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INVESTIGATING THE ANTIVIRAL EFFECTS OF VIG-3 FROM RAINBOW TROUT

by

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Honours BSc Biology, Wilfrid Laurier University, 2019

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

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Abstract

Rainbow trout is the most farmed fish in Ontario, and thus is economically important to the province. Despite this, there is a lack of understanding regarding fish innate immunity, specifically with regards to interferon-stimulated genes (ISGs) and their antiviral effector functions. ISGs are the workhorses of the innate antiviral response, operating together to limit each step of virus replication. The Viral Hemorrhagic Septicemia Virus (VHSV) induced gene (Vig)-3 is a newly identified ISG within many fish species and is homologous to ISG-15 in mammals. It is a small ubiquitin-like protein inducible by type I interferon (IFN-I), and is suggested to have antiviral effects within the cell. Vig-3 has been proposed to establish an antiviral response by acting both intracellularly through covalent modification of proteins, as well as extracellularly as a signaling molecule. It is for these reasons it was investigated in Rainbow trout. To do this, Rainbow trout gonadal cells (RTG-2) were infected with two fish viruses (infectious pancreatic necrosis virus; IPNV and VHSV), as well as treated with poly I:C, and the expression of vig-3 was monitored over 24- and 48h periods at the transcript, protein, and cellular level. The transcript level of expression was analyzed via quantitative real time polymerase chain reaction (q-RT-PCR) and demonstrated that *vig-3* expression was induced during treatment with VHSV, IPNV and poly I:C. Western blot analysis was used to analyze protein expression of Vig-3 during infection with the same viruses and treatment with poly I:C. It was found that during poly I:C treatment and viral infection Vig-3 protein expression was induced from 6h to 48h. It was also found that Vig-3 was able to bind to target proteins in a process known as ISGylation. Immunocytochemistry was used to determine the cellular expression of Vig-3 during viral infection with IPNV and VHSV and treatment with poly I:C. In this case it was determined that Vig-3 was upregulated at both 12h and 24h during all treatments, as well as is both localized to the cytoplasm and nucleus. These findings contribute to a better understanding of a poorly studied aspect of innate antiviral immunity in an economically valuable fish species.

Acknowledgements

If anyone knows the few years, I have had during my master's degree one may be shocked I am finally completing it. I would like to first thank my family for their support over these two years. I would like to acknowledge my girlfriend Samantha for absolutely everything she does, from bringing me coffee on busy days to watching Netflix in the research building while I wait for my gels to run, thank you for always supporting me and motivating me to be the best scientist I can be. Thank you for your encouragement and supporting me when I put school first. It means for than I can explain, and I cannot thank you enough. I cannot wait for what's next in store for us. Thank you to all my friends who know exactly when to give me a hug or give me a beer. Shout out to Alysha Burnett, Louis Pfeifer, Alex Anderson, James Bannister and Katarina Mandic for ALL your support. I have gotten to know each and every one of you in each special way. You have all had such a huge influence on not only my research, but my personal life and I thank you so much for putting up with all my grad office rants. And you are very welcome for keeping you laughing. I cannot forget to thank my dog Olive, as the crazy dog mom I am. Thank you for loving me as much as you do through the busy times. I don't know what I would have done without your wagging tail and puppy kisses through all the ups and downs that were my Masters.

Thank you to the staff at Wilfrid Laurier for keeping up a safe and clean working environment. And a special thank you to all my committee members, especially Dr. Alison Mcdonald for getting on board so last minute.

Thank you to all the present and past members of the DeWitte-Orr lab. You have all helped me through my research struggles and for that I thank you. A special thank you to Dr. Sarah Poynter for kick-starting this project for me and completing some of the tedious preliminary experiments. Most importantly I thank you, Dr. Stephanie DeWitte-Orr for not giving up on me. As I said these years have probably been two of the hardest of my life so far, and there is not a single person I can

thank more for helping me through than Dr. D. Thank you for encouraging me when I thought there was no hope left, and thank you for believing in me when I didn't even believe in myself. I don't know what I would have done without you. You truly are one in a million, and I strive to one day grow into half the intelligent, witty, successful & super bad-ass woman that is you. Thank you.

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List of Abbreviations

CFDA: 5-Carboxyfluorescein Diacetate Acetoxymethyl Ester

CMV14: Cytomegalovirus-14

DNA: deoxyribonucleic acid

dsRNA: double-stranded (ds) ribonucleic acid (RNA)

EPC: Epithelioma papulosum cyprini

EtBr: ethidium bromide

FBS: fetal bovine serum

GFP: green fluorescent protein

HRP: horseradish peroxidase

ICC: immunocytochemistry

IF: immunofluorescence

IFN: type I interferon

IFNAR: type I IFN-α/β receptor

IPN: Infectious Pancreatic Necrosis

IPNV: infectious pancreatic necrosis virus

ISG: IFN stimulated gene

ISGF3; interferon stimulated gene-factor 3

ISRE: interferon sensitive response element

JAK: Janus kinase

JAK-STAT: Janus Kinase/Signal Transducers and Activators of Transcription

LPS: lipopolysaccharide

MDA5: melanoma differentiation-associated protein 5

MOI: multiplicity of infection

NF-kB: nuclear factor kappa-B

OAS: 2’,5’-oligoadenylate synthetase (OAS)

PAGE: polyacrylamide gel electrophoresis

PAMP: pathogen associated molecular pattern

pi: post infection

POLY I:C: Polyinosinic:polycytidylic acid

PKR: Protein Kinase R

PRR: Pattern Recognition Receptor

PRR: pattern recognition receptor

P/S: penicillin/streptomycin

PVDF: polyvinylidene fluoride

qRT-PCR: quantitative reverse-transcription polymerase chain reaction

RIPA buffer: radioimmunoprecipitation assay buffer

RTG2: rainbow trout gonadal cell line

RTgill-W1: rainbow trout gill cell line

RTgutGC: rainbow trout gut cell line

RNA: ribonucleic acid

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

STAT: signal transducer and activator of transcription

TCID50/mL: 50% tissue culture infective dose per milliliter

TBS: tris buffered saline

VHS: Viral Hemorrhagic Septicemia

VHSV: viral hemorrhagic septicemia virus

VIG: viral hemorrhagic septicemia virus-induced gene

4. Introduction

4.1 Rainbow trout

Rainbow trout (*Oncorhynchus mykiss*) is a species native to North America and is distributed across all continents, therefore the species is highly adaptive to different climates and habitats[25,7]. The species was introduced to the Great Lakes in the 1800s and continues to play a crucial role in aquatic ecosystems[25,21]. Rainbow trout are at the top of their food chain and therefore are ecologically relevant to their environment[55,66]. Rainbow trout are also of major economic importance to Ontario, as they are the main freshwater fish being cultured[55,41]. To put this into perspective, over the last five years an annual average of 7000 tons of Rainbow trout was produced in Canada, 55% of this amount was from Ontario[,41]. Globally fish farming is on the rise, however this creates new opportunities for emerging viral pathogens and rapid spread of disease[25]. To this end, there are no treatments for viral infection in Rainbow trout, and the method of delivery for treatments in natural aquatic environments remains unknown[25]. Therefore, it is crucial that the immune system of this valuable species to Ontario is fully understood.

4.2 Important fish viruses

As mentioned, fish farming has exploded globally over the past two decades making the industry essential to Canada's economy[3,16.] Annually, fisheries employ approximately 200 million people world-wide, generating a net income of $80 billion[16,3]. Furthermore, 10% of the world's population relies on the aquaculture industry for their livelihood[16,3]. Therefore, aquaculture not only contributes as a food source for humans but also as a source of employment for many people. The increase in fisheries has allowed emerging viral pathogens to make their way into fish farms of different areas of the world where they would not normally be present[3,16]. Currently, viral diseases are the limiting factor to aquaculture production and biodiversity within ecosystems[3,16]. It is for this reason that the study and understanding of fish viruses is important. Two emerging pathogens of

Rainbow trout include infectious pancreatic necrosis virus (IPNV) and viral hemorrhagic septicemia virus (VHSV), both present in the Great Lakes of Ontario[8].

4.2.1 Infectious Pancreatic Necrosis Virus (IPNV)

IPNV is the etiological agent of infectious pancreatic necrosis (IPN), an important disease in the salmonid fish farming industry[3,8]. This virus has a non-enveloped bi-segmented dsRNA genome and belongs to the *Birniviradae* virus family[3,8]. IPNV has a wide host range, mainly infecting young salmonids such as trout or salmon, however adult fish may be carriers without showing symptoms[3,8]. These sub-clinical carriers can shed virus in their feces or urine that contributes to viral transmission, which is problematic in aquatic environment[9,8]. The broad host range of this virus has hindered advances in limiting its infectivity[9,8]. Using IPNV to investigate the immune system of Rainbow trout will not only shed light on important immune genes for this species, but also could give insight into the diversity of the virus, offering opportunities to protect the species further.

4.2.2 Viral Hemorrhagic Septicemia Virus (VHSV)

VHSV is a negative-sense single-stranded (ss)RNA virus of the *Rhabdoviridae* virus family and is the causative agent of viral hemorrhagic septicemia (VHS) [9,8]. The virus inhabits both the Atlantic and Pacific Oceans, as well as the Great Lakes [9,8]. To this end, four genotypes (I-IV) have been identified and many sub-species, including the IVb strain, which has been confirmed in the Ontario Great Lakes[3,8]. This virus is responsible for great losses in aquaculture, affecting not only salmonids but many other marine fish and aquatic species[9,8]. The host range for the species continues to grow, exceeding approximately 50 species to date[9,8]. This presents a difficulty for disease control programs[9,8]. It is for this reason that it is important to study antiviral genes induced by this virus,

specifically in Rainbow trout, to protect such a significant species to Ontario. To begin discussion of antiviral genes, fish innate immunity must first be explained.

4.3 Fish Innate Immunity

Innate immunity is the first line of defense against pathogen infection in fish[46,47,11]. The innate immune response in fish is triggered by host-derived pattern recognition receptors (PRRs) sensing pathogen associated molecular patterns (PAMPs) produced by the invading pathogen[47,48,11]. PRR activation triggers signalling cascades within the cell that activates a transcription factor, interferon regulatory factor (IRF)-3, which controls the production of type I interferons (IFN-I)[47,48,11]. The goal of IFNs is to establish an antiviral state, making cells refractive to virus infectivity and replication[47,48,11]. Once IFNs are secreted from the cell, they act on other cells by binding to their cognate receptor (IFNAR) in an autocrine or paracrine fashion, triggering the Janus-kinase – signal transduction and activator of transcription (JAK-STAT) signaling pathway[47,48,11]. Following this signalling, the transcription factor interferon-stimulated gene (ISG)-factor 3 is formed (STAT1/STAT2/IRF-9), which then binds to the IFN-stimulated regulatory element (ISRE) of numerous ISGs inducing the expression of many important antiviral genes[47,48,11]. A schematic of this process is outlined in Figure 1. As the masters of the innate antiviral response, ISGs work together to limit every step of a virus's replication and infectivity[47,48,11]. Important fish ISGs include the Mxs, PKR and Vig-3, also known as ISG-15 in mammals[47,48,2].

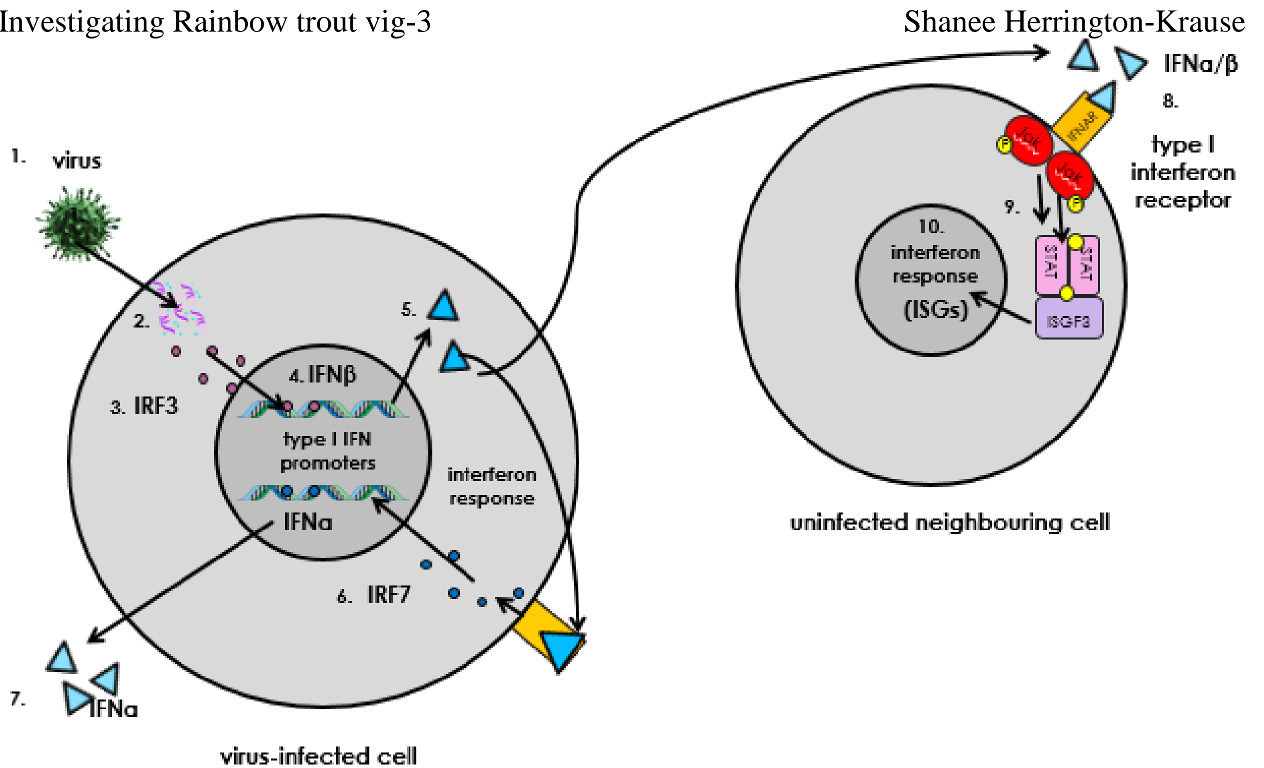

Figure 1: Schematic diagram of the innate immune system in mammals. The innate immune system is the first line of defense against viral infections. **1.** When a virus infects a cell, it enters and begins replicating. **2.** By-products of virus replication are PAMPs and are sensed by the cell's PRRs **3**. The sensing of the viral PAMPs triggers a signaling cascade resulting in the activation of interferon regulator factor-3 (IRF3). **4.** This up-regulates expression of interferon-beta (IFN-β), which will be **5.** secreted from the cell and act in both an autocrine and a paracrine fashion. **6.** IFN-β will bind to its cognate receptor on the surface of a cell and initiate the activation of interferon regulatory factor-7 (IRF-7), which will enter the nucleus to initiate expression of interferon-alpha (IFN-α).**7.** IFN- α will then be secreted from the cell and can also act in an autocrine or paracrine fashion to warn neighbouring cells of viral attack. **8.** IFN- β can also initiate a signalling cascade that results in the production of interferon-stimulated genes (ISGs). This occurs through the binding of IFN-α/β binding to their cognate receptor IFNAR, **9.** triggering the phosphorylation and signaling of the JAK-STAT pathway, **10.** which culminates in a series of enzymatic reaction, ultimately leading to the transcription of ISGs.

