Interferon Kinetics and its effect on DNA vaccine induced immunity in Channel Catfish (*Ictalurus punctatus*)

Except where reference is made to the work of others, the work described in this thesis is my own or was done in collaboration with my advisory committee. This thesis does not include proprietary or classified information.

Kendal Thomas Harder

Certificate of Approval:

Richard C. Bird, PhD
Professor
Pathobiology

Kenneth E. Nusbaum, Chair
Associate Professor
Pathobiology

Vicky L. van Santen
Professor
Pathobiology

Frederik W. van Ginkel
Associate Professor
Pathobiology

George T. Flowers
Interim Dean
Graduate School

Interferon Kinetics and its effect on DNA vaccine induced immunity in Channel Catfish (*Ictalurus punctatus*)

Kendal Thomas Harder

A Thesis

Submitted to

the Graduate Faculty of

Auburn University

in Partial Fulfillment of the

Requirements for the

Degree of

Master of Science

Auburn, Alabama
August 9, 2008

Interferon Kinetics and its effect on DNA vaccine induced immunity in Channel Catfish (*Ictalurus punctatus*)

Kendal Thomas Harder

_____
Signature of Author

_____
Date of Graduation

Vita

Kendal Dalton (Thomas) Harder, daughter of James Philip and Dalton (Robinson) Thomas, was born April 5, 1980, in Jackson, Mississippi. She graduated magna cum laude from The Woodlands High School in 1999. While attending Baylor University in Waco, Texas, she worked for two summers as a summer research assistant for Dr. Dorota Skowyra in the Edward A. Doisy Department of Biochemistry and Molecular Biology at St. Louis University of Medicine, and worked one semester as a research assistant for Dr. Myeongwoo Lee in the Department of Biology at Baylor University. She graduated with a Bachelor of Science degree in Biology with minors in Chemistry and Computer Science. She married Adam Wells Harder, son of Mike and Ann (Wells) Harder, on August 30, 2003. She worked for 4 months as a lab technician for Dr. Kenneth E. Nusbaum in the department of Pathobiology at Auburn University College of Veterinary Medicine before entering Graduate School at Auburn University. She currently lives in Melbourne, Florida with her husband and is a Software Engineer at Harris Corporation.

Thesis Abstract

Interferon Kinetics and its effect on DNA vaccine induced immunity in Channel Catfish (*Ictalurus punctatus*)

Kendal Thomas Harder

Master of Science, August 9, 2008
(B.S., Baylor University, 2003)

101 Typed Pages

Directed by Kenneth E. Nusbaum

Increasing the effectiveness of vaccination against channel catfish diseases, including channel catfish virus (CCV) is important economically and clinically for channel catfish farming. Catfish interferon (IFN), an innate, nonspecific immune protein, provides immediate nonspecific viral protection and can be induced with either CCV, UV-inactivated CCV or polyinosinic-polycytidylic acid, (poly I:C). Conditions and kinetics of the IFN response in channel catfish ovary (CCO) cells and in channel catfish are described. Vaccination of channel catfish with ORF 59, a cloned gene, increased total anti-CCV activity during 4 hours to 4 weeks post vaccination. When IFN is elicited concurrently with DNA vaccination, the effectiveness of the vaccine does not increase.

Acknowledgments

The author thanks Dr. Vicky van Santen and Natalia Petrenko for time and assistance with real time PCR. She thanks Patricia DeInnocentes for guidance, mentorship, counseling and friendship during the course of this study.

Style manual or journal used Fish & Shellfish Immunology (together with the style known as "aums"). Bibliograpy follows Fish & Shellfish Immunology.

Computer software used The document preparation package TEX (specifically LATEX) together with the departmental style-file `aums.sty`. Text composed in Google Docs.

Table of Contents

List of Figures

Chapter 1

Introduction

Farmed fish are unavoidably subjected to a variety of stresses, which will lower their innate defenses and will subsequently lead to outbreaks of viral or bacterial infections. These stresses are caused by handling, transportation, living in over-crowded conditions, poor water quality and exposure to pollutants. They are associated with an increased susceptibility of fish to diseases (reviewed in [1]). The innate immune system in fish is constantly challenged by pathogens and constitute the first line of defense (reviewed in [2]). Some investigators have suggested that the innate immune system is more important in fish than adaptive immune responses, because innate defenses respond quickly and are temperature independent. Therefore, they are able to respond to and slow down or halt viral and bacterial invasions before adaptive responses are generated (reviewed in [2]). However, the innate system in fish alone is not enough to protect fish against viral pathogens. In fact, viral diseases cause significant losses in aquaculture each year [3]. In aquaculture there is an increasing demand for effective and economical vaccines. Viral diseases in fish are of clinical and economic importance in farmed fish. Thus, many studies have focused on ways to efficiently vaccinate fish against viral diseases.

An example of a fish disease of economic consequence is channel catfish virus (CCV) disease. CCV, a herpesvirus (ictalurid herpesvirus 1, IHV-1), may cause fatal disease in 40-90% of channel catfish fry each year [3]. CCV is an α-like herpesvirus and has a double stranded 134 kbp genome that contains 79 open reading frames (ORF), with 14 of the genes present twice [4] [5]. Even though experimental data suggest that older fish

are susceptible to natural outbreaks of acute CCV, the disease occurs almost exclusively in fish that are less than 1 year of age, and generally less than 4 months of age [6]. Water temperature is a critical environmental factor for outbreaks. When water temperature rises above 27°C the mortality rate is high. The rate decreases as water temperature decreases, and there is no mortality below 18°C [6]. Diseased fish exhibit ascites, exophthalmia and hemorrhage in fins and musculature. Histologically, the most damage occurs in the kidney with extensive necrosis of renal tubules and interstitial tissue [6].

Fish that survive CCV infection may become latent carriers. Surviving fish display protective levels of CCV-specific antibodies (reviewed in [6]). During the latent state, the virus is undetectable by traditional culture or antigen-detecting methods, even in immunosuppressed spawning adults (reviewed in [6]).

The main reservoir of CCV consists of carrier fish. Infectious CCV can be detected in tanks of experimentally infected catfish, although the route of viral shedding has yet to be determined. CCV transmission occurs horizontally and vertically. Horizontal transmission may be direct or through water, the main abiotic vector. Vertical transmission occurs commonly, though the mechanism is not known (reviewed in [6]).

Currently, control of CCV is based on maintaining relatively low stocking densities of fish and avoiding stressful handling of young fish during the summer months. Facilities used for incubating eggs and rearing catfish fry and juveniles are separated from those used for carrier populations to prevent the occurrence of CCV in a CCV-free fish production site. Defining CCV-free populations has to be done largely from historical data or from identifying populations that are sero-negative to the virus, since virus is not detected in latent carrier states but only during active outbreaks. However, recent use of PCR and hybridization probes to detect latent CCV in fish shows that CCV is present

in many fish populations, including those that have no record of the disease (reviewed in [6]). CCV is cited as the cause of deaths in fewer than 3% of cases submitted to diagnostic laboratories (reviewed in [6]). However, due to the ubiquity of CCV in culturing channel catfish, the virus is an important economic consideration for the catfish industry.

CCV infected cells are most likely targeted by channel catfish NK-like cells, as reported in mammalian herpesviruses. NK cells efficiently lyse herpesvirus-infected cells in a MHC-unrestricted manner. The recognition of herpesvirus-infected cells by cytotoxic cells requires only early virus gene expression [7]; thus, it could be very beneficial to catfish to induce a rapid innate response against CCV. Vaccination against CCV is currently not being used due to a lack of efficient delivery of the available vaccines. Of the different vaccines, nucleic acid-based vaccines are the most promising. They induce rapid innate immune responses to viruses, as well as antigen-specific protection in fish [8] [9] [10] [11] [12] [13] [14] [15].

1.0.1 Nucleic acid vaccines

A typical DNA vaccine is composed of a bacterial plasmid that contains a complementary DNA (cDNA) encoding a protein antigen from a virus. Antigen presenting cells (APC), such as dendritic cells, are transfected by the plasmid and the cDNA is transcribed and translated into immunogenic peptides that are presented on cell surfaces and elicit specific immune, B and T cell responses. DNA vaccines provide strong protection because they produce viral proteins that are correctly folded and modified by the host cell so that viral peptides are presented by both class I and II MHC molecules,

thus providing long-lived humoral and cell-mediated immune protection of the host (reviewed in [8]). This protection is caused by specific immunity consisting of antibodies and CTL responses. DNA vaccines are able to induce a strong CTL response because the DNA-encoded proteins are synthesized in the cytosol of transfected cells and presented on MHC class I proteins on the cell membrane. Additionally, DNA vaccines provide strong protection because plasmid DNA is rich in unmethylated CpG motifs that are recognized by Toll-like receptor 9 (TLR9) on macrophages, T and B cells. The CpG motifs activate APCs and increase the expression of surface molecules that induce and enhance the adaptive immune response. These CpG motifs also induce release of interleukins IL-1, IL-12, IFN-γ and tumor necrosis factor alpha (TNF-α) (reviewed in [8]). Thus, plasmid DNA vaccines are effective and their CpG motifs may act as an intrinsic adjuvant (reviewed in [16]). Kim *et al.* [8], showed that CpG motif-containing DNA acts similarly in fish as has been reported in mammals.

1.0.2 Mass spectrometry to identify genes encoding structural proteins of CCV for vaccination

Davison and Davison [5] used mass spectrometry to compare herpes simplex virus type 1 (HSV-1) to herpesvirus CCV because of their common virion morphology. This commonality enabled Davison and Davison [5] to identify 12 genes, 11 viral and 1 cellular, which encoded 16 major structural proteins that could be used as possible antigenic determinants for CCV. Some of these proteins are late proteins which generally are large, stable and antigenic. These structural proteins made up the mature capsid, immature capsid, the tegument, and the envelope associated proteins.

Sequence analysis of the proteins found in CCV showed that CCV lacked envelope glycoproteins that are homologous to envelope glycoproteins found in mammalian or avian herpesviruses. Gene *59* encodes the principal envelope protein of CCV. The protein encoded by gene 59 contains four stretches of hydrophobic residues capable of spanning the membrane, and if it is folded in an orientation in which the termini are inside the envelope, this would position a loop containing three potential N-linked glycosylation sites on the external surface of the virion [5]. Although multiple hydrophobic proteins are a feature to all herpesviruses, CCV is unusual in that a protein with so many hydrophobic domains forms the main envelope protein. Some membrane proteins that are not major envelope proteins are encoded by seven other CCV genes, include genes *6*, *7*, *8*, *10*, *19*, *46*, and *51*. Gene *59* proteins are prime candidates for the major antigenic determinants of CCV [5] and [3]. In fact, Nusbaum *et al.* [3] selected CCV genes *59*, *6*, *7*, *8a*, *10*, *51* and *53* to be used in DNA vaccinations against CCV, because of the size, putative order of expression, and association with membrane structures as envelope glycoproteins, or function as viral capsid proteins of the proteins encoded by these genes.

1.0.3 Using nucleic acid vaccines in channel catfish

Nusbaum *et al.* [3] were able to induce protective immunity in channel catfish using a DNA vaccine. Seven full-length transcripts from the CCV genome were selected for the DNA vaccination. ORF 59 encodes a glycoprotein associated with the envelope; ORFs 6, 7, 10 and 51 encode membrane proteins; ORF 8a encodes a membrane-associated protein; and ORF 53 encodes the viral capsid protein. Channel catfish were vaccinated with either a single or a pair of ORF(s) inserted into an expression vector. Solutions of DNA containing one or two of the seven CCV ORFs, vector alone, or PBS were

injected intramuscularly into 6 to 10 month old catfish [3]. Four to six weeks post vaccination, the catfish were challenged by immersion with one LD_{50} of CCV/ml [3]. When compared to other single ORF vaccinations, fish vaccinated with ORFs 59 and 6 alone showed the strongest resistance to CCV challenge, with a 74% survival rate and a 39% survival rate, respectively. Non-injected, PBS injected, or vector injected fish showed only a 34% to 56% survival rate [3]. The combination of the vaccine pair ORF 59 and ORF 6 proved to be even more effective against challenge with a 78% survival rate. The enhanced protection suggests that CCV possesses multiple targets encoding neutralization antigens [3].

Two weeks after the challenge, surviving fish were bled to assay for neutralizing antibodies. All fish injected with vaccines, including the control group, showed neutralization activity, although barely detectable in control fish. All of the CCV ORF vaccinated groups developed an increase in neutralizing antibodies following sublethal viral challenge, however in fish vaccinated with ORFs 59 and 6, protection was seen and neutralizing antibodies increased by two-fold.

1.0.4 DNA vaccines induce IFN expression

DNA vaccines encoding viral glycoproteins not only provide long-term specific immunity, but have also been found to induce interferon (IFN) expression in rainbow trout [8]. Kim *et al.* [8] found that initial protection provided by DNA vaccines is due to IFN type I expression.

Previous experiments [17] showed that injection of a plasmid expressing a viral glycoprotein to infectious hematopoietic necrosis virus (IHNV) provided protection, although it was unclear whether this protection was due to a specific immune response.

The first appearance of the anti-glycoprotein antibody was around 8 weeks post vaccination, yet protection against IHNV challenge was observed as early as 3 to 4 weeks post vaccination. Also, virus-neutralizing antibodies at low titers only appeared in some fish around 6 weeks post vaccination. To further understand if glycoprotein-encoding DNA vaccines induce specific protection, Kim *et al.* [8] vaccinated fish against heterologous viruses and challenged the fish with another virus. The DNA vaccines encoded viral glycoproteins chosen from three serologically distantly related fish rhabdoviruses, including IHNV, spring viremia of carp virus (SVCV), and snakehead rhabdovirus (SHRV). The three different groups of vaccinated fish, plus two control groups injected with either PBS or an empty DNA plasmid, were all challenged with lethal doses of IHNV 30 days post vaccination (dpv) and later at 70 dpv. They found that significant heterologous protection existed against IHNV 30 dpv in fish vaccinated against SVCV and SHRV. However, this protection did not persist against IHNV challenge at 70 dpv for the same fish vaccinated against SVCV and SHRV. Kim *et al.* [8] believe their results suggest that DNA vaccination with glycoprotein genes induce a potent IFN response in fish producing heterologous protection [8].

At 30 dpv, the relative percent survival (RPS) of fish challenged with IHNV is 93% for fish vaccinated against IHNV, 98% for fish vaccinated against SHRV, and 95% for fish vaccinated against SVCV [8]. When the control groups were challenged 30 dpv, they had a cumulative percent mortality (CPM) of 55% for PBS injected fish, and 57% for plasmid-injected fish. At 70 dpv, the RPS value of fish challenged with IHNV was only 26% for fish vaccinated with SHRV and 17% for fish vaccinated with SVCV, whereas IHNV vaccinated fish had a RPS value of 87%. Again, the control groups demonstrated

little protection; PBS injected fish had a CPM value of 96% and the plasmid injected fish had a CPM value of 91%.

To confirm the induction of an IFN response, Kim *et al.* [8] measured Mx protein production in the fish from day 0 (30 dpv) to 7 days post challenge (dpc) with IHNV. In the fish that received vaccines, increased Mx expression was observed before challenge with IHNV. After challenge, Mx expression in fish vaccinated against IHNV ceased by 7 dpc, and Mx expression in fish vaccinated with SHRV also decreased over 7 dpc (although did not cease), while Mx expression in fish vaccinated against SVCV showed no change over the 7 dpc. In control fish samples, Mx expression was not observed at day 0. However, post challenge, Mx expression increased so that by day 7 there was strong expression of Mx proteins in the control fish [8]. Thus, Kim *et al.* [8] concluded that decreased Mx production upon viral challenge correlates with the specificity of the glycoprotein in the vaccine. They postulated that this could be due to the removal of the glycoprotein-expressing cells by specific immunity upon viral challenge [8] or it could be due to removal of the virus.

Kim *et al.* [8] concluded that the fish vaccinated against IHNV, SVCV, and SHRV were protected against IHNV challenge at 30 dpv due to induction of IFN-α/β. This conclusion was based on the fact that the protection SVCV and SHRV vaccinated fish had at 30 dpv was no longer present at 70 dpv. Kim *et al.* [8] believed that at 30 dpv IFN-α provided early and effective protection against virus infection, yet over time, the antiviral state provided by IFN-α diminished, as a result of decreasing glycoprotein production or as a result of some other down regulation event [8]. As the general antiviral state lessened, the long-term specific protection provided by the DNA vaccine became

the important player in immunity, so that by 70 dpv only the IHNV vaccinated fish maintained strong protection against IHNV challenge.

Other experiments (referenced unpublished data by Kim CH in [8]) helped to confirm their conclusion that IFN-α is the source of protection in glyprotein DNA vaccinated fish. In this study, (referenced unpublished data by Kim CH in [8]) when fish were injected with the DNA vaccine for IHNV, IFN-inducible Mx protein expression was found in the liver and kidney tissues of the fish. However, when fish were injected with formalin-killed vaccines, subunit IHNV vaccines, or vaccine controls, no Mx protein expression was found. Kim *et al.* [8] considered the possibility that the production of the Mx protein itself provided nonspecific protection for the heterologous vaccines at 30 dpv. However, cell culture experiments showed Kim *et al.* [8] that the over-expression of Mx proteins did not inhibit replication of IHNV, leaving Kim *et al.* [8] to conclude that IFN, not Mx proteins provided nonspecific protection.

Similarly, in their work with DNA vaccines in rainbow trout against viral hemorrhagic septicemia virus (VHSV) and infectious hemorrhagic necrosis virus (IHNV), McLauchlan *et al.* [12] showed that these vaccines not only stimulated antibody production in the fish, but the vaccines also induced the expression of Mx proteins in the muscle tissues of the fish at the site of injection of the vaccine. The control DNA plasmid, which lacked the viral glycoprotein gene insert, did not induce any of these responses in the fish. Protection against VHSV was found as early as 1 week after the DNA vaccination was administered even though antibodies to VHSV were not detected until 4 weeks post-vaccination. Protection was consistent with Mx protein expression. DNA vaccines seemed to induce a rapid protection against viruses mediated by the innate nonspecific

IFN responses, while long-term protection is mediated by the specific immune response [12].

Chapter 2

Literature Review

2.1 Introduction to fish immunity

Fish, like homeothermic vertebrates, have both innate and specific immunity. The innate defense mechanisms are nonspecific and can be pre-existing and/or inducible. This nonspecific innate defense system provides protection that prevents the attachment, invasion or replication of a pathogen on or in the tissue of fish. Some even believe fish depend more on their innate defense mechanisms because innate responses are essentially temperature independent [2] [20]. These characteristics are very important for ectothermic vertebrates because the specific immune defenses take more time to respond and are temperature dependent (reviewed in [2]). For channel catfish, a species living in a temperate climate, optimal antibody production takes 2 to 4 weeks, yet many pathogens can kill a fish within a few days of infection. For this reason it has been suggested that protection offered by specific immune responses is only more important for survival than innate responses in previously immunized fish [2]. Because fish are always in close contact with their environment, which can contain high concentrations of bacteria or viruses, it is possible that innate defenses are more important in fish than in endothermic vertebrates [2]. For this reason there have been several studies looking at non-specific protection in fish. Having a better understanding of the innate immune system in fish not only helps researchers find different ways to control fish diseases, but also provides insight into how the adaptive immune system evolved.

2.2 Introduction to IFN

Infection of cells with viruses induces the expression of a family of proteins that are known as interferons (IFNs), because they interfere with viral replication [21]. IFN was discovered and named by Isaacs and Lindenmann [21] as an antiviral factor secreted by chick cells after treatment with heat-inactivated influenza virus. Interferons induce an antiviral state in vertebrate cells and therefore play a major role in the defense against viral infections [22]. The first IFN genes cloned were human IFN-α and IFN-β in 1980 [23] [24]. IFN-like activity was detected in fish as early as 1965 [25], and has since been detected in number of fish species after viral infection or treatment with double-stranded RNA or poly I:C [26] [8] [27] [28], but fish IFN genes were not cloned until recently [29] [30] [31] [32].

