

## Vaccines for finfish aquaculture: What do we need to know to make them work?

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**Abstract** Aquaculture still faces serious economic impacts due to the loss of animals to disease. A conservative estimate of 5% losses due to disease means that the finfish aquaculture industry loses over \$1 billion annually on a global scale. One proven way to prevent costly disease outbreaks is to vaccinate fish against common or known pathogens. Current vaccination schemes still result in losses, however, and this may be due in part to vaccine design. Vaccines are currently designed using state of the art knowledge of immune responses, which is based primarily on mammalian studies. Just how applicable is this information to fish immunity and vaccine design, however? This review discusses what is currently known for teleost fish about two key processes that drive immune response: antigen presentation and cytokine regulation. In both cases many of the genes known to be involved have been identified; in the case of cytokines recent genome projects have added to the total rapidly in recent years. Most functional studies to date in these areas have focused on gene expression and mRNA levels, due to a lack of available antibodies that are required for studies at the protein level. These studies are confounded by the fact that in many cases the teleost equivalents of single copy mammalian genes are duplicated and are regulated in very different ways. This suggests that vaccines designed around mammalian immunological principles will not be as efficient as they could be. Future research goals for fish immunologists should be to develop the antibodies required for protein level functional studies in order to provide the true understanding of fish immunity that is required for the design of finfish aquaculture vaccines that are truly effective.

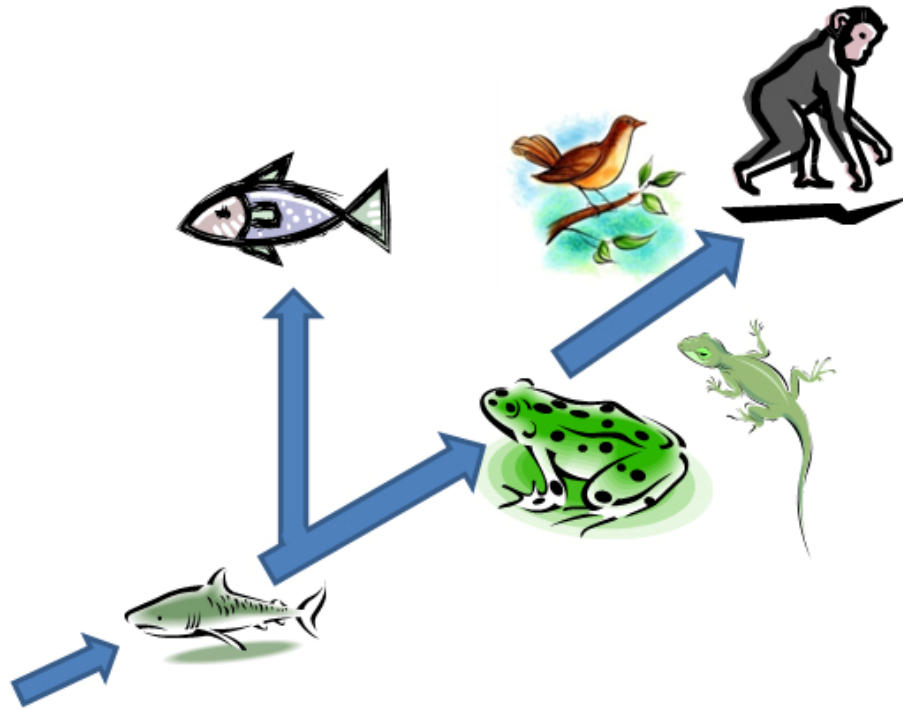
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### The need for finfish vaccines