4.4 Vig-3

Vig-3 is a recently discovered ISG in Rainbow trout, homologous to ISG-15 in mammals and other fish species[2]. It is a small gene (~762bp) encoding a putative small ubiquitin-like modifying (SUMO) protein (15kDa) composed of two ubiquitin-like domains (UBL), connected by a short linker sequence, and a conserved C-terminal RLRGG motif[2,13,51,57,48](Figure 2). This conserved RLRGG domain is required for conjugation to other proteins in mammalian studies in a process known as ISGylation[2,13,51,57,48]. ISG-15 has also been proven to be inducible by type I interferon (IFN)[57]- the master switch of innate antiviral immune responses. This finding suggests Vig-3 could have antiviral effects within the cell. Even though the mammalian homologue of Vig-3, ISG-15, has shown antiviral activities and mechanisms, such as ISG-15's ability to conjugate to host and viral proteins and act on neighbouring cells[31], there is little to no understanding of its actions in fish. Mammalian and fish ISGs are often homologous and display similar antiviral activities. Such ISGs include the myxovirus resistance genes (Mxs) and protein kinase R (PKR)[2], therefore, it is not implausible to anticipate the same for ISG-15. It is for these reasons that the antiviral activation and activity of Vig-3 was investigated, as it could hold promise to the future of fish innate immunity.

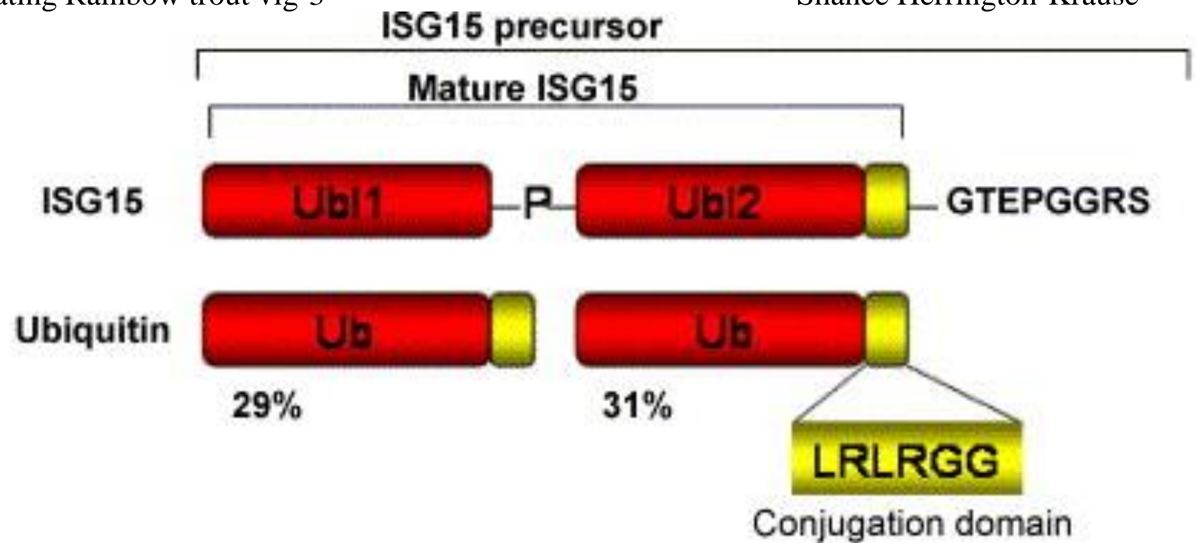

Figure 2: Schematic representation of ISG-15 in mammals in comparison to ubiquitin adapted from Andersen *et al*[1]. Note the LRLRGG domain on the C terminus is conserved between the two proteins. In mammals, this domain has been identified to be required for ISGylation- the covalent conjugation of ISG-15 to its target protein.

4.5 Induction & Expression of vig-3

As mentioned previously, when stimulated with stressful stimuli, fish cells respond via the innate immune system and the production of IFNs, culminating in the production of ISGs[1,2]. In mammalian studies, ISG-15 induction was found to be the result of interferon stimulated gene factor three (ISGF-3) binding to the interferon stimulatory response element (ISRE) of ISG-15, culminating in the expression of ISG-15[10,32]. This coincides with evidence that mammalian ISG-15 expression is directly influenced by IFN. ISRE was also found to contain a putative response element in the promotor of Zebrafish ISG-15[10]. This suggests that the mechanism of regulation of ISG-15 could also be conserved in fish. Despite this, the antiviral role and mechanism of regulation of fish ISG-15 has not yet been fully elucidated. To initiate the IFN response in fish cells, there must be a stressful stimulus present[1,2]. Stimuli include dsRNA (a viral PAMP), viruses and bacteria[15]. This section will discuss ISG-15 expression levels *in vitro* either constitutive expression or expression levels when prompted with various stressful stimuli.

4.6 Constitutive Vig-3 Expression

Healthy cells often express ISGs at low levels to be prepared for a potential viral attack[2]. In addition, many ISGs are produced in inactive forms and remain in this form until required, i.e. when a virus infects the cell[2]. This was found to be the case for ISG-15 in unstimulated rat hepatocytes, Chinese hamster ovary cells and human embryonic kidney cell lines analyzed via immunoblot analysis[12], which all showed low levels of endogenous ISG-15 expression. Similarly, ISG-15 is often transcribed in its inactive form, with an extra motif on the C terminus. This motif is required to be cleaved to reveal the active form of ISG-15[10]. This can be visualized in Figure 2 as the GETPGGRS domain. To this end, there have not been studies discussing endogenous ISG-15 expression in fish, however there are few studies investigating induced expression of ISG-15 via various stimuli, which are described below.

4.7 Double stranded RNA (dsRNA)

Double-stranded RNA (dsRNA) is a PAMP and potent inducer of the innate immune response in fish[1]. In a natural system, dsRNA sensed by a cell would be the product of a viral infection; as during replication, viruses produce genomic fragments and intermediates, which are sensed by the host cell[47,48]. These dsRNA molecules are differentiated from endogenous dsRNA molecules based on sequence, length, molecular modifications and localization, as these dsRNA molecules are foreign to a healthy cell[47,48]. A host cell senses dsRNA via different sensors depending on the location of dsRNA within the cell[47,48]. These range from toll-like receptors (TLRs) within the endosome, and various cytoplasmic DNA sensors (CDSs) such as RIG-I/MDA5[47,48]. Sensor activation triggers a signaling cascade via multiple adaptor proteins[47,48]. These adapter proteins include IFN-β promoter stimulator 1 (IPS-1) or TIR-domain-containing adapter-inducing interferon-β (TRIF), which activates transcription factors, such as interferon regulatory factor (IRF) 3/7, and ultimately lead to the production of IFNs[47,48]. As described above, once IFNs are secreted, they can

act in both an autocrine and paracrine fashion via their IFN-α/β receptor, initiating the JAK-STAT pathway, culminating in the expression of various ISGs[47,48]. This can be visualized in Figure 1. Even though dsRNA is a very potent inducer of the immune response, and therefore holds promise for antiviral therapies, it is difficult to collect virus-derived dsRNA in quantities large enough for *in vivo* or *in vitro* experiments[47,48]. Polyinosinic:polycytidylic acid (poly I:C) is a synthetic analogue of viral dsRNA and has been identified as a potent inducer of IFN in both mammalian and fish studies[1,2,39]. It is also incapable of evading the host immune response, which is a key strategy exploited by viruses for prolonging their infectivity[17]. It is for this reason that poly I:C has been used in many studies as a stimulant of ISG-15 to determine the expression pattern of the protein. It has been shown in teleost fish species that poly I:C induces a rapid and strong up-regulation of *ISG-15*. When gilthead seabream fibroblasts were treated with poly I:C over a 24 h period and the expression of *ISG-15* was measured at the transcript level, a 300-fold increase was observed[2]. Similarly, sea bass brain and spleen cells following similar treatment showed a 25-fold induction after 6 h[36]. Lastly, Atlantic salmon head kidney cells treated with poly I:C appeared to exhibit induced ISG-15 expression at the protein level as early as 6 h via Western blot analysis[54]. An interesting finding was that the expression of ISG-15 in teleost fish species stimulated with poly I:C varies over time. For example, Atlantic salmon head kidney cells treated with poly I:C over a 48h period showed induction as early as 6h, with the highest level in induction at 36h and a noticeable decrease in expression at 48h[54]. This finding is interesting as fish IFN-I works in a cyclic expression pattern over the course of induction[2], therefore this difference in *ISG-15* induced expression could be a result of cyclic IFN-I and the ISG following induction from the cytokine. These findings support previous findings in mammalian studies suggesting that ISG-15 is in fact inducible by dsRNA, and sheds light to the hypothesis of the ISG being IFN inducible. It is obvious that there are several studies elucidating dsRNA-induced antiviral mechanisms in fish; however, most studies focus on

identification of antiviral proteins, while functional or mechanistic insight for many of these antiviral pathways remain elusive. This is particularly the case for Vig-3.

4.8 Viruses

Although poly I:C treatment provides a clear understanding of ISG-15 induction, it is important to also investigate the differences in ISG-15 induction in response to viruses, which as mentioned, may have mechanisms to evade the host. This model also provides a better understanding of ISG-15 in a natural system. Viruses remain a target of IFN-induced antiviral proteins therefore many viruses adapt mechanisms to evade or to suppress host interferon responses[15]. Since the IFN response is the first line of defense of a cell on virus infection, it is common for viruses to evolve mechanisms of evasion for this pathway. Indeed, both viruses used in this study have immune evasion capabilities. Recently it was found that the NV protein of VHSV can suppress the type I IFN response in Epithelioma papulosum cyprini (EPC) cells[26,15,15], although the mechanism of action has yet to be elucidated. Additionally, IPNV infection in chinook salmon embryo cells (CHSE-214) inhibited IFN-induced Mx promoter activity therefore having an antagonistic effect on innate antiviral responses[56]. Thus, it is reasonable to hypothesize that VHSV and IPNV could manipulate ISG-15 expression. Studies to date demonstrate that immune evasion mechanisms for viruses, related to ISG-15, exist in fish cells. For example, *ISG-15* was observed to be induced most extensively at 12h post infection (pi) in gilthead seabream fibroblast cells treated with VHSV over a 48h period[2]. This induction was seen later than the treatment with poly I:C, which coincides with the previous hypothesis of IFN evasion strategies of VHSV; the virus may be shutting down IFN. In contrast, sea bass head kidney cells treated with red-spotted grouper nervous necrosis virus, a *Betanodavirus*, showed a significant downregulation of *ISG-15* expression at 24h pi, however expression was slightly upregulated from 48h onwards[36]. This difference in expression between viruses could be the result of different immune evasion mechanism strategies of different virus families, or perhaps could suggest that ISG-15 has both stimulatory and inhibitory effects during

different infections. For example, ISGylation of target proteins during hepatitis C virus (HCV) infection in human liver tissues promoted viral production, showing evidence of a stimulatory effect[11]. On the other hand, ISGylation of Ebola virus VP40 matrix protein was shown to inhibit viral budding from mammalian cells, indicating an inhibitory effect[43]. This could also suggest that ISG-15 may have a broad range of mechanisms of activation and activity, which will be discussed later. Although the scope of ISG-15 research is centered around viral infections and antiviral activities, it is important to note other stimuli that can induce ISG-15.

4.9 Other stressful stimuli

Although the focus of ISG-15 research has been on its antiviral activity and induction via dsRNA and viruses, its expression has also been found to be induced in many cell types by bacterial endotoxins[15]. Mammalian studies have observed ISG-15 induction during lipopolysaccharide (LPS) stimulation- a part of the outer membrane of Gram-negative bacteria[49]. For example, when treating rat microglia with LPS, a significant increase in ISG-15 expression was observed at the protein level 6h post treatment[49]. Similarly, experimental challenges with LPS displayed significant ISG-15 expression in red drum kidney cells as well as crucian carp liver cells[26,15]. Interestingly, it was also found that *ISG-15* was the most highly induced ISG expressed during *Mycobacterium tuberculosis* infection in *in vivo* mice studies, and several teleost fish species infected with *Mycobacterium* have also shown an increase in *ISG-15* expression[28,6]. However, to this end the mechanism by which ISG-15 is being induced in response to these bacteria and bacterial endotoxin is not fully understood. These findings suggest that ISG-15 may play a much larger role in innate immunity than simply an antiviral function, as previously suggested, and further supports the hypothesis of conserved function of ISG-15 between mammals and fish. It is obvious that ISG-15 has a broad range of induction parameters, and therefore is likely that the protein may be able to be activated via various mechanisms. Mammalian studies have discussed both an intra- and extracellular role for ISG-15 and

possible activation mechanisms including ISGylation, as mentioned, and cytokine-like activity. The next section will discuss activation of ISG-15 in both multiple cellular compartments based on mammalian studies.

4.10 ISG-15 Activity Effects

As mentioned previously, ISG-15 belongs to the family of ubiquitin-like modifiers (UBLs), resembling a tandem orientation of two ubiquitin domains[57]. Multiple studies have shown that ISG-15 is able to modify cellular proteins by conjugating its C-terminal glycine residues to lysine residue side-chain amino groups of various target proteins[59]. However, studies have also demonstrated that ISG-15 is able to exert various roles as a free intracellular molecule and can even be secreted thus functioning as a cytokine[59]. The proposed mechanisms by which ISG-15 acts can be found in Figure 3.