The three families of IFNs (type I and type II and IFN-III) can be distinguished on the basis of gene sequences, protein structure, and functional properties [22]. Although all three IFN types belong to the class II α-helical cytokine (HC) family, they have different 3-dimentional structures and bind to different receptors. Type I IFNs, including the classical IFN-α/βs, are antiviral effector molecules induced by viruses in most cells, encoded by intron-lacking genes, and bind to the single type I IFN receptor composed of two chains, IFNAR1 and IFNAR2. IFN-γ, which belongs to the type II IFN family, although not directly induced by viral infection, is produced by natural killer (NK) cells and T lymphocytes in response to interleukin-12 (IL-12), IL-18, and mitogenic and antigenic stimuli, and binds to a receptor composed of two chains, IFNGR1 and IFNGR2 (reviewed in [33] [22]). IFN-γs are quite distinct from type I IFNs and have a more important role in the adaptive immune response to intracellular pathogens (reviewed in

[22]). The human type III IFNs or IFN-λs share the same biological properties as type I IFNs, although they possess lower antiviral activity than IFN-α/β. Also, unlike type I IFNs, IFN-λs are encoded by intron-containing genes and bind to a receptor that is distinct from the type I IFN receptor [34]. This receptor consists of subunits IFN-λR1 and IFN-10R2 [34].

2.2.1 Type I IFN antiviral pathway in higher vertebrates

Mammalian type I IFNs make up a multigene family with at least 8 subclasses, IFN-α, IFN-β, IFN-ω, IFN-τ, IFN-κ, IFN-ϵ, IFN-δ, and IFN-ζ (limitin) (reviewed in [35] [36]). In humans, there are 13 IFN-α genes, 1 IFN-β gene, and 1 IFN-ω gene. Mononuclear phagocytes are the major source of IFN-α production, and IFN-β is produced by many cells, such as fibroblasts, epithelial cells, endothelial cells, lymphoid cells and astrocytes (reviewed in [35]). IFN-α, IFN-β and IFN-ω are expressed in several different species whereas the other type I IFNs have more exclusive expression. IFN-τ is only expressed in ruminant species during a specific stage in pregnancy [37]. IFN-κ is only expressed by human keratinocytes [38]. IFN-ζ, also known as limitin, is expressed only in murine cells and shares some homology with IFN-α, IFN-β, and IFN-ω [39]. IFN-δ is only produced by the trophoblasts of pigs [40].

Mammalian type I IFN vary in size (143 to 172 amino acids) and number of disulfide bonds (0 to 2), but crystal structures show that mammalian type I IFN share a common three-dimensional structure composed of 5 α-helices [41] [42] [43] [44]. Type I IFN genes encode secretory signal peptide sequences that are proteolytically cleaved before secretion from the cell (reviewed by [35]).

The most potent stimulator of type I IFN production is viral infection; specifically, it is the double-stranded RNA produced by viruses during their replication in infected cells (reviewed in [22]). Other microbial products such as glycoproteins can stimulate IFN production (reviewed in [1]). All type I IFNs bind to the same receptor containing the IFNAR-1 and IFNAR-2 subunits even though all the different type I IFNs are not structurally the same [35]. When IFNs bind to their receptors, a rapid and direct signaling pathway, known as the JAK-STAT signaling pathway, effects activation and deactivation of gene expression in the nucleus. Inactive enzymes called Janus kinases (JAKs) are loosely attached to the cytoplasmic domains of IFN receptors. When IFN binds its receptor, the two IFN receptor molecules are brought together and this dimerization of the receptor molecules activates the IFN receptor, bringing the associated cytoplasmic kinases, JAK1 and Tyk2, within close proximity of each other so that they are able to trans-phosphorylate each other (reviewed in [45]).

The activated receptor-associated JAKs phosphorylate tyrosine residues in the cytoplasmic portion of the clustered receptors, and the phophorylated tyrosines from the IFN receptors recognize and bind to the Src homology 2 (SH2) domains located in monomeric cytosolic transcription factor proteins called signal transducers and activators of transcription (STATs). When the STAT proteins attach themselves to the IFN receptors, they become phosphorylated by the receptor-associated JAK kinases. The SH2 domain of one STAT protein is able to bind to the phosphotyrosine residues of another STAT protein to form a dimer. Mostly STAT1 and STAT2 proteins, although to a lesser degree, STAT3 and STAT4 are phosphorylated. STAT1 can homodimerize, although it is mostly STAT1-STAT2 heterodimers that form. When two STAT proteins form a dimer, they dissociate from the interferon receptor and migrate to the nucleus where they bind

to IFN's promoter/enhancer regions and activate gene transcription (reviewed in [45]). Oddly enough, STAT2 cannot bind directly to DNA, so STAT1-STAT2 heterodimer complexes are recruited to DNA sequences through interaction with a DNA binding protein called interferon regulatory factor (IRF)-9. The three proteins form a multimeric transcription factor called ISGF3, which binds to interferon-stimulated response elements (ISRE) in the promoter of interferon-stimulated genes and activates their transcription (reviewed by [46][45]).

IFN type I induction in mammals includes the expression of close to one hundred genes and some of these gene products block transcription of viral RNA or DNA and viral replication by either activating or inhibiting protein synthesis and by degrading mRNA in virus-infected cells. The genes from three families of IFN-inducible genes that are best studied participate in inhibiting viral replication. They include: protein kinase (PKR), the 2′,5′-oligoadenylate synthetases (OAS), and the Mx proteins (reviewed by [45]).

PKR, when activated by viral double-stranded RNA (dsRNA) which it binds during viral infection, phosphorylates and inactivates the eukaryotic protein synthesis initiation factor eIF-2α, which leads to a general inhibition of protein synthesis. Thus, activation of PKR in virus-infected cells leads to the inhibition of protein expression and viral replication (reviewed in [45]).

OAS, also activated by viral dsRNA, catalyzes the synthesis of 2′,5′-oligoadenylates, which in turn bind to inactive monomeric RNase L, which induces dimerization and activation of RNase L. The activated RNase L degrades both viral mRNA and rRNA in the cytoplasm of the cell, which leads to the inhibition of protein expression (reviewed in [45]).

Mx proteins, only induced by type I IFN, (reviewed in [22]) are homologous to dynamins and can be located in the cytoplasm or nucleus. Not all Mx proteins have antiviral activity, although some Mx proteins when expressed in the cell offer some resistance to infection by several RNA viruses. The antiviral mechanism of Mx proteins is still not completely understood, although it is believed that Mx proteins interfere with trafficking and/or the transcriptional activity of the viral ribonucleoprotein complexes (reviewd in [47]).

2.2.2 Type II IFN function in higher vertebrates

Type II IFN, or IFN-γ, is also called immune IFN because unlike type I IFNs, IFN-γ is produced exclusively by cells of the immune system. IFN-γ plays a major role in adaptive cell-mediated immune responses because it is produced by $CD4^+$ T helper (T_H1) cells, and $CD8^+$ cytotoxic T lymphocytes (CTLs) in response to MHC-presented antigens. IFN-γ also plays a role in innate responses when mononuclear phagocytes and/or antigen presenting cells are infected with intracellular pathogens and release IL-12 and/or IL-18 stimulating NK cells to produce IFN-γ. (reviewed in [22]).

IFN-γ binds to a different receptor from type I IFNs and mediates signaling through a different, but overlapping JAK-STAT pathway. IFN-γ has some antiviral activity by inducing in most cells PKR and $2'$,$5'$-oligoadenylate synthetases, yet IFN-γ is not a potent antiviral interferon, and it functions mainly as an effector cytokine in immune responses (reviewed in [22]). Another difference between type II IFN and type I IFN is that mammalian and avian IFN-γ are encoded by a single gene containing four exons and three introns and show no sequence similarity to type I IFNs.

There are several unique functions of IFN-γ that are important in cell-mediated immunity against intracellular pathogens. One function of IFN-γ is that it is the macrophage-activating cytokine. This means that IFN-γ provides the means by which T lymphocytes and NK cells activate macrophages to kill phagocytosed microbes. IFN-γ enhances the microbicidal function of macrophages by stimulating the synthesis of reactive oxygen intermediates and nitric oxide (reviewed in [22]).

Another action of IFN-γ is that it stimulates expression of class I and class II MHC molecules and costimulators on APCs. IFN-γ also stimulates the synthesis of many proteins involved in antigen processing. Thus, IFN-γ boosts MHC-associated antigen presentation and strengthens the recognition phase of the immune response by enhancing the expression of the ligands that T cells recognize (reviewed in [22]).

An additional function of IFN-γ is that it promotes the differentiation of naïve $CD4^+$ T cells to the T_H1 subset and inhibits the proliferation of T_H2 cells. IFN-γ also maintains T_H1 effector cells by activating mononuclear phagocytes to produce IL-12, which in turn will produce more IFN-γ, the major T_H1-inducing cytokine (reviewed in [22]).

2.3 Antiviral pathways in fish

IFN genes have been recently cloned from different fish species including channel catfish [29], zebrafish [30], Atlantic salmon [32], the Japanese pufferfish *Takifugu rubripes* (Fugu), and the spotted green pufferfish *Tetraodon nigrovirides* [31]. Only one functional IFN gene has been cloned from zebrafish and pufferfish genomes. Two functional genes were found in Atlantic salmon and catfish, and it has been suggested that additional

IFN genes and pseudogenes exist in the Atlantic salmon genome [32], and at least 2 pseudogenes have already been found in the catfish genome.

The IFN gene for zebrafish (zfIFN) was cloned and characterized by Altmann *et al.* [30] by searching the Zebrafish Information Network EST database using BLAST with a gene for chicken IFN. Only one EST containing all but the $5'$ end of the zfIFN gene was found. Upstream RACE-PCR was used to expand the zfIFN gene.

Robertsen *et al.* [32] cloned two type I IFN cDNAs from Atlantic salmon TO cells which originate from Atlantic salmon head kidneys. They induced IFN mRNA by exposing Atlantic TO cells to poly I:C and used suppressive subtractive hybridization (SSH) techniques to generate cDNA from the cells. Using the subtracted cDNA as a template, and a degenerate forward primer from the conserved vertebrate type I IFN sequence motif YSACAW at the C-terminus, PCR amplification of the IFN-like cDNA was accomplished. These sequences allowed design of a specific gene primer that could be used to amplify the $5'$-end of the Atlantic salmon IFN by RACE cloning. Two $5'$-end IFN products were obtained using cDNA from the head kidney of poly I:C stimulated fish. Based on these new sequences, new primers were designed to amplify the whole ORF of both genes by $3'$ RACE cloning. Two putative IFN cDNAs were amplified, one 829 bp and the other 1290 bp. The two clones were called SasaIFN-α1 (829 bp) and SasaIFN-α2 (1290 bp). However, there is a possible third salmon IFN gene yet to be cloned [32].

Lutfalla *et al.* [31] used a strategy to find pufferfish IFN gene based on conserved amino acid sequence and gene structure to identify class II helical cytokines (HCII) and their receptors (HCRII) in the genome of pufferfish, *Tetraodon nigroviridis*. The

helical cytokine family includes interferons, most interleukins, LIF, CNTF, GCSF, GM-CSF, and thrombopoietin. These helical cytokines have no similarities at the level of primary amino acid sequences, but they are all structured around a similar four alpha helix bundle, and therefore share a common 3-D structure (as reviewed in [31]). The *T. nigroviridis* genome was searched for exons capable of coding for molecules structurally related to the IFNs and the IL10 related cytokines [31]. IFNs and IL-10 are not encoded by genes that share similar intron/exon structures; type I IFN genes do not contain introns, whereas type II IFN genes contain three introns, and IFN-λ and IL-10 related genes contain four common introns. Thus, the intron/exon structure to identify IFN and IL-10 cytokine homologs in distant species could not be used [31]. Their strategy identified three IL-10 related cytokines genes and a single IFN gene [31]. The Fugu genomic data was also examined and a Fugu IFN gene was identified [31].

To clone a channel catfish IFN cDNA, Long *et al.* [29] created an EST library and a single clone with sequence homology to rat and mouse IFN α was identified. However, this IFN-1 gene did not contain a classical signal sequence within the first 70 amino acids of the catfish IFN-1 amino terminus [29]. Since this gene provided protection against viral challenge, it was assumed IFN-1 was a valid expressed gene, not a pseudogene, and that the missing signal sequence was not important for function in catfish [29]. However, it has since been determined that without the expressed signal sequence, the IFN-1 channel catfish gene is in fact an expressed pseudogene and cDNAs representing three additional IFN genes have been cloned from channel catfish, where two of these genes, IFN-2 and IFN-4, are functional IFN genes including signal sequences [48].

2.3.1 Characterization of fish IFNs

All of the fish IFN genes translate into putative precursor proteins that contain between 175 to 194 amino acids and have signal peptides of 22 to 24 amino acids. The zfIFN gene codes for a protein that is 185 amino acids in length, with the first 22 amino acids representing a putative signal sequence [30]. Both translated Atlantic salmon IFN-α clones translate into 175-amino acid peptides that shows 95% amino acid sequence identity and 98% nucleotide sequence identity to each other. Both Atlantic salmon IFN-α genes encode a hydrophobic 23 amino acid signal sequence at the N-terminus so that the mature Atlantic salmon IFNs contain 152 amino acids. When the Atlantic salmon IFN-α1 precursor protein was aligned with 24 different type I IFNs from other vertebrates, Robertsen *et al* [32] found that the signal sequence from the salmon IFN gene showed hardly any homology with the signal sequences from higher vertebrates (0-9%), with the exception of pig IFN-δ showing 19% identity to Atlantic salmon IFN-α1 [32]. However, it was demonstrated that the signal sequences of fish IFNs display some homology amongst themselves (around 32% identity) [32].

The properties of putative mature fish IFNs were compared and it was found that fish IFNs are similar in size (between 152 to 170 amino acids) and similar to mammalian IFNs [49]. Catfish IFN-2 is the largest fish IFN with 170 amino acids [48] while Atlantic salmon IFN-α1 is the smallest with 152 amino acids [32].

The isoelectric points of catfish, zebrafish, and Atlantic salmon IFNs are basic [48], [30], [32]. However, Fugu and pufferfish IFNs both have acidic isoelectric points [31]. This difference in isoelectric points is not uncommon, as type I IFNs of higher vertebrates also show large differences in isoelectric points (reviewed in [49]).

Potential N-linked glycosylation sites were found in all fish IFNs except those of zebrafish [30]. The number of glycosylation sites varies between fish species with salmon and catfish IFN-1 genes encoding 1 [32] [29], catfish IFN-4 containing 2 [48], catfish IFN-2 and pufferfish IFN containing 3 [48] [31], and Fugu IFN containing 4 potential glycosylation sites [31]. Again, it is not uncommon for higher vertebrates to have a varied number of N-linked glycosylation sites between type I IFNs, from having none in most IFN-α proteins, to 3 in mouse IFN-β (reviewed in [49]). Salmon IFN contains its N-glycosylation motif at amino acid 124, whereas channel catfish IFN-1 contains its glycosylation site at amino acid positions 27-30, which is characteristic of mammalian IFN β [29]. Thus, it was argued that glycosylation does not appear to be conserved through evolution [49].

The salmon, pufferfish, Fugu and zebrafish all contain 2 cysteines in their mature putative IFNs [32] [31][30], while all catfish IFN proteins contain 3 cysteines [48]. Based on the number of cysteines, the mature fish IFN proteins seem to show similarities to IFN-alpha proteins from higher vertebrates.For example, the putative mature salmon IFNs have a cysteine as the first amino acid, which is similar to mammalian IFN-α. The two cysteine residues in zebrafish IFN are conserved among all IFN sequences except mammalian IFN-β [42] [43] [44]. However, mammalian and avian IFN-α genes generally code for four or more cysteine residues and the encoded proteins may form 2 or more disulfide bonds, whereas all the fish IFNs only have the potential to form 1 disulfide bond, which is similar to mammalian IFN-β [29] [48] [49]. Robertsen *et al.* [32] believe this demonstrates that the ancestral vertebrate IFN possessed only one pair of cysteines and that the other pair appeared at a later stage, but before the divergence of the birds.

All the cloned IFN genes from the different fish species contain five exons and four phase 0 introns [48] [30] [32] [31], as opposed to mammalian and avian type I IFN genes which do not contain introns (reviewed in [32] [49]). However, it is not uncommon for genes to lose introns in vertebrate evolution [18]. The fish type I IFNs have the same exon/intron structure as mammalian IL-10 and IFN-λ genes. This suggests that possibly type I IFN genes and IL-10 genes evolved from a common ancestor [31]. In fact, the three IL-10 related cytokines discovered in pufferfish by Lutfalla *et al.* [31] also each had four phase 0 introns. Long *et al.* [48] also showed that the locations of the second and third introns in teleost IFN genes are the same as those of mammalian IFN-λ. When Lutfalla *et al.* [31] aligned their *T. nigroviridis* IFN and zebrafish IFN genes with different mammalian type I IFNs and human IFN lambdas, they found that both fish IFNs were more closely related to human IFN-λs.

Atlantic salmon and zebrafish IFN genes share similar size exons, however the introns, for the most part, are smaller in the salmon than in the zebrafish [32] [30]. It was also noted that the trend for intron sizes to differ between fish, but exon sizes between the different fish species were similar, with the only exception of the catfish IFN-1 exon 1, which lacks a signal sequence [48].

Robertsen [49] showed that 17 amino acid positions appear to be conserved among type I IFNs from fish and higher vertebrates. These conserved amino acids might be important for the stabilization and/or activity of the IFNs. Leu30, Arg33 and Phe36 are three conserved residues in human IFN that are thought to be involved in binding to the IFN-$\alpha/\beta/\omega/\delta/\kappa/\tau$ group IFN receptor [50]. Leu30 and Arg33 do not appear in the fish or avian IFNs, while Phe36, shown to be important for biological activity, is conserved in zebrafish and salmon [30] [49].

Although mammalian type I IFNs vary in size and number of disulfide bonds, crystal structure determinations show that mammalian type I IFNs share a common three-dimensional structure composed of 5 alpha helices (reviewed in [32]). The helical cytokine family, which includes interferons, has similarities at the primary amino acid sequence level and is structured around a similar four alpha helix bundle [42] and, therefore, share a common 3-D structure. Robertsen *et al.* [32] analyzed the secondary structure of salmon IFN and found that salmon IFN has 5 alpha helices which overlap with the helices of other mammalian type I IFNs. In fact, Robertsen *et al.* [32] found that the most conserved amino acids in salmon IFNs appeared to occur in and adjacent to these 5 alpha helices, with the most conserved region of the salmon type I IFN gene being in the C-terminal-coding region.

2.3.2 Functional studies of type I IFN in fish

For each IFN gene cloned from the different fish species, studies have been performed to show that they have the same functional properties as mammalian type I IFNs.

Double-stranded RNA poly I:C, which is a well known inducer of type I IFNs in mammals, induced transcripts of type I IFNs at various times in channel catfish [29], zebrafish [30], and Atlantic salmon [32]. To determine the extent to which zebrafish IFN (zfIFN) could be induced by poly I:C *in vitro*, Altmann *et al.* [30] treated zebrafish liver (ZFL) cells with 25 μg of poly I:C/ml and RNA was extracted 6, 12, 24, 36 and 48 hours later. Quantitative qPCR results showed zfIFN expression at 6 hours and an increase in expression by 12 hours. By 24 hours zfIFN expression had ceased. It was found that there was a low level of constitutive expression at all times [30]. When channel catfish ovary cells (CCO) were induced with poly I:C, catfish IFN-1 mRNA peaked at 2 hours

and declined thereafter [29]. However, when Atlantic salmon head kidney TO cells were stimulated with poly I:C, IFN expression occurred 12 to 24 hours after poly I:C exposure, with the highest level of expression occurring at 24 hours post stimulation. It was also shown that IFN was expressed in salmon [32]. Investigators used northern blot analysis to show that two IFN transcripts of the correct sizes were expressed in the head kidney of Atlantic salmon 14 hours after injection of poly I:C at relatively low concentrations. The highest level of IFN expression was observed 12 hours after injection of poly I:C, and this expression diminished by 24 and 48 hours post injection. Robertsen *et al.* [32] concluded that IFN transcripts were induced by poly I:C more rapidly in live fish than in TO cells because cell populations in fish, perhaps leukocytes, are more reactive to poly I:C than the cell cultured head kidney TO cells.

Recombinant IFNs from pufferfish, channel catfish and Atlantic salmon have been expressed in eukaryotic cells or by *in vitro* translation [31] [29] [32] to show the protective activity of the fish IFNs. Mx protein was induced by salmon and pufferfish IFNs [32] [31]. To study whether the Atlantic salmon IFN-α1 gene encoded a biologically active IFN, Robertsen *et al.* [32] subcloned it into a eukaryotic expression vector pCR3.1 in forward and reverse orientation from the cytomegolovirus (CMV) promoter. The constructs were transfected into human embryonic kidney 293 (HEK293) cells. The media supernatants from the HEK293 cells were collected 48 hours later, acid treated over night, and then assayed for antiviral activity against infectious pancreatic necrosis virus (IPNV) in Atlantic salmon TO cells or in the Chinook salmon CHSE-214 cell line. Supernatants from HEK293 cells transfected with the SasaIFN-α1 gene in the correct orientation showed relatively high levels of antiviral activity when tested on TO cells and

CHSE-214 cells. The HEK293 cells that were transfected with the Atlantic salmon IFN-α1 gene in the reverse orientation gave no IFN activity in either cell system. Robertsen *et al.* [32] also demonstrated that supernatants of HEK293 cells transfected with the salmon IFN-α1 gene in the forward orientation induced the Mx protein in the CHSE-214 cells and TO cells.

