injected control). The fish were anesthetized (Metacain, Pharmaq) and i.p. injected with either 0.1 ml Aquavac® PD7 (MSD Animal Health) (hereafter called vaccinated) or 0.1 ml sterile saline (hereafter called saline-injected). Aquavac® PD7 vet is a commercial inactivated, multivalent injection vaccine for immunization of Atlantic salmon. The active components are two inactivated viral antigens, salmon pancreas disease virus (SPDV) and infectious pancreatic necrosis virus (IPNV), and five inactivated bacterial antigens: *Aeromonas salmonicida* subsp. salmonicida, *Vibrio salmonicida*, *Vibrio anguillarum* serotype O1, *Vibrio anguillarum* serotype O2a and *Moritella viscosa* and the oil-based adjuvant. The vaccine is administered to healthy Atlantic salmon of a minimum size of 30 g. The two groups were marked by cutting the left or right maxilla (250 fish per group) under the same anaesthesia and thereafter mixed and kept in a tank supplied with flow through freshwater from a natural water source. The temperature decreased slowly from 11° to 7 °C during the 35 days post vaccination (dpv) sampling regime (7th September to 11th October). In order to induce smoltification, the fish were subjected to 24 h light from 1 dpv. Smoltification status was monitored through seawater exposure test with chlorine testing according to standard procedure at VESO Vikan and the fish were confirmed ready for sea transfer at 43 dpv. Samples were collected 1 day prior to vaccination (0-samples), and thereafter at 1, 3, 7, 14, 21, 28 and 35 dpv. Except for the 0-samples, 10 vaccinated and 5 saline-injected fish were collected at each time-point and subjected to a lethal dose of anaesthetic. Blood was collected from the caudal vein, and plasma collected following immediate spinning of whole blood and frozen at −20 °C until analysis. Head kidney was collected in tubes with RNAlater (Thermo Fisher Scientific), stored for one day in the fridge before storage at −20 °C until analysis. The same type of samples were collected for the 0-samples, taken 1 day prior to vaccination from 10 fish netted from the same fish population. The fish were fed with regular Skretting® Nutura feed according to appetite throughout the freshwater period. Feed was withheld 48 h prior to vaccination and 24 h after vaccination.

2.2. Microarrays

RNA was extracted with Biomek NXP Laboratory Automation Workstation robot (Beckman Coulter) using the RNAdvance Tissue Kit Total RNA isolation (Agencourt) according to the manufacturer's instruction. The RNA concentration and quality were determined using the NanoDrop 8000 Spectrophotometer (Thermo Scientific) and 2100 Bioanalyzer (Agilent Technologies), respectively. RNA was stored at -80 °C until use. In all samples used for gene expression analyses RIN was higher than 8. Nofima's genome-wide Atlantic salmon microarray Salgeno with 44k 60-mer oligonucleotide probes was used. The platform was annotated with bioinformatics package STARS (Haugland et al., 2005). Samples included for microarray analysis were: 1 day prior to vaccination (0-samples, n = 3), vaccinated fish at 1, 3, 7, 14, 21, 28 dpv (n = 5) and 35 dpv (n = 3), and saline injected fish at 1, 7, 14 and 28 dpv (n = 4). Data from 28 dpv and 35 dpv were combined since they were nearly identical. Microarrays were manufactured by Agilent Technologies, and the reagents and equipment were purchased from the same provider. RNA amplification and labelling were performed with a One-Color Quick Amp Labelling Kit, and a Gene Expression Hybridization kit was used for fragmentation of labelled RNA. Total RNA input for each reaction was 500 ng. After overnight hybridization in an oven (17 h, 65 °C, rotation speed 0,01 g), arrays were washed with Gene Expression Wash Buffers 1 and 2 and scanned with Agilent scanner. Subsequent data analyses were carried out with STARS. Global normalization was performed by equalizing the mean intensities of all microarrays. Next, the individual values for each feature were divided to the mean value of all samples producing expression ratios (ER). The $\log_2$-ER were calculated and normalized with the locally weighted non-linear regression (Lowess). Differentially expressed genes (DEG) were selected by the following criteria: expression ratio > 1.75-fold and $p < 0.05$. Effects of vaccination were assessed by comparison between vaccinated and saline injected salmon. Samples collected before vaccination were included in analyses to examine the temporal changes. Enrichment analysis was carried out for the functional categories of GO, KEGG pathways and STARS (Krasnov et al., 2011) annotations. Numbers of genes associated with the terms were compared in the list of DEG and the entire microarray platform. Significance of enrichment was assessed by Yates' corrected chi test.

2.3. RT-qPCR

cDNA was synthesized from 1 µg of RNA using QuantiTect Reverse Transcription Kit (Qiagen) following the manufacturer's recommendations and stored at -20 °C until use. The RT-qPCR was performed on three to five individuals per group and time-point. Samples were

Table 1
PCR primers.

Gene	Accession no.	Sequence (5′–3′)	PCR eff.
EF1A	X70165.1	F – CCCCTCCAGGACGTTTACAAA R – CACACGGCCCACAGGTACA	107%
mIgM	BT059185	F - CCTACAAGAGGGAGACCGA R - GATGAAGGTGAAGGCTGTTTT	108%
sIgM	Y12457	F - CTACAAGAGGGAGACCGGAG R - AGGGTCACCGTATTATCACTAGTTT	107%
IgT	ACX50290	F - CAACACTGACTGGAACAACAAGGT R - CGTCAGCGGTTCTGTTTTGGA	107%

analyzed in duplicates in 20 ul reactions, and each plate included a negative control lacking the template and a positive control/calibrator for plate to plate variations. The RT-qPCR was performed using Maxima® SYBR Green/ROX qPCR Master Mix (Thermo Scientific, USA) according to the manufacturer's description and with the following set-up: 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 1 min at 60 °C. Samples were analyzed for transcripts of secreted immunoglobulin M (*sigm*), membrane bound IgM (*migm*) and *igt*. Elongation factor 1α (*ef1α*) was used as the reference gene. The primers used are listed in Table 1. The RT-qPCR was carried out with cDNA corresponding to 15 ng of RNA. Primer optimization was performed and a concentration of 450 nM F/R for each primer was used. PCR reaction efficiency for each gene assay was determined using 5-fold serial dilutions of randomly pooled cDNA in triplicates.

2.4. Bead coupling and multiplex immunoassay

For detection of specific vaccine-relevant antibodies, the A-layer protein from *Aeromonas (A.) salmonicida* (Stromsheim et al., 1994), in-house produced), and whole cell sonicate from *Moritella (M.) viscosa* (kindly provided by Liv-Jorun Reitan, Norwegian Veterinary Institute) were included. For detection of antibodies not specific to the vaccine components, the hapten-carrier DNP-keyhole limpet hemocyanin (DNP-KLH) (Calbiochem, Merck, Darmstadt, Germany) was used. Antigens were coupled to distinct MagPlex® -C Microspheres (Luminex Corp. Austin, TX, USA) of different bead regions and according to the manufacturer's protocol using the Bio-Plex amine coupling kit (Bio-Rad) as previously described (Teige et al., 2017). The DNP-KLH antigen was used at an amount of 10 µg per 1x scale coupling reaction, and A-layer and *M.viscosa* sonicate at an amount of 12 µg and 7 µg, respectively. Stock uncoupled beads and beads coupled with the protein carrier KLH alone (Sigma-Aldrich, Oslo, Norway) were also included. For the immunoassay, Bio-Plex Pro™ Flat Bottom Plates were used. Beads were diluted in assay buffer consisting of PBS with 0,5% BSA (Rinder-albumin; Bio-Rad Diagnostics GmbH, Dreieich, Germany) and 0,05% azide (Merck, Darmstadt, Germany), and 2500 beads per region were added to each well. Beads were washed three times with 200 µl assay buffer per well for 30 s in the dark and on a shaker at 800 rpm, then kept for 120 s in a Bio-Plex handheld magnetic washer before the supernatant was poured off. Plasma samples were diluted 1:200 in assay buffer, before 50 µl sample was added to each well in duplicates. The plate was incubated for 30 min at RT in the dark and on a shaker at 600 rpm. All subsequent washing –and incubation steps were performed similarly. Following incubation, beads were washed and all wells were added 50 µl of Anti-Salmonid-IgH monoclonal antibody (1:400, clone IPA5F12, Cedarlane, Burlington, Ontario, Canada). After incubation and washing, each well was added biotinylated goat Anti-Mouse IgG2a antibody (1:1000, Southern Biotechnology Association, Birmingham, AL, USA), and finally, after incubation and washing, Streptavidin-PE (1:50, Invitrogen). After the final incubation, beads were washed and resuspended in assay buffer. Plates were analyzed using a Bio-Plex 200 in combination with Bio-Plex Manager 5.0 software (Bio-Rad). The reading was carried out using a low PMT target value, the DD-gate was set to 5000–25000, and 100 beads from each region were read in each well. Each bead is classified by its signature fluorescent pattern and then analyzed for the mean fluorescence intensity (MFI) of the reporter molecule.

2.5. Ig-seq

Libraries for sequencing were produced with PCR of fragments containing CDR3. The cDNA was synthesized using SuperScript II (Thermo Fisher Scientific) and IGMR1 primer to the constant region of Atlantic salmon IgM heavy chain (TAAAGAGACGGGTGCTGCAG). Two consecutive PCR amplifications were performed in a 50 µl volume with Platinium Taq DNA polymerase (Thermo Fisher Scientific). The first PCR was performed with primers containing *igm* sequences and Illumina adaptors (underlined):

IGV5 TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGARGAC-WCWGCWGTGTATTAYTGTG

IGC3 GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGAACAA AGTCGGAGCAGTTGATGA.

IGV5 was designed to the 3'-ends of fifteen highly similar Variable regions of Heavy chain (VH) segments, which are present in more than 60% *igm* transcripts (Krasnov et al., 2017), and the reverse primer matched the 5'-end of the constant region. The second PCR (10 cycles) amplified 5 µl of PCR1 product with primers from Illumina Nextera Index kit. DNA concentrations were determined using Qubit fluorometer (Thermo Fisher Scientific). Aliquots from all samples were combined and purified twice with Qiagen PCR cleanup kit. Sequencing with Illumina MiSeq Reagent Kit v2 (read one, 200 cycles from the 3'-end) was performed according to manufacturer's instructions. After trimming primers, sequences were filtered (Illumina $q > 20$) and the J segments were identified using Smith-Waterman algorithm. Sequences were translated in three frames and CDR3 were determined by guidelines of IMGT (Brochet et al., 2008; Lefranc, 2014). Clonotypes were assigned by the amino acid sequences of CDR3 and the numbers of transcripts per clonotype (frequencies) were assessed. In each sample, clonotypes represented with at least two transcripts were used for further analyses. Clonotypes were denoted as unique if transcripts were detected in a single fish, or shared if transcripts were found in at least two individuals with a transcript frequency of at least 1 per 10,000. The hundred most abundant clonotypes were selected in each individual and cumulative frequencies of unique and shared clonotypes were calculated. Further, sequences were submitted to IMGT HighV-QUEST server (Brochet et al., 2008). Nucleotide sequences corresponding to each CDR3 were determined and the numbers of nucleotide substitutions were calculated.

2.6. Statistics

Data produced with RT-qPCR and Ig-seq methods were analysed with ANOVA followed with post hoc tests using Statistica 13.

3. Results

3.1. Microarrays and RT-qPCR

A total of 4928 genes of 44 k presented on the array were differentially expressed on at least one time-point in the head kidney of vaccinated and saline-injected control fish. The number of differentially expressed genes (DEG) was 836 at 1 dpv, increased to a maximum level of 3033 at 14 dpv, and decreased afterwards but remained relatively high to the end – 703 genes at the last time-point (Fig. 1). An overview of enriched functional categories of DEG is shown in Table 2.

Genes of innate immunity were divided into three groups based on their expression profiles after vaccination; constantly up-regulated, early up-regulated, and late up-regulated (Fig. 2). The constantly up-regulated group included genes with diverse immune functions, such as

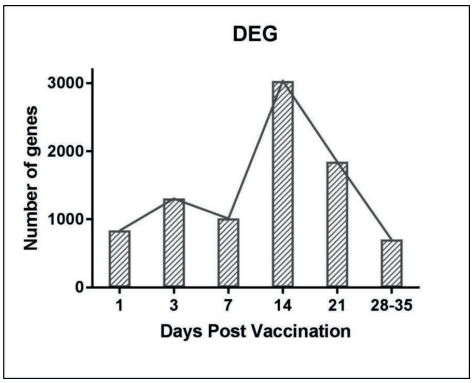

Fig. 1. Numbers of differentially expressed genes (DEG) in the head kidney between vaccinated and saline-injected fish (microarray analyses).

Table 2
Enrichment of functional categories and pathways.

Group_Name	DEG[1]	ALL	Annotation
Innate immune response	137	627	GO
Inflammatory response	102	544	GO
Cytokine-cytokine receptor interaction	68	380	KEGG
Chemokines	25	71	STARS
Eicosanoid metabolism	26	106	STARS
Lectins	46	200	STARS
Acute phase proteins	28	114	STARS
Complement classical pathway	31	80	GO
Scavenger receptor activity	42	209	GO
Neutrophil chemotaxis	28	89	GO
Cell adhesion molecules	65	388	KEGG
Hematopoietic cell lineage	44	133	KEGG
Myeloid cell differentiation	23	71	GO
T cells	54	282	STARS
B cell receptor signaling	50	230	KEGG
Lymphocytes	41	199	STARS

[1] For each term, the number of differentially expressed genes in all time-points (DEG) was compared with the total number of genes presented on the microarray platform (ALL) and enrichment was assessed with Yates' corrected chi square test.

antimicrobial and acute phase proteins (*saa, cath, differentially regulated trout protein, irg1*), genes involved in eicosanoid metabolism (*alox, loxe3, aloxe3*), pathogen recognition (*tlr8*) and regulation of signalling (*socs3b*). Strong expression changes were exhibited by genes with poorly explored or unknown (*l-serine dehydratase, saitotoxin binding protein*) immune roles. In the early up-regulated group, genes encoding for acute phase proteins, lectins and complement factors had increased expression already at 1 dpv and remained up-regulated until 14 dpv. Several chemokine genes showed an increase in expression from 1 dpv to 7 dpv. The late up-regulated genes, which had an increase from 14 dpv, include leukocyte markers, cytochromes and enzymes producing free radicals (*cyba, cybb, mpo, ncf2, ncf4*).

While innate responses were characterized by constant or prolonged changes in vaccinated fish, this was less common for genes that are associated with B cells (Fig. 3). Only *cd72-like* and two *blimp-like* genes had an increased expression level until 14 dpv. Several genes showed biphasic responses and a large group of genes were up-regulated only at 1 dpv or not later than 3 dpv. Among these, multiple structurally divergent genes from different orthology groups (Zdobnov et al., 2017) are denoted as *cd22-like* by the nearest matches. A suite of genes that can take part in B cells differentiation including *rag1*, *rag2* and *cd5* were up-regulated from 14 dpv. Of note is that multiple genes were differentially expressed only at 14 dpv, where up- and down-regulation was observed in respectively 438 and 654 genes. A notable feature was transient reduced abundance of genes associated with T cells, including *cd3*, *cd8* and *cd28*.

