Analysis of IgM sub-variants related to ancestral tetraploidy in salmonid fish

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This thesis is dedicated to my parents.

For their endless love, support, encouragement and prayers

My father, Sultan Muhammad did not only raise and nurture me but also taxed himself dearly over the years for my education and intellectual development. My mother, Siraj Begum has been a source of motivation and strength during moments of despair and discouragement. Her motherly care and support have been shown always in incredible ways.

Acknowledgements

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Abbreviations

2D	Two dimensional
Ab	Antibody
AEC	Anion exchange chromatography
AR	Agglutination reactions
AS	Ammonium sulfate
Asn	Asparagine
C	Constant
CDR	Complementarity determining region
CF	Complement fixation
CY	Cytoplasmatic tail
D	Diversity
DAMP	Damage associated molecular patterns
DC	Dendritic cells
ELISA	Enzyme linked immunosorbant assay
EST	Expressed sequence tag
FR	Framework regions
FSGD	Fish specific genome duplication
H	Heavy
IF	Isoelectric focusing
Ig	Immunoglobulin
IHC	Immunohistochemistry
IP	Immune-precipitation
IT	Ion trap
J	Joining
KDa	Kilo Dalton
Kg	kilo gram
L	Light
LRR	Leucine rich repeat
MBP	Mannan binding protein
MHC	Major histocompatibility complex
MS	Mass spectrometry

MYA	Million years ago
NMR	Nuclear magnetic resonance
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen associated molecular pattern
PRR	Pattern recognition receptor
SDS	Sodium dodecyl sulphate
TCR	T cell receptor
TLR	Toll like receptor
TM	Transmembrane
TOF	Time-of-flight
V	Variable
VLR	Variable lymphocyte receptor
WGD	Whole genome duplication

Abstract

Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*) possess two paralogous IgM heavy chain (µ) genes related to ancestral tetraploidy. Accordingly, IgM subpopulations of Atlantic salmon and brown trout can be separated by gradient anion exchange chromatography (AEC) into two distinct peaks. In contrast, IgM of arctic char (*Salvelinus alpinus*) and rainbow trout (*Oncorhynchus mykiss*) is eluted in a single peak. In the present study mass spectrometry analysis verified that IgM of peak 1 (subpopulation 1) have heavy chains previously designated as µB type whereas IgM of peak 2 (subpopulation 2) have heavy chains of µA type, in Atlantic salmon and brown trout. Salmon IgM of both peak 1 and peak 2 contain light chains of the two most common isotypes: IgL1 and IgL3. Two adjacent cysteine residues are present near the C-terminal part of µB, in contrast to one cysteine residue in µA. Most likely, the additional cysteine is involved in inter-chain disulfide bonding and influences the elution profiles of IgM-A and IgM-B on AEC. Molecular cloning of µ cDNA from arctic char revealed two sub-variants (µA-1 and µA-2), and hybrids of char/salmon expressed µA-1, µA-2, µA and µB, indicating that there are two paralogous µ loci in the haploid genome of char, like in Atlantic salmon. Neither of the µ sub-variants in arctic char have the additional cysteine, and char IgM, as well as salmon and brown trout IgM-A, show a lower degree of inter-chain disulfide bonding than IgM-B when subjected to denaturation and gel electrophoresis under non-reducing conditions. Surprisingly, a monoclonal antibody MAb4C10 against rainbow trout IgM, reacted with µA in salmon, whereas in brown trout it reacted with µB. MAb4C10 was conjugated to magnetic beads and used to separate cells, demonstrating that µ transcripts residing from captured cells were primarily of A type in salmon and B type in brown trout. It is plausible to assume that DNA has been exchanged between the paralogous A and B loci during evolution while maintaining the two sub-variants, with and without the extra cysteine. An analysis of amino acid substitutions in µA and µB of salmon and brown trout indicated that the third constant domain is essential for MAb4C10 binding. This was supported by 3D modeling and was finally verified by studies of MAb4C10 reactivity with a series of recombinant µ3 constructs. Substitution of a proline residue located in the loop between the B and C beta strands of salmon µA3 eliminated MAb4C10 reactivity. Accordingly, the reverse substitution in salmon µB restored MAb4C10 reactivity. Molecular cloning of MAb4C10 cDNA and mass spectrometry analysis confirmed that MAb4C10 is of IgG-1 subtype, and the VH sequence of MAb4C10 was determined. To reveal possible

differential expression of IgM-A and IgM-B, a broad spectrum of samples from previous and ongoing experiments (fresh water and salt water) and unvaccinated/vaccinated diploid and triploid fish were analyzed. The μA and μB genes appeared to be uniformly expressed in a series of tissues, whereas the AEC profiles of purified IgM from vaccinated fish indicated that the A:B ratio can be skewed in challenged fish.

List of publications

Paper 1

Kamil A., Falk K., Sharma A., Raae A., Berven F., Koppang E. O. and Hordvik I. (2011) A monoclonal antibody distinguishes between two IgM heavy chain isotypes in Atlantic salmon and brown trout: protein characterization, 3D modeling and epitope mapping. *Molecular Immunology* 48; 1859-67.

Paper 2

Kamil A., Raae A., Fjelldal P. G., Koppang E. O., Kari F. E. and Hordvik I. (2013) Comparative analysis of IgM sub-variants in salmonid fish and identification of a residue in μ3 which is essential for MAb4C10 reactivity. *Fish & Shellfish Immunoogy* 34; 667-672.

Paper 3

Kamil A., Fjelldal P. G., Hansen T., Raae A., Koppang E. O. and Hordvik I. (2013) Analysis of serum immunoglobulin and Ig heavy chain gene expression in vaccinated versus unvaccinated Atlantic salmon. *Manuscript*.

1. Introduction

1.1 Teleost fish

Fishes are the most diverse group of aquatic vertebrates, broadly divided into three major superclasses i.e. Agnatha (jawless fishes), Chondrichthyes (cartilaginous fishes) and Osteichthyes (bony fishes). The superclass bony fish consists of a large and diverse subclass called Actinopterygii (ray-finned fishes). The ray-finned fishes make up around 95 % of all existing fish species (Nelson, 1994). The majority of ray-finned fish (almost 99 %) are grouped together in the infraclass called teleost fish (Volff, 2005), exceeding 28,000 extant species (Alfaro et al., 2009). Thus most living fishes belong to this infraclass. To understand the classification of teleost fish, a schematic presentation has been drawn (Fig 1).
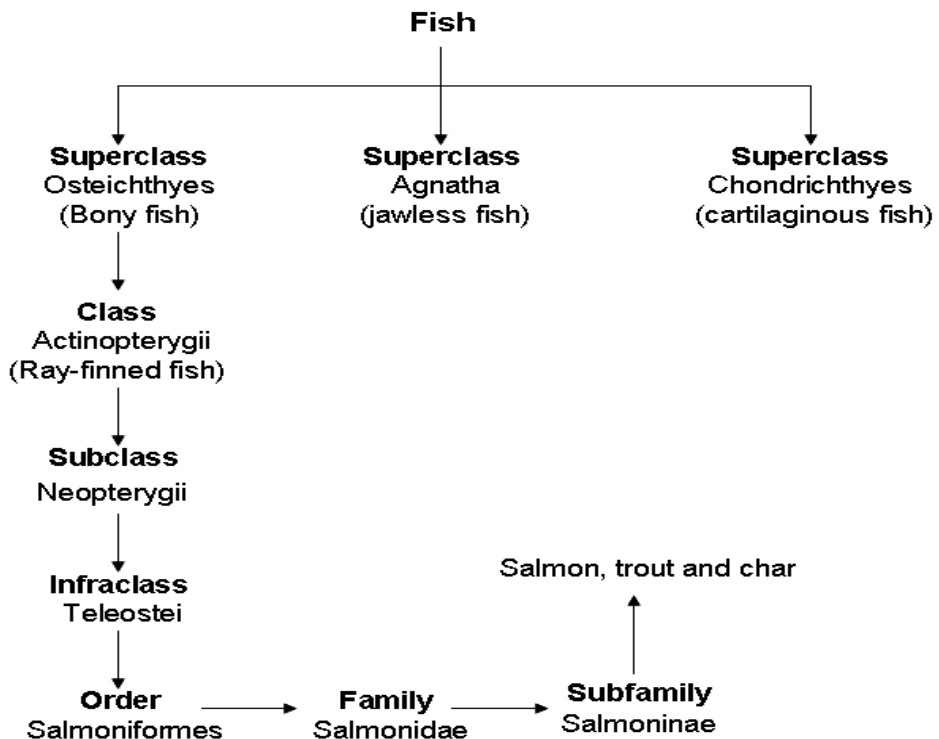
Figure 1. Schematic presentation of teleosts phylogeny. The subfamily Salmoninae is an important and diverse family of teleosts that includes species like Atlantic salmon, brown trout, rainbow trout and arctic char.

Teleost fish adapted modifications in their jaw muscular composition and thereby possess a movable maxilla and premaxilla to protrude its jaws from the mouth. Homocercal (upper and lower lobes are of equal size) caudal fin has been found in teleosts and its spine is extended till the caudal peduncle, in contrast to other fish groups where the spine extends into the upper lobe of the caudal fin. Teleosts are adapted to a wide variety of habitats ranging from cold arctic and antarctic oceans (less than 0 °C) to desert hot springs (more than 38 °C), implicating varied behaviors and life cycles.

Studies of teleost fish are considered very important due to their economical, ecological and cultural significance and for aspects related to evolution and biodiversity. Lately, several teleost genomes have been sequenced, leading to new insight into many aspects of their biology (Nelson, 1994; Volff, 2005).

1.2 Polyploidy

Organisms within a certain species usually possess a specified number of chromosomes in their body cells: e.g. human cells contain 46 chromosomes, horse cells contain 64 chromosomes and sheep cells contain 54 chromosomes. The majority of Metazoan species are diploid, i.e. their somatic cells contain a duplicated set of homologous chromosomes, while their gametes (sperm and ovum) are haploid i.e. contain a single set of chromosomes. The two types of cell division known as mitosis and meiosis play important roles in maintaining the diploid state of somatic cells and haploid state of gametes respectively. The first meiotic division produces two haploid gametes and the homologous chromosome pairs segregates equally between the two gametes. Thereby each haploid gamete receives one chromosome from each pair of homologous chromosomes. These haploid gametes are multiplied in the second meiotic division, generating four haploid gametes. The haploid gametes fuse together during fertilization and restore the diploid homologous chromosomes in somatic cells. The somatic cells divide and re-divide by mitosis and maintain the duplicated set of homologous chromosomes in all cells of the body parts. Thereby each organism maintains its duplicated set of homologous chromosomes in somatic cells and haploid chromosomes in their gametes, generation after generation. However, mistakes do happen in the equal separation of homologous chromosomes during first meiotic division and the resulting gametes receives un-equal chromosomes. After the fertilization of these gametes the resulting individuals are either deficient of some chromosomes or having additional sets of chromosomes.

The individual that adopt additional sets of chromosomes compared to the reported number for that particular organism is defined as polyploid. Individuals with one additional set of chromosomes (three sets of homologous chromosomes) are called triploids. The gametes that receive additional sets of chromosomes are abnormal, thereby when fused with a normal gamete result in polyploid individuals. The production of polyploid organisms by fertilization among individuals within the same species is known as autopolyploidy, whereas among the individuals from different species is called allopolyploidy. Autopolyploid individuals arise spontaneously through many different ways after chromosome multiplication in their gametes. Un-equal chromosome segregation happens during embryo cleavage due to errors in meiosis or mitosis. These errors include first or second meiotic division suppression as well as aged ova and poly-spermy (Aegerter and Jalabert, 2004; Aegerter et al., 2004; Aegerter et al., 2005; Cherfas et al., 1991; Ezaz et al., 2004; Flajshans et al., 2007; Grunina et al., 1995; Grunina et al., 2006; Piferrer et al., 2009; Varkonyi et al., 1998). The allopolyploid organisms are produced by the combination of abnormal gametes from related species. Such natural inter-generic hybridization results in modified but evolutionarily conserved chromosomal combinations. Hundreds of such hybrids adapting modified reproductive nature have been reported in lower vertebrates (Alves et al., 2001). The phenomenon of allopolyploidy has been reported in gynogenesis, hybridogenesis and parthenogenesis (Schlupp, 2005). Several hybrid asexual triploid fish have been reported all over Europe.

Advancement in the molecular genetic techniques made it possible to induce polyploidy in fish and shellfish by inhibiting the extrusion of polar body. The fish eggs are arrested after release at the metaphase stage of meiosis II (Colas and Dube, 1998) and once the sperm enter the arrested egg, further development of the egg is initiated from the point of arrest. Thereafter the physiochemical shock in meiosis II inhibits the second polar body extrusion while allowing the duplication process of chromosomes, thereby producing triploid fish. Likewise the physiochemical shock during first cleavage of zygote also suppresses cell division and permit chromosome duplication that lead to the production of tetraploid fish (Fig 2).

The triploid fish are usually sterile adapting vestigial or extremely late development of gonads. The aneuploid gametes (abnormal chromosome number) produced by the triploid fish are infertile due to random separation of trivalent chromosomes which are not capable of fertilization. Thereby triploid fish are considered as the dead ends in reproductive lineages,

which is not always correct. There are many examples of naturally occurring hybrid polyploid fish, where both triploid female and male fish were found fertile e.g. Prussian carp (Juchno and Boron, 2006; Vasil'ev et al., 2003). The eggs produced by these allotriploid females adopt varied polyploidy and are different in size, while the fertility of allotriploid sperms were found different for different fish species (Alves et al., 2004; Momotani et al., 2002; Oshima et al., 2005).
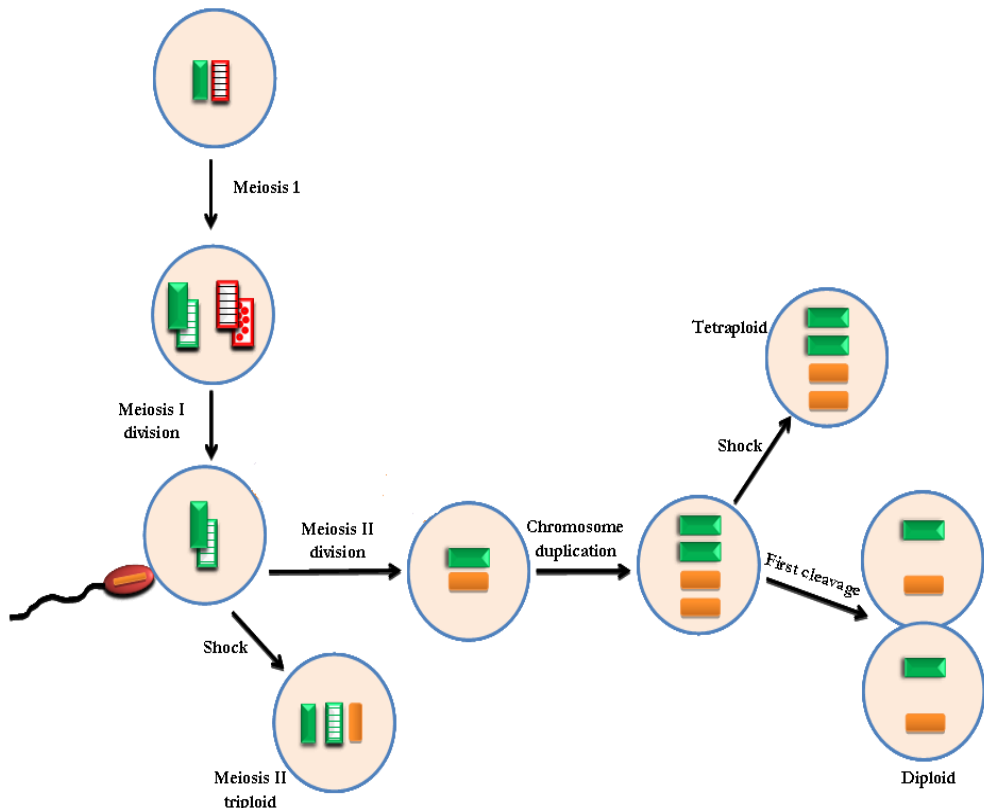
Figure 2. Artificial polypoidy induction in fish. *The arrested eggs at metaphase of meiosis II resumes meiotic division post fertilization. Physiochemical shock causing the suppression of meiosis II produces triploids while suppression of first cleavage in zygote produces tetraploids. Here each colored bar in the cell show one chromosome and overlapping bars show the sister chromatids post DNA duplication (modified from Piferrer et al., 2009).*

Among many phylogenetically distant orders of wild and farmed fish we find examples of spontaneous polyploid fish (Thorgaard and Gall, 1979). Polyploidy events during fish

evolution has played a major role in fish speciation and biodiversity (Le Comber and Smith, 2004). The growth rates of fish body decreases significantly due to sexual maturation as gonad development and maturation of fish consumes quite large amounts of energy. Sexual maturation is also quite often associated with increased incidence of diseases. Polyploidy is an efficient method of producing sterile fish that possess potentiality of genetic containment of aquaculture fish. The main issues associated with induction of triploidy in fish and its applications in aquaculture have been described previously (Maxime, 2008; Piferrer et al., 2009; Tiwary et al., 2004). The triploid fish adapt the gonad sterility, thereby reducing the risk to diseases, retarded rate of growth and several other organoleptic properties associated with sexual maturation. Triploid molluscs adapt superior growth in aquaculture. The technique of triploid generation is of great advantage to produce specific hybrid fish.

1.3 Genome evolution and biodiversity in teleosts

Genome duplication has been considered a crucial factor in the process of evolution (Bridges, 1936; Ohno, 1970; Stephens, 1951). The huge complexity and large genome size of vertebrates indicate that during early evolution of vertebrates two rounds of whole genome duplication (WGD) occurred (Ohno, 1970). Approximately 400 million years ago (MYA) during the early evolutionary stages of ray-finned fish, a process of WGD, called fish specific genome duplication (FSGD) occurred (Hoegg et al., 2004b; Hurley et al., 2007; Meyer and Van de Peer, 2005; Postlethwait et al., 2004; Taylor et al., 2001b). A huge morphological diversification found in teleosts might be causally related to the event of FSGD (Amores et al., 1998; Christoffels et al., 2004; Hoegg et al., 2004a; Taylor et al., 2003; Taylor et al., 2001a; Vandepoele et al., 2004). It has been shown that the process of speciation together with differential retention and loss of duplicated genes after WGD might prove a potential lineage-splitting force (Lynch and Conery, 2000). Several teleost genomes have been sequenced indicating that teleost genomes have preserved several duplicated genes as a result of the FSGD event. Thus, teleost fish represents a model for the study of retention and loss of duplicated genes and their evolutionary trajectory after WGD.

Most of the duplicated genes usually become non-fictional, i.e. become pseudogenes after some time (Jaillon et al., 2004; Wolfe and Shields, 1997). According to a recent survey, five teleost species showed around 90 % differential retention and loss of duplicated genes out of 1500 gene families, whereas around 10 % duplicated gene families were found in all five species, thereby indicating a relationship between differential gene retention and phylogenetic

position as well as relatedness between teleost species. Differential retention and loss of duplicated genes is a continuous process post FSGD, resulting in a huge diversification in teleosts.

In comparison to other species of ray-finned fish, teleost fish showed the most high diversity (more than 99 %) of all diversity found in ray-finned fish (Alfaro et al., 2009). Active transposable element families have also been reported in teleosts, which could have impact on the diversity of genomes and speciation. A huge diversity and species richness has been reported in the two groups Ostariophysi and Perciformes. Ostariophysi are freshwater species including piranhas, catfish, carps and danios, while Perciformes belongs to the spiny-rayed fish consisting most of the coastal and pelagic marine fish and few large freshwater fish such as perches and cichlids (Alfaro et al., 2009).

1.4 The *Salmoninae* subfamily

The teleost fish family *Salmonidae* includes three subfamilies: *Salmoninae, Coregoninae* and *Thymallinae*. The *Salmoninae* subfamily habitats the northern hemisphere and comprises the three genera S*almo* (including Atlantic salmon and brown trout), *Oncorhynchus* (including rainbow trout) and *Salvelinus* (including arctic char). Salmonid fish are of great interest in the aquaculture industry and huge efforts are made to cope with a wide variety of pathogens and diseases to improve the quality and production of these fish species (Klemetsen et al., 2003). Their current distributions are generally quite diverse overlapping in the European region, however, their natural distributions vary from one species to another.

Salmon, trout and char occur as both anadromous (fresh water/salt water) and non-anadromous (fresh water) forms (Maccrimmon and Gots, 1979; Shearer et al., 1992). The anadromous form starts their life cycle in fresh water and later on migrates to salt water. However, there exists variation in their life cycle and each phase is distinguishable with the specified alterations in behavior, physiology and habitat. Fertilization takes place externally in the fresh water.

Salmonid species are considered very important for comparative immunology due to its unique position in the phylogenetic tree and its recent WGD event (Koop et al., 2010; Yasuike et al., 2010). Significant genetic and phenotypic variations are found among the different

regional populations and traits within the species. Population of a specific locality is well adapted to their native habitat and show important conserved values.

1.4.1 Atlantic salmon

Atlantic salmon is mostly found in northern Atlantic Ocean as well as the east and west coasts of the northern Atlantic Ocean and the Baltic Sea. Atlantic salmon of European vs North America and Eastern Atlantic vs Baltic population show significant differences (Maccrimmon and Gots, 1979; Shearer et al., 1992). Atlantic salmon of weight more than 30 Kilo grams (Kg) has been reported in Norway. The Atlantic salmon genome consists of 58 chromosomes (Rees, 1967). Atlantic salmon show strong tendency towards natal areas for spawning. The female deposit her eggs in gravel nests in rivers. Juvenile salmon feed on small invertebrates while adult salmon feed on crustaceans and small fish. The different stages of life cycle have been presented schematically (Fig 3).
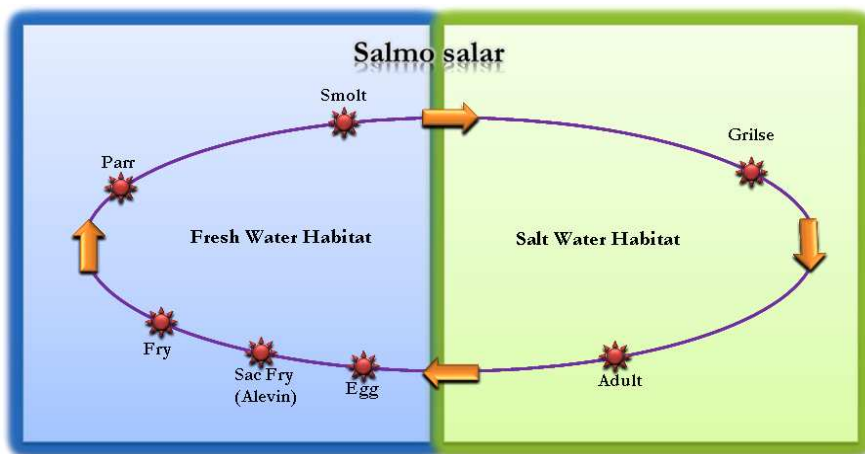
Figure 3: Life cycle of Atlantic salmon. Atlantic salmon habitats both freshwater and salt water ecosystems during their life cycle. The life begins in fresh water when the adult salmon hatches eggs and after few years they migrate to the sea water in the form of smolt. The smolt becomes adult salmon during the next feeding years in the salt water of sea. The adult salmon migrate back to the fresh water for hatching and thereby completes its life cycle.

