Superinfection fitness as a component of overall fitness for a vertebrate RNA virus

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Abstract

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To gain a greater understanding of the overall fitness of IHNV viral variants, this thesis describes the development of a novel in vivo superinfection fitness assay and the application of this assay to the IHNV-*O. mykiss* system. This assay is the first of its kind to be used to study RNA virus superinfection fitness using reciprocal experimental challenges of a natural vertebrate host with two genetic variants (referred to here as genotypes) of the same virus species to define the dynamics of superinfection fitness. In combination with the single infection, co-infection, and transmission fitness assays described in the introduction, we have applied the novel superinfection assay as well as an environmental stability assay to determine whether virulence is associated with increased superinfection fitness or whether viral genotype displacement events correlate with an increase in viral fitness. By examining many aspects of the virus infection cycle such as entry, in-host replication in co-infection and superinfection, virus transmission potential, and stability in the external environment, we present a more complete understanding of the fitness of the IHNV genotypes tested and the possible correlates of fitness such as virulence or displacement in the field.

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Introduction

It has often been said
there's so much to be read,
you never can cram
all those words in your head.

So the writer who breeds
more words than he needs
is making a chore
for the reader who reads.

That's why my belief is
the briefer the brief is,
the greater the sigh
of the reader's relief is.

And that's why your books
have such power and strength.
You publish with shorth!
(Shorth is better than length.)"
— Dr. Seuss

RNA virus evolution and fitness:

RNA viruses cause significant disease in humans and animals throughout the world and can pose serious health concerns for hosts. For many RNA viruses that infect humans, such as measles, dengue, and chikungunya viruses, research has begun to focus on understanding the evolution of these viruses within the human population and determination of the selective pressures driving the spread of these viruses around the world. In addition, the evolution of zoonotic RNA viruses such as eastern equine encephalitis, influenza, and West Nile viruses have also been explored for clues to explain how viruses jump from one host species to another and what viral components are necessary for replication and disease in diverse hosts. Much of this work has demonstrated that RNA viruses tend to be highly diverse both within and between virus species. This high level of diversity between members of the same virus species is attributed mainly to the error-prone replication machinery of RNA viruses. The RNA-dependent RNA polymerase is responsible for as many as one error in every $10^3 – 10^5$ nucleotides polymerized, depending on the virus examined. This relatively high mutation rate, combined with a generation time often calculated in hours, helps to explain the enormous level of within species diversity RNA viruses. For RNA viruses, this level of mutation results in a population of closely related viral variants called a "quasispecies".

Importantly, not all viral variants present in the quasispecies are viable for survival within the host environment and the members of the viral population compete for survival within the host. The term viral fitness has been used to define the ability of a viral variant to replicate and produce infectious progeny in a given environment (25, 44). A viral variant able to produce more progeny in a given environment may be more likely to be transmitted to the next susceptible host, and thus may establish more infections in the host population than other variants. The

overall fitness of a viral variant is a summation of it fitness at various stages of an infection cycle. A virus must be capable of entering a susceptible host and replicating within the target tissues of that host. For many viruses, the progeny must be released from the infected host and that virus must be capable of remaining in the external environment until it encounters another susceptible host to repeat the cycle. Each step in this simplified infection model represents a different environment. Thus at each step, selective pressures, such as the host immune response or environmental temperature changes, are being applied to the quasispecies. These pressures will favor certain viral traits over others and the variant sub-population that possesses the most cumulative traits selected by the various environments will successfully complete the infection cycle to be transmitted to a new susceptible host.

Importantly, not all viruses experience the same selective pressures based on their specific life cycles. For some viruses which are directly transmitted from host to host, such as human immunodeficiency virus (HIV) and hepatitis C virus (HCV), survival outside of the host is not of considerable importance and likely does not provide a significant fitness advantage. In such cases, localization to the site of transmission may be more important. For example, localization of HIV variants to sexual mucosal sites increases their ability to be transmitted to a susceptible host through sexual contact, whereas circulation of other variants in the bloodstream of infected individuals increases their likelihood of being transmitted through injection drug use. Localization to these different environments could select for different viral traits that could make a variant more fit in the acidic environment of the blood or the mucosal environment.

In addition, the host environment can be significantly variable both between hosts and within the same host over time. For example, each individual host may have a unique immune response to the same virus population and an individual's immune response will likely change

over time throughout the course of an infection. How a variant viral population responds to this changing environment will determine its overall success and fitness within that host population.

In a host population where multiple variants of the same virus species co-circulate, infection with multiple variants at the same time can sometimes occur. Theoretically, the likelihood of simultaneous infection with two variants in the field is low, while the likelihood of sequential infection is much higher in these cases. For the purposes of the research presented in this thesis, we have defined superinfection as concurrent infection of a host by two genetic variants of the same viral species resulting from sequential exposures to each variant. In such cases, the competitive interactions between viral variants may determine which variant is subsequently transmitted and may provide one variant with an advantage over the other within that host population. Observations of natural superinfection in humans with hepatitis C virus, human immunodeficiency virus, and dengue virus have been reported, with distinct virologic outcomes. Superinfection with hepatitis C virus leads to competitive exclusion of one genotype and dominance of the other in all but very few documented cases (62, 87, 99). In contrast, superinfection with dengue and human immunodeficiency viruses appears to result in co-existence of multiple genotypes within the host (88, 91). However, the clinical outcomes of superinfection are still not clear and the role of viral fitness in those outcomes is debated.

The ability of RNA viruses to quickly evolve and adapt to changing environments presents a challenge for a host population seeking to predict or prevent infection and disease. However, a greater understanding of the determinants of fitness for RNA viruses will provide clues into the evolution of virulence and may explain the spread of virus genotypes within the host population. The work presented in this thesis demonstrates that fitness measured for different stages of the virus life cycle can differ and two virus variants that appear to have equal

ability to establish an infection and replicate within the host can have significantly different rates of shedding from an infected host and thus different transmission potential. Thus, to be truly informative, an understanding of overall viral fitness must include fitness at multiple stages of the infection cycle so as to gauge the fitness for the entire life cycle.

Fitness defined in the laboratory

When explored in the laboratory, measurements of viral fitness most often involve measurements of within-host replication, either in vitro (cell culture) or in vivo (individual hosts). Through these studies, numerous viral traits have been associated in some way with viral fitness, including polymerase fidelity, immune evasion, and virulence (86, 101, 114, 115, 118, 120). Various assays have been developed to measure replication fitness in the laboratory and are commonly used with a variety of virus-host systems.

Single infection replication kinetics

First, fitness can be measured by the kinetics of replication in a single infection context. This technique has been used to describe fitness of field isolates, the fitness distribution of viral populations, and species specificity (81, 113, 126). By comparing the ability of different variants to replicate over time in cell culture or in individual hosts, differences in replication rate and total viral load can be measured if those differences are sufficiently large or the assay is sufficiently powerful. Lidbury et al. identified a Ross River virus (alphavirus) variant that replicated efficiently in macrophages and demonstrated that its ability to modulate innate immune responses in vitro was correlated to increased replication rate in a mouse model of infection when compared a parental strain of virus (65). An important drawback to comparing fitness between two variants using single infection kinetics is that, in order to exclude background noise

and random experimental variation, detection of statistically significant fitness differences requires that those differences be quite large.

Co-infection fitness

Another common assay for comparing in-host replication fitness between two variants is that of co-infection competition. For our purposes here, we have defined co-infection rather narrowly as the concurrent infection of a cell population or host as a result of *simultaneous* infection with two genetic variants of the same virus species. In a co-infection assay, two variants simultaneously initiate infection (in vitro or in vivo) and are allowed to replicate and compete within that environment for a period of time. The variant which produces more progeny following the replication period is determined to be more fit than the other variant. As with single infection replication kinetic assays, these studies have been performed in cell culture (17, 31, 32, 42, 70, 122) and in vivo for a variety of viruses and host systems (20, 23, 30, 37, 45, 112). Co-infection competition assays are regarded as more sensitive than comparison of single infection replication rates because the issue of experimental variation is removed and a head to head comparison of variants within the same environment can reveal subtle difference in replication (24).

Controlled studies of superinfection

Superinfection differs from co-infection in that one variant is allowed to establish an infection in a given environment and subsequently another variant is applied which has to compete in an entirely different environment than that which was encountered by the first variant. The environment is presumed to have changed because replication of the first variant will have potentially altered the availability of receptors or other cellular components required

for replication. In addition, an immune response may have been triggered by the primary infection which could prove to be a formidable impediment to replication of the secondary virus. Thus, superinfection does not provide a head to head comparison of variants in identical environments, but instead identifies variants that are capable of overcoming a competitive environment which includes productive replication of another variant and a primed immune response of the host. While there has been some work investigating the dynamics of viral superinfection, superinfection fitness has not been extensively explored.

Superinfection of a single cell has been intensively studied in the laboratory for many RNA viruses such as vesicular stomatitis virus, West Nile virus, and Sindbis virus (47, 105, 127). Such studies have defined various mechanisms for superinfection exclusion, defined as the phenomenon observed when prior infection interferes with secondary infection of a single cell. Mechanisms of exclusion include, but are not limited to, receptor downregulation or sequestration (42, 63), trans-acting viral protease to prevent subsequent genome replication (47), or interference with endocytic processes (105). Interestingly, these restrictions to secondary infection tend to function only against homologous viruses, either of the same species or very closely related species, and not against heterologous viruses of different species or genera. In addition, studies of cellular superinfection have identified mutants that can overcome superinfection exclusion mechanisms. For example, through sequential passage of West Nile virus on BHK-21 cells containing viral replicons that mimic a superinfection environment, Zou *et al*. identified mutations in structural genes that enhanced viral replication in both naïve and replicon-containing cells (127). Thus, replication in a superinfection-like environment can lead to the evolution of viral variants with increased fitness in that environment. While such studies performed in cell culture have added valuable knowledge to our understanding of viral

interactions, these systems do not fully mimic the environment within a natural host and in vivo laboratory systems for superinfection studies are generally lacking.

Plant systems have been used to study the clinical outcomes of superinfection by multiple strains of the same viral species. Research has demonstrated the ability of some viruses to inhibit subsequent superinfection while other viral infections can enhance subsequent infection and speed the onset of disease symptoms (59, 102). However, because the physiology and population structure of plants differ considerably from those of vertebrates, it is unclear whether these findings are translatable to vertebrate systems.

A small number of researchers have addressed questions regarding RNA virus superinfection and the interactions between viral variants within a vertebrate host. Superinfection of chickens with two strains of infectious bursal disease virus (IBDV) has recently been reported with the aim of determining the effect of superinfection on pathogenesis (6). Ashraf *et al*. found that infection of young chickens with a mild strain prior to infection with a more pathogenic strain resulted in suppression of disease compared to chickens infected with only the pathogenic strain or chickens infected simultaneously with both strains. The authors suggested that the mild virus interfered with the replication of the pathogenic virus during superinfection, but viral RNA was not quantified to verify this claim. It would also have been of interest to determine if this phenomenon was observed when the pathogenic virus was administered first followed by the mild strain. However, this experiment was not performed. In another important study, Yeh *et al*. measured viral replication following sequential challenges with heterologous simian immunodeficiency virus variants in rhesus monkeys and found that the superinfecting variant replicated to low levels (124). The time between primary and secondary virus challenge was not a controlled factor in this study, therefore conclusions could not be made about the importance of

time between infections on viral replication in superinfection. In addition to these, many studies have investigated the effect of superinfection with viruses of different species in natural and model hosts (1, 49, 52, 69, 117, 121). Thus, the dynamics of in vivo superinfection in controlled experimental studies with variants of the same viral species have yet to be extensively defined for a vertebrate RNA virus and could provide insights into the mechanisms behind many of the field observations of superinfection for other important vertebrate viruses.

Superinfection fitness

An important aspect lacking in superinfection studies, to date, is a more critical definition and assessment of viral fitness in superinfection. Overall superinfection fitness encompasses two distinct parameters: the ability to establish secondary infection and the ability to replicate in the presence of another viral variant within the same host. Theoretically, a virus which possesses greater ability for either parameter will have an advantage over other variants in an environment where superinfection occurs. Developing a laboratory system to study superinfection fitness requires careful consideration of the time interval between exposures to each variant, the order of exposures, and the time of sample collection to determine superinfection prevalence and viral load for each variant. Such assays are highly complex and, unless properly constructed, can be difficult to interpret. However, the information gained by such assays could propel the field forward by providing deeper understanding of viral interactions and the selective pressures driving the evolution of viruses. For these reasons, this thesis presents the development of a novel in vivo superinfection assay which was carefully designed to measure both parameters of superinfection fitness and address the questions proposed.

Theoretical models of superinfection

While experimental data may be limited in the field of viral superinfection fitness, a wealth of theoretical data exists which proposes theories of virus evolution shaped by competition in superinfection. The inclusion of superinfection competition into mathematical models of virus evolution began with Nowak and May in 1994 (77). Using the very simplistic model they developed for understanding the basic reproductive rate of parasites in the context of superinfection, the researchers concluded that superinfection selects for higher levels of virulence and population diversity than single infections. Since this seminal work, many more complicated models have been developed to incorporate host life-history traits, such as immunity or resistance, and multi-species dynamics into the analyses (11, 14, 33, 68, 73). These theoretical hypotheses of virus evolution lead to interesting avenues for experimental research to directly test the assumptions and conclusions of these models. Recent research using the model rhabdovirus, vesicular stomatitis virus (VSV), has demonstrated that superinfection competition can promote adaptation to novel environments *in vitro* (17). Thus, superinfection may in fact apply selective pressures on competing viral genotypes that lead to genetic diversity within the viral population. However, much more experimental research is needed to further validate or expand upon the theoretical models.

Transmission fitness

The ability of viruses to transmit from one host to the next is an important parameter for completing the life cycle in the field. Measurements of transmission fitness have been conducted mainly in arbovirus and plant virus systems. Transmission fitness in arboviruses, such as chikungunya virus and dengue virus, typically involve measurements of virus dissemination or

localization to the salivary glands in the mosquito vector host (5, 30, 38, 60, 74, 111, 112). Plant virus systems have the added advantage that multiple rounds of transmission are easily studied as is investigating the importance of various modes of transmission, such as direct or vector mediated transmission (9, 46, 71, 106). Influenza virus transmissibility has been investigated through measurements of direct transmission between mammalian model hosts (12). As will be discussed later in this introduction, the transmission potential of infectious hematopoetic necrosis virus was determined by measuring the quantity of virus in shed into water surrounding the infected host (120). Importantly, while transmission fitness is often assumed to correlate strictly with in-host replication, there are examples of the exception to this rule presented in the literature and in chapter 3 of this thesis (26).

Environmental Stability

The persistence of infectious particles in the environment outside the host is an important step to ensure completion of the infection cycle for many viruses. The length of time a virus must be stable in the external environment will depend largely on the host population structure and behavior. Within an environment with a dense host population and strong mixing, a virus may only have to survive for short periods outside of the host before it encounters a new susceptible host. In contrast, if hosts are widely dispersed or immunologically protected, survival outside the host becomes a stronger selective pressure on the virus.

Investigations into the survival of viruses in a variety of environments outside the host have led to findings that help to understand how epidemic viruses spread, how virus life cycles impact their environmental stability, and how to design laboratory protocols for effective decontamination of research equipment. Sagripanti et al. sought to determine the survival of an alphavirus, a Lassa virus and an Ebola virus on glass, metal, and plastic surfaces (103). These

surfaces were chosen to reflect the common materials found in hospitals where patients may be under care during an epidemic. The assay was also performed in darkness to determine a maximal persistence of the virus in the absence of the known inactivation by UV light. By comparing the inactivation rate and time to 90% decrease in viral detection, this group determined that the alphavirus was most stable, followed by the Lassa virus, and finally the Ebola virus strain. Interestingly, the authors noted that overnight deactivation of any of the tested viruses should not be expected and these results indicate that highly pathogenic enveloped viruses can persist on fomites for many days in temperate conditions. Similar work to investigate whether virion stability outside the host correlates with the evolved mode of transmission or with epidemiologic spread of different virus species has also been reported (36, 40). Much less research has focused on the comparison of viral variants from the same species in their ability to remain infectious outside the host in natural conditions (43, 89, 123). In two studies comparing different strains of fish rhabdoviruses, no significant differences have been noted between variants of the same virus species.

Overall virus fitness

It is important to note that measurement of any of these parameters is an important step in understanding overall virus fitness. But only by combining the fitness measurements of each step of the infection cycle will we gain a more full understanding of the overall fitness of a virus. Thus, because each component is important, assays to measure each of these parameters are necessary within the same host-virus system to gain a true understanding of the selective pressures driving the evolution of an RNA virus. This thesis demonstrates the importance of collecting measurements of fitness at each stage by describing differences in viral fitness between variants at some stages and not others. Taken alone, each assay may yield opposing

conclusions. But taken together, they provide a full understanding of fitness and indicate which steps may exert a stronger selective pressure on the virus.

Infectious hematopoietic necrosis virus

IHNV genome and structure

Infectious hematopoietic necrosis virus (IHNV) is a single-stranded, negative-sense RNA virus and a member of the Rhabdovirus family. The linear, non-segmented genome is encapsulated in a bullet shaped virion and is composed of 6 genes, with a total length of ~11,000 nucleotides (Figure 1.1A). The six open reading frames (ORFs) are separated by short intergenic junctions and transcribed as monocistronic mRNAs (55, 72). Within the family *Rhabdoviridae*, IHNV represents the type species of the genus *Novirhabdoviridae* (90).

The five structural proteins of IHNV function similarly to other rhabdoviruses and an image of a rhabdovirus virion is shown in Figure 1.1B (40). The viral nucleoprotein (N) forms the nucleocapsid core, tightly wrapping the genomic RNA. The matrix (M) protein lines the host-membrane derived envelope of the virion. In addition to a role in virion maturation, the IHNV matrix protein has been proposed to modulate host gene expression and apoptosis (18). The glycoprotein (G) forms trimers on the surface of the virion and functions in virus attachment and entry as well as being the major viral antigen and target for host immunity. The phosphoprotein (P) interacts with the RNA-dependent RNA polymerase (L) to form the viral RNA replication machinery. Finally, the non-virion (NV) protein is not present in other rhabdoviruses outside the genus and therefore distinguishes the members of the Novirhabdovirus genus from other rhabdoviruses. The NV protein is not found packaged within mature virions but is expressed within infected cells. While the NV protein is not required for replication in all cases, this protein

is required for pathogenicity in a natural host, rainbow trout (58, 107). The NV protein has also been implicated in roles to modulate the apoptotic pathway in infected cells and inhibit the interferon pathway, thereby evading a productive immune response by the host (2, 19, 80).

IHNV infection and host response

IHNV establishes acute, lethal infection in juvenile Pacific salmonids and results in significant losses to aquaculture industries every year. IHNV is transmitted horizontally, through shedding from an infected host into the surrounding water, or vertically, through egg-associated virus. Although vertical transmission can occur and may have some importance in wild populations, current disinfection treatments of eggs in rearing facilities has limited this route of infection in fish culture (7). In water-borne infection, the virus has been demonstrated to enter through a number of separate sites such as the gill tissues, the base of the pectoral fin, sites of abrasion on the skin, and through the digestive tract (15, 41). Thus, IHNV can potentially enter the host and establish productive infection through multiple sites.

Infection results in necrosis of the kidney and spleen with mortality occurring between five and 20 days post-infection. Field isolates of IHNV have been tested for virulence in natural hosts in the laboratory and demonstrate a wide range of virulence in various host species (8, 51, 61, 83, 84, 92, 108, 118, 120). This range in virulence likely results from virus specific traits and variance in the host immune response to infection. Survival of acute infection appears to depend largely on the interferon (IFN) system. Studies in a variety of hosts have demonstrated the strong activation of this system through measurements of the induction of IFN stimulated genes following infection with IHNV (39, 50, 79, 84, 94, 97, 104). Further, triggering the IFN system with injection of the TLR3 agonist poly:IC prior to challenge with IHNV protects against mortality and reduces the viral load in rainbow trout and sockeye salmon, (Peñaranda

unpublished data,(10)). The ability of virulent strains to replicate in the presence of the host innate immune response is thought to be in part due to the functions of the matrix and non-virion proteins to suppress these responses (18, 107). Recently, transcriptome analysis of the hematopoietic tissues in IHNV infected fish identified a number of interferon stimulated genes (ISGs) which were upregulated in response to infection (97). Further investigations into the immune profile of salmonids during IHNV infection will provide more clues into the mechanisms of protection and innate immunity during acute viral infection.

Survival of acute IHNV infection generally results in long-lasting protective immunity. Protective immunity can also be elicited through vaccination against IHNV in juvenile fish. Vaccines can take the form of inactivated virus, recombinant proteins, or DNA (3, 34, 56, 66). Generally, DNA vaccination results in broad protection against multiple strains of IHNV (66, 82). Adaptive immune protection is associated with the development of memory T and B lymphocytes which respond rapidly following subsequent infection (54). Highly effective DNA vaccines for IHNV have been developed, but are not licensed for use in the U.S. and logistical challenges associated with delivery by injection prevent wide-spread use in juvenile fish. Therefore, IHNV remains a constant threat to the health of salmon populations in the Pacific Northwest region.

IHNV phylogenetics

Phylogenetic analysis has established five genogroups of IHNV worldwide: U (upper), M (middle), L (lower), E (Europe), and J (Japanese). U, M, and L correspond to regions of the North American Pacific Northwest. Genogroup E is closely related to the North American M genogroup (29). Genogroup J is derived from North American U and is found primarily in Japanese and Korean rainbow trout (53). Both genogroups found outside of North America are

thought to have derived from importation of infected salmonid eggs from the endemic region of North America.

While not entirely species specific, the prevalence of viruses belonging to each genogroup does vary depending on host species. Specifically, genogroup U is predominantly isolated from Sockeye salmon (*Oncorhynchus nerka*), but also in Chinook salmon (*O. tshawytscha*), and occasionally steelhead trout (*O. mykiss*). Genogroup L is found mainly in Chinook salmon, but occasionally in steelhead trout as well. Finally, genogroups M, J, and E often cause epidemics and disease in rainbow and steelhead trout (freshwater and anadromous forms of the same species, *O. mykiss*). However, viruses belonging to the M genogroup have also been isolated from Chinook salmon in the Pacific Northwest. Phylogenetic analysis of a 303 nucleotide region of the glycoprotein gene from 323 isolates collected throughout North America demonstrated that genogroup M likely diverged from genogroup U (57). Further analyses including sequences from over 2000 isolates support this conclusion (27).

IHN disease epidemics were first identified in sockeye and Chinook salmon following the development of fish rearing facilities in the western U.S. beginning in the 1950s and were caused by genogroup U and L viruses respectively. The first IHNV epidemics known to be caused by M genogroup viruses were observed in 1978 at two rainbow trout farms in the Hagerman Valley, ID (57). From 1978-1980 IHNV quickly spread throughout the valley in rainbow trout farms where it has been endemic ever since. Thus, the emergence of the M genogroup occurred within the rainbow trout farming industry and M genogroup viruses have also spread within the Columbia River basin (CRB) region where it co-occurs with endemic U genogroup viruses (see Figure 1.2) (13, 35, 57, 109). The research presented in this thesis was performed exclusively with M genogroup viruses in rainbow and steelhead trout.

IHNV genotype displacement events

Since the emergence of IHNV in the Columbia River basin in the early 1980s, surveillance of the virus throughout the Pacific Northwest has been continuously conducted by various state, federal, and tribal fish health laboratories. Over the last 15 years genetic characterization of over 2000 virus isolates from the region has led to a greater understanding of the movement of this virus throughout the region (13, 28, 35, 57, 109, 110). Within the M genogroup of IHNV, a series of virus genotype displacement events were observed in the Columbia River basin and on the Washington state coast (Figure 1.2). Displacement events are characterized by the replacement of a previously dominant virus genotype with a novel genotype in a given region. Dominant genotypes were identified by the number of viral events, defined by features such as site or host species (13), from which a specific genotype was isolated within the region of interest. A genotype which was isolated from more sites, hosts, and years was determined to be the dominant genotype for the time period. During the surveillance period between 1980-1994, genotype mG007M (referred to hereafter as 007) was the only M genotype genotype isolated in the Columbia River basin (35). While other genotypes were isolated just once or twice over one or two consecutive years, genotype 007 persisted at multiple locations for several years and was last isolated in 1994. Genotype mG111M (referred to here as 111) was first isolated in 1994 and subsequently became the dominant genotype isolated from the lower Columbia River basin until 1999. A gap of about three years then occurred for M genogroup isolates in our database, but beginning in 2002, a novel genotype, mG110M (110) was isolated and emerged to strongly dominate the virus population in the Columbia River basin. This virus genotype remains the dominant type isolated in the lower Columbia river basin from wild and hatchery raised steelhead trout, despite the occasional detection of other IHNV M genogroup

variants throughout the basin, including mG139M (139). Genotype 110 was also responsible for a large emergence event of IHNV M in steelhead trout on the Washington coast beginning in 2007 (13). Interestingly, genotype 139 is now the dominant genotype isolated from the Washington coast, replacing genotype 110 in 2010 (13). Continuous surveillance efforts have added a great deal to our understanding of virus spatial paterns over the last 30 years and genetic typing has led to many investigations regarding the selective pressures driving the evolution of this virus in the field.

These displacement events observed for genogroup M viruses are similar to replacement events that have been observed for many other vertebrate RNA viruses including dengue, rabies, measles, West Nile, and chikungunya viruses (16, 21, 22, 64, 76, 111, 116). Numerous theories have been postulated to explain such events including, but not limited to, random genetic drift resulting from stochastic processes or bottleneck/founder events, antigenic variation to escape host immunity, changes in host specificity, and changes in viral replication or transmission fitness. While some groups have found positive correlations between displacement events and viral fitness (5, 38, 60, 74, 112), other groups have determined that differences in fitness could not explain these events (67, 75, 78). It is possible, if not probable, that different pressures, or a combination of pressures, have acted on all these viruses leading to a common phenomenon of genotype displacement. Therefore, it is necessary to investigate these displacement events independently to discern the unique selection pressures acting in each host-pathogen system and to apply that knowledge to inform disease prevention strategies that may differ between systems.

Fitness and IHNV

With the availability of numerous virus isolates from the field, a main research focus in the Kurath laboratory has been the development of novel, in vivo virus fitness assays to

investigate fitness and the evolution of IHNV in different natural host species. These virus isolates are hereafter referred to as genotypes, as they are used as representatives of known genotypes from the field. These assays have been used to understand the importance of fitness in virulence and to investigate host specificity of IHNV.

The first assay to be developed was an in vivo co-infection fitness assay described by Troyer *et al.* involving the competition of two M genogroup viruses of equal virulence, B and C, in juvenile rainbow trout (108). This assay involved the challenge of groups of rainbow trout in batch via immersion in water containing either a single genotype or a 1:1 mix of both virus genotypes, followed by separation of individual fish into individual tanks in static water conditions. This separation prevented cross-infection and allowed for each infection to remain independent. Because it was determined previously that peak viral load for both genotypes occurred at 3 days post-infection, individual fish were harvested at 72 hours post-challenge and analyzed for the presence and quantity of each genotype in individual, whole fish. To determine the relative quantity of each genotype in co-infected fish, a genotype-specific restriction digest assay was designed which amplified both genotypes but resulted in fragments of different length depending on the genotype present. Densitometric analysis allowed for relative quantification of each virus genotype present in the samples. They observed that these two genotypes, genotype B and genotype C, demonstrated equal relative fitness in co-infection in rainbow trout (108).

To follow these observations, two postdoctoral researchers in the Kurath laboratory, Kyle Garver and Andrew Wargo, performed a series of experiments to test the correlation between virulence and co-infection fitness in rainbow trout (118). Virulence in this system is defined as the mortality resulting from viral infection. To test virulence, triplicate groups of 20 fish were challenged with single genotypes and mortality was measured for 30 days post-exposure.

Virulence is then assessed by comparison of the kinetics and final cumulative percent mortality in each group. The co-infection fitness studies involved the use of two genotypes of unequal virulence: one genotype of high virulence (HV) and one genotype of low virulence (LV). Genotype HV has consistently been shown to have greater virulence than genotype LV, and typically induces approximately 80% mortality in exposed fish, whereas LV induces approximately 20% mortality, at the same challenge dosage (118). Dr. Wargo modified the original assay and developed genotype specific quantitative PCR (qPCR) assays to quantify each genotype present in individual whole fish and to distinguish these genotypes in mixed infection. Importantly, these qPCR assays included transcript standards for quantification of absolute viral RNA rather than relative quantities in mixed infections. The first study published by Wargo *et al.* demonstrated that, for the two genotypes tested, virulence correlated with replication fitness in both co-infection and in single infections (118).

To expand upon these observations, Dr. Wargo then developed novel assays to investigate whether fitness for host entry and transmission were also correlated with virulence in this system (120). Intraperitoneal (IP) injection of IHNV into salmonids can result in infection, disease and death similar to challenge by immersion. The difference is that IP injection bypasses the requirement for the virus to enter the host, breaking through the first barriers of defense. Quantification of virus following IP injection thus assesses only the in-host replication of each genotype. Viral quantification following immersion challenge assesses fitness associated with both entry and in-host replication. By comparing fitness assayed by immersion versus injection challenge, he could assess whether differences in host entry contributed to the overall fitness differences between HV and LV. Finally, by quantifying the concentration of viral RNA present in the water at the time of harvest, Dr. Wargo was able to quantify total virus shed from

individual fish of each genotype in single infections and in co-infection. From these assays, it was concluded that virulence was associated with increased fitness for each of the three steps of the infection cycle, with that increased fitness being greatest for in-host replication (120).

In continuation of this line of research, an expanded IHNV shedding assay has been developed to monitor the dynamics and rate of virus shedding by infected fish over 30 days post-infection. The previous shedding assay described above quantified the total virus shed over three days post-infection. In this previous assay, total virus shed was measured before mortality began at 5 days post-infection, so the true potential cost of virulence (death and thereby removal of an infected host from the system) would not have been measured. Theoretically, if the majority of hosts infected with a high virulence genotype (HV) perished early in infection, the total quantity of virus shed could be less than that from hosts infected with a low virulence genotype (LV) that persisted to be shed for a longer duration (4). The opportunity to empirically test this "trade-off" hypothesis was the motivation behind the development of this novel, long-term shedding assay (4). By holding individual fish in separate tanks and flowing water through the tanks at regular intervals to flush accumulated virus, Dr. Wargo has quantified the mean rate of virus shedding each day over 30 days following infection with each genotype or a 1:1 mix of both genotypes. Surprisingly, fish infected with the high virulence genotype (HV) shed significantly more virus over the 30 day period than those infected with the low virulence genotype (LV) regardless of mortality (Wargo, Scott, and Kurath, unpublished data). This difference was largely due to the clearance of LV from infected fish at a faster rate than in fish infected with HV, even though the peak viral shed early in infection with either genotype was equal. These results have been independently repeated with two other pairs of genotypes with unequal virulence, indicating that

a fitness trade-off for virulence, if it does exist for this system, is not based on long-term shedding.

IHNV fitness and host specificity

To investigate the mechanisms behind the host preference of virus variants belonging to genogroups M and U, a series of studies was undertaken both in vivo and in vitro. The in-host replication fitness of an IHNV genotype representative of genogroup U, which causes disease in sockeye salmon, was compared to the fitness of a genotype representing the genogroup M, which causes disease in rainbow trout. The previously designed co-infection assay was used by Dr. Michelle Peñaranda to compare these two virus genotypes in rainbow trout and sockeye salmon (83, 84). Using new genotype-specific RT-qPCR assays the M genotype was quantified at higher viral loads in rainbow trout compared to the U genotype. Conversely, the U genotype replicated to higher viral loads than the M genotype in sockeye salmon. These results were observed in both single infections and co-infections. Additionally, innate immune stimulation was observed to correlate with viral load and induction of IFN-stimulated genes lagged behind viral replication. Therefore, it was concluded from these studies that in-host replication fitness was host-specific, and correlated with host-specific virulence in each of these hosts. To expand upon this work, a series of in vitro studies conducted by Dr. J.W. Park demonstrated differential growth of the U and M IHNV variants in a rainbow trout cell line, ultimately suggesting that differential interactions with cellular protein kinases may be responsible for the specificity (80, 81). This work demonstrates that host specificity is associated with in-host replication and virulence.

Taken together, these assays represent a significant collection of tools for in vivo studies of RNA virus evolution using a natural vertebrate host-virus relationship. The findings described

above contribute to our knowledge of the viral determinants of fitness and the key steps in the infection cycle that impact overall fitness of virus genotypes. Such studies contribute to the field of virus evolution by allowing for rigorous experimental testing of theoretical hypotheses put forth over the last 30 years.

Contributions of this thesis:

Differences in viral in-host replication fitness are often hypothesized to explain the evolution and spread of viruses throughout a host population. However, in-host replication is only one aspect of the virus life-cycle and selective pressures can be applied at other stages such as entry or release from the host, stability within the environment, and replication in the face of immune pressure. Thus, to fully understand the selective pressures driving the evolution of a particular virus, a greater understanding of fitness at each stage of the infection cycle is necessary. The work presented in this thesis describes the overall viral fitness of divergent genotypes of infectious hematopoietic necrosis virus (IHNV) defined though in vivo assays to measure host-entry fitness, shedding, in-host growth kinetics, environmental stability, and in-host replication fitness in co-infection and superinfection contexts.

For this graduate thesis, I developed a novel, in vivo superinfection fitness assay to define the dynamics of IHNV superinfection in rainbow trout and to investigate the role of virulence in superinfection fitness (48). The work presented in chapters 3 and 4 describes the use of this assay with a genotype pair of equal virulence (B and C) and a genotype pair of unequal virulence (HV and LV). With superinfection fitness defined as the ability to establish a secondary infection and the ability to replicate in the presence of another genotype within the same host, we measured the frequency with which superinfection occurred and the viral load of each genotype in a

superinfection context and compared that to the viral load in the context of a single infection (48).

In addition to superinfection fitness, we examined the overall fitness of genotypes B and C through co-infection competition assays, presented in chapter 3. Immersion co-infection competition experiments allowed us to examine host entry and in-host replication fitness. Injection co-infection experiments provided an indication of the in-host replication fitness but by-passing the entry step. Finally, quantification of the virus shed following immersion co-infection provided and overall assessment of all three steps of the infection cycle.

