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The innate antiviral effects of extracellular viral dsRNA in rainbow trout cells

by

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HBSc Molecular Biology and Genetics, McMaster University, 2012

THESIS

Submitted to the Department of Biology

Faculty of Science

in partial fulfillment of the requirements for the

Master of Science in Integrative Biology

Wilfrid Laurier University

2014

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Abstract

Viral double-stranded RNA (dsRNA) molecules are a potent pathogen-associated molecular pattern and play a crucial role in the innate immune response. During a viral infection, extracellular and intracellular dsRNA can initiate pathways resulting in the production of type I interferons (IFNs) and interferon-stimulated genes (ISGs). The accumulation of ISGs within a cell results in a protective antiviral state. This study used both commercially available dsRNA (poly I:C) and *in vitro* transcribed dsRNA molecules, based on the viral hemorrhagic septicemia virus (VHSV) genome sequence, as stimuli to investigate the effects of these molecules on the innate immune response in rainbow trout cells. The goals of the present project were to elucidate the i) IFN ii) ISG and iii) antiviral responses of fish cells to both types of dsRNA molecules. Different lengths of poly I:C and *in vitro* transcribed dsRNA were used to determine potential length effects of dsRNA in fish cells. The aims of the project were achieved using a functional interferon assay, an ISG-promoter reporter system, an antiviral assay, and RT-PCR. It was found that extracellular dsRNA, either poly I:C or *in vitro* transcribed dsRNA, is able to induce innate antiviral responses in the fish cell line, RTG-2. Consistent with mammalian studies there was a greater magnitude of immune response when cells were stimulated with longer dsRNA molecules, demonstrating dsRNA length effects in fish cells.

Acknowledgements

I would like to acknowledge the many individuals who were involved with my MSc and those who helped to get me here. I would like to thank my supervisor Dr. Stephanie DeWitte-Orr for her support and mentoring. Her positive and optimistic personality has shaped my outlook on scientific research. I look forward to *many* more years of working together. I would also like to thank all the members of the DeWitte-Orr lab, past and present, for making the lab a fun and enjoyable environment, as well as the Bols lab, particularly the wonderful Nguyen Vo-Tran for all of his assistance. I would like to thank my committee members Dr. Joel Weadge and Dr. Matt Smith for their guidance and advice.

A gigantic thank you is due to my family and my friends. My parents have been the image of perfection with their encouraging words and their unwavering belief in me. I would like to thank my partner Cody for his patience and love, and for his ability to listen to hours of my scientific presentations and still pretend to be interested.

I would like to dedicate this thesis to my late Grandfather who passed away at the beginning of this year. He is the hardest working individual I have ever known and I hope that I have been able to embody even a sliver of his work ethic in my project.

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Commonly Used Abbreviations

2-5 A – 2-5 adenylates
acLDL – Acetylated low-density lipoprotein
bp – Base pair
cDNA – Complementary DNA
CFDA-AM – Carboxyfluorescein diacetate acetoxymethyl ester
CPE – Cytopathic effect
CSV – Chum salmon reovirus
dNTP – deoxynucleotide triphosphate
dsRNA – Double stranded RNA
eIF2α – Eukaryotic initiation factor
FBS – Fetal bovine serum
HMW – High molecular weight poly I:C
IFN – Interferon
IPS-1 – Interferon promoter stimulator
ISG – Interferon-stimulated gene
ISRE – Interferon-sensitive response element
kb- Kilobase pairs
L-15 – Leibovitz's L-15 media
LGP2 – Laboratory of Genetics and Physiology 2
LMW – Low molecular weight poly I:C
MDA5 – Melanoma differentiation-associated protein 5
OAS – Oligoadenylate synthetase
PAMP – Pathogen associated molecular pattern
PCR– Polymerase chain reaction
PKR – DsRNA-dependent serine/threonine protein kinase R
Poly I:C – Polyinosinic: polycytidylic acid
PRR – Pattern recognition receptor
RIG-I – Retinoic acid-inducible gene 1
RLR – RIG-I-like receptors
RT-PCR – Reverse transcriptase PCR
SR-A – Class A scavenger receptor
SR – Scavenger receptor
TCID50 – 50% Tissue culture infective dose
TIR– Toll or interleukin-1 receptor
TLR – Toll-like receptor
TICAM –TIR domain- containing adaptor molecule
VHSV – Viral hemorrhagic septicemia virus

CHAPTER 1: INTRODUCTION

1.1 Double Stranded (ds)RNA

Double-stranded RNA (ds)RNA was first identified in 1963 in encephalomyocarditis-infected mouse ascites tumor cells (Montagnier and Sanders, 1963*)*. The dsRNA structure has since been elucidated; dsRNA is comprised of two antiparallel strands in a right-handed double helix (DeWitte-Orr and Mossman, 2010). dsRNA has a 11-fold helical pitch and exhibits Watson-Crick base pairing (Tang and Draper, 1994). dsRNA has an A-helix motif and a narrow, deep major groove, and a shallow minor groove. The diameter of dsRNA is 30% larger than DNA, and the net charge of dsRNA is lower than DNA (Gast and Sanger, 1994; Van Den Hout *et al.,* 2010). The molar absorbance coefficient of dsRNA is lower than ssRNA and the melting temperature of dsRNA is higher than ssRNA or DNA (Libonati *et al.,* 1980*)*. DsRNA is stable in the extracellular space because of its high melting temperature and resistance to nuclease degradation. dsRNA is not digested by RNase A and B, or by RNase T1 under high salt conditions, but is digested by RNase III (Libonati *et al.,* 1980; DeWitte-Orr and Mossman, 2010).

1.1.1 Viral DsRNA

All viruses produce dsRNA at some point in their replication cycle (Jacobs and Langland, 1996). This includes viruses of all genome types; dsRNA is produced as a replicative intermediate from ssRNA viruses, genomic fragments from dsRNA viruses, and by overlapping convergent transcripts from DNA viruses (Jacobs and Langland, 1996). The dsRNA molecules produced by a viral infection are referred to as native

dsRNA. As dsRNA is a byproduct of replication, the length of the viral genome appears to correlate with the size of the dsRNA molecules produced; larger genomes have the capacity to generate larger dsRNA molecules (DeWitte-Orr *et al.,* 2009).

dsRNA is an effective signaling molecule for a number of reasons. As mentioned above it is resistant to nuclease degradation and as such is stable in the extracellular space (Takeuchi and Akira, 2009). It is also a potent signaling molecule; a single long dsRNA molecule within a cell is sufficient to induce a response (Marcus and Sekellick, 1977). Host cells in vertebrates do not produce endogenous dsRNA greater than ~30bp in length whereas viruses produce longer molecules (DeWitte-Orr and Mossman, 2010). This difference allows the immune system to discriminate self- and viral-dsRNA molecules (DeWitte-Orr and Mossman, 2010). Cells use endogenous dsRNA molecules in the RNAi pathway (Novina and Sharp, 2004). When an infected cell lyses the viral dsRNA is released into the extracellular space (Figure 1.1). These molecules can be recognized and brought into neighbouring cells by surface expressed class-A scavenger receptors (SR-As) through clathrin-mediated endocytosis (DeWitte-Orr *et al*., 2010). Viral dsRNA in the endosome activates endosomal dsRNA receptors; subsequently, dsRNA appears to escape the endosome by an undetermined mechanism, activating cytoplasmic receptors as well (Figure 1.1) (DeWitte-Orr *et al*., 2010).

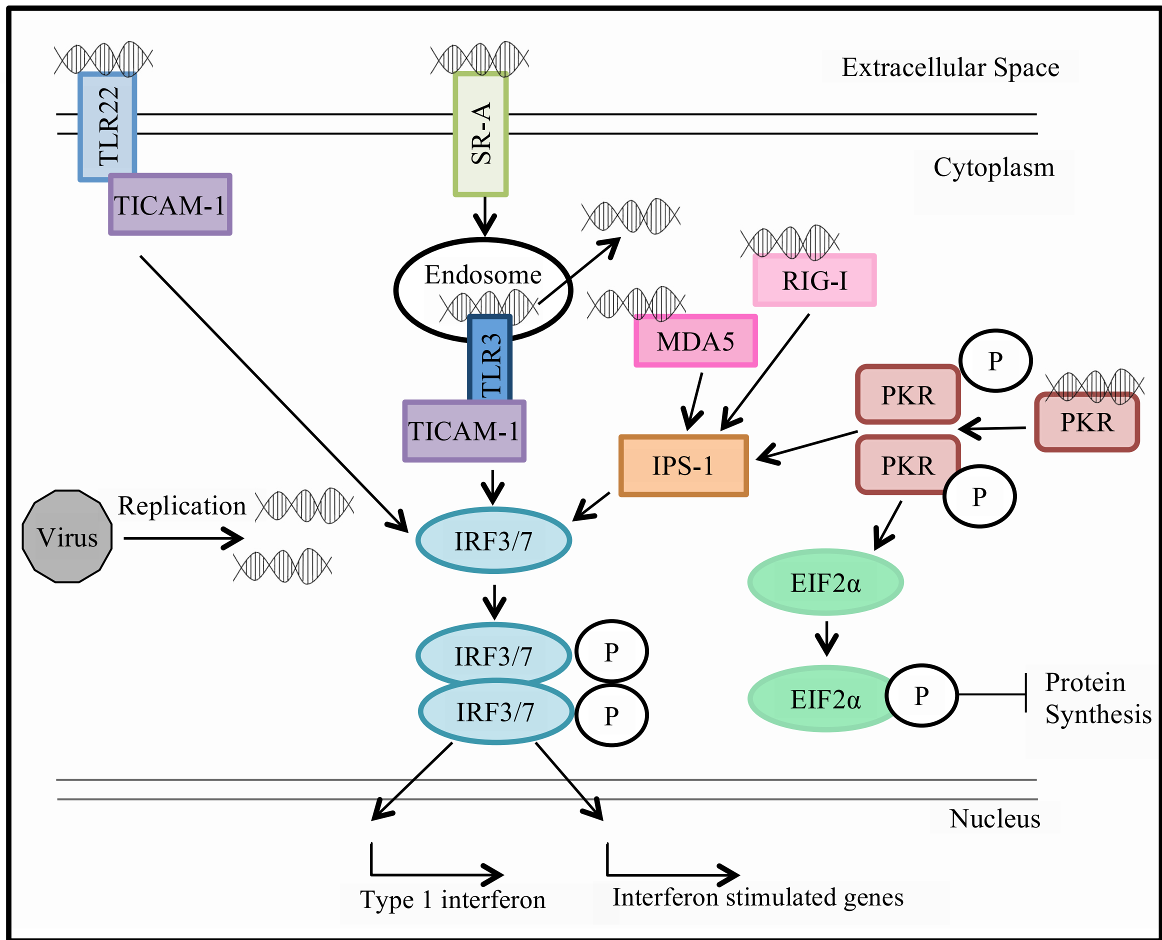

Figure 1.1 Viral dsRNA initiated antiviral pathways.
Extracellular viral dsRNA is recognized by scavenger receptors or TLR22, both located on the cell membrane. TLR22 recruits TICAM-1 and initiates ISG and interferon synthesis through IRF3 and IRF7. SR-As internalize the dsRNA through clathrin-mediated endocytosis, where dsRNA either remains in the endosome or escapes into the cytoplasm. Viral replication also results in cytoplasmic dsRNA. Endosomal dsRNA is recognized by TLR3, which recruits TICAM-1 and through IRF3/7 induces expression of IFNs and ISGs. Cytoplasmic dsRNA is recognized by multiple PRRs including the RLR family that contains MDA5 and RIG-I and upon binding to dsRNA and activation subsequently activates IPS-1, IRF3, and IRF7 leading to an antiviral response. PKR recognize cytoplasmic viral dsRNA and activates IRF3/7 through IPS-1 or halts protein synthesis by phosphorylating eIF2α.

1.1.2 Synthetic dsRNA: Poly I:C

It was determined in the late 1950s that polyinosinic and polycytidylic acid can form a helical complex with a 1:1 composition and the same ribose-phosphate backbone as a ribonucleic acid (Davies and Rich, 1958). By the mid-1960s it was found that dsRNA molecules were potent inducers of type I interferons (IFNs) (Lampson *et al*., 1967). Scientists from Merck were the first to show that polyinosinic: polycytidylic acid (poly I:C) could be used to induce IFNs in mammals (Carter and de Clercq, 1974), and now poly I:C is a commonly used viral dsRNA mimic or TLR3 agonist (Loseke *et al.,* 2006). Poly I:C is a potent immunostimulant, however it lacks the natural structures, base composition, and sequence variations of viral dsRNA (Loseke *et al.,* 2006). Because of these differences poly I:C does not accurately reflect the functional properties of natural dsRNA fragments (Loseke *et al.,* 2006). A practical example of this was seen in a study measuring phosphorylation of interferon-regulatory factor 3 (IRF3). It was determined that the kinetics of the dsRNA-induced phospho-IRF3 expression were different in response to poly I:C compared to *in vitro* transcribed dsRNA molecules based on a viral genome (Jiang *et al.,* 2011). Poly I:C has been investigated as a therapeutic agent in mammals but had toxic effects due to its potency (DeWitte-Orr and Mossman, 2010).

High- and low- molecular weight (HMW and LMW) poly I:C are commercially available products. HMW poly I:C has a range of 1.5bp-8kb fragments and LMW poly I:C has a range of 0.2-1kb. A previous study used HMW and LMW poly I:C to explore length effects within the immune response (Jiang *et al.,* 2011). Poly I:C is commonly

used in fish to study innate antiviral responses, both *in vitro* and *in vivo* (DeWitte-Orr *et al.,* 2007; Congleton and Sun, 1996; Chang *et al.,* 2011).

1.1.3 Synthetic dsRNA: *In Vitro* Transcribed dsRNA

In vitro transcribed dsRNA can act as a viral dsRNA mimic with a sequence that is more biologically relevant compared to poly I:C. The sequence of the dsRNA molecule can be selected by the researcher and be based on a viral genome sequence. *In vitro* transcribed dsRNA molecules represent relevant viral sequences of defined length which allow for exact molar amounts of dsRNA to be added to cells, the main concerns with poly I:C. *In vitro* transcribed dsRNA is made using a T7 RNA polymerase based transcription system (Jiang *et al.*, 2011). T7 RNA polymerase is produced by the T7 bacteriophage and is used for *in vitro* transcription of RNA due to its robust activity and promoter specificity (Sousa and Mukherjee, 2003). Previous studies have used *in vitro* transcribed dsRNA as a native viral dsRNA alternative to study the immune response. Studies in mice and human cells demonstrate similar but not identical innate immune responses when cells were treated with poly I:C or *in vitro* transcribed dsRNA (DeWitte-Orr *et al.,* 2009; Jiang *et al.,* 2011). These studies also utilized different lengths of *in vitro* transcribed dsRNA to look at length effects on innate immune responses (DeWitte-Orr *et al.,* 2009; Jiang *et al.,* 2011). Although not nearly as well studied as poly I:C, there is a single study where a variety of lengths of *in vitro* transcribed dsRNA were used to study length requirements for fish and human dsRNA receptor activation of immune pathways (Matsuo *et al.,* 2008).

1.2 Innate Immunity

dsRNA is sensed by the first line of defense against microorganism infection, the innate immune system (Akira *et al.,* 2006). Unlike the adaptive immune response that is specific to vertebrates, all multicellular organisms have an innate immune system (Medzhitov and Janeway, 1997). The innate immune system recognizes microorganisms through germline encoded pattern recognition receptors (PRRs) (Akira *et al.,* 2006). PRRs can be humoral macromolecules in the plasma, endocytic surface-expressed receptors, or signaling receptors expressed intracellularly or on the cell surface (Medzhitov and Janeway, 1997). PRRs share common characteristics; they are expressed constitutively within a host cell, are germline encoded, expressed on all cells of a given type, and are independent of immunological memory (Akira *et al.,* 2006). PRRs recognize pathogen-associated molecular patterns (PAMPs) (Akira *et al.,* 2006; Medzhitov and Janeway, 1997).

PAMPs are structures that are unique to pathogens and are required for the survival of the microorganism producing them (Akira *et al.,* 2006; Medzhitov and Janeway, 1997). The conservation of these microbial structures between large groups of pathogens allows for a small number of PRRs to recognize a wide variety of PAMPs (Medzhitov and Janeway, 1997). Some examples of PAMPs include bacterial lipopolysaccharide and flagellin, and viral nucleic acids (ssRNA, dsRNA and DNA) (Akira *et al.,* 2006).

PAMPs have potential applications as vaccine adjuvants. An immunological adjuvant is a substance used in combination with a vaccine antigen that accelerates, extends, or improves an antigen-specific immune response (Sasaki and Okuda, 2000).

Adjuvants stimulate the innate immune system to produce signals required for the adaptive immune response to become activated (Medzhitov and Janeway, 1997). While adjuvants are potent immune response stimulators, many fail to be clinically relevant due to adverse side effects (Medzhitov and Janeway, 1997). Poly I:C is a potential adjuvant target, however it has limited clinical applications in mammals due to its potency (DeWitte-Orr and Mossman, 2010). The toxicity seen in mammals does not seem to be as robust in fish. For example poly I:C has been used as an effective adjuvant in studies using Japanese flounder. However poly I:C has appeared to have some toxic effects in Atlantic Salmon at certain temperatures (Takami *et al.*, 2010; Salinas *et al.,* 2004).

The PRRs involved in dsRNA sensing in fish are described below and shown in Figure 1.1. What is known of their subsequent signaling pathways, the resulting induction of IFNs, interferon-stimulated genes (ISGs) and the antiviral state, are summarized below for mammals and, where known, in fish.

1.3 Pattern Recognition Receptors (PRRs)

1.3.1 Class-A Scavenger Receptors

There are a number of PRRs expressed by host cells, at different interfaces between the cell and its environment (surface, endosome, cytoplasm) that detect viral dsRNA. As mentioned, scavenger receptors, being surface expressed, are one of the first receptors to recognize dsRNA. Scavenger receptors were initially characterized for their ability to bind and internalize acetylated low-density lipoproteins (acLDL) (Goldstein *et al.*, 1979). Scavenger receptors are now known to bind a wide range of polyanionic molecules (Limmon *et al.,* 2008; Whelan *et al.,* 2012). There are 8 classes of scavenger

receptors (A-H) (Murphy *et al.,* 2005). Class-A scavenger receptors (SR-As) can bind and internalize dsRNA (Limmon *et al.,* 2008; Whelan *et al.,* 2012), delivering the dsRNA molecules to intracellular sensors (DeWitte-Orr *et al.,* 2010). SR-As in mammals include: SR-AI/II, MARCO, SCARA3, SCARA4 and SCARA5 (Murphy *et al.,* 2005; Whelan *et al.,* 2012). SR-As mediate dsRNA binding in murine and human fibroblasts (DeWitte-Orr *et al.,* 2010). Little is known about SR-As in fish, however the SR-As SCARA3, SCARA4, and SCARA5 have been identified in a small number of fish species through a bioinformatics search (Whelan *et al.,* 2012). A SCARA5 homolog has been cloned and demonstrated to recognize and internalize LPS in *Tetraodon nigroviridis* (Meng *et al.*, 2012). Rainbow trout cell lines appear to express SR-As that bind dsRNA and acLDL, based on binding/blocking studies and transcript analysis (unpublished data). Once dsRNA is internalized endoplasmic and cytoplasmic PRRs are able to recognize the molecule.