Expression of immunoglobulins (*ig,* 30 probes to different segments) changed in both the vaccinated and saline-injected group (Fig. 4A). Expression levels were higher in the vaccinated fish already at 1 dpv, and the difference reached a maximum at 14 dpv followed by a gradual decrease. At the end of the study, the *ig* levels were equalized. Microarray analysis detected changes of the entire pool of *ig* transcripts. The qPCR analysis separated *s (soluble)igm*, *m (membrane)igm* and *igt*, which comprised respectively 90.7%, 8.2% and 1.1% of total *ig*. Only *sigm* showed higher expression in vaccinated salmon, and the greatest difference between the groups was observed at 14 dpv (Fig. 5A). At the end of the trial the expression levelled out between the groups in concordance with the microarray results. Marked difference between the vaccinated and control group was shown by the *sigm / sigt* ratio suggesting a shift to production of IgM in immunized salmon (data not shown). The reduction of *ig* transcripts in the saline-injected control group was found in parallel with an increased expression of erythrocyte markers (Fig. 4B). This set, which was previously identified in a study of red blood cell differentiation in Atlantic salmon (Krasnov et al., 2013),

Gene	D1	D3	D7	D14	D21	D28-35
Constant up-regulation						
Serum amyloid A (saa)	3.7	11.2	12.6	18.1	1.9	3.0
Arachidonate 15-lipoxygenase B-like (alox15)		2.7		4.9	2.8	2.7
Carcinoembryonic antigen-related cell adhesion 20 (ceacam)	9.0	8.4	3.3	6.2	3.0	2.6
Cathelicidin antimicrobial peptide (cath, 2 genes)	11.4	17.3	14.8	21.6	9.8	3.9
Differentially regulated trout protein (3 genes)	2.4	5.2	4.0	2.8		
Epidermis-type lipoxygenase 3 (loxe3)		1.9		3.5	2.2	2.0
Hydroperoxide isomerase ALOXE3-like	2.0	2.9	2.0	5.0	2.9	2.6
Immunoresponsive 1 homolog (mouse) (irg1)	18.0	20.3	8.1	3.9	3.1	2.3
L-selectin-like (cd62l)	2.2	2.0	2.3	4.1	3.2	2.5
L-serine dehydratase (2 genes)	3.5	15.0	11.4	4.3	2.1	
Suppressor of cytokine signaling 3b (socs3b)	12.0	6.1	2.3	2.1		1.8
TNF receptor member 11B (tnfr11b)	15.0	22.8	10.1	3.9	2.3	
Toll-like receptor 8 (tlr8)	2.2	2.7	2.3	2.3	1.8	1.9
Early up-regulation						
Saxitoxin and tetrodotoxin-binding protein (psbp1)	5.8	10.9	9.3	1.9		
Complement component 3a receptor (c5ar)	2.2	4.2	3.4	2.3		
Leukemia inhibitory factor receptor-like (cd118)	3.4	5.0	3.9	1.8		
C type lectin receptor B (2 genes)	2.4	7.2	5.1	2.0		
C type lectin receptor A	3.8	3.7	2.0	1.9		
C-type lectin domain family 4 member E	1.9	1.8		1.9		
Complement component C8 gamma chain (c8g)		2.2	2.7	1.9		

Gene	D1	D3	D7	D14	D21	D28-35
Early up-regulation						
C-C motif chemokine 4-like	18.6	36.4	14.6			
C-C motif chemokine 19 -1	2.9	3.6	2.3			
C-C motif chemokine 4-like	2.8	2.4	1.9			
Haptoglobin-like (5 genes)	4.8	5.3	7.3			
Late up-regulation						
Angiogenin-1 / RNase ZF3 (rnh1, 4 genes)					2.7	2.8
Arachidonate 5-lipoxygenase-activating (alox5ap, 2 genes)				2.0	1.8	
CD209 antigen-like protein C				2.5	2.4	2.3
C-type lectin domain family 4 member E-like (clec4e)				2.7		2.2
C-type lectin domain family 4 member F-like (clec4f)				1.8	2.2	2.2
C-type lectin domain family 4 member M (clec4m, 2 genes)				3.0	2.2	2.6
C-X-C chemokine receptor type 4				2.5	2.3	2.0
Cytochrome b-245 light chain (cyba)				2.4	2.1	1.8
Cytochrome b-245, beta polypeptide (cybb)				2.6	2.5	1.8
Leukotriene A-4 hydrolase (lta4)				2.4	2.3	2.0
Leukotriene B4 receptor 1 (ltb4r)				2.2	1.8	2.1
Myeloperoxidase (mpo)				2.4	2.2	
Neutrophil cytosolic factor 2 (ncf2)				2.3	1.8	
Neutrophil cytosolic factor 4 (ncf4)				1.8	1.8	
Ornithine aminotransferase, mitochondrial (oat)				2.3	2.1	
P-selectin-like (selp)				2.3	1.9	

Fig. 2. Differentially expressed innate immunity genes in the head kidney. Data are vaccinated (n = 5) to saline-injected (n = 4) expression ratios (folds), all shown values are significantly different (t test, $p < 0.05$). Up and down-regulated genes are highlighted with respectively red and green colours (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Gene	D1	D3	D7	D14	D21	D28-35
B-cell lymphoma 3 protein (bcl3)	3.4					
B-cell receptor CD22-16	3.0					
B-cell receptor CD22-17	1.8					
B-cell receptor CD22-3	2.3	2.0				
B-cell receptor CD22-4	2.6	1.9				
B-cell receptor CD22-5	2.1	1.8				
B-cell receptor CD22-6 (2 genes)	1.8					
B-cell receptor CD22-9	1.8					
Phosphatidylinositol 4,5-bisphosphate 3-kinase (p13kca)	1.9					
Phosphoinositide 3-kinase regulatory subunit 5 (pi3krs6)	2.1					
PR domain containing 1a, with ZNF domain (blimp1-like)	2.5			1.9		
B-cell differentiation antigen CD72-like	*2.3*		*1.9*	*2.0*		
B-cell antigen receptor complex-associated (2 genes)	1.9				1.8	
B-cell CLL/lymphoma 6a (bcl6a, 6 genes)	1.9			1.8		1.8
Phosphoinositide 3-kinase regulatory subunit 5 (pi3krs5)	2.5			1.7	1.8	
PR domain containing 1a, with ZNF domain (blimp1, 3 genes)	2.5			1.9		
B-cell receptor CD22-10				1.8	1.8	
B-cell receptor CD22-8					1.8	1.8
DNA cross-link repair 1C (dclre1c)		-5.2	-3.2	2.9	1.9	1.9
Hematopoietic lineage cell-specific protein (hcls1)				*1.9*	*1.9*	
Phosphatidylinositol 4,5-bisphosphate 3-kinase (pi3kca, 3 genes)				2.2	2.0	
Phosphoinositide 3-kinase regulatory subunit 5 (pi3krs6)				2.0	2.0	
Rag1		0.5		1.9	1.7	
Rag2				2.6	2.1	
CD5 antigen-like				1.9	1.8	1.0
CD3gammadelta-B				-2.2		
CD28				-1.9		
CD8 alpha				-1.9		

Fig. 3. Differentially expressed genes involved in development of B cells. Data are vaccinated (n = 5) to saline-injected (n = 4) expression ratios (folds), all shown values are significantly different (t test, $p < 0.05$). Up and down-regulated genes are highlighted with respectively red and green colours (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

contains 52 erythrocyte related genes including *hemoglobins*, multiple components of the cytoskeleton and membrane, transporters, enzymes and transcription factors. Expression of these genes in vaccinated salmon was reduced to the minimum level at 3 dpv and slowly increased afterwards. Difference between the vaccinated and saline-injected group was greatest at 14 dpv and completely disappeared at 28 dpv.

3.2. Multiplex immunoassay

Titres of specific antibodies against two bacterial components of the vaccine, the A-layer of *A. salmonicida* (Fig. 6A) and *M. viscosa* whole-cell sonicate (Fig. 6B), were significantly different in vaccinated Atlantic salmon compared to the saline-injected controls from 14 dpv and their titres reached top levels at 28 dpv. Specific antibody titres in the saline-injected group remained low throughout the observation period. Both vaccinated and saline-injected fish showed high titres of non-vaccine specific antibodies, recognizing the hapten-carrier antigen DNP-KLH (Fig. 6C). Vaccinated salmon showed increased titres of non-vaccine specific antibodies compared to the saline-injected group from 14 dpv, and significant differences in titres from 21 dpv and until the end of trial. The overall dynamic range in MFI values varied depending on the coating antigen, with the greatest median MFI values observed for antibody titres against DNP-KLH, showing more than 30-fold higher titres than the titres against A-layer at 14 dpv. Comparison between titres of non-vaccine specific antibodies and A-layer specific antibodies (Fig. 7A) and between the two vaccine-specific antibodies (Fig. 7B) showed very low correlation: Pearson r respectively 0.1 and 0.19.

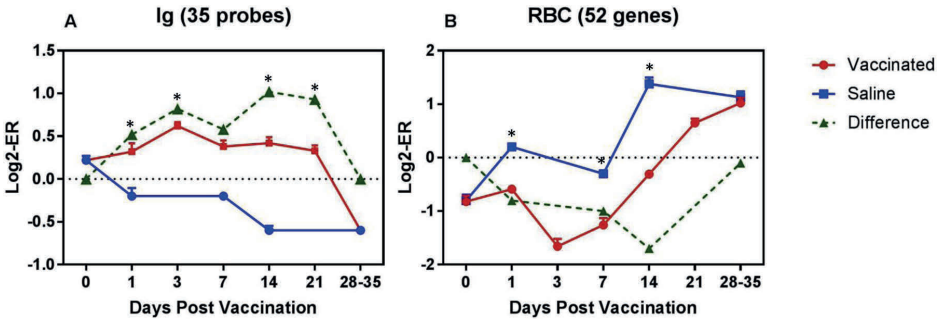

Fig. 4. Expression of *ig* (A) and markers of red blood cells (B), microarray data. Data are mean log_2-ER ± SE. Significant differences between vaccinated (n = 5) and saline-injected fish (n = 4) (t test, $p < 0.05$) are indicated with asterisks.

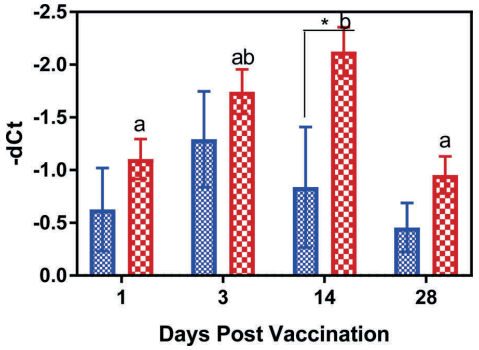

Fig. 5. RT-qPCR. Expression of secretory immunoglobulin M – *sigm*. Three to five fish were analyzed per group (vaccinated and saline) and time-point. Significant difference between the time-points within each group is indicated with letters (omitted when no significant difference was found), and significant difference between groups at given time-points is indicated with asterisks (ANOVA, Tukey test, $p < 0.05$).

3.3. Ig-seq

A total of 15 M high quality immunoglobulin sequences were produced. Two metrics were applied to characterize the IgM repertoire and the status of unique and shared clonotypes: cumulative frequencies of the most abundant clonotypes (CF) and variance of the CDR3 region sequences. The most abundant clonotypes (leaders), i.e. clones that encode a large fraction of antibodies, represent the greatest interest. Exposure to pathogenic antigens induces proliferation of B cells clones and / or increases their production of antibodies. Either way, a response to a recent immunization can be detected as an increase of CF due to enhanced transcription of the leaders. Here, CF were calculated for the hundred most abundant clonotypes and further divided into two fractions, shared or unique. CF of shared clonotypes showed no difference between vaccinated fish and saline-injected fish (not shown), while CF of unique clonotypes were significantly higher in vaccinated salmon at two time-points (Fig. 8A). Difference between vaccinated and saline-injected fish in the variance of the nucleotide sequences of CDR3 of unique clonotypes was observed at 14 dpv (Fig. 8B).

4. Discussion

The aim of this study was to expand the knowledge of immune processes taking place in Atlantic salmon shortly after vaccination, by analysing the production of circulating antibodies, and the IgM repertoire and gene expression in the head kidney. Vaccination induced a powerful and multifaceted immune reaction, which included communication and signalling via chemokines, cytokines and lipid mediators, activation of innate factors (acute phase proteins, complement, scavengers, lectins) and genes associated with both innate and adaptive cells. A synopsis of results produced with three independent methods pointing to 14 days post vaccination as the critical time-point is presented in Fig. 9.

Microarray results showed an upregulation of a number of genes with diverse immune roles as well as genes with unknown functions, which can be ranked by the magnitude and stability of expression changes after vaccination. Constant up-regulation was shown by

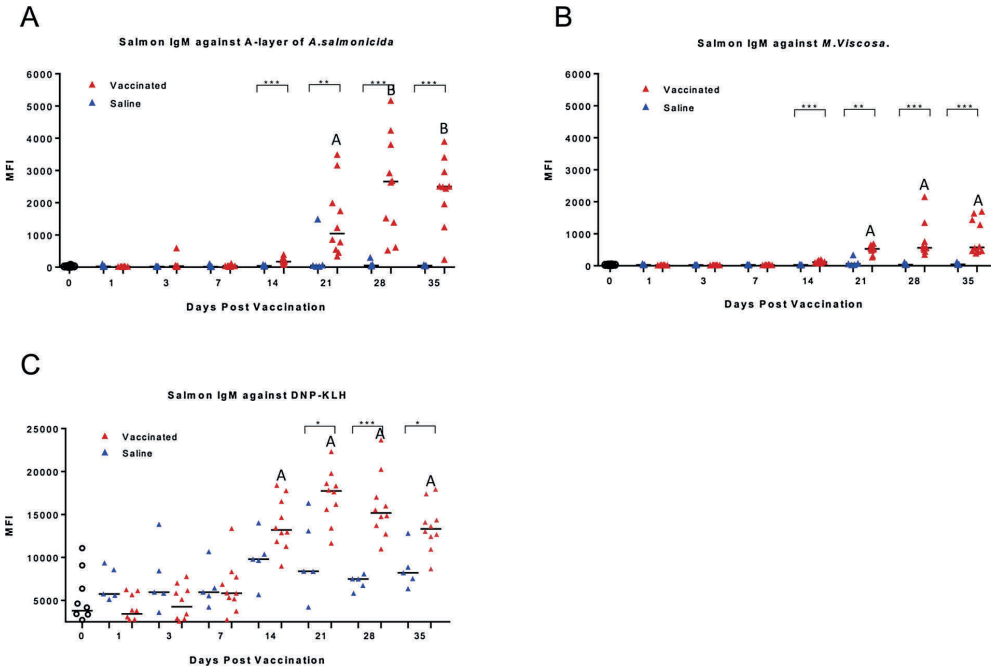

Fig. 6. Multiplex antibody assays. Salmon IgM antibodies against A-layer of *A. salmonicida* (A), *M. viscosa* (B) and DNP-KLH (C). All individual values of vaccinated (red triangles, n = 10), saline-injected (blue triangles, n = 5) and pre-vaccinated fish (black open circles, n = 10) are shown. The median value of each sample group is shown as a black line. Significant difference between the time-points and treatment groups are indicated with respectively letter and asterisks (ANOVA, Newman – Keuls test). MFI = Mean Fluorescent intensity (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

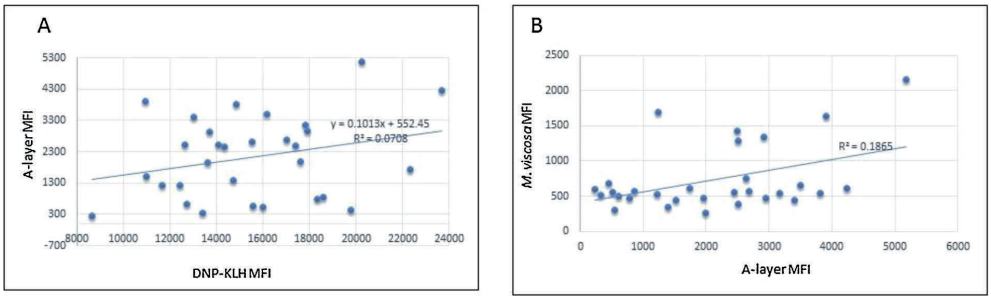

Fig. 7. Relationship between titres of antibodies to different antigens determined in multiplex assays. A: DNP-KLH and A-layer. B: A-layer and *M. viscosa*.

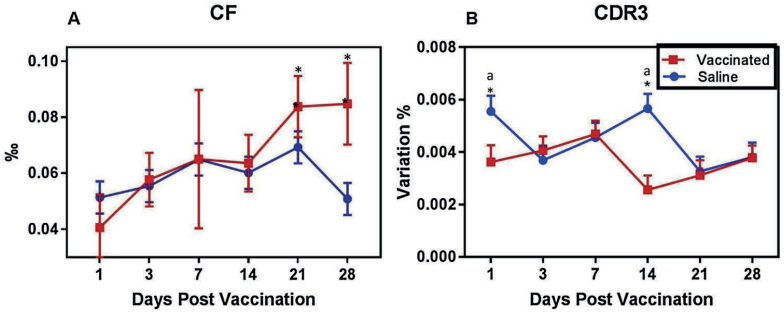

Fig. 8. Deep sequencing of the variable regions of IgM. Results for unique clonotypes are shown.
A: Cumulative frequencies (CF) of most abundant unique clonotypes in vaccinated and saline-injected fish (N = 5). B: Variance of nucleotide sequences of Complementarity Determining Region 3 (CDR3). Differences between vaccinated and saline injected fish are indicated with asterisks (ANOVA, LSD test, $p < 0.05$).

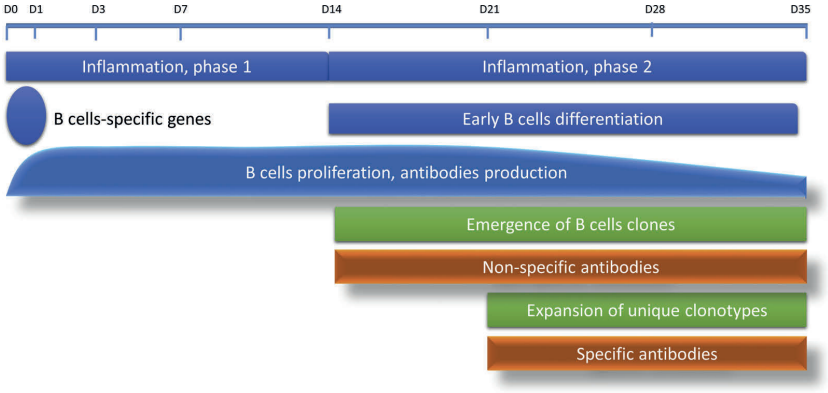

Fig. 9. Schematic presentation of processes taking place in the head kidney and plasma of Atlantic salmon after vaccination. Results produced with microarrays (blue), Ig-seq (green) and multiplex antibody assays (orange) are presented in different colours (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

cathelicidin and *serum amyloid A*, generic inflammatory markers with anti-microbial roles in Atlantic salmon (Chang et al., 2006; Jensen et al., 1997; Lee et al., 2017). *Irg1* or *cis-aconitate decarboxylase* contributes to the antimicrobial activity of macrophages (Michelucci et al., 2013), and mammalian *tnfrsf11b* is a regulator of lymphocyte development (Kong et al., 1999). The increased expression of innate response genes in vaccinated fish persisted for at least four weeks and many genes maintained an activated status to the end of observations. This immune stimulation is likely caused by the oil adjuvant providing prolonged stimulation and exposure of the bacterins in the vaccine, and is a wanted effect of immunization to enhance the innate immune response to the vaccine. A novel finding here, was switch of the character of inflammation after 14 dpv, which coincided with other events including changes in the population of B cells.