The biological activity of pufferfish IFN (TnIFN) was tested by assessing expression of the TnMX gene [31]. The TnIFN ORF was cloned into the same plasmid and used to produce recombinant TnIFN [31] . The recombinant TnIFN was used to challenge primary cultures of *T. nigroviridis* cephalic kidney cells. After a 6 hour exposure to recombinant TnIFN, total RNA was isolated from cells, and the amount of TnMX mRNA expressed was measured. When Lutfalla *et al.* [31] intraperitoneally injected poly I:C into the *T. nigroviridis*, a large amount of TnIFN was induced in the testis and kidney of the pufferfish. This treatment with poly I:C was used as a positive control to induce TnIFN in the pufferfish. Compared to poly I:C treatment, the recombinant TnIFN molecule induced a similar level of TnMX mRNA expression in pufferfish. Expression of the two PKR genes in *T. nigroviridis* was used to verify the results found with TnMX expression; both PKR genes were induced by the recombinant TnIFN just like the single TnMX gene [31].

When zebrafish cells were transfected with the zebrafish IFN gene, they showed increased resistance against infection by snakehead rhabdovirus and increased Mx gene expression [30]. Zebrafish ZF4 cells, chosen for their ability to form plaques when infected with snakehead rhabdovirus (SHRV), were transfected with zfIFN and 16 hours later were exposed to SHRV for 1 hour. Altmann *et al.* [30] were able to show that zfIFN can protect ZF4 cells against viral infection when the gene is transfected 16 hours prior to

viral challenge. There was a 31% reduction in plaque number in zfIFN-transfected cells compared to untransfected cells and a 36% reduction compared to cells transfected with the control pcDNA3 vector. An experiment was performed to determine if the zfIFN gene was capable of inducing the Mx gene [30]. This was done by cotransfections of ZF4 cells using the Mx promoter-pGL3 luciferase construct and the zfIFN gene. Expression from the Mx promoter induced by IFN expression is necessary to drive luciferase expression. ZF4 cells transfected with Mx promoter construct alone were induced with poly I:C as a positive control. The negative control was an Mx promoter construct cotransfected with control pcDNA3 vector to show that random DNA does not induce the Mx gene. ZF4 cells cotransfected with zfIFN and the Mx promoter construct had greatest luciferase activity at 5.6 X 10^3 relative light units (RLU), while poly I:C induction yielded an average of 4.6 X 10^3 RLU [30].

The catfish and salmon IFNs demonstrated antiviral activity against channel catfish virus (CCV) and infectious pancreatic necrosis virus (IPNV), respectively [29] [32].

2.3.3 Alignment analysis of fish IFNs

Using alignment analysis, it was demonstrated that a low homology between the fish IFN amino acid sequences and IFN sequences from higher vertebrates existed [49], [48], [32], [31], [30], [29]. It was also noted that fish IFNs, when compared to higher vertebrate IFN, showed better homology with IFNs from different fish species than vertebrates. For example, the catfish IFN-1 sequence showed only 28% identity and 41% similarity to rat IFN α and 30% identity and 52% similarity to mouse IFN α, whereas the catfish IFN-1 gene showed 39% identity and 55% similarity to the zebrafish IFN gene. The different fish IFN genes were aligned and percent identity was calculated [49]. It was found that

IFN sequences from catfish, zebrafish, and Atlantic salmon are most similar, showing 43-46% identity, whereas the pufferfish and Fugu IFNs showed 23-35% identity with the other fish IFNs. The highest percent identity/similarities are shown between IFN gene sequences from the same fish species. The fugu (*T. rubripes*) and pufferfish (*T. nigroviridis*) IFNs are more similar to each other (67.6% similar) and less similar to the other fish IFNs, and they share the least similarity with catfish IFNs (between 15.3 and 17.6% similarity) [48]. The two Atlantic salmon IFNs have 97% sequence identity [32] and the catfish IFNs show between 66.9 to 81.6% similarity [48]. Interestingly, zebrafish and goldfish IFNs share 66.1% similarity [48].

When pairwise comparisons of catfish IFN proteins with the different mammalian and avian IFNs was performed, the highest similarity was found to be with mammalian IFN-α at 12-19% similarity [48]. The other comparisons showed even lower similarity, with catfish having 10-14% similarity to mammalian IFN-λ, 14-15% similarity to mammalian IFN-β, 12-14% similarity to mammalian IFN-γ, and 12-16% similarity to avian IFN-α/β [48]. Similarly, the mature salmon IFN protein showed highest sequence identity with IFN-α sequences and lowest identity with mouse and human IFN-β [32]. On further analysis of the alignment of catfish IFNs with other fish IFNs, it was found that there are a limited number of sections of amino acid conservation, i.e. five, were identified among the six fish species [48]. Outside these conserved areas, amino acid identities are scarce. When the analysis was expanded to include mammalian and avian IFN genes or proteins, the highest similarity was found to be to mammalian IFN-α genes, although the identity/similarity was still very low [48]. Interestingly, IFN-αs from different mammals show about the same degree of homology as that shown between the salmon and zebrafish [32].

Since teleost IFN genes share a similar intron/exon structure with IFN-λ, it has been suggested that fish IFN and IL-10 genes evolved from a common ancestor gene that also had four phase 0 introns, similar to IFN-λ [31]. Furthermore, it has been suggested that all class II helical cytokine (HCII) ligands and receptors share a common ancestor [31]. The first duplication (creating ancestral IL-10 related genes and ancestral IFN) would have taken place before the osteichthyes (bony fish) radiation [31]. Lambda IFNs seems to have evolved from ancestral IFN, while type I IFNs evolved in a retroposition event that occurred during vertebrate evolution [31]. Thus, the key event in evolution of the IFN gene was a retroposition event that occurred after separation of sarcopterygians (lobe finned fish) from actinopterygians (ray finned fish) and this event created an intronless type I IFN gene [31]. In the mammalian lineage, this gene then underwent successive duplications to generate the alpha and the beta interferons. Later, during the mammalian radiation, numerous alpha IFN genes were generated (reviewed in [51]).

Whether IFN-λ genes truly represent ancestral IFN genes is uncertain because as has been pointed out, IFN-λ shares very little sequence similarity to the type I IFNs of fish or higher vertebrates [48], [49]. Even with the structure similarity between teleost IFNs and IFN-λ, phylogenetic trees suggests that teleost IFNs are most closely related to mammalian α and β IFNs than the other IFNs [48]. The fish IFN genes could be derived from a primordial gene that predated the divergence of the teleost and tetrapod lineages [48]. Perhaps the teleost IFN genes failed to expand or diversify, whereas after the divergence of tetrapods from bony fish, their IFN genes underwent major duplication and diversification, evolving into the three main classes of IFN genes observed today in mammals. Since the duplication that gave rise to the IFN α/β gene families happened

after the divergence of teleosts and mammals, it is possible that fish IFN genes have both IFN α and β-like properties [48].

The rather low level of similarity seen between fish IFNs may be due to the increased separation time between existing fish species and their last common ancestor [48]. The high percent similarity between zebrafish and goldfish (66.1%), which are members of the same taxonomic order, Cypriniformes, supports this idea that the low level of similarity seen between the other fish IFNs from different orders is due to increased time between these species and their last common ancestor. The low level of similarity between fish IFNs might otherwise be due to the possibility that the identified fish IFNs from the different species may not be members of the same subfamily (e.g., IFN-α and IFN-β) [48]. In mammals there are differences among type I IFN classes. For example, humans and mice have multiple IFN-α genes and only a single IFN-β gene, whereas pigs, horses, and cows have at least five interrelated IFN-β genes (reviewed in [48]). Within the same species, subclasses can be very different. For example, in humans, IFN-α genes only share 50% amino acid sequence identity, whereas IFN-αs only share 22% identity with human IFN-β and 37% identity with human IFN-ω [35]. It has been suggested that 300 million years ago, when the reptile/bird/mammal split occurred, the common ancestor genome probably contained a single class of type I IFN (reviewed in [35]). The IFN-α/β gene families are suspected to be the result of gene duplication in the mammalian ancestor 250 million years ago, which is supported by the presence of distinct clusters of IFN-α and IFN-β genes within different species (reviewed in [35]). The low level of sequence identity between fish and mammalian IFN gene products further suggests the emergence of different type I IFN gene classes [48]. The low level of sequence identity between fish and mammals suggests that catfish IFNs are members of a novel IFN class

not represented in mammals, or possibly this low level of sequence identity is due to the long separation between mammals and teleost species and their last common ancestor [48].

2.3.4 Function of type II IFN in fish

IFN-γ genes have recently been cloned from Fugu (pufferfish) [52], zebrafish (GenBank accession no. AB158361) and Atlantic salmon (GenBank accession no. AY795563). Fish IFN-γ genes, like the IFN-γ genes of higher vertebrates, all contain three introns and four exons [52], (GenBank accession no. AB158361), (GenBank accession no. AY795563), [49]. The fish IFN-γ precursors contain 180 to 189 amino acids and appear to contain signal peptides of 23 to 24 amino acids so that the mature fish IFN-γs contain 156 to 167 amino acids. As expected, fish IFN-γ shows no sequence similarity to fish type I IFNs. Zou *et al.* [52] believe the Fugu IFN-γ gene to be an ortholog to mammalian and avian IFN-γ for three reasons. First, comparative analysis showed homology using BLAST analysis and 3D modeling, second, the Fugu IFN-γ gene has the same genomic structure as other known IFN-γ molecules, and finally, the synteny of the IFN-γ gene cluster is conserved between humans and Fugu. Zou *et al.* [52] need further data to determine the cellular sources and biological activity of Fugu IFN-γ in terms of regulating cell mediated immunity in fish.

Robertsen [49] aligned the fish IFN-γ sequences from zebrafish, salmon and Fugu with mammalian and avian IFN-γ, and found that fish IFN-γ sequences displayed very low sequence identity with mammalian and avian IFN-γ at 17 to 31%. In fact, the fish IFN-γ sequences showed less identity between themselves at 21 to 33% than the

mammalian IFN-γ sequences showed between themselves at 53 to 78% [49]. The diversification rate of fish IFN-γs had just as high a rate as the diversification of type I IFNs [49].

Cloning of the T cell receptor (TCR), MHC class I and II genes from fish suggests that fish probably possess T cell mediated adaptive immune responses similar to higher vertebrates (reviewed in [53]). Recently, the TCR co-receptor CD8α gene was cloned from rainbow trout and the TCR co-receptor CD4 gene was cloned from Fugu, implying the presence of both CTLs and T_H1 cells in teleost fish [54] [55]. In fact, Nakanishi *et al.* [56] demonstrated through functional studies that rainbow trout possess CTLs [56], while it was demonstrated that catfish possess NK-like effector cells that are capable of killing virus-infected cells [7]. The homologues of the IFN-γ inducing cytokines, IL-12 and IL-18 have been cloned from Fugu and rainbow trout respectively [57] [58]. Thus, teleost fish seem to contain the major IFN-γ producing cell types and cytokines found in mammals. Although functional studies of cloned fish IFN-γ have yet to be carried out, previous work in fish has shown that rainbow trout leukocytes produce a macrophage activating factor with IFN-γ-like properties [59], and a gene of an IFN-γ induced enzyme, nitric oxide synthase (iNOS), has also been cloned from rainbow trout [60].

2.3.5 JAK-STAT pathway and members in fish

Although less is known about the IFN-signaling system in fish than in mammals, several mammalian JAK and STAT homologues have been found in fish. JAK1 cDNA has been cloned from carp [61] and from zebrafish [62], the JAK1 gene has been cloned from round-spotted pufferfish *Tetraodon fluviatilis* [63]. Leu *et al.* [64] cloned JAK2, JAK3 and TYK2 genes from the round-spotted pufferfish *T. fluviatilis* and believe that all four

genes are derived from a common ancestor gene. Amino acid sequence comparison of the four round-spotted pufferfish JAKs with carp and zebrafish show that JAK1 is the most conserved between the fish species with 67.9% and 70.2% sequence identity, respectively [64]. Round-spotted pufferfish JAK1 showed 57% amino acid identity to human and murine JAK1, and carp showed high homology to mammalian JAK1 in both the kinase-like (JH2) and kinase (JH1) domains. Similarly, the pufferfish JAK2 displays high amino acid identity at 66.8% with mouse JAK2 [64]. However, the round-spotted pufferfish JAK3 and TYK2 show lower amino acid identity with their mammalian counterparts than JAK1 and JAK2 suggesting that JAK1 and JAK2 are more conserved between fish and mammals than TYK2 and JAK3 [64]. Leu *et al.* [63] also found a putative STAT-binding sequence in the JAK1 gene. STAT1 and STAT3 genes have been cloned from zebrafish [65], STAT5 gene has been cloned from round-spotted pufferfish *T. fluviatilis* [66] and STAT1 cDNA has been cloned from crucian carp [67]. Oates *et al.* [65] showed that zebrafish STAT1 protein can rescue type I IFN-signaling functions in a STAT1-deficient human cell lines, suggesting that cytokine-signaling mechanisms are likely to be conserved between fish and mammals. Crucian carp STAT1 protein was induced by poly I:C, grass carp hemorrhagic virus (GCHV), and inactivated GCHV [67]. Zhang and Gui [67] also showed that carp Mx1 transcriptional activation was under control of the JAK-STAT pathway in carp, suggesting that the fish IFN signaling transduction pathway and antiviral mechanisms are similar to that in mammals.

2.3.6 Antiviral pathways induced by IFN in fish

Mx proteins appear to be present in all vertebrates and Mx genes or cDNAs have been cloned from a number of different fish species including perch [68], rainbow trout

[69] [70], Atlantic salmon [71], Atlantic halibut [72], Japanese flounder [73], Fugu [74], carp [75], channel catfish [76], gilthead sea bream [77], zebrafish [78] [31], and orange-spotted grouper [79]. As with higher vertebrates, fish Mx is expressed in the cytoplasm [80] or in the nucleus [81], and fish Mx transcripts and proteins are induced by poly I:C, IFN, and/or virus infection. Interferon-stimulated response elements (ISRE) were found in the promoter of rainbow trout [82] and zebrafish [78] Mx, similar to mammalian Mx, showing that the fish Mx protein is under control of a typical interferon-induced promoter.

Plant and Thune [76] cloned and sequenced a full-length cDNA of channel catfish Mx gene using degenerate primers. The initial amplified 600 bp channel catfish fragment was found to be highly homologous to existing mammalian and fish Mx sequences, although greatest homology was with other fish Mx genes. A comparison to zebrafish, Atlantic salmon, Atlantic halibut, rainbow trout, Japanese flounder, Fugu, and fragments of perch and turbot Mx genes resulted in nucleotide percent identities ranging from 78 to 84% and amino acid percent identities ranging from 72 to 80%. Using RACE PCR to amplify the full-length catfish Mx mRNA, Plant and Thune [76] were able to analyze the gene. They found that the amino terminus of the encoded protein contained a tripartite GTP binding motif and a dynamin family signature. The carboxy terminus contained a leucine zipper. BLAST analysis of the channel catfish Mx protein compared with other Mx proteins showed highest level of identity with perch Mx protein (79%), followed by turbot, Atlantic salmon Mx1 and Mx3, and rainbow trout Mx3, all at 74% identity. Human MxA and MxB were 53 and 51% identical, respectively. When a parsimony tree was created comparing all the fish Mx genes, it showed that the zebrafish and channel catfish Mx proteins are sufficiently different from the other fish Mx proteins to each

warrant their own clade [76]. The perch, turbot, Japanese flounder, Atlantic halibut and Fugu sequences clustered together, while the Atlantic salmon and rainbow trout sequences clustered together. Interestingly, the Mx protein is more conserved than IFN in fish. The greatest conservation between the catfish Mx sequence and other fish Mx sequences was found in the amino terminus. Southern blot analysis [76] showed that there is a minimum of three Mx genes in channel catfish, which is similar to rainbow trout and Atlantic salmon, which possess three Mx genes [70] [71] and Atlantic halibut, which possess two Mx genes [72].

Mx mRNA synthesis was induced *in vivo* by injecting catfish with either poly I:C or CCV and mRNA samples were collected 0, 1, 2, 4, 6 and 8 days after injection [76]. Interestingly, whenever an rt-PCR was performed on day 0, all catfish samples were synthesizing low levels of Mx mRNA [76]. Expression of Mx mRNA in healthy fish was also shown in Japanese Flounder [73]. This basal level of Mx expression is possibly associated with low levels of IFN circulating to protect against initial exposure to viral infection [76]. Atlantic salmon Mx protein was expressed in PBS-injected control fish as well as non-treated fish verifying that Mx expression was not a reaction to injection [83]. When catfish were injected with poly I:C they demonstrated the highest level of Mx mRNA expression on day 1, and this expression decreased slightly on days 2 and 4. By day 6, Mx mRNA expression had declined, although by day 8 there was some Mx mRNA expression detected again. Injection with CCV induced a total mortality of 15% and was found to increase Mx mRNA expression within 24 to 48 hours. The Mx mRNA expression level decreased after day 2 but mRNA production was maintained through day 8 (which was the last sampling point). CCO cells were also found to constitutively produce a low level of the Mx mRNA. Likewise, levels of Mx mRNA were enhanced in

CCO cells beyond basal levels in response to both 5 and 50 μg/ml of poly I:C. Mx mRNA transcripts showed expression by 24 hours and continued expression by 48 hours [76].

Mx mRNA expression in channel catfish in response to CCV occurred earlier and lasted longer than noted in any other fish species. For example, Mx mRNA could be detected from the first sampling point at day 2 to day 4 in rainbow trout infected with infectious hematopoietic necrosis virus (IHNV) [69] and likewise, Atlantic halibut infected with infectious pancreatic necrosis virus (IPNV) displayed a low level of Mx mRNA at 2 and 4 days post infection, yet by day 7 expression was not seen [72]. Similarly, Japanese flounder infected with hirame rhabdovirus (HRV) initially showed Mx mRNA expression at 2 days and this expression peaked by day 3 [73]. The prolonged *in vivo* expression of Mx mRNA in response to CCV may indicate that the catfish Mx protein has antiviral activity, although Plant and Thune [76] were not able to show the exact role of the Mx protein in catfish.

In fact, until recently, most Mx protein studies in fish were not able to demonstrate the antiviral activity of Mx proteins. For example, the effect of Atlantic salmon (AS)-IFN (the supernatants with IFN-like activity induced by poly I:C) and poly I:C on the Mx protein expression and antiviral activity against infectious salmon anaemia virus (ISAV) in the Atlantic salmon cell lines TO and SHK-1 was measured, in order to see if there was a correlation between Mx protein expression and virus inhibition [84]. This analysis did not show that Mx protein production in Atlantic salmon had antiviral properties [84]. Similarly, rainbow trout Mx proteins do not appear to inhibit replication of the rhabdovirus IHNV [70].

Only recently has antiviral activity of Mx proteins in fish been demonstrated. Knowing that a correlation between inhibition of IPNV and Mx expression in IFN-stimulated

salmon cells existed and had been demonstrated [28], the antiviral action of Atlantic salmon Mx proteins against replication of IPNV was uncovered [80]. To demonstrate antiviral activity of Mx, a Chinook salmon embryo (CHSE-214) cell line was established that expresses Atlantic salmon Mx1 (ASMx1) constitutively [80]. The CHSE-214 cell line was defective in producing IFN-like activity. The ASMx1-transfected CHSE-214 cells expressing ASMx1 showed a severely reduced IPNV-induced cytopathic effect, which was supported by a 500-fold decrease in virus yield. The antiviral activity for IPNV was further validated by the inhibition of virus protein synthesis, especially virus capsid protein VP2 [80]. IFN was not detected in the ASMx1-transfected CHSE-214 cells indicating that endogenous IFN was not contributing to the protective effect of ASMx1.