1.3.2 TLR3

Toll-like receptor 3 (TLR3) is a member of the Toll-like receptor family and is an endosomal sensor for dsRNA. TLR3 contains extracellular domain (ECD) monomers that bind cooperatively to dsRNA to form TLR3ECD dimers. Multiple dimers can bind the same dsRNA molecule (Leonard *et al.*, 2008). dsRNA binding by TLR3 requires a low pH such as what is found in an acidified endosome (Leonard *et al*., 2008). Once activated TLR3 recruits toll/IL-1 receptor (TIR) domain-containing adaptor molecule (TRIF/TICAM), which initiates downstream interferon signaling pathways through interferon regulatory factor 3 (IRF3) (Leonard *et al.,* 2008; Sen and Sarkar, 2005). TLR3 acts through the IRF3/7 pathway to induce type I IFN and ISG production (Figure 1.1)

(Holland *et al.,* 2008). TLR3 has been identified in multiple fish species including rainbow trout (Rodriguez *et al.,* 2005).

1.3.3 TLR22

In addition to TLR3, teleost fish express TLR22 (Matsuo *et al.,* 2008). TLR22 is a cell surface dsRNA receptor expressed only in aquatic animals (Workenhe *et al.,* 2010). Both TLR3 and TLR22 use the adapter protein TRIF/TICAM-1, however their pathways are thought to function independently of one another (Matsuo *et al.*, 2008). It is not known whether TLR22 requires a low pH for dsRNA binding, which is the case for TLR3. Once bound to dsRNA, TLR22 activates IRF3 to initiate the production of type 1 IFNs (Kasamatsu, 2013). TLR22 represents a key difference between mammalian and teleost fish/amphibian dsRNA mediated pathways.

1.3.4 RIG-I/MDA5

The retinoic acid-inducible gene I (RIG-I) – like receptors (RLRs) are a family of dsRNA PRRs that include RIG-I and melanoma differentiation-associated protein 5 (MDA5), as well as Laboratory of Genetics and Physiology 2 (LGP2). LGP2 lacks a functional caspase-recruitment domain (CARD) and therefore is not believed to be involved in downstream signaling (Takeuchi and Akira, 2008). RIG-I and MDA5 are helicases located in the cytoplasm of host cells (Takeuchi and Akira, 2008; Peisley and Hur, 2012). In its inactivated state the CARD of RIG-I is not exposed (Jensen and Thomsen, 2012). When dsRNA binds to the repressor domain of RIG-I the CARD domain is exposed allowing interactions with the adaptor molecule, interferon promoter stimulator (IPS-1), which when activated initiates IFN-mediated singling pathways via IRF3/7 (Figure 1.1) (Jensen and Thomsen, 2012).

Melanoma differentiation-associated antigen 5 (MDA5) is another member of the RLR family. MDA5 is homologous to RIG-I; containing the same domain structure. A main difference between RIG-I and MDA5 is MDA5 preferentially binds longer lengths of dsRNA (> 1000bp) and RIG-I preferentially binds shorter dsRNA (<1000 bp) (Jensen and Thomsen, 2012). RIG-I has yet to be identified in rainbow trout, but RIG-I-like proteins have been found in carp and salmon (Biacchesi *et al.*, 2009). MDA5 has been cloned from rainbow trout (Chang *et al.*, 2011). Activated MDA5 induces IFN and ISG transcript expression via the IPS-1, IRF3/7 pathway, similar to RIG-I (Holland *et al.,* 2008; Yoo *et al.,* 2008).

1.3.5 PKR

The dsRNA-dependent protein kinase (PKR) is a cytoplasmic PRR that functions as a serine/threonine protein kinase. PKR belongs to a family of stress-response kinases, including PKZ, GCN2, HRI and PERK (Lemaire *et al.,* 2008). The PKR regulatory module contains an N-terminal dsRNA-binding domain that has two tandem dsRNA binding motifs (Lemaire *et al.,* 2008). dsRNA binding to PKR brings multiple PKR molecules into close proximity with each other allowing for dimerization, autophosphorylation and subsequent PKR activation (Lemaire *et al.*, 2008). When active, PKR phosphorylates the eukaryotic initiation factor eIF2α resulting in global inhibition of protein synthesis by sequestering a component of the translation machinery (Garner *et al.*, 2003; Balachandran *et al.,* 2000). PKR is constitutively expressed in human and murine cells in an inactive form (Balachandran *et al.,* 2000). PKR-like genes have been characterized in fish, and PKR activity, particularly eIF2α phosphorylation, has been found in rainbow trout (Garner *et al.,* 2003).

1.3.6 OAS/RNase L System

Another cytoplasmic PRR involved in dsRNA binding in mammals is oligoadenylate synthetase (OAS). OAS expression is IFN sensitive and is upregulated in response to IFN treatment via the interferon sensitive response element (ISRE) within its promoter region (Silverman, 2007). OAS binds cytoplasmic dsRNA and is activated resulting in the production of 2-5 adenylates (2-5A) species from ATP (Silverman, 2007). These 2-5A molecules bind and activate the nuclease RNase L (Silverman, 2007). RNaseL degrades ssRNA molecules, including both host and viral mRNAs, effectively blocking protein translation in the cell (Silverman, 2007). In humans three OAS genes have been identified that encode for 8-10 different isoforms of OAS and in mice eight OAS1 genes have been identified (Silverman, 2007).

1.3.7 Length Dependence

dsRNA length appears to be a major influencer of immune response. In addition to meeting the minimal length required by each PRR for binding dsRNA (usually >30bp), long dsRNA molecules (1000-3000bp) have been shown to induce higher levels of IFN and ISGs in murine fibroblasts compared with shorter molecules (200-500bp) (DeWitte-Orr *et al.,* 2009; Kato *et al.*, 2008). Length effects of dsRNA on immune responses have been previously demonstrated in a number of mammalian species; however to our knowledge the present study is the first investigating the effects of different dsRNA lengths in fish cells. A study done in rabbits found that dsRNA of 661bp in length induced more potent acute-phase effects when compared to a 108bp molecule (Fang *et al.*, 1999). A human cell line (HUH7.5/RIG-I) with an ISG56 promoter reporter assay showed a significantly greater response to longer dsRNA (Binder *et al.,* 2011). A study

done in murine embryonic fibroblast cells demonstrated that longer dsRNA molecules induced a greater magnitude of antiviral, ISG, and interferon response (DeWitte-Orr *et al.,* 2009). There is a single report with TLR22 (a fish receptor) being expressed in human cells that showed length effects, suggesting that longer dsRNA molecules might also be more potent in fish as is observed in mammals (Matsuo *et al.,* 2008).

Length effects can be explained by the mechanisms by which PRRs are able to induce immune responses. Firstly, length dictates whether a PRR can bind a dsRNA molecule. RIG-I and MDA5 both are able to bind to dsRNA molecules of approximately 18bp in length, they require longer lengths for activation, 30bp for RIG-I and up to 2000bp for MDA5 (Berke and Modis, 2012). RIG-I recognizes short dsRNA strands (<1000bp) and MDA5 recognizes longer strands (>1000bp) (Kato *et al.,* 2008; Peisley and Hur, 2012). TLR22 preferentially recognizes long dsRNA (Matsuo *et al.,* 2008). The dsRNA-binding domain of PKR can bind to sequences 15-16bp in length, dsRNA lengths of >30bp are required to initiate dimerization, autophosphorylation, and activation of PKR (Heinicke *et al.*, 2009). OAS requires a minimal length of 18-20bp dsRNA for activation, but higher enzymatic activity is seen with longer dsRNA (Peisley and Hur, 2012). TLR3 requires a minimal dsRNA length of 39-48bp for activation (Leonard *et al.,* 2008).

Secondly, longer dsRNA molecules support the binding of more PRRs along their length. For example, RIG-I multimerizes along a dsRNA molecule, the longer the dsRNA molecule the more RIG-I can bind, and more bound RIG-I means a stronger activation of downstream signaling pathways (Binder *et al.,* 2011). TLR3 affinity for dsRNA increases with dsRNA length, and longer dsRNA molecules support the assembly

of more TLR3 dimers along its length, triggering a stronger downstream signaling pathway (Leonard *et al*., 2008).

Finally, in addition to multimerization, a contributor to the length effects might be translocation of certain PRRs along the dsRNA molecule (Binder *et al.,* 2011). RIG-I was found to translocate along dsRNA without unwinding the molecule. In the presence of 5`tripshophates, RIG-I uses ATP hydrolysis to translocate along one strand of dsRNA (Myong *et al.,* 2009). The duration of translocation increases with dsRNA length and is hypothesized to affect the strength of downstream signaling pathways (Myong *et al.,* 2009; Schmidt *et al.,* 2011). It is important to note that in the study done by Myong and colleagues long dsRNA molecules were only 50bp in length; more exploration into longer lengths of dsRNA would be beneficial (Myong *et al.,* 2009).

1.3.8 Sequence Dependence

Attempts to elucidate dsRNA sequence motifs necessary for PRR binding have been largely unsuccessful. Most PRRs appear to bind dsRNA based on its unique helical structure as opposed to its sequence (Peisley and Hur, 2012). PKR binds without sequence specificity (Lemaire *et al.,* 2008). An exceptions is OAS, where there appears to be four critical base pairs in the minor groove of the dsRNA which may be necessary for binding (Kodym *et al*., 2009). Additionally, RIG-I ligands generally have a uridine- or adenosine-rich ribonucleotide sequence (Saito and Gale, 2008). RNA sequences with 5`triphosphates are a powerful PAMP for RIG-I (Myong *et al.,* 2009). Despite what is known about dsRNA PRRs, the relationship between dsRNA-length and –sequence and immune response still remains largely unknown.

1.4 Type I Interferons

IFNs are glycoproteins that are responsible for activating both the innate and adaptive immune responses against both viral and microbial infections (Dorner *et al.,* 1973, Fensterl and Sen, 2009). The two key antiviral type I IFNs in mammals are classified as either IFNα or β with 15 IFNα subtypes and a single IFNβ subtype (Hertzog and Williams, 2013). Rainbow trout have at least 5 type 1 IFNs classified as two groups. Group 1 consists of IFNs containing 2 cysteine residues and a single disulphide bond. To date, three rainbow trout interferon genes have been classified as group 1: IFN1, IFN2, and IFN5 (Chang *et al.,* 2009). Group 2 type 1 interferons are composed of IFNs containing 4 cysteine residues and two disulphide bonds. In rainbow trout, two group 2 IFNs have been identified: IFN3 and IFN4 (Purcell *et al.*, 2009; Chang *et al.,* 2009).

Viral dsRNA is a potent inducer of type I IFN, one dsRNA molecule being sufficient to induce IFN production (Marcus and Sekellick, 1977). In mammals the dsRNA PRRs such as TLR3 and the RLRs activate transcription factors: IRF3/7 and NF-kB that bind to the positive elements of the IFN promoter regions inducing transcription. IFNs likely follow the same protein synthesis pathway as other cytokines; proteins are synthesized in the rough endoplasmic reticulum via the secretory protein synthesis pathway, modified in the Golgi apparatus, and secreted by exocytosis (Stanley and Lacy, 2010).

IFNs inhibit viral replication and infection independent of viral species, by inducing an antiviral state in virus infected cells and neighbouring uninfected cells (Fensterl and Sen, 2009). Type 1 IFN receptors are cell surface receptors that are ubiquitously expressed (Fensterl and Sen, 2009). Type 1 interferons signal through a

common heterodimeric receptor, IFNABR, which is comprised of subunits IFNAR1 and IFNAR2 (David *et al.,* 2012; Goodbourn *et al.,* 2000). IFNAR1 and IFNAR2 belong to class II helical cytokine receptor family (de Weerd *et al.,* 2007).

When IFN is bound to IFNABR the Janus tyrosine kinases JAK1 and TYK2 phosphorylate transcription factors STAT1 and STAT2 (Fensterl and Sen, 2009). The activated STATs form a heterodimer and recruit interferon-regulatory factor 9 (IRF9) to produce a STAT1-STAT2-IRF9 complex called ISGF-3 (Fensterl and Sen, 2009; David *et al.,* 2012). This complex translocates into the nucleus and activates interferon-stimulated genes (ISGs) through binding to the interferon-sensitive response element (ISRE) within the promoter of the ISGs (Fensterl and Sen, 2009).

I.5 Interferon-Stimulated Genes

ISGs are a varied group of proteins that have many different actions within the cell but all work together to inhibit virus replication (Fensterl and Sen, 2009). Many ISGs have been identified in teleost fish, including vig1-10 and Mx1-3 (O'Farrell *et al.*, 2002). The Mx family has been identified in a wide range of species and consists of dynamin-related members of the large GTPase super-family (DeWitte-Orr *et al.*, 2007). In rainbow trout, Mx1 and Mx3 are localized in the cytoplasm whereas Mx2 is localized in the nucleus (Leong *et al.*, 1998). In mammalian models Mx proteins have been shown to exhibit significant antiviral activity against many different viruses including vesicular stomatitis and the influenza virus (Leong *et al.,* 1998; Pavlovic *et al.,* 1990; Staeheli *et al.,* 1986). In mammals Mx proteins appear to block viral replication at an early stage. Human MxA protein has been shown to bind to the nucleocapsids of Thogoto virus blocking transport into the nucleus and preventing transcription of the viral genome

(Kochs and Haller, 1999). Atlantic salmon Mx1 protein was found to inhibit the replication of a fish virus, infectious pancreatic necrosis virus and Japanese flounder Mx was able to inhibit fish rhabdoviruses (Larsen *et al.,* 2004; Caipang *et al.,* 2003).

Virus-induced genes (vigs) are a group of proteins that are upregulated during virus infections. Rainbow trout vig1, a homologue to mammalian viperin or cig5, is proposed to be involved in the virus-induced synthesis of enzymatic cofactors in the nitric oxide pathway (Boudinot *et al.*, 1999). Vig2 has been characterized as an interferon-responsive gene, its function is currently not known (Boudinot *et al.*, 2001). Vig3 is an ubiquitin-like protein that has high similarity to the mammalian ISG15 (Verrier *et al.*, 2011). ISG15 has demonstrated antiviral activity against a variety of viruses and target proteins through an isgylation pathway (Verrier *et al.*, 2011). In Ebola virus infections ISG15 inhibits viral budding by preventing ubiquitination of a viral protein (Malakhova and Zhang, 2008). Vig4 is homologous to the mammalian ISG56/ISG58 and encodes a teinoic acid- and interferon-inducible protein that contains tetratricopeptide repeats. The mammalian protein homologue (ISG56) has been shown to interfere with protein synthesis (Verrier *et al.*, 2011; Robertsen 2008).

1.6 Antiviral State

The accumulation of ISGs within a cell results in the establishment of an antiviral state. The antiviral state inhibits the replication of DNA and RNA viruses (Samuel, 2001). This has been observed in both *in vitro* and *in vivo* studies (Samuel, 2001). Many virus infections that have been studied in cell culture systems are hindered at the primary step of the virus replication cycle, the synthesis of viral polypeptides (Samuel, 2001). Some viruses may be inhibited at different points in their multiplication cycles, for

example some myxoviruses and rhabdoviruses are inhibited before transcription (Samuel, 2001).

Previous studies have demonstrated the antiviral properties of poly I:C in fish and fish cell lines. Poly I:C transfected into a Chinook salmon embryo cell line resulted in an antiviral state against infectious pancreatic necrosis virus (Jensen *et al.,* 2002). 4 species of whole salmon were injected with poly I:C and demonstrated antiviral responses when challenged with infectious hematopoietic necrosis virus or erythrocytic necrosis virus (Eaton, 1990).

1.7 Fish Viruses

1.7.1 Viral Hemorrhagic Septicemia Virus

VHSV infects a wide range of freshwater and saltwater species of fish. It belongs to the *Rhabdoviridae* family of viruses, has an 11kb single-stranded negative-sense non-segmented RNA genome, and is a lytic virus whose cytopathic effect (CPE) is cell death (Purcell *et al*., 2012). The VHSV genome consists of 6 open reading frames encoding for 6 proteins. VHSV replicates in the endothelial cells of blood capillaries, leucocytes, haematopoietic tissues and nephron cells (Essbauer and Ahne, 2001).

There are four distinct genotypes of VHSV (I-IV) and it infects over 70 teleost fish species from many diverse aquatic environments (Kim and Faisal, 2010). Genotype IV encompasses isolates found in North America, Japan and Korea (Kim and Faisal, 2010), while the VHSV subgroup IVb, which is used in the present study, is specific to the Great Lakes (Cornwell *et al.,* 2011). The Great Lakes subgroup IVb is particularly virulent and has a wide host range (Pierce and Stepien, 2012; Kim and Faisal, 2010).

VHSV was first identified in the North American Great Lakes in 2003 (Great Lakes Commission, 2011; Pierce and Stepien, 2012). There are no treatments or vaccines available for VHSV-IVb (Kipp *et al.,* 2013) and to our knowledge nothing is known of the effects of VHSV-IVb produced dsRNA on the immune response. VHSV is an important pathogen in Canadian waters and requires further study to understand how the virus interacts with the host immune system.

1.7.2 Chum Salmon Reovirus

Chum salmon reovirus (CSV) is a dsRNA virus that was isolated from adult chum salmon (*Oncorhynchus keta*) in a Japanese hatchery (Winton *et al.,* 1981). CSV is a non-enveloped virus with an icosahedral double capsid and has a genome consisting of 11 segments (Winton *et al.,* 1987). CSV infection results in cell fusion to create large multinucleated cells called syncytia (DeWitte-Orr and Bols, 2007). This unique cytopathic effect (CPE) of the virus is easily observed under light microscopy. CSV replicates in many cell lines from many different species of fish including rainbow trout and Chinook salmon (DeWitte-Orr and Bols, 2007).

1.8 Rainbow trout

Rainbow trout (*Oncorhynchus mykiss)* is a fresh water teleost fish species often used in aquaculture. Rainbow trout are readily infected by VHSV in aquaculture, in a laboratory setting, and in the wild, and in Europe rainbow trout are considered one of the most susceptible species found in European fisheries (Snow *et al.*, 1999). Rainbow trout are an important contributor to the economy through both aquaculture and sport fishing; rainbow trout are one of the top five sport fishes in North America (Fisheries and Oceans

Canada, 2010). Rainbow trout farming occurs in almost every province in Canada and Ontario is the largest producer of trout (Fisheries and Oceans Canada, 2011). Rainbow trout, whole animals and cell lines, are often used as a model for immunological studies and there is an existing knowledge base of rainbow trout type 1 IFN and ISGs (Zou *et al.,* 2007; Tafalla *et al.,* 2008).

1.9 Cell Lines

Cell culture is frequently used in the study of virology (Leland and Ginocchio, 2007). Cell culture was utilized for the current study over whole animals for numerous reasons. Using fish cell lines as opposed to intact fish limits the need for sacrificing animals. Fish cell lines are easy to culture; they grow at room temperature and do not require some of the special maintenance required for mammalian cells (e.g. CO_2 or special incubators) (Fryer and Lannan, 1994). Using a cell line allows the researcher to focus on a single cell type. Results are attained faster than with whole animals and the cost is lower (Bols *et al.,* 2005). Four fish cell lines were used in this study: three from rainbow trout and one from Chinook salmon. RTG-2 was the main cell line used to characterize dsRNA responses, as its IFN and dsRNA-mediated immune response has been somewhat characterized; the remaining cell lines were utilized for specific reasons relating to an assay. A brief background on these four cell lines is described below.

