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

**Atif Kamil**

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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**.

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**Atif Kamil**

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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).

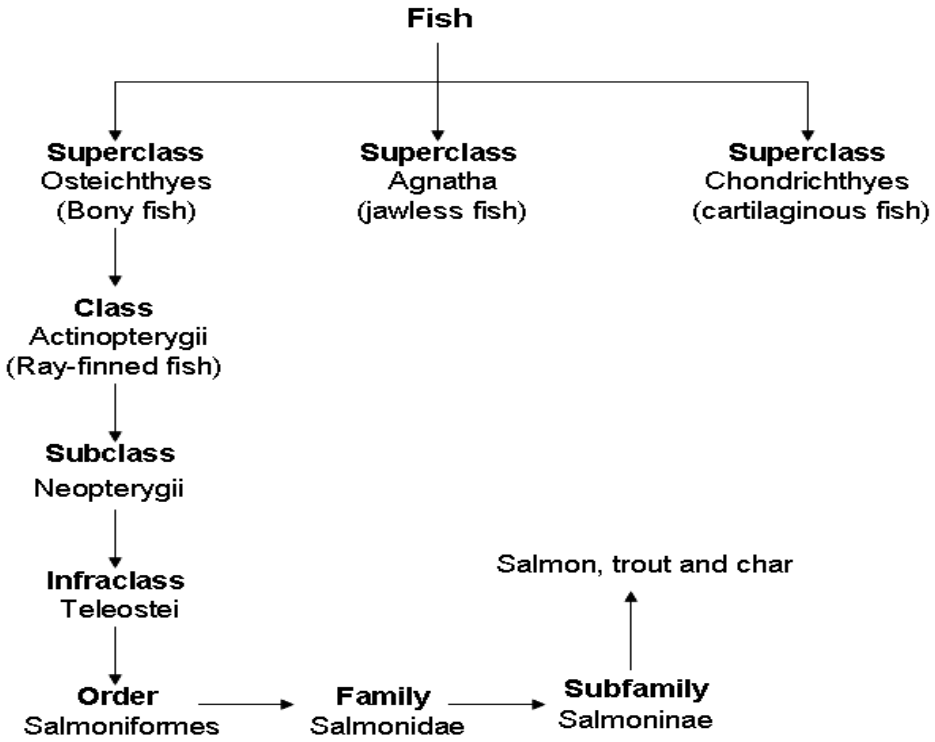


***Figure 1. Schematic presentation of teleosts phylogeny.*** *The subfamily Salmoninae is an important and diverse family of teleosts that includes species like Atlantic salmon, brown trout, rainbow trout and arctic char.*

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).