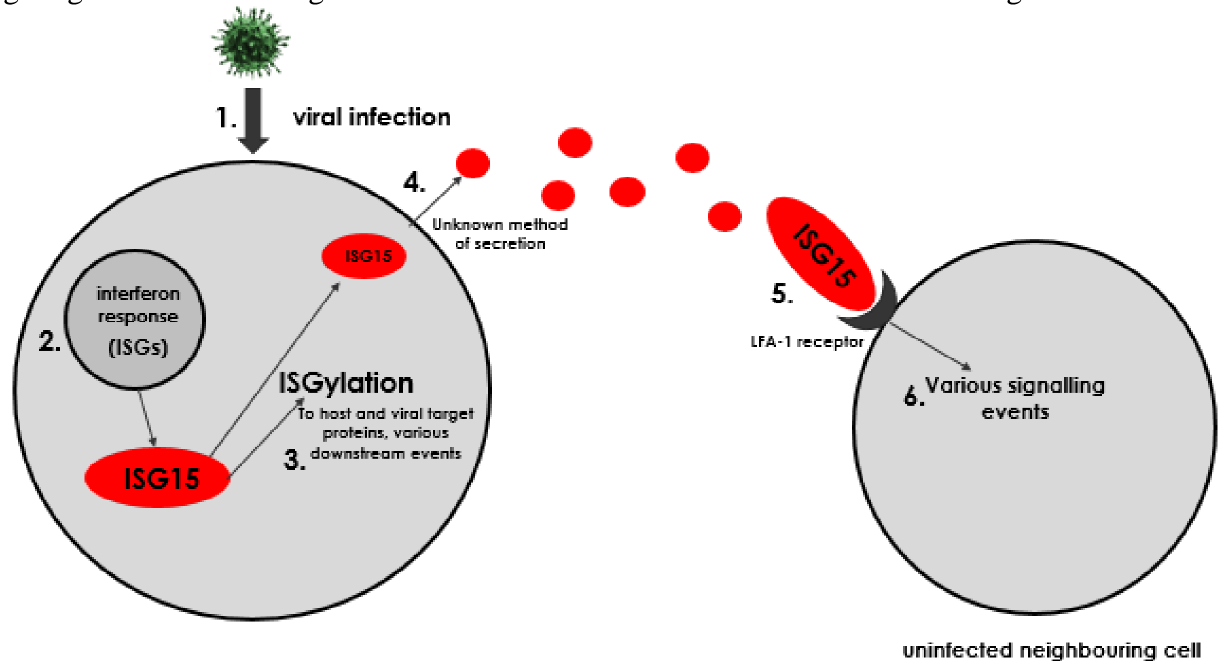

Figure 3: Proposed antiviral mechanism of vig-3 based on mammalian ISG-15. Although there is a lack of research regarding the mechanism of vig-3 in Rainbow trout, hypothesized mechanisms have been proposed based on the activities of ISG-15 in mammals. 1. Once a cell is infected with a virus and the interferon response is initiated (2), ISG-15 will be proteolytically cleaved into its active form, however the enzyme responsible for this is not known. From there, it is proposed that ISG-15 can *A.* act intracellularly, conjugating itself to a broad range of proteins with various downstream effects (3), or *B.* Exit the cell via an unknown method of secretion (4). 5. Once extracellular ISG-15 can bind to its cognate receptor (LFA-1 receptor) on uninfected neighbouring cells and exert various downstream effects (6).

4.11 Intracellular Activation: ISGylation

In mammals, ISG-15 can form conjugates with a wide range of target proteins in a process referred to as ISGylation[63,57]. ISGylation occurs via a step-by-step enzymatic process involving similar enzymes to the ubiquitin conjugation pathway[63,57]. In a natural system, ISG-15 is encoded as a 17-kDa precursor protein that is proteolytically cleaved at the start of a viral infection[2]. This cleavage process occurs at its C-terminus exposing a LRLRGG domain, identical to the C-terminus of ubiquitin[2,63] The processing enzyme, a specific unnamed protease[46], cleaves pro-ISG-15 specifically at the Gly^{157}-Gly^{158} peptide bond, generating the mature ISG-15 carboxyl terminus[2,63,46] (Figure 2). This domain has been demonstrated in mammals and fish to be required for binding[23,57,12]. The process of ISGylation begins with the cognate ISG-15 E1 activating enzyme, UbE1L, which

initiates the pathway by forming a thioester bond between the C-terminal glycine of ISG-15 and a cysteine residue of UbE1L[23,57,12]. This priming of ISG-15 allows it to be transferred to a cysteine residue of the E2 conjugating enzyme, UbcH8[23,57,12]. From here, the E3 ligase enzyme assists in the conjugation of ISG-15 to lysine residues on target proteins[23,57,12]. Herc5 and TRIM25 have both been identified as potential E3 ligases for ISG-15 in mammalian studies [23,57,12]. ISG-15's deconjugation from proteins is mediated by an ISG-15 specific protease called USP18[23,57,12]. A schematic of this process can be visualized in Figure 4. It has been found that the ISG-15 conjugating and deconjugating enzymes are strongly induced by IFN, like ISG-15 expression[23,57,12]. Remarkably, mammalian studies have also demonstrated that ISGylation of IRF3 is able to block the degradation of IRF3, furthermore enhancing continual IFN activation in a positive feedback loop during viral infection[23,57,12]. Studies have shown that ISGylation is non-specific, as the ISG-15+E3 ligase complex can bind to a wide variety of proteins, ranging from both host and viral[12]. It was also discovered that ISGylation targets newly synthesized proteins and occurs mainly within the ribosome inside the rough endoplasmic reticulum of a cell[12], as there is a prevalence of newly synthesized proteins in this area. Proteomic studies have identified numerous ISG-15 target proteins which are involved in various biological systems[12]. A recent study identified a total of 174 unique protein targets of ISG-15 via anti-FLAG immunoaffinity purification[13]. A subset of these proteins are outlined in Table 1. Although the biochemical conjugation of ISG-15 resembles the same pathway of ubiquitination, there are a few prominent differences between pathways. To start, there has been no evidence suggesting that ISG-15 is able to conjugate to itself forming poly-ISG-15 chains, which is a distinction from the poly-ubiquitin modification[12]. However, there is evidence suggesting that ISG-15 can ISGylate to different lysine residues on the same protein[12]. Likewise, ISGylation is not always a signal for proteasomal degradation as is the case with ubiquitin[12]. Instead, ISGylation can have various downstream effects on proteins, which will be further discussed in the next section.

Table 1: A subset of candidate substrate proteins for ISGylation organized based on cellular process; adapted from Durfee, *et al.* 2010[13].

Cellular Metabolism	Protein Folding and Trafficking	Defense and Immunity	Cell Cycle & Motility
Aldolase A (carbohydrate metabolism)	Clathrin – heavy chain	Complement component 1	Actin-β/α/γ
Destrin (protein metabolism)	Hsp 70	Macrophage migration inhibitory factor	Myosin – heavy chain
Lactate dehydrogenase (carbohydrate metabolism)	Ribosomal protein 40S/60S	MxA/B	Tubulin-α/β
Enolase 1 (carbohydrate metabolism)	Annexin	STAT1	Cytokeratin
UBE1L (protein metabolism)	Destrin	IRF3	Gelsolin

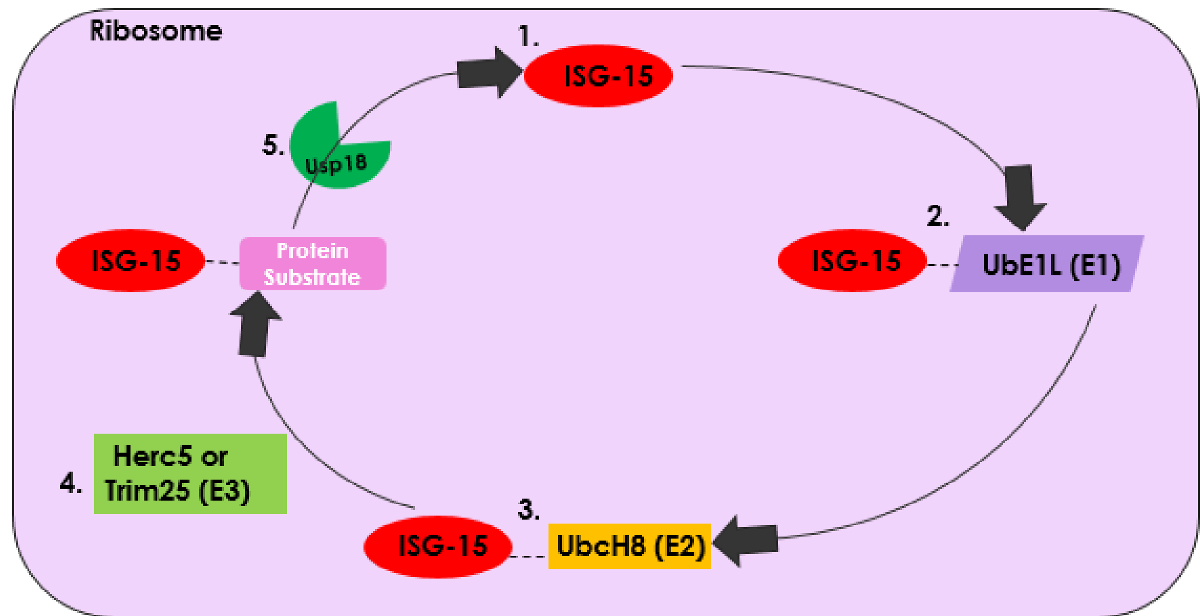

Figure 4: Schematic view of ISG-15 ISGylation system adapted from Skuag *et al*, 2010[57]. 1. ISG-15 exists as a free protein in the intracellular environment. 2. During infection, the ISG-15 E1 activating enzyme, UbE1L, initiates the pathway by forming a thioester bond between the C-terminal glycine of ISG-15 and a cysteine residue of UbE1L, likely near the ribosomal complex due to the presence of newly synthesized proteins. 3. Once primed by UbE1L, ISG-15 is transferred to a cysteine residue of an E2 conjugating enzyme, UbcH8. 4. Finally, an E3 ligase facilitates the conjugation of ISG-15 to lysine residues of target proteins. 5. If the role of ISG-15 conjugation is no longer required, its deconjugation is mediated by an ISG-15 specific protease called USP18. ISG-15 may then return to a free molecule within the cytoplasm of the cell.

4.12 Extracellular Activation: Cytokine-like Activity & Surface Receptors

Mammalian studies have demonstrated that ISG-15 could be found in the extracellular space[40,67,35,39]. However this finding brings up more questions, such as: 1) how did ISG-15 enter the extracellular space? and 2) what is ISG-15's function within the extracellular space? and 3) what is ISG-15's cognate receptor? There is evidence for ISG-15 in both gelatinase and secretory granules of granulocytes, which suggests that this could be a mechanism of ISG-15 entering the extracellular space[6,5,14]. Many other immune cell types such as monocytes, lymphocytes and neutrophils have also demonstrated the ability to release ISG-15, however the mechanism of release has not been identified[6,5,14]. Suggested mechanisms of release include ISGylated proteins being delivered to the cell surface, cell death allowing spillage of whole cell contents into the extracellular space, or the

incorporation of ISG-15 into surface proteins of various pathogens released from the cell[1,23]. All these sources of extracellular ISG-15 have been shown to have the potential to engage cell surface receptors on neighboring cells[1,40,23]. Recent studies have identified a cell surface receptor for ISG-15 to be leukocyte function-associated antigen-1 (LFA-1) on lymphocyte cells[58,35]. ISG-15 was demonstrated to act directly on LFA-1 without prior activation from other proteins[58,35]. The identification of this ISG-15 cell surface receptor on leukocytes provides a significant insight to its various function in mammals. Future work on the vig-3 surface receptor in Rainbow trout could be done in the Rainbow trout monocyte-macrophage cell line RTS11, as it is a leukocyte[20]. As mentioned, ISG-15 has been demonstrated to bind to many immune cells, having various downstream effects to be later discussed.

4.13 Activity

To this end, ISG-15's protein characteristics and method of activation in mammalian systems have been discussed, however the downstream effects that the ISG has on various cellular proteins and processes has been overlooked. In mammalian studies, ISG-15 has been shown to have a wide variety of effects which vary based on its mechanism of activation; namely ISGylation or cytokine-like activity. An overview of these activities can be observed in Figures 5 (ISGylation) and 6 (cytokine-like activity). This section will discuss the wide range of effects that ISG-15 activation has demonstrated in mammalian and fish species.

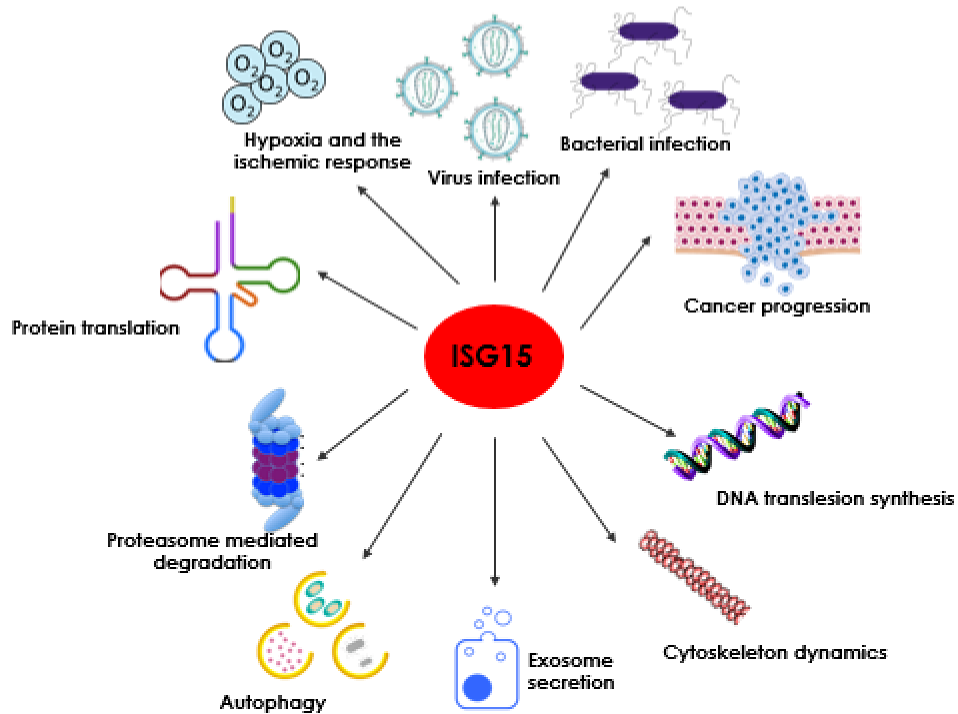

Figure 5: Overview of ISG-15 functions via ISGylation. ISG-15 restricts viral infections by conjugating to cellular proteins or to viral proteins. ISGylation restricts bacterial infection by modifying proteins. ISG-15 can also promote and inhibit the progression of different cancers via ISGylation. ISGylation is also involved in DNA translesion synthesis (DNA repair) termination as well as actin cytoskeleton dynamics. ISGylation can also inhibit exosome secretion and promote autophagy. ISGylation can both inhibit and initiate proteasome-mediated degradation and inhibit protein translation. ISG-15 is also involved in induction of hypoxia via ISGylation. Figure adapted from Villarroya-Beltri, *et al*. 2017[63].