1.9.1 RTG-2

RTG-2 is a rainbow trout gonadal cell line derived from both male and female gonads (Wolf and Quimby, 1962). RTG-2 is readily infected by VHSV and responds to extracellular poly I:C (DeWitte-Orr *et al.*, 2007; Tafalla *et al.,* 2008). Previous studies

found unstimulated RTG-2 cells constitutively express low levels of IFN2 but no detectable amounts of IFN1; however, both IFN1 and IFN2 are strongly upregulated in response to poly I:C (Zou *et al.,* 2007). IFN3/4 is expressed in rainbow trout ovaries but has not been detected in RTG-2 (Zou *et al.,* 2007). The ISGs Mx1-3 and vig1 are upregulated in response to poly I:C and Chum salmon reovirus in this cell line (DeWitte-Orr *et al*., 2007).

1.9.2 RTG-P1

RTG-P1 is RTG-2 stably transfected with a luciferase gene controlled by the Mx1 promoter (Collet *et al.*, 2004). RTG-2 cells were transfected with a vector (pGL3-prMx1-Basic-Neo) that contains an Mx1-promoter upstream of a firefly luciferase gene (Collet *et al.,* 2004). RTG-P1 has been shown to produce luciferase upon stimulation with poly I:C (Collet *et al.,* 2004). In this study, RTG-P1 was used as a part of a quantifiable reporter assay to measuring ISG-promoter activation in response to different dsRNA molecules.

1.9.3 RTgutGC

RTgutGC is an epithelial rainbow trout (*Oncorhynchus mykiss*) cell line developed from the intestine (Kawano *et al.,* 2010). Little is known with regards to the type 1 interferon response in RTgutGC cells; rainbow trout gut tissue expresses IFN2 constitutively (Zou *et al.,* 2007). RTgutGC was utilized in this study in the functional interferon assay because it was found to be a better IFN producer than RTG-2 in response to dsRNA.

1.9.4 CHSE-214

CHSE-214 is an epithelial Chinook salmon (*Oncorhynchus tshawytscha)* embryo cell line (Lannan *et al.,* 1984). The cell line is effective for use in both detecting and propagating salmonid fish viruses (Lannan *et al.,* 1984). Chum salmon reovirus (CSV) infects and replicates within CHSE-214 cells (Winton *et al.,* 1981; DeWitte-Orr *et al.,* 2007). It appears that CHSE-214 lacks cell surface dsRNA receptors as it cannot respond to extracellular dsRNA (Jensen *et al.,* 2002). The cell line is able to respond to extracellular interferon (Nygaard *et al.*, 2000). CHSE-214 was used in the functional interferon assay in this study, as it can respond to interferon but not the extracellular dsRNA (Jensen *et al.,* 2002; Nygaard *et al.,* 2000).

1.10 Cell Viability Assay

Carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) is a cell viability indicator that has been used with fish cells (DeWitte-Orr *et al.,* 2005; Schreer *et al.,* 2005). It is a non-toxic dye that is retained within viable cells and cleaved by enzymatic esterases to a fluorescent product. Fluorescent CFDA is a measure of membrane integrity and can be measured using a multi-well plate reader (Schreer *et al.,* 2005). RTG-2 and CHSE-214 cell viability has previously been monitored using CFDA-AM (DeWitte-Orr *et al.,* 2005). In this study this cell viability assay was used to measure virus-induced cell death.

The functional interferon assay and antiviral assay both measured the effectiveness of a stimulant, interferon or dsRNA, in establishing a protective antiviral state. To measure the antiviral state cells were challenged with a virus and cell viability was measured to demonstrate any protection against the virus. If the stimulant was able

to produce an antiviral state, fewer cells would be killed and cell viability would be higher in these groups. Other methods of measuring viral infection or replication include using a green fluorescent protein virus vector, plaque assays, and quantitative PCR (Ferreira *et al.,* 2013; Purcell *et al.,* 2006). CFDA-AM is ideal for this study as it is a quantitative assay without the subjectivity of counting plaques. CFDA-AM effectively measures cell viability in any virus infected cell and does not have the viral specificity of a viral-reporter vector, such as GFP, or PCR, which was important to this study as two different viruses were used.

1.11 Project Aims

The goal of the present study was to better understand dsRNA induced innate immune responses in rainbow trout cell lines. There is a basic understanding as to how fish cells respond to poly IC; however, nothing is known of the effects of more relevant dsRNA molecules such as *in vitro* transcribed dsRNA, and the effects of length, whether with poly I:C or *in vitro* transcribed dsRNA, are is largely unexplored in fish cells as well. The main hypothesis for the present project was that dsRNA, either synthetic or *in vitro* transcribed based on a viral genome, would induce a robust immune response within rainbow trout cells. Specifically it was hypothesized that both dsRNA molecule would induce the production of type 1 IFN, ISGs, and an antiviral state in rainbow trout cells. Based on what was observed in mammals, it was hypothesized that the dsRNA would induce responses in a length-dependent manner with longer molecules inducing a greater magnitude of response compared to shorter molecules, and that sequence would have no effect on the immune response. Finally, it was hypothesized that poly I:C would induce a stronger response than the *in vitro* transcribed molecules. To address these hypotheses

four main objectives were explored:

1. To characterize the IFN response to dsRNA in rainbow trout cells using RT- PCR and a functional IFN assay

2. To characterize the ISG response to dsRNA in rainbow trout cells using RT- PCR and a Mx1-reporter assay

3. To characterize the dsRNA-induced antiviral state in rainbow trout cells using an antiviral assay

4. To identify any potential length effects of dsRNA on the magnitude of the innate immune response using tools developed in objectives 1-3.

CHAPTER 2: MATERIALS AND METHODS

2.1 Cell Lines

Four cell lines were used in this study, Chinook salmon embryo (CHSE-214), rainbow trout gut (RTgutGC), rainbow trout gonad (RTG-2), and the transgenic RTG-2 (RTG-P1). CHSE-214, RTgutGC and RTG-2 were obtained from N. Bols (University of Waterloo) and RTG-P1 was obtained from B. Collet (University of St. Andrews). All cell lines were grown in 75cm^2 plastic tissue culture flasks (BD Falcon, Bedford, MA, USA) at room temperature in Leibovitz's L-15 media (HyClone, Logan, UT, USA) supplemented with 10%v/v fetal bovine serum (FBS) (Corning, Manassas, VA, USA) and 1%v/v penicillin/streptomycin (P/S) (10mg/mL streptomycin and 10000U/mL penicillin) (Fisher Scientific, Fair Lawn, NJ, USA). RTG-P1 media was supplemented with an additional 100μg/mL of G418 neomycin (Sigma-Aldrich, St Louis, MO, USA).

2.2 Virus Propagation

Viral hemorrhagic septicemia virus (VHSV) was propagated on monolayers of Epithelioma papulosum cyprini (EPC), and chum salmon reovirus (CSV) was propagated on CHSE-214. Virus containing media (5%v/v FBS (Corning)) was collected 4-7 days post-infection, filtered through a 0.45μm filter (Nalgene, Rochester NY, USA) and kept frozen at -20°C for short-term storage and -80°C for long-term storage.

50% Tissue culture infective dose (TCID50)/mL values were estimated using the Reed and Muench calculator (Reed and Muench, 1988). To estimate the TCID50/mL the cell line used to propagate the virus was seeded into 96-well plates (BD Falcon, Franklin Lakes, NJ, USA) at 3x10^{4}cells/well. Serial dilutions of the virus were used to infect 6

wells each of the host cell line. After a 7-day incubation wells were monitored for cytopathic effects and the Reed and Muench calculator was used (Appendix A1).

2.3 dsRNA

2.3.1 Poly I:C

Poly I:C was stored in stock solutions of 10 mg/mL for regular (Sigma-Aldrich, St Louis, MO, USA) and low molecular weight (LMW) poly I:C (InvivoGen, San Diego, CA, USA) and 1mg/mL for high molecular weight (HMW) poly I:C (InvivoGen, San Diego, CA, USA) diluted in phosphate buffered saline (PBS) (HyClone, Logan, UT, USA) based on manufacturers suggestions. Average length was estimated based on the smear produced by running 1µg of poly I:C on a 1% agarose (Fisher Scientific, Fair Lawn, NJ, USA) gel with 5µg of O'GeneRuler 1kb PLUS DNA Ladder (Fermentas, Carlsbad, CA, USA) The gel was stained with 0.5µg/mL ethidium bromide (Fisher Scientific, Fair Lawn, NJ, USA). Poly I:C aliquots were stored at -20°C.

2.3.2 *In Vitro* Transcribed dsRNA

dsRNA was transcribed *in vitro* using the MegaScript RNAi kit (Ambion by Life Technologies, Carlsbad, CA, USA) following the manufacturers instructions summarized in Figure 2.1. Fragments of the VHSV-IVb genome were amplified by RT-PCR using sequence specific primers linked 5` to a T7 RNA polymerase promoter (**3`-**TAATACGACTCACTATAGGG-**5`**) (Figure 2.2, Table 2.1). T7 RNA polymerase synthesized complimentary RNA strands from the template that were annealed to form dsRNA. The fragments created were 200bp (v200) and 1264bp (v1200) in length and based off a randomly chosen section of the VHSV genome (Figure 2.2).

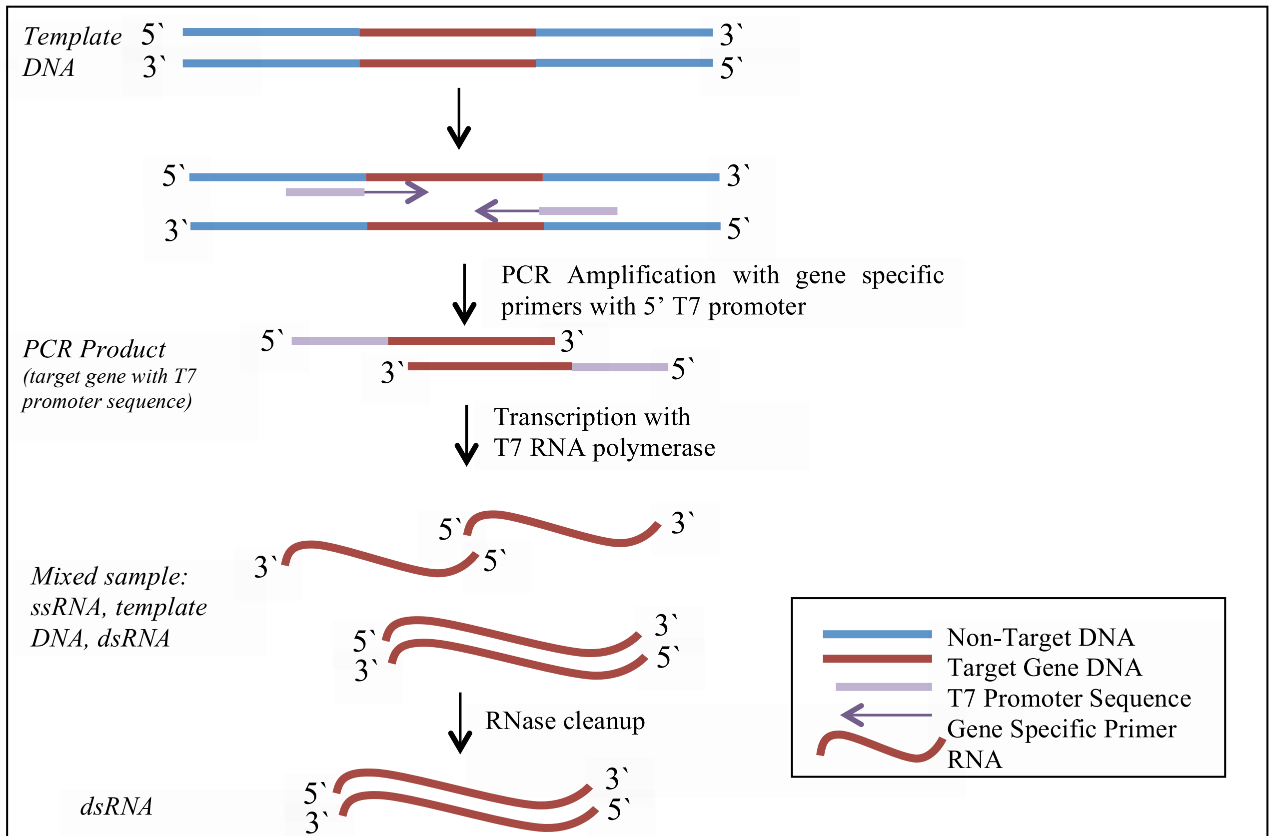

Figure 2.1 *In vitro* transcription of dsRNA.
Using an RNAi Megascript kit (Ambion) dsRNA can be synthesized from template DNA. This process makes use of T7 RNA polymerase that is capable of synthesizing RNA from DNA. In order for T7 polymerase to recognize the DNA template, T7 promoter sequences must be added to the target sequence, on both the sense and antisense strand. This is done using gene specific primers (forward and reverse) with the T7 promoter sequence attached to the 5` end. After T7 transcription the product is a mix of nucleic acids, a DNase and RNase treatment exploits the differential degradation of ssRNA and DNA to end up with a pure dsRNA product.

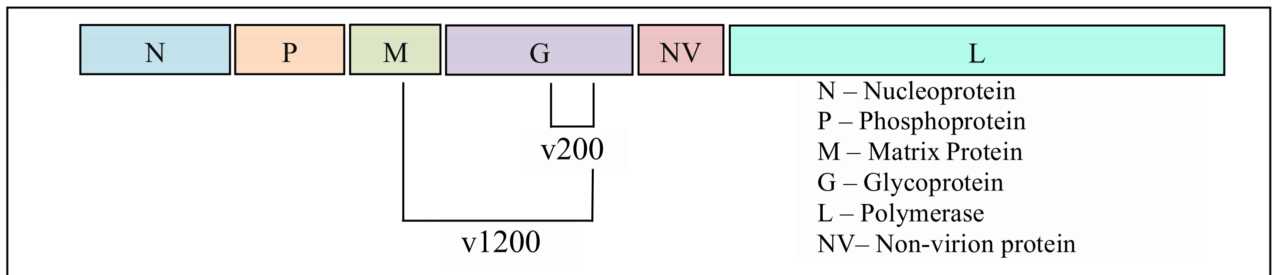

Figure 2.2 VHSV genome and dsRNA fragments.
A simplified schematic of the VHSV genome showing two random fragments chosen for amplification for dsRNA synthesis. The v200 is 200bp in length and the v1200 is 1264bp length. Adapted from Ammayappan, A., Vakharia, V.N. (2009).

2.3.3 Native dsRNA

RTgill-W1 cells were infected with VHSV-IVb (3.16×10^3 TCID50/mL). After a 4-day incubation total RNA was extracted. Native dsRNA was isolated using a lithium chloride (LiCl) (Sigma-Aldrich, St Louis, MO, USA) precipitation method and gel extraction method. In both cases total RNA was extracted using TRIzol (Life Technologies, Carlsbad, CA, USA) following manufacturers instructions. The LiCl extraction has been previously described (Diaz-Ruiz and Kaper, 1978). 4M LiCl was added to total RNA to a final concentration of 2M. The solution was incubated at -20^oC for 30 min and centrifuged at 17000xg for 30min at 4^oC. 8M LiCl was added to the supernatant to a final concentration of 4M. The solution was incubated at -20^oC for 30 min and centrifuged at 17000xg for 30min at 4^oC. The resultant pellet was air-dried, suspended in DNA quality H_2O, and quantified using a NanoDrop lite Spectrophotometer at 260nM (Thermo Scientific, Waltham MA, USA).

For the gel extraction method 10-40µL of total RNA was run on a 1%w/v agarose (Fisher Scientific) gel and stained with 0.5µg/mL ethidium bromide (Fisher Scientific). The dsRNA band was excised and extracted using the Qiagen gel extraction kit (Qiagen, Hilden, Germany). The dsRNA was quantified using a NanoDrop lite Spectrophotometer at 260nM (Thermo Scientific).

2.3.4 Acridine Orange Staining

A novel method was developed based on Pichlmair and colleagues 2009. Total RNA and dsRNA extractions were run on a 1%w/v agarose (Fisher Scientific) gel. The resulting gel was stained with 30µg/mL acridine orange in MilliQ water (Farrel, 2005)

(Sigma-Aldrich, St Louis, MO, USA) in H_2O for 10min. The gel was run under hot running tap water for 20min to facilitate destaining (Farrel, 2005), then under cool running tap water for 5min to counteract the slight softening from the hot water. The gel was placed in room temperature H_2O overnight. The gel was visualized using a VersaDoc Imager (Bio-Rad, Hercules, CA, USA) set to 2 channels, 695BP Blue LED and 530BP Blue LED.

2.4 RT-PCR

2.4.1 dsRNA Stimulation

RTG-2 cells were split 1:2 into 75cm^2 flasks (BD Falcon) or seeded at 5x10^5 cells/well into 6-well plates (BD Falcon, Franklin Lakes, NJ, USA) and allowed to attach overnight. Cells were treated with growth media (10% FBS (Corning)) containing 50μg/mL regular poly I:C (Sigma-Aldrich), 1.5nM HMW poly I:C (InvivoGen), 1.5nM LMW poly I:C (InvivoGen), 1.5nM v200 or 1.5nM v1200 *in vitro* transcribed dsRNA, or untreated control media. After 24h total RNA was extracted.

2.4.2 RNA Extraction

Total RNA was extracted using TRIzol (Life Technologies) according to the manufacturers instructions. TRIzol (Life Technologies) reagent was added to attached cells and the growing surface was scraped to facilitate cellular material collection. The TRIzol/cell mixture was collected and mixed with chloroform (Sigma-Aldrich, St Louis, MO, USA). Centrifugation at 16100xg for 15min at 4°C resulted in phase separation. RNA phase was collected and combined with 100% isopropanol (VWR, West Chester PA, USA). Centrifugation at 16100xg for 10min at 4°C was used to precipitate and pellet

RNA. RNA was washed using 80% ethanol (Sigma-Aldrich, St Louis, MO, USA) and centrifuged at 16100xg for 5min at 4°C. RNA pellet was dried briefly to remove residual ethanol and resuspended in DNA quality water (Fisher Scientific). RNA was quantified using a NanoDrop Lite Spectrophotometer (Thermo Scientific) and stored at -80°C until use.

2.4.3 cDNA Synthesis

cDNA was synthesized using GoScript reverse transcriptase (Promega, Madison, WI, USA). Briefly, 2µg of RNA were incubated with 0.5µg oligo-$(dT)_{23}$ primer (Sigma-Aldrich, St Louis, MO, USA) for rainbow trout genes or random hexamer (Sigma-Aldrich, Canada) for viral gene fragments at 70°C for 5min and immediately placed on ice for 5min. A reaction mix containing 1x GoScript Reaction Buffer (Promega, Madison, WI, USA), 1.5mM $MgCl_2$ (Promega, Madison, WI, USA), 0.5mM each deoxynucleotide triphosphate (dNTP) (Sigma-Aldrich, St Louis, MO, USA), and 160U GoScript reverse transcriptase (Promega, Madison, WI, USA) was added to the RNA and oligo-(dT) (Sigma-Aldrich) mix. The reaction was incubated at 25°C for 5min, 42°C for 1h, and held at 4°C. Half the resulting cDNA was diluted 1:3 in DNA quality water (Fisher Scientific, Fair Lawn, NJ, USA) and stored at 4°C for short-term storage or -20°C for long-term storage.