Antiviral activity of Japanese flounder Mx protein (JFMx) was also demonstrated [85]. A hirame natural embryo cell line (HINAE) was transfected to stably express the recombinant JFMx. The JFMx-expressing cells were infected with hirame rhabdovirus (HIRRV) and viral hemorrhagic septicemia virus (VHSV). A lower expression level of the viral nucleoprotein transcripts in the JFMx-transfected cell line was observed [85]. Kinetics of the two viruses showed that reduced levels of glycoprotein and nucleoprotein transcripts were due to JFMx blocking replication by interfering with transcription of the viral subgenomic mRNAs and fewer viral particles [85].

Although the duration of Mx mRNA expression is relatively short lived in the different fish species, detection of Mx proteins lasts much longer. It was shown that the halibut Mx protein expression in the liver reached a maximum at 3 days and Mx protein levels in the liver remained elevated for 14 days after poly I:C treatment [81]. Likewise, it was shown that IPNV infection increased halibut Mx protein expression in the liver

from 4 to at least 35 days [81]. After vaccinating rainbow trout against different viruses, Mx protein levels are elevated 30 days post vaccination [8].

The eIF2α gene from rainbow trout and zebrafish has been characterized and the predicted amino acid sequences were found to be 93 and 91% identical to the human eIF2α protein suggesting that fish eIF2αs will exhibit the same function and regulation as mammalian eIF2α [86]. To show that the fish eIF2αs are substrates for known eIF2α-kinases, phosphorylation of recombinant rainbow trout and zebrafish eIF2α *in vitro* by human PKR was demonstrated [86]. It was also demonstrated that rainbow trout and zebrafish eIF2α could be phosphorylated *in vivo* [86]. When rainbow trout gonad cells (RTG-2) were exposed to poly I:C or infected with IPNV, an increased phosphorylation of eIF2α was noted, which suggests the presence of a PKR gene in rainbow trout [86]. Thus, similar to mammals, fish are able to regulate protein synthesis in response to cellular stresses through phosphorylation of eIF2α [86].

The PKR-like gene from crucian carp *Carassius auratus* blastulae embryonic cells (CAB) was cloned and sequenced following exposure to UV-inactivated grass carp hemorrhagic virus (GCHV) [87]. The carp PKR-like gene showed a high homology to all family members of eIF2α kinases, although it shared the highest homology with human PKR, and it up-regulated expression in response to active GCHV, UV-inactivated GCHV and carp IFN [87]. Investigators found that induction of the carp PKR-like gene (also called *CaPKR-like*) also required the production of cellular IFN. The *Ca*PKR-like protein is similar in amino acid length to mammalian PKRs, has a NH_2-terminal regulatory domain and a C-terminal protein kinase catalytic domain similar to mammalian PKR proteins, and this catalytic domain shares high sequence conservation with the catalytic domain from mammalian PKRs [87].

2.3.7 IFN activity demonstrated in fish - two viruses used to demonstrate protection

In early work done in fish, researchers could only detect the antiviral protection of IFN without clearly understanding their mechanisms. In several studies [26], [27], two viruses were used to demonstrate induction of antiviral protection due to viral interference. In these studies, the first virus provided protection against subsequent infection of the second virus. The first virus, while inhibiting host cell macromolecule synthesis, also inhibited replication of co-infecting heterologous or homologous viruses. Viral interference is a result of several different mechanisms occurring within the cell. One possible mechanism is that the infection with one virus may inhibit replication of a second virus by blocking virus entry into the cell. The second possible mechanism is that when the first virus infects the host cell, it may inhibit host cell functions required by the super-infecting virus. The third possible mechanism is that the first virus blocks virus replication directly. Finally, the first virus may block the replication of a second virus by inducing IFN or other anti-viral factors [26].

Prior exposure to an avirulent reovirus stimulated innate host defense to the infectious rhabdovirus hematopoietic necrosis virus (IHNV) in rainbow trout [27]. Previous experiments had demonstrated that rainbow trout pre-exposed to cutthroat trout virus (CTV) were less susceptible to subsequent challenges with IHNV [19]. Four weeks of protection against viral infection as observed following CTV exposure [19]. Additionally, the concentration of anti-IHNV neutralizing antibodies in the serum was significantly higher among fish previously exposed to CTV [19]. The mechanism of anti-viral protection was

unclear, although it resembled interferon-like activity [19]. Thus, in subsequent experiments, a reovirus was used to induce host defense against a rhabdovirus [27], because reoviruses are known to be potent stimulators of IFN (reviewed in [27]). Rainbow trout (*O. mykiss*) was exposed to chum salmon reovirus (CSV) for one hour, which resulted in strong protection for up to 8 weeks post-exposure to CSV [27]. Survival rates ranged from 68 to 100% when fish were challenged with IHNV over an 8 week period. Similar to previous work, higher neutralization titers in IHNV exposed fish were observed, when previously exposed to CSV [27]. It was concluded that nonspecific immune functions, including IFN production and the activation of macrophages and/or NK cells, protected the fish [27].

Similar work was done wherein a double-stranded RNA virus, channel catfish reovirus (CRV), was used to inhibit channel catfish herpes-virus (CCV) following either productive infection or exposure to UV-inactivated CRV. CRV inhibited CCV replication by two different mechanisms. First, CRV inhibited CCV replication directly, as a consequence of its own replication [26]. In cells infected with CRV alone, cellular translation was stopped due to gradual replacement of cellular gene expression by reovirus translation. In cells co-infected with CRV and CCV, CRV infection appeared to suppress both host and heterologous virus (CCV) gene expression. Inhibition of cellular protein synthesis probably prevented the synthesis of any anti-viral factors [26].

Secondly, CRV was able to block CCV replication indirectly due to the synthesis (or release) of an anti-viral factor [26]. Experiments in which CCO cells were treated with UV-inactivated CRV led to induction of an anti-viral factor rather than to viral interference. CCO cells were infected with UV-CRV and the culture medium was harvested at

24 hours and tested for its ability to block CCV replication [26]. The cells were monitored for the development of CPE for 48 hours and for the production of infectious CCV. UV-CRV infected cultures showed little CPE, and the reduction of CPE in UV-CRV infected CCO cells correlated with a reduction in sustaining CCV replication. Also, CCO cells infected with UV-CRV synthesized or secreted an anti-viral factor that peaked in protective ability within 24 to 48 hours after infection and did not increase significantly over the next 2 days. This protection was short-lived, and 72 hours after CCV infection, UV-CRV-treated cultures showed a marked increase of CPE. Alternatively, mock-treated CCO cells were readily susceptible to CCV infection and showed CPE within 48 hours of infection. No anti-viral activity was detected in medium harvested from CCO cells before UV-CRV infection, or from medium harvested from mock-infected cultures during the 4 days of the experiment [26].

The kinetics of the induced anti-viral factor produced by the CCO cells was compared with the kinetics of mammalian IFN and found to be similar, suggesting that IFN is a first line of defense following virus infection in catfish [26]. However, protection of the cells provided by the anti-viral factor was incomplete and could be overcome by increasing the amount of challenging virus. Although cultures that produced the anti-viral factor were protected from CCV challenge for up to 48 hours, during the next 24 hours "residual" virus replicated and caused extensive CPE, which may have been caused by the decline of the anti-viral state over time [26].

In addition to anti-viral activity being produced in CCO cells infected with UV-CRV, anti-viral activity was also found constitutively in medium harvested from several long-term catfish T cell-like and macrophage lines. Levels of anti-viral activity were similar to those seen following UV-CRV infection and experiments showed that this anti-viral

factor was a heat liable protein, which suggested an interferon. However, B cell clones and fibroblast cell lines did not constitutively produce the anti-viral factor. In previous experiments, newly established T cell and macrophage lines were non-permissive for CCV replication, which may be due to the generation of an anti-viral factor [26].

2.3.8 IFN activity demonstrated in fish - IFN detection using Mx protein expression induced by poly I:C

Mx protein expression was used to measure IFN expression [28]. After stimulating Atlantic salmonid cells with poly I:C, an IFN-like activity in the cells was observed at 24 hours and Mx protein expression at 48 hours [28]. It was found that double-stranded RNA induced Mx protein expression via induction of type I IFN production, similar to mammalian systems [28]. It was also demonstrated that Atlantic salmon macrophages were induced by poly I:C to produce IFN-like activity and Mx protein expression [28]. Atlantic salmon macrophages were chosen to express IFN-like activity (pooled supernatants labeled AS-IFN) because mammalian macrophages are potent expressers of type I IFN (reviewed in [28]). It was found that IFN antiviral activity in macrophages peaked at 24 hours and then declined after 36 hours and this peak and decline in IFN production was independent of the poly I:C concentration used [28].

Atlantic salmon macrophages were cultured in the presence or absence of poly I:C for 24 and 48 hours. When the macrophages were cultured in the absence of poly I:C, they did not produce Mx proteins. In the presence of poly I:C at 48 hours, the macrophages expressed the Mx protein, while at 24 hours the macrophages showed very weak Mx protein expression. Atlantic salmon fibroblast cells were cultured in the presence or absence of either poly I:C or AS-IFN. In the presence of either poly I:C or AS-IFN the

fibroblast cells were able to express Atlantic salmon Mx protein. CHSE-214 cells were also cultured in the presence or absence of either poly I:C or AS-IFN and analyzed for Mx protein expression. Only the CHSE-214 cells cultured in the presence of AS-IFN expressed the Mx protein [28]. Mammalian type I IFN was compared with the Mx protein inducing activity of AS-IFN from the Atlantic salmon macrophage supernatants [28]. The pH sensitivity was tested and the AS-IFN Mx inducing ability was highly resistant to low pH. This AS-IFN activity also diminished with trypsin or heat treatment [28]. Based on analogy to the mammalian system, these findings suggested that the acid stability of the Mx protein inducing activity of AS-IFN was due to type I IFN activity rather than type II IFN activity [28]. Furthermore, in humans, type II IFN-γ induced only 1% of the MxA proteins expression when compared with type I IFN [88].

Thus, several experiments indicated that induction of Mx proteins by double-stranded RNA in salmon cells occured via induction of IFN [28]. First, AS-IFN, not poly I:C, induced expression of Mx proteins in CHSE-214 cells, which lack the ability to make their own IFN. Second, Atlantic salmon IFN induced maximal Mx protein expression 24 hours earlier than poly I:C in macrophage cell lines. These observations suggested that poly I:C induced Mx protein expression indirectly through the induction of type I IFN, which is consistent with observations in mammalian and avian systems [28]. Moreover, studies of the chicken Mx promoter in monkey cells that lack IFNα and β genes have shown that the promoter is only responsive to type I IFN, but not to poly I:C [89]. This is further supported by studies in which IFN-containing supernatant induced Mx transcripts more rapidly than poly I:C in rainbow trout gonad cells [82]. Furthermore, the Mx promoter in rainbow trout [82] and zebrafish [78] contained an IFN-stimulated response element (ISRE).

2.4 Conclusion

It is clear that teleost fish have a type I IFN system similar to mammals. This is based on the facts that several type I IFN-inducible genes have been cloned and sequenced from several different fish species [67]. Thus, it is believed that fish have a complete IFN system with similar antiviral mechanisms as mammals [87]. IFN signal transduction was also conserved between mammals and teleost [67]. Although fish IFN genes show a low degree of similarity with mammalian homologues, some of the other molecules important in IFN activities are more highly conserved when compared to mammalian homologues, such as the Mx protein [76]. Knowing more about how the innate immune system works in fish will help in future vaccination designs to optimize induction of the innate IFN system.

Chapter 3

Materials and Methods

3.1 Maintaining cell culture and virus propagation

Channel catfish ovary (CCO) cells were obtained from John Plumb, PhD, Department of Fisheries and Allied Aquacultures, Auburn University. Cells were grown at 28°C in HEPES-buffered Eagles minimum essential medium (MEM) plus 10% fetal calf serum (Hyclone Biologics, Logan, UT) plus antibiotic/antimycotic. The Auburn -1 strain of CCV (ATCC VR-665) was grown by removing medium from the flasks and inoculating CCV directly onto cells at a multiplicity of infection (MOI) of 0.5. After two hours, MEM was added to the cells and incubated at 28°C until widespread cytopathic effects (CPE) could be seen. Cells and medium were decanted and sonicated on ice twice at 400W peak electrical power for 15 sec, and the suspension was cleared by slow speed centrifugation on Beckman TJ-6 tabletop centrifuge. The supernate was aliquoted into 1 ml units and frozen at -80°C. Virus was titrated by serial dilution using the Karber method [90].

3.2 Induction and detection of IFN by poly I:C in CCO cells

CCO cells were incubated with 1 μg/ml, 10 μg/ml, or 50 μg/ml polyinosinic-polycytidilic acid (poly I:C) in MEM to induce expression of interferon (IFN). After 1 h, the solution was decanted, the cells washed with fresh MEM, and new medium was added to the cells, and incubation continued for times ranging from 1 to 50 h. Culture

medium was then harvested and frozen at -20°C and RNA was extracted from the cells and stored at -80°C.

CCO cells were removed from the flasks using a sterile cell scraper and resuspended in 1 ml RNase-free 1% PBS and concentrated by centrifugation. The pellet was resuspended in 200 μl 1% PBS and RNA extracted using High Pure RNA Isolation kit (Roche, Penzberg, Germany). Total RNA was frozen at -80°C.

Culture medium from poly I:C treated cells, was diluted two fold seven times and added to fresh CCO cells in 96-well plates as described by Chinchar et al. [26]. Medium from two different control flasks was used as an experimental control (labeled T0) to gauge the difference in constitutive IFN production and induced IFN production. The culture in the first control flask was 2 days old and confluent (labeled T0-2). The culture in the second control flask was 1 day old and not completely confluent (labeled T0-1). Cells were incubated for 1 h at 28°C, the medium was removed, and 100 μl of fresh MEM plus 100 $TCID_{50}$ CCV added to each well. Two different controls were used on the 96-well plates. One control that served as the positive control contained CCO cells that did not receive culture medium from poly I:C treated cells and were not infected with CCV. The second control that served as the negative control contained CCO cells that did not receive culture medium from poly I:C treated cells, but were infected with CCV. Plates were sealed with paraffin wrap to prevent evaporation of medium, and the plates incubated for 24 or 48 hours at 28°C. Medium was then removed, the plates were fixed and stained with 1% crystal violet in 70% ethanol, and immediately rinsed. Protection against CCV infection was assessed by determining which titer on the 96-well plate the cells no longer showed 50% protection.

3.3 Induction of IFN expression by CCV in CCO cells

CCO cells were infected with CCV for 1 h at 28°C with an MOI of 1.0. Cells were washed with MEM after an hour, refed with fresh MEM, and harvested at times from 1 to 6 hours post infection. RNA was extracted from cells and frozen at -80°C as described above.

3.4 Induction of IFN expression by ultraviolet (UV) inactivated CCV in CCO cells

CCV were inactivated by using 2.4x10^5 μJoules of UV in a Stratalinker 1800 (Stratagene, LaJolla, CA). Inactivation was confirmed by serial dilution of virus on cells and infection was measured. CCO cells were treated the same as when induced by poly I:C, and at time intervals 1 through 6 hours, RNA was harvested from the cells and frozen at -80°C as described in that experiment.

3.5 Primers and conditions for rt-PCR

rt-PCR was used to detect expression of the IFN genes. Primer set 1, designed to detect IFN-1 [29] actually detects expression of 4 different catfish IFN genes [48]. The forward primer (5′ GCCAGTACAGAGCAAGAACA 3′) and the reverse prime (5′ CCATTCCTGATTCGCTCCA 3′) span nucleotides 151 through 558 of the channel catfish IFN-1 cDNA in Genbank (accession number AY267538) resulting in a 408 bp amplicon. Previously described optimized conditions for rt-PCR [29] were 1 cycle of 48°C for 45 min; 1 cycle of 94°C for 3 min; 30 cycles of 94°C for 30 sec, 55°C for 45 sec, and 72°C for 1 min; 1 cycle of 72°C for 5 min. The primer set for IFN-2 mRNA (Genbank accession

number AY 847295) (forward primer 5′ CCAATGACAGGAAACACCTCTTTCCCA 3′; reverse primer 5′ CAGTGGATGAACGTCTTGCTCTTTCAGC 3′) spans 265 bp of the IFN-2 gene. Optimum conditions were 1 cycle of 48°C for 45 min; 1 cycle of 94°C for 3 min; 35 cycles of 94°C for 1 min; 56.6°C for 1 min, 72°C for 3 min, and 1 cycle of 68 C for 10 min. Amplicons were detected using 2 μg of RNA electrophoresed in 1.5% (IFN-1-4) or 2% (IFN-2) agarose with ethidium bromide. Amplicons were harvested from the gel and purified using QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA), cloned using TA cloning kit (Invitrogen), and sequenced by the Auburn University Genomics and Sequencing Laboratory.

3.6 Induction of IFN in channel catfish

Three groups of 12 yearling channel catfish, 10-15 cm in length, were held in 55 L of aerated dechlorinated tap water at 20 +/- 2°C. Group 1 fish were injected intraperitoneally (IP) with 50 μg poly I:C. Group 2 fish were bathed in 10^4 $TCID_{50}$ CCV/ ml for 1 h and then moved into a tank comparable to that housing group 1 fish. Control fish were untreated. Times ranging from 1 to 7 hours post initiation of treatment, sera and head kidneys were collected from 3 fish from each group. Head kidneys were frozen in liquid nitrogen, stored at -80°C, and RNA was extracted using RNA STAT-60 and stored at -80°C.

3.7 Effect of IFN induction at the time of vaccination

Six groups of 30 yearling channel catfish, 5-8 cm in length were held in 55 L flow-through aquaria with aerated dechlorinated water at 20 +/- 2°C. Group 1 fish served as the control population. Group 2 fish were injected IP with 10 μg of poly I:C. Group

3 fish were injected intramuscularly with 23.3 μg of CCV ORF 59 DNA as previously described [3]. Group 4 fish were injected with poly I:C and vaccinated with ORF 59 DNA simultaneously; Group 5 were injected with poly I:C one hour before vaccination with ORF 59 DNA; and Group 6 were injected with poly I:C one hour after vaccination with ORF 59 DNA.

At 4, 24, and 48 hours post treatment, serum and RNA from head kidneys were harvested from 5 fish in each group and treated as previously described. Sera were also harvested from 5 fish from each group 2 and 4 weeks after treatment.

3.8 Antiviral assays

Sera from fish either treated with poly I:C, CCV vaccination, or infected with CCV, were diluted two fold seven times and added to fresh CCO cells in 96-well plates. Sera were not pooled; each column represented a single fish. Cells were incubated with sera for 1 h at 28°C, before 100 μl of medium containing 100 $TCID_{50}$ CCV were added to each well. Two controls groups were included on the 96-well plate. The cells that served as the positive control did not receive sera and were not infected with CCV. The cells that served as the negative control did not receive sera and were infected with CCV. Plates were sealed with paraffin wrap to prevent evaporation of medium, and the plates incubated for 24 or 48 hours at 28°C. Medium was then discarded, the plates were fixed and stained with 1% crystal violet in 70% ethanol, and immediately rinsed. Antiviral activity was evaluated by looking at which titer the cells received less than 50% protection from virus.

3.9 Virus neutralization assays

An insufficient amount of serum was collected from the fish injected only with poly I:C or ORF 59 to use in experiments. Sera from individual fish in the other groups were initially diluted 1:5 in MEM, and then serially diluted 1:2 in MEM. 100 μl of the dilution were mixed with 100 μl of medium containing 1000 $TCID_{50}$ CCV and incubated at 28°C for 2 h. 100 μl of the serum-virus mixtures were then transferred to wells of a 96-well plate containing confluent monolayers of CCO cells. Plates were sealed, incubated, and stained as described above.

Chapter 4

Results

4.1 Inducing IFN expression in CCO cells

4.1.1 Optimization and kinetics of poly I:C

As measured by CCO protection, optimum induction of IFN occurred when either 1, 10, or 50 μg/ml poly I:C were used (Fig. 4.1 and not shown). Peak generation of IFN protection occurred at 8 hours following incubation with poly I:C (Fig. 4.1) and increased IFN mRNA production in cells occurred 1 through 7 hours (Fig. 4.2). The primers used for rtPCR in these experiments were created by Long *et al.* [29] and were later confirmed by Long *et al.* [48] to anneal to a sequence shared by all four catfish IFN genes (Fig. 4.2). Samples collected from control groups always showed a low background level of mRNA IFN expression. Within the first 5 hours post incubation with poly I:C, all three concentrations of poly I:C had the same effect on IFN expression; they all caused an increase in expression of IFN mRNA (data not shown). However, after 5 hours post incubation with 1 μg/ml of poly I:C, the level of IFN mRNA expression dropped when compared to IFN mRNA levels induced by 10 μg/ml and 50 μg/ml poly I:C, which continued to show increased expression of IFN mRNA up to 7 hours post incubation with poly I:C. Thus, we chose to continue experiments using 10 μg/ml or 50 μg/ml of poly I:C. Under these conditions, CCO cells expressed IFN mRNA within the first 12 hours post treatment with poly I:C, although, after 7 hours, the cells no longer expressed maximum levels of IFN mRNA, but a lesser amount that was still greater than background levels of IFN expression (Fig. 4.2). After 13 hours, repeated experiments

showed that induced IFN mRNA expression stopped or greatly decreased, with IFN levels equal to or less than background expression levels of IFN mRNA expressed by non-induced CCO cells. Overall, there was not much of a difference between using 10 μg/ml and 50 μg/ml poly I:C to induce IFN expression in CCO cells.