In 2007 the global market for aquaculture was worth US\$ 60 billion, of which US\$ 20 billion was the value of finfish aquaculture. Losses in finfish aquaculture can cause massive economic losses. A Canadian example was the recent outbreak of IPN virus in the Mainstream Dixon Bay farm in British Columbia which resulted in the destruction of 500,000 fish, a loss of well over a million dollars in revenue for that one company in one year. There are 375 marine finfish and 822 freshwater finfish licenses currently in Canada, for a total of 1,197 finfish licenses (<http://www.dfo-mpo.gc.ca/aquaculture/faq-eng.htm>). A typical farm has between 6 to 24 cages that each contain 35,000 to 50,000 fish, so the 500,000 animals at the Dixon Bay site noted above is average. That means that there are an approximate total of nearly 600 million fish grown annually in Canada. If 5% of those farms suffer severe outbreaks that cause the destruction of stock, which is approximately 30 million fish, it will cost of 60 million dollars in losses. In fact the data suggest 5% loss to parasites and diseases is conservative. The data for Norwegian farms over a 14 year period ([http://www.bellona.org/aquaculture/artikler/Lost\\_production/](http://www.bellona.org/aquaculture/artikler/Lost_production/)) suggest an average of 8.6% losses, of which 85% or 7.31% was due to fish deaths, the majority of which is caused by pathogens and parasites. If we use the 2007 numbers from above, 7% losses to disease would have a value of 1.4 billion dollars. Thus by far the biggest target area for preventing very costly losses to the finfish

aquaculture industry is to prevent fish from dying of diseases. Better vaccines could prevent a large proportion of those losses as treatment will prevent the destruction of animals.

What do we need to know to design better vaccines for fish? The most important piece of knowledge needed here is detailed information about way fish immunity develops and eliminates pathogens. This will tell us what types of pathogenic molecules should be included in vaccines and exactly what type of response they will elicit. Additionally, this detailed knowledge will inform us of the appropriate choices for adjuvants which are the critical component of vaccines that initiate innate immune responses, without which our peptide or protein will not be recognized. While teleost immune systems possess most of the same cells and molecules that mammalian immune systems do, we cannot rely on knowledge of mammalian immunity to guide the production of vaccines for teleost fish. The main reason for this is, as René Stet and I argued in 2003, that while teleost fish possess all of the same components as our immune systems, they use them very differently. Indeed the data we have accumulated since then has only confirmed our argument that teleost fish and mammalian immune systems have evolved along divergent pathways from the same starting point (Figure 1).



**Fig. 1 A schematic representation of the evolution of immune systems.** The immunoglobulin superfamily protein based immune system found in all vertebrates arose in an ancestor of the sharks after its divergence from the lineage of lampreys and hagfish, which possess an adaptive immune system based on leucine rich repeat proteins. Teleost fish underwent a series of genome duplications during their evolution, which allowed them to use the same basic set of genes and cells in a completely different manner from the lobe-finned fishes which were the ancestors of amphibians and hence the whole tetrapod lineage.

### Current knowledge of fish immune system genes

Fish immune systems have been studied at the molecular level for over 20 years. Molecular biology allowed the cloning of immunoglobulin genes starting in 1989, with the cloning of the catfish genes (Ghaffari and Lobb, 1989). Cloning of the carp Major Histocompatibility (MH) genes, which encode receptors essential for the initiation of adaptive immune responses, was first reported in 1990 (Hashimoto et al. 1990). In both these cases, numerous papers detailing the cloning of these both of these genes from other species followed over the following five years (using MH genes as an example, see Dixon et al. (1995) for a review of the numerous species studied between 1990 and 1995).