Microarray results highlighted a number of genes whose mammalian orthologues are known for major roles in differentiation and maturation of B cells. Biphasic or early responses were found in genes encoding B cell markers expressed on mature B cells (*cd22, cd72*) that are lost with differentiation into plasma cells (Poe and Tedder, 2012; Li et al., 2006; Wu and Bondada, 2009). Their early rise and subsequent decline may thus reflect B cell proliferation followed by differentiation into plasma cells. *Blimp1* is an essential regulator of plasma cell functions (Tellier et al., 2016), and accordingly, we find expression sustained for a longer period. Of special interest was the late up-regulation of *rag1* and *rag2* genes (Hansen and Kaattari, 1996; Hansen, 1997). In mice, late *rag* expression in lymphoid tissues was observed following immunization, representing newly recruited immature but non-proliferating B cells mainly assigned to the adjuvant (Nagaoka et al., 2000). *Rag* expressing B cells outside germinal centres cells were later shown to edit their receptors and concomitantly express *cd5* which are hallmarks of B1 cells in mammals and likely also in fish (Abos et al., 2018; Hillion et al., 2005). In the present study, *cd5* was moderately

upregulated at day 14–21, along with *rag1* and *rag2*, and might suggest a presence of cells of this type that could be a cellular source of non-specific antibodies, discussed below. However both *cd5* and *rag* are also expressed in T-cells, and although *cd3* and *cd8* were downregulated at this time, the contribution of T-cells cannot be excluded.

Changes in the antibody repertoire were analysed by multiplex immunoassay and immunoglobulin sequencing. Antibodies in sera of naïve fish are often referred to as natural by analogy with natural antibodies of mammals. Another frequently used term is polyreactive antibodies, which strictly would require the demonstration of multiple binding capacities. For the present study, we prefer an assumption-free term: non-vaccine specific antibodies or non-specific antibodies (NSABs) due to profound differences in observations made in fish and mammals. *Sensu stricto*, natural antibodies are found in the absence of antigenic challenge, and their presence should be demonstrated in a germ-free animal. Natural antibodies are produced by specialized populations of B cells, such as murine B1, are characterized with a close to germline structure, and their responses to antigens are rapid (reviewed by (Panda and Ding, 2015)). In contrast, NSABs are commonly found in relatively high titres in naïve fish that have been exposed to diverse endogenous and environmental microflora (Kachamakova et al., 2006). In a number of independent studies we have observed a strong increase in NSAB titres following immunization. In mammals, natural or non-specific IgM has been shown to contribute to antimicrobial defence in part by complement activation, and is regarded to be important in early antiviral defence, especially prior to establishment of specific immune responses (reviewed in (Baumgarth, 2013; Racine and Winslow, 2009)). In fish, the mechanisms of production of NSABs are still poorly understood, and their role in immunity to vaccination and infection needs to be further elucidated.

The presence of shared clonotypes with complex structure in multiple individuals and their structural features (Krasnov et al., 2017) suggest selection of B cell clones by exposure to antigens, as part of an antigen-driven response. Until now, we have not found highly abundant clonotypes with a simple structure, as all CDR3 have insertions and deletions (Krasnov et al., 2017). In the present study, we found an increased production of NSABs binding to the hapten-carrier antigen DNP-KLH from 14 days after vaccination. Increase in the cumulative frequency of immunoglobulin repertoire was found only for unique clonotypes simultaneously with an increase in systemic specific antibodies to the bacterial antigens delivered with the vaccine. In parallel, a decrease of CDR3 sequence variance coinciding with the increase in the expansion of unique clonotypes suggests a rapid expansion of recently emerged B-cell clones. The short-term character of the observed decrease may indicate egress of B cells to spleen, a secondary lymphatic organ of Atlantic salmon (Ma et al., 2013; Bromage et al., 2004), and migration of cells might also explain decrease of *ig* transcripts starting from 28 dpv. Of note is that the approach used in this study underestimates divergence, since only silent nucleotide substitutions are taken into account.

Large parts of the mechanisms of initiation of adaptive responses in fish remain unclear. S. Kaattari et al. described seven cell populations corresponding to the stages of B cells differentiation, each characterized with a set of gene markers (Ye et al., 2011; Bromage et al., 2004; Zwollo et al., 2005). We here observed marked stimulation of NSAB in vaccinated salmon, which preceded detection of markedly elevated levels of specific antibodies against A-layer and *M. viscosa*. Increase of NSABs titres could be due to an indirect stimulation of multiple B cell clones via the cytokine and chemokine network. It is plausible to think that at least part of these NSABs are encoded with highly abundant shared clonotypes, which have been selected by recognition of common endogenous or external antigens (Krasnov et al., 2017). However, if these NSAB producing cells are not proliferating and start editing their receptors upon stimulation (Hillion et al., 2005) as the late *rag* expression might suggest, they may appear as unique clonotypes, thus explaining the lack of vaccination effect on frequency of shared clonotype in the present study. Perhaps independently of this, the vaccine antigen gives rise to a slower and much more specific B-cell expansion and production of antigen-specific antibodies and unique clonotypes. However, it is important to emphasize that trends observed in the current study regarding the maturation and trafficking of B cells should be taken with caution. Subsequent analysis into this field should include the analysis of gene expression and immunoglobulin repertoire in additional lymphatic organs. Moreover, there is an urgent need for antibodies that can target key cell markers of B cells in salmon.

In summary, this study presented parallel analyses of multiple gene expression, immunoglobulin sequencing and plasma antibody titers in the first few weeks after injection of a commercially used multivalent vaccine to Atlantic salmon. Particularly intriguing are findings pointing to a contribution of non-specific antibodies in the early vaccine response. Further studies are needed to reveal the origin and functions of such antibodies, including their role in homologous and heterologous disease protection.

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Paper II

II

vaccines

MDPI

Article

IgM+ and IgT+ B Cell Traffic to the Heart during SAV Infection in Atlantic Salmon

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Abstract: B cells of teleost fish differentiate in the head kidney, and spleen, and either remain in the lymphatic organs or move to the blood and peripheral tissues. There is limited knowledge about piscine B cell traffic to sites of vaccination and infection and their functional roles at these sites. In this work, we examined the traffic of B cells in Atlantic salmon challenged with salmonid alphavirus (SAV). In situ hybridization (RNAScope) showed increased numbers of immunoglobin (Ig)M$^+$ and IgT$^+$ B cells in the heart in response to SAV challenge, with IgM$^+$ B cells being most abundant. An increase in IgT$^+$ B cells was also evident, indicating a role of IgT$^+$ B cells in nonmucosal tissues and systemic viral infections. After infection, B cells were mainly found in the *stratum spongiosum* of the cardiac ventricle, colocalizing with virus-infected myocardial-like cells. From sequencing the variable region of IgM in the main target organ (heart) and comparing it with a major lymphatic organ (the spleen), co-occurrence in antibody repertoires indicated a transfer of B cells from the spleen to the heart, as well as earlier recruitment of B cells to the heart in vaccinated fish compared to those that were unvaccinated. Transcriptome analyses performed at 21 days post-challenge suggested higher expression of multiple mediators of inflammation and lymphocyte-specific genes in unvaccinated compared to vaccinated fish, in parallel with a massive suppression of genes involved in heart contraction, metabolism, and development of tissue. The adaptive responses to SAV in vaccinated salmon appeared to alleviate the disease. Altogether, these results suggest that migration of B cells from lymphatic organs to sites of infection is an important part of the adaptive immune response of Atlantic salmon to SAV.

Keywords: Atlantic salmon; B cells; salmon alphavirus; in situ hybridization; IgM sequencing; transcriptome

1. Introduction

The adaptive immune system of modern bony fish includes B cells, which participate in specific targeting and elimination of pathogens. Teleost B cells produce immunoglobulins (Ig) of three isotypes, i.e., IgM, IgD, and IgT (the latter being equivalent to IgZ in zebrafish) [1–3]. While IgM and IgD isotypes are evolutionary conserved and present in all teleost species, IgT is only found in some

teleosts, including Atlantic salmon (*Salmo salar*) [4]. The arrangement of the Ig locus prevents isotype switching, which ensures the exclusive expression of membrane-bound IgM, IgM/IgD, or IgT [1,5].

The most abundant immunoglobulin in Atlantic salmon is tetrameric IgM [6]. IgM is found in serum, indicating a prominent role in systemic immunity, but is also secreted to the mucus of mucous membranes [7,8]. The level of IgT in serum of Atlantic salmon is 100–1000 times lower than the level of IgM [9]. IgT is present as a monomer in serum and as a tetramer in gut mucus. IgT consists of three different subclasses in rainbow trout (*Oncorhynchus mykiss*), which differ between organs at the transcriptional level [10]. The ratio of IgT to IgM in rainbow trout is much higher in mucus, and several studies have suggested a primary role of IgT in mucosal immunity of salmonid fish, especially after infection with parasites [11–13]. Pathogen-specific responses of IgT were also shown to be present in the internal organs of rainbow trout, namely, the spleen [1] and kidney [14], after infection with viruses and parasites, respectively.

In teleost fish, B cells are believed to originate from lymphopoietic stem cells in the head kidney, making it analogous to mammalian bone marrow [15]. They mature in the head kidney and migrate to sites of activation, such as the spleen or more posterior parts of the kidney [16]. As there are no lymph nodes in teleost [17,18], the spleen constitutes the main secondary lymphatic organ [18]. In addition to head kidney and spleen, populations of B cells with distinct properties are located in different tissues and organs, including mucosa-associated lymphoid tissues (MALTs) [8]. The B cell populations are highly heterogenous, as seen by differential expression of surface cell markers [19] and genes [20,21]. The presence of B cells in peripheral tissues in response to infection is known, e.g., IgM^+ B cells are recruited to the peritoneum of rainbow trout after injection of bacterium (*Escherichia coli*), *E. coli*-derived lipopolysaccharide, or viral hemorrhagic septicemia virus [22], with increased observation of IgM^+ and IgT^+ cells in the pyloric caeca of orally vaccinated rainbow trout [23] and in the skeletal muscle of rainbow trout [24] and Atlantic salmon [25,26] after DNA vaccination. The lack of lymph nodes suggests that terminal differentiation of B cells can occur at various sites, possibly also at the site of inflammation. At present, little is known about the routes and dynamics of B cell traffic and their functional roles under various conditions, including vaccination and viral infection. The recent development of parallel sequencing of the variable region of immunoglobulins (repertoire sequencing (Rep-seq) or immunoglobulin sequencing (Ig-seq)) has opened up novel research possibilities [27,28]. A unique complementarity-determining region (CDR3) or clonotype marks B cells derived from the same ancestor cell, which allows tracking of cell movement between organs and tissues. Each unique CDR3 sequence is a marker of clonal B cells. The presence of a clone in two different tissues indicates a relatively recent migration from one organ to another, with the largest clonotypes indicating recent expansion. Furthermore, RNAscope [29] markedly improved the accuracy and sensitivity of *in situ* hybridizations, helping to overcome one of the main obstacles in fish immunology, i.e., the limited selection of specific antibodies targeting key cell markers. This technique enables visualization of transcripts of both immune and virus genes within Atlantic salmon tissues [30–33].

Salmonid pancreas disease virus (SPDV), also known as salmonid alphavirus (SAV), is the causative agent for pancreas disease (PD) [34,35]. PD is characterized by necrosis and loss of exocrine pancreatic tissue, in addition to necrosis and inflammation of the heart and skeletal muscle [36]. Increased Ig transcripts were previously observed in the hearts of salmon infected with SAV [37], suggesting recruitment of B cells. This makes challenge with SAV an attractive model to investigate B cell traffic and their roles in disease protection. Belonging to the genus *Alphavirus*, SAV is a single-stranded positive-sense RNA virus that genetically clusters into six subtypes. Subtypes 2 and 3 (SAV2 and SAV3) are currently found in farmed salmon in Norway with various geographical distribution [36]. The genome of SAV includes nonstructural and structural proteins, with the different subtypes classified on the basis of both structural and nonstructural gene nucleotide sequence [38,39]. Although SAV infections affect multiple tissues, a tissue tropism for the heart (ventricle) was previously demonstrated [40], and tissue from heart samples is commonly used in routine RT-qPCR testing for the virus. Successful vaccination [41–43], studies of the adaptive immune responses to SAV [44], and comparison of Atlantic salmon strains with different resistance levels to infection [45,46]

point to an important role of immunoglobulins in the protection against pancreas disease. Of note is that the quantitative trait locus (QTL) of Atlantic salmon with higher resistance to PD includes the B locus of the immunoglobulin heavy chain [47].

The aim of the present study was to describe the spatial and functional B cell distribution in the target organ following SAV infection in Atlantic salmon and to investigate B cell traffic by comparing the presence of clonotypes in a major secondary lymphoid organ (SLO) (the spleen) and a target organ (the heart), as well as to assess ensuing heart function by measuring gene expression changes. We observed that SAV challenge enriched IgM^+ and IgT^+ in cardiac compartments and found a clonal B cell relationship indicative of virus-induced traffic from the spleen to the heart. Gene expression analysis seemed to show a strong inflammatory response and compromised cardiac physiology in the infected heart of the unvaccinated control group, which was alleviated in the vaccinated fish.

2. Materials and Methods

2.1. Animal Study

The experimental fish were Atlantic salmon parr/smolt. The animal study was carried out at Veso Vikan Hatchery and Veso Vikan Research Facility (Namsos, Norway). The fish were acclimatized for a minimum of 1 week and starved for a minimum of 48 h prior to vaccination. A total of 51 fish with a mean weight of 62 g were anesthetized (Metacain, Pharmaq) and marked by Passive Integrated Transponder (PIT) tagging prior to vaccination. Vaccination was carried out by intraperitoneal injection with 0.1 mL Aquavac PD7 (MSD Animal Health). The control group ($n = 52$), hereafter named the unvaccinated, were injected with 0.1 mL sterile 0.9% NaCl. Aquavac PD7 vet is a commercial inactivated, multivalent injection vaccine for the immunization of Atlantic salmon. The active components are two inactivated viral antigens, namely, salmon pancreas disease virus (SPDV) and infectious pancreatic necrosis virus (IPNV), and five inactivated bacterial antigens, namely, *Aeromonas salmonicida* subsp. *salmonicida*, *Vibrio salmonicida*, *Vibrio anguillarum* serotype O1, *Vibrio anguillarum* serotype O2a, and *Moritella viscosa,* alongside the oil-based adjuvant. Fish were subjected to 24 h of light stimulus following vaccination. Vaccinated and unvaccinated fish were kept in the same tank with 12 °C flow through fresh water for 47 days before transfer to the research facility. Following transport and prior to challenge, fish were acclimatized to 25‰ salinity at 12 °C for 14 days. SAV3 challenge was performed according to standard procedures at Veso Vikan research facility using a cohabitation challenge model. SAV was provided by The Norwegian Veterinary Institute (Oslo, Norway), Isolate 4 SAV3 210916, 3 passes CHSE-214, titer 106,0TCID50 [48]. The SAV3 virus was diluted 1:5, and 0.1 mL was injected in each shedder. SAV shedders ($n = 30$) were placed into the challenge tank (approximately 450 L) with the cohabitants (vaccinated, $n = 51$; unvaccinated $n = 5$ 2) at 9 weeks post-vaccination. Samples were taken at 0, 21, 35, and 42 days post-challenge (dpc). Shedders were marked by removing the right maxilla. Tissue samples were collected in tubes with RNAlater™ (Sigma Aldrich, St. Louis, MI USA), which was kindly provided by PatoGen AS (Ålesund, Norway), and stored for 1 day at 4 °C before storage at −20 °C until analysis. Tissues were fixed in formalin (4% formalin, 0.08 M sodium phosphate, pH 7.0), processed in a Thermo Scientific Excelsior® tissue processor (Thermo Fisher Scientific, Waltham, MA USA), and embedded in paraffin Histowax using a Tissue-Tek®, TEC 5 (Sakura Finetek, Alphen aan den Rijn, The Netherlands) embedding center. The challenge trial was approved by the Norwegian Food Safety Authority (permit number 13160).

2.2. In Situ Hybridization

Three individuals from each sampling point were subjected to in situ hybridization targeting IgM, IgT, and SAV transcripts using RNAscope 2.5 HD Assay RED (Advanced Cell Diagnostics (ACD), Newark, CA, USA). RNAscope is an *in situ* hybridization (ISH) method enabling detection of target RNA within tissue or intact cells with high sensitivity and specificity and low background noise due to specific probe design. This is achieved through the use of multiple short probes containing a tail coupling preamplifier, forming a tree-like structure that binds a fluorescent label to

produce a strong signal. Briefly, paraffin-embedded tissue sections (4 μm) of heart and pancreas/pyloric caeca were dewaxed at 60 °C for 90 min in ACD HybEZ II, followed by hydrogen peroxide treatment for 10 min while being incubated at room temperature. Samples were boiled in RNAscope target antigen retrieval reagent for 15 min and each section was incubated with RNAscope protease plus at 40 °C for 15 min in a HybEZ oven. Each section was hybridized with the respective RNAscope target probe for 2 h at room temperature. Custom ZZ RNAscope probes were designed to target IgM, IgT, and SAV transcripts (Table 1). The IgM and IgT probes were designed to detect both secretory and membrane-bound forms and all subvariants of each immunoglobulin. SAV inhabits four nonstructural proteins (nsP) named nsP1–nsP4, respectively. Single-stranded, positive-sense RNA can serve both as genomic and mRNA nucleic acid during replication, making it impossible to distinguish between genomic and mRNA target. The nsP1 ISH probe was designed to cover all SAV subtypes. Fast Red chromogenic substrate was used to detect the signals amplified, following the manufacturer's instructions. Counterstaining was done with 50% Gill's hematoxylin solution and mounted with EcoMount (BioCare Medical, Pacheco, CA, USA). Imaging was performed by bright field microscopy (Leica microsystems, LM2500, Wetzlar, Germany).

Table 1. Target and control probes for in situ hybridization.

	Probe	Accession No.	Target Region (bp)	Catalogue No.
Target	IgT	GQ907003	3–883	532171
	IgM	XM_014203125	219–1157	532181
	SAV	NC_003930	410–1279	844631
Control	DapB (negative)	EF191515	414–862	310043
	PPIB (positive)	NM_001140870	20–934	494421

The head kidney was used as the positive control for the IgM and IgT probes. Material from pre- and post-infection served as positive control material for the SAV probe. A probe targeting peptidylpropyl isomerase B (PPIB) in Atlantic salmon was used as a reference target gene to test RNA integrity. A negative control probe (targeting the DapB gene of *Bacillus subtilis*, universal negative control probe) was used to assess cross-reactivity. Both positive and negative control probes were obtained from the manufacturer.