Atlantic salmon migrate through North Atlantic Ocean from coastal rivers of several regions including Europe, Russia, Iceland and North America. The European and North American salmon get mixed with each other at the sea, because they share Greenland grounds for

feeding in summer. Numerous populations of Atlantic salmon have been reported residing at several rivers and fjords in Norway (Berg et al., 2001). The Norwegian aquaculture and fisheries industry relies mainly on Atlantic salmon for its exports of seafood. The Norwegian Sea food Export Council report from 2010 showed that export value of Atlantic salmon was higher than the total value of all other seafood products.

1.4.2 Brown trout

The brown trout (*Salmo trutta*) raised in Europe resemble salmon. Brown trout show significant intraspecific genetic and phenotypic diversity (Bernatchez, 2001). The adult brown trout ranges in size up to 20 Kg. Brown trout contain 80 chromosomes (2n) in their somatic cells (Garciavazquez et al., 1995). The maximum life span of brown trout is usually around 20 years, however, most male trout and salmon die after spawning. Due to opportunistic feeding nature in the sea, trout usually remain active by day and night, whereas in rivers they usually feed on invertebrates, small fish and insects. In contrast to Atlantic salmon, hybrid fish of brown trout usually remain consistently infertile, such as hybrid of brown trout/brook trout. Advancements in the brown trout farming industry led to successful production of infertile triploid fish that grow faster and become much bigger than diploid fish. Another advantage of infertile triploid brown trout includes its harmless introduction (inability of cross-breeding) into environment of wild brown trout. However, the aggressive mode of triploid trout could disturb spawning behavior of diploids as well as may certainly compete with diploids for food, space and other resources (Dannewitz et al., 2004; Elliott, 1994).

1.4.3 Arctic char

Arctic char (*Salvelinus alpinus*) is a cold water species, widely distributed in northern hemisphere, in several Arctic and sub-Arctic lakes. They are found in several variable forms and colors within the same lake (Johnson, 1980). Two eco-morphological distinct char (pelagic and benthic) have been reported (Gardner et al., 1988; Walker and Greer, 1988). The somatic cells of char contain 78 chromosomes (Hartley, 1989). Its phenotypic appearance has close resemblance to salmon but genetically it is closely linked to trout. Typically arctic char range in weight from 0.2 to 4.5 kg but char with heavy weight have been reported. The commercial production of arctic char is increasing in Norway, Iceland, Canada and other European countries (Gross et al., 2004). Arctic char spend the first four to five years in freshwater followed by migration into the sea. Due to their high sensitivity towards environmental inability, char migrate back into fresh water in the late summer and fall.

Thereby char spend brief period in the sea, when conditions are adequate for temperature and food (Elliott and Baroudy, 1995).

1.5 The adaptive immune system in teleost fish

The immune system of an organism is usually described as two parts, the innate immune system (or non-specific) and the adaptive immune system (or specific). The innate and adaptive immune systems are integrated into each other. To cope with the invading micro-organisms, the innate immune system represents the first line of defense while the adaptive immune system works as a second line of defense. Both the innate and adaptive immune systems utilize cellular as well as humoral components for the protection against invading pathogens. The innate immune system possesses special features working as physical barriers to infection. The innate immune system cleans up the pathogens through cell phagocytosis and secretion of soluble antimicrobial molecules. Microbes share common motifs called pathogen associated molecular patterns (PAMPs), recognized by innate immune receptors called pattern recognition receptors (PRR). In this context toll like receptors (TLRs), contribute a key role (Janeway and Medzhitov, 2002). The adaptive immune system is a highly evolved system of specific responses. The components of the adaptive immune system possess the ability to not only recognize but also to "remember" specific pathogens so as to produce a faster and stronger response after second exposure of the same pathogen. This feature of the adaptive immune system is known as memory.

The immune organs of teleost fish include kidney, spleen, thymus and mucosa-associated lymphoid tissues i.e. skin, gut and gill. Fish do not possess bone marrow and lymph nodes which are important immune organs in mammals. The thymus of teleost is located close to the gill cavity, showing distinct morphological features of well shaped medulla and cortex in some species (Press and Evensen, 1999). The kidney; the analog of the bone marrow in mammals, represents a complex organ consisting of several district systems such as lymphoid, endocrine, reticulo-endothelial and excretory systems. The spleen is composed of a fibrous capsule and small trabeculae that is usually extended into the parenchyma. The parenchyma can be divided into red (occupy most of the organ) and white pulp (Grace and Manning, 1980; Secombes and Manning, 1980). The spleen represents a secondary lymphoid organ showing thrombopoietic and erythropoietic activity (Rombout et al., 2005). The gut, skin and gills contain mucus layer as well as array of nonspecific immune defenses. These organs are exposed to the external environment and represents the first line of defense to invading

pathogens (Dalmo et al., 1997). Recently new interbranchial lymphoid tissue was discovered close to gill in Atlantic salmon, showing high number of T lymphocytes (Haugarvoll et al., 2008; Koppang et al., 2010).

The adaptive immune system consists of B lymphocytes, T lymphocytes, and antigen presenting cells. Both B and T lymphocytes express surface molecules for binding with antigen called B cell receptors (BCR) and T cell receptors (TCR), respectively. Somatic recombination result in generation of diverse B and T cells receptors for the recognitions of large variety of pathogens (Danilova and Amemiya, 2009; Schatz et al., 1992). T cells are of two types. Helper T cells which express surface CD4 receptors, play a role in cytokine secretion and B cell activation. Cytotoxic T cells which express surface CD8 receptors, kill target cells infected with pathogens. B cells binds directly with the invading antigens, whereas, the TCR usually recognize those antigens which are processed and presented by the major histocompatibility complex (MHC) antigen. B cells are activated by direct interaction with antigen and by cytokine (e.g. interleukins) released by helper T cells (Clark and Ledbetter, 1994).

The characteristic adaptive immune system consisting of B cell receptors and antibodies (immunoglobulins), TCR and MHC antigens is present in jawed vertebrates (Litman et al., 2004; Pancer and Cooper, 2006). In jawed vertebrates the diverse repertoire of B and T cell receptors are generated by rearrangement of variable-diversity-joining (V-D-J) gene segments (Bassing et al., 2002; Schlissel, 2003). In jawless vertebrates, another type of variable lymphocyte receptors (VLRs) are generated through recombination of leucine-rich repeat (LRR) modular units (Cooper et al., 2004; Cooper et al., 2005; Pancer et al., 2005).

Genetic recombination of V, D and J segments and somatic hyper-mutation make the adaptive immune system dynamic. A limited number of genes produce a large number of antigen receptors, creating a basis for clonal expansion of lymphocytes carrying appropriate receptors during an adaptive immune response. However, varying temperature might affect the adaptive immune system to a greater extent than the innate immune system, due to the ectothermic physiology of the fish, indicating that adaptive immune responses are not as efficient in fish as in mammals (Afonso et al., 1998; Ellis, 2001; Magnadottir, 2006; Yoder, 2004).

1.6 Immunoglobulin (Ig) molecules in teleost fish

1.6.1 Composition of the Ig monomer

A typical Ig monomer consists of two identical heavy (H) chains and two identical light (L) chains encoded by the IgH and IgL loci, respectively. Usually the H chains are connected to each other and to L chains through disulfide bonds. The H and L chains consist of characteristic folds known as Ig-domains. The H and L chains possess a variable (V) region towards the N-terminal and a constant (C) region towards the C-terminal. The L chain consists of one V domain and one C domain while the H chain consists of one V domain and 2 to 16 C domains in fish Igs. The V domain consists of complimentary determining regions (CDR) i.e. CDR1, CDR2 and CDR3 flanked by framework regions (FR) i.e. FR1, FR2 and FR3 (Fig 4).
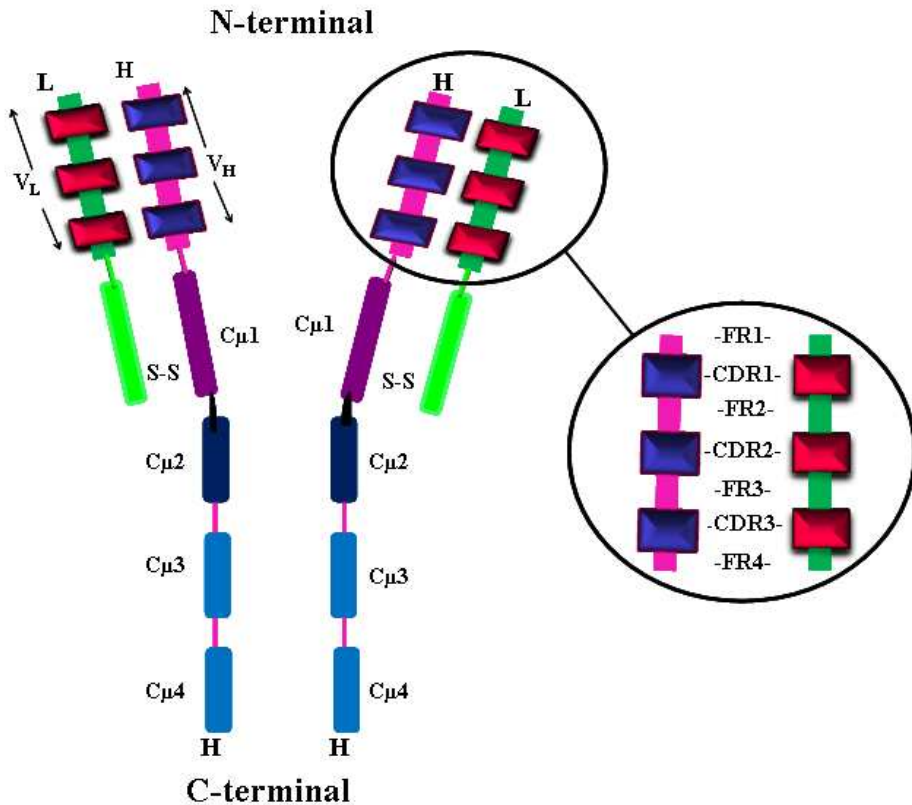
Figure 4. Schematic presentation of an Ig monomer. *The arrangement of H and L chains and their connectivity through s-s bond is shown. Variable domains of both H and L chains are towards the N-terminal and contain the antigen binding region.*

The CDRs are highly diverse, and mediate highly specific recognition of epitopes expressed on different antigens. Igs can be in membrane bound form, expressed on B-lymphocytes, or in secreted form in the serum and mucus (Pilstrom and Bengten, 1996). The membrane anchored form of Ig consists of a transmembrane (TM) part and a cytoplasmatic tail (CY). Igs in the serum and mucus may occur in different polymer forms like monomer, dimer, trimer, tetramer, pentamer and hexamer. The Ig heavy chain gene complex in teleost fish encodes three antibody classes i.e. IgM (μ), IgD (δ) and IgT (τ) (or IgZ), in contrast to the five antibody classes (IgM, IgD, IgG, IgE and IgA) reported in mammals (Danilova et al., 2005; Hansen et al., 2005).

1.6.2 IgM

IgM is the predominant serum antibody in teleost fish. IgM is present in all jawed vertebrates and plays a major role during primary antibody response, and is the first Ig that arises in the mammalian fetus. The overall structure of the IgM monomer is well-conserved during vertebrate evolution (Andersson and Matsunaga, 1993; Bengten et al., 1991; Fellah et al., 1992; Lee et al., 1993; Rosenshein et al., 1985). However, IgM primary structure varies considerably and membrane anchored IgM in teleosts lack the forth constant domain (μ4) (Fig.5) due to a special mRNA splicing pattern (Andersson and Matsunaga, 1993; Bengten et al., 1991). IgM has been reported in distinct polymer forms in different species i.e. hexamer in certain amphibians (Hsu and Du Pasquier, 1984), pentamer in higher vertebrates and cartilaginous fish (Kobayashi et al., 1984), tetramer in teleost fish (Acton et al., 1971) and monomer in certain species (Clem and McLean, 1975). The J-chain responsible for the polymerization of mammalian IgM has not been found in teleost fish (Sanchez et al., 1989; Weinheim et al., 1971; Weinheim.Pf et al., 1971). There exists significant variation in the number and position of the s-s bonds holding the H chains together (Partula and Charlemagne, 1993; Whittington, 1993). Variation has also been reported for the carbohydrate content (Acton et al., 1971; Lee et al., 1993; Magnadottir et al., 1997). Serum IgM concentration in teleosts was found quite variable in different life stages as well as under different physiochemical conditions like age, weight, sex, disease, vaccination, challenge and environmental conditions (Fuda et al., 1991; Havarstein et al., 1988; Ingram, 1979; Israelsson et al., 1991; Olesen and Jorgensen, 1986; Sanchez et al., 1993a; Voss et al., 1980). Some of the variation of IgM level reported might be attributed to the purification strategy as well as quantification assay.

1.6.3 IgT

A unique Ig class, IgT/IgZ present in teleost fish, has now been characterized in several species including zebrafish (Danilova et al., 2005), rainbow trout (Hansen et al., 2005), fugu (Sakai et al., 2005b), carp (Sakai et al., 2005a) and Atlantic salmon (Tadiso et al., 2010), but seems to be absent in channel catfish (Bengten et al., 2002a). Both secreted and membrane anchored IgT has been reported in rainbow trout and zebrafish (Danilova et al., 2005; Hansen et al., 2005; Zhang et al., 2009). In contrast to IgM, the secreted as well as membrane bound IgT comprise four constant Ig domains in Atlantic salmon i.e. $\tau 1-\tau 4$ (Fig. 5). Recent analysis of rainbow trout IgT showed that it exists as monomer in serum and tetramer in gut mucous. However, native SDS-PAGE (denaturing but non-reducing) showed only monomer IgT in gut mucous samples, indicating that non-covalent interactions held together the tetramer IgT. Reverse transcription quantitative PCR (RT-qPCR) analysis of Atlantic salmon revealed that μ transcripts are 20x as abundant compared to τ (Tadiso et al., 2010). Recent findings indicate that IgT might be involved in mucosal immunity similar to IgA in mammals (Zhang et al., 2010b; Zhang et al., 2011).

1.6.4 IgD

Teleost IgD was first discovered in channel catfish (Wilson et al., 1997), and subsequently in a series of other teleosts (Hirono et al., 2003; Hordvik, 2002; Hordvik et al., 1999b; Stenvik and Jorgensen, 2000; Suetake et al., 2004). The translated polypeptide sequence showed some similarity to human IgD, the δ gene was located immediately downstream of the μ gene and both IgM and IgD could be expressed in the same lymphocyte, regulated by alternative mRNA splicing. Thus, IgD which was previously considered to have emerged relatively recent in evolution showed to be an ancient Ig class. This has now been confirmed by the discovery of IgD homologs in most jawed vertebrates, with a few exceptions (Bengten et al., 2002a; Edholm et al., 2010; Sun et al., 2011). The heavy chain of teleost IgD is a chimeric molecule including $\mu 1$ in addition to the VDJ region and the δ chain (Aoki et al., 2003; Hordvik et al., 1999b; Stenvik and Jorgensen, 2000; Suetake et al., 2004; Wilson et al., 1997). Considerable variation has been observed in the size and composition of IgD molecules (Bengten et al., 2002a; Stenvik and Jorgensen, 2000), e.g. mouse IgD consists of two δ domains while the zebrafish IgD consists of sixteen δ domains. Transcripts encoding membrane bound IgD appear to be dominant in all species examined except channel catfish. Minor amounts of a special mRNA splicing variant of IgD were found in Atlantic salmon, but it is uncertain whether these transcripts are translated into a functional product (Hordvik,

2002). Recently three secretory IgD isoforms of 165 KDa, 125 KDa and 100 KDa were found in rainbow trout (Ramirez-Gomez et al., 2012). In channel catfish as well as in humans IgD showed innate immune responses against certain pathogens (Chen et al., 2009).
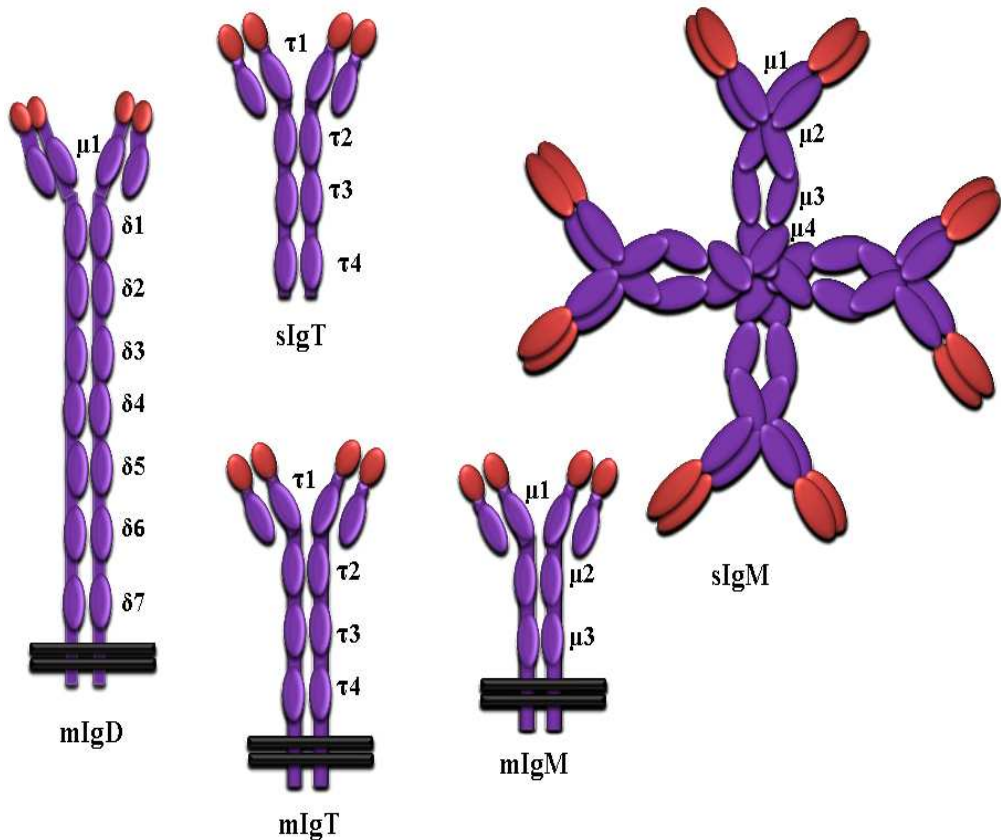
Figure 5. Schematic presentation of Ig classes in Atlantic salmon. *The three Ig classes i.e. IgM, IgT and IgD, both secretory and membrane anchored form found in Atlantic salmon are shown.*

1.6.5 Organization of the Ig heavy chain genes

The Ig heavy chain genes appear in two distinct types of genomic organization. In cartilaginous fish such as sharks and skates, closely linked clusters of V-D-J-C gene segments are repeated 100 - 200 times (Flajnik and Dooley, 2006; Hsu et al., 2006), while a translocon organization like (V)n-(D)m-(J)x-(C)y (or modified versions of this) has been reported in the

majority of bony vertebrates, including teleost fish (Amemiya and Litman, 1990; Ghaffari and Lobb, 1992; Hordvik et al., 1997; Warr, 1995; Yasuike et al., 2010).

The organization of the IgH genes in Atlantic salmon is shown in Fig 6. The Cτ genes of Atlantic salmon (and most other studied species of teleosts) are found scattered in the VH region upstream of the Cμ and Cδ genes (Yasuike et al., 2010). The τ genes have been reported to possess its own D and J segments in zebrafish (Danilova et al., 2005; Hansen et al., 2005) and fugu (Sakai et al., 2005b).
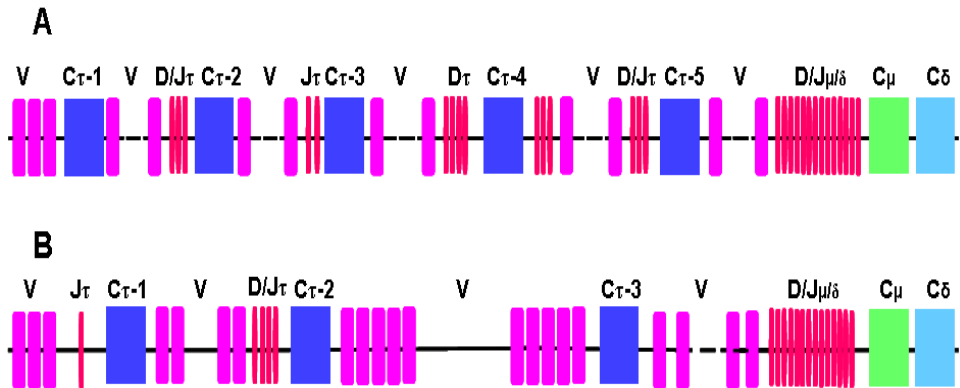
Figure 6. Schematic presentation of Ig H chain gene organization in Atlantic salmon. *The duplicated H chain gene complexes are named A and B. The μ and δ genes are* located downstream of the V/D/J-segments*, while the τ genes are scattered in the VH region (modified version of Yasuike et al., 2010).*

The τ gene segments were not found in catfish (Bengten et al., 2006b). Instead, three pairs of linked μ and δ genes, including one functional μ gene and three functional δ genes have been found (Bengten et al., 2006a; Bengten et al., 2006b; Bengten et al., 2002b). Thus, there is a considerable variability with regard to IgH gene organization in teleosts.

The L chain loci in teleosts showed a cluster organization, similar to shark (Bengten et al., 1994; Daggfeldt et al., 1993a; Ghaffari and Lobb, 1993). However the V segments showed an opposite transcriptional orientation to the J and C segments. Atlantic cod and rainbow trout share 55 % identity in their CL domain, whereas both share 30 % - 37 % identity with mammals (Daggfeldt et al., 1993b). Distinct L chain variants with different molecular mass

and structure have been identified (Lobb et al., 1984; Sanchez and Dominguez, 1991a; van der Heijden et al., 1995). Atlantic salmon possess at least three isotypes of IgL (Solem and Jorgensen, 2002). The most abundantly occurring light chain isotypes in salmon are IgL1 and IgL3 (Solem and Jorgensen, 2002).

1.6.6 Carbohydrate content of Igs

Glycosylation is a crucial post-translational modification of proteins exerting great impact on the structure and function of proteins. The present advancement in the field of protein engineering explored several common and specific roles of protein glycosylation (Svanes et al., 2010). There are two main types of protein glycosylation i.e. N-linked and O-linked oligosaccharides (Zhang et al., 2010a). The N-linked oligosaccharide is covalently bonded through nitrogen to asparagine (Asn), while the O-linked oligosaccharide is covalently bonded through oxygen to serine and threonine. N-glycosylation has been found to be most common in fish Igs. The oligosaccharide adapts massive structural diversity and numerous N-linked oligosaccharide subgroups are found sharing similar core structure but different composition and complexity (Castro-Giner et al., 2009; Friguls et al., 2009; Zhang et al., 2010a).