Cytokines, generally small soluble proteins that are secreted by one cell in order to send a signal to another, play many roles in the physiological functioning of an animal. For example, Interleukin-1 $\beta$  (IL- $\beta$ ), which is a key cytokine expressed early in immune responses by macrophages and endothelial cells in order to activate the immune response by initiating the fever response and activating both T cells and macrophage. However, it also plays a role in communicating between the endocrine system and the immune system during the suppression of immune response due to stress while also exhibiting a host of other properties, including, stimulating myeloid cell production in the bone marrow, angiogenesis (Bar et al. 2003) and blood vessel dilation (Jiménez-Altayó et al. 2006). Tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) shares many of the same immunological properties as IL1 $\beta$ . Other key cytokines are Interferon  $\gamma$  (IFN $\gamma$ ) which is released by T cells and NK cells in order to activate macrophages, and IL-6, which is produced by T cells, macrophages and endothelial cells in order to activate the acute phase/fever response, while also inducing the growth of B and T cells. Despite their importance, the first fish cytokine genes took longer to isolate, with the first being TGF-beta in 1998 (Hardie et al. 1998) followed by several others, including IL-1b in 1999 (Zou et al. 1999), and the first chemokine, chemoattractants that traffic lymphocytes from one location to another for example to sites of injury and infection, in 1998 (Dixon et al. 1998). One interesting feature of cytokines in teleost fish is that most of those cloned to date exist as duplicated genes in the genome of most species. For example, both type I interferons (Chang et al. 2009) and type II interferons are present as duplicate genes. It is not entirely clear at this point what the functional significance of this gene duplication is as both are usually upregulated upon immune stimulation, for example IFN- $\gamma$ , however there is differential regulation of these genes, suggesting that each isoform may have different functions (Purcell et al. 2009).

Molecular techniques such as subtractive hybridization (SSH) added numerous cloned genes for all classes of immune molecules to the list of those isolated throughout the 1990's (for example just from my own work see: three forms of invariant chain: Fujiki et al. 2003a, IL-6: Fujiki et al. 2003b, CD9: Fujiki et al. 2002, C5a receptor: Fujiki et al. 2003c). Finally in the 2000's there were genome projects for model species such as zebrafish and pufferfish, as well as large scale EST projects for key aquaculture species such as Atlantic Salmon and Cod. Thus, large lists of the genes which make up the immune system of several fish species are now available including those that encode receptors, cytokines and effector molecules. These lists are limited however, either due to poor annotation or a lack of identification of a significant proportion of the sequences produced. In some cases over 30% of the sequences in the databases are unknown. Additionally, the one key piece of information that has emerged from data mining of these databases is that many key immune system genes, which are single copy in mammals, are duplicated in teleost fish (invariant chain (Fujiki et al. 2003a; Chang et al. 2009; Purcell et al. 2009) complicating the task of developing an understanding of teleost immune systems. But what functional data is available regarding those genes?

### **Knowledge of fish immune system gene expression patterns**

The early discoveries of fish immune system genes, either by targeted cloning or by selective techniques such as SSH, were often accompanied by the characterization of gene expression patterns by northern blot or reverse transcriptase PCR analysis of various fish tissues in unstimulated and immune stimulated conditions (again just drawing from my own work, some examples can be seen in the Fujiki et al. references (Fujiki et al. 2002; Fujiki et al. 2003a; Fujiki et al. 2003b; Fujiki et al. 2003c) or Kales et al. (2004). Thus basic expression patterns and conditions for some genes are known. For the large numbers of genes produced by genome and EST projects however, the information is less reliable. This may be due to the gene duplication and lack of annotation issue noted above, or for technical reasons, such as the use of oligonucleotide based microarrays for expression analysis, upon which the oligonucleotides used fail to differentiate between alternate forms of the genes (for example between isotypes of antibody or duplicates of interleukin genes). That being said some useful information can be mined from these databases regarding the expression pattern of some specific genes from microarray studies or lists of EST derived from specific tissues before and after immune stimulation.