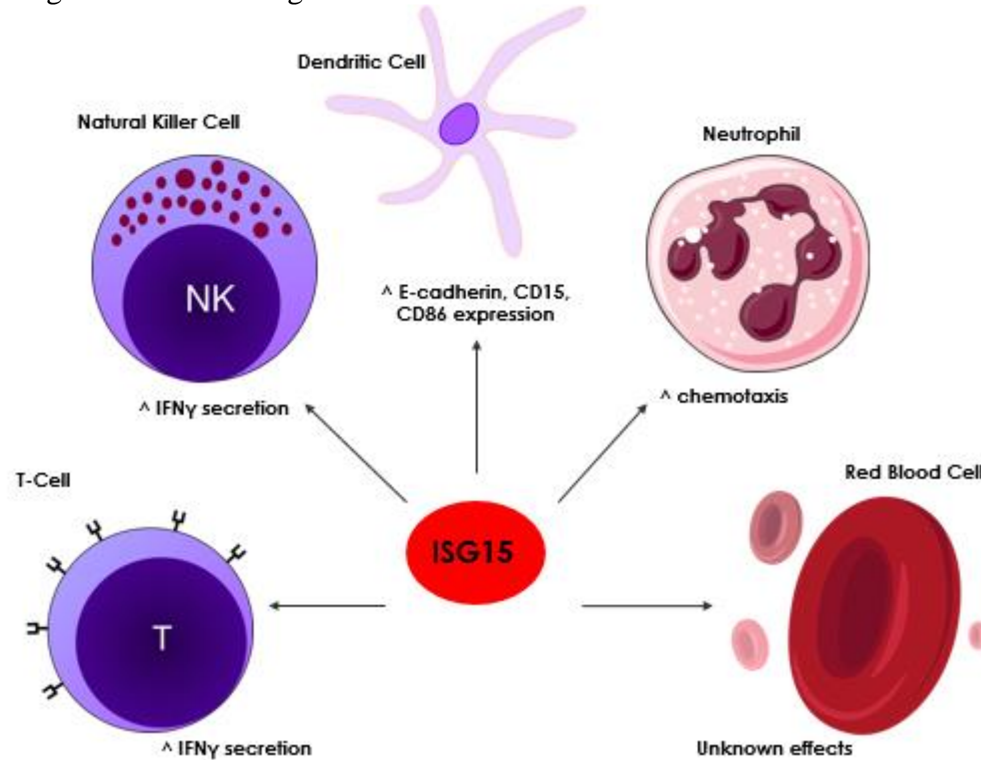

Figure 6: Overview of free ISG-15 cytokine-like activity in the extracellular space via binding to cell surface receptors. Activities range from increasing chemotaxis, increasing IFN gamma secretion, increasing expression of E-cadherin (cell adhesion), CD15 (cell adhesion) and CD86 (T cell co-stimulatory and survival molecule) expression. Figure adapted from Villarroya-Beltri, *et al*. 2017[63]

4.14 Antiviral Activity

ISG-15's antiviral activity was first observed while identifying antiviral ISGs in mice during Sindbis viral infection[26,6,33]. In this experiment, mice expressing ISG-15 were infected with Sindbis virus and showed complete protection from the virus[45,33]. This finding sparked a magnitude of reports of ISG-15's antiviral activity. Replication of influenza virus, vesicular stomatitis virus, Sendai virus, Japanese encephalitis virus, Newcastle disease virus, avian sarcoma leukosis virus, human papilloma virus, human immunodeficiency virus 1, West Nile virus, and Ebola virus like particles are all suppressed by ISG-15 in cell culture[28,1,38]. To determine antiviral activity of ISG-15 *in vivo* ISG-15-deficient mice were generated[45,33]. As predicted, when these mice were infected with Sindbis virus they were found to be more susceptible than wild-type (WT) mice[45,33]. This increase

in susceptibility in the ISG-15 deficient mice was reversed when the mice were treated with recombinant WT-ISG-15, however not when the recombinant ISG-15 was mutated along the LRLRGG binding domain (required for ISGylation)[45,33]. Other viruses which are limited *in vivo* by ISG-15 include human cytomegalovirus, human respiratory syncytial virus, chikungunya virus, west Nile virus and hepatitis C virus[14]. The range in different virus families which ISG-15 has been found to act on suggests that the mechanisms through which ISG-15 acts may also be diverse. Specifically, Vig-3's antiviral activity. However, research into ISG-15's activities has started using Zebrafish, seabass and flounder. The fish ISG-15 homologue has been demonstrated to both inhibit virus replication as well as be secreted from cells and exhibit cytokine-like activity. Therefore, antiviral function of ISG-15 has been shown to be conserved between species. Two antiviral functions which have been already introduced are ISGylation and cell-signaling.

4.14.1 Antiviral activity via ISGylation

ISGylation of ISG-15 has been proven to aid in innate immunity in response to many virus infections in mammals. To this end, it has been determined that ISG-15 is able to conjugate to both host and viral proteins[32]. This suggests that perhaps the antiviral activity may be non-specific and may venture further than targeting newly synthesized viral proteins[17,28]. Interestingly, it has been found that ISGylation may have both stabilizing and destabilizing effects on different target proteins, i.e. up- or downregulating expression of the protein during different infections, however the mechanisms of these changes in expression are unknown[2,17,28]. As mentioned, antiviral activity as a result of protein ISGylation has been found for both DNA and RNA viruses[13,17,28]. Studies have provided evidence into the mechanisms by which ISG-15 may function as an innate antiviral molecule with a wide-range of activity[17,28]. It was found that during influenza B virus infection, ISG-15 conjugates to the nonstructural protein 1 of the virus in order to protect the cell[17,24,12]. As mentioned above ISG-15 knockout mice were more susceptible to Sindbis virus infection[45,33]. What

is interesting is that not only was the conjugation domain required for this protection, but a similar study using mice deficient in the ISGylation E1 enzyme UbE1L were more susceptible to Sindbis infection compared to WT, demonstrating that it was ISGylation of ISG-15 that was providing the protection[45,33,19]. Based on this it can be concluded that the antiviral activity of ISG-15 against influenza B virus in this model was largely dependent on ISGylation[17,24,12]. More recently, a study on human papilloma virus (HPV) discovered that ISGylation of the HPV capsid protein caused alterations in the geometry of the viral capsid structure, inhibiting the infectivity of the released virus[12]. Although there is much focus on ISG-15 targeting viral proteins to aid in antiviral activity, it has also been found that it is capable of conjugating to host proteins to protect the cell. For example, ISGylation of IRF3 was demonstrated to sustain activated IRF3, therefore positively regulating the type I IFN response in a positive feedback loop during viral infection[12,62]. In contrast, recent studies have demonstrated that ISGylation may also aid in viral infection. For example, ISG-15 and its ability to conjugate to other proteins was exploited by the hepatitis B virus; ISG-15 overexpression and ISGylation significantly increased levels of HBV DNA within the cytoplasm of infected cells suggesting that the virus was using the protein to stimulate its own viral replication[11]. It is quite clear that ISGylation plays a major role in ISG-15s antiviral function and viral infectivity, however recent evidence in mammalian models suggests that free ISG-15 may also be an important innate immune antiviral effector in response to viral infections[12].

4.14.2 Antiviral activity via cytokine-like activity

ISG-15 has been demonstrated to have a role in the extracellular space, acting in a cytokine-like fashion[58]. Although ISG-15 does not have recognized signal peptides for release, several immune cells have been shown to release ISG-15 after IFN stimulation[58,6]. The treatment of T-cells with active ISG-15 resulted in explicit proliferation of natural killer (NK) cells[58,6,5]. This proliferation was determined to be the result of induction of IFNγ by T-cells due to the treatment of

ISG-15[5]. As discussed previously, ISG-15 can bind to the LFA-1 receptor of different immune cells[58,35]. Recent studies have confirmed this, as ISG-15 binding to the LFA-1 receptor triggers activation of SRC family kinases, promoting IFNγ and IL-10 (interleukin-10; anti-inflammatory cytokine) secretion in NK cells[58,6,35]. It was also found that ISG-15 and IL-12 (interleukin-12; cytokine capable of inducing IFNγ) have a synergistic relationship with respect to the cellular release of IFNγ[58]. This novel finding provides a specific role for free ISG-15 as an innate immune stimulant in mammals. However, the proteins signaling roles extend further than an IFN-gamma stimulant. Okumura *et al.*[43] demonstrated that ISG-15 expression blocked the ubiquitination process which usually promotes HIV-1 release from a cell[43],[42,4]. Interestingly, ISG-15 had no effect on the synthesis of HIV-1 proteins, and the usual ISGylation involved in viral budding was absent, suggesting the antiviral effects in this model were due to free ISG-15[42,43,44]. A recent study using ISG-15 deficient neonatal mice demonstrated that these mice were highly susceptible to viral infection with Sindbis virus, however mice lacking the E1 enzyme UbE1L showed no increase in lethality, indicating that ISG-15 conjugation played no role in protecting mice from infection, or perhaps suggesting that there is a similar E1 enzyme involved[45,33,19]. These studies propose that ISG-15 may play an antiviral role during infection not only through ISGylation but also as a free molecule. Although the antiviral activity of ISG-15 has received much attention, it is also important to understand the effects ISG-15 has on "non-antiviral effector" proteins involved in pivotal biological processes as well, as described next.

4.15 Mitochondria

Although ISG-15 has been proven to play a huge role in mammalian innate immunity, it has also been found to play a crucial role in mitochondrial homeostasis[7,3]. In a study using murine bone marrow-derived macrophages (BMDMs) deficient of ISG-15 and treated with IFN-I, it was found that the mitochondria of these cells were extremely damaged, specifically with respect to

mitochondrial dysfunction and alterations in the oxidative phosphorylation (OXPHOS) pathways[7,1,3]. This led to a decrease in oxygen consumption and ATP production when compared to WT BMDMs, two pivotal cellular processes systems rely on for survival[7,1,3]. Disruptions in the OXPHOS pathway furthermore lead to a decrease in reactive oxygen species, having effects on macrophage polarization and even mitophagy initiation[7,1,3]. Together this evidence suggests that ISG-15 plays an important role in the mitochondrial processes in murine BMDMs, which could extend to other mammalian and fish species[7,23,3]. As the powerhouse of the cell, the implications of mitochondrial function to cell survival are enormous. Therefore, it is remarkable that ISG-15 could play such a huge role in the organelle's homeostasis. This finding provides further insight into ISG-15 as an endogenous cellular protein of mammals and broadens its physiological role beyond that of an antiviral ISG.

4.16 Cancer

To this end it is quite clear that ISG-15 and ISGylation have been implicated in a magnitude of vital cellular processes. More recently, studies have focused on the role of ISG-15 in cancer pathogenesis within mammals, including humans[1,64,4]. It was found that ISG-15 as well as the conjugating and de-conjugating enzymes involved in ISGylation are deregulated in various cancers including bladder, breast, and prostate cancers[64,68,66,4]. However the roles that ISG-15 has in each case, whether anti- or pro-tumoral, are unclear. Similarly, studies have revealed that when comparing ISG-15 expression in breast cancer cells to normal mammary tissue, there was a significant increase in ISG-15 expression in cancer tissue[64,4]. Furthermore, it was found that ISG-15 expression increased during the progression of cancer; when comparing grade 1 and 3 mammary tumors, the grade 3 tumors showed higher ISG-15 expression than the latter[22,4]. This gives insight to the role of ISG-15 in perhaps attempting to limit the progression of cancer, or the opposite: aiding in the progression of cancer. It also provides a potential biomarker for breast cancer studies, as ISG-

15 expression and protein levels are up-regulated during the progression of disease[64,4]. Recent studies have focused in on a vital cancer protein, p53[64]. It has been demonstrated that ISGylation of p53 is able to increase its activity in tumor suppression which could hold promise to the future of p53-based cancer therapies and p53-induced cell death[64]. However, these experiments are novel, and much investigation needs to be done before this process is fully understood. It is remarkable that such a small protein has shown numerous effects on physiological processes in both mammals and fish, which provides further justification to dig deeper into investigating this protein.

4.17 Significance and Importance

Rainbow trout are a main contributor to Ontario both economically via fish farming and aquaculture and ecologically via regulating ecosystems[7]. One of the main areas for Rainbow trout production in Ontario is the Great Lakes[5]. Virus infections in general can cause catastrophic effects to both farmed and wild fish populations[41] and there are currently no antiviral therapies available [7]. This presents an urgent need to understand antiviral response in Rainbow trout in order to develop novel, antiviral therapeutics. The antiviral mechanisms of Vig-3's homologue ISG-15 in mammals are likely conserved with Vig-3 in Rainbow trout[41]. A well-rounded understanding of Vig-3's antiviral activities contributes to the protection of these economically and ecologically important Ontarian fish species, as well as other important aquatic animals. At present, the sequence of *vig-3* has been identified and cloned from Rainbow trout, however no mechanistic properties of Vig-3 have been investigated in this fish species. The goal of the current research project is to explore the antiviral mechanisms of Vig-3, in hopes of obtaining an understanding of its full antiviral potential.

4.18 Objectives & Hypotheses

This project will determine Vig-3's role in innate antiviral immunity in virus infected Rainbow trout cells, specifically the Rainbow trout gonad cell line, RTG-2[35]. The RTG-2 cell line was chosen because it is relatively well studied with regards to its innate antiviral processes and

responses[20,35,47,48]. To study the induction patterns of *Vig-3*, three stimulants were chosen, poly I:C, IPNV and VHSV. Poly I:C was chosen as a positive control, as it is a relatively well studied synthetic dsRNA analogue. It is also abiotic and therefore does not have immune evasion mechanisms that many viruses possess. VHSV and IPNV were chosen as they are both important fish pathogens, but come from different virus families, therefore can affect the host cell in different ways. Based on the literature, it is hypothesized that *Vig-3* will be up-regulated during treatment with poly I:C and infection with IPNV and VHSV to aid in the establishment of an antiviral state. Secondly, it is hypothesized that induced Vig-3 will ISGylate proteins within the infected cell. This research will give insight into the antiviral effects of Vig-3 in Rainbow trout; contributing to the development of better antiviral therapeutics. The proposed project will contain 2 objectives:

1) Investigate *Vig-3* expression patterns following infection with IPNV and VHSV and dsRNA (poly I:C) treatment and the levels of transcript, protein and cellular location.
2) Determine Vig-3's ISGylation capabilities during an innate antiviral response.

An overview of the aims of this research project are summarized in Figure 7.

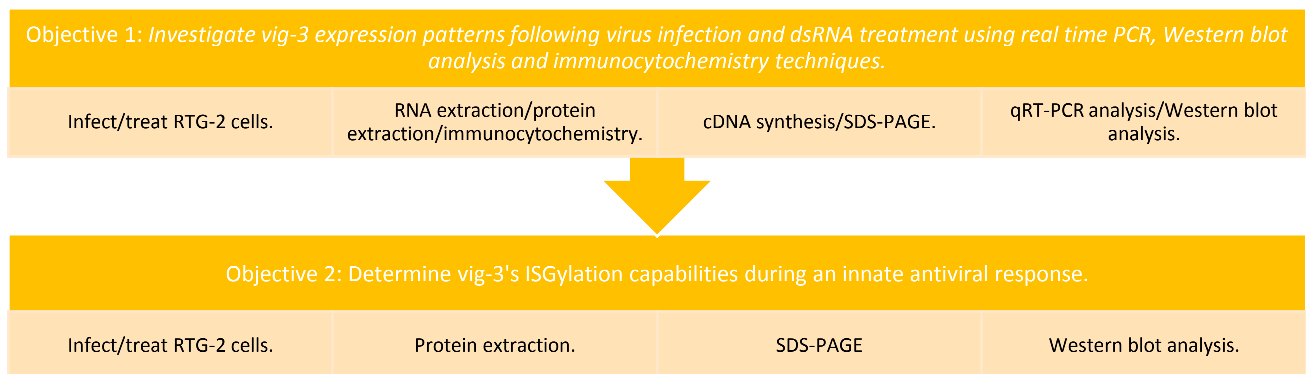

Figure 7: Schematic representation of experimental design which is discussed in full within the Materials and Methods section.