Additionally, Chapter 5 explores the hypothesis that IHNV genotype field displacement events were associated with increased fitness in the natural host steelhead trout. Four IHNV isolates from the M genogroup were selected to represent the four virus genotypes involved in three major genotype displacement events observed in Washington state. Fitness was measured using single infection replication kinetics, in-host replication in co-infection, and superinfection competition (Kell *et al.*, manuscript in preparation). While the observations varied slightly between genotype pairs, we found no evidence to support the hypothesis that the virus genotype observed to displace a previously dominant genotype possessed greater fitness by any measurement.

This thesis describes the quantification of overall viral fitness through the use of multiple in vivo virus competition assays to determine fitness at multiple steps in the infection cycle. By determining the role of viral fitness in virulence or genotype displacement events, this thesis contributes significantly to our understanding of the selective pressures which drive virus evolution in the field and demonstrate the complexity of factors that may be involved in selection of virus variants in a single host and in the larger host population. Further research into overall

viral fitness using other virus-host systems will be necessary to build upon this foundation and inform the field about the potential generality of these findings.

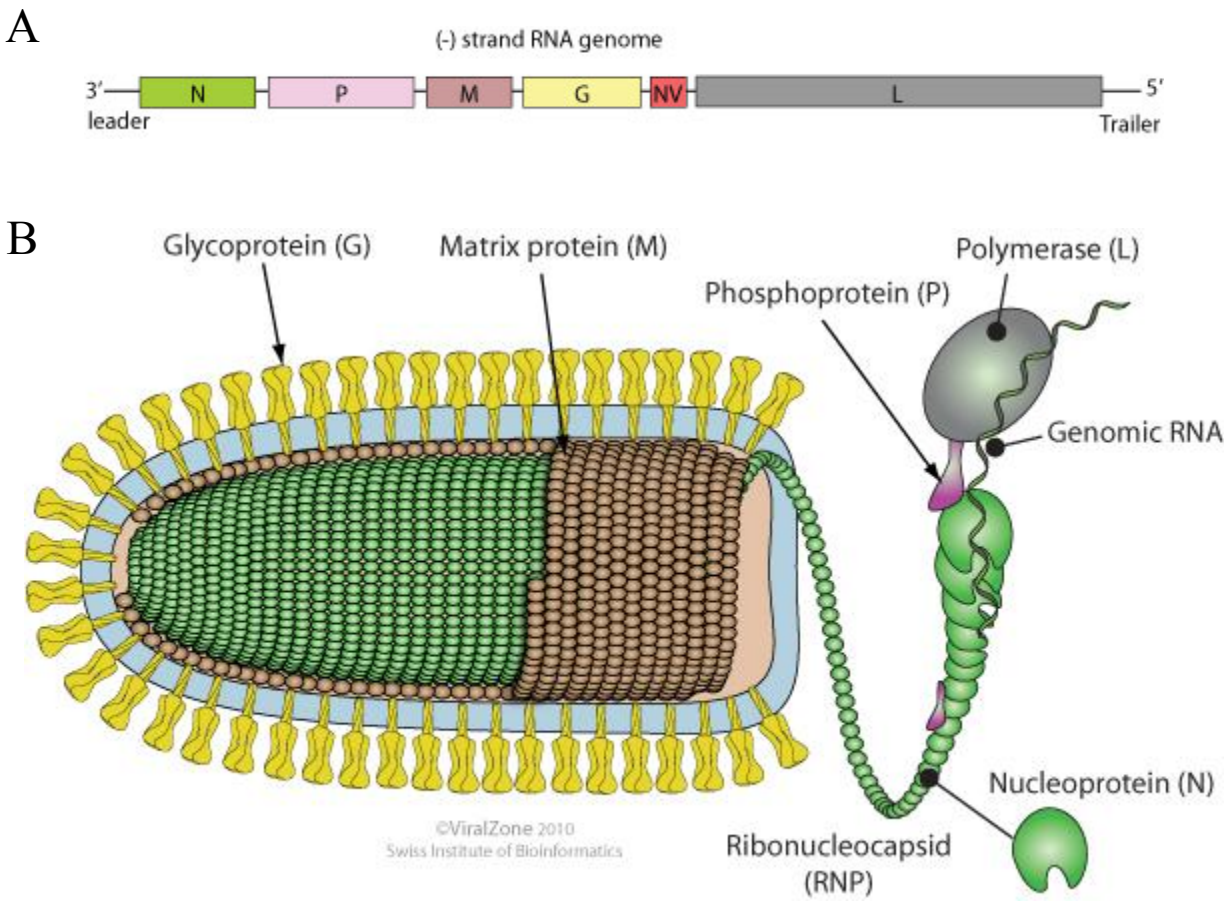

Figure 1.1. Rhabdovirus genome organization and virion structure. A) Genome organization of a general novirhabdovirus. B) Cartoon representation of structure of a general rhabdovirus. (Swiss Institute of Bioinformatics, http://www.ViralZone.com, 2010)

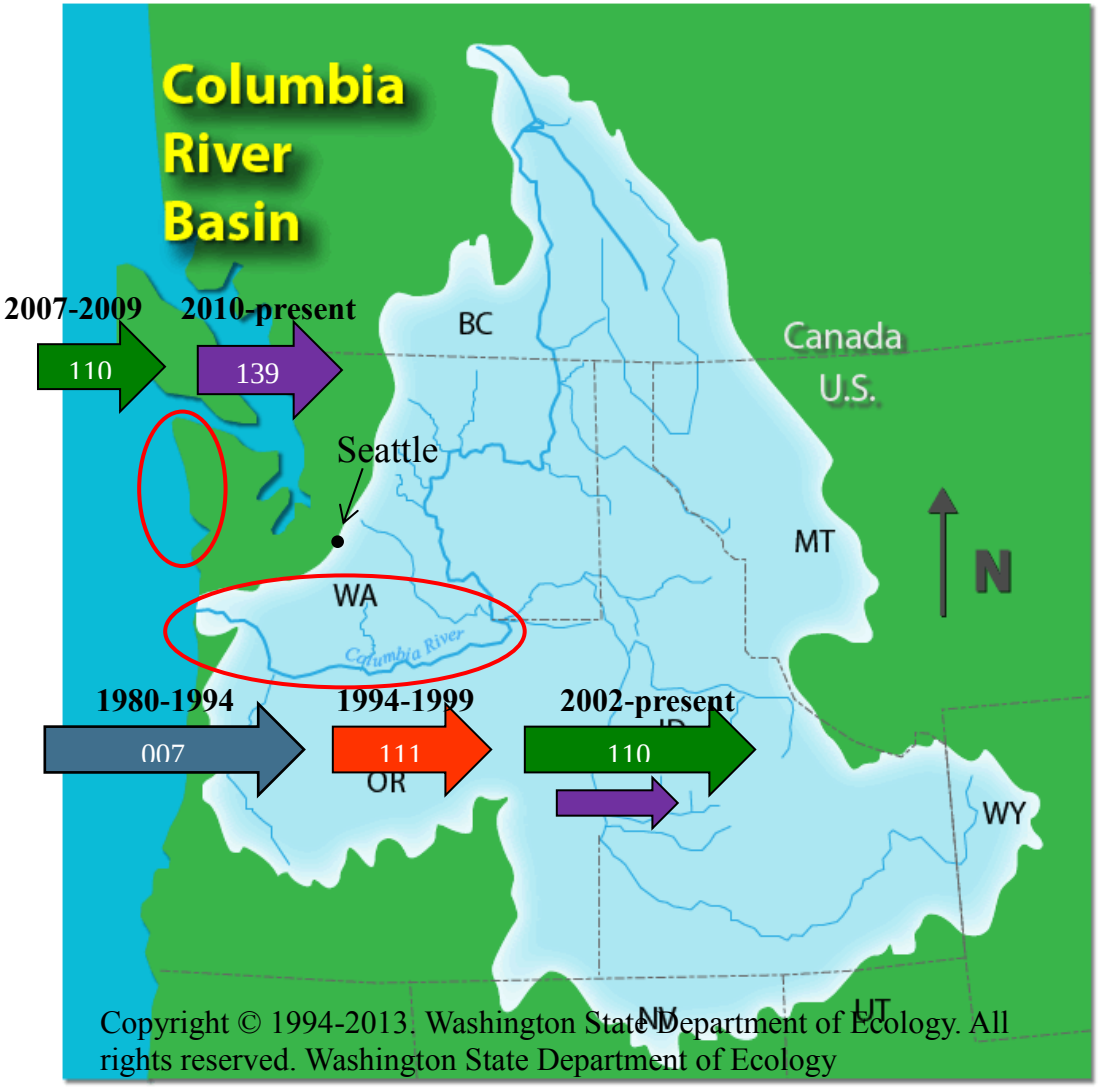

Figure 1.2 Genotype displacement events in the lower Columbia River basin and Washington state coast. Arrows represent dominant genotypes present in each region. Dates of dominance are shown above respective arrows. Map modified with permission from Office of Columbia River.

Chapter 2

Materials and Methods

“If you want to catch beasts you don't see every day,
You have to go places quite out of the way,
You have to go places no others can get to.
You have to get cold and you have to get wet, too.”
— Dr. Seuss

Ethics Statement

All animal procedures were completed in accordance with recommendations by the American Association for Laboratory Animal Science and approved under a University of Washington IACUC protocol (UW 3042-12, 3042-13, 3042-15). All effort was taken to minimize suffering.

Virus and host

Four genetically distinct isolates (genotypes) of IHNV, herein labeled B, C, HV, and LV were used in studies to examine the role of virulence in superinfection fitness. These genotypes were originally obtained from farm cultured rainbow trout in the field and subsequently characterized for virulence (35, 108, 118). All four genotypes belong to the M genogroup of IHNV and thus have evolved host specificity for rainbow trout (57). Genotypes HV and LV were previously referred to as 220-90 and WRAC/039-82 respectively (110). Genotypes B and C were previously described as FF020-91/MB20 and FF030-91/MC30, respectively (108). All virus genotypes were maintained through propagation, between 4-9 passages, on cyprinid fish epithelioma papulosum cyprini (EPC) cells and stored at −80°C as described elsewhere (118). Preparation of working stocks of virus and high accuracy quantification by plaque assay was performed as previously described (118). The genotype pairs used here were found to have equal stability for the duration of 12 hour challenge conditions (Wargo AR, data not shown).

All experiments in the study to examine the role of virulence in superinfection fitness were performed using juvenile, 1-2 gram, research-grade rainbow trout (*Oncorhynchus mykiss*) obtained from Dr. Scott LaPatra, Clear Springs Foods, Inc. Fish were maintained in sand-filtered, UV-irradiated, virus-free water at 15°C. Multiple lots of fish were used for the experiments

described here. Each lot of juvenile rainbow trout used for experimentation was held in a single tank as one population prior to use. Experiments with genotypes B, C, HV, and LV were performed at 15°C.

Four genetically distinct isolates (genotypes) of IHNV were used in the study to examine the role of viral fitness in genotype displacement events observed in the field. These isolates are herein labeled 007, 111, 110, and 139. All genotypes were originally obtained from naturally infected steelhead trout (*O. mykiss*). All virus genotypes were maintained through propagation on cyprinid fish epithelioma papulosum cyprini (EPC) cells and stored at −80°C as described elsewhere (118). Preparation of working stocks of virus and quantification by plaque assay was performed as previously described (118).

The displacement studies were performed using juvenile, 1-2 gram, steelhead trout belonging to the Lake Quinalt steelhead stock, obtained as eyed eggs from the Quinalt National Fish Hatchery located on the Olympic Peninsula in Washington State, USA. Fish were maintained in pathogen-free water at 10°C and the population of fish was held in a single tank prior to use. All experiments were performed using eggs from brood year 2012, with the exception of the repeated superinfection experiment performed with genotypes 111 and 110 with a 48 hour interval, which was performed with eggs from brood year 2013. All experiments using steelhead trout were conducted at 10°C.

Virulence Assays

Triplicate groups of 20 fish were challenged in batch with each virus genotype and monitored daily for mortality for a total of 30 days as previously described (108). Briefly, individual fish were randomly assigned to groups of 20 fish and held in 1 liter of static water with aeration for 1 hour with a challenge dose of $2x10^5$ plaque forming units/milliliter (pfu/mL).

Following challenge, fish were held for 30 days in flowing water with aeration. Mortality was recorded each day and deceased fish were removed from the tanks. One set of triplicate tanks of 20 fish was exposed to virus-free minimum essential media (Flow Laboratories, Rockville, MD) during the challenge period and maintained as a mock infected control group.

Co-infection fitness assays

Co-infection fitness was assessed as previously described (108, 118). Briefly, for immersion challenge, groups of 28 randomly sampled fish were challenged in batch with either a single genotype at a challenge dose of $1x10^4$ pfu/mL, with a 1:1 ratio of two genotypes at a total challenge dose of $2x10^4$ pfu/mL, or mock challenged with virus-free media. Following 12 hour static challenge with aeration, fish were rinsed in flowing water for one hour, and individual fish were then separated into individual beakers filled with 400mL static water and held for 72 hours following the end of the 12 hour challenge. One milliliter of water from each beaker was sampled at 72 hours following the end of challenge to be used to quantify virus shed by each individual fish following immersion challenge. All fish were euthanized at 72 hours following the end of 12 hour challenge and stored at -80°C.

For injection challenges, individual fish were anesthetized with buffered MS-222 and IP injected with 100 pfu of each virus genotype, or a 1:1 mix of both genotypes for a total dose of 200 pfu. After recovery from anesthesia, individual fish were placed into individual beakers and held in static water for 72 hours following injection. All fish were euthanized at 72 hours and stored at -80°C.

Superinfection Assay Development

Because this was the first in vivo assay designed to utilize a stand-alone tower tank system for *O. mykiss* infection (see Figure 2.1), we first needed to establish an infection protocol and determine the minimum infectious dose at which 90-100% of exposed fish would be infected with IHNV. We began by measuring the percent of fish with detectable viral load three days following exposure to IHNV genotypes B and C under a variety of experimental conditions shown in Table 2.1. Harvest at 72 hours post-infection was chosen because peak viral load was observed for all IHNV genotypes to be between three and four days post-infection. Based on these pilot experiments, we decided on a preliminary experimental infection protocol wherein juvenile rainbow or steelhead trout were exposed to a virus dose of $5x10^4$ plaque forming units (pfu) per milliliter via static immersion in a total volume of 400 mL for 12 hours. However, upon application of this assay to study superinfection using genotypes HV and LV, we observed a decreased infection frequency in fish exposed to genotype LV at the same dose of $5x10^4$ pfu/mL (data not shown). Therefore, a second series of conditions was tested using genotype LV, as shown in Table 2.2. Based on the results of these experimental infections, we chose to increase the dose of infectious virus to administer to $2x10^5$ pfu/mL and to decrease the volume of water in which the individual fish were immersed to 200mL for both primary and secondary challenge. Thus, all superinfection experiments presented here and used for analysis were performed with this latter infection protocol.

The experimental design was developed to maximize the power of statistical analyses and to maximize the potential for biologically relevant comparisons, within the overall limit of 79 fish per stand-alone tower system (Aquatic Habitats) (Figure 2.1). For each experiment, 79 individuals were randomly selected from a single lot of fish (one population) and netted into

individual 1.5L tanks each containing 200 mL of static water. Each treatment group was randomly assigned 9-15 fish, with the exception of the mock control group, which was assigned 4 fish (scheme shown in Figure 2.2A). Tanks were arranged in the tower system in a blocked design so that each row contained individuals from all treatments to control for potential row effects. Water temperature was held at a constant 15°C for rainbow trout, or 10°C for steelhead trout, for the entirety of the experiment. A visual representation of the assay can be found in Figure 2.2B. Challenge was initiated by adding 1mL of water containing $4x10^7$ pfu/mL of a single genotype to each individual tank (final concentration $2x10^5$ pfu/mL). Fish were then held in static challenge for 12 hours. Fish undergoing a mock challenge were exposed to 1mL water containing minimum essential media (Flow Laboratories, Rockville, MD) in place of virus. To maintain constant water temperature during the static challenge, each row of tanks was held in a flowing water bath containing water at either 10°C or 15°C. Following 12 hour static immersion challenge, water lines were connected to each tank and water flow was initiated and allowed to flow for at least one hour at the full tank volume of 1.5 liters to remove the challenge inoculum from the tank. Previous experiments have demonstrated that 1 hour of water flow through the tanks is sufficient to remove any virus detectable by RT-qPCR from the water in the tanks (120). Fish were held in flow-through water conditions for the desired interval time before secondary challenge. Interval time was calculated to be the time between start of each challenge. For example, for the 24 hour challenge interval, fish were held for 12 hours in static water with the primary virus genotype, then 12 hours in flow-through conditions, and then challenged for 12 hours in static conditions with the secondary virus genotype (equals a total of 24 hours between initiation of primary challenge and initiation of secondary challenge). All fish were held in flowing water conditions for 72 hours following the completion of secondary 12 hour challenge,

and then all the fish in the experiment were euthanized by adding an overdose of MS-222 solution buffered with sodium bicarbonate directly to each tank. Each individual fish was placed into an individual collection bag and stored at -80°C until RNA extraction described below.

For this assay, we defined 8 treatment groups as shown in Figure 2.2A. Groups 1 and 2 are designated as reciprocal superinfection groups that were sequentially exposed to both viral genotypes. Groups 3 and 4 were exposed to just one genotype during the primary exposure period and then subsequently exposed to virus-free media (mock-exposed) during the secondary exposure period. Groups 5 and 6 were exposed to virus-free media during the primary exposure period and then subsequently exposed to only one of the genotypes during the secondary exposure period. Groups 3-6 served as single exposure controls and were used to inform the interpretation of observations from the superinfection groups. For most experiments, we included a simultaneous co-infection group exposed to a 1:1 ratio of both genotypes during the secondary exposure period (total exposure $4x10^5$ pfu/mL). Finally, for all experiments, we included a group exposed to virus-free media during each exposure period to serve as mock controls.

Fish challenge for in-host viral replication kinetics and Mx-1 gene expression

For each experiment, 114 individual fish were randomly selected from a single lot of fish (one population) and netted into individual 1.5L tanks in a stand-alone tower system (Aquatic Habitats). Tanks were filled with 200mL static water. Each treatment group was randomly assigned 45 fish, with the exception of the mock control group, which was assigned 27 fish. Individual fish were challenged with $2x10^5$ pfu/mL of a single virus genotype, or mock challenged, and held in static conditions for 12 hours. Water flow was then initiated and continued for the entirety of the experiment. At sampling time points 0, 13, 24, 48, 72, 96, 120, 144, 168 hours post-initiation of challenge, 5 fish from each virus-challenged group and 3 fish

from the mock-challenged group were euthanized and stored at -80°C until RNA extraction and qPCR analysis as described below.

Viral load quantification

Total RNA was extracted from individual, homogenized, whole fish using guanidine-thiocyanate as described previously (118). All samples then underwent a general cDNA synthesis reaction using random hexamer and oligo-dT primers that transcribed all viral genotypes and host RNA (118). For quantification of viral load, cDNA was diluted 1/10 and then subjected to quantitative PCR using genotype-specific assays targeting either the virus glycoprotein (G) gene or the non-virion (NV) gene. Briefly, the qPCR reactions consisted of 5uL diluted cDNA, 200nM genotype-specific, minor grove binder (MGB) probe, and 900nM of each genotype-specific primer in a 12uL total reaction volume as described elsewhere (119). A control reaction containing RNA transcripts of the G or NV genes of each genotype was included in each set of cDNA synthesis reactions and then utilized in each qPCR run to generate an 8-step, 10-fold dilution series standard curve for each assay (118). Genotype-specific quantitative PCR assays for genotypes B and C and HV and LV were based on the viral G gene and have been previously described (118, 119). Genotype-specific quantitative PCR assays for the displacement genotypes were based on the G or NV genes and are described below. Since the RT-qPCR methodology quantifies RNA copies, it provides a measure of total viral RNA (both genome and message) of each genotype and not a direct measure of infectious particles (94). Therefore, we use the term "viral load" to refer to total viral genome copies plus viral mRNA per gram of host tissue at the time of harvest. A consistent relationship between viable virus quantities determined by plaque assay and qPCR viral load has been reported for IHNV in rainbow trout (94).

Measurement of Mx-1 induction

cDNA derived from individual whole fish used for the viral replication kinetic experiments was also used for qPCR analysis of rainbow trout Mx-1 transcription in IHNV infected versus mock infected fish. The primer and probe sequences for this assay have been previously described (94). The expression level of Mx-1 was normalized against the expression level of a housekeeping gene, acidic ribosomal phosphoprotein P0 (ARP) (94). Analysis and calculation of fold change relative to mock-infected control groups has also been previously described (94).

Quantification of viral RNA shed into water

To determine the quantity of virus shed, viral RNA was extracted from 550 µl of each of the water samples taken at the time of fish harvest, using the QIAamp MinElute virus spin kit (Qiagen) scaled up to the sample volume (120). At the end of the protocol, the extracted water samples were eluted in 20 µl diethyl pyrocarbonate (DEPC)-treated nuclease-free water (Growcells) and stored at −80°C. After RNA extraction, all samples underwent a general cDNA synthesis reaction. The cDNA derived from these samples was diluted 1:5 and genotype specific qPCR analysis was performed as described above.

Environmental stability of virions

Stability of virus isolates in water at various temperatures and salinity was measured as the decay rate of plaque forming units in water over time post inoculation. For freshwater assays, water was collected from Lake Washington at the boat dock in Magnuson Park, Seattle. The water temperature at the time of collection was 10.8°C. For saltwater assays, water was collected off the North Jetty in Ocean Shores, WA. The temperature at the time of collection was 8.6°C.

Effort was made during collection to avoid turbid or highly particulate water. Two liters of water was placed at 4°C overnight to allow for particulate materials to settle to the bottom of the collection container. Triplicate tubes of 30mL water were inoculated with each virus genotype at a final concentration of $1x10^5$ pfu/mL and placed at either 10°C and 15°C for freshwater, or 10°C and 4°C for saltwater. 1 mL of water from each replicate sample tube was collected at regular 12 or 24 hour time points for up to 10 days post-inoculation and used for titration and plaque assay to quantify the infectious particles present over time. Plaque assay was performed on EPC cells according to protocol previously described (7). The detection limit of this assay was determined to be 0.56 log pfu/mL, i.e. the concentration that would yield one plaque in one of three replicate wells.

Development of genotype-specific RT-qPCR assays for displacement genotypes

In order to accurately quantify each virus genotype present in a mixed genotype infection in a single host fish, we have developed genotype-specific qPCR assays for multiple genotype pairs. These assays have been designed to distinguish between two IHNV genotypes that are used in the competition assays described above. Quantification of viral load was possible through the development and use of RNA transcript standards for each genotype assayed. These transcripts underwent the reverse transcription step alongside samples and known quantities of these transcripts were diluted ten-fold to create a standard curve for each qPCR assay performed. Specificity and sensitivity were very important considerations in the design and optimization of these assays, therefore they have all undergone rigorous testing in artificial mixtures of known quantities of target genotype templates with non-target templates as described below.

Transcript development

RNA transcripts of the full viral glycoprotein (G) gene (containing target of qPCR) were developed for genotypes 007 (strain LR80), 111 (strain Mer95), and 110 (strain Qts07) and used as qRT-PCR standards at known concentrations. The development of these transcripts is outlined here. Due to significant similarity between genotype 110 strain Qts07 and genotype 139 strain DW09, the G gene could not be used as a target and the NV gene was used instead. The development of those transcripts is described below. As a template for the G plasmid, viral RNA was extracted from a 400 µl volume of each virus in cell free media with 500 µl of Tri® Reagent (Sigma-Aldrich) plus 100 µl EPC cells as a carrier according to the manufacturer's protocol and as previously described (93). The viral RNA was converted to cDNA using SuperScript II™ reverse transcriptase (Invitrogen) as suggested by the manufacturer, with 1.6 µg RNA and 2 pmol of a G gene specific primer (5′-CACTTTTGTGCTTTTAGACAG-3′). The cDNA was then PCR amplified to produce the full 1.6 kb G gene product using Taq DNA polymerase (Promega) in a 100 µl reaction with: 10 µl 10× PCR buffer, 0.2 mM dNTPs, 2.5 mM MgCl2, 25 U Taq, 7.5 mg RNase A (Promega), 5 µl cDNA, 1 pmol primers (forward: 5′-CACTTTTGTGCTTTTAGACAG -3′; reverse: 5′-ATCTCTTGGCTGGAATCA-3′). Cycling conditions were 94 °C 2 min, 30 cycles: 94 °C 30 s, 50 °C 30 s, 72 °C 2 min; followed by 72 °C 10 min, 4 °C hold.

The PCR product was gel purified using the QIAquick® gel extraction kit according to the manufacturer's protocol (Qiagen) and then inserted into pSTBlue-1 vector using the AccepTor® vector kit as outlined by the manufacturer (Novagen). Candidate transformant colonies were sequenced to ensure full G gene insertion and a stock of plasmid was prepared

using the EndoFree® Plasmid Maxi kit (Qiagen). Sequencing also allowed us to determine which direction the gene insert had been placed.

To obtain negative-sense transcript RNA, 20 µg of each plasmid was digested with either HindIII or BamH1 to linearize the plasmid and RNA transcripts were synthesized with either T7 RNA polymerase or Sp6 RNA polymerase (Promega). Linearized plasmids and synthesized RNA transcripts were purified by phenol-chloroform-isoamyl alcohol extraction. The polymerase used for synthesis was chosen based on the direction of the transcript within the plasmid. An orientation in which the T7 promoter follows the gene insertion will warrant transcription with T7. The opposite would be true if the Sp6 promoter followed the target gene sequence. Residual DNA was digested with RQ1 DNase and quantity of RNA was measured with a nanodrop spectrophotometer. The G gene of IHNV is 1610 nucleotides (nt) in length which equals a molecular weight of 546,906.6, 553,159.4, and 552,939.4 g/mol for 007, 111, and 110, respectively. Using these values, it was estimated that 1 µg of G transcript contained 1.101 x 10^{12}, 1.088 x 10^{12}, and 1.089 × 10^{12} gene copies, respectively.

Due to the very high similarity of the G gene sequences (6 nucleotide differences across 1610 total nucleotides) between genotype 110 (strain Qts07) and genotype 139 (strain DW09), an appropriately divergent region within the glycoprotein gene could not be identified. Thus, the quantitative PCR assay to distinguish these two virus genotypes was developed against a region of the non virion (NV) gene. RNA transcripts of the full NV gene of each strain were developed and used as RT-qPCR standards at known concentrations. Plasmids containing the NV genes of each genotype were developed as above, except that primers in the flanking G and L genes (forward: 5′- CCCACAATCTCCGCACTG -3′; reverse: 5′-TTCTCTGTGAGTCGCGC-3′) were used to amplify the entire NV gene. Purified PCR product of 595 base pairs was then inserted

into pSTBlue-1 vector as described above and bacterial colonies were screened by PCR and sequenced for presence of plasmids containing correct PCR product. Note that the amplicon length is larger than the 371 nucleotide length of the NV gene. This is a result of using primers for amplification that sit within the G gene and L gene and therefore the amplicon includes portions of those genes as well as the full length NV gene (total length is 595 nt). This amplicon includes 179 nucleotides of the L gene and 45 nucleotides of the G gene flanking the NV gene sequence.

To obtain negative-sense NV transcript RNA, 20 µg of plasmids, prepared as described above, were digested and linearized with XhoI and purified by phenol-chloroform-isoamyl alcohol extraction. RNA transcripts were synthesized with T7 RNA polymerase (Promega) and purified by phenol-choroform-isoamyl alcohol extraction. Residual DNA was digested with RQ1 DNase. The NV gene transcript is 701 nucleotides (nt) in length which equals a molecular weight of 225,855.2 and 225,784.4 g/mol for DW09 and Qts07 respectively. Using these values, it was estimated that 1 µg of NV transcript contained 9.74x10^11 and 1.12x10^12 gene copies, respectively.

For all transcripts developed, purified RNA was diluted into 50uL of RNase-free water and the concentration of RNA was measured using a nanodrop spectrophotometer (Thermo scientific). Transcripts were then diluted to 3.636x10^8 copies per uL and individual aliquots of 11uL were made and stored at -80C for single use as RT-qPCR standards.

Quantitative PCR primer/probe design

We developed genotype-specific quantitative PCR assays to distinguish between specific pairs of genotypes present in a single fish sample, at times in mixtures with a high excess of non-

target genotype RNA. This need for high levels of sensitivity and specificity required careful development of genotype specific qPCR assays using genotype specific primer sets and genotype specific MGB probes. Assays were designed to detect individual genotypes in the selected displacement pairs 007:111, 111:110, and 110:139.

Effort was made to identify regions (50-200 nucleotides in length) of sequence heterogeneity between the relevant virus genotype pairs and to design primer and probe sets containing at least one heterogeneous nucleotide in each primer and in the probe to maximize specificity. With this consideration, primers and probes were manually designed in accordance with the ABI Primer Express Software version 2.0 user manual. While not all of the specific recommendations were followed for the design of all genotype specific assays, care was taken to satisfy as many requirements as possible. Primary considerations for primer design were length (13-25 nucleotides), avoidance of four or more guanidine repeats, and melting temperature between 58-60°C. Primary considerations for probe design were length (13-18 nucleotides), avoidance of four or more guanidine repeats, avoidance of 5' guanidine residues, and melting temperature between 68-70°C. Multiple primer sets for each genotype were selected and tested as described below. Based on these recommendations and subsequent testing for sensitivity and specificity, primer and probe sets were tested as described below.

Testing for specificity and sensitivity

These assays were designed to distinguish each genotype from another genotype in mixtures within individual fish and to accurately quantify the amount of each genotype even in a mixture containing a large excess of another genotype. Therefore, for each assay, many primers,

and in some cases more than one probe, were designed and tested for both qualities: high specificity and high sensitivity.

We began by testing genotype specific primer and probe sets in reactions containing high concentrations ($1x10^8$ and $1x10^7$ copies/reaction) of either target or non-target cDNA derived from reverse transcription of standard transcript RNA of known concentration. Primer/probe sets that amplified any non-target cDNA were excluded from further use. Of the sets that amplified no non-target cDNA the set with the lowest Ct value for amplification of target cDNA was chosen for further evaluation. We next tested genotype specific primer and probe sets for their efficiency using a 10 fold cDNA dilution series with target gene copies ranging from 1×10^8 to 1×10^1 per reaction. An efficiency determined to be ≥90% (slope between 3.3-3.6) was required for all assays. See Table 2.3. Where possible, effort was made to match efficiencies between assays that would be used together for analysis within individual fish. Finally, each assay was tested for both sensitivity and specificity in artificial mixtures of cDNA spanning ratios of 10,000:1-1:1-1:10,000 target:non-target. The specificity and sensitivity of these assays can be seen visually in Figure 2.3.

The primer and probe sets chosen for use are described in Table 2.3. The number of nucleotide differences between target sequences varied between the assays from as many as 8 nucleotide differences between 3' primers in the 007 and 111 assays to 1 nucleotide difference in each primer and probe for the 110 and 139 assays.

<u>G gene qPCR assays designed to distinguish between 007 and 111:</u>

These assays were designed and tested by Dr. Andrew Wargo and Jake Scott. Within the target region of the 5' primer there are 4 nucleotide mismatches between the two genotypes.

Within the target region of the 3' primer there are 8 nucleotide mismatches. Within the target region of the probe, there is one mismatch. The primer sets chosen do not share the same start and end points. The 5' primer for 111 sits on the gene sequence six nucleotides before the 5' primer for 007. The 007 5' primer continues for 7 nucleotides further than the 111 primer. The 3' primer for 007 continues for one more nucleotide than the 111 3' primer but begins seven nucleotides after the 111 primer. The probe sequences differ by only one nucleotide near the 3' end of the sequence.

G gene qPCR assays designed to distinguish between 111 and 110:

Within the target region of the 5' primer there is one nucleotide mismatch between the two genotypes. Within the target region of the 3' primer there are 2 nucleotide mismatches. Within the target region of the probe, there are 2 nucleotide mismatches. These two mismatches within the probe target region are located on either end of the probe sequence. The amplicon length is 180 nucloetides. For these assays, the technique of adding a "wobble" nucleotide was used. A single nucleotide (conserved between the two genotypes) was chosen near the 3' end of the primer and changed to a different base for one primer. In the case of 110, the 5' primer chosen for use in the assay has a single mismatch with the 110 target sequence at the second to last nucleotide in the primer where a guanine has been changed to adenine. Similarly, for 111, the 3' primer chosen for use in the assay contains a single mismatch with the 111 target sequence at the base located fifth from the 3' end where a cytosine has been changed to a thymine. In total 3 forward primers and 4 reverse primers were tested for the 111 assay, and 3 forward primers and 4 reverse primers were tested for the 110 assay.

NV gene qPCR assays designed to distinguish between 110 and 139:

Within the target region of the 5' primer there is one nucleotide mismatch between the two genotypes. Within the target region of the 3' primer there is one nucleotide mismatch. Within the target region of the probe, there is one nucleotide mismatch. Amplicon length is 167 nucleotides. For these assays, the wobble technique was again tried but neither assay used a wobble primer. For the 110 assay 4 forward primers were tested and 4 reverse primers were tested. For the 139 assay, 2 forward primers were tested and 2 reverse primers were tested. The 139 assay was developed and tested using a probe with a VIC® reporter dye but upon amplification of RNA extracted from samples, a mysterious "double waterfall" effect occurred in the amplification curve, which is thought to occur due to an incorrect baseline. According to Life Technologies technical support, if the baseline is set too early, it will subtract real signal from the plot and thus makes the amplification curve drop down only to rise again after sufficient signal is reached again. The probe was re-ordered with a 6FAM™ reporter dye and the artifact was not seen again. A satisfactory explanation for this issue has never been provided.

Statistical analyses

All statistical tests were carried out in R (version 2.11.1).

Virulence

Differences in the kinetics and final mortality in rainbow and steelhead trout over 30 days post-exposure to each of the genotypes were assessed by comparison of Kaplan-Meier survival curves with a Weiball distribution in R. Significance ($p < 0.05$) indicates that survival differs between

groups depending on the virus to which each group was exposed. Each tank was represented as an independent infection event and all data was analyzed together with three replicate events for each treatment group.

