Review article

The immune response of Atlantic cod, *Gadus morhua* L.

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ABSTRACT

Interest in cod aquaculture has been growing over the last few decades, resulting in varied research on cod aimed at optimising conditions for both growth and survival. Cultural conditions may increase the danger of infections. Disease control by vaccination has proved successful in the farming of several fish species. However, cod do not respond well to vaccination. Recently it was shown that the fish lacks an antigen presenting mechanism (MHC class II molecules) and T helper cells; these are considered critical elements for developing a specific antibody response and, hence, successful vaccines. The immune response of cod to immunisation, infection and other stimuli is reviewed in this paper. Cod has an effective innate immune system and elements of the adaptive system that show unusual diversity and abundance. The defect in the cod's immune system is of great interest in comparative and evolutionary immunology and calls for a new approach to vaccine development and vaccine strategies in cod aquaculture.

Keywords: Atlantic cod, *Gadus morhua* L., vaccination, immune response, MHC, T-cells, B-cells

Abbreviations: Va: *Vibrio anguillarum, Ass: Aeromonas salmonicida ssp. salmonicida, aAs: atypical Aeromonas salmonicida, Asa: Aeromonas salmonicida ssp. achromgenes.*

YFIRLIT

Ónæmisviðbrögð þorsks (Gadus morhua L.)
Vaxandi áhugi hefur verið á þorskeldi á síðustu áratugum sem hefur leitt til margs konar rannsókna á þorski í þeim tilgangi að efla vöxt og heilbrigði í eldi. Þéttleiki fisks í eldi eykur hættu á sýkingum. Bólusetningar hafa gefið góða vörn gegn sjúkdómum í eldi margra fisktegunda. Hins vegar, kom í ljós að þorskurinn svarar ekki vel bólusetningu. Nýlega var sýnt fram á að þorskinn vantaði ákveðnar vefjaflokkasameindir (MHC af flokki II) og T-hjálparfrumur sem eru lykilþættir við myndun mótefna og góðs árangurs bólusetningar. Í þessari yfirlitsgrein er gert grein fyrir ónæmisviðbrögðum þorsks við bólusetningu, sýkingu og öðru ónæmisáreiti. Þorskurinn hefur öflugt meðfætt ónæmiskerfi og þættir í áunna kerfinu sýna óvenju mikla fjölbreytni. Gallinn í ónæmiskerfi þorsk er áhugaverður með tilliti til samanburðar og þróunarsögulegra rannsókna en, ekki síst, kallar á nýja nálgun á þróun bóluefna og aðferða við bólusetningar í þorskeldi.

INTRODUCTION

Cod in aquaculture

Atlantic cod, *Gadus morhua* L. has, for centuries, been an economically important fish species to the countries around the North Atlantic (Olafsdottir et al. 2014). It is still the mainstay of Iceland's economy. Catches of wild cod in the North Atlantic during the past four decades have greatly diminished, falling from approximately 3.9 million tons in 1968 to under 840 thousand tons in 2005, and the same trend has been observed in Icelandic waters (ICES 2006). The reason is primarily overfishing but climatic changes may also play a part (Myers et al. 1997, Van Leeuwen et al. 2008). Over the past 20 – 25 years interest in cod aquaculture has been growing and several experimental and commercial cod farms have been set up in Norway, Scotland, Canada and Iceland. As with the farming of other fish species, disease and other problems associated with the intensive cultural conditions are difficult to avoid. This calls for preventive measures including vaccination strategies, which have proved essential in the aquaculture of other commercially important species like the Atlantic salmon (*Salmo salar* L.; Dixon 2012, Gudding & Van Muiswinkel 2013).

In cod aquaculture vaccination against important pathogens has proved to be of limited value (Samuelsen et al. 2006, Gudmundsdottir & Bjornsdottir 2007, Dixon 2012, Brudeseth et al. 2013). This imposes a possible limitation on the continuous growth and success of the cod farming industry and considerable research activity has been aimed at understanding the reason for this apparent anomaly or defect in the cod's immune system.

The anomaly in the cod's immune system

Since the first published studies of the immune system of cod by Lars Pilstrom and co-workers in Uppsala, Sweden and Trond Jorgensen and co-workers in Tromso, Norway, it was clear that the immune system of cod was very different from that of other teleosts (Espelid et al.

1991, Pilstrom & Petersson 1991, Schroder et al. 1992).

The most noticeable difference that emerged from these early studies was the fact that unlike other fish species, cod did not produce high levels of specific antibodies following immunisation. It was also established in these early studies that the immunoglobulin concentration in cod serum was relatively high or approximately 10 – 20 times the IgM serum concentration seen in Atlantic salmon (Israelsson et al. 1991, Pilstrom & Petersson 1991, Magnadottir 1998) and the natural antibody activity, when expressed as a function of anti-hapten activity, was high (Pilstrom & Petersson 1991, Magnadottir et al. 2001).

In spite of this evident irregularity cod are not susceptible to common fish diseases and vaccination against certain bacterial diseases, like vibriosis (caused by the bacterium *Vibrio anguillarum*, Va), has resulted in satisfactory protection (Espelid et al. 1991).

Concurrent with varied studies on the immune response of cod, investigators have looked for the possible genetic basis of this poor antibody response. The conclusion drawn from these studies was that the genetic diversity and expression of cod antibody (IgM) was comparable to that of other teleost species and could not explain the poor antibody response (Daggfeldt et al. 1993, Bengten et al. 1994, Stenvik et al. 2001, Wermenstam & Pilstrom 2001, Solem & Stenvik 2006). However, some peculiarities in the antigen presentation mechanism of cod, i.e. in the major histocompatibility classes (MHC class I and class II), did emerge. An unusually high sequence variation was seen in the MHC class I molecules (Persson et al. 1999), while the presence of MHC class II molecules could not be demonstrated in cod (Wermenstam & Pilstrom 2001).