5. Materials and Methods

5.1 Cell Lines and Tissues

The cell lines used in this study are the Rainbow trout gonadal cell line (RTG-2) and epithelioma papulosum cyprinid (EPC). Both cell lines were obtained from N. Bols (University of Waterloo). The cell lines were grown in 75 cm^2 plastic tissue culture flasks (BD Falcon, Bedford, MA) at room temperature in Leibovitz's L-15 media (HyClone, Logan, UT) supplemented with 10% v/v fetal bovine serum (FBS) (Fisher Scientific, Fair Lawn, NJ) and 1% v/v penicillin/streptomycin (P/S) (10mg/mL streptomycin and 10000U/mL penicillin) (Fisher Scientific). All 10 tissues used for the tissue panel were extracted from 3 separate fish obtained from B. Dixon (University of Waterloo). An RNA extraction of the tissues was done using the TRIzol method as described in section 7.4.2.

5.2 Virus propagation

Viral hemorrhagic septicemia virus (VHSV)-IVb (strain U13653) was propagated on monolayers of EPC cells. Virus containing media (L-15 with 5% v/v FBS; Fisher Scientific) was collected post-infection and filtered through a 0.45um filter (Nalgene, Rochester NY, USA). Virus preparations were kept frozen at -20°C for short term storage and -80 ^{o}C for long term storage. The 50% tissue culture infective dose (TCID50)/mL values were calculated according to the method of Reed and Muench.

Infections pancreatic necrotic virus (IPNV) was propagated on monolayers of Chinook salmon embryonic cell line (CHSE-214) cells. Virus containing media (L-15 with 5% v/v FBS; Fisher Scientific) was collected post-infection and filtered through a 0.45µm filter (Nalgene, Rochester NY, USA). Virus preparations were kept frozen at -20°C for short term storage and -80 ^{o}C for long term storage. The 50% tissue culture infective dose (TCID50)/mL values were estimated according to the method of Reed and Muench.

5.3 Poly I:C

Polyinosinic: polycytidylic acid (poly I:C) (Sigma-Aldrich, St Louis, MO, USA) was resuspended in the appropriate volume of phosphate buffered saline (PBS) (HyClone) to a concentration of 10μg/mL, heated for 10 min at 65°C and then cooled to room temperature to allow annealing. The stock solution was diluted 10mg/mL and stored at -20°C.

5.4 RT-PCR

5.4.1 Primer design

Primers for amplifying the Rainbow trout vig-3 sequence were designed using NCBI Primer-BLAST using the predicted Atlantic salmon (*Salmo salar*) ISG-15 sequence (XM_014190663.1). The full-length Rainbow trout vig-3 was determined to be ~762bp. Primer re-suspension followed Sigma-Aldrich protocol (100 μM) and working concentrations were created of 10μM using DNA quality water for dilution.

5.4.2 RNA Extraction/cDNA Synthesis

RNA samples from RTG-2, RT-Gill, RTS-11 and RT-Gut were collected using TRIzol (Ambion by Life Technologies, Carlsbad, CA) as per manufacturer's instructions. RNA samples were quantified using a NanoDrop Lite Spectrophotometer (ThermoScientific) and stored at -80 degrees Celsius until needed. RNA samples were DNase treated using Invitrogen DNA-free DNA Removal Kit (Fischer Scientific, Massachusetts, USA) following manufacturers instructions. cDNA was synthesized from 2μg of DNase treated RNA mixed with 0.5μg Oligo(dT) primer (Sigma-Aldrich, St Louise, MO), 1X GoScript reaction buffer (Promega, Madison WI), 1.5mM $MgCl_2$ (Promega), 0.5mM each deoxynucleotide triphosphate (dNTP; Fischer Scientific) and 160U of GoScript Reverse Transcriptase (Promega). Reactions were performed according to the manufacturer's instructions via the GoScript Protocol.

5.4.3 PCR/Gel Electrophoresis

Using cDNA prepared as described above, PCR reactions were performed using 2µL of undiluted cDNA, 1x Green GoTaq Flexi Buffer (Promega), 1.25 GoTaq Flexi DNA Polymerase (Promega), 0.2mM dNTP mix (Fischer Scientific), 1.5mM $MgCl_2$ (Promega), and 0.5mM of each forward and reverse primer (Sigma-Aldrich). Cycle conditions were: 95 degrees Celsius for 3 minutes, 34 cycles of: 95 degrees Celsius for 3 minutes, gene-specific annealing temperature (Ta) for 3 minutes, a temperature gradient of 45 degrees to 60 degrees Celsius for 1 minute, 6-minute elongation time, and followed by 10 minutes at 72 degrees Celsius. 10µL of the final PCR products were run on a 1% w/v agarose (Thermo Fisher Scientific) gel with 5µL of O'GeneRuler 1kb Plus DNA Ladder (Thermo Fisher Scientific). The resulting gel was stained with 0.5 mg/mL ethidium bromide (Sigma Aldrich) for visualization using a VersaDoc Imager (Bio-Rad) set to UV transillumination. Putative successful PCR products were then run on an agarose gel stained with GelGreen Nucleic Acid Gel Stain (Biotium, California, USA) to allow for visualization under a blue light to avoid UV cross linking, and a gel extraction was performed using High Pure PCR Product Purification Kit (Roche, Laval QC, Canada) or a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The purified products were then sequenced by the Molecular Biology Unit within Laboratory Services of the University of Guelph (Guelph, ON) to confirm the identity of the product.

5.5 qPCR reactions

All PCR reactions contained: 2µL of diluted cDNA, 2x SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA), 0.2µM forward primer (Sigma Aldrich), 0.2µM reverse primer (Sigma-Aldrich) and nuclease-free water to a total volume of 10µl (Fisher-Scientific). qPCR reactions were performed using CFX Connect Real-Time PCR Detection System (Bio-Rad). The program used for all qPCR reactions was 98ºC 2 min, 40 cycles of 98ºC 5 s, 55ºC 10s, followed by 95ºC with a read every 5s. Product specificity was determined through single PCR melting peaks. Data were analysed using

the $\Delta\Delta C_t$ method. Specifically, gene expression was normalized to the housekeeping gene (β-actin) and expressed as fold change over the control group.

5.6 Western blotting

5.6.1 Sample preparation

RTG-2 cells were seeded at 2 x 10^6 cells/well in a 6-well plate and allowed to attach over night. Cells were treated with either 5µg/mL poly I:C, or infected with either VHSV-IVb (MOI=1) or IPNV(MOI=1) for 6 hours, 12 hours, 24 hours, 48 hours. Cells were then washed twice with PBS (Fisher Scientific) and protein was collected using 350µL RIPA buffer (50 mM Tris, 150mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, pH 8.0) with 2µL protease inhibitor cocktail (Sigma-Aldrich). The lysate was incubated on ice for 30 minutes to allow for adequate cell lysis. Immediately after lysis, the tubes were centrifuged for 13 minutes at 11,600xg. Following centrifugation, the supernatant was removed and either quantified immediately or stored at -80°C for future use. Protein samples were quantified using the Pierce BCA Protein Assay Kit (Thermo Fischer Scientific) following the manufacturers instructions. Absorbance readings were analyzed at 595 nm using a Synergy HT plate reader (BioTek, Winooski, VT) with Gen5 software.

5.6.2 Western blot analysis

Following protein quantification, 15µg of protein samples were mixed 1:1 with 5X SDS-PAGE protein loading sample buffer prepared following the Cold Springs Harbor Protocol, and run on a 12% acrylamide SDS-PAGE gel. Gels were cast using the TGX stain-free FastCast kit (Bio-Rad). Of each protein sample, 15ug was run on the gel along with 4µL of the PageRuler Prestained protein ladder (Thermo Scientific), after which proteins were transferred onto a methanol-activated polyvinylidene fluoride (PVDF) membrane (BioRad) using the Trans-Blot Turbo system (Bio-Rad) and the pre-set program for mixed molecular weight proteins (1.3A; up to 25V for 7 min). Blots were then rinsed in Tris-buffered saline (TBS) with 0.5% Tween-20 (TBS-T) and transferred into blocking solution (5% skim milk powder in TBS-T) for 1 h. After blocking, the blots were rinsed in

TBS-T and probed with a 1:2000 dilution primary antibody, rabbit anti-vig-3 prepared by Abclonal Logistics, Abclonal Science Inc, Woburn MA, overnight at 4 degrees Celsius. Blots were rinsed three times in TBS-T and probed with 1:2000 secondary antibody, goat anti-rabbit conjugated with horseradish peroxidase (HRP) for one hour at room temperature. Blots were then rinsed in TBS-T followed by detection. Protein was detected on blots using the chemiluminescent Clarity Western ECL Substrate (Bio-Rad). Images were captured using a VersaDoc Imager (Bio-Rad).

5.7. Immunocytochemistry

RTG-2 cells were seeded at 2 x 10^5 on glass coverslips in a 12-well plate and incubated overnight in Leibovitz's L-15 media (HyClone, Logan, UT) supplemented with 10% v/v fetal bovine serum (FBS) (Fisher Scientific, Fair Lawn, NJ) and 1% v/v penicillin/streptomycin (P/S) (10mg/mL streptomycin and 10000U/mL penicillin). The following day, media was aspirated and replaced to remove residual TryplE. Cells were then treated with 5µg poly I:C, VHSV (MOI=1) or IPNV (MOI=1) for 6, 12 and 24h. Following treatment or infection, cells were fixed with 10% buffered formalin (Fisher Scientific) for 10 minutes, permeabilized with a 0.1% Triton X-100 (Thermo Fisher) PBS solution for 15 min and blocked in blocking buffer (3% goat serum (Sigma-Aldrich), 3% bovine serum albumin (BSA; Sigma-Aldrich) and 0.02% Tween 20 (Thermo Fisher) in PBS) for 1 h at room temperature. Coverslips were then incubated with the anti-vig-3 antibody at a 1:2000 dilution for 1 h at room temperature, followed by FITC-labelled anti-rabbit secondary antibody conjugated with FITC, at 1:200 dilution for 1 h in a humidified chamber. All antibody dilutions were done in blocking buffer. Nuclei were stained with a concentration of 1µg/mL of DAPI for 5 minutes and coverslips were mounted using SlowFade Gold Antifade (Thermo Fisher) mountant. Mounting media was then cured by incubating slides overnight at room temperature in the dark. Cells were imaged at 200x magnification with an inverted fluorescence microscope (Nikon Eclipse TiE with Qi1 camera).

5.8 Statistical Analysis

All data represent at least three independent trials and are presented with the standard error of the mean (SEM). Data were analyzed using GraphPad Prism version 7.00 for PC, GraphPad Software La Jolla, CA USA, www.graphpad.com. Where indicated, a one-way ANOVA with a Dunnett's multiple comparisons test was performed to indicate significant differences between treatments and the control group. A 95% confidence interval was used and a p value of <0.05 was considered significant. An Asterix system was used to indicate significant difference between means.

6. Results

Objective 1: Investigate *Vig-3* expression patterns, both constitutively and following infection with IPNV, VHSV and dsRNA treatment.

6.1 Constitutive vig-3 transcript expression in Rainbow trout tissue and cell lines.

To begin characterizing *vig-3* transcript in Rainbow trout, its presence was investigated in Rainbow trout tissues and cell lines using RT-PCR. It was found that *vig-3* transcript was constitutively expressed in all ten tissues (liver, brain, kidney, spleen, muscle, gut, gill, head kidney, gonad and heart) (Figure 8A). Constitutive *vig-3* transcript presence was also found in four Rainbow trout cell lines: RTG-2, RTgutGC, RTgill-W1 and RTS11 (Figure 8B). In order to not only detect the presence of transcript but also to quantify transcript levels between tissues, the ten Rainbow trout tissues were also analyzed via RT-qPCR. The data are the average of 3 separate fish (Figure 9). Constitutive *vig-3* expression was highest in gonad, followed by gut and liver (Figure 9).

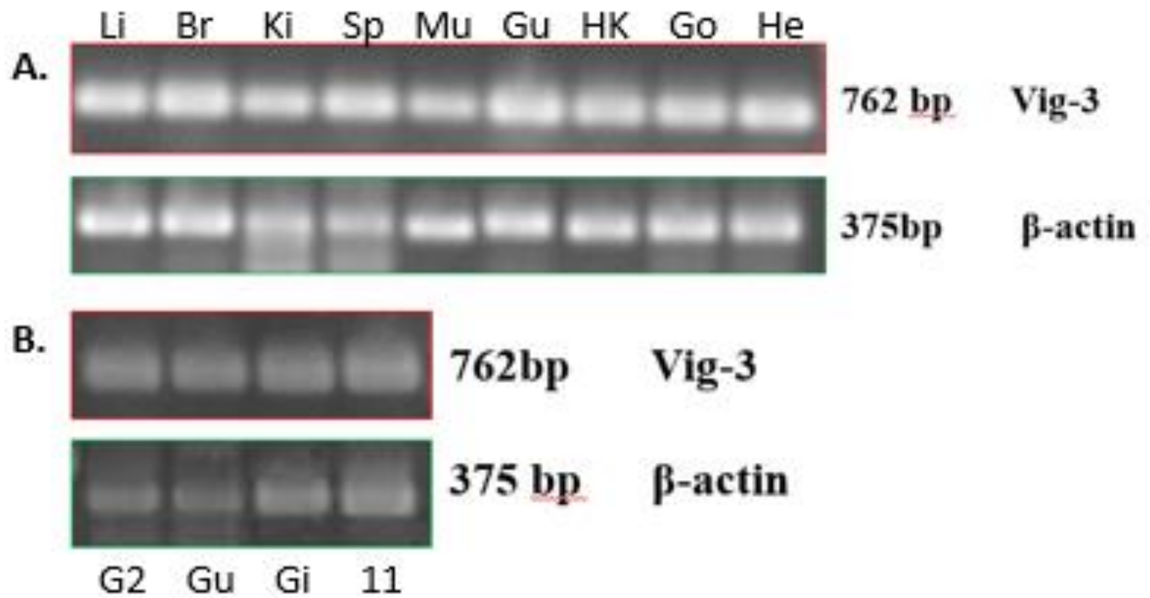

Figure 8. Detection of *vig-3* transcript expression in tissues and cell lines derived from rainbow trout as measured by RT-PCR. A: Agarose gel electrophoresis of *vig-3* expression in untreated Rainbow trout tissues: from left to right tissues are liver(Li), brain(Br), kidney(Ki), spleen(Sp), muscle(Mu), gut(Gu), head kidney(HK), gonad(Go) and heart(He). Tissues were obtained from live fish specimens and a TRIzol RNA extraction was performed, cDNA was created and amplified using specific *vig-3* primers. The PCR product was then run on an agarose gel. **B**: Agarose gel electrophoresis of *vig-3* transcript expression in untreated Rainbow trout cell lines: from left to right cell lines include RTG-2(G2), RTGutGC(Gu), RTGill-W1(Gi) and RTS-11(11). Cell lines were obtained from characterized cell lines a TRIzol RNA extraction was performed, cDNA was created and amplified using specific *vig-3* primers. The PCR product was then run on an agarose gel. Rainbow trout vig-3 is approximately 762bp in size. Beta-actin is 375bp in size.

