Genetic diversity, evolution, and fitness of infectious hematopoietic necrosis virus within an endemic focus in rainbow trout aquaculture

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A dissertation submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy

University of Washington

2002

Program Authorized to Offer Degree: Pathobiology

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Abstract

GENETIC DIVERSITY, EVOLUTION, AND FITNESS OF INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS WITHIN AN ENDEMIC FOCUS IN RAINBOW TROUT AQUACULTURE

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The rhabdovirus infectious hematopoietic necrosis virus (IHNV) is the most significant viral pathogen of salmon and trout in North America. The virus infects wild, hatchery, and farmed fish populations from Alaska to California and inland to Idaho, causing disease with up to 90% mortality. In the 41 mile Hagerman Valley of Idaho, IHNV is endemic among numerous rainbow trout farms and resource mitigation hatcheries. In this work, the genetic diversity of IHNV in the Hagerman Valley was characterized at multiple levels using RNase protection assays (RPA) of the entire viral glycoprotein (G) gene and nucleotide sequencing of a 303 nucleotide region of the G gene. Characterization of 122 virus isolates from 18 rainbow trout farms and three state fish hatcheries demonstrated the presence of multiple virus lineages which appeared to co-circulate among all types of facilities throughout the geographic region. This demonstrated five-fold greater genetic diversity than has been found in other geographic regions, suggesting that conditions specific to rainbow trout aquaculture have accelerated the evolution of IHNV. Virus populations within two individual outbreaks in aquaculture rearing ponds and within four individual rainbow trout had levels of heterogeneity similar to IHNV populations in other environments, suggesting that the forces responsible for increased viral diversity in this environment do not act at the intra-outbreak or intra-host level. In order to assess the relative fitness of IHNV variants in rainbow trout, a novel *in vivo* competition system was

developed. Methods were developed for challenging rainbow trout with two different IHNV genotypes, allowing infection and virus competition within the host to occur, and then subsequently analyzing the virus progeny populations present within each infected fish to determine whether one or both of the genotypes were present and in what proportions. Challenge of 16 fish with two IHNV genotypes from the Hagerman Valley demonstrated the ability of the system to generate and characterize mixed infections, and indicated that the two genotypes were of equal fitness.

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ACKNOWLEDGEMENTS

I wish to express sincere appreciation to the Department of Pathobiology and the Western Fisheries Research Center for their long-term support and especially to my graduate mentor Dr. Gael Kurath for her knowledge, patience, guidance, and unique perspective on life. This dissertation would not have been possible without the collaboration of Dr. Scott LaPatra and colleagues in Idaho, the guidance of my supervisory committee, and the encouragement and devotion of my family and friends.

INTRODUCTION

RNA VIRUS DIVERSITY, FITNESS, AND EVOLUTION

Mutation and the virus quasispecies concept

RNA viruses use the same mechanisms of genetic variation which operate with cellular DNA: mutation, recombination (homologous and nonhomologous) and genetic segment reassortment. However, no recombination or reassortment has ever been observed for a non-segmented negative-sense RNA virus, such as infectious hematopoietic necrosis virus (IHNV) [58]. Therefore, this introduction focuses on mutation as a source of RNA virus genetic diversity. Replication of RNA viruses occurs in the absence of the editing 3'→5' exonuclease activity found in cellular DNA polymerases [94]. Thus RNA viruses maintain error rates which have been estimated at 10^{-4} to 10^{-5} mutations per nucleotide copied [65-67]. These high mutation rates are 10^{3} to 10^{7} times higher than the average mutation rates for cellular DNA replication (10^{-8} to 10^{-11} mutations per nucleotide copied) [64] and appear to approach the tolerable error threshold for maintenance of genetic information [114]. The consequence of such high mutation rates is that RNA viruses can exist as heterogeneous mutant swarms, rather than as identical copies of one dominant sequence. The concept of "quasispecies" was first formulated as an evolutionary model for early RNA replicons [72]. This model describes a heterogeneous distribution of genomes ordered around one or a few "master" sequences in which the mutants act cooperatively. The entire quasispecies, rather than individual genomes, is the subject of selection and evolution [58, 74]. The term "quasispecies" originally referred to this specific evolutionary model, but has since become used widely by virologists to describe the genetically heterogeneous mutant swarms formed by RNA viruses [63, 118].

The first evidence that RNA viruses form quasispecies distributions came from the observation of Qβ phage T1 RNase fingerprint patterns [54]. Since then, many other RNA viruses have been shown to exist as heterogeneous mutant swarms, notably vesicular stomatitis virus (VSV) [244], foot-and-mouth disease virus (FMDV) [55], rabies virus (RABV) [18], human immunodeficiency virus (HIV) [256], and hepatitis C virus (HCV) [160]. The diversity of the viral quasispecies *in vivo* varies depending on many factors including the virus [62, 233], host [133, 213, 234], and the type and history of infection [62, 273, 285, 286]. An important factor in the generation of quasispecies diversity is the rate of virus turnover in the host. The high diversity generated in HIV quasispecies is likely partially due to the enormous number of new virions (10^{10}) produced each day [236], which provide the virus ample opportunity to explore new sequence combinations. The diversity of viral quasispecies forms the basis for virus diversification and adaptation (reviewed in [56-58]) and has been implicated in viral disease progression [85] and in response to treatment [86, 207]. It is very important to recognize that the mutation frequency found in the quasispecies *in vivo* is not solely determined by the mutation rate of the viral polymerase. Instead, the mutation frequency is a function of both the error rate of the polymerase and the number of rounds of replication the virus has undergone, as well as the effects of selection and bottleneck events on the variant genomes within the quasispecies [62, 125].

RNA virus fitness and evolution

Fitness is a complex parameter which, in general terms, defines the reproductive adaptation of an organism to its environment (reviewed in [58, 61]). A generally accepted approximation for viral fitness is the relative ability to produce stable, infectious progeny in a given environment [58, 61, 62, 217]. Within a viral quasispecies, every individual genome has its own fitness for the particular environment in which the virus is replicating [58, 61, 71]. Thus, genomes in which

deleterious mutations occur are eliminated by negative selection, while genomes which harbor beneficial mutations increase in frequency in the population due to positive selection [56-58, 71]. Examples of mutations which might be positively selected include those that allow the virus to infect different cell types, increase replication efficiency, or avoid the host immune response [61, 204]. Under conditions in which the virus reaches a state of equilibrium with its host, the average or consensus sequence of the virus quasispecies may remain unchanged despite the constant production of new mutants [57]. However, as the environment in which the virus is replicating changes, the "fitness landscape" [275] changes, initiating a process of viral adaptation to new fitness peaks [58]. A classic example of this is avian influenza A virus, which is genetically stable in the avian host, but undergoes rapid evolution when introduced into humans [263]. Thus, the diversity of the virus quasispecies endows RNA viruses with a remarkable ability to rapidly adapt to a changing environment. Examples of adaptation facilitated by quasispecies heterogeneity include the development of drug-resistance [21, 59], antigenic escape mutants [60, 93], host cell-specific or tissue-specific variants [13, 170], and host-range changes [200].

A number of studies have made use of controlled laboratory infections in cell culture systems, primarily with VSV and FMDV, to define and understand the parameters affecting viral fitness and the evolution of the quasispecies (reviewed in [62]). One highly important feature of RNA virus evolution is the effect of extreme reductions in the virus population size, or "bottleneck" events, which are likely to commonly occur in many virus populations, particularly upon transmission to a new host or invasion of new tissues, where a small number of virus particles may found the subsequent virus population [1, 51, 71]. One or several virus genomes sampled from a heterogeneous RNA virus quasispecies are likely to harbor deleterious mutations relative to the average or consensus sequence, so virus bottlenecks are expected to decrease viral fitness [56, 74]. Experimentally, serial plaque-to-plaque transfers of bacteriophage [45], VSV [69, 70], FMDV [82, 83], and HIV [282, 283] have resulted

in significant fitness losses, which occurred in a stochastic (random) manner in each case [57], demonstrating the fitness loss predicted by the population biology theory of "Muller's ratchet" [185]. The effect of bottlenecks on virus diversity is typically a reduction in heterogeneity [57], demonstrating that stochastic events may profoundly affect viral diversity as well as viral fitness. The fitness loss occurring upon bottleneck transfer is counteracted by virus adaptation through multiple rounds of replication in which competition among variants results in fitness gains [47, 169, 198]. In contrast to plaque-to-plaque transfer, large population transfers result in increased fitness [198]. Continuous replication in a cell line from one host has been demonstrated to consistently increase fitness in that host [115], while decreasing fitness in cell lines from other hosts [197]. However, alternating passage of virus between cell lines from two hosts has been shown to result in fitness gains in both hosts [199, 262].

Virus competition and fitness in vivo

Recently, the importance of genetically diverse infections in influencing disease severity, as well as epidemiology and evolution, has come under increasing attention [171, 172, 218, 219]. Probably the most significant example of this is the enormous amount of research that has been conducted on the within-host evolution of HIV-1 [48]. While numerous studies on viruses such as rabies virus, HIV, and HCV have characterized patterns of genetic diversity in the host [39, 85, 86, 133, 273, 285, 286], very few controlled infection studies have addressed the role of viral fitness *in vivo*. Numerous studies have used *in vitro* cell culture-based competition assays to investigate the relative fitness of viruses which have different phenotypes *in vivo*, such as drug-resistant variants [50, 106, 135, 158]. Recently, an *ex vivo* system for determining the fitness of HIV-1 primary isolates in competition in cultured peripheral blood mononuclear cells was developed [217]. This study demonstrated a strong correlation between *ex vivo* fitness and disease progression.

Despite the recognition that virus competition and fitness *in vivo* is likely to be highly complex and may depend on selection pressures which differ from *in vitro* conditions [58, 61, 218, 219], few studies have been conducted *in vivo* because of concerns about performing experiments in mammals and limits on using large numbers of experimental animals. We are aware of only three *in vivo* virus competition studies conducted in vertebrate hosts. A pair-competition study of three FMDV variants, with each pair of viruses co-injected into two pigs, demonstrated *in vivo* fitness variations among members of the quasispecies mutant spectrum [44]. Competition between two lymphocytic choriomeningitis virus (LCMV) variants in three mice demonstrated tissue-specific tropism [53]. A competition between wild-type hepatitis B virus (HBV) and a cytopathic variant in groups of nine ducklings, indicated reduced fitness of the cytopathic variant [153]. While these studies demonstrated important differences between phenotypic variants in their replicative ability within the host and their ability to infect different tissues, the low number of animals per group and the artificial means of inoculation (injection) limit the applicability of these systems to study virus population biology phenomena. Other relevant literature on *in vivo* competition includes virus competitions conducted in plants [105, 139], bacteriophage competition studies [252, 253], and competitions of bacteria and parasites in mammals [218, 219]. Numerous controlled *in vivo* competitions of malarial *Plasmodium sp.* parasites in mice and mosquitoes have been conducted [218, 219].

RNA virus diversity, distribution, and evolution in host populations

The study of virus diversity and evolution has blossomed in the era of molecular biology with the application of genotyping techniques, the polymerase chain reaction, and nucleotide sequencing to viruses. These techniques have permitted the characterization of the relationships among viruses at much greater resolution than was possible with serological techniques alone [116]. Molecular epidemiology has led

to a better understanding of viral ecology and epidemiology, including insight into what forces impact virus emergence, disease occurrence, transmission and spread, host associations, and reservoirs. Many different patterns of virus circulation have been revealed, including multiple co-circulating lineages, sequentially predominating lineages, geographic clustering of lineages, and the restriction of lineages to specific reservoir species [130]. The enormous amount of data that has been generated on RNA virus genetic diversity precludes a comprehensive review of the literature in this introduction, and therefore this introduction will focus on what is known about the closest relatives of IHNV, the rhabdoviruses (vesicular stomatitis virus, rabies virus, and the fish rhabdovirus viral hemorrhagic septicemia virus), with comparison to several paramyxoviruses and other well-characterized RNA viruses of animals, including human immunodeficiency virus, hepatitis C virus, and influenza virus.

Vesicular stomatitis virus (VSV) is an insect-transmitted rhabdovirus which causes disease in farm animals, but is thought to infect additional unidentified wild reservoir species [226]. Studies of the genetic diversity within the two major serotypes of VSV, New Jersey and Indiana, have found up to 20% divergence among isolates in the glycoprotein [24, 191] with similar levels in the nucleoprotein (N) and phosphoprotein (P) genes [25]. Phylogenetic analysis of VSV isolates shows no relationship between tree position and host or date of isolation [191, 192, 225]. Instead, the phylogeny suggests an association between geographic origin and position on the tree, with lineages from endemic regions of Central America clustering separately from lineages in the United States and Mexico, which are primarily associated with sporadic disease [191, 192, 225]. There are a number of different lineages which co-circulate across Central America [24, 25, 191, 192, 224, 225], but lineages are highly conserved within endemic foci over long periods of time [192, 224, 254]. A detailed phylogenetic study of virus isolates in Costa Rica showed that the two major phylogenetic clades of VSV in the country correlate with two distinct ecological zones, highland premontane tropical forest and lowland tropical dry forest, suggesting that the virus is maintained by specific vectors and/or reservoirs in each

ecological zone [224]. That this relationship is ecological rather than just due to geography is indicated by identical virus types originating at very distant geographic locations, but in the same ecological zone. The ability of VSV to persist despite the presence of neutralizing antibodies in cattle suggested the possibility of antigenic variation, and clusters of nonsynonymous mutations were initially taken as evidence of immune selection [24, 191]. However, diverse isolates display little antigenic variation and there is very little sequence divergence within endemic foci [192, 254]. A recent quantitative analysis of the rates of evolution of 50 RNA viruses estimated that VSV has one of the lower overall rates of evolution found among RNA viruses [125]. However, there is evidence that while the virus may undergo long periods of stasis, it can evolve rapidly in short periods of time, which may correspond to changes in selection pressure [192].

Rabies virus is the type species of the lyssavirus genus of rhabdoviruses [258]. The virus is neurotropic and infects a wide variety of mammalian species, causing lethal encephalitis in some species including humans [187]. Dog rabies has disappeared from much of the developed world, but is still endemic to most of the developing world. However, rabies still persists in wildlife populations such as the red fox, skunk, raccoon, and bat [187]. Rabies virus isolates from around the world have up to 17% divergence in the N gene [132]. The pattern of evolution of rabies virus is highly complex and varies greatly with host species [239]. While the majority of lineages are associated with a particular host species within a particular geographic range [187, 239], host-species boundaries are not absolute, and in Europe geography may be a stronger influence than host species [30, 119]. It is unclear whether the species-specific phylogenetic groups observed are due to virus strains being adapted to particular animal species or whether rabies lineages circulate in a series of "epizootiological compartments" [119], defined as geographic clusters where virus types can infect a variety of species, even if they are typically found in a single species [30, 119]. Morimoto et al. [170] found that passage in different cell lines and in mice could reproducibly select for specific glycoprotein variants within the quasispecies,

suggesting that changes in the host environment may rapidly result in shifts in the dominant variant. Similarly, laboratory passage of a rabies strain in several different hosts indicated an elevated rate of nonsynonymous substitutions in the G gene, consistent with host adaptation [133]. However, phylogenetic studies have indicated that there are few host-specific amino acid changes observed, suggesting that few changes are needed for host adaptation [30]. In general, rabies virus appears to be highly evolutionarily constrained at the amino acid level, with very low rates of nonsynonymous mutations in the G and N genes ($5.1x10^{-5}$ and $2.9x10^{-5}$ nonsynonymous substitutions per site, respectively) [30, 119]. The estimated rates of synonymous substitution are also at the low end for RNA viruses ($4\text{-}5x10^{-4}$ synonymous substitutions per site) [119].

Viral hemorrhagic septicemia virus (VHSV) is a rhabdovirus of fish which, along with IHNV, is a member of the novirhabdovirus genus [258]. VHSV causes disease in European rainbow trout with up to 90% mortality, and infects a number of different marine reservoir species in Europe and North America [129, 168, 173, 240, 245]. The phylogeny of VHSV indicates three major clades associated with geographic origin located in North America, continental Europe, and the British Isles [19, 245]. The North American clade is the most divergent with up to 15-18% G gene divergence from the European clades [19]. The phylogeny does not indicate a relationship between tree position and date of isolation, host species, or virus serotype [19, 240, 245]. In several cases, similar nucleotide sequences have been found in VHSV marine isolates and isolates associated with outbreaks in rainbow trout [240, 245], suggesting an epidemiologic link between the marine reservoir and the occurrence of disease in fresh water fish. It appears that barriers associated with transmission between species are low and it was demonstrated that 5 serial passages of a marine VHSV isolate in rainbow trout resulted in increased virulence for rainbow trout [241].

Phylogenetic analysis of VSV, rabies virus, and VHSV demonstrates that the evolutionary pattern of each of these rhabdoviruses is at least partially determined by

geography. It is intuitive that virus lineages would typically correlate with geographic origin, but this is not always the case. Different rabies virus lineages circulate in the same geographic region, associated with different host species [187, 239]. A more extreme example is the paramyxovirus, human respiratory syncytial virus (HRSV), in which viruses from divergent lineages can be present in the same epidemic (co-circulating lineages), while closely related isolates can be isolated in distant parts of the world in different years [42, 96, 270]. It is striking that all three of the example rhabdoviruses are able to infect a wide range of species and appear to have relatively low barriers to crossing species boundaries. However, there appears to be the potential for host-specificity (rabies virus) and possible vector-specificity (VSV).

The three example rhabdoviruses all have maximum levels of genetic divergence between field isolates of 17%-20%. While levels of maximum divergence are highly dependent on the somewhat arbitrary concept of virus species, they do provide an indication of the evolutionary divergence which has occurred among a defined set of phylogenetically related viruses. Maximum divergence varies widely among RNA viruses. Wild-type measles virus is an example of low diversity, with a maximum of 7% divergence [229]. HIV-1 is an example of high diversity, with up to 35% divergence among isolates within the M group [186] and much more between the M, N, and O groups [165]. Full-length HCV virus genomes may vary by up to 30% while variable genes may vary up to 50% [117]. The causes of differences among viruses in rates of evolution and levels of divergence are likely to be highly complex. One common factor shared by several high-diversity viruses such as HIV, HCV, and HRSV, is the strong evidence that host immune selection of viral immune-escape variants plays a major role in the evolution of these viruses [42, 93, 96, 204, 270]. Of the three example rhaboviruses, there is not strong evidence that immune selection of viral variants occurs for any, although some authors have speculated on the possibility [24, 191]. Additionally, accumulation of mutations may be favored during HIV and HCV infection largely due to the fact that these viruses typically establish long-term chronic infection with high levels of virus replication [48, 236].

Another commonality between the three example rhabdoviruses is the general lack of a correlation between position on the phylogenetic tree and date of isolation, termed molecular clock. The presence of a molecular clock is typically taken as evidence that evolution is proceeding at a predictable rate, with substitutions of neutral selective value predominating [98], while the absence of a molecular clock likely indicates that the rate of evolution is not regular, instead proceeding through periods of stasis (as has been demonstrated for VSV [254]) and periods of more rapid evolution. The classic example of the presence of a molecular clock is human influenza virus A, in which substitutions accumulate at a predictable rate [108, 263], although there has been disagreement over whether neutral evolution or positive selection is the dominating feature of influenza A virus evolution [37, 90, 124]. Another factor with the potential to influence virus diversity, which may often be overlooked, is the total size of the host population. Using phylogenetic methods, Zanotto et al. [284] demonstrated that the number of reconstructed dengue virus lineages mirrors the size of the world's human population, with an exponential increase in both beginning approximately 200 years ago.

INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS

Virus taxonomy, structure, genome, and proteins

Infectious hematopoietic necrosis virus (IHNV) is the type species of the genus *Novirhabdovirus* within the family *Rhabdoviridae* [258]. The novirhabdovirus genus also includes two other fish rhabdoviruses: viral hemorrhagic septicemia virus (VHSV) and Hirame rhabdovirus (HIRRV). Phylogenetic analyses clearly separate the novirhabdoviruses from other rhabdovirus genera [15, 27, 140, 174]. The other major rhabdovirus genera which infect vertebrates are the vesiculovirus genus, typified by vesicular stomatitis virus (VSV), and the lyssavirus genus, typified by rabies virus (RABV). The virions of IHNV are bullet-shaped and measure

approximately 110 nm by 70 nm [112]. The genome of the virus is a single minus-sense RNA strand of 11,131 nucleotides [174]. This includes a 60 nt 3' leader, a 101 nt 5' trailer, and six genes in the following order: nucleocapsid gene, N; phosphoprotein gene, P; matrix protein, M; glycoprotein gene, G; non-virion gene, NV; and polymerase gene, L [137]. The NV gene is unique to the members of the genus *Novirhabdovirus* [140, 258]. Six mRNAs, one corresponding to each gene, have been identified [138] and all are currently considered to be monocistronic. However, it is possible that additional transcripts and/or proteins exist.

The five structural proteins include nucleocapsid protein (N), phophoprotein (P), matrix protein (M), glycoprotein (G), and polymerase (L) [162]. The non-virion (NV) protein is present in infected cells, but is not detected in virions [138]. The 413 amino acid N protein is the most abundant protein during infection [154]. It is associated with the IHNV genome [78, 79] and is phosphorylated [120, 162]. The 230 amino acid P protein is very basic, phosphorylated, and thought to be associated with the N and L proteins [29]. M is a highly basic protein of 195 amino acid [29]. The 508 amino acid G protein forms spikes on the virus surface and is thought to be responsible for virus attachment [162]. It possesses a 20 amino acid hydrophobic N-terminal signal peptide and a hydrophobic domain near the C-terminus, thought to be the transmembrane domain [29]. The glycosylation sites and deduced structure of the IHNV glycoprotein are similar to those of other rhabdoviruses [75, 257] and several epitopes have been identified near the middle of the glycoprotein which bind to monoclonal antibodies generated in mice [122]. The 111 amino acid NV protein has no significant homology to other proteins and its function is not known [15, 193]. The L protein is 1986 amino acids [26, 174], and has six conserved amino acid blocks which are conserved among all negative-sense RNA virus polymerases [212]. The L protein likely functions in conjunction with the P and N proteins as the RNA-directed RNA polymerase for viral transcription and replication [26]. The recent development of reverse genetics for generating infectious IHNV molecular clones [22] will likely

lead to a better understanding of the function and role in pathogenesis of viral genes and proteins [23].

The disease: infectious hematopoietic necrosis

The primary signs of infectious hematopoietic necrosis (IHN) include increased fish mortality, darkening of the body, exophthalmia, pale gills, distended abdomen, fecal casts, petechial hemorrhaging, and erratic swimming [2, 272, 279]. Additionally, survivors of outbreaks frequently have spinal deformities [2, 35, 230, 272]. Studies of steelhead trout, rainbow trout, and coho salmon, challenged with IHNV by immersion, have indicated that virus enters fish through the gills, oral cavity, skin, and esophagus-cardiac stomach region [68, 109, 110, 278]. An immunohistochemical study of immersion-challenged steelhead trout demonstrated that the virus progressed through the gills to the circulatory system and also through the oral cavity to the gastrointestinal tract and then into the circulatory system [68]. Once in the circulatory system, the virus became widely disseminated to many organs [68, 278]. The primary infected organs are the hematopoietic organs; kidney, spleen, and to a lesser extent, liver; with primary pathologic changes including significant degeneration and necrosis [2, 272]. The disease primarily affects young salmonid fishes, with cumulative mortalities in outbreaks among fingerlings and fry as high as 90%, but outbreaks have also been reported in adult fish [34, 248, 280]. The disease course is typically acute, especially in young salmonids, with mortality beginning at around five to six days postexposure in laboratory immersion challenges and continuing for up to one month [278].

Host range and geographic distribution

The current geographic range of IHNV in North America includes most of the watersheds in which anadromous Pacific salmon reside, and extends along the Pacific

coast from Alaska through northern California and inland to Idaho. Within this geographic range, the host range of IHNV includes the five species of Pacific salmon, commercially-reared Atlantic salmon, and several species of trout [29]. While the historic geographic range and host species of IHNV prior to the impact of humans are not known with certainty, it is highly likely that the virus originated in the Pacific Northwest of North America. Historically, the first reported outbreaks of IHNV occurred in sockeye salmon (*Oncorhynchus nerka*) fry at Washington state fish hatcheries from 1951-1953 [104, 230, 260]. The first reported IHNV outbreaks in other locations were: Oregon state sockeye salmon hatcheries, 1954 and 1958 [266]; California chinook salmon (*O. tshawytscha*) hatchery fingerlings, 1960 [228]; British Columbia hatchery rainbow trout (*O. mykiss*), 1967; Alaska hatchery sockeye salmon fingerlings, 1974 [100]. However, in hindsight it appeared that the virus may have been the cause of unexplained high mortalities in California as early as 1941 [228], and in British Columbia from 1925 to 1936 [2, 92].

Following the development of cell culture and assays to identify the virus, a survey showed that IHNV was already widespread by the mid-1970's in adult fish throughout Alaska [100]. However, surveys indicated that the virus was not widespread in Washington and Oregon through the 1970's, instead persisting in several localized sites, with only sporadic occurrence elsewhere [4, 183, 209]. The virus may have been spread inadvertently by the historically common practice of salmon transplantation [272], such as the shipments of Alaskan sockeye eggs and fry to numerous sites in Washington and Oregon during the 1920's and 1930's [33, 227] or the practice of feeding raw salmon viscera to fish throughout the Pacific Northwest of North America in the 1940's and 1950's.

Several viral emergence events have altered the geographic range and host range of IHNV. The virus was introduced to Japan in 1968, likely through a shipment of sockeye eggs from Alaska to Hokkaido Island, and the virus was later found on Honshu Island, but the source was not determined [231]. In Japan, the virus has caused disease in rainbow trout, as well as several additional salmonid species

including amago salmon (*O. rhodorus*) and yamame salmon (*O. masou*) [231, 272]. IHNV emerged and became endemic in rainbow trout throughout the Hagerman Valley trout farming industry in south-central Idaho between 1977 and 1980 [35](see the "infectious hematopoietic necrosis virus in Idaho aquaculture" section in this Introduction). IHNV also emerged in hatchery salmonids in the middle and lower Columbia River basin in the early 1980's, causing high levels of mortality [102]. Isolation of IHNV from fish in the Columbia River basin is now common [29, 272]. In the mid-1990's, the virus spread among Atlantic salmon (*Salmo salar*) reared in marine net-pen aquaculture on the coast of British Columbia and caused numerous outbreaks [243]. This epidemic eventually subsided, likely due to management efforts, but the virus has recently begun to infect and cause outbreaks in British Columbian net-pen raised Atlantic salmon once again (G.S. Traxler, Canadian Department of Fisheries and Oceans, personal communication). The virus was also reported in Taiwan in 1985 [259], Korea in 1990 [206], and Western Europe in 1987 where it has caused multiple outbreaks [12, 17, 31]. In addition to these emergence events, the virus has been spread through the transport of infected fish or eggs to other locations where it did not become established, including Australia and a number of locations throughout the United States (reviewed in [272]).

IHNV infections and disease are commonly reported in rainbow trout (*O. mykiss*), steelhead trout (anadromous *O. mykiss*), sockeye salmon (*O. nerka*), kokanee salmon (landlocked *O. nerka*), chinook salmon (*O. tschawytscha*), and chum salmon (*O. keta*) (reviewed in [272]). Other species in which natural outbreaks have occurred in North America include Atlantic salmon (*Salmo salar*), cutthroat trout (*O. clarki*), brook trout (*Salvelinus fontinalis*), and brown trout (*Salmo trutta*) (reviewed in [151]). Coho salmon (*O. kisutch*), pink salmon (*O. gorbuscha*), arctic grayling (*Thymallus arcticus*), lake trout (*Salvelinus namaycush*), and artic char (*Salvelinus alpinus*) are resistant to disease, but have low susceptibility to infection (reviewed in [29]).

Salmonid fishes are a genetically-diverse group of species, each with its own unique and complex life history [103]. All salmonids spawn in fresh water, but those

species which are anadromous migrate to the ocean for a portion of their life and then return to fresh water to spawn. Anadromous fish home to their native stream, but a certain amount of normal fish straying occurs [214], which can be exacerbated by certain conditions such as El Niño events. For the purposes of simplicity and clarity, the fish populations discussed in this dissertation may be divided into three categories based on the level of human-interaction in their life history: wild fish, hatchery fish, and farmed fish. Wild fish spawn naturally, independent from direct human interaction (this definition includes feral fish and fish which spawn in artificial channels). Hatchery fish are typically spawned under human control in a confined setting, fed by humans until they reach a predetermined lifestage or size, and then released into the environment. By contrast, farmed fish produced for human consumption are confined for their entire life history in either land-based ponds or large net-pens placed in marine waters.

Transmission and epidemiology

IHNV outbreaks primarily occur in young fish, although adult fish may become infected and disease can occur in other life stages including adults [35, 248, 280]. The source of virus for outbreaks in young fish could be vertical transmission from spawning adults, water-borne transmission from other populations of fish, or water-borne transmission from an unidentified reservoir [29]. In hatchery and farm settings, there is the additional possibility of cross-contamination from other fish within the facility or handling practices. Vertical transmission is thought to occur mostly due to IHNV-contaminated eggs (reviewed in [29, 272]), although IHNV can be isolated from milt [167, 180] and so male fish could play a role in vertical transmission as well. Eggs from IHNV-infected spawning females clearly can have virus associated with the surface of the egg [181, 271], but there is some controversy as to whether virus can be present within the egg. The effectiveness of egg iodination in controlling egg-associated virus spread suggests that virus is primarily present on

the surface of the egg [272]. While vertical transmission from infected spawning adults to progeny fish clearly can occur [29, 181, 268, 271, 272], some studies which have analyzed the prevalence of IHNV among the progeny of infected parents have suggested that the frequency of vertical transmission in nature may be low in some cases [5, 80, 142, 145, 250].

IHNV survives in 15°C fresh water for approximately 25 days and in 15°C saline water for approximately 14 days [14, 247], although adherence to clay has been demonstrated to preserve infectious virus for nine months [281]. The virus does not likely survive over the winter [177]. No alternate fresh water hosts or reservoirs for the virus have been identified. Although the virus has been isolated from several invertebrate species including leeches [182, 277] and copepods [182] associated with infected sockeye, as well as mayflys in Idaho (virus was detected after two blind passages in cell culture) [237], virus replication or persistence in these species has not been demonstrated.

Within an outbreak, virus is readily spread horizontally from fish-to-fish in the water [177, 277] and virus has been isolated from fish up to 50 days after virus exposure [5, 36, 107, 167]. Interestingly though, the virus typically can not be reisolated from fish after this initial window of time, but often can be isolated from sexually mature fish at spawning [36, 91, 107, 167]. In naturally spawning sockeye salmon, virus is typically not isolated during their migration from ocean to spawning grounds, but virus appears in several adults in the spawning grounds. As the spawning migration continued, the prevalence and titers in fish increases [176, 178, 184]. Two hypotheses have been proposed to explain this phenomenon. One is that a few fish become infected with virus from other fish populations or an unknown source in the ocean or during their spawning migration, and then the virus is transmitted among fish in the densely populated spawning grounds. The other is that some juvenile fish develop low-level latent or carrier infection, which becomes activated during their spawning migration, and then the virus is transmitted to other fish in the spawning grounds. Studies in rainbow trout have indicated that when fish surviving outbreaks

were held in virus-free water for long period of time, virus could again be isolated from a low percentage of fish when they reached sexual maturity [5, 36]. This led to the proposal that IHN-survivors develop a latent viral infection which may become reactivated upon sexual maturation [5]. A recent study also demonstrated carrier infection of adult chinook salmon [242]. These studies suggest that at least some salmonid species can develop carrier infections at a low rate, but the frequency of the carrier state in nature is not known. Sockeye salmon captured over 3 years from a population which normally had 98-100% prevalence of IHNV did not become infected when held to sexual maturity in virus-free water [8]. With either hypothesis, horizontal spread of the virus between spawning adults may be facilitated by a weakened immune response [178], although spawning fish have been shown to produce antibodies in response to infection [250].

If there are additional sources/reservoirs of IHNV which can infect adult fish, they have eluded identification [29]. The finding of IHNV in sockeye salmon during the ocean phase of their life history [129, 250] and the presence of IHNV in Atlantic salmon in salt water [242] suggest the potential for an IHNV ocean reservoir. A survey for pathogens in marine fish from the Pacific Ocean off the coast of British Columbia found IHNV in three non-salmonid species including herring (*Clupea pallasi*), shiner perch (*Cymatogaster aggregata*), and tube-snout (*Aulorhynchus flavidus*) [129]. While these fish comprised a very small number of the total sampled, it is possible that a reservoir remains to be identified.

Factors affecting pathogenicity and virulence

Factors affecting the pathogenicity of IHNV have been reviewed in detail by LaPatra [151] and others [29, 272]. Factors which have been shown to influence the pathogenicity of IHN include, but are not limited to, fish host species, viral strain, temperature, fish population density, fish life stage/size/age, stress, nutritional status, the presence of other pathogens, and other environmental factors such as water quality

and salinity [29, 151]. A major factor governing the pathogenicity of IHNV is temperature. Outbreaks of IHNV occur most frequently when water temperatures are between 10-12°C [194] and naturally occurring outbreaks have not been reported at temperatures above 15°C, although rainbow trout fry in laboratory challenges held at 3°C to 18°C died due to IHN [111]. In general rainbow trout, steelhead trout, and chinook salmon typically become more resistant to IHNV as they increase in age and weight [143, 146, 150, 272] but this relationship is not necessarily true for kokanee and sockeye salmon [7, 143] or Atlantic salmon [243], and may depend on virus type [143]. Also, high fish density correlates with the occurrence of IHN outbreaks [202, 249].