2.4.4 PCR Reactions

PCR reactions were carried out using GoTaq Flexi DNA Polymerase (Promega, Madison, WI, USA). All PCR reactions contained: 2µl of cDNA, 1x Green GOTaq Flexi Buffer (Promega, Madison, WI, USA), 0.2mM each dNTP (Sigma-Aldrich, St Louis, MO, USA), 1.25U GoTaq Flexi DNA Polymerase (Promega, Madison, WI, USA),

1.5mM $MgCl_2$ (Promega, Madison, WI, USA), 0.5µM forward primer (Sigma-Aldrich, St Louis, MO, USA), 0.5µM reverse primer (Sigma-Aldrich, St Louis, MO, USA) and up to 25µL nuclease-free water (Fisher Scientific). The primer sequence, cDNA dilution, and annealing temperature for each gene-specific primer set can be found in Table 2.1. The PCR reactions were conducted in a T100 Thermal Cycler (Bio-Rad, Hercules CA, USA) at the following cycle conditions: 95°C for 2min, 27 cycles of: 95°C for 45s, specific annealing temperature for 45s, 72°C for 45s followed by 72°C for 10min and held at 4°C. For the amplification of viral fragments for dsRNA synthesis elongation time was 1min 30s and cycle number was 34. β-Actin was included as a positive control. 5-10µl of product was run on a 1%w/v agarose (Fisher Scientific) gel and stained with 0.5µg/mL ethidium bromide (Sigma-Aldrich) for visualization with a VersaDoc Imager (Bio-Rad) set to UV transillumination. Data shown are representative of three independent experiments.

2.5 Functional Interferon Assay

A novel functional interferon assay was developed. RTgutGC was seeded at $7x10^5$cells/well into 6-well plates (BD Falcon); cells were allowed to attach overnight. Media was removed from wells and cells were treated with one of: 3nM v1200, 3nM v200, 3nM LMW poly I:C (InvivoGen), 3nM HMW poly I:C (InvivoGen) or control media (10%v/v FBS (Corning)) and incubated for 24h. CHSE-214 was seeded at $3x10^4$ cells/well in 96-well plates (BD Falcon) and allowed to attach overnight. Conditioned media from RTgutGC cells was collected and centrifuged at 10xg for 5min to remove debris. CHSE-214 cells were treated with RTgutGC-conditioned, media for 24h. Cells were rinsed with PBS (HyClone) and infected with CSV (TCID50/mL $1.21x10^5$) in

1%v/v FBS (Corning) containing media. After a 5-day incubation at 17°C cell viability was measured using CFDA-AM (Invitrogen, Carlsbad, CA, USA) (Figure 2.3). Data from five independent experiments were statistically analyzed using a one-way ANOVA and Tukey post-hoc test performed using Kaleidagraph software.

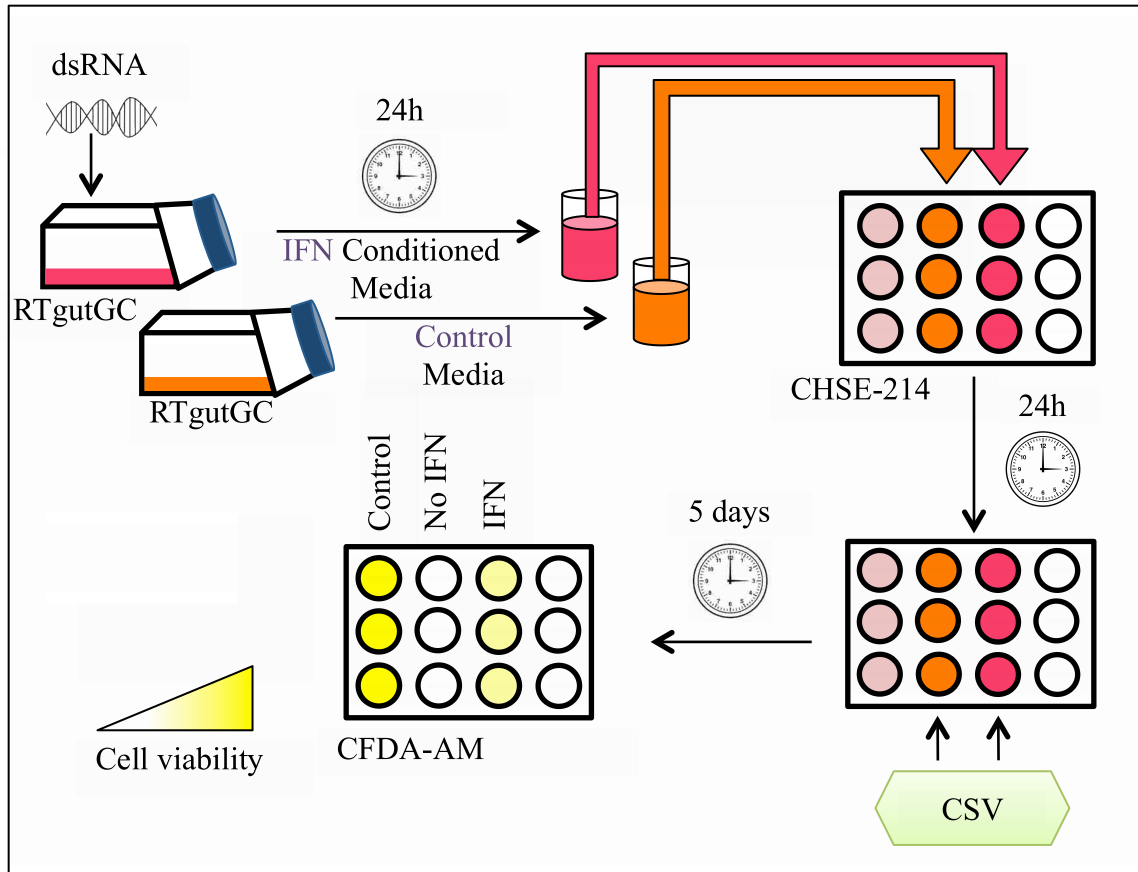

Figure 2.3 Functional interferon assay.
Rainbow trout cells were stimulated for 24h with dsRNA, to allow for IFN to accumulate in the media. The conditioned media was then collected and used to treat CHSE-214 cells for 24h. During this time any present interferon would be stimulating an antiviral state within the CHSE-214 cells. Afterwards the CHSE-214 cells were infected with chum salmon reovirus (CSV). 5 days post-infection cell viability was measured using CFDA-AM. If functional interferon was present in the media, treated cells would have a protective, antiviral state and more cells would survive the virus infection. Assay was completed in a 96-well plate; a 12-well plate was shown for simplicity.

Table 2.1 RT-PCR primers.
Primer sequences, including product size, annealing temperature (T_A), and reference for primers or accession number. *indicates undiluted cDNA was used, all other products were amplified from cDNA diluted 1:3.

Gene or fragment	Primer Sequence 5`-3`	Product size (bp)	T_A (^oC)	Reference or Accession Number
β-actin	**F**-ATCGTGGGGCGCCCCAGGCACC **R**-CCTCCTTAATGTCACGCACGATTTC	514	53	DeWitte-Orr *et al.*, 2007
Mx1	**F**-ATGCCACCCTACAGGAGATGATATGA **R**-AAAAAGGATAACAAAGGACT	452	54.4	DeWitte-Orr *et al.*, 2007
Mx2	**F**-TTGGTAGACAAAGGCACAGAGGA **R**-AAGTTCTTTCCAGAGCGATCCA	500	65	DeWitte-Orr *et al.*, 2007
Mx3	**F**-ATGCCACCCTACAGGAGATGATT **R**-CCACAGTGTACATTTAGTTG	380	53	DeWitte-Orr *et al.*, 2007
Vig1*	**F**-CAGTTCAGTGGCTTTGACGA **R**-ACAAACTCCTCAAGGTATGG	232	65	DeWitte-Orr *et al.*, 2007
Vig2	**F**-GCATTGGTACACGCGTAG **R**-TTGAGGAGAATTAGCTACACA	279	55	Boudinot *et al.*, 2001
Vig3	**F**-TGCTTGCCCTCGTGAATCAG **R**-CATCAGTCTCAGCGATGATTCAAA	225	55	O'Farrell *et al.*, 2002
Vig4	**F**-CATTCCTTTGCCTAGGGCATCT **R**-TGTCTCCTGATGTGCCTCAC	613	55	O'Farrell *et al.*, 2002
rtIFN1*	**F**-AATTCCTGTGTATCACCTGCCA **R**-GATGATCAGTACATCTGTGCTG	182	57	Tafalla *et al.*, 2008
rtIFN2*	**F**-TGGATCCAACACCACTTCGG **R-**GCTCAGTACATCTGTCCCACA	467	58	AJ580911
rtIFN3/4	**F-** TGGTTGAGCATTTGCCTGAC **R-** TCAGAGTAACCGAGCCTCCA	400	55	AJ829673.1
v200*	**F** - TTCAGATGAGGGGAGCCACA **R** - TCGCATGATCTGGCCATCAA	200	62	GQ385941
v1200*	**F** - ACGGACAAGCGAAGGACTAC **R** - TCGCATGATCTGGCCATCAA	1200	52	GQ385941

2.6 Mx1-Promoter Reporter Assay

The Mx1-promoter reporter assay was performed as shown in Figure 2.4. The novel assay was modified from Collet and colleagues (2004). RTG-P1 cells were seeded at a density of $5x10^5$ cells/well in a 6-well plate (BD Falcon). Cells were allowed to attach overnight. Media was removed from all wells, cells were treated with media containing 1.5nM of either regular poly I:C (Sigma-Aldrich), HMW poly I:C (InvivoGen), LMW poly I:C (InvivoGen), v1200, or v200 *in vitro* transcribed molecules or untreated control media. After 24h cells were removed from the growing surface with trypLE (Life Technologies, Grand Island, NY) and collected through centrifugation at 1400xg for 5min. The cell pellet was re-suspended in 100µl of Steady-Glo luciferase substrate (Promega, Madison, WI, USA) and incubated for 40min in the dark. 90µl of the substrate/cell mixture was plated into a 96-well plate (BD Falcon) and luminescence was measured in relative luminescent units (RLUs) using a Synergy HT plate reader (BioTek, Winooski, VT, USA). Data from three independent experiments were statistically analyzed using a one-way ANOVA and Tukey post-hoc test performed using Kaleidagraph software.

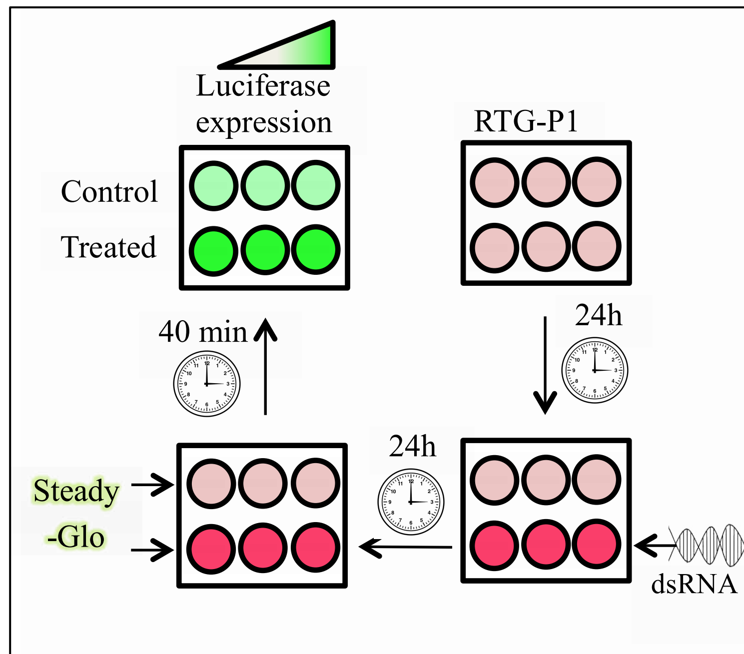

Figure 2.4 Mx1-promoter reporter assay protocol.
RTG-P1 cells were plated in 6-well plates and allowed to attach overnight. Treatment wells were treated with dsRNA for 24h. After 24h cells were removed from wells, a Steady-Glo luciferase substrate was added, incubated, transferred to a new microphage and luminescence was measured. Wells with more Mx1-promoter activation expressed more luciferase and therefore more luminescence.

2.7 Antiviral Assay

A novel antiviral assay was developed to measure the establishment of the antiviral state (Figure 2.5). RTG-2 cells were seeded at $3x10^4$ cells/well in 96-well plates (BD Falcon). Cells were allowed to attach overnight. Media was removed from wells and replaced with media containing 3nM of either v1200, v200, LMW poly I:C (InvivoGen), or HMW poly I:C (InvivoGen) or control media (all with 10%v/v FBS (Corning)). This concentration of dsRNA was determined to be optimal based on an antiviral assay concentration gradient using 1.5nM to 30nM poly I:C (Appendix A2). After 6h or 24h the media was removed and the cells were rinsed with PBS (HyClone). Cells were infected with VHSV ($3.16x10^5$ TCID50/mL) in 1%v/v FBS (Corning) media. After a 7-day incubation at 17^oC cell viability was measured using CFDA-AM (Invitrogen). Data from three independent experiments were statistically analyzed using a one-way ANOVA and Tukey post-hoc test performed using Kaleidagraph software.

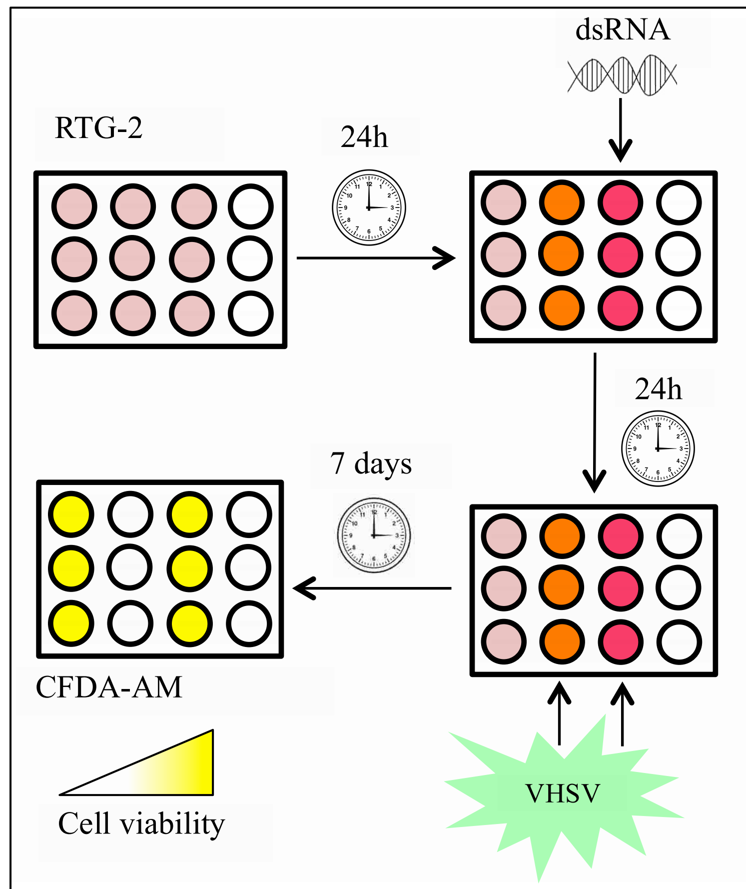

Figure 2.5 Antiviral assay protocol.
RTG-2 cells were treated with dsRNA or growth media alone for 24h to allow dsRNA to mount an antiviral state. Afterwards cells were challenged with VHSV. 7-days post infection a CFDA-AM cell viability assay was used to measure dsRNA-induced cell protection. A 96-well was use for the assay; a 12-well plate is shown for simplicity.

2.8 Cell Viability Assay

After incubation with the virus, the media was removed and the cells were rinsed with PBS (HyClone). A solution containing 4μM CFDA-AM (Invitrogen) was added to wells. The plate was incubated in the dark for 1h. Fluorescence was measured in a Synergy HT plate reader (BioTek) at Ex 485nM Em 530nM. Data from three independent experiments were statistically analyzed using a one-way ANOVA and Tukey post-hoc test performed using Kaleidagraph software.

CHAPTER 3: RESULTS

3.1 Synthetic dsRNA characterization

To confirm dsRNA lengths, 1μg of each type of poly I:C was run on an agarose gel and stained with ethidium bromide (Figure 3.1). All poly I:C produced a smear of mixed length nucleic acids. LMW poly I:C was estimated to have an average length of 300bp, HMW poly I:C to have an average length of 3000bp, and regular poly I:C to have an average length of 500bp.

In vitro transcribed dsRNA was synthesized using a DNA template amplified from the VHSV genome. Resulting dsRNA fragments were 200bp and 1264bp long and called v200 and v1200 respectively. The dsRNA products were confirmed as single bands of the appropriate length by agarose gel electrophoresis (Figure 3.2).

3.2 Native dsRNA isolation characterization

Total RNA collected from uninfected and VHSV infected RTgill-W1 cells were run on a 1%w/v agarose gel and visualized using an acridine orange stain and VersaDoc Imager (BioRad). The infected cells showed a robust high molecular weight green dsRNA band that was not present in uninfected control cells (Figure 3.3). The dsRNA band was larger than the 20kb band found in the DNA ladder. Both uninfected and infected cells produced 2 red ssRNA bands, likely 28S and 18S rRNA.

Two methods were used to isolate the dsRNA from the total RNA samples. Firstly, the dsRNA was isolated using a gel extraction method and run on a gel to check integrity and purity (Figure 3.4). The dsRNA band appears of similar size to the original dsRNA band and to be free of ssRNA contamination. The final concentration of the

dsRNA extracted from four infected 75cm^2 flasks of cells was 14.5ng/µL in 50µL. The second method used to isolate native dsRNA used a lithium chloride based extraction. The dsRNA product from this method was visualized on an acridine orange stained agarose cell (Figure 3.4). The resulting dsRNA product appeared to have some contaminating ssRNA present. In addition the dsRNA did not run on an agarose gel in a manner that was consistent with the pre-extracted sample. As sufficient amounts of native dsRNA to dose cells with were unable to be achieved with the methods described, the induced IFN, ISG and antiviral states of fish cells were evaluated using poly I:C and in vitro transcribed dsRNA. Native dsRNA-induced cell responses will be measured in future studies once better methods of extraction are developed.

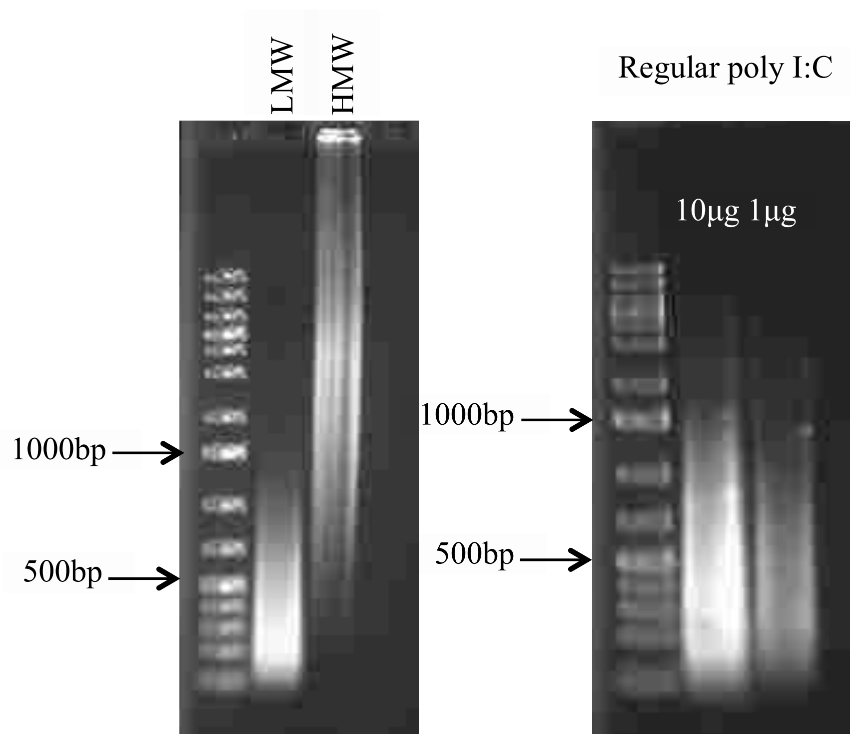

Figure 3.1 Characterizing length of different types of poly I:C.
1μg of low- (LMW), 1μg of high- (HMW) molecular weight poly I:C and 1μg or 10μg of regular poly I:C were run on a 1%w/v agarose gel, stained with ethidium bromide and visualized by UV transillumination. The average length was estimated to be 300bp for LMW, 3000bp for HMW, and 500bp for regular poly I:C.