Viral growth kinetics

Differences in kinetics of viral replication in single infections were determined by GLM analysis of all fish with quantifiable viral load at the time of harvest. The dependent variable in this analysis was viral load with the independent factors being genotype and day collected. The test performed was: viralload~genotype*day collected. To control for inflated type I error due to multiple tests we utilized a Bonferroni adjustment (alpha = 0.05 ÷ 3 = 0.01667). The GLM analysis only includes fish that were virus-positive by qPCR. This resulted in exclusion of most fish collected at time 0.

Co-infection fitness for injection, immersion, and shedding

To determine if there were statistical differences in viral load between treatment groups, general linear models were conducted (GLM). The dependent variable was viral load with the independent factors being genotype (B, C, 007, 111, 110, or 139) and competition (co-infection or single infection). We initially included experiment as a factor (levels = 1 and 2) but it was not found to be significant so it was dropped from all further analyses. Data collected from duplicate experiments was combined and the GLM analysis was performed wherein the test performed was: viralload~competition*genotype. To control for inflated type I error due to multiple tests we utilized a Bonferroni adjustment (alpha = 0.05 ÷ 3 = 0.01667). For interaction terms that were found significant by GLM, Tukey HSD tests were conducted to determine the precise treatment groups (levels of factors) that differed. Here we focused on biologically relevant comparisons.

Viral load data was log transformed to meet test assumptions of normality and homogeneity of variances. The GLM analysis only includes fish positive by qPCR.

Ratio shed virus : viral load

A simple linear model was used to determine the correlation between individual in-host viral load and the quantity of virus shed into the surrounding water by individual fish infected with genotypes B or C via immersion challenge as described in (120). Statistical analysis was performed on data derived from all fish (single and mixed infections) but separated by genotype. A p value of less than 0.05 indicates a significant correlation between viral load and virus shed.

Superinfection

Superinfection frequency data were analyzed in two ways: exact multinomial goodness of fit and Fisher exact tests. To examine whether the ability of a virus genotype to infect a fish was influenced by the presence of another virus genotype in the host, we conducted a multinomial goodness of fit analysis. This analysis tests whether or not the frequency of falling into each of the infection status categories (superinfected, primary virus only, secondary virus only, uninfected) is significantly different than the frequency expected based on the single exposure treatments. In other words, it is testing whether or not the observed frequency of fish in the four infection categories is independent of exposure type (superinfection vs. single infection). This test utilizes the observed frequencies in single exposure to calculate expected frequencies in superinfection exposure by the following formula: [expected = (ratio infected:uninfected for relevant primary single infection control * ratio infected:uninfected relevant secondary single infection control)* number of fish in superinfection group]. For example, the expected number of fish to be superinfected when B is the primary virus = (observed ratio infected:uninfected in B ->

mock single infection group * observed ratio infected:uninfected in mock -> C single infection group)*total fish challenged in superinfection group B -> C. This analysis was conducted for each experiment at each interval, and each superinfection group (B first, C second, etc…). This resulted in 20 total tests per genotype pair. To control for inflated type I error due to multiple tests we utilized a Bonferroni adjustment (alpha = 0.05 ÷ 20 = 0.0025) (125). We used this correct alpha value to determine statistical significance $p<0.0025$).

To examine if the ability of a genotype to infect a fish was dependent on the order of exposure in superinfection, Fisher exact tests were utilized. This test compares the frequency of fish falling into the four infection categories (superinfected, primary only, secondary only, uninfected) between the two reciprocal superinfection exposure treatments (Genotype X first, Y second, and genotype Y first, X second) in each experiment. The null hypothesis is that the distribution of fish among the four categories is independent of which genotype was used for the primary exposure. We carried out this test for each experiment and genotype pair (HV vs LV, B vs C). This resulted in 10 tests total. To correct for inflated type I error due to multiple tests, we utilized a Bonferroni adjustment alpha = 0.05 ÷ 10 = 0.005) (125). We used this correct alpha value to determine statistical significance $p<0.005$).

To determine if there were statistical differences in viral load between treatment groups, general linear models were conducted (GLM). The dependent variable was viral load with the independent factors being genotype (B or C, and HV or LV), competition (superinfection or single infection), order (first or second) and interval period (12, 24, 48, 96, or 168 hours). We initially included experiment as a factor (levels = 1 and 2) but it was not found to be significant so it was dropped from all further analyses. The experiments were designed to control for variation in viral load due to timing of exposure, i.e. single infections were conducted at both the

primary and secondary time points. This made it possible to compare the viral load attributed to a genotype in the superinfection treatment group to its corresponding single infection control (i.e. secondary superinfection to secondary single infection) and control for variation which might be induced by exposure timing, thus avoiding non-biologically relevant comparisons (single primary to superinfection secondary and vice versa). As such, the GLM analysis was split into four tests per genotype pair to minimize these non-biologically relevant comparisons. Test 1 = competition*genotype*interval, for virus administered at the primary infection period. Test 2 = competition*genotype*interval, for virus used at the secondary infection period. Test 3 = order*competition*interval, for a single genotype in the pair of interest. Test 4 = order*competition*interval, for the other genotype in the pair of interest. This resulted in 8 separate tests. To control for inflated type I error due to multiple tests we utilized a Bonferroni adjustment (alpha = 0.05 ÷ 8 = 0.00625). For interaction terms that were found significant by GLM, Tukey HSD tests were conducted to determine the precise treatment groups (levels of factors) that differed. Here we focused on biologically relevant comparisons. Viral load data was log transformed to meet test assumptions of normality and homogeneity of variances. The GLM analysis only includes fish positive by qPCR and, in the case of superinfection treatments, those which were positive for both genotypes. The analysis was also conducted setting all negative fish to the minimum detection threshold of qPCR (100 copies/g fish). This alternative analysis did not change the overall conclusions for any genotype pair.

Environmental stability

Triplicate wells were averaged for plaque number and mean titer was calculated for each replicate water sample. Mean log titer was then averaged for triplicate water samples to provide a mean log titer of each virus for each time point sampled. Percent survival at a given time point

was calculated by dividing the titer at that time point by the titer determined at the original time 0 and multiplied by 100. These values were plotted as percent survival over time and a linear regression equation was determined in Microsoft Excel for the best fit. This equation was applied to calculate the time expected to reach 90% decay (T_{90}), 99% decay (T_{99}), and below detection (T_{BD}). y=mx+b, x=(y-b)/m, where y = 10, 1, or 0.00361, respectively. Detection limit was defined in methods section describing stability assay. Inactivation rate (IR) was determined by plotting the log inactivation at each time point [Log_{10}(original titer/current titer)] and plotting a best fit linear regression line to the data. The slope of the best fit line represents the inactivation rate in log plaque forming units over days (log pfu/day). The r^2 value of the line represents how well the regression fit the observed data.

Table 2.1 – Pilot superinfection assays using IHNV genotypes B and C.

Experiment	Infection type / genotype	Dose (pfu/mL)	Volume static water*	Interval period	Number of fish	Total infected	Total superinfected
1	Single / B	$1x10^4$ pfu/mL	1.46 liters	N/A	10	6	
1	Single / C	$1x10^4$ pfu/mL	1.46 liters	N/A	10	7	
1	mock	0	1.46 liters	N/A	4	0	
2	Single / B	$1x10^4$ pfu/mL	400 mL	N/A	6	6	
2	Single / B	$5x10^4$ pfu/mL	400 mL	N/A	6	6	
2	Single / C	$1x10^4$ pfu/mL	400 mL	N/A	6	6	
2	Single / C	$5x10^4$ pfu/mL	400 mL	N/A	6	6	
2	mock	0	400 mL	N/A	4	2	
3	Superinfection / B then C	$5x10^4$ pfu/mL	400 mL	24	8	7	3
3	Superinfection / C then B	$5x10^4$ pfu/mL	400 mL	24	8	8	3
3	Superinfection / B then C	$5x10^4$ pfu/mL	400 mL	48	8	8	2
3	Superinfection / C then B	$5x10^4$ pfu/mL	400 mL	48	8	8	2

* Volume indicates water level in liters (L) or milliliters (mL) during 12 hour static challenge period, after which all tanks were filled to 1.5L of flowing water for the 72 hours prior to harvest.

Table 2.2. Pilot superinfection assay using IHNV genotype LV.

Infection type / genotype	Dose (pfu/mL)	Volume static water*	Number of fish	Total infected
Single / genotype LV	$5x10^4$ pfu/mL	400 mL	8	3
Single / genotype LV	$2x10^5$ pfu/mL	400 mL	8	7
Single / genotype LV	$5x10^4$ pfu/mL	200 mL	8	4
Single / genotype LV	$2x10^5$ pfu/mL	200 mL	8	8

* Volume indicates water level in liters (L) or milliliters (mL) during 12 hour static challenge period, after which all tanks were filled to 1.5L of flowing water for the 72 hours prior to harvest.

Table 2.3 - Genotype specific quantitative PCR assays.

Target genotype	Non-target genotype	Target gene	Primer/probe sequences[a]	Efficiency	Reliable quantification limit	Detection limit	Reliable quantification[b]
007	111	glycoprotein (G)	5' - CATGATCACCACTCCGCTCAT 3' - CTTGCGGTGTCGGGTTTG probe - 6FAM-CTGCGGAGCAAACA-MGBNFQ	93.10%	< 4 log virus copies / g fish	100 virus copies	Log(observed quantity) = Log(expected quantity)*(0.996±0.007) – 0.013 ± 0.012, $F_{1,163}$ = 27750, P<0.001, R^2 = 0.994
111	007	glycoprotein (G)	5' - GGACACCGTGACAACCACCT 3' -TTGGTCTGACTCACTTGTGGTGTT probe - 6FAM-CCTGCGGAGCAGACA-MGBNFQ	93.10%	< 4 log virus copies / g fish	100 virus copies	Log(observed quantity) = Log(expected quantity)*(1.001±0.007) + 0.009 ± 0.037, $F_{1,66}$ = 24430, P<0.001, R^2 = 0.997
111	110	glycoprotein (G)	5' - CGTCAAACCCAACACCACAAGT 3' - GACCGGATGAATGTGG**T**ATGG probe - 6FAM-CTCTCTTCACCTATCCC-MGBNFQ	94.50%	< 4 log virus copies / g fish	100 virus copies	Log(observed quantity) = Log(expected quantity)*(1.006±0.033) – 0.102 ± 0.162, $F_{1,52}$ = 890.8, P<0.001, R^2 = 0.944
110	111	glycoprotein (G)	5' - CGTCAAACCCAACACCACCAAC 3' - GACCGGATGAATGTGGGATAAG probe- VIC- CGCTCTTCACCTATCCT-MGBNFQ	98.03%	< 4 log virus copies / g fish	100 virus copies	Log(observed quantity) = Log(expected quantity)*(0.97±0.006) + 0.175 ± 0.035, $F_{1,38}$ = 20930, P<0.001, R^2 = 0.998
110	139	non-virion (NV)	5' - ATGGACCACCGCGACATAAACA 3' - AGATCAGTGCGTTGGCGATG probe - 6FAM-ACCTGGTATGGCGTGA-MGBNFQ	90.94%	< 4 log virus copies / g fish	100 virus copies	Log(observed quantity) = Log(expected quantity)*(0.99±0.006) – 0.038 ± 0.028, $F_{1,108}$ = 25330, P<0.001, R^2 = 0.99
139	110	non-virion (NV)	5' - ATGGACCACCGTGACATAAACA 3' - AGATCAGTGCGTTGGCGACT probe - 6FAM-ACCTGGCATGGCGTGA-MGBNFQ	89.50%	< 4 log virus copies / g fish	100 virus copies	Log(observed quantity) = Log(expected quantity)*(1.011±0.011) – 0.08 ± 0.054, $F_{1,45}$ = 8393, P<0.001, R^2 = 0.99

[a] Underlined bases represent mismatches between genotypes in pairs. Bold, highlighted bases represent "wobbles" which do not match the target sequence.

[b] Reliable quantification was assessed through application of linear models in which observed quantity of viral RNA (qPCR quantification) was the dependent variable and expected quantity of viral RNA (known transcript input quantity). P value < 0.05 indicates strong correlation.

Figure 2.1. Photograph of stand-alone tower tank system used for superinfection experiments.

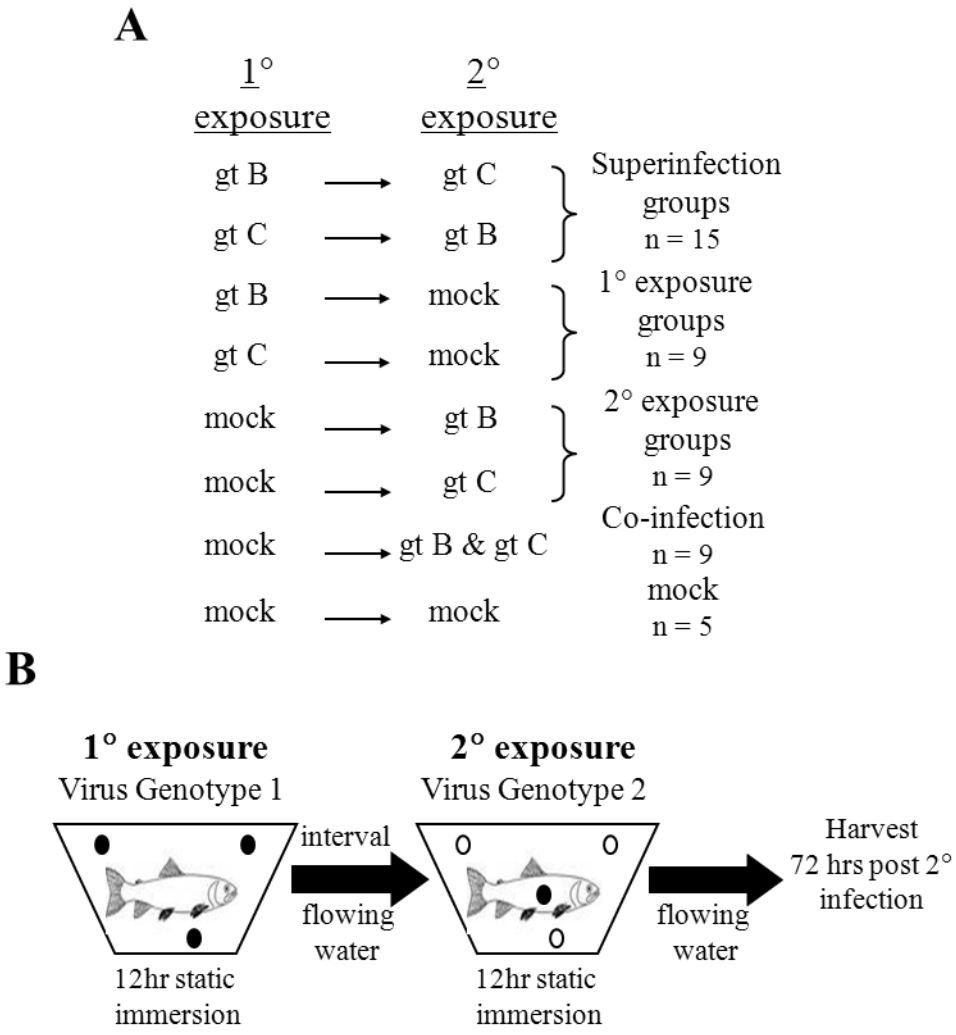

Figure 2.2. In vivo superinfection assay design. A) Treatment groups for each superinfection experiment, illustrated for assays using genotypes B and C. Assays with genotypes HV and LV were performed with the same design. "gt" denotes genotype. "n" indicates numbers of individual fish used in each group. B) Diagram describing the in vivo superinfection assay.

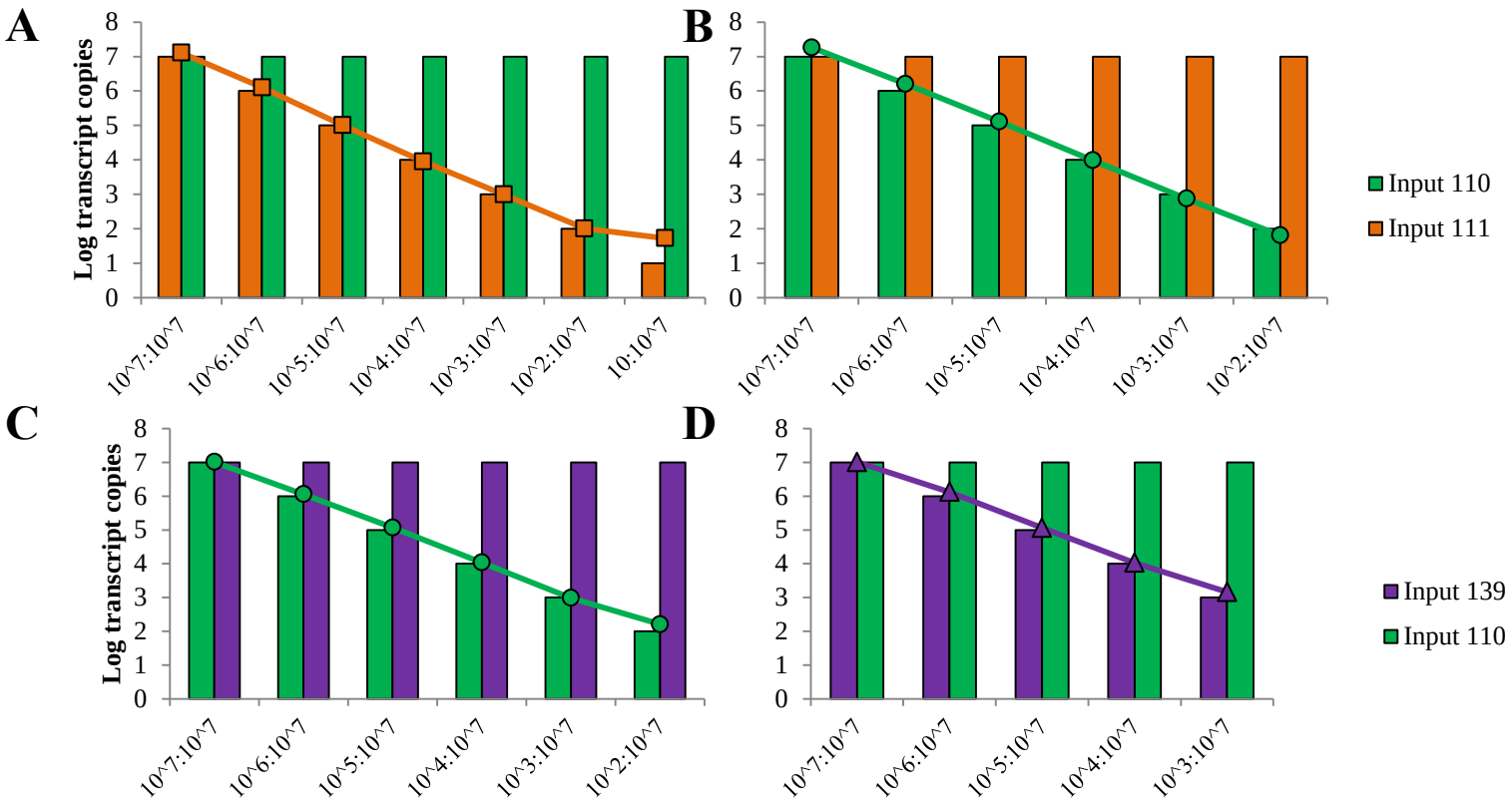

Figure 2.3. Quantitative PCR assay validations for sensitivity and specificity. Quantification of viral transcripts for two genotype displacement pairs is shown for each set of genotype specific qPCR assay developed A) assay to detect genotype 111 in mixtures with 110, B) assay to detect genotype 110 in mixtures with 111, C) assay to detect genotype 110 in mixtures with 139, D) assay to detect genotype 139 in mixtures with 110. Bars represent quantity of transcript input also denoted on x-axis as ratio oftarget:non-target genotypes in artificial mixtures. Points represent quantity of target template measured by each assay in each respective mixture.

Chapter 3

Fitness of IHNV genotype pair of equal virulence, B and C

"You'll miss the best things if you keep your eyes shut."
— Dr. Seuss, *I Can Read With My Eyes Shut!*

Introduction

As described in chapter 1 of this thesis, the first IHNV in vivo co-infection fitness assay was developed by Troyer et al. and was performed using IHNV genotypes B and C to infect juvenile rainbow trout (108). In this assay, groups of rainbow trout were challenged via immersion in water containing either genotype B, genotype C, or a 1:1 mix of both virus genotypes followed by separation into individual beakers and harvest at 3 days post-challenge. The fitness of each genotype was determined by calculating relative quantity of each genotype in co-infected fish through restriction digest and densitometric analysis. This study concluded that genotypes B and C had equal replication fitness in rainbow trout following immersion co-infection challenge.

In order to expand upon this result, we questioned whether the equal fitness observed in the previous study was representative of the overall fitness of these genotypes which includes host entry, in-host replication, virus shedding, and fitness in superinfection. In addition, genotype-specific RT-qPCR assays were developed to quantify the viral load attributed to each genotype in single and mixed infections, to more precisely determine fitness for each genotype. We, therefore, applied previously developed co-infection fitness assays to determine the competitive fitness of genotypes B and C, of equal virulence, at three stages of the infection cycle: entry into the host, in-host replication, and shedding into the surrounding environment. Fitness in superinfection was also explored using the novel superinfection fitness assay developed for this thesis work. These different measures of competitive fitness were used to determine the fitness at each of these stages and, thus, the overall fitness of these genotypes.

The quantity of each genotype present in fish challenged by intraperitoneal (IP) injection of each genotype or a one-to-one mixture of both genotypes indicates the ability of each genotype to replicate within the target tissues of the host. The quantity of each genotype present in fish challenged by immersion in static water containing a single genotype or a one-to-one mixture of each genotype indicates the ability to enter the host and replicate in the target tissues of the host. Because infection by injection bypasses the entry step, differences in mean log viral load measured between groups of fish infected either through immersion or injection challenge can be attributed to differences in the abilities of the two genotypes to enter the host and replicate. Finally, quantities of each viral RNA detected in the water surrounding fish infected via immersion indicates the ability to complete the entire cycle from entry, in-host replication and shedding into the environment. Each of these assays provided insight into the importance of each step in determining overall fitness of these genotypes. This work was presented as part of a larger data set to explore host genetic diversity and viral entry as sources of variation in viral replication in rainbow trout (119).

To determine the dynamics of IHNV superinfection in rainbow trout, we first performed experiments with genotypes B and C using the superinfection fitness assay described in chapter 2. We tested variable times between sequential exposures and measured the frequency with which superinfection occurs and quantified the viral load of each genotype in individual fish exposed to just one genotype or individual fish sequentially exposed to both genotypes. The time intervals between sequential exposures were 12, 24, 48, 96, and 168 hours. We defined superinfection fitness as having two distinct components: the ability to establish, or restrict the establishment of, secondary infection in single hosts, measured by the frequency of superinfection; and the ability to replicate in the context of superinfection, measured by the viral

load of each genotype in single infections compared to superinfection contexts. Using this assay, we determined that genotypes B and C have equal fitness in superinfection. This superinfection work was described in Kell *et al.*, 2013 (48).

Results

Virulence of genotypes B and C in rainbow trout

To demonstrate the virulence of genotypes B and C in juvenile rainbow trout, mean cumulative percent mortality following batch challenge of triplicate groups of 20 fish with either IHNV genotype B or genotype C at a concentration of 1×10^4 pfu/mL is shown in Figure 3.1. Mortality began on day 3 for both genotypes and continued until day 27 for genotype B and day 16 for genotype C. Tanks challenged with genotype B experienced only 2 mortalities between days 12 and 27. For genotype B the mean day to death was 9.2. For genotype C the mean day to death was 7.1. The genotypes had mean survival times of 17 (B) and 15 (C) days. This suggests that the kinetics of infection are quite similar for the two genotypes. While the mean cumulative percent mortality was slightly higher in groups exposed to genotype C during the course of the 30 day monitoring period (70% for genotype C and 63% for genotype B), survival analysis determined that genotypes B and C do not differ significantly in virulence in rainbow trout ($p > 0.05$). These results are consistent with previous descriptions of the virulence of genotypes B and C in juvenile rainbow trout (108).

Co-infection fitness following immersion challenge

Immersion challenge with either genotype B, genotype, C, or a 1:1 mix of both genotypes was conducted for 12 hours at a concentration of 2×10^5 pfu/mL. Challenge at this dosage has previously been demonstrated to result in 95-100% infection of juvenile rainbow trout in these

conditions. Following challenge, individual fish were separated into individual tanks and held in static conditions for 72 hours to allow for in-host replication. Again, previous work demonstrated that genotypes B and C reach peak viral load 72 hours post-challenge (108). Following harvest, the quantity of each genotype was measured in individual whole fish using genotype-specific qPCR assays to quantify the entry and replication of each genotype in vivo in a single infection context compared to a co-infection context. Two independent experiments were performed and the quantity of each genotype for individual fish in each group is shown in Figures 3.2 (Experiment 1) and 3.3 (Experiment 2). Similar levels of variation in viral load within the groups of 28 fish were observed independent of the genotype used for challenge (119). Mean log viral loads for each group for both experiments is shown combined in Figure 3.8A and Table 3.1. Statistical analysis revealed no significant differences between the genotypes in their ability to replicate within the host in single infection or in co-infection after immersion challenge (ANOVA, genotype*competition interaction, $F_{1,215} = 1.85$, $p = 0.42$). Therefore genotypes B and C demonstrate no significant differences in the ability to enter and replicate within the host in this assay.

Co-infection fitness following injection challenge

For the injection challenges, 28 individual fish per group were injected with 100 pfu of either genotype B, genotype C, or a 1:1 mix of both genotypes (200 pfu total). This challenge dose was chosen following previous work demonstrating 95-100% infection of juvenile rainbow trout by this method (83). Following injection, individual fish were separated into individual tanks and held under static conditions for 72 hours to allow the genotypes to replicate within the host. Following harvest, each genotype in individual whole fish was quantified to determine the replication fitness of each genotype in vivo in single infection and co-infection contexts. Two

independent experiments were performed and the quantity of each genotype in each group for individual fish is shown in Figures 3.4 (Experiment 1) and 3.5 (Experiment 2). Similar levels of variation in viral load within the groups of 28 fish were observed independent of the genotype used for challenge (119). Mean log viral loads for each group for both experiments are shown in Figure 3.8B and Table 3.1. Statistical analysis revealed no significant differences between the genotypes in their ability to replicate within the host in single infection or in co-infection (ANOVA, genotype*competition interaction, $F_{1, 216} = 1.87$, $p = 0.89$). These results demonstrate that genotypes B and C have equal replication fitness after injection challenge, which measures only in-host replication. Because both genotypes were observed to have equal fitness following both immersion and injection challenges, we can also conclude that the abilities of each genotype to enter the host did not differ significantly.

Virus shed into surrounding water following immersion challenge

For those fish challenged via immersion as described above, 1.7mL of water was sampled from the static holding water at 72 hours post challenge, corresponding to the time of harvest. We determined the total quantity of virus shed from infected fish into the surrounding water as a measure of the transmission potential of each genotype in single infection as well as in co-infection competition (Figures 3.6 and 3.7). In single genotype infections in both experiments, a statistically significant difference in the total virus shed by fish infected with either genotype B or genotype C was observed, with genotype C being shed at higher mean levels than genotype B (Figures 3.6D, 3.7D, and 3.8C). For the combined data from experiments 1 and 2, fish infected, either in single or co-infections, with genotype C shed on average log 5.07 virus copies/mL of water. Whereas, for fish infected with genotype B, either in single or co-infections, the mean shed was log 4.53 virus copies/mL. This statistically significant difference was also observed in

both experiments in co-infected fish, with genotype C again shed at higher levels than genotype B from co-infected fish than from singly infected fish (ANOVA with combined data, genotype, $F_{1, 205} = 0.338$, $p < 0.0001$). Interestingly, mean levels of virus shed attributed to genotype C in co-infection were not significantly different than in single infection ($p = 0.84$). In contrast, levels of genotype B shed from co-infected fish were significantly lower than that from fish infected with genotype B alone ($p = 0.0008$). Importantly, the results were consistent between the two experiments performed. This observation indicates that shedding of genotype B is significantly affected by competition while shedding of genotype C is not.

Because we observed no significant difference in the abilities of genotypes B and C to replicate within the host, we hypothesized that shedding does not always correlate with viral load. A simple linear model determined that for genotype B the quantity of virus shed was not correlated with the total viral load in individual fish ($F_{1,99} = 0.0747$, $p = 0.79$, $R^2 = -0.009$) (Figure 3.9). In contrast, for fish infected with genotype C, total viral load was found to correlate positively with total virus shed into the surrounding water ($F_{1,105} = 35.05$, $p < 0.001$, $R^2 = 0.243$). Thus, although the two genotypes replicate to relatively equal levels within the host, shedding of genotypes C correlates with in-host viral load, whereas shedding of genotype B does not.

Frequency of superinfection

To assess the frequency with which superinfection occurred following sequential exposure to both genotypes B and C, individual fish were analyzed for the presence or absence of each virus genotype. Superinfection was concluded to have occurred when a fish sample was found to be positive for the presence of both viral genotypes at the time of harvest. Figure 3.10 shows the mean percentage of fish from superinfection groups with detectable amounts of both

viral genotypes at the time of harvest for two independent experiments performed for each time interval. In experiments with 12 and 24 hour intervals between exposures, the mean percentage of fish that were superinfected ranged from 60-73%. For interval periods from 24 to 96 hours, as the time interval between exposures to each genotype increased, the percentage of fish found to be superinfected decreased. However, with an interval of 168 hours between exposures to each viral genotype, the percentages of fish infected with both genotypes increased compared to the 96 hour interval. For all intervals, the majority of fish that were not superinfected at the time of harvest were infected with only the virus used for the primary infection.

A central goal of this work was to determine whether restriction of secondary infection occurs in rainbow trout already infected with IHNV. Thus, our null hypothesis was that, if the restriction of secondary infection does not occur, the probability of observing superinfection in individual fish sequentially exposed to both genotypes would be equal to the probability of observing single infections in individual fish exposed only to one genotype at the relevant exposure times. To test this hypothesis, we used the observed infection frequencies in the single infection control groups to determine the expected numbers of fish from the superinfection treatment groups that should fall into each of four infection categories: superinfected, infected with primary genotype, infected with secondary genotype, and uninfected. These data for genotypes B and C are shown in Table 3.2. These observed and expected frequencies were then used for analysis in an exact multinomial goodness of fit test to determine the probability of observing a given distribution among infection categories for fish in the superinfection groups. For each of two experiments performed at each time interval, we found that the distribution of individual fish observed within the infection categories differed significantly from what would have been expected under the null hypothesis (exact multinomial goodness of fit, $p < 0.05$

Bonferroni adjusted) (Table 3.2). We found that superinfection in this system occurred less often than would be expected, and single infection with only the genotype used to establish the primary infection occurred more often than expected under the null hypothesis. This result indicates that primary infection significantly restricts subsequent infection at all intervals tested, beginning at 12 hours post-infection.

In addition, we were interested in whether the distribution of individual fish into the four infection status categories was dependent on which genotype was used first (i.e. B or C). For all experiments with all intervals examined, the distribution of fish across the four infection categories was not found to depend on the order of exposure to each genotype (Fisher exact test, $p<0.05$ Bonferroni adjusted) (Table 3.2). This result suggests that the observed restriction of secondary infection did not depend on the genotype used for primary infection and that genotypes B and C of equal relative virulence have equal ability to restrict, or establish, secondary infection.

Impact of superinfection on viral load

To determine the impact of superinfection on the ability of each genotype to replicate within the host, we compared the mean viral load of each genotype in superinfected fish with the mean viral load in single infection groups of fish exposed to that same genotype during the corresponding exposure period (Figure 3.11). Experiments performed with a 96 hour interval between exposures were not included in this analysis because no fish were found to be superinfected in groups exposed to genotype C before genotype B in both experiments performed. We used general linear models (GLM) to determine the statistical significance of these trends.

For genotype B, there was a significant effect of superinfection on viral load that was dependent on the order of exposures to each genotype (competition:order interaction, $F_{1,223}$ = 13.56, p = 0.0046). Using a post-hoc Tukey Honestly significant difference test (HSD), we determined that this significance was likely driven by a decrease in the viral load of genotype B in superinfected fish compared to fish singly infected with genotype B during the secondary exposure period. This decrease in viral load during superinfection was not observed when genotype B was used to establish the primary infection. When this same analysis was performed for genotype C, we found that the observed greater replication of the primary infection was statistically significant prior to a Bonferroni adjustment for multiple tests, but was not significant following adjustment (competition:order interaction, $F_{1,228}$ = 5.78, p = 0.0169 unadjusted, p = 0.204 adjusted). Based on this difference, we performed a separate GLM analysis to test whether the replication of each genotype might be differently affected by superinfection competition. However, this analysis determined no significant dependence of the superinfection effect on genotype during the secondary exposure period (competition:genotype interaction, $F_{1,222}$ = 0.065, p = 1). This suggests that the genotypes do not differ significantly in their ability to replicate in the presence of another genotype within the same host. However, for both genotypes B and C, the secondary virus demonstrated a significant replication disadvantage in superinfection compared to its performance in single infection whereas the primary virus did not experience this disadvantage. This pattern was consistently observed for both experiments performed across all delay intervals between exposures, indicating that time between exposures did not significantly influence the impact of superinfection on viral replication.

Viral growth and Mx-1 gene expression

As a preliminary investigation into the mechanism of the observed superinfection restriction, we performed single infections with either genotype B or genotype C and measured both viral load and Mx-1 gene expression in five individual, whole fish at each time point corresponding to the timing of secondary exposures (13, 24, 48, 96, and 168 hours). As an interferon-alpha stimulated gene, Mx-1 gene expression is known to be induced, along with many other interferon stimulated genes, as part of a strong innate immune response following IHNV infection in rainbow trout (94, 95, 97). Mean viral load of fish infected with either genotype B or genotype C sampled at points up to 168 hours (7 days) post-infection are shown in Figure 3.12A. These viral growth curves demonstrated that these two genotypes had similar replication kinetics within the host. As Figure 3.12B demonstrates, Mx-1 expression was up-regulated beginning at 72hrs following exposure to each genotype and continued to increase as the infections progressed. However, superinfection restriction was observed beginning at 12 hours post-exposure to the primary genotype and therefore the kinetics of measurable Mx-1 response did not correlate with the onset of restriction.