The concept of a relationship between fish host species, IHN virus strain, and pathogenicity was established soon after the identification of IHNV, with the finding that an IHNV isolate from sockeye salmon was virulent for sockeye but not other species [230] and the finding that an isolate from chinook salmon was most virulent for chinook [205, 228]. Using the virus classification system developed by Hsu et al. [121], based on the electrophoretic mobility of proteins, several studies have demonstrated a correlation between electropherotype and virulence in different host species. Type 1 virus isolates were generally most virulent in kokanee and sockeye salmon and type 2 isolates were more virulent in rainbow trout and steelhead trout than types 1 and 3 [143, 144, 147]. Type 3 isolates were generally most virulent for chinook salmon [46, 147]. It is important to note that this correlation was not absolute and virulence was shown to vary within individual electropherotypes [46, 147, 150]. These data suggest the possibility of some host-specific viral adaptation, but the virus clearly is not highly host-specific since virus type has been shown to correlate with geography rather than host species [121, 193, 203]. Fish genetics has some influence on IHN susceptibility, as differences between individual fish stocks and families within species have been demonstrated [7, 128, 166, 276].

Immune response

Innate immunity is likely to act as a first line of defense against IHNV, and interferon-like activity has been detected in response to IHNV infection [52]. The Mx gene, which is interferon-stimulated in other vertebrates, is upregulated in fish in response to IHNV infection [251]. Antibodies are readily detectable in fish following infection with IHNV. Several studies have shown low prevalence and titer of anti-IHNV antibodies in rainbow trout by one week postexposure, with significant levels of neutralizing antibodies by 21-28 days postexposure at 15°C [40, 148]. A survey of adult salmonid fishes demonstrated that humoral immune responses in other species are similar to those in rainbow trout [81]. In studies of antibody specificity, the majority of sera have been found to react only with the IHNV P (formerly termed M1) and G proteins by Western blot [148, 175, 223]. Immersion of fish in purified IHNV glycoprotein has demonstrated that G protein is both necessary and sufficient for protection of fish against virus challenge [78, 79]. In a study of plasmid DNA vaccines expressing the IHNV N, P, M, G, and NV proteins, only the vaccine expressing G protected fish against challenge [49]. Cellular immune responses do occur in fish [159] and may also be involved in IHNV resistance, but many of the tools used to study cellular immunity in other vertebrates are in the process of development for fish.

Control strategies

Currently, salmonid aquaculture facilities utilize a number of different management strategies to prevent the spread of IHNV and reduce the incidence of IHN. These include rearing fish in virus-free water, using virus-free broodstock (sexually mature adult fish used for egg production), and treating eggs with iodophor, which has been shown to destroy 99.98% of IHNV on the surface of the egg [3, 99]. In cases where the water source is not inherently virus-free, disinfection of water

supplies by ozonation, ultraviolet light, chlorination/dechlorination, or other means has shown some success in controlling virus spread [268]. These practices, when employed, significantly reduce virus spread and disease. However, outbreaks of IHN still occur [29, 272]. Numerous antiviral compounds have been identified and several have demonstrated efficacy, but costs and/or toxicity are prohibitive to the use of these compounds to treat IHN (reviewed in [29, 268]).

The likelihood of erradicating IHNV by immunizing fish in aquaculture is low since the virus is present in wild fish populations [34, 178, 184, 248]. However, development of a vaccine which elicits long-lasting protective immunity and is cost-effective would be tremendously beneficial to fish farms and resource enhancement hatcheries, as well as in reducing the transmission of virus between hatchery or farmed fish and wild fish. Vaccines for IHNV have been reviewed by several authors [155-157, 268, 269]. It was demonstrated that immersion of steelhead trout in purified IHNV glycoprotein from one virus isolate (RB1) protected against challenge with five isolates [78] representing each of the five IHNV electropherotypes identified by Hsu et al. [121]. This work indicated that the fish immune system reponded with cross-protective immunity against diverse virus isolates and suggested that antigenic variability is not likely to be a limiting factor in developing an effective vaccine. Indeed, a number of IHNV vaccines have been developed which are limited in their application by cost, inconsistent efficacy, lack of efficient vaccine delivery methods, safety concerns, and regulatory concerns. Briefly, vaccines have been developed using five different strategies: attenuated virus [95], killed virus [6, 195], purified IHNV glycoprotein [78], recombinant IHNV glycoprotein produced in several expression systems [41, 97, 134, 196, 201], and DNA vaccines encoding the IHNV glycoprotein gene under the control of a cytomegalovirus promoter [9, 10]. Despite success in generating protective immunity, no licensed, cost-effective, protective vaccine for IHNV is currently commercially available. The only vaccines available are local autogenous vaccines based on killed virus.

INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS DIVERSITY

Phenotypic differences among IHNV isolates from different sources have been observed ever since the identification of the virus in the 1950's [230], including differences in pathogenicity [46, 143, 144, 147, 205, 228] and cell culture plaque size [179]. In a study by Hsu et al. [121], 71 IHNV isolates were divided into five groups based on differences in the electrophoretic mobility of viral proteins in polyacrylamide gels. The three primary electopherotypes; types 1, 2, and 3; were shown to correlate with the geographic origin of the virus isolate rather than the host species the virus was isolated from or the year of isolation. The first suggestion of antigenic differences among virus isolates came from neutralization assays using polyclonal antisera [164]. A monoclonal antibody was later identified by Ristow and Arnzen de Avila [221] which reacted only with electropherotype 2 IHNV isolates. This antibody has been subsequently used for many years by fish health management personnel to differentiate between type 2 and non-type 2 IHNV isolates. Additional studies have shown differences among isolates in reactivity to monoclonal and polyclonal antibodies [150, 193, 222, 267]. LaPatra et al. [150] used two monoclonal and two polyclonal antibodies to examine the heterogeneity of serum neutralization profiles among 106 IHNV isolates from rainbow trout (*Oncorhynchus mykiss*) at four aquaculture facilities in Idaho. Ten different serum neutralization groups were found, with three groups representing the majority (91%) of the isolates.

The genetic diversity of 26 IHNV isolates obtained from fish throughout the endemic range of the virus was investigated by Oshima et al. [203], using T1 RNase fingerprint analysis. The results of this study indicated that virus type correlated with geographic origin, in agreement with the findings of Hsu et al. [121]. Additionally, the results suggested low overall genetic diversity of less than 5% [203]. Phylogenetic analyses of the glycoprotein and non-virion genes for 12 isolates from California, Oregon, Washington, and Idaho, confirmed both the correlation of virus genetic type

with geographic origin and low overall virus diversity, with a maximum of 3.6% divergence in the G gene and a maximum of 4.4% divergence in the NV gene [193].

To examine the genetic diversity and phylogenetic relationship among IHNV isolates throughout the geographic range of the virus at a finer level of resolution, we have used the RNase protection assay and nucleotide sequence analysis in localized and regional studies of IHNV isolates. A detailed analysis of a study site on the Deschutes River in Oregon demonstrated virus evolution and complex virus traffic on a local scale, with virus transmission from wild to hatchery fish and successive replacement of dominant virus types over time occurring through both virus evolution and the introduction of new virus types [11]. Despite the notable differences in RNase protection patterns observed in this study, the overall nucleotide divergence was estimated at 2.6% by nucleotide sequencing of partial glycoprotein gene sequences for selected isolates [11]. Three regional studies in Alaska [76], British Columbia (E.D. Anderson, G. Traxler, and G. Kurath, unpublished data), and Washington coastal watersheds [77], each analyzing more than 40 IHNV isolates over about 20 years, elucidated complex epidemiological relationships and also found less than 2% genetic divergence in each study.

INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS IN IDAHO AQUACULTURE

The state of Idaho accounts for 75% of U.S. foodsize trout production (USDA Economic Research Service, Aquaculture Outlook, March 2001). The majority of this industry is located along a stretch of the mid-Snake River in south-central Idaho associated with an abundance of water flowing from the Eastern Snake River Aquifer through numerous natural springs. For the purposes of simplicity and clarity, this region of intensive *Onchorynchus mykiss* (rainbow trout) aquaculture, encompassing the Twin Falls to Hagerman reach of the mid-Snake River from river mile 610 to river mile 569, is referred to in this dissertation as the "Hagerman Valley," although it

exceeds the technical boundaries of what is commonly known as the Hagerman Valley. Rainbow trout have been farmed continuously in this region since 1928 and the area also has federal and state hatcheries that primarily raise rainbow trout and steelhead trout [32]. Currently there are three state fish hatcheries, one national fish hatchery, and approximately 100 trout farms of various sizes (G. Fornshell, University of Idaho Extension, personal communication). These facilities are served by a variety of water sources which include: first-use spring water piped directly to the facility, first-use spring water channeled via unprotected waterway, and surface water with previous uses (such as irrigation or fish culture). Prior to 1977, fish culture facilities in the valley did not experience significant levels of disease associated with IHNV. However, acute IHN began to appear in the Hagerman Valley with two epidemics at independent sites in 1977 [35]. These outbreaks did not appear to be associated with marked changes in aquaculture practices, leading some to speculate that the manifestation of acute IHN at this time represented an adaptation of virus already present in the Hagerman Valley [35]. Alternatively, the appearance of acute IHN may have resulted from a new introduction of virus from an exogenous source. From 1978 through the early 1980's, IHN appeared progressively at additional facilities throughout the valley [35]. Since that time, the virus has been endemic in the valley, although some facilities do not experience disease and may be free of virus. IHN-associated mortality and deformities in surviving fish result in serious economic losses to the Idaho trout industry.

PURPOSE

Viruses are important components of every ecosystem, yet few are well understood from a genetic diversity perspective. In this dissertation, the infectious hematopoietic necrosis virus (IHNV) was investigated as a model aquatic virus for the study of virus genetic diversity with the aim of understanding how virus populations mutate, compete, adapt, and evolve. IHNV is an important pathogen of salmonid

fishes and as such is a threat to a vital natural resource as well as to rainbow trout aquaculture – an important food source. Investigation of the genetic diversity of IHNV contributes to our understanding of virus epidemiology and virus evolution, which may aid in risk management and disease prevention. From a more basic perspective, development of IHNV as a model system for studying virus fitness and competition contributes to our understanding of virus population biology.

This body of work was aimed at two major objectives which collectively investigated IHNV genetic diversity at multiple levels and attempted to correlate the patterns observed with possible causes. First, we characterized the genetic diversity of IHNV at four levels: within a localized endemic focus in four rainbow trout farms (Chapter 1), within aquaculture facilities throughout an endemic geographic region (Chapter 2), within individual outbreaks (Chapter 3), and within individual fish (Chapter 4). The geographic region investigated in this proposal was a localized region of intensive trout aquaculture in south central Idaho, referred to as the Hagerman Valley. This region is important both as a site of high IHNV prevalence and as a novel environment for the virus. The fish host populations characterized included rainbow trout from 18 different fish farms and several fish species from 3 Idaho state hatcheries. Second, an in-host competition system was developed for determining relative virus fitness (Chapter 5). This system involved water-borne challenge of rainbow trout with mixtures of two IHNV virus genotypes. Initially, this system allowed us to compete two viruses from Hagerman Valley rainbow trout aquaculture and establish quantifiable mixed infections.

CHAPTER 1

IHNV diversity within an endemic focus

INTRODUCTION

Previous investigations of IHNV field isolates showed that numerous strains exist which differ in many protein based characteristics [121, 150, 222, 267]. Oshima et al. [203] differentiated 26 IHNV isolates by T1 ribonuclease fingerprint patterns, and Nichol et al. [193] produced a phylogenetic tree of 12 isolates from the northwestern U.S. based on sequence analysis of the glycoprotein and non-virion genes. These studies demonstrated that IHNV relatedness generally correlates with geographic origin. To characterize the overall and region-specific genetic diversity of IHNV field isolates, our laboratory is studying genetic types of the virus throughout its range in North America. Surveys of Alaskan, British Columbian, and Washington coastal IHNV have documented relatively low genetic diversity (less than 2.0% nucleotide divergence) in these large geographic areas [76, 77] (E.D. Anderson, G.S. Traxler and G. Kurath, unpublished data). In apparent contrast, LaPatra et al. [146, 150] found a high level of antigenic diversity among virus isolates at four aquaculture facilities along a twelve mile stretch of the Snake River in the Hagerman Valley, Idaho. These studies used two monoclonal and two polyclonal antibodies to examine the heterogeneity of serum neutralization profiles among 106 IHNV isolates taken from rainbow trout (*Oncorhynchus mykiss*) at four rainbow trout culture facilities between 1990 and 1992. Ten different serum neutralization groups were found, with three groups representing the majority (91%) of the isolates.

Due to the development of high resolution IHNV strain typing techniques and the previous indication of unusually high antigenic diversity in this region, we re-examined the genetic diversity of IHNV isolates from the same study site (four trout farms in the LaPatra et al. [150] study) over an expanded time period. A total of 84 virus isolates were characterized representing three discrete time periods within a 20 year span (1978-1998) dating back to the emergence of IHNV-associated disease in

this region. Due to the nature of the virus isolates available, slightly different selection criteria were used for virus isolates from each of the three time periods. From the 1990-1992 isolates analyzed serologically by LaPatra et al. [150], 42 were selected to include each of the ten identified antigenic groupings, thus presumably representing the most diverse types of the 106 isolates examined. Isolates dating from 1997-1998 were examined in order to characterize the more recent virus types present at the same four facilities and to assess possible epidemiological trends. Since there were no neutralization group profiles on the numerous isolates available from these years, 32 isolates were selected by taking four isolates from each of the four facilities from both of the years 1997 and 1998. Information on isolation dates and locations within each facility was used to attempt to choose the most diverse set of isolates from this time period. During the years 1978-1988, few isolates of IHNV were saved and hence, only 10 isolates were available. Six of these isolates were recovered from facilities within the Hagerman Valley other than the four trout farms represented in the 1990-1992 and 1997-1998 sample sets.

The RNase protection assay (RPA) was used to provide rapid characterization of genetic differences over a large genomic region. A probe complementary to the entire glycoprotein (G) gene was chosen for the analysis because the glycoprotein forms the antigenic surface of IHNV and thus presumably reflects the serological variation observed by LaPatra et al. [150]. In addition, sequencing of a 303 bp region in the middle of the G gene (referred to as mid-G) was undertaken for selected isolates in order to estimate nucleotide divergence and potentially infer virus lineage based on phylogenetic analyses. The mid-G region was previously shown to be variable in a study of 12 geographically dispersed IHNV strains [193]. However, in a subsequent study of 42 IHNV isolates from throughout Alaska, the mid-G sequences were too low in diversity to generate an informative phylogenetic analysis [76]. Thus, RPA and mid-G sequence analyses were carried out in order to provide a high level of confidence in the characterization of IHNV genetic heterogeneity in this study.

METHODS

Virus isolates and RNA extraction

The majority of virus isolates were provided by S.E. LaPatra and three historical isolates were generously provided by D. Ramsey, Rangen Aquaculture, Hagerman, ID. Isolates were generally recovered from single, acutely infected farm-reared rainbow trout fry. Viruses were isolated by standard methods [149] and stored as frozen aliquots of cell culture supernatant. Seventy-eight out of 84 isolates were sampled from fish at four aquaculture facilities (referred to as Facilities I-IV) along a twelve mile stretch of the Snake River in south-central Idaho (Table 1.1). The remaining six isolates were recovered from fish at other aquaculture facilities within the 96-mile Hagerman Valley. Virus isolates were inoculated onto confluent monolayers of *epithelioma papulosum cyprini* (EPC) cells [89] and total RNA was extracted from infected cells using a guanidinium thiocyanate procedure as previously described [11]. RNA aliquots of 1-3 μg/μL were used as viral target in each RPA reaction.

RNase protection assay

IHNV isolates were characterized by the RNase protection assay (RPA) using a ^{32}P-UTP radiolabelled minus-sense RNA probe (prGF) complementary to the entire glycoprotein gene of IHNV strain RB1 (accession no. U50401). Probe synthesis and the RPA protocol were previously described by Anderson et al. [11]. Briefly, for each reaction 10^5 cpm of GF probe was hybridized to viral target RNA overnight, followed by RNase digestion of mismatches, electrophoresis of cleavage products, and autoradiography. Four controls reactions were included with each set of assays: 1. viral RNA from strain RB1 prepared from infected cells as above, 2. plus-sense RNA synthesized *in vitro* which was complementary to the GF probe, 3. GF probe with no

target RNA and no RNases in the digestion step, 4. GF probe with no target RNA. Each of the 84 isolates were analyzed by RPA with the GF probe a minimum of two times. Banding patterns for all the isolates were compared visually and those isolates with identical banding patterns were considered to have the same haplotype. A haplotype was defined as a specific banding pattern of cleavage fragments. Isolates with at least one band difference were considered to have different haplotypes.

Sequence analyses

A 303 nt region of the IHNV glycoprotein gene, from nucleotide 686 to 988 (numbering as in GenBank accession U50401) (mid-G region), was amplified and sequenced for 49 isolates. Cell culture supernatants containing virus were thawed on ice, diluted 1:20 in water, heated at 95°C for 2 min and cooled on ice. Reverse transcription-polymerase chain reaction (RT-PCR) amplification was performed using 5 µl of the heated 1:20 dilution in a 50 µl reaction using 4.5 units AMV reverse transcriptase (Promega) and 2.5 units Taq DNA polymerase (Promega) with 2.5 mM magnesium chloride, 0.2 mM dNTPs, and 7.5 units RNasin (Promega). Sense primer 517+ (5'-AGA-GAT-CCC-TAC-ACC-AGA-GAC-3') and antisense primer 1209- (5'-GGT-GGT-GTT-GTT-TCC-GTG-CAA-3') were used in the initial RT-PCR at final concentrations of 1 µM. The reaction conditions included reverse transcription at 50°C for 60 min and denaturation at 95°C for 2 min, followed by 25-30 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 60 s, followed by a final extension at 72°C for 7 min. The RT-PCR product was then used as a template for a 50 µl nested PCR reaction with 2 µl of the initial RT-PCR product, sense primer 577+ (5'-TCA-CCC-TGC-CAG-ACT-CAT-TGG-3'), and antisense primer 1059- (5'-ATA-GAT-GGA-GCC-TTT-GTG-CAT-3') under identical PCR reaction conditions and primer concentrations, without the initial 50°C reverse transcription step, reverse transcriptase, or RNasin. Resulting PCR products were purified with the StrataPrep PCR purification kit (Stratagene) and used as a template for synthesis of fluorescently

labelled DNA with the BigDye terminator cycle sequencing kit (Applied Biosystems) according to manufacturer specifications using the second set of primers listed above. The resulting labelled DNA was purified using Centri-Sep columns (Princeton Separations) and analyzed on an ABI-PRISM 310 genetic analyzer (Applied Biosystems).

Sequence files were edited and analyzed using MacVector 6.0 and AssemblyLIGN 1.0.9 applications (Oxford Molecular Group). In order to minimize the effect of random polymerase errors in the RT-PCR, those sequences which contained unique nucleotide substitutions within this set of sequences were confirmed by an independent repeat of RT-PCR sequence analysis from cell culture supernatant. Mid-G nucleotide sequences of selected IHNV isolates from each of the four virus lineages are available in Genbank (accession nos. AF237983-AF237992), and all sequences obtained are available from the author upon request. Synonymous and nonsynonymous mutation analysis was performed using SNAP (http://hiv-web.lanl.gov/SNAP/WEBSNAP/SNAP .html) according to the method of Nei and Gojobori [189]. Phylogenetic analyses were performed with PAUP* v. 4 [246] and Phylip v. 3.5 [87]. The Sacramento River Chinook Virus isolate of IHNV (SRCV, isolated in California in 1966, [193]) was used as an outgroup root. SRCV is the oldest isolate on the tree and it is well established that this isolate, as well as all California isolates analyzed to date, are genetically distinct from the rest of the range of IHNV [76, 121, 193, 203]. The cluster of sequences to the right of a branch with a significant bootstrap value constitute a "clade." The intrapopulational nucleotide diversity (π) was calculated according to the method of Nei [190] utilizing the Kimura two-parameter model [131] as applied in the Arlequin v. 1.1 software package [232].

Virus virulence assays

Virulence determinations [150] were performed by S.E. LaPatra and colleagues on virus isolates selected by the author and G. Kurath. Briefly, duplicate

16-24 fish groups of two sizes of *O. mykiss* (rainbow trout) juveniles (mean weight, 0.8 g and 3.0 g) were challenged by immersion in 10^5 pfu/mL of representative IHNV isolates for one hour with aeration in a volume of water that was 10x the total weight of the fish (g). Mock-infected control groups were exposed to cell culture media only. Experimental groups were held separately in 19 L aquaria at 15 °C and monitored for 21 d. A minimum of 20% of each day's mortalities were examined for virus by plaque assay procedures [141]. Proportions of mortalities (equivalent to cumulative percent mortality) were compared using the chi-square test.

RESULTS

RNase protection assay confirms heterogeneity among study site IHNV isolates

RPAs were performed on 84 IHNV isolates (Table 1.1) using the GF probe, which is complementary to the entire glycoprotein gene. These analyses identified a total of 46 different haplotypes. Isolates from the three time periods were chosen based on slightly different selection criteria so the number of haplotypes found within each time period is not directly comparable. In the 1990-1992 sample set there were 25 haplotypes out of 42 isolates (Figure 1.1a). Isolates from 1978-1988 included 10 haplotypes out of 10 isolates (Figure 1.1b). Isolates from 1997-1998 included 14 haplotypes out of 32 isolates (Figure 1.1c).

Analyses of the banding pattern similarity between haplotypes revealed four haplotype groups based on a qualitative visual assessment of the number of shared bands between patterns (Figure 1.1). Based on these analyses, haplotypes were designated by a capital letter denoting their group (Haplogroups A-D), followed by a number to specify individual haplotypes within the group, e.g. haplotype A1. Several of the haplotypes from 1978-1988 isolates did not share enough bands with any of the other haplotypes to be placed into a group, so they were assigned the letter 'N' denoting their lack of group designation.

Previous characterization of the 1990-1992 isolates [150] classified 106 isolates into 10 antigenic profile types while the RPA classified 42 of these isolates into 25 haplotypes. A comparison of the RPA groups of the 1990-1992 isolates with antigenic types revealed a correlation between RPA haplotype group and antigenic reactivity (data not shown).

RT-PCR sequence and phylogenetic analyses

Sequence analyses of the 303 nt mid-G region were performed on representative isolates from all 46 individual haplotypes along with two additional isolates from haplotype A2 and one additional isolate from haplotype C9. In all, 49 isolates were analyzed, yielding 32 unique sequence types. The sequences of the three isolates of haplotype A2 were all identical to each other, as were sequences of the two isolates from the haplotype C9. The maximum pairwise nucleotide divergence among the isolates was 7.6%, which occurred within the 1997-1998 isolates. The maximum divergence within the 1990-1992 isolates was 5.9% and within the 1978-1988 isolates it was 3.6%. This trend of an increase in diversity over the three time periods examined was also observed in the intrapopulational nucleotide diversities (π) of the Idaho study site isolates (Figure 1.2a). Although these differences in intrapopulational diversity have overlapping error bars, the trend observed is the opposite of what might be explained by the differences in selection criteria for the isolates from the different time periods, suggesting that the increased heterogeneity with time may be real. When the same analysis was applied to compare the overall intrapopulational nucleotide diversity in the Idaho study site with that from the Alaska study [76], a clearly significant difference was found in that the Idaho study site diversity was six-fold greater than that found throughout Alaska (Figure 1.2b).

The predicted translation products of the 32 nucleotide sequences included 24 different amino acid sequences (data not shown). Several hotspots for amino acid substitutions were noted. There were four different amino acids present at

glycoprotein positions 252 and 277 and five different amino acids were present at positions 256 and 270 (numbering as in accession no. U50401). These codons, as well as codons 275, 276, 284, and 285, had an excess of nonsynonymous mutations relative to synonymous mutations, suggesting that these sites may be under particularly strong positive selection. These codons are located relatively near to previously identified linear neutralizing epitopes mapped to amino acids 230-231 and 272-276 [122].

Phylogenetic analyses included the 32 non-identical mid-G sequences obtained in this Idaho IHNV study, along with the mid-G sequences of 12 isolates from the Northwestern U.S. [193] and single representative sequences of isolates from Alaska [76] and British Columbia (E.D. Anderson, G.S. Traxler & G. Kurath, unpublished). The isolates from Idaho formed a strongly supported monophyletic clade that was separate from virus isolates which represent the rest of the North American range of IHNV examined to date (Figure 1.3). Within the Idaho clade, four bootstrap-supported subclades were present, indicating four distinct virus lineages. The grouping of isolates into these four lineages matched the independently determined RPA haplogroups A-D isolate-for-isolate. Therefore the four groups identified by both RPA and sequence phylogeny are referred to hereafter as lineages A-D. The four 1978-1988 isolates not assigned to an RPA haplogroup (RPA-N) did not fit into any of the four lineages. Branch lengths in Figure 1.3 reflect genetic distance and thus it is directly observable that the genetic distance among the study site isolates is greater than that currently found within the rest of the North American range of IHNV. Within three of the four lineages (B, C, and D), there was a temporal trend with a correlation between more recent time of isolation and greater genetic distance from the trunk of the tree (Figure 1.3). The mid-G branching order of isolates sequenced by Nichol et al. [193] shown in Figure 1.3 was equivalent to the phylogenetic branching order generated by Nichol et al. [193] for the entire glycoprotein and non-virion genes. This provided validation for phylogenetic analyses based on sequence from the mid-G region.

Spatial and temporal distribution of RNase protection haplotypes

Analysis of the distribution of RPA haplotypes by date of isolation and aquaculture facility showed that multiple haplotypes were present at individual facilities during the same year (Table 1.2). Detailed analysis of the exact dates of virus isolation showed that in many cases multiple haplotypes were present at the same time or within several weeks at a single facility (data not shown). The four virus lineages identified by RPA grouping and sequence phylogeny were all present at multiple facilities. However, individual haplotypes within these lineages were generally confined to a single facility with the exception of haplotypes C1, A2, and D4, which were observed at multiple facilities. Of these exceptions, haplotype A2 was first observed in 1982, detected again in 1991, and by 1997 was observed at all four facilities, while haplotype D4 also was first observed at all four facilities during 1997. A number of interesting phenomena were observed within facilities. At facility I, only IHNV lineage B was observed during 1990-1992, three lineages (A, B, and D) were present by 1997, and lineage C was not observed. At facility II, lineage C was highly prevalent from 1990-1992, but in 1997-1998 lineages A, B, and D were all present and lineage C was undetected. At facility III, lineage C remained prevalent both during 1990-1992 and 1997-1998. Lineages A and D were first detected at facility III in 1997 while the previously detected lineage B was not found during 1997-1998. Facility IV was found to have all four lineages from both 1990-1992 and 1997-1998. The 1978-1988 time period isolates revealed the early presence of lineages A, B, and D as well as several haplotypes which were not placed in lineages.

Virulence

To determine if a change in the virulence of isolates from the four IHNV lineages had occurred between 1990-1992 and 1997-1998, S.E. LaPatra and colleagues challenged groups of two sizes of naive rainbow trout fry with eight virus

isolates from 1997-1998 as well as four virus isolates from 1990-1992. These isolates were selected by the author and G. Kurath to represent the four virus lineages found in this study during the two most recent time periods examined. Three of the four 1990-1992 isolates had been previously assayed for virulence [150] and these represented the least virulent isolate (FF001-82), the most virulent isolate (FF003-90), and an isolate of intermediate virulence (FF002-90). Virus challenge experiments with these isolates resulted in relative levels of cumulative percent mortality (CPM) similar to that observed by LaPatra et al. [150]. In both sizes of fish, the virulence of 1997-1998 isolates as measured by mean CPM was not statistically different from the virulence of 1990-1992 isolates (Table 1.3). It was noted that the two isolates of lineage A were of lower virulence than all other lineages in both sizes of fish.

DISCUSSION

Previous investigations of IHNV isolates from throughout North America have suggested that IHNV genetic diversity is less than 5% [76, 77, 193, 203]. This level of diversity is much lower than that reported for field isolates of other rhabdoviruses such as VSV and rabies virus [24, 30, 132, 191]. A related rhabdovirus of salmonids, viral hemorrhagic septicemia virus (VHSV), has high genetic diversity between isolates from distant geographic regions, with up to 18% nucleotide diversity between European and North American virus isolates [19]. Within regions however, VHSV has diversity similar to IHNV, with 3.4% divergence within continental Europe and 0.9% divergence within North America [19]. Thus, the observation of up to 7.6% divergence within the highly localized Idaho study site is novel among fish rhabdoviruses characterized to date. This is especially significant for IHNV since it exceeds the previous estimate of divergence for the entire North American range of the virus. We considered the possibility that the high level of diversity observed within the Idaho study site might be due to hypervariability of the G gene. However, RPAs of a limited number of Idaho isolates using probes for the NV gene and the 3'

910 nucleotides of the N gene showed variability of patterns similar to those obtained with the G gene probe (data not shown).

The picture that emerged of IHNV within this rainbow trout culture study site was the presence of four virus lineages of unusually high diversity which co-circulated among the four aquaculture facilities examined and which appeared to diverge over time. This pattern of evolution is quite different from the apparent genetic stasis of the virus in Alaska [76] and in coastal Washington watersheds [77]. By phylogeny and RPA, lineage A exhibited relative genetic stasis while lineages B and C showed clear temporal trends consistent with directional evolution. Lineage D showed an intermediate pattern of evolution in that it exhibited a temporal trend in the phylogeny but was more conserved than lineages B and C by RPA (see Figure 1.1c). Additionally lineage D exhibited a pattern of distribution similar to lineage A in that it was present at only one facility (IV) in 1990-1992 but had spread to all facilities by 1997. Phenomena at individual facilities demonstrate that virus traffic and micro-evolution are highly dynamic and complex at this site. At individual facilities we observed the apparent acquisition of lineages, spread of relatively stable lineages throughout the facilities (lineages A and D), the apparent loss of a lineage (lineage C at facility II), and the persistence of high diversity (Facility IV).

The phylogenetic analysis presented here suggested four IHNV lineages radiating from a single source. This was in agreement with the known history, which suggests that isolates in the Hagerman Valley likely have a more recent common ancestor than the rest of the IHNV North American range [35]. Our data indicated that it is unlikely that there were multiple introductions from different sources. However, the possibility of multiple introductions from a single source cannot be ruled out. Also, this is a partial gene phylogeny, rather than a true virus phylogeny. However, due to the absence of intergenomic recombination in rhabdoviruses, it has been suggested that any genome region should reflect the virus phylogeny, provided that the data are sufficiently robust [187].

The observation that multiple haplotypes and multiple lineages persist within the study site suggests that the haplotypes present are of relatively similar fitness, and may indicate that there is a lack of purifying selection (genetic bottlenecks). There are several major biological features of rainbow trout farm aquaculture that may contribute to the different evolutionary pattern of IHNV seen in this study compared with IHNV from hatchery and wild salmonid populations. Within this trout farm study site fish are held in captivity throughout their lives, while both hatchery-produced and wild salmonids migrate to the ocean for a major portion of their life cycles. In the Idaho study site, photoperiod manipulation of fish is used to modify the annual spawning cycle and provide year-round fish production. This leads to a key difference in disease dynamics; there is no seasonal bottleneck in which susceptible fish are at low density. Instead, the frequent introduction of naive fish at a susceptible life stage into a high density situation provides a year-round supply of susceptible host populations. Thus there is no density restriction on virus transmission [220] and so there is little opportunity for purifying selection to occur. Additionally, the virus potentially may undergo a greater number of rounds of replication per time in this environment in which naive hosts are available year-round. The use of a geographically separate virus-free adult broodstock for egg production eliminates potential virus selection pressure on fish host evolution, so in the farm environment fish do not develop virus resistance in successive generations. Additionally, the compartmentalization of the host population within the study site into separate facilities, and further into individual rearing units, may allow simultaneous amplification of viral variants with no competitive selection of one variant over the other. This partitioning of the virus population into multiple niches may also contribute to a high level of diversity. The restriction of most individual haplotypes to single facilities shows that there is some level of localized micro-evolution underlying the larger phylogeny. However, isolates within lineages do not phylogenetically cluster together by facility. Geographic separation of facilities and physical separation of rearing units within facilities likely allows a limited amount of localized evolution

although clearly co-circulation of virus types occurs. Other unique aspects of aquaculture at the study site should not be overlooked, including the presence of a single host species (rainbow trout) and a constant water temperature of 15°C as opposed to river and ocean temperatures which are typically lower and which vary seasonally, ranging from 0°C-22°C [28]. The constant 15°C temperature may allow fish to generate a stronger antibody response that fish at lower temperatures [127], which could result in stronger immune selection.

Co-circulation of the virus lineages between facilities implies a vector. The water supplies for each of the four rainbow trout farm facilities are independently spring-fed and are considered virus-free. Susceptible eggs and young fry, which are kept in an enclosed and carefully controlled hatch-house and share the same water supply as the outdoor rearing units, very rarely experience IHNV-related mortality. Likewise, the eggs that produce the rainbow trout populations are routinely tested for virus and are considered virus-free. In addition, there is generally no transfer of fish, personnel, or equipment between facilities. Thus, additional vectors may be involved in IHNV traffic. Examples may include resident fish, wind dispersion by aerosol, or birds. Facility IV maintained the highest lineage diversity observed throughout the different time periods examined. The presence of lineages A and D at Facility IV in 1991-1992 and subsequent presence of these lineages at the three remaining facilities in 1997 suggests that facility IV may potentially serve as a reservoir of virus that spreads to the other facilities.

It is important to note that the high and potentially increasing diversity of IHNV in trout farms did not appear to be associated with a change in virulence over the time periods examined. The introduction of naive fish into a high density situation with apparently efficient waterborne transmission between fish may be likely to favor extensive replication in the host with little fitness cost for this replication [84]. There has been some suggestion that virus strains present in Hagerman Valley rainbow trout aquaculture have elevated virulence for rainbow trout compared to other strains of the virus [143, 144, 147]. However, here we show that the virulence of IHNV at four

aquaculture facilities in the Hagerman Valley did not change significantly between 1990-1992 and 1997-1998. It is possible that IHNV virulence reached a threshold level for rainbow trout in the study site environment prior to 1990. While the two isolates of lineage A were of lower virulence than all other isolates in both sizes of fish, an association of lineage with virulence would require further study with a greater number of replicates.