2.3. RNA Isolation

Heart and spleen samples (5–10 mg) were placed in tubes with 400 μL lysis buffer (Qiagen), and 20 μL proteinase K (50 mg/m) was added into each tube. Samples were homogenized in FastPrep 96 (MP Biomedicals, Eschwege, Germany) for 120 s at maximum shaking, then centrifuged and incubated at 37 °C for 30 min. RNA was extracted on Biomek 4000 robot using Agencourt RNAdvance Tissue kit (Qiagen Norway, Oslo, Norway) according to the manufacturer's instructions. RNA concentration was measured with NanoDrop™ One (Thermo Fisher Scientific, Waltham, MA USA) and quality was assessed with Agilent Bioanalyzer 2100.

2.4. Sequencing of the Variable Region of the IgM Heavy Chain

Samples collected at 0, 21, and 42 days post-challenge (dpc) from vaccinated fish (n = 8) with a multivalent vaccine (including inactivated SPDV) and unvaccinated controls (n = 8) were included in the immunoglobulin sequencing (Ig-seq) analysis. Synthesis of complementary DNA was primed with an oligonucleotide to the constant region (CH) of Atlantic salmon IgM (TAAAGAGACGGGTGCTGCAG) using SuperScript IV reverse transcriptase (Thermo Fisher Scientific, Waltham, MA USA), according to the manufacturer's instructions. Libraries were prepared with two PCR reactions. The first PCR amplified the cDNA with a degenerate primer, TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTGARGACWCWGCWGTGTATTAYTGTG,

which aligned to the 3′-end of all Atlantic salmon heavy chain variable (VH) genes, with the primer GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGAACAAAGTCGGAGCAGTTGATGA annealing to the 5′-end of CH. Both primers were complementary to Illumina Nextera adaptors. Reaction mixtures (20 μL) included 10 μL 2x Platinum Hot Start PCR Master Mix (Thermo Fisher Scientific, Waltham, MA USA), 0.5 μL of each primer (10 pmol/μL), 8 μL of water, and 1 μL of template. The second PCR used Illumina Nextera XT Index Kit v2, and the reaction included 2 μL of each primer and 2 μL of the product of the first PCR. The PCR program included heating for 1 min at 94 °C, amplification for 10 s at 94 °C, 20 s at 53 °C, and 20 s at 72 °C (30 cycles in the first PCR and 9 cycles in the second PCR), and extension for 5 min at 72 °C. DNA concentration was measured with Qubit (Thermo Fisher Scientific). Aliquots of the libraries were combined and purified twice with the Qiagen PCR clean-up kit. Sequencing was carried out using a Illumina MiSeq Reagent Kit v3 (150-cycle) (Illumina, Inc., San Diego, CA USA). The libraries were diluted to 4 nM, and PhiX control was added to reach 0.8 nM. After trimming of Illumina adaptors and primers and removal of low-quality reads, sequences were transferred to a database and translated into amino acids. The frequency of each unique sequence (clonotype) was calculated. This study focused on the traffic of B cells between the spleen and the heart. Sharing of IgM repertoires was assessed by the co-occurrence of the largest clonotypes (leaders). Heart–spleen (HS100) and spleen–heart (SH100) metrics were determined as the 100 largest clonotypes of the heart (spleen) that were also detected in the spleen (heart) at a frequency of $>10^{-4}$. Differences were assessed with ANOVA followed by Tukey's test (Statistica 13).

2.5. Microarrays

Analyses were carried out on heart samples from unvaccinated and vaccinated salmon before challenge (0 dpc) and 21 dpc using Nofima's Atlantic salmon genome-wide 44 k DNA oligonucleotide microarray Salgeno-2 (GPL28080), with a total of 24 microarrays used. The platform was annotated using the bioinformatic pipeline STARS [49]. Microarrays were manufactured by Agilent Technologies (Santa Clara, CA USA), and the reagents and equipment were purchased from the same provider. RNA amplification and labeling were performed with a One-Color Quick Amp Labeling Kit and a Gene Expression Hybridization kit was used for fragmentation of the labeled RNA. Total RNA input for each reaction was 500 ng. After overnight hybridization in an oven (17 h, 65 °C, rotation speed 0.01 g), arrays were washed with Gene Expression Wash Buffers 1 and 2 and scanned with an Agilent scanner. Subsequent data analyses were performed with STARS. Global normalization was performed by equalizing the mean intensities of all microarrays. Next, the individual values for each feature were divided by the mean value of all samples, thereby producing expression ratios (ER). The log2-ER values were calculated and normalized with locally weighted nonlinear regression (Lowess). Differential expression between vaccinated salmon and control, SAV-challenged, and intact fish was assessed by using an expression ratio of >1.75-fold and $p < 0.05$ (*t*-test). STARS annotations were used for comparison of the functional groups of genes (mean log2-ER, *t*-test).

3. Results

3.1. IgM⁺ and IgT⁺ B Cell Transcripts Increased in the Heart after Viral Challenge

In situ hybridization targeting IgM (Figure 1) and IgT (Figure 2) transcripts was performed on heart and pancreas/pyloric caeca samples collected at 0, 35, and 42 days post-challenge (dpc) from unvaccinated fish. Prior to challenge (0 dpc), many IgM-transcribing cells were present in the atrium, while only a few scattered positive cells were detected in the ventricle and pancreas (Figure 1A–C, respectively). At 35 dpc, substantial amounts of IgM-transcribing cells remained present in the atrium (Figure 1D), while a marked increase of IgM transcripts was evident in the ventricle and pancreas (Figure 1E, F, respectively). The highest amounts of IgM-transcribing cells were detected in the atrium and ventricle at 42 dpc (Figure 1G, H, respectively), while in the pancreas, the quantity and distribution of IgM transcripts at 42 dpc (Figure 1I) were similar to 35 dpc (Figure 1F). The

distribution of IgM transcripts differed within the compartments of the heart. Most IgM transcripts were detected in the atrium and in the *stratum spongiosum* of the heart ventricle.

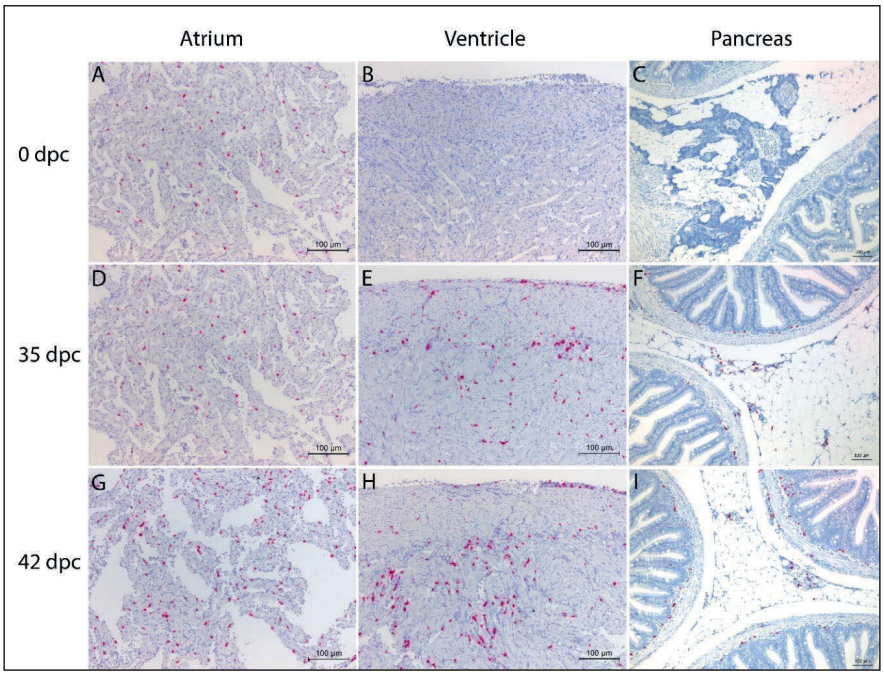

Figure 1. *In situ* hybridization of immunoglobin (Ig)M transcripts in salmonid alphavirus (SAV)-naïve Atlantic salmon at 0, 35, and 42 days post-SAV challenge (dpc). Images taken from representative individuals within each group. (**A–C**) Many IgM-positive cells in the atrium at 0 dpc. Few and scattered positive cells in the ventricle and pancreas. (**D–F**) Many IgM-positive cells in the atrium at 35 dpc. Substantial amounts of IgM-positive cells in the ventricle and in the exocrine pancreas. (**G–I**) High amounts of IgM-positive cells evident in both the atrium and the ventricle at 42 dpc. IgM-positive cell infiltrates are mainly seen in the *stratum spongiosum* and in the transition zone between the *stratum compactum* and the *stratum spongiosum*. Positive cells also occur in the epicardium. Similar quantities and distributions of IgM-positive cells observed in the pancreas at 42 dpc and 35 dpc.

Only occasional IgT-transcribing cells were detected in the atrium and ventricle prior to challenge (Figure 2A,B, respectively), while no IgT-transcribing cells were detected in the pancreas/pyloric caeca (Figure 2C). The presence of IgT-transcribing cells was evident in both the atrium and ventricle as well as the pancreas/pyloric caeca at 35 dpc (Figure 2D–F, respectively). A further increase in IgT transcripts was observed in both the atrium and ventricle at 42 dpc (Figure 2G,H), showing a similar distribution within the compartments of the heart as that observed for the IgM transcripts (Figure 1). An increase in IgT-transcribing cells was evident in the pancreas/pyloric caeca at 42 dpc (Figure 2I), but to a lesser degree than what was seen in the heart.

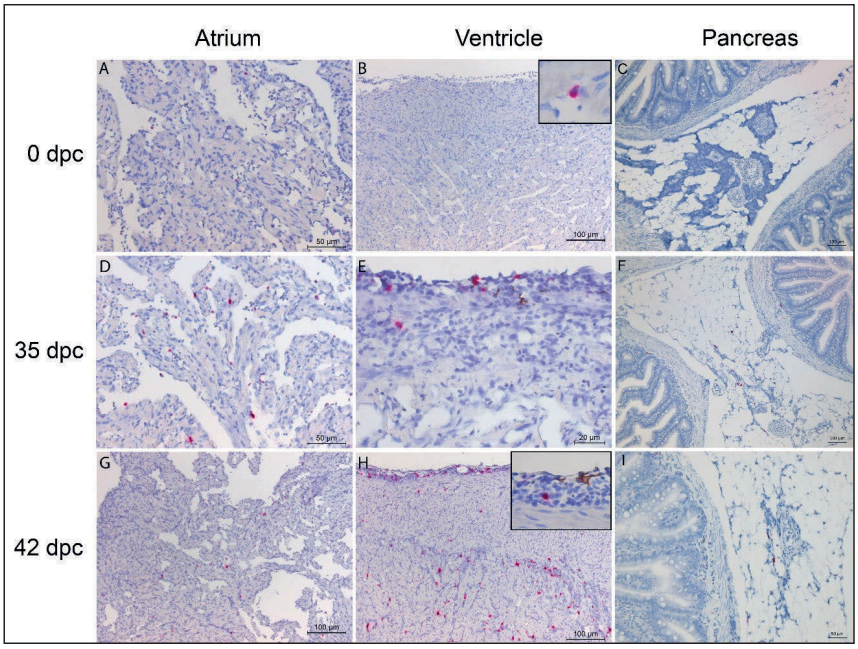

Figure 2. *In situ* hybridization of IgT transcripts in SAV-naïve Atlantic salmon at 0, 35, and 42 days post-SAV challenge (dpc). Images are taken from representative individuals within each group. (**A**–**C**) Few scattered IgT-positive cells evident in the atrium, ventricle, and pancreas at 0 dpc. (**D**–**F**) Presence of IgT-positive cells in all tissues evident at 35 dpc. (**G**–**I**) IgT-positive cell infiltrates mainly seen in the *stratum spongiosum* and in the transition zone between the *stratum compactum* and the *stratum spongiosum* at 42 dpc. Few scattered IgT-positive cells evident in the atrium and pancreas.

3.2. Distribution of SAV Transcripts in Target Organs

To study the distribution of virus replication in target organs after challenge, we performed in situ hybridization for SAV nsP1 transcripts on heart samples from unvaccinated fish collected at 0, 35, and 42 dpc. Additional RT-PCR was performed on heart samples from a total of eight individuals at 21 dpc, confirming all individuals as positive for SAV (data not shown). Using *in situ* hybridization, no viral transcripts were detectable at 0 dpc (Figure 3A). Samples from 35 dpc showed the highest occurrence of viral transcripts. At this time, a few positive cells were observed in the border between the ventricle and the atrium, but viral transcripts were generally mainly detected in the *stratum spongiosum* of the cardiac ventricle (Figure 3B) and within myocardial-like cells of the heart ventricle (Figure 3C). At 42 dpc, the signal was still only detectable in the heart ventricle, and to a lesser degree than 35 dpc (Figure 3D).

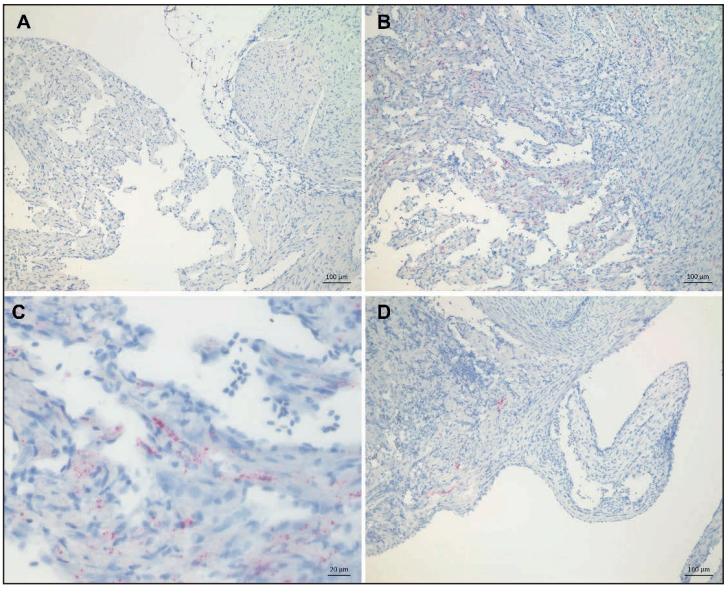

Figure 3. *In situ* hybridization of nonstructural protein (nsP)1 transcripts in the hearts of SAV-naïve Atlantic salmon at 0, 35, and 42 days post-SAV challenge (dpc). Images taken from representative individuals within each group. Localization of SAV transcripts shown by *in situ* hybridization of nsP1 in the hearts of unvaccinated fish. (**A**) No positive signal at 0 dpc; (**B**,**C**) virus was mainly detected in the *stratum spongiosum* of the ventricle at 35 dpc; (**D**) virus detected sparingly, mainly in the *stratum spongiosum* of the ventricle, at 42 dpc.

3.3. Ig-seq Revealed Movement of B Cells from Lymphoid Tissue to the Heart in Response to Infection

To evaluate the traffic of B cells, we looked for co-occurrence of B cell clonotypes, which appear in different organs following migration of clonally expanded cells. We estimated the co-occurrence of leaders (the 100 largest cardiac clonotypes) in the heart that are also found in the spleen (HS100) and, reciprocally, the leaders from the spleen simultaneously present in the heart (SH100). Presuming that B cell expansion occurs in a major SLO (the spleen), and that B cells move from the SLO toward the infected organ (the heart), we used HS100 to detect spleen-to-heart traffic, with SH100 indicating recently expanded clones recruited to the heart. At 21 dpc, HS100 in vaccinated salmon was double that of the unvaccinated controls, indicating larger accumulated migration (Figure 4A), while the higher SH100 in vaccinated salmon compared to unvaccinated fish indicated a significantly larger fraction of recently expanded splenic clonotypes directed to the heart (Figure 4B). Three weeks later (42 dpc), traffic increased in unvaccinated salmon and both metrics equalized in the unvaccinated and vaccinated groups.

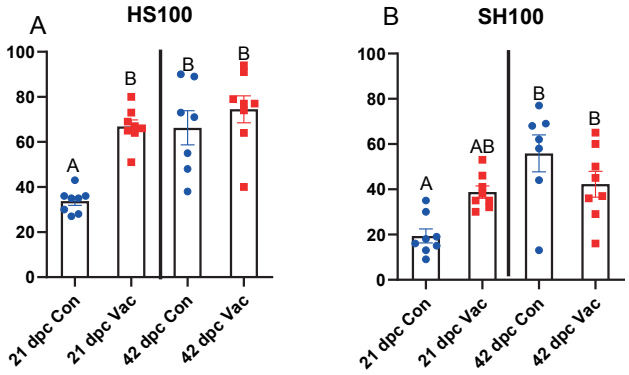

Figure 4. Immunoglobulin sequencing data showing an overlap of repertoires in the heart and spleen of unvaccinated (Con) or vaccinated (Vac) Atlantic salmon 21 and 42 days post-challenge (dpc). (**A**) Occurrence of the 100 most abundant clonotypes in the heart that are also found in the spleen (HS100). (**B**) Occurrence of the 100 most abundant clonotypes in the spleen that are also found in the heart (SH100). Columns not sharing a common letter are significantly different (individual for each figure) ($n = 8$, ANOVA, Tukey's test, $p < 0.05$).