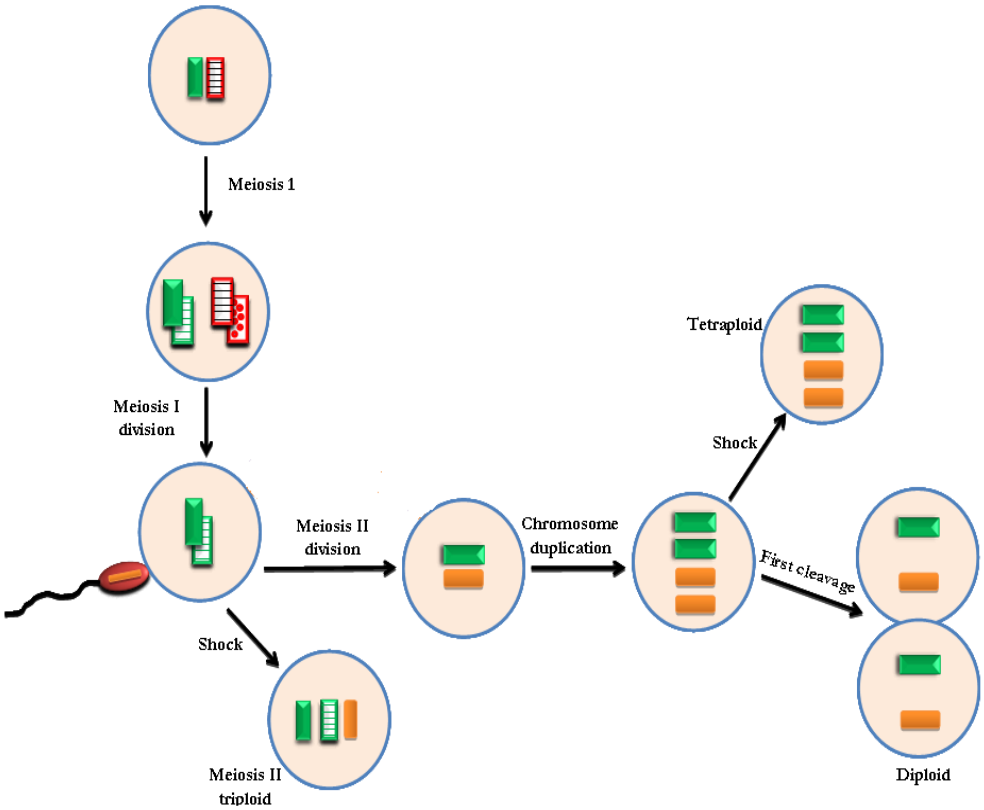


***Figure 2. Artificial polypoidy induction in fish.*** *The arrested eggs at metaphase of meiosis II resumes meiotic division post fertilization. Physiochemical shock causing the suppression of meiosis II produces triploids while suppression of first cleavage in zygote produces tetraploids. Here each 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).

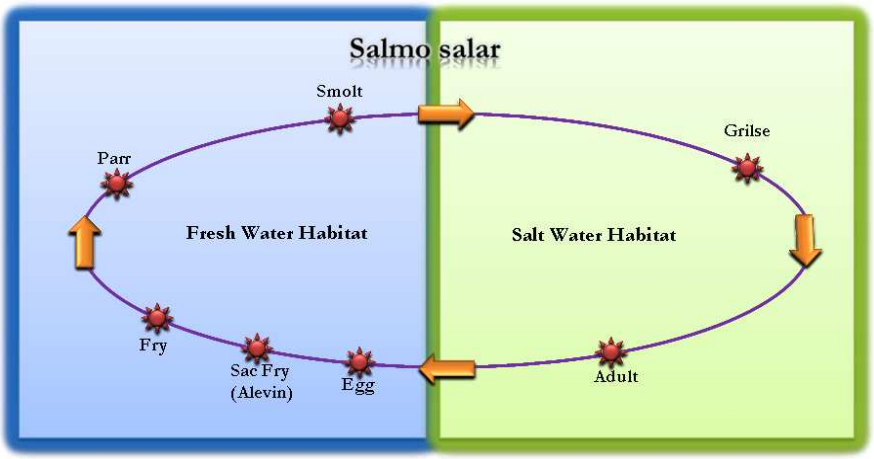


***Figure 3: Life cycle of Atlantic salmon.*** *Atlantic salmon habitats both freshwater and salt water ecosystems during their life cycle. The life begins in fresh water when the adult salmon hatches eggs and after few years they migrate to the sea water in the form of smolt. The smolt becomes adult 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).