Igs possess different forms of carbohydrates bond to the H and L chain, such as galactose, sialic acid, glucosamine or mannose. Both type and amount of carbohydrate bound to each Ig class vary considerably. A comparative analysis of the carbohydrate moiety of IgM was conducted among four species of teleost (Atlantic salmon, halibut, haddock and Atlantic cod). This investigation reported that N-oligosaccharide bound to the H chain ranged from 7.8-12.5 % of the total IgM molecular weight (Magnadottir, 1998; Magnadottir et al., 1997). In mammals, numerous specified roles have been suggested for the carbohydrate content of Igs, some of which are summarized here. Protection against protease enzymes and structural stability (Cerveri et al., 2009), influence of avidity (Liu et al., 2010), IgM polymerization (Orriols et al., 2009; Zhang et al., 2010a), function as an effector e.g. recognition of Fc receptor and complement fixation. The carbohydrate content of Igs was found variable under certain circumstances such as pregnancy, ageing or diseases (Axford, 1993; Bond et al., 1993). Accordingly, the N-oligosaccharide bound to fish Igs has great impact on the structural stability, protection against protease digestion and various biological functions (Magnadottir et al., 2002).

1.7 Purification strategies for Igs of teleost fish

Purification of proteins is a crucial requirement for research and development in the field of biomedical science. There is a strong need for new purification strategies which could efficiently isolate and enrich a target from a complex mixture as well as from very dilute solutions. A large variety of techniques are available for this purpose, such as chromatography, electrophoresis, ultra-filtration, precipitation and magnetic affinity purification (AC). Anion exchange chromatography (AEC) separate protein molecules according to its net ionic interaction strength with a solid material, thereby applying high or low ionic strength buffers for the association or disassociation of proteins with the solid material. Each of these techniques has some advantages and drawbacks.

Serum from several teleosts, have been subjected to Ig purification (Table 1), applying a variety of strategies, such as molecular sieving, AC, AEC and precipitation through saturated ammonium sulfate (Acton et al., 1971; Fuda et al., 1991; Havarstein et al., 1988; Ingram and Alexander, 1979; Israelsson et al., 1991; Olesen and Jorgensen, 1986; Sanchez et al., 1993a; Voss et al., 1980). The affinity columns have advantages of specificity and simplicity due to the bound ligands like IgG, mannan binding protein (MBP) and Protein A (Al-Harbi et al., 2000; Bromage et al., 2004; Crosbie and Nowak, 2002; Suzuki et al., 1990; Watts et al., 2001). IgM from African catfish (*Clarias gariepinus*) was purified by affinity chromatography (Rathore et al., 2006). The binding affinity of IgM to protein A was found to be very low for Atlantic salmon, brown trout, arctic char and rainbow trout, whereas high amounts were purified from Mosambique tilapia and barramundi (100 % and 80 % respectively) (Bromage et al., 2004; Estevez et al., 1993; Suzuki et al., 1990). IgM from Atlantic salmon, brown trout, rainbow trout and arctic char has been purified by gel filtration followed by ion-exchange chromatography (Havarstein et al., 1988; Hordvik et al., 2002). Ammonium sulphate precipitation followed by gel filtration has been implemented for carp IgM purification (Rombout et al., 1993).

Table 1: *A literature survey of serum IgM purification techniques and serum IgM concentration in teleost species. The serum IgM concentrations varies quite much according to different physiochemical parameters such as body weight and length, water temperature, immunized or diseased, farm fish, aquarium fish or free living stream fish. The additional factors include experimental methodologies such as purification strategy and IgM concentration measurement approach.*

Teleost specie	Purification strategy	Method for protein con. measurement	Weight of fish	Fish Physiological status	IgM Con. (mg/ml)	IgM Con. (% of serum proteins)	Reference
Brown trout	AS precipitation followed by gel filtration and then AEC	immunodiffusion	-	-	7.3 mg/ml	10 %	(Ingram & Alexander, 1979)
Coho salmon	AS precipitation followed by gel filtration	Radiul diffusion test	50-200 g	Healthy Adult	2.1 mg/ml	-	(Voss et al., 1980)
Rainbow trout	AS precipitation followed by gel filtration and then AEC	Biuret Assay	-	Healthy Adult	1.5-10.9 mg/ml	3-34 %	(Olesen and Jorgensen, 1986)
	Affinity Chromatography	ELISA	20 g-1 Kg	Healthy Adult	0.67-9.36 mg/ml	3.8-17.4 %	(Sanchez et al., 1993)
Atlantic salmon	Gel filtration and then AEC	Bradford Assay (BioRad)	-	Healthy Adult	0.8-1.3 mg/ml	2 %	(Håvarstein et al., 1988)
Masu salmon	AS precipitation followed by gel filtration	Bradford Assay (BioRad)	-	Larvae and fry	0.47-0.69 mg/ml	-	(Fuda et al., 1991)
Atlantic cod	Gel filtration	ELISA	954 g	Healthy Adult	5.62 mg/ml	17.2 %	(Israelsson et al., 1991)
Antarctic teleost	AS precipitation followed by gel filtration and then AC	Bradford Assay (BioRad)	189-382 g	Healthy Adult	1.0-4.6 mg/ml	9.6 %	(Pucci et al., 2003)
Asian sea bass	Affinity Chromatography	ELISA	-	Immunized Adult	3.6-7.2 mg/ml	16.7 %	(Choudury and Prasad, 2011)

1.8 IgM subpopulations in salmonid fish

Purification of serum immunoglobulin from Atlantic salmon, by gel filtration followed by AEC, revealed two distinct sub-populations with similarity to IgM (Havarstein et al., 1988). The molecular weight was estimated to be 1000 kDa when examined by gel filtration on a Superose 6 column, whereas gel electrophoresis indicated a molecular weight of approximately 800 KDa. The heavy and light chain subunits were determined by SDS-PAGE to be 72 and 27 KDa, although the light chain bands appeared to vary to some degree in size (Havarstein et al., 1988; Lobb et al., 1984). In agreement with the initial characterization of salmon IgM, molecular cloning of cDNA revealed two distinct μ genes, both of which were present in haploid embryos (Hordvik et al., 1992). Further characterization of the genes showed that there are two paralogous IgH gene loci (named A and B) in Atlantic salmon as a result of ancestral tetraploidy (Hordvik, 1998; Hordvik, 2002; Hordvik et al., 2002; Hordvik et al., 1997; Hordvik et al., 1999a; Solem et al., 2001; Tadiso et al., 2010; Yasuike et al., 2010). A comparative study revealed an equivalent situation in brown trout, and the molecules were named accordingly, i.e. IgM-A and IgM-B. In contrast to Atlantic salmon and brown trout, IgM of rainbow trout and char was eluted in a single peak by AEC (Hordvik et al., 2002).

1.9 Implementation of proteomic tools in fish immunology research

Characterization of immunological changes in response to different challenges as well as diseases represents an important step towards effective vaccine and drug development. Since immune cells migrate rapidly from one place to another in the body, and since immune responses involve many humoral components in serum and other body fluids, proteomic analyses are very important for this research field, e.g. by identifying key markers for monitoring immune responses. Owing to the implementation of high throughput sequencing many fish genomes (and pathogens) are now sequenced completely, favoring identification of proteins by mass spectrometry (MS) analysis. Advancements and commercialization of mass spectrometry analyses promote the implementation of proteomics in many laboratories at the moment. Within the field of fish immunology this is evident by several publications during the last decade (Booth and Bilodeau-Bourgeois, 2009; Bromage et al., 2006; Hordvik et al., 2002; Huang and Chen, 2012; Rajan et al., 2011; Rathore et al., 2006; Swain et al., 2004), including the characterization of secretory IgD in catfish (Bengten et al., 2002a), IgT in rainbow trout (Zhang et al., 2010b) and IgD in rainbow trout (Ramirez-Gomez et al., 2012).

2. Aim of the present project

Atlantic salmon production has increased rapidly, from around 50,000 tons in 1986 to 1006,000 tons in 2011 in Norway alone. The introduction of vaccines has been a key factor to this achievement, but diseases and development of new vaccines are a continuous challenge for the aquaculture industry.

Antibodies are key players in mounting protective responses. However, although comprehensive genetic information is available now, there is still very limited information on the structure and function of antibody isotypes in fish. The main objective of the present project was to study the sub-variants of the major serum immunoglobulin, i.e. IgM in Atlantic salmon. To this aim closely related species of salmonid fish were included for comparative analysis.

Subgoals
1. Further characterization of IgM-A and IgM-B in Atlantic salmon and brown trout
2. Development of tools to distinguish between IgM sub-variants and the genes that encodes these in Atlantic salmon and brown trout
3. Molecular cloning of arctic char μ cDNA and comparative analysis of corresponding sequences from representative species of salmonid fish
4. Analysis of possible differential expression of IgM sub-variants in Atlantic salmon

A major undertaking of the present project was to develop tools and implement modern proteomics to gain further insight into IgM heterogeneity of salmonid fish. Analysis of possible differential expression of IgM sub-variants was done on samples from previous experiments and in collaboration with researchers at Matre research station (Institute of Marine Research). This collaboration gave us access to diploid and triploid un-vaccinated and vaccinated salmon, as well as hybrids of char/salmon.

3. Results

3.1 IgM heavy chain sub-variants in salmonid fish

Two IgM subpopulations in Atlantic salmon and brown trout which are separable by AEC were, by MS analysis confirmed to correspond to µA and µB, respectively. The MS analysis also revealed that the most common L chain isotypes, IgL1 and IgL3, are present in both IgM-A and IgM-B of Atlantic salmon (**Paper 1**). Molecular cloning revealed two slightly different µ subvariants µA-1 and µA-2, in arctic char, and mRNA encoding both sub-variants, plus salmon µA and µB, were expressed in hybrids of Atlantic salmon/arctic char. *In silico* analysis revealed two types of µ ESTs from grayling. A comparison of representative µ sequences from salmonid fish was performed (**Paper 2**).

3.2 Tools to distinguish between IgM sub-variants

A mouse monoclonal antibody (MAb) 4C10 originally raised against rainbow trout IgM, reacted with IgM-A in Atlantic salmon and IgM-B in brown trout, whereas it did not react with salmon IgM-B or brown trout IgM-A. cDNA analysis showed that magnetic beads coated with Mab4C10 captured primarily µA producing lymphocytes from Atlantic salmon, whereas the unbound cells expressed mainly µB transcripts, and *vice versa* in brown trout. Magnetic beads were also used to isolate IgM-A from salmon (confirmed by MS analysis). Subcloning of µ3 variants from Atlantic salmon, brown trout and rainbow trout into a eukaryotic expression vector, followed by transfection and immunostaining, indicated that MAb4C10 recognizes the µ3 domain (**Paper 1**). Subsequently, it was shown that mutation of a proline residue at the 251 position (to threonine) caused loss of salmon µA3 binding, and *vice versa* a T251P mutation in salmon µB3 restored binding with MAb4C10 (**Paper 2**). Molecular cloning of MAb4C10 cDNA and MS analysis confirmed that MAb4C10 is of IgG-1 subtype, and the VH sequence of MAb4C10 was determined (**Appendix**). Different RT-PCR assays were attempted to evaluate the ratio of µA/µB transcripts in tissue samples from salmon and brown trout. However, due to high sequence similarity between the sub-variants it was difficult to reach the required experimental precision by these methods (**Paper 3**).

3.3 Analysis of IgM inter-chain disulfide bonding

MS analysis confirmed that IgM-B of Atlantic salmon and brown trout contain an additional cysteine residue in the C terminal part of µ4 (**Paper 1**), as previously proposed (Hordvik et

al., 2002). Native-PAGE and immunoblotting showed one major and clear band of tetrameric IgM-B, whereas IgM-A showed additional bands of the expected sizes of trimers, dimers and monomers. The stability of IgM-B was distinctly higher compared to IgM-A when subjected to extreme heating conditions (**Paper 2**).

3.4 Expression analysis of Ig isotypes, with focus on IgM sub-variants

Reverse transcription real time PCR showed that vaccinated salmon had a higher abundance of μ transcripts in spleen than unvaccinated fish. ELISA analysis also showed that vaccinated salmon maintained higher IgM concentrations in serum. IgT heavy chain (τ) gene expression in spleen did not correlate with vaccination status and the abundance of IgD (δ) mRNA was too low for comparative analysis. Higher IgM expression was observed in males compared to females in vaccinated as well as unvaccinated fish, but confirmation of this needs a more comprehensive study. The exact ratios of μA and μB transcripts could not be estimated by reverse transcription real time PCR or conventional PCR followed by restriction enzyme analysis. However, the AEC profile of purified IgM in vaccinated fish indicate that the A:B ratio can be skewed in challenged fish (**Paper 3**).

4. Discussion

The present work has provided answers to key subjects addressed in the aims of the project, and at the same time raised several new questions, especially regarding the functional impact of IgM heterogeneity in salmonid fish. Sequence data from representative salmonid fishes strongly indicate that the sub-variant named IgM-B emerged in evolution prior to the radiation of the species Atlantic salmon and brown trout, and after the radiation of the three genera *Salmo, Oncorhynchus* and *Salvelinus* (**Paper 2**). A monoclonal antibody which distinguishes between IgM-A and IgM-B sub-variants in Atlantic salmon and brown trout has now been characterized, and can be used for further studies. In the context of recent discoveries in rainbow trout (Costa et al., 2012; Ye et al., 2010), showing that high affinity antibodies have a higher degree of disulfide bonding and a longer half life time, Atlantic salmon and brown trout should be ideal model fish to gain further insight into this phenomenon, since these species possess distinct (and distinguishable) subpopulations of IgM in contrast to rainbow trout.

The present work confirmed that the IgM-A and IgM-B subpopulations in Atlantic salmon and brown trout correspond to the products of the paralogous µA and µB gene loci. An additional cysteine residue at the C terminal part of µB is the only characteristic difference between IgM-A and IgM-B (**Paper 1**). In agreement with previous analyses, comparison of representative salmonid µ sequences showed that the third constant domain (µ3) diverges most rapidly followed by µ1 and µ2. The fourth constant domain (µ4) represents the most conserved domain with very few substitutions (**Paper 2**). Interestingly, MAb4C10, as well as three newly developed monoclonal antibodies (Hedfors et al., 2012), showed to react with salmon µ3. It might be questioned if antibodies in mice are preferably raised against structures that are most diverged in comparison to mouse IgM or if this domain of the molecule is exposed in a favorable manner to the immune system in mice.

Based on sequence analysis and 3D structure prediction a defined region of µ3 was supposed to be involved in the interaction with MAb4C10. The exposed charged amino acid residues in µA3 of salmon (K225, T227, E247) were thought to play a key role in the interaction (**Paper 1**). However, transfection analysis did not show any effect on binding when these residues were mutated.

Figure 7. Amino acid substitutions for µ sub-variants of Atlantic salmon and brown trout.
The potential amino acid hypothesized to be the key residue for binding to Mab4C10 is indicated by a red arrow (modified version from paper 1).

Later on we found a critical mistake in the sequence entry for µB3 of brown trout (Fig. 7) in Genbank (251T instead of 251P), probably due to PCR jumping during the cloning of this cDNA. Thus, 251P was ignored in the initial predictions. *In vitro* mutagenesis indicated that 251P, located in the loop between the B and C beta strands of the Ig domain, is the key amino acid involved in the interaction with MAb4C10 (**Paper 2**). The H chain of MAb4C10 was characterized by molecular cloning of cDNA from MAb4C10 hybridoma cells, whereas both H and L chains were partially determined by orbitrap MS analysis (**Appendix**).Unfortunately, the functional L chain cDNAwas not obtained in the course of the present project and further three dimensional (3D) structure prediction and docking analysis must await further studies in the future.

The importance of characterizing MAb4C10 is obvious regarding studies of IgM sub-variants in Atlantic salmon and brown trout. Most monoclonal antibodies raised against native protein react with both IgM-A and IgM-B (and cross-react with other salmonid species) due to high similarity between the primary structures of µ sub-variants. Monoclonal antibodies that recognize exclusively one sub-variant are rare and valuable. MAb4C10 has been used by many research groups and the report describing the development of this antibody (Thuvander et al., 1990b) has been cited in more than 108 research reports (ISI Web of knowledge). Some of these studies have used MAb4C10 to identify lymphocytes in Atlantic salmon without knowing that it only recognizes the IgM-A expressing lymphocytes. This fact underline the importance of critically evaluating specific antibodies and other tools used in research.

Although MAb4C10 is a valuable tool for further dissection of the immune system in salmonid fish, the usage of this antibody is not straight forward for all applications. In Western blots the conditions were easy to optimize, providing distinct signals. Immunostaining of µ3 transfected cells also gave clear-cut answers (**Paper 1 and Paper 2**). Using MAb4C10 for immunostaining of salmon lymphocytes was more challenging, with relatively weak specific staining and often with a diffuse background staining. Double staining with MAb4C10 and a polyclonal antibody against Atlantic salmon IgM was attempted in the course of the present project, but needs further optimization to be a routine procedure (Fig 8). However, flow cytometry analysis of Atlantic salmon leukocytes was successfully performed with MAb4C10 by another research group recently (Hedfors et al., 2012).
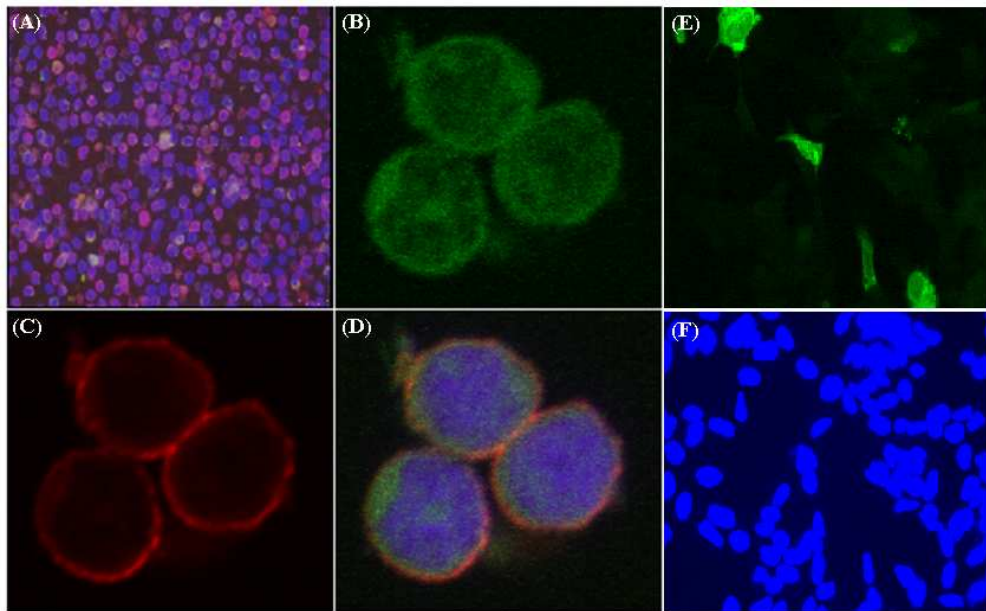
Figure 8. Immunolabeling of B-lymphocytes expressing IgM from Atlantic salmon and SHSY5Y cells transfected with µA3 construct. *Lymphocytes from Atlantic salmon expressing IgM were stained with both polyclonal and monoclonal antibodies against IgM (A-D). The double stained lymphocytes were imaged by confocal microscope for monoclonal staining (B), polyclonal staining (C) and merged (D), whereas the florescence microscope image is shown as merged of monoclonal and polyclonal staining. The human neroblastoma cell lines (SHSY5Y), transfected with µA3 construct of Atlantic salmon were stained with MAB4C10 (E) and nuclei were stained with DAPI (F).*

Whereas weak signal is a common drawback associated with monoclonal antibodies, possible cross-reaction with non-IgM molecules is a disadvantage when using polyclonal antibodies against IgM. It is plausible to assume that carbohydrate structures shared by different receptors interfere, and epitopes on the Ig folds of IgM might resemble other members of the super-immunoglobulin family, causing cross-reaction. To overcome problems associated with weak signals of monoclonals, mixtures of two or more monoclonal antibodies can be used. Due to IgM heterogeneity in salmonid fish, the composition of these mixtures must be carefully evaluated. The present study has provided new insight into the heavy chain of salmonid IgM molecules, founding a basis for evaluating specific antibodies against the heavy chain. Regarding the Ig light chains in salmonid fish the situation is probably more complex.

SDS-PAGE and 2D gel electrophoresis of IgM from salmonid fish has shown that samples vary considerably with respect to the molecular weight of the light chain (Sanchez and Dominguez, 1991b; Sanchez et al., 1993b), and molecular cloning of cDNA has revealed extensive diversity of light chain sequences (Lobb et al., 1984; Sanchez and Dominguez, 1991b; Solem and Jorgensen, 2002; Tomana et al., 2002; van der Heijden et al., 1995).

As previously shown by translation of cDNA sequences, and in this thesis by MS analysis of purified IgM subunits, the only characteristic residue of µB is an extra cysteine in µ4, the most conserved constant domain of IgM. The corresponding residue in rainbow trout has been substituted to an R and in char µA-1 to an N (**Paper 2**). Non-reducing SDS-PAGE analysis of native IgM samples showed a major band of 800 KDa, corresponding to a tetramer, for both IgM-B and IgM-A, whereas IgM-A showed additional bands presumably corresponding to trimers (600 KDa), dimers (400 KDa) and monomers (200 KDa), i.e. a less tightly connected polymer structure of IgM-A, like in several other species of teleosts (Evans et al., 1998; Kaattari et al., 1998; Lobb and Clem, 1981; Warr, 1983). Several experiments were conducted, using different denaturing buffers and conditions. Consistently, IgM-B showed a higher degree of stability under extreme heating conditions in comparison to IgM-A (results not shown). The fact that IgM-B exhibits a higher degree of disulfide bonding raises questions regarding their half life time and if IgM-B dominate more during secondary antibody responses. In the present study we observed atypical AEC profiles of IgM purified from vaccinated fish which could indicate that the abundance of IgM-B might be higher than normally observed. However, verification of this requires a more comprehensive and systematic approach.

It is reasonable to believe that evolution eventually lead to a single IgH locus (by complete/partial degeneration and deletion/fusion events) after a WGD event (as occured after the FSGD). Thus, the situation with two paralogous IgH loci in several species of salmonid fish is probably an intermediate situation in evolutionary terms. As discussed in **Paper 2**, a partial somewhat more diverged µ sequence of grayling *(Thymallus thymallus)* could indicate that this species is in the process of re-establishing a one-locus organization. It might be questioned if Atlantic salmon and brown trout have some advantage of having IgM-A and IgM-B subpopulations today. The IgM-B isotype appears to be more stable. However, if the IgM-B isotype was generally favorable compared to IgM-A it should be expected to dominate

more. The two IgH loci might complement each other to some degree, e.g. by broadening the repertoire of VH specificities. However, if this is true, the IgM-A subpopulation should also be expected to increase in response to certain antigens. The present study indicates that both loci are relatively uniformly expressed throughout the different life stages of the fish (**Paper 3**). Gene expression is presumably regulated by allotypic/isotypic exclusion leading to a continuous production and balanced ratio of μA and μB mRNA. A skewed ratio of IgM-A/IgM-B, as indicated by some AEC profiles, could be a result of IgM-B accumulation due to a longer half life time associated with a more stable structure. Alternatively, it could be a result of clonal expansion of lymphocytes producing this subtype, but this topic needs further investigation. Unfortunately, PCR approaches were not precise enough to monitor the exact ratio of μA/μB transcripts in the course of the present study.