3.4. Transcriptome Responses: Immune and Cardiac Functions

Microarray analyses performed on heart samples found minor differences between unvaccinated and vaccinated salmon before challenge (Supplementary File). After challenge, infected fish showed transcriptome responses to SAV similar to those reported in previous studies [37,46,47]. The changes in relation to uninfected fish seemed to be significantly greater in unvaccinated than in vaccinated salmon, as seen in the numbers of differentially expressed genes (6626 versus 3595 genes), in the functional groups (Figure 5) and individual genes (Figure 6). The strongest responses to SAV and the highest difference between the groups were observed in virus-responsive genes (VRG), a large set of genes activated in response to viruses, double-stranded RNA, and bacterial DNA [26,50], with their expression reflecting the pathogen load. *Isg15* and *viperin* are characterized by the greatest upregulation in infected tissues. Similar, albeit smaller expression changes were indicated in the functional groups of genes associated with signaling via chemokine and cytokine networks, antigen presentation, inflammation, and adaptive immunity. Unvaccinated fish seemed to show higher expression of a suite of genes known as markers of acute inflammation in Atlantic salmon, including the antibacterial peptide *cathelecidin*, *arginase ii,* and immune effectors, all with different modes of action (Figure 6A). This was paralleled by apparently reduced cardiac expression of genes encoding the components of myofiber and proteins that regulate muscle contraction (*ryanodine receptor 1b,* among others) and supply it with energy (sugar metabolism, mitochondria) (Figure 6B). Decreased levels of transcripts for *globins* and erythrocyte markers, such as *rhag,* indicated reduced blood circulation. Reduced expression of *collagens* and proteins specific for epithelium and endothelium was also suggested in the unvaccinated group.

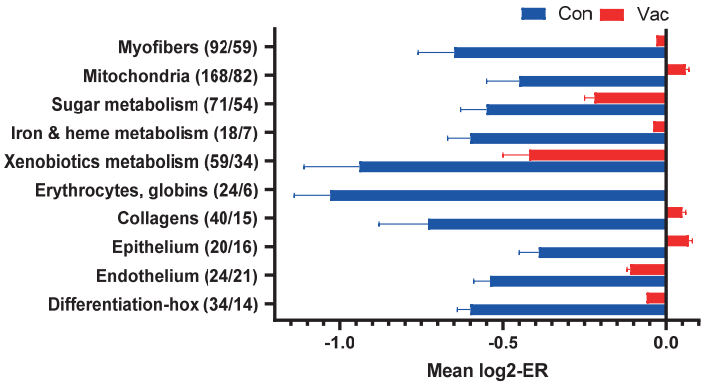

Figure 5. Functional groups of genes with coordinated expression changes in hearts of SAV-challenged Atlantic salmon at 21 dpc in vaccinated fish (vac) versus unvaccinated controls (con). The numbers of differentially expressed genes are in parentheses (saline control/vaccinated). Data are mean log2-expression ratios (infected to intact control) ± Standard Error. All differences between vaccinated fish and saline control are significant.

A number of lymphocyte-specific genes were upregulated only in the control group, including genes involved in activation, signaling, and differentiation of lymphocytes (transcription factor *mafb*, *lymphoid-specific helicase, cd166, cd209,* and *lymphocyte cytosolic protein*), B cells (several *cd22-like* genes, *tnf receptor 11b, pu.1, btk tyrosine protein kinase,* and *pi3k regulatory subunit 6*), and T cells (*cd276, cd226,* and *cd4*) (Figure 6B). Two genes involved in V(D)J recombination [51] (*artemis* and *dna cross-link repair 1c*) were downregulated in unvaccinated fish and showed a slight increase in vaccinated fish.

A

Role	Genes	Con	Vac	Con / Vac
Antiviral	ISG15-like	***185.7***	***14.3***	3.5
Antiviral	Viperin	***80.9***	***8.0***	3.4
Inflammation	Cathelicidin	***23.6***	***3.2***	***6.6***
Antigen presentation	Antigen peptide transporter	***7.1***	1.0	***6.3***
Effector	Arginase, type II	***6.4***	***2.5***	***2.7***
Effector	Cytochrome b-245 light chain	***4.4***	1.6	***2.7***
Effector	Neutrophil cytosolic factor 2	***4.3***	1.4	***3.4***
Effector	Bactericidal permeability-increasin	***3.9***	1.3	***3.3***
Effector	Neutrophil cytosolic factor 1	***3.8***	1.7	***3.0***
Endocrine	Neuropeptide Y	***16.3***	2.3	***6.7***
Myofiber	Ryanodine receptor 1b	***-8.9***	-1.2	***-8.0***
Myofiber	Myozenin 2	***-4.1***	1.0	***-4.4***
Calcium metabolism	Calsequestrin 2	***-2.9***	-1.3	***-2.3***
Sugar metabolism	Glycogen phosphorylase,	***-15.3***	-1.9	***-7.2***
Sugar metabolism	Glycogen debranching enzyme	***-4.1***	-1.4	***-2.8***
Endothelium	Endothelial differentiation factor 1	***-7.8***	***-2.9***	***-2.3***
Erythrocytes	Rhag	***-4.0***	-1.2	***-3.0***
Erythrocytes	Hemoglobin subunit alpha	***-3.5***	-0.8	***-4.4***

B

Role	Gene	Con	Vac	Con / Vac
B cells	BTK tyrosine protein kinase	***2.4***	1.2	***2.3***
B cells	PI3K regulatory subunit 6	***2.6***	1.1	***2.2***
B cells	Transcription factor PU.1	***3.9***	1.0	***4.0***
B cells	B-cell receptor CD22-1	***2.4***	1.2	***2.0***
B cells	B-cell receptor CD22-2	***3.5***	1.3	***3.0***
B cells	TNF receptor member 11B	***7.2***	-1.2	***6.4***
Lymphocytes	Transcription factor MafB	***8.0***	1.6	***5.5***
Lymphocytes	CD166 antigen homolog	***4.7***	1.3	***3.2***
Lymphocytes	CD209 antigen-like protein C	***8.2***	***2.0***	***3.9***
Lymphocytes	Lymphocyte cytosolic protein 2	***3.7***	1.4	***2.8***
Lymphocytes	Fermitin family	***7.0***	***2.0***	***3.9***
Lymphocytes	Cytohesin 1b	***4.4***	1.3	***3.7***
Lymphocytes	Lymphoid-specific helicase	1.3	***3.7***	1.3
T cells	CD226 molecule	***2.6***	1.2	***2.4***
T cells	CD276 antigen	***3.2***	1.0	***2.6***
T cells	CD4-like	***2.2***	-1.3	***3.2***
Lymphocytes	Artemis-like	***-2.6***	***2.2***	***-5.8***
Lymphocytes	DNA cross-link repair 1C	***-2.7***	***2.2***	***-6.1***

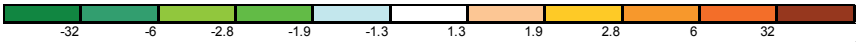

Figure 6. Differentially expressed genes at 21 days post-challenge. Data are the expression ratios (folds) of infected salmon to intact control (first two columns) and saline control to vaccinated fish (third column), highlighted with color scale. Differential expression (>1.75-fold; $p < 0.05$) is indicated with underlined, italic, bold font. (**A**) Inflammation, functional disorders, and heart pathology; (**B**) lymphocyte-specific genes.

4. Discussion

In the present study, the distribution of IgT$^+$ and IgM$^+$ B cells within the heart of Atlantic salmon before and after SAV infection was examined using *in situ* hybridization. Furthermore, sequencing

of the IgM variable region (Ig-seq) was used to study B cell traffic between the spleen and heart after viral challenge, while the functional consequences of SAV-induced myocarditis in control and vaccinated salmon were assessed using microarray gene expression analyses.

In situ hybridization showed that, prior to infection, the most abundant B cells in the heart were IgM^+, which were primarily present in the cardiac atrium and less in the ventricle. A previous work showed only a sparse increase in IgM in the heart 1 to 3 weeks after SAV infection by IHC using a polyclonal antibody to recognize salmon IgM [52]. However, in the present study, using *in situ* hybridization, we detected a large increase in IgM transcripts from 21 to 42 days post-challenge in both the atrium and the ventricle, suggesting a role of B cells in the immune response against SAV in the heart, either by infiltration or by clonal expansion of B cells in the heart. Only a few IgT transcripts were detected in the heart prior to infection, but an increase was evident after SAV challenge. Thus, while IgT was mainly associated with mucosal immunity in fish, these results, as well as previous reports of IgT^+ cells in the heart [53] and IgT transcripts in the head kidney [14,54] and spleen [1] of salmonids after infection, indicate a role of IgT+ B cells in nonmucosal tissues and in systemic viral infections in salmonids.

IgM^+ and IgT^+ B cells were mainly detected in the *stratum spongiosum* and the transition zone between the *stratum compactum* and *stratum spongiosum* of the heart, in addition to some positive cells in the epicardium. This coincided with the localization of nsP1 transcripts corresponding to salmonid alphavirus (SAV) in the heart. Alphaviruses are known to infect muscle cells in other species [55,56], and it was shown that SAV2 targets muscle stem cells in rainbow trout [57]. In Atlantic salmon, virus particles in myocardial cells were indicated by applying electron microscopy [58]. In the current study, viral transcripts were found mainly within myocardial-like cells in the heart ventricle and, to a lesser degree, in equivalent cells in the atrium, suggesting tropism of SAV to ventricular cells in the heart.

As the name pancreas disease suggests, the pancreas is the first organ to develop lesions following infection with SAV, which were detectable at only 2 weeks post-challenge [34]. However, the pancreatic tissue with associated pyloric caeca was previously shown to be unsuitable for RT-PCR detection of SAV [40] due to low levels of viral RNA. In our study, ISH revealed scattered IgM^+ cells present in the pancreas prior to infection, but low levels after infection in comparison to the heart. Likewise, the levels of IgT transcripts in the pancreas/pyloric caeca were low, and all intestinal layers and smooth muscle, as well as the endocrine and exocrine pancreas, were negative for SAV nsP1 transcripts. These results confirmed that the pancreas is an unreliable source of detecting SAV presence and the ensuing immune response.

While *in situ* hybridization documented the distribution of B cells within the infected heart, Ig-seq and transcriptome analyses elucidated their origin and possible roles in the defense against SAV infection. IgM sequencing performed in several previous studies revealed a relatively small overlap of the repertoires of the lymphatic and peripheral tissue repertoires under basal conditions [27,28]. A part of newly emerged clonotypes migrate from the lymphatic organs to the target tissues and can be detected at both sites for a limited period of time. A twofold higher co-occurrence of large clonotypes in the hearts and spleens of vaccinated salmon at 21 dpc reflected enhanced traffic of B cells at this time point. B cell traffic to the target organ should not be confused with the rapid, transient, and nonspecific influx of B cells to the sites of vaccination and infection, which is most likely due to their innate and antigen-presenting immune cell roles [59,60] as chemotactic responders to mediators of inflammation [61]. In this study, due to increased traffic of B cells to SAV-infected hearts, we speculate that the delivery of antibody-producing B cells directly at the infection site may increase the effectiveness of the humoral immune response. In vaccinated fish, recruitment of B cells into the infected heart was stimulated much earlier than in control fish.

Our hypothesis of a protective humoral response role in vaccinated individuals was supported by the transcriptomic profiling. At 21 dpc, signs of cardiac dysfunction were detected in the unvaccinated group, including downregulation of genes involved in muscle contraction and energy metabolism, which is typical for PD [37,62]. Decreased levels of transcripts for *globins* and erythrocyte markers, *collagens*, regulators of differentiation, and proteins specific for epithelium and endothelium

suggested a lower ability for tissue repair. The upregulation of *arginase ii* and *neuropeptide y* in combination with the downregulation of *calsequestrin* observed in unvaccinated salmon was proposed as a diagnostic symptom of heart pathology caused by PD [37,62]. The differential expression of lymphocyte-specific genes in the infected heart resembled early responses to vaccination in the head kidneys of Atlantic salmon [63]. It is likely that B cells entering the heart of unvaccinated salmon had not yet reached the developmental stage when the surface receptors (BCR) were fully substituted with the secreted IgM. *Pu1*, *mafb*, *pi3k regulatory subunit 6*, and *lymphoid-specific helicase* are essential for the differentiation and survival of lymphocytes, with *btk* considered a master regulator of B cell development [64]. *Cd166*, *cd209*, *cd226*, *fermitin family protein*, and *cytohesin 1b* control adhesion and interactions between lymphocytes and the lymphatic tissue environment. The Atlantic salmon genome includes up to 37 *cd22-like* genes, with 19 exhibiting upregulation in the head kidneys of vaccinated salmon, as well as *tnf receptor 11b* [63]. *Artemis* and *dna cross-link repair 1c* proteins, known to be involved in V(D)J recombination [51], were downregulated in the unvaccinated group, which was also previously observed in the head kidneys and spleens of vaccinated salmon. Increased cardiac expression of genes that control differentiation of lymphocytes in Atlantic salmon was observed in previous studies with PD [37,46], suggesting that the terminal stages of lymphocyte maturation can take place at infection sites. In this work, the higher expression of these genes in the unvaccinated group most likely reflected an earlier developmental stage of the lymphocytes, with the B cells of vaccinated salmon possibly reaching more complete differentiation in the spleen or migrating to the heart at an earlier time point than 21 dpc.

5. Conclusions

The results of this work suggest that SAV has a tropism for cardiomyocytes in the ventricles of Atlantic salmon. IgM^+ and IgT^+ B cells were recruited to the heart in response to SAV challenge, with IgM^+ B cells being more abundant than IgT^+ B cells. However, an increase in IgT^+ B cells was evident, indicating a role of IgT^+ B cells in nonmucosal tissues and systemic viral infections in salmonids. The viral challenge substantially increased the fraction of splenic B cells directed to the heart, and previous vaccination accelerated this traffic. Gene expression profiling at 21 dpc suggested suppression of various cardiac functions in the control group, which was relieved in the vaccinated fish. Altogether, B cell trafficking to the heart, with subsequent sustainment of cardiac function and reduction of inflammation, may alleviate the severity of this disease. We conclude that traffic of B cells is an essential part of the adaptive humoral responses to SAV in Atlantic salmon.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Differentially expressed genes.

Author Contributions: Conceptualization, A.F.B., A.K., P.B., P.F., and H.L.; methodology, A.F.B., H.B., E.O.K., A.K., and H.L.; software and data curation, S.A.; writing—original draft preparation, A.F.B., A.K., H.B., P.F., and H.L; writing—review and editing, A.K., P.F., and P.B.; supervision, P.B. and H.L. project administration and funding acquisition, P.F. and AK. All authors have read and agreed to the published version of the manuscript.

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Paper III

III

Effect of two constant light regimens on antibody profiles and immune gene expression in Atlantic salmon following vaccination and experimental challenge with Salmonid Alphavirus

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Abstract

Before seawater transfer, farmed Atlantic salmon are subjected to treatments that may affect the immune system and susceptibility to pathogens. E.g., exposure to constant light (CL) stimulates smoltification, which prepares salmon to life in sea water, but endocrine changes in this period are associated with suppression of immune genes. Salmon are vaccinated towards end of the freshwater period to safeguard that adequate vaccine efficacy is achieved by the time the fish is transferred to sea. In the present study, we investigated how the responses to vaccination and viral infection varied depending of time of CL onset relative to vaccination. The salmon were either exposed to CL two weeks of prior to vaccination (2-PRI) or exposed to CL at the time of vaccination (0-PRI). A cohabitant challenge with salmonid alphavirus, the causative agent of pancreatic disease, was performed 9 weeks post vaccination. The immunological effects of the different light manipulation were examined at 0 and 6 weeks post vaccination, and 6 weeks post challenge. Antibody levels in plasma were measured using a serological bead-based multiplex panel as well as ELISA, and 92 immune genes in heart and spleen were measured using an integrated fluidic circuit-based qPCR array for multiple gene expression. The 2-PRI group showed a moderate transcript down-regulation of genes in the heart at the time of vaccination, which were restored 6 weeks after vaccination (WPV). Conversely, at 6WPV a down-regulation was seen for the 0-PRI fish. Moreover, the 2-PRI group had significantly higher levels of antibodies binding to three of the vaccine components at 6WPV, compared to 0-PRI. In response to SAV challenge, transcription of immune genes between 2-PRI and 0-PRI was markedly dissimilar in the heart and spleen of control fish, but no difference was found between vaccinated salmon from the two CL regimens. Thus, by using labor-saving high

throughput detection methods, we demonstrated that light regimens affected antibody production and transcription of immune genes in non-vaccinated and virus challenged salmon, but the differences between the light treatment groups appeared eliminated by vaccination.

1 Introduction

Farmed Atlantic salmon are kept under controlled environmental conditions and protected from many infections during the freshwater period. Pathogen pressure combined with stress from transportation, adaptation to seawater and marine environment substantially increase the risk and occurrence of diseases after seawater transfer (SWT). The Atlantic salmon industry in Norway experienced a loss of 59,3 million farmed salmon during the seawater phase in 2019, where Pancreas disease and deaths related to smoltification were on the top ten list of reported causes of death [1]. To mitigate losses, Atlantic salmon are routinely vaccinated in the freshwater phase, substantially reducing morbidity, mortality and losses from reduced growth and quality [2-5].

Readiness to life in seawater and overall quality of smolts is of paramount importance for the success of Atlantic salmon aquaculture. Smoltification is a process that takes place during the transition period between the freshwater and seawater phases of the salmon life. Critical factors that initiate smoltification are the size of juveniles and the photoperiod. Protocols to trigger the smoltification process of farmed salmon thus include, among others, the changing of artificial light from to short day exposure (winter) to constant light (CL) (summer). The smoltification process triggers profound endocrine changes, which shift the osmotic balance, induce anadromous migration and change the entire lifestyle [6-8]. Considering concurrent alterations of coloration, body shape, nutritional preferences, and metabolism, smoltification is compared with metamorphosis. Possible immune suppression during smoltification has been indicated with changes in the composition of leukocytes [9], reduced serum levels of IgM [10] and decreased transcription of multiple genes in different tissues, which may be maintained for several months after transfer to seawater [11-13]. Down-regulated genes include both innate and adaptive immune responses. The magnitude of changes and the composition of differentially expressed genes vary significantly, suggesting that suppression is rather a side effect of smoltification with a strong stochastic component, than an integral part of the developmental program. Vaccination takes place towards the end of the freshwater stage to safeguard an adequate vaccine response prior to SWT and mounting of vaccine responses may thereby occur simultaneously with smoltification processes. Despite potential consequences for salmon health and disease resistance, only a few studies have investigated the interactions between vaccination responses and smoltification in Atlantic salmon [14-16].