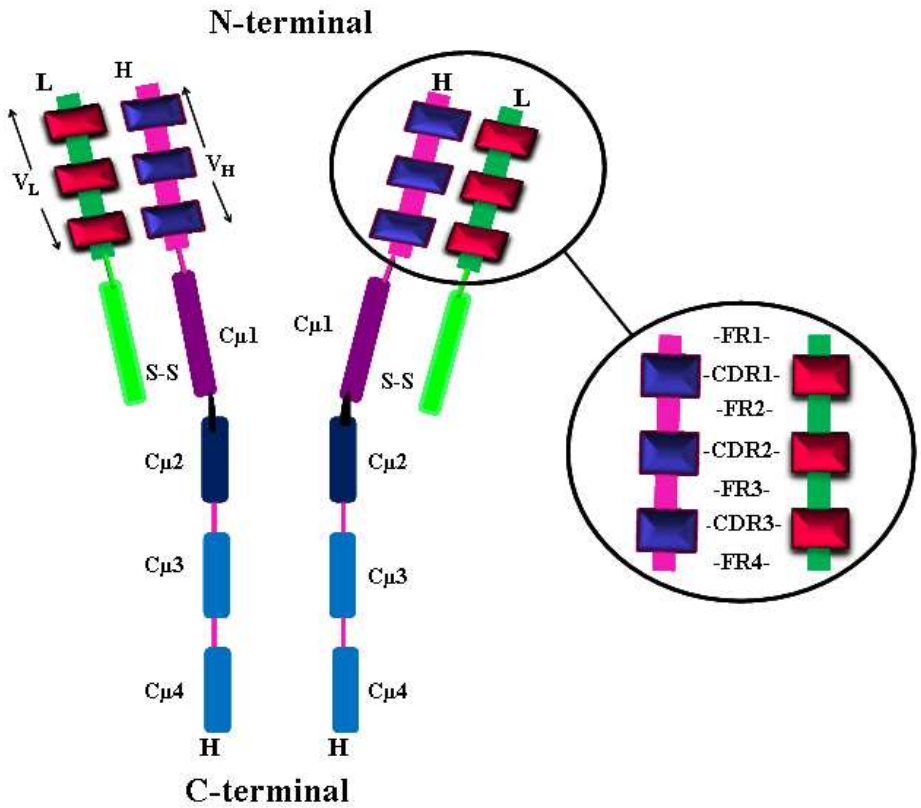


***Figure 4. Schematic presentation of an Ig monomer.*** *The arrangement of H and L chains and their connectivity through s-s bond is shown. Variable domains of both H and L chains are towards the N-terminal and contain the antigen binding region.*

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. τ1– τ4 (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 μ1 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).

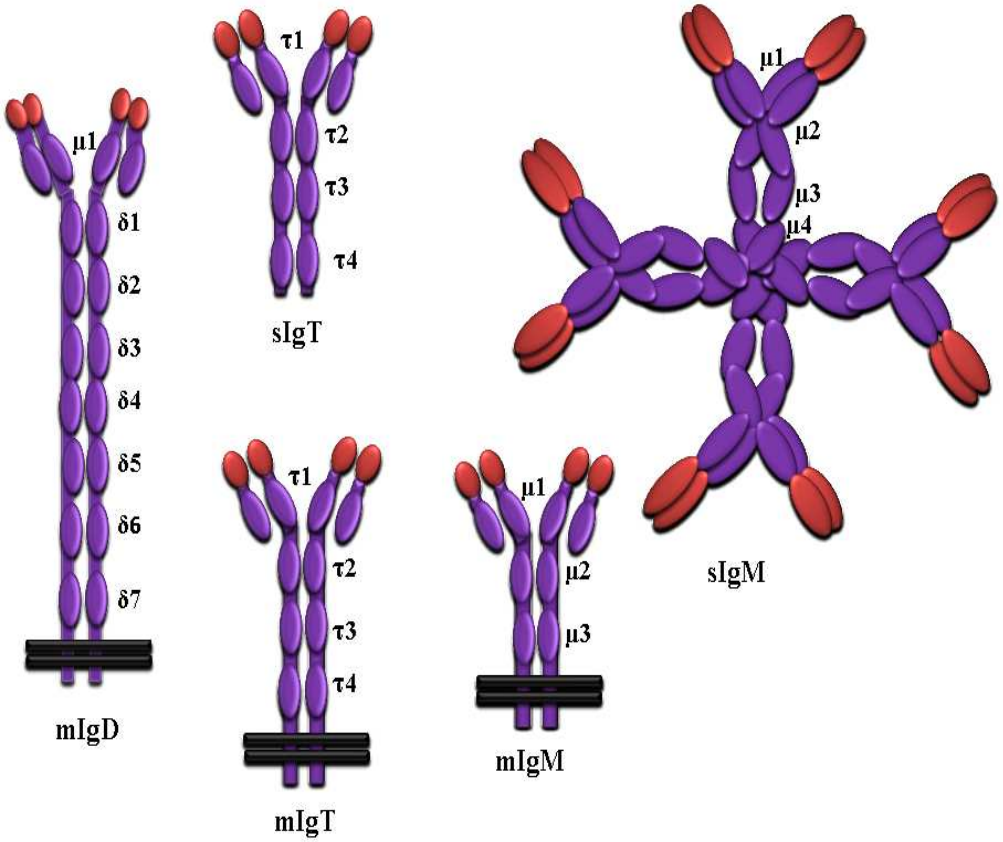


***Figure 5. Schematic presentation of Ig classes in Atlantic salmon.*** *The three Ig classes i.e. IgM, IgT and IgD, both secretory and membrane anchored form found in Atlantic salmon are shown.*