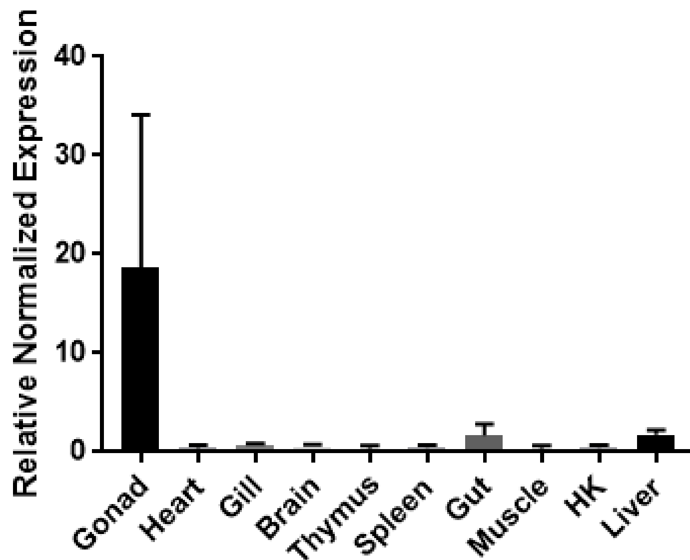

Figure 9: Visual representation of *vig-3* induction at the transcript level in ten different untreated Rainbow trout tissues (gonad, heart, gill, brain, thymus, spleen, gut, muscle, head kidney and liver). averaged from three fish (n=3). Tissues were obtained from freshly sacrificed fish specimens and a TRIzol RNA extraction was performed, cDNA was created and amplified using specific *vig-3* primers and then diluted and a RT-qPCR cycle was performed. Statistical analysis was then completed using GraphPad Prism- the SEM of all tissues is presented. A 95% confidence interval was used and a p value of <0.05 was considered significant.

6.2 Induction of vig-3 at the transcript level

Induction patterns of *vig-3* were investigated at the transcript level in RTG-2 cells treated with dsRNA, recombinant IFN or infected with either VHSV or IPNV over a 48h time course. IFN-1 was included in these measurements as a positive control, indicating stimulation of innate antiviral immunity within the cell following specific treatment.

6.2.1 Poly I:C

RTG-2 cells were treated with 5µg/mL of poly I:C for 6h, 12h, 24h and 48h. Untreated cells were used as a control group. *Vig-3* and *IFN-I* induction were measured at the transcript level via RT-qPCR (Figure 10). Induction of *vig-3* transcript was observed to increase from 6-12h before decreasing in expression to 48h. The most significant induction of *vig-3* was at the 12h time-point, but 12h, 24h and 48h showed significant induction compared to control cells. *IFN-I* induction followed a similar pattern, albeit faster, with 6h being the timepoint with the highest *IFN-I*

expression, followed by a decrease in expression to 24h. Interestingly, *IFN-I* expression was found to be cyclical and increase again at 48h.

6.2.2 VHSV-IVb

RTG-2 cells were infected with VHSV-IVb (MOI=1) for 6h, 12h, 24h and 48h. Uninfected cells were used as a control group. *Vig-3* and *IFN-I* induction were measured at the transcript level via RT-qPCR (Figure 10). *Vig-3* expression was significantly induced at 24h and 48h pi. *IFN-I* expression was very low throughout the experiment with only the 24h timepoint being significantly higher than control cells.

6.2.3 IPNV

RTG-2 cells were infected with IPNV (MOI=1) for 6h, 12h, 24h and 48h and uninfected cells were used as a control group. *Vig-3* and *IFN-I* induction were measured at the transcript level via RT-qPCR (Figure 10). *Vig-3* transcript levels showed an induction trend from 12h-48h; however, due to variation between experiments, there was no statistically significant increase detected. During the same treatment, *IFN-I* transcript induction was significantly different from uninfected cells at the 24h and 48h timepoint.

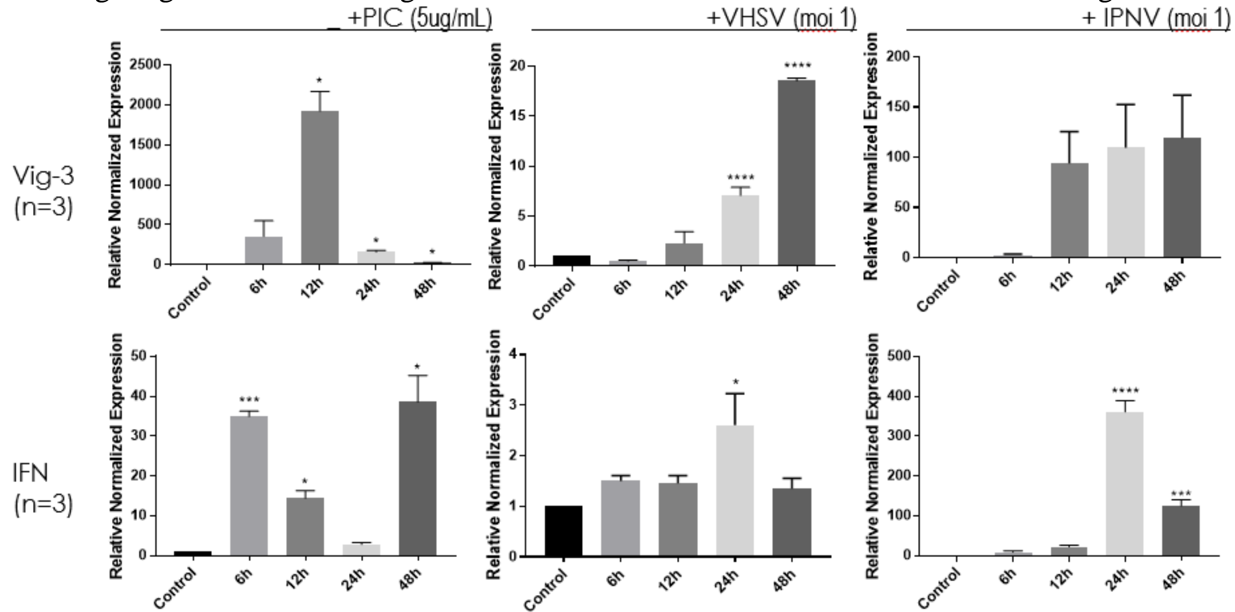

Figure 10: *Vig-3* and *IFN-I* induction at the transcript level in RTG-2 cells treated with poly I:C (5ug/mL), VHSV (MOI=1) or IPNV (MOI=1) for 6h, 12h, 24h and 48h as measured by RT-qPCR. RTG-2 cells were treated with 5ug/mL of poly I:C and infected with VHSV and IPNV both at an MOI of 1. After which, RNA was extracted, cDNA synthesized, and qPCR performed for vig-3 and IFN-I. Data is an average of 3 independent experiments. Data analyzed by a one-way ANOVA and a Dunnett's post hoc statistical analysis; *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, ns$p>0.05$(not shown by any indicator).

6.2.4 Recombinant IFN-I

If Vig-3 is indeed an ISG, then it would be induced by interferon directly. As such, RTG-2 cells were treated with a recombinant rainbow trout IFN-I protein prepared by Tania Rodriguez Ramos of Dr. Brian Dixon's lab at the University of Waterloo. RTG-2 cells were treated with two concentrations of recombinant IFN protein for 24h; 500ng/mL and 5µg/mL (Figure 11). These concentrations were chosen based on optimizing the volume of recombinant protein available to reach the highest concentration over three replications, as well as a low concentration. *Vig-3* transcript induction increased significantly at both concentrations. RTG-2 cells treated with 5 µg/mL poly I:C were included as a positive control.

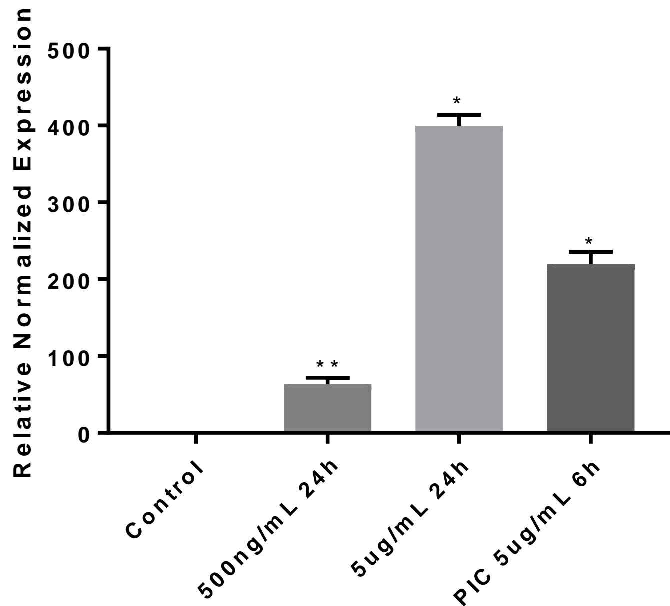

Figure 11: *Vig-3* induction at the transcript level in RTG-2 cells treated with recombinant IFN-I protein for 24h as measured by RT-qPCR. RTG-2 cells were treated with 500ng/mL or 5 μg/mL recombinant Rainbow trout interferon 1 for 24h. After which, RNA was extracted, cDNA synthesized, and qPCR performed for vig-3. RTG-2 cells treated with 5 μg/mL poly I:C were included as a positive control. Data is an average of 3 independent experiments. Data analyzed by a one-way ANOVA and Dunnett's post hoc statistical analysis; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, nsp>0.05(not shown by any indicator).

6.3 Induction of vig-3 at the protein level

Vig-3 expression levels were investigated at the protein level using Western blot analysis.

6.3.1 Poly I:C

RTG-2 cells were treated with 5 μg/mL of poly I:C for 6h, 12h, 24h and 48h. Untreated RTG-2 cells were included as a negative control. Protein samples were analyzed by Western blot analysis. A distinct band can be observed at 15kDa representing Vig-3 at all treatment time points. Although not quantitative, there appears to be the most Vig-3 induced at 12h post treatment, which correlates with the transcript data in Figure 10. There is additional higher weight banding in all samples, including the control, that appear to accumulate over the 48h. This banding pattern could be indicative of ISGylation (Figure 12). β-tubulin is included as a loading control.

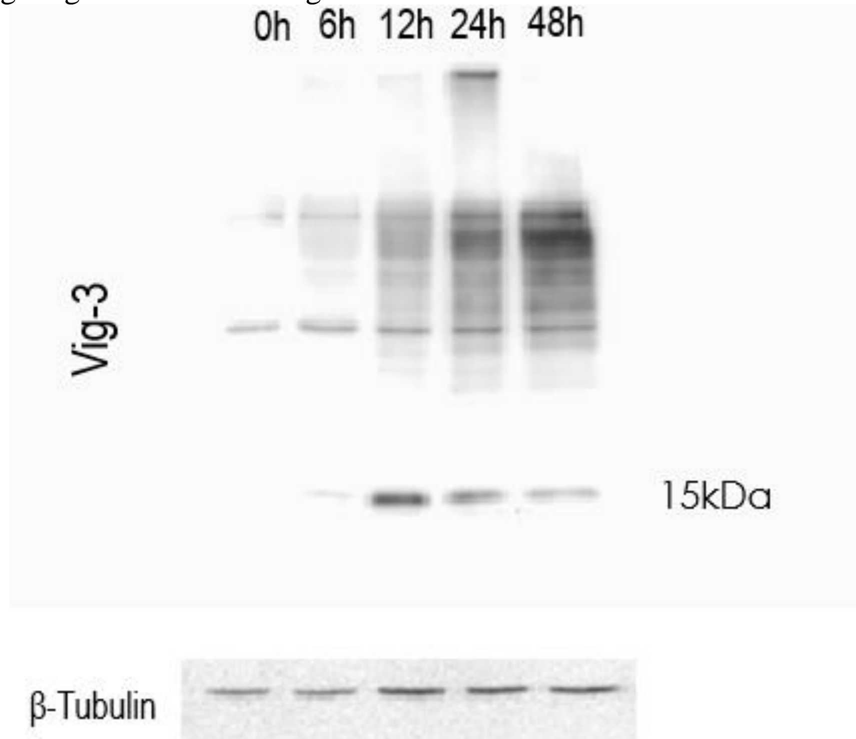

Figure 12: Visual representation of Vig-3 induction at the protein level over a 48h time course during treatment with poly I:C. RTG-2 cells were treated with 5µg/mL of poly I:C for 6h, 12h, 24h and 48h. Protein was then extracted and quantified, and 15µg/mL was loaded into a SDS-PAGE gel and ran at 120V for 90minutes. An equal amount of protein was run for each treatment. The gel was then transferred to a PVDF membrane and incubated in blocking buffer for one-hour, primary anti-RT-vig-3 antibody overnight at 4°C and then goat-anti-rabbit-secondary antibody conjugated with HRP for one hour, then imaged on a VersaDoc Imager. A distinct band at 15kDa is present at all timepoints which is indicative of Vig-3. Banding above the 15kDa size is indicative of ISGylation. The same protein samples were proved with anti-beta tubulin as an equal loading control.

6.4 Visualization of Vig-3 induction by immunocytochemistry (ICC).

ICC was used to visualize the intracellular induction and localization of Vig-3 induction in RTG-2 cells. RTG-2 cells were treated with 5 µg/mL poly I:C or infected with VHSV-IVb (MOI=1) or IPNV (MOI=1) and Vig-3 protein localization and induction was visualized by ICC using the rabbit anti-rainbow trout-vig-3 antibody. Untreated cells are included as a negative control, as well as cells probed with the secondary antibody alone. The blue staining indicates the nuclei and the green staining indicates the expression of Vig-3 (Figure 13). Protein localization in all treatments appears to be both cytoplasmic and nuclear (Figure 14). Vig-3 protein expression is clearly induced

following treatment and infection; however further analysis is required to determine whether Vig-3 expression increases between 24h and 48h pi.