Future Perspectives

A complete salmon genome sequence, which will be available soon, represents an important step forward for research on this species. Accordingly, as a result of the application of new sequencing technology, a steadily increasing amount of data on corresponding gene transcripts is generated, representing a useful resource regarding possible differential expression of paralogous genes, for example.

Although high throughput technology is of essential importance there is still a need to develop tools to identify subpopulations of cells and individual components of the immune system. In human, studies of leukocyte surface molecules and development of specific antibodies against CD (Clusters of Differentiation) markers is organized through HCDM (Human Cell Differentiation Molecules), representing a crucial driving force in immunology research. Till now, very few antibodies against immune components in fish are commercially available (most are against IgM of different fish species), but initiatives have been done to stimulate a more coordinated effort to establish a broader set of tools to study the immune system in fish and other animals, e.g. (http://www.umass.edu/vetimm/catfish/index.htm).

Implementation of proteomic tools might be the key to identify markers that can be used for monitoring immune responses during challenge experiments and vaccine development. Further elucidation of the lymphoid tissue in the gills of salmon will be of great interest, both with regard to further understanding of the evolution of adaptive immune responses and more practical issues related to alternative vaccine composition and delivery strategies.

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Appendix

Characterization of MAb4C10 heavy and light chain

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1. Introduction

MAb4C10 was originally raised against affinity purified rainbow trout *(Onchorhynchus mykiss)* IgM, and was shown to cross-react with IgM from Atlantic salmon *(Salmo salar)* and brown trout *(Salmo trutta)* (Thuvander et al., 1990). Later, it has been shown that MAb4C10 distinguishes between IgM subpopulations in Atlantic salmon and brown trout, i.e. it reacts exclusively with IgM-A in Atlantic salmon and IgM-B in brown trout. This was shown by different approaches, by direct affinity purification of IgM (utilizing magnetic beads coated with MAb4C10) followed by mass spectrometry analysis, and by immunomagnetic purification of lymphocytes followed by molecular genetic analysis (Kamil et al., 2011).

A proline residue in salmon µA3 was found to be essential for the reactivity withMAb4C10, and reactivity with salmon µB3 could be restored by *in vitro* mutagenesis, changing a threonine to proline in the actual position. MAb4C10 also reacted with arctic char *(Salvelinus alpinus)* IgM which has a proline in this position (Kamil et al., 2013). Mab4C10 reacts in Western blots and against deglycosylated protein (Kamil et al., 2011).

2. Materials and Methods

2.1 Isolation of RNA and synthesis of cDNA

RNA was isolated by use of Trizol Reagent (Life Technologies, USA). First strand cDNA was synthesized by use of MMLV reverse transcriptase (Promega, Madison, USA) and an oligo-dT primer.

2.2 Polymerase chain reaction (PCR)

PCR was performed with Accuprime (Invitrogen). Following profile was repeated 25-40 cycles: 94 ºC, 30 sec, 55 ºC, 30 sec, and 72 ºC, approximately 1 min per kb of the expected length of the PCR-product.

2.3 Sequencing and analysis of DNA

DNA sequencing was performed by use of BigDye Sequencing kit (Amersham Life Science, Cleveland, USA). DNA and peptide sequences were analyzed with BLAST (www.ncbi.nih.nlm.gov) and CLUSTAL (www.ebi.ac.uk/services).

2.4 Mass Spectrometry protein analysis

The pure sample of MAb4C10 was separated by 4–10 % SDS-PAGE. The protein bands corresponding in mass to the heavy and light chains of MAb4C10 were excised from the gel, and the proteins in the gel piece were reduced/alkylated and digested by trypsin as described elsewhere (http://www.uib.no/filearchive/ingel-proteindigestion.pdf).The peptide sample was loaded and desalted on a pre-column (Acclaim PepMap 100, 2cm x 75µm i.d. nanoViper column, packed with 3µm C18 beads) at a flow rate of 5µl/min for 6 min using an isocratic flow of 0.1% FA (formic acid, vol/vol) with 2% ACN (acetonitrile, vol/vol). Peptide separation and elution were accomplished on an analytical column (Acclaim PepMap 100, 15 cm x 75µm i.d. nanoViper column, packed with 2µm C18 beads) using a biphasic ACN gradient from two nanoflow UPLC pumps (flow rate of 280 nl /min). Solvent A and B was 0.1% FA (vol/vol) with 2% ACN or 90% ACN (vol/vol) respectively. The gradient composition was 8-40%B over 30.5 min, then 40-90%B over 3 min. Elution of very hydrophobic peptides and conditioning of the column were performed during 5 minutes isocratic elution with 90%B and 12 minutes isocratic elution with 5%B respectively. The eluting peptides were ionized in the electrospray and analyzed by the LTQ-Orbitrap Velos Pro. The mass spectrometer was operated in the DDA-mode (data-dependent-acquisition) to automatically switch between full scan MS and MS/MS acquisition. Instrument control was through Tune 2.7 and Xcalibur 2.2. Survey full scan MS spectra (from 300 to 2000 m/z) were acquired for 60 min in the Orbitrap with a resolution R = 60000 at 400 m/z (after accumulation to a target value of 1E6 in the linear ion trap with maximum allowed ion accumulation time of 500 ms). The 7 most intense eluting peptides above ion threshold value of 1000 counts and charge states 2 or higher, were sequentially isolated in a back-to-back analysis of same-precursors using two different fragmentation techniques, (i) CID (Collision-Induced Dissociation) and (ii) HCD (Higher-Energy Collision Dissosiation). The slower HCD fragmentation gives better resolution and mass accuracy, and enables DeNovo sequencing for peptides not found in protein databases. Thereafter procedure (i) or (ii) was implemented. (i) Ions were isolated to a target value of 1E4 at a maximum ion accumulation time of 200 ms, and fragmented in the high-pressure linear ion trap by low-energy CID with normalized

collision energy of 35% and wideband-activation enabled. The maximum allowed accumulation time for CID was 200 ms, isolation width maintained at 2 Da, activation q = 0.25, and activation time of 10 ms. The resulting fragment ions were scanned out in the low-pressure ion trap at normal scan rate, and recorded with the secondary electron multipliers. (ii) Ions were isolated in the high-pressure linear ion trap to a target value of 5e5 at a maximum allowed accumulation time of 1000 ms, and isolation width maintained at 3 Da. Fragmentation in the HCD cell was performed with a normalized collision energy of 40%, and activation time of 0.1 ms. Fragments were detected in the Orbitrap at a resolution of 7500 with first mass fixed at m/z 160. Two MS/MS spectra of a precursor mass were allowed before dynamic exclusion for 10 s. Lock-mass internal calibration was not enabled.

3. Results

3.1 Molecular cloning of Ig heavy chain cDNA from MAb4C10 producing cells

IgG1 cDNA from MAb4C10 was amplified by use of one mixed sense primer derived from the signal peptide (Larrick and Fry, 1991) and one antisense primer from the constant region (IgGa1 or IgGa2, respectively, Table 1). Cloning and sequencing confirmed that MAb4C10 was of IgG1 isotype and the variable region of the heavy chain was revealed (Fig 1).

3.2 Mass spectrometry analysis of MAb4C10 heavy chain

MS analysis confirmed that the heavy chain of MAb4C10 was of IgG1 isotype (>64% coverage), and matches with the variable region was in accordance with the translated cDNA sequence (EVQLQQSGPELVKPGASVK and ATLTVDKSSSTAYMQLNSLTSEDSAVYYCAR, respectively).

3.3 Molecular cloning of Ig light chain cDNA from MAb4C10 producing cells

Ig light chain cDNA of MAb4C10 was amplified by use of the primers VK1 and CK, from the variable and constant region of kappa light chain cDNA (Table 1). BLAST searches in GenBank, using the amplified fragment as query showed that the amplified fragment was produced from cDNA of a processed pseudo-IgK chain mRNA, previously reported from several hybridoma cell lines (e.g. acc.nos. U5641, L02345, M35669, X05184, JF412705, JF412706, FN422002, FJ233898).

3.4 Mass spectrometry analysis of MAb4C10 light chain

MS analysis showed that the light chain of MAb4C10 is of kappa isotype (>63% coverage).
Matches with the variable region are listed in Table 2.

Acknowledgement

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Table 1 Primers used for amplification of MAb4C10 heavy and light chain cDNA

Primer name	sequence	reference
Signal-P2	ATGRAATGSASCTGGGTYWTYCTCT	Larrick and Fry, 1991
IgG1a2	CCAGGGGCCAGTGGATAGAC	Acc.no. HM627495
IgG2a1	GAGGTCAGACTGCAGGACAGC	Acc.no. HM627495
CK1	ACTGGATGGTGGGAAGATGG	Eswarakumaret al., 1997
VK1	ATGGAGACAGACACACTCCTGCTAT	Eswarakumaret al., 1997

R=A+G, S=G+C, Y=C+T, W=A+T

Table 2. MS analysis of MAb4C10

Acc.no	Peptide matches with IgK variable region
P01631	DVVMTQTPLSLPVSLGDQASISCR
P01631	VSNRFSGVPDRFSGSGSGTDFTLK
P01636	ASENIYSYLAWYQQK
P01636	FSGSGSGTQFSLK

Figure 1. Mab4C10 heavy chain cDNA. The IgG1 constant part is indicated in yellow.

```
ctcttgtcaggaactgcaggtgtccactctgaggtccagctgcaacagtctggacctgag
 L  L  S  G  T  A  G  V  H  S  E  V  Q  L  Q  Q  S  G  P  E
ctggtgaagcctggggcttcagtgaagatgtcctgtaaggcttctggatacacattcact
 L  V  K  P  G  A  S  V  K  M  S  C  K  A  S  G  Y  T  F  T
gacttctacatggactgggtgaagcagagccatggagaaagctttgagtggattggacgt
 D  F  Y  M  D  W  V  K  Q  S  H  G  E  S  F  E  W  I  G  R
gttaatccttacaatggtggtactggctacaaccagaagttcaagggcaaggccacattg
 V  N  P  Y  N  G  G  T  G  Y  N  Q  K  F  K  G  K  A  T  L
actgttgacaagtcctccagcacagcctacatggagctcaacagcctgacatctgaggac
 T  V  D  K  S  S  S  T  A  Y  M  E  L  N  S  L  T  S  E  D
tctgcggtctattactgtgcaagatggttactcccttatgctatggactactggggtcaa
 S  A  V  Y  Y  C  A  R  W  L  L  P  Y  A  M  D  Y  W  G  Q
ggaacctcagtcaccgtctcctcagccaaaacgacacccccatctgtctatccactggcc
 G  T  S  V  T  V  S  S  A  K  T  T  P  P  S  V  Y  P  L  A
cctggatctgctgcccaaactaactccatggtgaccctgggatgcctggtcaagggctat
 P  G  S  A  A  Q  T  N  S  M  V  T  L  G  C  L  V  K  G  Y
ttccctgagccagtgacagtgacctggaactctggatccctgtccagcggtgtgcacacc
 F  P  E  P  V  T  V  T  W  N  S  G  S  L  S  S  G  V  H  T
ttccca
 F  P
```

Figure 2. Mab4C10 pseudo-IgK chain cDNA.

```
gtactgctgctctggggttccaggttccactggtgacattgtgctgacacagtctcctgct
 V  L  L  L  W  V  P  G  S  T  G  D  I  V  L  T  Q  S  P  A
tccttagctgtatctctggggcagagggccaccatctcatacagggccagcaaaagtgtc
 S  L  A  V  S  L  G  Q  R  A  T  I  S  Y  R  A  S  K  S  V
agtacatctggctatagttatatgcactggaaccaacagaaaccaggacagccacccaga
 S  T  S  G  Y  S  Y  M  H  W  N  Q  Q  K  P  G  Q  P  P  R
ctcctcatctatcttgtatccaacctagaatctggggtccctgccaggttcagtggcagt
 L  L  I  Y  L  V  S  N  L  E  S  G  V  P  A  R  F  S  G  S
gggtctgggacagacttcaccctcaacatccatcctgtggaggaggaggatgctgcaacc
 G  S  G  T  D  F  T  L  N  I  H  P  V  E  E  E  D  A  A  T
tattactgtcagcacattagggagcttacacgttcggaggggggaccaagctggaaataa
 Y  Y  C  Q  H  I  R  E  L  T  R  S  E  G  G  P  S  W  K  -
aacgggctgatgctgcaccaactgtat
 N  G  L  M  L  H  Q  L  Y
```

Papers

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A monoclonal antibody distinguishes between two IgM heavy chain isotypes in Atlantic salmon and brown trout: Protein characterization, 3D modeling and epitope mapping

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ABSTRACT

Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*) possess two distinct subpopulations of IgM which can be separated by anion exchange chromatography. Accordingly, there are two isotypic μ genes in these species, related to ancestral tetraploidy. In the present work it was verified by mass spectrometry that IgM of peak 1 (subpopulation 1) have heavy chains previously designated as μB type whereas IgM of peak 2 (subpopulation 2) have heavy chains of μA type. Two adjacent cysteine residues are present near the C-terminal part of μB, in contrast to one cysteine residue in μA. Salmon IgM of both peak 1 and peak 2 contain light chains of the two most common isotypes: IgL1 and IgL3. In contrast to salmon and brown trout, IgM of rainbow trout (*Oncorhynchus mykiss*) is eluted in a single peak when subjected to anion exchange chromatography. Surprisingly, a monoclonal antibody MAb4C10 against rainbow trout IgM, reacted with μA in salmon, whereas in brown trout it reacted with μB. It is plausible to assume that DNA has been exchanged between the paralogous A and B loci during evolution while maintaining the two sub-variants, with and without the extra cysteine. MAb4C10 was conjugated to magnetic beads and used to separate cells, demonstrating that μ transcripts residing from captured cells were primarily of A type in salmon and B type in brown trout. An analysis of amino acid substitutions in μA and μB of salmon and brown trout indicated that the third constant domain is essential for MAb4C10 binding. This was supported by 3D modeling and was finally verified by studies of MAb4C10 reactivity with a series of recombinant μ3 constructs.

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1. Introduction

IgM is the primary systemic antibody in teleost fish. Teleost IgM is typically a tetramer (Acton et al., 1971), and each monomer consists of two identical heavy chains and two identical light chains. The heavy chain (μ) of secreted IgM consists of one variable Ig domain and four constant Ig domains (μ1, μ2, μ3 and μ4). The membrane anchored form of IgM, i.e., the B-cell receptor, is one Ig domain shorter than the secreted form as a result of a special splicing pattern in teleosts which excludes μ4 (Ross et al., 1998).

A J-chain homolog has been revealed in representatives of all vertebrates except cyclostomes and bony fish (Klimovich et al., 2008). Thus, presence of a J-chain appears to correlate with the ability to form IgM pentamers; in mammals, amphibians, reptiles and cartilaginous fishes.

Purification of serum IgM from salmonid fish is usually performed by a combination of anion exchange chromatography and gel filtration, or by affinity chromatography employing specific antibodies against the IgM of interest (Kobayashi et al., 1982; Haavarstein et al., 1988; Fuda et al., 1991; Sanchez et al., 1993, 1995; Magnadóttir et al., 1996, 1997). Only a small proportion of

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rainbow trout IgM was found to bind to Staphylococcal protein-A (Estevez et al., 1993). In addition to being the major antibody in serum, IgM has also been detected in skin mucus and eggs of salmonid fish (Hatten et al., 2001; Olsen and Press, 1997).

Early studies in our laboratory showed that IgM of Atlantic salmon (*Salmo salar*) can be separated into two distinct subpopulations by anion exchange chromatography (Haavarstein et al., 1988). Accordingly, two distinct types of cDNAs were isolated and shown to represent isotypic genes named μA and μB (Hordvik et al., 1992, 1997). A comparative study of brown trout (*Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*) and Arctic char (*Salvelinus alpinus*) showed that only IgM of brown trout was separated into two peaks by anion exchange chromatography, like IgM of salmon (Hordvik et al., 2002).

As in most teleost fishes the Ig heavy chain gene complex in Atlantic salmon encodes three main types of heavy chains: μ, δ and τ, corresponding to the classes IgM, IgD and IgT (Hordvik et al., 1992, 1997, 1999; Tadiso et al., 2011; Yasuike et al., 2010). Atlantic salmon belong to the family *Salmonidae*. Due to ancestral tetraploidy, members of this family of fishes very often possess two similar sub-variants of proteins encoded by paralogous loci. The general view is that salmonid fish descend from a tetraploid ancestor and that members of this fish family are still going through a diploidisation process (Allendorf and Thorgaard, 1984). It has been suggested that the genera *Salmo*, *Oncorhynchus* and *Salvelinus* radiated 12–16 million years ago (Andersson et al., 1995) and that the tetraploid event occurred about 25–100 million years ago (Allendorf and Thorgaard, 1984). As a result of ancestral tetraploidy there are two Ig heavy chain gene complexes, A and B, in Atlantic salmon, encoding highly similar sub-variants of IgM, IgD and IgT (Hordvik, 1998, 2002; Solem et al., 2001; Tadiso et al., 2011; Yasuike et al., 2010).

Like in Atlantic salmon, two μ isotypes in brown trout were designated as A and B type, respectively (Hordvik et al., 2002). Since IgM subpopulations of salmon and brown trout showed highly similar elution profiles from anion exchange chromatography we expected that they had similar pI features. Somewhat unexpected, the IgM heavy chains in brown trout differed by only 0.14 pI units (theoretically), while in Atlantic salmon the difference was 0.67. Isoelectric focusing of IgM from Atlantic salmon and brown trout was in agreement with the theoretical values (Hordvik et al., 2002). Only one common residue is characteristic for the B type in brown trout and Atlantic salmon; this is an extra cysteine residue near the C-terminal part of the heavy chain (Hordvik et al., 2002). Atlantic salmon possess at least three isotypes of immunoglobulin light chains (IgL). The most abundant transcripts encode IgL1 and IgL3, respectively (Solem and Jorgensen, 2002).

A molecule homologous to the polymeric immunoglobulin receptor (pIgR) is present in teleost fish, and can be bound to mucosal IgM (Feng et al., 2009; Hamuro et al., 2007; Rombout et al., 2008). Characterization of a pIgR homolog in salmon is in progress (Tadiso and Hordvik, unpublished data). In mammals, the pIgR has a fundamental role in the transport of IgA (and IgM) across the epithelial cell layer into the mucus. A part of the pIgR (secretory component) is bound to the antibody and protects it from degradation in the hostile mucosal milieu. A pIgR homolog in rainbow trout was found to be associated with polymeric IgT in gut mucus, and the concentrations of gut IgT were double those in serum, indicating that this Ig class is specialized in mucosal immunity (Zhang et al., 2010).

The aim of the present study was to characterize IgM subpopulations in Atlantic salmon and brown trout in more detail. A monoclonal antibody MAb4C10, originally raised against rainbow trout IgM (Thuvander et al., 1990) showed to be useful as it reacted exclusively with μA in salmon and exclusively with μB in brown trout. MAb4C10 has been applied for various purposes by several research groups and has been used in at least 55 of 100 studies referring to Thuvander et al. (1990).

2. Materials and methods

2.1. Fish

Atlantic salmon were obtained from The Industrial and Aquatic Laboratory at the High Technology Center in Bergen. Rainbow trout were provided from the marine research station at Matre (Institute of Marine Research). Brown trout were caught in a mountain lake near Bergen (Bergsdalen).

2.2. Purification of IgM from Atlantic salmon, brown trout and rainbow trout

IgM from serum were purified essentially as described in Haavarstein et al. (1988). Salmon IgM was first partly purified by gel filtration (Superdex 200 16 60). The IgM rich low-through fraction was loaded onto an anion exchanger (Mono Q) and IgM was subsequently separated into two separate peaks.

2.3. Monoclonal antibody against rainbow trout IgM

MAb4C10: a mouse IgG1 antibody against rainbow trout IgM has been described previously (Thuvander et al., 1990). In the present study, supernatant was used if not otherwise stated. ProteinG-purified MAb4C10 was applied in some experiments.

2.4. Immunomagnetic purification of salmon IgM

IgM was purified from gel filtrate fractions of Atlantic salmon serum using Dynabeads® M-450 Epoxy coated with MAb4C10 according to the provided manual (Invitrogen).

2.5. Precipitation and up-concentration of protein samples

Protein samples were precipitated with $3\times$ vol ice cold acetone over night at $-20\,^{\circ}$C and centrifuged at $15,000 \times g$ at $4\,^{\circ}$C for 20 min to pellet the proteins. Acetone was removed and pellets were air-dried and re-suspended in $1\times$ SDS sample buffer. Protein samples were up-concentrated with Amicon® Ultra-15 10,000 MWCO centrifugal filter devices (Millipore).

2.6. Protein deglycosylation

Approximately 3 μg protein was dissolved in 15 μl of denaturation solution (5% SDS with 10% 2-mercaptoethanol) and heated at $100\,^{\circ}$C for 5 min. After cooling, 1.5 μl of $10\times$ PNGase F reaction buffer was added (500 mM ammonium bicarbonate with 10% NP-40). Deglycosylation was performed with 1 unit PNGase F (Sigma–Aldrich) per 2 μg of protein sample at $37\,^{\circ}$C overnight.

2.7. SDS-PAGE, Western blot and immunodetection

SDS-PAGE was performed according to the method described by Laemmli (1970). Protein samples mixed with $1\times$ SDS loading buffer were boiled for 5 min at $95\,^{\circ}$C before loading on the polyacrylamide gel (4% stacking gel and 12.5% separating gel). The gel electrophoresis was carried out at 180 V for approximately 1 h. The gel was either preceded for Coomassie Brilliant Blue R-250 (Sigma) staining and de-staining, or Western blotting; 100 V for 1 h at $4\,^{\circ}$C (BioRad system and Amersham Hybond™-P PVDF Membrane). After electro-blotting, the PVDF membrane was blocked at room temperature for 1 h in 5% dry milk in $1\times$ PBST, and incubated overnight with 1:50 dilution of MAb4C10 at

4 °C. The next day the membrane was washed four times with $1\times$ PBST, each for 5 min at room temperature on a rocker, and was incubated for 1 h with HRP-conjugated anti-mouse IgG in 1:3000 dilution at room temperature. The membrane was washed again four times with $1\times$ PBST each for 5 min at room temperature and developed using ECL reagents as described by the manufacturer (ECL Plus Western Blot Detection, GE Healthcare Life Sciences).