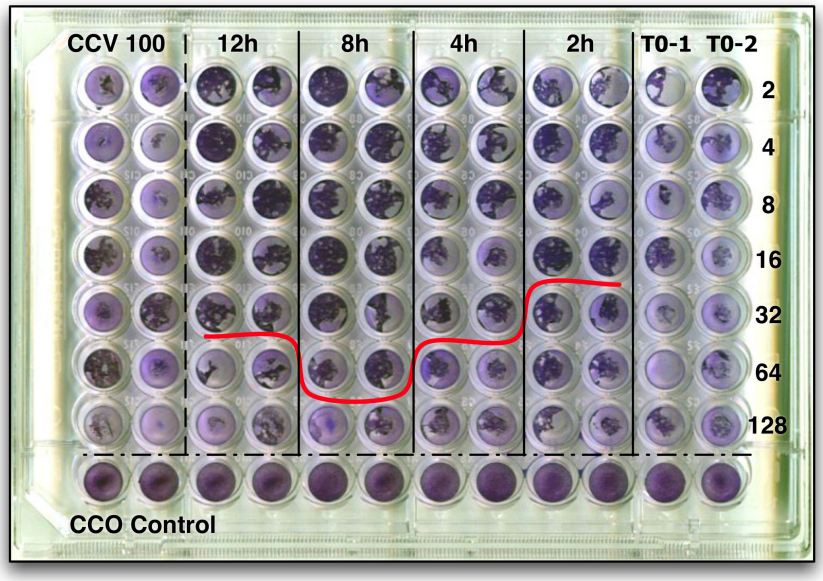

Figure 4.1: Antiviral activity of culture medium collected from CCO cells at 2, 4, 8 and 12 hours post induction with 50 μg/ml poly I:C. The CCO cells in the assay were challenged with 100 $TCID_{50}$. The highest dilutions of culture medium collected at indicated times that show antiviral activity are as follows: at 2 hours, 1:16; 4 hours, 1:32; 8 hours, 1:64; and at 12 hours, 1:32.

4.1.2 Comparison of expression of IFN genes

Two different sets of primers were used to examine mRNA expression of catfish IFN. When we cloned and sequenced an IFN cDNA expressed in CCO cells sequencing results showed that this cloned cDNA was very similar to the CF IFN-1 posted in Genbank,

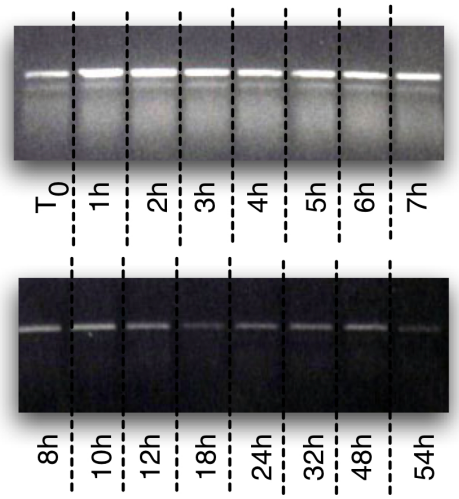

Figure 4.2: IFN mRNA expression in CCO cells. 10 μg/ml poly I:C induced IFN mRNA expression in CCO cells. Poly I:C was incubated 1 hour on cells before removal. RNA was extracted from the cells up to 54 hours post incubation. General IFN primers were used for rt-PCR, which show expression of all four CF IFN genes. Expression is strongest within the first 7 hours, although there was continued expression through 12 hours.

although not 100% identical (showed only 87% similarity). The other three catfish IFN genes had not yet been found or released to Genbank. However, months later, after new data was released to Genbank, we found that our IFN clone showed 99.3% similarity to the CF IFN-2 gene (Genbank accession number AY847295) except at the ends of the cloned sequence where the primer, which was designed specifically for CF IFN-1 sequence, had annealed to the CF IFN-2 gene (Fig. 4.3). From the CF IFN-2 gene, new primers were designed to compare the differences between CF IFN-2 cDNA expression and IFN expression using the nonspecific primers. This second set of primers amplified a 265 bp fragment unique to the catfish IFN-2 gene.

```
catfishIFN AY847295 (IFN2)   GCCAGTACAGAGCGAAGAACGACTACTGTTTGTCACTGCTGAATGAAATGGGTGGAGAGATTGTTCCAATGACAGGAAAC
IFN 24h 10 µg/µl m13 Rev     GCCAGTACAGAGCGAAGAACAACTACTGTTTGTCACTGCTGAATGAAATGGGTGGAGAGATTGTTCCAATGACAGGAAAC
IFN 24h 10 µg/µl T7 Forward  GCCAGTACAGAGCGAAGAACAACTACTGTTTGTCACTGCTGAATGAAATGGGTGGAGAGATTGTTCCAATGACAGGAAAC

catfishIFN AY847295 (IFN2)   ACCTCTTTCCCACGTCGGGCATACCATGAAATCGAGAAGGCCGAGGTACAGGCAGAAGATCAGGTGAGGTTTCTGGCTGT
IFN 24h 10 µg/µl m13 Rev     ACCTCTTTCCCACGTCGGGCATACCATGAAATCGAGAAGGCCGAGGTACAGGCAGAAGATCAGGTGAGGTTTCTGGCTGT
IFN 24h 10 µg/µl T7 Forward  ACCTCTTTCCCACGTCGGGCATACCATGAAATCGAGAAGGCCGAGGTACAGGCAGAAGATCAGGTGAGGTTTCTGGCTGT

catfishIFN AY847295 (IFN2)   GGCCACAAACGAGATCATCATTCTCTTCAGTGCTGTGTCTCATGTGGATGATGTAAAATGGGACAGCAGGACACTGGATA
IFN 24h 10 µg/µl m13 Rev     GGCCACAAACGAGATCATCATTCTCTTCAGTGCTGTGTCTCATGTGGATGATGTAAAATGGGACAGCAGGACACTGGATA
IFN 24h 10 µg/µl T7 Forward  GGCCACAAACGAGATCATCATTCTCTTCAGTGCTGTGTCTCATGTGGATGATGTAAAATGGGACAGCAGGACACTGGATA

catfishIFN AY847295 (IFN2)   ATTTCCTGAACATACTTAGTACTCGGCAGTTATCAGAGCTTAGAAATTGTACATCAACATATGCTGAAAGAGCAAGACGT
IFN 24h 10 µg/µl m13 Rev     ATTTCCTGAACATACTTAGTACTCGGCAGTTATCAGAGCTTAGAAATTGTACATCAACATATGCTGAAAGAGCAAGACGT
IFN 24h 10 µg/µl T7 Forward  ATTTCCTGAACATACTTAGTACTCGGCAGTTATCAGAGCTTAGAAATTGTACATCAACATATGCTGAAAGAGCAAGACGT

catfishIFN AY847295 (IFN2)   TCATCCACTGAGAAAAAACTGAGAAAACACTTCAAGGATTTGAGGAAATACCTGAAAAACTCTAACTACAGCGCAGACTC
IFN 24h 10 µg/µl m13 Rev     TCATCCACTGAGAAAAAACTGAGAAAACACTTCAAGGATTTGAGGAAATACCTGAAAAACTCTAACTACAGCGCAGACTC
IFN 24h 10 µg/µl T7 Forward  TCATCCACTGAGAAAAAACTGAGAAAACACTTCAAGGATTTGAGGAAATACCTGAAAAACTCTAACTACAGCGCAGACTC

catfishIFN AY847295 (IFN2)   TTTGGAGCAAATCAGGAGTGTGG
IFN 24h 10 µg/µl m13 Rev     TTTGGAGCGAATCAGGAATGTGG
IFN 24h 10 µg/µl T7 Forward  TTTGGAGCGAATCAGGAATGTGG
```

Figure 4.3: Sequence of an IFN cDNA expressed in CCO cells treated with 10μg/μl poly I:C to induce IFN expression. cDNA was amplified using primers designed by Long *et al.*[29], which anneal to all four catfish IFN genes. The clone was completely identical to the catfish IFN-2 gene, except near the ends of the sequence where the general primers anneal.

Subsequent experiments compared expression of all four IFN genes (amplified using the general IFN-1 primers) to IFN-2 gene expression using the two different sets of primers in rt-PCR reactions. Maximum IFN-2 mRNA induction was reached within the first 3 hours at both 10 μg/ml (Fig. 4.4) and 50 μg/ml poly I:C concentrations (data not shown). This differed from the results generated with general IFN-1 primers created by Long *et al.* [29] showing that results generated with IFN mRNA expression was noted up to 7 hours for both concentrations of poly I:C in rt-PCR reactions (compare Figures 4.2 and 4.4). By 4 hours, increased IFN-2 gene expression ceases. Interestingly, however, over the 54-hour period, strong IFN-2 expression appeared sporadically at 6 and 10 hours when 10 μg/ml poly I:C was used and 18 and 48 hours when 50 μg/ml poly

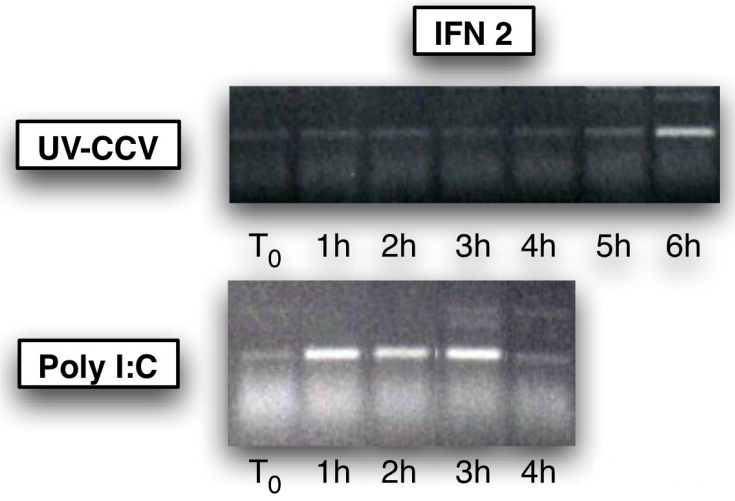

Figure 4.4: IFN-2 gene expression induced by 10 μg/ml poly I:C in CCO cells with the strongest expression within the first 3 hours post induction. IFN-2 gene expression was also induced by UV-inactivated CCV, with strongest expression at 6 hours post induction.

I:C was used (data not shown). Bands double in size of the expected 265 bp amplicon also occurred sporadically, although, again, not at the same time for the different poly I:C concentrations. These bands showed greatest expression at 18 hours post induction at 50 μg/ml of poly I:C; cloning and sequencing confirmed these to be doublets.

4.1.3 Inducing IFN expression using CCV

Experiments were done to see if CCV induced IFN expression in CCO cells. Using the general IFN-1 primers, the results indicated that CCV induced IFN expression in CCO cells within the first 5 hours post infection (Fig. 4.5). The strongest expression was seen at 1, 4 and 5 hours post infection with CCV. By 6 hours post infection with

CCV, increased IFN mRNA expression ceased. Using the IFN-2 primers to see if CCV induced IFN-2, no evidence of any expression of this gene was found (data not shown).

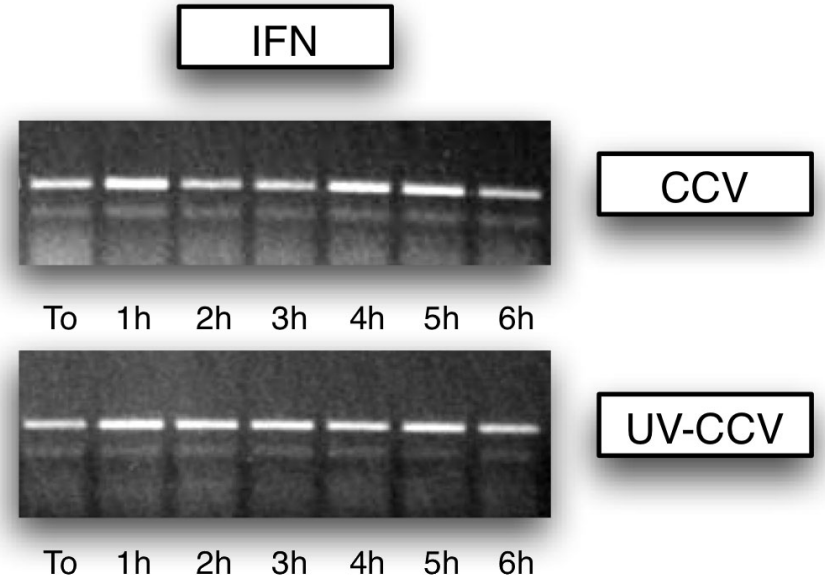

Figure 4.5: IFN mRNA expression over 6 hours induced in CCO cells by infection with CCV or UV-inactivated CCV over 6 hours. General IFN-1 primers were used.

4.1.4 Inducing IFN expression using inactivated CCV

UV-inactivated CCV was also used to induce IFN mRNA expression, and was found to be able to induce IFN expression in CCO cells (Fig. 4.5). This was a qualitative conclusion that will need to be tested quantitatively to confirm this conclusion. Using the general IFN-1 primers in an rt-PCR showed that IFN genes were expressed within the first 6 hours post infection with inactivated CCV. While live CCV did not induce the IFN-2 gene in CCO cells, inactivated CCV induced expression of the IFN-2 gene, with a very low level of mRNA expression beginning at 5 hours post infection and increasing by

6 hours post infection (Fig. 4.4). These experiments showed that CCV and inactivated CCV induce different levels of IFN mRNA expression in CCO cells, especially in regard to the ability of CCO cells to express the IFN-2 gene in the presence of inactivated CCV.

CCV	0 hr	1 hr	2 hrs	3 hrs	4 hrs	5 hrs	6 hrs
IFN general	-	+	+	+	+	+	-
IFN-2	-	-	-	-	-	-	-
ORF 6	-	+	+	-	-	-	-
ORF 53	-	-	-	+	+	+	+
UV-CCV	0 hr	1 hr	2 hrs	3 hrs	4 hrs	5 hrs	6 hrs
IFN general	-	+	+	+	+	+	+
IFN-2	-	-	-	-	-	+	+
ORF 6	-	-	-	-	+	+	+
ORF 53	-	-	-	-	-	+	-

Table 4.1: Expression (+) or no expression (-) induced by either CCV or UV-inactivated CCV in CCO cells using the general primers that anneal to all 4 IFN mRNA genes, primers specific for IFN-2 mRNA, primers specific to early gene CCV ORF 6 mRNA, and primers specific to late gene ORF 53 mRNA.

CCV gene expression in cells infected with CCV or UV-inactivated CCV was also examined by rt-PCR. In CCV infected cells, ORF 6, a membrane protein expressed early in the infection of CCV, expression happens within the first 2 hours post infection with expression ceasing by 3 hours post infection. In contrast, in UV-inactivated CCV infected cells, early gene ORF 6 expression happens at 5 and 6 hours post infection with inactivated CCV, as compared to expression being reached within the first 2 hours post infection with CCV. In CCV infected CCO cells, late gene ORF 53, encoding a capsid protein, begins to show expression by 3 hours and continues expression up to 6 hours post infection with CCV. Dissimilarly, in UV-inactivated CCV infected cells, the ORF 53 gene only shows expression at 5 hours post infection.

4.2 Challenge Assays using Culture Medium from induced CCO cells

Culture medium was collected from the experiments in which confluent CCO cells were induced by poly I:C to produce IFN. This culture medium was collected from the cells and used to measure antiviral activity. The cells incubated with poly I:C and induced to produce IFN provided protection against CCV infection (Fig. 4.1). This protection did not completely align with the times of IFN mRNA expression from rt-PCR assays (compare Figures 4.2 and 4.1). However, our results were similar to work by Long *et al.* [29], which showed correlation between protection and IFN mRNA expression. In their results, poly I:C induced mRNA expression only at 2 hours, whereas challenge assays showed strongest protection at 2, 4 and 7 hours post treatment with poly I:C, with 4 hours having the strongest protection [29]. Our assays showed that antiviral titers were, at 2 hours, 1:16; 4 hours, 1:32; 8 hours, 1:64; and at 12 hours, 1:32 (Fig. 4.1). The protection against CCV in the challenge assays seemed to increase with time up to 8 hours. At 8 and 12 hours, as seen from the rt-PCR reactions, IFN expression begins to decline.

4.3 Inducing IFN expression in channel catfish with poly I:C and ORF 59 vaccination

In these experiments, one hundred eighty channel catfish 5 to 8 cm in length were divided into six groups of 30 fish each, and each received intraperitoneal injection with poly I:C and/or intramuscular injection of CCV ORF 59 DNA vaccine.

Using the primers designed by Long *et al.* [29] to look at IFN expression, results showed that no significant increase in the amount of IFN expressed in the catfish. In

fact, at 4 hours, fish injected with either poly I:C or poly I:C and ORF 59 did not show increased expression (Fig. 4.6). At 24 hours, fish injected with only poly I:C showed slightly decreased expression and fish injected with both poly I:C and ORF 59 vaccine showed increased IFN mRNA expression (Fig. 4.6). At 48 hours, fish from groups either injected with only poly I:C or injected with both poly I:C and ORF 59 expressed IFN mRNA, although this expression was slightly above the approximated background level of IFN mRNA expression in the experimental control fish (Fig. 4.6). The fish injected with both the ORF 59 vaccine and poly I:C expressed more IFN mRNA at 24 hours than at 48 hours.

4.4 Antiviral activity at 4, 24, and 48 hours from poly I:C/ORF 59 vaccinated fish

Catfish Groups	4 hrs	24 hrs	48 hrs
experimental control	16	16	16
Poly I:C	64	32	32
ORF 59	32	64	64
ORF 59 + Poly I:C together	16	32	64

Table 4.2: Endpoint dilution of serum-derived IFN protection of CCO cells challenged with 100 $TCID_{50}$ CCV. Serum was collected at 4, 24 and 48 hours after channel catfish were injected with either poly I:C, ORF 59 or both.

The serum collected from the different groups of catfish at 4, 24 and 48 hours was used in interferon protection assays. At all three time points in the experimental control assay, protection stopped at 1:16 dilution (Table 4.2). At four hours post injection, serum collected from fish injected with poly I:C showed the greatest protection (detectable at 1:64 dilution); serum collected from fish injected with the ORF 59 vaccine showed less

protection (detectable at 1:32 dilution); and serum collected from fish injected with both ORF 59 and poly I:C showed least protection (detectable at 1:16 dilution), which was the same level of protection seen in control fish (Table 4.2). At 24 hours, serum collected from fish injected with only poly I:C showed decreased protection (detectable at 1:32 dilution), serum collected from fish injected with only the ORF 59 vaccine showed increased protection (detectable at 1:64 dilution), and serum collected from fish injected with both ORF 59 and poly I:C at the same time showed increased protection from background levels (detectable at 1:32 dilution) (Table 4.2). At 48 hours, protection stayed the same for fish groups in which serum was collected from fish injected with only poly I:C and from fish injected with only the ORF 59 vaccine, whereas protection increased to 1:64 for fish groups in which serum was collected from fish injected with both ORF 59 and poly I:C at the same time (Table 4.2). The rt-PCR results at 24 hours show IFN mRNA expression from fish injected with both ORF 59 and poly I:C, while fish injected with only poly I:C do not show any increased IFN expression and yet the antiviral titers are the same for both groups. Fish injected with both poly I:C and ORF 59 expressed more IFN mRNA at 24 hours than 48 hours, yet antiviral protection increased between 24 and 48 hours.