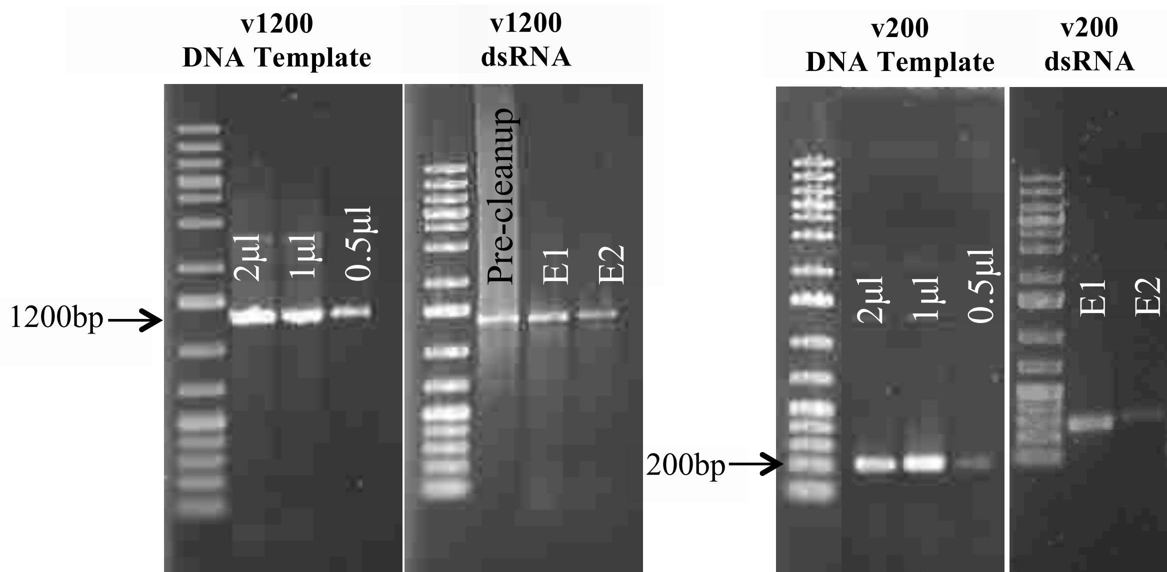

Figure 3.2 ***In vitro*** **dsRNA synthesis products.**
Two fragments, 1200bp and 200bp, of the VHSV genome were amplified using RT-PCR to create a DNA template. The DNA template was used in a T7 RNA polymerase reaction to create complementary strands of ssRNA. The ssRNA strands annealed to create dsRNA of known length and sequence. Different amounts of DNA templates were run on a gel for quantification. The purified, synthesized dsRNA was also run on a gel to confirm size and quantity (E1 and E2 represent two separate elutions of the final dsRNA product from the same column). 1/400th of the final product was run on a gel. The "pre-cleanup" lane shows the dsRNA prior to digestion with nucleases and column cleanup, it was shown for v1200 as a representation of the effectiveness of the cleanup.

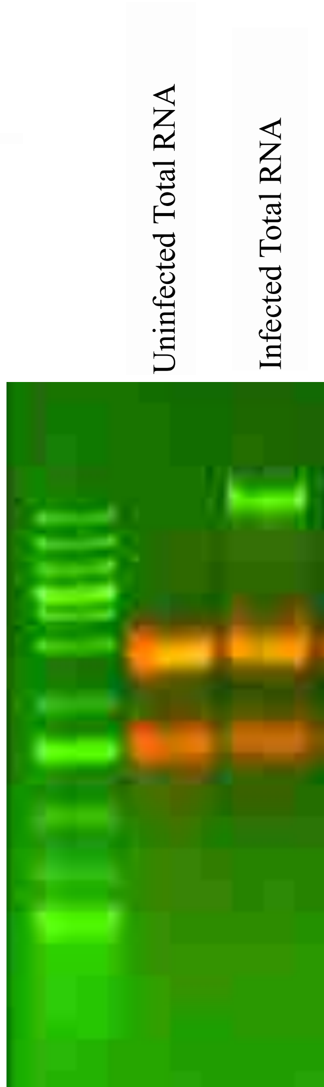

Figure 3.3 Native dsRNA produced by VHSV.
RTgill-W1 cells were infected with VHSV for 4 days. Total RNA was extracted and run on a 1%w/v agarose gel then stained with acridine orange. Acridine orange stains single-stranded nucleotides red and double-stranded nucleotides green. The two ssRNA bands present in both uninfected and infected samples are likely 28S and 18S RNA.

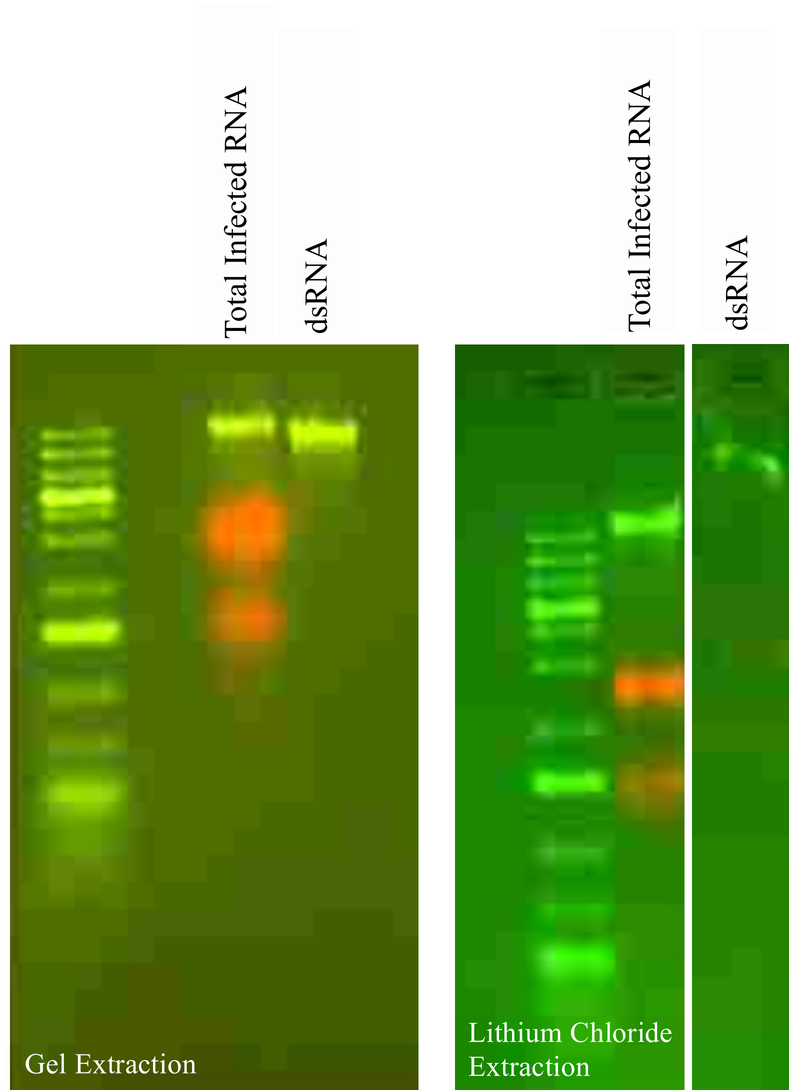

Figure 3.4 Native dsRNA isolation products.

RTgill-W1 cells were infected with VHSV for 4 days. Total RNA was extracted and dsRNA was isolated using a gel extraction method, or a lithium chloride based extraction method. The total RNA and extracted dsRNA, ~1µg of each, were run on an agarose gel then stained with acridine orange and visualized by UV transillumination. Acridine orange stains single-stranded nucleotides red and double-stranded nucleotides green.

3.3 RT-PCR analysis of IFN and ISG production in response to extracellular dsRNA

The expression profile of type 1 IFN and ISG transcripts was determined in dsRNA-stimulated and unstimulated RTG-2 cells. In response to 50μg/mL (~151.5nM) poly I:C for 24h, IFN1 and IFN2 and all ISGs (Mx1-3 and vig1-4) were upregulated (Figure 3.5). In response to 1.5nM HMW poly I:C, v200, or v1200 for 24h, IFN1 and IFN2 were upregulated compared to controls however less upregulation was seen compared to the higher concentration of poly I:C. The greatest upregulation for IFN1 and IFN2 appeared to be in response to HMW poly I:C. There was little to no detectable IFN1 or IFN2 transcript production seen after treatment with LMW poly I:C. All ISGs were clearly upregulated with low levels of constitutive expression, with the exception of vig1, which was not detected in response to 1.5nM treatments and vig2 which was strongly upregulated by 50μg/mL poly I:C but very little by 1.5nM v200, v1200 and HMW treatments. LMW poly I:C did not result in detectable amounts of vig2 transcripts. β-actin was included as a positive control, constitutive expression was detected across all groups.

	A	B	C
	Ctrl Poly I:C	Ctrl LMW HMW	Ctrl v200 v1200
β-Actin			
IFN1			
IFN2			
Vig1			
Vig2			
Vig3			
Vig4			
Mx1			
Mx2			
Mx3			

Figure 3.5 IFN and ISG transcript expression following dsRNA treatment as measured by RT-PCR.

RTG-2 cells were stimulated with (A) 50μg/mL poly I:C or (B) 1.5nM high- (HMW) or low- (LMW) molecular weight poly I:C or (C) 1.5nM *in vitro* transcribed dsRNA 200bp (v200) or 1200bp (v1200) for 24h. After the incubation period RNA was extracted, cDNA was synthesized and RT-PCR was performed using gene specific primers. Most IFNs and ISGs were upregulated in response to extracellular dsRNA. β-Actin was included as a housekeeping gene control. Results are representative of three independent trials.

3.4 Functional interferon production in response to extracellular dsRNA

Functional interferon activity was measured using an assay developed in lab. IFN-containing media was collected from RTgutGC cells stimulated with extracellular 3nM dsRNA and used to treat CHSE cells. An uninfected control given fresh media and an infected control treated with conditioned media from unstimulated RTgutGC cells were also included. The cells were challenged with CSV for 5-days and CFDA-AM was used to determine cell viability. The average relative fluorescent units (RFUs) obtained were as follows: uninfected control 1103.26 +/- 24.15 RFUs, infected control 973.86 +/- 20.97 RFUs, LMW poly I:C 1015.62 +/- 21.57, HMW poly I:C 1024.26 +/- 27.54 RFUs. This increase in cell viability indicates decreased viral effectiveness and thus protective IFN activity (Figure 3.6). This demonstrates a protective effect provided by the functional interferon produced by stimulated RTgutGC but no apparent length effects

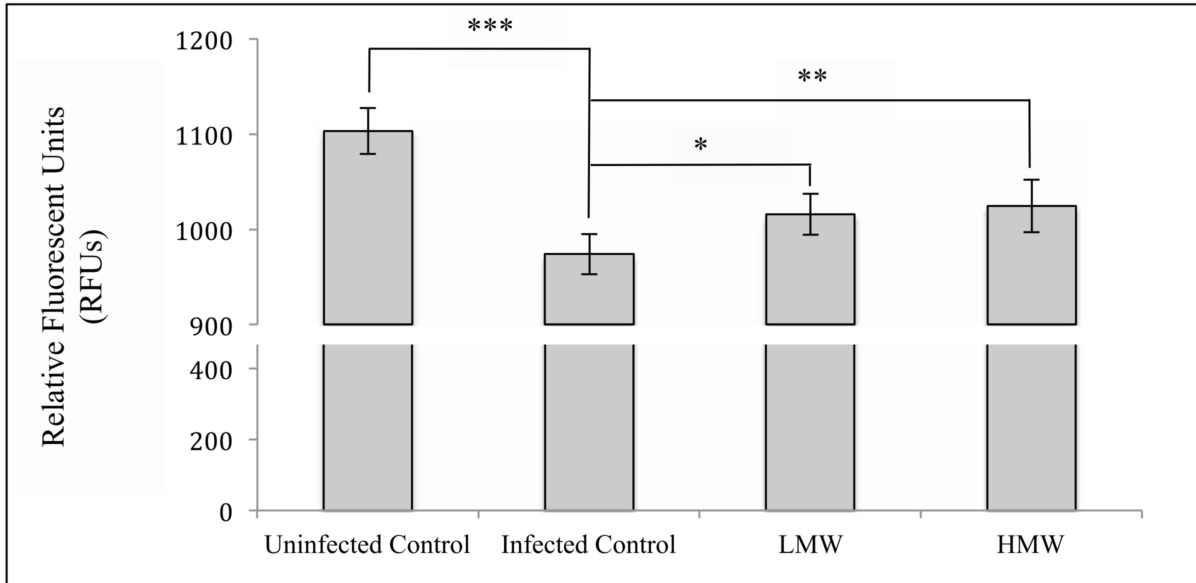

Figure 3.6 HMW and LMW poly I:C induced interferon results in a protective state. Interferon-conditioned media from RTgutGC cells stimulated with 3nM LMW or HMW poly I:C for 24h was used to treat CHSE-214 cells. Cells were challenged with chum salmon reovirus (TCID50/mL 1.21×10^5). 5 days post-infection cell viability was measured using a fluorescent cell viability indicator, CFDA-AM. An uninfected control with fresh media and an infected control with conditioned media from unstimulated RTgutGC cells were included. Statistical analysis was performed by a one-way ANOVA with Tukey post-hoc test (*$p<0.05$, ** $p<0.001$, ***$p<0.0001$), n=5.

3.5 Mx1-promoter activation in response to extracellular dsRNA

ISG promoter activity was evaluated using an Mx1-promoter reporter assay. After RTG-P1 cells were stimulated with 1.5nM of dsRNA luciferase expression was quantified. With either poly I:C or *in vitro* transcribed dsRNA there was a significantly greater amounts of luminescence seen in the dsRNA treated groups compared to the untreated control. In the HMW/LMW poly I:C experiment the relative luminescence units (RLUs) measured were as follows: control 5884.12 +/-1972.63 RLUs, LMW poly I:C 35812.67 +/-5198.50 RLUs, HMW poly I:C 51964.33 +/-3916.58 RLUs. In the v200/v1200 experiment the RLUs measured were: control 1212.21 +/-213.23 RFUs, v200 14988.83 +/-2246.13, v1200 23068.42 +/-3059.18 RLUs, poly I:C 19268.54 +/-3752.494 RLUs. The longer dsRNA molecules, HMW poly I:C and v1200, were able to induce a significantly greater magnitude of response compared to the shorter molecules, LMW poly I:C and v200 respectively (Figure 3.7, Figure 3.8). Regular poly I:C was used in the same experiments as the v200 and v1200, the poly I:C produced a greater response than the v200 and a slightly (not significantly) lower response compared to the v1200. There was variability between the two experiments, however there was consistency within an experiment. This is seen quite clearly between the controls (5884.12 RLUs and 1212.21 RLUs) and one possible explanation is the luminescent substrate reagent losing efficacy over time.

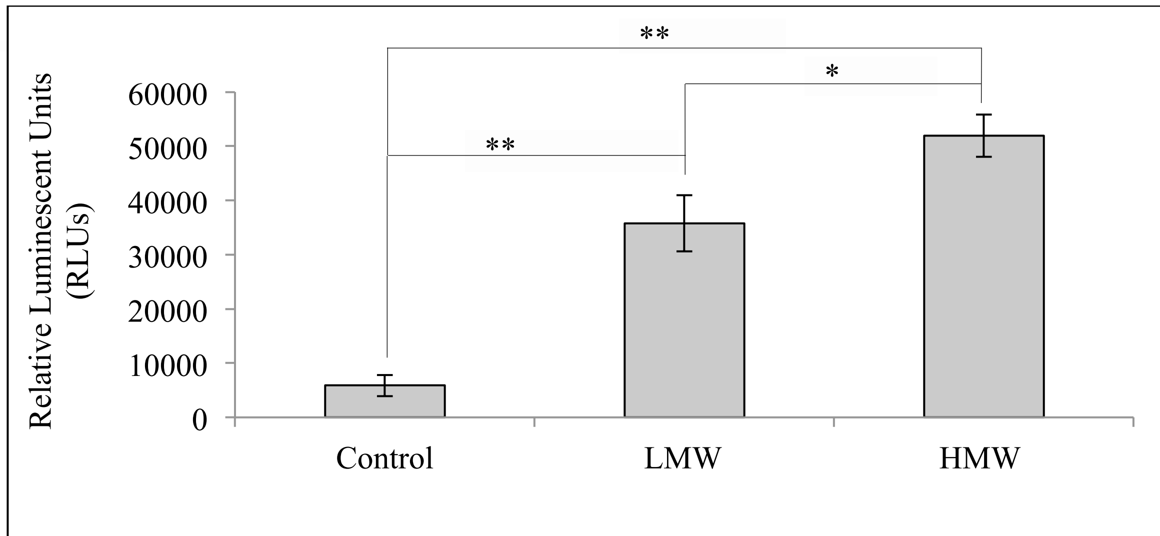

Figure 3.7 Mx-promoter activity in response to high molecular weight (HMW) or low molecular weight (LMW) poly I:C.

RTG-P1 cells were treated with 1.5nM of either HMW or LMW poly I:C for 24h. After 24h a luciferase substrate was added to cells and luminescence was measured using a plate reader. These data are an average of three independent experiments. Statistical analysis was performed by a one-way ANOVA with Tukey post-hoc test (*$p<0.01$, **$p<0.0001$), $n=3$.

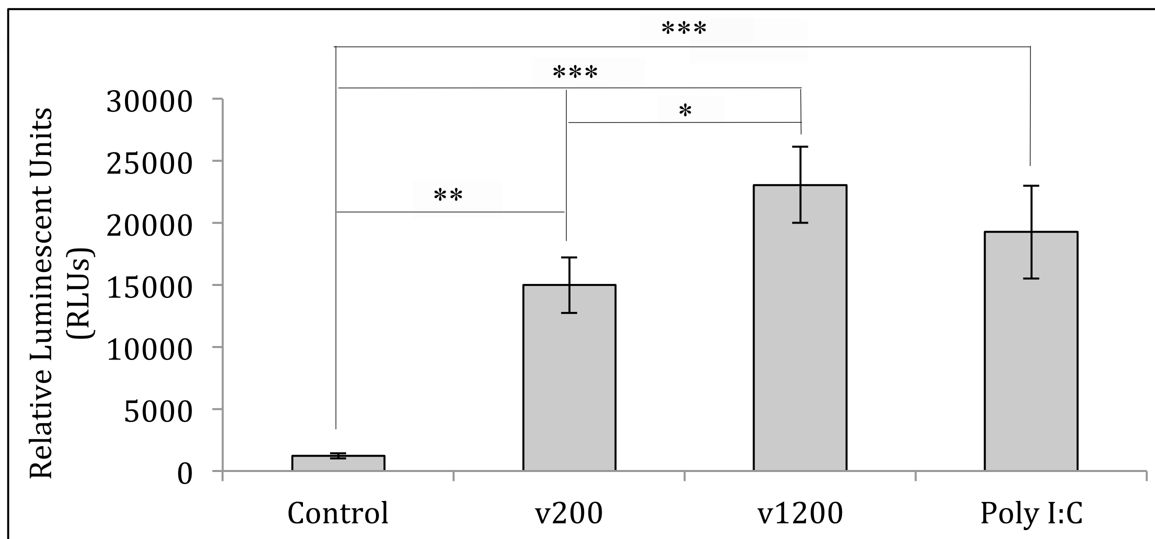

Figure 3.8 Mx1-promoter activity in response to two lengths of *in vitro* transcribed dsRNA or poly I:C.