In 2005 Pilstrom et al. put forward the theory that the MHC class II were absent in cod and discussed how this could explain the poor antibody response observed (Pilstrom et al. 2005). This hypothesis was finally accepted

in 2011 after Kjetel Jacobsen's group in Oslo, Norway, confirmed this experimentally by analysing the genomic sequence of cod (Star et al. 2011). They showed that cod had lost the genes expressing MHC class II, as well as the CD4 (cluster of differentiation 4) marker of T helper cells and the invariant chain (Ii), a component of the MHC class II binding site. These factors are essential elements in the adaptive response of jawed vertebrates (the gnathostomata), including all teleosts hitherto studied. The absence in cod presents an interesting anomaly in the immune defence of a teleost species.

The two arms of the immune system of vertebrates

A key role of the immune system is to identify molecular patterns associated with foreign (pathogenic) invaders or the organism's own altered cells (e.g. virally infected, apoptotic or tumour cells). Various pattern recognition proteins or receptors (PRP/R) are involved in this identification, which in turn activates different immune processes that either stimulate or regulate the response and lead to the destruction of the infective agent or altered cell (Du Pasquier 2001, Janeway Jr 2001).

The immune system of vertebrates is divided into the innate (non-specific) arm and the adaptive (specific or acquired) arm. The innate arm is an evolutionary ancient defence system present in all animal phyla, while the adaptive arm is only present in jawed vertebrates and, hence, first seen in primitive fish (Du Pasquier 2001).

The innate immune system identifies foreign agents with the help of a varied but predetermined number of germ lines encoded PRP/Rs and is the first and immediate line of defence. In theory (there are exceptions to this, at least amongst invertebrates) the response has no memory, i.e. on repeated encounters the response is always the same as regards specificity, efficiency and magnitude (Du Pasquier 2001). The innate system usually succeeds in the eradication of most invaders or harmful cellular changes, but can also activate the second line of defence, the adaptive immune response.

A key characteristic of the adaptive system is the near unlimited variety of its receptors to recognize non-self antigens. The diversity of the receptors is created somatically by a variety of processes, including somatic mutation and genetic recombination. Hence, the receptors of the adaptive system are acquired during the lifetime of the organism and their specificity varies from one individual to another. The ability to develop specific immunological memory and enhanced response to repeated encounters with a pathogen is the central element of acquired immunity and the basis of successful vaccination.

The adaptive arm of the immune response can follow three paths:

1) The MHC class I molecules, which are present on all nucleated cells, present endogenous (altered self) antigens to specific T cell receptor (TCR) of CD8 cytotoxic T cells. This leads to the activation and expansion of the specific cytotoxic T cell and formation of cytotoxic memory T cells. This path is involved in the destruction of virally infected, tumour or otherwise altered cells.

2) The MHC class II molecules are present on special professional antigen presenting cells, like macrophages, dendritic cells and B cells, which take up and digest pathogens. The MHC class II present the exogenous (foreign) antigens to, and stimulate CD4 helper T cells (Th). As in mammals, several subsets of CD4 helper cells have been identified in fish like Th1, Th2, Th17/22 and the regulatory T (Treg) cell subset (Scapigliati 2013). Functional activity comparable to the mammalian counterparts has also been identified in some fish species like rainbow trout and zebra fish. The activation of T helper cells is a critical element in "cell mediated immunity" and the establishment of CD4 memory T cells as well as in "humoral immunity" inducing B cell activation, specific antibody production and the crea-tion of memory B cells. The Treg-Th

cells are important in regulating and controlling the immune response, as also in fish (Scapigliati 2013).

3) Certain types of antigens, so called T-independent antigens, can stimulate B cells directly and induce antibody response without the help of T cells or presentation by the MHC molecule. Examples of T-independent antigens include bacterial surface lipopolysaccharide (LPS), cross-linking polysaccharides like ficoll or phosphorylcholine and synthetic immunostimulants, e.g. a single stranded bacterial DNA motive like CpG or a viral RNA motive like Poly I:C.

Vaccination activates primarily path 2, resulting in long lasting protection and the magnitude of the antibody response generated correlates with the protection afforded, i.e. the vaccine efficacy.

The type of immune response evoked is dictated by the nature and dosage of the pathogen or antigenic stimuli and by the mode of entry. In poikilothermic animals, like fish, the environmental temperature is also influential (Bowden 2008). Various cytokines and other factors released during the immune response, as well as the Treg cells, control and regulate the overall response (Wang & Secombes 2013).

The immune pathways and cod
Several studies have shown that the innate immune defence of cod is effective and can be enhanced by various methods. On the other hand, as shown by Star et al. (2011), there are defects in the adaptive system. Path 2 is apparently missing in cod since it lacks the genes expressing the MHC class II and the CD4 marker of T helper cells. However, path 1, cellular immunity induced by viral or self-antigens presented by MHC class I molecules to CD8 T cytotoxic cells, is present in cod. Indeed, an unusual abundance of expressed MHC class I sequences has been demonstrated in cod compared to both other fish and mammals. There is also a great diversity seen in the CD8 marker of the cytotoxic T cells of cod

(Wermenstam & Pilstrom 2001, Star et al. 2011). This suggests that the cytotoxic pathway plays a major role and possibly a novel role in the cellular immune defence of cod compared to other fish or mammalian species. The importance of cellular or systemic defence is also reflected by the characteristic granuloma formation in cod in response to bacterial infections (Magnadottir et al. 2002a).

An antibody response induced by path 3 above is also available to the cod. The antibody response induced by T independent antigens in mammals is generally weak and of poor specificity but may be long lasting (Obukhanych & Nussenzweig 2006). The cod's antibody response seems to reflect this situation (Pilstrom et al. 2005). An unusually high serum concentration of IgM and natural antibody activity may augment this pathway in cod.