Due to the sequential timing of IHN virus emergence throughout the Hagerman Valley in 1977-1980 and throughout the Columbia River Basin in 1980-1982, it has been suggested that the Hagerman Valley was a source of virus for fish downstream throughout the rest of the Columbia River Basin [101]. Here we have characterized thoroughly the IHNV genetic types from a study site in the Hagerman Valley. However, comparisons with IHNV present in the Columbia River Basin downstream are premature at this time because very few isolates from the Columbia River Basin have been genetically characterized. To thoroughly address the question of relatedness between Hagerman Valley IHNV and IHNV downstream, representative isolates from numerous locations in the Columbia River Basin within the years 1990-1998 will be examined.

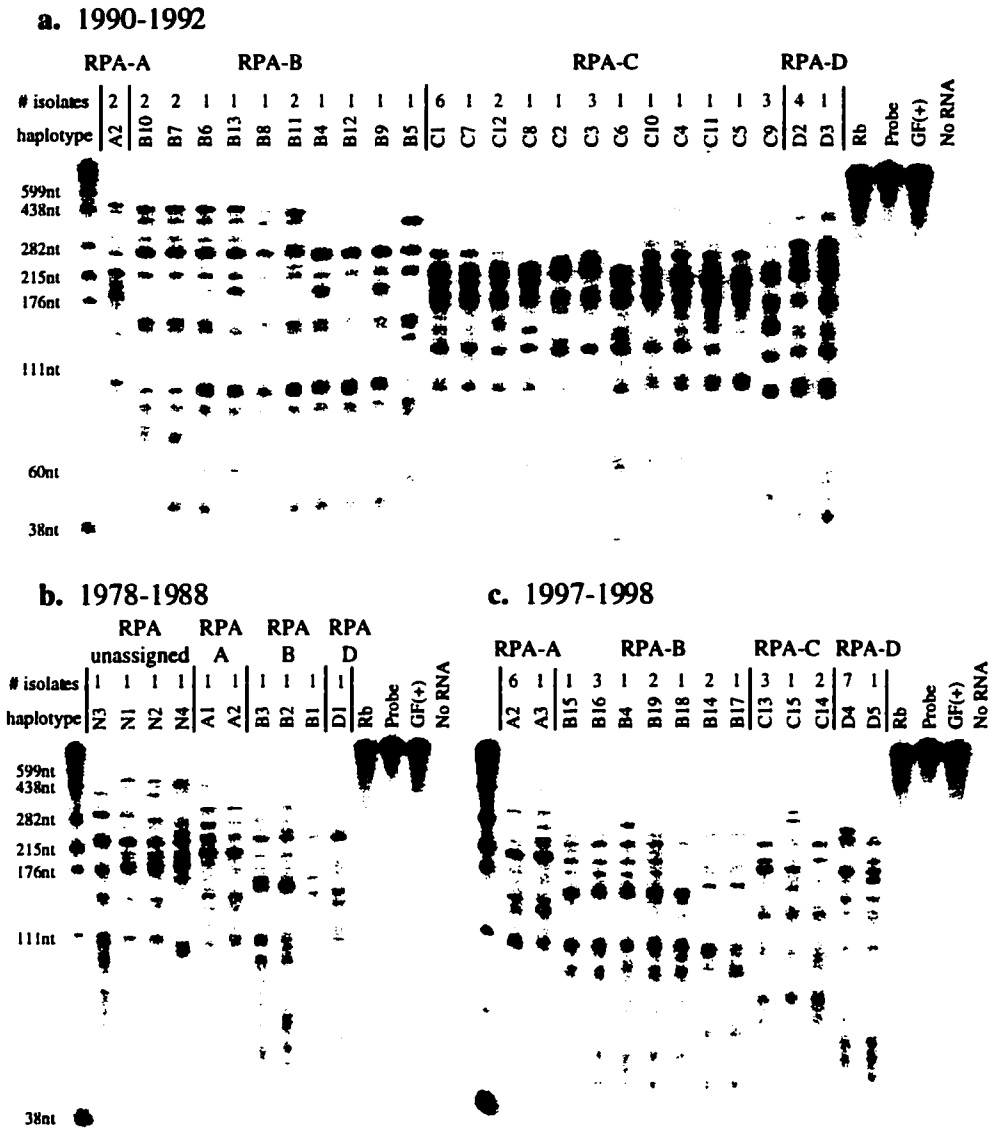


Figure 1.1 RPA haplotypes generated for 84 IHNV isolates from (a) 1990 to 1992, (b) 1978 to 1988, (c) 1997 to 1998 using probe GF, which is complementary to the full-length of the glycoprotein gene (1610 nt). Each lane represents a unique pattern of cleavage fragments, referred to as a haplotype, for a particular virus isolate. The number of isolates which share each haplotype is listed above the haplotype name. Rb, target RNA extracted from cells infected with the RB1 strain, which is complementary to the probe; probe, control with no RNase or target RNA; GF(+), plus-sense RNA transcript synthesized *in vitro*, which is complementary to the probe; no RNA, no target RNA.

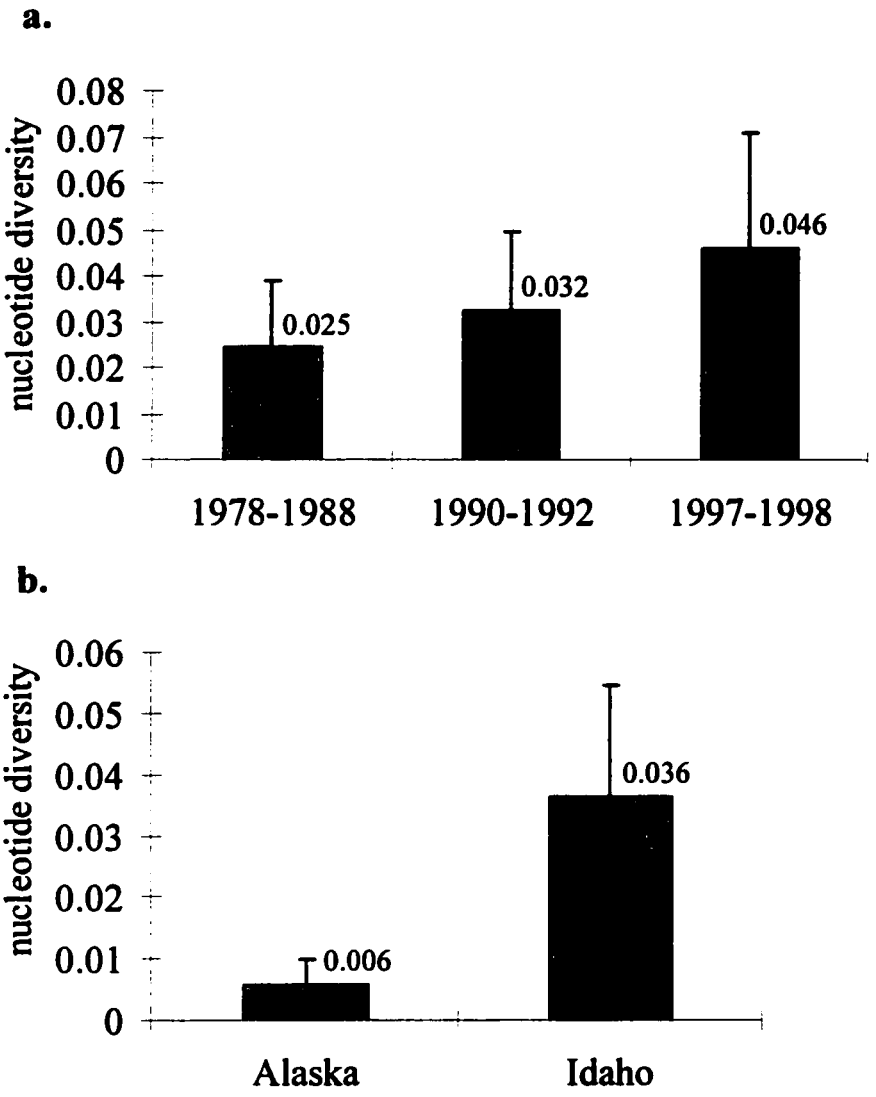


Figure 1.2 Intrapopulational nucleotide diversity (π). (a) π within time periods at the Idaho study site was calculated utilizing all 49 mid-G sequences generated in this study. (b) Overall π within the Idaho study site and Alaska [76] were calculated using populations that included one representative mid-G sequenced isolate from each GF probe RPA (46 in Idaho and 21 in Alaska). It was necessary to randomly select one isolate to represent each Alaskan RPA haplotype so that the selection criteria for the Alaskan population matched that for the Idaho study site population. Intrapopulational nucleotide diversity is defined here as the probability that two randomly chosen homologous nucleotides are different within the population.

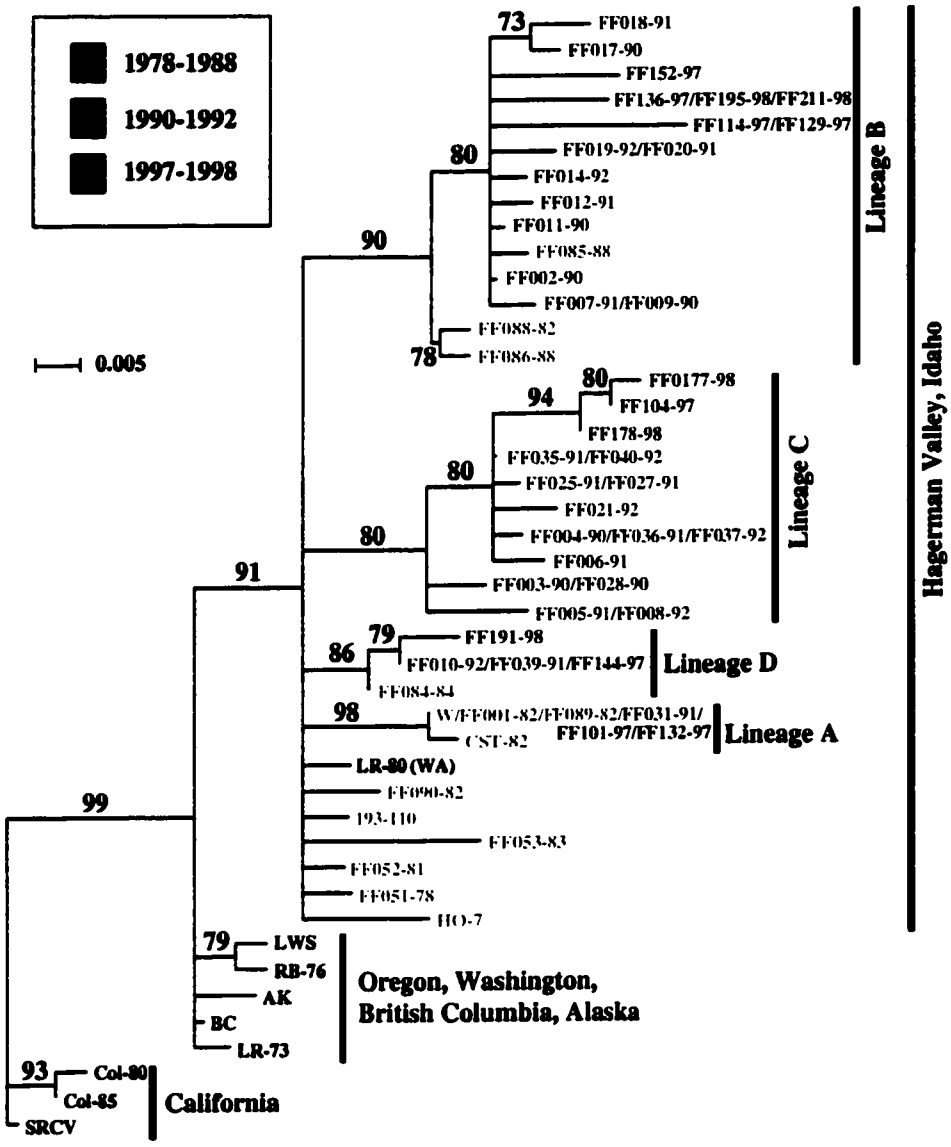


Figure 1.3 Phylogenetic tree constructed from the mid-G sequences (303 nt) of Idaho and selected non-Idaho IHNV isolates. This neighbor-joining tree was generated with PAUP* [246] using SRCV as an outgroup root. The significance of the branching order was assessed by bootstrap resampling of 1000 replicates. Branches with values of > 70% that correspond to a confidence interval of > 95% [113] are indicated on each branch. Branches with values of < 70% were collapsed. H-07, LR-80, 193-110, CST-82, W, LWS, CRS89, RB-76, LR-73, SRCV, Col-80, and Col-85 were sequenced by Nichol et al. [193] (accession nos L40871-L40882). BC represents a major IHNV type found in British Columbia (E.D. Anderson, G.S. Traxler and G. Kurath, unpublished data). AK represents a major IHNV type found in Alaska [76]. All other numbered isolates were sequenced in this study. Isolates from the Hagerman Valley, ID are shown in color to indicate the time period of isolation. Phylogenetic lineages A-D matched RPA haplotypes A-D exactly. Maximum parsimony and neighbor-joining analyses of 1000 bootstrap resampled trees generated from the same sequence data using PHYLIP [87] produced a phylogeny with identical branching order at significant nodes and highly similar bootstrap values throughout (data not shown).

Table 1.1 Isolate list

Isolate name	Case number^	Isolation Date	Location	Tissue†	RPA‡
FF001-82*	039-82	2/12/82	Snake River	PKS	A
FF002-90*	220-90	7/15/90	Crystal Springs	GKS	B
FF003-90*	HV-90	–	Rim View	PKS	C
FF004-90*	382-90	12/14/90	Snake River	Brain	C
FF005-91*	428-91	12/20/91	Snake River	GKSL	C
FF006-91*	426-91	12/19/91	Clear Lake	KSL	C
FF007-91*	201-91	7/3/91	Research	KS	B
FF008-92*	030-92	1/27/92	Snake River	Mucus	C
FF009-90*	267-90	8/24/90	Snake River	Brain	C
FF010-92*	033-92	1/29/92	Box Canyon	Brain	D
FF011-90*	260-90-1	8/20/90	Crystal Springs	Brain	B
FF012-91*	270-91-4	8/27/91	Crystal Springs	Brain	B
FF013-91	270-91-1	8/27/91	Crystal Springs	GKS	B
FF014-92*	096-92	3/19/92	Crystal Springs	GKS	B
FF015-90	267-90-4	8/24/90	Snake River	Brain	B
FF016-91	148-91-12	5/20/91	Snake River	GKS	B
FF017-90*	250-90-2	8/15/90	Clear Lake-1A	KS	B
FF018-91*	132-91-4	5/2/91	Clear Lake	GKS	B
FF019-92*	024-92-13	1/21/92	Box Canyon	KSL	B
FF020-91*	146-91-1	5/16/91	Box Canyon	Brain	B
FF021-92*	108-92-4	3/27/92	Snake River	Mucus	C
FF022-91	163-91-10	6/10/91	Snake River	GKS	C
FF023-91	163-91-7	6/10/91	Snake River	Brain	C
FF024-91	166-91-1	6/11/91	Snake River	GKS	C
FF025-91*	390-91-3	11/21/91	Snake River	KS	C
FF026-92	106-92-3	3/26/92	Snake River	Mucus	C
FF027-91*	403-91-3	12/2/91	Clear Lake	GKS	C
FF028-90*	350-90-2	11/12/90	Box Canyon	Fin	C
FF029-90	350-90-3	11/12/90	Box Canyon-20A	Fin	C
FF030-91	057-91-5	2/18/91	Box Canyon	Brain	C
FF031-91*	292-91-11	9/11/91	Box Canyon	Brain	A
FF032-91	404-91-3	12/2/91	Box Canyon	KS	A
FF033-90	267-90-1	8/24/90	Snake River	KS	C
FF034-92	059-92-11	2/18/92	Snake River	KS	C
FF035-91*	156-91-1	6/3/91	Snake River	GKS	C
FF036-91*	155-91-5	5/31/91	Clear Lake	GKS	C
FF037-92*	034-92-2	1/29/92	Clear Lake	KS	C
FF038-91	302-91-127	9/20/91	Box Canyon	KS	D

continued on the following page

Table 1.1 continued

Isolate name	Case number^	Isolation Date	Location	Tissue†	RPA‡
FF039-91*	302-91-137	9/20/91	Box Canyon	KS	D
FF040-92*	010-92-4	1/7/92	Box Canyon	KSL	C
FF041-92	033-92-22	1/29/92	Box Canyon	KSL	D
FF042-92	125-92-1	4/9/92	Box Canyon-12A	GKS	D
FF043-91	430-91	12/23/91	Snake River	KSL	D
FF051-78*	068-78	3/13/78	–	–	N
FF052-81*	067-81	3/1/81	–	–	N
FF053-83*	005-83	1/5/83	–	–	N
FF084-84*	204-84	10/17/84	Riley Creek	KSP	D
FF085-88*	154-88	5/13/88	Clear Lake	K	B
FF086-88*	132-88	4/21/88	Box Canyon	KSP	B
FF088-82*	181-82	10/17/82	Snake River	K	B
FF089-82*	128-82-5	6/22/82	Springfield	OF	N
FF090-82*	118-82	–	Niagara Springs	–	N
FF101-97*	069-97-14	2/26/97	Clear Lake-28A	KSL	A
FF104-97*	118-97-1	4/15/97	Clear Lake-6A	KSB	C
FF110-97	385-97-5	12/12/97	Clear Lake-12A	KSB	D
FF112-97	276-97-3	9/9/97	Clear Lake-15A	KSB	C
FF114-97*	013-97-1	1/14/97	Box Canyon-14C	KSL	B
FF118-97	094-97-2	3/19/97	Box Canyon-HH	KSB	A
FF122-97	239-97-14	8/14/97	Box Canyon	KSL	D
FF129-97*	395-97-6	12/29/97	Box Canyon-5C	KSB	B
FF132-97*	052-97-2	2/12/97	Crystal-4WHH	KSL	A
FF136-97*	144-97-6	5/7/97	Crystal-3E	KSB	B
FF139-97	272-97-12	9/9/97	Crystal-12EH	KSL	D
FF144-97*	389-97-1	12/16/97	Crystal-5G	KSL	D
FF146-97	010-97-1	1/13/97	Snake-3HH	KSL	A
FF152-97*	155-97-22	5/20/97	Snake	Brain	B
FF156-97	229-97-5	8/1/97	Snake-6HH	KSL	D
FF161-97	351-97-1	11/10/97	Snake-8A	KS	A
FF163-98	004-98-10	1/5/98	Clear Lake-20B	KSB	D
FF166-98	043-98-7	2/12/98	Clear Lake-22C	KSB	C
FF171-98	090-98-5	3/25/98	Clear Lake-24A	KSB	C
FF177-98*	183-98-18	6/23/98	Clear Lake-19A	KSB	C
FF178-98*	100-98-2	3/27/98	Box Canyon-4A	KSB	C
FF186-98	150-98-9	5/11/98	Box Can-20HH	KSB	A
FF190-98	199-98-1	7/14/98	Box-13,14HH	KSB	B

continued on the following page

Table 1.1 continued

Isolate name	Case number^	Isolation Date	Location	Tissue†	RPA‡
FF191-98*	246-98-4	8/21/98	Box Canyon-3D	KSB	D
FF195-98*	030-98-7	1/29/98	Crystal-10F	KSB	B
FF203-98	093-98-2	3/26/98	Crystal-8C	KSB	D
FF205-98	156-98-18	5/21/98	Crystal-7E	KSL	B
FF209-98	231-98-5	8/6/98	Crystal-23EHH	KSB	B
FF211-98*	027-98-3	1/26/98	Snake-1A	KSB	B
FF217-98	109-98-6	4/7/98	Snake-3HH	KSLB	A
FF220-98	119-98-1	4/14/98	Snake-13A	KSB	B
FF224-98	135-98-2	4/27/98	Snake-9A	KSB	B

* Isolates for which sequencing of the mid-G region was performed

^ Numbers designated by the person or group which originally isolated the virus

† B, brain; G, gill; K, kidney; L, liver; OF, ovarian fluid; P, pyloric caeca; S, spleen

‡ RPA haplotype group

Table 1.2 RPA haplotype distribution by year and facility for 84 IHNV isolates

Each haplotype entry represents one or more isolates; the numbers in parentheses indicate the number of isolates with a particular haplotype. Facilities I-IV are located along 12 river miles of the Snake River and are supplied by independent spring-fed water sources.

Year	Facility I	Facility II	Facility III	Facility IV	Other / unknown*
1978	–	–	–	–	N1
1981	–	–	–	–	N2
1982	–	A1	–	–	A2
	–	B1	–	–	–
	–	–	–	–	N3
1983	–	–	–	–	N4
1984	–	–	–	–	D1
1988	–	–	B3	B2	–
1990	B4, B6	B7(2)	B5	–	–
	–	C1, C4	–	C2, C3	C3
1991	–	–	–	A2(2)	–
	B11(2)	B10	B8	B9	B10
	–	C1(3), C6,	C1, C5, C8	C3	–
	–	C7, C9(2)	–	D2(2)	–
1992	B13	–	–	B12	–
	–	C1, C9,	C11	C10	–
	–	C12(2)	–	D2(2), D3	–
		–			
1997	A2	A2(2)	A3	A2	–
	B15	B16	–	B14, B17	–
	–	–	C13(2)	–	–
	D4(2)	D4	D4	D4	–
1998	–	A2	–	A2	–
	B4, B19(2)	B16(2), B18	–	B14	–
	–	–	C13, C14(2)	C15	–
	D4	–	D4	D5	–

*Isolates from within the Hagerman Valley, ID but not necessarily within the 12 mile study site

Table 1.3 Virulence of selected IHNV isolates[a]

Total cumulative percent mortality (CPM) values represent data pooled from duplicate tanks of 16-24 fish (in parentheses). Virus was isolated from 218 of the 231 (94%) dead fish examined from this experiment. Fish in the mock-infected group were handled identically to other groups but were exposed to minimal essential media with no virus as negative controls. No virus was detected in any mock-infected control fish. Virus concentrations detected in tissue homogenates ranged from $10^{3.2}$ pfu/g to more than $10^{7.3}$ pfu/g (mean, $10^{6.5}$ pfu/g).

			0.8 gram Fish		3.0 gram Fish	
Isolate #	Lineage	Year	Total CPM	Replicate CPM	Total CPM	Replicate CPM
FF001-82	A	1982	48.8	(50.0, 47.6)	6.1	(5.9, 6.3)
FF002-90	B	1990	72.5	(75.0, 70.0)	21.9	(37.5, 6.3)
FF003-90	C	1990	82.9	(76.2, 90.0)	44.8	(47.1, 41.7)
FF010-92	D	1992	64.3	(68.2, 60.0)	15.6	(25.0, 6.3)
FF186-98	A	1998	11.4	(0.0, 20.8)	0.0	(0.0, 0.0)
FF190-98	B	1998	55.0	(40.0, 70.0)	9.7	(0.0, 20.0)
FF195-98	B	1998	90.2	(95.0, 85.7)	33.3	(35.3, 31.3)
FF211-98	B	1998	80.0	(85.0, 75.0)	12.5	(25.0, 0.0)
FF177-98	C	1998	81.1	(73.7, 88.9)	30.0	(35.7, 25.0)
FF178-98	C	1998	89.5	(89.5, 89.5)	35.5	(33.3, 37.5)
FF122-97	D	1997	75.0	(60.0, 90.0)	12.5	(18.8, 6.3)
FF191-98	D	1998	50.0	(37.5, 60.0)	6.3	(0.0, 12.5)
mock			0.0	no replicate	4.8	no replicate

[a] data generated by collaborator S.E. LaPatra and colleagues

CHAPTER 2

IHNV diversity within aquaculture facilities throughout the Hagerman Valley

INTRODUCTION

The data presented in Chapter 1 provided a detailed picture of IHNV diversity and evolution within four trout farms, all owned by the same producer. However, the Hagerman Valley region, as we have defined it, includes approximately 100 rainbow trout farms and additional state and federal resource mitigation hatcheries culturing both trout and anadromous salmonids. Thus, we sought to test the hypothesis that high IHNV genetic diversity and co-circulating lineages are characteristic of aquaculture facilities throughout the region. Since nucleotide sequence analyses of the mid-G region were highly informative in the original study (Chapter 1), we chose to use this technique to characterize 73 additional IHNV isolates: 59 isolates from 14 private trout farm facilities (not including the four farms previously studied) and 14 isolates from the three state hatcheries located in the Hagerman Valley (Table 2.1). These viruses were isolated between 1978 and 1999.

METHODS

Virus isolates

IHNV isolates from fish reared at privately-owned rainbow trout farms were obtained from D. Ramsey of Rangen Aquaculture, Hagerman, ID and S.E. LaPatra of Clear Springs Foods Inc., Buhl, ID. These isolates were originally obtained from dead or moribund rainbow trout with clinical IHN, except for ten isolates that were obtained from asymptomatic adult fish as noted in Table 2.1. Virus isolates from fish reared at state hatchery facilities were obtained from S. Landin and K. Johnson of the Eagle Fish Health Laboratory, Idaho Dept. of Fish & Game, Eagle, ID and the stock

collections of the Western Fisheries Research Center, Seattle, WA. These isolates were obtained from several salmonid species (Table 2.1) and were taken from dead or moribund fish with clinical IHN or from asymptomatic adult fish surveyed at spawning. State and private facility isolates were obtained from either individual fish or several pooled fish within a single rearing unit, although specifics of this information were not available for many individual isolates. The majority of viruses were isolated as described by LaPatra [149] and stored as frozen (–75°C) aliquots of cell culture supernatant. Several pre-1986 isolates were stored lyophilized at room temperature. These isolates were re-hydrated with Eagle's minimum essential medium containing 10% fetal calf serum and other components as described by Anderson et al. [11]. Typically, the exact passage history of each isolate was not available, but the great majority were passaged only one to three times in cell culture. The 73 isolates sequenced in this study are labeled "Hg" for Hagerman Valley, followed by a three digit isolate number, and then the last two digits indicate the year of isolation (19XX).

Sequence analyses

A 303 nt region (mid-G) of the IHNV glycoprotein (G) gene, from nt 686 to 988 (numbering as in accession no. U50401), was amplified and sequenced for 73 isolates. RT-PCR amplification and nucleotide sequencing were carried out as described in Chapter 1. Phylogenetic analyses were performed with PAUP* 4.0 [246]. The Sacramento River chinook virus (SRCV) isolate of IHNV was used as an outgroup for display of the tree as described in Chapter 1. The significance of the branching order was assessed by bootstrap resampling of 1000 replicates. Branches with values of >70% correspond to a confidence interval of >95% [113].

The nucleotide diversity (π) within the IHNV population was calculated according to the method of Nei [190] utilizing Kimura's two-parameter model [131] as applied in the Arlequin 1.1 software package [232]. For evolutionary rate calculations the genetic distance of the sequence of each virus isolate from the inferred ancestor of

the Hagerman Valley clade was calculated by summing the horizontal branch lengths of the neighbor-joining tree illustrated in Figure 2.1. The genetic distance for each virus isolate was then plotted against the virus isolation dates. Linear regression of the data points produced a trendline, the slope of which is an estimate of the rate of evolution of the population expressed in mean number of mutations per nucleotide site per year. The significance of the positive slope of this line was assessed using InStat v. 3.01 (GraphPad Software). The number of nonsynonymous mutations per nonsynonymous site (dn) and the number of synonymous mutations per synonymous site (ds) were calculated using the method of Nei and Gojobori [189] with the Jukes-Cantor correction [126] as implemented in the MEGA 2.1 software package [136].

RESULTS

RT-PCR sequence and phylogenetic analyses

When the mid-G regions of 73 IHNV isolates were sequenced and compared, 41 unique sequences were obtained. These sequences were then compared by phylogenetic analyses to each other and to sequences of previously characterized IHNV isolates from the Hagerman Valley (Chapter 1; [193]). Seven sequences representing IHNV isolated from outside the Hagerman Valley were also included for comparison. In the resulting phylogenetic tree, the 73 isolates sequenced in this study are displayed in color (Figure 2.1). All of the isolates from the Hagerman Valley formed a major cluster or clade which was separate from those clades containing isolates from other geographic regions. This Hagerman Valley clade is supported by a high confidence bootstrap value of 84 (Figure 2.1). Within the Hagerman Valley clade, the majority (70%) of the 73 isolates analyzed in this study were members of the previously described subclades A-D (Table 2.2). Two additional subclades, labeled E (five isolates) and F (six isolates), were also identified. Among the newly characterized isolates, eight sequences representing 11 isolates from before 1989 did

not cluster into subclades A-F but instead fell on single branches linked directly to the ancestral node of the clade. These were referred to as "N" isolates, denoting their lack of subclade designation.

The isolates in this study were obtained from fish in two different environments: private rainbow trout farms and Idaho state hatcheries culturing anadromous salmon as well as rainbow trout. State and private facilities maintained similar virus types, with subclades B, C, and E present at both (Table 2.2). In three instances, isolates from state facilities had identical mid-G sequences to isolates from private facilities (note three branch tips of the tree in Figure 2.1 which include both state and private facility isolates).

The majority of isolates in this study were obtained from fish at the fry and juvenile lifestages. However, ten virus isolates from sexually-mature adult rainbow trout at facility #14 (Table 2.1) were also analyzed. The sequences of these isolates grouped with the A, B, and D subclades and were either identical or highly similar in sequence to virus isolates from other state hatchery and production farm facilities throughout the valley (Figure 2.1). These isolates included the only subclade A virus found in this study. However, #14 is the only facility in this study within the same production operation as the original four facilities, where subclade A was prevalent among fish from all lifestages (Chapter 1).

The intrapopulational nucleotide diversity parameter π [190] indicates the average number of nucleotide differences per nucleotide site between all pairs of sequences in a defined population, thus providing a measure of the overall genetic heterogeneity. In our previous four farm study, π was 0.036 (Chapter 1). With the addition of the 73 isolates in this study, the π of IHNV isolates from throughout the Hagerman Valley was 0.034. This is five-fold higher than the nucleotide diversity for IHNV isolates from throughout Alaska (π 0.006, [76]) and coastal Washington (π 0.007, [77]).

In the phylogenetic analysis there was a trend toward greater divergence with time, i.e. older isolates in general tend to be positioned closer to the ancestral root of

the Hagerman Valley clade (Figure 2.1), while newer isolates tend to be located toward the tips of the tree (Figure 2.1). We estimated a rate of evolution for the mid-G sequence region of all characterized IHNV in the Hagerman Valley by plotting the genetic distance (number of mutations per nucleotide site) from the phylogenetically inferred ancestor of the Hagerman Valley clade versus the year of virus isolation for each isolate (Figure 2.3). The rate observed was 1.2×10^{-3} mutations per nucleotide site per year. While it may often be inappropriate to assume that RNA viruses evolve in a clock-like manner (reviewed in [62]), this rate is in the middle of the range of evolutionary rates estimated for many other RNA viruses [62, 125]. The positive slope in Figure 2.3 is significantly different from zero ($p<0.0001$) and serves to illustrate a general association between year of isolation and the number of mutations which have accumulated in each sequence ($r^2=0.50$).

The presence of an excess of nonsynonmous mutations (those which result in a predicted amino-acid change) compared to the number of synonymous mutations (those which are silent) in a gene or region of a gene has been interpreted as evidence for positive selection acting on that region [123, 189, 235]. In order to examine the extent of positive selection acting on the mid-G region of IHNV in the Hagerman Valley, we compared the number of nonsynonymous mutations per nonsynonymous site (dn) to the number of synonymous mutations per synonymous site (ds) for all characterized IHNV in the Hagerman Valley. We found a dn/ds ratio of 0.43, indicating an overall excess of synonymous mutations. However, alignment of the amino acid sequences of all isolates in this study showed several hotspots for nonsynonymous mutation. In particular, glycoprotein amino acids 252, 256 and 270 each had an excess of nonsynonymous mutations.

Geographic distribution of IHNV subclades

The geographic distribution of IHNV subclades throughout the Hagerman Valley region of the mid-Snake River is shown diagrammatically in Figure 2.2. This

figure includes all 73 isolates analyzed in this study as well as the 84 isolates analyzed in Chapter 1. Individual facilities were found to maintain multiple subclades. In fact, at each site for which more than one isolate was analyzed, more than one subclade was found. Among the 21 facilities in Figure 2.2, isolates representing the most prevalent subclades, B and C, were obtained from 13 and 16 different facilities, respectively, and thus were the most widely distributed (Figure 2.2, Table 2.2). Subclade D was also found at 7 facilities throughout the valley (Figure 2.2). The prevalences of subclades B, C, and D among the 17 facilities examined in this study were similar to those found in the original four trout farms (Table 2.2). Examination of virus from private farms and state hatcheries showed that both of these types of facilities harbor similar virus subclades (Figure 2.2, Table 2.2). The complex distribution of subclades illustrated in Figure 2.2 does not support a correlation between virus subclade and geographic location within the valley. Analysis of individual sequences within subclades also does not support a correlation between virus genetic type and geographic location. For example, the most prevalent single sequence identified in this study (underlined in Figure 2.2) was found in 11 isolates at eight different sites throughout the valley.

Temporal distribution of IHNV subclades

The distribution of isolation dates from the 73 isolates examined in this study (Table 2.1) and the 84 isolates analyzed in Chapter 1 showed two clear gaps in the data, with no isolates in 1986-1987 or in 1995. Therefore, we chose to divide the isolates into three temporal groups (1978-1985, 1988-1994, and 1996-1999) for the purpose of examining changes in the IHNV subclade distribution over time. Isolates from the early emergence period of the virus (1978-1985) are marked in Figure 2.1 with an asterisk. The two earliest isolates, FF051-78 and Hg201-78, differed by 5 nucleotides (1.7%). The 24 isolates taken during the early emergence of the virus within the valley included a number of variant genotypes represented by 16 different

sequences. Collectively, these sequences fit within the major Hagerman Valley clade of virus. However, nearly all (20/24) of these isolates either did not group with any subclade (listed as "N") or fell within the F subclade (Table 2.2). Only one (1/24) of the early isolates grouped with subclade B, and none grouped with subclade C, despite the fact that these two subclades represented the majority of the virus population present in the valley at later time points (Table 2.2). Lineage F was restricted to isolates from three facilities in 1983-1985 (Figure 2.4) and thus it may have subsequently disappeared from the valley, possibly being outcompeted by other lineages. Similarly, lineage E was found only at three sites from 1990-1991 (Figure 2.4) and may have subsequently disappeared from the valley.