### **Knowledge of the function of fish immune system gene products**

The area where there is a big gap in functional knowledge between fish and mammals is at the level of protein function. Even for some of the earliest genes to be discovered, there are still no antibodies or functional tests available, although in recent years this has become more of a focus for researchers. Antibodies to immunoglobulin genes from fish had been produced several years before the cloning of the genes (Etlinger et al. 1977; Warr et al. 1979), due to the fact that isolating the immunoglobulins

from blood was not too onerous (Raison et al. 1978). Indeed, antibodies to fish immunoglobulins for various species are one commercial product that has emerged from this line of research, with companies such as Cedarlane Labs in Burlington Ontario and Aquatic Diagnostics in Stirling, Scotland, marketing these. Antibodies to other immune system genes such as MH receptors were isolated relatively quickly after the discovery of the genes (Van Erp et al. 1996; Rodrigues et al. 1998; Kales et al. 2004; Nath et al. 2006) while antibodies to fish cytokines have been slower to emerge. These have been used for some functional studies where possible, but the lack of antibodies to most available proteins has been a limiting factor in true understanding of immune function in teleosts.

### **What we need I: Understanding of how antigen presentation starts responses**

One of the key specific pieces of knowledge we need to know in order to develop better finfish vaccines is how fish immune systems recognize pathogens and target them for their B and T cells. This will require knowing what particular protein fragments of pathogens are recognized by teleost T cells and antibodies in order to assess their general nature, as well as knowledge of the specific epitopes from key pathogens that are recognized. This will require functional knowledge of the accessory molecules involved in antigen presentation such as TAP, Tapasin, and the other molecules of the antigen presentation pathways.

### **Knowing the peptides targeted by the immune system is essential**

The immune system recognizes pathogens and starts specific responses-antibodies or T cells targeted to short specific peptide sequences of the pathogen only when T cells have been activated to produce the cytokine signals which start the response (see below). T cell activation requires both peptides of the pathogen presented in the groove of the MH receptor to be recognized by the T cell receptor and a confirmatory signal from the antigen presenting cell that confirms that the peptide is indeed pathogenic in origin.

For vaccine development, one key piece of knowledge would be the actual sequence of the peptides which can activate the T cells. This knowledge can be used to include such peptide sequences within the vaccines, guaranteeing activation of T cells specific for the pathogen. The technology for doing this has been worked out for mammalian systems (Barber et al. 1996; Gleimer et al. 2011), but challenges remain for performing this in teleost systems. Firstly one needs to know which particular MH receptor alleles are capable of binding peptides of the pathogens. This is achieved by characterizing the MH alleles of several different families of fish to assess the diversity of MH alleles present (one species of fish can contain hundreds of different alleles: for example, my lab has discovered over 100 different MH class I beta alleles in 200 arctic char individuals that we have typed to date). One then runs a disease trial with these families using the pathogen of interest and sequences the MH alleles present in the survivors (and mortalities if possible). The specific MH alleles present in high frequency in the survivors carries the peptides that protective T cells can recognize, and conversely if there are alleles present at high frequency in the mortalities, these are not effective and causes susceptibility to the pathogen. This approach was used by (Grimholt et al. 2003) to characterize Atlantic salmon MH alleles that were protective against the pathogens *A. salmonicida* and Infectious Salmon Anemia Virus (ISAV), and the process has been started for other countries fish stocks, such as the lab of Dr Sergio Marshall which has looked at the alleles in Chilean Atlantic salmon which protect against ISAV and *P. salmonis* (Gómez et al. 2010). This information can be used to breed protective MH alleles into the broodstock, but one must be careful to keep a diversity of alleles present as well as new pathogens emerge and it is difficult to predict which MH alleles will be protective for those diseases.

However once the main protective MH alleles have been identified and bred into the population, it should be possible to design a vaccine specific for those key MH alleles that will provide the peptides it binds and allow enhanced T cell activation. The process involves cloning the genes for the MH class II alpha and beta chain alleles of interest into mammalian expression vectors and using those vectors to express the complete MH receptor on the surface of a cell line which does not normally express that receptor (for example expressing fish MH receptors on the surface of mammalian cells). In order to be expressed on the surface the alpha and beta chain must bind to a peptide which fits into the unique groove of the receptor. One then acid washes the receptors from the surface of the cell, purifies them on a column containing MH specific antibodies under non-denaturing conditions so the alpha-beta-peptide trimer remains intact and then dissociates the peptide from the alpha and beta chains using mild detergent, purifies the peptide from the MH chains using the same chromatography column and