Bearing in mind this flaw in the cod's immune system the immune response of cod following immunisation, infection or other immune stimuli will be reviewed below. In the first section studies of the humoral and mucosal response will be examined. This includes the specific and natural antibody response to vaccination and other immune stimuli, vaccine efficacy and the effects on innate humoral and mucosal immune parameters. In the second section studies of the systemic or cellular immune response of cod will be reviewed. The main emphasis will be on the granuloma formation and gene expression studies of various immune parameters in cells and organs of cod.

RESPONSE OF COD TO IMMUNIZATION, INFECTION OR OTHER STIMULI

I. HUMORAL AND MUCOSAL RESPONSE
The specific antibody response of cod; vaccination and vaccine efficacy
Numerous vaccination and/or challenge studies have been carried out on cod in the past two decades. The main emphasis has been on stud-

ying the cod's immune response and vaccine efficacy against the most problematic pathogens in cod aquaculture: *Vibrio anguillarum* (Va), causing vibriosis in cod, *Aeromonas salmonicida ssp salmonicida* (Ass) causing typical furunculosis in salmonids, and atypical *Aeromonas salmonicida* (aAs) subspecies, including *Aeromonas salmonicida ssp. achromogenes* (Asa), causing atypical furunculosis (Espelid et al. 1991, Mikkelsen et al. 2004, Schroder et al. 2006, Lund et al. 2007, Lund et al. 2008, Gudmundsdottir et al. 2009, Lund et al. 2009, Mikkelsen et al. 2011).

The response to various other bacterial pathogens has also been studied, including *V. salmonicida* (Schroder et al. 1992), *Francicella* sp. (Schroder et al. 2009, Ellingsen et al. 2011) and *Moritella viscosa* (Gudmundsdottir et al. 2006, Gudmundsdottir & Bjornsdottir 2007). Studies of the immune response of cod to viral and parasitic infections have been limited and have focused more on systemic rather than humoral response (Samuelsen et al. 2006).

Vaccination trials of cod have mostly involved vaccines made from inactivated whole bacterial preparations, with or without mineral oil adjuvant, and the administration has normally been either by dipping or by intra-peritoneal injection. In most cases the antibody response and specificity is then monitored (using ELISA and immunoblotting) and/or a challenge test with live bacterium carried out to test the vaccine efficacy. A common observation in these vaccination trials is that there is generally no correlation between the antibody activity and vaccine efficacy.

Vaccination trials have shown that the immune system of cod will distinguish between different serotypes of *Vibrio* sp. (Espelid et al. 1991, Schroder et al. 1992) and different subspecies of *Aeromonas* sp. (Lund et al. 2008). Experiments have also shown that vaccination against Va can induce satisfactory and relatively long lasting protection (Gudmundsdottir et al. 2009). However, the specific antibody response induced by vaccination against *Vibrio*

sp. is poor and variable (titre <400) and is primarily against the LPS components of the bacteria. Similarly, an elevated anti-Va antibody titre of a group of vaccinated cod can generally be attributed to only a few individuals. Commercial vaccines against Va are available for cod which contain a mixture of different Va serotypes. The fact that vaccination against Va has in some instances been less successful than the experimental trials is attributed to the possible occurrence of pathogenic strains of Va in the field not contained in the vaccine preparation (Mikkelsen et al. 2007).

The vaccination and challenge trials against typical or atypical furunculosis (*Aeromonas* ssp.) have resulted in more variable outcomes, both as regards the immune response of cod and the vaccine efficacy (Mikkelsen et al. 2004, Lund et al. 2006, Lund et al. 2008, Lund et al. 2009). Higher antibody titres are induced by As (titre up to 3200 (anti-aAs)) than by Va and more individuals show a positive response (Lund et al. 2006). As in the case of the Va trials the antibody response is primarily against the LPS surface component. The efficacy of the experimental vaccines against *Aeromonas* ssp. infections in cod has on the whole been disappointing and no commercial vaccines are as yet available for cod aquaculture.

It has been shown that the A-layer protein of As attached to the LPS surface component of the bacteria is one of many protective antigens against atypical furunculosis, although not always inducing specific antibody response (O'Dowd et al. 1999, Lund et al. 2009, Arnesen et al. 2010). However, no specific antibody response was seen in cod when injected with purified A-layer protein or LPS and these preparations do not induce protection against the disease (Lund et al. 2009).

Schroder et al. (2009) compared the antibody response of cod to different isolates and subspecies of Va, As and *Francisella* sp. and found that *Francisella* induced the highest antibody response and, unlike the response to Va and As, all individuals showed a relatively

high antibody titre. The antibody specificity was limited to anti-LPS activity but the protectivity of this experimental *Francisella* vaccine in cod was not examined. *Francisella*, unlike Va and As, is an intracellular pathogen and, hence, would be expected to induce a different path of immune response, probably using the MHC class I – cytotoxic T cell pathway. The relatively high anti-LPS antibody response also seems to indicate stimulation of the T independent antibody response pathway.

The few studies that have looked at the antibody response of cod when immunized with protein antigens show that cod, in agreement with its lack of MHC class II and Th2 helper cells (path 2 above), can probably not produce specific anti-protein antibodies. It also seems that LPS may have to be attached to the bacterium to induce anti-LPS antibody response in cod (Magnadottir et al. 2001, Lund et al. 2009).

The natural antibody response of cod
Natural antibodies are antibodies present in the serum of vertebrates without any apparent antigenic stimulation. Their activity is commonly measured against haptenated proteins like TNP-BSA (2,4,6-Trinitrophenyl bovine serum albumin; Boes 2000). Natural antibodies have received some attention in studies of fish immunology and have, for example, been shown to take part in the immune defence of rainbow trout and goldfish against both viral and bacterial diseases (Gonzalez et al. 1989, Sinyakov et al. 2002).