### 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).

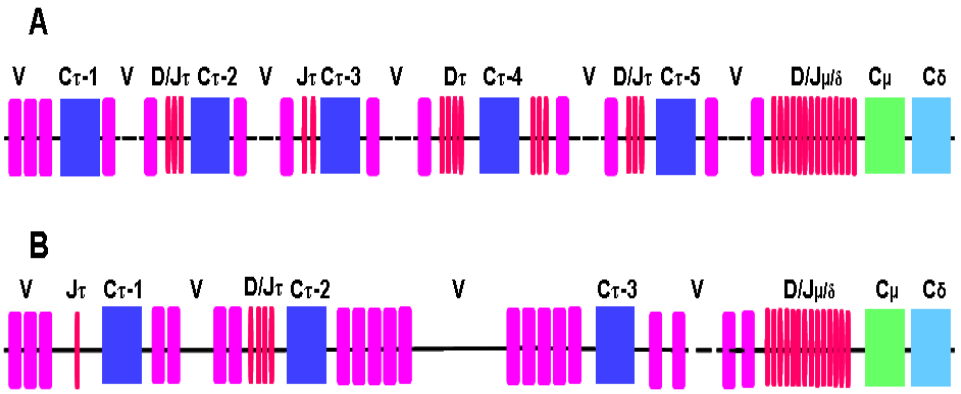


***Figure 6. Schematic presentation of Ig H chain gene organization in Atlantic salmon.*** *The duplicated H chain gene complexes are named A and B. The μ and δ genes are* located downstream of the V/D/J-segments*, while the τ genes are scattered in the VH region (modified version of Yasuike et al., 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.