Salmon Pancreas Disease Virus, also referred to as Salmonid alphavirus (SAV), the causative agent of pancreatic disease, is a major pathogen in salmonids and a target for vaccination [17, 18]. SAV provokes strong immune responses in the heart [19-22], which is also a seat for immune suppression during smoltification [13], thus presenting itself as a key organ for the study of smoltification/vaccine interactions. Another organ of relevance is the spleen, considered a secondary lymphatic organ of Atlantic salmon playing a key part in mounting of acquired immune responses and the seat of a large fraction of antibody production [23]. Antibody responses in teleosts include vaccine-specific as well as non-specific antibodies (NSAB), the latter constitutively produced at high levels by Atlantic salmon [24]. The functional roles of NSABs are unknown, but recent studies have shown strong responses of these antibodies to immunization [24, 25].

We here aimed to investigate how the timing of CL relative to vaccination affects antibody production and gene expression after vaccination and following a viral challenge. Two groups of Atlantic salmon were vaccinated with a multivalent commercial vaccine including inactivated SAV, and either exposed to CL at the time of vaccination (0-PRI) or 2 weeks prior to vaccination(2-PRI). Both groups were challenged with SAV 9 weeks post vaccination (WPV). A bead-based multiplex immunoassay and ELISA were applied to study vaccine specific, non-vaccine specific and total immunoglobulin (Ig) in circulation. Moreover, we report the use of a newly developed multigene qPCR-based gene expression array for the assessment of immune competence of Atlantic salmon.

2 Material and methods

2.1 Experimental design

Fish, vaccination, challenge trial and sampling

The experimental fish were Atlantic salmon parr/smolt. The animal study was carried out at Veso Vikan Hatchery and Veso Vikan Research Facility (Namsos, Norway). The trial was approved by the Norwegian Food Safety Authority (permit number 13160). Fish were kept at at 12 °C throughout the entire trial and automatically fed with 1% biomass per day of Skretting Nutra Olympic 3.0 (Skretting, Norway) throughout the day. At Veso Vikan Hatchery fish were kept in flow-through freshwater, but following transport and prior to challenge, fish were acclimatized to 25 ‰ salinity for 14 days. After acclimatizing the challenge was conducted, and fish were kept at 25 ‰ salinity (brackish water) for the remaining time of the trial.

One group of fish (n=103) was subjected to the 24h constant light (CL) from two weeks prior to vaccination (2-PRI), while the other group (n=103) was subjected to CL from the time of vaccination (0-PRI). 2-PRI and 0-PRI group were kept at 12:12 light prior to the CL exposure for 7 and 9 weeks, respectively. At the time of vaccination fish had a mean weight of 62g and 65g in 0-

PRI and 2-PRI, respectively. The total number of fish used in the trial were n=266, including 60 shedder fish.

Fish were anesthetized (Metacain, Pharmaq) and marked by PIT-tagging two weeks prior to vaccination. Both groups consisted of vaccinated and unvaccinated saline injected control fish and were named according to the time of CL exposure prior to vaccination, 2-PRI and 0-PRI, respectively (Figure 1). Vaccination was carried out by i.p. injection with 0.1ml Aquavac® PD7 (MSD Animal Health). Control fish were i.p. injected with 0.1ml sterile 0.9% NaCl. Aquavac®PD7 vet is a commercial inactivated, multivalent injection vaccine for immunization of Atlantic salmon. The active components are two inactivated viral antigens; SAV and infectious pancreatic necrosis virus (IPNV), and five inactivated bacterial antigens; *Aeromonas salmonicida subsp. salmonicida*, *Vibrio salmonicida*, *Vibrio anguillarum* serotype O1, *Vibrio anguillarum* serotype O2a and *Moritella viscosa* and the oil-based adjuvant. Vaccinated and unvaccinated fish in each group were kept in the same tank with 12°C flow through fresh water for 47 days (~ seven weeks) before transfer from the hatchery to the research facility challenge zone. SAV challenge was performed according to standard procedures at Veso Vikan research facility using a cohabitation challenge model. SAV was provided by The Norwegian Veterinary Institute (Oslo, Norway) - Isolate 4 SAV3 210916, passage level 3 on CHSE-214 cells, titer 10^6 CID_{50} pr. ml [26]. SAV was diluted 1:5 and 0.1 ml was i.p. injected in each shedder. At 9 WPC the SAV shedders (n=30) were added to each of the challenge tanks (approx. 450L) with the cohabitants. Shedders were marked by removing the right maxilla. Before sampling, fish were anesthetized with 10mL of Bezocain chloride (5% solution in propylene glycol) in 10L water prior and double dose was used for euthanizing. Blood and tissue (spleen and heart) samples were collected at 0, 6 and 15 WPV (Equal to 6WPC) (Figure 1). Blood was collected from the caudal vein, and immediately centrifuged. Serum was kept at -20 °C until analysis. Tissue samples were placed in tubes with RNAlater and stored for one day at 4°C before storage at −20°C until analysis. Vaccination and sampling were performed during normal daytime work hours.

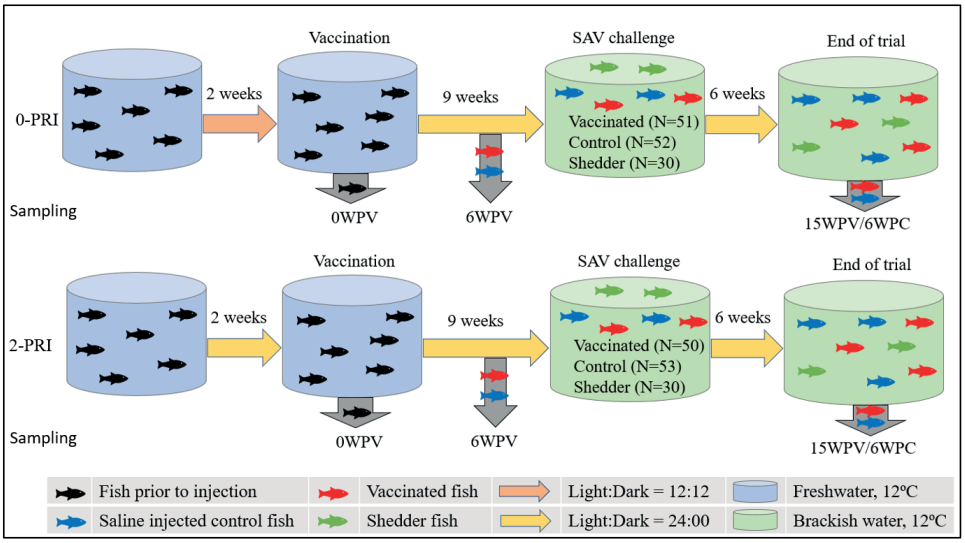


Figure 1. Overview of experimental design and sampling. Salmon were exposed to constant light at the time of vaccination (0-PRI) or two weeks prior to vaccination (2-PRI). Duration of light manipulations, time of sampling, vaccination and SAV challenge are indicated in the figure.

Initiation and monitoring of smoltification processes

The light source used to initiate the smoltification process were florescent tubes placed 2,5-3,5m above the tank, giving a water surface light intensity of 200-500 lux at both the hatchery and the research facility. It has been suggested that the light intensity has less effect on smoltification processes than the photoperiod [27]. Based on database records from a large number of experiments over several years (PatoGen, unpublished results), it was estimated that the smoltification process would take place over a period of 4 weeks at the water temperature in this trial (12°C). Furthermore, this led us to choose 2 weeks as an adequate duration of constant light exposure prior to vaccination to obtain fish vaccinated during smoltification. A commercially available method termed SmoltTimer® supplied by PatoGen AS was used to monitor the process of smoltification. SmoltTimer® includes the quantification of expressed freshwater ATPase-genes using qPCR[28] , and the results are presented as SmoltTimer®-values calculated on the basis of PatoGens proprietary technology. A SmoltTimer® value <10 is preferable before seawater transfer and correlated with fish that perform better in sea. SmoltTimer® values at 0wpv and 7wpv is shown in figure 2.

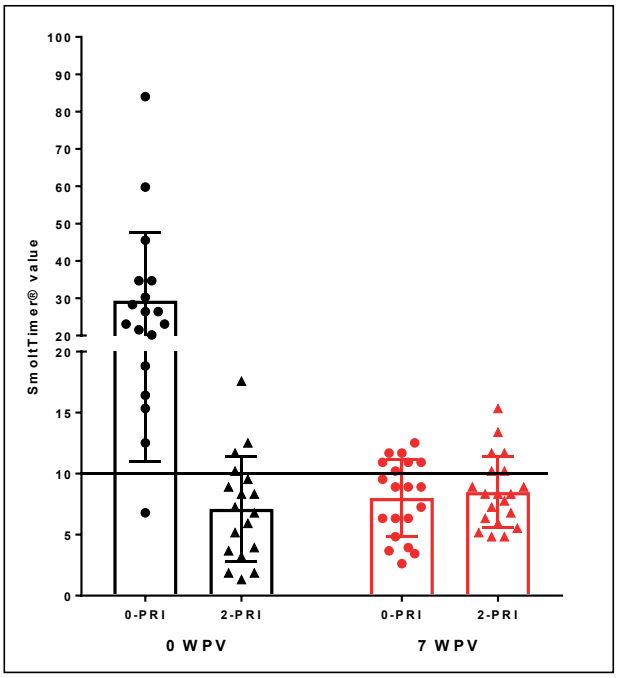


Figure 2. Difference in SmoltTimer® values between the 0-PRI and 2-PRI group at 0 (n=18) and 7 wpv (n=20). Plot shows individual values and a mean with standard deviation. Unvaccinated fish were tested at 0wpv, while only vaccinated fish were tested at 7wpv.

2.2 **Indirect** ***Enzyme linked immunosorbent assays (ELISA)***

ELISA was used to measure total immunoglobulin (Ig) in plasma. Prior to measurement of all samples, several samples were selected (different time-points and treatments), and an end-point titration was performed to identify the linear part of the sample dilution curve. Sample dilution of 1:12,500 and 1:50,000 were both within the linear portion of the sample dilution curve and were selected for analysing all samples. All solutions were at room temperature. Wash buffer (R&D systems) was diluted from 25× to 1× prior to use. Wash buffer + 4% horse serum was used as blocking buffer, while wash buffer + 1% horse serum was used as sample diluent buffer. Nunc maxisorp plates (Thermo Scientific) were coated with anti-salmonid Ig (Heavy chain) monoclonal antibody supernatant (CLF004 from Cederlane) diluted in carbonate buffer pH=9.6 to an end concentration of 0.3 µg/ml, 200 µl were added to each well and plates were incubated 48h at 4°C. Plates were washed 3 times with 400 µl before 200 µl blocking buffer was added to each well and plates were incubated for 2h at room temperature (RT). Following washing 3 times with 400 µl, 100µl salmon plasma (diluted 1/ 12,500 and 1/50,000) were added to each well and plates were incubated overnight at 4°C. Plates were washed four times with 400µl before 100 µl primary antibody (rabbit-anti-salmonid Ig, CLF003AP from Cedarlane) diluted 1:3500 were added to each well and plates were incubated for 2h at RT. Plates were washed four times with 400µl before 100 µl

substrate (3, 3', 5, 5' Tetramethylbenzidine Liquid Substrate, TMB, T4444-100ml from Sigma-Aldrich) were added to each well. Plates were wrapped in foil and incubated for 20 min at RT. Fifty µl stop-solution (1M H_2SO_4) were added to each well before plates were gently shaken. Plates were read at 450nm at Multiscan FC (Thermo Scientific).

Concentrations of total Ig in plasma samples were estimated using the 1:50,000 sample dilution. Data is presented as a Tukey's boxplot for n=8 individuals at each time-point and treatment group. All samples are from individual fish.

2.3 Bead coupling and multiplex immunoassay

Multiplex immunoassay was used to measure target (vaccine) specific and non-specific antibodies from serum samples. Analyses were performed as described previously [24]. Optimal antigen concentrations for bead conjugation and optimal sample dilutions for the multiplex immunoassay has previously been established in our lab by initial titrations of selected samples. For detection of target (vaccine) specific antibodies, the A-layer protein from *A. salmonicida subsp. salmonicida* [29] (in-house produced, essentially by using the method described by Phipps and colleagues [30]), and whole cell sonicate from *M. viscosa* type strain NCIMB 13584, recombinant E2-protein (rE2) (prepared as reported in [31]) were included in the multiplex assay. For detection of NSAB, i.e. not specific to the vaccine components, dinitrophenylated-keyhole limpet hemocyanin (DNP- KLH, Calbiochem, Merck) was used. Antigens were coupled to distinct MagPlex-C Microspheres (Luminex Corp.) of different bead regions according to the manufacturer's protocol using the Bio-Plex amine coupling kit (Bio-Rad). DNP-KLH was used at an amount of 10 µg per 1× scale coupling reaction, and A-layer protein at an amount of 12 µg, *M. viscosa* sonicate and rE2-protein at an amount of 7 µg and 10 µg, respectively. Beads were diluted in assay buffer (PBS with 0.5 % BSA and 0.05 % azide), and 5,000 beads per region were added to each well. Beads were washed three times with assay buffer (30 s in the dark and on a shaker at 800 rpm), then kept for 120 s in a Bio-Plex handheld magnetic washer before the supernatant was poured off. Serum samples (HSS=9, non-HSS=7) were diluted 1:200 in assay buffer and added in duplicates on the plate. The plate was incubated for 30 min at RT in the dark and on a shaker at 800 rpm. All subsequent incubations and washing steps were performed similarly. Following incubation and washing, beads were incubated with anti Salmonid-IgH monoclonal antibody (1:400, clone IPA5F12, Cedarlane). After incubation and washing, biotinylated goat anti-mouse Ig group2a antibody (1:1000, Southern Biotechnology Association,) was added in each well, and finally, after incubation and washing, Streptavidin-PE (1:50, Invitrogen) was applied. Plates were analyzed using a Bio-Plex 200 in combination with Bio-Plex Manager 6.1 software (Bio-Rad). Each bead is classified by its signature fluorescent pattern and then analyzed for the median fluorescent intensity (MFI) of the reporter molecule. Data is presented as a Tukey's boxplot, 0-PRI n=7-8 and 2-PRI=10-15 individuals at each time-point and treatment group. For simplicity, statistical analysis was only performed between light treatment group (0 and

2-PRI) in vaccinated and control group, respectively. All samples are from individual fish, meaning no fish were sampled more than once. Bio-plex immunoassay data were analysed with GraphPad Prism 7 software, using the non-parametric Mann-Whitney test ($p < 0.05$).

2.4 Multigene transcription assays

Primer design

The assay was developed on the BioMark HD platform (Fluidigm). The assay included two reference genes (*eef1a1b* and *rps20* [32]) and 92 genes of immune and stress responses. The assay was designed based on results of 115 experiments with >5000 microarray analyses stored in the Nofima's gene expression database STARS [33]. More than half of experiments included exposure of fish and cells to pathogens (viruses, bacteria and parasites) and inflammatory agents, vaccination and other treatments affecting the immune system. The genes were selected considering the stability of responses (the proportion of experiments with differential expression), the scale of transcription changes and the functional roles to represent the main pathways and functional groups of the immune system. Nine genes encoding chaperones, enzymes and transcription factors involved in responses to stress and DNA damage were included in the assay because they are consistently co-activated with immune genes and form an important part of defense against infections. Atlantic salmon-specific primers were designed to simultaneously detect the various paralogs of the respective target genes using the Pyrosequencing Assay Design software v.1.0.6 (Biotage). In most cases, either the sense or the antisense primer were placed on exon-exon boundaries. The LightCycler96 Real-Time PCR System (Roche) was used to generate assay-specific standard curves based on serial 10-fold dilutions starting from $1\times10^2 – 1\times10^8$ copies of the individual amplicons [34]. These standard curves served determining the efficiency of the primer pairs, which ranged between 90.7% and 110.3% ($R^2 = 0.999$). The list of genes and primers is in Supplementary file 1.

qPCR analyses

Small pieces of heart and spleen (5-10 mg) were placed in tubes with 400 µl lysis buffer (Qiagen) and beads, 20 µl proteinase K (50 mg/ml) was added in each tube. Samples were homogenized in FastPrep 96 (MP Biomedicals) for 120 s at maximum shaking, centrifuged and incubated at 37°C for 30 min. RNA was extracted on Biomek 4000 robot using Agencourt RNAdvance Tissue kit according to the manufacturer's instructions. RNA concentration was measured with NanoDrop One (Thermofisher Scientific) and quality was assessed with Agilent Bioanalyzer 2100. One microliter of the extracted RNA was reverse-transcribed using the Reverse transcription master mix (Fluidigm). Subsequently, the individual cDNA samples were adjusted at 10 ng/5 µl, added to the aforementioned 96 primer pairs (100 µM) and the PreAmp master mix (Fluidigm) and subjected to 12 pre-amplification cycles in a standard thermocycler (TAdvanced, Biometra). The pre-amplified

products were treated with exonuclease I (New England BioLabs) and diluted in a SoFast EvaGreen supermix with Low ROX (Bio-Rad) and 20× DNA-binding dye sample loading reagent. The sample and primer mixes were transferred to the respective inlets of two 48.48 dynamic array IFC chips. These chips were individually primed in the BioMark IFC controller MX (Fluidigm) according to the Load mix 48.48 GE script. The loaded array chips were then placed in the BioMark HD system (Fluidigm) to proceed with the qPCR according to the GE 48×48 Fast PCR+Melt v2.pcl cycling program. Fluidigm RealTime PCR analysis software v. 3.0.2 was used to retrieve raw qPCR results. Results were transferred in a relational database. The geometric means of two reference genes (*ef1f* and *rps20*), which showed stability across samples were used for calculation of ΔCt values. -ΔCt values for all genes and samples are shown in Supplementary file 1. ΔΔCt values were calculated by using the grand mean ΔCt of each gene either before (Figure 4) or after (Figure 5) challenge. Gene expression data were analysed with ANOVA followed by post hoc tests ($p < 0.05$) using Statistica 13. The numbers of DEGs were counted as statistical different genes (t-test, $p < 0.05$) between pairs of treatment groups based on ΔCt values, (0-PRI and 2-PRI), time-points (0WPV and 6WPV), vaccinated and saline injected control salmon (Vac and Con), before and after SAV challenge (6WPV and 6 weeks post challenge, WPC) (Figure 6).