The natural (and specific) antibody response has been measured in some trials at Keldur. When cod was immunised with protein antigen no specific antibody response was detected while the natural antibody (anti-TNP-LPH) response, induced by the adjuvant, imitated a specific antibody response of fish (Magnadottir et al. 2001). In another vaccination and challenge trial of cod against Va using different bacterial preparations and vaccination protocols it was shown that both specific anti-Va and natural antibody response was influenced by the vaccination regime. However, neither specific nor natural antibody response could be used to predict vaccine efficacy (Gudmundsdottir et al. 2009).

Further studies on the natural antibodies of cod by Magnadottir et al. (2009) have indicated varied individual specificity and heterogeneous but relatively high binding affinity for haptenated proteins.

IgM of cod
The antibody of cod (specific or natural) is of the tetrameric IgM type, as in other teleosts, and is probably the only secreted immunoglobulin of cod. Cod has also a unique IgD-like B-cell receptor (Stenvik & Jorgensen 2000, Stenvik et al. 2001). Cod IgM shows some features (i.e. redox forms) that probably increase its flexibility and, hence, ability to bind to or capture T independent antigens (Magnadottir 1998, Dacanay et al. 2006). The molecule is about 10% glycosylated and the carbohydrate moiety has been shown to influence the hapten binding property of IgM as well as protecting the molecule against proteolysis (Magnadottir et al. 2002b).

The IgM concentration in cod serum is relatively high compared to other teleost species and has been shown to increase with increasing age and increasing temperature, and seasonal variations have also been indicated (Magnadottir et al. 1999a, Magnadottir et al. 1999b, Magnadottir et al. 2001). Infection and acute phase stimulation, on the other hand, have limited effect on the IgM serum level. (Magnadottir et al. 2002a, Magnadottir et al. 2010, Magnadottir et al. 2011). This seems to indicate that the IgM globulin fraction, which can be as high as about 85% of the serum protein in individual fish (Magnadottir et al. 1999b), may have other, possibly more physiological, functions in the blood than purely immunological.

Innate humoral response of cod
Innate immune parameters include a diversity of pattern recognition receptors and proteins,

cell associated or secreted (Magnadottir 2006, Magnadottir 2010, Ye et al. 2013). Although it is generally assumed that the innate defence system is of great importance to cod, bearing in mind the flaw in its adaptive immune system, studies of the innate humoral response of cod following infection, immunisation or other stimuli have been rather limited. In such studies at Keldur some of the following serum parameters have been monitored and will be briefly reviewed here: The concentration of pentraxins and the haemolytic, anti-protease and lysozyme activity has been monitored in some instances as well as iron-binding parameters.

Substantial individual variation in these parameters seems to be a characteristic feature of cod. In addition, many of these parameters have been shown to be influenced by such factors as size (age), temperature and season (Magnadottir et al. 1999a, Magnadottir et al. 1999b, Magnadottir et al. 2001). This makes comparison between different studies and statistical analysis problematic.

However, certain trends have been observed. Haemolytic activity (HA), which is attributed to the alternative complement pathway, showed seasonal variation and reduced activity with increasing environmental temperature and with increasing size. In cod experimentally infected with Asa the HA varied, both depending on the bacterial dosage and on the route of infection (Magnadottir et al. 2002a). Some peculiarities were observed in the HA activity of cod, which will be discussed in the final section.

The lysozyme activity was low (< 10 units ml^{-1}) or absent in the serum of all experimental cod at Keldur. On the other hand some lysozyme activity was detected in cod mucus (< 100 units ml^{-1}, unpublished data). Short-term stress induction has been shown to increase lysozyme activity in cod serum, which was still relatively low (< 4 units ml^{-1}; Caipang et al. 2009a). In comparison, halibut and sea bass have been shown to have relatively high serum lysozyme activity or about 400 and 1000 units ml^{-1} respectively (Lange et al. 2001).

The serum anti-protease activity, attributed to the α2macroglobulin component of serum (Ellis 2001), shows seasonal variation in cod and is reduced at raised environmental temperatures (Magnadottir et al. 1999a, Arnason et al. 2013). Infection and acute phase induction, on the other hand, have limited effects on the anti-protease activity of cod serum (Magnadottir et al. 2002a, Magnadottir et al. 2010, Magnadottir et al. 2011).

Cod has two types of pentraxins, CRP-PI and CRP-PII (Gisladottir et al. 2009). Both infection and acute phase stimulation had slightly reducing effects on the pentraxin concentration in cod serum (Magnadottir et al. 2010, Magnadottir et al. 2011). These results and studies of the gene expression of the two pentraxin types (see below) suggest that pentraxins are not classical acute phase proteins in cod as described in mammals and some fish but may be more closely linked to the complement activation pathways (Bayne & Gerwick 2001).

The iron binding capacity of serum is an important parameter in withholding iron from bacterial pathogens that need iron for their pathogenicity (Ellis 2001). Studies at Keldur have indicated that infection by Asa may initially stimulate iron binding capacity, but at later stages of infection the binding capacity is reduced (Fazio et al. 2013). The injection of heat-killed bacteria or stress has been shown to increase the bactericidal activity of cod serum, probably through the stimulation of some of the alternative or lytic complement cascade and lysozyme activity (Caipang et al. 2008a, Caipang et al. 2008c).

Mucosal immune response
Humoral immune parameters are also present in the external mucus of cod as well as the mucosal surfaces of the alimentary canal and gills. The mucus is an important first barrier to infection and provides a physical protection by trapping and immobilizing the pathogen.

Immune relevant components that have been identified in cod mucus and epidermal tissue include anti proteases, lysozyme, antimicrobial peptides (AMPs), lectins and immunoglobulins (Schroder et al. 1998a, Subramanian et al. 2007, Inami et al. 2009, Ruangsri et al. 2010, Rajan et al. 2011, Brinchmann et al. 2013). Bactericidal activity has been demonstrated in the external mucus against various bacteria, including known pathogens of cod (Bergsson et al. 2005). However, the activity of the mucus extract was relatively low compared to the bactericidal effects of tissue extracts from immune organs like head kidney and spleen (Ruangsri et al. 2010). The monitoring of changes in the immune parameters of cod mucus following or during infection, immunisation or other immune stimuli has primarily involved expression studies of selected genes in the mucosal cell layer. Changes in the gene expression of antimicrobial protein have, for example, been observed in the skin of cod following experimental infection with Va (Kitani et al. 2013). Recently a proteome reference map for skin mucus of Atlantic cod was established, which will help with future comparative analysis (Rajan et al. 2011).