4.5 Antiviral assays from poly I:C/ORF 59 vaccinated fish at 2 and 4 weeks

At 2 and 4 weeks post injection with poly I:C and/or ORF 59, serum was collected for antiviral assays from 5 fish from each of the 6 groups mentioned in the above experiments. The results were expressed using the Geometric Mean Titer (GMT) (Table 4.3). Nonspecific antiviral protection was greatest in fish that received the ORF 59 vaccination 1 hour prior to poly I:C, although the combination of having the ORF 59 vaccine with

Catfish Groups	2 weeks	4 weeks
Control	6	3
Poly I:C	20	9
ORF 59	17	40
ORF 59 + Poly I:C together	113	35
Poly I:C 1h before ORF 59	80	92
ORF 59 1h before Poly I:C	160	160

Table 4.3: GMT from antiviral activity serum collected from fish groups at 2 and 4 weeks post treatment

Catfish Groups	4 weeks
Control	3
ORF 59 + Poly I:C together	10
Poly I:C 1h before ORF 59	10
ORF 59 1h before Poly I:C	13

Table 4.4: GMT from viral neutralization assays of serum collected from groups at 4 weeks post experiment

poly I:C injection seemed to produce stronger antiviral results than either poly I:C or the ORF 59 vaccine alone (Table 4.3).

4.6 Viral neutralization assays from poly I:C/ORF 59 vaccinated fish at 4 weeks post vaccination

At 4 weeks post injection with poly I:C and/or ORF 59, serum was collected for viral neutralization assays from 5 fish from each of the fish groups injected with both poly I:C and the ORF 59 DNA vaccine. The results were expressed using the Geometric Mean Titer (GMT) (Table 4.4). No significant neutralizing activity was detected by 4 weeks. Results for all three combinations of ORF 59 and poly I:C were similarly low.

4.7 Inducing IFN expression in channel catfish with poly I:C and CCV

One group of twelve 5 to 8 cm yearling channel catfish each were intraperitoneally injected with 500 μl of 100 μg/ml (50 μg) of poly I:C to ensure induction of IFN mRNA expression *in vivo*, and another group of twelve yearling catfish were bathed for 1 hour in 1 L of water with 30 ml of CCV at $10^{5.5}$ LD_{50}/ml. A third group, which did not receive any treatment was set aside as a control. IFN mRNA expression was detected in channel catfish using the general IFN-1 primers. The rt-PCR results from this CCV/poly I:C experiment suggest that the increased concentration of poly I:C injected into the fish is able to induce better IFN mRNA expression in channel catfish (compare Figures 4.6 and 4.7). Results showed increased IFN mRNA expression collected from channel catfish at all four time points, 1, 3, 5 and 7 hours post injection with poly I:C. IFN mRNA expression was also induced in catfish exposed to CCV, although for a shorter period of expression than catfish induced with poly I:C (Fig. 4.7). Results showed increased IFN mRNA expression at 3 and 5 hours post exposure to CCV. Interestingly, only rt-PCR with the primers designed by Long *et al.* [29] was able to show increased catfish IFN expression *in vivo*. The primers designed to amplify the IFN-2 mRNA did not detect any IFN-2 mRNA expression *in vivo* at all in fish induced by either poly I:C or CCV (data not shown). This suggests that one or more of the other three IFN genes (IFN-1, IFN-3, or IFN-4) was induced *in vivo*.

4.8 IFN protection in serum from CCV and poly I:C induced channel catfish

The serum collected from the above experiment was used in interferon protection assays. Serum from fish injected with 50 μg of poly I:C showed the most antiviral protection 1 hour post injection (GMT of 102), protection decreased 3 hours post injection (GMT of 81), and protection decreased further and stayed the same for both 5 and 7 hours post injection (GMT of 64) (Fig. 4.7). Serum from catfish exposed to CCV for 1 hour showed equal protection at 1, 5 and 7 hours post CCV exposure with a GMT of 51 (Fig. 4.7). The greatest antiviral activity was seen in fish 3 hours post CCV exposure with a GMT of 64 (Fig. 4.7). This antiviral activity data for the poly I:C injected fish correlates with rt-PCR results, with strongest IFN mRNA expression and antiviral protection within the first 3 hours. However, the data from the antiviral activity assay for fish exposed to CCV showed strong protection at all time points, while the rt-PCR data showed that IFN mRNA was induced only at 3 and 5 hours post exposure to CCV.

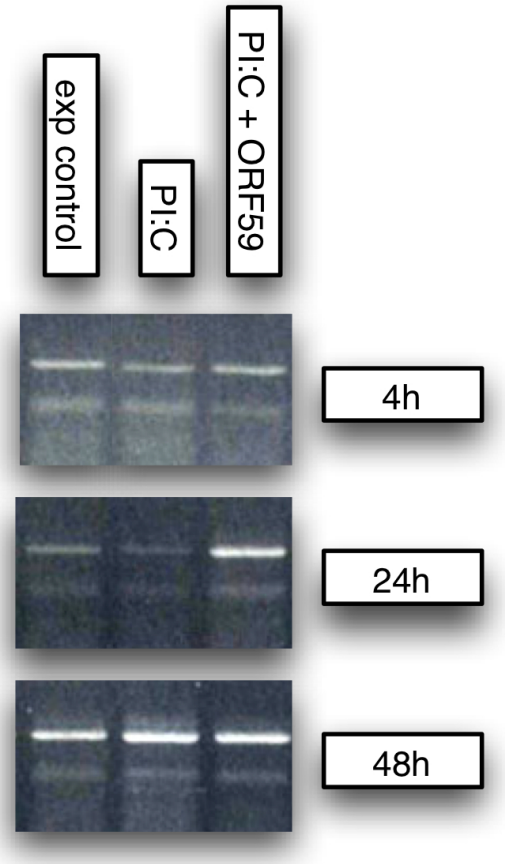

Figure 4.6: IFN mRNA expression collected at 4, 24 and 48 hours post treatment from experimental control fish, fish injected with 10 μg poly I:C, or fish injected with both poly I:C and ORF 59 vaccine. The general IFN-1 primers were used for the rt-PCR reactions shown.

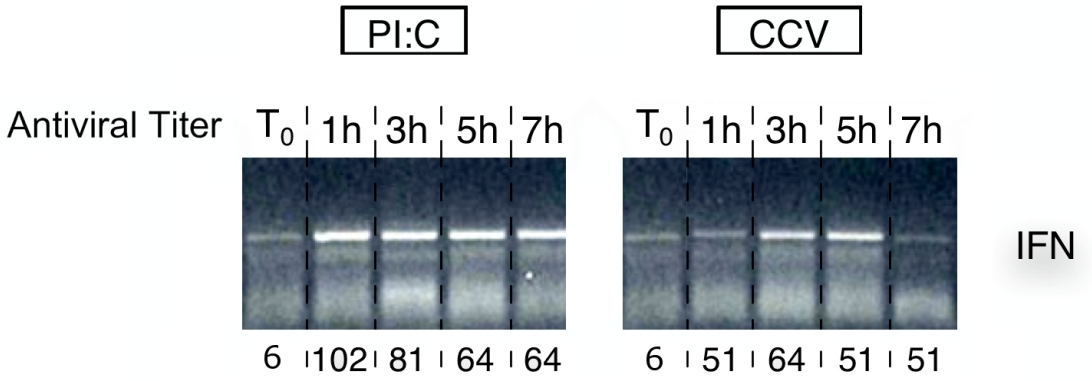

Figure 4.7: IFN expression, as determined by rt-PCR using general IFN-1 primers, and interferon protection, as determined by antiviral activity assays. RNA collected from fish either injected with 50 μg poly I:C or induced with CCV at 1, 3, 5 or 7 hours post challenge. Serum was collected from fish at each time point shown above the lanes and the antiviral titer is shown below each lane.

Chapter 5

Discussion

Based on the available information, teleost fish have a type I IFN system very similar to mammals. Many type I IFN-inducible genes have been cloned and sequenced from a variety of fish including pufferfish, Japanese flounder, rainbow trout, Atlantic salmon, channel catfish, zebrafish, and Atlantic halibut. These include type I and II IFN genes, [29], [30], [31], [32], [52], Mx proteins, [70], [71], [72], [73], [76], [77], [78], [79], interleukin genes, [31], IFN receptor genes [31], interferon regulatory factor IRF genes, [98], and JAK/STAT signaling genes [61], [63], [64], [66], [67]. Furthermore, it was shown that poly I:C induces type I IFN, which subsequently induces Mx proteins, ultimately providing protection against viral challenge similarly as seen in mammalian systems [28]. The type I IFN innate immune response and other innate responses play a major role in the elimination of replicating virus during the early phase of viral infection. They also help the specific immune response become more effective. This biphasic response (early nonspecific protection succeeded by a later specific response) has also been observed following DNA vaccination in fish. It was demonstrated that DNA vaccines provide nonspecific protection due to IFN production and Mx expression and Mx protein protection persisted for the following 4 weeks [8]. It was also demonstrated that non antigen-specific protection by Mx protection in trout persisted for 4 weeks post vaccination with a DNA vaccine [12]. Early and innate immune responses in fish immunized with DNA vaccines possibly help trigger an efficient, long-lasting specific immune response (as discussed in [13]). The lack of or inefficient stimulation of the innate immune system could restrict

induction of an effective immune response to a vaccine [93]. The use of different genetic adjuvants would help generate a more effective immune response [93]. In fact, Leitner *et al.* [95] were able to increase the efficacy of DNA vaccines in mice by providing stronger adjuvants generating "danger signals" to the innate immune system. Also, IFN induction in fish by poly I:C provided protection against viral challenge [29] [83]. In fact, it was found that injecting poly I:C into fish induced IFN mRNA expression and ultimately Mx protein expression which protected fish against viral challenge for up to 2 weeks [83]. It is anticipated that inducing IFN expression with poly I:C during vaccination would enhance antigen-specific protection in vaccinated catfish. Wowever, we found that inducing IFN by poly I:C did not increase the effectiveness of vaccination with DNA vaccines based on virus-neutralization titers (Table 4.4), although some evidence of nonspecific protection was observed. Our results, although limited, show similarities to work by others with viral protection offered by Mx proteins [91] [83], although further tests would need to confirm this notion.

Others demonstrated that the lowest concentration of poly I:C to induce IFN mRNA expression *in vitro* was 1 μg/ml in Atlantic salmon head kidney macrophage cells [28]. Our results confirmed this; within the first hours, IFN mRNA expression can be induced with as little as 1 μg/ml poly I:C or as much as 50 μg/ml. However, using the higher doses of 10 μg/ml or 50 μg/ml poly I:C to induce IFN mRNA expression in CCO cells caused increased IFN expression to last longer than with 1 μg/ml poly I:C. There was not a visible difference observed between the ability of 10 μg/ml and 50 μg/ml poly I:C to induce IFN expression *in vitro*. However, our *in vivo* results differ from the *in vitro* findings. Using 10 μg poly I:C *in vivo* is too low of a concentration to induce consistent IFN mRNA expression in channel catfish. Injection of 400 μg of poly I:C

into fish has been shown to be successful [83]. In fact, when we increased the amount of poly I:C injected into fish to 50 μg poly I:C, more consistent results were obtained. The *in vivo* kinetics from using 50 μg poly I:C were also consistent with kinetics seen in *in vitro* experiments. In fact, in CCO cells, increased IFN mRNA expression was seen at 1 hour post induction, (our first sampling point), with 10 μg/ml poly I:C, continues up to 12 hours post induction, and is gone by 24 and 48 hours. Similarly, when 50 μg poly I:C is used in catfish IFN mRNA expression was detected as early as 1 hour post induction with poly I:C, and continued up to 7 hours post induction. The two different *in vivo* experiments demonstrate that IFN mRNA expression is poly I:C dose dependent (compare IFN expression in figures 4.6 and 4.7 in which fish received different amounts of poly I:C).

In the DNA vaccine experiment in which only 10 μg poly I:C was used, at 4 hours post vaccination or injection with poly I:C there was no measurable increased in amount of IFN mRNA detected (Fig. 4.6). In fish injected with poly I:C, there was no increase in IFN mRNA detected likely because there was not enough poly I:C injected into fish to induce expression. In fish vaccinated with ORF 59, no IFN mRNA was detected 4 hours after vaccination likely because insufficient vaccine glycoprotein was produced to induce IFN expression.

Fish that received coincidental vaccination and poly I:C injection showed increased IFN mRNA expression at 24 hours, whereas fish injected with only poly I:C did not (Fig. 4.6). This is similar to previous findings [8], with a DNA vaccine. The results demonstrated that increased IFN mRNA expression in fish injected with both poly I:C and the vaccine was probably not due to poly I:C because fish that were only injected

with poly I:C did not show increased expression of IFN mRNA at 24 hours. In addition, CCO cells do not express increased IFN mRNA at 24 hours after poly I:C induction.

IFN mRNA expression was increased somewhat at 48 hours in fish injected with poly I:C and those receiving both ORF 59 vaccination and the poly I:C injection. These results seem inconsistent with IFN mRNA expression kinetics in CCO cells, which showed that IFN expression was the highest within the first 12 hours after induction with poly I:C and disappeared by 48 hours.

Even in the absence of detectable increased IFN mRNA expression at 4 hours there was limited nonspecific antiviral protection against CCV challenge (Table 4.2). At 4 hours post injection, serum from fish injected with only poly I:C showed the best protection against CCV with a protection titer of 64. This protection was stronger than protection seen in fish that injected with the ORF 59 DNA vaccine (protection titer 32). Since the amount of poly I:C used gave inconsistent results with IFN mRNA expression, it is hard to know whether the antiviral nonspecific protection shown in the titers for fish induced with poly I:C is due to the nonspecific effects of IFN induction or some other antiviral component. Experiments performed by Harbottle *et al.* [91] did not show Mx expression this early after vaccination. So, it is unclear what is causing the nonspecific protection in ORF 59 vaccinated fish. At 4 hours, serum from fish that were both vaccinated and injected with poly I:C did not show nonspecific antiviral protection against CCV above the constitutive levels of antiviral protection seen in control fish. Our results may be due to competition for receptor sites, or spontaneous binding of vaccine and poly I:C.

Antiviral protection increased 24 hours after fish were vaccinated with ORF 59 and in fish groups that received the ORF 59 vaccination and poly I:C simultaneously (Table

4.2). This helps support the notion that IFN expression at 24 hours could be elicited by the vaccine [8], [12]. In fact, the early IFN response in fish vaccinated with DNA vaccines leads to Mx protein expression [12]. It was also noted that Mx protein expression occured 1 day after vaccination with DNA in fish [91]. In fish that only received poly I:C injections, antiviral protection decreased, which is what our *in vitro* time kinetics would predict; IFN mRNA expression ceases before 24 hours.

Fish injected with both ORF 59 and the poly I:C were the only fish group with increased antiviral protection at 48 hours after injection, while antiviral protection from fish injected with either only poly I:C or only ORF 59, did not change (Table 4.2). Most likely the stronger nonspecific antiviral protection seen in fish groups that received the DNA vaccine is due to the additive stimulation of the vaccine to induce innate anti-viral molecules in the fish.

At four weeks post vaccination and/or poly I:C injection, the virus neutralization titers were measured. The results were similar for all three fish groups; no neutralizing antibodies were detected. New data suggests that specific antibody protection does not usually show up as early as 4 weeks post vaccination [92], [91], [12]. In fact, McLauchlan *et al.* [12] demonstrated that the smaller the fish and DNA vaccine dose used, the longer it takes for neutralization antibodies to appear. Based on a previous publication by McLauchlan *et al.* [12], it is expected that neutralization antibodies will show up no earlier than 5 weeks after immunization based on fish size and vaccine dose. Even so, DNA vaccination against CCV has not been effective at 5 or 6 weeks post vaccination in channel catfish with very low neutralization antibody titers detected at these time points [91]. In fact, DNA vaccines have shown the most success against rhabdoviral diseases in fish, including infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic

septicemia virus (VHSV), [9], [11], [12], [13], snakehead rhabdovirus (SHRV) and spring viremia of carp virus (SVCV) [8]. Attempts to use DNA vaccines against other fish diseases has been limited (reviewed in [15]). Fish rhabdoviruses are presently the only pathogens where consistent and significant levels of protection have been reported following DNA vaccination (reviewed in [15]). None of the DNA vaccines for infectious salmon anaemia virus, an aquaorthomyxovirus, have provided protection to fish (created by E. Anderson and cited in [15]). Likewise, DNA vaccination against Atlantic halibut nodavirus also did not provide protection for fish [93].

Although our studied catfish had low neutralization titers, we did note nonspecific protection in antiviral assays with the fish serum. We do not have RNA evidence to support our data, but similar experiments have reported non antigen-specific protection in the form of Mx protein expression after DNA vaccination [8] [91]. Mx protein expression is only induced by IFN expression (reviewed in [22]). In fact, even with low neutralization results and poor protection results following CCV challenge, Mx protein expression was noted in catfish in all treatments and all doses as early as 1 day post vaccination and through 35 days post vaccination [91]. In our assays, the highest nonspecific protection was seen in fish 2 and 4 weeks after they received both the ORF 59 vaccination and poly I:C injection. We did not perform immersion challenge on the catfish, but we can speculate that they would not have survived CCV challenge based on low neutralization titers and previous studies [91] [93].

In our antiviral assays, fish that received the ORF 59 vaccine 1 hour before poly I:C injection had a GMT of 160, while the fish that received the ORF 59 vaccine and poly I:C injection at the same time had the next highest antiviral titer with GMT 113. The fish that received the poly I:C injection 1 hour before the ORF 59 vaccine had an

antiviral titer of GMT 80. Even lower antiviral titers were seen in fish that were only vaccinated with ORF 59 or fish that only received the poly I:C injection, at GMT of 17 and 20 respectively. These results are similar to a salmon study in which salmon received nonspecific protection up to 2 weeks after induction with poly I:C [83]. In another study, Mx proteins induced by poly I:C in catfish maintained protection up to 8 days (their last sampling point), even though Mx mRNA expression began to decline after 2 to 4 days post injection with poly I:C [76]. Possibly poly I:C elicits Mx mRNA expression within the first several days, but Mx protein expression lasts much longer, as seen with Jensen *et al.* [83]. Kim *et al.* [8] showed that at 2 weeks, nonspecific protection exist in vaccinated trout due to glycoprotein expression and Harbottle *et al.* [91] also showed Mx protein expression in vaccinated catfish. In these studies, fish were induced either by poly I:C or by DNA vaccines to produce Mx proteins and provided protection by innate immune mechanisms.

At 4 weeks antiviral titers from fish that received only the poly I:C injection decreased from GMT 20 to GMT 9 (Table 4.4). Similarly, it was suggested that nonspecific protection decreased 2 weeks after induction with poly I:C, because the half-life of Mx proteins were only around 2 weeks [83]. Jensen *et al.* [83] did not show whether protection persisted after 2 weeks. Our results suggest that without a strong antiviral titer at 4 weeks, the fish would not have antiviral protection against lethal viral challenge. Also, our virus neutralization tests showed that there would be no protection in these fish upon challenge. Our results suggest that although catfish IFN induces some antiviral protein to provide nonspecific protection 2 weeks post injection, this antiviral protein decreases between 2 and 4 weeks, so that almost no protection exists by 4 weeks. At 4 weeks, fish vaccinated with ORF 59 showed an increased antiviral titer from GMT 17 to GMT

40. It is unclear why nonspecific protection would increase at 4 weeks, especially when neutralization titers were so low. Nonspecific protection and Mx protein expression were seen in fish as long 4 weeks or 35 days [8] [91], but generally, specific protection begins around this time.

The results for fish that received the ORF 59 DNA vaccination and injection with poly I:C varied. In the group that received poly I:C 1 hour before the ORF 59 vaccine, there was a slight increase in the antiviral titer from 2 weeks GMT 80, to 4 weeks GMT 92. In the group where fish first received ORF 59 vaccination 1 hour before poly I:C injection, the antiviral titer stayed the same at GMT 160. However, in the group where ORF 59 and poly I:C were administered at the same time, there was a significant decrease in the antiviral titer from GMT 113 to GMT 35. It is unclear why there would be a strong decrease in this group while the other two groups that received both the vaccine and poly I:C do not show a decrease. These results suggest that the most effective way to vaccinate catfish against CCV is to administer the vaccine and poly I:C at different times.