3 Results

3.1 Effect of CL regimen on antibody levels

We compared two groups of fish vaccinated at two different time-points relative to light regimens: Either exposed to CL two weeks prior to vaccination (2-PRI) or put on CL at the time of vaccination (0-PRI). The total amount of Ig was measured by ELISA at three time-points: 0WPV (just prior to vaccination), 6WPV and 15WPV (equal to 6 weeks post challenge (WPC)). In vaccinated fish, the total Ig increased after vaccination in both light regimen groups (Figure 3). The total Ig in plasma remained stable from 0 to 6WPV in control fish irrespective of light regimen. Both vaccinated and control fish showed an increase in total Ig after challenge with SAV (15WPV/6WPC). No effect of light regimen on total Ig was found.

We next assessed levels of antibodies towards antigens delivered with the vaccine using multiplex immunoassay. Antigens included the A-layer of *A.salmonicida* subsp. *salmonicida, M.viscosa* whole-cell sonicate and recombinant SAV E2-protein (rE2) (Figure 4 A, B and C, respectively). Prior to vaccination (0WPV), antibody levels against vaccine components were at background levels in both light treatment groups, except a slightly higher signal in the 2-PRI group for recombinant E2-protein (rE2).

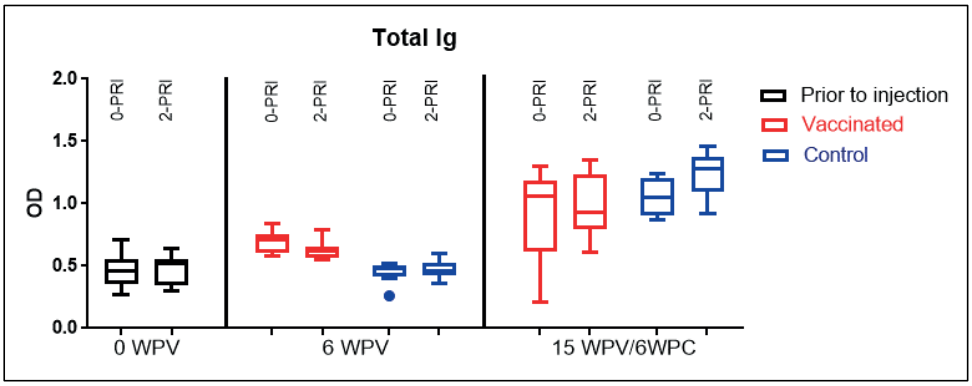


Figure 3. Effects of combinations of constant light regimens and vaccination on total immunoglobulin (Ig) in Atlantic salmon plasma were examined at three time-points; 0 WPV (just prior to vaccination), 6WPV and 15WPV/6 weeks post challenge (WPC). Salmon were exposed to constant light at the time of vaccination (0-PRI) or two weeks prior to vaccination (2-PRI). Data is presented as a Tukey's boxplot, n=8 individuals at each time-point and treatment group. Dots outside the box are outliers.

Levels of antibodies binding to vaccine-specific antigens increased in both light regimen groups after vaccination. The 2-PRI fish had significantly higher levels of antibodies against *A.salmonicida*, *M.viscosa* and rE2 at 6WPV compared to the 0-PRI group. A further increase in antibody levels against all measured vaccine antigens was seen from 6 to 15 WPV, reaching equal levels for both of the light treatment groups at 15WPV, except for *M.viscosa* antibodies that were significantly higher in the 2-PRI group. Following SAV challenge, the levels of antibodies to rE2 increased at 6WPC in both vaccinated and control fish, and in both light regimen groups (Figure 4C). In control fish, a small increase in antibodies against the other two vaccine specific antigens (not related to SAV) was also observed (Figure 4A and B).

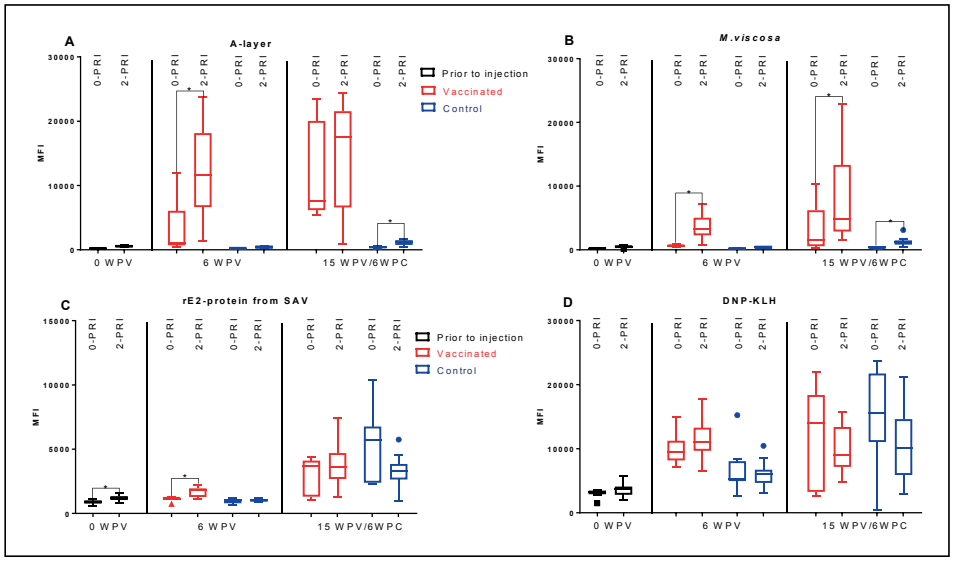


Figure 4. Effects of combinations of constant light regimens and vaccination on levels of specific and non-specific antibodies in Atlantic salmon, measured by a multiplex immunoassay. Levels of antibodies binding to beads coupled with A-layer from *A.salmonicida* (A), *M.viscosa* whole cell sonicate (B), recombinant E2-protein from SAV (C) and non-specific antibodies binding to DNP-KLH (D) are displayed as median fluorescent intensity (MFI). Three time-points were examined; just prior to vaccination (0 weeks post vaccination (0WPV)), 6WPV and 15WPV/6 weeks post challenge (WPC). Salmon were exposed to constant light at the time of vaccination (0-PRI) or two weeks prior to vaccination (2-PRI). Data is presented as a Tukey's boxplot, 0-PRI n=7-8 and 2-PRI=10-15 individuals at each time-point and treatment group. Dots outside the box are outliers. For simplicity, significant differences are shown only between light treatment group (0 and 2-PRI) in vaccinated and control groups, respectively.

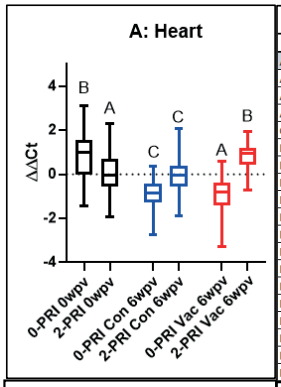
To determine the levels of NSABs we measured binding to a vaccine-irrelevant antigen frequently used for this purpose, DNP-KLH [24, 35] (Figure 4D). Both light treatment groups had DNP-KLH binding antibodies present already prior to vaccination, as illustrated by a consistently higher MFI at 0 WPV than observed for vaccine-relevant antigens. Following vaccination, levels of NSABs increased in vaccinated salmon of both light regimen groups. In the control group, an increase of DNP-KLH binding antibodies was first observed after challenge. No significant difference in NSAB levels between light treatment groups was observed at any time point.

3.2 *Effect of CL regimen on gene transcription*

Transcription of immune genes in the heart and spleen of fish from all groups was measured before (Figure 5) and after challenge with SAV (Figure 6), using the Fluidigm Biomark HD multiplex qPCR-based gene expression array. The total numbers of differentially expressed genes (DEG) are shown in Figure 7, ΔCt gene-data and the mean values for all time-points are presented in Supplement 1. At the time of vaccination (0WPV) fish in the 2-PRI group showed a downregulation of genes in the heart (Figure 5A). Genes with lower expression in the heart were from various

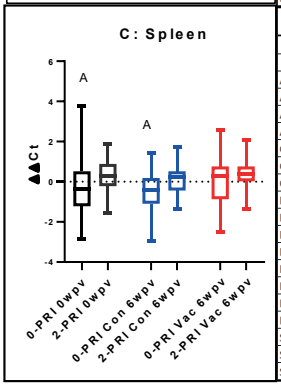
functional groups including virus-responsive genes (VRG [36]), effectors and lymphocyte-specific genes (Figure 5B). When counting significant heart DEGs at 0WPV, we found that 15 genes were downregulated in 2-PRI fish compared to 0-PRI fish, while only three genes were upregulated (Figure 7A blue print). At 6WPV the difference between the two light treatment groups was reversed, as immune genes in the hearts of both vaccinated and control 0-PRI fish were downregulated compared to their corresponding 2-PRI groups at 6WPV (Figure 5A,B; Figure 7A blue and purple print). When comparing between time points, vaccinated fish showed a markedly induced cardiac gene transcription from 0WPV to 6WPV, while the same genes had been downregulated in the vaccinated 0-PRI fish (Figure 5B; Figure 7A black print). In the spleen, transcriptional differences between the two light regimen groups were smaller compared to heart; at the time of vaccination only minor differences in gene transcription was seen between light regimens (Figures 5C, D; Figure 7B blue print). At 6WPV a small set of splenic immune and stress genes were slightly lower in the 0-PRI fish compared to 2-PRI fish, both true for control fish and for vaccinated fish (Figure 5D).

Challenge with SAV caused strong transcription changes, typical for virus-infected Atlantic salmon [19]. In all heart samples, from 52 to 68 genes showed increased transcription, and from 0 to 7 genes were downregulated (Figure 7 red print, Supplement 1). In addition, responses to challenge in heart assessed as mean $\Delta\Delta$Ct were equal across the study groups except significantly lower in control of 2-PRI (Figure 6 A,E). A similar tendency was seen in the spleen (Figure 6 C,F). In the spleen, the transcriptional changes were greatest in control salmon from the 0-PRI group, similar or equal in vaccinated fish from both groups and lowest in control salmon from the 2-PRI group (Figure 6F). Of note, transcription of eight VRG, which as a rule reflects the pathogen load [19, 36], was lowest in the spleen of vaccinated 0-PRI fish, followed by vaccinated 2-PRI fish, while in control fish from both groups the VRG transcription was significantly higher (Figure 6D). No difference in VRG were found in the heart (Figure 6B).



B: Heart

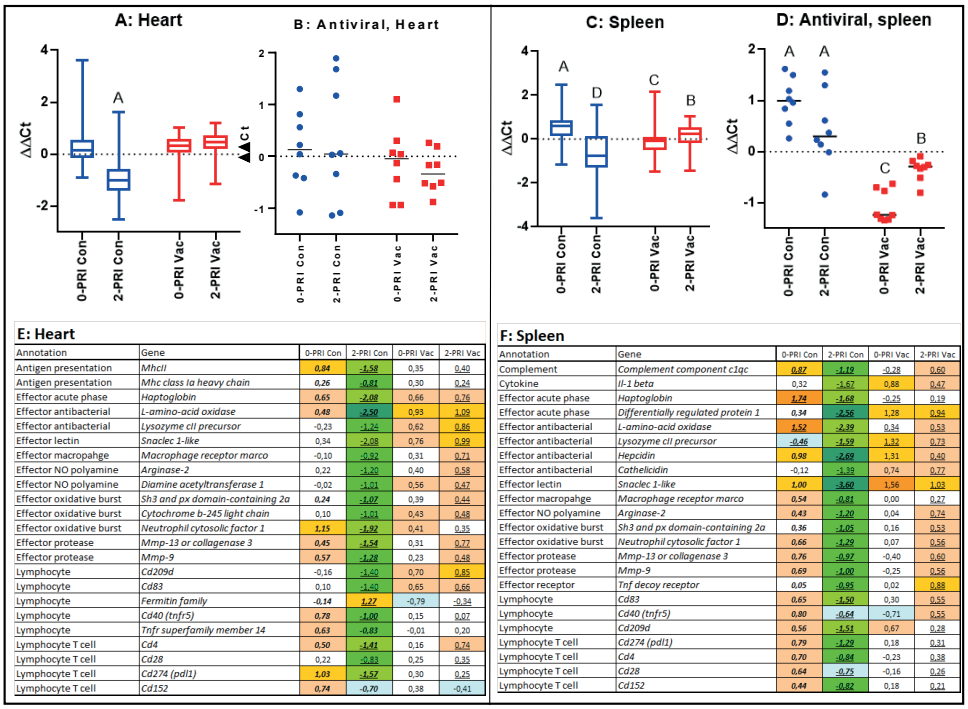
		0 WPV		6 WPV			
Annotation	Gene	0-PRI	2-PRI	0-PRI Con	2-PRI Con	0-PRI Vac	2-PRI Vac
Antiviral	*Gig2*	***2,48***	***-0,59***	-0,96	0,76	***-1,14***	-0,55
Antiviral	*Sacsin*	***1,31***	***-1,08***	-0,23	0,26	-0,11	-0,15
Antiviral	*Receptor transporting protein 2*	***1,69***	***-0,76***	0,29	-0,97	0,48	-0,73
Complement	*Complement component c1qc*	***1,35***	***-0,94***	-0,64	-0,29	-0,63	1,15
Effector antibacterial	*Lysozyme cII*	1,01	0,57	-1,43	-0,22	***-1,72***	***1,79***
Effector antibacterial	*L-amino-acid oxidase*	0,70	0,36	***-1,41***	***1,45***	***-2,27***	***1,17***
Effector macropahge	*Macrophage receptor marco*	***2,72***	***0,65***	***-2,36***	***0,75***	***-3,28***	***1,52***
Effector NO polyamine	*Ornithine decarboxylase 1*	***-0,20***	***1,43***	***-1,25***	***0,40***	***-1,33***	***0,96***
Effector NO polyamine	*Arginase-2*	-0,54	-0,03	***-0,92***	***0,51***	***-0,79***	***1,78***
Effector oxidative burst	*Neutrophil cytosolic factor 1*	***2,81***	***-0,02***	-0,15	-1,05	-2,54	0,95
Effector oxidative burst	*Sh3 and px domain-containing 2a*	***1,45***	***-0,07***	-0,92	-0,52	-0,82	0,88
Effector protease	*Mmp-13 or collagenase 3*	2,48	0,05	-1,13	-1,16	-0,93	0,69
Effector protease	*Mmp-9*	1,90	-0,69	-0,60	-0,74	-0,87	1,00
Lymphocyte	*Sh2 domain-containing 1a*	***3,10***	***-0,53***	-1,92	-0,98	-1,47	1,79
Lymphocyte	*Cd83*	***1,43***	***0,43***	-1,38	0,42	***-1,85***	***0,95***
Lymphocyte	*Sam and sh3 domain 3*	***1,86***	***-0,36***	-0,91	-0,46	-0,54	0,41
Lymphocyte T cell	*Cd28*	***1,21***	***-0,15***	-1,12	-0,14	-0,21	0,42
Lymphocyte T cell	*Cd4*	1,19	-0,54	-0,86	-0,72	-0,82	1,75



D: Spleen

		0 WPV		6 WPV			
	Gene	0-PRI	2-PRI	0-PRI Con	2-PRI Con	0-PRI Vac	2-PRI Vac
Antiviral	*Receptor transporting protein 2*	0,42	-0,67	1,25	-0,52	***0,90***	***-1,38***
Antiviral	*Barrier-to-autointegration factor*	-1,39	-0,21	1,41	0,44	***1,09***	***-1,34***
Antiviral	*Ifit5-2*	-0,36	-0,39	0,68	0,28	***0,76***	***-0,98***
Antiviral	*Sacsin*	-0,56	-0,65	0,81	0,47	***0,77***	***-0,84***
Chemokine	*C-c motif chemokine 19-4*	-1,53	-0,58	***1,04***	***0,24***	***1,08***	***-0,26***
Complement	*Complement component c4*	0,23	0,48	***-1,10***	***0,12***	-0,01	0,27
Complement	*Complement component c1qc*	-2,41	-0,96	0,70	1,15	1,33	0,18
Effector acute phase	*Plasminogen activator inhibitor 1*	0,37	0,39	***-1,86***	***0,70***	***-1,42***	***1,81***
Effector acute phase	*Serum amyloid a-5*	-2,83	0,02	-0,49	-1,34	2,56	2,09
Effector antibacterial	*L-amino-acid oxidase*	***2,46***	***0,06***	***-2,45***	***1,62***	***-2,48***	***0,79***
Effector antibacterial	*Lysozyme cII precursor*	-2,81	0,44	-0,46	0,19	1,07	1,58
Lymphocyte	*Cd83*	0,17	0,67	***-1,19***	***0,67***	***-0,91***	***0,57***
Lymphocyte	*Cd209d*	-0,38	1,44	***-1,34***	***0,95***	-0,87	0,20
Lymphocyte	*Tnfr superfamily member 14*	***-2,01***	***-0,07***	0,18	0,17	1,22	0,51
Lymphocyte T cell	*Cd274 (pdl1)*	0,68	1,04	***-1,73***	***0,52***	***-1,28***	***0,77***
Stress protein	*Heat shock 70 kda protein*	0,97	1,44	***-1,83***	***0,26***	***-1,47***	***0,61***
Stress protein	*Heat shock protein 30*	1,40	0,79	***-1,32***	***0,55***	***-1,83***	***0,42***
Stress transcriotion factor	*Immediate early response 2-2*	1,22	1,85	***-1,93***	***-0,16***	***-1,38***	***0,40***

Figure 5. Effects of constant light regimens on transcription of immune genes in vaccinated (Vac) and saline injected control (Con) salmon at 0 and 6WPV. Multigene transcription assay in heart and spleen of salmon exposed to constant light two weeks prior to vaccination (2-PRI) and at vaccination (0-PRI). Data (ΔΔCt) were normalized so that the mean value for each gene is equal to zero. The mean values for all time-points are in Supplement 1. A and C: All analyzed genes in heart and spleen, respectively. Columns not sharing common letter are significantly different (ANOVA, Tukey's test, $p < 0.05$). B and D: Individual genes in heart and spleen, respectively. Significant differences between groups are indicated with bold italics and differences between vaccinated and control salmon are underlined.