2.8. Mass spectrometry protein analysis

The samples enriched in IgM after anion exchange chromatography were acetone precipitated and solubilised in $1\times$ SDS loading buffer, boiled for 5 min at 95 °C, and loaded and separated on a Nu PAGE 4–12% Bis Tris gel. The protein bands corresponding in mass to the heavy and light IgM chains were excised from the gel, and the proteins in the gel piece were reduced/alkylated and digested by trypsin as described elsewhere (http://www.uib.no/filearchive/in-gel-proteindigestion.pdf). The resulting peptides extracted from the gel piece were dissolved in 0.1% FA, and injected into a nano-HPLC system. The settings for the LC separation were: trap column: 2% ACN, 0.1% FA with a flow rate of 25 μl/min. Analytical column: the analytical column was a fused-silica capillary column (15 cm long, 75 μm i.d.) packed with Reprosil–Pur 3 μm C18 resin (Dr. Maisch, Ammerbuch-Entringen, Germany). Solvent A was 0.1% FA and Solvent B was 90% ACN, 0.1% FA. The flow rate was 0.300 μl/min with the following gradient: 5–10% Solvent B in 2 min, 10–40% Solvent B in 43 min, 40–95% Solvent B in 1 min, 95% Solvent B was kept constant for 5 min, 95–5% Solvent B in 3 min, and regeneration of the column in 21 min. The nano-HPLC system (Dionex, Ultimate, Sunnyvale, CA, USA) was coupled online to an Ultima Global ESI-Q-TOF mass spectrometer (Waters, Wilford, MA, USA), and the peptides were analyzed by the mass spectrometer during continuous elution from the analytical column. The scan area for the MS survey scan was m/z 300–1500 with automatic fragmentation of the three ions with highest intensity. All the data was acquired in data dependent mode. The resulting data was searched against the NCBIn database using Mascot. Taxonomy chosen for the search was Metazoa (animals), with carbamidomethylation of cysteine as fixed modification and oxidation of methionine as variable modification.

2.9. Isolation of lymphocytes

The fish were killed by a sharp blow to the head. Blood were immediately collected using a syringe with 5 ml vacutainer tubes (containing heparin) and kept on ice for a maximum of three hours before further use. The amount of blood isolated from each fish varied from 2 to 8 ml depending on the fish size and the success of the blood collecting. One ml of blood was mixed with 3 ml Balanced Salt Solution (Solution A: Anhydrous D-glucose; 0.1%, $CaCl_2 \times 2H_2O$; 5.0×10^{-5} M, $MgCl_2 \times 6H_2O$; 9.8×10^{-4} M, KCl; $5,4 \times 10^{-3}$ M, Tris; 0.145 M, Solution B: NaCl; 0.14 M). 3 ml Ficoll-Paque Plus (Amersham Biosciences) were added to 10 ml centrifuge tubes. The diluted blood was carefully layered on top of the Ficoll-Paque Plus. Centrifugation was carried out in a temperature range between 8 and 15 °C for 40 min at $400 \times g$. After centrifugation the plasma layer was drawn off before collecting the band of lymphocytes. Lymphocytes from the same fish but from different tubes were pooled and washed twice in 3 volumes of Balanced Salt Solution with centrifugation at $100 \times g$ for 20 min to collect the cells in between each wash. The cells were finally suspended in 500 μl of PBS/0.1% BSA.

2.10. Immunomagnetic separation of cells

Dynabeads M-450 Goat anti-Mouse IgG (Dynal) were utilized for separation of cells using the direct technique by pre-coating the Dynabeads with MAb4C10. 1×10^7 Dynabeads M-450 were washed twice in PBS/0.1% BSA and re-suspended in 500 μl PBS/0.1% BSA before adding 1.5 μg of rinsed MAb4C10. The mixture was incubated by gentle tilting and rotation for 60 min at room temperature. The coated Dynabeads were washed $4\times$ in PBS/0.1% BSA utilizing a magnetic particle concentrator and finally suspended in PBS/0.1% BSA. Cells suspended in PBS/0.1% BSA were mixed with the pre-coated Dynabeads in a total volume of 1 ml and subsequently incubated at 4 °C on a rotor for 20 min. Estimation of number of cells was done utilizing a Brinkmann cell chamber. The ratios of Dynabeads/cells varied (between 1:9 and 3:1). After the incubation of the Dynabeads/Cell mix the Dynabeads were washed $5\times$ in PBS/0.1% BSA.

2.11. Scanning electron microscopy (SEM)

Leukocytes fixed with Karnowsky were washed in 0.2 M sodium phosphate buffer, followed by 1 h fixation in 1% aqueous solution of osmium tetroxide (OsO_4). The cells were washed in PBS and dehydrated with cold acetone; (1) 60% acetone, (2) 90% acetone, and (3) 100% acetone. The cells were finally attached to an object glass and coated with gold–palladium (Polaron SC502 Sputter Coater, Fison Instruments). The cells were examined by scanning electron microscopy (ZEISS Supra 55VP).

2.12. Isolation of RNA and synthesis of cDNA

RNA was isolated using Trizol Reagent (Life Technologies, USA). First strand cDNA was synthesized by oligo-dT priming on total RNA with MMLV reverse transcriptase (Promega, Madison, USA).

2.13. Polymerase chain reaction (PCR)

Chemicals and *Taq* polymerase for PCR were purchased from Pharmacia. Following profile was used: 94 °C, 30 s, 55 °C, 30 s, 72 °C, 1 min, 35 cycles, linked to 72 °C, 10 min.

2.14. Relative abundance of μA and μB mRNA in cells captured by MAb4C10/Dynabeads

cDNA descending from the cell fraction captured by the MAb4C10/Dynabeads as well as cDNA descending from cells that were not captured by the MAb4C10/Dynabeads, were used as template in PCR with primers J-sense (TTTGACTACTGGGGGAAAGG) and μ3-antisense (CCCATTGCTCCAGTCCTCAT). A characteristic *Eco*RI site in salmon μA1, which is lacking in μB1 cDNA, was employed to decide whether the cells captured by the MAb4C10/Dynabeads had transcripts for either μA or μB. A characteristic *Sau*3A site in brown trout μB3, which is lacking in μA3, was employed to decide whether cells captured by MAb4C10/Dynabeads had transcripts for either μA or μB. PCR-products were purified by the use of QIAquick spin columns (Qiagen) before being subjected to restriction digestion. Selected PCR products were cloned into TA-vector (Invitrogen).

2.15. Construction of μ3 expression plasmids

DNA-fragments encoding salmon μA3, salmon μB3, brown trout μA3, brown trout μB3 and rainbow trout μ3 were generated

```
μ1
Consensus  ASSTAPTLFPLAQCGSGTGDMMTLGCIATGFTPASLTFKWNEEGGNSLTDFVQYPAVQTGGSYMGVSQLRVKRADWDSKxFECAVEHSAGSKxVPVKKQ
Salmon A   ---------------------------------------------Q--------------S-----------------I------------T------
Salmon B   -------------------V-------------------------Q--------------S-----------------I------------T-L--
Trout A    ---------------------------------------------Q--------V------T-----------------I------------T------
Trout B    ---------------------------------------------Q--------------T-----------------I------------T------
Rainbow    ---------------------------------------------D----------K---------------------K----K

μ2
Consensus  xEYLQHPSLYVMTPSKEEMAENKTASFACFANDFSPRTHTIKWMRMEKGTExEVVSDFKSSCESEKKSEKTLYSTTSYLRVNESEWKSEEVTFTCVFENKAGNVRRTVGYTSSD
Salmon A   A----------------------M----------------Q-I-K------------------D-----------------K-------------------
Salmon B   V---------------------------------------I-K-----------------------------------S----K-----------
Trout A    V---------------------------------------I-K-----------------------------------S---------------
Trout B    V---------------------------------------I-K-----------------------------------S---------------
Rainbow    P----Q-----------S--------------------Q------------T--------------------------------------------

μ3
Consensus  AGPVHxHSVVIxIxPPSLEDMLMNKKAELVCDVxELVPGFMSVKWENDNGKTLTSRKGVTDKIAILDITYEDWSNGTVFYCAVDHLENLGxLVKKAYKRETG
Salmon A   -----K-T------------------E------------------------R-------------------------S----P------
Salmon B   -----A---N-I----------------K---T----------------M------------------------------S----P------
Trout A    V----N-I----------------K-----------------------------------------------------T----P-----
Trout B    -----A--K-T----S---------E-----------------A--R------------------------------T----P------
Rainbow    -----G----T-IE-----------Q-----N------L-----------------------------------M---D-------

μ4
Consensus  GDPQRPSVFLLAPAEQTSDNTVTLTCYVKDFYPKDVLVAWLVDDEPVERTSSSALYQFNTTSQIQSGRTYSVYSQLTFSNDLWKNEEVVYSCVVYHESMIKSTKILMRTIDRTS
Salmon A   ---------------K------------E-------I------------T--------------K-----------
Salmon B   ---------------K-----------E-----I----------------------------K----------
Trout A    -------P-----------------E------I--L-----------------------------
Trout B    --------------------------E-----I-----------------K------------
Rainbow    -V------------------------E-----I--------------K----------------N-I--------

Consensus  NQPNLVNLSLNVPQSCKAQ
Salmon A   ---Y---------------
Salmon B   --------------C---
Trout A    -------------------
Trout B    --------------C---
Rainbow    --------------R-M--
```

Fig. 1. Alignment of μ sequences from Atlantic salmon (Hordvik et al., 1992), brown trout (Hordvik et al., 2002) and rainbow trout (Lee et al., 1993; Hansen et al., 1994) showing peptides identified by mass spectrometry. Trypsin cleavage sites are in bold and the peptides that were identified by mass spectrometry are indicated with grey.

by reverse transcription (RT)-PCR, utilizing sense primer *Eco*RI-IgMs (GGAATTCACTGTGGGCTACACTTCATCA) and reverse primer *Eco*RV-IgMa (GATATCATCATTTCACCTTGATGGCAGT). The PCR fragments were cloned into TA-vector (Invitrogen) and sequenced. Subsequently, plasmid preparations were digested with *Eco*RI and *Eco*RV and inserts were purified from agarose gel before being ligated into pcDNASp FLAG vector as described in (Koppang et al., 2010).

2.16. Transfection and immunostaining

Approximately 35% confluent SH-SY5Y cells were transfected in DMEM media (Sigma–Aldrich) with plasmids using Lipofectamine 2000 (Invitrogen) or Metafectin PRO (Biontex, Planegg, Germany) transfection reagent according to the manufacturer's protocols. After 6 h the medium was changed to DMEM containing ampicillin/streptomycin and 10% serum, and was incubated for further 48 h. When the cells were approximately 80% confluent they were fixed on cover slips by incubation for 20 min at room temperature in 2% formaldehyde in PBS, and thereafter washed three times with PBS. For permeabilization the cells were incubated with 0.2% Triton X-100 in PBS for 10 min and thereafter washed. Then the cells were blocked for 1 h at room temperature with 10% BSA in PBS. Cells were immunostained overnight at 4 °C using primary antibody mouse monoclonal anti-Flag (1:1000) or MAb4C10 (1:40) in PBS with 3% BSA. After the cells were washed, they were incubated for 1 h in dark at room temperature, with secondary antibody FITC anti-mouse (1:5000) in PBS with 3% BSA. The cells were washed again and the cover slips were mounted on an object glass with a drop of mounting solution ProLong® Gold antifade with DAPI (Invitrogen).

2.17. Sequencing and analysis of DNA

DNA sequencing was performed by use of the BigDye Sequencing kit (Amersham Life Science, Cleveland, USA). Sequences were analyzed with Vector NTI Suite (Informax, Inc.), CLUSTALW (www.ebi.ac.uk/services) and BLAST (www.ncbi.nih.nlm.gov).

2.18. Homology modeling and structural analysis

Primary sequence analysis was done using BLAST (www.ncbi.nih.nlm.gov) and CLUSTALW (www.ebi.ac.uk/services). Modeller (http://www.salilab.org/modeller/) and SWISS-MODEL (http://swissmodel.expasy.org) were used to model the 3D structures. Cysteine bridge analysis was done using WHAT-IF (http://swift.cmbi.ru.nl/servers/html/listcys.html). Visualization and presentation of the models were done using Rasmol (http://openrasmol.org/) with command-line interaction.

2.19. Bioinformatics analysis and 3D structure prediction

PDB ID 2W59 chain A with structural resolution of 1.75 angstrom was found to be a good template (ID% ∼ 25%) for modeling using BLAST and SWISS-MODEL. This template satisfied the additional constraint of possible disulfide bond between the cysteine residues in the studied protein sequence. In the modeled structures, WHAT-IF analysis showed the possibility of cysteine bridges. The Anolea and Gromos scores were in favorable negative range for most of the modeled residues with final energy ∼1900 KJ/mol. Further the Modeller showed mean DOPE score of ∼8000 and GA341 ∼0.5 for all the models. The models were further processed using RasMol 2.7.5 windows.

Peak 1 Atlantic salmon

```
AF273014-L1    ------IFIWIFALHLQESRGQVTVTQTPAVKTISVGDLVSLSCKTSSAVYSD-RHGQRLAWYQQKPGGAPELLIYLAKTLQSGIPSRFSGSGTGSD
AF273017-L1    -MTFITIFIWTLALCLQESRGQVTVTQTPAVKSVSVGNSVSLSCKTSSAVYSD-SNGHYLHWYQQKPGGAPELLIYWAKTLQSGTPSRFSGSGSGSD
AF273020-L1    -MTFIMSFVWILMSLIHESRGQVTVTQTPAVKAVLTGQTVPLNCKTSSDVYQAGTSSPRLAWYQQKPGEAPKLLIYYATTLQSGTPSRFSGSGTHSD
AF273012-L1    -MTFITITIFIWTLAFCFQESRGQITVTQTPTVKAVVSGQTVSLNCKTSSDVHAN----VYVAWYQQKPGGAPELLIYTATSLQSGTPFRFSGSGSGSD
AF406957-L3    MMMSLTLLLGTLGLLVQESSGDIILTQSPKSQSVRPGETVSISCTASSSTYNN------LQWYLQKPGEAPKLLVYSTTNRQSGIPGRFSGSGSGSS
ACI68649-L3    MMMSLILLLWTLGLLVQESSADIILTQSPKSQSVRLGETVSISCTASSSTGNN------LHWYLQKPGEAPKLLVYSTTSRQSGIPGRFSGSGSGSS
ACI70011-L3    -MTFITIFIWTLVCYTQGSWGQYVLTQS-AAQSVQPGQTVSIDCSASSKVNQYSGSRYYLAWYHQTFGEAPKLLIYYTSDRFTGVSTRFSGSGRGNG


AF273014-L1    ---FTLTISGVQAEDAGDYYCQSLHNPNSVWVFTFGSGTRLDVGSNSAPTLTVLPPSSEELSSTTTATLMCLANKGFPSDWTMSWKVDGNSKK----
AF273017-L1    ---FTLTISGVQAEDAGDYYCQSYHSG---YVYTFGSGTRLDVGSNSAPTLTVLPPSSEELSSTTTATLMCLANKGFPSDWTMSWKVDGTSKK----
AF273020-L1    ---FTLTISGVQAEDAGDYYCQSFHYPNSKYVFTFGSATRLDVGSNSAPTLTVLPPSSEELSSTTTTTLMCLANKGFPSDWTMSWKVDGNSKK----
AF273012-L1    ---FTLTISGVQAEDAGDYYCQSLHNPNSVWVYTFGSGTRLDVGSNSAPTLTILPPSSEELSSTTTTATLTCLANKGFPSDWTMSWKVDGTSKN----
AF406957-L3    YTQFTLTISGVQAEDAGDYYCQQGYS----TPYTFGGGTRLDIGSDVRPTLTVLPPSSVELQ--QGKATLMCLANKGFPSDWKLGWKVDGSSSS-TWE
ACI68649-L3    YTHYTLTISGVQAEDAGDYYCQQGNS----SPWTFGGGTKLSVGSDVRPTLTVLPPSSVELQ--QGKATLMCLANKGFPSDWKLSWKVDGSSSSNTWE
ACI70011-L3    -IDFTLTISKVQAEDTGVYYCQSYHSG---TVLTFGGGTKLSVGSDVRPTMTVLPPSSVELQ--QEKATLMCLANKGFPSDWKLSWKVDGSSSS-TWE


AF273014-L1    QEASPGVLEKDGLYSWSSTLTLTAQEWTKAGEVTCEAQQISQTPVTKTLRRADCSG 236
AF273017-L1    QEASPGVLEKDGLYSWSSTLTLTAQEWTKAGEVTCEAQQISQTPVTKTLRRADCSG 238
AF273020-L1    QEASPGVLEKDGLYSWSSTLTLTAQEWTKAGEVTCEAQQISQTPVTKTLRRADCSG 242
AF273012-L1    QKTSPGVLEKDRLYSWSSTLTLTGQEWTKAGEVTCEAQQNSQTPVTKTLRRADCSG 238
AF406957-L3    VTGSPGVLEKDGHYSWSSTLTFFPVDQWKKVGSVVCEATQGSQSPLSETLRRDQCSD 238
ACI68649-L3    VTGSPGFQEKDGHYSWSSTLTLPVDQWKKVGSVVCEATQGSQSPLSETLRRDQCSD 239
ACI70011-L3    VTGSPGVLEKDGHYSWSSTLTLPVDQWRKVGSVTCEATQGTQTPLSETLRRDQCSD 242
```

Peak 2 Atlantic salmon

```
AF273020-L1    -MTFIMSFVWILMSLIHESRGQVTVTQTPAVKAVLTGQTVPLNCKTSSDVYQAGTSS-----PRLAWYQQKPGEAPKLLIYYATTLQSGTPSRFSGSGTHSD
AF273018-L1    -MTFIMSFVWILMSLIHESRGQVTVTQTPAVKAVLTGQTVPLNCKTSSDVYQAGTSS-----PRLAWYQQKPGEAPKLLIYYATTLQSGTPSRFSGSGTHSD
ACI66923-L1    -MTFIMSFVWILMSLIHESRGQATVTQTPAVKAVLTGQTVPLNCKTSSDVYQAGTSS-----PRLAWYQQKPGEAPKLLIYYATTLQSGTPSRFSGSGTHSD
AF273014-L1    ------IFIWIFALHLQESRGQVTVTQTPAVKTISVGDLVSLSCKTSSAVYSDRHG------PRLAWYQQKPGGAPELLIYLAKTLQSGTPSRFSGSGTGSD
AF273017-L1    -MTFITIFIWTLALCLQESRGQVTVTQTPAVKSVSVGNSVSLSCKTSSAVYSDSNG------HYLHWYQQKPGGAPELLIYWAKTLQSGTPSRFSGSGSGSD
ACI68406-L1    -MTSITIFIWTLALCFKDSRGQITVTQTPAVKAVLPGQAVYLTCKTSSSVFGDCHNGQSWGHQCLSWYQQKPGENPKLMMLPGNTLYSGTPSRFSGSGSGSD
AF273012-L1    -MTFITIFIWTLAFCFQESRGQITVTQTPTVKAVVSGQTVSLNCKTSSDVHAN----VYVAWYQQKPGGAPELLIYTATSLQSGTPFRFSGSGSGSD
ACI67959-L1    -MTFITIFIWTLVSCFQEARGQYVLTQTPAVKAVVPGQQVSLNCKTSSDVYND---------NCLAWYQQKPGGAPELLIYYATTLQSGTPSRFSGSGSRSD
AF406957-L3    MMMSLTLLLGTLGLLVQESSGDIILTQSPKSQSVRPGETVSISCTASSSTYNN-----------LQWYLQKPGEAPKLLVYSTTNRQSGIPGRFSGSGSGSS
ACI68649-L3    MMMSLILLLWTLGLLVQESSADIILTQSPKSQSVRLGETVSISCTASSSTGNN-----------LHWYLQKPGEAPKLLVYSTTSRQSGIPGRFSGSGSGSS
ACI70011-L3    -MTFITIFIWTLVCYTQGSWGQYVLTQS-AAQSVQPGQTVSIDCSASSKVNQYSGSR-----YYLAWYHQTFGEAPKLLIYYTSDRFTGVSTRFSGSGRGNG


AF273020-L1    ---FTLTISGVQAEDAGDYYCQSFHYPNSKYVFTFGSATRLDVGSNSAPTLTVLPPSSEELSSTTTTTLMCLANKGFPSDWTMSWKVDGNSKK----QEASP
AF273018-L1    ---FTLTISGVQAEDAGDYYCQSFHYPNSKYVYTFGSATRLDVGSNSAPTLTVLPPSSEELSSTTTATLMCLANKGFPSDWTMSWKVDGNSKK----QEASP
ACI66923-L1    ---FTLTISGVLAEDAGDYYCQSYHYINSKNVYTFGSATRLDVGSNSAPTLTVLPPSSEELSSTTTATLMCLANKGFPSDWTMSWKVDGNSKK----QEASP
AF273014-L1    ---FTLTISGVQAEDAGDYYCQSLHNPNSVWVFTFGSGTRLDVGSNSAPTLTVLPPSSEELSSTTTATLMCLANKGFPSDWTMSWKVDGNSKK----QEASP
AF273017-L1    ---FTLTISGVQAEDAGDYYCQSYHSG---YVYTFGSGTRLDVGSNSAPTLTVLPPSSEELSSTTTATLMCLANKGFPSDWTMSWKVDGTSKK----QEASP
ACI68406-L1    ---FTLTISGVQAEDAGDYYCQSWHSGN---VFTFGSATRLDVESNSAPTLTVLPPSSEELSSTTTATLMCLANKGFPSDWTMSWKVDGNSKK----QEASP
AF273012-L1    ---FTLTISGVQAEDAGDYYCQSLHNPNSVWVYTFGSGTRLDVGSNSAPTLTILPPSSEELSSTTTTATLTCLANKGFPSDWTMSWKVDGTSKN----QKTSP
ACI67959-L1    ---FTLTISRVQAEDAGDYYCQSFHPNYKYVYTFGSGTRLDVGSNSAPTLTVLPPSSEELSSTTTATLTCLANKGFPSDWTMSWKVDGTSKN----QETSP
AF406957-L3    YTQFTLTISGVQAEDAGDYYCQQGYS----TPYTFGGGTRLDIGSDVRPTLTVLPPSSVELQ--QGKATLMCLANKGFPSDWKLGWKVDGSSSS-TWEVTGSP
ACI68649-L3    YTHYTLTISGVQAEDAGDYYCQQGNS----SPWTFGGGTKLSVGSDVRPTLTVLPPSSVELQ--QGKATLMCLANKGFPSDWKLSWKVDGSSSSNTWEVTGSP
ACI70011-L3    -IDFTLTISKVQAEDTGVYYCQSYHSG---TVLTFGGGTKLSVGSDVRPTMTVLPPSSVELQ--QEKATLMCLANKGFPSDWKLSWKVDGSSSS-TWEVTGSP


AF273020-L1    GVLEKDGLYSWSSTLTLTAQEWTKAGEVTCEAQQISQTPVTKTLRRADCSG 242
AF273018-L1    GVLEKDGLYSWSSTLTLTAQEWTKAGEVTCEAQQISQTPVTKTLRRADCSG 242
ACI66923-L1    GVLEKDGLYSWSSTLTLTAQEWTKAGEVTCEAQQISQTPVTKTLRRADCSG 242
AF273014-L1    GVLEKDGLYSWSSTLTLTAQEWTKAGEVTCEAQQISQTPVTKTLRRADCSG 236
AF273017-L1    GVLEKDGLYSWSSTLTLTAQEWTKAGEVTCEAQQISQTPVTKTLRRADCSG 238
ACI68406-L1    GVLEKDGLYSWSSTLTLTAQEWTKAGEVTCEAQQISQTPVTKTLRRADCSG 244
AF273012-L1    GVLEKDRLYSWSSTLTLTGQEWTKAGEVTCEAQQNSQTPVTKTLRRADCSG 238
ACI67959-L1    GVLEKDGLYSWSSTLTLTGQEWTKAGEVTCEAQQKSQTPVTKTLRKADCSG 238
AF406957-L3    GVLEKDGHYSWSSTLTFFPVDQWKKVGSVVCEATQGSQSPLSETLRRDQCSD 238
ACI68649-L3    GFQEKDGHYSWSSTLTLPVDQWKKVGSVVCEATQGSQSPLSETLRRDQCSD 239
ACI70011-L3    GVLEKDGHYSWSSTLTLPVDQWRKVGSVTCEATQGTQTPLSETLRRDQCSD 242
```

Fig. 2. Alignment of light chain sequences identified within the Atlantic salmon IgM populations eluted in peak 1 and peak 2, respectively. Trypsin cleavage sites are in bold and the peptides that were identified are indicated with grey. GenBank accession numbers are shown in addition to which isotype family the sequence belong to. L1 and L3 are the most abundant light chain isotypes in Atlantic salmon (Solem et al., 2001).