Some successful commercial ventures are based on the immune properties of cod mucus, e.g. the production of anti-cold spray and wound healing bandages (http://www.zymetech.com and http://www.kerecis.com).

II. SYTEMIC (CELLULAR) RESPONSE
Studies of the systemic or cellular immune response of cod have proved difficult to carry out. The reason is the paucity of reagents for identifying specific cell markers, which is still a practical problem affecting most fish immunologists.

Histology, flow cytometry and associated analysis of phagocytic activity, e.g. respiratory burst activity, have been used for studying the leukocyte population and systemic immune response of cod (Ronneseth et al. 2007, Pérez-Casanova et al. 2008, Overland et al. 2010, Magnadottir et al. 2011, Vestvik et al. 2013).

In recent years analysis of the expression of immune-related genes in different tissues following immune-stimulation has been the favoured tool for studying systemic immune response in cod (Olsvik 2008, Caipang et al. 2009b).

This section will concentrate on the histological examination of granuloma formation in cod tissue during bacterial infection and on some gene expression studies in cod tissues and cells following varied in vivo or in vitro immune stimuli of cod.

Granuloma formation
A characteristic feature of bacterial infection in cod is the widespread formation of granuloma in various tissues and organs. These have, for example, been described in cod infected by *Aeromonas* (Asa), *Yersinia* and *Francicella* (Magnadottir et al. 2002a, Olsen et al. 2006, Ellingsen et al. 2011, Gudmundsdottir et al. 2013, Magnadottir et al. 2013). Granuloma form by the gradual segregation of bacterial colonies and cellular debris, which become surrounded by epithelioid cells, macrophages and lymphocytes with an outer zone of connective tissue (Gudmundsdottir et al. 2013). In cod experimentally infected by intramuscular injection of Asa, fully formed granuloma were observed in the muscle tissue within 4 weeks (Magnadottir et al. 2002a).

In two recent papers the involvement of several immune parameters with advanced granuloma structures was studied in cod naturally infected with Asa and *Yersinia ruckeri* (Yr) with the help of immunohistochemistry (Gudmundsdottir et al. 2013, Magnadottir et al. 2013). The specific antibodies used were against cod IgM, complement component C3, Apolipoprotein A1 (ApoLP-A1), pentraxins (CRP-PI and CRP-PII) and g-type lysozyme as well as specific anti-Asa and anti-Yr antibodies. Apart from the anti-bacterial antibodies only the anti-C3 antibody reacted strongly with the bacterial core, while the other antibodies reacted strongly (compared to the tissue outside the granuloma) with the surrounding

matrix of the granuloma, within the epithelioid and connective tissue boundary of the granuloma. This points to the involvement of both the alternative and classical complement pathways as well as lysozyme-aided bacterial killing in the granulomatosis of cod.

Gene expression studies of systemic immune response

In the last decade a genetic approach has become more important and several immune relevant genes have been identified and cloned from cod. Studies of the change in expression of these genes following immune stimuli has been monitored in a number of experiments, the favoured method being reverse transcription followed by the quantitative polymerase chain reaction (RTqPCR) technique. The use of other techniques like *in situ* hybridization (Rise et al. 2008) and microarray analysis (Bowman et al. 2011) has added to the diversity of gene markers tested. The recently released sequence of the cod genome (Star et al. 2011) is expected to be a valuable resource for further gene expression studies of cod and the identification of important cell markers of the cod's cellular immune system.

With the application of the RTqPCR method the emphasis has been on immune-related genes which may be roughly divided into the following five categories:

1) Inflammatory cytokines like Interleukin (IL)-1β, IL-8 and IL-10; 2) Anti-viral agents like like interferon (IFN)-γ and interferon stimulating gene (ISG) 15; 3) Anti-bacterial agents like the bactericidal/permeability-increasing protein (BPI) and lipopolysaccharide-binding protein (LBP; BPI/LBP), g-type lysozyme, transferrin and antimicrobial peptides (AMP); 4) Other innate parameters like non-specific cytotoxic cell receptor (NSCCR), complement factor C3, the pentraxins (CRP-PI and CRP-PII), ApoLP A1 and toll-like receptors (TLR); and 5) Adaptive parameters like IgM, IgD and other lymphocyte cell markers (CD8, CD3, TCR). The analysis has been carried out on leukocytes isolated from various tissues including whole blood, head kidney, spleen, liver and gills, following infection or *in vivo* or *in vitro* treatment with bacterial, viral or other immune stimulants. The results of some of these RTqPCR studies are reviewed below.

Inflammatory cytokine gene expression

Cytokines are important activating and regulating molecules of immune response and inflammatory reaction. Seppola et al. (2008) sequenced three important cytokine genes interleukins (IL-1β, IL-8, and IL-10) from cod. The effects of viral and bacterial infections as well as the effects of stimulants like the viral mimic Poly I:C, bacterial LPS or CpG motive or general mitogens (PHA) on the gene expression of cod interleukins have been studied in different tissues and varied experiments of cod.