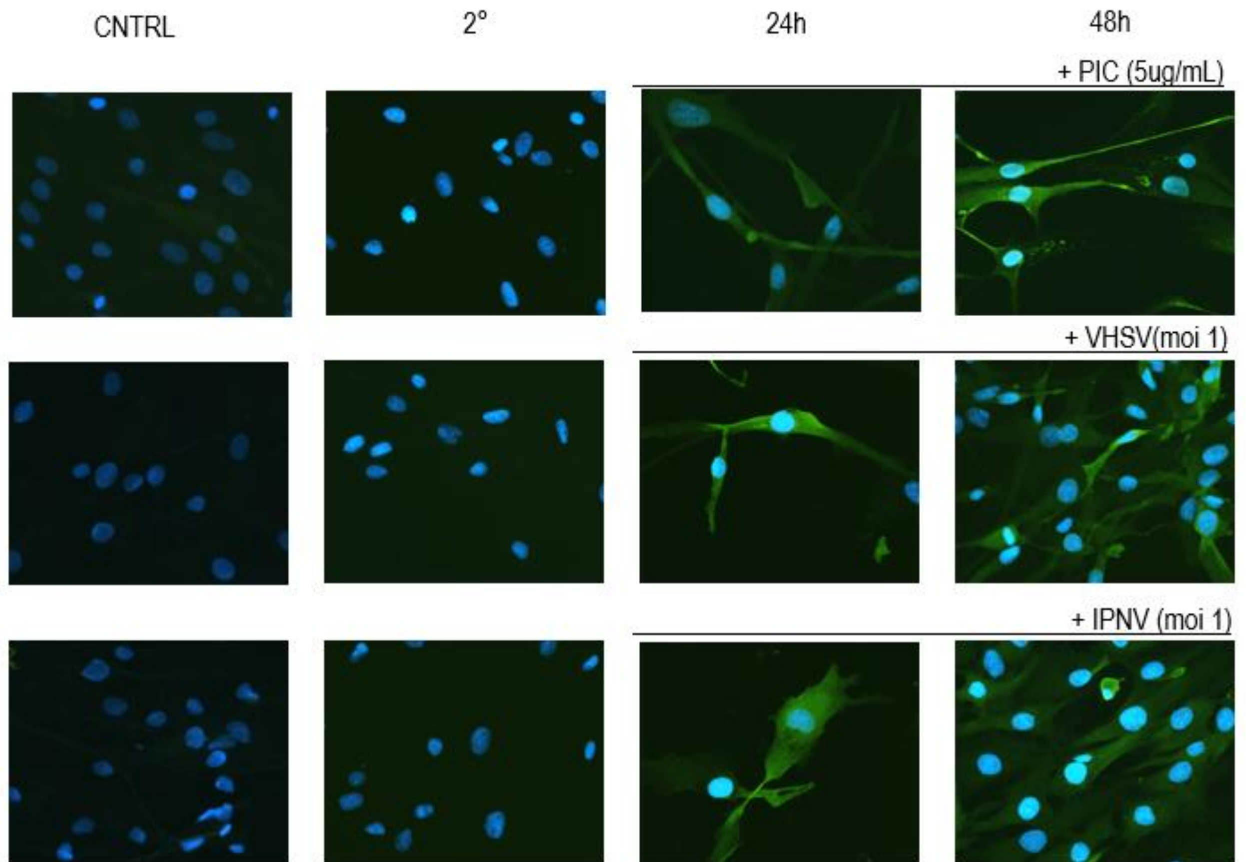

Figure 13: Visual representation of Vig-3 induction and localization at the cellular level during treatment with 5µg/mL of poly I:C and infection with VHSV and IPNV at an MOI of 1. RTG-2 cells were plated on coverslips and then were treated or infected for 24h and 48h. They were then incubated for one hour at room temperature with blocking buffer, then with anti-RT-vig-3 primary antibody for one hour at room temperature and finally for one hour with goat-anti-rabbit-secondary conjugated with FITC. Coverslips were then mounted onto glass slides and images were taken on a Nikon Eclipse microscope. Green = Vig-3, blue = DAPI stained nuclei. Magnification = 400x.

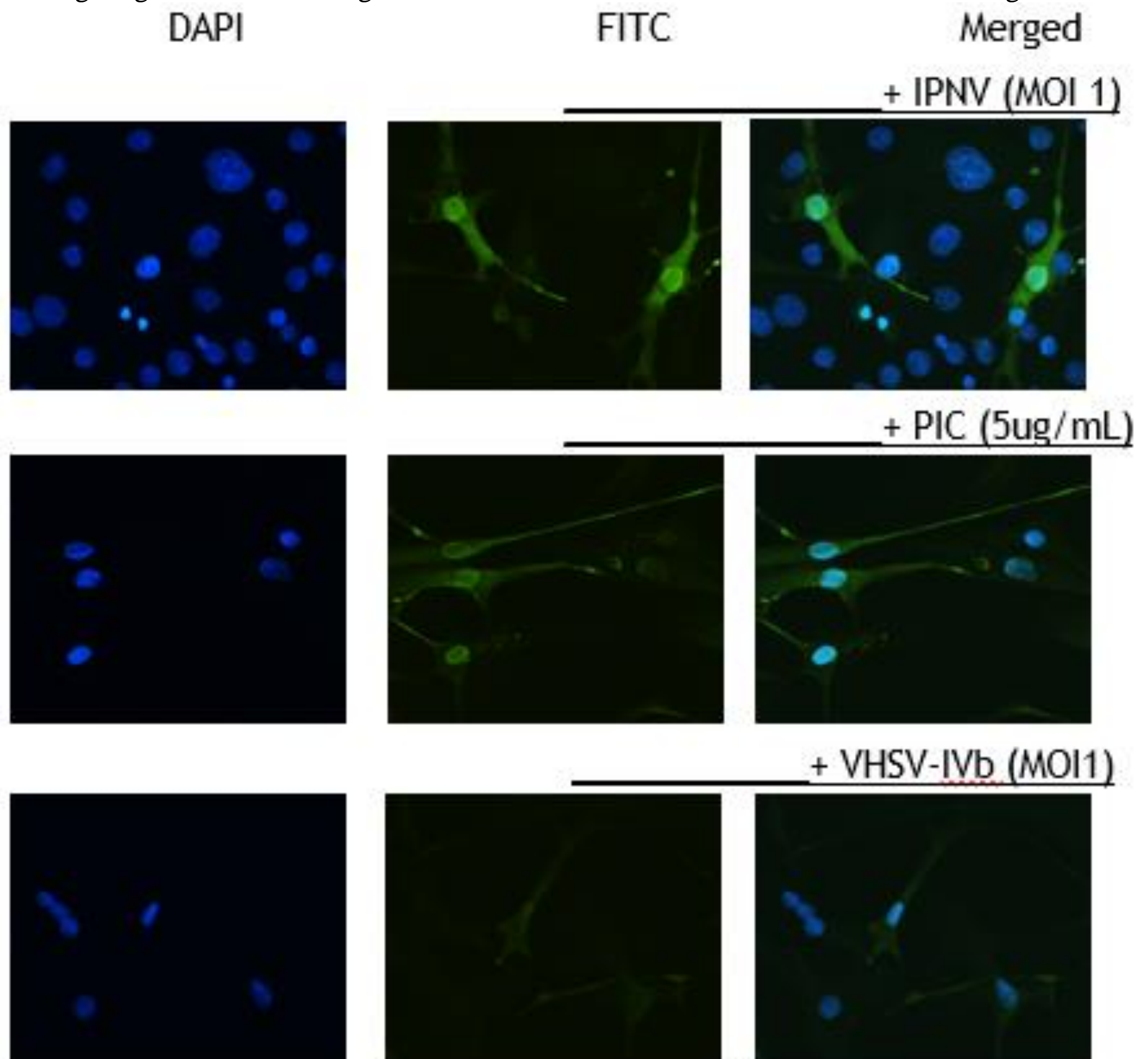

Figure 14: Cellular localization of Vig-3. Localization of vig-3 at the protein level during infection with IPNV and VHSV at an MOI of 1. RTG-2 cells were plated on coverslips and then were treated or infected for 48h. They were then incubated for one hour at room temperature with blocking buffer, then with anti-RT-vig-3 primary antibody for one hour at room temperature and finally for one hour with goat-anti-rabbit-secondary conjugated with FITC. Coverslips were then mounted onto glass slides and images were taken on a Nikon Eclipse microscope. Green = Vig-3, blue = DAPI stained nuclei. Magnification = 400x.

6.5 ISGylation via Virus Infection

RTG-2 cells were infected with either VHSV-IVb (MOI=1) or IPNV (MOI=1) for 6h, 12h and 24h. Uninfected RTG-2 cells were included as a negative control. Protein was extracted from the samples and Western blot analysis performed using the rabbit anti-Rainbow trout vig-3 antibody (Figure 15). For the VHSV-IVb infection, Vig-3 was detectable at all infection time points with increasing band intensity over time. For IPNV infected cells, Vig-3 was detectable at 12h and 24h pi. The high banding pattern was present in all samples, including uninfected control cultures and the poly I:C treated positive control.

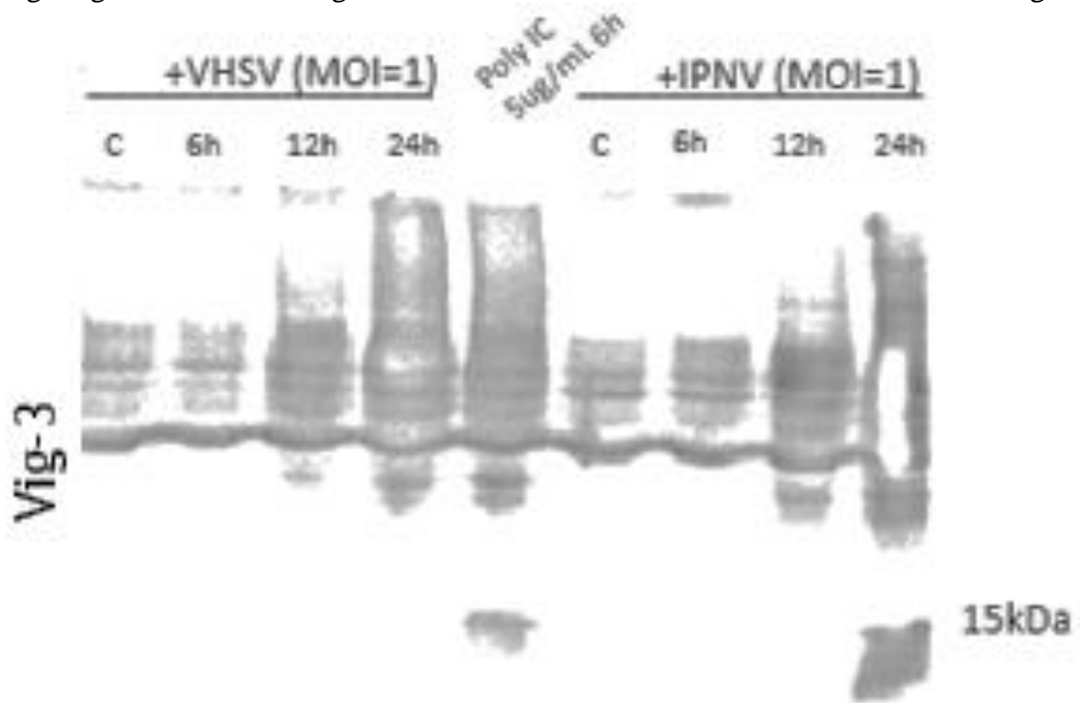

Figure 15: Visual representation of Vig-3 induction at the protein level over a 24h time course during infection with VHSV and IPNV. RTG-2 cells were infected with VHSV and IPNV both at an MOI of 1 for 6h, 12h and 24h. Protein was then extracted and quantified, and 15µg/mL was loaded into a SDS-PAGE gel and ran at 120V for 90minutes. An equal amount of protein was ran for each treatment. The gel was then transferred to a PVDF membrane and incubated in blocking buffer for one hour, primary anti-RT-vig-3 antibody overnight at 4°C and then goat-anti-rabbit-secondary antibody conjugated with HRP for one hour, then imaged on a VersaDoc Imager. A distinct band at 15kDa is present at all timepoints which is indicative of Vig-3. Poly I:C treated RTG-2 protein was used as a positive control. Banding above the 15kDa size is indicative of ISGylation.

7. Discussion:

ISG-15 has become a protein of interest for many fish and mammalian researchers in the immunological field, showing evidence of acting as an anti-viral protein. As mentioned previously, ISG-15 has been found to be capable of conjugating to both host and viral proteins to contribute to the hosts antiviral response, as well as being secreted from cells in order to stimulate immune responses in neighbouring cells. Rainbow trout are of both economical and ecological importance to Ontario. However, there are many fish pathogens which pose a danger to this valuable species. It is for this reason that ISG-15 was investigated in this organism to examine vig-3 transcript levels and the protein's expression and induction during viral infection, in the hopes of providing insight to further protect Rainbow trout, and other aquatic species from viral pathogens.

ISG expression is highly inducible following viral infection; however, their constitutive expression can vary between individual proteins and cell types. Thus, to begin the investigation on Vig-3 transcript expression patterns in Rainbow trout, constitutive expression was analyzed in four Rainbow trout cell lines via RT-PCR. The four cell lines tested included: RTG-2, RTS-11, RT-GutGC and RTgill-W1. These cell lines are derived from the gonad, spleen intestine and gill respectively and represent three different cell types: epithelial (RTgutGC and RTgill-W1), monocyte/macrophage (RTS11) and fibroblast (RTG-2). It was anticipated that RTG-2 and RTS-11 would have a higher constitutive expression in comparison to the latter, as RTG-2 is derived from the gonad and RTS11 from the spleen. The gonad is an immune privileged site and therefore can tolerate the introduction of different antigens without inducing an inflammatory immune response[67]. The spleen monitors blood therefore ISGs may be heightened in this tissue to protect against infection[67]. However, all four cell lines appeared to have similar constitutive expression of vig-3 based on RT-PCR.

Next, ten tissues (liver, brain, kidney, spleen, muscle, gut, gill, head kidney, gonad and heart) were analyzed to determine constitutive expression via RT-PCR and qRT-PCR. Based on the RT-PCR data, all ten tissues constitutively express vig-3 transcript. To investigate levels of expression, q-RT-PCR analysis was performed with the ten tissues in three independent fish. It was anticipated that the brain, spleen, head kidney and gonad would have the highest levels of expression as these tissues play important roles as lymphoid organs (head kidney, spleen) or as immune privileged sites (brain, gonad)[67]. The gonad had the highest level of *vig-3* transcript expression, however the standard error for this tissue was very high. This could perhaps be explained by the variance in sex of the fish; the fish used for the experiment were juvenile and we were unable to determine sex. The other tissues with significant *vig-3* expression included the gut and liver. It is interesting that both the gut cell line and tissue showed constitutive expression. As an aquatic species, it can be postulated that it benefits the Rainbow trout to have relatively higher levels of ISG expression in the gut to protect it from invading pathogens. Complimentary to the present study, a similar study reported elevated *ISG-15* transcript levels in the gonad, gut and liver of healthy fish. In addition to these tissues, *ISG-15* is constitutively elevated in the gill, kidney, muscle and spleen.[30] Differences in constitutive *ISG-15/vig-3* expression may be due to species variation and requires further study.