RTG-P1 cells were treated with 1.5nM *in vitro* transcribed dsRNA of 200bp (v200), 1200bp (v1200) lengths or poly I:C for 24h. After 24h a luciferase substrate was added to cells and luminescence was measured using a plate reader. These data are an average of three independent experiments. Statistical analysis was performed by a one-way ANOVA with Tukey post-hoc test (*p<0.01, **p<0.001, ***p<0.0001), n=3.

3.6 Antiviral activity in cells stimulated with extracellular dsRNA

To look at the antiviral effects of dsRNA RTG-2 cells were stimulated with 3nM dsRNA for 24h or 6h and then challenged with VHSV. To asses potency of dsRNA a range of molar concentrations of poly I:C were used in a preliminary study (Appendix A2). Cells treated with dsRNA demonstrated higher levels of cell viability after the 7-day infection as measured using CFDA-AM. Treatments with either poly I:C or *in vitro* transcribed dsRNA significantly protected cells against the VHSV infection compared with untreated controls (Figure 3.9, Figure 3.10). Near complete protection was observed following 6h and 24h dsRNA treatments, and no length effects were observed. The 24h treatment experiment cell viability results were as follows: uninfected control 1211.97 +/- 23.37RFUs, infected control 692.17 +/-122.76 RFUs, v200 1237.28 +/- 221.63 RFUs, v1200 1244.55 +/-160.55 RFUs. The 6h treatment experiment cell viability results were as follows: uninfected control 1082.7 +/-131.70 RFUs, infected control 734.67 +/-129.40 RFUs, v200 1091.28 +/-71.12 RFUs, v1200 1073.00 +/-100.54 RFUs, LMW poly I:C 1049.64 +/-75.76 RFUs, HMW poly I:C 1060.33 +/-134.96 RFUs.

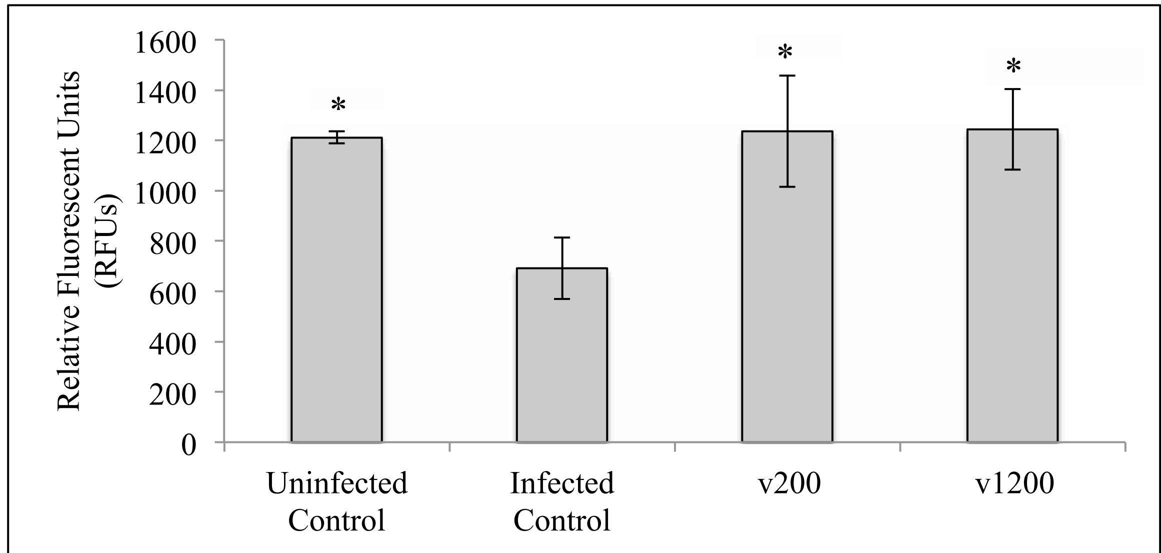

Figure 3.9 I*n vitro* transcribed dsRNA-induced antiviral state following 24h treatment

RTG-2 cells were treated with 3nM *in vitro* transcribed dsRNA of 200bp (v200) or 1200bp (v1200) lengths for 24h. Cells were challenged with VHSV (3.16 x 10^5 $TCID_{50}$/mL). 7-days pi cell viability was measured using the fluorescent indicator dye, CFDA-AM. An uninfected control was included as was an infected control that was not pretreated with dsRNA. These data are an average of three independent experiments. Statistical analysis was performed by a one-way ANOVA with Tukey post-hoc test * = significant difference from infected control (*$p<0.01$), n=3. No significant difference was found between dsRNA treatment groups.

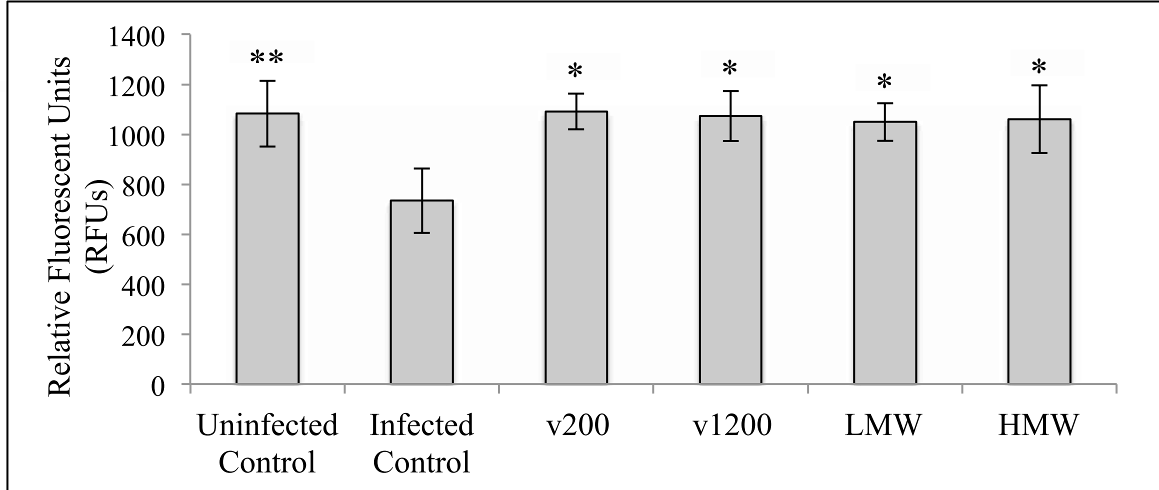

Figure 3.10 Poly I:C or *in vitro* transcribed dsRNA-induced antiviral state following 6h treatment.
RTG-2 cells were treated with 3nM *in vitro* transcribed dsRNA of 200bp (v200) or 1200bp (v1200) lengths or HMW or LMW poly I:C for 24h. Cells were challenged with VHSV (3.16×10^5 $TCID_{50}$/mL). 7-days post infection cell viability was measured using a fluorescent indicator dye, CFDA-AM. An uninfected control was included as was an infected control that was not pretreated with dsRNA. These data are an average of three independent experiments. Statistical analysis was performed by a one-way ANOVA with Tukey post-hoc test * = significant difference from infected control (*$p<0.05$, **$p<0.01$), n=3. No significant difference was found between dsRNA treatment groups.

CHAPTER 4: DISCUSSION

The main hypothesis of this project was that dsRNA, both synthetic and *in vitro* transcribed dsRNA based on a viral genome, would induce a robust immune response within rainbow trout cells. A secondary hypothesis was that rainbow trout cells would respond to dsRNA in a length dependent manner; longer molecules will produce a greater magnitude of response. A third hypothesis was that poly I:C would produce a stronger response than the *in vitro* transcribed dsRNA. To test these hypotheses three aspects of the immune response were measured in response to dsRNA, the IFN response, ISG response, and the antiviral state. The DeWitte-Orr lab has previously shown that fish viruses produce dsRNA in fish cells, validating the use of fish cells and viruses for this study (unpublished data). The majority of the work done on fish cells using dsRNA has relied on the synthetic form of dsRNA, poly I:C. This is the first time low- and high-molecular weight poly I:C have been used in fish cells to explore innate immunity and length effects. To increase biological relevancy dsRNA molecules were synthesized *in vitro* with a more natural sequence and defined length and sequence. dsRNA was successfully generated in the lab from a template derived from the VHSV genome. This is the first time a VHSV based dsRNA molecule has been synthesized and used to treat fish cells.

Many of the protocols used in this study were novel and designed as part of the study. The functional interferon assay was the first of its kind utilizing established rainbow trout cell lines as the interferon producer, CHSE-214 as the responding cell, and cell viability measured using CFDA-AM as an end point. The antiviral assay is similar to previous antiviral assays (DeWitte-Orr *et al.,* 2005), the protocol required optimization

for a VHSV infection within RTG-2 pretreated with molar concentrations of extracellular dsRNA and using the CFDA-AM cell viability dye as an endpoint measurement. RT-PCR has previously been used to identify ISG transcripts in rainbow trout cells, however the gene-specific primer set for IFN2 and the v200 and v1200 VHSV dsRNA templates were novel and optimized accordingly. The primers for mx1-3 and vig1 had previously been used with RTG-2 cells; the primers for vig2-4 came from previously completed studies, however, had not been optimized for use in RTG-2 (Table 2.1). None of the primers had been used in the DeWitte-Orr lab and required optimization. The mx1-promoter reporter assay required adjustment for nanomolar concentrations of dsRNA and it required a novel protocol to be developed to accurately measure luciferase expression using a Steady-Glo reagent (Promega).

4.1 dsRNA characterization

As mentioned above, this is the first time dsRNA has been made using a fish virus genome template. Two different lengths of dsRNA were produced, 1264bp (v1200) and 200bp (v200). The lengths were chosen as representative short and long dsRNA molecules. While the v200 was considered the 'short' molecule in this study compared to the v1200, when compared to endogenous dsRNA (<30bp) v200 is still a long dsRNA molecule that is capable of being sensed by host cell PRRs. To avoid number of molecules acting as a confounding variable, cells were treated in nanomolar concentrations as opposed to weight concentrations. This ensured an equal number of molecules/cell for each dsRNA length; thus differences in responses can be attributed to length as opposed to differences in number of molecules/cell. Previous studies used molar concentrations of *in vitro* transcribed dsRNA molecules when exploring length effects

(DeWitte-Orr *et al.,* 2010; Binder, 2011). For poly I:C, which is of mixed length, the approximate average length was used for molarity calculations. This is one way *in vitro* molecules are more relevant for studying length effects compared to poly I:C, as the length of the molecules is homogenous and not mixed, this allows for accurate molar dosing. A concentration gradient was used to determine the optimal dsRNA molarity for the antiviral assay, 3nM was chosen as a molarity sufficient to induce a strong response and low enough to conserve dsRNA. The same molarity was used for the functional interferon assay. 1.5nM was found to be sufficient for the RT-PCR panel and the Mx1-promoter assays; as these assays required greater volumes of dsRNA the reduced concentration was preferable.

To isolate and characterize dsRNA an acridine orange staining protocol was optimized from Pichlmair colleagues 2009. Acridine orange is a useful tool as it stains double stranded nucleic acids green and single stranded nucleic acids red. Acridine orange is an incredibly robust stain and destaining was difficult. If there are suboptimal staining conditions single stranded nucleic acids stain green instead of red, making them indistinguishable. Once optimized acridine orange was used to detect dsRNA production in virus infected rainbow trout cells (Figure 3.3). The dsRNA molecules produced by VHSV were large, which is consistent with the genome size of VHSV (11.2kb). The dsRNA on the gel appears to be approximately 20kb in length; however, the size could be more accurately determined using a DNA ladder with larger bands and running the samples on a lower percent agarose gel. Regardless, the isolated dsRNA band was large, likely larger than the virus genome. The reason for this is yet unknown and is currently being investigated One possible explanation is that the genome is self-annealing to

create the long fragments. Similar results were found in one study that demonstrated encephalomyocarditis virus, a positive sense ssRNA virus with a genome size of approximately 7.7kp, produced dsRNA molecules larger than 12kb, longer than the genome size (Pichlmair *et al.,* 2009; Shi *et al.,* 2010). Positive sense ssRNA viruses have been shown to produce long dsRNA fragments, >3000bp and ~8000bp (Kato *et al.,* 2008; DeWitte-Orr *et al.,* 2009). Interestingly, it has been suggested that negative sense ssRNA viruses, one of which is VHSV, do not produce dsRNA (Weber *et al.,* 2006), however studies have found VHSV and vesicular stomatitis virus (VSV), both negative sense ssRNA viruses, produce dsRNA (unpublished data; Kato *et al.,* 2008). This is the first time that fish virus native dsRNA has been observed using an acridine orange staining method.

Two different techniques were used to isolate the native dsRNA and the extraction was checked for effectiveness using the acridine orange stain. A lithium chloride extraction method and an agarose gel extraction method were attempted (Figure 3.4). After the LiCl extraction the dsRNA sample contained a small amount of ssRNA contamination. Also, the dsRNA no longer ran the same way on a gel as before it was extracted, possibly because of residual salts. Attempts to elucidate the effects of native dsRNA would be hindered if the dsRNA molecules were modified during the extraction process. The gel extraction was successful in isolating dsRNA with no visible ssRNA contamination and the dsRNA appeared similar to the pre-extraction dsRNA molecule; however, the resulting dsRNA concentrations were too low to be practical for dosing cells, even when the protocol was scaled up to use more starting material. Unfortunately most protocols for isolating dsRNA have been developed for extracting plant pathogen

genomic dsRNA, which would be more abundant in the infected cells as compared to what is expected with replicative by-product viral dsRNA (Morris and Dodds, 1979; Li *et al.,* 2007). The LiCl based extraction completed in this study was developed for use with plant viruses (Diaz-Ruiz and Kaper, 1979). While a promising beginning it is evident more work is needed to fully optimize the protocols and isolate workable concentrations of viral dsRNA.

4.2 dsRNA induced antiviral responses

The central hypothesis of this study was that dsRNA molecules would induce innate immune responses, more specifically the production of IFN, ISGs, and an antiviral state. A variety of assays were utilized to test this hypothesis, the results of which are discussed below.

4.2.1 IFN response

The present study provided several different lines of evidence supporting the hypothesis that dsRNA induces an immune response in rainbow trout cells. The first step in the dsRNA-mediated pathway is the production of type 1 interferon. While RT-PCR is an important assay to measure transcript presence within a cell, it cannot be assumed that transcript production means functional protein is being produced. There are post-transcriptional modifications that could influence whether or not the transcript becomes a functional protein. For example many interferons undergo post-translational modifications, such as glycosylation (Fensterl and Sen, 2009). The functional interferon assay was developed in the current study and differed from previous assays similar in nature. Previous studies have used interferon-conditioned media from rainbow trout cells

to treat other rainbow trout cells (DeWitte-Orr *et al.,* 2007; Saint-Jean and Perez-Prieto, 2006). In these studies the conditioned media from dsRNA treated cells is transferred to new cells to measure IFN activity. Even though the dsRNA treated cells are washed prior to IFN accumulation in the media, dsRNA is still likely to be present and would induce IFN in the recipient cells, masking the effects of the IFN as rainbow trout cells can respond to both IFN and extracellular dsRNA. The assay developed for this study is similar to that developed by Congleton and Sun (1996) in that it utilizes CHSE-214 cells that cannot respond to extracellular dsRNA. Congleton and Sun transferred conditioned media from rainbow trout anterior kidney cells stimulated with poly I:C or the fish virus infectious hemapoetic necrosis virus onto CHSE-214. After allowing time for the IFN to induce a response, the cells were infected with the virus, incubated to allow for virus replication, and then fixed and stained with crystal violet. The optical density was measured to quantify virus-induced cytolysis (Congleton and Sun, 1996). The assay developed for the current study was novel in its use of RTgutGC cells as the producer of interferon conditioned media, and the use of a fluorescent indicator dye, CFDA-AM, as the end point cell viability measure.

There was a protective state in CHSE-214 cells treated with IFN-conditioned media from dsRNA-stimulated RTgutGC (Figure 3.6). This suggests that functional interferon is being produced and is able to initiate immune pathways resulting in a protective state within the cell. This is the first study to demonstrate the ability of RTgutGC to produce functional interferon in response to any type of dsRNA. The ability for *in vitro* transcribed dsRNA to stimulate the production of functional IFN has not yet been tested but will be in future experiments.

RTgutGC was used for this assay as opposed to RTG-2 because it appears that CHSE-214 mounts a stronger antiviral state in response to the IFN produced by RTgutGC cells (unpublished data). The mechanism behind this is currently unknown, however it is possible that RTgutGC either produces a type one IFN that RTG-2 does not, or produces the same IFNs but in greater amounts. Another hypothesis is that RTgutGC interferon may peak closer to 24h (the time at which media was collected) than the RTG-2 cells, producing more accumulated IFN in the media. Preliminary results using RT-PCR suggest at 24h RTgutGC cells stimulated with poly I:C express IFN1 and IFN2 robustly, and that unstimulated cells express IFN2 constitutively (Appendix A3). It is also notable that the cell death observed in the functional interferon assay virus control was not as advanced as might be expected after a 5-day infection. Previous experiments in the lab have shown that when CHSE-214 cells are untreated and infected with CSV for 5-days there is between 50-40% cell death compared to the uninfected control. The experiments performed in the present study yielded approximately 15% cell death 5-days post infection. The hypothesized explanation is the conditioned media from unstimulated RTgutGC contains constitutively expressed IFN. Thus cells treated with unstimulated RTgutGC conditioned media are still being exposed to accumulated IFN within the media that appears to have a protective state, however media from dsRNA-stimulated cells appear to produce more IFN and more than one type.

Interestingly, the expression profile of IFNs in CHSE-214 has been preliminarily investigated using rainbow trout IFN primers (Appendix A4). It appears as though CHSE-214 itself produces three type 1 IFN-like transcripts that align similarly to the rainbow trout IFNs IFN1, IFN2, and IFN3/4, and the IFN expression in CHSE-214

appears to be constitutive as all IFNs were seen in unstimulated cells. The identity of each band was confirmed by sequencing. The CHSE sequence obtained using rainbow trout IFN1 primers had a 90% similarity to rainbow trout IFN1 (121/134 identities), rainbow trout primers for IFN2 resulted in a sequence with 87% similarity to rainbow trout IFN2 (263/303 identities), and IFN3/4 primers resulted in an 85% similarity to rainbow trout IFN3 (542/636 identities). This would suggest CSV must have mechanisms of overcoming IFNs produced by CHSE-214, a fascinating area for future research.