Discussion

Here, we assessed the fitness of two IHNV genotypes of equal virulence in co-infection and superinfection competition assays. Co-infection fitness was determined by the ability to enter the host, replicate within the host, and shed virus into the surrounding environment. No significant differences were observed in the viral load between genotypes B and C following

either immersion or injection challenge. Because challenge via injection bypasses the requirement for entry of the virus into the host, these observations indicate that these two virus genotypes have equal fitness for entry and in-host replication in both single infections and co-infection competition. Interestingly, we did observe a significant difference in the quantity of genotypes B and C which was shed into the surrounding water following immersion challenge, with C being shed in significantly higher quantities. This difference was observed to be consistent for both experiments performed. Because there was no significant difference in the in-host viral load between fish infected with genotype B or genotype C, this observed difference in virus shed indicates that genotype C has an advantage over genotype B in the release of virus from the host and thus potentially an advantage in transmission to new hosts. However, we must note that shed virus was measured by RT-qPCR of water samples to identify virus RNA and presence of infectious particles in this water was not measured. This observed difference could also be attributed to differences in the stability of each genotype in water which was not measured here. Thus, we cannot rule out the possibility that genotype C does not shed more infectious progeny than genotype B. The work presented here supports previous reports using different methods. We conclude that genotypes B and C have equal replication fitness in rainbow trout and demonstrate that these two genotypes also have equal fitness for entry into the host. However, the observation that significantly more of IHNV genotype C is shed into the surrounding environment demonstrates the importance of investigating multiple steps in the infection cycle to determine overall viral fitness.

These same genotypes of equal virulence were also used to define the dynamics of IHNV superinfection in rainbow trout and to determine whether they possess equal superinfection fitness. Superinfection was observed in most groups at each time interval between exposures, but

with decreasing frequency as the time between exposures increased. This trend was observed for groups of fish exposed to genotype B first and groups exposed to genotype C first, indicating that these two genotypes have equal fitness to establish, or restrict establishment of, secondary infection. The observed restriction of superinfection suggests that competition, either direct or apparent, occurs between the two genotypes in a superinfection context. Direct competition could be responsible for restriction if primary infection results in saturation of sufficient target cells within the host such that the secondary, superinfecting virus does not have access to susceptible cells and thus cannot establish a productive, detectable infection. Biacchesi et al. demonstrated that superinfection of virus-susceptible EPC cells with IHNV and a closely related rhabdovirus species, Viral Hemorrhagic Septicemia virus, is restricted (10). This refractory state is hypothesized to be a result of either competition for host entry receptors or competition for endocytotic machinery (10). The latter mechanism has been reported for cellular superinfection exclusion by another rhabdovirus, Vesicular Stomatitis Virus, in cultured mammalian cells (105).

However, direct competition within the host seems unlikely in our system when considering results from the co-infection studies presented here and previously (84, 118, 120). Neither genotype demonstrated a significant reduction in absolute levels of replication in co-infection competition when compared to single infection controls. This was observed even though fish in co-infection groups were initially challenged with twice the total virus compared to fish in single infection groups, and total measured viral load was, at times, doubled in co-infected fish compared to those singly infected (118, 120). These results suggest that host tissues and target cells for replication were not limited in co-infection. However, this possibility cannot be ruled out for the case of superinfection. Target cells required for entry into the host may be saturated or killed during the primary infection, inhibiting a secondary virus from entering.

Similarly, as a primary infection progresses within the target hematopoietic tissues of an individual fish, viral spread and necrosis may leave very few naïve, susceptible cells for a secondary incoming virus to establish a productive and detectable infection. However, if this were true, one might expect to see a continual decrease in viral load of the secondary virus as the interval between primary and secondary infection increases, which was not observed in our system.

Apparent competition has been described as a refractory state resulting from immune stimulation following primary viral infection (100). For IHNV infection in rainbow trout, it has been thoroughly documented that, within the first few days of infection, the host response is dominated by a strong induction of numerous interferon-stimulated genes, including Mx-1 (95). In addition, 24 hour pre-treatment of rainbow trout with injection of Poly(I:C), a TLR-3 agonist, offers significant protection from infection and mortality following challenge with IHNV (52). We therefore conducted a preliminary study to determine whether innate immune stimulation following primary infection might correlate with restriction of secondary infection. However, we found that the timing of systemic Mx-1 transcription up-regulation following infection with each IHNV genotype lagged behind the first time point at which we see significant superinfection restriction. Although this suggests a mechanism other than apparent competition, it does not exclude the possibility that innate immune signaling may play a significant role in the restriction observed. Our observations are based on the analysis of Mx-1 transcripts from whole fish and not specifically from target tissues, such as kidney and spleen, or from potential entry sites, such as gills, skin, or intestine. Therefore, it is possible that biologically relevant, local induction of Mx-1 occurs earlier in these tissues but is not yet detectable by our methods until a strong systemic response has been elicited. Additionally, many interferon-induced genes other than Mx-1 are up-

regulated in response to IHNV infection and may play a role in defense against secondary infection (92, 94, 96-98). Ultimately, the mechanism underlying superinfection restriction in our system is of great interest and future work will be directed at identifying and characterizing the mechanism(s) in rainbow trout.

A strength of this in vivo superinfection assay is the ability to quantify the viral load of each genotype in individual superinfected hosts and compare the mean level of viral replication to that found in individuals from groups exposed to just one of the genotypes at the same exposure period. This analysis allows us to determine the ability of each genotype to replicate in a superinfection context compared to a single infection and to determine whether there is a cost to viral replication in superinfection. We observed a clear disadvantage for the superinfecting, or secondary, virus with regard to viral replication, while the primary infecting virus was unaffected by subsequent infection. These results support the hypothesis put forward by Ashraf *et al*. that reduced secondary viral replication drove the observed reduction in pathogenesis of IBDV superinfection in juvenile chickens when first infected with a mild strain followed by challenge with a virulent strain of the virus (6). In addition, superinfection studies using dengue virus in mammalian and mosquito cell cultures demonstrated a reduction in the viral titer of the superinfecting virus when compared to single infection controls (85). However, we observed no significant increase in the disadvantage of the secondary virus with increasing time between exposures, while Pepin et al. observed that increased time between incubation of mosquito cells with the primary virus and exposure to a superinfecting variant was correlated with a greater decrease in the titer of the secondary virus (85). Additionally, significant differences were observed between dengue virus strains with regard to replication during superinfection in a cell

culture system, whereas no differences in superinfection replication fitness between IHNV genotypes were observed in this in vivo system.

Taken together, these results demonstrate the importance of strictly defining the environment in which measurements of viral fitness are being made. Previously, relative viral fitness for genotypes B and C was reported to be equal when fitness was assessed after immersion challenge, which assessed the ability to enter the host and replicate within the host (108). Our work supports those results, but also demonstrated that the relationship between B and C changes as the virus moves through the infection cycle from in-host replication to viral shedding into the surrounding environment. By this fitness measure, genotype C has a significant advantage over genotype B. Thus, if viral fitness were defined as the ability to enter, establish productive infection, and shed progeny virus into the environment, genotype C would have significantly greater fitness than genotype B in co-infection of rainbow trout. However, if viral fitness were measured as the ability to establish a superinfection or replicate in the context of superinfection, these two genotypes would again be determined to have equal fitness in rainbow trout. Ultimately, the environment within which viral fitness is measured must be considered and clearly defined before the outcome of viral competition can be understood.

A point of note regarding these experiments is that, although statistically significant differences between genotypes B and C were not found in most experiments, there was a consistent trend of higher mortality and higher viral load attributed to genotype C in single infections and co-infections. Additionally, while not statistically significant, a trend was observed with genotype C having a greater ability to establish or prevent the establishment of secondary infection in superinfection with 24 or 48 hours between exposures. These observations, combined with the statistically significant difference in virus shedding, may signify

a biological difference between these genotypes that is not large enough to be statistically significant but may have biological relevance in the field. The finding that these trends of higher fitness in genotype C correlate with a trend of higher virulence in genotype C is consistent with previous reports that virulence correlates with viral fitness for two genotype of unequal virulence in rainbow trout (118, 120). These observations again support the utility of multiple assays to measure overall viral fitness because, when combined, these observations make a strong case for a biological difference between genotypes B and C when a single assay would have provided insufficient evidence for such a difference.

Table 3.1 Mean log viral load in fish and water for each co-infection experiment

		B (log pfu/g fish) (±SEM)		C (log pfu/g fish) (±SEM)	
Experiment	**Measurement**	**Single**	**Mixed**	**Single**	**Mixed**
Co-infection, immersion (1)	In-host viral load	8.63 (±0.19)	8.06 (±0.18)	8.74 (±0.22)	8.64 (±0.19)
Co-infection, immersion (2)	In-host viral load	6.81 (±0.18)	6.32 (±0.18)	6.98 (±0.22)	6.75 (±0.19)
Co-infection, injection (1)	In-host viral load	8.38 (±0.21)	8.27 (±0.18)	8.92 (±0.23)	8.28 (±0.31)
Co-infection, injection (2)	In-host viral load	7.61 (±0.24)	7.36 (±0.22)	7.79 (±0.31)	7.95 (±0.26)
Co-infection, immersion (1)	Virus shed	4.55 (±0.12)	4.07 (±0.11)	5.32 (±0.16)	4.91 (±0.09)
Co-infection, immersion (2)	Virus shed	4.93 (±0.07)	4.58 (±0.08)	4.95 (±0.12)	5.14 (±0.08)

Table 3.2 Distribution of fish in infection status categories for genotypes B and C.

	Genotype Exposure		Observed Infection Status (Expected Infection Status)[a], in numbers of fish				adjusted p value[b]	
Interval (Experiment)	**1°**	**2°**	**Super-infection**	**1° only**	**2° only**	**un-infected**	**Goodness of fit[c]**	**Fisher exact[d]**
12 hour (1)	B	C	4 (10)	4 (0)	1 (0)	1 (0)	0.000	1.000
	C	B	5 (8)	3 (0)	0 (0)	0 (0)	0.000	
	B	mock		6	0	0		
	C	mock		3	0	0		
	mock	B		0	6	0		
	mock	C		0	6	0		
12 hour (2)	B	C	13 (15)	0 (0)	2 (0)	0 (0)	0.000	0.367
	C	B	9 (13.3)	5 (1.7)	1 (0)	0 (0)	0.000	
	B	mock		9	0	0		
	C	mock		9	0	0		
	mock	B		0	8	1		
	mock	C		0	9	0		
24 hour (1)	B	C	10 (15)	5 (0)	0 (0)	0 (0)	0.000	1.000
	C	B	9 (13.3)	5 (1.7)	1 (0)	0 (0)	0.000	
	B	mock		9	0	0		
	C	mock		9	0	0		
	mock	B		0	8	1		
	mock	C		0	9	0		
24 hour (2)	B	C	12 (13.3)	3 (0)	0 (1.7)	0 (0)	0.000	0.769
	C	B	7 (11.8)	8 (1.5)	0 (1.5)	0 (0.18)	0.006	
	B	mock		8	0	1		
	C	mock		8	0	1		
	mock	B		0	8	1		
	mock	C		0	9	0		
48 hour (1)	B	C	7 (10.5)	4 (0)	4 (4.5)	0 (0)	0.000	0.315
	C	B	2 (15)	10 (0)	1 (0)	2 (0)	0.000	
	B	mock		7	0	3		
	C	mock		9	0	0		
	mock	B		0	8	0		
	mock	C		0	9	0		
48 hour (2)	B	C	3 (11.7)	10 (0)	1 (3.3)	1 (0)	0.000	1.000
	C	B	0 (11.7)	15 (0)	0 (3.3)	0 (0)	0.000	
	B	mock		8	0	1		
	C	mock		3	0	6		
	mock	B		0	8	1		
	mock	C		0	9	0		

96 hour (1)	B	C	7 (13.3)	6 (0)	0 (1.7)	2 (0)	0.000	0.388
	C	B	8 (11.7)	4 (3.3)	2 (0)	1 (0)	0.000	
	B	mock		7	0	2		
	C	mock		7	0	2		
	mock	B		0	9	0		
	mock	C		0	9	0		
96 hour (2)	B	C	0 (10)	9 (0)	4 (5)	2 (0)	0.000	0.367
	C	B	0 (11.7)	14 (3.3)	0 (0)	1 (0)	0.000	
	B	mock		8	0	1		
	C	mock		9	0	0		
	mock	B		0	7	2		
	mock	C		0	9	0		
7 days (1)	B	C	2 (15)	9 (0)	1 (0)	2 (0)	0.000	0.950
	C	B	0 (13.3)	14 (1.7)	0 (0)	0 (0)	0.000	
	B	mock		6	0	3		
	C	mock		7	0	2		
	mock	B		0	9	0		
	mock	C		0	9	0		
7 days (2)	B	C	4 (9.1)	6 (2.6)	3 (2.6)	2 (0.7)	0.000	1.000
	C	B	3 (11.8)	11 (1.5)	0 (1.5)	1 (0.2)	0.000	
	B	mock		9	0	0		
	C	mock		9	0	0		
	mock	B		0	8	1		
	mock	C		0	9	0		

[a] Expected infection status is calculated as proportion of fish likely to be in each category based on the observed infection frequencies of single infection groups multiplied by total number of fish in the superinfection group.

[b] p values following Bonferroni adjustment

[c]Exact multinomial test was used to determine whether observed distribution of fish in infection status categories is equal to expected distribution based on single infection controls (null hypothesis). Analysis was performed for each superinfection group for each experiment. $p < 0.05$ indicates support to reject null hypothesis, i.e. observed distribution does not equal expected.

[d]Fisher exact test was used to determine whether distribution of fish in infection status categories was independent of genotype used for primary infection (null hypothesis). $p < 0.05$ indicates support to reject null hypothesis, i.e. distribution is not independent of genotype used first.

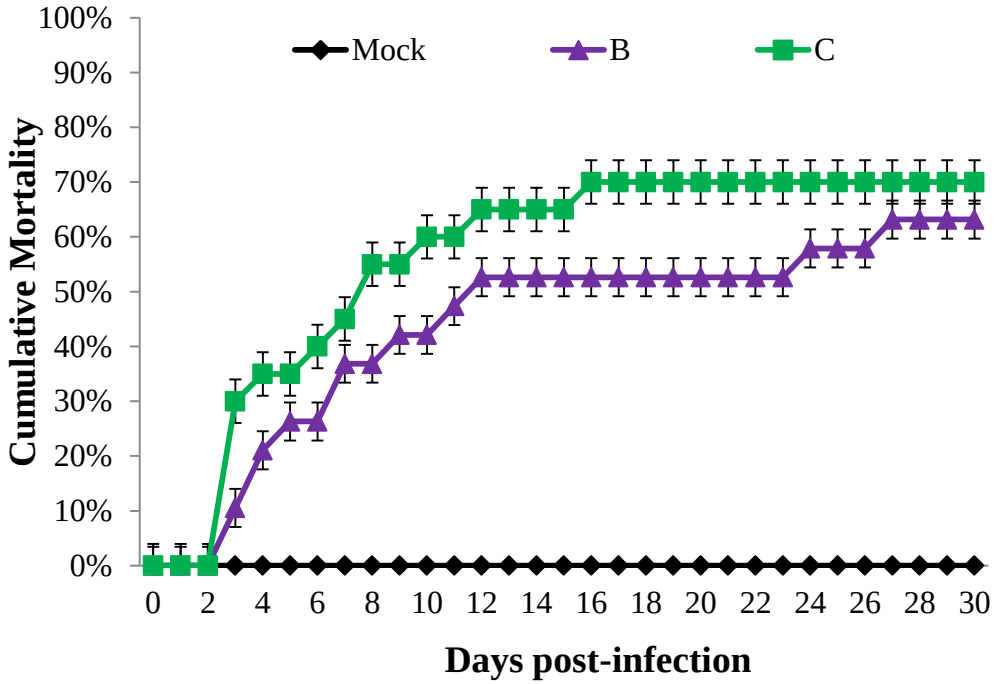

Figure 3.1. Virulence of genotypes B and C in juvenile rainbow trout. Experimental batch immersion challenge defining virulence of IHNV genotypes B (purple triangles) and C (green squares) in triplicate groups of 20 juvenile rainbow trout. Lines are daily mean cumulative percent mortality (± 1 S.E.M) over 30 days of infection. Mock exposed fish showed no mortality (black diamonds).

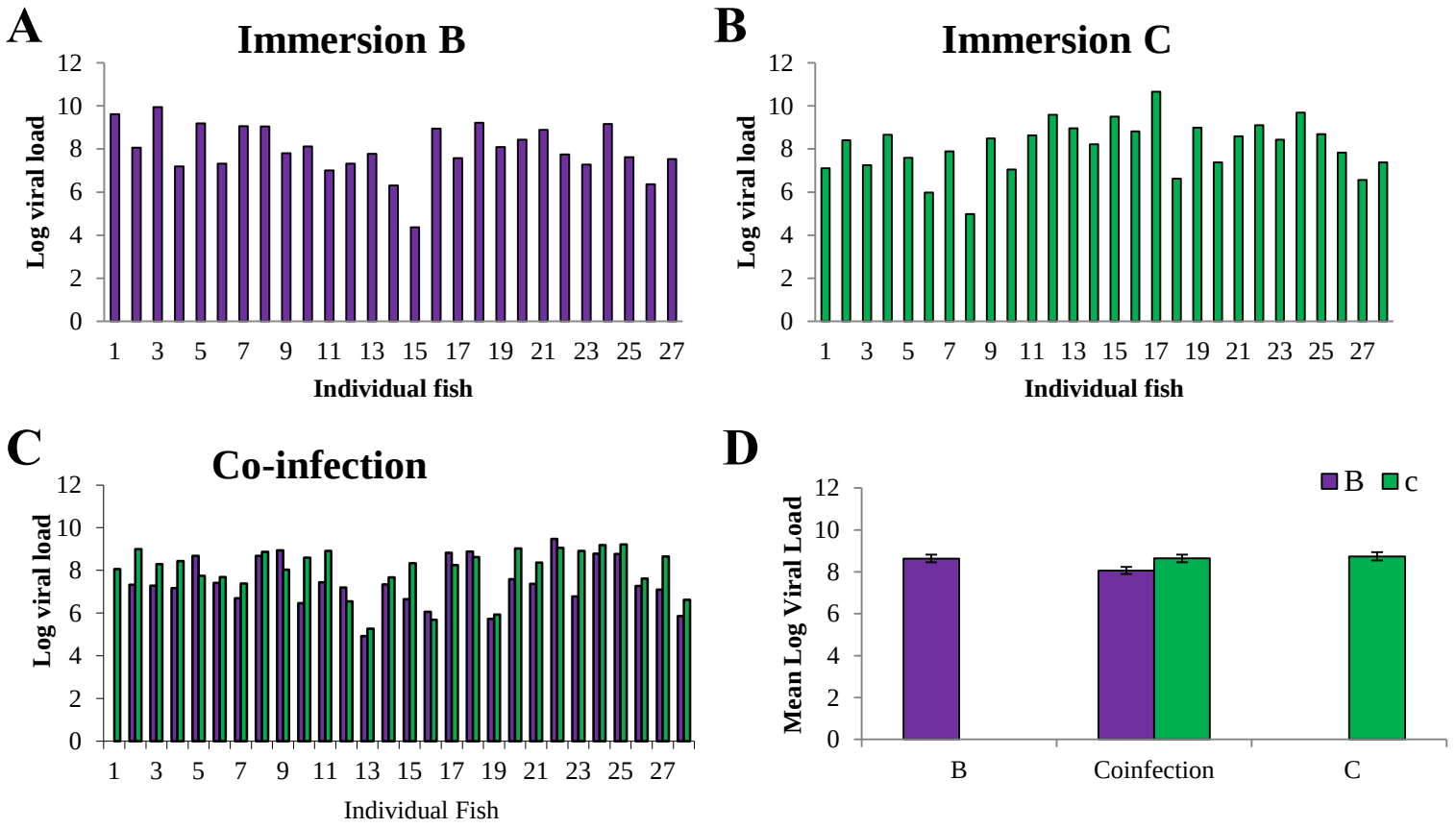

Figure 3.2. In-host replication of genotypes B and C following immersion co-infection challenge, Experiment 1. Viral loads of genotypes B (purple bars) and C (green bars) determined by genotype-specific qRT-PCR in individual fish from experiment 1, three days after exposure to A) B alone, B) C alone, C) a 1:1 mixture of B:C. D) Mean log viral loads for each experimental group (±SEM).

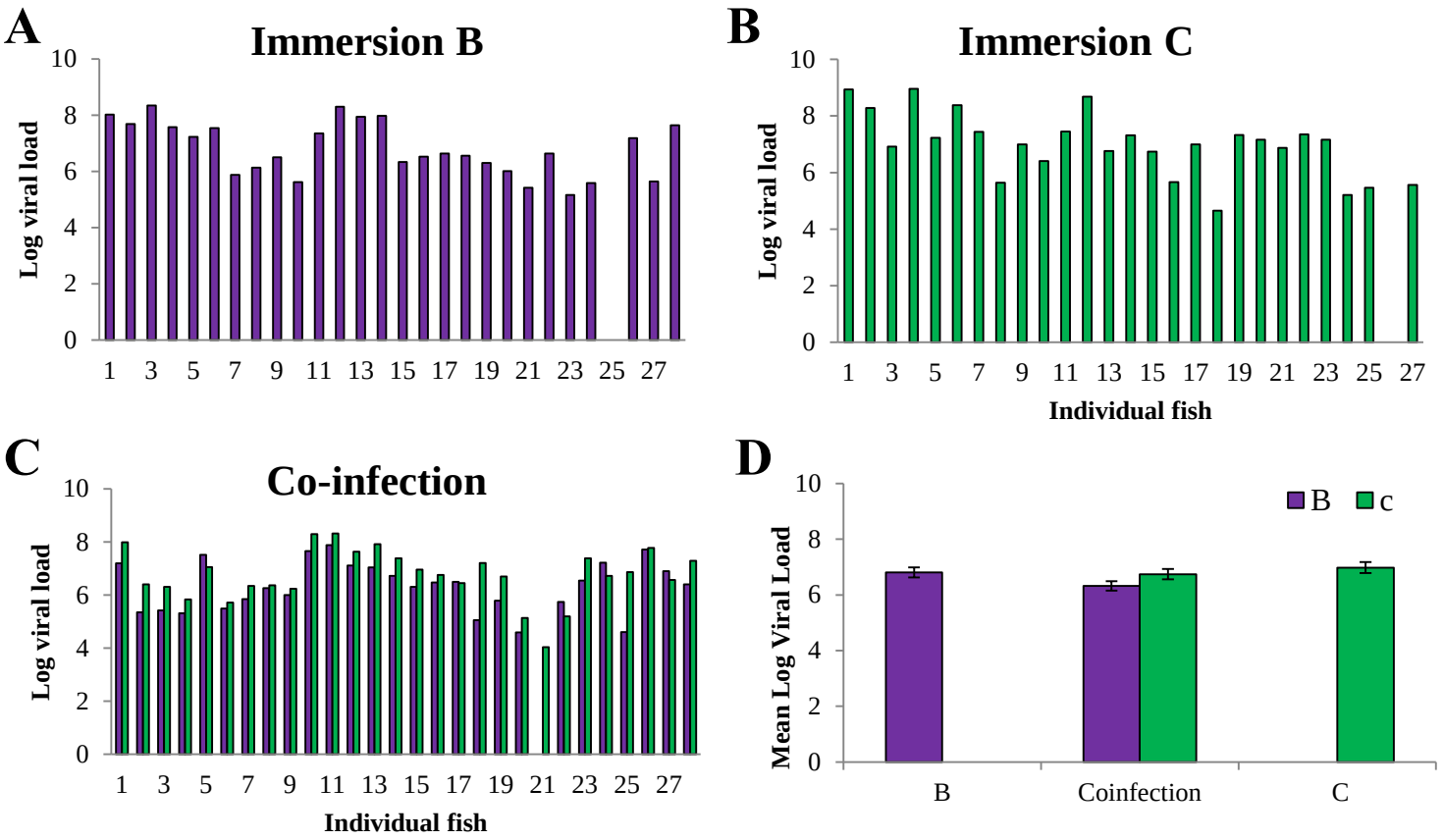

Figure 3.3. In-host replication of genotypes B and C following immersion co-infection challenge, Experiment 2. Viral loads of genotypes B (purple bars) and C (green bars) determined by genotype-specific qRT-PCR in individual fish from experiment 2, three days after exposure to A) B alone, B) C alone, C) a 1:1 mixture of B:C. D) Mean log viral loads for each experimental group (±SEM).

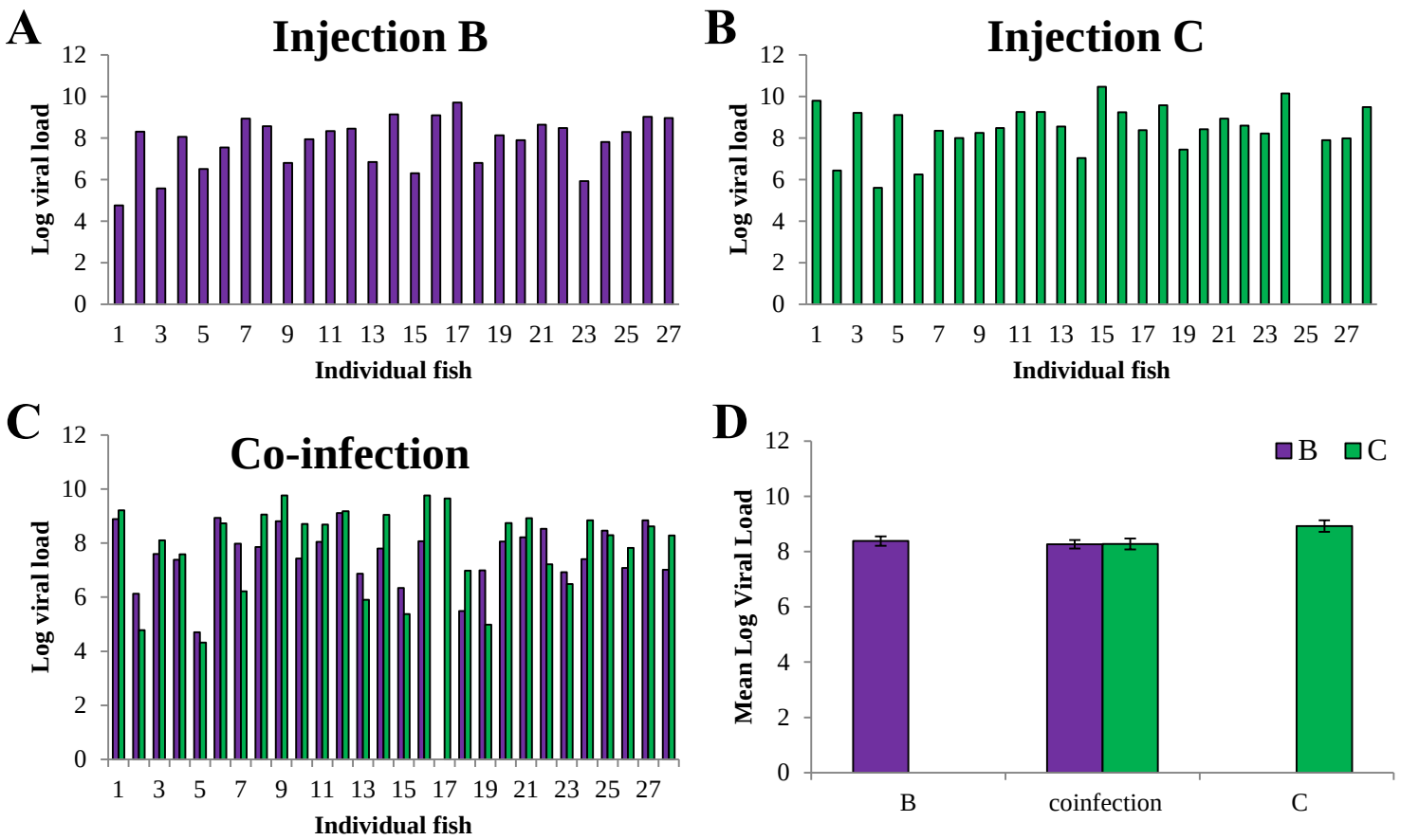

Figure 3.4. In-host replication of genotypes B and C following injection co-infection challenge, Experiment 1. Viral loads of genotypes B (purple bars) and C (green bars) determined by genotype-specific qRT-PCR in individual fish from experiment 1, three days after injection with A) B alone, B) C alone, C) a 1:1 mixture of B:C. D) Mean log viral loads for each experimental group.

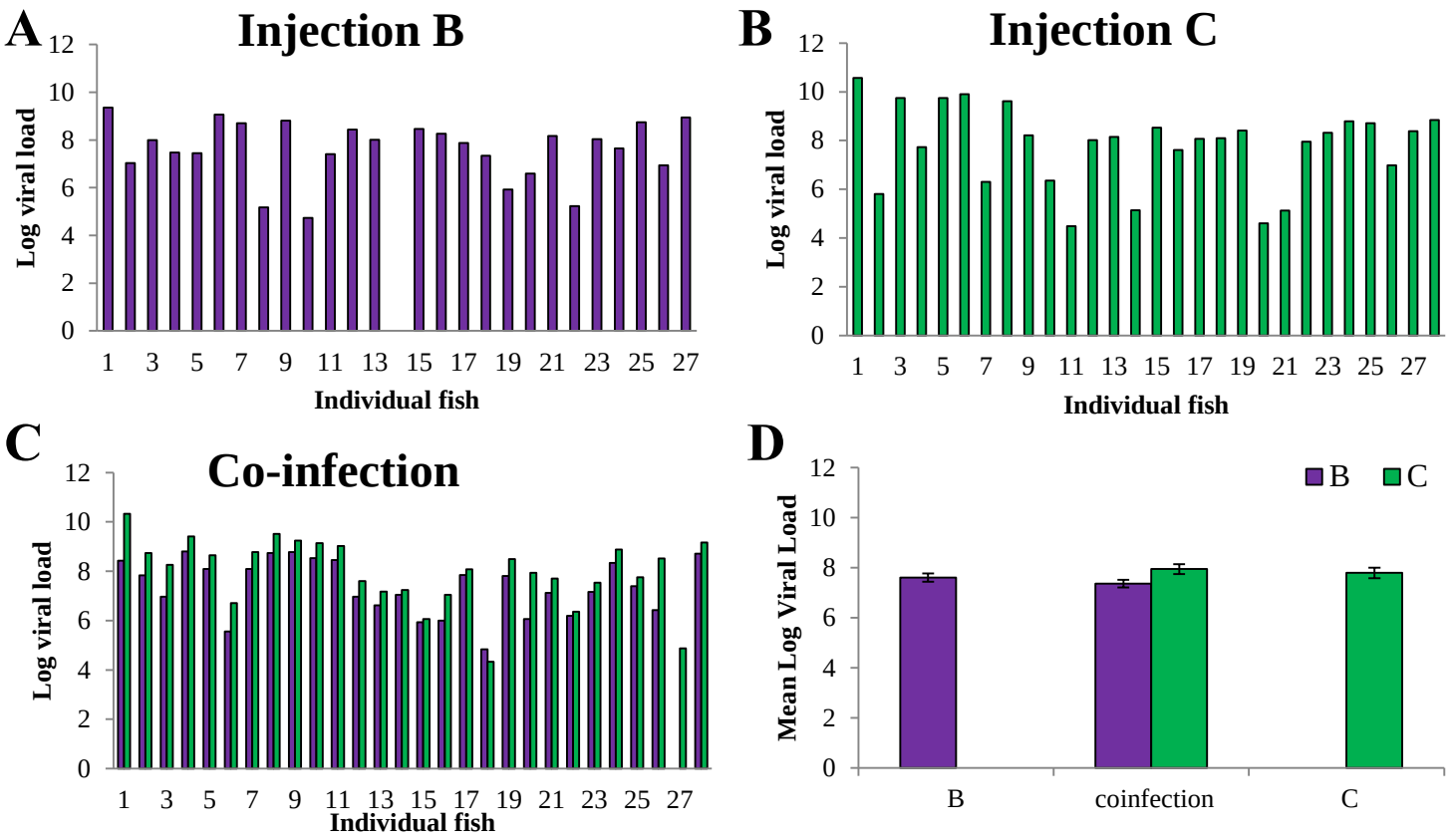

Figure 3.5. In-host replication of genotypes B and C following injection co-infection challenge, Experiment 2. Viral loads of genotypes B (purple bars) and C (green bars) determined by genotype-specific qRT-PCR in individual fish from experiment 2, three days after injection with A) B alone, B) C alone, C) a 1:1 mixture of B:C. D) Mean log viral loads for each experimental group.