Adding poly I:C to our vaccination did seem to increase nonspecific protection when compared to the ORF 59 vaccination given alone, although it remains unclear whether this nonspecific protection is due to the poly I:C acting as an adjuvant, or if it was due to the CpG motif in the plasmid of the vaccine, or if it was due to expressed viral glycoproteins. Our negative controls also show some antiviral nonspecific protection, but the protection is much lower than the positive samples. Likewise, it was shown that a low amount of Mx protein expression was detected in buffer injected control fish as well as non-treated fish verifying that low Mx expression was not a reaction to the poly I:C IP injection [83]. Viral glycoproteins are known inducers of IFN in mammals such as mice

[94], while CpG motifs in oligodeoxynucleotides have also been shown to provide protection shortly after administration to mice [16]. Krieg [16] speculates that CpG motifs, found in plasmid-based DNA vaccines, induce an innate response that subsequently promotes a specific response, acting as an intrinsic adjuvant. However, Lorenzen *et al.* [13] argue that CpG motifs alone are unlikely responsible for the early non-specific antiviral protection seen in DNA vaccination. In fact, most studies did not show protection in fish that received an empty DNA vaccine [8], [14], [11]. LaPatra *et al.* [10] would even go as far to argue that the early protection seen in DNA vaccinated fish is not unique to DNA vaccination, but is a feature of the natural response to endogenous expression of the rhabdo-viral G protein in somatic fish cells. However, Harbottle *et al.* [91] noted very low levels of Mx protein expression in their vector-only negative controls, whereas McLauchlan *et al.* [12] did not see Mx expression in vector-only negative controls. We did not include a vector-only negative control in our experiment. Without RNA evidence, CCV challenge data, or vector-only negative controls, we do not know if the nonspecific protection we saw in our antiviral titers was the result of the CpG motif found in plasmid-based DNA vaccines, or if the nonspecific protection was the direct result of the glycoprotein expressed in the vaccine.

In the poly I:C/CCV experiments in which we injected 50 μg poly I:C into fish, we detected a significant amount of IFN mRNA expression in the head kidneys. IFN mRNA expression in catfish induced with 50 μg poly I:C was seen as early as 1 hour post induction and lasted 7 hours post induction (our last sampling point), with the strongest IFN mRNA expression within the first 3 hours (Fig. 4.7). These results are also consistent with *in vitro* kinetics, which showed IFN expression occurred rapidly

within the first 7 hours after induction before decreasing almost completely by 12 hours and ceasing by 24 and 48 hours (Fig. 4.2).

The IFN mRNA expression in fish induced with 50 μg poly I:C correlated with their IFN titers (Fig. 4.7). The strongest antiviral protection was seen at 1 and 3 hours post injection with poly I:C (GMT 102 and GMT 81 respectively). At 5 and 7 hours post injection, antiviral protection decreased slightly, although it was still strong (GMT 64). Interestingly, this was also seen in the previous experiment in which fish were injected with only 10 μg poly I:C. In those assays, the strongest protection was within the first 4 hours at a titer of 64, before decreasing slightly to GMT 32 by 24 and 48 hours. The antiviral assays for the poly I:C/CCV experiments showed antiviral protection decreased at 5 hours to 64, which was equivalent to the poly I:C/ORF 59 antiviral titer at 4 hours (Table 4.2). In both experiments, fish induced with poly I:C showed strongest antiviral protection within the first several hours and this antiviral protection slowly decreased over the next 48 hours. This decrease in antiviral protection from both experiments correlated with the decrease in expression of IFN mRNA in the catfish, as seen when 50 μg poly I:C was used. Results from the poly I:C/ORF 59 experiment suggest that protection would continue to decrease over the next 2 weeks, so that at 2 weeks post injection with poly I:C, there is still some nonspecific innate proteins that would probably provide protection as seen by Jensen *et al.* [83].

Even though IFN mRNA expression in catfish induced with 50 μg poly I:C correlated with IFN mRNA expression in CCO cells (compare Figures 4.2 and 4.7), the antiviral titer taken from *in vitro* samples did not correlate with the antiviral titers taken from the *in vivo* samples (compare Figures 4.1 and 4.7). The strongest antiviral protection from the *in vivo* assays was within the first several hours, which was also the time

the strongest IFN mRNA expression occurred. However, antiviral activity from culture medium collected from the CCO cells exposed to poly I:C showed that antiviral activity continued to increase after IFN mRNA expression ceased. Our *in vitro* results were similar to work by Long *et al.* [29], which showed correlation between protection and IFN mRNA expression. In their results, poly I:C induced IFN mRNA expression only at 2 hours, whereas protection in challenge assays induced strongest protection at 2, 4 and 7 hours post treatment with poly I:C, with 4 hours having the strongest protection [29]. In our *in vitro* results 8 hours post induction with poly I:C, culture medium collected from CCO cells showed the strongest antiviral activity at a titer of 1:64, and this only slightly decreased by 12 hours post induction to 1:32, even though increased IFN mRNA expression had ceased. Possibly, soluble IFN in the culture medium accumulated at these time points rather than at earlier time points because maximum IFN mRNA production by the cells had occurred and IFN was released into the culture medium. Due to the incubation with poly I:C, cells released expressed IFN that offered protection against viral challenge to cells. However, Long *et al.* [48] found that two of the channel catfish IFN genes lack signal sequences, which they believe causes most of the IFN mRNA product to remain in the cytoplasm of the cell. Also, the original primers used, (created by Long *et al.* [29]), for the CF IFN-1 gene, were able to anneal to the other CF IFN genes. We were able to show with our IFN-2 specific primers that *in vitro* the CF IFN-2 gene, which is not a pseudogene, was being expressed and contributed to providing protection. Our work agreed with others [29] who demonstrated that the IFN gene was the factor responsible for antiviral activity and protection in challenge assays. However, it still remains to be determined whether catfish IFN inhibits viral replication through the induction of antiviral proteins, although previous work [83] [28], suggests that when

most fish are induced with poly I:C to produce IFN, the fish also induces antiviral proteins.

We also infected channel catfish with CCV to compare the kinetics of IFN mRNA expression induced with poly I:C or CCV and the protection offered by each (Fig. 4.7). IFN mRNA expression induced by CCV was comparable to poly I:C induction. Poly I:C induced IFN expression from 1 to 7 hours post induction, whereas CCV only induced IFN expression from 3 to 5 hours post infection. Antiviral titers showed that CCV infection induced less protection than that induced in fish with poly I:C. At 1 hour post infection with CCV, serum protection was at GMT of 51 and this protection increased slightly to GMT of 64 at 3 hours. This is the time when an increase in IFN mRNA expression was first seen. Subsequently, the protection decreased slightly to GMT of 51 and stayed there for 5 to 7 hours. The strongest antiviral protection was seen at 3 hours (GMT 64), which correlated with increased IFN mRNA expression. Poly I:C is possibly a better inducer of IFN mRNA expression and nonspecific antiviral protection. Although no increased IFN mRNA expression was observed in fish at 1 and 7 hours post CCV infection, protection was still strong, indicating some innate defense was at work.

The kinetics of IFN induction following CCV infection *in vivo* in channel catfish were compared with those following CCV infection *in vitro* in CCO cells (compare Figures 4.7 and 4.5). When the CCO cells were infected with CCV, they expressed incrased levels of IFN mRNA at 1, 4 and 5 hours post infection with CCV, although not at 2, 3 and 6 hours. Similarly, when channel catfish were infected with CCV, they showed increased IFN mRNA expression at 3 and 5 hours, although not at 1 and 7 hours. We did not collect medium from CCO cells infected with CCV to run in an antiviral assay, however,

previous studies by Chinchar *et al.* [26] indicate that virally infected cell medium can provide protection in challenge assays due to the induction of IFN.

Also worth noting was that IFN-2 mRNA was expressed in *in vitro* studies with CCO cells (Fig. 4.4). We designed IFN-2 primers unique to the catfish IFN-2 mRNA and used them to detect differences in kinetics of IFN-2 expression and expression of other catfish IFN genes detected with the original primers designed by Long *et al.* [29]. The primers designed by Long *et al.* were later found to anneal to all four IFN genes [48]. We were able to induce IFN-2 mRNA expression by both poly I:C and UV inactivated CCV in CCO cells. The UV irradiation did not completely inactivate all of mRNA from the CCV, as seen by the ORF 6 and ORF 53 rt-PCR results, although IFN-2 mRNA synthesis was enabled along with expression of all the catfish IFNs as seen with using the general primers that anneal to all four catfish genes.

Live CCV did not induce IFN-2 mRNA expression in CCO cells or channel catfish. A possible explanation for this is that CCV has evolved a way to elude the IFN system, specifically activation of the IFN-2 gene. It could be that CCV genome, as with other large DNA viruses, may encode proteins that inhibit IFN induction [26]. We would need to perform more experiments to further understand why live CCV did not induce IFN-2 expression in our cells or catfish. Long *et al.* [48], found that the catfish IFN-2 gene was upregulated due to live virus infection in cells, however they used catfish reovirus (CRV). They also found that catfish IFN-2 gene expression was induced in response to UV-CRV exposure.

Unlike *in vitro* experiments in CCO cells, we were unable to show induction of IFN-2 mRNA expression by poly I:C in channel catfish. Possibly, the IFN-2 gene is not expressed in the head kidneys of channel catfish. Alternatively, we could have been

unable to find IFN-2 mRNA expression because the amount of poly I:C used was too low to induce expression. Catfish IFN mRNA expression was only detected *in vivo* when the general IFN-1 primers, which anneal to all four genes were used (designed by [29]). This could be due to the expression of pseudogenes. We would need to re-run these experiments and clone our rt-PCR products to know for certain which IFN genes were being expressed. Also, at the time these experiments were being performed, we did not know that the primers were annealing to all four genes and that IFN-1 was a pseudogene.

In all experiments, controls were used to measure constitutive IFN expression in rt-PCRs and challenge assays. What is interesting in these experiments is the amount of IFN mRNA the experimental control expressed, since this is the gauge to compare induced to non-induced CCO cells and catfish IFN mRNA expression. Long *et al.* [29] showed that fibroblast and T cell lines constitutively synthesize low levels of IFN mRNA, while Nygaard *et al.* [28] found that unstimulated macrophages produced Mx proteins because of constitutive IFN production that was up-regulated by culturing of the cells. In contrast, Chinchar *et al.* [26] found that newly established T cell and macrophage cell lines did not allow CCV replication, which they believe to be due to the generation of an anti-viral factor [26]. As the T cell/macrophage lines are maintained in cell culture, they gradually became more permissive for CCV infection. This increased susceptibility was caused by the loss of synthesis of "non-essential proteins" (or cytokines) [26]. Other studies have confirmed this finding, showing that long-term catfish lymphoid cell lines, which initially secrete compounds functionally homologous to IL-2 and IL-4, eventually lose their ability to synthesize and/or secrete these "luxury proteins" after they have been passed for a while [26]. In other words, the cells begin to lose their differentiation. In our *in vitro* experiments with CCO cells, cells in culture for more than 4 days and

nearly confluent monolayers were more likely than less confluent cells to express IFN, and displayed higher background level of IFN mRNA expression, as seen in some of our rt-PCR and challenge assay experiments. Thus, the age and confluency of the cultures contribute to IFN mRNA expression. Possibly, older confluent cells become "stressed" when they run out of room to grow and divide in flasks, and therefore, induce a low level of IFN protection. Also, in CCO cells it is possible that constitutive levels of IFN mRNA expression showed up because we used a larger number of amplification cycles in rt-PCR protocols. Long *et al.* [29] found that by increasing the number of amplification cycles in rt-PCR protocols to greater than 15, their seemingly negative fibroblast CCO lines constitutively expressed low levels of IFN mRNA. Our protocols always used greater than 15 amplification cycles. IFN is expressed within the first 12 hours of induction and after 12 hours, IFN mRNA is expressed at levels less than or equal to constitutively expressed control levels. We also saw that live catfish express a low level of constitutive IFN as well. All of the experimental control fish expressed some small amount of IFN mRNA and showed some level of antiviral protection. Even when fish were not injected or vaccinated, they expressed a low background level of IFN mRNA. An explanation for IFN expression in control fish is that IFN is constitutively expressed or continuously induced due to exposure to viral particles present in water [83]. Likewise, low levels of IFN-α mRNA are known to be constitutively produced in organs and in peripheral blood monocytes of healthy people [96] [97].

Chapter 6

Overall Conclusions

We found that both 10 and 50 μg/ml poly I:C act similarly in CCO cells, inducing IFN mRNA expression within the first 7 hours post incubation with poly I:C. The IFN-2 gene was cloned and sequenced from CCO cells, and we found that this gene is strongly expressed within the first 3 hours after induction with poly I:C, and that CCV infection prevents IFN-2 mRNA expression. The minimum amount of poly I:C that can be used to provide consistent IFN mRNA expression *in vivo* is 50 μg poly I:C, because less poly I:C does not induce IFN mRNA expression consistent with *in vitro* results. We were unable to induce the INF 2 gene *in vivo* with poly I:C, although poly I:C does induce IFN-2 mRNA expression *in vitro* in CCO cells. CCV was also able to induce IFN mRNA expression *in vivo*, although not as well as poly I:C.

Our vaccination project showed that inducing IFN expression with poly I:C along with ORF 59 vaccination does not increase the effectiveness of vaccination, although there was some evidence of nonspecific protection. Our neutralization virus results were consistent with Harbottle *et al.* [91] who also found that neutralization titers are low 4 to 6 weeks post vaccination.

Bibliography

[1] Robertsen B. Modulation of the non-specific defense of fish by structurally conserved microbial polymers. *Fish & Shellfish Immunology*, 9:269–290, 1999.

[2] Ellis AE. Innate host mechanisms of fish against viruses and bacteria. *Developmental & Comparative Immunology*, 25:827–839, 2001.

[3] Nusbaum KE, Smith BF, DeInnocentes P, and Bird RC. Protective immunity induced by DNA vaccination of channel catfish with early and late transcripts of the channel catfish herpervirus (IHV-1). *Veterinary Immunology and Immunopathology*, 6545:1–18, 2001.

[4] Davison AJ. Channel catfish virus: a new type of herpesvirus. *Virology*, 186:9–14, 1992.

[5] Davison AJ and Davison MD. Identification of structural proteins of channel catfish virus by mass spectrometry. *Virology*, 206:1035–1043, 1995.

[6] Diagnostic Manual for Aquatic Animal Diseases. Channel Catfish Virus Disease. *http://www.oie.int/eng/normes/fmanual/A_summry.htm*, (accessed on June 30, 2003), 2000.

[7] Hogan RJ, Stuge TB, Clem LW, Miller NW, and Chinchar VG. Anti-viral cytotoxic cells in the channel catfish (*Ictalurus punctatus*). *Developmental & Comparative Immunology*, 20:115–127, 1996.

[8] Kim CH, Johnson MC, Drennan JD, Simon BE, Thomann E, and Leong JC. DNA vaccines encoding viral glycoproteins induce nonspecific immunity and Mx protein synthesis in fish. *Journal of Virology*, 74:7048–7054, 2000.

[9] LaPatra SE, Corbeil S, Jones GR, Shewmaker WD, and Kurath G. The dose-dependent effect on protection and humoral response to a DNA vaccine against infectious hematopoietic necrosis (IHN) virus in subyearling rainbow trout. *Journal of Aquatic Animal Health*, 12:181–188, 2000.

[10] LaPatra SE, Corbeil S, Jones GR, Shewmaker WD, Lorenzen N, Anderson ED, and Kurath G. Protection of rainbow trout against infectious hematopoietic necrosis virus four days after specific or semi-specific DNA vaccination. *Vaccine*, 19:4011–4019, 2001.

[11] Lorenzen E, Einer-Jensen K, Martinussen T, LaPatra SE, and Lorenzen N. DNA vaccination of rainbow trout against viral hemorrhagic septicemia virus: a dose-response and time-course study. *Journal of Aquatic Animal Health*, 12:167–180, 2000.

[12] McLauchlan PE, Collet B, Ingerslev E, Secombes CJ, Lorenzen N, and Ellis AE. DNA vaccination against viral haemorrhagic septicaemia (VHS) in rainbow trout: size, dose, route of injection and duration of protection–early protection correlates with Mx expression. *Fish & Shellfish Immunology*, 15:39–50, 2003.

[13] Lorenzen N, Lorenzen E, Einer-Jensen K, and LaPatra SE. DNA vaccines as a tool for analysing the protective immune response against rhabdoviruses in rainbow trout. *Fish & Shellfish Immunology*, 12:439–453, 2002.

[14] Lorenzen N, Lorenzen E, Einer-Jensen K, and LaPatra SE. Immunity induced shortly after DNA vaccination of rainbow trout against rhabdoviruses protects against heterologous virus but not against bacterial pathogens. *Developmental & Comparative Immunology*, 26:173–179, 2002.

[15] Lorenzen N and LaPatra SE. DNA vaccines for aquacultured fish. *Rev. sci. tech. Epiz.*, 24:201–213, 2005.

[16] Krieg AM. The role of CpG motifs in innate immunity. *Current Opinion in Immunology*, 12:35–43, 2000.

[17] Anderson ED, Mourich DV, Fahrenkrug SC, LaPatra S, Shepherd J, and Leong JA. Genetic immunization of rainbow trout (*Oncorhynchus mykiss*) against infectious hematopoietic necrosis virus. *Molecular Marine Biology and Biotechnology*, 5:114–122, 1996.

[18] Roy SW, Fedorov A, and Gilbert W. Large-scale comparison of intron positions in mammalian genes shows intron loss but no gain. *Proceedings of the National Academy of Sciences of the USA*, 100:7158–7162, 2003.

[19] Hedrick RP, LaPatra SE, Yun S, Lauda KA, Jones GR, Congleton JL, and de Kinkelin P. Induction of protection from infectious hematopoietic necrosis virus in rainbow trout *Oncorhynchus mykiss* by pre-exposure to the avirulent cutthroat trout virus. *Diseases of Aquatic Organisms*, 20:111–118, 1994.

[20] Robertsen B, Engstad R, and Jorgensen JB. β-Glucans as immunostimulants in fish. *In Modulators of Fish Immune Response*, pages 83–99, 1994.

[21] Isaacs A and Lindenmann J. Virus interference I. The interferon. *Proceedings of the Royal Society London B: Biological Sciences*, 147:258–267, 1957.

[22] Samuel CE. Antiviral actions of interferons. *Clinical Microbiology Reviews*, 14:778–809, 2001.

[23] Taniguchi T, Mantei N, Schwarzstein M, Nagata S, Muramatsu M, and Weissmann C. Human leukocyte and fibroblast interferons are structurally related. *Nature*, 285:547–549, 1980.

[24] Taniguchi T, Fujii-Kuriyama Y, and Muramatsu M. Molecular cloning of human interferon cDNA. *Proceedings of the National Academy of Sciences USA*, 77:4003–4006, 1980.

[25] Gravell M and Malsberger RG. A permanent cell line from the fathead minnow (*Pimephales promelas*). *Annals of the New York Academy of Sciences*, 126:555–565, 1965.

[26] Chinchar VG, Logue O, Antao A, and Chinchar GD. Channel catfish reovirus (CRV) inhibits replication of channel catfish herpesvirus (CCV) by two distinct mechanisms: viral interference and induction of an anti-viral factor. *Diseases of Aquatic Organisms*, 33:77–85, 1998.

[27] LaPatra SE, Lauda KA, and Jones GR. Aquareovirus interference mediated resistance to infectious hematopoietic necrosis virus. *Veterinary Research*, 26:455–459, 1995.

[28] Nygaard R, Husgard S, Sommer A, Leong JC, and Robertsen B. Induction of Mx protein by interferon and double stranded RNA in salmonid cells. *Fish & Shellfish Immunology*, 10:435–450, 2000.

[29] Long S, Wilson M, Bengten E, Bryan L, Clem LW, Miller NW, and Chinchar VG. Identification of a cDNA encoding channel catfish interferon. *Developmental & Comparative Immunology*, 28:97–111, 2004.

[30] Altmann SM, Mellon MT, Distel DL, and Kim CH. Molecular and functional analysis of interferon gene from the zebrafish, *Danio rerio*. *Journal of Virology*, 77:1992–2002, 2003.

[31] Lutfalla G, Crollius HR, Stange-Thomann N, Jaillon O, Mogensen K, and Monneron D. Comparative genomic analylsis reveals independent expansion of a lineage-specific gene family in vertebrates: the class II cytokine receptors and their ligands in mammals and fish. *BMC Genomics*, 4:29, 2003.

[32] Robertsen B, Bergan V, Rokenes T, Larsen R, and Albuquerque A. Atlantic salmon interferon genes: cloning, sequence analysis, exression, and biological activity. *Journal of Interferon & Cytokine Research*, 23:601–612, 2003.

[33] Pestka S, Langer JA, Zoon KC, and Samuel CE. Interferons and their actions. *Annual Review of Biochemistry*, 56:727–777, 1987.