Once an understanding of *vig-3* constitutive expression was established, induction patterns were explored using various stimuli and RT-qPCR analysis. All stimulant studies were performed using RTG-2 cells as, these cells have a relatively well studied innate immune response[47,48], are easy to grow and are sensitive to all stimuli used in the study. The first stimulant studied were poly I:C, a synthetically manufactured dsRNA analogue. Poly I:C was used because it is a potent type I IFN inducer yet has no mechanisms to evade the host cell immune response, unlike viruses. Over the 48h of poly IC treatment, *vig-3* expression began to rise at 6h however it was induced significantly at 12h post treatment. It then began to decline to baseline at 24 and 48h. These data supports the previous experiments done with sea bass and Atlantic salmon cell cultures where *ISG-15* induction

increased over time[8,36,54.] Sea bass fibroblast cells demonstrated significant *ISG-15* induction at 48h where as RTG-2 *vig-3* expression had peaked and was already back to baseline by 48h[8,36,54]. Interestingly, sea bass brain and spleen cells demonstrated significant increase in *ISG-15* expression at 6h. As these are the tissues that either are reproductive tissue or immune tissue, these tissues may have quick antiviral responses to maintain homeostasis[8,36,54]. Atlantic salmon head kidney cells treated with poly I:C showed variance in a cyclic pattern of *ISG-15* expression from 6h to 48h[8,54]. This finding coincides with the Rainbow trout data, and perhaps could be explained by the cyclic nature of expression of IFN-I, as mentioned previously.

As vig-3 is hypothesized to be interferon inducible, *IFN-1* expression was measured alongside *vig-3* induction, to determine if *IFN* transcript induction preceded that of *vig-3*. *IFN-1* induction was observed to spike at 6h post treatment, begin to decline at 12h and 24h, and then spiked again at 48h post treatment. The induction of *IFN-I* was faster than vig-3, suggesting that *IFN-I* could indeed be inducing vig-3 expression in this system. The *IFN-I* induction pattern is also interesting, as it appears to be cyclic. A similar study in RTG-2 treated with 100 µg/mL of poly I:C did not observe the same cyclical pattern; however, the study evaluated *IFN-I* expression at 24h and 72h but not 48h, so it is possible the down-regulation was missed. Thus, it is possible that the cyclical induction of *IFN-I* is a novel observation and therefore requires further investigation.

Next, the induction of *vig-3* in response to VHSV-IVb infection was explored, by infecting RTG-2 cells with the virus (MOI=1) over a 48h period. During the infection, an upwards trend of *vig-3* induction was observed from 6h to 48h, with significance at 24h and 48h. This trend was expected, as the expression of *vig-3*, an ISG, should be increasing over the course of a viral infection. However, when comparing the level of induction between poly I:C treatment and VHSV-IVb infection, the induction of *vig-3* is much lower in the virus infected cells; *vig-3* expression reached an almost 2000-fold increase at 12h post poly I:C treatment; however, its expression did surpass 20-fold during the VHSV-IVb infection at 48h post treatment. This result suggests that there are

immune evasion mechanisms at play in the VHSV-IVb infected cells. VHSV-IVb is a *Rhabdovirus*, and there have been studies suggesting that rhabdoviruses may have ways in combating the host immune response, specifically by targeting the IFN system[52]. For example, neurotropic rabies virus (RV) a human rhabdovirus, has a phosphoprotein that inhibits the IFN response by interfering with IRFs and STATI signalling[52]. It is therefore possible that VHSV-IVb is producing proteins that inhibit *IFN-1* induction, *IFN-1* transcript levels did not appear to increase substantially at any time point pi, with only a slight induction at 24h. However, the small amount of *IFN* that is induced appears sufficient to induce low levels of vig-3.

A second virus, the *Birnavirus* IPNV, was next explored. RTG-2 cells were infected with IPNV (MOI=1) over a 48h period. During this infection, an upwards trend in *vig-3* expression was observed from 6h to 48h, most significantly at 48h. When comparing the level of *vig-3* expression between viral infections, it is notable that IPNV was able to induce higher levels of *vig-3* compared to VHSV-IVb (~100 fold increase at 12h-48h post treatment), but not as high as poly I:C. IPNV infected cells also demonstrated *IFN-1* transcript expression at 24h and 48h pi at levels that were much higher than those observed in VHSV-IVb cells. This suggests that IPNV could delay but not inhibit the *IFN-1* induction in these cells. Infectious bursal disease virus, a different member of the *Birnaviridae* family, is capable of blocking *IFN-I* induction at the dsRNA detection step in chickens. Therefore, it is possible something similar is happening with IPNV in the RTG-2 cells.

For each stimulant studied, *IFN-1* induction either preceded or occurred at the same time as vig-3 induction, suggesting that vig-3 was being directly induced by IFNs. To pursue this further, RTG-2 cells were treated with a recombinant IFN-1 protein, at both 500ng/µL and 5µg/mL over a 24h period. Recombinant IFN-1 was able to induce significant *vig-3* expression at both timepoints, reaching a maximum of 400-fold increase in expression. This expression level surpassed levels of *vig-3* induction in either virus infection. This novel finding suggests that IFN-I is in fact able to induce *vig-3* transcript expression in the absence of pathogenic stimuli, and furthermore suggests

that Vig-3 is in fact an ISG. Although ISG-15 has been reported to be IFN inducible, there is little to no research exploring this finding within fish. This brings great value to this experiment in Rainbow trout cells.

Once the level of *vig-3* induction was analyzed at the transcript level, the next step was to investigate Vig-3 induction at the protein level. Protein levels of Vig-3 expression were measured using a polyclonal antibody raised in rabbits against recombinant Rainbow trout Vig-3 produced by, Abclonal Science Inc, Woburn MA. RTG-2 cells were treated with either 5µg/mL of poly I:C or infected with VHSV-IVb (MOI=1) or IPNV (MOI=1) over a 48h period and Vig-3 cellular localization was measured by immunocytochemistry (ICC), while induction levels and ISGylation status were determined by Western blot analysis. For ICC, untreated and uninfected cells showed little to no detectable Vig-3 expression. Vig-3 was present at both 12h and 24h post poly I:C treatment and virus infection with both VHSV-IVb and IPNV. This corelates well with the RT-qPCR data. It does appear as though fluorescence is more intense at the 24h time point for all treatments/infections; however, this would need to be quantified in future work. Additionally, it appears as though some cells exhibit more intense fluorescence at the surface of the cells. As mentioned in the introduction of this thesis, it has been suggested that Vig-3 can be secreted from the cell and/or bind to neighbouring cells in order to elicit an enhanced immune response[57]. Perhaps the intense fluorescence at the cell surface is vig-3 preparing to be secreted or perhaps the protein recently entered the cell via interactions with LFA-1[35,58]. Although the mechanism of Vig-3 movement in and out of a cell has not yet been elucidated, many hypotheses have appeared in the literature including movement in secretory vesicles[6,5,14]. ISG-15 was recently found sequestered in gelatinous and secretory granules of human cells, which provides evidence for this finding[6,5,14]. However further analysis is required to determine if this hypothesis holds true.

Protein expression, as evaluated by Western blot analysis, can determine not only Vig-3 presence and induction but also the presence of ISGylation. During poly I:C treatment, a distinct

band at 15kDa was observed from 6h to 48h in the treated groups but not in the untreated control. This 15kDa band would be indicative of free Vig-3, meaning Vig-3 not bound to other proteins. This was to be expected, as it is the appropriate size of the active protein. It is also interesting to note the most prominent band was at 12h, which coincides with the peak in *vig-3* transcript as measured by RT-qPCR. The higher molecular banding pattern is interpreted as ISGylation based on previous literature[29,59,60]. Though not quantitative, it appears as though the ISGylation pattern increases in intensity during the 48h treatment, which coincides with results in mammalian studies testing ISG-15 ISGylation in response to viruses[29,59,60]. Similar results were observed in virus infected RTG-2 cells. In both VHSV-IVb and IPNV infected cells 15kDa Vig-3 expression was induced slightly at 6h and increased in intensity over the 24h infection. The ISGylation bands also appeared to increase in intensity of this timeframe. It is interesting to note that the ISGylation pattern does not appear to be negatively affected by virus infection, as the banding intensity appears to increase over time with both virus infections. It would be interesting to identify the proteins in which Vig-3 is binding to, specifically to see if they differ between viral infections and pathogenic stimuli. Also of interest is that, although *vig-3* was detectable constitutively at the transcript level, it was not detectable in untreated or uninfected cells at the protein level. This may be due to difference in detection limits between assays.

8. Conclusions

Although ISGs are the cornerstone for establishing an antiviral state, nothing had been known regarding *Vig-3*'s induction and expression in Rainbow trout at the transcript and protein levels. Rainbow trout is both economically and ecologically important to the province of Ontario therefore it is essential that there is a well-rounded understanding of the innate immune system of Rainbow trout to protect this valuable species. To this end, inductions patterns of vig-3 were investigated in RTG-2 cells using both poly I:C and virus infections, and ISGylation patterns were also identified. The novel findings of this thesis are as follows: (1) *vig-3* transcript is constitutively expressed at

high levels in gonad, gut and liver, (2) *vig-3* transcript can be induced by poly I:C, VHSV-IVb, IPNV treatments, (3) *vig-3* is an authentic ISG, as it is capable of being induced by IFN-1 directly, (4) Vig-3 protein cell localization patterns are cytoplasmic & nuclear, and (5) evidence of protein ISGylation can be evaluated in rainbow trout cells by western blot analysis. Further research of Vig-3 will elucidate these mechanisms in Rainbow trout which will facilitate the establishment of novel treatments and protection strategies for cultured and wild fish species respectively.

Although this thesis determined much novel information regarding Vig-3, there is much more research to fully understand the proteins antiviral functions. Future experiments for this protein include 1) determining target proteins for ISGylation, 2) determining if Vig-3 is secreted from Rainbow trout cells and 3) is there a cell surface receptor for Rainbow trout cells? Suggested experiments include 1) using ELISAs or pulldown assays, blotting with a different antibody, 2) using molecular weight centricons with the cellular media and blotting for Vig-3 using Western blot analysis and 3) using RTS-11 a Rainbow trout monocyte/macrophage cell line, which is the same cell type in which LFA-1 was recently found as a receptor for ISG-15 in mammals, respectively.

Although this research has been largely focused on cellular biology, there are several biological aspects that took part in the project truly making it integrative. Through the elucidation of Rainbow trout immunity at the cellular level, it shed light on the species at the ecological level, as a loss in this species could interrupt ecosystems and furthermore several other aquatic species. Furthermore, the it wouldn't be without the use and knowledge of chemistry methods such as Western blot analysis to make the research possible. Several different backgrounds of research from immunology, chemistry and microbiology came together to formulate new ideas and troubleshoot problems. Furthermore, ISG-15 has proven to play a role not only in immunity but also mitochondrial function, cancer, and several other roles named throughout. The knowledge of DNA structure and characteristics made it possible to identify the vig-3 sequence as well as explore its expression patterns in response to stressful stimuli. Furthermore, immunology and virology

knowledge made it possible to explore various viruses alongside this pivotal antiviral protein. Environmental and ecological studies shed light on the species of Rainbow trout and signified its importance as a species in Ontario. Amino acid research and protein dynamics allowed analysis of Vig-3 as a protein using Western blotting as well as immunocytochemistry techniques. This research goes to show how integrative biology truly is.

9. Summary

Innate immunity constitutes the first line of defense against virus infections. During their replicative cycles, viruses produce nucleic acids, both RNA and DNA. These nucleic acids are foreign to healthy cells and are sensed by pattern recognition receptors (PRRs), based on their type (RNA or DNA), their strandedness (ss or ds) and their location (endosomal, extracellular or cytoplasmic). When a virus infection occurs, PRRs activate signalling cascades that culminate in the production of type I interferons (IFNs) and the induction of an antiviral state. Interferon stimulated genes (ISGs) are the workhorses of the innate immune system as they establish an antiviral state and stop virus replication. ISGs, such as Mx1-3, have begun to be characterized in many fish species, but almost nothing is known of ISG-15, known as virus-induced gene 3 (Vig-3) in Rainbow trout. ISG-15 is a small protein with many roles in mammalian immunity; it is a ubiquitin-like protein that covalently binds to its target protein in a process known as ISGylation, but it also has roles that appear to be cytokine-like. ISG-15 has been identified in many mammalian species and few fish species and has recently been found to be induced by type I IFNs. As in mammals, the LRLRGG motif of fish ISG-15 is crucial for its antiviral activity, however the mechanism by which it acts is still unknown. This project has elucidated the transcript and protein expression patterns of *Vig-3* in Rainbow trout cells. It was determined that RTG-2 cells can constitutively express *vig-3* at low levels, perhaps as a protection mechanism to prepare for viral infection. It was also found that *Vig-3* was induced by poly I:C treatment and VHSV-IVb and IPNV infection in RTG-2 cells, at both the transcript, protein, and cellular level. Future research will focus on the antiviral activity of Vig-3 during these infections, specifically if the protein is sufficient to protect the cell during viral infections and dampen viral titres. Furthermore, specificity in the activation of vig-3 can be studied, as well as its ability to be secreted, as suggested by mammalian research. This work will significantly further our understanding of vig-3 (ISG-15) in Rainbow trout.

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Appendix I

i. Rainbow trout vig-3 sequence performed by Dr. Sarah Poynter

atggaactca ctataacact tttgaatggg
gactcacatc ccctgacggt tcagccacac accactctgg ggccgctcaa
gagtctgatc
aaccaacact ttggagtggc catggaaagg cagaggctgt caggtgtcaa
tgggaacaac
atcagtctca gcgatgattc aaaaactttg agtgactatg gcctgcattc
aggatccaaa
gtgatggtgc tgattacaga acccactcat atccaggtgt tcctgaaaaa
cgaaaagggc
cagacgcaca catatgaggt ggtgtcaggt gagactgtaa cccagttcaa
agccaaggtc
caaaacaagg agggagtccc agccgaccag cagaggctga ttcacgaggg
caagcagctc
gatgatagaa agaaactgga agactatggt gtcagaaatc taagcactat
tc***acctgacg***
ctccgtctaa ggggaggctg a

ii. Primers used for this study as designed by Dr. Sarah Poynter.

Vig-3 forward primer: TACCTTGAGTGATATTGTGA
Vig-3 reverse primer: TCAGCCTCCCCTTAGACG