Examination of the middle time period (1988-1994) showed that a general shift occurred from the previous period, with subclade C representing 53.7% of the isolates examined, subclade B representing 26.8%, and subclades A, D, and E also present (Table 2.2). Comparison of the middle time period with the most recent period (1996-1999) demonstrated another shift, with subclade C declining in prevalence from 53.7% to 17.6%, accompanied by increases in the prevalence of subclades A, B, and D (Table 2.2). This decline in the prevalence of subclade C was observed independently in both the original 84 isolates (Chapter 1) and the 73 isolates examined in this study (data not shown). Subclade B was the most prevalent in the most recent time period. The shifts observed indicated that the virus population in the Hagerman Valley changes with time.

DISCUSSION

The data presented here clearly establish that aquaculture facilities throughout the Hagerman Valley maintain multiple co-circulating lineages of IHNV. These lineages are shared among private trout farms and state hatchery facilities alike, regardless of rainbow trout life stage. Within the Hagerman Valley over a period of less than thirty years, the virus has evolved to a greater level of diversity than that

found among isolates from much larger regions of the virus' geographic range. The presence of a general trend toward divergence over time suggests that the virus is actively evolving in the valley rather than exhibiting the relative genetic stasis observed in Alaska and the Washington state coastal region [76, 77].

We hypothesize that the generation of this diversity was facilitated by conditions specific to Hagerman Valley aquaculture. Year-round trout production with the constant introduction of immunologically naïve fish may allow more rounds of viral replication per year than in anadromous hatchery or wild fish, where low-level chronic or carrier infection may be more common. In addition, partitioning of fish populations into numerous facilities, each with numerous rearing units may result in a lack of competition and purifying selection, allowing multiple variants to be simultaneously maintained. Rapid evolution of IHNV may also have been initiated by the process of virus adaptation to the unique Hagerman Valley environment which includes the rainbow trout host and constant 15°C water temperature. The constant 15°C temperature might potentially allow fish to generate a stronger antibody response than fish at lower temperatures [127], which could result in stronger immune selection. The finding of several hotspots for nonsynonymous mutation in the mid-G region suggests that host immune selection at several codons might also play a role in the generation of diversity, although the overall excess of synonymous mutations is evidence that immune selection is likely not the dominant selective force for this genomic region.

The presence of identical, or highly similar, virus types at different facilities throughout the valley indicates that virus traffic is very likely occurring between facilities within the valley at a rate which is sufficient to widely distribute the major virus lineages. The mechanism of this traffic is unknown. Some of this traffic may be due to the movement of fish between facilities owned by the same producer. However, this does not explain why state and private facilities or private facilities owned by different producers have the same virus types. At many facilities, spring-water is transported directly to the facility in enclosed pipes, so it is unlikely that water

source is a mechanism of virus spread between facilities in these cases. However, in the case of facilities which receive water from unenclosed sources such as open channels, resident fish above the facility or other unknown hosts or vectors may play a role as sources of virus. In fact, indoor facilities for rearing eggs and very young fry have typically experienced high levels of IHN disease if fed by an open water source (G. Fornshell, University of Idaho Extension, personal communication). This is strong anecdotal evidence that IHNV is present in open water sources. However, most of the facilities analyzed in this study receive water from sources that are independent of each other, suggesting additional vectors. Birds have been implicated as a potential vector of fish pathogenic viruses [163, 208] and the application of bird-netting around facilities in the Hagerman Valley appears to have reduced the incidence of IHN disease in some facilities (D. Ramsey, Rangen Aquaculture, personal communication). Aerosols may also potentially serve to transfer fish pathogens [274], especially in the Hagerman Valley which experiences strong winds and a significant amount of heavy mist in the winter because the spring water is 15°C while air temperatures can commonly be –4°C (S.E. LaPatra, Clear Springs Foods, personal communication). In general, personnel and equipment are not shared between fish producers, and farms practice hygiene for disease control. However, even rare or occasional events such as fish transfers or common use of equipment could serve to transfer IHN virus.

It is particularly significant that private trout farm and state hatchery facilities have the same virus types, since these facilities have very different purposes and fish culture practices. Private trout farms engage in high intensity (fast growth rates and relatively high fish densities), year-round production of rainbow trout for human consumption, with fish being contained within the farm for their lifespan (approximately one year). State resource mitigation hatcheries receive fish stocks (primarily rainbow trout and steelhead) as eggs or juveniles from a variety of sources around the state, raise the fish at facilities within the Hagerman Valley for limited periods and at relatively lower density, and then outplant these fish to regions around the state. Thus, fish raised within the Hagerman Valley might potentially serve as

vectors of IHNV to other areas outside the Hagerman Valley. However, it is important to acknowledge that fish management personnel monitor fish health and consider the potential for the spread of pathogens when choosing to move fish from one location to another. It is also possible that trout farms could potentially spread virus outside of the valley in farm effluent, although approximately 99% of virus becomes inactivated in Snake River water within 24 hours [152] and the concentrations of virus exiting facilities appear to be less than 7 pfu/mL (S.E. LaPatra, unpublished data). The genetic types of IHNV in fish throughout the Columbia River Basin including the rest of Idaho are currently being investigated in our laboratory.

It is not known whether the emergence of IHNV in the Hagerman Valley was the result of adaptation of the virus to the unique environment of the Hagerman Valley, an introduction of virus from an exogenous source, or a change in the host or environment. Relative to the IHNV that was endemic in Alaska and Columbia River basin sockeye salmon by 1974 [4, 100], the emergence of IHNV in the Hagerman Valley would certainly have involved both adaptation to the rainbow trout host and to replication at the higher temperature of 15°C. Interestingly, the two earliest isolates in this study (both 1978) had five nucleotide differences in the mid-G region. This suggests that either multiple related viruses were responsible for the early outbreaks or that very rapid evolution from a single progenitor occurred under new selection pressures. The early isolates of undefined subclade (classified as 'N') may represent IHNV lineages that were not successfully maintained in the valley or were outcompeted by other lineages. Alternatively, some of these isolates may represent ancestors of the observed subclades A-F. Regardless of the early origin of IHNV in the Hagerman Valley, rainbow trout aquaculture appears to have allowed the virus to evolve multiple lineages which co-circulate in a complex manner throughout both state hatchery and trout farm facilities.

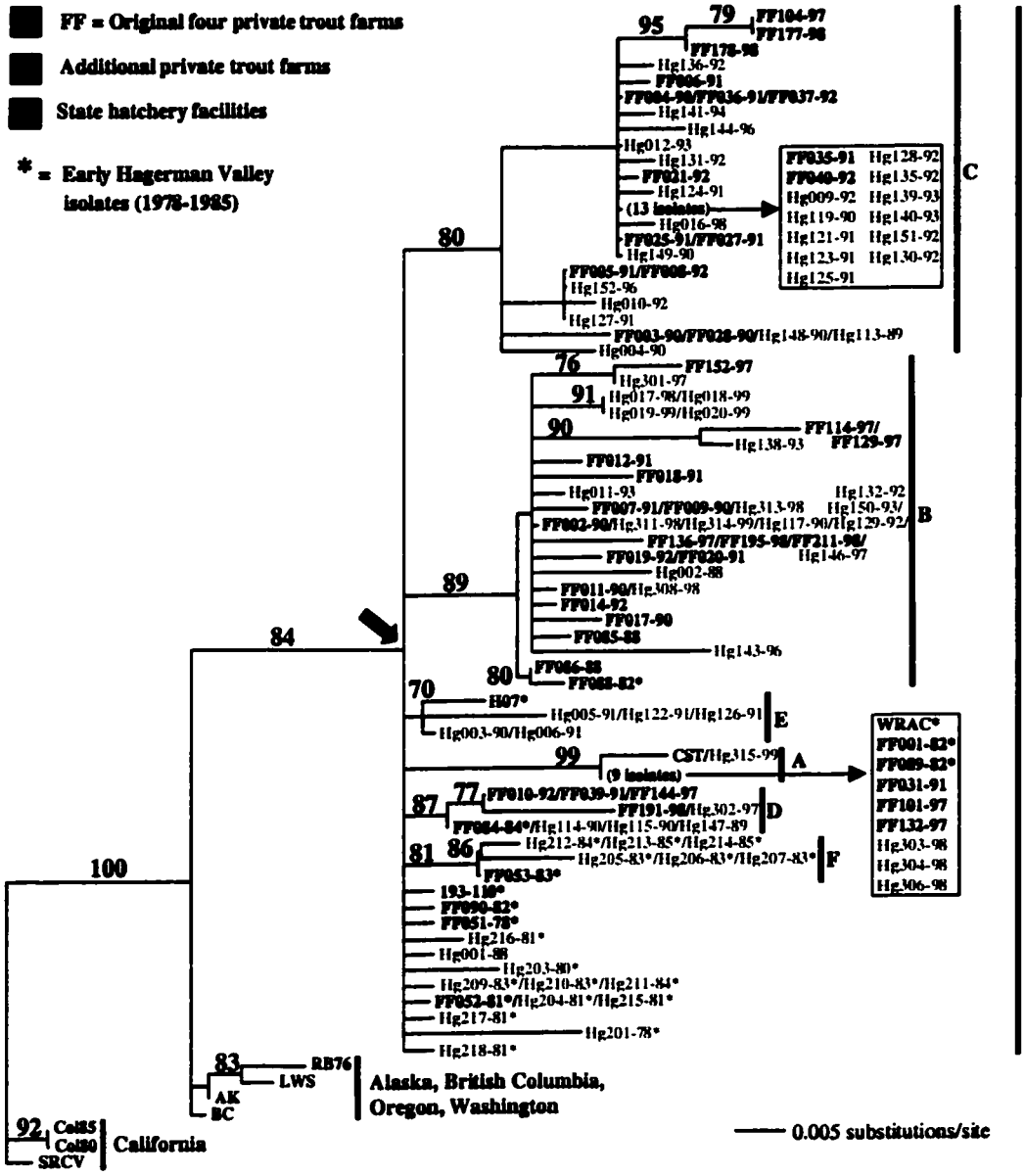


Figure 2.1 Phylogenetic tree of IHNV isolates within the Hagerman Valley, ID based on mid-G sequences (303 nt). The 73 isolates analyzed in this study are shown in color: blue for state hatchery facility isolates and red for private farm isolates. The 49 isolates from four private trout farms sequenced in Chapter 1 are shown in black, starting with an "FF" designation. Isolates from the years 1978-1985 are indicated with an asterisk. This neighbor-joining tree was generated with PAUP* [246] using SRCV as an outgroup for display of the tree. The significance of the branching order was assessed by bootstrap resampling of 1000 replicates. Branches with values of >70% correspond to a confidence interval of >95% [113]. Branches with values <60% were collapsed and only bootstrap values of 70 or above are shown. Sequences of isolates labeled H07, 193-110, CST, RB76, LWS, SRCV, Col80, and Col85 were from Nichol et al. [193]. BC represents a major IHNV type found in British Columbia (E.D. Anderson, G.S. Traxler, and G. Kurath, unpublished data). AK represents a major IHNV type found in Alaska by Emmenegger et al. [76]. Maximum parsimony analysis of 1000 bootstrap resampled trees generated from the same sequence data produced a phylogeny with identical branching order at significant nodes and highly similar bootstrap values throughout (data not shown). The ancestral node of the Hagerman Valley clade is indicated with a green arrow.

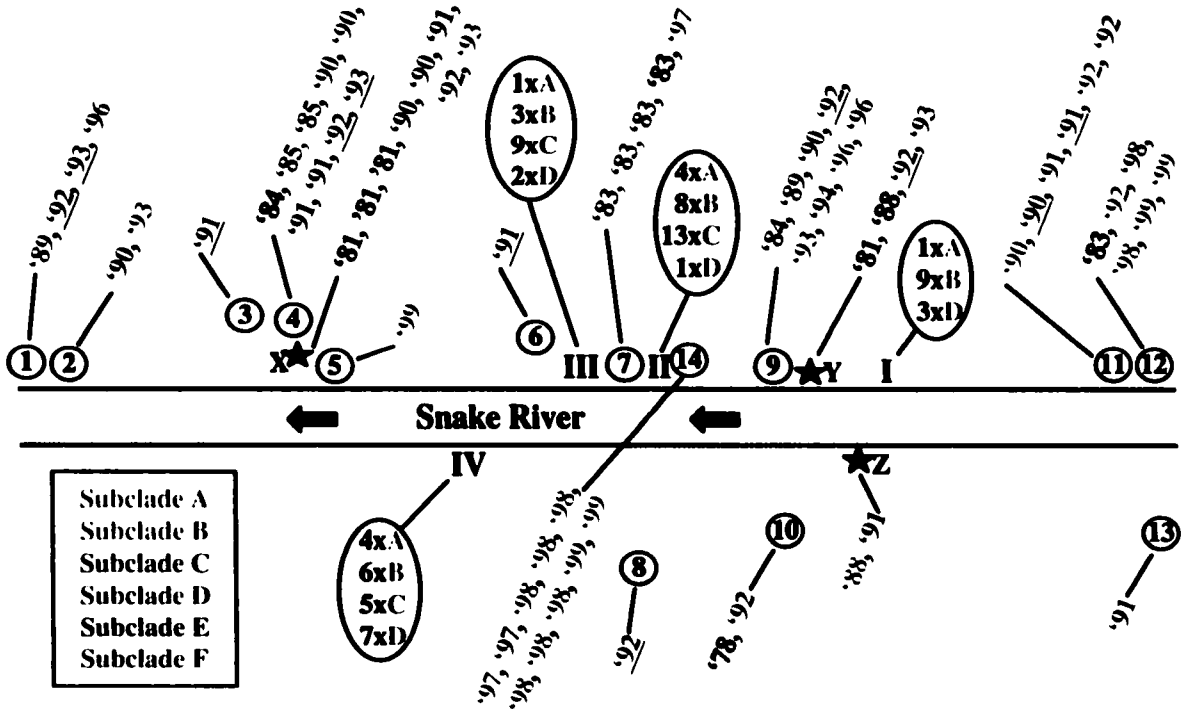


Figure 2.2 Distribution of IHNV subclades by facility and year of isolation. This diagram is intended to show the relative location of all facilities in this study along the Twin Falls to Hagerman reach of the Snake River from mile 610 to mile 569 for the purpose of simple epidemiological analyses. Distances are not precisely to scale and the Snake River is shown as straight and flowing from right to left for ease of illustration and interpretation. Numbers within circles indicate private trout farms #1-14. Roman numerals I-IV indicate the four private trout farms previously described (Chapter 1). Stars labelled X, Y, and Z indicate the three Idaho state hatcheries. Each year shown (e.g. '91) indicates the phylogenetic subclade (by color) and year of isolation for one IHNV isolate, with lines to its site of isolation. The number of isolates belonging to each subclade found in the original four trout farms described in Chapter 1 are shown in ovals. The underlined isolates indicate those which share the most prevalent identical sequence (see Figure 2.1, subclade C).

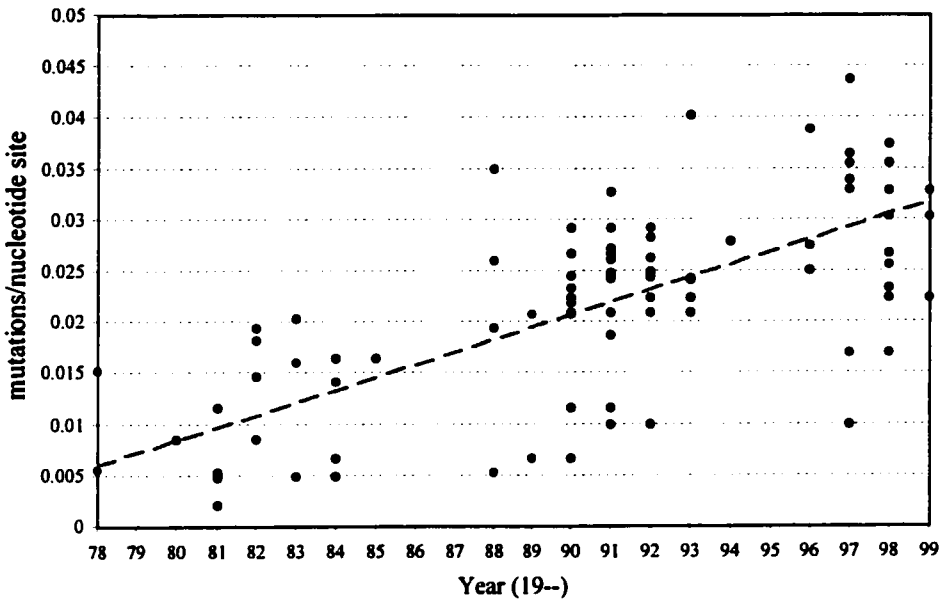


Figure 2.3 Rate of evolution of IHNV in the Hagerman Valley. For each virus isolate the year of virus isolation was plotted against the genetic distance (number of mutations per nucleotide site) from the inferred ancestor of the Hagerman Valley clade (green arrow in Figure 2.1).

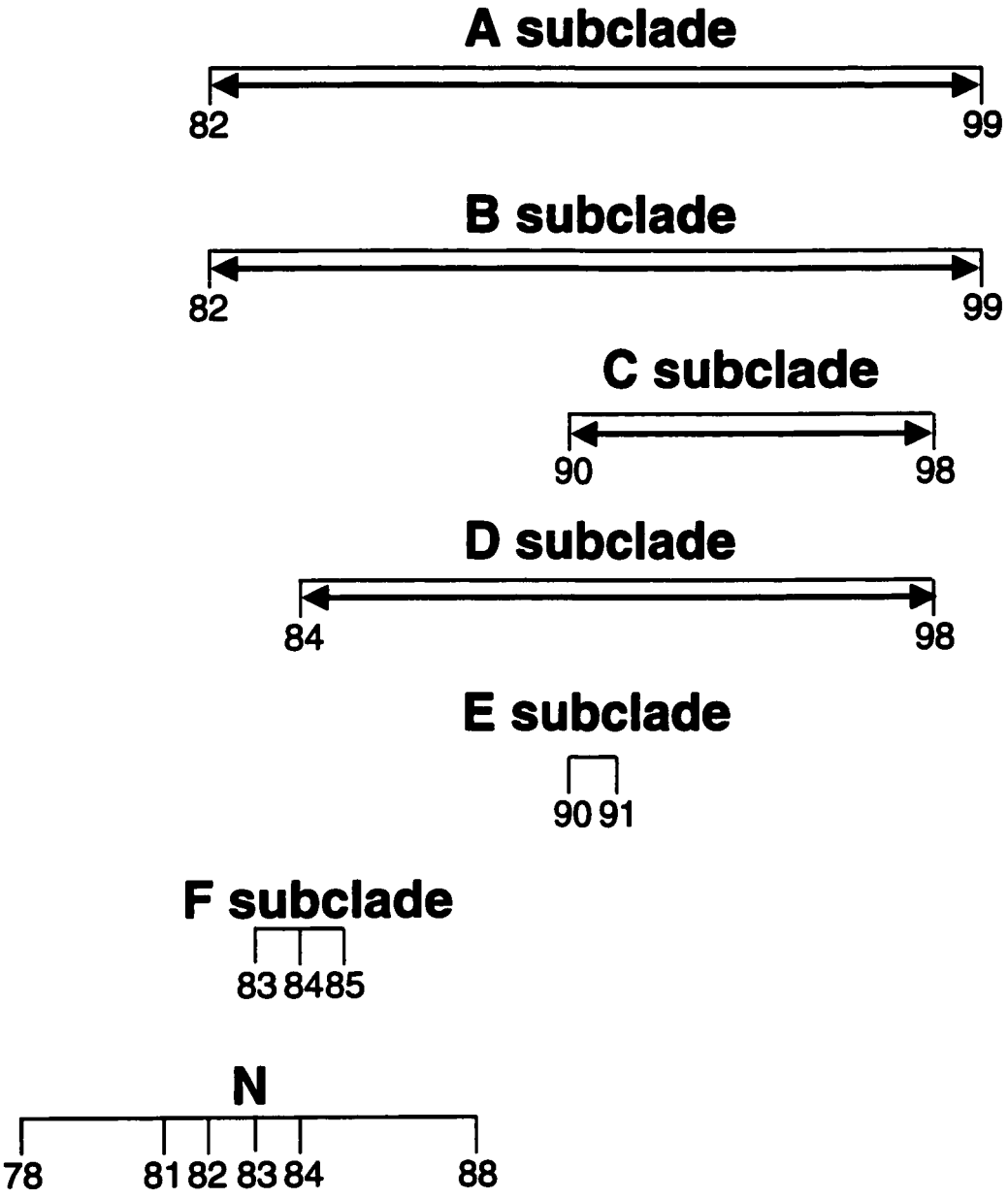


Figure 2.4 Timeline of the IHNV subclades found within the Hagerman Valley. This figure indicates the years in which viruses of each subclade were isolated at sites within the Hagerman Valley. "N" indicates viruses of unclassified subclade.

Table 2.1 IHNV isolates and their sites of isolation, dates of isolation, and subclade designations

Isolate designation[a]	Case #[b]	Site[c]	Isolation Date	Subclade[d]
Hg001-88 (St)	88-90	Y	7/13/88	N
Hg002-88 (St)	89-42	Z	3/22/88	B
Hg003-90	90-17	X	1/16/90	E
Hg004-90 (R/St)	90-233	X	9/5/90	C
Hg005-91 (R/Ct)	91-200	X	7/1/91	E
Hg006-91 (St)	91-203	Z	7/10/91	E
Hg009-92 (St)	92-285	Y	7/23/92	C
Hg010-92	92-293	X	7/28/92	C
Hg011-93 (Coho)	93-122	X	4/9/93	B
Hg012-93 (St)	93-292	Y	7/21/93	C
Hg016-98	278-98-5	12	9/29/98	C
Hg017-98	297-98-6	12	10/15/98	B
Hg018-99	007-99-1	12	1/8/99	B
Hg019-99	338-99-1	12	12/6/99	B
Hg020-99	048-99-6	5	2/17/99	B
Hg113-89	075-FY90	9	12/11/89	C
Hg114-90	097-FY90	4	1/11/90	D
Hg115-90	112-FY90	4	1/31/90	D
Hg117-90	216-FY90	11	6/11/90	B
Hg119-90	088-FY91	11	11/27/90	C
Hg121-91	146-FY91	6	2/12/91	C
Hg122-91	148-FY91	4	2/13/91	E
Hg123-91	154-FY91	3	2/25/91	C
Hg124-91	157-FY91	11	3/12/91	C
Hg125-91	230-FY91	11	5/21/91	C
Hg126-91	250-FY91	4	6/13/91	E
Hg127-91	256-FY91	13	6/24/91	C
Hg128-92	141-FY92	9	3/10/92	C
Hg129-92	033-FY93	12	9/15/92	B
Hg130-92	044-FY93	8	10/12/92	C
Hg131-92	046-FY93	10	10/12/92	C
Hg132-92	048-FY93	11	10/15/92	B
Hg135-92	058-FY93	1	12/2/92	C
Hg136-92	059-FY93	11	12/2/92	C
Hg138-93	111-FY93	9	3/16/93	B
Hg139-93	047-FY94	1	10/27/93	C
Hg140-93	062-FY94	4	12/20/93	C

continued on the following page

Table 2.1 continued

Isolate designation[a]	Case #[b]	Site[c]	Isolation Date	Subclade[d]
Hg141-94	142-FY94	9	5/26/94	C
Hg143-96	163-FY96	9	5/13/96	B
Hg144-96	015-FY97	1	9/16/96	C
Hg146-97	073-FY97	7	4/7/97	B
Hg147-89	101-FY89	1	4/11/89	D
Hg148-90	210-FY90	9	6/6/90	C
Hg149-90	049-FY91	2	10/2/90	C
Hg150-93	025-FY94	2	8/16/93	B
Hg151-92	061-FY93	4	12/3/92	C
Hg152-96	005-FY97	9	7/15/96	C
Hg201-78	237-78	10	6/?/78	N
Hg203-80	011-80	NA	1/?/80	N
Hg204-81	054-81	NA	3/?/81	N
Hg205-83	003-83	7	1/5/83	F
Hg206-83	006-83	7	1/5/83	F
Hg207-83	007-83	7	1/5/83	F
Hg209-83	009-83	7	1/5/83	N
Hg210-83	018-83	12	1/13/83	N
Hg211-84	080-84	4	1/4/84	N
Hg212-84	134-84	9	5/21/84	F
Hg213-85	H85-09	4	2/1/85	F
Hg214-85	H85-20	4	3/11/85	F
Hg215-81 (St)	NR[e]	Y	8/?/81	N
Hg216-81	NR[e]	X	12/27/81	N
Hg217-81 (Ct)	NR[e]	X	7/14/81	N
Hg218-81	NR[e]	X	7/14/81	N
Hg301-97 (Adult)	061-97-2	14	2/18/97	B
Hg302-97 (Adult)	376-97-5	14	12/2/97	D
Hg303-98 (Adult)	079-98-1	14	3/16/98	A
Hg304-98 (Adult)	088-98-31/32	14	3/24/98	A
Hg306-98 (Adult)	173-98-3	14	6/10/98	A
Hg308-98 (Adult)	181-98-23	14	6/23/98	B
Hg311-98 (Adult)	277-98-37	14	9/29/98	B
Hg313-98 (Adult)	330-98-1	14	11/19/98	B
Hg314-99 (Adult)	251-99-2	14	9/22/99	B
Hg315-99 (Adult)	298-99-2	14	11/4/99	A

[a] Each isolate originated from a rainbow trout host unless otherwise indicated. (St) = steelhead trout, (Ct) = cutthroat trout, (Coho) = coho salmon, (R/St) = rainbow trout x steelhead hybrid, (R/Ct) = rainbow trout x cutthroat trout hybrid. The (Adult) designation indicates a mature rainbow trout adult. All other fish are fry, juvenile, or unknown lifestage.

[b] Numbers designated by the person or group which originally performed the virus isolation

[c] 1-14 indicate private rainbow trout farm facilities #1-14. X, Y, and Z indicate Idaho state hatchery facilities. NA indicates that the site of isolation is not known

[d] Subclade designation was determined by phylogenetic analyses as described in the Methods

[e] Not recorded. Isolate was logged by date.

Table 2.2 IHNV subclade characteristics

Subclade	Number of Isolates[a]	Number of Facilities[b]	Private Facility	State Facility	Mature Adult	Number of isolates per year-group[c]		
						1978-1985	1988-1994	1996-1999
A	4 (11)	1 (4)	+	–	+	2 (8.3%)	2 (2.4%)	11 (21.6%)
B	18 (27)	9 (4)	+	+	+	1 (4.2%)	22 (26.8%)	22 (43.1%)
C	25 (28)	13 (3)	+	+	–	0	44 (53.7%)	9 (17.6%)
D	4 (14)	3 (4)	+	–	+	1 (4.2%)	8 (9.8%)	9 (17.6%)
E	5 (0)	3 (0)	+	+	–	0	5 (6.1%)	0
F	6 (1)	3 (0)	+	–	–	6 (25.0%)	0	0
N[d]	11 (3)	6 (0)	+	+	–	14 (58.3%)	1 (1.2%)	0
Total	73 (84)	17 (4)				24	82	51

[a] This number indicates the total number of isolates which group into each subclade. Numbers in parentheses represent previous data from the original four facilities examined in Chapter 1, and are included to facilitate comparison.
[b] This number indicates the number of different facilities which were found to have isolates matching each subclade. Numbers in parentheses refer to the original four facilities examined (Chapter 1).
[c] This number indicates the total number of isolates from each subclade observed during the given time periods. This data includes all 73 isolates from this study and all 84 isolates from the original four facilities (Chapter 1).
[d] isolates which do not group with subclades A-F

CHAPTER 3

IHNV genetic diversity within individual outbreaks

INTRODUCTION

In Chapters 1 and 2, the genetic heterogeneity within rainbow trout farm and state hatchery facilities in the Hagerman Valley was characterized by comparing a total of 157 IHNV isolates. Each of these isolates was obtained by sampling one or several fish within a single rearing unit experiencing an outbreak of IHN. Because these isolates were nearly all obtained from separate outbreaks, the data essentially represent inter-outbreak genetic diversity. This data demonstrated that multiple genetically heterogeneous variants co-circulated within facilities. In order to examine the genetic diversity of IHNV in rainbow trout aquaculture at a finer level of resolution, we chose to analyze intra-outbreak diversity by obtaining virus isolates from multiple individual fish within the outbreak. In parallel studies, the genetic heterogeneity of IHNV from multiple individual fish within outbreaks in hatchery and wild host populations has also been characterized by other researchers in our laboratory (G. Kurath, unpublished data). Thus this study, in conjunction with data from other researchers in our laboratory, allows comparison of the within-outbreak genetic diversity found in Hagerman Valley rainbow trout farms to that found in other populations. We hypothesize that the diversity within individual IHNV outbreaks in Hagerman Valley rainbow trout aquaculture is greater than that found in other populations. This may be the basis for the significantly higher inter-outbreak diversity described in Chapters 1 and 2.

We sought to characterize virus genetic diversity within two outbreaks that occurred simultaneously in separate sections of rainbow trout farm facility IV (see Chapters 1 and 2) in 1998. S.E. LaPatra and colleagues sampled a total of 20 fish from each outbreak over a period of two weeks, and obtained individual virus isolates from each fish. We then used the RNase protection assay (RPA) to generate

fingerprint patterns for the IHNV isolate from each individual fish. We chose the RPA because it allowed us to probe a larger portion of the IHNV genome (3278 nt.) than could be reasonably analyzed by nucleotide sequencing. The definition of a "single outbreak" is not always completely clear because fish within multiple rearing units at IHNV-endemic facilities may simultaneously show disease signs and it is generally not known if these incidences of disease are part of a single or multiple chains of virus transmission within the facility. Given this difficulty in defining an outbreak, we chose to instead define two "populations" of fish which were located in different rearing units in separate portions of the same facility and both began to show disease signs at approximately the same time. IHNV outbreaks in rainbow trout farms occur in rearing units that may contain 100,000-200,000 fish. An outbreak in a 100,000-fish rearing unit which causes 25% mortality must therefore involve infection of at least 25,000 fish (mortality in rearing units may range from a negligible level up to as high as 90%, and 25% would not be considered atypical in many facilities). This is actually an underestimate, since some fish survive infection. The sheer scale of these host populations underscores the need to characterize intra-outbreak heterogeneity in order to obtain a more complete picture of the diversity of IHNV within rainbow trout aquaculture.

METHODS

Rainbow trout populations

The two separate populations of rainbow trout in this study were located in rearing unit 8C (population HV1) and rearing unit 23C (population HV2) at rainbow trout farm facility IV (Chapters 1 and 2) in the Hagerman Valley, ID. The beginning of each outbreak was defined by the first occurrence of IHN-associated mortalities in the population and the end was defined as the day on which daily mortalities within the population returned to a low background level of mortality similar to fish with no

disease. The fish of population HV1 were 5.7g (mean weight) at the beginning of the outbreak on 9/11/98 and 9.9g (mean weight) by the end of the outbreak on 10/8/98 (day 28). The fish of population HV2 were 15.7g (mean weight) at the beginning of the outbreak on 9/12/98 and 20.6g (mean weight) by the end of the outbreak on 10/2/98 (day 21). Thus population HV1 consisted of fish weighing 6-10 g which underwent a 28-day IHN outbreak that resulted in 25% cumulative mortality. Population HV2 consisted of fish weighing 16-21 g which underwent a 21-day IHN outbreak that resulted in 54% cumulative mortality.

During the course of the outbreaks, individual fish which showed disease signs were sampled from these populations by S.E. LaPatra and colleagues. Individual virus isolates from each fish were prepared according to the method of LaPatra [149] and stored as frozen (-75°C) aliquots of cell culture supernatant. Two fish were sampled from each population on 9/18/98 (day 8 of the outbreak for pop. HV1; day 7 for pop. HV2), nine fish were sampled from each population on 9/25/98 (day 15 of the outbreak for pop. HV1; day 14 for pop. HV2), and nine fish were sampled from each population on 10/2/98 (day 22 of the outbreak for pop. HV1; day 21 for pop. HV2). Virus isolates were then transported to the Western Fisheries Research Center for genetic analyses.

RNase protection assay (RPA)

IHNV isolates were characterized by the RPA as described in the Methods section of Chapter 1 with the exception that four ^{32}P-UTP radiolabelled minus-sense RNA probes were used in this study. These probes (prN5, prN3, prGF, and prNV) were complementary to sequences of IHNV strain RB1 (Genbank accession nos. U50401, U50402, U47846) and their locations in the IHNV genome are shown in Figure 3.1. Briefly, probes N5 and N3 are complementary to the 5' and 3' two-thirds (plus-sense) of the IHNV N gene respectively, and they overlap in the central portion of the gene. Probe GF is complementary to the entire glycoprotein gene and probe

NV is complementary to the entire NV gene. In total, these probes cover 3,278 of the ~11,000 nucleotides of the IHNV RNA genome. As in Chapter 1, a haplotype was defined as a specific banding pattern of cleavage fragments. Isolates with one or more differences were considered to have different haplotypes.

RESULTS

RPA demonstrates near homogeneity of IHNV haplotypes within farmed rainbow trout populations

RPAs were performed on 20 IHNV isolates from each of the fish populations HV1 and HV2 using 4 probes (prN5, prN3, prGF, prNV) that are complementary to different portions of the IHNV genome (Figure 3.1). Each host population included virus isolates from individual fish sampled at three different timepoints during the outbreak. Figure 3.2 shows all prGF RPA patterns for populations HV1 and HV2. In population HV1, all 20 isolates had an identical haplotype referred to as the consensus. In population HV2, 18 of 20 isolates had an identical haplotype, referred to as the consensus, and two isolates had the same variant haplotype (varGFa, asterisk lanes in Figure 3.2b), which differed from the consensus pattern by 4 out of 12 bands. In comparison to the consensus haplotype, the variant haplotype, designated varGFa (asterisk lanes in Fig 3.2b) lacked two high molecular weight bands, had a band at approximately 55 nt, and had an enhanced band at approximately 90 nt. Comparison of the consensus prGF haplotypes of populations HV1 and HV2 showed that they differed by multiple bands.