On the whole the results have shown similarities to other teleosts, IL-1β and IL-8 being pro-inflammatory while IL-10 seems to have a suppressive and controlling role. For example, an up-regulation of IL-1β and IL-8 (but not of IL-10) was seen in different tissues of cod following Va and As infection (Caipang et al. 2008c, Caipang et al. 2010). Seppola et al. (2008) studying the expression of IL-1β, IL-8 and IL-10 in cod showed that the response varied depending on the stimulant used, the tissue under study and whether the stimulation was in *vivo* or in *vitro*. A relatively long lasting change in the expression of inflammatory genes in kidney, spleen and intestinal tissues was seen in cod both naturally and experimentally infected with *F. noatunensis* bacterium (Ellingsen et al. 2011). Bakkemo et al. (2011) showed that this intracellular bacterium induces an up-regulation of the anti-inflammatory gene IL-10, suggesting that this might be a virulence mechanism of the bacterium. Acute phase stimulation has also been shown to induce IL-1β gene expression in cod (Audunsdottir et al. 2012) as well as a gradually increased temperature and an exposure to polluted water (Pérez-Casanova et al. 2008, Pérez-Casanova et al. 2010).

Anti-viral gene expression

(IFN-γ) is a cytokine critical for both innate and adaptive defence against viral and intracellular bacterial infections, inducing the expression of a variety of interferon stimulating genes (e.g. ISG15). Both genes (IFN-γ and ISG15) have been cloned and characterised in cod (Furnes et al. 2009a, Furnes et al. 2009b, Seppola et al. 2007b).

Furnes et al. (2009b) showed that IFN-γ was up-regulated in cod injected with Poly I:C and a weaker stimulation was seen in cod injected with bacteria (Va), indicating a role in innate immune response against both virus and bacteria. Seppola et al. (2007b), who cloned the interferon stimulating gene (ISG15), also demonstrated that the expression of the cod ISG15 gene was strongly induced following injection with Poly I:C and also by an injection by formalin-killed Va while bacterial LPS did not induce ISG15 expression (*in vitro* study). In 2009 Furnes et al. (2009a) demonstrated three homologues of ISG-15 in cod, which may have different functions in the anti-viral innate defence of cod. Vaccination of cod with heat-killed Va similarly induced an up-regulation of ISG15 in spleen leukocytes (Caipang et al. 2009b) and both a Va and As *in vitro* infection of gill tissue leukocytes increased the gene expression of IFN-γ (Caipang et al. 2010). In cod infected with infections pancreatic necrosis virus the expression of the viral transcripts and ISG15 was monitored in the liver. The highest level of viral transcripts was found to coincide with the onset and duration of mortality and there was an early (within 3 days) increase in ISG15 expression, which persisted for the duration of the experiment (21 days; Das et al. 2007). This suggests that a relatively long lasting stimulation of innate viral (and bacterial) defence may be induced by vaccination.

Anti-bacterial gene expression

An up-regulation of genes involved in anti-bacterial activity has been demonstrated in several experiments of cod vaccinated with, for example, inactivated Va and As and other stimulants. Stenvik et al. (2004) cloned and characterised the gene expressing BPI/LBP in cod and showed that intraperitoneal injection of Va culture suspension (bacterin) induced high levels of BPI/LBP expression in peripheral blood cells and spleen. This was believed to support the idea that the BPI/LBP protein of cod was involved in bactericidal or permeability activity rather than LPS binding. Similar results were obtained by Solstad et al. (2007) who also showed by *in situ* hybridisation that in the head kidney BPI/LBP was produced by neutrophil-like cells.

Although lysozyme activity is low or absent in cod serum it is expressed in various tissues of cod. Larsen et al. (2009) have identified two types of goose-type (g-type) lysozyme in cod (codg1 and codg2) showing differential expression depending on the tissue under study. An up-regulation of codg2 was seen in the peritoneum and gills after Va injection, indicating a role for g-type lysozyme in the innate defence. A relatively long lasting (10 days) up-regulation of g-type lysozyme gene expression in different tissues was also demonstrated in cod vaccinated with inactivated Va (Caipang et al. 2008c, Caipang et al. 2009b, Caipang et al. 2010).

Transferrin is an iron-binding protein which reversibly binds iron and can create low-iron conditions and which restricts the growth of some pathogenic bacteria. The structure and function of cod transferrin is similar to that of other fish species, like salmon, but its gene expression shows a different tissue distribution. Unlike, for example, Atlantic salmon, transferrin was expressed in the brain of cod but to a lesser extent in the head kidney (Denovan-Wright et al. 1996). An up-regulation of transferrin expression has been demonstrated in cod injected with inactivated Va or As (Caipang et al. 2010). Audunsdottir et al. (2012) demonstrated a constitutive expression of transferrin in head kidney and spleen and an up-regulation following acute phase induction.

The role of AMPs in the innate immunity of cod has received increasing attention in recent

years. These are small cationic peptides with bacterial killing capacity. Some of the AMPs characterised in cod include hepcidin (Solstad et al. 2008), cathelicidin (Maier et al. 2008, Broekman et al. 2011), piscidin-like AMP called gaducidin (Browne et al. 2011) and piscidins (Fernandes et al. 2010, Ruangsri et al. 2012). The gene expression of these AMPs has been studied in some experiments on cod. In general these AMPs are constitutively expressed in several immune related tissues of cod, including the head kidney and spleen, and increased expression has been demonstrated following both bacterial, viral and acute phase stimulation (Solstad et al. 2008, Browne et al. 2011, Audunsdottir et al. 2012, Ruangsri et al. 2012, Broekman et al., 2013). The exceptions were the cod piscidins, which were shown to have anti-parasitic activity rather than anti-bacterial, and gene expression was down-regulated in Va infected cod (Ruangsri et al. 2012).

Gene expression of other innate parameters (NSCCR, C3, CRP-PI, CRP-PII, ApoLP A1 and TLR)
Seppola et al. (2007a) have characterised the NCCRP-1 gene in cod, which is a cell receptor involved in the activation of non-specific cytotoxic cells of teleosts. It was shown that the constitutive expression was higher in head kidney and spleen than in other organ tissues tested. The NCCRP-1 gene expression was not, however, up-regulated *in vitro* in kidney cells by Poly I:C or bacterial LPS or following *in vivo* injections with the viral mimic or formalin killed Va (Seppola et al. 2007a). An increase in the gene expression of NCCRP-1 was seen in peripheral blood cells following vaccination with heat killed Va (Caipang et al. 2008c).