finally sequences the peptide using mass spectrometry. Even for a single MH allele this should yield a large number of peptides as usually only 2 anchor amino acids are needed to bind the peptide to the MH receptor groove and peptides bound to MH class I grooves are usually around 9 amino acids in length, while MH class II bound peptides can be around 20 amino acids long. The number and types of peptides which can be bound by at least one Atlantic salmon allele have been modelled (Cárdenas et al. 2010). By searching the main amino acid sequences through sequence databases containing pathogen genomes or ESTs, one can identify pathogenic peptide sequences which match those bound by the protective MH alleles. Inclusion of the pathogenic proteins which include these target peptide sequences should ensure a robust immune response, thus improving vaccine efficacy in a species population-specific, indeed population-specific, manner.

What do we need to actually carry this out for finfish? For salmonids, the tools are available now as studies linking MH alleles to specific diseases have already been carried out and my laboratory has antibodies against salmonid MH receptors. All that remains is to surpass the technical hurdles involved in expressing salmonid MH alleles on the surface of MH negative cell lines. Dr. Sergio Marshall's laboratory and mine are currently collaborating on that process and hope to achieve this shortly. For other aquaculture species, studies linking MH alleles to disease as well as the development of MH receptor specific antibodies are still needed, but these are both easily achievable.

The placement of specific peptides in the groove of MH receptors does not depend solely on the MH receptor itself, however. The process of peptide loading for both MH class I and MH class II receptors is guided by accessory molecules (Saunders and Van Endert, 2011; Schulze and Wucherpfennig, 2012). In the case of peptide loading into the MH class I receptor, comprising MH class I heavy chain and beta-2 microglobulin, the peptides which are eventually bound are edited by the proteasome in the cytoplasm, only selectively transported into the ER by TAP and must be actively loaded into the groove by tapasin. The chaperones calreticulin and ERP57 are bound to the MH class I: beta-2 microglobulin: tapasin: TAP complex and may also exert influence on which specific peptides are inserted into the MH receptor groove (Wearsch and Cresswell, 2008). For MH class II peptide loading, the DM accessory molecule loads peptides into the groove and may influence the nature of the peptides bound. All of these protein:protein interactions have been characterized mammalian systems, but for not teleost fish as while most of the genes have been cloned, no antibodies which can be used for immunoprecipitation or microscopic co-localization studies are commercially available. However understanding these interactions and the way they ultimately shape the peptide repertoire bound by MH class I and II receptors could reveal key information needed for understanding the types of pathogenic proteins included as targets in vaccines: choosing a protein that will be processed with high efficiency so that peptides from it are presented with high frequency by MH receptors will substantially increase vaccine efficacy.

In order to understand how MH class I receptors bind to and carry peptides to the cell surface in teleosts, if the same process in mammals is a guide, one would have to have antibodies to at least eight of these different proteins (MH class I, beta-2 microglobulin, calreticulin, calnexin, ERP57, Tapasin and both TAP subunits) just to examine the molecular interactions between proteins within the ER. For MH class II one would need to examine the interaction between the MH class II receptor, MH class II associated invariant chain within the ER and the interaction of MH class II receptors and DM in the MH class II compartment vesicles. For salmonids, the genes encoding all of the accessory proteins known to be involved in the MH class I antigen presentation pathway have all been cloned (Grimholt et al. 2002; Fuller et al. 2004; Kales et al. 2004; Landis et al. 2006), including ERP57 for which we have just submitted a manuscript (Sever Bols and Dixon, unpublished). We have developed antisera to all of these proteins and we are currently trying to characterize the protein:protein interactions involved in teleost (well, at least salmonid) MH class I presentation. For the salmonid MH class II pathway, which at first glance appears less complicated due to the fact that fewer proteins are involved, the study of the process is not quite as advanced. In part this is due to the fact that a teleost homologue of DM has not yet been identified, despite all of the recent genome projects. DM is evolutionarily related to MH class II receptors and is similar in structure and sequence. Given that teleost MH receptors are only roughly 35% identical in protein sequence to their mammalian equivalents, it may be that the teleost DM equivalent sequences are sitting in a database identified as aberrant MH class II receptor sequences. The other obstacle to the study of this process is the complicated nature of the other chaperone molecule involved in this pathway - MH class II associated invariant chain (Ii). In mammals, this molecule, while encoded by a single gene, produces four different polypeptides using alternative translation start sites and alternative splicing (Strubin et al. 1986). Genes encoding zebrafish (Yoder et al. 1999) and rainbow trout (Fujiki et al. 2003c) equivalents of Ii have been isolated, but in this case