RPA patterns generated with probes N5, N3, and NV also demonstrated near-homogeneity within each population, similar to the results observed for prGF. Figure 3.3 shows representative consensus prN5, prN3, and prNV haplotypes along with all variant haplotypes observed for populations HV1 and HV2. Probe N5 detected two different variant haplotypes, varN5a and varN5b, for isolates in population HV1, and

no variants within population HV2 (Figure 3.3a). Probe N3 detected no variants within population HV1, and one variant haplotype, varN3a, present in three isolates in population HV2 (Figure 3.3b). Probe NV detected no variants within population HV1 and one variant haplotype, varNVa, present in two isolates in population HV2 (Figure 3.3c). Comparisons clearly demonstrated that the consensus haplotype for population HV1 differed from that of HV2 with all three additional probes (Figure 3.3).

Distribution of RPA haplotype variants

The distribution of the genetic variation detected in IHNV isolates from fish population HV1 is shown in Table 3.1. No genetic diversity was detected with the N3, GF, or NV probes. Two variant haplotype patterns were observed with probe N5 which differed from each other as well as the consensus pattern. Pattern varN5a was present in one of nine isolates at day 15 of the outbreak and pattern varN5b was present in one of nine isolates at day 22 of the outbreak.

The distribution of the genetic variation detected in IHNV isolates from fish population HV2 (Table 3.2) indicated variant haplotypes with probes N3, GF, and NV. A single variant prN3 haplotype pattern was detected in one of nine isolates sampled at day 14 of the outbreak and two of nine isolates sampled at day 21 of the outbreak. Two isolates, one sampled at day 7 of the outbreak (#31) and one sampled at day 14 of the outbreak (#44), each had the variant prGF pattern, varGFa, and the variant prNV pattern, varNVa. Thus, isolate #44, sampled at day 14 of the outbreak, had all three variant patterns detected in this population.

DISCUSSION

It was shown in Chapter 1 that rainbow trout farm facilities in the Hagerman Valley harbored multiple viral variants with divergent RPA haplotypes. The data presented here indicate that IHNV isolates from individual fish within each of two

separate outbreaks at a rainbow trout farm had relatively homogeneous RPA patterns with occasional variant haplotypes observed in both populations (0-3 out of 20). Each probe detected haplotype variants: prN5 variants were found in population HV1, and prN3, prGF, and prNV variants were found in population HV2. Thus, there was genetic diversity detected in each of the genes assayed (N, G, and NV genes) and there was no observed bias for mutation in particular genomic regions. Additionally, the varNVa pattern and varGFa pattern were detected in the same two isolates (Table 3.2), suggesting that the responsible mutations may be linked, although sequence analysis would be necessary to confirm this. It is very interesting that isolate #44 in population HV2 had all three of the variant haplotypes detected in this population (Table 3.2). If the isolates with identical variant patterns in fact carried the same mutation or mutations in all three cases (varN5a, varGFa, varNVa), the possible explanations for the presence of all three variants in one isolate are either convergent evolution or recombination. Given that no recombination has ever been detected for a negative-sense RNA virus in nature, a convergent event (the same mutation occurring independently in two separate viruses) would be suggested.

Both populations HV1 and HV2 appeared to have a dominant IHNV consensus haplotype with occasional haplotype variants. Although these variants made up a relatively minor portion of the population (0%-15%), this corresponded to an enormous number of infected fish. For instance, population HV2 likely consisted of >100,000 fish (S.E. LaPatra, Clear Springs Foods, personal communication), so with 54% cumulative mortality, at least 54,000 fish were infected. Thus, variant IHNV haplotypes varGFa and varNVa, which were present in 10% of the population, were likely present in >5,000 fish. This example illustrates that the host population size was so large that the finding of a particular variant haplotype in more than one fish out of the 20 sampled, likely indicates that the variant was present in thousands of fish, while the finding of a haplotype variant in one fish could represent a sporadic mutation. So, in population HV1, the two nonidentical haplotype variants (varN5a and varN5b) could be explained by a low (5%) frequency of random mutation.

However, in population HV2, the observation of a single variant haplotype found in more than one fish at different timepoints (varN3a, varGFa, and varNVa) likely indicates that the mutations were in the process of fixation in the population due to selection or stochastic events.

The consensus RPA haplotypes for populations HV1 and HV2 differed with each of the four RPA probes, suggesting multiple genetic differences between the viruses associated with the two outbreaks. The disease progression in the two outbreaks also differed in that HV2 had more acute mortality (21 day outbreak with 54% cumulative mortality), while HV1 had somewhat more chronic mortality (28 day outbreak with 25% cumulative mortality). Thus, the two distinct virus consensus haplotypes found in the two populations were associated with different disease progression in the host population. However, it is very important to note that many other factors have been demonstrated to play a role in the severity of IHN (reviewed in [151]) including, but not limited to, fish density, life stage, nutritional status, water quality, and the presence of other pathogens. Viral, host, and environmental factors could have accounted for the difference in mortality.

In Chapters 1 and 2, it was demonstrated that there is a greater overall level of IHNV genetic diversity present within trout farm facilities in the Hagerman Valley, Idaho than throughout much larger geographic regions including Alaska and British Columbia. In these larger geographic regions, IHNV infects a number of different salmonid host species in wild, hatchery, and spawning channel environments. As part of a larger study by other researchers in our laboratory, the genetic diversity of IHNV isolates from individual fish within five other INHV-infected populations, that differ in host species and environment, has been characterized by RPA with probes N5, N3, GF, and NV (G. Kurath, unpublished data). The results of these studies are summarized in Table 3.3 along with the results for the trout farm populations presented here. Comparison of the number of isolates in each population which have variant RPA patterns by at least one probe indicates that two wild fish populations (OS, wild kokanee and CK, wild steelhead) had a greater percentage of variant isolates

than were observed in trout farm populations HV1 and HV2, while three populations (MP, wild steelhead; RB, hatchery steelhead; and PK, spawning channel sockeye) had no detectable variants. Thus, the hypothesis that the greater level of IHNV diversity observed within the Hagerman Valley would also be reflected in the diversity within individual host populations was not supported.

Here we have shown that two simultaneously-occurring IHNV outbreaks within a rainbow trout farm facility were associated with quite different virus haplotypes. This is a confirmation of the results of Chapters 1 and 2, which suggested that multiple co-circulating virus lineages were present within individual facilities. However, within each of these two outbreaks, the virus present in multiple individual fish appeared relatively homogeneous over the two week period studied, with occasional relatively minor haplotype variants. The similarity of these variant haplotypes to the consensus haplotype suggested that the mutations responsible for variation have arisen recently, quite possibly within the outbreak under study, although the possibility of multiple viral variants being introduced into the host population cannot be excluded. It is likely that these minor variants in the population represent the raw material on which evolutionary forces operate in this trout farm system through a process in which some variants are amplified either by selection or neutral (chance) events.

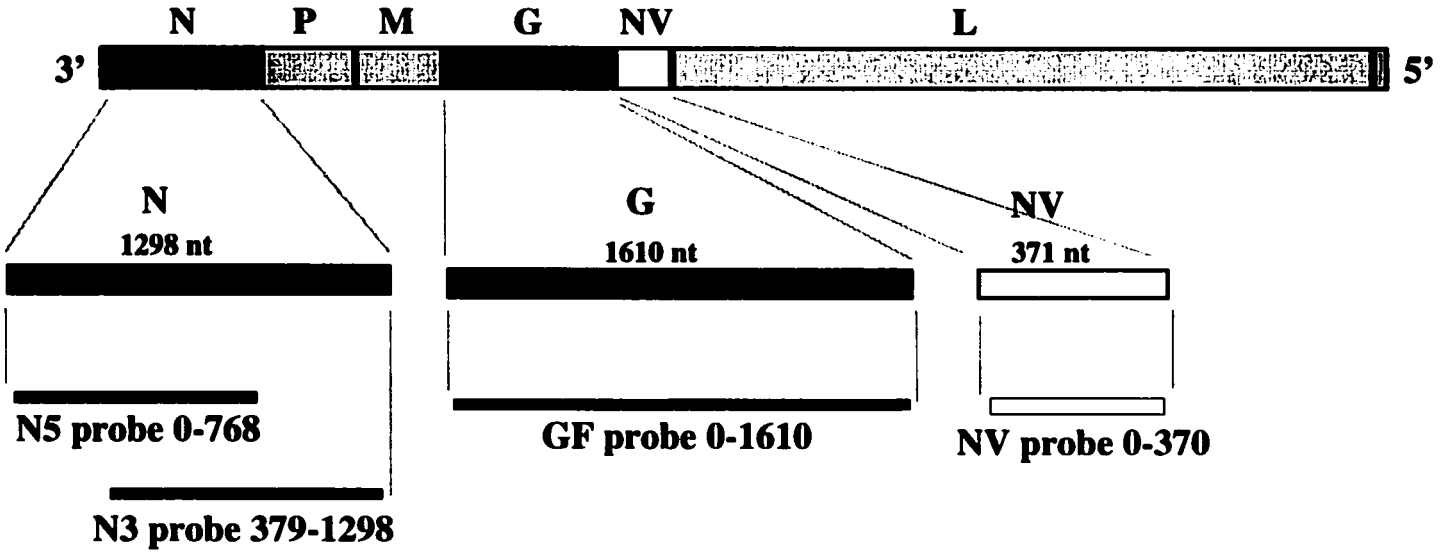


Figure 3.1 Illustration of the four RPA probes used in this study. These minus-sense probes are complementary to the RB1 strain of IHNV (GenBank accession nos. U50401, U50402, U47846).

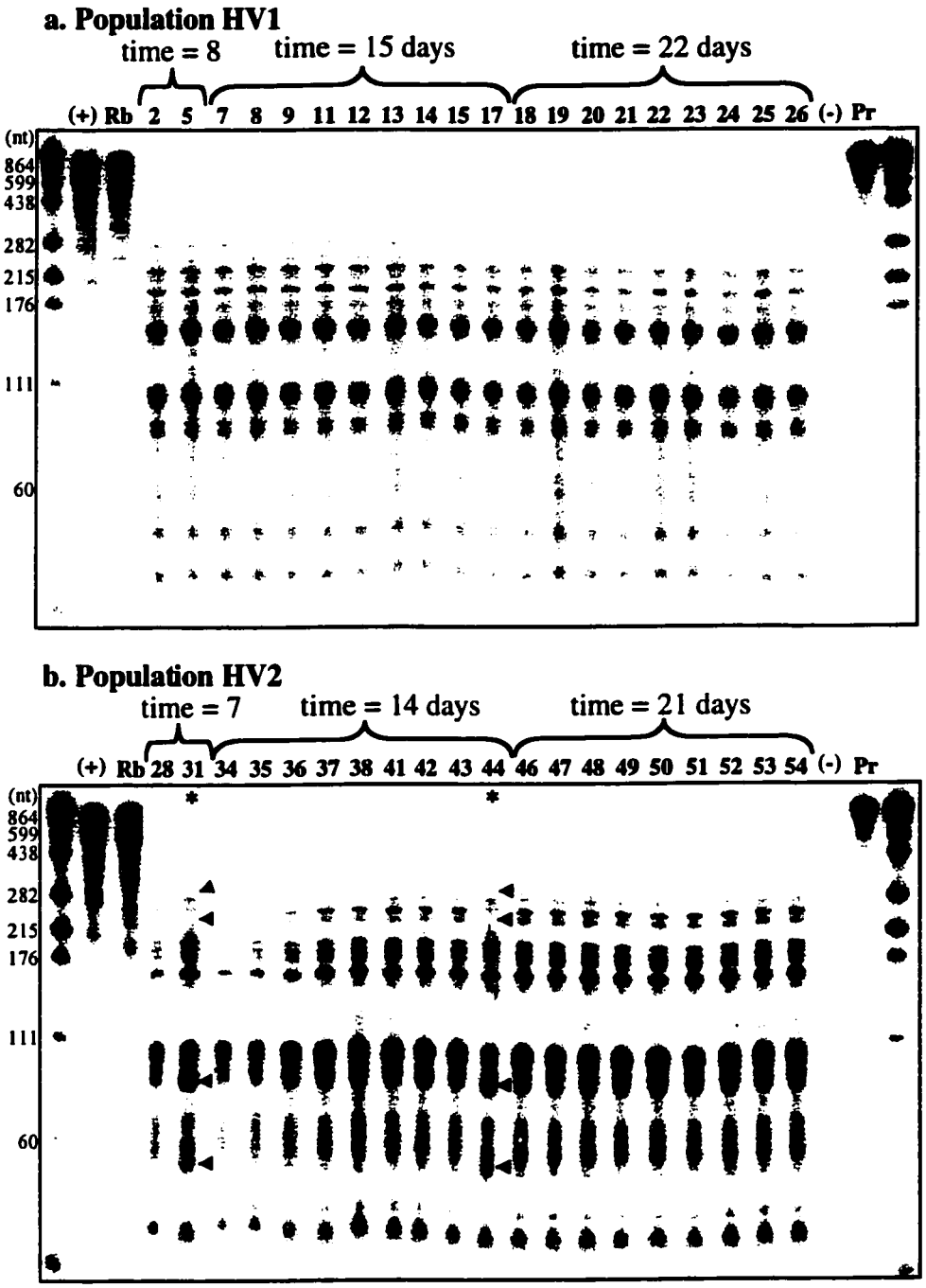


Figure 3.2 RPA haplotypes generated with the GF probe for virus isolates from two simultaneous IHNV outbreaks in populations HV1 (a) and HV2 (b). Each lane represents a pattern of cleavage fragments, referred to as a haplotype, for a particular virus isolate. Patterns which vary from the consensus (most common) haplotype are marked with an asterisk and bands which vary from the consensus haplotype are marked with arrows. RPA control lanes are indicated as follows: Rb, target RNA extracted from cells infected with the RB1 strain, which is complementary to the probe; Pr, control with no RNase or target RNA; (+), plus-sense RNA transcript synthesized *in vitro*, which is complementary to the probe; (-), no target RNA.

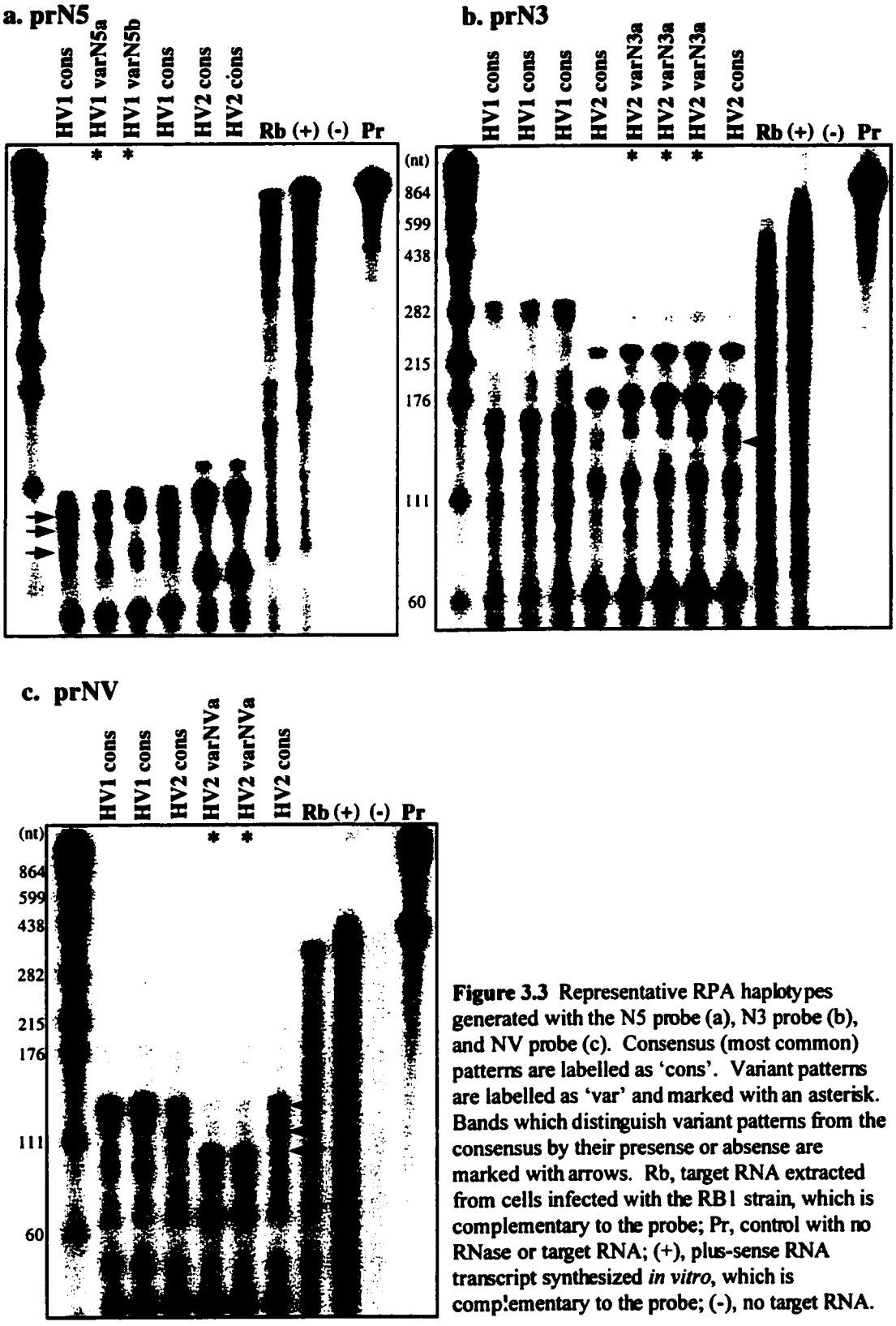


Figure 3.3 Representative RPA haplotypes generated with the N5 probe (a), N3 probe (b), and NV probe (c). Consensus (most common) patterns are labelled as 'cons'. Variant patterns are labelled as 'var' and marked with an asterisk. Bands which distinguish variant patterns from the consensus by their presense or absense are marked with arrows. Rb, target RNA extracted from cells infected with the RB1 strain, which is complementary to the probe; Pr, control with no RNase or target RNA; (+), plus-sense RNA transcript synthesized *in vitro*, which is complementary to the probe; (-), no target RNA.

Table 3.1 RPA haplotypes of IHNV isolates from population HV1. Isolates which have the consensus (most common) RPA pattern for population HV1 are noted with a dash, while those with variant patterns are noted by a 'var' designation.

Day of isolation	Isolate number	N5 probe	N3 probe	GF probe	NV probe
8	02	–	–	–	–
8	05	–	–	–	–
15	07	–	–	–	–
15	08	–	–	–	–
15	09	varN5a	–	–	–
15	11	–	–	–	–
15	12	–	–	–	–
15	13	–	–	–	–
15	14	–	–	–	–
15	15	–	–	–	–
15	17	–	–	–	–
22	18	–	–	–	–
22	19	varN5b	–	–	–
22	20	–	–	–	–
22	21	–	–	–	–
22	22	–	–	–	–
22	23	–	–	–	–
22	24	–	–	–	–
22	25	–	–	–	–
22	26	–	–	–	–
Total	–	2/20	0/20	0/20	0/20

Table 3.2 RPA haplotypes of IHNV isolates from population HV2. Isolates which have the consensus (most common) RPA pattern for population HV2 are noted with a dash, while those with variant patterns are noted by a 'var' designation.

Day of isolation	Isolate number	N5 probe	N3 probe	GF probe	NV probe
7	28	–	–	–	–
7	31	–	–	varGFa	varNVa
14	34	–	–	–	–
14	35	–	–	–	–
14	36	–	–	–	–
14	37	–	–	–	–
14	38	–	–	–	–
14	41	–	–	–	–
14	42	–	–	–	–
14	43	–	–	–	–
14	44	–	varN3a	varGFa	varNVa
21	46	–	–	–	–
21	47	–	varN3a	–	–
21	48	–	–	–	–
21	49	–	–	–	–
21	50	–	–	–	–
21	51	–	–	–	–
21	52	–	–	–	–
21	53	–	varN3a	–	–
21	54	–	–	–	–
Total	–	0/20	3/20	2/20	2/20

Table 3.3 IHNV genetic heterogeneity within host populations.

Pop.[a]	Host	Environment	# Timepoints	# Variant isolates	Variation in:
HV1	rainbow trout	farm	3	2/20 (10%)	N5
HV2	rainbow trout	farm	3	4/20 (20%)	N3, G, NV
OS[b]	kokanee salmon	wild	1	10/14 (71%)	N5, N3, G
MP[b]	steelhead trout	wild	2	0/9 (0%)	–
CK[b]	steelhead trout	wild	2	4/12 (33%)	N5, N3, G
RB[b]	steelhead trout	hatchery	3	0/20 (0%)	–
PK[b]	sockeye salmon	spawn. channel	1	0/20 (0%)	–

[a] Population
[b] G. Kurath, unpublished data

CHAPTER 4

IHNV genetic diversity within individual fish: the virus quasispecies

INTRODUCTION

In Chapter 1-3, the genetic diversity of IHNV in farmed rainbow trout was investigated at two different levels: among isolates from numerous outbreaks at many facilities (Chapter 1 and 2) and among isolates from individual fish within outbreaks (Chapter 3). These analyses were based on the consensus virus sequence or haplotype within each fish. In order to further characterize the genetic diversity of IHNV at a finer level of resolution, we chose to investigate the genetic diversity among IHNV genomes within the infected host. As discussed in the introduction to this dissertation, RNA viruses replicate by means of error-prone polymerases which do not make use of cellular proofreading mechanisms. Thus RNA viruses inherently have the potential to rapidly develop heterogeneous populations, frequently termed "quasispecies" in the virology literature, referring to populations of highly related but distinct virus genomes [58, 73]. A quasispecies may develop as the virus population within a single organism, within a particular organ, within a cell culture flask, et cetera. Since polymerase errors tend to occur randomly, RNA viruses may develop heterogeneous quasispecies with no change in the consensus sequence within the infected host [57, 244]. Once polymerase errors have been incorporated, they are subject to competition, selection, and neutral (chance) events. Thus the virus quasispecies diversity in the host reflects these forces as well the inherent error rate of the polymerase and the number of rounds of replication undergone by the virus.

Previous studies characterizing quasispecies diversity in natural hosts have found a wide range in levels of diversity depending on the virus [62, 233], host [133, 213, 234], and the type and history of infection [62, 273, 285, 286]. Estimates of quasispecies diversity as low as one mutation per genome have been made [43] while up to 100 mutations per genome have been estimated for long-term HIV-1 infection of

a single host [48, 188]. The within-host diversity of IHNV has never been characterized and there is only one report of quasispecies diversity for an aquatic rhabdovirus [19]. Thus, in order to characterize the within-host genetic diversity of IHNV present in Hagerman Valley farmed rainbow trout, we cloned and sequenced the 303 nucleotide mid-G region of multiple IHNV genomes from within four fish sampled from two IHNV outbreaks. Two rainbow trout fry of <1 gram each were sampled from an acute IHN outbreak, while two rainbow trout juveniles of 16.5 grams each were sampled at the end of a more chronic IHN outbreak. These populations were chosen in order to reflect the typical fish populations in which IHN outbreaks occur in the Hagerman Valley. This is part of a larger study in which the within-host IHNV diversity present in six other fish populations which differ in virus type, host species, and host environment have been characterized in our laboratory (E.J. Emmenegger unpublished data). This study design allowed us to evaluate the hypothesis that differences in quasispecies diversity correlate with differences in virus host species and environment. Specifically, we sought to evaluate whether the greater overall virus genetic diversity observed in the Hagerman Valley is reflected at the quasispecies level.

METHODS

Populations

The four within-host virus populations under study included virus from two rainbow trout fry, IFa and IFb, and two rainbow trout juveniles, IJa and IJb. The labels IFa, IFb, IJa, and IJb are used in this chapter to refer both to the host fish (Idaho fry or Idaho juvenile) and the virus population (a or b) within the fish since the two are inherently linked. Fry IFa and IFb were sampled by S.E. LaPatra and colleagues from an acute outbreak of IHN and were 0.80 and 0.94 grams, respectively. Whole fish were immediately frozen and shipped frozen directly to the author. Since RNA was

extracted from the whole fish, the virus titers within IFa and IFb were not determined. However, two other fish sampled at the same time from this outbreak had titers of $8.4x10^6$ pfu/g and $>2.8x10^7$ pfu/g. Juveniles IJa and IJb were sampled by S.E. LaPatra and colleagues near the end (the 18th day) of an IHN oubreak which resulted in 37% cumulative mortality. These fish were both approximately 16.5 grams in size and were greater than 4 months old (measured from the eyed-egg stage). Whole kidneys were extracted from each fish and divided in half – one half of each was frozen and shipped to the author (IJa=0.32g, IJb=0.20g) and the other half was assayed for virus titer. The kidneys of IJa and IJb had virus levels of $8.0x10^5$ pfu/g and $3.4x10^6$ pfu/g respectively. Both outbreaks occurred during the year 2001.

RNA extraction

RNA was extracted directly from IHNV-infected fish and fish tissues with no passage in cell culture. Whole IHNV-infected fry IFa and IFb were placed in 10mL of denaturing solution (described in [11]) and homogenized using a Stomacher Lab-Blender 80 (Tekmar). IHNV-infected kidney tissue from juveniles IJa and IJb were placed in 2mL denaturing solution and homogenized using the Tissue Tearor 985-370 (Biospec Products). Total RNA was then extracted following the procedure described by Anderson et al. [11] and resuspended in water (40µl for IFa and IFb, 30µl for IJa and IJb).

Reverse transcription and polymerase chain reaction amplification

We used a single-round PCR protocol modified from [133], which was designed to limit PCR-induced sequence errors. Reverse transcription (RT) of minus-sense genomic viral RNA was carried out using 2 µl of total RNA (1.0-2.0 µg), diluted with 3 µl water and heated at 95°C for five minutes, in a 20 µl reaction containing 200 units MMLV-RT (Amersham), 1X 1st strand buffer (Amersham), 1 mM dNTPs, and

20 units RNasin (Promega). Glycoprotein gene sense primer 517+ (5'-AGA-GAT-CCC-TAC-ACC-AGA-GAC-3') was used in the reaction at a final concentration of 1 μM. The reaction was incubated at 42°C for 45 minutes, followed by 95°C for five minutes.

Polymerase chain reaction (PCR) amplification of a 303 nt region (mid-G) of the IHNV glycoprotein (G) gene, from nt 686 to 988 (numbering as in GenBank accession no. U50401), was carried out with 10 μl of the RT reaction in a 100 μl reaction using 5 units PfuTurbo polymerase (Stratagene), 1X cloned Pfu buffer (Stratagene), 7.5 μg RNase A, and 0.11 mM dNTPs. An additional 11 pmol of sense primer 517+ was added along with 22 pmol of antisense primer 1209 (5'-GGT-GGT-GTT-GTT-TCC-GTG-CAA-3'). The reaction conditions included denaturation at 95°C for 1 min; followed by 25 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 20 s; followed by a final extension at 72°C for 10 min. Alternatively, RT and PCR amplification of viral RNA from fry IJa was conducted with primers 558+ (5'-TGG-AGG-AAA-ATG-CAC-CAA-ATC-3') and 1014- (5'-ATG-TGG-AGA-TCG-GAA-CTT-GGA-3'). Resulting PCR product DNA was isolated from a 3% low-melt NuSieve GTG agarose (BMA) gel in 1X TAE using the Wizard PCR Preps kit (Promega).

Cloning and nucleotide sequencing

PCR products from each virus population were subcloned into a plasmid, pSTBlue-1, using the Perfectly Blunt Cloning Kit (Novagen). The mid-G regions (303 nt) of >30 clones from each virus population were sequenced in both directions, as described in Chapter 1, using dye terminator chemistry and an ABI 310 automated sequencer. The primers used for dye terminator reactions were 577+ (5'-TCA-CCC-TGC-CAG-ACT-CAT-TGG-3') and 1059- (5'-ATA-GAT-GGA-GCC-TTT-GTG-CAT-3'). Sequence files were edited and compared as described in Chapter 1.

RESULTS

The mid-G regions of 32-40 clones from each of four within-host virus populations (IFa, IFb, IJa, and IJB) were sequenced. Virus mid-G sequences from rainbow trout fry IFa and IFb were highly homogeneous. For virus population IFa, 35 of 36 clones had identical sequences (consensus), while one clone possessed a single mutation (Figure 4.1). For virus population IFb, 30 of 32 clones had an identical consensus sequence, while two clones possessed a total of three mutations (Figure 4.2). The consensus sequences of the virus populations IFa and IFb were identical to each other and phylogenetically clustered with the B subclade described in Chapters 1 and 2 (data not shown).

Virus sequences from rainbow trout juveniles were also highly homogeneous. In virus population IJa, 33 of 37 clones had an identical consensus sequence while four clones each possessed a single mutation (Figure 4.3). In virus population IJb, 38 of 40 clones had an identical consensus sequence while two clones each possessed a single mutation (Figure 4.4). The consensus sequences of IJa and IJb were identical to each other and phylogenetically clustered with the IFa/IFb consensus sequence in subclade B (data not shown). There were four nucleotide differences between the IFa/IFb and IJa/IJb consensus sequences.

The characteristics of the mutations detected and the mutation frequency for each population are summarized in Table 4.1. In total, 145 clones were sequenced and nine mutant clones were found, bearing a total of 10 mutations. These 10 mutations were spread throughout the mid-G region and none occurred at the same nucleotide site (Table 4.1). Nine of 10 mutations were transitions while six of 10 mutations were nonsynonymous (coded for a predicted amino acid change). The mutation frequencies (number of mutations per base sequenced) for the four populations were: IFa, 0.92×10^{-4}; IFb, 3.1×10^{-4}; IJa, 3.6×10^{-4}; and IJb, 1.7×10^{-4}. The number of mutations detected in each population per 10^4 bases sequenced is approximately equivalent to the number of mutations per genome size, since the IHNV genome is ~11,000 nucleotides. The

background mutation frequency inherent in the methods was determined by E. Emmenegger by performing the exact RT-PCR, cloning, and sequencing protocol described above using a homogeneous RNA population transcribed from a plasmid as the template for reverse transcription. In that analysis, sequencing of 40 clones detected three mutations for a background mutation frequency of 2.5×10^{-4} mutations/base sequenced (E. Emmenegger unpublished data).

DISCUSSION

The mutation frequencies detected for the four virus populations studied here ranged from 0.92×10^{-4} to 3.6×10^{-4} mut./base. However, these levels were not notably different from the estimated background mutation frequency of 2.5×10^{-4} mut./base. Therefore, we cannot reject the null hypothesis that all of the mutations detected in this study were due to reverse transcription or polymerase errors inherent in the methods. We must therefore conclude that the mutation frequencies in the four virus populations from Hagerman Valley farmed rainbow trout were equal to, or less than, the background. The six virus populations from IHNV-infected sockeye salmon fry and adult fish analyzed by E.J. Emmenegger also had mutation frequencies which were not significantly different from the background mutation frequency (0-2.7×10^{-4} mut./base, Table 4.2), suggesting that, in general, IHNV infection in nature results in low within-host diversity for the mid-G genomic region such that no detectable genetic heterogeneity exists. Studies of a second genomic region are ongoing (E.J. Emmenegger, unpublished data).

While RNA virus within-host diversity is likely to depend on many different factors and may vary greatly for the same virus, other studies of rhabdovirus G gene within-host diversity have shown an order of magnitude higher levels of diversity when compared to IHNV (Table 4.2). These include a study of rabies virus [133] and a study of the fish rhabdovirus viral hemorrhagic septicemia virus (VHSV) [19]. There is an enormous body of literature focused on the within-host diversity of HIV

[48, 165, 186, 188, 213, 215, 216, 236, 273, 285, 286] and a rapidly accumulating amount of information on HCV [38, 39, 160, 161]. HIV can generate levels of within-host nucleotide diversity in excess of 1% (>100 mutations per genome)[188, 215, 216], likely due to long-term high levels of virus replication and strong positive selection [48, 204, 236]. While HIV and HCV may represent extreme cases of high within-host diversity, studies of a number of other RNA viruses [88, 210, 211, 233, 234, 255] have typically demonstrated higher levels of within-host diversity than the levels indicated for IHNV (Table 4.2), suggesting that IHNV infections generate unusually low levels of within-host diversity.

The causes for the particularly low IHNV quasispecies diversity during infection of rainbow trout are not clear. It is possible that the glycoprotein is under particularly strong selective constraint. However, it has been clearly demonstrated in Chapters 1 and 2 that the mid-G region possesses genomic plasticity, i.e. not all mutation which occur in this region are deleterious. We also considered the possibility that the virus populations might have been put through a bottleneck in the RT-PCR protocol, resulting in artificially-low diversity. However, based on the virus titers for the fish samples described in the methods, we estimated that RNA from a minimum of 40,000 virus pfu, an underestimate of the true amount of virus, was present in each reverse transcription reaction making it unlikely that DNA was amplified from a single, or very few copies of viral RNA. It is also not likely that the IHNV RNA polymerase has exceptionally high fidelity, as all RNA viruses studied to date possess error-prone polymerases [62]. A potential explanation for these results may be that the IHNV populations examined in this study, which were all from relatively typical natural IHNV infections, represented infections in which the virus had not undergone enough rounds of replication since its last bottleneck event to generate the high levels of diversity observed in some viruses which establish longer-term infection with high levels of virus replication [48, 236]. In fact, long-term infection with IHNV is controversial and is thought to occur in the form of a very low-level or carrier state [29]. Regardless of the causes of this low within-host diversity,

the data suggest that the causes for the greater overall diversity of IHNV within Hagerman Valley rainbow trout are not forces which affect the diversity of the quasispecies within individual fish.