Limited studies have included the RTqPCR analysis of the genes expressing the complement component C3, ApoLP-A1 and the pentraxins in response to immune stimuli of cod. C3 is the key factor in all three complement pathways of cod (Lange et al. 2005). Furthermore, ApoLP-A-1 is believed to be closely

associated with C3 in cod, possibly inhibiting the lytic pathway (Magnadottir & Lange 2004). The pentraxins, although not typical acute phase proteins in cod as seen in mammals and some fish, are important pattern recognition proteins and also linked to the complement system (Gisladottir et al. 2009). The change in gene expression of these three parameters was studied in cod head kidney and spleen leukocytes following *in vivo* injection with turpentine, a typical acute phase inducer (Audunsdottir et al. 2012). All were constitutively expressed in both organs but increased gene expression following the induction was restricted to the head kidney cells. It was suggested that the pentraxins and ApoA-I could be early mediators of acute phase responce in cod, possibly stimulating C3 (and IL-1β) response (Audunsdottir et al. 2012).

The publication of the cod genome has revealed an unusual TLR repertoire. Cod have lost all surface TLRs including those recognising bacterial molecular patterns (TLR 2, 4, 5 and 11), retaining intracellular mammalian homologues (TLR 3, 7, 8 and 9) and the only surface TLR receptors being the teleost specific TLR 21, 22 and 23. At the same time these, in particular the TLR 22 type, show multiple paralogues. Some of these will recognise bacterial molecular patterns, making up for the absence of a mammalian-like TLR system, and are constitutively expressed by immune-related organs and tissues of cod (Star et al. 2011, Sundaram et al., 2012). Sundaram et al. (2012) have shown that the expression of the different teleost specific TLRs changed following challenge of cod with Va and that the change varied, depending both on the organ studied and the TLR paralogue.

Gene expression of adaptive parameters (IgM, IgD CD8, CD3 and TCR).
Changes in the gene expression of adaptive parameters have been recorded in a few studies. For example, Mikkelsen et al. (2011) observed no change in the expression of IgM and IgD in spleen and head kidney in their

study on the efficacy of a dip vaccine against vibriosis, in agreement with the absence of specific antibody response. However, environmental changes, i.e. exposure to increasing temperature or polluted water, resulted in some up-regulation of MHC Class I and IgM H- and L-chains genes (Pérez-Casanova et al. 2008, Pérez-Casanova et al. 2010). Using an *in situ* hybridization technique and probes for the IgM and IgD surface receptors and the secretory IgM Stenvik et al. (2001) showed that immunization with haptenated protein did not increase or change the expression of these parameters or change their tissue distribution. In other words the antibody response at genetic level seems to be in agreement with the observed poor protein expression of IgM in cod serum following immunisation. However, Krasnov et al. (2013), using the microarray technique, have recently demonstrated a relatively long lasting up-regulation of genes involved in adaptive immunity like B and T cell markers in the brain of cod during viral infection as well as of various innate immune related genes (Krasnov et al. 2013a, Krasnov et al. 2013b).

DISCUSSION
It is well known that cultural conditions put considerable pressure on the immune system of fish and the threat of infection is magnified compared to the conditions in the wild. Vaccination has been very successful in the eradication of diseases in the farming of several important fish species like the salmonids. Hence, with the growing interest in cod aquaculture in the early 90's, a parallel increase was seen in research of the immune system of cod. One of the goals was to improve disease control and develop vaccines for this new species in aquaculture. At the same time a new candidate in the studies of the immune system in evolutionary context presented itself in the cod.

As described in the introduction it soon became apparent that cod's immune response was unusual, antibody response being limited or absent and vaccines comparable to those developed in the farming of other fish species were of limited or no value for cod. In consequence commercial vaccines against some important pathogens are still not available in cod aquaculture.

Various ideas and theories have been put forward to explain this apparent defect in the cod's immune system, i.e. the poor antibody response. Finally, in 2005 the idea was put forward that the antigen presenting mechanism of cod might be flawed (Pilstrom et al. 2005). The wide acceptance of this hypothesis in 2011 (Star et al. 2011) was a major breakthrough and put a new perspective on everything that was known about the immune system and immune response of cod.

The fact that, in spite of this defect, wild cod is a very successful teleost and not particularly disease susceptible also made this discovery extremely interesting in an evolutionary context.

The missing MHC Class II and T helper cell genes are key elements in specific antibody response and vaccine efficacy in mammals and most fish species. Losing this important defence mechanism leaves cod with two paths of adaptive immunity: the antigen presentation of the MHC class I molecules and systemic response and the limited T independent antibody response.

The large body of research on the immune system and immune response of cod that has been carried out in the past 2 – 3 decades, and is (partly) recounted in this review, has demonstrated that in spite of these limitations the immune response of cod is surprisingly specific. Both the weak antibody response and the protection induced by vaccination will distinguish between closely related bacterial serotypes. At the same time cross protection has been induced by vaccines against unrelated bacteria like Va and As and vaccine adjuvants will commonly induce as good a protection as the bacterial vaccine. The immune response of cod is therefore a mixture of broad and specific defence elements, involving active innate para-

meters, adaptive cellular response and T independent antibody response of varied specificity. Individual variation in immune parameters and immune response seems to be a characteristic feature of cod. This may be a defence tool in itself for the species as a whole or it may relate to the previous immune experience of each fish.

The cod's response to vaccination varies depending on many factors including the nature of the pathogen, dosage, route of entry, adjuvant, the cod's age and phenotype as well as environmental factors. However, a clear picture of the duration of vaccine-induced protection in cod is difficult to determine from the data currently available. Protection against Va induced by vaccination was shown to last for at least 7 months (Schroder et al. 2006) and in another experiment anti-LPS antibodies were shown to last for at least 11 months (Lund et al. 2007).