E: Heart

Annotation	Gene	0-PRI Con	2-PRI Con	0-PRI Vac	2-PRI Vac
Antigen presentation	*Mhcll*	***0,84***	***-1,58***	0,35	0,40
Antigen presentation	*Mhc class Ia heavy chain*	***0,26***	***-0,81***	0,30	0,24
Effector acute phase	*Haptoglobin*	***0,65***	***-2,08***	0,66	0,76
Effector antibacterial	*L-amino-acid oxidase*	***0,48***	***-2,50***	0,93	1,09
Effector antibacterial	*Lysozyme cII precursor*	-0,23	-1,24	0,62	0,86
Effector lectin	*Snaclec 1-like*	0,34	-2,08	0,76	0,92
Effector macropahge	*Macrophage receptor marco*	-0,10	-0,92	0,31	0,71
Effector NO polyamine	*Arginase-2*	0,22	-1,20	0,40	0,58
Effector NO polyamine	*Diamine acetyltransferase 1*	-0,02	-1,01	0,56	0,47
Effector oxidative burst	*Sh3 and px domain-containing 2a*	***0,24***	***-1,07***	0,39	0,44
Effector oxidative burst	*Cytochrome b-245 light chain*	0,10	-1,01	0,43	0,48
Effector oxidative burst	*Neutrophil cytosolic factor 1*	***1,15***	***-1,92***	0,41	0,35
Effector protease	*Mmp-13 or collagenase 3*	***0,45***	***-1,54***	0,31	0,77
Effector protease	*Mmp-9*	***0,57***	***-1,28***	0,23	0,48
Lymphocyte	*Cd209d*	-0,16	-1,40	0,70	0,85
Lymphocyte	*Cd83*	0,10	-1,40	0,65	0,66
Lymphocyte	*Fermitin family*	***-0,14***	***1,27***	-0,79	-0,34
Lymphocyte	*Cd40 (tnfr5)*	***0,78***	***-1,00***	0,15	0,07
Lymphocyte	*Tnfr superfamily member 14*	***0,63***	***-0,83***	-0,01	0,20
Lymphocyte T cell	*Cd4*	***0,50***	***-1,41***	0,16	0,74
Lymphocyte T cell	*Cd28*	0,22	-0,83	0,25	0,35
Lymphocyte T cell	*Cd274 (pdl1)*	***1,03***	***-1,57***	0,30	0,25
Lymphocyte T cell	*Cd152*	***0,74***	***-0,70***	0,38	-0,41

F: Spleen

Annotation	Gene	0-PRI Con	2-PRI Con	0-PRI Vac	2-PRI Vac
Complement	*Complement component c1qc*	***0,87***	***-1,19***	-0,28	0,60
Cytokine	*Il-1 beta*	0,32	-1,67	0,88	0,47
Effector acute phase	*Haptoglobin*	***1,74***	***-1,68***	-0,25	0,19
Effector acute phase	*Differentially regulated protein 1*	***0,34***	***-2,56***	1,28	0,94
Effector antibacterial	*L-amino-acid oxidase*	***1,52***	***-2,39***	0,34	0,53
Effector antibacterial	*Lysozyme cII precursor*	***-0,46***	***-1,59***	1,32	0,73
Effector antibacterial	*Hepcidin*	***0,98***	***-2,69***	1,31	0,40
Effector antibacterial	*Cathelicidin*	-0,12	-1,39	0,74	0,77
Effector lectin	*Snaclec 1-like*	***1,00***	***-3,60***	1,56	1,03
Effector macropahge	*Macrophage receptor marco*	***0,54***	***-0,81***	0,00	0,27
Effector NO polyamine	*Arginase-2*	***0,43***	***-1,20***	0,04	0,74
Effector oxidative burst	*Sh3 and px domain-containing 2a*	***0,36***	***-1,05***	0,16	0,53
Effector oxidative burst	*Neutrophil cytosolic factor 1*	***0,66***	***-1,29***	0,07	0,56
Effector protease	*Mmp-13 or collagenase 3*	***0,76***	***-0,97***	-0,40	0,60
Effector protease	*Mmp-9*	***0,69***	***-1,00***	-0,25	0,56
Effector receptor	*Tnf decoy receptor*	***0,05***	***-0,95***	0,02	0,88
Lymphocyte	*Cd83*	***0,65***	***-1,50***	0,30	0,55
Lymphocyte	*Cd40 (tnfr5)*	***0,80***	***-0,64***	-0,71	0,55
Lymphocyte	*Cd209d*	***0,56***	***-1,51***	0,67	0,28
Lymphocyte T cell	*Cd274 (pdl1)*	***0,79***	***-1,29***	0,18	0,31
Lymphocyte T cell	*Cd4*	***0,70***	***-0,84***	-0,23	0,38
Lymphocyte T cell	*Cd28*	***0,64***	***-0,75***	-0,16	0,26
Lymphocyte T cell	*Cd152*	***0,44***	***-0,82***	0,18	0,21

Figure 6. Effects of constant light regimens on transcription of immune genes in vaccinated (Vac) and saline injected control (Con) salmon after challenge with SAV. Multigene transcription assay in heart and spleen of salmon exposed to constant light two weeks prior to vaccination (2-PRI) and at vaccination (0-PRI). Data (ΔΔCt) were normalized so that the mean value for each gene is equal to zero. The mean values for all time-points are in Supplement 1. All data presented are from 6 weeks post SAV-challenge A: all analyzed genes in heart, B: VRG in the heart (8 genes), C: all analyzed genes in spleen, D: VRG in the spleen (8 genes). Boxes not sharing common letter are significantly different (ANOVA, Tukey's test, $p < 0.05$). E, F: individual genes in heart and spleen, respectively. Significant differences between groups are indicated with bold italics and differences between vaccinated and control salmon are underlined.

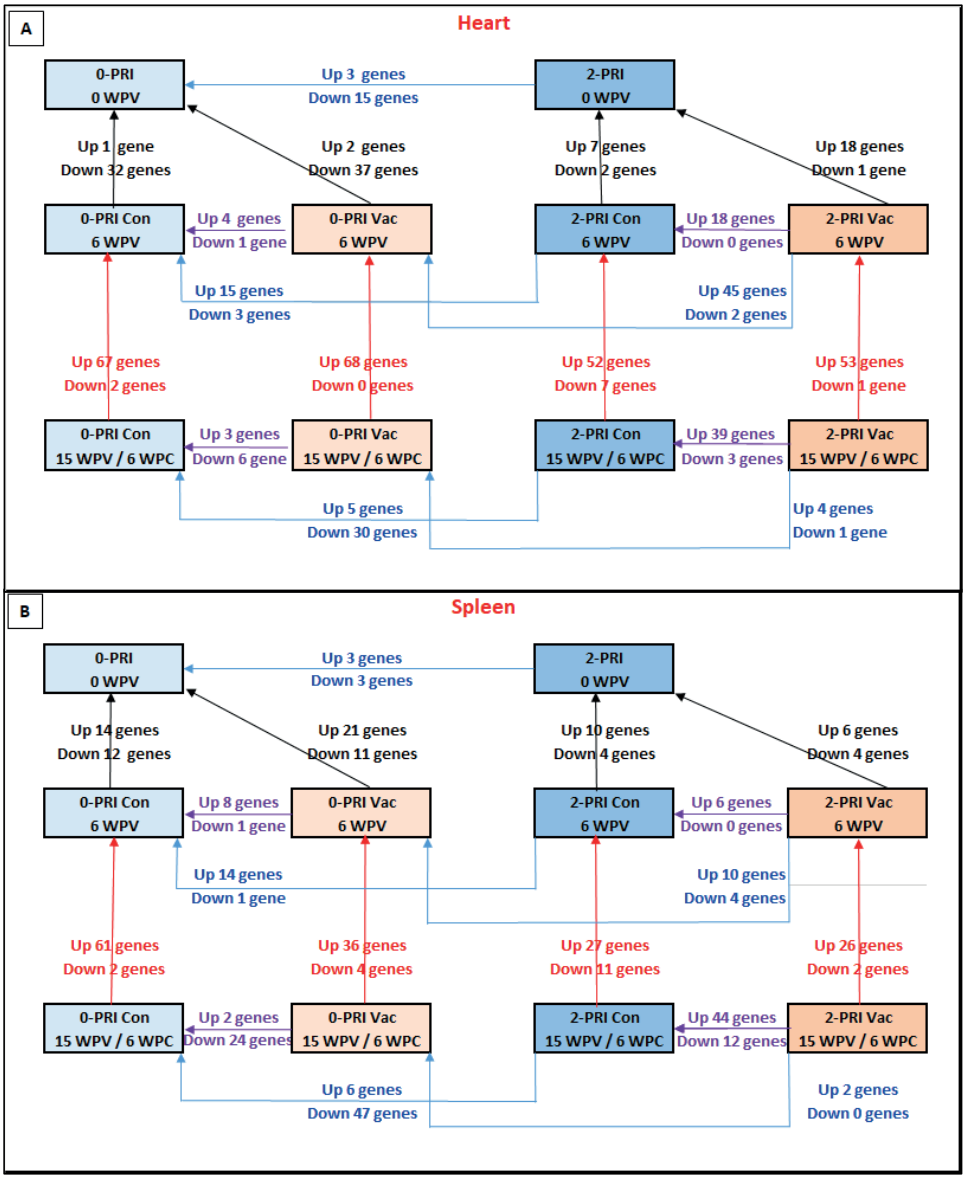


Figure 7. Summary of multigene transcription assay in heart (A) and spleen (B) in salmon put on constant light two weeks prior to vaccination (2-PRI) and at vaccination (0-PRI). Contrasts and numbers of differentially expressed genes are shown in the figure. The numbers of DEGs were counted as statistical different genes (t-test, $p < 0.05$) between pairs of treatment groups based on ΔCt values. Comparisons are indicated with colors (text): blue (timing of vaccination, 0-PRI and 2- PRI), black (time-points before challenge, 0 and 6 weeks post vaccination (WPV)), purple (effects of vaccination, Vac – vaccinated, Con – saline injected) and red (responses to challenge with SAV, 6 weeks post challenge(WPC) versus last time-point before challenge (6 WPV vac).

4. Discussion

Exposure to CL is a common regimen in Atlantic salmon aquaculture for inducing the smoltification process and enable transfer to saltwater. Salmon are often vaccinated towards the end of the freshwater phase to ensure an adequate vaccine response by the time the fish is transferred to sea. We have previously shown down-regulation of multiple immune genes during smoltification [11] and upregulated after viral challenges in Atlantic salmon [19, 20, 37, 38]. Thus, a point of concern is that endocrine changes induced with CL may interfere with the mounting of a vaccine response. However, in contrast to transcriptome responses to bacterial and especially viral pathogens that are well reproduced among these challenge trials, the gene transcription changes of immune genes during smoltification have been variable and called for further studies.

In the present study, the immune competence of Atlantic salmon in terms of antibody production and immune gene expression was compared between fish undergoing two different CL regimens, either initiated at the time of vaccination (0-PRI) or two weeks prior to vaccination (2-PRI). SmoltTimer® values at the time of vaccination showed two groups at different stages in the smoltification process (Figure 2). At the first time point included in the analysis (0WPV), the expression of immune genes was lower in the heart of salmon from the 2-PRI group that were exposed to CL for two weeks. Six weeks later, the situation was reversed as the 2-PRI group showed a stimulated cardiac gene transcription, while there was a down-regulation of immune genes manifested in hearts of both vaccinated and control fish in the 0-PRI group. By aligning the two CL treatment groups, the present experiment might indicate the following sequence of major gene transcription events: Immune suppression began no later than two weeks into CL, lasted no less than six weeks, and the immune system then recovered no later than eight weeks after onset of CL (Figure 7 or supplementary figure). The difference in the immune response of the two groups of vaccinated salmon was also reflected in the serological data. Although the 2-PRI group was pre-exposed to CL and vaccinated when many immune genes were downregulated, our results showed that an effective production of antibodies took place during the recovery period. Indeed, at six weeks after vaccination the 2-PRI group had significantly higher levels of antibodies against all vaccine-relevant antigens compared to the 0-PRI group.

Previous studies with viral challenges have demonstrated that even small differences in the life history of salmon may significantly affect the immune responses [39, 40]. An intriguing finding in the present study was that the vaccinated fish from the two treatment groups had almost identical transcriptional profiles after challenge, despite a large transcriptional difference before challenge. The control salmon from the 0-PRI group showed a similar transcriptional profile after challenge as

vaccinated individuals, but with a significantly higher VRG transcription, which is shown to reflect a higher viral load [36, 41]. In contrast, the 2-PRI controls showed an overall markedly lower transcription of immune genes in spleen and heart after challenge. Similar observations were made in a recent study of interactions between light regimens, in which Atlantic salmon were vaccinated and challenged with IPNV [42]. The authors showed that control fish exposed to CL for six weeks showed much higher mortality in comparison with groups with shorter CL exposure, in concordance with the higher susceptibility of smolt to this pathogen, compared to parr. However, vaccination eliminated this difference, and the authors found that vaccine-mediated protection to IPN was equal irrespective of the smolt status at challenge. The results in the present study might indicate a similar tendency: Vaccinated fish from 0-PRI and 2-PRI appeared to have similar cardiac and splenic immune responses to SAV challenge, in addition to a lower transcription of VRG in the spleen compared to unvaccinated fish, suggesting an effective clearance of infection in vaccinated salmon irrespective of previous light treatment regimen.

Teleost antibodies reacting to targets not delivered by a vaccine or a specific infection have often been noted, and given different names such as “non-specific (NSABs), natural, heterologous, or polyreactive antibodies” depending on context [24, 31, 35]. The majority of responding antibodies in the circulation of teleosts are IgM, which have higher avidity and often less clear specificity when compared e.g. to mammalian IgG. In the present study we observed high levels of NSAB and a strong increase after SAV challenge, especially in the unvaccinated controls of both light regimens. This may suggest a compensatory role of NSABs in the absence of an adaptive recall response. Furthermore, we found a significant presence of antibodies binding to a specific (vaccine-relevant) antigen at baseline (rE2 at 0WPV) and to non-SAV antigens in unvaccinated fish after SAV challenge (*A.salmonicida*, *M.viscosa* at 15WPV). It is likely that in addition to vaccine-specific antibodies, NSABs may have bound to these antigens to a certain degree, in line with our previous observation that the SAV-specific recombinant E2-protein to some extent bound antibodies in a non-specific fashion [31]. Altogether, this illustrates that NSAB contribution must be considered in serological assays of salmon, and that the role of NSABs in different contexts requires further studies.

To conclude, our results indicate that although CL treatment of salmon produces a transient immune suppression, fish vaccinated in this period seem to respond to the vaccine by producing adequate levels of antibodies, return to normal immune gene expression by the time of SWT, and seem to respond to challenge with SAV well despite the initial immune suppression. These findings may contribute to decision making over production protocols and should be explored further with different risk scenarios in the field.

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Erratataliste:			
Side	**Linje**	**Endret fra**	**Endret til**
.i	124-127	A special thanks to Elisabeth Furuseth Hansen, Lene Hammerlund Teige, Karla Meza Parada, Stine Braaen, Ingvild Berg Nyman, Grethe M. Johansen, Ingrid Mo, Ida Aksnes and Marianne Hansen at Nofima for all the advice, support, technical help, workouts, discussions etc. Thank you!	A special thanks to Elisabeth Furuseth Hansen, Lena Hammerlund Teige, Karla Meza Parada, Stine Braaen, Ingvild Berg Nyman, Grethe M. Johansen, Ingrid Mo, Ida Aksnes, Aud Kari Fauske, Espen Rimstad and Marianne Helén Selander Hansen for all the advices, support, technical help, workouts, discussions etc. Thank you!
,I	139	Anne Bakke	Anne Flore Bakke
,iii	158-160	**Constant light regimen affects immune responses of Atlantic salmon following vaccination and challenge with salmonid alphavirus**	**Effect of two constant light regimen on antibody profiles and immune gene expression in Atlantic salmon following vaccination and challenge with Salmonid alphavirus**
.iii	162-163	Anne Flore Bakke[1], Alexander Rebl[2], Sergey Afanasyev[3], Petter Frost[4*], Tina Søfteland[4], Hege Lund[1], Preben Boysen[1], Aleksei Krasnov[5]	**Anne Flore Bakke**, Alexander Rebl, Petter Frost, Sergey Afanasyev, Tina Søfteland, Hege Lund, Preben Boysen, Aleksei Krasnov
,iii	143	Paper 1	Paper I
,iii	150	Paper 2	Paper II
,iii	157	Paper 3	Paper III
20	747-749	**Constant light regimen affects immune responses of Atlantic salmon following vaccination and challenge with salmonid alphavirus.**	**Effect of two constant light regimen on antibody profiles and immune gene expression in Atlantic salmon following vaccination and challenge with Salmonid alphavirus**.
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