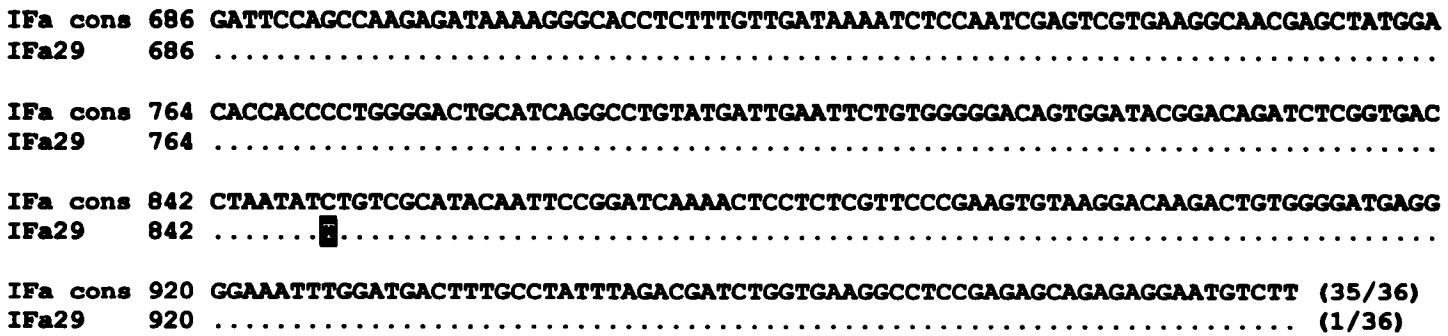


Figure 4.1 IFa quasispecies sequence alignment. The consensus sequence is shown on top. The variant sequence is shown on a second line, with only nucleotides that differed from the consensus displayed. Numbers at the end of each sequence indicate the number of clones possessing that sequence, divided by the total number of clones analyzed. Nucleotide numbering as in the entire G gene (GenBank accession no. U50401).

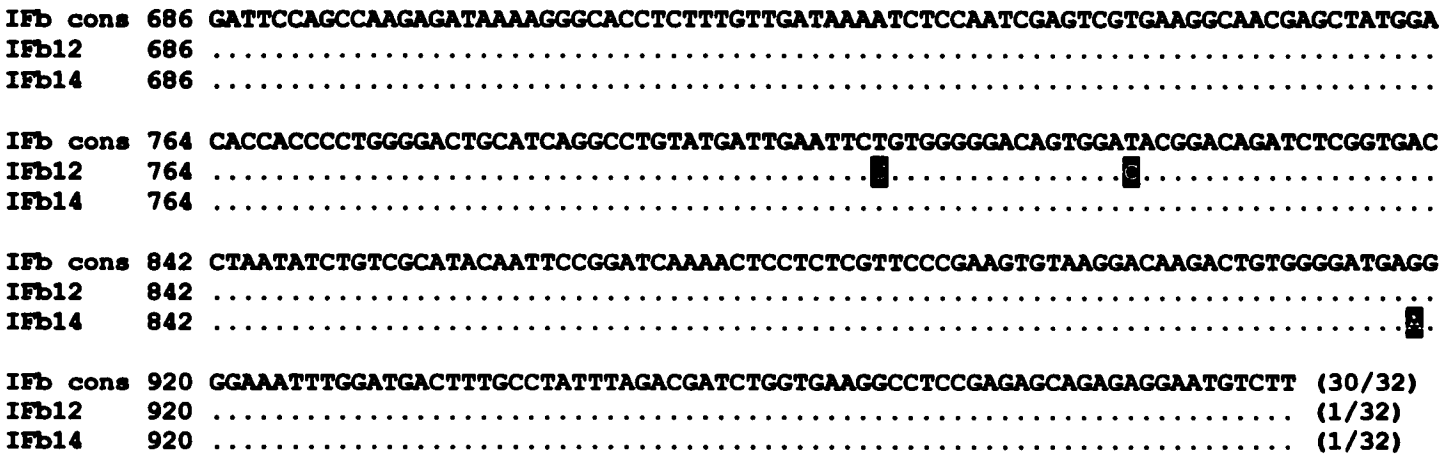


Figure 4.2 IFb quasispecies sequence alignment. The consensus sequence is shown on top. Variant sequences are shown on additional lines, with only nucleotides that differed from the consensus displayed. Numbers at the end of each sequence indicate the number of clones possessing that sequence, divided by the total number of clones analyzed. Nucleotide numbering as in the entire G gene (GenBank accession no. U50401).

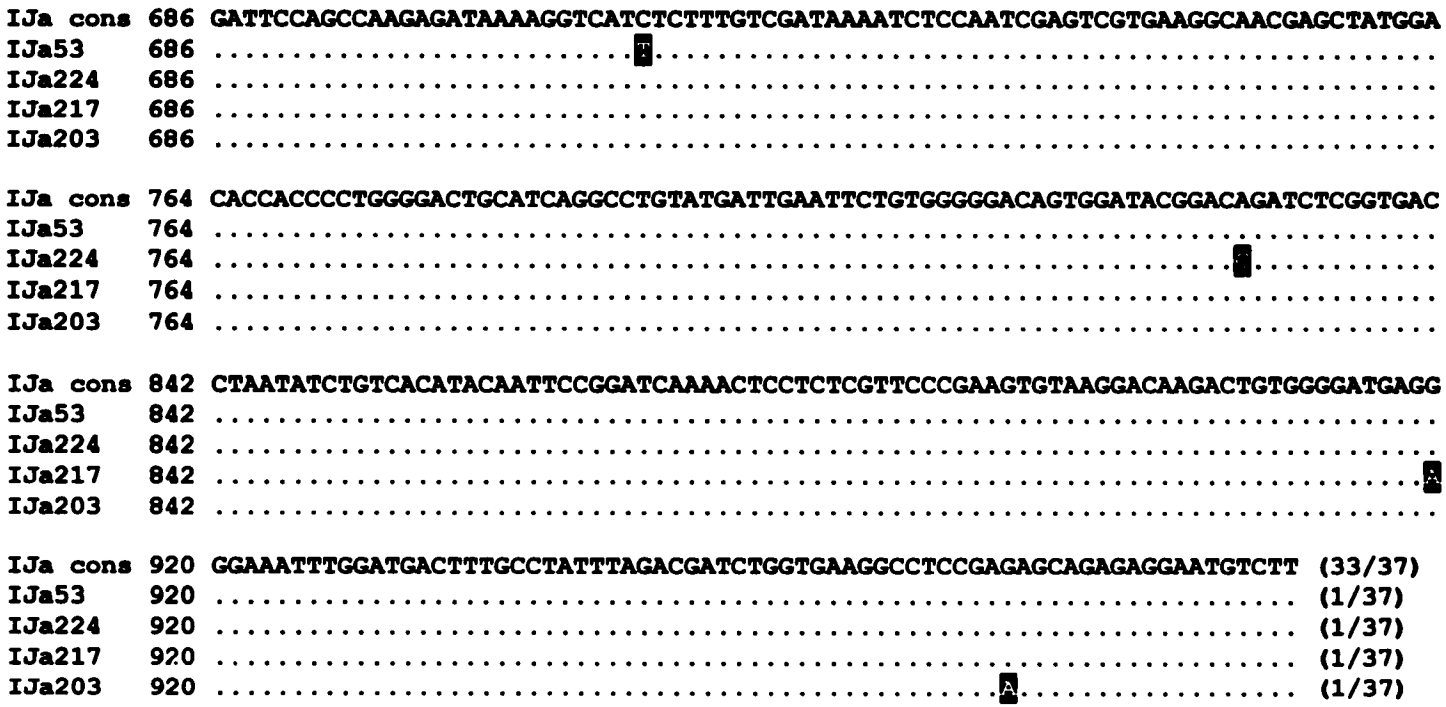


Figure 4.3 IJa quasispecies sequence alignment. The consensus sequence is shown on top. Variant sequences are shown on additional lines, with only nucleotides that differed from the consensus displayed. Numbers at the end of each sequence indicate the number of clones possessing that sequence, divided by the total number of clones analyzed. Nucleotide numbering as in the entire G gene (GenBank accession no. U50401).

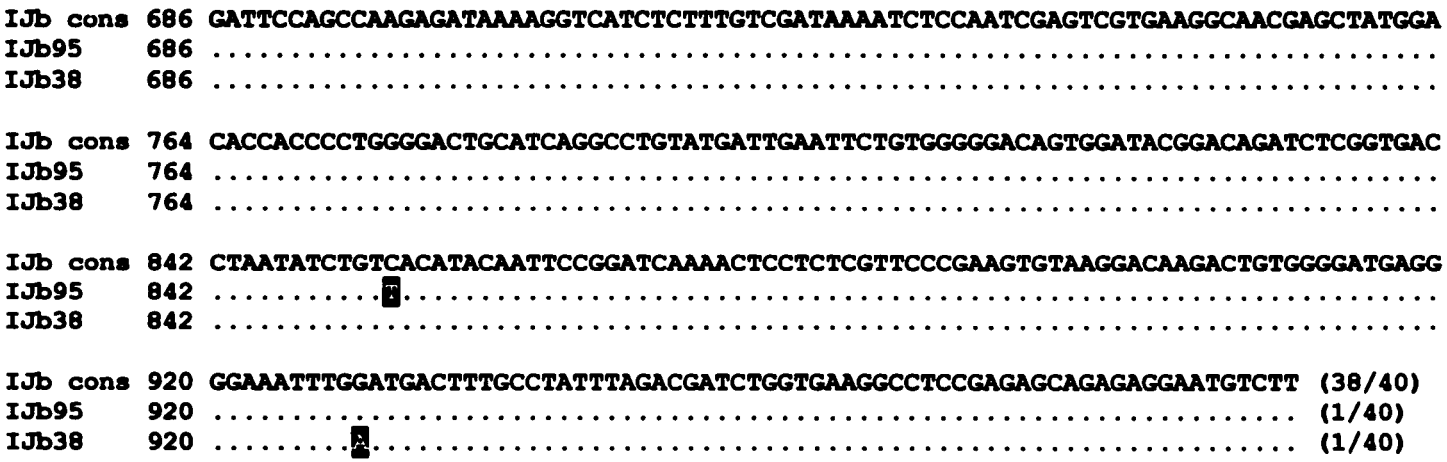


Figure 4.4 IJb quasispecies sequence alignment The consensus sequence is shown on top. Variant sequences are shown on additional lines, with only nucleotides that differed from the consensus displayed. Numbers at the end of each sequence indicate the number of clones possessing that sequence, divided by the total number of clones analyzed. Nucleotide numbering as in the entire G gene (GenBank accession no. U50401).

Table 4.1 Summary of Hagerman Valley within-host IHNV quasispecies populations.

Virus population	Number of clones sequenced	Clones with a mutation	Nucleotide substitutions[a]	Amino acid substitutions[b]	Mutations per base sequenced	Mutation frequency (mut./base)
IFa	36	IFa29	#849 C→T	#268 Ser→Phe	1/10,908	0.92×10^{-4}
IFb	32	IFb12 IFb12 IFb14	#806 T→C #822 T→C #918 G→A	#254 Cys→Arg #259 Ile→Thr #291 Arg→Lys	3/9696	3.1×10^{-4}
IJa	37	IJa53 IJa224 IJa217 IJa203	#713 C→T #829 A→C #919 G→A #970 G→A	#223 Leu→Phe synonymous synonymous synonymous	4/11,211	3.6×10^{-4}
IJb	40	IJb95 IJb38	#853 C→T #929 G→A	synonymous #295 Asp→Asn	2/12,120	1.7×10^{-4}
Total	145	9 clones	9/10 transitions	4/10 synonymous	10/43,935	2.3×10^{-4}

[a] Nucleotide numbering as in the entire glycoprotein gene (GenBank U50401).
[b] Amino acid numbering as in the entire glycoprotein (GenBank AAA97894).

Table 4.2 In-host mutation frequencies of RNA viruses

Virus	Host	Gene	Background mut. frequency $x10^{-4}$	Mutation frequency $x10^{-4}$
IHNV[a]	IF rainbow trout fry	G	2.5	0.9, 3.1
	IJ rainbow trout juveniles	G	2.5	3.6, 1.7
	BC sockeye fry	G	2.5	0.8, 0.9
	AK sockeye fry	G	2.5	0, 2.0
	CR sockeye adults	G	2.5	2.7, 0
VHSV[b]	Rainbow trout adult	G	0.7	12.0
Rabies[c]	Fox, dog, mouse	G	9.3	21.9
HIV[d]	Human	env, pol	1.2	5.0-200
HCV[e]	Human	E2/NS2, NS3	ND[j]	17.2-188.7
HGV/ GBV-C[f]	Human	NS5b	7.0	113
TUL, PUU[g]	European common vole	S/N	2.0	7.9-29
SNV[h]	wild mice and rats	G1	5.1	18-58
CCMV, TMV, CMV[i]	*N. benthamiana*, tobacco, squash, tomato, pepper	coat and flanking regions	0.5	0.7-18

[a] infectious hematopoietic necrosis virus, (E.J. Emmenegger and G. Kurath, unpublished data)
[b] viral hemorrhagic septicemia virus [19]
[c] rabies virus [133]
[d] human immunodeficiency virus [215, 216]
[e] hepatitis C virus [38, 39, 160, 161]
[f] hepatitis G virus / GB virus-C [255]
[g] Tula hantavirus and Puumala hantavirus [210, 211]
[h] Sin Nombre hantavirus [88]
[i] cowpea chlorotic mottle virus, tobacco mosaic virus, cucumber mosaic virus [233, 234]
[j] not determined

CHAPTER 5

Development of an in-host competition system for determining relative virus fitness

INTRODUCTION

As discussed in the Introduction to this dissertation, fitness is one of the most important parameters thought to influence virus diversity, virus distribution, and viral evolution. Viral fitness is typically measured as the relative ability of two competing viruses to produce infectious progeny in a given environment [58, 61, 62, 217]. However, the vast majority of literature on RNA virus fitness has been generated in cell culture-based systems due to limitations on doing experiments in mammalian hosts and limits on using large numbers of experimental animals. *In vitro* systems have provided a great deal of information on the features of RNA virus fitness and population biology [45, 47, 62, 69-71, 82, 83, 115, 169, 197-199, 244, 252, 253, 262] but their relevance in the host remains to be demonstrated. Therefore, in order to study the relative fitness of IHNV variants, we sought to develop an *in vivo* competition system to measure virus fitness in the fish host. This system involved challenging rainbow trout, a natural host for the virus, with two different IHNV genotypes, allowing infection and virus competition within the host to occur, and then subsequently analyzing the virus progeny populations present within each infected fish to determine whether one or both of the genotypes were present and in what proportions. This allowed calculation of the relative fitness of the two genotypes being competed.

IHNV GENOTYPES USED IN VIRUS COMPETITION

To perform virus competitions, it was necessary to demonstrate that we could detect and quantify mixed infections in fish (both genotypes present in the virus

progeny population), if mixed infections occur. Therefore we chose to initially compete genotypes which were hypothesized to be of similar relative fitness, to determine whether mixed infections occur and to demonstrate the ability to detect and quantify the genotypes present in a mixed infection. The genotypes chosen for the initial study included three virus isolates (FF020-91, FF030-91, and FF039-91; see Table 1.1 for isolate information) which were present within the same facility (facility IV) in the same year (1991) and so were hypothesized to be of relatively similar fitness. These isolates were members of the three most prevalent and widely distributed IHNV lineages in the Hagerman Valley; B, C, and D; and are referred to throughout this chapter as genotypes B (FF020-91), C (FF030-91), and D (FF039-91) or abbreviated as gtB, gtC, and gtD.

These viruses were passaged in cell culture (EPC) at an MOI of 0.001 to produce large, high-titer stocks which were aliquoted into 1.5 mL Eppendorf tubes and frozen at 70°C. Virus stocks were titered extremely carefully by plaque assay [16], with a minimum of seven replicate dilution series, each counted in triplicate wells. Titers were: gtB, $2.79 \pm 0.20 \times 10^{-8}$ pfu/mL; gtC, $2.14 \pm 0.18 \times 10^{-8}$ pfu/mL; gtD, $1.44 \pm 0.22 \times 10^{-8}$ pfu/mL. Since gtB and gtC were representatives of the most prevalent and widely-distributed subclades present in the Hagerman Valley, we chose to focus the majority of effort on these two genotypes for the initial development of the system.

VIRULENCE OF IHNV GENOTYPES USED IN VIRUS COMPETITION

As an initial rough estimate of whether the chosen genotypes had similar replication kinetics in fish, we challenged groups of fish with genotypes B, C, and D to generate mortality curves for each virus (a kinetic measure of virus virulence). Currently it is not known if IHNV virus virulence correlates with virus replication kinetics. However, gross differences in virulence between genotypes may indicate differences in replication which might reflect fitness. We also included a fourth genotype, A (FF031-91), which represented subclade A. Triplicate groups of 25

rainbow trout (75 fish total per genotype, mean weight 1.34 g) were challenged in batch for 12 hours at $1x10^5$ pfu/mL in 1 L of static 15°C water. Following challenge, fish were held in batch in 4 L tanks with flowing 15°C water for 30 days, with mortalities recorded daily. The variance in mortality between replicate tanks was low and so the replicates were pooled for each genotype. The resulting cumulative mortality curves for genotypes B, C, and D all had very similar slopes (Figure 5.1) with similar total percent mortality: gtB, 74.7%; gtC, 82.4%; gtD, 80.3%. Challenge with genotype A produced 6.7% total mortality (Figure 5.1) prompting us to eliminate this genotype from further comparisons at this time. Fish which were mock-challenged with cell culture media alone had 4.0% total mortality (Figure 5.1).

MODIFIED CHALLENGE CONDITIONS: THE MISFIT SYSTEM

Standard IHNV challenge of fish is typically conducted by immersing a group of fish in a static suspension of virus for one hour, followed by resuming water flow and holding the fish in batch for 21-30 days [278]. This method produces a strong dose-response between the challenge titer and the resulting cumulative mortality. However, a disadvantage of this method for in-host competition experiments is that the interpretation of results is confounded by the multiple cycles of infection which occur when infected fish shed virus which then initiates subsequent rounds of infection in other fish. Therefore we developed a modified protocol that involved initially challenging fish in batch and then subsequently separating them into individual isolation units. This modified protocol is referred to as the "MISFIT" system for Multiple Isolated Fish Infection Test.

Duration of immersion challenge

To define challenge conditions for the MISFIT system, we performed challenges to determine whether the standard one hour challenge would be sufficient

to establish infection. All rainbow trout for this and subsequent experiments were Clear Springs strain. Groups of fish were typically the result of single pair matings and so groups of fish for each experiment were siblings. Groups of 8 rainbow trout fry (2.5 g mean weight) were challenged with one of four viruses: gtB, gtC, gtD, or WRAC (IHNV type strain, positive control). A group of 8 fish was also mock-challenged with virus-free cell-culture supernatant. Preliminary conditions for the MISFIT system were as follows. Fish were restricted from feeding for 24 hours prior to challenge in order to reduce stress [264]. Fish were then challenged by immersion in a static suspension of $1x10^5$ pfu/mL of virus in 15°C water for one hour with aeration. The total amount of static water in each challenge was standardized to 50 mL/fish with a minimum volume per tank of 1 L. After the one hour challenge, water flow to each tank was restarted and allowed to flow for four hours to rinse out excess virus. Fish were placed into individual tanks with 4 L 15°C flowing water with aeration and held there for 25 days. Mortalities were recorded on a daily basis. After 25 days, the number of mortalities per group were: mock-challenged negative control, 0/8; WRAC positive control, 0/8; gtB, 2/8; gtC, 2/8; and gtD, 2/8. Based on previous experience with WRAC strain challenges, we expected at least 50% mortality in the WRAC positive control. This result and the relatively low percent morality with genotypes B, C, and D (25%) likely indicated a low percentage of infected fish.

In an effort to increase the percentage of infected fish, we repeated the above challenge but extended the duration of immersion challenge to 12 hours. Also, immediately after challenge, fish were transferred in batch to a separate four liter tank with actively flowing aerated 15°C water for a one hour "wash" period to remove excess virus. This replaced the previous procedure above, which included a four-hour "wash" in the same tank used for challenge. This change was necessary in order to keep the total time that fish were held in batch to less than 16 hours, which we estimated as the earliest time that fish might potentially begin to shed virus [278](B. Busch, personal communication). Additionally, fish were held after challenge in individual beakers with 150 mL static water, rather than in 4 L tanks with flowing

water. The beakers were kept at a constant temperature of 15°C by circulating water around the outside of the beaker. This protocol change allowed up to 60 fish to be isolated in individual beakers on a single laboratory challenge table. Groups of approximately ten fish (mean weight 1.3 g) were challenged with gtB, gtC, gtD, or mock-challenged. Fish were held after challenge for 7 days, sacrificed by adding MS-222 to each beaker, and titered by virus plaque assay on EPC cells. All mock-challenged fish were negative for virus while all fish challenged with gtB, gtC, or gtD were positive for virus (gtB, 10/10; gtC, 9/9; gtD, 9/9). Virus titers ranged from 800 pfu/g up to $6.5x10^6$ pfu/g and there were no notable differences in virus titer between fish challenged with different genotypes. In summary, a 12 hour challenge, along with the other changes incorporated in this experiment, allowed a 100% rate of productive infection. Thus, these procedures were incorporated into the MISFIT protocol.

Challenge titer

In setting up competition experiments, it was very important to establish a challenge protocol that produced infection in as close to 100% of fish as possible since, by definition, in-host competition requires both viruses to be present in the fish. However, in order to most accurately model what occurs in natural IHNV outbreaks, it was also desirable to keep the immersion challenge titer as close to what has been observed in IHNV outbreaks as possible. Typical titers of virus in the water during outbreaks of IHNV within rearing units at a Hagerman Valley fish farm were measured at between 0 and 43 pfu/mL (S.E. LaPatra, unpublished data) but titers as high as $1.6x10^3$ pfu/mL have also been reported [177]. We performed an initial experiment to determine the challenge dose necessary to establish infection in a high percentage of fish when fish were held in individual isolation units after challenge. Fish were challenged using the MISFIT conditions described above. Nine groups of 10 rainbow trout (mean weight 1.0 g) were challenged with genotypes B, C, and D at three different titers: $1x10^2$ (low), $5x10^3$ (medium), and $1x10^5$ (high) pfu/mL. Fish

were held for 7 days and sacrificed by adding MS-222 to each beaker. Virus plaque assays were conducted on each fish to determine virus titer. The resulting titers ranged from below the minimum detectable titer of 200 pfu/g up to a maximum of $5.6x10^6$ pfu/g and there were no notable differences in titer between fish infected with different genotypes. The number of virus-positive fish per group for the low dose were: gtB-low, 4/10; gtC-low, 2/10; gtD-low, 0/10. For the medium dose, the results were: gtB-med, 7/8; gtC-med, 8/10; gtD-med, 9/10. And for the high dose, the results were: gtB-high, 9/9; gtC-high, 9/10; gtD-high, 9/10. Thus, there were no notable differences between gtB, gtC, and gtD. When the data from groups challenged with genotypes B, C, and D was pooled, the low challenge dose ($1x10^2$ pfu/mL) produced detectable infection in 6/30 fish (20%), the middle challenge dose ($5x10^3$ pfu/mL) produced detectable infection in 24/28 fish (86%), and the high challenge dose ($1x10^5$ pfu/mL) produced detectable infection in 27/29 fish (93%). Based on this data, we chose $1x10^4$ pfu/mL as the challenge titer for future experiments in order to assure that >86% of challenged fish would have productive infections.

Duration of the in-host competition period

Selection of a timepoint at which to sacrifice fish for assay of the in-host competition involved two main factors. First, it was desirable to end the competition before mortality began in order to avoid complicating the analysis with the factor of fish death. The IHNV genotypes used in this study began to cause mortality in rainbow trout fry at day 6 of batch challenge (Figure 5.1). Second, it was important that the period for holding the fish be sufficiently long that the viruses being competed would have both reached their respective maximum titers if challenged singly. Otherwise, the competition result could be artificially biased toward a virus with initially faster replication kinetics. Because of this concern, we performed in-host growth curves for genotypes B, C, and D in rainbow trout fry. These growth curve experiments were performed twice for each virus genotype following the MISFIT

protocol. At daily timepoints, three fish challenged with each genotype were sacrificed, frozen, and then titered by plaque assay (fish were assigned to each timepoint based on a random numbering scheme).

In the first experiment (mean fish weight 0.8 g), the first timepoint was 16 hours after the initiation of challenge and then subsequently 24 hour timepoints were taken. In this challenge, there were significant titers (3.5 to 4 log pfu/g) in fish at the first (16 hour) timepoint. In order to characterize the initial increase in virus titer within the fish, the experiment was repeated under the same conditions (mean fish weight 1.4 g), but including earlier timepoints at one hour, six hours, and 12 hours after initiation of challenge. There was fish-to-fish variation at individual timepoints, but typically growth curves showed no detectable virus (detection limit = 2.3 log pfu/g) through 12 hours after initiation of challenge, then a quickly rising titer level which peaked around 70 hours and then a plateau or gradual decline (Figure 5.2). A plot of the mean growth curves for all three genotypes in both experiments (Figure 5.3) showed that the genotypes had relatively similar growth kinetics, with peak titers occurring by 72 hours after initiation of challenge. Based on this data, 72 hours (three days) was chosen as the timepoint at which fish would be sacrificed and assayed in the initial competition experiments.

METHODS FOR ANALYSIS OF MIXED-GENOTYPE VIRUS POPULATIONS

For analysis of mixed genotype virus progeny populations within each fish, we chose two methods that involved a region of the glycoprotein gene which included the mid-G region (303 nt) described in previous chapters. This region was chosen because nucleotide sequencing of genotypes B, C, and D showed that these viruses had a number of nucleotide differences which could be used as markers to distinguish them in mixed infections. The basic strategy for analysis of virus progeny populations (Figure 5.4) involved two methods: genotype-specific restriction enzyme analysis to determine the relative proportions of each genotype in a population, and nested

genotype-specific PCR for determining the presence or absence of genotypes with maximum sensitivity.

First round amplification of partial glycoprotein gene cDNA fragment for analysis

Fish were thawed, homogenized, and total RNA was extracted from each entire fish using the procedure described in Chapter 4. Briefly, whole IHNV-infected fry were placed in 10 mL denaturing solution (described in [11]) and homogenized using a Stomacher Lab-Blender 80 (Tekmar). Total RNA was then extracted following the procedure described by Anderson et al. [11] and resuspended in 40 μL water. Reverse transcription of minus-sense genomic viral RNA was carried out using 3 μl of total RNA, diluted with 2 μl water and heated at 95°C for five minutes, in a 20 μl reaction containing 200 units MMLV-RT (USB), 1X MMLV buffer (USB), 1 mM dNTPs, and 20 units RNasin (Promega). Glycoprotein gene sense primer 558+ (5'-TGG-AGG-AAA-ATG-CAC-CAA-ATC-3') was used in the reaction at a final concentration of 1 μM. The reaction was incubated at 42°C for 45 minutes, followed by 95°C for five minutes. The nucleotide sequence of primer 558+ was conserved among genotypes B, C, and D (Figure 5.5).

The cDNA produced in this reaction was then used as a template for PCR with primers (558+ and 1014-, Figure 5.5) that are complementary to all three genotypes and therefore amplify a 483 bp DNA product from all genotypes present in the virus population. This reaction was carried out with 10 μl of the RT reaction in a 100 μl reaction using 5 units Taq polymerase (Promega), 1X PCR buffer (Promega), 7.5 μg RNase A, and 0.11 mM dNTPs. An additional 11 pmol of sense primer 558+ was added along with 22 pmol of antisense primer 1014 (5'-ATG-TGG-AGA-TCG-GAA-CTT-GGA-3'). The reaction conditions included denaturation at 95°C for 2 min, followed by 25 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 60 s, followed by a final extension at 72°C for 7 min This reaction produced amplified DNA which represented the whole virus population present in each fish. Two different strategies,

to be described below, were then employed to analyze the genotypes that were present in each virus population (Figure 5.4).

Genotype-specific restriction enzyme digest (gt-spec RE digest)

To assay for and quantitate the relative proportion of each genotype in RNA populations of genotype pairs B:C, C:D, and B:D, we used restriction enzymes which were specific for sites within the mid-G region of only one genotype from each competing pair. In order to generate sufficient amounts of DNA for the analysis from each virus progeny population, the primary conserved PCR product was used as a template in a second nested round of PCR using conserved primers 619+ (5'-GAT-GCA-GGG-ATA-CCA-GCC-TGT-3') and 975 (5'-ACT-GTT-TGT-TGA-TAT-TAT-CTC-3') to produce a 357 bp product (Figure 5.5). To do this, the PCR products (483 bp) generated in the initial conserved reaction were purified using the StrataPrep PCR Purification Kit (Stratagene). Then 2 μL of this product was used as a template in a 50 μL PCR containing 2.5 units Taq DNA polymerase (Promega) with 2.5 mM magnesium chloride, and 0.2 mM dNTPs. Primers 619+ and 975 were used in the reaction at final concentrations of 1 μM. The reaction conditions included denaturation at 95°C for 2 min, followed by 25 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 60 s, followed by a final extension at 72°C for 7 min. PCR products were purified using the StrataPrep PCR Purification Kit (Stratagene).

To initially test the ability of the restriction enzymes to differentiate between genotypes, digestions were performed with ~400 ng of DNA amplified by nested PCR from RNA of each genotype alone, with five restriction enzymes: EcoRI (20 units, 37°C for 2 hours), XbaI (20 units, 37°C for 2 hours), BciVI (20 units, 37°C for 2 hours), BsmI (10 units, 65°C for 2 hours), and TaqI (20 units, 65°C for 2 hours) (Figure 5.6). The goal was to identify enzymes which distinguish between genotypes in genotype pairs B:C, C:D, and B:D. Genotype (gt) pair B:C and pair B:D could be distinguished using EcoRI or XbaI. EcoRI cut gtB only, while XbaI cut gtC and gtD

but not gtB (Figure 5.6). Pair C:D could be distinguished using BciVI, BsmI, or TaqI. BciVI cut gtC but not gtD while BsmI and TaqI cut gtD but not gtC (Figure 5.6).

With each of the enzymes tested, there was a small level of residual uncut DNA (357 bp) in lanes in which cutting occurred (Figure 5.6). Increased levels of enzyme and increased incubation times did not eliminate this uncut DNA. We considered the possibility that genetic heterogeneity within the virus stocks (quasispecies) at the restriction sites might be responsible. To address this possibility, we PCR-amplified partial glycoprotein DNA from a plasmid (pWG) containing a copy of the glycoprotein gene with both the EcoRI and XbaI restriction sites. We then performed restriction digests with EcoRI and XbaI, and found that in both cases the residual uncut band remained, despite the fact that the DNA was amplified from a presumably non-heterogeneous plasmid template (Figure 5.7, lanes 4 and 8). This lack of complete cutting would complicate quantitation of the genotype proportions if only one restriction enzyme were used, because the genotype which remains uncut could be artificially favored. However, if two enzymes that each cut opposing genotypes were used in a double digest, then the residual uncut DNA would not need to be considered in the analysis as long as the cutting efficiency for each enzyme was similar in single-genotype controls. Alternatively, for single-enzyme analysis, we could mathematically correct each analyzed proportion for the expected level of residual uncut DNA based on the proportion of uncut DNA in single-genotype controls. We chose to pursue the former strategy for analyzing genotype pair B:C.

We chose to focus on genotypes B and C for development of the competition system and so all additional control experiments for quantitation of virus progeny populations were performed using this genotype pair. We performed a digestion reaction including both enzymes that cut gtB and gtC in a single reaction. This reaction was performed by digesting a gtB and gtC DNA mixture (~400 ng) with EcoRI (20 units) and XbaI (20 units) at 37°C for 2 hours using buffer #2 (NEB). The reaction produced gtB-specific bands (219 bp and 138 bp) and gtC-specific bands (281 bp and 76 bp) (Figure 5.7). In addition, we used densitometry to determine the

proportion of uncut DNA versus cut DNA for single-genotype restriction digests using both enzymes (lanes 9 and 10, Figure 5.7). This was used to test for possible differences in cutting efficiency between EcoRI and XbaI in double digests. For genotype B alone, 9% of the DNA remained uncut and for genotype C alone, 10% of the DNA remained uncut.

In order to determine the accuracy of the gt-spec RE digest quantitation of virus progeny populations, we prepared mixtures of genotypes B and C in known ratios based on stock virus titers and analyzed these mixtures by gt-spec RE digest. Mixtures of gtB and gtC virus stocks were prepared so that the total titer of each sample was $1x10^8$ pfu/ml. Five µl of each sample were then diluted with 95 µl water, vortexed, heated at 95°C for 2 minutes, and placed on ice. An aliquot of 3 µl of this dilution was then used as a template in an RT reaction. The RT, nested PCR, and gt-spec RE digest were performed as described above. The levels of genotypes B and C in each reaction were determined by densitometry of the 281 bp (C-specific) and 219 bp (B-specific) bands in each lane with adjustment of the raw readings to control for DNA length (Figure 5.8). The smaller molecular weight bands (138 bp and 76 bp) were not used in determining the levels of gtB and gtC due to less linear staining by ethidium bromide. The levels of genotypes B and C were then calculated as relative percentages within each lane. Because these values were relative within each lane, there was no need to precisely standardize the amount of DNA loaded from lane to lane. Densitometry suggested that the total DNA did not vary from lane to lane by more than two-fold. Genotype B alone and genotype C alone showed only the expected B and C-specific bands (Figure 5.8). For the genotype B and C mixtures, the analyzed (quantitated) ratios of gtB and gtC reasonably faithfully reproduced the input ratios, with consistently less than 10% difference between the input and analyzed ratios (Figure 5.8). In addition, these results demonstrated that gt-spec RE digest was able to detect genotypes that comprised only 5% of the total in each lane (Figure 5.8). Thus the system could quantitate up to a 20-fold difference between the levels of genotypes B and C.