3. Results

3.1. Mass spectrometry protein identification of heavy and light chains in IgM samples purified from Atlantic salmon, brown trout and rainbow trout

Sera were purified by gel filtration followed by anion exchange chromatography. IgM subpopulations of Atlantic salmon and brown trout were eluted in two distinct peaks, whereas IgM of rainbow trout was eluted in one peak. Protein identification by mass spectrometry confirmed that IgM of peak 1 contained heavy chains of μB type whereas IgM of peak 2 contained heavy chains of μA type; in both Atlantic salmon and brown trout, as previously hypothesized (Hordvik et al., 2002). Analysis of light chain bands from peak 1 and peak 2 of salmon showed that both fractions contained the two most common light chain isotypes; IgL1 and IgL3 (Solem et al., 2001). BLAST matches are indicated in Figs. 1 and 2.
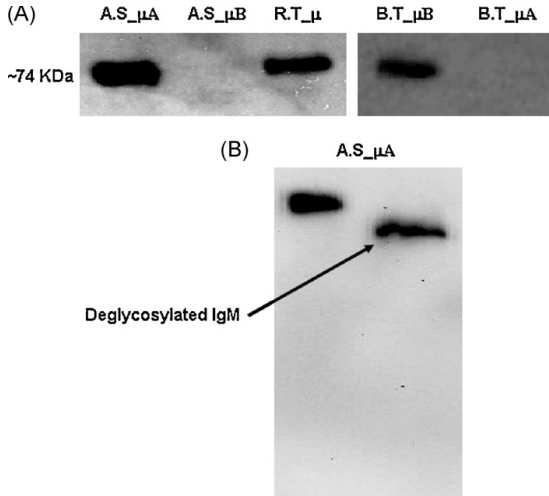
Fig. 3. Cross-reactivity between MAb4C10 and IgM of salmon, brown trout and rainbow trout. (A) Mab4C10 reacted in Western blots with rainbow trout μ, salmon μA, brown trout μB, but not with salmon μB or brown trout μA. (B) MAb4C10 reacted also with deglycosylated protein in Western blots; the reaction with salmon μA is shown.

3.2. MAb4C10 reacts with μA in Atlantic salmon whereas it reacts with μB in brown trout

MAb4C10 was found to react in Western blots with salmon μA, but not with salmon μB. In brown trout it was opposite: it reacted with μB, but not with μA. MAb4C10 also reacted with the deglycosylated polypeptide (Fig. 3). Identification of the immunopurified native salmon IgM showed that it was the IgM-A subpopulation that was captured by magnetic beads coated with MAb4C10 (results not shown).

3.3. MAb4C10 capture of lymphocytes from Atlantic salmon and brown trout

As illustrated in Fig. 4 A, MAb4C10/Dynabeads captured leukocytes with the expected size and form of B-cells. The abundance of μA versus μB transcripts in cells captured by MAb4C10/Dynabeads was analyzed by a PCR approach. An *Eco*RI restriction site in μ1 is unique for isotype μA in salmon, allowing a rough estimate of which transcripts are most abundant. *Eco*RI restriction of PCR products that originated from cDNA of cells that had been captured by the MAb4C10/Dynabeads indicated that the μA transcripts were predominant (Fig. 4B). Cloning and sequencing of PCR products were done to verify the findings. For brown trout a *Sau*3A restriction site in μ3 is unique for μB. The procedure was repeated for brown trout, showing that MAb4C10/Dynabeads captured cells had primarily transcripts for μB (results not shown).

3.4. Searching for a possible MAb4C10 binding epitope

The fact that MAb4C10 reacts with membrane bound IgM strongly indicates that the reactive epitope must be located in μ1-μ2-μ3, since μ4 is not part of the B-cell receptor. Alignment of amino acid substitutions in μA and μB of Atlantic salmon compared to μA and μB of brown trout shows that the only common and characteristic residue in μB is an extra cysteine near the C-terminal part of μ4. Interestingly, three substituted positions in μ3 were
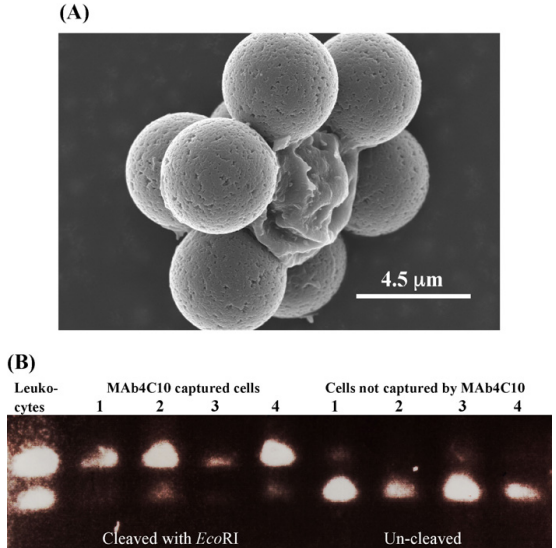
Fig. 4. Capture of lymphocytes with MAb4C10/Dynabeads, and restriction analysis of μ RT-PCR products. (A) EM picture of lymphocyte captured by MAb4C10. (B) RT-PCR products digested with *Eco*RI. Lymphocytes from four salmon individuals were captured by MAb4C10/Dynabeads. RNA was purified from captured cells and from cells that were not captured. The samples were subjected to RT-PCR, followed by restriction with *Eco*RI. Untreated leukocytes were used as a reference (left). Cleavage of the major fraction of the PCR products indicated that μA is most abundant in the captured cells whereas μB is most abundant in the cells that were not captured.

the same in salmon μA and brown trout μB, pointing to a possible region for interaction with MAb4C10 (Fig. 5). Modeling of μ3 gave further support for this hypothesis: the three residues are exposed on the surface of the Ig fold which could account for the reactivity against native IgM (Fig. 6). However, MAb4C10 reactivity in this part of μ3 is not obvious since the corresponding residues in rainbow trout are different. Still, as discussed below, μ3 was thought to be the best candidate for MAb4C10 reactivity when considering the physiochemical properties of the actual amino acids.

3.5. Experimental evidence for binding between MAb4C10 and μ3

The effect of DNA-fragments encoding salmon μA3, salmon μB3, brown trout μA3, brown trout μB3 and rainbow trout μ3 were analyzed after transfection into SH-SY5Y cells. In accordance with the hypothesis discussed in Section 3.4, MAb4C10 showed reactivity with salmon μA3, brown trout μB3 and rainbow trout μ3, but no reactivity with salmon μB3 and brown trout μA3 (Table 1). Although a subjective observation, the reactivity in transfected cells appeared to be somewhat stronger with salmon μA and brown trout μB compared to rainbow trout μ3.

Table 1
Transfection and immunostaining.

pcDNA plasmid	Reactivity against Flag Ab	Reactivity against MAb4C10
Atlantic salmon μA3	+	+
Atlantic salmon μB3	+	−
Brown trout μA3	+	−
Brown trout μB3	+	+
Rainbow trout μ3	+	+

	μA		μB	
	salmon	trout	salmon	trout
μ1				
22	M	M	V	M
56	A	V	A	A
60	S	G	S	G
64	M	T	M	T
96	V	V	L	V
μ2				
100	A	V	V	V
122	M	K	K	K
147	Q	K	K	K
168	D	E	E	E
191	A	S	S	S
197	E	E	K	E
μ3				
219	A	V	A	A
225	K	N	N	K	←
227	T	I	I	T	←
237	N	N	N	S	←
247	E	K	K	E	←
251	P	P	T	P
270	K	K	M	K
272	V	V	V	A
275	R	R	K	R
304	S	T	S	T
μ4				
325	L	P	L	L
331	K	Q	K	Q
361	P	L	P	P
381	T	S	S	S
401	K	E	K	K
433	Y	N	N	N
444	S	S *	C	C

Fig. 5. Amino acid substitutions in μA and μB of Atlantic salmon and brown trout: search for a possible Mab4C10 reactive epitope. Residues that are identical in salmon μA and brown trout μB, and at the same time different from salmon μB and brown trout μA are indicated with arrows.

4. Discussion

The present study has shown that two IgM subpopulations in Atlantic salmon and brown trout correspond to μA and μB, previously characterized by cDNA cloning (Hordvik et al., 1992; Hordvik et al., 2002). The IgM fraction which was eluted first on the anion exchanger contained μB, defined by an extra cysteine residue near the C-terminal end of the polypeptide. The extra cysteine residue in μ4 is the only residue that is common for salmon and brown trout μB and at the same time is different from both salmon and brown trout μA.

Somewhat unexpected we found that the monoclonal antibody MAb4C10, originally raised against rainbow trout IgM, reacted exclusively with μA in Atlantic salmon and exclusively with μB in brown trout. The μB transcripts in salmon were previously estimated to constitute about 60% of total μ mRNA in leukocytes and immune organs of healthy fish. This estimate is also in agreement with the ratio of IgM-B versus IgM-A predicted from the anion exchange elution profiles (Hordvik et al., 2002). During many years we have observed that both IgM-A and IgM-B are present in every examined individual. Thus, it is plausible to assume that

MAb4C10 reacts with 40–60% of the IgM population in Atlantic salmon and brown trout, and accordingly, with 40–60% of the IgM + lymphocytes in healthy fish.

As illustrated in Fig. 5, substitutions in μ3 might explain MAb4C10 reactivity with salmon μA and brown trout μB, and absence of reactivity with salmon μB and brown trout μA. The pattern of substitutions indicates that recombination has occurred between the paralogous A and B loci in either salmon or brown trout after the introduction of the relevant mutations. In rainbow trout, the corresponding positions are occupied by amino acids T_{225}, I_{227} and N_{247}, which are different from those in salmon μA and brown trout μB, i.e., K_{225}, T_{227} and E_{247}. The 227 position is occupied by an isoleucine in rainbow trout, corresponding to that in salmon μB and brown trout μA (N_{225}, I_{227} and K_{247}).

In general, the teleost μ3 sequences show higher divergence rates than μ1, μ2 and μ4. Two slightly different allelic variants of rainbow trout μ, differing in position 247 (N versus I, respectively) have been reported to the databases (Andersson and Matsunaga, 1993; Lee et al., 1993; Hansen et al., 1994). However, allelic differences are not relevant in the present study since transfection constructs were sequenced and verified to be identical to the previously reported sequences shown in Fig. 1. When considering features of the amino acids in rainbow trout, the pattern of MAb4C10 reactivity is still reasonable. It is tempting to speculate on the possibility that the negatively charged E_{247} in salmon μA and brown trout μB is compatible with MAb4C10 reactivity whereas the positively charged K_{247} in salmon μB and brown trout μA might repel the interaction with MAb4C10.

Whereas the putative MAb4C10 binding epitope must be exposed on the surface, a putative N-glycosylation site is located on the opposite site of the Ig fold, i.e., towards the apparent core of the IgM monomer (Fig. 6F). This site is conserved in salmon, brown trout, rainbow trout and char (Hordvik et al., 2002) and could represent a binding site for carbohydrate moieties involved in stabilization of the molecule. An exact definition of the epitope–MAb4C10 interaction might provide useful information with regard to further experiments and understanding of the IgM-A versus IgM-B structure. The present study did not reveal any associated molecules that could possibly explain why IgM-A and IgM-B are eluted in two distinct peaks by anion chromatography. Thus, we still hold to the hypothesis that the extra cysteine in the C-terminal part of μB has some major impact on the polymer structure and that this leads to the characteristic elution profile of IgM-A and IgM-B (Hordvik et al., 2002).

The present study has not addressed functional aspects related to the presence of IgM-A and IgM-B in Atlantic salmon and brown trout. However, since μA and μB have continued to exist over a long time during evolution it is likely that this variability has some biological significance. A recent study showed a connection between greater antibody affinity and increased disulfide polymerization in rainbow trout: it was demonstrated that high affinity B-cells produce more highly polymerized IgM, and that the high-affinity, highly polymerized antibodies possess longer half-lives than lower-affinity antibodies (Ye et al., 2010). Variability in inter-heavy chain polymerization of the IgM tetramer is common among teleost fish (Kaattari et al., 1998; Bromage et al., 2006; Ye et al., 2011). In channel catfish, an additional cysteine residue in the C-terminal sequence of μ was found to be essential for establishing a series of covalently inter-bonded forms of IgM (Ghaffari and Lobb, 1989; Getahun et al., 1999). In this context it is striking that the μA and μB variants of salmon and brown trout differ with respect to the presence of an extra cysteine near the C-terminal part. Recombination between the paralogous loci could easily allow one of the variants to take over, but both variants have been maintained during evolution. Thus, there are reasons to believe that the presence of both IgM-A and IgM-B is beneficial. Among others, an interest-
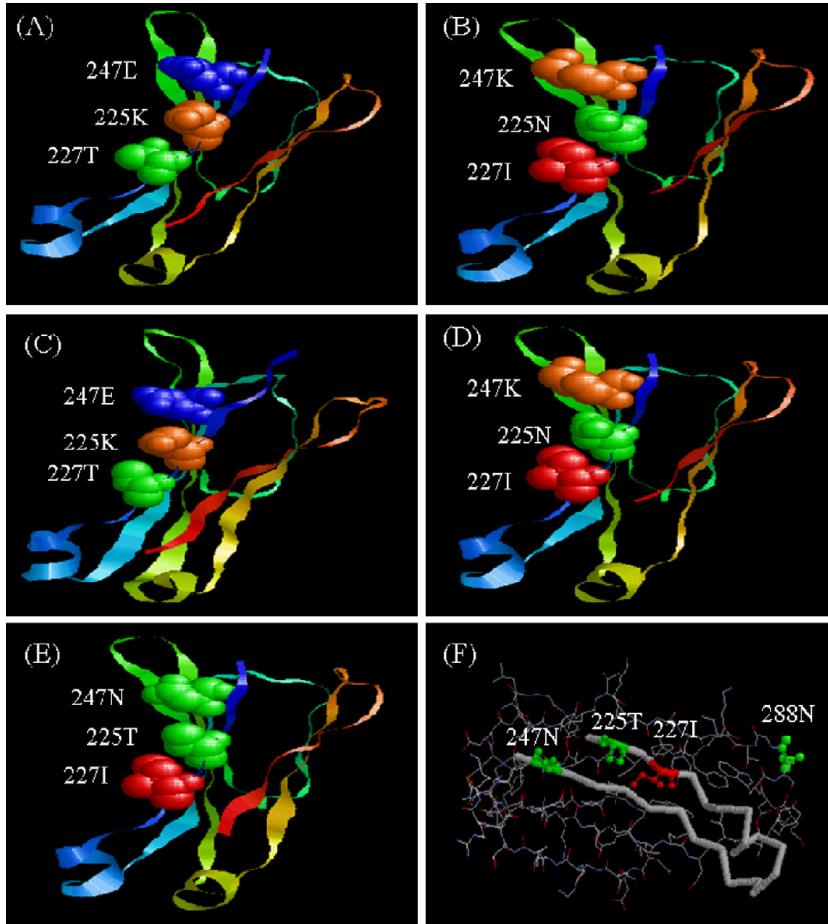
Fig. 6. Modeling of μ3 variants in Atlantic salmon, brown trout and rainbow trout reveals surface exposure of putative Mab4C10 reactive amino acids. Key residues indicated with arrows in Fig. 5 are depicted in red (hydrophobic), blue (negatively charged), brown (positively charged) and green (neutral), respectively. (A) Atlantic salmon μA3, (B) Atlantic salmon μB3, (C) Brown trout μB3, (D) Brown trout μA3, (E) Rainbow trout μ3 and (F) Rainbow trout μ3; a conserved N-glycosylation site in rainbow trout, Atlantic salmon and brown trout (N_{288}) on the opposite side of the Ig fold and the putative Mab4C10 binding site is indicated.

ing topic for a follow up study will be to find out whether there is a difference the inter-heavy chain polymerization of IgM-A and IgM-B, and if this can be correlated to high affinity versus low affinity antibodies.

Acknowledgements

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II

Comparative analysis of IgM sub-variants in salmonid fish and identification of a residue in μ3 which is essential for MAb4C10 reactivity

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ABSTRACT

In rainbow trout (*Onchorhynchus mykiss*) it has been shown that high affinity IgM antibodies have a higher degree of disulfide polymerization and a longer half life time. In the present study, distinct IgM sub-variants related to ancestral tetraploidy in salmonid fish were analyzed to reveal possible characteristic differences between these. A monoclonal antibody (MAb4C10) which distinguishes between IgM-A and IgM-B in Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*) was further characterized. It was shown that substitution of a proline located in the loop between the B and C beta strands of the third constant domain (μ3) of salmon μA eliminated MAb4C10 reactivity. Accordingly, the reverse substitution in salmon μB restored MAb4C10 reactivity. Molecular cloning of μ cDNA from arctic char (*Salvelinus alpinus*) revealed two sub-variants (μA-1 and μA-2), i.e. a similar situation as in Atlantic salmon and brown trout. However, arctic char IgM eluted in one peak by anion exchange chromatography, in contrast to salmon and brown trout IgM that are eluted in two peaks. The only characteristic residue of salmon and brown trout μB is an additional cysteine in the C-terminal part of μ4. Most likely, this cysteine is involved in inter-chain disulfide bonding and influences the elution profiles of IgM-A and IgM-B on anion exchange chromatography. Neither of the μ sub-variants in arctic char have the additional cysteine, and char IgM, as well as salmon and brown trout IgM-A, showed a lower degree of inter-chain disulfide bonding than IgM-B when subjected to denaturation and gel electrophoresis under non-reducing conditions. Hybrids of char/salmon expressed μA-1, μA-2, μA and μB, indicating that there are two paralogous Ig heavy chain gene complexes in the haploid genome of char, like in Atlantic salmon. A comparison of salmonid μ sequences is presented, including representatives of *Salmoninae* (trout, salmon and char), *Thymallinae* (grayling) and *Coregoninae* (whitefish).

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1. Introduction

The salmonid fish family (*Salmonidae*) comprises three subfamilies: *Salmoninae* (trout, salmon and char), *Coregoninae* (ciscos and whitefish) and *Thymallinae* (grayling). Salmonid fish are in a pseudotetraploid state as a result of a whole genome duplication event that occurred in the common ancestor of salmonids. It has been suggested that the whole genome duplication occurred through autotetraploidization about 25–100 million years ago [1]. Based on comparative analysis of partial IgM genomic sequences it

was estimated that the three genera *Salvelinus*, *Salmo* and *Onchorhynchus* radiated in short successions 10–18 million years ago [2].

Different *Salmoninae* species can be crossed to give viable offspring. Hybrids between salmon and brown trout are known to occur naturally [3], while hybrids between salmon and char, and char and brown trout have been produced artificially [4]. The first attempt to make inter species hybrids within the *Salmoninae* subfamily dates back to 1865 [5].

As a result of ancestral tetraploidy, the genome of Atlantic salmon (*Salmo salar*) contains two paralogous gene complexes A and B encoding IgM, IgD and IgT heavy chains [6–9]. Two subpopulations of IgM which are separable by anion exchange chromatography [10] correspond to the IgM heavy chain sub-variants μA and μB [11–13]. The situation in brown trout (*Salmo*

trutta) is equivalent to that in Atlantic salmon, and the molecules have been named μA and μB in both species [12]. Comparison of the translated cDNA sequences and mass spectrometry analysis of the native proteins has shown that only a single amino acid position is characteristic for the IgM-B subtype in Atlantic salmon and brown trout; namely an additional cysteine near the C-terminus of μB [12,13].

In rainbow trout (*Onchorhynchus mykiss*) only one μ gene (and allotypes of this) appears to be expressed, although the Ig heavy chain locus might be duplicated in this species as well [14]. A partial genomic sequence comprising μ1–μ4 has been reported from arctic char (*Salvelinus alpinus*), but questions regarding sub-variants were not addressed [2]. To our knowledge IgM sequences from grayling and whitefish have not been characterized.

Fish immunoglobulin responses differ from higher vertebrates by the lack of a class switch mechanism. It has been suggested that post translational diversity of IgM in teleosts might compensate to some degree for the absence of for example IgG during secondary immune responses [15]. In rainbow trout it has been shown that high affinity IgM antibodies have a higher degree of disulfide polymerization and a longer half life time [16].

A monoclonal antibody (MAb4C10) raised against rainbow trout IgM [17], showed to react with salmon IgM-A and brown trout IgM-B, but not with salmon IgM-B and brown trout IgM-A [13]. It is plausible to assume that this pattern of reactivity is a result of crossover events between the A and B loci during evolution since parts of the sequences show contradictory relationships; for example that salmon μA is most similar to brown trout μB in a defined region [12]. However, both sub-variants, with and without the additional cysteine, have been maintained for a long period of evolutionary time since salmon and brown trout radiated and appear to be present in every individual of salmon and brown trout (Hordvik, personal observations).

Based on comparative analysis we postulated that μ3 is involved in MAb4C10 reactivity. This hypothesis was confirmed by transfection of a series of tagged plasmid constructs into eukaryotic cells, followed by immunostaining with MAb4C10 (and an anti-tag antibody as control). MAb4C10 reacts in Western blots, implying that it recognizes a linear epitope [13]. Alignment of amino acid sequences from rainbow trout, salmon and brown trout indicated that a defined region of μA3 might be involved in the interaction with MAb4C10 [13]. In contrast to MAb4C10, three newly published monoclonal antibodies showed reaction with both salmon μA3 and salmon μB3 [18].