The functional interferon assay suggested that interferons were being produced in rainbow trout cells in response to dsRNA stimulation, however the assay does not provide any information as to what type 1 IFN subtype are being produced. To verify the functional interferon results and delineate which type 1 IFN subtypes are produced RT-PCR was used. Three type 1 IFNs (IFN1, IFN2, IFN3/4) were surveyed, although only IFN1 and IFN2 were identified in RTG-2 cells, IFN3/4 has been shown to not be expressed in RTG-2 (Zou *et al.,* 2007). Both IFN1 and IFN2 were upregulated in RTG-2 in response to poly I:C (Figure 3.5), which was expected based on previous studies where IFN1 expression was upregulated in RTG-2 cells stimulated with poly I:C, less robustly than IFN2 but not at concentrations lower than 1μg/mL poly I:C (Zou *et al.,* 2007). Though not quantitative, our study also shows IFN1 being induced at lower levels compared with IFN2 in response to poly I:C. To our knowledge no one has investigated the effects of LMW or HMW poly I:C and *in vitro* transcribed dsRNA in IFN expression. The *in vitro* transcribed dsRNA and LMW poly I:C did not seem to be as effective at inducing IFNs as the HMW poly I:C, inducing trace amounts compared with untreated

controls. This could be because the HMW poly I:C has an average length more than double the length of the v1200 and ten times greater than LMW poly I:C. These results need to be pursued using quantitative RT-PCR methods to confirm differences in levels of IFNs. Also, different time points should be considered as IFN transcript production can be cyclical, and by 24h the peak of IFN production for the *in vitro* transcribed molecules may be missed.

The present study investigated the expression of IFN1, 2 and 3/4 at the transcript level; however, rainbow trout also express IFN5. IFN5 transcript has been previously found in poly I:C stimulated RTS-11 and RTG-2 cells (Chang *et al.,* 2009). This has yet to be repeated and attempts to repeat the study as part of the current study and identify IFN5 transcripts in RTG-2 after poly I:C stimulation were unsuccessful. IFN3/4 was not detected, which corresponds with previous studies that were unable to detect IFN3/4 in RTG-2 cells (Zou *et al.,* 2007). The results seen in this study suggest that fish respond to *in vitro* transcribed dsRNA somewhat differently compared to mammalian models. In mice cells *in vitro* transcribed dsRNA was able to robustly induce type 1 interferon, the response appeared much stronger compared to the results seen in fish (DeWitte-Orr *et al.,* 2009). This may be due to the different species or different cell types, the mammalian work was done in embryonic fibroblasts and this study utilized gonadal fibroblasts.

4.2.2 ISG response

The next stage in the innate immune pathway occurs when functional IFN leaves the cell and acts through its cognate receptor to induce the expression of a series of genes, ISGs (Robertsen, 2006). Western blot analysis would be ideal for measuring ISG expression; however, few antibodies exist against fish ISGs. Thus RT-PCR was

performed, which has the benefit of making it possible to assay multiple ISGs quickly. The panel consisted of vig1-4 and Mx1-3 and was optimized using 50μg/mL of poly I:C, which is a concentration commonly used in the literature; however it is a high dose for this study where 50μg/mL of regular poly I:C with an average length of 500bp is approximately 150nM. Vig1 and mx1-3 were chosen as they have previously been shown to be upregulated in RTG-2 cells in response to poly I:C (DeWitte-Orr *et al.,* 2007; Tafalla *et al.,* 2007). The ISGs vig2, vig3, and vig4 were chosen because they have been identified in rainbow trout cells and Vig3 and vig4 have important human homologues. Vig2, vig3, and vig4 have been identified in virus-infected rainbow trout leukocytes from head-kidney (O'Farrell *et al.*, 2002; Boudinot *et al.,* 2001) but this is the first time they have been measured in a dsRNA stimulated cell line. Thus the induction of vig2, vig3, and vig4 in RTG-2 cells following poly I:C treatment is a novel finding. This is also the first time *in vitro* transcribed dsRNA has been shown to induce any ISGs in rainbow trout cells.

Both poly I:C and *in vitro* transcribed dsRNA molecules were able to induce a variety of ISGs in RTG-2 cells (Figure 3.5). Vig3, vig4, and Mx1-3 were robustly upregulated in all treatment groups. Vig1 was only induced at higher concentrations of poly I:C, higher concentrations of HMW, LMW poly I:C or *in vitro* dsRNA were not tested. This is not inconsistent with previous experiments involving vig1 and RTG-2, where it was shown that vig1 expression was upregulated after 24h in RTG-2 cells treated with 50μg/mL poly I:C (DeWitte-Orr *et al.,* 2007). It is notable that ISG expression between treatments looks to be of similar strength despite the IFN transcripts appearing substantially less upregulated in RTG-2 treated with *in vitro* transcribed treatment groups

or LMW poly I:C. One possible explanation is that IFN transcripts peak earlier than 24h. To test this hypothesis a time course could be performed to measure IFN transcript over time. Another hypothesis is that there are other IFNs being produced that have not yet been measured in the current study, such as IFN5, a novel type 1 interferon subtype, or an interferon of a different type such as type III interferon. It also is possible that the ISGs are being induced by an IFN-independent mechanism, as has been seen with mammalian ISG56 and ISG15 (Chew *et al.,* 2009). VHSV has been shown to induce vig1 in an interferon-independent fashion (Boundinot *et al.,* 1999).

Although not quantitative, the RT-PCR panel suggests that there are potential length effects seen in transcript production, as longer molecules produced more ISG transcripts, particularly between LMW and HMW poly I:C. The difference between HMW and LMW looks to be larger than the v200 and v1200, the HMW (~3000bp) and LMW (~300bp) molecules are further apart in length compared to v200 and v1200 that could explain this trend. The RT-PCR is valuable for insights into what ISGs are being produced in response to dsRNA; however, quantitative assays are needed to demonstrate significant length effects on the production of ISGs.

One such quantitative assay is the Mx1-promoter reporter assay. RTG-P1 has previously been used to demonstrate the ability of poly I:C to activate the Mx1-promoter (Collet *et al.,* 2004). The present study used the RTG-P1 in a similar manner, treating the cells with poly I:C but also with *in vitro* transcribed dsRNA. All extracellular dsRNA was able to significantly activate the Mx1-promoter (Figure 3.7; Figure 3.8). Longer dsRNA molecules stimulated the promoter more strongly compared with shorter lengths. These data will be discussed further in section 4.3.1.

4.2.3 Antiviral response

The RT-PCR and Mx1-promoter reporter assay demonstrated the production of ISG transcripts and promoter activation, however it was unclear whether this was leading to a protective antiviral state. An antiviral assay was developed to see if dsRNA was able to protect the rainbow trout cells from viral infection. Since the accumulation of ISGs results in an antiviral state, the antiviral state assay is in effect a functional ISG assay. The results demonstrate a protective state was induced following poly I:C or *in vitro* transcribed dsRNA treatment (Figure 3.9; Figure 3.10), as treated cells were viable following VHSV infection while those without dsRNA treatment died. Poly I:C has been used in other studies to stimulate an antiviral state within fish cells (Jensen *et al.,* 2002; Jensen and Robertsen, 2002; DeWitte-Orr *et al.,* 2005). A main difference between previous assays and the assay used in the present study was the concentration of poly I:C. Previous studies have used poly I:C concentrations such as 10μg/mL and 50μg/mL, whereas this assay was adjusted for low nanomolar concentrations of dsRNA, between 0.396μg/mL – 5.94μg/mL (Jensen and Robertsen, 2002; DeWitte-Orr *et al.,* 2005). *In vitro* transcribed molecules have not previously been shown to induce an antiviral state in fish cells.

dsRNA was applied to cells in low amounts and there was still a robust antiviral state established; this reinforces how potent dsRNA is as an immunomodulating molecule (Marcus and Sekellick, 1977). With 24h of dsRNA treatment there was complete protection, no cell death was detected. These high levels of cell viability could have been hiding potential dsRNA length effects. It was hypothesized that 24h was too long an incubation to elucidate length effects and 6h was tested. At 6h there were no differences

seen between the v1200 and v200 *in vitro* transcribed molecules and the LMW and HMW poly I:C, as once again dsRNA induced complete protection of the monolayer. Shorter treatment times are currently being tested to attempt to explore length effects. This protocol may not be optimal for measuring length effects as there is a 7-day infection with VHSV, over the course of the 7-days the effects of different dsRNA treatments may equalize. A shorter assay such as the Mx1-promoter assay that only involves a single 24h incubation, was better at capturing length effects.

4.3 dsRNA features influencing immune responses

In addition to exploring the rainbow trout immune response to dsRNA, the characteristics of dsRNA that might influence the magnitude of the immune response were also examined. Length and sequence are two features of dsRNA that were examined. In addition the differences between commercially available poly I:C and *in vitro* transcribed dsRNA based on a fish viral genome sequence were also explored. The findings are discussed in the following sections.

4.3.1 Length effects

It was hypothesized that longer dsRNA molecules would induce a greater magnitude of immune response compared to shorter molecules. The Mx1-promoter assay demonstrates that fish cells have a more robust immune response to longer dsRNA molecules. This is the first study to suggest the dsRNA-mediated innate immune response in fish is length dependent. The Mx1-promoter assay showed statistically more activation in response to long dsRNA molecules compared to their shorter counterparts.

The RT-PCR indicated a similar trend for HMW and LMW poly I:C. These length effects were also suggested in the RT-PCR assay with both IFNs and ISGs.

The reason for length effects could be the PRR expression. Many PRRs have been shown in mammalian models to show increased activation in response to longer lengths of dsRNA (Binder *et al.,* 2011; Lemaire *et al.,* 2008; Peisley and Hur, 2012). RIG-I is known to preferentially bind shorter dsRNA (< 1000 bp) however it has not yet been cloned in rainbow trout. If RIG-I is not expressed then another receptor is binding the short dsRNA molecules such as v200, LMW poly I:C and regular poly I:C. A human cell line transfected with Fugu TLR3 was treated with different lengths of *in vitro* transcribed dsRNA and 200bp created the most robust response (Matsuo *et al.,* 2008). TLR3 could be compensating for the potential lack of RIG-I and be binding the v200. In the same study human cells transfected with Fugu TLR22 responded to poly I:C and shorter lengths of poly I:C moderately, however the 1000bp *in vitro* transcribed dsRNA generated the most IFN-β activity (Matsuo *et al.,* 2008). If the these PRRs act in rainbow trout as they do in fugu, the *in vitro* transcribed dsRNA lengths used in the present study would target TLR22 (v1200) and TLR3 (v200) respectively. Both receptors are hypothesized to trigger the same downstream pathways via the adaptor molecule TICAM-1 (Kasamatsu, 2013). MDA5 is also present in rainbow trout and is able to bind poly I:C and mediate up-regulation of Mx1 (Chang *et al.,* 2011). It is unclear at this time which PRRs are involved in the IFN, ISG and antiviral states monitored in the present assay. What is clear; however, is that longer dsRNA molecules are able to induce Mx1-promoter activity significantly more than shorter dsRNA molecules. This is similar to what has been previously seen in mammalian studies where longer dsRNA molecules

induced a greater magnitude of antiviral, ISG, and interferon response (DeWitte-Orr *et al.*, 2009).

4.3.2 Sequence effects

It was hypothesized that sequence would not have an effect on the magnitude of the innate immune response. The main emphasis of the current study was investigating the effects of *in vitro* transcribed dsRNA in rainbow trout cells with a comparison of two lengths, sequence was not fully investigated but some conclusions can be drawn for the role of sequence in the dsRNA mediated immune pathway. *In vitro* transcribed dsRNA which had a more natural sequence and poly I:C which is not naturally produced by viruses both seemed to have similar effects on the rainbow trout cells, with length being the possible dictator of response intensity, not sequence. Poly I:C (~500bp) induced Mx1 promoter activity somewhere between v200 and v1200, suggesting effect of length irrespective of sequence. To fully elucidate the role of sequence more *in vitro* molecules would need to be produced with different sequences but the same length. Aside from the minor sequence requirements, such as the 4 critical base pairs for OAS or uridine- or adenosine-rich ribonucleotide sequences for RIG-I, there has been little evidence to suggest dsRNA sequence influences receptor binding (Kodym *et al.*, 2009; Saito and Gale, 2008). PKR and TLR3 bind without sequence specificity (Lemaire *et al.,* 2008; Liu *et al.,* 2008). However the effect of dsRNA sequence on innate immune responses has not been explored as well as dsRNA length in any animal model.

4.3.3 Poly I:C vs. *in vitro* transcribed dsRNA

Previous studies in mammals have suggested that poly I:C and *in vitro* transcribed dsRNA produce similar but not identical innate immune responses (DeWitte-Orr *et al.,*

2009; Jiang *et al.,* 2011). Studies in mammals have shown differences between poly I:C and *in vitro* transcribed dsRNA molecules of similar length. A study in murine cells examined the expression of a panel of ISGs in response to poly I:C and similar length *in vitro* molecules (DeWitte-Orr *et al.,* 2009). It was found that poly I:C induced some of the ISGs within the panel more strongly than *in vitro* molecules, however some ISGs were induced more greatly in response to *in vitro* transcribed molecules (DeWitte-Orr *et al.,* 2009). A different study in murine cells looked specifically at IFN-β production found greater production in response to *in vitro* molecules of similar length to poly I:C (Kato *et al.,* 2006). It is difficult to compare the poly I:C and *in vitro* transcribed data in many studies as the length of the poly I:C is often not reported.

At the receptor level a notable difference has been identified between fish and humans. When human TLR3 and a fish (Fugu) TLR3 were treated with poly I:C and *in vitro* transcribed dsRNA, the human TLR3 responded most strongly to the poly I:C whereas the fish TLR3 responded most strongly to *in vitro* transcribed dsRNA (Matsuo *et al.,* 2008). The hypothesis for the current study was that fish cells would show a greater immune response to poly I:C compared to *in vitro* molecules. The data from the quantitative Mx1-promoter assay showed the poly I:C response was not significantly different from either *in vitro* transcribed treatment (Figure 3.8). The poly I:C treatment fell between the v200 and v1200 *in vitro* transcribed dsRNA response. The regular poly I:C stimulated slightly less promoter activity compared to the v1200 group and more than v200. While the average length is 500bp for the poly I:C, longer molecules are present within the group, possibly contributing to the higher expression which was closer to the v1200.

4.4 Conclusions

This study investigated the effects of extracellular dsRNA on the innate immune system in rainbow trout cells. Two types of dsRNA, poly I:C and *in vitro* transcribed dsRNA, were used to treat rainbow trout cells and the response was measured at each major step in the innate antiviral pathway. The novel findings from this study are twofold; firstly this was the first study to measure the effects of *in vitro* transcribed dsRNA on fish cells. It was determined that rainbow trout cells are able to respond to extracellular in vitro transcribed dsRNA through the induction of type 1 interferons, interferon-stimulated genes, and an antiviral state. This response did not vary dramatically from the poly I:C induced response. Secondly, this was also the first study to investigate the effects of dsRNA length on the antiviral response in fish cells. Different lengths of dsRNA were used to show that rainbow trout cells show similar length effects to those seen in mammalian models, in which longer dsRNA molecules produce a greater magnitude of immune response. *In vitro* transcribed dsRNA molecules are potent inducers of innate immune responses.

The results from this study contribute to a better understanding of the host response to viral infection. As dsRNA has the potential to be an antiviral therapy or vaccine adjuvant (DeWitte-Orr and Mossman, 2010), this data produces a knowledge base for possible future therapeutic agents. Also, the innate immune system is well conserved between species and therefore understanding the fish innate immune pathway will provide insight into other species.

4.5 Future directions

The next step to explore length effects is to expand the variety of lengths of *in vitro* dsRNA molecules. Two different lengths of *in vitro* transcribed dsRNA were used in this study, expanding the number of lengths to include both longer and shorter molecules would further elucidate length effects. In addition to length, sequence effects could be explored using the methods in this study. One way to investigate sequence effects would be to create *in vitro* molecules of the same length and different sequences. It is unknown whether fish species other than rainbow trout respond to dsRNA in a length-dependent manner. Other cell lines from rainbow trout and other species will be stimulated with dsRNA to see if there are differences in the dsRNA-mediated immune responses between fish species.

Another future direction for this project is to extract native dsRNA molecules produced from a viral infection and stimulate cells with these naturally occurring molecules. Some possible methods of extracting native dsRNA include using cellulose, lithium chloride or ammonium acetate extractions, immune-precipitation, or differential digestion (Attoui *et al.,* 2000; Okuda and Hanada, 2001; Kaneko *et al.,* 2011). Many protocols for extracting dsRNA are designed for extracting dsRNA genome viruses from plants. These protocols provide a good starting point for extracting viral dsRNA however they likely will require optimization. After extraction, dsRNA molecules could be characterized for length, sequence, and abundance. In addition, proteins associated with native dsRNA could be identified. The dsRNA molecules could be used to stimulate cells so the natural immune response can be characterized.

Once native molecules are extracted and characterized it would provide the information for how to best mimic virally produced dsRNA. It is hypothesized that the dsRNA produced by viruses will differ depending on the genome of the virus. It is possible that a mix of *in vitro* transcribed molecules will be able to closely mimic the natural viral molecules and be able to be used in place of native molecules, which may be necessary if extracting native dsRNA proves to be difficult, costly, or results in very low yields of dsRNA or if researchers are looking to alter an aspect of the native dsRNA-induced response.

4.6 An integrative approach

A more integrative biological understanding of a pathway can be obtained by looking at multiple levels of a pathway. This study integrates the cellular and molecular mechanisms of the innate immune response with the whole cell response. By studying different levels of response, a broader understanding of the cellular reaction is gained. This study was a cross-disciplinary and trans-disciplinary research project. Techniques used were from numerous fields of biology, including cellular biology, molecular biology, virology, and immunology. Aspects of the project that transcended biology included many of the methods that had chemistry components.

The DeWitte-Orr lab is an integrative environment; many different projects come together for a common goal. Other students in the lab are studying the extracellular dsRNA receptors, properties of cell lines, and other fish viruses; all of these projects relate back to the present project and results from this project impact other studies in the lab as well. In addition the DeWitte-Orr lab collaborates with many different labs around Canada and the world. It is very valuable to the lab to be able to work with other groups

of scientists. Being able to integrate ideas and techniques from different areas strengthens our research and provides new directions for related studies.