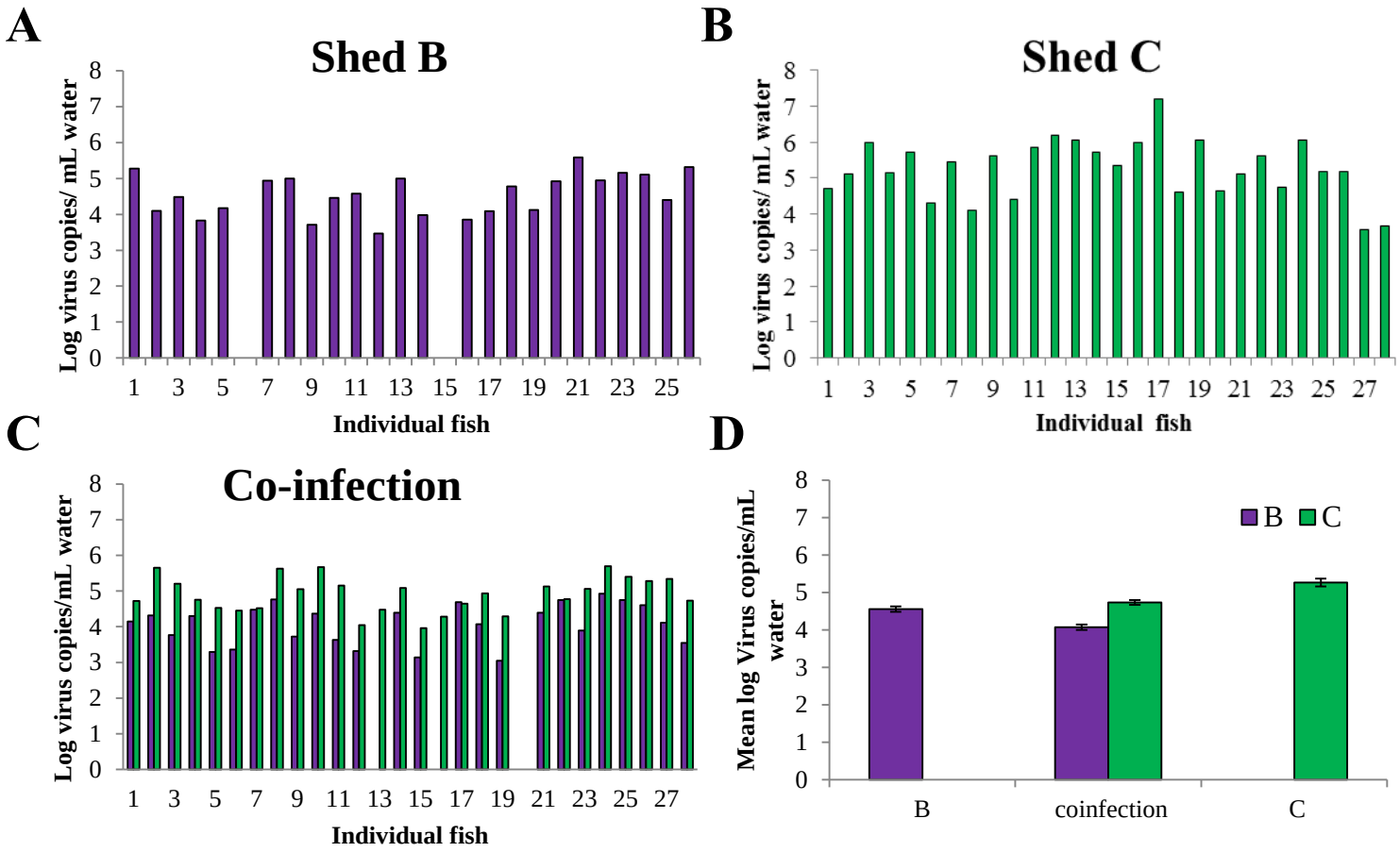

Figure 3.6. Virus shed into water following immersion co-infection challenge, Experiment 1. Shed virus for genotypes B (purple bars) and C (green bars) determined by genotype-specific qRT-PCR in individual fish from experiment 1, three days after exposure to A) B alone, B) C alone, C) a 1:1 mixture of B:C. D) Mean log viral loads for each experimental group.

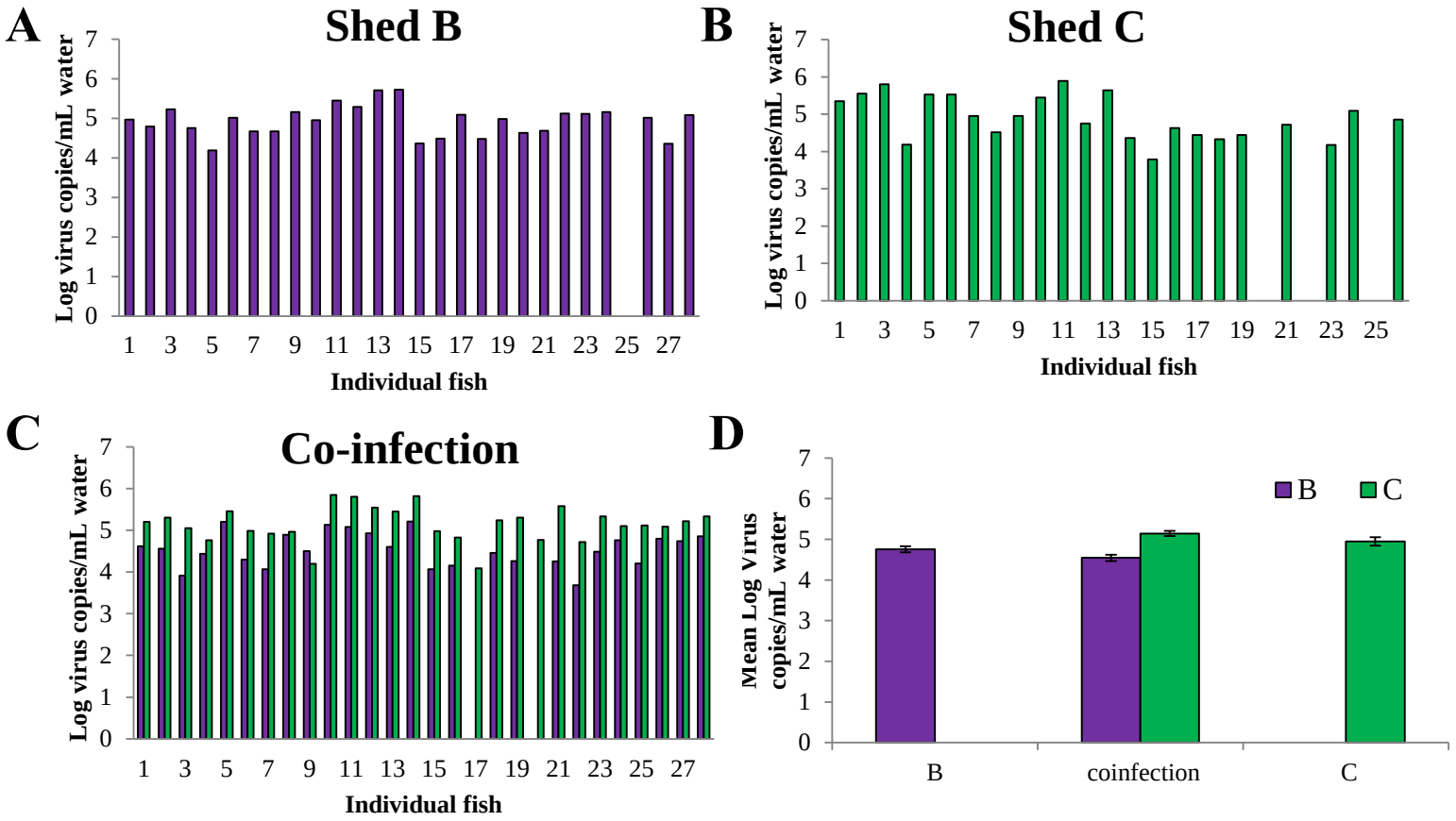

Figure 3.7. Virus shed into water following immersion co-infection challenge, Experiment 2. Shed virus for genotypes B (purple bars) and C (green bars) determined by genotype-specific qRT-PCR in individual fish from experiment 2, three days after exposure to A) B alone, B) C alone, C) a 1:1 mixture of B:C. D) Mean log viral loads for each experimental group.

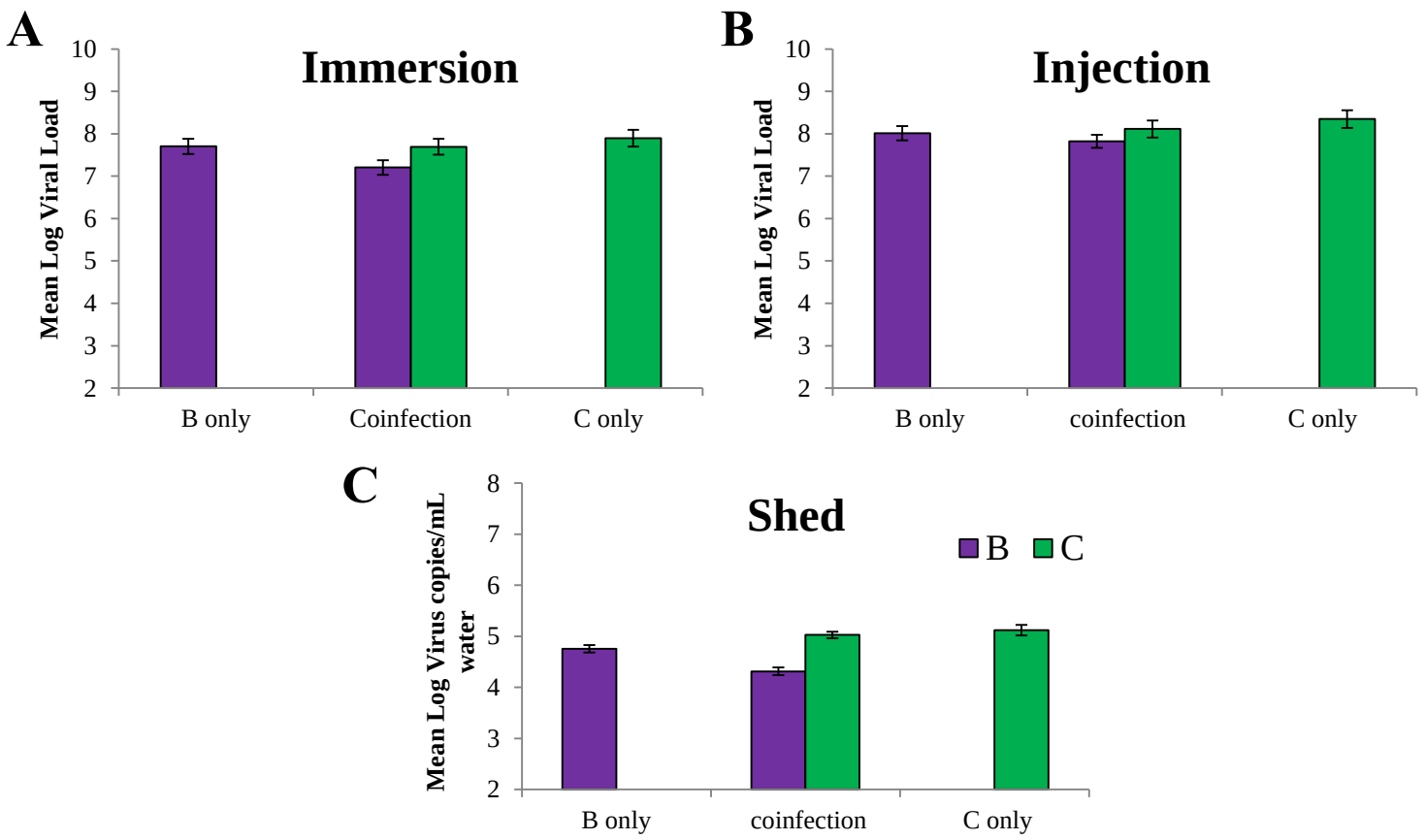

Figure 3.8. Mean viral load for all co-infection challenges for IHNV genotypes B and C. Mean viral loads for two experiments for each treatment group from A) immersion challenge or B) injection challenge. C) Mean virus shed from immersion challenged fish for two experiments (±SEM).

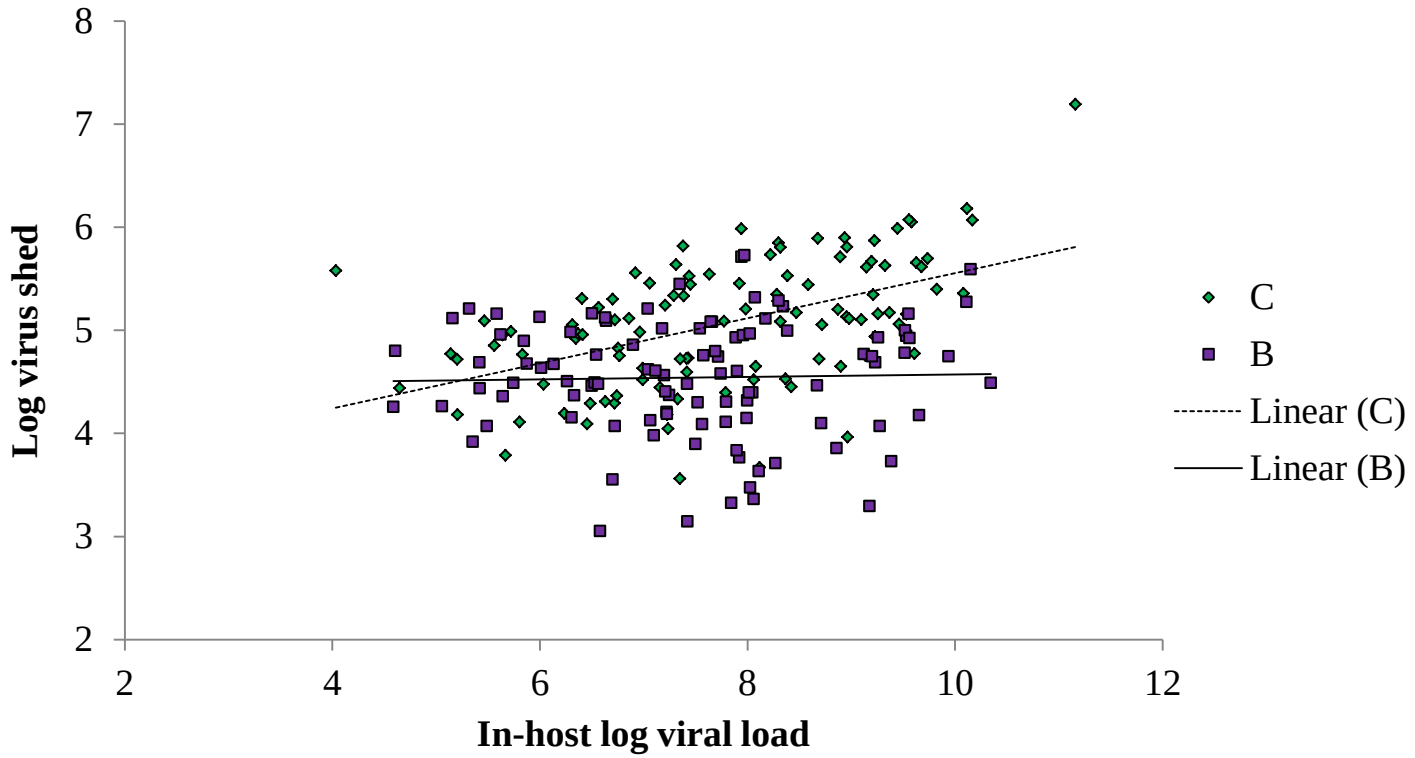

Figure 3.9. Correlation of viral loads in individual fish with viral loads in the water for genotypes B and C. Each point represents a single fish in either single infection or co-infection groups in experiment 1 and 2. All samples positive for virus in both the fish and water were plotted. The best-fit lines for B [log(virus copies/ml H_2O) = log(virus copies/g fish) × (0.012 ± 0.04) + (4.45 ± 0.32)] and C [log(virus copies/ml H_2O) = log(virus copies/g fish) × (0.219 ± 0.04) + (3.37 ± 0.29)] are shown.

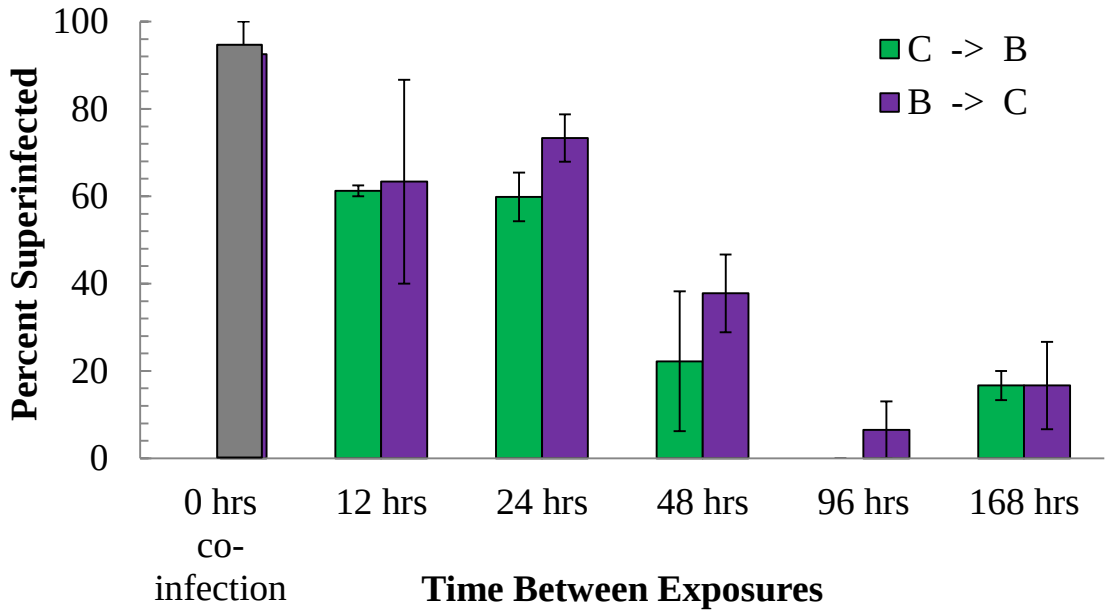

Figure 3.10. Frequency of superinfection in groups exposed to genotypes B and C (equal virulence pair) with increasing time intervals between exposures. Green bars represent the percentage of superinfection in groups of fish exposed to genotype C before genotype B. Purple bars represent the percentage of superinfection in groups of fish exposed to genotype B before genotype C. The grey bar represents the percentage of fish infected with both genotypes following exposure to both genotypes at a 1:1 ratio during the secondary exposure period (co-infection control). Percentages are an average of two independent experiments (±SEM).

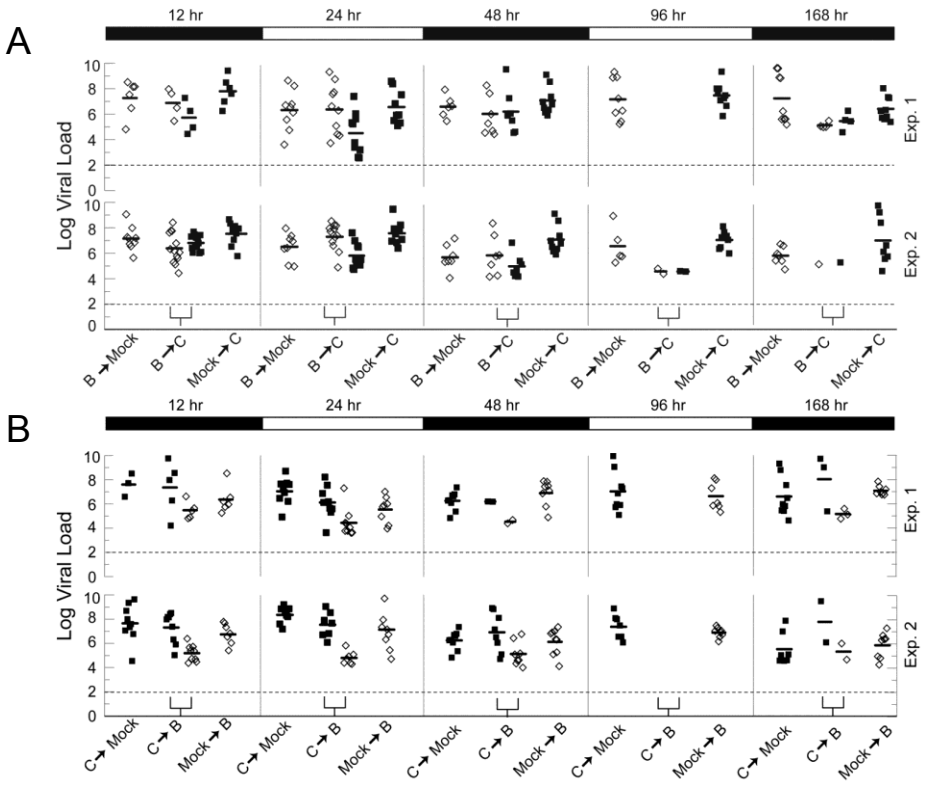

Figure 3.11. Impact of superinfection on viral load for all experiments performed with genotypes B and C, where B is used for primary infection(A) and where C is used for primary infection (B). Viral load attributed to genotype C (black squares) and genotype B (white diamonds) in individual fish from each treatment group (x-axis) is shown for replicate experiments for each time interval between exposures as indicated. Horizontal dotted lines indicate detection levels for the genotype-specific RT-qPCR assays. Differences between groups were analyzed by ANOVA with Bonferroni correction.

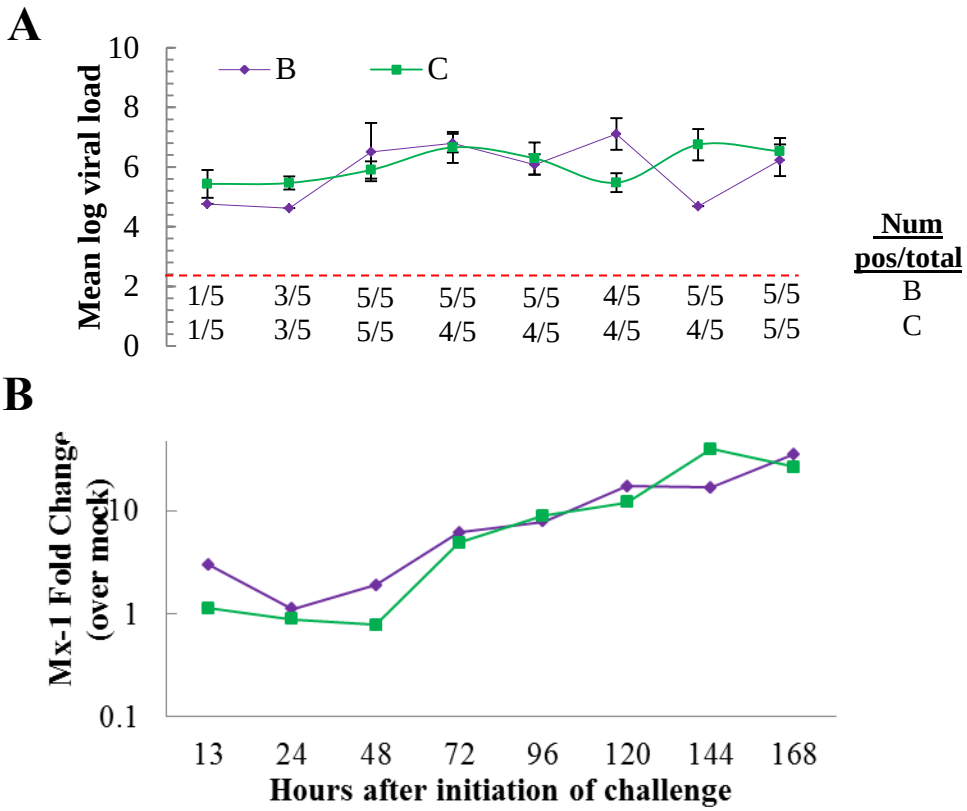

Figure 3.12. Viral growth kinetics and *Mx-1* induction in single infections with genotypes B and C. A) Mean log viral load in 5 fish sampled at each time point, excluding virus-negative samples, in rainbow trout following single exposure using our superinfection protocol conditions with genotype B (purple diamonds) or genotype C (green squares) (±SEM). Horizontal dotted line indicates detection levels for the genotype-specific RT-qPCR assays. "Num pos/total" indicates prevalence of virus-positive fish out of total fish sampled at each time point for each genotype. B) Mean normalized *Mx-1* transcription fold changes, relative to the mock-control group, in the same fish shown in the viral load curves (4.12A). Fish exposed to genotype B (purple diamonds) and genotype C (green squares).

Chapter 4

Superinfection fitness of IHNV genotype pair of unequal virulence, HV & LV

"One fish, two fish, red fish, blue fish!"
- Dr. Seuss, *One fish, two fish, red fish, blue fish*

Introduction

As discussed and demonstrated in Chapter 3, in vivo competition between two virus genotypes of equal virulence (genotype B and genotype C) revealed that these viruses are of equal entry and in-host replication fitness in co-infection and superinfection contexts, while genotype C has a significant advantage in shedding virus into the surrounding environment. Interestingly, previous in vivo co-infection competition experiments with variants displaying high virulence (genotype HV) and low virulence (genotype LV), demonstrated that observed differences in virulence correlated with significant differences in in-host replication, as well as host entry and virus shedding (118, 120). Importantly, higher virulence was consistently associated with greater viral fitness in multiple traits associated with co-infection. We therefore hypothesized that virulence would also be positively associated with fitness in superinfection.

To test this hypothesis, we applied our in vivo superinfection assay to genotypes HV and LV using methods described in previous chapters. We performed duplicate superinfection assays with each of five time intervals between exposures using genotypes HV and LV. This work comprises the first detailed investigation into superinfection fitness and virulence using controlled in vivo assays in a natural, vertebrate host-virus system, and was published as Kell *et al.*, 2013 (48).

Results

Frequency of superinfection

The mean percentage of fish found to be superinfected following exposures using the low and high virulence IHNV genotypes (HV and LV, respectively) with variable intervals between

exposures is shown in Figure 4.1. As seen with the equal virulence genotype pair B and C, superinfection frequency decreased as time between exposures increased. Additionally, we determined that, for groups with greater than 12 hours between exposures, the frequency of superinfection observed differed significantly from the frequency that would have been expected based on single infection control groups, indicating that superinfection restriction occurs with this genotype pair (exact multinomial goodness of fit, $p<0.05$ Bonferroni adjusted). Infection status data from these experiments are shown in Table 4.1.

To test whether high virulence was associated with greater ability to establish a secondary infection for these genotypes, we compared the frequency of superinfected fish in groups exposed to genotype LV prior to genotype HV with the frequency of superinfection when exposed to HV prior to LV. For some experiments, there was a significant difference in the distribution of fish into the four infection categories when comparing the reciprocal superinfection groups (Fisher exact, $p<0.05$ Bonferroni adjusted) (Table 4.1). The frequency of superinfection in groups of fish exposed to LV first followed by HV was significantly greater than in the reciprocal group for one of two experiments performed at intervals of 24 hrs, 48 hrs, and 7 days between exposures. These findings were reversed for one of two experiments with a 96hr interval between exposures (Figure 4.1).

Impact of superinfection on viral load

To test the ability of HV and LV to replicate during superinfection, we again compared the viral load of each genotype in single infections to the viral load of the same genotype in the context of a superinfection (Figure 4.2). As was observed for the equal virulence pair, there was a significant superinfection effect that was dependent upon the order of exposures to each

genotype (competition:order interaction, $F_{1,247} = 31.23$, $p = 0.001$ and $F_{1,228} = 24.19$, $p < 0.001$, HV and LV respectively). Again, while the primary virus appeared unaffected, the virus genotype used during the secondary exposure period in superinfection groups replicated to significantly lower levels than in groups exposed to single genotypes during the secondary exposure period (Figure 4.2).

To address the question of whether virulence was associated with the ability to replicate in superinfection for genotypes HV and LV, we compared the impact of superinfection on viral load of the two genotypes and found no significant difference (competition:genotype interaction, $F_{1,236} = 0.0043$, $p=1$). Thus, measured viral load of both genotypes were equally impacted by superinfection and differences in virulence did not correlate with viral replication in superinfection. As seen for the equal virulence pair, similar patterns were observed across intervals and, as such, the interval period did not significantly influence the magnitude of the impact of superinfection on viral replication.

Viral growth and Mx-1 gene expression

We performed single infections with either genotype HV or genotype LV and measured both viral load and Mx-1 gene expression in five individual, whole fish at each time point corresponding to the timing of secondary exposures. In addition to the time points assayed for genotypes B and C, fish were sampled at time 0 which corresponds to the beginning of the challenge period. As observed for genotype pair B and C, the replication of HV and LV over time was very similar (Figure 4.3B). Expression of Mx-1 was significantly induced beginning 48 hours after infection (Figure 4.3B). Thus, we again observed a lag in Mx-1 up-regulation relative

to the onset of superinfection restriction, which began at 24 hours following primary infection (Figure 4.1).

Discussion

Our previous analyses discussed in chapter 3 determined that for the genotypes of equal virulence, B and C, there were no significant differences in the frequencies of superinfection between groups exposed to genotype B before C, or genotype C before B, for any experiments with all intervals between exposures. Although similar results were obtained for most experiments with genotypes HV and LV, the superinfection frequency was significantly different in some experiments at some intervals. The frequency of superinfection was significantly higher in groups exposed to genotype LV before genotype HV when compared to the reciprocal group for one of two experiments in three of the four intervals tested. Interestingly, for the 96 hour interval experiments, one experiment demonstrated the opposite effect. While we conclude from these experiments that virulence alone does not determine overall superinfection fitness, based on the three experiments that did find significant difference in superinfection fitness, we cannot exclude the possibility that HV may have some advantage over LV in this system.

Although we found that virulence was not associated with an advantage in replication in superinfection as we originally hypothesized, the trend toward increased capability to establish superinfection or prevent establishment of a secondary infection may endow a high virulence variant a discrete competitive advantage in a region where multiple variants co-circulate and infection rates are high. Such an advantage could result in a selection for increased virulence within a viral population and could have profound impacts on the host population, particularly when this advantage is combined with the entry, replication, and shedding advantages previously

documented for the virulent genotype (120). It is important to note that our observation that virulence plays a very limited role, if any, in superinfection fitness is based on the investigation of only one pair of IHNV variants of high or low virulence, and investigations with different variants may yield different results. Although additional studies will be necessary to make conclusions about the full role of virulence, our observations do suggest that virulence does not determine superinfection fitness in this system.

Table 4.1. Distribution of fish in infection status categories for genotypes HV and LV.

	Genotype Exposure		Observed Infection Status (Expected Infection Status)[a], in numbers of fish				corrected p value[b]	
Interval (Experiment)	1°	2°	Super-infected	1° only	2° only	uninfected	Goodness of fit [c]	Fisher exact [d]
12 hour (1)	HV	LV	10 (15)	4 (0)	1 (0)	0 (0)	0.000	1.000
	LV	HV	13 (15)	2 (0)	0 (0)	0 (0)	0.000	
	HV	mock	0	9	0	0		
	LV	mock	0	9	0	0		
	mock	HV	0	0	9	0		
	mock	LV	0	0	9	0		
12 hour (2)	HV	LV	7 (6.7)	6 (8.3)	0 (0)	2 (0)	0.000	1.000
	LV	HV	10 (10)	5 (0)	0 (5)	0 (0)	0.000	
	HV	mock	0	9	0	0		
	LV	mock	0	6	0	3		
	mock	HV	0	0	9	0		
	mock	LV	0	0	4	5		
24 hour (1)	HV	LV	6 (10)	9 (5)	0 (0)	0 (0)	1.000	1.000
	LV	HV	10 (15)	5 (0)	0 (0)	0 (0)	0.000	
	HV	mock	0	9	0	0		
	LV	mock	0	9	0	0		
	mock	HV	0	0	9	0		
	mock	LV	0	0	6	3		
24 hour (2)	HV	LV	2 (15)	13 (0)	0 (0)	0 (0)	0.000	0.000
	LV	HV	12 (15)	1 (0)	1 (0)	0 (0)	0.000	
	HV	mock	0	9	0	0		
	LV	mock	0	9	0	0		
	mock	HV	0	0	9	0		
	mock	LV	0	0	9	0		
48 hour (1)	HV	LV	1 (13.3)	12 (0)	0 (1.7)	2 (0)	0.000	1.000
	LV	HV	3 (15)	11 (0)	1 (0)	0 (0)	0.000	
	HV	mock	0	8	0	1		
	LV	mock	0	9	0	0		
	mock	HV	0	0	9	0		
	mock	LV	0	0	9	0		
48 hour (2)	HV	LV	0 (13.3)	15 (1.7)	0 (0)	0 (0)	0.000	0.000
	LV	HV	6 (15)	2 (0)	5 (0)	2 (0)	0.000	
	HV	mock	0	9	0	0		

	LV	mock	0	9	0	0		
	mock	HV	0	0	9	0		
	mock	LV	0	0	8	1		
96 hour (1)	HV	LV	1 (13.3)	8 (1.7)	2 (0)	3 (0)	0.000	1.000
	LV	HV	1 (8)	3 (1)	5 (5.3)	5 (0.7)	0.000	
	HV	mock	0	8	0	0		
	LV	mock	0	6	0	4		
	mock	HV	0	0	8	1		
	mock	LV	0	0	8	1		
96 hour (2)	HV	LV	7 (9.1)	7 (2.6)	0 (2.6)	1 (0.7)	0.308	0.043
	LV	HV	0 (10)	13 (0)	0 (5)	2 (0)	0.000	
	HV	mock	0	7	0	2		
	LV	mock	0	6	0	3		
	mock	HV	0	0	9	0		
	mock	LV	0	0	7	2		
7 days (1)	HV	LV	1 (13.3)	11 (0)	0 (1.7)	2 (0)	0.000	1.000
	LV	HV	1 (8.3)	9 (0)	1 (6.7)	4 (0)	0.000	
	HV	mock	0	8	0	1		
	LV	mock	0	5	0	4		
	mock	HV	0	0	9	0		
	mock	LV	0	0	9	0		
7 days (2)	HV	LV	0 (13.3)	15 (1.7)	0 (0)	0 (0)	0.000	0.008
	LV	HV	3 (15)	6 (0)	5 (0)	1 (0)	0.000	
	HV	mock	0	9	0	0		
	LV	mock	0	9	0	0		
	mock	HV	0	0	9	0		
	mock	LV	0	0	8	1		

[a] Expected infection status is calculated as proportion of fish likely to be in each category based on the observed infection frequencies of single infection groups multiplied by total number of fish in the superinfection group.

[b] p values following Bonferroni adjustment

[c]Exact multinomial test was used to determine whether observed distribution of fish in infection status categories is equal to expected distribution based on single infection controls (null hypothesis). Analysis was performed for each superinfection group for each experiment. $p < 0.05$ indicates support to reject null hypothesis, i.e. observed distribution does not equal expected.

[d]Fisher exact test was used to determine whether distribution of fish in infection status categories was independent of genotype used for primary infection (null hypothesis). $p < 0.05$ indicates support to reject null hypothesis, i.e. distribution is not independent of genotype used first.