there are multiple gene copies instead of alternative splicing, each of which encodes a variant form, some of which are missing key features of the mammalian Ii protein. Each of these isoforms, however, is very similar in sequence to each other, making it difficult for the polyclonal antibodies to distinguish between them. Thus elucidating the steps of the antigen presentation pathway, particularly which Ii isoforms are binding to the MH class II receptor at particular time points or under different conditions, will prove difficult. My laboratory is currently looking at strategies needed to clone the teleost DM gene and producing monoclonal antibodies which can distinguish the various Ii isoforms.

### **What we need II: Cytokines are key players**

Once immune responses are initiated, the type of response that follows is guided by the cytokines that are produced in the early phases of the immune response. Depending on these initial signals, the immune response can upregulate mechanisms that response to viruses, extracellular bacteria or allergens and parasites such as worms. In mammals, these three pathways develop separately and in particular the extracellular bacteria (TH1) and allergen/worm (TH2) pathways are mutually exclusive (Hall et al. 2011; Bolon, 2012). Thus in developing vaccines it is important to ensure that the correct pathway is triggered and this could be achieved by assaying the cytokines expressed during the response to the vaccine. How does this translate to fish immunity? Many cytokine genes have been discovered, but the gene for the key cytokine that initiates the TH2 response has not yet been isolated. Additionally, while gene discovery has provided knowledge of fish cytokines at an academic level, it has not yet translated into very many practical products for assessing the health or immune status of fish in either a commercial or research settings. Such assays would ideally measure the levels of the protein products of specific genes, perhaps even specific isoforms of these genes as post-transcriptional modifications may be required for functionality of these proteins and are quite common mechanisms of regulating expression in mammalian immune system genes, especially the cytokine genes.

Thus assays which could detect specific amounts of cytokines in the blood or other tissues of fish or the number of cells from peripheral blood or a cell culture could be used not only for academic research into fish immune responses, which is currently becoming limited due to the lack of those assays, but also for many other aspects of fish physiology as well. Indeed with sufficient knowledge as to the exact role of these cytokines in initiating immune responses, sufficiently inexpensive and simple assays for quantifying production of these cytokines could be used commercially as a screening tool for diseases in aquaculture situations. This would be particularly useful for novel pathogens for which there is not yet a diagnostic test.

### **Assays for cytokine concentrations are needed**

A common assay used for the assessment of cytokine levels in mammalian tissue is a quantitative or direct Enzyme Linked ImmunoSorbent Assay (ELISA), sometimes called a sandwich ELISA. This assay requires two antibodies to the target cytokine, one to coat the 96 well plates for “capturing” the target cytokine from the sample, and a second, usually conjugated to a flouochrome or enzyme, for detection. The “capture” antibody is coated onto the plate first, and after washing to remove any excess, the sample containing the unknown amount of the target compound is added. The target compound is bound by the capture antibody, while everything else in the sample is washed away. Finally the detection antibody is added and binds to the target cytokine. The assay usually comes with a set of standards of known concentration so that when the absorbance or fluorescence value of the unknown are plotted on a curve, the actual concentration of the target cytokine in the unknown can be determined.