To determine the reproducibility of the gt-spec RE digest quantitation, we performed the analysis using three different virus progeny populations to be described below (see "pilot virus in-host competitions with genotypes B and C"). These virus progeny populations consisted of total RNA extracted from fish which had detectable mixed infections with both gtB and gtC. Analysis of these populations from reverse transcription through gt-spec RE digest was performed four times independently and the percent gtB and gtC were calculated by densitometry scans of the gtB-specific 219 bp band and the gtC-specific 281 bp band. The quantitation of virus progeny populations was highly reproducible, with the four replicates for each virus progeny population varying by 3.1% or less (Figure 5.9).

Genotype-specific PCR (gt-spec PCR)

In performing competition experiments, it was desirable to have a method which could detect the presence or absence of genotypes with high sensitivity in order to establish whether a fish was infected with one or both genotypes. Therefore we developed genotype-specific PCR primers which were complementary to one genotype (gtB, gtC, or gtD) but had at least one substitution relative to the other genotypes (Figure 5.5). These primers bind to the same sites within the mid-G region of IHNV but were designed to be slightly different in length in order to equalize primer melting temperature (Tm) at 60°C. Primer sequences were as follows: gtB+(5'-ATG-ATT-GAA-TTC-TGT-GGG-GGA-3'), gtB-(5'-TCC-CCA-CAG-TCT-TGT-CAC-3'); gtC+(5'-G-ATG-ACA-TTC-TGT-GGG-GGG-3'), gtC-(5'-TCC-CCA-CAG-TCT-TGC-CCT-3'); gtD+(5'-T-ATG-ATT-GCA-TTC-TGT-GGG-AAA-3'), gtD-(5'-CA-TCC-CCA-CAG-TAT-TGT-CCT-3'). To initially show that these primers amplified only their complementary genotype, RT-PCR and nested PCR reactions were performed as described in Chapter 1 using high-titer stock virus of genotypes B, C, and D in cell culture supernatant. The genotype-specific primers were substituted for the second round PCR primers listed in Chapter 1. With each set

of primers, these reactions very clearly amplify only the complementary genotype at an annealing temperature of 60°C (Figure 5.10).

In order to perform genotype-specific PCR (gt-spec PCR) on virus progeny populations from fish challenges, the primary conserved PCR product (483 bp) described above, which represents cDNA from all genotype pairs present in the fish, was used as a template for gt-spec PCR. Two μL of this PCR product was used in a 50 μL reaction containing 2.5 units Taq DNA polymerase (Promega) with 2.5 mM magnesium chloride, and 0.2 mM dNTPs. Gt-spec primers were used in the reaction at final concentrations of 1 μM. The reaction conditions included denaturation at 95°C for 2 min, followed by 25 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 60 s, followed by a final extension at 72°C for 7 min. Reactions performed using virus progeny population cDNA as a template produced a ~120 bp product which was genotype-specific (Figure 5.12, control lanes).

PILOT VIRUS IN-HOST COMPETITIONS WITH GENOTYPES B AND C

Challenge with a 1:1 input ratio of genotypes B and C demonstrated establishment and detection of mixed infections

A pilot competition experiment was performed by challenging 16 fish with a 1:1 ratio of gtB and gtC. As controls, 4 fish were challenged with gtB only, 4 fish with gtC only, and 4 fish were mock-challenged with cell culture supernatant containing no virus. The challenge was performed using the MISFIT system described above. Following the standard three day in-host growth competition period, total RNA was extracted from each fish and these virus progeny populations were analyzed by both gt-spec RE digest and gt-spec PCR as described above.

Gt-spec RE digest of the virus progeny populations from the four control fish challenged with gt B alone and the four control fish challenged with gt C alone produced only gtB-specific bands (219 bp and 138 bp) and gtC-specific bands (281 bp

and 78 bp) respectively, while the four mock-challenged fish showed no detectable virus populations. Representatives of these controls are shown in Figure 5.11. Lanes 1-16 in Figure 5.11 show virus progeny populations from 16 fish challenged with a 1:1 ratio of gtB and gtC. The percentage of gtB and/or gtC within each population was determined by densitometry scans of the gtB-specific 219 bp band and the gtC-specific 281 bp band as described above. In this competition the "fitness" of each genotype was estimated as its relative abundance within each fish compared to the other genotype. So, for each virus progeny population, the "fitness ratio" was calculated as the percent of the more abundant genotype divided by the percent of the less abundant genotype. The fitness ratio therefore indicated the fold difference in quantity between the two genotypes (Figure 5.11). We showed that 5% of the total virus population was detectable by gt-spec RE digest, so any population with only one genotype visible was considered to have a fitness ratio of greater than 20-fold. Of the 16 populations, 13 had mixed infections with both genotypes visible, while three had only one genotype detectable by gt-spec RE digest (marked with asterisks in Figure 5.11). Of the 16 populations, eight had a higher percentage of gt B and eight had a higher percentage of gt C (Figure 5.11). The fitness ratios for each population ranged from 1.0 (essentially equal levels) to greater than 20 (detection limit). The majority (12/16) of populations had fitness ratios of 4.1 or less. In order to determine the mean fitness ratio of gt B to gt C, the percentages of gtB and gtC present in each of the 16 progeny populations were summed and divided by 16. The resulting fitness ratio showed gtC having an extremely slight advantage with a gtB:gtC fitness ratio of 1:1.02.

Gt-spec PCR of the virus progeny populations from the pilot equimolar challenge with genotypes B and C was performed as described earlier. Gt-spec PCR using gtB-specific primers amplified progeny populations from the four control fish challenged with gtB alone, but not the four control fish challenged with gtC alone (Figure 5.12). Gt-spec PCR using gtC-specific primers amplified progeny populations from the four control fish challenged with gtC alone but not the four control fish

challenged with gtC alone (Figure 5.12). All 16 fish challenged with a 1:1 ratio of genotypes B and C were positive by gt-spec PCR for both gtB and gtC (Figure 5.12). This indicated that all 16 fish had mixed infections with both genotypes, providing the opportunity for virus competition within each fish. It is important to note that gt-spec PCR was able to detect low levels of minor genotypes which were not detectable with gt-spec RE digest. Thus, the dual techniques of gt-spec PCR and gt-spec RE digest allowed both a high level of sensitivity to detect mixed infections (gt-spec PCR) and the ability to quantitate relative proportions in those mixed infections (gt-spec RE digest).

Modified challenge ratios tested the limits of our ability to establish dual infections

Challenge of fish with a 1:1 ratio of genotypes B and C produced 100% (16/16) mixed infections. In order to define the limits of our ability to establish mixed infections in fish, we performed competitions using gtB and gtC with modified challenge ratios of approximately 9:1, 99:1, and 999:1. This was a practical question because future competitions between unequally fit variants may result in infection with consistently only one genotype, requiring skewed challenge ratios in order to establish dual infections that may be quantified [217]. In a pilot scale study, six groups of four or five fish were each challenged at different ratios of gtB to gtC. Three of the groups were challenged with greater amounts of gtB at ratios of ~9:1, ~99:1, and ~999:1, while three groups were challenged with greater amounts of gtC at similar ratios. The total virus titer in each case was $1x10^4$ pfu/ml. The challenges were performed exactly as described in the previous section and virus progeny populations were assayed by gt-spec PCR as previously described. The results of the gt-spec PCR for each virus progeny population at each modified challenge ratio are shown in Table 5.1. All fish challenged at ~9:1 were positive for both genotypes (8/8), indicating that challenge at this ratio produced consistent mixed infection (Table 5.1). Of the fish challenged at ~99:1, 56% (5/9) were positive for both genotypes,

indicating that this challenge ratio inconsistently produced mixed infection (Table 5.1). At a ratio of ~999:1, only one of eight fish were positive for both genotypes, indicating that challenge at this ratio only rarely produced dual infection (Table 5.1). In order to quantitate the levels of genotypes B and C present in the dual infections established after challenge with modified ratios, gt-spec RE digest was performed as described previously for each virus progeny population in which dual infection was detected by gt-spec PCR. In nearly all cases, only the higher input-level genotype was detected by gt-spec RE digest (Figure 5.13). However, of the four fish challenged with a ratio of 8 gtB to 1 gtC, two fish showed significant levels of gtC with one fish actually showing a higher level of gtC than gtB (Figure 5.13).

DISCUSSION

In summary, a novel system has been developed which allows determination of IHNV relative fitness values directly in the rainbow trout host. The advantages of this system include the fact that IHNV infection of rainbow trout is a natural host-pathogen relationship, large numbers of experimental animals can be used, fish can be challenged by immersion which mimics the natural horizontal route of infection with IHNV, and individual fish may be held in separate tanks to prevent cross-infection. The dual techniques of gt-spec PCR and gt-spec RE digest provide both a high level of sensitivity to detect mixed infections (gt-spec PCR) and the ability to quantitate the relative proportions within those mixed infections (gt-spec RE digest). The gt-spec RE digest quantitation is highly reproducible (within 3%) and also accurate (within 10%).

The initial application of this system was to perform a pilot-scale competition study between two virus genotypes which co-circulated in Hagerman Valley, Idaho aquaculture. The data in Chapters 1-4 suggest that multiple IHNV lineages co-circulate among the aquaculture facilities of the Hagerman Valley and that virus traffic frequently occurs within and between facilities. This suggests that viral variants likely

must compete with each other in order to be maintained within this system and that the virus types from the most prevalent and widely distributed subclades B and C are likely to have similar fitness. The results indicate that mixed infection is the typical outcome of challenge with representative virus genotypes gtB and gtC under the MISFIT conditions developed for the in-host competition system.

We anticipated the fish-to-fish variability in the gtB:gtC ratio observed in this pilot experiment for several reasons. Viral infection of a host organism is a complex process which is likely to involve many stochastic events which may allow one or a few virus particles to found the progeny population observed within the host [20, 58, 62, 285, 286]. These bottleneck events may occur upon transmission or during spread and replication within the host [71]. One contributing mechanism for bottlenecks within the host may be the phenomenon of viral interference, which has been demonstrated for the rhabdoviruses VSV and rabies virus [238, 265]. Stochastic events are expected to contribute to the variability of results from fish-to-fish. In fact, previous virus competition studies conducted in animal and plant hosts have shown significant variability between individual host organisms [53, 105, 139, 153]. Thus we expected to observe significant fish-to-fish variation in the levels of gtB and gtC in competition. This is exactly what was observed, with two fish having >95% gtC, one having >95% gtB, and the 13 others having various levels in between these extremes. This variability is strong evidence for the existence of stochastic events at some point in the transmission and replication cycle in this system, an observation that would not be appreciated in cell culture-based competitions. The unlikely alternative explanation for these results is that each fish has a different selective environment, with gtB being more fit in some fish and gtC more fit in others. Regardless of the mechanism, the wide variation seen between progeny of competitions in individual host is likely to reflect the reality of competition processes in nature. This emphasizes the need to do these studies with high numbers of animals to get a mean that indicates true population-scale fitness differences.

Despite the fish-to-fish variability in levels of gtB to gtC, preliminary results indicated that genotypes B and C were of similar fitness with an overall mean fitness ratio of 1.02 for the 16 fish challenged at 1:1 with gtB and gtC. This experiment will be repeated independently with a similar number of fish at least once more. We cannot formally claim that the outcome of this competition between gtB and gtC represents relatively equal fitness until it is demonstrated that this system can distinguish between genotypes of unequal fitness. This work is currently being conducted by K.A. Garver using pairs of isolates which are hypothesized to be of unequal fitness because they were associated with displacement events in the field. An experiment to be conducted in the immediate future will address the effect of virus entry into the host on the variability of fitness ratios from fish-to-fish by challenging fish via immersion and also via injection in parallel. In addition, competitions between viruses which have different levels of virulence will be conducted. With this system we also plan to determine whether viral fitness varies with host species, determine whether a time delay between challenge with one genotype and challenge with a second genotype limits superinfection, measure fitness at different stages of the virus transmission and amplification cycle (entry, in-host replication, shedding), and determine whether cell culture competition systems accurately reflect in-host fitness.

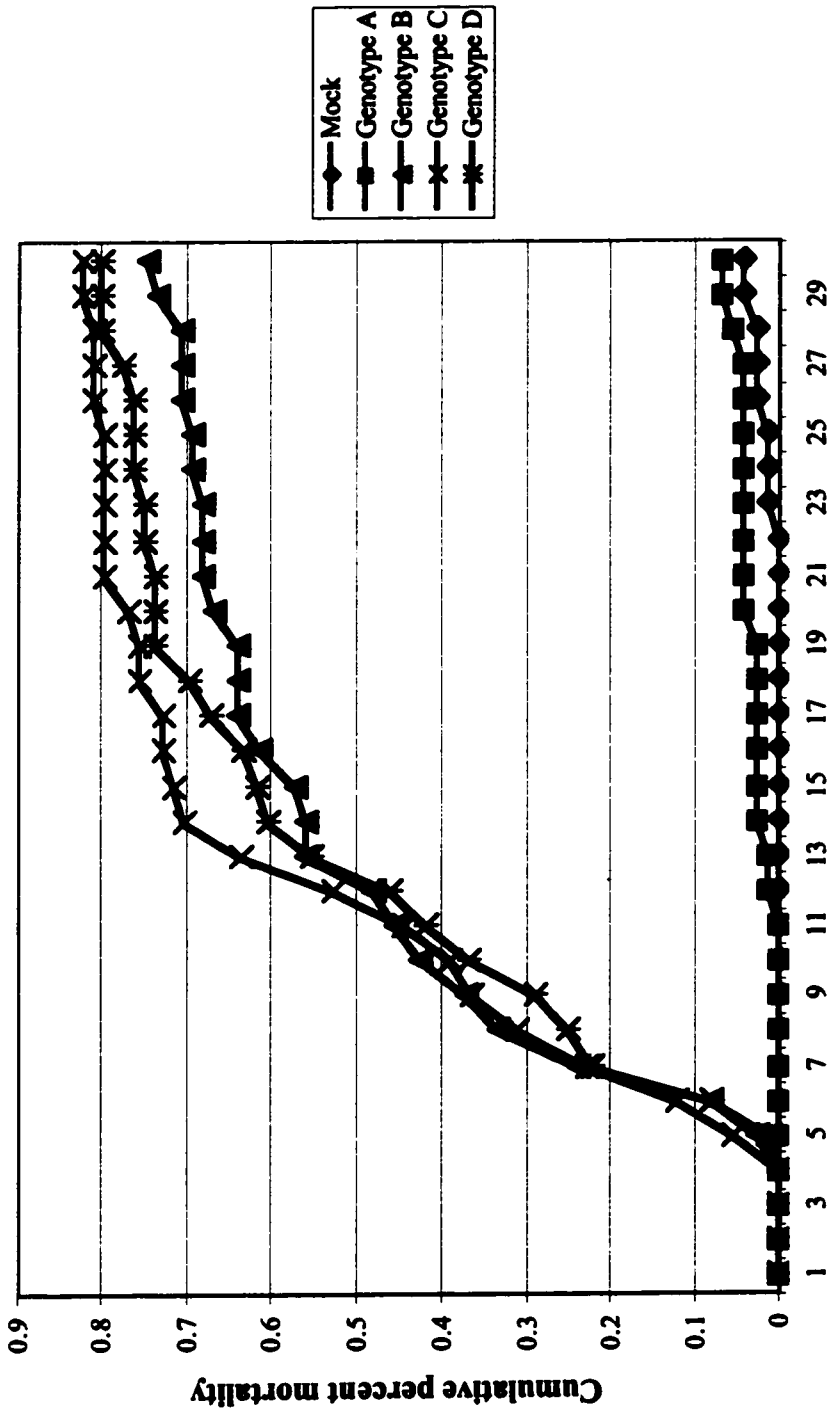


Figure 5.1 Virulence of IHNV genotypes chosen for virus competition experiments. The cumulative percent mortality for each genotype represents data pooled from three 25-fish replicate batch challenges (75 fish total per genotype). Groups of fish (1.34 g mean weight) were challenged at 10^5 virus pfu/mL for 12 hours at 15°C, then water flow was restored and fish were held for 30 days in batch at 15°C with mortalities removed and counted daily. "Days post-challenge" indicates the number of days since the initiation of the challenge.

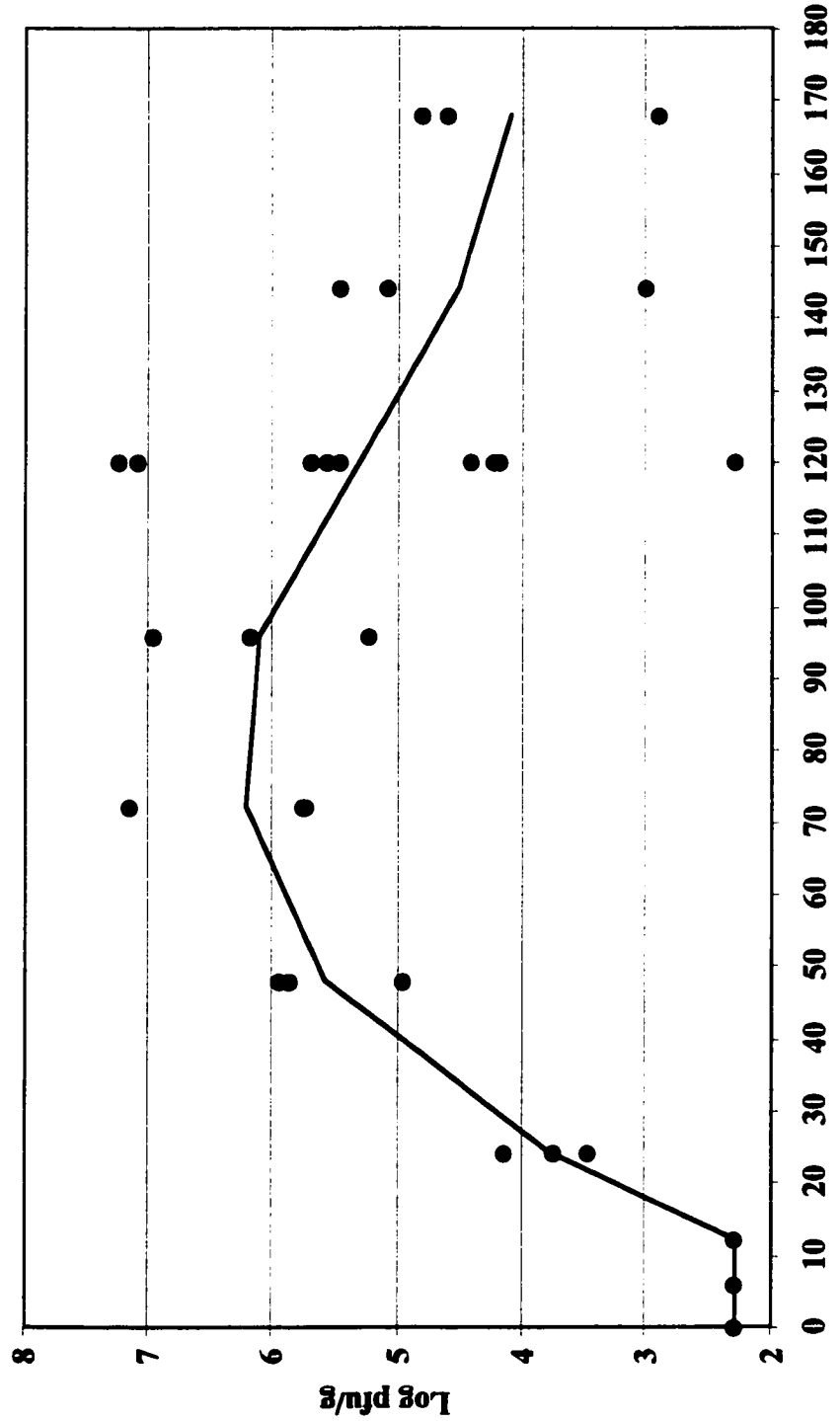


Figure 5.2 Example of an IHNV growth curve in fish: genotype C. Rainbow trout were challenged at 10^4 pfu/mL for 12 hours with genotype B, C, or D. Fish were then placed in a 4 L tank with flowing water for one hour to wash off excess virus. Fish were then held in individual buckets with 150 mL of 15°C water. At each timepoint, three fish were randomly chosen, sacrificed, frozen, and titered by plaque assay. The points on this graph represent titers for individual fish with a minimum detection limit of 2.3 log pfu/g. Ten fish were titered at the 120 hour timepoint.

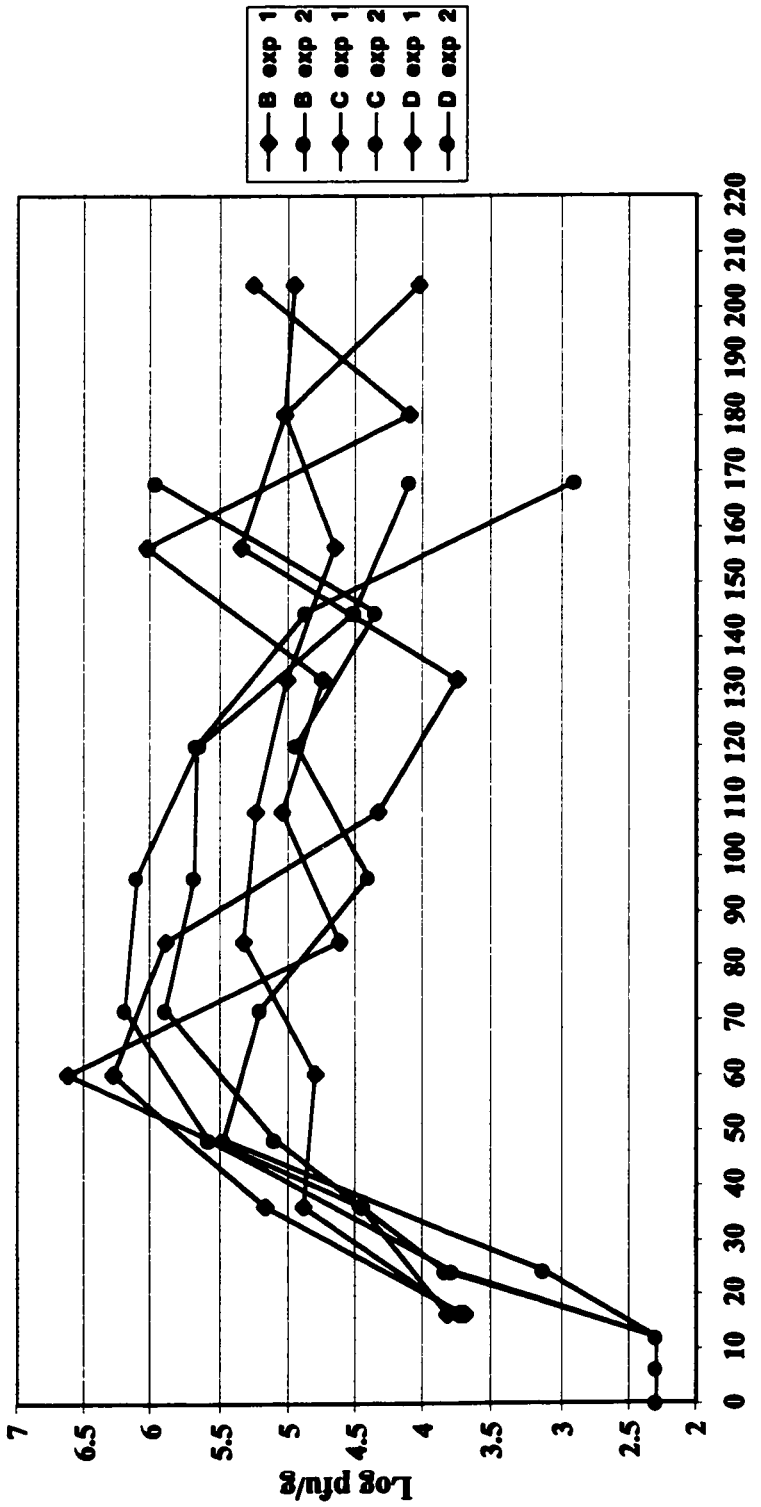


Figure 5.3 IHNV growth curves in fish: mean data. Rainbow trout were challenged and held as described in Figure 5.2. This graph shows mean titers in log pfu/g, calculated based on the titers of three individual fish sampled at each timepoint for each genotype. Two separate challenges were conducted. The results of experiment one are shown with diamonds and the results of experiment two are shown with circles.

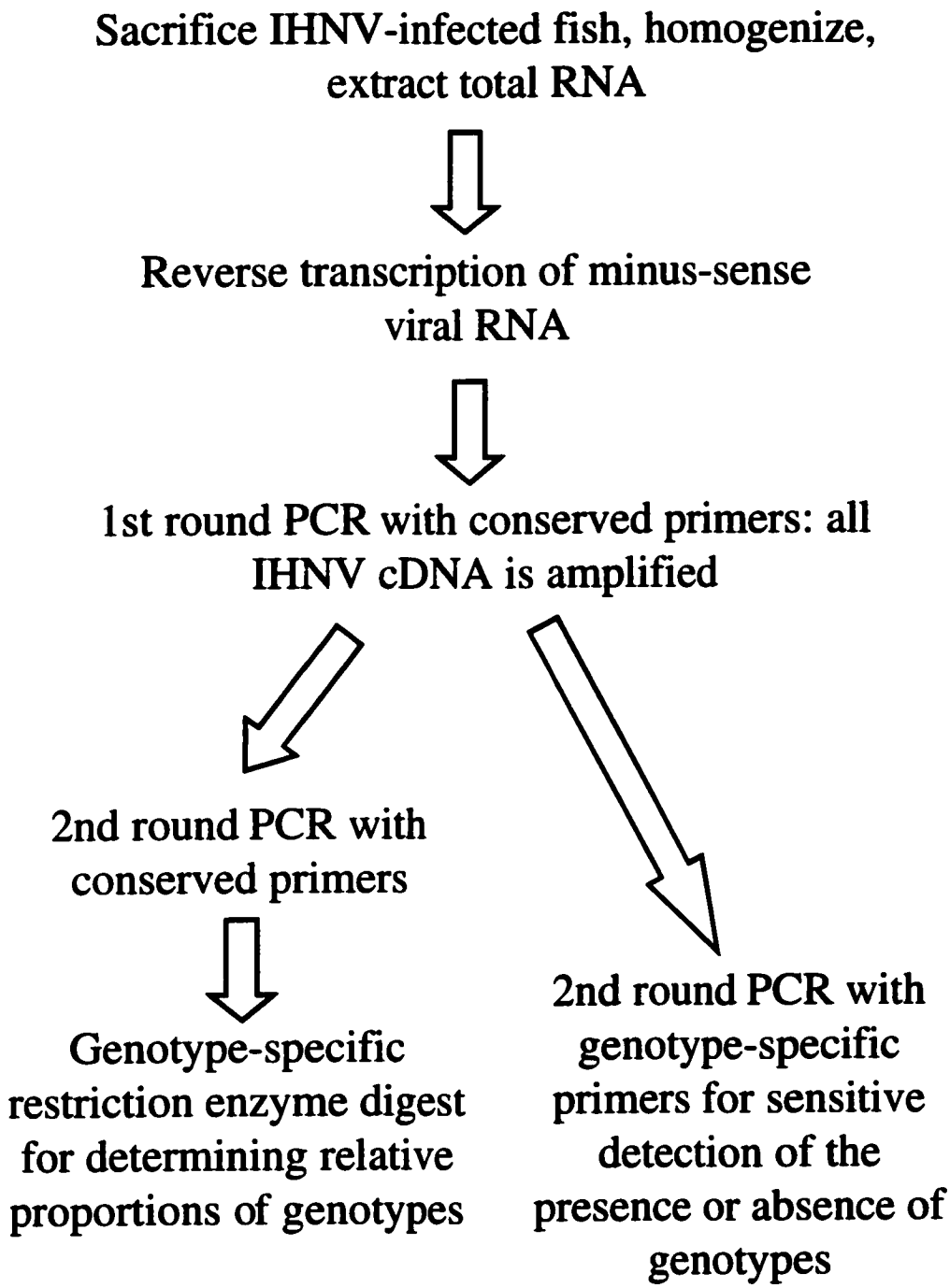


Figure 5.4 Flowchart of the analysis strategy for virus progeny populations.

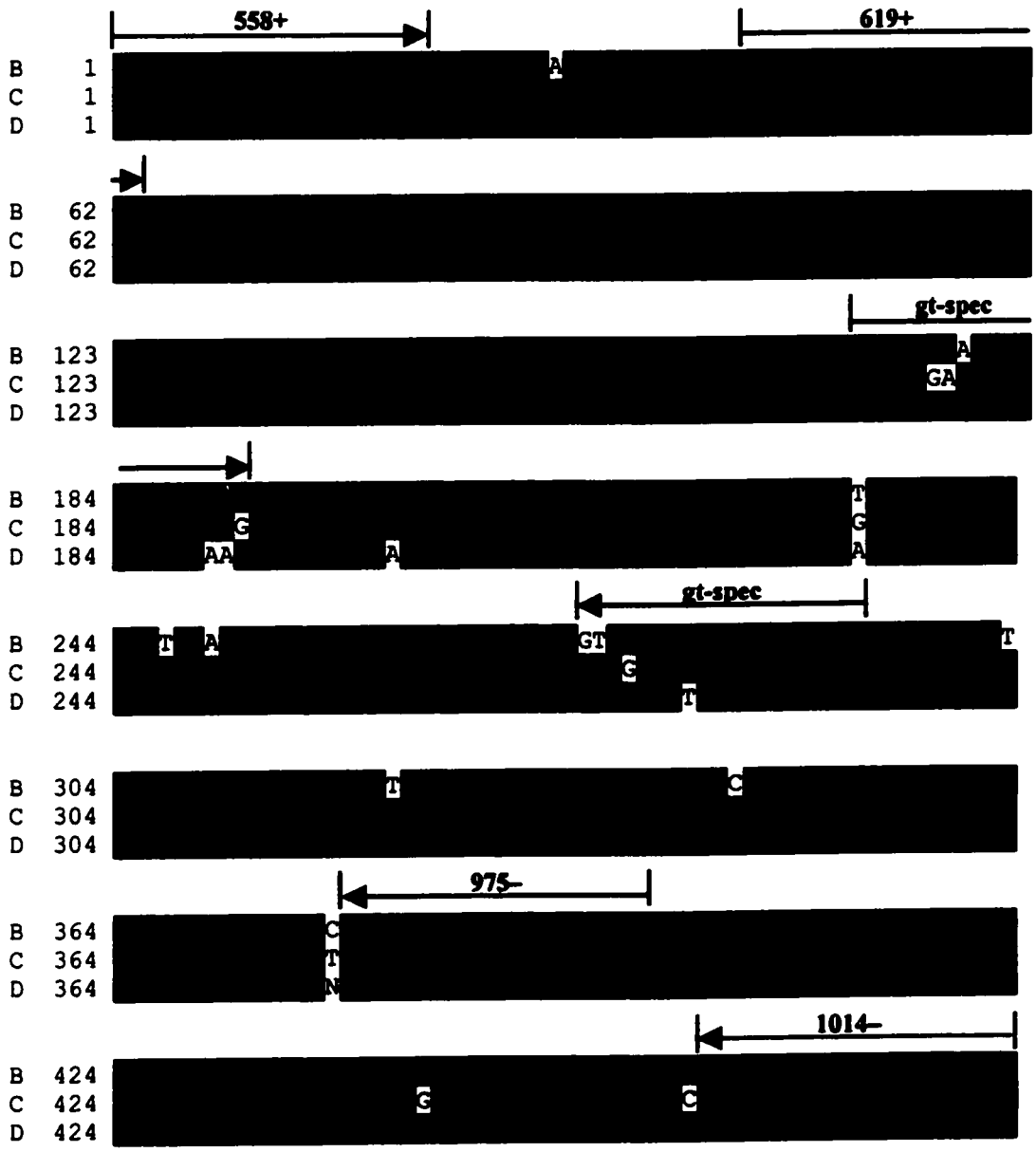


Figure 5.5 Location of the PCR primers used in this study. Primer pairs are matched by color: 558+:1014– (green) are conserved between the genotypes and amplify a 483 bp product, 619+:975– (red) are conserved and amplify a 357 bp product, and the genotype-specific (gt-spec, blue) PCR primers for genotypes B, C, and D amplify only their respective genotype. The gt-spec primers are aligned, but vary in length at their 5' ends (products between 119 and 124 bp).

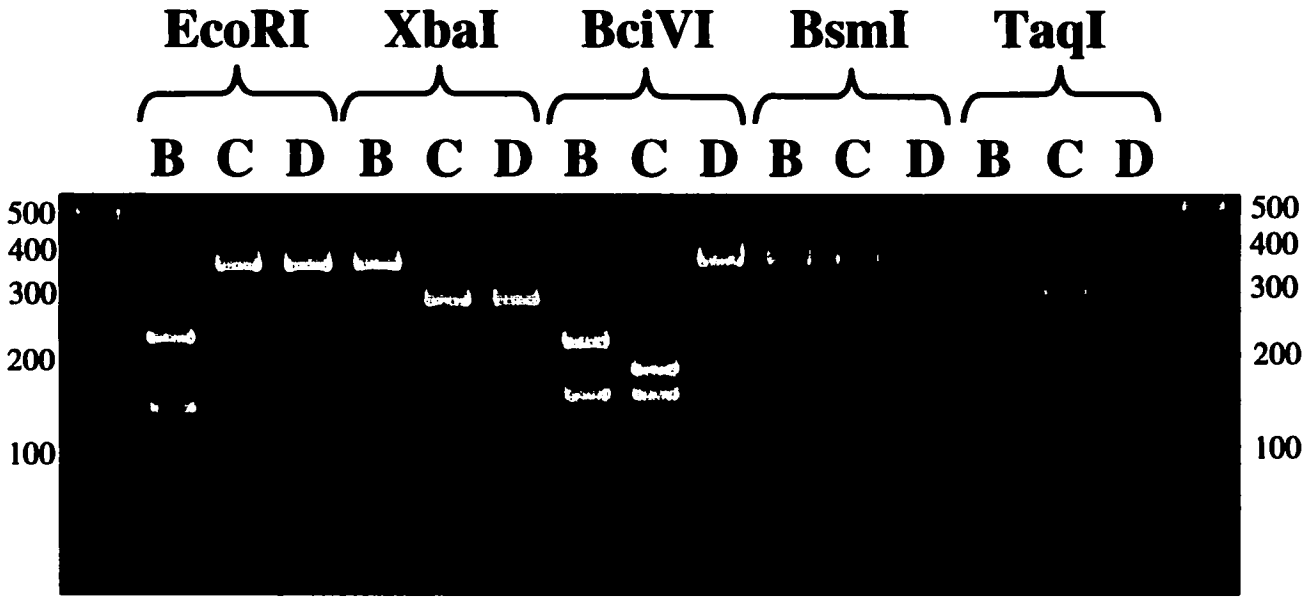


Figure 5.6 Genotype-specific restriction enzymes differentiate genotypes B, C, and D. The letter above each lane indicates the genotype of the DNA used in each restriction digest. The restriction enzyme used in each reaction is indicated above. EcoRI and XbaI differentiate genotype pairs B-C and B-D. BciVI, BsmI, and TaqI differentiate genotype pair C-D.