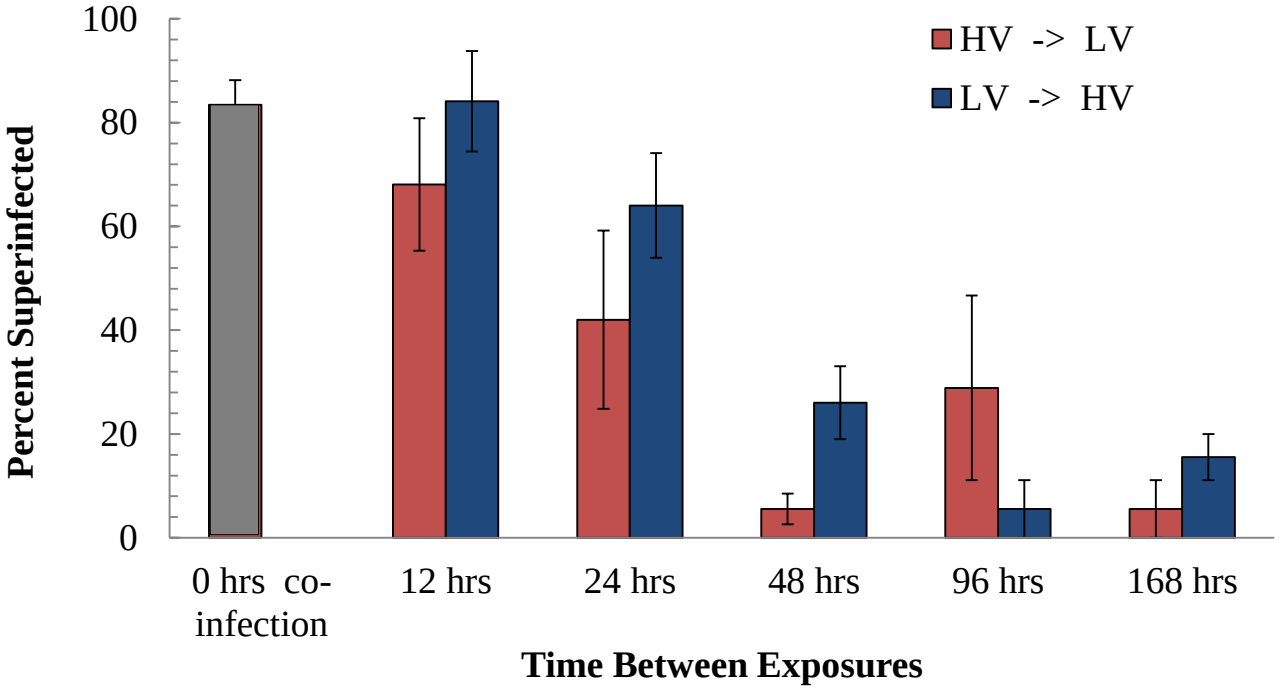

Figure 4.1. Frequency of superinfection in groups exposed to genotypes HV and LV (unequal virulence pair) with increasing intervals between exposures. Red bars represent the percentage of superinfection in groups of fish exposed to genotype HV before genotype LV. Blue bars represent the percentage of superinfection in groups of fish exposed to genotype LV before genotype HV. The grey bar represents the percentage of fish infected with both genotypes following exposure to both genotypes at a 1:1 ratio during the secondary exposure period. Percentages are an average of two independent experiments (±SEM).

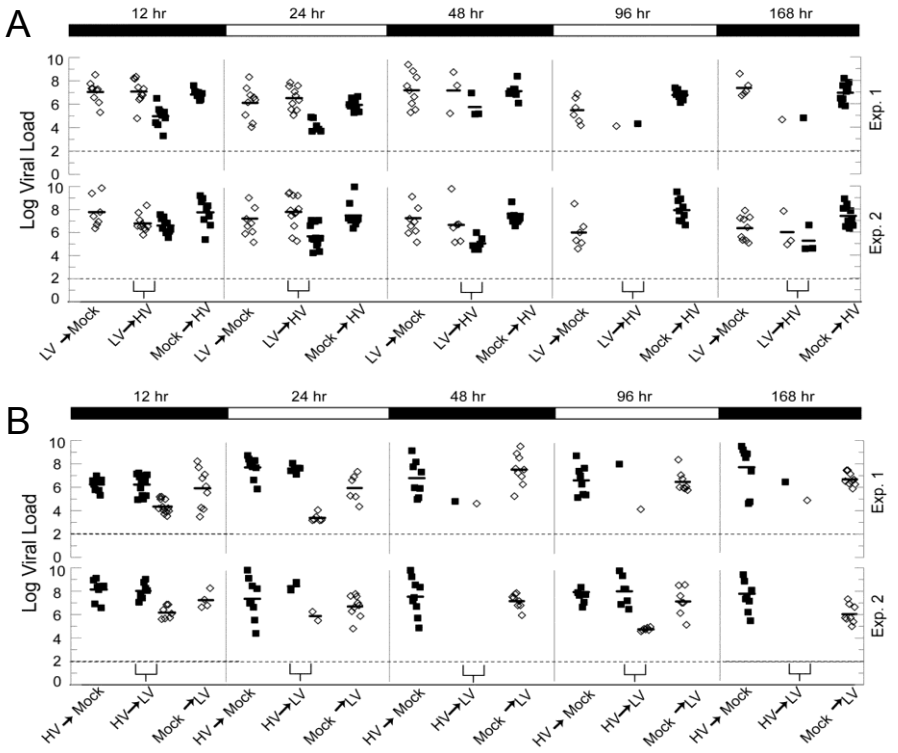

Figure 4.2. Impact of superinfection on viral load for all experiments performed with genotypes HV and LV, where LV is used for primary infection (A) and where HV is used for primary infection (B). Viral load attributed to genotype HV (black squares) and genotype LV (white diamonds) in individual fish from each treatment group (x-axis) is shown for replicate experiments for each time interval between exposures as indicated. Horizontal dotted lines indicate detection levels for the genotype-specific RT-qPCR assays. Differences between groups were analyzed by ANOVA with Bonferroni correction.

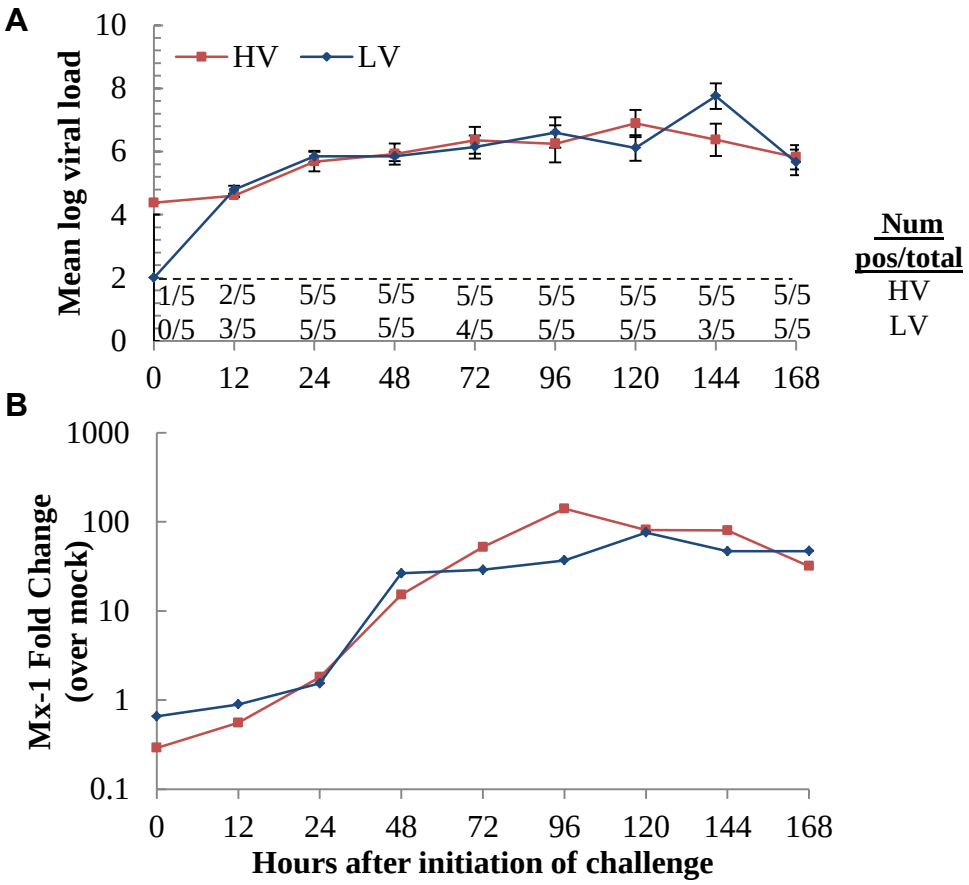

Figure 4.3. Viral growth kinetics and *Mx-1* induction in single infections with genotypes HV and LV. A) Mean log viral load in 5 fish sampled at each time point, excluding virus-negative samples, in rainbow trout following single exposure using our superinfection protocol conditions with genotype LV (blue diamonds) or genotype HV (red squares) (±SEM). Horizontal dotted line indicates detection levels for the genotype-specific RT-qPCR assays. "Num pos/total" indicates prevalence of virus-positive fish out of total fish sampled at each time point for each genotype. B) Mean normalized *Mx-1* transcription fold changes, relative to the mock-control group, in the same fish shown in the viral load curves (5.3A). Fish exposed to genotype LV (blue diamonds) and genotype HV (red squares).

Chapter 5

Role of viral fitness in three major IHNV displacement events

"Sometimes the questions are complicated and the answers are simple."
— Dr. Seuss

Introduction

As described in the introduction chapter, three major virus genotype displacement events have been observed for M group IHNV in the Pacific Northwest region in the last 30 years (Figure 1.2). The evolutionary processes which shape the genetic diversity of IHNV in nature are unknown and the causal relationship between displacements of IHNV genotypes in the Pacific Northwest region have not been demonstrated. Here, we hypothesized that differences in viral fitness might explain these replacement events observed in the lower Colombia River basin and on the Washington state coast.

To test this hypothesis we chose four specific virus isolates collected from the field to serve as representatives of each genotype involved in the displacement events. Genotype 007 is represented by strain LR80, isolated in 1980 from a fish in the Lewis River in Washington State. Genotype 111 is represented by strain Mer95, isolated from a sample in 1995 from the Merwin Fish Hatchery in Washington State. Genotype 110 is represented by strain Qts07, isolated in 2007 from a sample in the Queets River. Finally, genotype 139 is represented by DW09, isolated in 2009 from a sample located at the Dworshak fish hatchery in Idaho. Some considerations when choosing representative isolates included source location, source species, involvement in juvenile epidemics, and year of isolation. We sought isolates that were found in or very near Washington State, from steelhead trout, and responsible for juvenile epidemics at the time of isolation. These isolates, referred to hereafter as genotypes, were used in laboratory experiments as displacement pairs with 007 competing against 111, 111 competing against 110, and 110 competing against 139.

To assess fitness differences between the virus genotypes, we measured the fitness of the representative virus isolates from each of four genotypes using in vivo co-infection and superinfection competition assays with the natural host, steelhead trout. For each co-infection assay, a pair of genotypes was used to challenge groups of fish in either single infections or co-infections to determine the ability of each genotype to enter the host and replicate in vivo. Each co-infection assay was performed twice for each genotype pair. The superinfection competitions were carried out as described in chapter 2, with interval periods between exposures of 24, 48, and 96 hours. In addition, we measured the virulence, in vivo kinetics of viral replication in single infections, and the interferon-induced Mx-1 expression during single infections, as previously described in chapters 2, 3 and 4. Finally, we measured the stability of each genotype in freshwater and saltwater conditions. Differences between the four genotypes were assessed as three pairs involved in each displacement event in order to determine whether differences in overall viral fitness correlate with the sequential displacements observed in the field.

Results

Virulence

Virulence of each virus genotype in juvenile steelhead trout was assessed by measuring cumulative percent mortality over 30 days post infection by immersion (Figure 5.1). Triplicate tanks of 20 fish each were challenged with each single genotype and mortality was recorded over 30 days post-challenge. Mortality began between 5 and 7 days post-challenge and continued to 29 days for some genotypes. Mean day to death was calculated as 11.9 days, 11.6 days, 9.9 days, and 13.4 days for fish exposed to genotypes 007, 111, 110, and 139 respectively. During the period from 10-30 days post-exposure, the mean cumulative percent mortality (CPM) for each

treatment group differed consistently, with fish exposed to genotype 110 experiencing the highest level of cumulative mortality, followed by groups exposed to genotype 111, then 139, and finally 007. Final CPM was higher than 55% for all treatment groups. Pairwise comparisons of survivorship were made between genotypes involved in each displacement event. Kaplan-Meier survivorship analysis indicated that the first two genotype displacement pairs (007 versus 111, 111 versus 110) did not differ statistically in virulence ($p > 0.05$). For the third displacement pair, significantly higher mortality was observed in fish challenged with genotype 110 compared to those challenged with genotype 139 ($p = 0.0096$). While most differences were not statistically significant, there was an observed trend of increasing virulence in steelhead trout over the first two successive displacement events, with the exception of the third displacement pair.

In-host virus replication and host Mx-1 gene expression

In order to determine the in-host replication dynamics of each genotype, we measured the total viral load found in individual fish infected with single genotypes over seven days post infection (Figure 5.2A). Each day, five fish infected with each virus genotype were sampled and total viral load and Mx-1 gene expression were measured. The four genotypes were quantified independently but the data is presented as relevant pairs for comparison in Figure 5.2. Genotypes in pairs 1 (007 and 111) and 3 (110 and 139) replicated to comparable levels throughout the 7 day course of acute infection. However, fish exposed to genotypes 111 and 110 were observed to have significantly different viral loads during the first four days following infection ($p = 0.0024$), with genotype 111 replicating to higher levels than genotype 110. Peak viral load in fish exposed to genotype 111 was 7.5 log virus copies/ gram of fish on day 3. Viral load reached a peak of 7.5 log virus copies/gram of fish on day 6 for fish infected with genotype 110. Therefore, the

kinetics of viral replication are significantly different between these two genotypes during the early infection period.

Because the kinetics of viral growth could be affected by the level of immune stimulation induced by each genotype, we examined the induction of the interferon-α stimulated gene, Mx-1 in the same fish for which viral load was quantified (Figure 5.2B). Mx-1 expression in fish exposed to genotype 007 differed significantly from that in fish exposed to genotype 111 at day four post-infection, but not on any other day. Groups exposed to genotype 111 demonstrated a stronger Mx-1 induction at days 1-3 than fish exposed to genotype 110. Mx-1 induction following infection with genotype 110 or 139 did not differ over the 7-day early infection period measured (Figure 5.2B).

Co-infection fitness

In order to determine co-infection fitness of the displacement pair isolates, we challenged groups of fish by immersion in water with either single genotypes or a 1:1 mix of two virus genotypes representing each of the three displacement pairs. Data from individual fish for each group for each of two experiments with each genotype pair is shown in Figures 5.3, 5.4, and 5.5. We then determined mean viral load of each genotype in each of these groups at three days post-infection (Figure 5.6). For the first displacement pair, we observed no statistically significant differences between the replication of genotypes 007 and 111 in either single or co-infection contexts ($F_{1,163} = 3.33$, $p = 0.1884$) (Figure 5.6A). However, a significant competition effect was observed for this pair, indicating that the replication of both genotypes was significantly suppressed in co-infection ($F_{1,163} = 8.24$, $p = 0.011$). For the second genotype pair we again observed no difference in the levels of replication between the two genotypes 111 and 110 ($F_{1,108}$

= 0.6408, p = 1), but there was again a statistically significant reduction in viral load for both genotypes in co-infection compared to single infection ($F_{1,108}$ = 28.625, p = 1.49e-6) (Figure 5.6B). For the third genotype pair, total viral load for genotype 110 was significantly higher than the viral load for genotype 139 in both the single infection and co-infection contexts ($F_{1,210}$ = 23.89, p = 6.06e-6) (Figure 5.6C). In addition, a statistically significant reduction in viral load was observed for both genotypes in co-infection compared to single infection ($F_{1,210}$ = 15.34, p = 3.66e-4). As such, for all three genotype pairs competitive suppression was observed where genotypes performed worse in co-infection then alone. However the level of this competitive suppression did not differ between the genotypes (competition*genotype interaction), suggesting that the impact of competition did not differ between genotypes. For all three genotype pairs examined, the genotype which was displaced in the field replicated to higher mean levels than the more recently dominant genotype. While this trend was only statistically significant for the third genotype pair, it directly contradicts the hypothesis that genotype displacement events are correlated with higher co-infection fitness for the displacing virus genotype.

Superinfection fitness

Because superinfection is theoretically more likely to occur in the field than simultaneous co-infection, we examined the fitness of each genotype in a superinfection context. The relative ability of each genotype to establish secondary infection in single hosts was measured by determining the frequency of superinfection in groups of fish sequentially exposed to each genotype pair using the superinfection assay described in chapter 2 (Figure 5.7, Table 5.1-5.3). For all genotype pairs, we observed a decrease in the percent of fish superinfected as the time interval between exposures increased. In addition, in those fish which were not superinfected, almost all were infected only with the virus genotype used for the primary infection. Again, we

tested whether restriction of secondary infection occurred in this system by performing an exact multinomial test, as described in chapter 2. We hypothesized that if superinfection restriction did occur, the frequency of observed superinfection would be less than that which would be expected based on the frequencies of single infections with each genotype at each time interval. For almost all groups at all time intervals tested, superinfection was observed to occur at a significantly different frequency than expected if superinfection occurred at the same frequency with which single infections occurred within the same experiment (Tables 5.1 – 5.3). Importantly, for experiments performed with a 24 hour interval between exposures, significant differences were a result of either greater observed frequency than expected (pair 1, genotypes 007 and 111) or a deviation of just one or two individuals from that expected value. Therefore, the biological relevance of this statistical difference at the 24 hour interval may be debatable. However, for experiments performed with 48 and 96 hours between exposures, the statistically significant difference observed was a result of lower than expected observed superinfection frequency for all genotype pairs, independent of the order of exposures. Thus, superinfection restriction was observed in fish exposed to each genotype.

For genotypes 007 and 111, no statistical differences were observed in the frequencies of superinfection between reciprocal exposure groups (Table 5.1). In other words, the frequency of superinfection did not depend on which virus genotype was used for primary infection. For genotypes 111 and 110, a statistically significant difference was observed between reciprocal exposure groups when an interval of 48 hours between exposures was used (Fisher exact test, $p < 0.001$) (Table 5.2). However, this difference was not observed for groups with 24 or 96 hours between exposures. As for the first genotype pair, the frequency of superinfection in groups

exposed to genotype 110 first and then exposed to 139 did not differ significantly from the reciprocal group for all time intervals tested (Table 5.3).

We next examined the ability of each genotype to replicate in superinfected fish by comparing the viral load of each genotype in superinfection to the viral load measured in fish singly infected with that same genotype at the same exposure period. The values obtained for all experiments are shown in Figure 5.8 – 5.10 and were analyzed for statistical differences using general linear models. For all genotypes and intervals tested, the genotype used for primary infection reached similar viral loads in superinfected fish compared to the viral loads reached in single infection where that genotype was used during the primary exposure period. In contrast, the genotype used to establish secondary infection reached significantly lower viral loads in superinfection than in the relevant single infection (competition*order interaction, $p < 0.001$). The effect of superinfection on the ability of the secondary virus to replicate within a superinfected host was not different between any of the reciprocal pairs of genotypes tested (genotype:competition, $p > 0.05$).

Overall, the superinfection fitness of the IHNV genotypes involved in displacement was not observed to increase with each displacement. The genotypes examined here demonstrated few differences in superinfection fitness, but those differences observed did not support the hypothesis that fitness was increasing with each displacement.

Environmental Stability

As a water-borne virus, the ability to remain infectious during periods outside the host is likely an important pressure faced by IHNV in the field, and as such may contribute to overall viral fitness. Therefore, we investigated whether differences in stability of these IHNV genotypes

correlated with the observed field displacement events. Stability was tested in four separate environments designed to mimic the aquatic environment likely encountered by this virus. Freshwater was collected and held at either 10 or 15 degrees Celsius and saltwater was collected and held at either 4 or 10 degrees Celsius. The water collected was not treated or filtered so the natural microbiota remained present for the assay. The temperatures chosen for each water type represent temperatures that would be expected in freshwater streams or lakes and below or at the surface of the Pacific Ocean. Each genotype began at a concentration of $1x10^5$ pfu/mL in each of the four conditions, and the rate of viral decay was measured over time by plaque assay. The effect of UV exposure on fish rhabdovirus stability is well known (4, 10, 23) and thus we sought to extend the potential stability of the virions to maximize the potential of measurable difference between genotypes by holding the samples in dark incubators. The mean titers obtained from each condition for each genotype are shown in Figure 5.11.

As seen in Figure 5.11, titers at time 0 (T_0) differ dramatically between some of the genotypes tested. Although we attempted to inoculate equivalent plaque forming units for each genotype in each replicate water sample, large variations in the titers calculated at this first time point were observed, even though variation between replicate samples was relatively low. This variation between T_0 titers for different genotypes may prevent us from accurately comparing inactivation rate and half-life for these genotypes. While we did perform this analysis as described below, we hesitate to put our full weight behind the conclusions until these experiments are repeated.

Half-life (T_{50}), time to 90% inactivation (T_{90}) , and time to 99% inactivation (T_{99}) for each genotype was calculated for each water condition (salinity and temperature) (Table 5.4). Significant differences between genotypes were determined by GLM analysis using the half-life

calculations. In saltwater held at 4°C, the half-life of both genotypes 007 and 111 were significantly greater than the half-life of either 110 or 139 (halflife~genotype, $F_{3,8} = 39.19$, $p < 0.0001$). However, genotype 007 did not differ significantly from 111, nor did genotype 110 differ significantly from 139. The same observation was made for the stability of these genotypes in saltwater held at 10°C, with genotypes 007 and 111 remaining infectious for significantly longer than genotypes 110 and 139 (halflife~genotype, $F_{3,8} = 66.51$, $p < 0.0001$). In freshwater held at 15°C, only genotype 111 differed significantly in half-life from the other three genotypes (halflife~genotype, $F_{3,8} = 11.59$, $p = 0.011$), with genotype 111 having greater stability. In freshwater held at 10°C, no statistically significant differences between the genotypes were observed. In sum, these results indicate that genotypes 007 and 111 may be able to remain infectious in saltwater conditions for longer periods of time than genotypes 110 and 139, and 111 may be more stable under some freshwater conditions. However, these results do not suggest that differences in stability between these genotypes consistently correlate with displacement.

Discussion

Observed genotype displacement events in the lower Columbia River basin and Washington state coastal region led us to investigate here whether differences in viral fitness could explain such events. Virus fitness measured here included replication kinetics in single infections, competitive fitness in co-infection and superinfection, and stability of infectious particles in water of varying salinity and temperatures. A preliminary investigation of the virulence at a single high challenge dose of each of these genotypes in steelhead trout was also included to determine whether overall virulence of the virus is changing with successive displacement events.

The first IHNV displacement event observed in the lower Columbia River basin occurred when genotype 111 displaced genotype 007 to become the dominant viral genotype isolated from 1994-1999 in this region. We therefore hypothesized that genotype 111 would demonstrate greater fitness for in-host replication in the context of single infection, co-infection, and/or superinfection. Contrary to this hypothesis, we observed no differences in replication kinetics or the ability to establish secondary infection or replicate in the presence of another virus genotype either in co-infection or superinfection contexts. Therefore we conclude that an increase in in-host replication fitness does not correlate with this first displacement event.

The second observed displacement event occurred when IHNV genotype 110 replaced 111 to become the dominant genotype isolated in the lower Columbia River basin from 2002 to the present. In-host replication fitness was found to differ significantly between genotype 110 and the previously dominant genotype 111 at some early time points. The mean viral load in fish infected with genotype 111 was observed to be significantly greater than that of fish infected with genotype 110 for the first four days post-infection. In addition, mean viral load reached a peak in fish infected with genotype 110 on day 6, three days after peak viral load was reached in fish infected with genotype 111. Consistent with higher viral loads in fish infected with genotype 111, the induction of Mx-1 gene expression in these same fish was observed to be higher than that in fish infected with genotype 110 at early time points. Expression of Mx-1, an alpha interferon-stimulated gene, has been demonstrated to correlate with viral load in IHNV infection in *O. mykiss* (21). Interestingly, a fitness difference between 111 and 110 was not observed when examining replication fitness in co-infection and superinfection. However, the relative ability to establish or restrict secondary infection was significantly higher for genotype 111 than for genotype 110 when there was a 48 hour interval between exposures. Because this was the only

experiment for which a statistical difference was observed between genotypes in superinfection, this experiment was repeated and the results were consistent (data not shown). This trend was also observed in the experiment performed with a 24 hour interval between exposures but was not found to be a statistically significant difference. The observed increased restriction of secondary infection following primary infection with genotype 111 correlates with the temporarily higher Mx-1 gene expression in fish infected with genotype 111 for the first few days post-infection. While the mechanism behind superinfection restriction is still unknown, these results represent the first indication that innate immune stimulation may correlate with the timing of superinfection restriction. Future research will be needed to fully elucidate the possible role of the innate immune response in superinfection restriction, including the gene expression analysis of other interferon stimulated genes and investigations into tissue specific responses within the host. Despite the mechanisms, these results overall demonstrate that, contrary to our hypothesis that displacement correlated with increased fitness, the displaced genotype 111 was observed to have higher fitness than genotype 110 by some measures.

The final observed field displacement event occurred with the displacement of genotype 110 by genotype 139, in 2009 on the Washington State Pacific coast. While no significant differences in in-host replication kinetics, Mx-1 gene induction, or superinfection fitness were observed for these two genotypes, a significant difference in fitness in co-infection was observed. Genotype 110 replicated to greater viral loads than genotype 139 in groups of fish infected with each genotype alone and in groups co-infected with a one-to-one mix of both genotypes. Overall, these results suggest that the displaced genotype 110 has greater in-host replication fitness than genotype 139. Again, these results are contrary to the original hypothesis

that displacement would be associated with increased fitness in a co-infection or superinfection context, because in this case, the displaced genotype had higher fitness.

With regard to virulence, measured as cumulative percent mortality, we observed, in general, a suggestive trend of increasing virulence with successive displacements, with the exception of the third displacement event. However, these suggestive increases in virulence were not statistically significant for the first two displacement pairs. Furthermore, for the one pair where the difference in virulence was significant, the displaced genotype had higher virulence. While there appears to be no general trend of increasing or decreasing virulence for IHNV in the field, the correlation between virulence and fitness observed here is consistent with previous studies of IHNV in co-infection. Wargo *et al*. demonstrated that high virulence correlates with higher in-host replication fitness in O. mykiss (33, 34). The observed higher virulence of genotype 110 over 139 also correlated with greater replication fitness in co-infection (Figure 5.6C). This difference in replication between genotypes 110 and 139 was not observed on day three of the single infection growth kinetics experiments (Figure 5.2A). This seeming contradiction is likely a result of the increased statistical power of the co-infection experiments due to much higher n values (14 fish versus 5) such that small but significant differences could be detected in the co-infection experiments. The correlation between virulence and in-host replication fitness is also supported by a lack of difference in replication fitness observed for genotype pairs 007 and 111 and 111 and 110, for which virulence did not differ significantly.

In addition to in-host replication, we investigated the stability of the four genotypes in various water conditions to determine whether the displacement events could be explained by an increased stability in the environment. Infectivity over time was assessed in untreated freshwater and seawater each held at two different temperatures. Overall, we observed significant

differences when comparing the early genotypes (007 and 111) to the more recent genotypes (110 and 139). Contrary to our hypothesis, these assays demonstrated that stability of the early genotypes was actually greater than that of the more recent genotypes in saltwater conditions. However, this difference was not seen in freshwater assays, except for genotype 111 at 15°C. The differences seen between water conditions are consistent with previous reports examining the stability of IHNV or the related novirhabdovirus viral hemorrhagic septicemia virus (VHSV) (4, 10, 23, 35). It has been previously shown that IHNV and VHSV are significantly more stable in freshwater than in seawater (4, 10, 35). This difference may be in part due to differences in water hardness. Pietch *et al.* demonstrated that VHSV particles formed aggregations more readily in water conditions with greater hardness, potentially contributing to their stability (23). While we did not measure water hardness for the experiments presented here, seawater has been demonstrated to have greater hardness than most freshwater sources. Thus, aggregation and sedimentation of virus particles may have contributed to the reduction in infectious particles measured. In addition to water hardness, the presence of other microorganisms in untreated water may also be a strong determinant of viral survival. The presence of proteolytic enzymes produced by resident bacteria has been demonstrated to reduce survival of VHSV in both freshwater and saltwater conditions (23). Temperature may determine the activity of such bacteria and therefore the stability of infectious virus.

One important caveat to all of these experiments is the use of a single virus isolate to represent each genotype involved in the displacement events. We cannot rule out the possibility that these specific isolates chosen do not accurately represent the genotypes examined. However, it should be noted that, the isolates used here were carefully chosen as representatives from

major IHNV epidemic events and therefore represent important events for detection and management.

While the results presented here do not support the hypothesis that increased viral fitness is associated with IHNV displacement events, further investigations into other aspects of viral fitness are warranted to fully evaluate the fitness of these genotypes in steelhead trout. For example, viral shedding was not assessed, so we cannot rule out the possibility that genotypes responsible for displacement have an increased ability to be released from the host and shed into the surround water environment, increasing the transmission potential of the virus. A more thorough analysis of virulence using multiple challenge doses to determine an LD_{50} for each displacement genotype would provide further insight into the evolution of virulence in these events. In addition, it is possible that genotypes responsible for displacement exhibit an increased potential for long-term persistence within the infected host, potentially increasing the duration of transmission or the distance the virus can travel to infect new susceptible hosts. The development of genotype-specific host resistance or herd immunity also cannot be ruled out as explanations for these observed displacement events. Thus, these investigations are currently underway to determine other potential correlates to these displacement events and to gain a greater understanding of the selection pressures driving the evolution of this virus in the field.

The specific circumstances surrounding each displacement event may suggest that differences in viral fitness would not be sufficient to explain these events. Genotype 110 emerged in the lower Columbia River basin in 2002. Between 1999 and 2001, none of the viruses isolated from this region were found to belong to the M genogroup of IHNV (7). This lull in detection could indicate that direct competition between genotypes 111 and 110 may not have occurred and that, instead, genotype 111 disappeared as a result of other factors and genotype

110 was able to establish its dominance in the region at a time where no genotype had filled this niche. It is, therefore, possible that the disappearance of genotype 111 and the emergence of 110 are two independent events within the region and therefore, viral fitness might not be expected to correlate with displacement. The specific conditions associated with the third displacement pair were different. Genotype 139 was detected in the Columbia River basin at two hatchery facilities between 2003 and 2005, however genotype 139 never reached dominance over genotype 110 in the region and has not been detected in the basin since 2005 (7). Thus, the displacement of 110 by 139 that occurred on the Washington coast did not happen in the Columbia River basin, indicating that external factors may be responsible for the displacement on the coast, while observed differences in viral fitness support the lack of displacement in the lower Columbia River basin. Further investigations will be necessary to determine the many potential external factors that may have played a role in these displacement events such as host population structure changes, changes in animal husbandry practices, or large-scale ecosystem changes that affect the movement of hosts within the environment.

The results presented here do not support the hypothesis that displacement is associated with increased viral fitness for in-host replication in either co-infection or superinfection, suggesting instead the importance of other non-viral factors in driving RNA virus evolution for IHNV displacement. While differences in viral fitness have helped to explain emergence and displacement of other viral species in the literature such as dengue virus and West Nile virus (2, 9, 12, 16, 17, 29, 30, 32), the finding that fitness does not always correlate with such events is also important (18, 19). This finding indicates that other selective pressures exist that have not been examined thoroughly and could help to explain such events, eventually leading to future predictions or modifications in disease management to limit spread and emergence of novel virus

strains. Further research exploring displacement events in other virus-host systems will be essential to determine whether the findings presented here represent the exception or the rule in nature.

Table 5.1 - Distribution of fish in infection status categories for genotypes 007 and 111.

	Genotype Exposure		Observed Infection Status (Expected Infection Status[a]), in numbers of fish				adjusted p value [b]	
Interval	1°	2°	Super- infection	1° only	2° only	uninfected	Goodness of fit [c]	Fisher exact [d]
24 hour	007	111	15 (12)	0 (1)	0 (1)	0 (2)	0	1
	111	007	15 (13)	0 (2)	0 (0)	0 (0)	0	
	007	mock	0	8	0	1		
	111	mock	0	9	0	0		
	mock	007	0	0	8	1		
	mock	111	0	0	8	1		
48 hour	007	111	9 (15)	5 (0)	1 (0)	0 (0)	0	0.714
	111	007	8 (15)	7 (0)	0 (0)	0 (0)	0	
	007	mock	0	9	0	0		
	111	mock	0	9	0	0		
	mock	007	0	0	8	0		
	mock	111	0	0	7	0		
96 hour	007	111	2 (13)	12 (2)	1 (0)	0 (0)	0	0.597
	111	007	1 (13)	14 (0)	0 (2)	0 (0)	0	
	007	mock	0	9	0	0		
	111	mock	0	8	0	1		
	mock	007	0	0	8	0		
	mock	111	0	0	7	1		

[a] Expected infection status is calculated (for superinfection groups) as proportion of fish likely to be in each category based on the observed infection frequencies of single infection groups multiplied by total number of fish in the superinfection group.

[b] p values following Bonferroni adjustment, significance reached when $p < 0.05$

[c] Exact multinomial test (Goodness of fit) was used to determine whether observed distribution of fish in infection status categories is equal to expected distribution based on single infection controls (null hypothesis). Analysis was performed for each superinfection group for each experiment. $p < 0.05$ indicates support to reject null hypothesis, i.e. observed distribution does not equal expected.

[d] Fisher exact test was used to determine whether distribution of fish in infection status categories was independent of genotype used for primary infection (null hypothesis). $p < 0.05$ indicates support to reject null hypothesis, i.e. distribution is not independent of genotype used first.

Table 5.2 - Distribution of fish in infection status categories for genotypes 111 and 110.