In the present work, site directed mutagenesis of salmon μA3 and μB3 was performed to identify essential residues in the interaction with MAb4C10. The reactivity between char IgM and MAb4C10 was tested, and the degree of inter-chain disulfide bonding was examined for IgM-A and IgM-B of salmon and brown trout, and IgM of char. Furthermore, we cloned μ cDNAs from arctic char, performed an *in silico* assembly of corresponding ESTs from grayling and whitefish, and carried out a comparative analysis with previously characterized μ sequences from salmon, brown trout and rainbow trout. Hybrids of char/salmon were used to reveal possible duplicated μ genes in the haploid genome of char.

2. Materials and methods

2.1. Fish

Atlantic salmon (*S. salar*) were obtained from The Industrial and Aquatic Laboratory at the High Technology Center in Bergen. Brown trout (*S. trutta*) and arctic char (*S. alpinus*) were caught in a mountain lake near Bergen (Bergsdalen). In addition, samples were taken from arctic char caught in Skogseidsvatnet near Bergen and reared in tanks at Institute of Marine Research, Matre Research Station, and salmon (female, Aquagen strain) − char (male, caught in Hopsvatnet in Masfjorden western Norway) hybrids made and raised at Institute of Marine Research, Matre Research Station.

2.2. Purification of IgM

Blood was sampled from Atlantic salmon, brown trout, rainbow trout, arctic char and salmon/char hybrids, respectively, and kept for 2–15 h at 4 °C before centrifugation. 1–3 ml of serum (fresh or stored at −80 °C) was purified by gel filtration followed by anion-exchange chromatography [10,13].

2.3. Mutagenesis of μ3 transfection constructs

The QuikChange Lightening Site-Directed Mutagenesis Kit (Stratagene) was utilized to substitute amino acids in μ3 transfected plasmids described previously [13]. For μA3 mutants the following primers were used; SalA-F1/SalA-R1 (E247K), SalA-F2/SalA-R2 (K225N) and SalA-F3/SalA-R3 (P251T). For μB3 mutants the following primers were used: SalB-F1/SalB-R1 (K247E), SalB-F2/SalB-R2 (N225K) and SalB-F3/SalB-R3 (T251P). Primer sequences are listed in Table 1.

2.4. Transfection and immunostaining

HEK293 cells were grown in DMEM media (Sigma−Aldrich) and transfected using CalPhos Mammalian transfection kit (Clontech) according to the manufacturer's protocol at approximately 35% confluency. After 6 h the medium was removed and cells were washed once with PBS before adding DMEM containing ampicillin/streptomycin and 10% serum and thereafter incubated for further 24 h. When the cells were approximately 80% confluent they were fixed on cover slips by incubation for 30 min in 4% formaldehyde solution at RT. The cells were washed after each step with 1× TBS. Cells were permeabilized with 0.2% Triton X-100 for 10 min. Blocking was performed for 1 h at RT with 10% BSA. Cells were immunostained overnight at 4 °C using mouse-monoclonal anti-Flag (1:1000) or MAb4C10 (1:40) with 3% BSA. Thereafter cells were incubated with FITC anti-mouse (1:500) with 3% BSA for 1 h in dark at RT. The cover slips were mounted on an object glass with a drop of mounting solution ProLong® Gold antifade with DAPI (Invitrogen).

2.5. SDS-PAGE, western blotting and immunodetection

A 4–12.5% denaturing and reducing SDS-PAGE was performed according to Laemmli [19]. Denaturing, but non-reducing, native-PAGE was performed using NativePAGE™ Novex® 4-16 Bis-Tris gels (Invitrogen) with slight modifications. The Laemmli buffer without reducing agent was added to protein samples and boiled at 95 °C

Table 1
Primers used for site directed mutagenesis.

SalA-F1	CTTGTGTGCGATGTCAAGGAACTAGTTCCTGGC
SalA-R1	GCCAGGAACTAGTTCCTTGACATCGCACACAAG
SalB-F1	CTTGTGTGCGATGTCGAAGAACTAGTTACTGGC
SalB-R1	GCCAGTAACTAGTTCTTCGACATCGCACACAAG
SalA-F2	CATTCAGTAGTCATTAACATCACCCCGCCGTCT
SalA-R2	AGACGGCGGGGTGATGTTAATGACTACTGAATG)
SalB-F2	CATTCAGTGGTCATTAAGATCATCCCGCCGTCT
SalB-R2	AGACGGCGGGATGATCTTAATGACCACTGAATG
SalA-F3	GTCGAGGAACTAGTTACTGGCTTCATGAGTGTC
SalA-R3	GACACTCATGAAGCCAGTAACTAGTTCCTCGAC
SalB-F3	GTCAAAGAACTAGTTCCTGGCTTCATGAGTGTC
SalB-R3	GACACTCATGAAGCCAGGAACTAGTTCTTTGAC

for 20 min and 40 min, respectively. The gel was run at 150 V for 4 h at 4 °C. Western blotting was performed at 50 V or 25 V for 1 h at 4 °C (BioRad system and Amersham HybondTMP PVDF Membrane). Thereafter the PVDF membrane was blocked at RT for 1 h in 0.5% dry milk and incubated overnight with either mouse monoclonal MAb4C10 (1:200) or rabbit polyclonal IgM (1:1000) at 4 °C. The membrane was washed 4× with 1× TBST, each for 5 min at RT on a rocker before and after incubating with either HRP-conjugated anti mouse IgG or HRP-conjugated anti rabbit IgG (1:5000) for 1 h at RT. The membrane was developed using ECL reagents as described by the manufacturer (ECL Plus Western Blot Detection, GE Healthcare Life Sciences).

2.6. Isolation of RNA and synthesis of cDNA

RNA was isolated by use of Trizol Reagent (Life Technologies, USA). First strand cDNA was synthesized by use of MMLV reverse transcriptase (Promega, Madison, USA) and an oligo-dT primer.

2.7. Polymerase chain reaction (PCR)

PCR was performed with Accuprime (Invitrogen). Following profile was repeated 25–40 cycles: 94 °C, 30 s, 55 °C, 30 s, and 72 °C, approximately 1 min per kb of the expected length of the PCR-product.

2.8. Sequencing and analysis of DNA

DNA sequencing was performed by use of BigDye Sequencing kit (Amersham Life Science, Cleveland, USA). DNA and peptide sequences were analyzed with BLAST (www.ncbi.nih.nlm.gov) and CLUSTAL (www.ebi.ac.uk/services). 3D structures of polypeptide sequences were predicted by use of PHYRE software (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index).

2.9. Molecular cloning of arctic char μ cDNA

RNA was purified from spleen and head kidney of arctic char and reverse transcribed. cDNA fragments were amplified by different combinations of primers previously used for PCR on salmon and trout (Table 2). Selected PCR-fragments were cloned into TOPO cloning vector (Invitrogen). Products generated by following primer combinations were sequenced: J-sense1/μ3-anti, J-sense2/μ3-anti, J-sense1/μ4-anti, μ2-sense/TM2-anti, J-sense1/TM2-anti. Sequencing of a number of clones revealed two distinct μ sequences, named μA-1 and μA-2. cDNA fragments encoding the constant part of secreted and membrane form of each variant were reported to GenBank (Acc. nos. KC012596, KC012597, KC012598 and KC012599).

2.10. In silico assembly of grayling and whitefish μ sequences

Grayling (*Thymallus thymallus*) and whitefish (*Coregonus clupeaformis*) ESTs encoding the IgM heavy chain were identified by BLAST searches in GenBank using char μ peptides as queries. Eight

Table 2
Primers used for PCR of char μ cDNA.

J-sense1	TTTGACTACTGGGGGAAAGG
J-sense2	TGGGGGAAAGGNACMATGG
μ3-anti	CCCATTGCTCCAGTCCTCAT
μ2-sense	TAATGACCCCCTCTAAAGAG
μ4-anti	ACACAACACAACCTCTACTG
TM2-anti	GATATCATCATTTCACCTTGATGGCAGT

ESTs were identified among grayling ESTs whereas only one was found for whitefish (Accession no. EV368531). The grayling ESTs were assembled to a full-length μ cDNA sequence encoding the secreted form of IgM (Fig. 1).

3. Results

3.1. A proline residue in salmon μA3 is essential for MAb4C10 binding

To test positions that were essential for binding between MAb4C10 and μ3, a series of transfection mutants were constructed by site-directed mutagenesis of plasmids previously used to confirm reactivity with salmon μA3 and lack of reactivity with salmon μB3. The mutants are aligned with relevant μ3 sequences in Fig. 2. Substitution of two charged amino acids (K-225 and E-247) that we previously suspected to have impact on MAb4C10 reactivity [13] did not appear to have any effect (mutants 1, 2, 3, 5, 6, 7). However, MAb4C10 reactivity with μA3 was abolished when P was substituted with a T in position 251 (mutant 4). Accordingly, reactivity between MAb4C10 and μB3 was restored by substituting T with a P in position 251 (mutant 8).

3.2. IgM-B exhibits a greater degree of disulfide bonding than IgM-A

The degree of inter-chain disulfide bonding in IgM populations purified from salmon, brown trout and char was analyzed by denaturation (heating), followed by SDS-PAGE analysis without reducing agents. After denaturation, one major band corresponding to a tetramer (800 kDa), one band corresponding to a monomer (200 kDa), one band corresponding to a trimer (and some very weak bands presumably corresponding to dimers and half-mers), could be seen in salmon and brown trout IgM-A, and char IgM, whereas a single 800 kDa band was dominant in salmon and brown trout IgM-B samples (Fig. 3).

3.3. Arctic char μ sub-variants

Molecular cloning of IgM heavy chain cDNA from arctic char revealed two distinct sub-variants, named μA-1 and μA-2. IgM heavy chain cDNA amplified from different strains of arctic char ("Bergsdalen" and "Skogseidvatnet") showed some allelic differences, but μA-1 and μA-2 were present in all fish examined. Analysis of char/salmon hybrids showed that these fish express four μ sub-variants: char μA-1, char μA-2, salmon μA and salmon μB. Char IgM was eluted in a single peak by anion exchange chromatography, as previously shown [12]. The heavy chain of char IgM reacted in Western blots with MAb4C10 (Fig. 3).

3.4. Grayling and whitefish μ sequences

In silico assembly of grayling ESTs revealed two different μ sequences. The identity index of the two polypeptides is 85%. Only a partial μ sequence of whitefish (*Coregoninae*) was identified in the

Fig. 1. Assembly of μ ESTs from grayling. A slightly different μ EST is indicated with a dotted line.

```
                               225                    247 251
rainbow trout  TVGYTSSDAGPVHGHSVVITIIEPSLEDMLMNKKAQLVCDVNELVPGFLSVKWENDNGKTLTSRKGVTDKIAILDITYEDWSNGTVFYCAVDHMENLGDLVKKAYKRET
Atl. salmon A            A     K TP              E       E       M                                           L    S    P
brown trout B           A     K TP         S  E         E       M                          A  R             L    T    P
Atl. salmon B           A     N  P              E       K  T  M                 M                            L    S    P
brown trout A           V     N  P              E       K  S  M                        R                     L    T    P
mut1 salmon A           A     K TP              E       K       M                          R                 L    S    P
mut2 salmon A           A     N TP              E       E       M                          R                 L    S    P
mut3 salmon A           A     N TP              E       K       M                          R                 L    S    P
mut4 salmon A           A     K TP              E       E  T  M                            R                 L    S    P
mut5 salmon B           A     N  P              E       P  T  M                 M                            L    S    P
mut6 salmon B           A     K  P              E       K  T  M                 M                            L    S    P
mut7 salmon B           A     K  P              E       P  T  M                 M                            L    S    P
mut8 salmon B           A     N  P              E       K       M               M                            L    S    P
arctic char 1           L E   N TP              E       V V   M                      D                       L    S
arctic char 2           P V   I K TP            E       D V   M           V     A  D                         L    S
```

Fig. 2. Salmon μ3 mutants. Residues in Atlantic salmon, brown trout and arctic char μ sequences which are different from rainbow trout are indicated, and substituted residues in the mutants are shown with white letters on black background (the T251P substitution in mutant 8 is underlined). Sequences that do not react with MAb4C10 are indicated with grey.

databanks. Table 3 shows identity scores between representatives of salmonid fishes based on comparison of fragments comprising a part of μ3, plus μ4 (the aligned fragments are indicated on Fig. 4).

4. Discussion

The objectives of this study were (1) to discover how the monoclonal antibody MAb4C10 distinguishes between IgM-A and

Fig. 3. Non-reducing SDS-PAGE of IgM samples from Atlantic salmon, brown trout and arctic char. Stained protein is shown only for Atlantic salmon. (A) Reactivity with MAb4C10. (B) Polyclonal antiserum against salmon IgM (the membrane was first incubated with MAb4C10 and then with the polyclonal antibody without stripping between).

IgM-B in Atlantic salmon and brown trout, and (2) to gain further insight into IgM heterogeneity related to pseudotetraploidy in salmonid fish. The interaction between MAb4C10 and μ3 was narrowed down to a proline residue which is located in the loop between the B and C beta strands of the Ig domain [20]. In accordance with this, char IgM (with a proline in the comparable position of *S. salar*) reacted with MAb4C10 in Western blots. Prior to the present study, the proline residue was ignored since one of two brown trout μB cDNAs reported to GenBank had a T instead of P in this position. It was regarded as an allotypic difference, but in the course of the present work this discrepancy was found to be attributed to "PCR jumping", i.e. formation of μA/μB hybrids by partial extension on one sub-variant followed by annealing and continued extension on the opposite sub-variant.

Molecular cloning revealed two distinct cDNAs encoding IgM heavy chains in arctic char (μA-1 and μA-2) although the protein is eluted in one peak by anion exchange chromatography [12 and present study]. As shown in Fig. 4, neither of the two char μ sub-variants has the additional cysteine residue near the C-terminus which characterizes the μB type in Atlantic salmon and brown trout. Thus, the present study support our previous hypothesis that IgM-A and IgM-B of Atlantic salmon and brown trout are eluted in distinct peaks by anion exchange chromatography as a result of structural features related to inter-chain disulfide bonding [12,13]. Accordingly, it was shown that the IgM-B exhibits a greater degree of disulfide bonding than IgM-A (Fig. 3). As estimated by non-reducing SDS-PAGE, the size of IgM-A is comparable to IgM-B, in agreement with the tetramer structure in teleost fish [21,22]. Many different approaches were attempted to reveal the "building blocks" of the IgM samples, i.e. tetramers, trimers, dimers, monomers and halfmers (results not shown). In agreement with a study of halibut IgM [23], protein staining showed that the majority of the IgM molecules were covalently linked tetramers, whereas immunestaining could give a somewhat misrepresenting picture with a disproportionately strong dimer band (Fig. 3).

The finding of four μ sub-variants in hybrids of char/salmon strongly indicates that there are two paralogous Ig heavy chain

Table 3
Identity scores obtained by multiple alignment of μ sequences from grayling (g), whitefish (wf), rainbow trout (rt), salmon (sal), brown trout (bt) and char.

	g-1	g-2	rt	sal-A	sal-B	bt-A	bt-B	char-1	char-2
wf	89	79	61	61	61	60	61	61	61
g-1		85	62	64	64	64	63	64	64
g-2			54	54	54	55	54	55	55
rt				93	94	93	95	94	93
sal-A					98	95	97	93	93
sal-B						96	98	94	93
bt-A							97	94	93
bt-B								94	93
char-1									98

```
μ1
                                                                                                        100
sal-A      ASS*TAPTLFPLAQCGSGTGDMMTLGCIATGFTPASLTFKWNEQGGNSLTDFVQYPAVQTSGSYMGVSQLRVKRADWDSKIFECAVEHSAGSKTVPVKKQ
sal-B      ----*-----------------V-------------------------------------------------------------------------L---
trout-A    ----*---------------------------------------------------------V------T-------------------------------
trout-B    ----*--------------------------------------------------------------T---------------------------------
rainbow    ----*---------------------------------DE--------------G--------------------K----------K-----
char-1     --TP-------V-----------------E--K-------------------------------T----------E-S----
char-2     ----*---------------------------*E-----------------------T-----------K-----
grayling-1 --E*----V---M-----A---L--------------A---K-A-EIA--------V----EK------I-------NK***-NPFKCA---TE-EIAPK

μ2
                                                                                                        114
sal-A      AEYLQH*PSLYVMTPSKEEMAENMTASFACFANDFSPRTHTIKWMRMEQGIEKEVVSDFKSSCESEKKSDK*TLYSTTSYLRVNESEWKSEEVAFTCVFENKAGNVRRTVGYTSSD
sal-B      V-----*-----------------K------------------K-------------------E-*----------------S-----K---------------
trout-A    V-----*-----------------K------------------K-------------------E-*------------------S---------------
trout-B    V-----*-----------------K------------------K------------------E-*----------------S-----------------
rainbow    P----Q*--------------S--K----------------------ET*----------------T-------------------
char-1     M-----*-------------K--------H---------T-T-K-T-R----------------E-*----S-----------T-----K-N-----------
char-2     L-----*-------------K-------H-----------T-T-K-T-------------------E-*----S----------T-----K---------------
grayling-1 V-RPPVS--F----------QD-Q------L-R----KD-E-T-LKNNIP-T-G-TN***-F-QD---AEHN-M--A--F---G-T--T*DGSKY--Q-VH-S--QTK--KFS-GE

μ3
              225              247 251                        >μ3/μ4 phylogenetic analysis      102
sal-A      AGPVHAHSVVIKITPPSLEDMLMNKKAELVCDVEELVPGFMSVKWENDNGKTLTSRKG***VTDRIAILDITYEDWSNGTVFYCAVDHLENLGSLVKKPYKRETG
sal-B      -----------N-I--------------------K---T------------------M---***--K----------------
trout-A    -----V-----N-I--------------------K---S--------------***---------------------------T---------
trout-B    ---------------------------S--------------------------***A-------------------T---------
rainbow    G----G-L---T-IE----------Q----N------L------------***-K----------------M---D---A------
char-1     ---L-E-----N-----------------V-V--------------------***D--K-----------------------A------
char-2     ----P-V-I---------------N-D-V---------------***A--K-D-----------------------A------
grayling-1 ***-CDTD-EFQFIE-TP-NLFLHE-VS-E-R-TSKQS--Q-I--MTEDK-L-FEEYDDREARSIT-T--IS----R---T-I-I-E-KDL---PEE-S-R--N-
grayling-2                                             -PIF-S--E-RE--T-I-I-EPKDF-*-PEE-S-R-GN-
whitefish                                               -KIL-S--E-R---T-I-S-E-KDLP*-PEEES-R-QN-

μ4
sal-A      GDPQRPSVFLLAPAEKTSDNTVTLTCYVKDFYPKEVLVAWLIDDEPVERTSSSALYQFNTTSQIQTGRT*YSVYSQLTFSNDLWKNKEVVYSCVVYHES
sal-B      ------------------------------------------------------S---*------------------
trout-A    ---------P-----Q-----------------------L----------------S---*------------E----------
trout-B    --------------Q-----------------------------------------S---*-----------------
rainbow    --V-----------Q--------D-----V--------------------------S---*----------------
char-1     ----------------Q-----------D------V---I-----------------S---*------------E----------
char-2     ----------------Q-----------D------V----I-G-------------S---*------------E----------
grayling-1 EREK-----Q-T-E--SGM--------------VN--L-T**MD-TK----A-E----Y----G---MGM-D-MVDGE--------F-T
grayling-2 EREKG----QGLT-E--SGM------WS-------------VN--L-T**MD-PKD----P-A-GA-AYC----G---MGM-D-MVDGE---------F--T
whitefish  EIEK-------T-E--SGM-----------V------VN--L-T**MD-TK--Y----A-E----*---G---MGM-D-MVDGE---------T

                                  *
sal-A      MIKSTKILMRTIDRTSNQPYLVNLSLNVPQSCKAQ 133
sal-B      --------------------N--------C----
trout-A    --------------------N-----------
trout-B    --------------------N--------C----
rainbow    --------------------N--------R-M---
char-1     ------MI-----------N--------N-----
char-2     ------MI-----------N--------S-----
grayling-1 IP---M-V--L-----K-N-----FDI--S----
grayling-2 IP--P-M-V--LG----K-N-----------I-LS----
whitefish  IP--I-M-V--H-----K-N---------*--P-
```

Fig. 4. Alignment of μ sequences from salmonid fish (GenBank acc. nos. in brackets): rainbow trout (AY870258), Atlantic salmon (Y12456, Y12457, Y12392), brown trout (AF228580, AF228581), arctic char (KC012596 and KC012598), grayling (Fig. 1) and whitefish (acc. no. EV368531). Residues that are identical to salmon μA are indicated by hyphens. Positions which were mutated are in bold (and with numbers above the sequences). The additional cysteine in Atlantic salmon and brown trout μB4 is indicated with an asterisk above the sequences.

gene complexes in arctic char, like in Atlantic salmon [6,9]. The presence of two types of grayling μ ESTs in GenBank might be attributed to ancestral tetraploidy as well (Fig. 1). However, the vari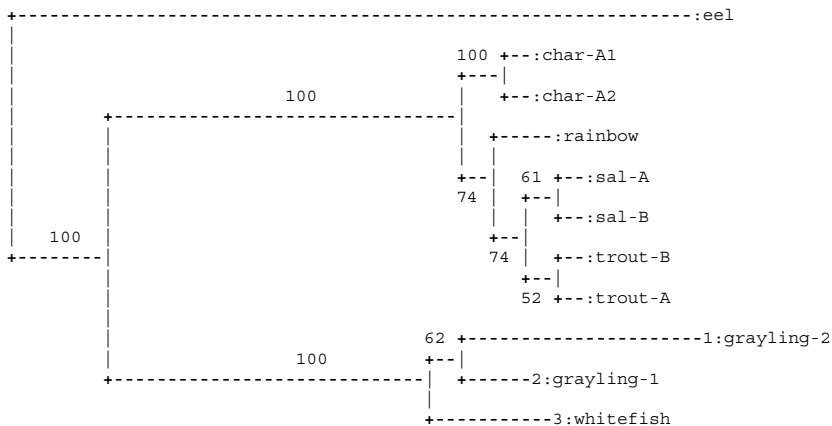ant named grayling-2 (represented by one EST) shows significantly lower identity indices than grayling-1 (represented by 7 ESTs) when compared to other salmonid species (Table 3). Evolution can act asymmetrically on paralogs, allowing one of the pair to diverge at a faster rate [24]. Thus, grayling Ig heavy chain genes might be in the process of re-establishing a situation with one functional μ gene. In Atlantic salmon and brown trout cross-over events between A and B loci might have homogenized the μ genes to some degree, and the paralogous gene complexes are still very similar in salmon [9,13].