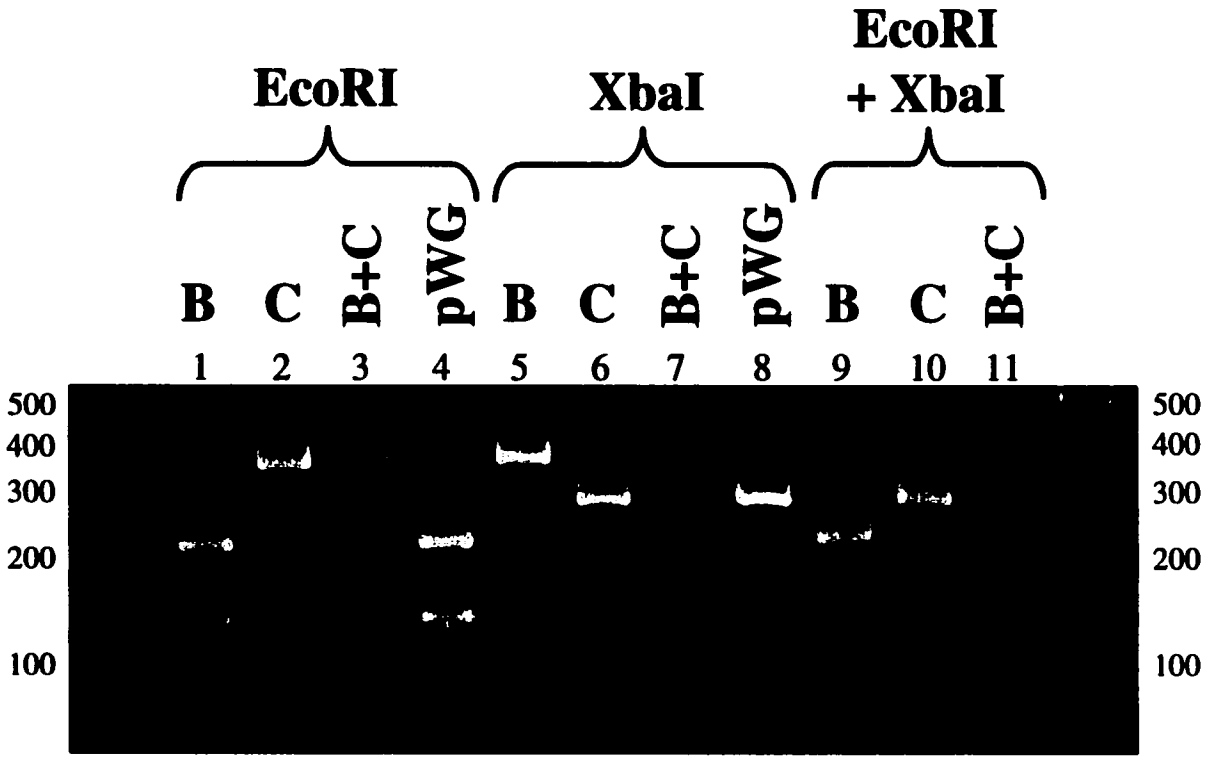


Figure 5.7 EcoRI and XbaI double digest (lanes 9-11) discriminates genotypes B and C in a single reaction. Lanes 1-8 are single restriction digests of DNA amplified from genotype B, genotype C, or both genotype B and C. Lanes 4 and 8 are digests of DNA amplified from a plasmid bearing the glycoprotein gene of the WRAC strain of IHNV, which contains both the EcoRI restriction site and the XbaI restriction site.

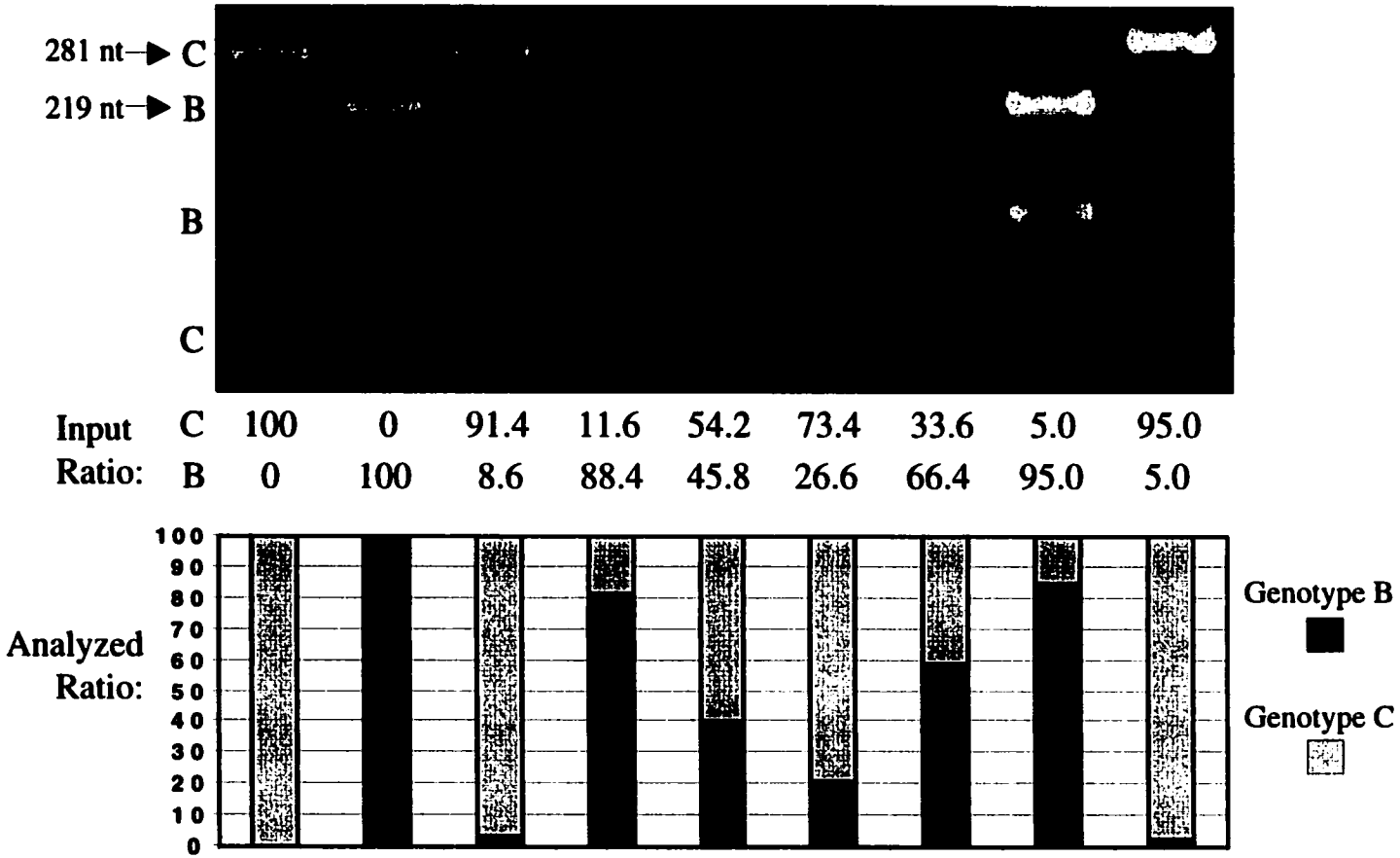
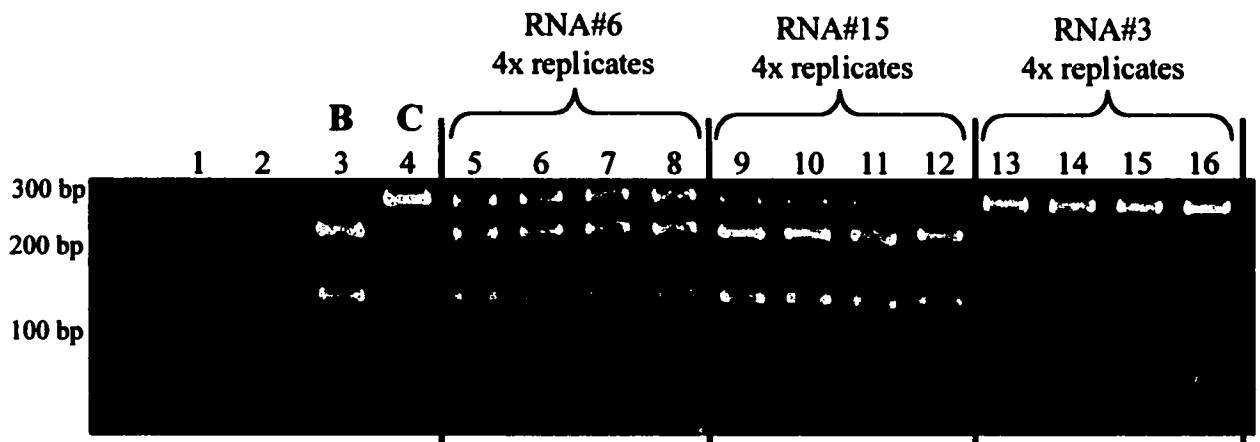


Figure 5.8 Quantitation of controlled mixtures of genotypes B and C. Titered virus stocks of genotypes B and C were mixed in the input ratios listed above. These mixtures were then RT-PCR amplified and analyzed by gt-spec RE digest as described. The gel panel shows the restriction digest patterns and arrows at left indicate the bands used for densitometry. The results, in terms of the observed percent of each genotype (anayzed ratios), are shown in the graph above.



Percent B	59.4	56.3	57.2	56.6	77.0	77.8	77.6	76.1	28.9	28.6	27.9	26.6
Percent C	40.6	43.7	42.8	43.4	23.0	22.2	22.4	23.9	71.1	71.4	72.1	73.4

Figure 5.9 RT-PCR followed by gt-spec RE digest provides highly reproducible quantitation of virus progeny populations. The proportion of genotypes B and C in each of three virus progeny populations (RNA#6, RNA#15, RNA#3) was quantitated in four independent replicates (lanes 5-8, 9-12, 13-16). Each RNA was subjected to reverse transcription, two rounds of PCR with conserved primers, and digestion with gt-spec restriction enzymes EcoRI and XbaI. Lane 1 is an RT-PCR negative (no template) control. Lane 2 shows analysis results using RNA extracted from an uninfected control fish. Lanes 3 and 4 indicate analysis of control B and C RNAs.

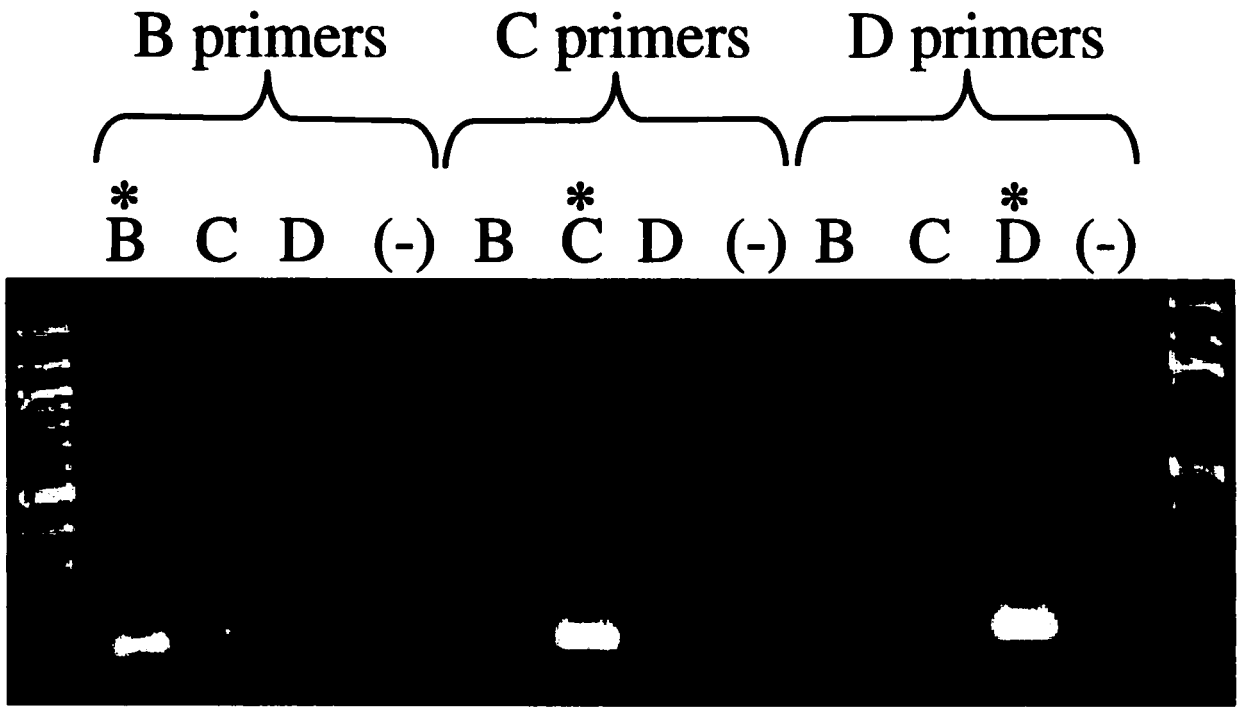


Figure 5.10 Genotype-specific PCR primers (gt-spec PCR). PCR reactions using gt-spec PCR primers for genotypes B, C, and D only amplify the complementary genotype. The letter above each lane indicates the genotype used as a template in the PCR reaction. The (–) indicates a control reaction with no template DNA

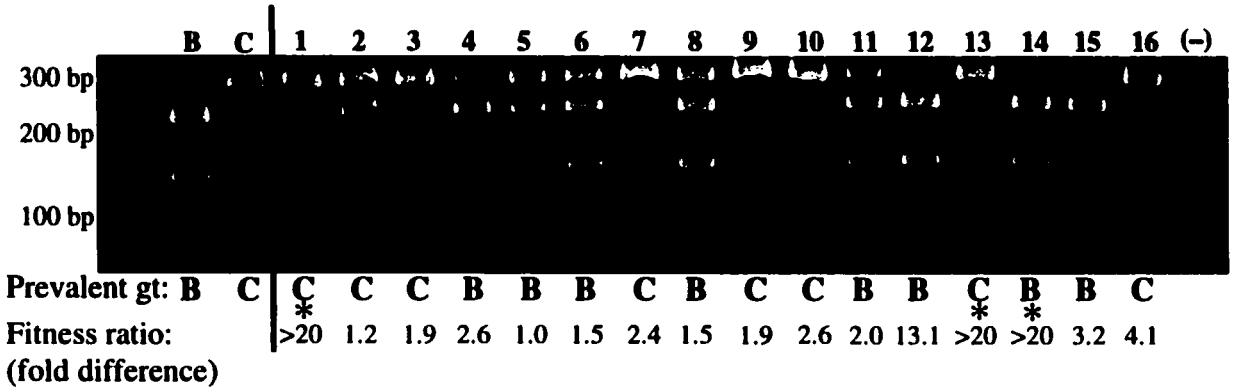


Figure 5.11 Genotype-specific RE digest of virus progeny populations from 16 fish (lanes 1-16) challenged with genotypes B and C at a 1:1 ratio. The genotype which was determined to make up a greater proportion of the total virus progeny population is listed underneath each lane. The fitness ratio is expressed as the percent of the more abundant genotype divided by the percent of the less abundant genotype and therefore indicates the fold difference in quantity between the two genotypes. The detection limit was <5% and so a fitness ratio of up to 20-fold was detectable. Asterisks indicate virus progeny populations with only one genotype detectable (<5% of the total). All other progeny populations have detectable mixed infections. The lanes marked "B" and "C" indicate single genotype controls.

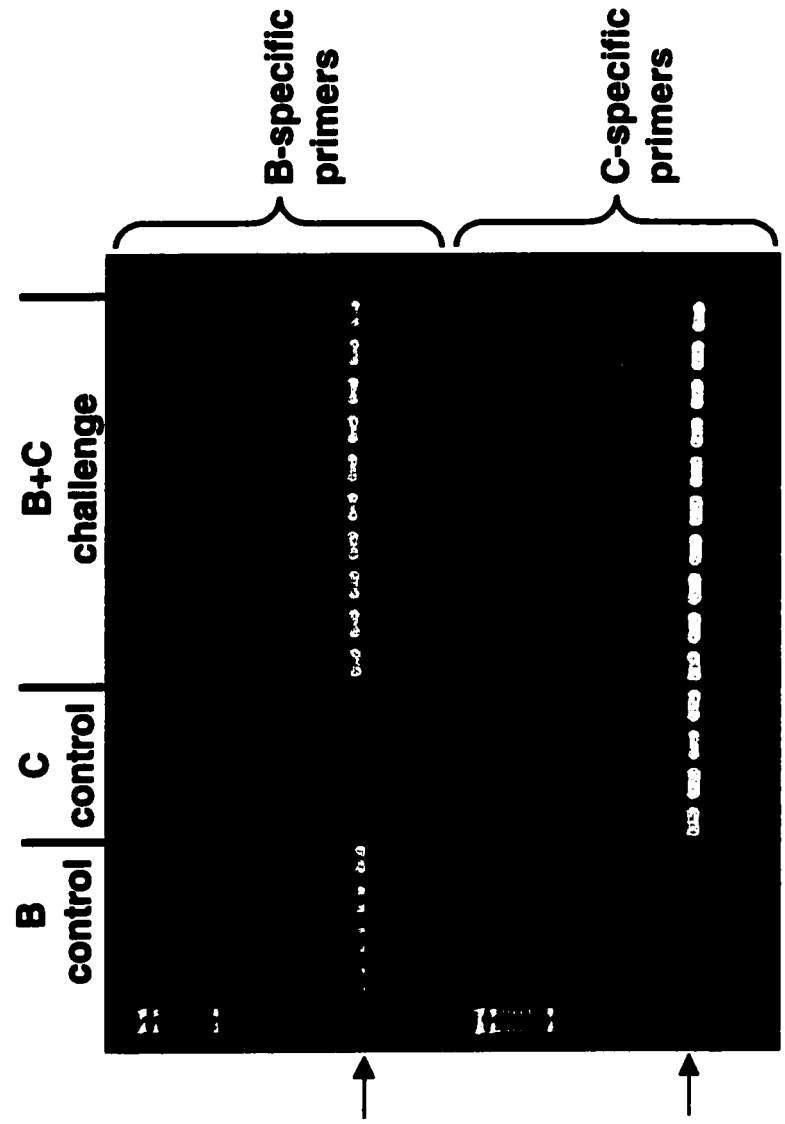


Figure 5.12 Genotype-specific PCR of virus progeny populations from fish challenged with either genotype B alone, genotype C alone, or a 1:1 mixture of genotypes B and C. Each lane indicates the result for one fish. Arrows mark the location of bands which indicate a positive result (~120 bp). The results for ten representative fish challenged with genotypes B and C are shown, but all 16 fish challenged with B and C were gt-spec PCR positive for both B and C.

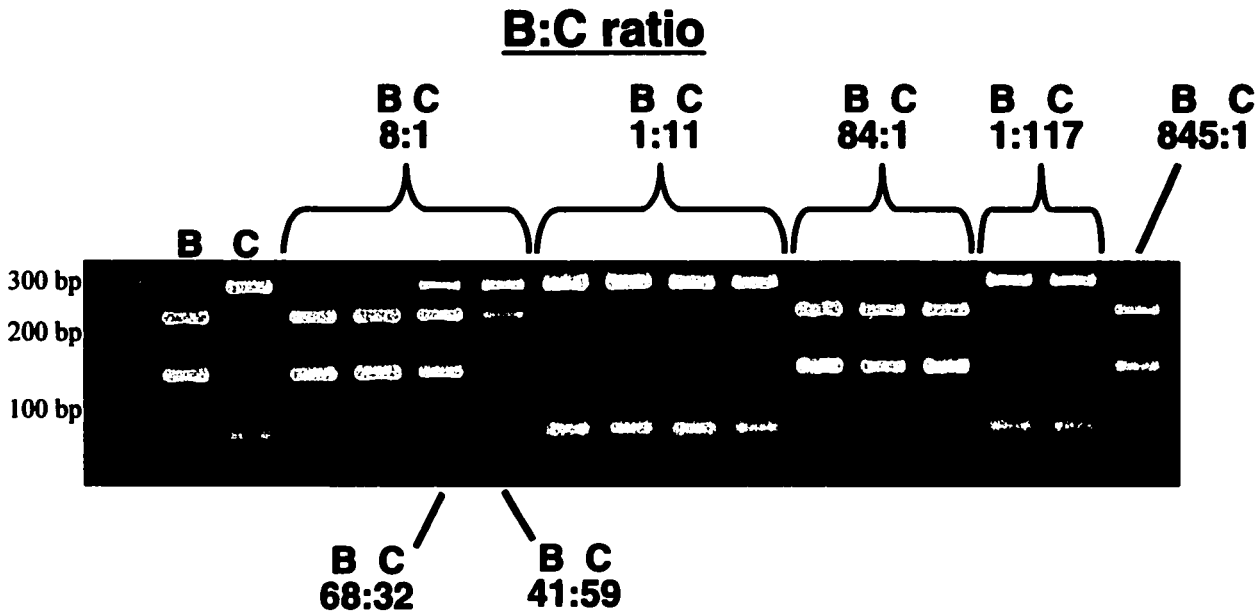


Figure 5.13 Genotype-specific RE digest of virus progeny populations in a modified-input ratio challenge. Fish were challenged with mixtures of genotypes B and C at ratios between 1:8 and 1:1181. Those fish which were positive for both genotypes by gt-spec PCR were quantified by gt-spec RE digest with EcoRI and XbaI, producing the results shown above. In the two cases above in which there was a detectable amount of the lower input-level genotype, the quantitated percent of each genotype in the progeny population is shown below lanes. The minimum detection limit is <5% of the total progeny population.

Table 5.1 In-host competition assays with modified input ratios of genotypes B and C. Genotype-specific PCR was used to determine the presence or absense of genotypes B and C in fish after challenge with modified ratios of B and C. Each row indicates the results for one fish.

B>C	B	C
8:1	+	+
	+	+
	+	+
	+	+
84:1	+	+
	+	+
	+	–
	+	–
	+	+
845:1	+	+
	+	–
	+	–
	+	–

C>B	B	C
11:1	+	+
	+	+
	+	+
	+	+
117:1	+	+
	–	+
	–	+
	+	+
1181:1	–	+
	–	+
	–	+
	–	+

CHAPTER 6: Summary Discussion

In summary, the genetic diversity of IHNV was characterized at four different levels within an endemic focus in rainbow trout aquaculture, and a novel *in vivo* virus competition system for IHNV genotypes in a rainbow trout host was developed. At the level of the Hagerman Valley trout farming region, the observation of up to 7.6% virus divergence within such a localized geographic region is novel among fish rhabdoviruses. To put these results in context, the Kurath laboratory has recently completed a study of the genetic diversity present among IHNV isolates throughout the North American range of the virus. A phylogenetic analysis of 323 IHNV isolates, including isolates from Chapter 1 of this dissertation, revealed that there are three major bootstrap-supported phylogenetic clades of IHNV, referred to as U, M, and L (G. Kurath, K.A. Garver, R.M. Troyer, E.J. Emmenegger, K. Einer-Jensen, and E.D. Anderson, submitted for review). The U clade comprises isolates which range geographically from Alaska through the Columbia River basin in Washington, northern Oregon, and Idaho; while the L clade comprises isolates from California and southern Oregon. With the exception of two isolates, there is no geographic overlap between the U and L clades. The M clade includes all isolates from the Hagerman Valley (characterized in this dissertation), as well as approximately one third of the IHNV isolates from the Columbia River basin. Detailed analysis of M clade isolates found in the Columbia River basin outside of the Hagerman Valley suggests that these viruses likely resulted from sporadic virus traffic from the Hagerman Valley, indicating that the Hagerman Valley is likely the reservoir for the entire M clade (K.A. Garver, R.M. Troyer, and G. Kurath, submitted for review). When the intrapopulational diversity within each of these three clades is compared, the M clade has a three to four-fold higher level of intra-clade diversity than the U and L clades. These data strongly support the hypothesis that conditions specific to Hagerman Valley rainbow trout aquaculture have accelerated the evolution of IHNV relative to the stasis observed in the rest of the North American range of the virus.

The maximum level of nucleotide diversity found within the entire study of 323 IHNV isolates was 8.6%. This is significantly lower than the 17-20% divergence observed among VSV, rabies virus, or VHSV isolates, suggesting that IHNV exhibits low genetic diversity compared to other rhabdoviruses. However, the M clade isolates alone had up to 7.6% divergence, significantly higher diversity than isolates in the other clades. The level of diversity found among M clade IHNV isolates does not approach the total divergence observed among other rhabdoviruses. However, these example rhabdoviruses have likely been evolving for hundreds, or even thousands of years, while IHNV has likely only been evolving within the Hagerman Valley since the mid-1970's. This approximate date of origin for the Hagerman Valley M clade is supported by the finding that isolates in the Hagerman Valley from the years 1978-1985 were positioned a very short genetic distance from the inferred ancestor of the clade. In fact, the temporal trend observed in this clade may be relatively unique among rhabdoviruses, for which there typically is not a clear relationship between tree position and date of isolation (see Introduction). The estimated rate of mid-G evolution of IHNV within the Hagerman Valley was 1.2×10^{-3} substitutions per nucleotide site per year (13.4 substitutions per IHNV genome length per year). This is three-fold higher than the estimated rate of evolution of the rabies virus glycoprotein gene at synonymous sites (the overall rate was not provided in this study, but would likely have been lower) [119]. Benmansour et al. [19] estimated rates of evolution for one presumed VHSV lineage, indicating a "fast phase" of 4.5×10^{-3} substitutions per nucleotide site per year, and a "slow phase" of 1.6×10^{-4} substitutions per nucleotide site per year. However, these calculations were, in each case, based on the genetic distance between a single pair of isolates presumed to be part of the same direct lineage.

The genetic diversity of IHNV in the Hagerman Valley was characterized at the intra-outbreak and intra-host (quasispecies) levels in order to obtain a higher-resolution picture of the diversity of IHNV in this unique environment. We speculated that by characterizing these populations and comparing them to other IHNV

populations in wild and hatchery fish, we might begin to understand the mechanism(s) by which conditions specific to Hagerman Valley rainbow trout aquaculture have accelerated IHNV evolution and allowed the co-circulation of multiple lineages. Taken together, the intra-outbreak and intra-host results suggest that at these levels, IHNV populations in Hagerman Valley rainbow trout look similar to IHNV populations in wild and hatchery fish in terms of genetic diversity. This finding does not support the hypothesis that the increased diversity is the result of forces which would be expected to act at the intra-host or intra-outbreak level, such as a higher mutation frequency of the viral polymerase at 15°C, selection of immune escape variants, or a lack of bottlenecks within a single outbreak (maintenance of diverse lineages within the outbreak). Instead, these findings lend support to the hypothesis that the increased diversity is the result of forces acting at a higher level. Perhaps a clue lies in the finding that the two different simultaneously-occurring outbreaks studied had two very different haplotypes. Partitioning of the enormous fish population present in the Hagerman Valley into multiple rearing units and multiple facilities may play a role in reducing virus competition and allowing localized micro-evolution and multiple co-circulating lineages. The preliminary finding that two major co-circulating genotypes within Hagerman Valley rainbow trout aquaculture have relatively equal fitness suggests that these viruses are relatively equally adapted to replication in rainbow trout and that competition among these genotypes in rainbow trout aquaculture, when it occurs, may not result in displacement of either genotype.

Virus diversity must originate at the intra-host level, but perhaps what differentiates the Hagerman Valley from other aquaculture environments is the lack of a density restriction on virus transmission and the lack of a seasonal virus bottleneck in the system. Although the transmission cycle of IHNV in nature is not well defined, it is possible that under natural conditions, virus lineages may spend significant portions of the year in a latent or carrier state. In contrast, replication of virus lineages in the Hagerman Valley might essentially involve movement from outbreak to outbreak, with varying levels of disease occurring, depending on host and

environmental parameters. This could result in virus lineages in the Hagerman Valley accumulating more mutations per year than virus lineages present in other environments, despite similar levels of diversity within single outbreaks. Additionally, there is evidence that the evolution of some vector-borne viruses is constrained by the requirement for replication in different species [125, 261, 262]. Thus, the diversity of IHNV in nature could be constrained by replication in an unidentified alternate host or vector species. It is also possible that virus adaptation to the rainbow trout host, 15°C temperature, or other condition within the Hagerman Valley may also play a role in the generation of diversity.

The novel in-host competition system developed in this dissertation will be an invaluable tool for exploring the biology of IHNV. At present we know that controlled mixed infections can be established in the laboratory, that the proportion of each virus type in these mixed infection can be characterized, that we can derive quantitative fitness values from these proportions, and that a preliminary competition between two Hagerman Valley IHNV genotypes resulted in fitness values that varied from fish-to-fish but were essentially equal when averaged across all 16 infections. In the future this system will allow us to assess the role of viral fitness in virulence, viral displacement events, host-specificity, and viral superinfection, as well as determining which stages of transmission are responsible for differences in fitness and determining whether cell culture competitions model what is observed *in vivo*. Additionally, by modifying virus input ratios in competition, we will be able to determine the bottleneck size associated with IHNV virus transmission. Understanding the size of this genetic bottleneck will be critical to designing serial passage experiments which will allow us to serially passage IHNV in fish under different selection pressures in order to determine what conditions are responsible for virus diversity in fish populations.

The results of the work in this dissertation have several important practical implications for fish health management. The co-circulation of IHNV lineages among all types of facilities within the Hagerman Valley implies that virus traffic occurs with

some frequency among facilities through an unknown vector and/or reservoir. This reinforces the importance of identifying the vector(s) for control of the virus, and suggests that elimination of the virus will only be possible through joint efforts between all facilities within the region. Now that we have thoroughly characterized the virus types present within the Hagerman Valley, it is possible to determine with some confidence whether the Hagerman Valley is a source of virus for wild and hatchery fish throughout the rest of the Columbia River basin. A recently completed analysis of virus types throughout this region suggests that in two cases (once in the early 1980's and recently in the late 1990's), an M clade virus type has become established in the Lower Columbia River basin, while sporadic cases of M clade virus isolation have occurred throughout the rest of the state of Idaho (K.A. Garver, R.M. Troyer, and G. Kurath, submitted for review). The epidemiologic patterns observed suggest that the Hagerman Valley is likely not a constant source of water-borne virus for regions downstream, but instead is a sporadic source of virus, likely through the transport of infected fish from hatcheries located within the Hagerman Valley (K.A. Garver, R.M. Troyer, and G. Kurath, submitted for review).

It is not known whether the ongoing evolution of IHNV within the Hagerman Valley presents a risk for rainbow trout farms or wild and hatchery fish. In the view of this author, as the virus continues to diverge and explore additional "sequence space" [73], the possibilities increase for the development of new phenotypes which could present problems for disease management. Examples of potential phenotypes could include immune escape variants, variants with increased pathogenicity, or variants with increased neurotropism (hence causing greater spinal deformities in survivors – a major loss to trout producers). All of these possibilities are highly speculative, but should not be overlooked, given the evolutionary capacity of IHNV in this environment. Although IHNV appears to maintain a single serotype [78], it will be prudent that those with an interest in vaccine design continue to periodically monitor new Hagerman Valley virus types which arise, to assure that immunity generated to a single virus isolate can cross-protect against other isolates. While we

could not detect a change in virus virulence between the two time periods examined in Chapter 1 (1990-1992 and 1997-1998), this does not exclude the possibility of future increases or decreases in virulence. The continued introduction of susceptible fish into high density rearing units suggests that there is not likely to be a high fitness cost to the virus associated with virulence in this system [84], which suggests that virulence is not likely to decrease significantly over time. With the recent re-appearance of IHNV epidemics in Atlantic salmon marine net-pen aquaculture in British Columbia, there is the possibility that if IHNV were allowed to become endemic within the industry it might begin a similar process of rapid evolution under conditions of intensive aquaculture. So far virus in the Atlantic salmon marine net-pen industry is only epidemic and the industry has instituted a disease management plan that includes quarantine programs, culling, and fallowing of sites [243]. This proved successful at eliminating the IHNV types associated with outbreaks in 1992-1997 (G. Kurath and G.S. Traxler, unpublished data). In terms of the potential for generating high levels of virus genetic diversity, another key difference from Hagerman Valley rainbow trout aquaculture is that there is no partitioning of the virus population between different Atlantic salmon net pens since these pens are suspended in the same water. Thus, facilities may not be as likely to maintain diverse viral genotypes.

In conclusion, the finding of high virus genetic diversity within the Hagerman Valley relative to other environments suggests that conditions specific to intensive rainbow trout aquaculture have resulted in increased IHNV evolution. This increase in genetic diversity is evident at the inter-outbreak level, but not at the intra-outbreak or intra-host levels. In order to move beyond observational studies, a novel in-host virus competition system was developed which will allow a thorough analysis of the role of virus fitness in the biology of IHNV.

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