	Genotype Exposure		Observed Infection Status (Expected Infection Status[a]), in numbers of fish				adjusted p value [b]	
Interval	1°	2°	Super-infection	1° only	2° only	uninfected	Goodness of fit [c]	Fisher exact [d]
24 hour	111	110	11 (12)	4 (0)	0 (3)	0 (0)	0	0.3295
	110	111	14 (15)	1 (0)	0 (0)	0 (0)	0.139	
	111	mock	0	7	0	2		
	110	mock	0	9	0	0		
	mock	111	0	0	9	0		
	mock	110	0	0	9	0		
48 hour	111	110	0 (15)	15 (0)	0 (0)	0 (0)	0	0.00019
	110	111	10 (15)	5 (0)	0 (0)	0 (0)	0	
	111	mock	0	10	0	0		
	110	mock	0	8	0	0		
	mock	111	0	0	8	0		
	mock	110	0	0	8	0		
96 hour	111	110	1 (10)	14 (1)	0 (3)	0 (1)	0	1
	110	111	1 (15)	14 (0)	0 (0)	0 (0)	0	
	111	mock	0	7	0	2		
	110	mock	0	9	0	0		
	mock	111	0	0	9	0		
	mock	110	0	0	8	1		

[a] Expected infection status is calculated (for superinfection groups) as proportion of fish likely to be in each category based on the observed infection frequencies of single infection groups multiplied by total number of fish in the superinfection group.

[b] p values following Bonferroni adjustment, significance reached when $p < 0.05$

[c] Exact multinomial test (Goodness of fit) was used to determine whether observed distribution of fish in infection status categories is equal to expected distribution based on single infection controls (null hypothesis). Analysis was performed for each superinfection group for each experiment. $p < 0.05$ indicates support to reject null hypothesis, i.e. observed distribution does not equal expected.

[d] Fisher exact test was used to determine whether distribution of fish in infection status categories was independent of genotype used for primary infection (null hypothesis). $p < 0.05$ indicates support to reject null hypothesis, i.e. distribution is not independent of genotype used first.

Table 5.3 - Distribution of fish in infection status categories for genotypes 110 and 139.

	Genotype Exposure		Observed Infection Status (Expected Infection Status[a]), in numbers of fish				adjusted p value [b]	
Interval	1°	2°	Super-infection	1° only	2° only	un-infected	Goodness of fit [c]	Fisher exact [d]
24 hour	110	139	13 (12)	1 (3)	1 (0)	0 (0)	0	1
	139	110	13 (15)	0 (0)	1 (0)	0 (0)	0	
	110	mock	0	9	0	0		
	139	mock	0	9	0	0		
	mock	110	0	0	8	0		
	mock	139	0	0	4	1		
48 hour	110	139	5 (15)	10 (0)	0 (0)	0 (0)	0	0.816
	139	110	9 (15)	6 (0)	0 (0)	0 (0)	0	
	110	mock	0	9	0	0		
	139	mock	0	7	0	0		
	mock	110	0	0	9	0		
	mock	139	0	0	7	0		
96 hour	110	139	1 (13)	14 (2)	0 (0)	0 (0)	0	1
	139	110	1 (15)	12 (0)	0 (0)	1 (0)	0	
	110	mock	0	9	0	0		
	139	mock	0	9	0	0		
	mock	110	0	0	9	0		
	mock	139	0	0	7	1		

[a] Expected infection status is calculated (for superinfection groups) as proportion of fish likely to be in each category based on the observed infection frequencies of single infection groups multiplied by total number of fish in the superinfection group.

[b] p values following Bonferroni adjustment, significance reached when $p < 0.05$

[c] Exact multinomial test (Goodness of fit) was used to determine whether observed distribution of fish in infection status categories is equal to expected distribution based on single infection controls (null hypothesis). Analysis was performed for each superinfection group for each experiment. $p < 0.05$ indicates support to reject null hypothesis, i.e. observed distribution does not equal expected.

[d] Fisher exact test was used to determine whether distribution of fish in infection status categories was independent of genotype used for primary infection (null hypothesis). $p < 0.05$ indicates support to reject null hypothesis, i.e. distribution is not independent of genotype used first.

Table 5.4 - Stability of displacement genotypes in environmental conditions.

Water	Temperature	Genotype	T_{50}[a]	T_{90}[b]	T_{99}[c]
Freshwater	10° C	007	0.77	2.57	5.13
		111	1.00	3.31	6.63
		110	0.67	2.21	4.42
		139	0.76	2.52	5.04
		Average[d]	0.80	2.65	5.30
Freshwater	15° C	007	0.51	1.70	3.41
		111	0.94	3.13	6.25
		110	0.54	1.79	3.57
		139	0.40	1.32	2.64
		Average[d]	0.60	1.98	3.97
Saltwater	4° C	007	1.01	3.35	6.70
		111	1.11	3.68	7.36
		110	0.61	2.05	4.11
		139	0.77	2.57	5.13
		Average[d]	0.87	2.91	5.83
Saltwater	10° C	007	0.38	1.27	2.54
		111	0.30	1.01	2.02
		110	0.16	0.52	1.05
		139	0.19	0.63	1.26
		Average[d]	0.26	0.86	1.71

[a] T_{50} corresponds to the average time (days) to 50% inactivation (half-life) of three replicate samples for each virus genotype, determined by ln(0.5)/slope of exponential regression.
[b] T_{90} corresponds to the average time (days) to 90% inactivation.
[c] T_{99} corresponds to the average time (days) to 99% inactivation.
[d] Average of all genotypes for specified condition.

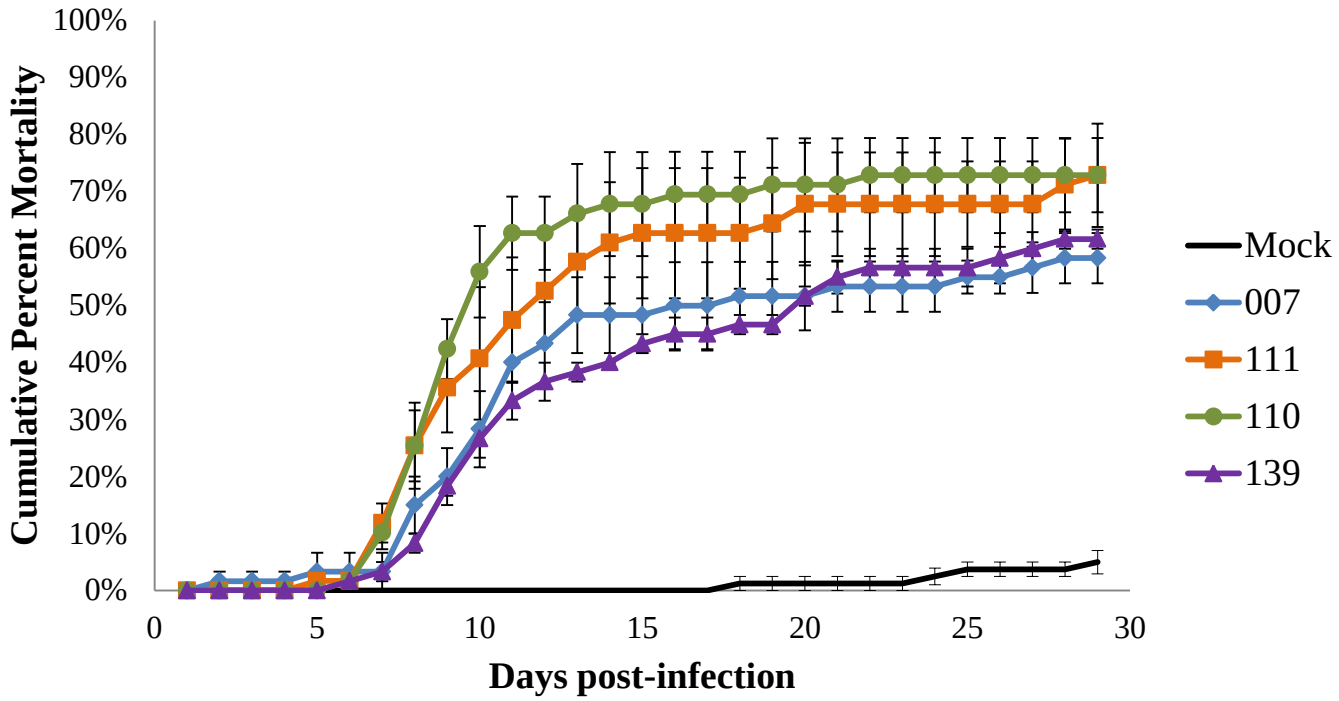

Figure 5.1. Virulence of IHNV displacement genotypes in juvenile steelhead trout. Experimental batch immersion challenge defining virulence of IHNV displacement genotypes in triplicate groups of 20 juvenile steelhead trout. Lines are daily mean cumulative percent mortality (± 1 S.E.M) over 30 days of infection.

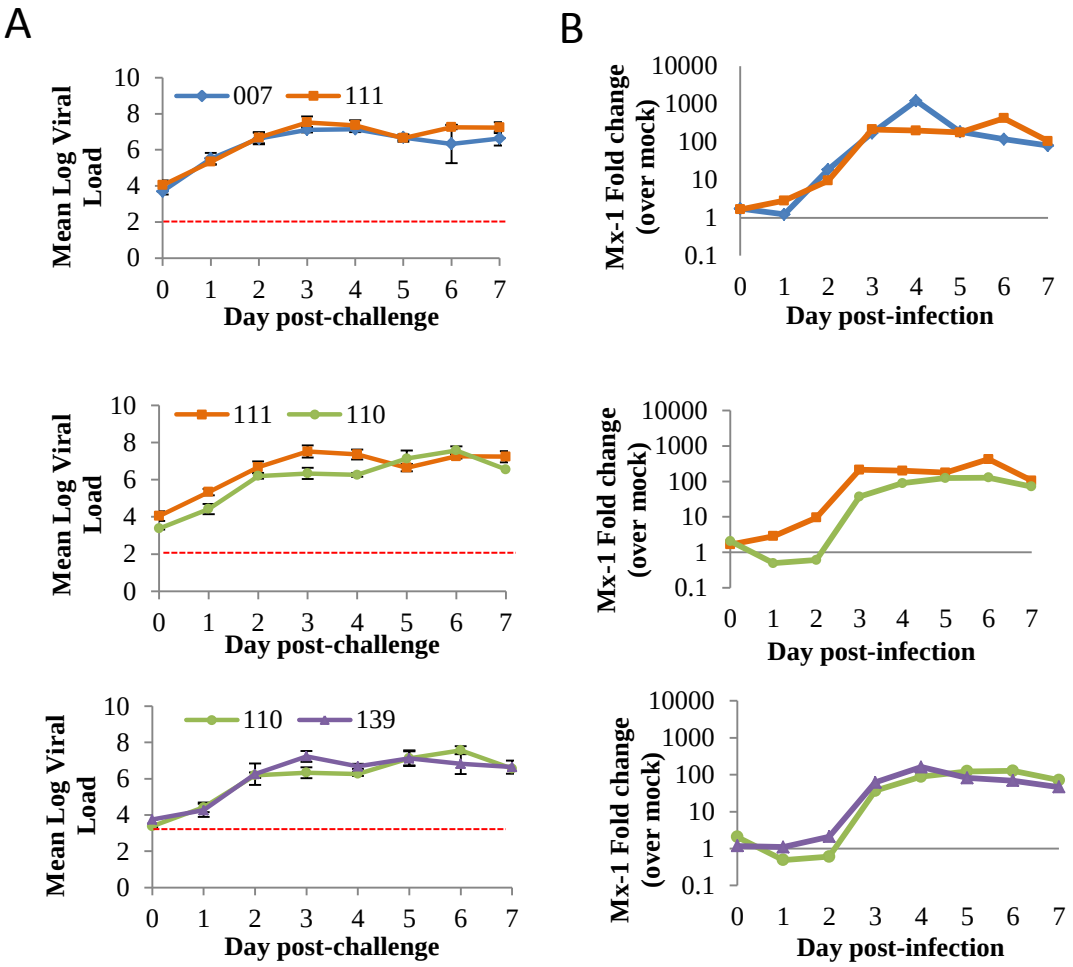

Figure 5.2. Viral growth kinetics and *Mx-1* induction in single infections with displacement genotypes. A) Mean log viral load in 5 fish sampled at each time point, excluding virus-negative samples, in rainbow trout following single exposure with genotype 007 (blue diamonds) 110 (orange squares), 111 (green circles), or 139 (purple triangles). (±SEM). Horizontal dotted line indicates detection levels for the genotype-specific RT-qPCR assays. Four genotypes were tested and analyzed independently, but data is shown here as pairwise comparisons. B) Mean normalized *Mx-1* transcription fold changes, relative to the mock-control group, in the same fish shown in the viral load curves (5.2A).

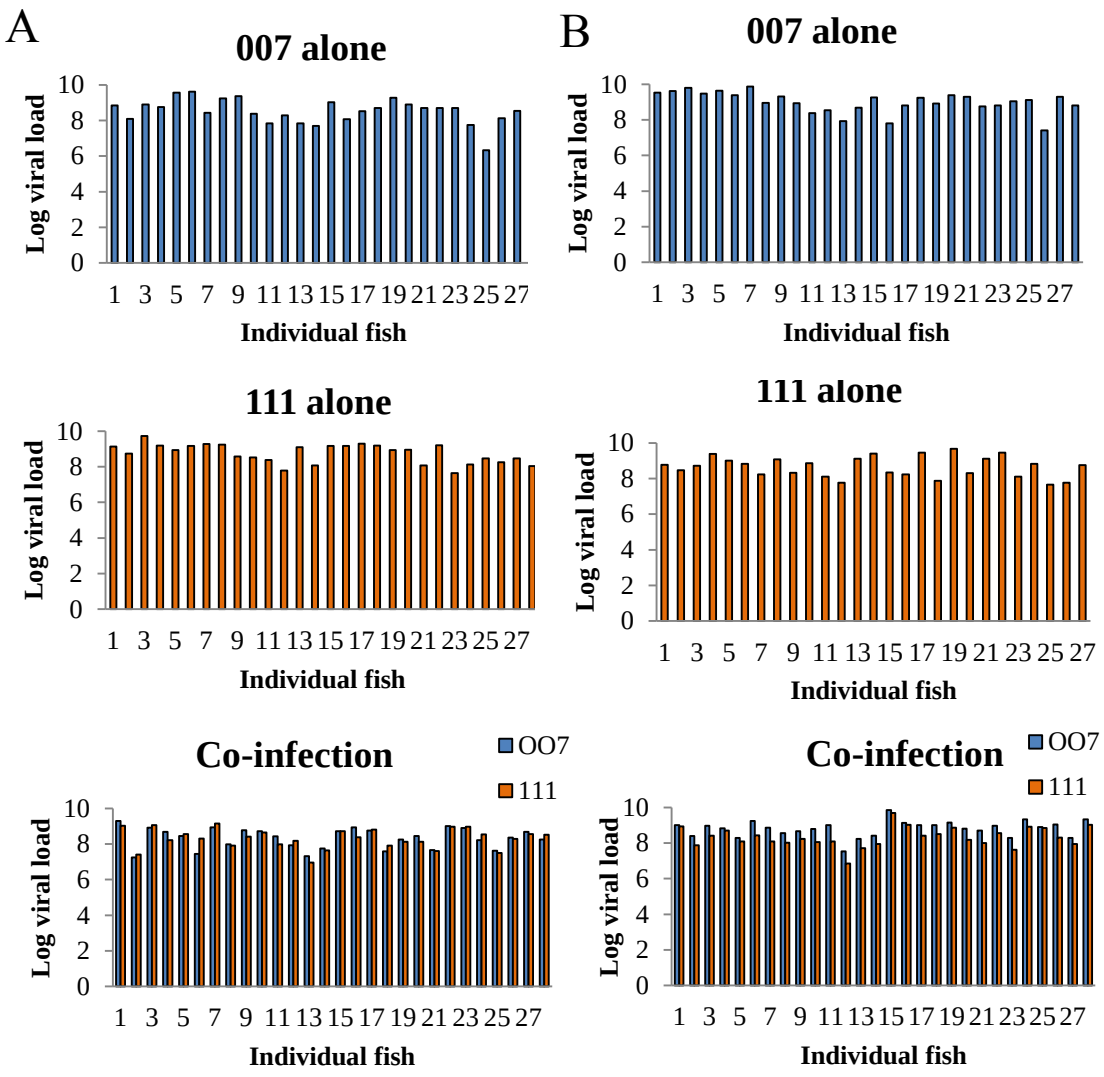

Figure 5.3. In-host replication of genotypes 007 and 111 following immersion co-infection challenge. Viral loads of genotypes 007 (blue bars) and 111 (orange bars) determined by qRT-PCR in individual fish from two independent experiments (A and B), three days after exposure to 007 alone, 111 alone, or 1:1 mix of both genotypes, co-infection.

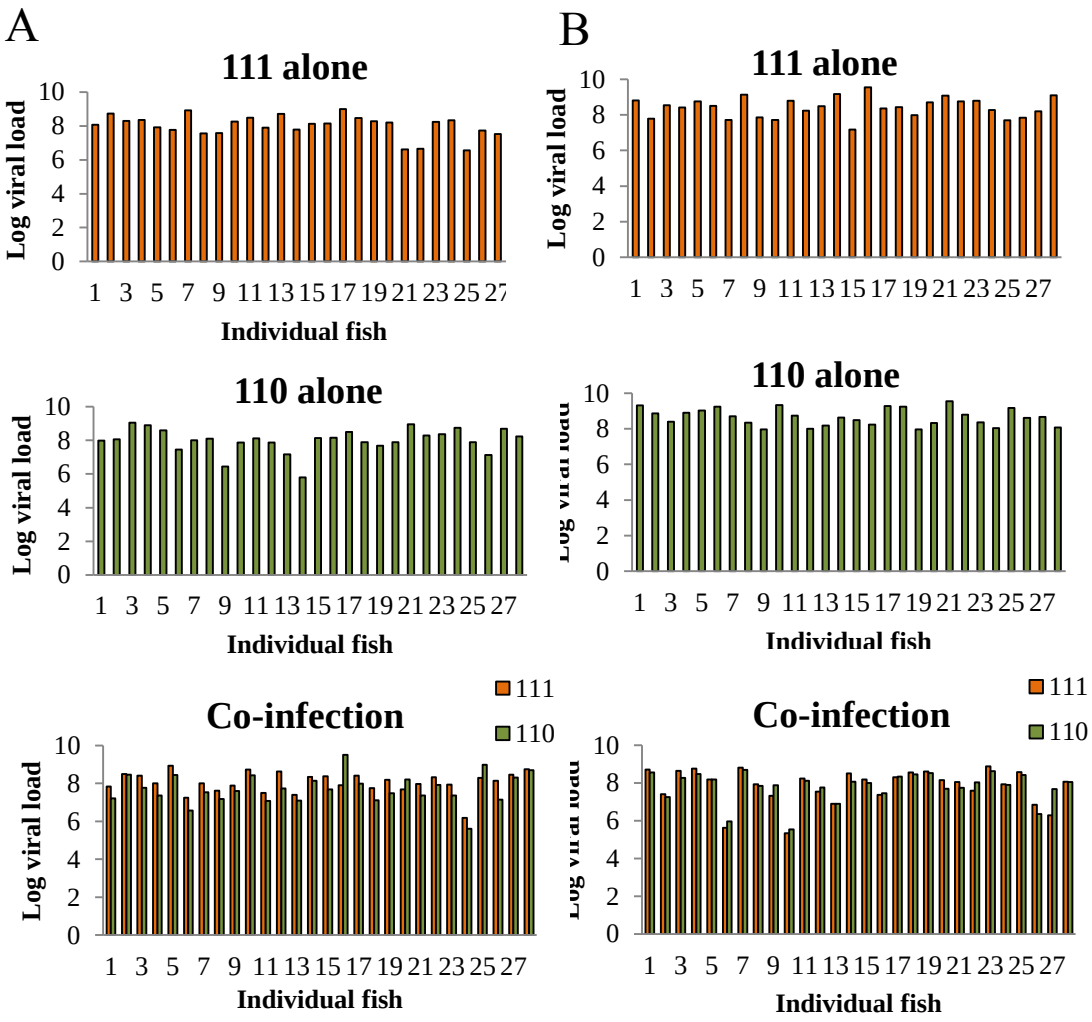

Figure 5.4. In-host replication of genotypes 111 and 110 following immersion co-infection challenge. Viral loads of genotypes 111 (orange bars) and 110 (green bars) determined by qRT-PCR in individual fish from two independent experiments (A and B), three days after exposure to 111 alone, 110 alone, or 1:1 mix of both genotypes, co-infection.

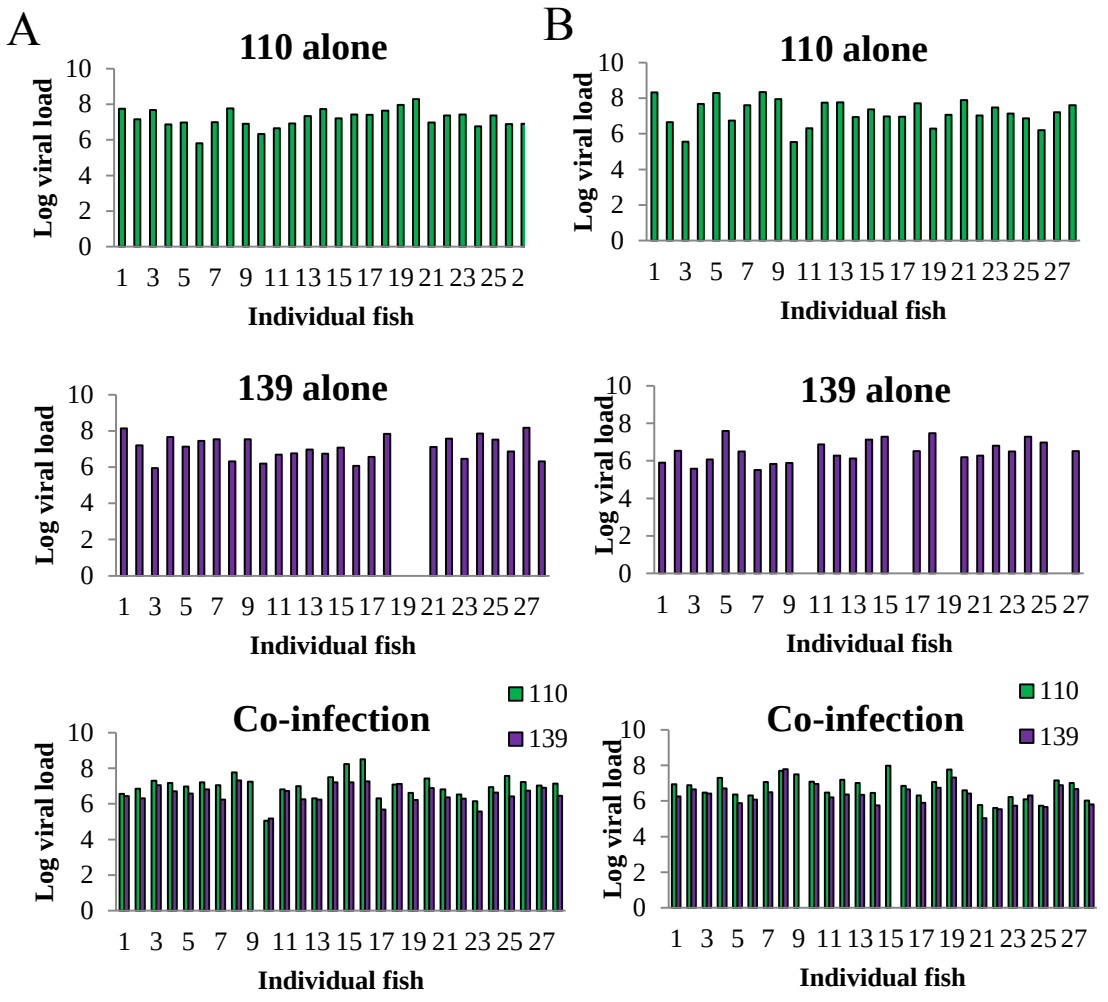

Figure 5.5. In-host replication of genotypes 110 and 139 following immersion co-infection challenge. Viral loads of genotypes 110 (green bars) and 139 (purple bars) determined by qRT-PCR in individual fish from two independent experiments (A and B), three days after exposure to 110 alone, 139 alone, or 1:1 mix of both genotypes, co-infection.

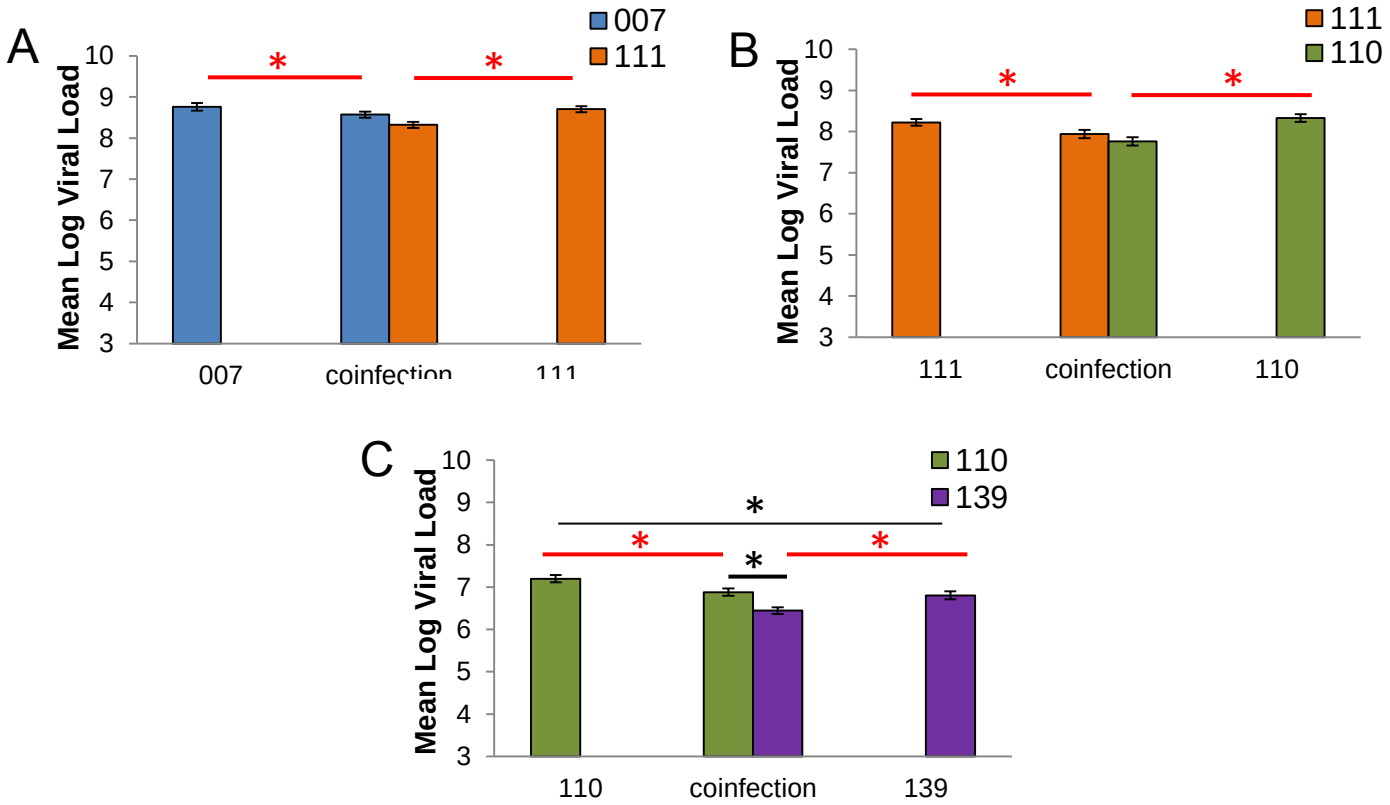

Figure 5.6. **Mean viral loads for single and co-infection challenges using IHNV displacement genotypes.** Mean viral loads (log virus copies/gram fish) for two experiments for each treatment group from A) genotypes 007 and 111, B) genotypes 111 and 110, and C) genotypes 110 and 139. Differences between groups were analyzed by ANOVA with Bonferroni correction, asterisk (*) indicates $p < 0.05$.

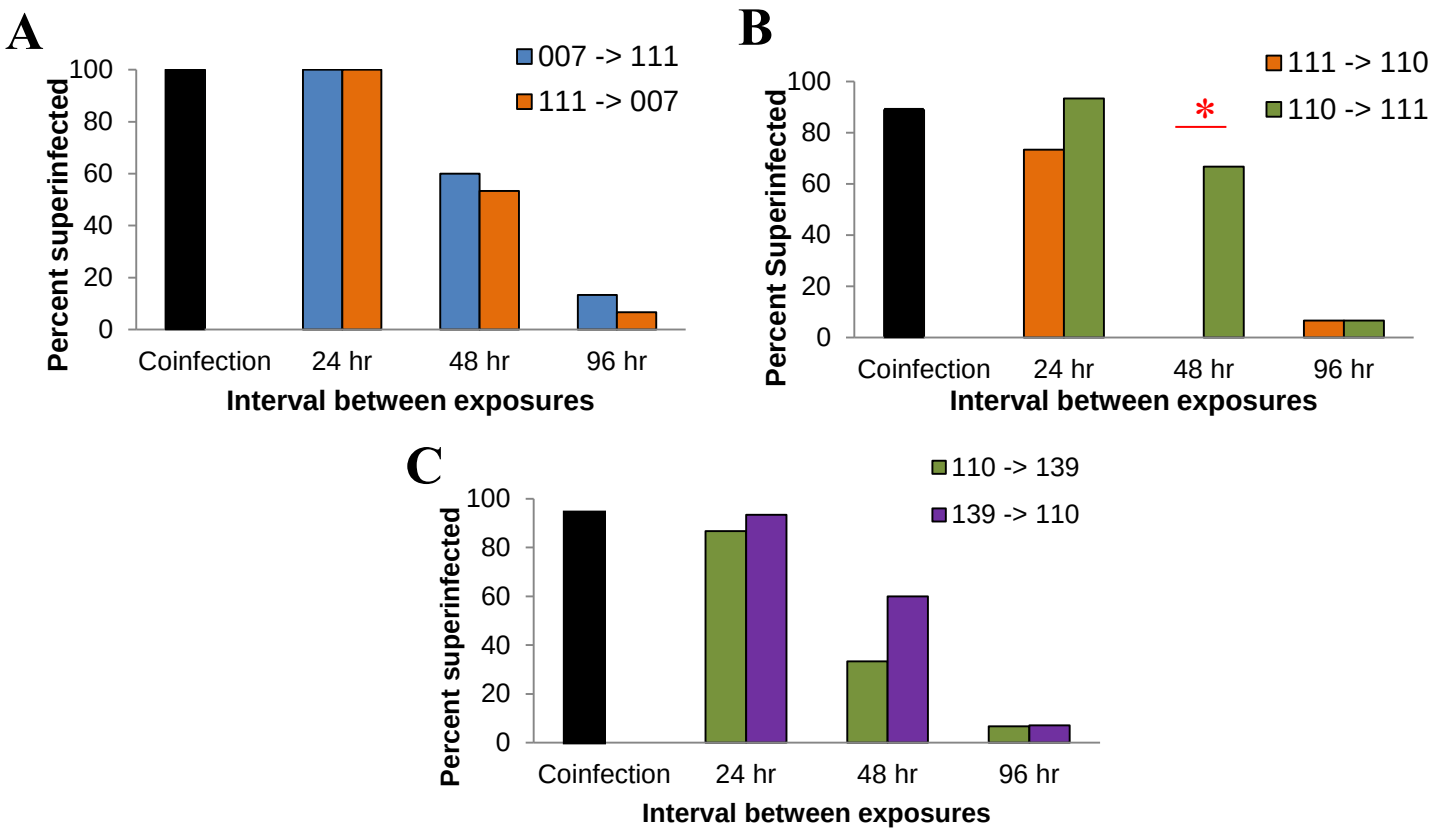

Figure 5.7. Frequency of superinfection in groups exposed to displacement genotype pairs with increasing time intervals between exposures. Colored bars represent the percentage of fish superinfected following exposure to 007 (blue bars), 111 (orange bars), 110 (green bars), or 139 (purple bars) during the primary exposure period. The black bar represents the percentage of fish infected with both genotypes following exposure to both genotypes at a 1:1 ratio during the secondary exposure period (co-infection control).

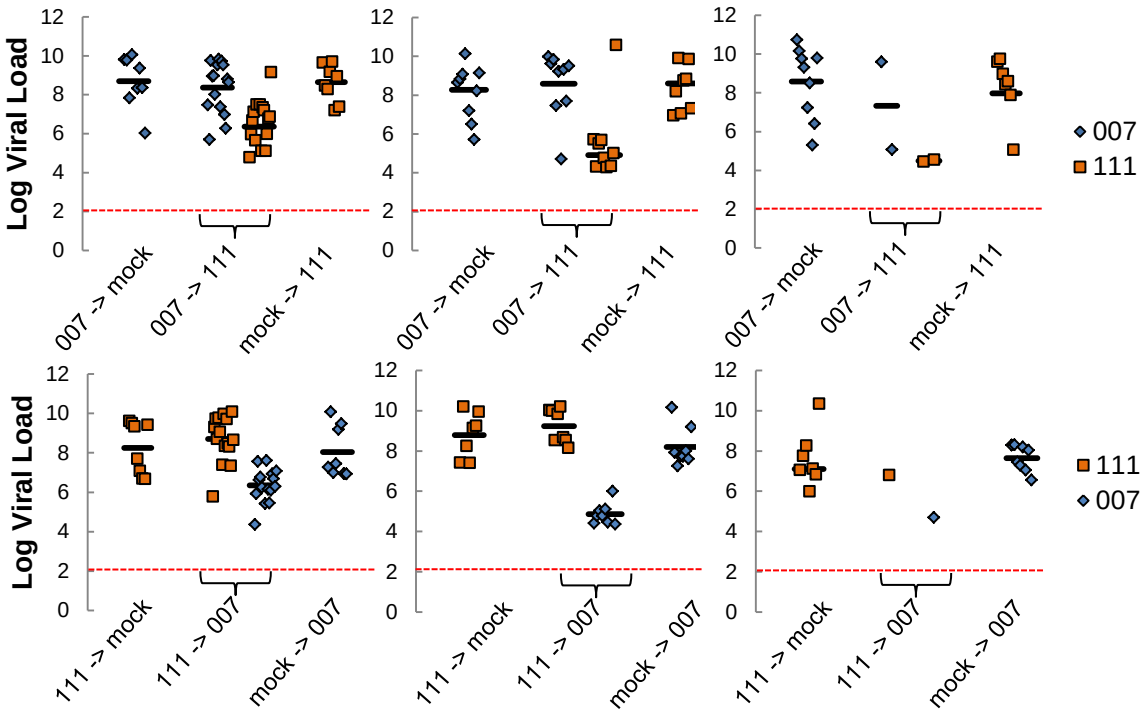

Figure 5.8. Impact of superinfection on viral load for all experiments performed with genotypes 007 and 111. Viral load (log virus copies/gram fish) attributed to genotype 007 (blue diamonds) and genotype 111 (orange squares) in individual fish from each treatment group (x-axis) is shown for experiments performed with each time interval between exposures as indicated. Black lines represent group mean log viral load. Horizontal dotted lines indicate detection levels for the genotype-specific RT-qPCR assays. Differences between groups were analyzed by ANOVA with Bonferroni correction.