Typically, the fourth constant domain (μ4) show the highest degree of conservation. The first constant domain (μ1) is also relatively highly conserved, whereas the third constant domain (μ3) diverges more rapidly. The identity indices between grayling-1 and rainbow trout, for example, are: μ1: 62%, μ2: 46%, μ3: 32% and μ4: 67%. Comparison of μ sequences (part of μ3, plus μ4: indicated on Fig. 4) from grayling (subfamily *Thymallinae*) and whitefish (subfamily *Coregoninae*) with those from trout, salmon and char, respectively (subfamily *Salmoninae*), showed similar identity indices (60–64%), supporting previous analyses which indicated that the genera *Salmo*, *Oncorhynchus* and *Salvelinus* radiated in relatively short successions [2]. The corresponding identity score of grayling-1 and whitefish is 89%, whereas the identity indices within *Salmoninae* are above 93%. The present study illustrates that both *Coregoninae* and *Thymallinae* are distantly related to *Salmoninae*. The relationship of the sequences is indicated in Fig. 5. Comprehensive studies of salmonid phylogeny have been published previously, e.g [25–27].

The present study strongly indicates that the IgM-B sub-variant is present only in salmonid fish belonging to the genus *Salmo* (i.e. Atlantic salmon and brown trout). However, an IgM heavy chain with an additional cysteine near the C-terminus (and with proven effect on the inter-heavy chain bonding of the IgM tetramer) has evolved independently in catfish [22]. A connection between antibody affinity and increased disulfide bonding of the IgM molecules has been documented in rainbow trout [16]. Accordingly, it is plausible to assume that the emergence of the μB variant in Atlantic salmon and brown trout has functional implications.

```
+-------------------------------------------------------:eel
|
|                                         100 +--:char-A1
|                                             +---|
|                              100            |  +--:char-A2
|               +-----------------------------+
|               |                             |   +-----:rainbow
|               |                             | +-|
|               |                             | | 61 +--:sal-A
|               |                           +--|  +--|
|               |                           74 |  |  +--:sal-B
|               |                             +--|  |
|               |                           74 |  +--:trout-B
|    100        |                             +--|
+---------------|                             52 +--:trout-A
|               |
|               |                        62 +--------------------1:grayling-2
|               |          100           +--|
|               +--------------------------|  +------2:grayling-1
|                                          |
|                                          +----------3:whitefish
```

Fig. 5. Phylogenetic relationships of salmonid μ sequences based on comparison of polypeptides comprising a part of μ3, plus μ4 (accession numbers and the aligned polypeptides are shown in Fig. 4, except eel: EU551243). The neighbor joining tree generated by ClustalW (default parameters) was bootstrapped 100 times.

Acknowledgment

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III

Analysis of serum immunoglobulin and Ig heavy chain gene expression in vaccinated versus unvaccinated Atlantic salmon

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Abstract

Relative quantification of total IgM in serum and corresponding heavy chain (μ)gene expression in spleen showed that Atlantic salmon *(Salmo salar)* which had received oil-adjuvanted commercial vaccine maintained about 2-fold higher levels of IgM protein and μ mRNA compared to unvaccinated fish kept in the same cage. Similar results were obtained (by ELISA and RT-qPCR) from two samplings, with average fish sizes of 3,0 kg and 4,7 kg, respectively; 14 and 17 months after intraperitoneal injection. IgT heavy chain (τ) gene expression in spleen did not correlate with vaccination status and the abundance of IgD (δ) mRNA was too low for comparative analysis. Purification of serum IgM by anion exchange chromatography indicated that prolonged triggering of the immune system can lead to a skewed ratio of IgM-A and IgM-B. The A and B subpopulations of IgM are encoded by two paralogous genes, μA and μB, related to ancestral tetraploidy. The typical A:B ratio is approximately 40:60. The IgM-B tetramer exhibits a higher degree of disulfide bonding than IgM-A, presumably due to an extra cysteine near the C-terminus of the heavy chain. In the context of recent results from rainbow trout *(Onchorhynchus mykiss)*, showing that high affinity antibodies are more highly polymerized and have a longer half life time, Atlantic salmon is an interesting model to elucidate these aspects further since tools are available to distinguish IgM-A and IgM-B in this species.

1. Introduction

The major form of serum immunoglobulin in teleost fish is IgM, whereas two other classes of antibodies; IgT and IgD, are present in relatively low quantities. In Atlantic salmon *(Salmo salar)*, the abundance of IgM (μ) transcripts was estimated to be up to 20 times higher than IgT (τ) and up to 200 times higher than IgD (δ) in a series of tissues from healthy fish (Tadiso et al., 2011). The IgT isotype, which is unique for teleosts, appears to be specialized to mucosal immune responses (Zhang et al., 2010). Transcripts encoding membrane bound IgD seem to be dominant in most teleosts, but recently it was shown that alternative mRNA usage generates secretory IgD in rainbow trout (Ramirez-Gomez et al., 2012). In catfish, a separate gene encodes secretory IgD, but this is probably not typical for teleost fish (Bengten et al., 2002). Teleosts constitute a heterogenous group of vertebrates with several examples of unique Ig structures, gene duplications and splicing variants (Stenvik and Jørgensen, 2000; Hordvik, 2002; Savan et al., 2005; Coscia et al., 2011; Quiniou et al., 2011). In contrast to higher vertebrates, the expression of Ig isotypes in teleost fish is not regulated by a class switch mechanism.

The IgM concentration in fish varies to a large extent: from species to species, between males and females, by age, and from one individual to another (Israelsson et al., 1991; Bromage et al., 2005). Furthermore, there are reproduction related immunoglobulin changes, and seasonal changes presumably related to water temperature (Suzuki et al., 1997). The IgM serum concentration in a series of examined fish species listed in Israelsson et al. (1991) ranged from 0.63 to 17.2 mg/ml, and the proportion of Ig protein in the sera varied from 2% to 47%. Atlantic salmon are among teleost fish with a relatively low serum IgM concentration (~0.8-1.3 mg/ml serum), constituting about 2% of the total serum protein (Haavarstein et al., 1988; Magnadottir, 1998). In comparison, Atlantic cod has a high IgM concentration (~5.6-11.5 mg/ml), constituting up to 50% of the total serum protein (Israelsson et al., 1991; Magnadottir, 1989).

Typical serum concentrations in human are, for IgM: 1.5 mg/ml, IgG: 13.5 mg/ml, and IgA: 3.5 mg/ml. Serum immunoglobulin levels (of IgM, IgG and IgA) are higher in adults than in children, and IgM levels are higher in females than males. IgG and IgA continue to increase throughout adult life (Stoop et al., 1969; Kalff, 1969). In general, variations among humans are small compared to those in fish.

It has been suggested that post translational diversity of IgM in teleosts might compensate to some degree for the absence of for example IgG during secondary immune responses. In rainbow trout *(Onchorhynchus mykiss)* high affinity IgM antibodies have a higher degree of disulfide polymerization and a longer half life time (Ye et al., 2010, Costa et al., 2012).

As a result of a tetraploid event in the ancestor of the salmonid fish family there are two paralogous Ig heavy chain gene complexes (or remnants) in the genome of several species belonging to *Salmonidae* (Hordvik et al., 1992; Hordvik, 1998; Solem et al., 2001; Hansen et al., 2005; Yasuike et al., 2010; Kamil et al., 2013). IgM heavy chain sub-variants have been characterized in Atlantic salmon, brown trout and arctic char (Hordvik et al., 2002; Kamil et al., 2013). In members of the genus *Salmo* (Atlantic salmon and brown trout) an additional cysteine residue has been introduced in the C-terminus of the IgM-B heavy chain (Hordvik et al., 2002; Kamil et al., 2013). In agreement with this, the IgM-B tetramers hold a higher degree of disulfide bonding than the accompanying sub-variant IgM-A (Kamil et al., 2013). Thus, Atlantic salmon and brown trout may be promising models to study functional aspects related to IgM heterogeneity since IgM-A and IgM-B have distinct features and can be distinguished by use of a monoclonal antibody (Kamil et al., 2011). The objective of the present study was to reveal possible effects of vaccination on serum IgM concentration, abundance of μ, τ and δ mRNA in spleen, and the ratio of IgM-A/IgM-B. To estimate the relative abundance of the highly similar μA and μB mRNAs different approaches were attempted and evaluated. Both diploid and triploid fish were included in the study to reveal possible ploidy effects on Ig expression.

2. Material and Methods

2.1 Fish

Atlantic salmon *(Salmo salar)* were provided by The Industrial and Aquatic Laboratory (ILAB) at the High Technology Center in Bergen for initial analyses of Ig gene expression. Subsequently, sampling of unvaccinated and vaccinated Atlantic salmon (diploid and triploid fish) was performed at Matre research station (Institute of Marine Research). Hatching of these fish started on 05.02. 2010 and was completed by 18.02.2010. Intra-peritoneal vaccination was performed 25.11.2010. In total the experiment included 3120 fish and represents the source for several studies. More detailed information on the fish can be found in (Cantas et al., 2011; Fraser et al., 2012). Our samplings were performed on 19.01.2012 and 30.04.2012, respectively. During sampling the fish were treated in accordance with the regulations for euthanasia of fish in aquaculture issued by the Norwegian Directorate of Fisheries. The fish were pit-tagged and the status of each individual (diploid versus triploid, and vaccinated versus unvaccinated) were recorded before killing. During the first sampling (n=20) 5 diploids and 5 triploids were selected from unvaccinated and vaccinated fish, respectively. The weight of the fish was from 1945 g to 4850 g and the length was from 53 cm to 71 cm. Gender was not recorded during the first sampling. During the second sampling (n=24) 6 diploids and 6 triploids were selected from unvaccinated and vaccinated fish, respectively. The weight of the fish varied from 2830 g to 6535 g and the length from 60 cm to 76 cm. Among the unvaccinated fish there were 5 females and 7 males. Among the vaccinated fish there were 7 females and 5 males.

2.2 Isolation of RNA and synthesis of cDNA

RNA was isolated by use of Trizol Reagent (Life Technologies, USA). First strand cDNA was synthesized by use of MMLV reverse transcriptase (Promega, Madison, USA) and an oligo-dT primer.

2.3 Conventional and real time PCR

Reverse transcription quantitative PCR (RT-qPCR) was performed as described in (Tadiso et al., 2011). Real time assays utilized in the present study are listed in Table 1. Conventional PCR followed by restriction enzyme analysis was performed as described in (Kamil et al., 2011). Primers J.s (TTTGACTACTGGGGGAAAGG) and µ3.anti (CCCATTGCTCCAGTCCTCAT) were used for amplification of µ mRNA.

2.4 ELISA

Salmon serum in several different dilutions (1/10, 1/20, 1/100, 1/200, 1/300 and 1/500) in coating buffer (carbonate-bicarbonate buffer from Sigma Aldrich, Product No: C3041) were used for coating flat bottom 96-well plates overnight at 4 °C. The plate was washed three times with PBST after each step. The plate was blocked with 5 % dry milk in PBS for 2 hours at room temperature. Thereafter the plate was incubated with polyclonal rabbit anti-salmon IgM (1:3000) or monoclonal mouse anti-trout IgM (MAb4C10) antibodies (1:500) overnight at 4 °C. Horseradish peroxidase (HRP)-conjugated anti-rabbit (1:10000) or anti-mouse (1:5000) respectively was applied for 2 hours at room temperature. For colorimetric measurement liquid substrate for HRP (Sigma-Aldrich Product No: T 0440) 3,3′,5,5′-tetramethylbenzidine (TMB) was applied to all the wells for 30 min in dark at room temperature. Then equal volume of TMB stop reagent (Sigma-Aldrich Catalog No: S5814) was added to all wells and the absorbance of the resulting yellow color was measured at 450 nm.

2.5 Purification of IgM

Blood was kept for 2-15 hours at 4 ºC before centrifugation. 1-3 ml of serum (fresh or stored at -80 ºC) was purified by gel filtration followed by anion-exchange chromatography as described previously (Haavarstein et al., 1988; Kamil et al., 2011).

3. Results

3.1 Immunoglobulin gene expression in unvaccinated and vaccinated fish

Atlantic salmon (unvaccinated and vaccinated, diploid and triploid fish) were sampled at two time points; 14 and 17 months after vaccination. At both samplings, the abundance of μ transcripts was higher (>2 fold) in the spleen of vaccinated fish compared to unvaccinated fish (Table 2). The abundance of τ transcripts was low (>5 CT values higher than μ) and did not show correlation to vaccination status (Table 3). The δ expression was too low to give reliable results (data not shown). The μ gene expression was somewhat higher in males than in females (Table 3), but confirmation of this variation requires a more comprehensive study. Prior to the examination of samples from the vaccination experiment, a series of tissues from healthy fish were subjected to reverse transcription real time PCR to evaluate the relative abundance of μA and μB transcripts. The main conclusion of this analysis was that both sub-types are uniformly expressed in healthy fish, but minor deviations might have been ignored due to methodological limitations (see discussion). Regarding the A:B ratio of μ transcripts in the spleen of vaccinated versus unvaccinated fish differences could not detected by PCR/restriction analysis (Fig 1).

3.2 Serum immunoglobulin in vaccinated versus unvaccinated Atlantic salmon

In accordance with the RT-qPCR analysis, ELISA showed that the percentage of serum IgM in total protein of serum was higher (1,8-1,9 fold) in vaccinated fish versus unvaccinated fish, as measured by use of the monoclonal antibody MAb4C10 (Table 2). Fold increase of IgM was somewhat lower when a polyclonal antiserum was employed instead of Mab4C10. However, it is plausible to assume that this discrepancy was attributed to cross-reaction with other proteins in serum. In a comprehensive study of fish of similar size it was found that the total IgM concentration was 3-4 fold higher in vaccinated fish (Koppang et al., 2008). Correlation to ploidy status was not found in the present study. However, males and females were not uniformly distributed in the diploid and triploid groups (Table 3), and males appeared to have a somewhat higher abundance of IgM transcripts than females. Thus, ploidy effects could have been overshadowed by other variables. Interestingly, gradient anion exchange chromatography of salmon IgM indicated that vaccination can lead to a skewed ratio of IgM-A/IgM-B (Fig 2).

4. Discussion

The present work supports a previous comparable study showing that oil-adjuvanted commercial vaccine triggers a prolonged increase in serum IgM concentration (Koppang et al., 2008). In our study, real time PCR also showed that the abundance of μ transcripts in

spleen was higher in vaccinated fish versus unvaccinated (whereas τ transcription did not correlate with vaccination status). Gradient anion exchange chromatography profiles obtained from fish used in this study (and IgM purifications during many years) indicate that the IgM-A/IgM-B ratio can be skewed in vaccinated fish. In accordance with the typical serum IgM profile, the µA/µB mRNA ratio was previously estimated to be approximately 40/60 in Atlantic salmon (Hordvik et al., 2002). In agreement with this, a recent study based on immunostaining and flow cytometry reported 40/60, 36/64 and 29/71 ratios of IgM-A/IgM-B expressing cells in peripheral blood, spleen and head kidney leukocytes of Atlantic salmon (Hedfors et al., 2012).

Exact ratios of µA and µB transcripts could not be obtained by the present PCR approaches. Since µA and µB mRNAs are highly similar it is difficult to design real time PCR assays that distinguish 100% between the variants. The choice of amplicon becomes more restricted because nucleotides that are different in µA and µB are not uniformly distributed. Reducing the region from where primers/probes can be selected may result in less optimal PCR, and in the next hand to difficulties in the interpretation of the results. The µ3 assays in this study were designed from the most diverged (coding) region of µA and µB, covering 5 different nucleotides. By use of plasmid clones it was shown that both assays cross-react with the opposite sub-variant to some degree although standard conditions and recommended melting temperatures for probes and primers were utilized. It may be possible to develop more precise qPCR tools to distinguish between sub-variants, but the precision and appropriateness of real time PCR can be questioned. We decided to switch to a RT-PCR/restriction enzyme based approach to reveal possible differences in the proportion of µA and µB mRNA (Hordvik et al., 2002; Kamil et al., 2011), but could not obtain the required resolution with this method either (Fig 1). Application of high throughput sequencing will allow more detailed quantification of the different Ig transcripts in future studies.

Based on the present knowledge it is plausible to assume that expression of the paralogous Ig heavy chain loci in Atlantic salmon are subjected to a random selection process equivalent to allotypic exclusion in mammals. Presumably, this process occur in the head kidney and results in a mixed population of IgM-A and IgM-B expressing lymphocytes with a broad spectrum of specificities contributed from both gene complexes (Solem et al., 2001; Yasuike et al., 2010). The typical ratio of µA/µB transcripts might reflect the number of successfully recombined variable genes from each locus and/or distinct features associated with the constant region. A skewed IgM-A/IgM-B ratio in serum of vaccinated fish, as illustrated in Fig 2 could indicate that there has been a clonal expansion of cells expressing a favorable fraction of the IgM-B subpopulation and/or that the expanded IgM-B population is more robust with a longer half life time, resulting in a dominance of these antibodies. Notably, the IgM-B tetramer in Atlantic salmon exhibits a higher degree of inter-chain disulfide bonding than IgM-A (Kamil et al., 2013). In rainbow trout it has been shown that the fraction of fully cross-linked IgM tetramers increases during the antibody response against the pathogen *Streptococcus iniae*, as well as other defined antigens (Ye et al., 2010; Costa et al., 2012).

As described in Materials and Methods, fish analyzed in the present study were part of a large trial setup, including both diploid and triploid fish. Triploid salmon are commercially produced in several countries, but very few studies have been done on the immune system of these fish (Piferrer et al., 2009). Triploid fish are expected to have fewer and larger immune cells compared to diploids. Thus, at the outset of this study, we thought that including fish with an extra set of chromosomes could possibly reveal relevant information regarding the ratio of IgM-A/IgM-B, e.g. that triploid fish could show a more pronounced skewed A:B ratio after a prolonged triggering of the immune response. However, as mentioned in the Results section a more comprehensive study is needed to study possible impact of ploidy on Ig expression in Atlantic salmon.

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Table 1. Real time PCR assays

	Forward primer (exon)	Probe (exon)	Reverse primer (exon)
μA3	ATTAAGATCACCCCCCGTC (μ3)	TTCCTCGACATCGCACACAA (μ3)	GGTCTTTCCATTGTCATTTTCCCATTT (μ3)
μB3	TCATTAACATCATCCGCCGTC(μ3)	TTCTTTGACATCGCACACAAG (μ3)	GGTCTTTCCATTGTCATTTTCCCATTT (μ3)
IgM	TGAGGAGAACTGTGGGCTACACT (μ2)	CATCAGATGCAGGTCC (μ2/μ3)	TGTTAATGACCACTGAATGTGCAT (μ3)
IgT	CAACACTGACTGGAACAACAAGGT (τ2)	AGTACAGCTGTGTGGTGCA (τ2)	CGTCAGCGGTTCTGTTTTGGA (τ2/τ3)
IgD	CCAGGTCCGAGTGGGATCA (δ6)	CACTGAGCCAAATAC (δ6/δ7)	TGGAGCAGGGTTGCTGTTG (δ7)
EF1A	CCCCTCCAGGACGTTTACAAA (E5)	CACACGGCCCACAGGTACA (E5/E6)	CACACGGCCCACAGGTACA (E6)

Table 2. Fold increase in μ gene expression (RT-qPCR) and IgM concentration (ELISA) in vaccinated (V) versus unvaccinated (UV) fish

Sampling	19.01.2012		30.04.2012	
Fish	UV (n = 10)	V (n = 10)	UV (n =12)	V (n = 12)
Average weight	3306 g	2747 g	4799 g	4513 g
Average length	63,8 cm	60,1 cm	71,3 cm	69 cm
RT-qPCR	1	2,7	1	2,1
ELISA*	1	1,8	1	1,9

* measured by use of MAb4C10

Table 3. Relative (fold) μ expression in individual fish sampled 30.04.2012

Fish	weight	length	gender	Fold μ expression	Fold τ expression
Diploid UV	5190	71	female	0.91	1,06
	3935	68	female	0.92	2,01
	3610	64	female	0.91	1,97
	5150	71	female	1.05	1,36
	4955	73	male	1.25	0,92
	4960	71	female	1.14	1,91
Triploid UV	6535	76	male	not determined	Not determined
	3600	66	male	1.56	2,39
	3705	69	male	2.12	3,13
	4750	74	male	1.24	0,58
	4900	72	male	0.82	1,26
	6300	78	male	0.83	3,48
Diploid V	5575	74	male	2.40	3,96
	5240	73	male	2.47	2,19
	5405	72	female	1.79	1,60
	4355	69	female	3.72	1,31
	4060	69	male	3.62	0,98
	3680	65	male	4.32	1,02
Triploid V	3885	66	female	1,00	1,00
	4285	70	female	0.91	1,43
	5060	72	female	1.80	1,28
	4900	72	male	4.61	3,18
	4880	70	female	1.39	2,15
	2830	60	female	5.82	1,06

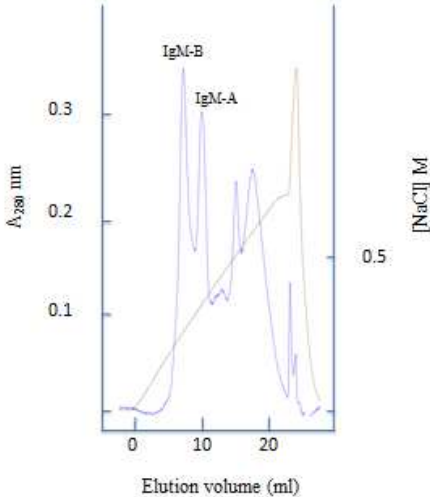**Figure 1. PCR/restriction analysis of µ cDNA from unvaccinated and vaccinated fish.**
Representative samples of unvaccinated and vaccinated fish from the first sampling
19.01.2012. A PCR fragment including the first constant exon (µ1) was amplified, and a
fraction of each sample was subjected to restriction enzyme digestion. A restriction site in
µA1 is not present in µB1. Thus, if the abundance of µA and µB cDNA is the same, the
amount of un-cleaved PCR-fragment in lane 1 should equal the applied amount of untreated
PCR-fragment in lane 2 (half volume of this was loaded on the gel), and so on for each
examined individual (1-4 of unvaccinated and 1-4 of vaccinated fish).
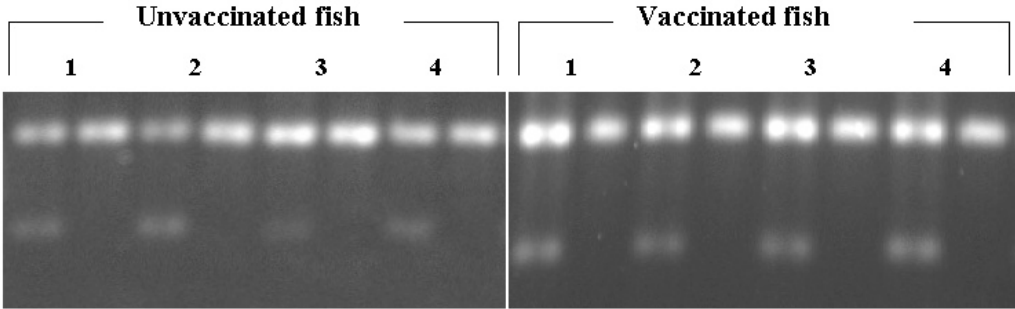
Figure 2. Anion exchange chromatography profiles of IgM from Atlantic salmon.

(A) typical profile (B) profile from a vaccinated triploid individual

(A)

(B)