[34] Kotenko SV, Gallagher G, Baurin VV, Lewis-Antes A, Shen M, Shah NK, Langer JA, Sheikh F, Dickensheets H, and Donnelly RP. IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. *Nature Immunology*, 4:69–77, 2003.

[35] Chen J, Baig E, and Fish E. Diversity and relatedness among the type I interferons. *Journal of Interferon & Cytokine Research*, 24:687–698, 2004.

[36] Pestka S, Krause CD, and Walter MR. Interferons, interferon-like cytokines, and their receptors. *Immunological Reviews*, 202:8–32, 2004.

[37] Martal JL, Chene NM, Huynh LP, L'Haridon RM, Reinaud PB, Guillomot MW, Charlier MA, and Charpigny SY. IFN-tau: a novel subtype I IFN1. Structural characteristics, non-ubiquitious expression, structure-function relationships, a pregnancy hormonal embryonic signal and cross-species therapeutic potentialities. *Biochimie*, 80:755–777, 1998.

[38] LaFleur DW, Nardelli B, Tsareva T, Mather D, Feng P, Semenuk M, Taylor K, Buergin M, Chinchilla D, Roshke V, Chen G, Ruben SM, Pitha PM, Coleman TA, and Moore PA. Interferon-kappa, a novel type I interferon expressed in human keratinocytes. *Journal of Biological Chemistry*, 276:39765–39771, 2001.

[39] Oritani K, Medina KL, Tomiyama Y, Ishikawa J, Okajima Y, Ogawa M, Yokota T, Aoyama K, Takahashi I, Kincade PW, and Matsuzawa Y. Limitin: an interferon-like cytokine that preferentially influences B-lymphocyte precursors. *Nature Medicine*, 6:659–666, 2000.

[40] Lefevre F and Boulay V. A novel and atypical type one interferon gene expressed by trophoblast during early pregnancy. *Journal of Biological Chemistry*, 268:19760–19768, 1993.

[41] Karpusas M, Nolte M, Benton CB, Meier W, Lipscomb WN, and Goelz S. The crystal structure of human interferon beta at 2.2-A resolution. *Proceedings of the National Academy of Sciences USA*, 94:11813–11818, 1997.

[42] Radhakrishnan R, Walter LJ, Hruza A, Reichert P, Trotta PP, Nagabhushan TL, and Walter MR. Zinc mediated dimer of human interferon-α_{2b} revealed by X-ray crystallography. *Structure*, 4:1453–1463, 1996.

[43] Radhakrishnan R, Walter LJ, Subramaniam PS, Johnson HM, and Walter MR. Crystal structure of ovine interferon-τ at 2.1 A resolution. *Journal of Molecular Biology*, 286:151–162, 1999.

[44] Senda T, Shimazu T, Matsuda S, Kawano G, Shimizu H, Nakamura KT, and Mitsui Y. Three-dimentional crystal structure of recombinant murine interferon-β. *EMBO Journal*, 11:3193–3201, 1992.

[45] Levy DE and Garcia-Sastre A. The virus battles: IFN induction of the antiviral state and mechanisms of viral evasion. *Cytokine & Growth Factor Reviews*, 12:143–156, 2001.

[46] Collet B and Secombes CJ. Type I-interferon signaling in fish. *Fish & Shellfish Immunology*, 12:389–397, 2002.

[47] Haller O and Kochs G. Interferon-induced Mx proteins: dynamin-like GTPases with antiviral activity. *Traffic*, 3:710–717, 2002.

[48] Long S, Milev-Milovanovic I, Wilson M, Bengten E, Clem LW, Miller NW, and Chinchar VG. Identification and expression analysis of cDNAs encoding channel catfish type I interferons. *Fish & Shellfish Immunology*, 21:42–59, 2006.

[49] Robertsen B. The interferon system of teleost fish. *Fish & Shellfish Immunology*, 20:172–191, 2006.

[50] Waine GJ, Tymms MJ, Brandt ER, Cheetham BF, and Linnane AW. Structure-fuction study of the region encompassing residues 26-40 of human interferon-α4: identification of residues important for antiviral and antiproliferative activities. *Journal of Interferon Research*, 12:43–48, 1992.

[51] Roberts RM, Liu L, Guo Q, Leaman D, and Bixby J. The evolution of the type I interferons. *Journal of Interferon & Cytokine Research*, 18:805–816, 1998.

[52] Zou J, Yoshiura Y, Dijkstra JM, Sakai M, Ototake M, and Secombes C. Identification of an interferon gamma homologue in Fugu, *Takifugu rubripes*. *Fish & Shellfish Immunology*, 17:403–409, 2004.

[53] Charlemagne J, Fellah JS, De Guerra A, Kerfourn F, and Partula S. T-cell receptors in ectothermic vertebrates. *Immunology Reviews*, 166:87–102, 1998.

[54] Hansen JD and Strassburger P. Description of an ectothermic TCR coreceptor, CD8 alpha, in rainbow trout. *Journal of Immunology*, 164:3132–3139, 2000.

[55] Suetake H, Araki K, and Suzuki Y. Cloning, expression, and characterization of fugu CD4, the first ectothermic animal CD4. *Immunogenetics*, 56:368–374, 2004.

[56] Nakanishi T, Fischer U, Dijkstra JM, Hasegawa S, Somamoto T, Okamoto N, and Ototake M. Cytotoxic T cell function in fish. *Developmental & Comparative Immunology*, 26:131–139, 2002.

[57] Yoshiura Y, Kiryu I, Fujiwara A, Suetake H, Suzuki Y, Nakanishi T, and Ototake M. Identification and characterization of fugu orthologues of mammalian interleukin-12 subunits. *Immunogenetics*, 55:296–306, 2003.

[58] Zou J, Bird S, Truckle J, Bols N, Horne M, and Secombes C. Identification and expression analysis of an IL-18 homologue and its alternatively spliced form in rainbow trout *Oncorhynchus mykiss*. *European Journal of Biochemistry*, 271:1913–1923, 2004.

[59] Graham S and Secombes CJ. The production of a macrophage-activating factor from rainbow trout Salmo gairdneri leucocytes. *Immunology*, 65:293–297, 1988.

[60] Wang T, Ward M, Grabowski P, and Secombes CJ. Molecular cloning, gene organization and expression of rainbow trout (*Oncorhynchus mykiss*) inducible nitric oxide synthase (iNOS) gene. *Biochemical Journal*, 358:747–755, 2001.

[61] Chang MS, Chang GD, Leu JH, Huang FL, Chou CK, Huang CJ, and Lo TB. Expression, characterization, and genomic structure of carp JAK1 kinase gene. *DNA and Cell Biology*, 10:827–844, 1996.

[62] Conway G, Margoliath A, Wong-Madden S, Roberts RJ, and Gilbert W. Jak1 kinase is required for cell migrations and anterior specification in zebrafish embryos. *Proceedings of the National Academy of Sciences of the USA*, 94:3082–3087, 1997.

[63] Leu JH, Chang MS, Yao CW, Chou CK, Chen ST, and Huang CJ. Genomic organization and characterization of the promoter region of the round-spotted pufferfish (*Tetraodon fluviatilis*) *JAK1* kinase gene. *Biochimica et Biophysica Acta*, 1395:50–56, 1998.

[64] Leu JH, Yan SJ, Lee TF, Chou CM, Chen ST, Hwang PP, Chou CK, and Huang CJ. Complete genomic organization and promoter analysis of the round-spotted pufferfish *JAK1, JAK2, JAK3,* and *TYK2* genes. *DNA and Cell Biology*, 19:431–446, 2000.

[65] Oates AC, Wollberg P, Pratt SJ, Paw BH, Johnson SL, Ho RK, Postlethwait JH, Zon LI, and Wilks AF. Zebrafish *stat3* is expressed in restricted tissues during embryogenesis and *stat1* rescues cytokine signaling in a *STAT1*-deficient human cell line. *Developmental Dynamics*, 215:352–370, 1999.

[66] Sung SC, Fan TJ, Chou CM, Leu JH, Hsu YL, Chen ST, Hsieh YC, and Huang CJ. Genomic structure, expression and characterization of a STAT5 homologue from pufferfish (*Tetraodon fluviatilis*). *European Journal of Biochemistry*, 270:239–252, 2003.

[67] Zhang Y and Gui J. Molecular characterization and IFN signal pathway analysis of *Carassius auratus CaSTAT*1 identified from the cultured cells in response to virus infection. *Developmental & Comparative Immunology*, 28:211–227, 2004.

[68] Staeheli P, Yu YX, Grob R, and Haller O. A double-stranded RNA-inducible fish gene homologous to the murine influenza virus resistance gene *Mx*. *Molecular and Cellular Biology*, 9:3117–3121, 1989.

[69] Trobridge GD and Leong JAC. Characterization of a rainbow trout Mx gene. *Journal of Interferon & Cytokine Research*, 15:691–702, 1995.

[70] Trobridge GD, Chiou PP, and Leong JC. Cloning of the rainbow trout (*Oncorhynchus mykiss*) Mx2 and Mx3 cDNAs and characterization of trout Mx protein expression in salmon cells. *Journal of Virology*, 71:5304–5311, 1997.

[71] Robertsen B, Trobridge G, and Leong J. Molecular cloning of double-stranded RNA inducible Mx genes from Atlantic salmon (*Salmo salar* L.). *Developmental & Comparative Immunology*, 21:397–412, 1997.

[72] Jensen V and Robertsen B. Cloning of an Mx cDNA from Atlantic halibut (*Hippoglussus hippoglossus*) and characterization of Mx mRNA expression in response to double-stranded RNA or infectious pancreatic necrosis virus. *Journal of Interferon & Cytokine Research*, 20:701–710, 2000.

[73] Lee JY, Hirono I, and Aoki T. Cloning and analysis of expression of Mx cDNA in Japanese flounder, *Paralichtys olivaceus*. *Developmental & Comparative Immunology*, 24:407–415, 2000.

[74] Yap WH, Tay A, Brenner S, and Venkatesh B. Molecular cloning of the pufferfish (*Takifugu rubripes*) and functional characterization of its promoter. *Immunogenetics*, 54:705–713, 2003.

[75] Zhang YB, Li Q, and Gui JF. Differential expression of two *Carassius auratus* Mx genes in cultured CAB cells induced by grass carp hemorrhage virus and interferon. *Immunogenetics*, 56:68–75, 2004.

[76] Plant KP and Thune RL. Cloning and characterisation of a channel catfish (*Ictalurus punctatus*) Mx gene. *Fish & Shellfish Immunology*, 16:391–405, 2004.

[77] Tafalla C, Aranguren R, Secombes CJ, Figueras A, and Novoa B. Cloning and analysis of expression of a gilthead sea bream (*Sparus aurata*) Mx cDNA. *Fish & Shellfish Immunology*, 16:11–24, 2004.

[78] Altmann SM, Mellon MT, Johnson MC, Paw BH, Trede NS, Zon LI, and Kim CH. Cloning and characterization of an Mx gene and its corresponding promoter from the zebrafish, *Danio rerio*. *Developmental & Comparative Immunology*, 28:295–306, 2004.

[79] Chen YM, Su YL, Lin JH, Yang HL, and Chen TY. Cloning of an orange-spotted grouper (*Epinephelus coioides*) Mx cDNA and characterisation of its expression in response to nodavirus. *Fish & Shellfish Immunology*, 20:58–71, 2006.

[80] Larsen R, Rokenes TP, and Robertsen B. Inhibition of infectious pancreatic necrosis virus replication by Atlantic salmon Mx1 protein. *Journal of Virology*, 78:7938–7944, 2004.

[81] Bergan V and Robertsen B. Characterization of Atlantic halibut (*Hippoglossus hippoglossus*) Mx protein expression. *Developmental & Comparative Immunology*, 28:1037–1047, 2004.

[82] Collet B and Secombes CJ. The rainbow trout (*Oncorhynchus mykiss*) Mx1 promotor. Structural and functional characterization. *European Journal of Biochemistry*, 268:1577–1584, 2001.

[83] Jensen I, Albuquerque A, Sommer AI, and Robertsen B. Effect of poly I:C on the expression of Mx proteins and resistance against infection by infectious salmon anaemia virus in Atlantic salmon. *Fish & Shellfish Immunology*, 13:311–326, 2002.

[84] Jensen I and Robertsen B. Effect of double-stranded RNA and interferon on the antiviral activity of Atlantic salmon cells against infectious salmon anemia virus and infectious pancreatic necrosis virus. *Fish & Shellfish Immunology*, 13:221–241, 2002.

[85] Caipang CM, Hirono I, and Aoki T. In vitro inhibition of fish rhabodviruses by Japanese flounder, *Paralichthys olivaceus* Mx. *Virology*, 317:373–382, 2003.

[86] Garner JN, Joshi B, and Jagus R. Characterization of rainbow trout and zebrafish eukaryotic initiation factor 2α and its response to endoplasmic reticulum stress and IPNV infection. *Developmental & Comparative Immunology*, 27:217–231, 2003.

[87] Hu CY, Zhang YB, Huang GP, Zhang QY, and Gui JF. Molecular cloning and characterisation of a fish PKR-like gene from cultured CAB cells induced by UV-inactivated virus. *Fish & Shellfish Immunology*, 17:353–366, 2004.

[88] von Wussow P, Jakschies D, Hochkeppel HK, Fibich C, Penner L, and Deicher H. The human intracellular Mx-homologous protein is specifically induced by type I interferons. *European Journal of Immunology*, 20:2015–2019, 1990.

[89] Schumacher B, Bernasconi D, Schultz U, and Staeheli P. The chicken Mx promoter contains an ISRE motif and confers interferon inducibility to a reporter gene in chick and monkey cells. *Virology*, 203:144–148, 1994.

[90] Schmidt NJ and Emmons RW. Calculation of the LD50 by the Karber method. in Diagnostic Procedures for Viral, Rickettsial, and Chlamydial Infections. *American Public Health Association*, 6:20–21, 1989.

[91] Harbottle H, Plant KP, and Thune RL. DNA vaccination against channel catfish virus results in minimal immune response and is not efficacious against challenge. *Journal of Aquatic Animal Health*, 17:251–262, 2005.

[92] Bromage ES, Kaattari IM, Zwollo P, and Kaattari SL. Plasmablast and plasma cell production and distribution in trout immune tissues. *The Journal of Immunology*, 173:7317–7323, 2004.

[93] Sommerset I, Skern R, Biering E, Bleie H, Fiksdal U, Grove S, and Nerland AH. Protection against Atlantic halibut nodavirus in turbot is induced by recombinant capsid protein vaccination but not following DNA vaccination. *Fish & Shellfish Immunology*, 18:13–29, 2005.

[94] Ito Y. Induction of interferon by virus glycoprotein(s) in lymphoid cells through interaction with the cellular receptors via lectin-like action: an alternative interferon induction mechanism. *Archives of Virology*, 138:187–198, 1994.

[95] Leitner WW, Hwang LN, deVeer MJ, Zhou A, Silverman RH, Williams BRG, Dubensky TW, Ying H, and Restifo NP. Alphavirus-based DNA vaccine breaks immunological tolerance by activating innate antiviral pathways. *Nature Medicine*, 9:33–39, 2002.

[96] Tovey MG, Streuli M, Gresser I, Gugenheim J, and Blanchard B. Interferon messenger RNA is produced constitutively in the organs of normal individuals. *Proceedings of the National Academy of Sciences USA*, 84:5038–5042, 1987.

[97] Lallemand C, Lebon P, Rizza P, Blanchard B, and Tovey MG. Constitutive expression of specific interferon isotypes in peripheral blood leukocytes from normal individuals and in promonocytic U937 cells. *Journal of Leukocyte Biology*, 60:137–146, 1996.

[98] Collet B, Hovens GC, Mazzoni D, Hirono I, Aoki T, and Secombes CJ. Cloning and expression analysis of rainbow trout *Oncorhynchus mykiss* interferon regulatory factor 1 and 2 (IRF-1 and IRF-2). *Developmental & Comparative Immunology*, 27:111–126, 2003.

[99] Alonso M, Johnson M, Simon B, and Leong J. A fish specific expression vector containing the interferon regulatory factor 1A (IRF1A) promoter for genetic immunization of fish. *Vaccine*, 21:1591–1600, 2003.

[100] Alonso M and Leong J. Suppressive subtraction libraries to identify interferon-inducible genes in fish. *Marine Biotechnology*, 4:74–80, 2002.

[101] Aparicio S, Chapman J, Stupka E, Putnam N, Chia JM, Dehal P, Christoffels A, Rash S, Hoon S, Smit A, Gelpke MDS, Roach J, Oh T, Ho IY, Wong M, Detter C, Verhoef F, Predki P, Tay A, Lucas S, Richardson P, Smith SF, Clark MS, Edwards YJK Doggett N, Zharkikh A, Tavtigian SV, Pruss D, Barnstead M, Evans C, Baden H, Powell J, Glusman G, Rowen L, Hood L, Tan YH, Elgar G, Hawkins T, Venkatesh B, Rokhsar D, and Brenner S. Whole-genome shotgun assembly and analysis of the genome of (*Fugu rubripes*). *Science*, 297:1301–1310, 2002.

[102] Awad MA, Nusbaum KE, and Brady YJ. Preliminary studies of a newly developed subunit vaccine for channel catfish virus disease. *Journal of Aquatic Animal Health*, 1:233–237, 1989.

[103] Bazzigher L, Pavlovic J, Haller O, and Staeheli P. Mx genes show weaker primary response to virus than other interferon-regulated genes. *Virology*, 186:154–160, 1992.

[104] Brierley MM and Fish EN. IFN-α/β receptor interactions to biolgic outcomes: understanding the circuitry. *Journal of Interferon & Cytokine Research*, 22:835–845, 2002.

[105] Grunewald K, Desai P, Winkler DC, Heymann JB, Belnap DM, Baumeister W, and Steven AC. Three-dimensional structure of herpes simplex virus from cyro-electron tomography. *Science*, 302:1396–1398, 2003.

[106] Hedrick RP and McDowell T. Passive transfer of sera with antivirus neutralizing activity from adult channel catfish protects juveniles from channel catfish virus disease. *Transactions of the American Fisheries Society*, 116:277–281, 1987.

[107] Hedrick RP, Groff JM, and McDowell T. Response of adult channel catfish to waterborne exposure of channel catfish virus. *The Progressive Fish-Culturist*, 49:181–187, 1987.

[108] Jordan IK, Makarova KS, Spouge JL, Wolf YI, and Koonin EV. Lineage-specific gene expressions in bacterial and archaeal genomes. *Genome Research*, 11:555–565, 2001.

[109] Kochs G and Haller O. Interferon-induced human MxA GTPase blocks nuclear import of Thogoto virus nucleocapsids. *Proceedings of the National Academy of Sciences of the USA*, 96:2082–2086, 1999.

[110] LaPatra SE, Lauda KA, Jones GR, Shewmaker WS, and Bayne CJ. Resistance to IHN virus infection in rainbow trout is increased by glucan while subsequent production of serum neutralizing activity is decreased. *Fish & Shellfish Immunology*, 8:435–446, 1998.

[111] Lunde H and Robertsen B. HP8 The complement component C3 is produced by hepatocytes and behaves as an acute-phase protein in Atlantic salmon, *Salmo salar* L., after injection of β-glucan. *Developmental & Comparative Immunology*, 21:150, 1997.

[112] Noga EJ and Hartmann JX. Establishment of walking catfish (*Clarius batrachus*) cell lines and development of a channel catfish (*Ictalurus punctatus*) virus vaccine. *Canadian Journal of Fisheries and Aquatic Sciences*, 38:925–930, 1981.

[113] Shen L, Stuge TB, Zhou H, Khayat M, Barker KS, Quiniou SM, Wilson M, Bengten E, Chinchar VG, Clem LW, and Miller NW. Channel catfish cytotoxic cells: a mini-review. *Developmental & Comparative Immunology*, 26:141–149, 2002.

[114] Stark GR, Kerr IM, Williams BR, Silverman RH, and Schreiber RD. How cells respond to interferons. *Annual Review of Biochemistry*, 67:227–264, 1998.

[115] Zhang HG, Hanson LA, and Kancharla SR. Channel catfish virus recombinants display reduced pathogenesis yet induce specific immunity in channel catfish fingerlings. *Proceedings of the International Symposium on Aquatic Animal Health*, 1994.

[116] Abbas AK, Lichtman AH, and Pober JS. *Cellular and Molecular Immunology*. W.B. Saunders Company, 5 edition, 2003.

[117] Janeway C, Shlomchik MJ, Walport M, and Travers P. *Immunobiology*. Garland, 6 edition, 2005.