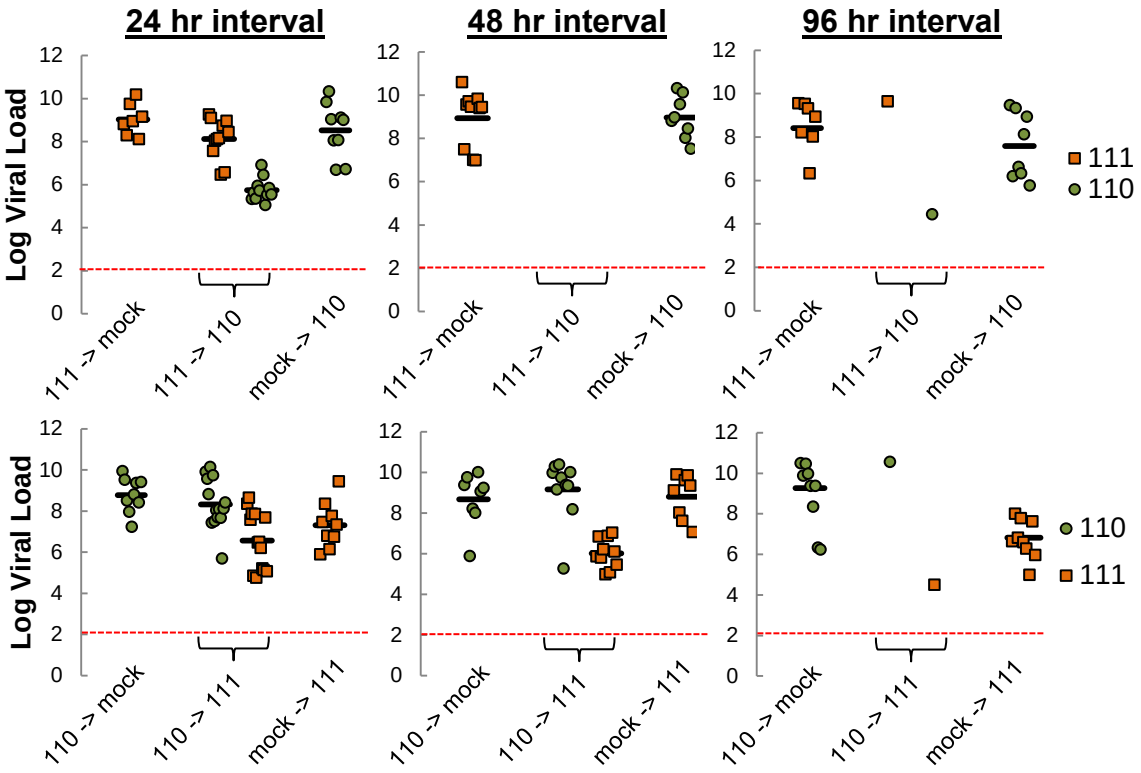

Figure 5.9. Impact of superinfection on viral load for all experiments performed with genotypes 111 and 110. Viral load (log virus copies/gram fish) attributed to genotype 111 (orange squares) and genotype 110 (green circles) in individual fish from each treatment group (x-axis) is shown for experiments performed with each time interval between exposures as indicated. Black lines represent group mean log viral load. Horizontal dotted lines indicate detection levels for the genotype-specific RT-qPCR assays. Differences between groups were analyzed by ANOVA with Bonferroni correction.

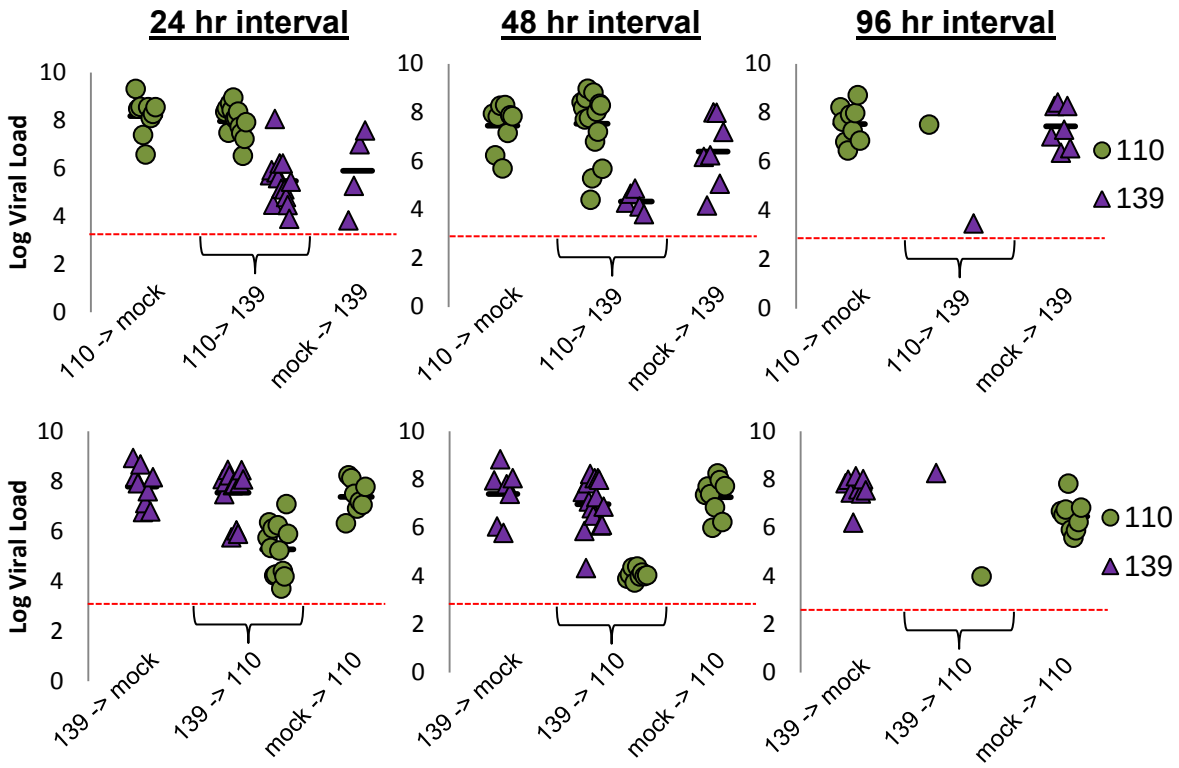

Figure 5.10. Impact of superinfection on viral load for all experiments performed with genotypes 110 and 139. Viral load (log virus copies/gram fish) attributed to genotype 110 (green circles) and genotype 139 (purple triangles) in individual fish from each treatment group (x-axis) is shown for experiments performed with each time interval between exposures as indicated. Black lines represent group mean log viral load. Horizontal dotted lines indicate detection levels for the genotype-specific RT-qPCR assays. Differences between groups were analyzed by ANOVA with Bonferroni correction.

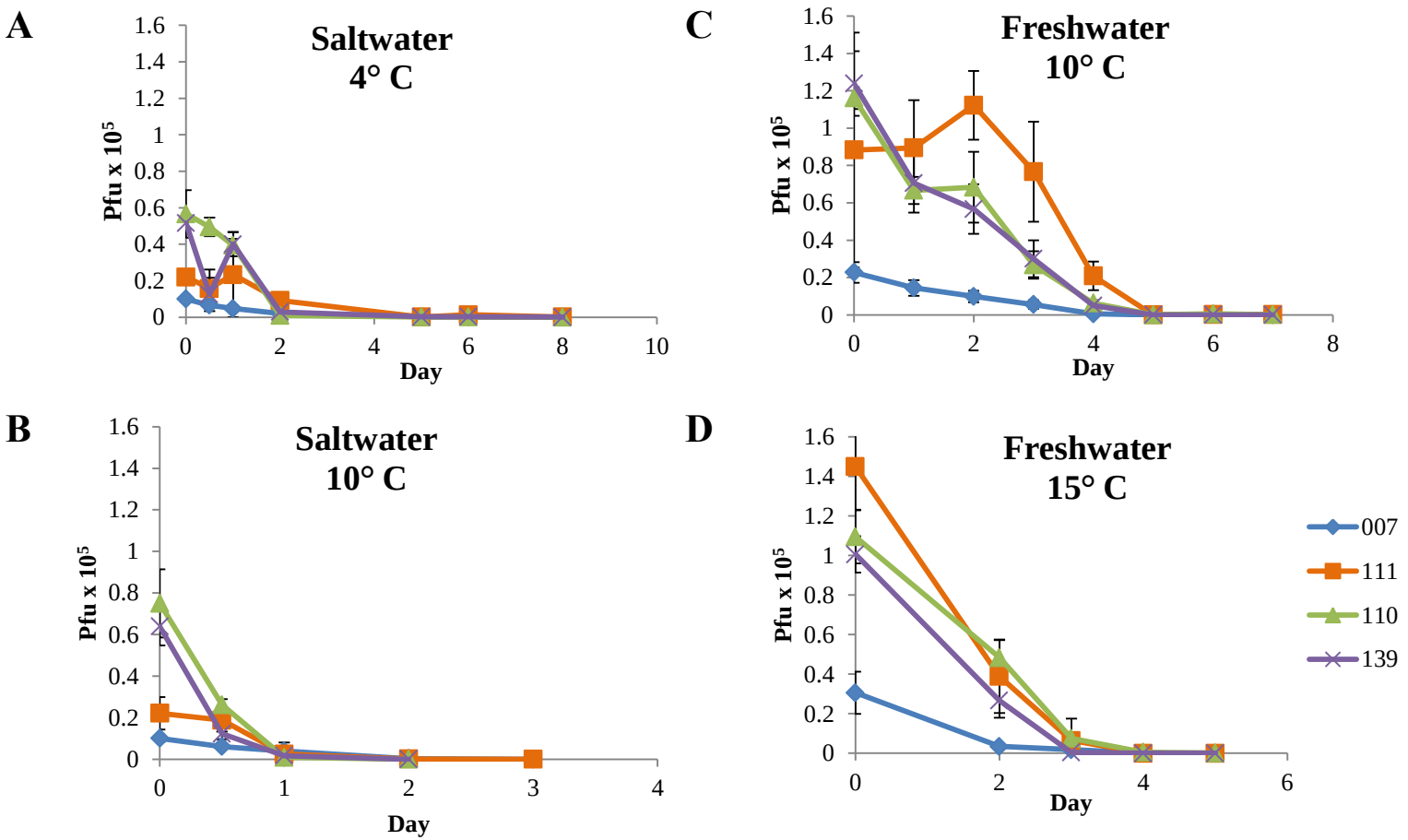

Figure 5.11. Stability of displacement genotypes in water with various salinity and temperature. Average plaque forming units (pfu) per mL of water quantified for each genotype held in A) saltwater at 4°C, B) saltwater at 10°C, C) freshwater at 10°C, or D) freshwater at 15°C. Each point represents average titer for three replicate wells for three replicate water samples for each genotype (±SD).

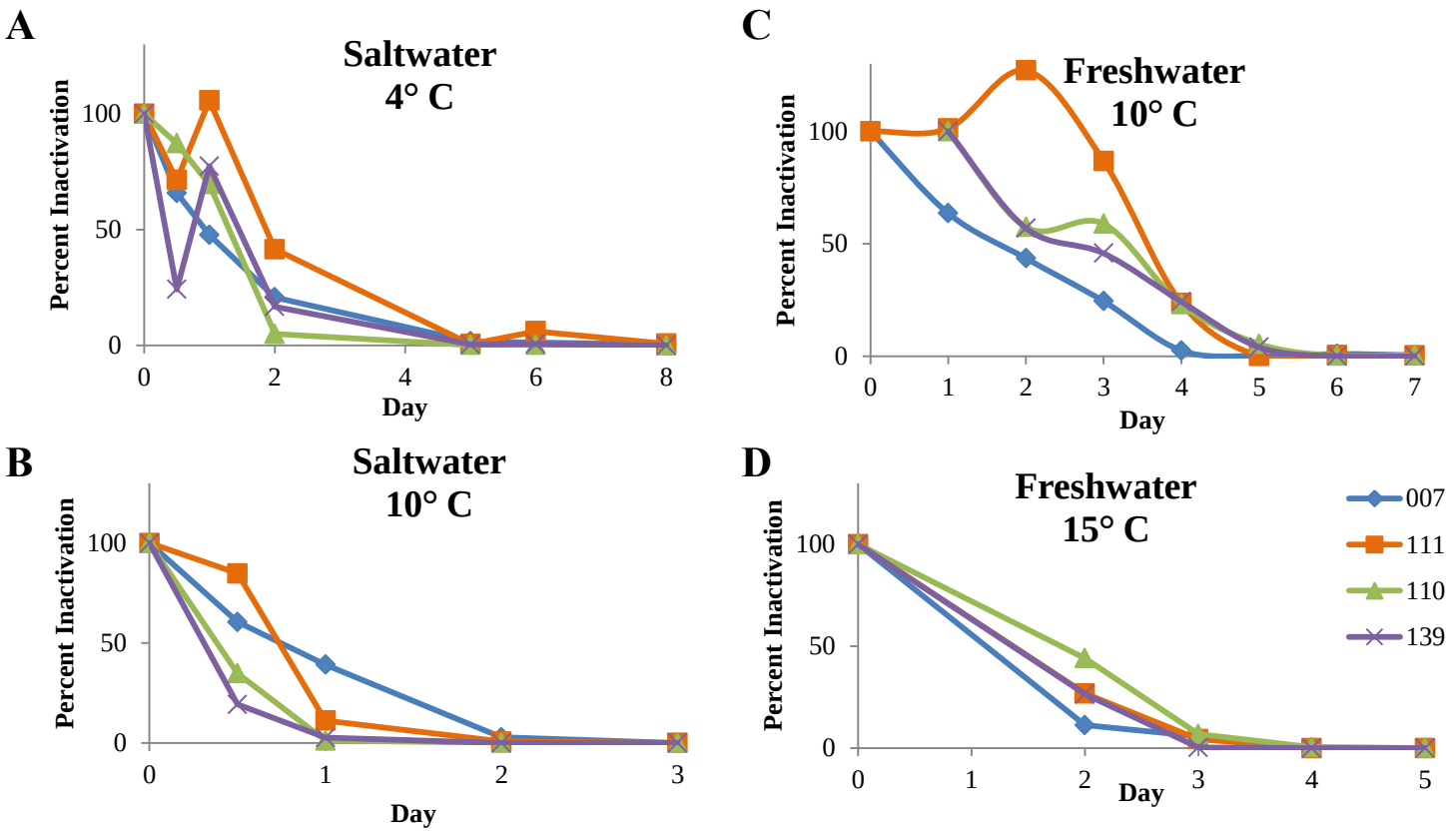

Figure 5.12. Percent inactivation over time of displacement genotypes in water with various salinity and temperature. Percent of each genotype remaining over time in A) saltwater at 4°C, B) saltwater at 10°C, C) freshwater at 10°C, or D) freshwater at 15°C. Each point represents average percent of T_0 titer (100%) remaining for three replicate wells for three replicate water samples for each genotype.

Chapter 6

General Discussion

"You have brains in your head. You have feet in your shoes.
You can steer yourself any direction you choose.
You're on your own. And you know what you know.
And YOU are the one who'll decide where to go..."

— Dr. Seuss, *Oh, the Places You'll Go!*

Summary

This thesis presents the development of a novel, in vivo superinfection assay to investigate both the dynamics of viral superinfection and the correlates of viral fitness in superinfection. Using this assay, we have demonstrated that virulence does not correlate with superinfection fitness in this host-pathogen system. By defining the dynamics of superinfection using an IHNV genotype pair of equal virulence, we observed that these two genotypes have equal fitness in both superinfection frequency and in-host replication. Similar dynamics were observed when two IHNV genotypes of unequal virulence were tested in the assay, indicating that virulence does not correlate with superinfection fitness in this system. In addition, we observed that increased superinfection fitness was not associated with genotype displacement events observed in the Pacific Northwest region. In fact, these displacement events also did not correlate with increased co-infection fitness, faster replication kinetics within the host, or the stability of virions in water. While the results for these two studies were counter to our original hypotheses, this work demonstrates the importance for critical examination of many host-pathogen systems to fully understand the evolution of RNA viruses. As with many novel systems, the results presented here also lead to numerous unanswered questions for future investigation, some of which will be discussed in this chapter.

Superinfection and pathogenesis

An important aspect of superinfection which has been investigated using other host-pathogen systems is that of pathogenesis resulting from superinfection. A remaining question is whether superinfection with IHNV alters the pathogenicity of different virus strains or genotypes or changes the outcome of infection. This could have a potential impact on virus spread if for

example the mortality were higher in fish infected with both a high and low virulence genotype compared to fish infected with just the low virulence genotype. In this example, the low virulence genotype would theoretically be at a disadvantage if transmission potential were lost due to early mortality caused by superinfection. In contrast, prior infection with a low virulence strain could protect against pathogenesis associated with a high virulence, superinfecting strain. Such protection was observed by researchers investigating the effect of superinfection on pathogenesis for chickens infected with IBDV discussed in the introduction (3). Based on the data presented in this thesis, one could hypothesize that prior infection with a low virulence strain would weaken the pathogenesis of a superinfecting high virulence strain due to the decrease in viral replication of a secondary virus in superinfection. This decreased replication may be associated with decreased virulence. Such dynamics have yet to be explored using this system and the results would be of significant interest to the field of virulence evolution and trade-off theorists.

Mechanism of Superinfection Restriction

A significant finding of this study was the observation that in vivo superinfection was significantly restricted between 24 and 96 hours following primary infection. This observation was consistent across all genotypes tested here and indicates a significant and somewhat universal superinfection restriction mechanism for IHNV. As discussed in the introduction, the mechanism behind cellular superinfection exclusion has been defined in other rhabdovirus systems including VSV and heterologous superinfection with IHNV and VHSV(5, 26). However, the mechanism behind in vivo superinfection restriction for IHNV remains unknown. Here, we conducted a preliminary investigation into the mechanism for IHNV superinfection restriction in rainbow and steelhead trout by measuring Mx-1 gene transcription following

infection with each viral genotype. Mx-1 expression was measured at regular intervals that correspond to the times at which fish were challenged with a secondary virus. We hypothesized that interferon stimulated gene expression would be elevated significantly at the time before or corresponding to secondary challenge when restriction was observed. In other words, we hypothesized that elevated gene expression would be observed prior to the onset of restriction. Contrary to this hypothesis, we observed a lag between the time at which restriction was first observed and induction of Mx-1 gene expression in individual whole fish. The potential reasons for this observation were discussed in the summary section of chapter 5. However, identification of the mechanism of restriction would provide important insight into the dynamics of superinfection and also potentially identify important factors in determining the outcome of infection with IHNV.

To address this question, a combination of in vivo and in vitro techniques would be desired. Previous work by Purcell et al. demonstrated a number of genes that were differentially regulated in rainbow trout kidney tissues at 48 hours post infection with IHNV (24). Based on this investigation, specific genes such as the VHSV-induced genes (Vig-1, Vig-2, Vig-3, Vig-4, Vig-5, Vig-9, and Vig-10) and genes involved in the interferon pathway such as IRF1 and IRF7 should be investigated further at multiple time points following infection to determine whether induction of these factors correlates with superinfection restriction. Additionally, analysis of gene expression in multiple tissues will be necessary to identify the tissues important for establishment of a secondary productive IHNV infection in rainbow and steelhead trout. It is possible that upregulation of immune response genes is only necessary in a specific tissue type to protect against secondary infection. Thus, an analysis of the expression profiles of specific immune response genes in various tissues such as skin, gills, kidney, spleen, and serum at

multiple time points post-infection will further elucidate whether there is a role for innate immune response in restricting secondary infection with IHNV in rainbow and steelhead trout.

However, it is also possible that the immune response does not play a significant role in the superinfection restriction observed here. Another potential form of competition that could explain the observed results is that of competitive exclusion. Competitive exclusion describes competition between two related viruses for the materials required for infection (cellular components for entry, replication, or release, presence of susceptible cells) resulting in the apparent exclusion of one genotype over another within an infected host (27). To test whether the presence of a replicating virus was sufficient to restrict secondary infection with IHNV, one may design an experiment in which immunocompromised *O. mykiss* are challenged with one genotype and subsequently challenged with another genotype 24 and 48 hours later. Such immunocompromised fish would lack the innate immune response that could play a role in the observed restriction. Therefore, if superinfection were restricted with the same overall pattern in immunocompromised fish compared to immunocompetent fish, one could conclude that innate immunity is not primarily responsible for superinfection restriction.

Further, investigations into the dynamics of cellular superinfection would also be interesting. It is likely that superinfection exclusion may occur for IHNV cellular superinfection based on reports of superinfection exclusion between IHNV and the related novirhabdovirus species VHSV (5). One potential method for studying this would be to sequentially expose a single culture of cells with two otherwise identical IHN viruses expressing two different fluorescent proteins and screen for cells containing both fluorescent colors using a fluorescent activated cell sorter (FACS). This would allow for quantification of superinfected cells. If a large percentage of cells ($\geq$ 70%) were found to express both colors, we could conclude that

superinfection exclusion does not occur at a high rate. In contrast, if the majority of cells express just one fluorescent protein, this would demonstrate exclusion and further investigations would be warranted to identify the potential mechanism. For example, if IHNV, like VSV, competes for the machinery involved in endocytosis but not receptor binding, application of a secondary virus to a culture already infected with a separate strain would result in accumulation of secondary virus on the outside of the cell. If that secondary virus were fluorescently labeled, again the FACS machine could be used to quantify the cells with fluorescent virus attached to the outside of the cell. An absence of virus bound to the outside of infected cells may suggest down regulation of cellular entry receptors and could be investigated through antibody-mediated labeling of receptors on the cell surface before and after viral infection. Unfortunately, although fibronectin has been implicated (14), the specific receptor required for IHNV entry is still not completely characterized and would need to be explicitly defined prior to this work.

Regardless of the mechanism, an important question is whether certain virus strains have evolved to overcome this exclusion. From an evolutionary perspective, a virus strain with an increased ability to establish superinfection would have a distinct advantage in regions of high host density and frequent viral infection. Through further experimentation using more IHNV genotypes for superinfection, the viral determinant(s) of superinfection may be elucidated.

IHNV superinfection in the field

Evolutionary theory suggests that superinfection, if it occurs, should have significant impact on the evolution of RNA viruses. Experimentally, we have shown that individual fish can be co-infected or superinfected with two genotypes of IHNV. However, a detailed understanding of the prevalence of superinfection in the field is lacking for IHNV in *O. mykiss*. Through genotyping efforts, we have identified samples which may represent examples of superinfection

in the field. Specifically, of over 2000 isolates, 27 (1.35%) were identified as having nucleotide sites within the 303 nucleotide mid-glycoprotein region that were heterogeneous upon sequencing. These heterogeneities are not thought to be an artifact of the reverse transcription reaction because these same sites can be detected in both sequencing directions and confirmed upon re-sequencing. These heterogeneous sequences may represent diverse genomes within a single infected fish as a result of diversification of the virus during a single infection with one variant, or they may represent multiple genomes resulting from co-infection or superinfection with one or more variants from the field. Further studies will be necessary to better determine the prevalence of multiple infection in the field.

One potential way to address this question would be to perform intense sampling and surveying of a single aquaculture facility before, during and after an epidemic. In an aquaculture facility, juvenile fish are housed at very high densities and previous research has demonstrated that viral diversity in certain types of facilities can be very high, with multiple strains circulating within a single facility at the same time (28). Assuming the probability of multiple infection in the field is quite rare, for example 0.1%, a sample size of roughly 250 would be required to identify a single case of multiple infection. To determine whether multiple infections were responsible for heterogeneous sequences as opposed to generation of in-host diversity within a single infection, a broader and more complete understanding of all of the variants circulating within the facility would be required. The identification of a heterogeneous isolate would warrant single copy PCR and sequencing to determine the true sequences of both genomes. Then comparisons of each sequence to the known sequences previously circulating within the facility would suggest whether the two genomes established independent infections or whether one grew out of a single circulating strain. Continuous sampling before, during, and after an epidemic

would provide a full picture of the variants circulating throughout the epidemic. While the absence of heterogenous samples from a study such as this would not prove that superinfections do not occur in the field, if such isolates are found, it would provide very clear evidence in support of superinfection in the field. Logic suggests that the probability that multiple infection would be caused by co-infection (simultaneous infection) would be quite low, allowing one to conclude that superinfection would be more likely. While we have demonstrated here that IHNV superinfection is possible in rainbow and steelhead trout, the implications of this possibility for virus evolution in the field is still unclear if we can provide no direct evidence for its occurrence.

Evolution of Virulence

The development of the in vivo superinfection assay added to previous research investigating the correlation between virulence and in-host replication fitness for IHNV in rainbow trout. While previous work in the laboratory investigated fitness in the context of co-infection, the superinfection assay allowed us to investigate the potential importance of the host immune response or resource availability for fitness and virulence in this system. It is important to note, however, that the studies in rainbow trout were all performed using a single genotype pair of unequal relative virulence and a single genotype pair of equal relative virulence. It is therefore possible that different genotype pairs may yield different results within the same fitness assays. For this reason, it would be worthwhile to complete similar such studies using multiple virus genotypes of varying virulence in rainbow trout. Interestingly, of the genotypes involved in the IHNV displacement events, only two differed significantly in virulence in steelhead trout, genotypes 110 and 139, with 110 having higher virulence. These two genotypes also differed significantly in their co-infection fitness but not superinfection fitness, with the genotype of higher virulence demonstrating greater co-infection fitness in steelhead trout. These results

support the previous demonstration that, while virulence may be associated with an increase in fitness for co-infection, virulence is not correlated with superinfection fitness.

While consistent within our system, these results lead one to wonder what advantage must a low virulence virus have in order to compete in the field. If there appears to be no in-host fitness cost to high virulence, why does the virus not continuously evolve towards ever higher virulence in the field? The trade-off hypothesis posits that high virulence may be selected against in the field because higher virulence leads to shorter potential transmission time for the virus due to early death of the infected host when compared to less virulent variants (1). Eventually, according to this theory, the virus will evolve to accommodate a balance between high replication fitness leading to higher virulence, but not so high that the host dies before transmission to sufficient susceptible hosts. IHNV isolates of variable virulence within the natural host have been collected over many years, indicating that the virus has not plateaued at an "optimal" virulence but instead maintains diversity. As described in chapter 1, measurement of shed virus as the transmission potential of genotypes HV and LV in single and co-infections indicated that the high virulence virus had an advantage (34). Slightly more of the high virulence virus was shed into the surrounding water of an infected fish than was shed by fish infected with the low virulence virus, even when mortalities were excluded from the analysis (Wargo, unpublished data). Thus, even the potential cost of mortality for higher virulence is smaller than the advantage of HV for in-host replication and shedding.

These same experiments are being repeated with other genotype pairs to determine if the previous results represent the exception or the rule for this system. In addition, it is important to note that shedding was quantified by RT-qPCR which measures virus RNA present in the water and does not determine infectious particles. It is possible that while viral RNA copies are present

at greater quantities, infectious particles may be less prevalent. Technical and logistical difficulties have prevented such measurement of infectious particles shed into surrounding water using this experimental system, but this remains a critical determination to be made. Going one step further, actual transmission studies would go a long way to demonstrating a true link between virulence and transmission by measuring which genotype is most often transmitted over many in vivo serial passages.

An alternative method for exploring the potential influence of virulence on evolution in the field is the use of mathematical models. A recent publication explored the potential impact of culling on the evolution of agricultural viruses using a susceptible-infected mathematical model approach (6). In this model, populations of hosts were culled during an epidemic when mortality reached a given threshold which was above that typically reached by a low virulence virus, but below the level typically reached by a high virulence virus. In an environment in which superinfection can occur, these researchers determined that a strict culling practice would, in fact, decrease the prevalence of high virulence viruses while offering an advantage to the low virulence viruses that evaded culling though low mortality. These researchers therefore concluded that, while high virulence was selected against in this system, culling would actually result in an increase in infections and virus diversity. This increased circulation of diverse viral variants could potentially lead to a greater incidence of spillover into human populations by viruses that infect agricultural species, even if it leads to less disease in the intended host species. Similar mathematical models are currently being developed to apply to the IHNV-trout system using much of the experimental data presented in this thesis.

Genotype displacement and viral fitness

The research presented in this work demonstrates that, for the isolates tested, IHNV genotype displacement events observed in the field do not correlate with increased in-host replication fitness in the context of single infection, co-infection, or superinfection. An important caveat to this work is that only one representative isolate was tested for each genotype examined. Again, it is possible that the isolates tested do not adequately represent the genotype as a whole and the testing of more isolates may provide different results. In addition, other measurements of fitness at different stages of the infection cycle such as entry and shedding may yield additional insights into the overall fitness of these virus strains.

The displacement events described here occurred within the Columbia River basin region and the Washington state Pacific coast region. The fish host used to test the comparative fitness of these genotypes was a stock acquired from the Washington coast and not from the Columbia River basin region. Therefore, it may be worthwhile to perform these same experiments using a separate host stock from the Columbia River basin which may have evolved in response to challenges from these virus genotypes in the past.

Again, the absence of experimental evidence to support the hypothesis that increased virus fitness is associated with and potentially responsible for the observed IHNV genotype displacement events indicates that other factors may be involved in driving the evolution and movement of this virus throughout the region. Such factors include but are not limited to changes in host disease management practices, changes in host population structure, and development of genotype-specific host resistance. Once more, mathematical models may be very useful in

exploring these possibilities in silico, with experimental validation of specific hypotheses that arise from the modeling efforts. This avenue of research is currently being pursued by our laboratory in collaboration with Dr. Shannon LeDeau of the Carey Institute of Ecosystem Studies in New York.

IHNV and Viral Fitness

The body of work presented here demonstrates the power of the natural host-virus system of IHNV infection in *O. mykiss* for studying RNA virus evolution. The assays already developed in the Kurath laboratory have potential uses to investigate numerous aspects of virus evolution including the impact of herd immunity, population level selective pressures, interactions between multiple pathogen species within the same host, and the importance of viral quasispecies diversity on viral fitness. Such research can be pursued with in vivo, in vitro, and in silico tools already developed for this system.

Herd immunity can be investigated by administration of a very effective IHNV DNA vaccine to groups of fish at varied ratios of vaccinated:unvaccinated. Passage of virus strains through populations of fish at various ratios of vaccinated:unvaccinated could determine what adaptations are required for the virus to overcome such immunity and whether certain viral phenotypic traits are associated with outgrowth. Such research could also provide insight into vaccination strategies that could prevent such outgrowth or explain epidemics in the face of wide-spread vaccination.

Understanding how multiple pathogen species interact in the field is important for managers of both cultured and wild host populations and can provide insight into why certain infections manifest differently in the field than under laboratory conditions. Numerous pathogens

have been reported to co-circulate with IHNV in the field including *Flavobacterium psychrophilum* and *Renibacterium salmoninarum* (11, 15, 20, 25). It has been suggested by hatchery managers and fish health experts that infection with IHNV exacerbates bacterial cold water disease, caused by *Flavobacterium psychrophilum*, and vice versa. Controlled laboratory experiments are still needed to support these claims of increased disease severity. Co-infection followed by mortality and histopathology assessments would contribute to the knowledge of how these two pathogens interact. In addition, the fitness of different viral strains may be altered dramatically in the presence of other pathogen species. Thus, a broader understanding of how multiple pathogens alter the selective pressures on one another within the host will be essential for designing better management strategies in the field to manage epidemics and prevent disease.

The importance of the viral quasispecies in determining the fitness of a particular strain of RNA virus has been demonstrated in numerous systems (13, 22, 31). While the diversity of the virus population in a natural IHNV infection of rainbow trout has been investigated in our laboratory in the past(8), new technologies, including next generation sequencing, may allow for more precise evaluation of the diversity present in infection by various strains of IHNV. Of particular interest would be to test the hypothesis that differences in virus diversity may correlate with differences in virulence in the natural host. Prominent work from the Andino and Kirkegaard laboratories demonstrated the importance of viral diversification during infection for virulence of poliovirus in a rodent model of infection (22, 31). To test this in the IHNV system, one could perform deep sequencing on samples of fish infected with either the LV or HV strains. It is possible that virulence is associated with increased viral diversity during infection to allow for outgrowth of virus variants that can escape immune pressure. However, without a deeper

understanding of the viral quasipecies of various strains of IHNV, this correlation cannot be defined.

Thus, the IHNV-salmonid host system is uniquely suited for study of RNA virus evolution and dynamics of host pathogen relationships and future work using this system will provide important insights to the field.

Final Summary

In this thesis, I have described the development of a novel, in vivo superinfection assay to be used in combination with previously developed in vivo virus fitness assays to investigate the phenotypic correlates of overall fitness for a vertebrate RNA virus. The work presented demonstrates the importance of investigating overall viral fitness through the use of multiple assays to assess fitness at specific stages in the infection cycle as well as within specific competition types such as co-infection and superinfection. The novel superinfection assay was used to determine that virulence plays a minor, if any, role in superinfection fitness for IHNV in rainbow trout, even though virulence was found to correlate with co-infection fitness. This assay is the first published assay used to investigate the dynamics of superinfection for a vertebrate virus in vivo in controlled, reciprocal challenges. In addition, single infection, co-infection and superinfection fitness assays were used to investigate the overall fitness of IHNV genotypes involved in sequential genotype displacement events in the field. This work demonstrated that increased fitness did not correlate with the displacement events, and therefore, viral fitness may not have played a significant role in these events. This work demonstrates the importance of developing in vivo tools to test viral fitness and describes a very useful model system for exploring additional research questions regarding RNA virus evolution in a vertebrate host.

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