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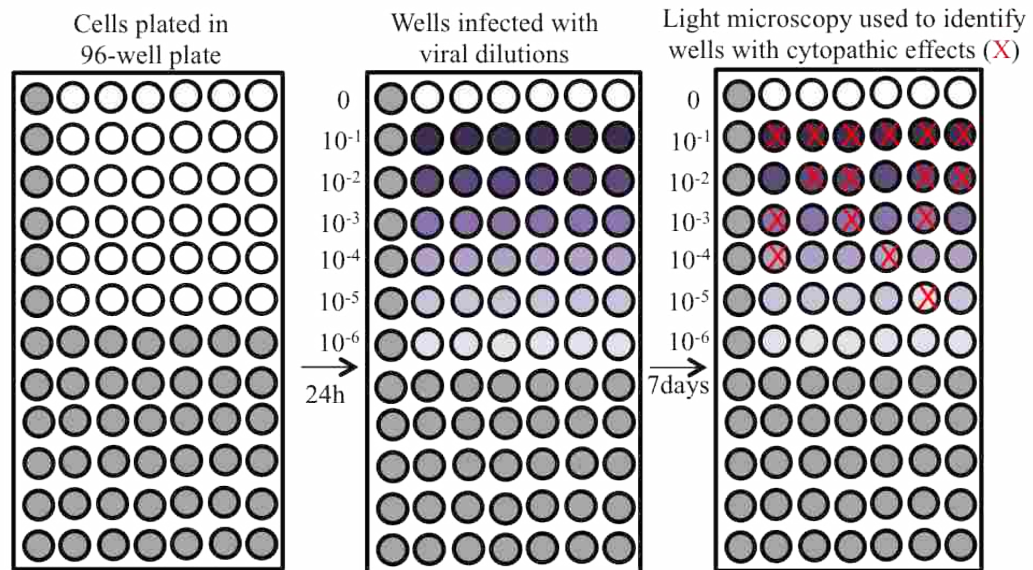
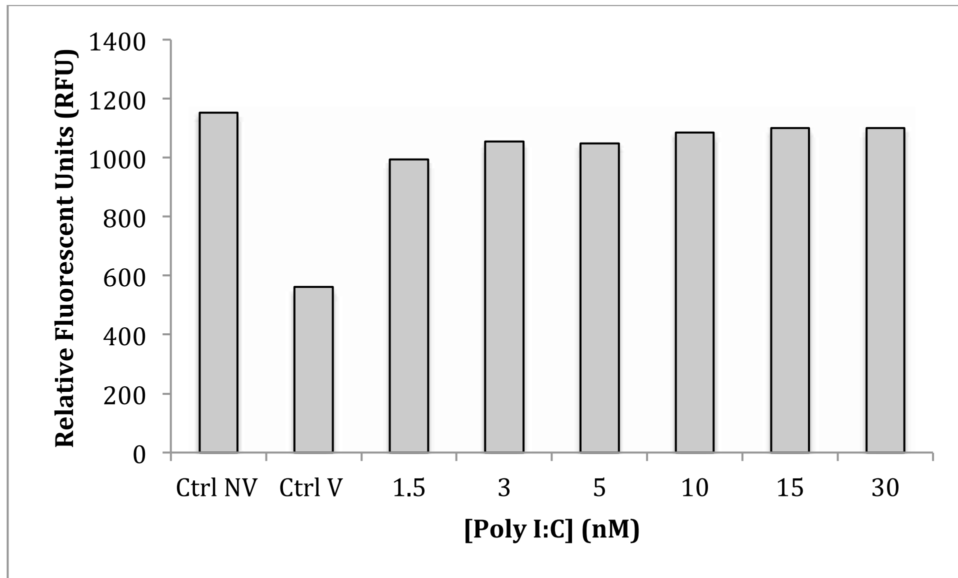
Appendix A. Supplementary Figures

A1 TCID50/mL Calculation Protocol.



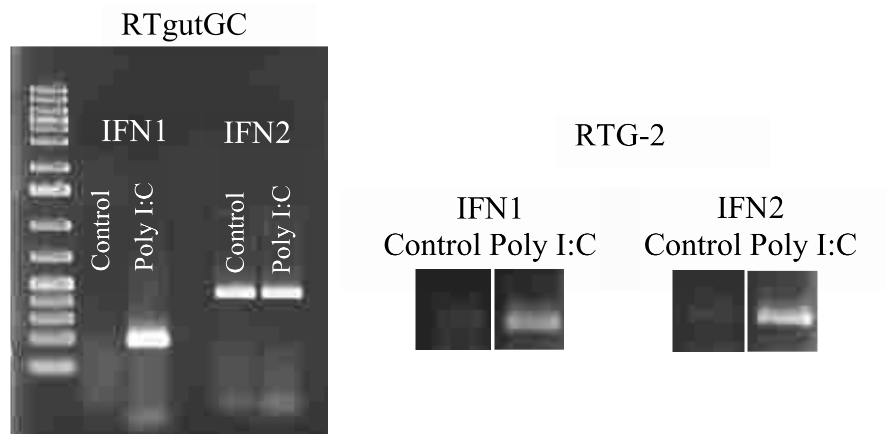
Appendix A1 Protocol for calculation of TCID50/mL for viral titres.
The cell line used to propagate the virus was plated into a 96-well plate and allowed to attach overnight. The virus stock was serially diluted 10-1- 10-6 dilutions. 100µL of virus was used to infect each well. After a 7-day incubation cells are observed and the wells with cytopathic effects are counted, in this figure cytopathic effect is denoted with a red X. The resulting numbers are used in the calculation of the TCID50 value (Reed and Muench, 1988).

A2 Determining the optimal dsRNA concentration to induce an antiviral state in RTG-2



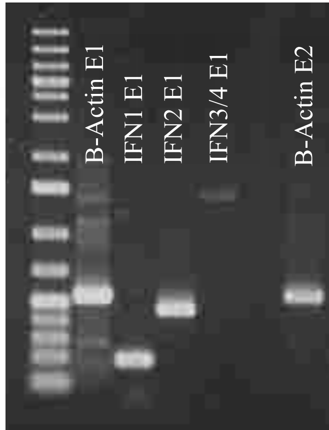
Appendix A2 Antiviral state established in RTG-2 in response to a range of poly I:C concentrations.
RTG-2 cells were stimulated with a range of concentrations of poly I:C for 24h (1.5-30 nM). And then challenged with VHSV for 7-days. Cell viability was measured using the fluorescent indicator dye CFDA-AM. Ctrl NV = uninfected control; Ctrl V = infected control not pretreated with dsRNA.

A3 Interferon expression in a rainbow trout gut cell line (RTgutGC).



Appendix A3 Interferon expression in a rainbow trout gut cell line (RTgutGC) as determined by RT-PCR.
RTgutGC cells were left untreated or stimulated with 3nM HMW poly I:C for 24h. RT-PCR was used to examine expression of IFN1 and IFN2. B-Actin (not shown) was also measured and found to be equally expressed between treated and control cells. As a comparison the RT-PCR for RTG-2 stimulated with 1.5nM HMW poly I:C for 24h was included.

A4 Interferon expression in a Chinook salmon embryo cell line (CHSE-214)



Appendix A4 Interferon expression in Chinook salmon embryo cell line (CHSE-214) as determined by RT-PCR. Rainbow trout primers for IFN1, IFN2, and IFN3/4 were used for RT-PCR with CHSE-214 cDNA. PCR products were purified using a PCR clean-up kit and sent for sequencing. The top alignment completed with NCBI's nucleotide BLAST was reported. RT = rainbow trout CS = Chinook salmon.

CHSE - IFN1

Description	Accession	Query cover	Identity
Oncorhynchus mykiss type 1 interferon (IFN1), mRNA	NM_001124531.1	99%	90%

```
CHSE IFN1  1     TACTTC-NGAAGTTG-ANAGGNAGGTTCTGannaaaaTGAACTACAGTACACANGCGNGG  58
                 ||||||  ||||||| | ||| |||||||||  ||||||||||||||| |||| ||| ||
RT IFN1    445   TACTTCAAGAAGTTGAATAGGAAGGTTCTGAGAAAAATGAACTACAGTGCACAGGCGTGG  504

           59    GAGCTCATCANGAAAGAGACGAAACGTCGTCTGCAAAGATTGGATATCCTTGCAGCACAG  118
                 |||||||||| ||||||||||||||||| |||||||||||||||||||||||||||||||
           505   GAGCTCATCAGGAAAGAGACGAAACGTCATCTGCAAAGATTGGATATCCTTGCAGCACAG  564

           119   ATGTACTGNTCATC  132
                 |||||||| |||||
           565   ATGTACTGATCATC  578
```

CHSE - IFN2

Description	Accession	Query cover	Identity
Oncorhynchus mykiss type 1 interferon 2 (IFN2 mRNA, complete cds	FJ184359.1	72%	87%

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CHSE IFN2  1     CTGGAGCAGATGGGAGGAGATATCACAAAGCAGAATGCCCCTGTCCTTTTCCCAACATCA  60
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
RT IFN2    160   CTGGAGCAGATGGGAGGAGATATCACAAAGCAGAATGCCCCTGTCCTTTTCCCAACATCA  219

           61    CTTTACAGACACATATATGATGCCGAGTTTGAGGACAAAGTCAGATTCCTGAATGAGACC  120
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||
           220   CTTTACAGACACATATATGATGCCGAGTTTGAGGACAAAGTCAGATTCCTGAACGAGACC  279

           121   ATCTATCAAATCATAAAACTGTTTGATGGGAATAGGAATACGAACTCCTGCACCTGGAAC  180
                 |||||||||||||||||||||||||||||||||||||||| ||| ||   ||||||| ||
           280   ATCTATCAAATCATAAAACTGTTTGATGGGAATAGGAATAGGAAGTCAGTCACCTGGGAC  339

           181   AAGAAGAACCAGTTCCACTTCCTTCTCATTCTCCAACTCCAATTTCATAACCTTAGTGCC  240
                 ||||| |||| |  | | |||||   ||||||| | | ||||||| | ||||||||  ||
           340   AAGAAAAACCTGGACGATTTCCTCAACATTCTCGATCGCCAATTTGAGAACCTTAGCTCC  399

           241   TGTGTTGCTGCTGCTGCG-G-AACCTAAAAAGAAACTGAACTTCTACTTCTTGAATTTGA  298
                 |||||  |  ||| | || | ||||| | |||| ||||||    ||||||  ||||||||
           400   TGTGTATCACCTG-T-CGTGAAACCTGAGAAGAGACTGAAACGGTACTTCAGGAATTTGA  457

           299   ATA  301
                 |||
           458   ATA  460
```

CHSE - IFN3/4

Alignment results:

Description	Accession	Query cover	Identity
Oncorhynchus mykiss IFN3 gene for type 1 interferon 3, exons 1-5	AM489417.1	63%	85%

```
CHSE IFN3/4 9    GGTGGNCGCTGTTTANGTTGGGGAAGCTGAACGATGTGAGCATAAACCTGCTCTCAGATA  68
    RT IFN3 76   GGTGGACGCAGTTTAGGTTGGGGAAGCTGAACGATGTGAGCATAGACCTGCTCTCAGATA  135

            69   TGGTGAGGAATTTAAACTGCACCAGTCCGCTATTTGATGTGCACAAATTGATGACCAATA  128
            136  TGGTGAGGAGTTTAAACTGCACCAGTCCGCTATTTGATGTGCACAAATTTATGACGAAAA  195

            129  TTCGCAATGATATTCANATTTCTAttttt--ttAANTATCACNTTGTGTTTTTAAGCAAT  186
            196  TTCGCAATAATATTTCTATTTCTATTTTGCATTA--TAGCTAATCTTATTTTTAAGCAAT  253

            187  ACTTTTTCTTTCTGTACCACACANTGTCATTTTTGGTTATTAGCACAATATANTTTACTT  246
            254  ACTTTCACTTTCTGTATCACAAAGTTTCATTTAAGGTTACTATCACAATATAGTTTACTT  313

            247  CTTGATATTGATATGAATTCTATTTCAGGGTGGANTCTTTCCCCTTATGTGTGCAGAAGA  306
            314  CTTGATTTTGATATGAATTCTATTTCAGGGTGGACTCTTTCCACTTATGTGTGCAGAAGA  373

            307  AAACGTCGAACAANTGTTTCNNGAGGATCNTTTN-AGAACNNAGAGGTAGGATACNCTTC  365
            374  AAACGTCGAACAAATGTTTCCAGAGGATCTTTACAAGAACACAGAGGTAGGATACACTTC  433

            366  ANNATATTGATAANCTAAAGTACAGNATGGTAAATTGGAAGTTTAACCATTTATTTAAAC  425
            434  AGTATATTGATAAACTAAAGTACAGTATGGTAAATTGGAAGTTTAACCATTTATTTAGAC  493

            426  ACCCANTGCCGTTTACCCTGTTTTATTTTATCNANCCNGAGNCCGTCTCTGTGGGTGCCT  485
            494  ACCCAATGCCTAAAACCCTGTTTTATTTTATCCAGGGTGAGGACGTCTCTGTGGTTGCAT  553

            486  TGGNNTCTATGCTATATGTGGAACAANNATATNACAACANAACTGACATCTGTCAACTGT  545
            554  TGGAGGCTATGCGATATGTGGACCAATTATATAACAACAGT-CTGACGTCTGTCACGTGG  612

            546  AACAANGTAAANCTTAACATGTTCCAAAAACGTCATATATCGTCAAGTTCAACACTTANA  605
            613  AACAAAATAAAACTTAACATGTTCC-AAAACGTCATATATCGTCAAGTTCAAAACTTAGA  671

            606  GTTATGTGTAAATATGATCCCTCNTGTN-AGTCTAT  640
            672  GTTATGTGTAAGTACGATCCCTCTTGTGTAGTCTAT  707
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Appendix B. Standard Operating Procedures

B1 RT-PCR – RNA extraction and cDNA synthesis

RNA Extraction

1. Aspirate media from flask using Pasteur pipette. For wells remove media by inverting the plate.
2. Add 400µL (for a 75cm^2 flask) of TRIzol reagent. 200µL for a 25cm^2 flask. 100µL for a well in a 6-well plate. *All subsequent reagent values are for 400µL TRIzol, adjust volumes as necessary if starting with less TRIzol.*
3. Spread TRIzol around flask until entire growing surface is coated. Reagent will turn a milky-pink color.
4. Use cell scraper to scrape down sides of the flask, scrape in the same direction.
5. Transfer solution to 1.5mL Eppendorf tube.

This stage can be stored at -80°C if necessary.

6. Add 80µl chloroform to tube and shake vigorously for 15s. Add chloroform in the fume hood.
7. Incubate at room temperature for 3min.
8. Centrifuge at max speed for 15min at 4°C.
9. At this stage there should be a clear phase (RNA), white layer (DNA) and pink phase (protein and cell debris). Remove clear top layer, transfer to new Eppendorf tube. White and pink layer need to be disposed of in a appropriate waste container.
10. To precipitate RNA add 200µl 100% isopropanol, shake for 15s.
11. Incubate at room temperature for 10 min.

12. Centrifuge at max speed for 10 min at 4^oC. A very small white pellet will form.
13. Carefully remove supernatant and discard.
14. To wash the pellet add 400µl 80% ethanol. Do not re-suspend pellet.
15. Centrifuge at max speed for 5min at 4^oC.
16. Remove supernatant and discard.
17. Invert tube and allow pellet to air dry over for approximately 10min.
18. Add 10µl DNA quality H_2O, re-suspend pellet.
19. Incubate at 55^oC for 10min.
20. Store at -80^oC.

cDNA Synthesis

1. Combine 2µg RNA (quantified using Nanodrop spectrophotometer) with 1µL primer (Oligo-dT_{23} or random hexamer). Add DNA quality H_2O to a final volume of 5µL.
2. Using thermocycler, heat at 70^oC for 5min, place in ice for at least 5min.
3. Centrifuge briefly to prevent loss of solution.
4. To the RNA and primer mixture add a reaction mixture containing:

    4µL GoScript 5x Buffer

    1.2µL $MgCl_2$

    1µL GoScript Reverse Transcriptase

    1µL 10mM each dNTP mixture

    7.8µL DNA quality H_2O

5. Mix thoroughly. Heat at 25^oC for 5min, followed by 42^oC for 1h.

6. Dilute cDNA as desired and store cDNA at 4°C for short term or -20°C for longer periods.

B2 Functional interferon assay

Used to identify functional interferon present in media of stimulated cells. RTgutGC was used as source of interferon however other cell lines could be used. Cell density may need to be optimized for different cell lines.

1. Plate RTgutGC cells at a density of $7x10^5$ cells/well in a 6-well plate. One well for each treatment and one for a control.
2. Allow to attach overnight.
3. Aspirate media. Treat cells with 3nM of treatment, or fresh media for control.
4. Incubate at room temperature for 24h.
5. On same day as step 3 plate $3x10^4$ CHSE-214 cells/well into 96-well plate. 6-wells/treatment, 6-wells for control, and 6-wells for a non-infected control.
6. Collect media from RTgutGC in microcentrifuge tubes. Centrifuge at 10xg for 5min.
7. Remove media from 96-well plate, treat appropriate wells with 50µl of conditioned or control media.
8. Incubate at room temperature for 24h.
9. Remove media from 96-well plate, rinse cells with 100µL PBS.
10. To all wells but no-virus control add 70µl 10^{-1} dilution of CSV made in 1%FBS media. Add 1% FBS media to the control wells.
11. Incubate at 17°C for 5 days.
12. Measure cell viability using CFDA-AM.

B3 RTG-P1 luciferase reporter assay

An assay for Mx1-promoter activity within RTG-P1 cells. Steady-Glo reagent creates bubbles readily, take care when pipetting the reagent.

1. Plate $5x10^5$ RTG-P1 cells into wells of 6-well plate, 3 wells for control and 3 wells/treatment.
2. Allow overnight to attach. Observe under light microscope to ensure all cells are attached and not over confluent.
3. Remove media, either by aspiration or inverting the plate. Treat cells with 500µl/well of 3nM treatment media or control media, 10% FBS for treatment and control.
4. Incubate at room temperature overnight.
5. Remove media, either by aspiration or inverting the plate. Dissociate cells using 250µL trypLE. Once all cells are detached add 1mL media and transfer mixture to 1.5mL Eppendorf tube.
6. Pellet cells by centrifugation at 1400xg for 5min. Carefully remove supernatant.
7. Add 100µL reconstituted Steady-Glo luciferase substrate to each tube. Steady-Glo substrate is very bubbly so take extra care not to introduce bubbles. Resuspend pellet and break up clumps by pipetting up and down gently.
8. Incubate tubes in the dark for ~40min.
9. Plate 90µL of each sample/substrate into wells of a 96-well plate. 90µL accounts for loss of volume due to bubbles.
10. Read using BioTek Synergy HT plate reader programmed for luminescence. A plug is used in place of a filter.

B4 Antiviral assay

Used to characterize antiviral activity of a molecule of interest. Concentration of treatment was optimized for dsRNA.

1. Plate $3x10^4$ RTG-2 cells/well into appropriate number of wells in 96-well plate. 6-wells for each treatment, 6-wells for an infected control, and 6-wells for an uninfected control.
2. Allow overnight to attach. Observe under light microscope to ensure all cells are attached and not over confluent.
3. Remove media, either by aspiration or inverting the plate. Add 50µl 3nM treatment or control media to each well, use media with 10% FBS for treatment and control.
4. Incubate at room temperature for 24h. Check cells after incubation to ensure they are still attached and have not come off at any point in the treatment.
5. Remove media, either by aspiration or inverting the plate. Infect all wells except uninfected control with 70µl of a 10^{-1} dilution of VHSV in 1% FBS media. Add 1% FBS media without virus to the control.
6. Incubate at 17°C for 7-days. Monitor for cytopathic effects. If significant cell death is not observed in untreated infected control consider extending incubation period.
7. Measure cell viability using CFDA-AM.

B5 CFDA-AM cell viability assay

Used to measure cell viability as an endpoint for many different experiments. Can be scaled up from 96-well plate. CFDA-AM is a light sensitive.

1. Remove media from wells by inverting the plate. Rinse with 100µl PBS/well.
2. Make CFDA-AM: 10mL PBS and 10.4µL 4nM CFDA-AM. (Solution mixture is for an entire 96-well plate, scale down solution if for fewer wells).
3. Add 100µl of CFDA-AM solution to each well. Plate an extra row of solution to use as a blank.
4. Incubate the plate in the dark for 1h.
5. Using the BioTek Synergy HT plate reader and Gen 5 1.11 software read the plate at Ex485 Em530.