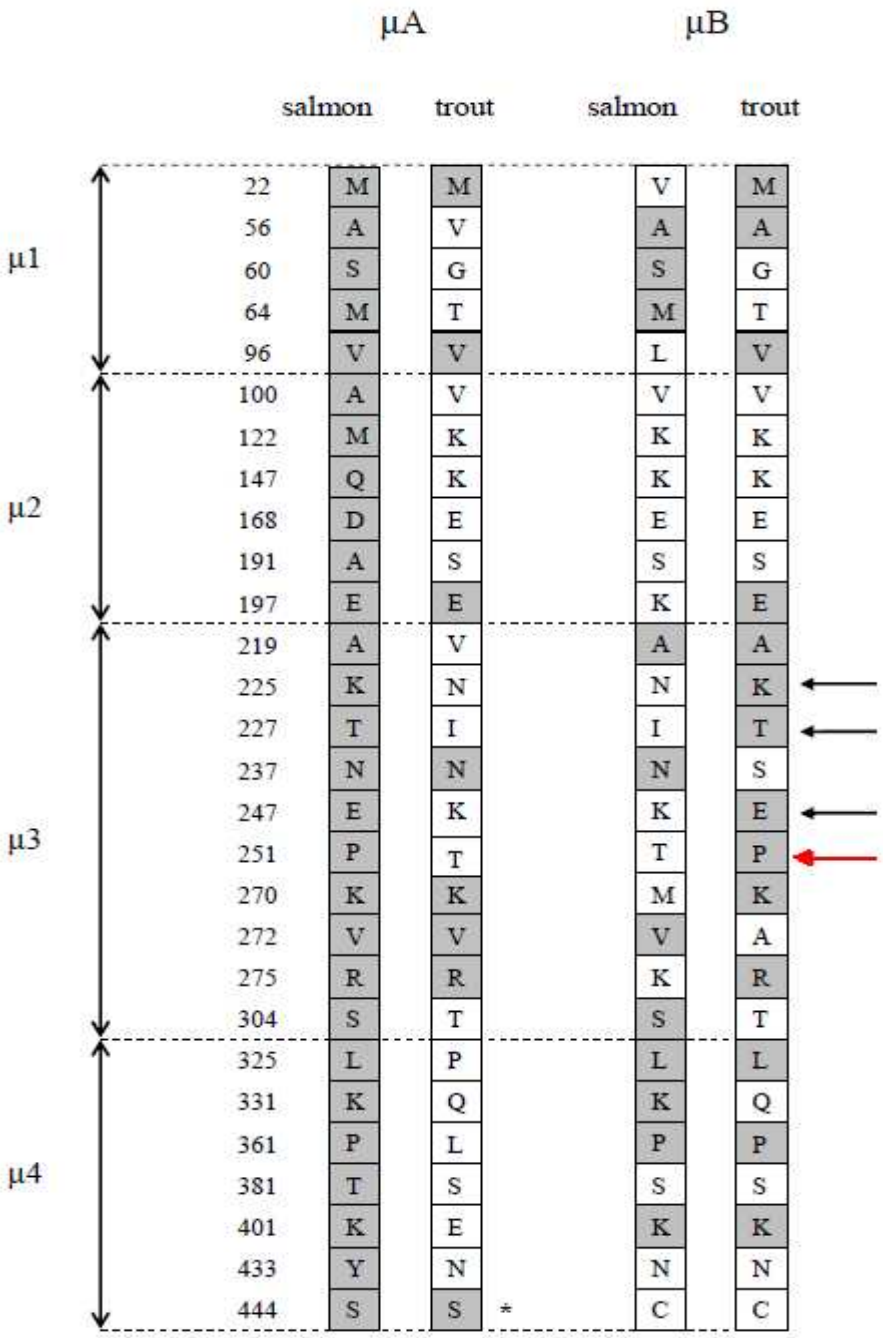


***Figure 7. Amino acid substitutions for μ sub-variants of Atlantic salmon and brown trout.*** *The potential amino acid hypothesized to be the key residue for binding to Mab4C10 is indicated by a red arrow (modified version from paper 1).*

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).

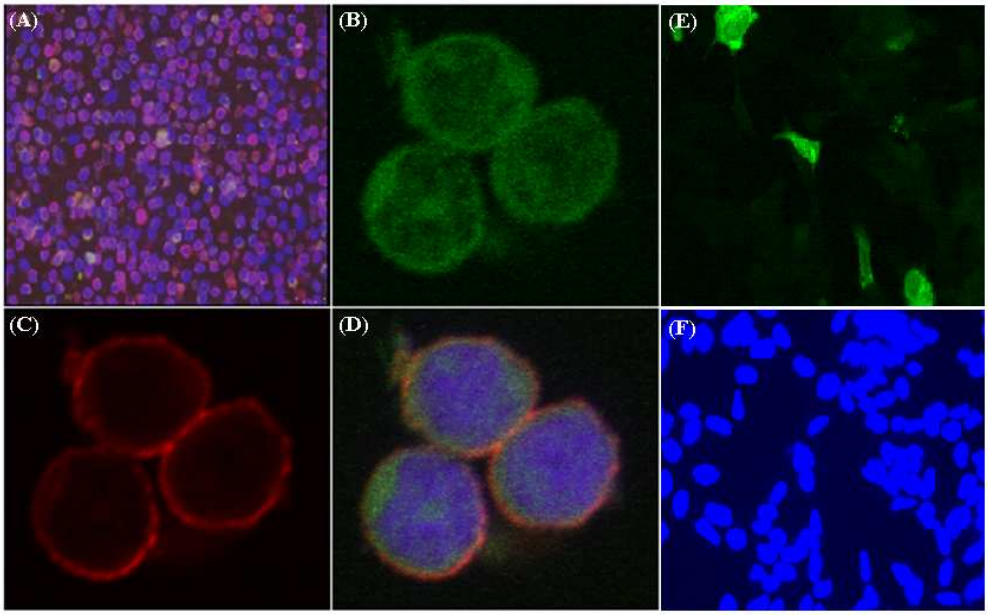


***Figure 8. Immunolabeling of B-lymphocytes expressing IgM from Atlantic salmon and SHSY5Y cells transfected with μA3 construct.*** *Lymphocytes from Atlantic salmon expressing IgM were stained with both polyclonal and monoclonal antibodies against IgM (A-D). The double stained lymphocytes were imaged 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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