It is clear that, with the improved knowledge of the immune system of cod now at hand, vaccination of cod has to be approached in a new way with emphasis on engaging the MHC Class I and cytotoxic pathway and the T independent as well as the innate arm of the immune system.

The gene expression studies that have been carried out have indicated that many, both humoral and systemic parameters, are enhanced during infection or vaccination and this has to be reappraised. The number of immune related genes studied so far is limited and does not cover all immune defence pathways. The understanding of the conversion of genetic response into protein expression is also still restricted. With the genome of cod now available and improved proteomic technology, further progress is to be expected in this area. In addition, advances in peptide and protein synthesis and specific antibody production will also provide new tools for research in this field. A newly available cod cell line is another important tool now available (Broekman et al. 2013, Jensen et al. 2013). The varied response to vaccination and vaccine efficacy depending on the pathogen involved leads to another area of research, which is important in vaccine development, i.e. a better understanding of the interaction between the different pathogens and the different paths of the cod's immune system. Concurrent with further research of how cod could be best stimulated for long lasting protection is the requirement to be able to monitor the response and also to determine vaccine efficacy, preferably without challenge experiments involving live fish.

The unusual and significant anomaly in the cod's immune system is interesting in an evolutionary context. There has been some speculation as to how this defect came about and why the immune system of cod is so different from other teleost species so far studied.

A likely explanation has been put forward by Star and Jentoft (2012) suggesting that during early evolution of the gadoid clade (*circa* 65 million years ago), as an adaptation to a cold deep-sea habitat, which has low microbiological load, these elements of the adaptive system became of limited value and energy expensive to express and, through selective pressure, the innate and systemic defences were positively selected for (Star and Jentoft 2012). As cod evolved into a more pelagic fish further diversification and adaptation of its immune system to new and more intensive changes were selected for. These adaptations have resulted in a major expansion of some of the cod's key immune parameters like the MHC class I molecules, the cytotoxic T cells receptors and some of the TLRs, even to the extent of showing the potential for neofunctionalisation, as suggested by Sundaram et al. (2012)

It is still not known if this cod "scenario" is unusual among teleosts. Other gadoid species like haddock and whiting probably also lack these elements of the adaptive system and for the same reason (Corripio-Myar et al. 2007). Recently a defective or limited MHC class II gene expression has also been indicated in *Syngnathinae* species, the pipefish and the seahorse (Bahr & Wilson, 2012, Haase et al,

2013). However, one could speculate that the underlying reason in this case was not the deep sea adaption but the loss of the jaw during the evolutionary history of these fish and consequent filter feeding. Other types of variations in the immune system may well be common in fish, bearing in mind that only a small proportion of the nearly 30 thousand known teleost species have been subjected to intensive analysis of their immune systems and immune responses.

During research at Keldur some other peculiarities have been noted in the cod's immune system. One example is the change observed in the haemolytic activity (HA) of the cod's serum. In the initial studies (*circa* pre-2000), the HA activity of wild cod and cod obtained from the Marine Institute's Experimental Station, Stadur, Grindavik, showed some unusual characteristics like heat tolerance and apparent enhanced activity induced by the "classical" complement inhibitor EDTA (ethylene-diamine-tetra-acetic acid), suggesting that a non- (or at least unusual) complement factor was involved (Magnadottir 2000). Since about 2000 very low or no HA activity has been detected in the serum of cultured cod from the same source.

It has been speculated that the disappearance of the HA activity may be attributed to gradual changes that took place over a period of time before and after 2000 at the Experimental Station both in the origin of the cod. i.e from wild to selected families of farm hatched cod, and also in the feeding regimes, i.e. from capelin to commercial feed. Further studies seemed to indicate that a possible increase in ApoLP A1 in the cod serum was associated with the low HA activity of the farmed cod (Magnadottir and Lange 2004). In this context, it is also of interest that Caipang et al. (2008b) have showed that serum from wild cod has a broader bacterial killing effect than serum from cultured cod (Caipang et al., 2008b).

Another unusual feature of the cod's immune system is evident in the individual variations in the CRP PII pentraxin described by Gisladottir

et al. (2009). Up to 12 different forms have been demonstrated, each individual apparently possessing a characteristic pentraxin protein pattern which is not influenced by age or immune stimulation (Gisladottir et al. 2009).

Finally, rather intriguing is the marked expression of certain immune parameters in the brain of the cod which can show relatively long lasting up-regulation following infection (Denovan-Wright et al. 1996, Krasnov et al. 2013b). However, since this organ is not commonly included in gene expression studies of fish, this may not be unusual.

This review has concentrated on studies of the immune response of cod in aquaculture to vaccination, immunisation, infection or other immune stimuli. Several other aspects of the immune system of cod that have been examined in the past decades, both at Keldur and elsewhere, have been left out due to limited space. This includes, for example, studies of the ontogenic development of the immune system of cod and the effects of various pro- or prebiotic treatments on the immune system and disease survival (Lange et al. 2004, Lange et al. 2005, Magnadottir et al. 2004, Sveinsdottir et al. 2009, Schroder et al. 1998b, Lauzon et al. 2010, Magnadottir et al. 2006, Broekman et al. 2011).

Research on the cod's immune system is now at a critical point in time. The recent genetic discoveries call for a novel approach in vaccine development for cod aquaculture. These discoveries are also of significant importance in understanding the evolution of the immune system in vertebrates and may prove useful in understanding the consequences of genetic defects in the immune system of higher animals.

It is unfortunate that at this point in time there is waning interest in the farming of cod due to lower prices caused by an increase in commercial catches of wild fish in the North Atlantic in recent years. As is often the case, the commercial demand is what stimulates the best funding environment for research and,

hence, interest in the funding of basic research of the cod's immune system seems to be diminishing and many cod immunologists have turned their attention to other species. Hopefully, this is only a temporary situation.

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