For determining the number of cells in a culture dish or in the diverse population of in peripheral blood lymphocytes (PBL) that produce a target cytokine, the best assay is an ELISpot assay. This is a variation on the common ELISA, in which it is the cells thought to be producing the target cytokine that are added to a 96 well plate which has been lined with a nylon membrane (indeed cells which have been stimulated can be added to one set of wells, while control unstimulated cells can be added to another set of wells, usually in triplicate or quadruplicate). The nylon membrane has been pre-coated with an antibody specific for the target cytokine. Thus as soon as the cells produce any target cytokine it is immediately immobilized on the solid phase of the nylon membrane. The target cytokine can then be detected using an enzyme conjugated second antibody specific for that target cytokine. Enzymatic detection such a nitro blue tetrazolium is used so that dark spot develops on the nylon membrane

where each target cytokine producing cell was located. The membrane can then be examined using a microscope to count the number of dark spots in each well. A comparison of the number of spots between stimulated and unstimulated cell cultures, for example, can inform the researcher whether or not the stimulus induced cytokine production. Or alternatively PBL from fish suspected to be infected could be compared to fish from a disease free environment. One advantage of the ELIspot assay is that once the image is acquired, the users can utilize any of numerous computer graphic programs that can measure the number of spots to derive numerical values.

For both the above assays, monoclonal or polyclonal antibodies can be used as either the capture or detection antibodies, although it is common to use monoclonal antibodies for capture as they have high specificity for the target cytokine. If the detection antibody is directly conjugated to the fluorescent tag or enzyme used for visualization, the two antibodies can be from the same species, but if an enzyme or fluorescently linked secondary antibody is needed, then the capture and detection antibodies must be from different species in order to prevent false positives.

It is possible that the specificity of the polyclonal antibodies for a particular cytokine might not be sufficient for accurate quantification, in which case the applicants might want to develop monoclonal antisera to that particular cytokine. This would however reduce the target species range for which the kit could be used, but it may be possible to design peptides for protein production which would react at least to both rainbow trout and Atlantic salmon.

While knowledge of the multiple isoforms of most fish cytokines is limited, it is clear that in most cases, both isoforms are upregulated during immune responses there are also differences in the degree by which these duplicate genes are upregulated (for example see interferon gamma up regulation in Purcell et al. 2009). If future research shows that there are indeed different functions for these duplicate cytokines, these assays could be modified by the inclusion of isoform specific monoclonal antibodies which would allow differentiation between the two forms.

## CONCLUDING REMARKS

The discussion above makes it clear that the “Road Not Taken” hypothesis advanced by Rene Stet and myself clearly holds water: teleosts and tetrapods have the same components in their immune systems, but evolution has set them on different paths causing them to regulate and use those components in very different ways (Stet et al. 2003). This means that in order to make vaccines that are truly effective for teleost fish, we need to understand immune processes as they happen in teleost fish and knowledge of mammalian immune processes can only be a loose guide at best. In most cases this means moving into the post genomic era and starting the painstaking process of examining the tens of thousands of sequences in the databases in functional studies, making recombinant proteins and antibodies in order to truly understand their function in a teleost context. Current attempts at explaining teleost immune responses based solely on mRNA expression patterns using genes annotated based on sequence similarities of less than 40%, not taking into account teleost gene duplications and radically different gene regulation patterns, for example the recent microarray study of Canadian sockeye salmon (Miller et al. 2011), are doomed to be wildly inaccurate until we have the necessary teleost-specific knowledge of immune regulation and function required to understand the significance of changes in gene expression. That knowledge, however, will be hugely beneficial in designing truly effective teleost vaccines, provided it can be incorporated into vaccine production technology in a cost effective manner.

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